UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Unité Propre du CNRS 9022

THÈSE présentée par :

Samantha HALLER

soutenue le : **18 septembre 2014**

pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : Sciences du Vivant

Aspects Moléculaires et Cellulaires de la Biologie

Etude des interactions hôte-pathogène entre

Pseudomonas aeruginosa* et *Drosophila melanogaster

dans un modèle d'infection intestinale

THÈSE dirigée par :
M. FERRANDON Dominique Docteur, UPR9022 CNRS

RAPPORTEURS :
M. JASPER Heinrich Professeur, Buck Institute for Research on Aging
M. FILLOUX Alain Professeur, Imperial College London

AUTRES MEMBRES DU JURY :
Mme BLANDIN Stéphanie Docteur, INSERM

Acknowledgements

First of all I wish to thank Professor Jules Hoffmann and Professor Jean-Marc Reichhart for having made the laboratory one among the best in the field of innate immunity and a very fruitful environment for young scientist.

I deeply thank Professor Heinrich Jasper, Professor Alain Filloux and Doctor Stephanie Blandin for taking from the time to read and evaluate my work and to come to Strasbourg to attend at my PhD defense.

Dear Dominique, the last five years you were always present for discussions and help whenever I needed it. I friendly thank you for all you taught me in Science but not only.

Steffi, I need to thank you for the establishment of the *Pseudomonas aeruginosa* intestinal infection model in our laboratory and even more for all you taught me during my first year in the lab.

I do not forget neither my current and formers colleagues and lab mates for their help and support the last five years…Moreover I also thank the ESBS team and especially Olivier for all his support.

I am grateful to my friends and to my family for their permanent support and encouragements.

ABBREVIATIONS

AHL	Acyl-homoserine lactone
AMP	Antimicrobial peptide
CCR	Copper cell region
CF	Cystitic fibrosis
CrPV	Cricket Paralysis Virus
Crq	Croquemort
DAP-type PGN	*meso*-diaminopimelic acid-type peptidoglycan
DCV	Drosophila C Virus
DD	Death domain
DIF	Dorsal-related immunity factor
DSCAM	Down syndrome cell adhesion molecule
DsRBP	DsRNA binding protein
DsRNA	Double-stranded RNA
DUOX	Dual oxidase
EB	Enteroblast
EC	Enterocyte
EE	Enteroendocrine cell
Gal4	Galactose4
Gal80ts	Galactose80 thermo-sensitive
GNBP	Gram-negative binding protein
HCN	Hydrogen cyanide
HHQ	4-hydroxy-2-heptylquinoline
Imd	Immune deficiency
IRAK	IL-1 receptor-associated kinase
IRD5	Immune response deficient 5
ISC	Intestinal stem cell
JAK	Janus kinase
JNK	Jun kinase
Key	Kenny
LRR	Leucine-rich repeat
Lys-type	PGN Lysine-type peptidoglycan
Mcr	Macroglobulin-complement related
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PO	Phenoloxidase
PQS	3,4-dihydroxy-2-heptylquinoline
ProPO	Pro-phenol oxidase
PRR	Pattern recognition receptor
PRR	Pattern recognition receptor
Psh	Persephone
QS	Quorum sensing
RHIM	RIP homotypic interaction
RIP	Receptor interacting protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species

Sec	Secretory pathway
siRNA	Small interfering RNA
SPE	Spaetzle-processing enzyme
SR-CI	Scavenger receptor CI
STAT	Signal transducer and activator of transcription
TAB2	TAK1-associated binding protein 2
TAK1	TGF-beta-activated kinase 1
Tat	Translocation pathway
TCT	Tracheal cytotoxcin
Tep	Thioester-containing protein
TIR	Toll/interleukin-1 receptor
TISC	Toll-induced signaling complex
TLR	Toll-like receptor
UAS	Upstream activator sequence
Upd3	Unpaired3
VSR	Viral suppressor of RNAi
VSV	Vesicular Stomatitis Virus

Table of content

Table of content

Preface

In their environment, multicellular organisms are permanently in contact with potential microbial invaders, a situation already encountered by the common ancestor to metazoans.

On one side, these organisms have developed different strategies to protect themselves and to destroy the pathogens. Even though the first layer of defense against invaders is a physical barrier, the stronger arm is the immune system. In all vertebrates, the immune system is composed by the innate and the adaptive immune response.

With regards to insects, the immune system is composed of only an innate immune system that is however sufficient to ensure a strong protection against a variety of microorganisms. Strong homologies between mammalians and insects innate immunity exist as has been shown with the identification of the Toll-like receptor, homologous to the *Drosophila* Toll receptor. Indeed, *Drosophila melanogaster* is a powerful genetic organism model that carry many avantages compared to mammalian model.

On the other side, invaders co-evolu with their hosts and constantly develop novel virulence strategies. One of these invaders, *Pseudomonas aeruginosa*, is an opportunistic Human pathogen and the fourth most commonly isolated nosocomial pathogen. Even though, *P. aeruginosa* infection are mostly curable, an acute fulminant infection like pneumonia, burn wound infection or sepsis, leads to a very strong mortality rate. Treatments against *P. aeruginosa* are principally based on antibiotics which become progressively less efficient with the apparition of resistant strains.

New therapeutics are under investigation, like molecules that could function as quorum sensing inhibitors. Some compounds have already been identified but their curative effect on a *P. aeruginosa* remains to be confirmed. Moreover, there is a need to deeper understand the bacteria virulence in order to better target specific strains.

Therefore the goal of my PhD was to use the power of the *Drosophila* model organism to study *P. aeruginosa* virulence systems.

General Introduction

.

I. *Drosophila melanogaster*

A. A genetic model organism

The fruit fly *Drosophila melanogaster* is an insect from the order *Diptera*. *Drosophila* have a short life-cycle (10 days at 25°C), a small size (2 mm) and thus small infrastructural requirements are needed to culture them.

Following a suggestion from Charles Woodworth, William E. Castle started studying *Drosophila melanogaster* and published a monograph in 1906 in PNAS : « The Effects of Inbreeding, Cross-Breeding, and Selection Upon the Fertility and Variability of *Drosophila* ». Following this work, Thomas H. Morgan isolated his first *Drosophila* mutant in 1909 and later on developed “The chromosomal theory of heredity” (Nobel Prize, 1933).

Thomas H. Morgan's students constituted the next generation of *Drosophila* researchers, Drosophilists. His students, especially A. H. Sturtevant, C. B. Bridges and H. J. Muller developed the *Drosophila* organism model by developing the balancers chromosomes, identification of larval salivary glands giant polytene chromosomes and mapping of genes on the chromosomes. In 1927, Hermann J. Muller showed that ionizing radiation (x-rays) causes genetic damage including chromosomal rearrangements (Nobel Prize, 1946). More recently, Edward B. Lewis developed a chemical mutagenesis that allowed to generate point mutations and thus ushered the first genome-wide saturating mutagenesis screens (Nobel Prize, 1995, together with Christian Nüsslein-Volhard and Erich Wieschaus).

In the time of a few decades, *Drosophila melanogaster* became a model organism of choice in the field of genetic. Today, physical methods, chemicals, and even transposons can be used to generate mutants and genome-wide analysis.

Associated to the generation of *Drosophila* mutants, balancer chromosomes are of particular importance. Because they carry a lethal mutation, they allow the maintenance of lethal or sterile mutant stocks by preventing the loss of these mutations that are "equilibrated" by the balancer lethal chromosome, hence their name. They possess multiple inverted

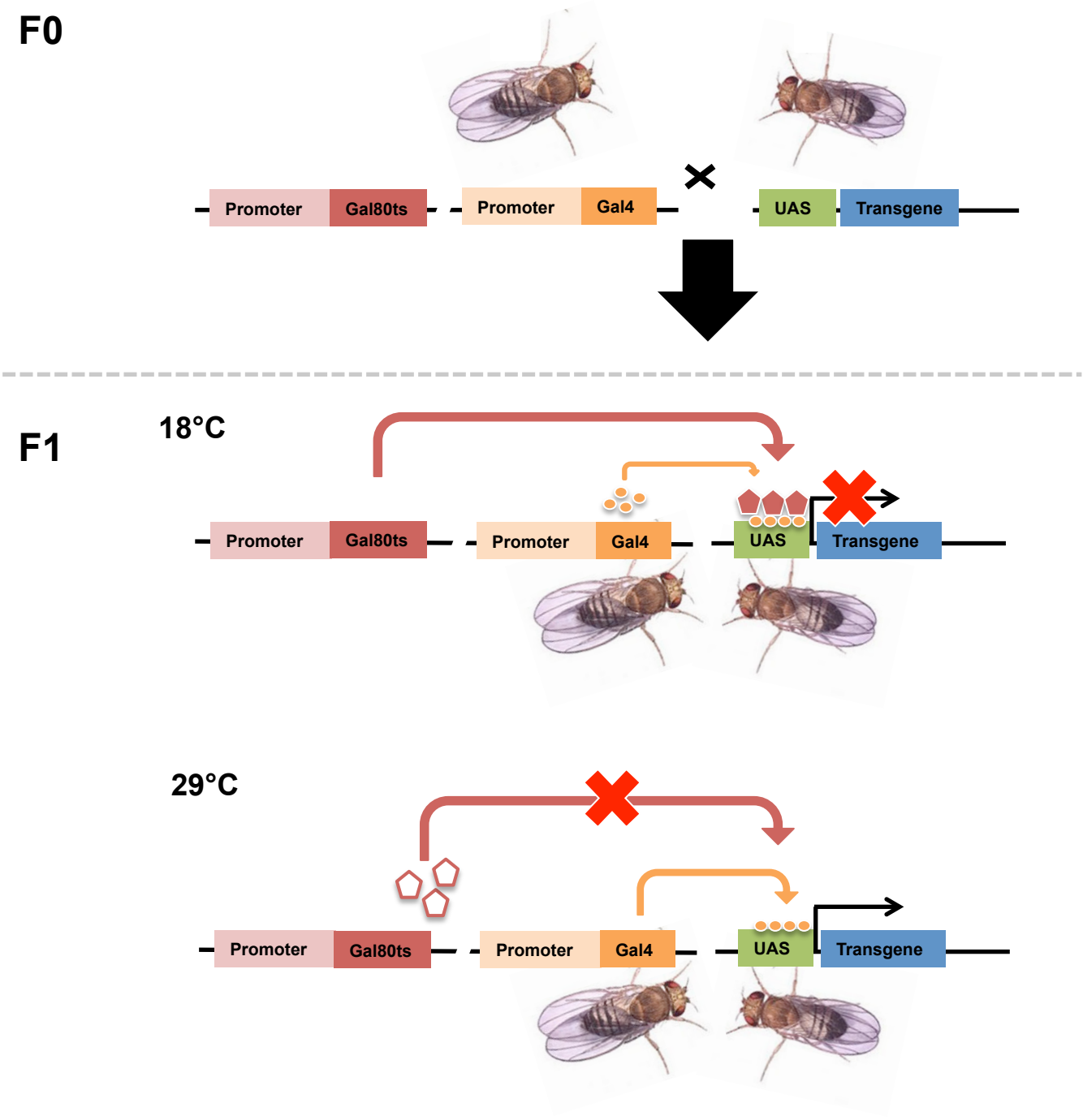


Figure 1. The thermosensitive UAS-Gal4 tissue- or cell type-specific expression system.

A fly line carrying the *Gal4* driver and the *Gal80ts* sequences under promoters (ubiquitous for *Gal80ts* and in the tissue or cell type of choice for *Gal4*) are crossed with a fly line harboring a transgene of choice expressed under the control of *UAS* sequences (F0). Crosses are done at 18°C. At that temperature, all three sequences are present in the progeny flies (F1) and a functional Gal4 repressor, Gal80 is expressed. Gal4 is inhibited thus blocking the transcription of the transgene. When F1 flies are transferred to 29°C, the conformation of Gal80 changes and it is unable to bind to Gal4, thus allowing the binding of Gal4 to *UAS* sequences and the recruitment of the RNA polymerase.

sequences that prevent meiotic recombination between homologous chromosomes. In addition, they contain marker genes allowing its tractability through fly generations.

Recently, the introduction of the yeast Upstream Activation Sequence (UAS) associated with the *galactose4* gene (Gal4) permits the spatial control of transgene expression. Later on, the development of a thermo-sensitive Gal80 (Gal80ts), in addition to the UAS-Gal4 system, allowed the temporal and the spatial control of the transgene expression (Fig. 1).

In parallel to the UAS-Gal4 system, the RNA interference (RNAi) has been developed using transgenic technology. A transgenic construct allows the expression under the control of Gal4 of a hairpin or a miRNA designed to target the transcripts of a given gene. It is a powerful tool to silence the expression of target genes. A library of more than 10,000 transgenic *Drosophila* strains covering more than 88% of the fly genome is kept at the Vienna *Drosophila* Research Center and is available to the *Drosophila* community (Dietzl et al., 2007). Other resources are available in the US (TRiP) or in Japan (NIG-Fly).

B. *Drosophila* innate immunity

The *Drosophila* innate immunity is composed of two branches of defense: the systemic humoral response and the cellular response. The systemic response involves mainly the Toll and the Immune deficiency (Imd) pathway while the cellular immune response corresponds predominantly to the phagocytosis ability of specialized macrophage-like cells referred to generically as hemocytes. The Toll and Imd pathways induce the expression of different genes like those encoding antimicrobial peptide (AMPs), opsonins, components of the melanization or clotting system, among others.

1. Cellular response

Like all insects, *Drosophila melanogaster* uses a tracheal respiratory system and its circulatory system is open. At the adult stage, the dorsal vessel drives a continuous flow of the hemolymph in the general cavity of the fly. In addition, hemocytes, use this flow to patrol the whole body.

Hemocytes are a phagocytic cell type that can be divided in two categories at the adult stage: “free-floating” hemocytes (around 10% of the total) and sessile hemocytes (around

90% of the total) (Meister, 2004). The latter cells are fixed directly on the inner side of the cuticle.

a) *Drosophila* hematopoiesis

Hemocytes present at the adult stage of *Drosophila* originate from two periods of hematopoiesis separated in a spatial and temporal manner (Holz et al., 2003).

The first wave of hematopoiesis takes place at the embryonic stage, in the head mesoderm. This pool of hemocytes constitutes all mature hemocytes found at the larval stage in the absence of a immune challenge (Tepass et al., 1994).

The second wave of hematopoiesis occurs in the larva in a specialized organ called the lymph gland (Jung et al., 2005). This set of hemocytes is only released shortly before metamorphosis (in the absence of a immune challenge) and participates in the remodeling of the tissue during metamorphosis. Their phagocytic function is especially activated by the peak of ecdysone hormone that induces metamorphosis. It has been shown *in vivo* that hemocytes unable to receive the ecdysone signal are unable to efficiently phagocytose bacteria and apoptotic corpses during metamorphosis (Regan et al., 2013).

During the adult stage, no hematopoietic activity has been detected thus far. Therefore, in adult *Drosophila*, there is a defined number of hemocytes (in the order of two thousand per fly) that cannot be replaced (Meister, 2004).

b) Different types of blood cells

At larval stages, blood cells or hemocytes are composed by three cell types: crystal cells, plasmatocytes and lamellocytes. All three cell types originate from prohemocyte cells and exhibit different functions.

Most hemocytes are plasmatocytes (90-95%) that phagocytose apoptotic corpses and microbes. These cells are either “free-floating” in the hemolymph or sessile and connected to ventral nerve cord. It has recently been shown that the “free-floating” plasmatocytes are also able to localize to the gut proventriculus and exert there their phagocytic function (Zaidman-Rémy et al., 2012). Upon infection, they are also involved in the secretion of AMPs by the fat body and have been described to secrete Spätzle (Charroux and Royet, 2009; Shia et al., 2009).

Lamellocytes are easily recognizable with their large and flat form. They are involved in an anti-parasitic activity called encapsulation, which consists in surrounding large structure like parasite eggs with a cellular insulating layer that becomes melanized. Lamellocytes represent a very low number of hemocytes in healthy larvae and this number dramatically increases upon parasitic infection such as the injection of eggs from a parasitoid wasp.

The last type, crystal cells, are present as a low number (5%). They are round cells recognizable by the large proPO (pro-phenol oxidase) crystals present in their cytoplasm. Upon activation, crystal cell membrane is disrupted and releases the proPO crystals which then initialize the melanization process.

At the adult stage, only one blood cell type has been identified, the plasmatocytes, thus often referred to as hemocytes at this stage. Both lamellocytes and crystal cells seem to be eliminated during metamorphosis. Similarly to the larval stage, these hemocytes are either "free floating" in the hemolymph or sessile.

These "free-floating" hemocytes are also able to localize to the gut proventriculus (Zaidman-Rémy et al., 2012). Moreover it has been recently shown that the phagocytic abilities of hemocytes decrease with aging in adult flies (Horn et al., 2014). Besides their phagocytic function, hemocytes have been described to secrete cytokines such as Upd3 (Agaisse et al., 2003).

c) Phagocytic receptors and opsonins

At larval or adult stages, the phagocytic function is accomplished by plasmatocytes. However, this function requires a detection of the elements that need to be phagocytozed by the plasmatocytes. This recognition involves specific receptors.

So far, a few phagocytic or potential phagocytic receptors have been identified and only some of them have been well-characterized. Among them, the Eater receptor is the most studied one.

The Eater receptor has been shown to bind directly to diverse live Gram-positive bacteria like *Staphylococcus aureus* and *Enterococcus faecalis*. It is required for controlling several systemic or intestinal infections (Kocks et al., 2005; Nehme et al., 2011). However, Eater receptor binding to Gram-negative bacteria (*Escherichia coli*, *Serratia marcescens* and

Pseudomonas aeruginosa) required a partial disruption of the bacterial membrane, which can be fulfilled by AMPs like the Cecropin A (Chung and Kocks, 2011).

Other phagocytic or potential phagocytic receptors like Down Syndrome Cell-Adhesion Molecule (DSCAM), Scavenger Receptor CI (SR-CI) and Croquemort (CRQ) have, so far, only been identified as being required for phagocytosis in *Drosophila* cultured cell models and will not be described any further.

In addition to the phagocytic receptors, six genes homologous to the complement/α2-macroglobulin family of genes, *thioester-containing protein* (*Tep*) have been identified in *Drosophila* (Lagueux et al., 2000), although one of them, *Tep5*, is likely a pseudo-gene. They could potentially play a role in microbial opsonisation and would thus facilitate the engulfment of microbes by phagocytes, like complement molecules in mammals.

Indeed, *Anopheles gambiae* Tep1 has been shown to function as an opsonin against some bacteria (Levashina et al., 2001). It also plays a major role in the defense against *Plasmodium* infections (Blandin et al., 2008).

Among the *Drosophila* Tep proteins, four possess a canonical thioester motif hence the name of the family. The last protein lacks the thioester motif and is referred as either Tep6 or Mcr (Macroglobulin-complement related). Unexpectedly, it has been shown to be a component of *Drosophila* septate junctions in epithelia of ectodermal origins (Bätz et al., 2014; Hall et al., 2014).

The Tep1-Tep4 proteins are potentially secreted in *Drosophila* hemolymph as they all possess a signal peptide. Moreover at least three of them (Tep1, Tep2, and Tep3) are up-regulated upon a mixed Gram-positive (*Micrococcus luteus*) and Gram-negative (*E. coli*) challenge (Lagueux et al., 2000). Furthermore, with a RNAi assay in *Drosophila* S2 cells, Tep2 and Tep3 were shown to be required for efficient phagocytosis of *E. coli* and *S. aureus* respectively (Stroschein-Stevenson et al., 2006). In the same study, *Candida albicans* was significantly less phagocytosed in *tep6* down-regulated cells than in wild-type. However, on the contrary to *A. gambiae*, no demonstration of a role *in vivo* for Tep proteins in *Drosophila* immunity has been achieved so far (Bou Aoun et al., 2011).

d) Host-pathogen interactions in phagocytosis

Following the detection of bacteria by phagocytic receptors, the cell membrane driven by the cytoskeleton invaginates around the particle. This invagination goes deeper until the bacteria is fully engulfed in a vesicle, the phagosome (Fairn and Grinstein, 2012; Ismail et al., 2002). From this step, host cells developed mechanisms to destroy these bacteria while some bacteria have evolved to escape or resist against these mechanisms.

The first mechanism is the acidification of the phagosome by successive fusions of the phagosome with mature endosomes and in finally with lysosomes. Acidification of the phagosome mainly requires the activity of the vacuolar H+ATPase (vATPase) and activates some proteases and lipases (Soldati and Neyrolles, 2012). Some pathogen like *Leishmania donovani* evolved to resist to this acidic and hostile environment (Peltan et al., 2012). Others (*Mycobacterium marinum*, *Chlamydia trachomatis* and *Francisella tularensis*) inhibit the phagosome-lysosome fusion by diverting the host ubiquitin ligase CDC27 (Akimana et al., 2010; Dionne et al., 2003; Elwell and Engel, 2005).

The second mechanism is to highly increase the concentration of toxic molecules like reactive oxygen species and reactive nitrogen intermediates inside the phagosome (Soldati and Neyrolles, 2012). It has been shown that among *Francisella novida* virulence factors some were involved in oxidative stress resistance like for instance *oxyR* (Ahlund et al., 2010; Moule et al., 2010).

Moreover, some bacteria have developed a mechanism that allows them to escape from the phagosome into the cytoplasm of the host cell. For instance, one study in *Drosophila* S2 cells demonstrated that the well studied *Listeria monocytogenes* uses its pore forming toxin Listeriolysin O to escape from the phagosome and this mechanism is vATPase dependent (Cheng and Portnoy, 2003).

2. The humoral immune response

Upon a septic injury, 80% of the genes induced are regulated by the Toll or the Imd pathway showing the importance of these two pathways in the immune defense of *Drosophila*. One major difference between the Imd and the Toll pathway is the kinetic of activation (Ferrandon et al., 2007; Ganesan et al., 2010; Lemaitre and Hoffmann, 2007). The Imd pathway is much faster activated than the Toll pathway. Imd pathway activation takes places within the first hours of the infection and the peak of Diptericin expression is reached at around 6 hours and then decreases to the normal state at around 30 hours. On the contrary

Toll pathway activation needs more time and the peak of Drosomycin expression is reached at around 24-30 hours and then decreases slowly (Lemaitre et al., 1996, 1997; Rutschmann et al., 2000a).

The *Drosophila* humoral immune response involves the expression of several genes in the fat body, a functional equivalent of a composite of the mammalian liver and adipose tissue, under the activation of the Toll and/or the Imd pathway. Among these genes, 20 encode antimicrobial peptides (AMPs). These AMPs are cationic peptides with different anti-bacterial or anti-fungal activities and can be grouped into seven classes (Imler and Bulet, 2005). Diptericin, Drosocin and Attacin are all three efficient against Gram-negative bacteria. Defensin is the only AMP active against Gram-positive bacteria. Drosomycin and Metchnikowin are efficient anti-fungals and Cecropin is active against both bacteria and some fungi.

a) Microbial recognition

Both Gram-positive and Gram-negative bacteria are able, in various proportions, to induce a humoral immune response. These microbes are detected or recognized by a variety of host proteins referred to as Pattern Recognition Receptor (PRR). These PRRs are positioned upstream of the Toll and the Imd pathway and are either transmembrane or circulating receptors. They belong to the families of peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs).

These PRR mainly recognize specific forms of microbe peptidoglycan (PGN). PGN is a composite polymer, a highly complex and fast evolving molecule, with marked differences from one bacterium to the other and restricted to the cell wall of both Gram-negative and Gram-positive bacteria. It consists of long chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues that are cross-linked to each other by short peptidic bridges (Leulier et al., 2003; Stenbak et al., 2004).

A major difference in the PGN of most Gram-positive and Gram-negative bacteria is the presence of lysine residue (Lys-type PGN) instead of meso-diaminopimelic acid (DAP-type PGN) at the third position in the peptide chain of PGN from some Gram-positive bacteria. Classically, DAP-type PGN is known to activate the Imd pathway and Lys-type PGN is known to activate the Toll pathway (Leulier et al., 2003). One exception is the Bacillus species (Gram-positive bacteria), which contain an amidated-DAP-type PGN, and

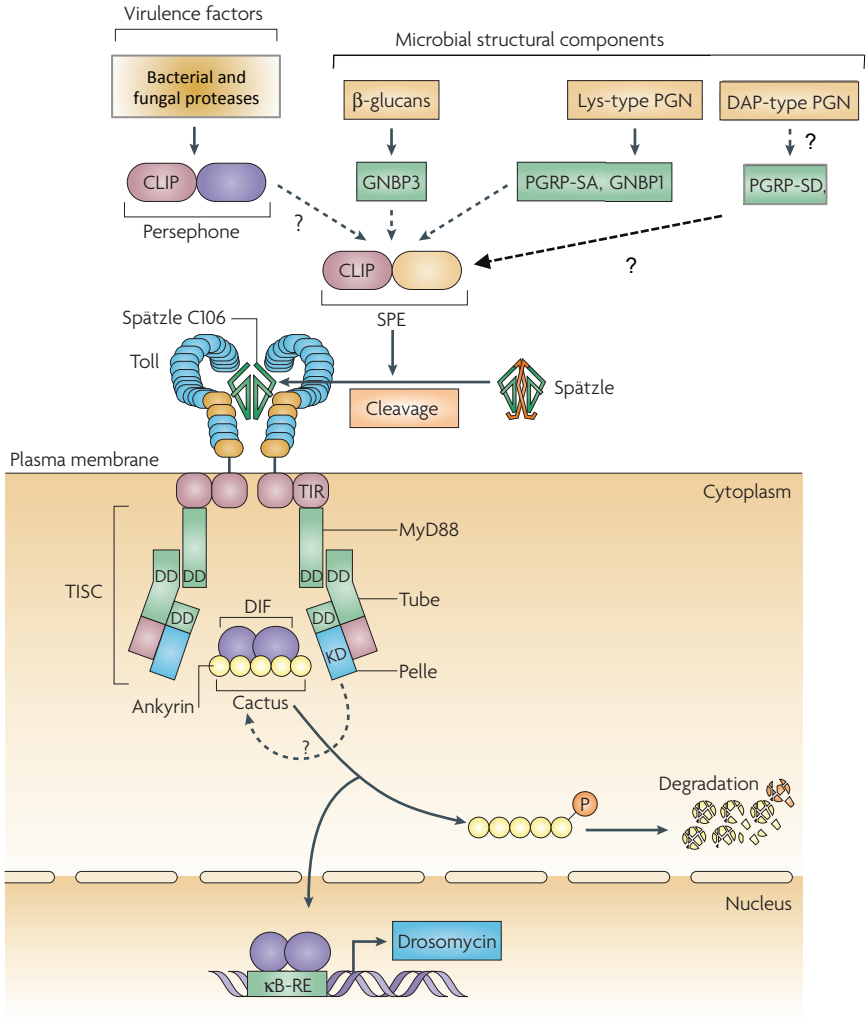


Figure 2. The Toll pathway in immunity

There are two ways to activate the Toll pathway, either through the recognition of microbial cell wall components by PRR like GNBP3 (β-glucans from fungi), PGRP-SA and GNBP1 (Lys-type PGN from Gram-positive bacteria), and PGRP-SD (could potentially bind to DAP-type PGN from Gram-negative bacteria), or through the detection by Persephone of virulence factor enzymatic activity (some bacterial or fungal proteases). These recognition events activate a proteolytic cascade involving different serine proteases until SPE ultimately cleaves pro-Spätzle into an active ligand able to bind the Toll receptor, which then activates the intracellular part of the pathway involving Myd88, Tube and Pelle. DIF, the NF-κB transcription factor of the Toll pathway, is anchored by Cactus in the cytoplasm and thus preventing its translocation to the nucleus. Upon Toll pathway activation, Cactus is phosphorylated (possibly by Pelle) and then polyubiquitylated, leading to its degradation. “Free” DIF can then translocate to the nucleus to regulate the expression of Toll pathway target genes.

The pathway is described in more details in the main text.

(Scheme adapted from Ferrandon et al. 2007).

that activated both the Imd pathway and the Toll pathway. However, these Bacillus species fail to induce the Imd pathway at least in an oral ingestion of the PGN (Zaidman-Rémy et al., 2006).

Another strong difference in the PGN between Gram-positive and Gram-negative bacteria is the location of the PGN. For Gram-positive bacteria, there is a multi-layered PGN surrounding the cell-wall and directly exposed at the surface of the bacteria. Gram-negative bacteria have a thinner layer of PGN inside of the periplasm, located between the inner cell membrane and the outer cell membrane. In this case the PGN is not directly exposed at the surface and small PGN fragments are released during the cell wall remodeling that accompanies bacterial growth and division.

PGRPs are highly conserved from insects to mammals and contains the PGRP domain (160 amino acid), a zinc-dependent N-acetylmuramoyl-L-alanine amidase domain, which shares similarities to the bacteriophage T7 lysozyme. In *Drosophila*, 13 PGRPs have been identified and they can be divided into two subgroups with either recognition or enzymatic properties (Werner et al., 2000). On one side, the first subgroup is composed by PGRP-SA, SD, LA, LC, LD, LE, and LF. These molecules lack zinc-binding residues necessary for amidase activity but are still able to recognize and bind PGN to function as PRR. On the other side, the second subgroup is composed by PGRP-SC1, SC2, LB, SB1 and SB2. These molecules are catalytic PGRPs as they have a zinc-dependent amidase activity (demonstrated for PGRP-SC1, LB and SB1 and predicted for PGRP-SC2 and SB2) that removes peptides from the glycan chains, thereby reducing or eliminating the immune elicitor activity of PGN. In addition, some PGRPs are able to modulate the immune response by scavenging PGN (Basbous et al., 2011; Bischoff et al., 2006; Maillet et al., 2008; Mellroth and Steiner, 2006; Mellroth et al., 2003; Zaidman-Rémy et al., 2006).

b) The Toll pathway

It is an evolutionary conserved signaling pathway. The Toll intracellular signaling pathway shares significant similarities with the signaling pathways activated downstream of Interleukin-1 and some TLRs, suggesting a common ancestry. However, one major difference between vertebrates and *Drosophila* is that in the former the Toll receptor does not work as a pattern recognition receptor (as the Toll-like receptors are in vertebrates) but is activated by the binding of a cytokine, Spätzle. The *Drosophila* genome encodes 9 Toll proteins and only

one is clearly implicated in *Drosophila* immunity (Ooi et al., 2002; Tauszig et al., 2000). Moreover, in *Drosophila*, the Toll pathway functions both during embryonic development (formation of the dorso-ventral axis) and in innate immunity.

The Toll pathway is activated upon some Gram-positive bacterial and fungal infections (Fig. 2). Two types of receptors, PGRPs and GNBPs, are involved in the activation of the Toll pathway. PGRP-SA is a secreted protein, detected in the hemolymph and involved in the recognition of Lys-type PGN from Gram-positive bacteria, together with GNBP1 (Gobert et al., 2003; Michel et al., 2001). PGRP-SA and GNBP1 form complexes in the hemolymph (Gottar et al., 2006; Pili-Floury et al., 2004). GNBP1 might hydrolyze Gram-positive PGN into small fragments detectable by PGRP-SA (Filipe et al., 2005; Wang et al., 2006), which however does not explain how the overexpression of both genes is sufficient to induce the Toll pathway in the absence of any infection (Gobert et al., 2003). Moreover, PGRP-SD is another secreted PRR that would function in partial redundancy with the PGRP-SA/GNBP1 complex but which would rather bind to DAP-type PGN than Lys-type PGN (Bischoff et al., 2004; Leone et al., 2008).

Another GNBP protein, GNBP3 is a circulating PRR that shares homologies with bacterial glucanases (Kim et al., 2000). GNBP3 contains an N-terminal domain that binds to the fungal β(1,3)-glucan (Mishima et al., 2009) and a C-terminal domain that is homologous to the catalytic domain of β-glucanases; however, the absence of conserved key residues in the catalytic site suggests that this domain is not functional. This PRR has a key role in the detection of fungal infections and subsequent activation of the Toll pathway and melanization cascades (Gottar et al., 2006; Matskevich et al., 2010).

An alternative mechanism of Toll pathway activation has been identified with entomopathogenic fungi but can also be triggered by some bacteria. Entomopathogenic fungi enter the *Drosophila* hemocoel by boring a microscopic hole through the cuticle. To this end, they secrete proteases such as PR1, as well as chitinases. The PR1 protease has been shown to cleave a host protease, Persephone, which upon cleavage functions as a sensor and activates Spätzle maturation through a downstream proteolytic cascade (Gottar et al., 2006). Persephone may also be activated by bacterial proteases that are released as virulence factors (El Chamy et al., 2008). Thus, this branch of Toll pathway activation relies on sensing the enzymatic activities of microbial virulence factors. Persephone self-activation is inhibited by the serpin Necrotic (Levashina et al., 1999; Ligoxygakis et al., 2002a)

After the detection of the microbe either by recognition of PGN or β-glucans, a proteolytic cascade is activated consisting of several serine proteases (ModSP, Grass…) that undergo zymogen activation (Buchon et al., 2009a). This protease cascade allows an amplification of the activating signal and can be down regulated by specific inhibitors such as serpins, for instance in the case of inappropriate activation or to terminate signaling (Ligoxygakis et al., 2002b).

On the other side of Toll pathway activation, it is not yet clear if Persephone is able to cleave directly or not SPE (an immune-regulated protein). At the end of the activation cascade, the serine protease SPE cleaves pro-Spätzle in the activated form of Spätzle able to bind to the Toll receptor (Jang et al., 2006; Kambris et al., 2006). Once clived, Spätzle binds with a high affinity to the N-terminal part (extracellular) of the Toll receptor (Weber et al., 2003). This extracellular part of the receptor contains multiple leucine-rich repeats (LRR) and the intracellular part is referred as the TIR domain (homologous to the intracytoplasmic signaling domain of the mammalian interleukin-1 receptor and to all TLRs).

Spätzle binding to the Toll receptor triggers conformational changes of the Toll receptor and signaling (Weber et al., 2005). In the intracellular space, the Toll-induced signaling complex (TISC) is composed by three proteins that interact with each other via their Death-Domain (DD). Myd88 (myeloid differentiation primary-response gene 88) is directly linked to Toll receptor via its TIR domain (Tauszig-Delamasure et al., 2002). Linked to Myd88, there is first Tube and then Pelle (a member of the IL-1R-associated kinase (IRAK) family of serine proteases), all three interacting through their DD (Sun et al., 2004).

The NF-κB transcription factor DIF (dorsal-related immunity factor) is retained in the cytoplasm by the Cactus inhibitor (an homologue of the mammalian inhibitor of NF-κB, IκB) (Belvin et al., 1995; Lu et al., 2001; Rutschmann et al., 2000a). Toll pathway activation induces the phosphorylation and cleavage of Cactus, and its subsequent degradation (probably through polyubiquitylation) (Nicolas et al., 1998). However the mechanism through which Cactus is phosphorylated remains unknown. In *Drosophila* embryos, it has been reported that the complex Dorsal/Cactus can interact with Tube (associated to the TISC) and therefore Cactus could potentially be phosphorylated by Pelle (Edwards et al., 1997; Yang and Steward, 1997).

Cleavage and degradation of Cactus leads to the release of DIF that than translocate to the nucleus to modulate the expression of Toll pathway target genes. However, it is likely that DIF activation requires some post-translational modifications as it has been shown that Dorsal phosphorylation is required for its nuclear import (Drier et al., 1999).

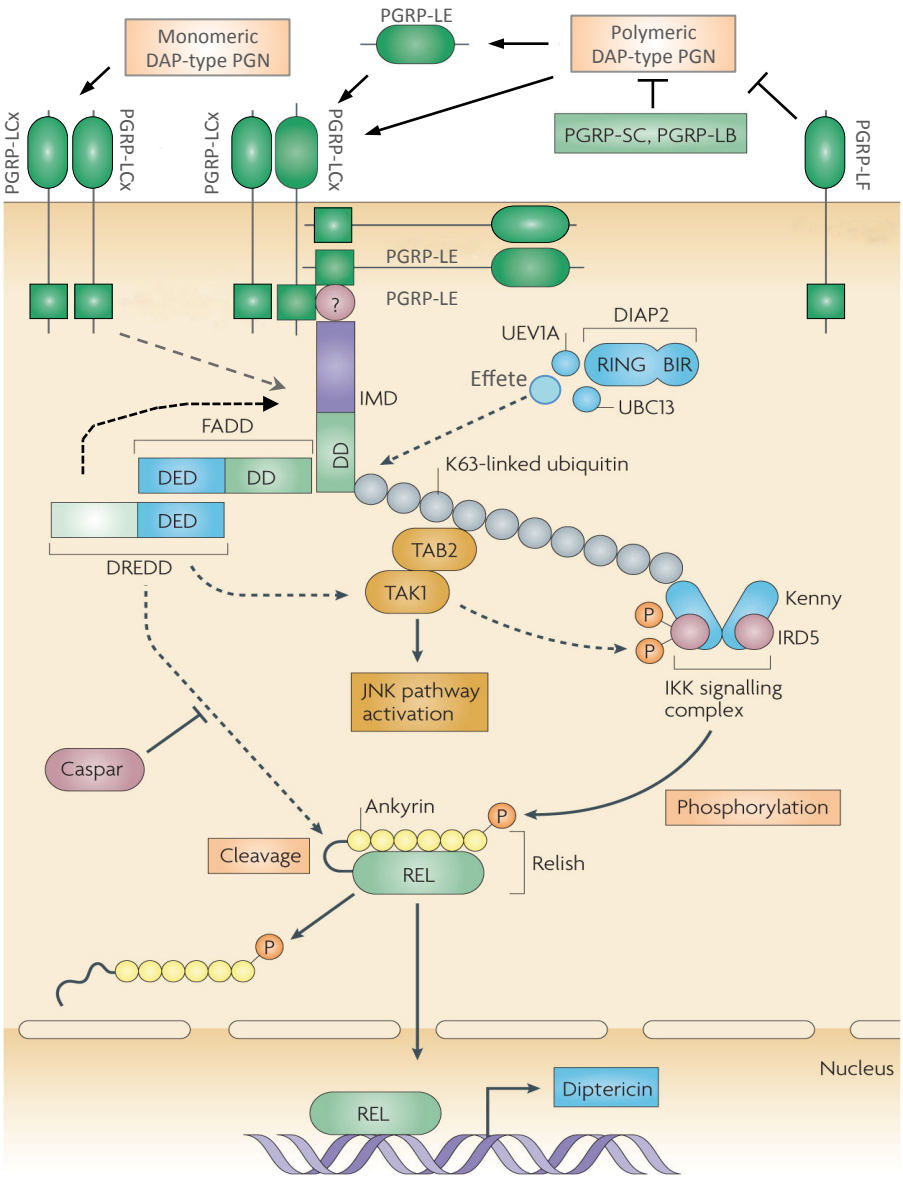


Figure 3. The IMD pathway

The *Drosophila* IMD pathway is activated through the detection of DAP-type PGN (Gram-negative bacteria) by PRR like PGRP-LC and PGRP-LE. Binding of PGN to the receptor directly activate the intracellular part of IMD pathway through the recruitment at the receptor of Imd followed by Fadd and Dredd. Dredd cleaves Imd which is polyubiquitinated by the complex formed with DIAP2, UEV1A, Effete and Bendless (UBC13). Next, Imd recruits both TAK1/TAB2 and IRD5/ Kenny. TAK1 phosphorylates IRD5 that then phosphorylates the NF-κB transcription factor Relish. Relish is likely cleaved by DREDD and the N-terminal part of Relish translocates to the nucleus to regulate the expression of Imd target genes.

The pathway is described in more details in the main text.

(Scheme adapted from Ferrandon et al. 2007).

c) The Imd pathway

In contrast to the Toll pathway, no developmental role has been identified for the *Drosophila* Imd pathway. This pathway shares similarities with the TNF-R pathway of vertebrates.

The *Drosophila* Imd pathway is activated by polymeric and monomeric DAP-type PGN (Fig. 3). A specific monomer, GlcNAc-MurNAc(anhydro)-L-Ala-γ-D-Glu-meso-DAP-D-Ala, also known as tracheal cytotoxin (TCT), was shown to be the minimal PGN motif able to induce efficiently the Imd pathway (Chang et al., 2006; Kaneko et al., 2004; Lim et al., 2006; Stenbak et al., 2004). TCT is generated from the ends of the PGN strands and is released during bacterial cell growth and division.

PGRP-LC is a transmembranar protein and is the major receptor of the Imd pathway (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). Alternative splicing of the mRNA can produce three proteins PGRP-LCa, LCx and LCy. These three proteins share the same intracellular domain (involved in signaling) but have different extracellular domains (involved in sensing) (Kaneko et al., 2004; Werner et al., 2000). PGRP-LCx seems to be involved in sensing of polymeric PGN whereas PGRP-LCa and LCy would be involved in recognition of monomeric PGN. PGRP-LE presents affinity to DAP-type PGN and is supposedly expressed both in extra and intra-cellular compartment. In the extra-cellular compartment, PGRP-LE seems to enhance PGRP-LC recognition of PGN, whereas in the intra-cellular compartment, PGRP-LE permits the induction of the Imd pathway without the recognition of PGN by PGRP-LC (Bosco-Drayon et al., 2012; Kaneko et al., 2006; Takehana et al., 2004).

The binding of PGN to the PGRP-LC receptor and dimerization of the receptor induce the direct recruitment of the Immune deficiency (Imd) adaptor through their RHIM (RIP (receptor-interacting protein) homotypic interaction motif)-like domain (Choe et al., 2005; Georgel et al., 2001; Kaneko et al., 2006; Takehana et al., 2004). On one side, Imd possesses a death domain (DD) through which it recruits *Drosophila* FAS-associated death domain (dFADD) that contains also a death effector domain (DED) through which it recruits the caspase death-related ced-3/Nedd2-like protein (Dredd) (Hu and Yang, 2000; Leulier et al., 2000). Once recruited, Dredd cleaves Imd, which is then polyubiquitinated by the complex

Bendless/Effete/DIAP2/UEV1A. UEV1A is the ubiquitin-conjugating enzyme E2 variant 1 and DIAP2 is the *D. melanogaster* inhibitor-of-apoptosis protein 2 (Paquette et al., 2010).

Polyubiquinated Imd is thought to recruit both TAK1/TAB2 (TGFβ-activated kinase 1/ TAK1-binding protein 2) and the IKK complex.

This last complex is composed by a catalytic subunit IRD5 (immune-response deficient 5) and the regulatory Kenny, (homologue to IKKγ) (Lu et al., 2001; Rutschmann et al., 2000b). TAK1 phosphorylates IRD5 that in turn phosphorylates the NF-κB transcription factor Relish. Phosphorylated Relish is then cleaved (possibly by Dredd) into N-Relish (N-terminal Relish) and C-Relish (C-terminal Relish) and the N-Relish domain translocates into the nucleus to regulate the expression of Imd pathway target genes, the phosphorylated sites promoting transcriptional activation, and not Relish cleavage as thought for a long time (Erturk-Hasdemir et al., 2009). So far, no function has been identified for C-Relish.

As described earlier, some PGRPs, especially PGRP-LB and SC have an amidase activity. This amidase activity is involved in the modification of PGN, reducing its recognition by PGRPs and therefore setting the threshold of Imd pathway activation (Paredes et al., 2011). PGRP-LB is active specifically on DAP-type PGN, whereas PGRP-SC modifies both DAP-type PGN and Lys-type PGN (Mellroth et al., 2003; Zaidman-Rémy et al., 2006). PGRP-LB protein is secreted in the hemolymph. Other negative regulators of Imd pathway act at the level the cytoplasmic cascade of activation, like Pirk (also referred as Pims) that impede the interaction Imd/PGRP-LC and remove the receptor from the membrane (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). *PGRP-LB, PGRP-SC* and *pirk* gene expressions are controlled by the Imd pathway. Other Imd pathway regulators acting at different level of Imd pathway have been identified but will not be described here.

The control of the Imd pathway activation seems to be critical to avoid the tissue damages caused by a strong and prolonged immune reaction (Bischoff et al., 2006; Lee and Ferrandon, 2011).

3. Intestinal immunity

a) The structure of the *Drosophila* intestine

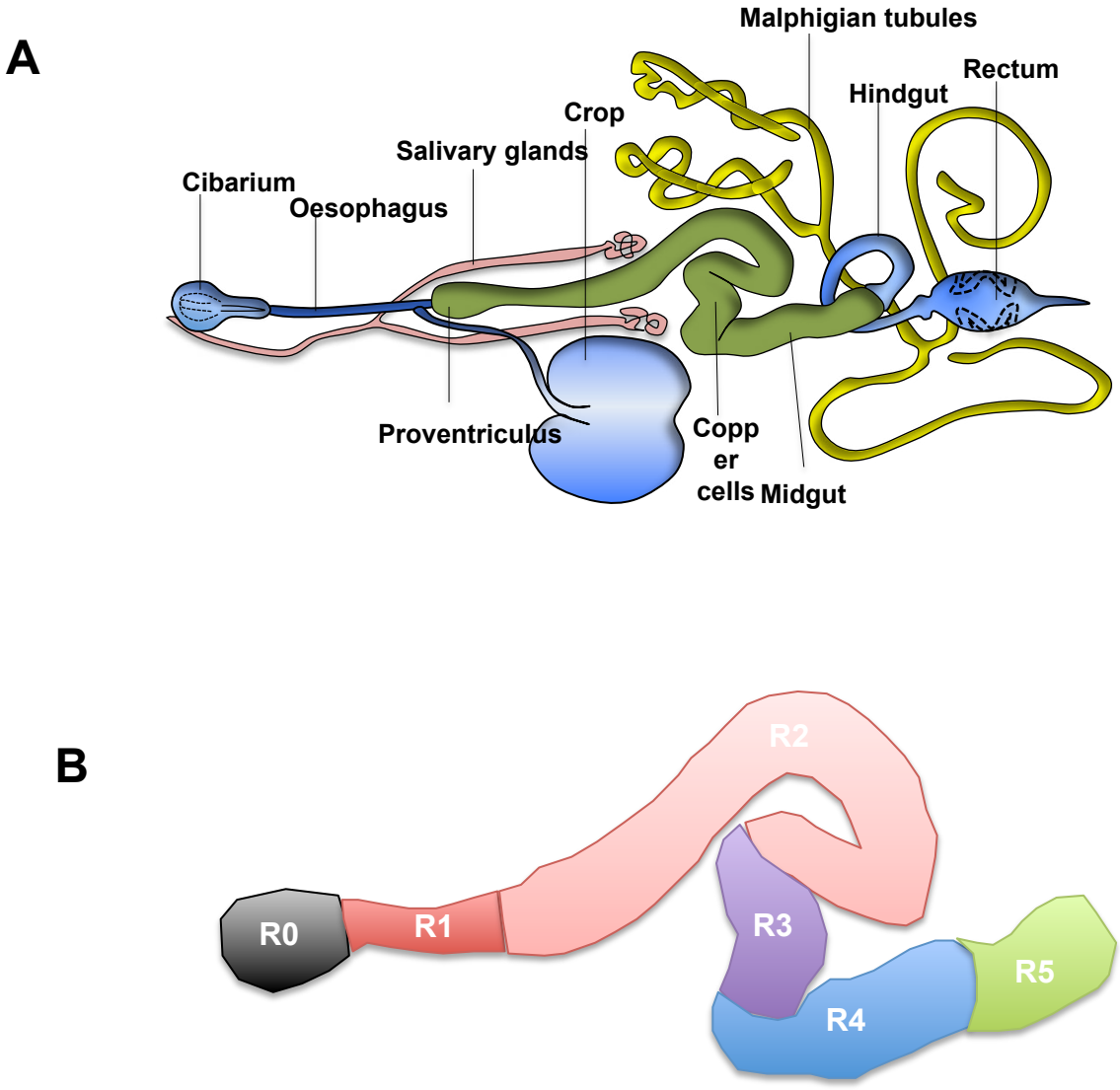


Figure 4. The digestive tract of *Drosophila melanogaster*.
(A) A schematic representation of the whole digestive tract of Drosophila. At the anterior part of the oesophagus is the cibarium, to which the salivary glands connect. At its posterior part, the crop branches away from the digestive tract and represents a food storage organ. The foregut is followed by the midgut with the proventriculus forming a valve at the border between fore-and midgut. It is also the place where the peritrophic matrix is initially synthetized, the anterior midgut, the Copper cell region and the posterior midgut. The Malphigian tubules(that filters the hemolymph, equivalent to the mammalian kidney) connect with the gut at the junction between the posterior midgut and the hindgut. Then there is the hindgut and at the end of the digestive tract, the rectum. (B) The midgut can be subdivided in different regions referred to as R0 to R5. R0: the proventriculus, R1 and R2: anterior midgut, R3: Copper cell region, and R4 and R5: posterior midgut.

The digestive tract is one of the most important structure for *Drosophila*. It is composed by the crop (a storage organ), the foregut (equivalent to the mammalian oesophagus), the midgut (equivalent to the mammalian small intestine), the hindgut (equivalent to the mammalian big intestine) and the rectum (Fig. 4A). The crop, the foregut and the hindgut are originating from the ectoderm embryonic layer while the midgut has an endodermic origin. Moreover, according to the pattern of gene expression, the midgut can be subdivided in six main regions referred to as R0 to R5 while R0 corresponds to the proventriculus (at the anterior extremity of the midgut) and R5 represents the very last posterior part of the midgut. In the middle of the midgut, the R3 region corresponds to the Copper cell region (CCR) which is an acidic compartment while the anterior and the posterior midgut are more alkaline regions (Buchon et al., 2013) (Fig. 4B).

The first and most effective protection against invaders is a physical barrier. The crop, foregut, and hindgut epithelia are covered by a cuticle layer, which strongly decreases potential exchanges between the lumen and the epithelial cells. The midgut, where digestion occurs, is protected by a semi-permeable membrane, the peritrophic matrix. It is a noncellular matrix synthesized by the proventriculus and the midgut epithelium, which is composed of chitin and glycoprotein fibrils. It lines the invertebrate midgut and separates the food bolus from the epithelium. (Lemaitre and Miguel-Aliaga, 2013)

Two major cell types have been identified so far in the *Drosophila* midgut: enterocytes (EC) (more than 95%) and enteroendocrine cells (EE) (less than 5%) (Fig. 5). Enterocytes are columnar, octoploid epithelial cells that secrete digestive enzymes and absorb nutriments. So far, little is known about EE functions but they have been reported to secrete hormones and are involved in the control of the gut physiology (Amcheslavsky, *et al,* Cell Reports, *in press*).

Moreover, the adult *Drosophila* midgut is capable of regeneration and intestinal homeostasis is controlled by the activation of the JAK/STAT pathway in ISCs (Beebe et al., 2009; Lin et al., 2010). ISC niche maintenance has been reported to involve Wingless pathway from intestinal visceral muscles (Lin et al., 2008). In addition, a gradient of Dpp signaling released from the anterior and posterior part of the posterior midgut is involved in the determination between ISCs and gastric stem cell fate choice. It has been shown that Dpp signaling is required for the maintenance of the Copper cell region and is sufficient to promote the copper cell fate in the anterior midgut (Li et al., 2013). More recently, a role of EE in ISCs homeostasis through the Bursicon/DLGR2 signaling has been described. *Drosophila* deficient for the Bursicon ligand (secreted by EE) or its receptor DLGR2 (in

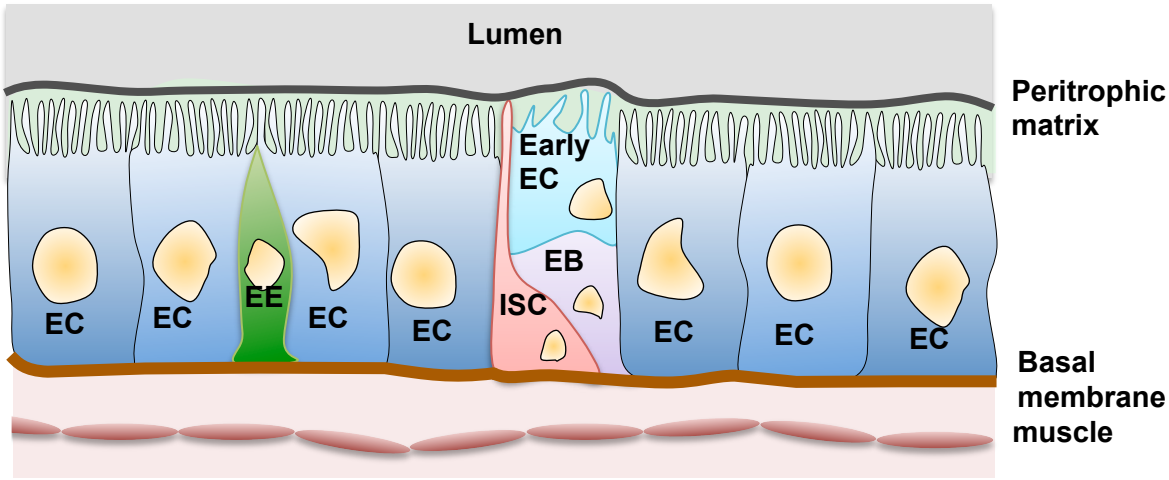


Figure 5. Constitution of the *Drosophila* midgut epithelium

The intestinal barrier in the midgut is composed by the peritrophic matrix, which protect the epithelial cells from a direct contact with the elements inside of the gut lumen, and the epithelium. This epithelium is a cellular mono-layer constituted by two distinct cell types: polyploid enterocytes (EC) and enteroendocrine cells (EE) both derived from intestinal stem cells (ISCs). ISCs may undergo asymetric division which results in the formation of one ISC and one enteroblast (EB). The EB cell will differentiate either into an EE or an EC. Before the EC stage, there will be an intermediate stage referred to as early-EC during which the cell undergoes a few endonuclear replications.

visceral muscles) present a hyperproliferation of ISCs (Scopelliti et al., 2014). Tachykinin expressed by a subset of EEs also functions in a similar manner (Amcheslavsky, *et al,* Cell Reports, *in press*).

On a regular basis, intestinal stem cells (ISCs) undergo asymmetric divisions (Fig. 5). After division, one daughter cell is maintained as ISC. Depending on the level of Notch pathway activation, the second daughter cell, referred to as an enteroblast (EB), differentiates either into EC or EE (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). Moreover, it has been recently reported that EE are involved in fate decision EC/EE of EB through the Slit/Robo2 signaling in the posterior midgut. The ligand Slit was shown to be secreted by EE while its receptor Robo2 is localized in ISCs. *Drosophila* down-expressing Robo2 specifically in ISCs displayed an increased number of Prospero positive cell (a marker for EE) suggesting that the fate decision takes place in ISC and that this Slit/Robo2 signaling acts upstream of the Notch signaling (Biteau and Jasper, 2014).

Upon infection or aging, EC damages induce JNK pathway in these cells, which in turn secrete Upd cytokine that over-activate JAK/STAT pathway in ISCs and lead to an increased ISC proliferation to repair gut damages (Biteau et al., 2008; Buchon et al., 2009b; Cronin et al., 2009; Jiang et al., 2009). Moreover other pathways like EGF, Dpp and Hippo have been shown to be required for a tight control of ISCs proliferation and regulation (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang and Edgar, 2009; Jiang et al., 2010; Mathur et al., 2010). Recently, Bursicon/DLGR2 signaling was shown to repress EGF signaling in ISCs by down-regulating EGF ligand Vein in visceral muscle (Scopelliti et al., 2014).

The *Drosophila* intestinal lumen is colonized by an abundant commensal flora referred as microbiota. In mammals and Human, strong alterations in the intestinal microbiota composition are linked to various pathologies like obesity, inflammatory bowel diseases and even cancer. Moreover it has been shown that in Human and *Drosophila* the microbiota is directly linked to the diet composition and evoluates during aging (Claesson et al., 2011, 2012). Indeed, there is not a real colonization as the microbiota needs constant replenishment: flies fed sterile food ultimately lose their microbiota (Blum et al., 2013).

The intestinal commensals of laboratory and wild strains *Drosophila* has been extensively studied (Broderick and Lemaitre, 2012). They have a relative simple microbiota constituted by only a few bacterial families like *Lactobacillaceae*, *Enterococcaceae*, *Acetobacteriaceae* and *Enterobacteriaceae*. Moreover it has been shown that, in the majority of cases, four to eight bacterial species are present in the intestinal commensals (Chandler et

al., 2011; Corby-Harris et al., 2007; Wong et al., 2011a). Given its relative simple microbiota composition, its features as a genetic organism model including the possibility to raise axenic flies that do not carry any commensals in the gut, *Drosophila* has become also an interesting organism model to study intestinal commensalism.

At larval stages, *Drosophila* microbiota has beneficial effects on development (Shin et al., 2011; Storelli et al., 2011). However, so far, the microbiotal potential beneficent or detrimental effects on adult *Drosophila* fitness remains to be demonstrated. It has been shown that intestinal commensals community varies in size during aging as only a few bacteria are present in young flies gut while a much larger amount of bacteria are retrieved from old flies gut (Guo et al., 2014). Moreover, two recent studies reported that microbiota modulates intestinal gene expression and influences intestinal homeostasis (Broderick et al., 2014; Combe et al., 2014). Axenic flies presented a decrease of ISCs proliferation, even more pronounced in the posterior midgut, compared to conventionally raised flies. Axenic flies displayed an increase of EE and a decrease of EB in both anterior and posterior midgut.

b) Intestinal defense mechanisms

Upon intestinal infection, the *Drosophila* midgut is able to secrete divers molecules like AMPs and reactive oxygen species (ROS) to kill pathogenic microbes.

The presence of some Gram-negative bacteria in the midgut induces a localized activation of the Imd pathway (Limmer et al., 2011a). This activation has been shown to be effective against the pathogenic bacteria as flies deficient for Imd pathway in the midgut are more susceptible to the infection (Liehl et al., 2006; Nehme et al., 2007; Ryu et al., 2006). In the intestine, Gram-negative bacteria are detected by two PRR, PGRP-LC (membrane bound) in the anterior midgut and PGRP-LE (intracellular) in the posterior midgut (Bosco-Drayon et al., 2012; Neyen et al., 2012).

Moreover, it has been shown that the homeobox Caudal that can induce the expression of *cecropin* and *drosocin* in some epithelia, is a negative regulator of the Imd pathway in the posterior midgut (Ryu et al., 2004, 2008). In addition, other negative regulators of the Imd pathway have been described in the *Drosophila* midgut, like Pirk, PGRP-LB1 and PGRP-SC2 (Aggarwal et al., 2008; Guo et al., 2014; Kleino et al., 2008; Lhocine et al., 2008; Paredes et

al., 2011; Zaidman-Rémy et al., 2006). This negative regulation of the pathway is essential to avoid an over-activation of the Imd pathway by commensals bacteria leading to **dysbiosis**[1].

So far, any efficient Toll pathway activation failed to be detected in the midgut. However, upon intestinal infection with *Erwinia carotovora*, one Drosomycin-like peptide gene was reported to be induced under the control of the JAK/STAT pathway, in the anterior midgut (Buchon et al., 2009b).

Upon intestinal infection by pathogenic bacteria, reactive oxygen species (ROS) are released in the midgut lumen by EC. Surprisingly, ROS are nearly exclusively produced in response to non-commensal bacteria infection (Ha et al., 2009a). These results suggest that the mechanism required for ROS production is able to distinguish commensal (non-pathogenic) and non-commensal (potentially pathogenic) bacteria. ROS are synthetized by the dual oxidase (DUOX) enzyme which is a member of the nicotinamide adenine dinucleotide phosphate (NADP)H oxidase family. *Drosophila* down-regulating the DUOX enzyme present a reduced lifespan and are more susceptible to pathogenic bacteria intestinal infection demonstrating the importance of this ROS response (Ha et al., 2005, 2009b). Moreover, ROS seem to be active on bacteria and intestinal cells inducing massive intestinal cell death (Buchon et al., 2009b, 2010).

Recently, it has been demonstrated that uracil is the ligand that induce DUOX activation. Uracil is nearly specifically produced by non-commensal bacteria and only rarely and at a low level released by commensal bacteria (*e.g.*, *Gluconobacter morbifer* and *Lactobacillus brevis*) (Lee et al., 2013). However, why commensal bacteria do not produce uracil, while non-commensal do, remains unclear.

4. Coagulation and Melanization

Coagulation and melanization are both activated immediately after a physical disruption of the arthropod cuticle (Theopold et al., 2004). Coagulation provokes the thickening of the hemolymph while melanization results in the formation of melanin and requires the activation of proPO. Coagulation is independent of melanization as it is still present in proPO deficient *Drosophila*.

[1] **Dysbiosis:** a dramatic modification of microbiota in term of composition and population size.

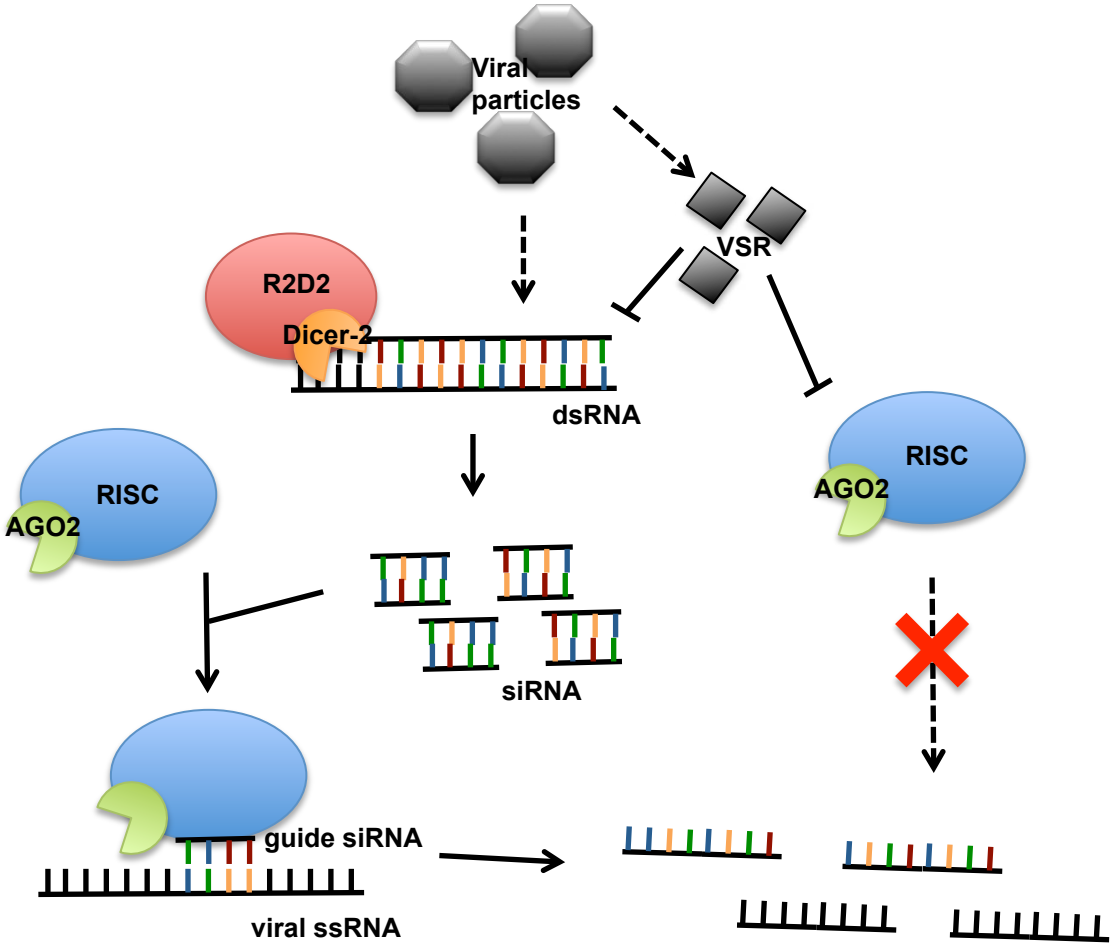


Figure 6. The *Drosophila* anti-viral RNA interference pathway.
Upon infection with RNA viruses, a complementary strand to their single strand RNA genome is synthesized by the viral-dependent RNA-polymerase contained in the viral particle and thus produces a long double stranded RNA (dsRNA). This dsRNA is recognized by the R2D2/Dicer-2 complex that will cleave the dsRNA into small interfering RNA (siRNA). One strand of siRNA will be taken up by the RISC complex and serves as a guide siRNA to sense other viral RNA. In the case of a positive recognition event, the core element of the RISC complex, Argonaute-2 (AGO-2) will slice the newly detected viral RNA. Some virus express proteins that contain a viral suppressor of RNAi (VSR)domain that can target different element of the RNAi pathway (dsRNA sequestration, inhibition of Ago2) to block this pathway and avoid the slicing of viral RNA.

These mechanisms induce the formation of a clot at the wound site and avoid massive loss of hemolymph and entry of microbes inside the general cavity of the fly (Scherfer et al., 2004). This clot is formed by the concentration, at the wound site, of hemolectin fibers that trap hemocytes and the invading microbes. Moreover, there is some evidence that enzymes like proPO and transglutaminases are required in hardening the clot (Bidla et al., 2009; Karlsson et al., 2004; Lindgren et al., 2008).

Specifically, melanization is the first immune reaction on the surface of invading parasites. It requires the cleavage of proPO by the serine protease prophenoloxidase-activating enzyme. Active phenoloxidase (PO) induces the oxidation of mono- and diphenols into orthoquinones. These orthoquinones then polymerize to form melanin.

Moreover, recently a function of PO has been reported in *Drosophila* defense against some microbes (the Gram-positive *Bacillus subtilis*, *Enterococcus faecalis* and *Staphylococcus aureus* and the fungi *Aspergillus fumigatus*, *Beauveria bassiana* and *Metarhizium anisopliae*) (Binggeli et al., 2014). Interestingly, melanization triggered by PGRP-SA/GNBP1 appears to be the main defense effective against *S. aureus*, as it appears to be resistant against the action of the Toll pathway (Bischoff et al., 2006; Nehme et al., 2011).

5. *Drosophila* antiviral response

In the field, viruses are major threats of *Drosophila* species. Around 25 virus species (RNA virus) have been identified in *Drosophila*. Moreover, around 40% of all flies are infected with viruses (Lemaitre and Hoffmann, 2007). *Drosophila* viral infection can occur either through a vertical or a horizontal transmission.

The *Drosophila* immune response against viruses is totally different from immune response against bacteria or fungi.

a) The RNAi pathway

Similarly to plants, the RNAi machinery is the most efficient anti-viral response in *Drosophila* through the detection and the destruction of viral RNA.

Basically, double-stranded RNA (dsRNA) are recognized and cleaved by Dicer-2 (an RNaseIII enzyme) into small interfering RNA (siRNA) (Fig. 6). Dicer-2 forms a complex with the dsRNA binding protein (dsRBP) protein R2D2. Then one strand of siRNA is

incorporated in the RNA-induced silencing complex (RISC). This RNA serves as a guide to recognize through bases complementarity other viral RNA. Upon positive base complementarity between the guide RNA and another RNA, Ago-2, associated to the RISC complex, cleave that RNA (Kemp and Imler, 2009).

In parallel, some viruses have developed a resistance mechanism against the RNAi pathway. These viruses express a viral suppressor of RNAi (VSR) that block the activity of the RNAi pathway. For instance it has been reported that Cricket Paralysis Virus (CrPV) express a VSR that suppress the activity of Ago-2 (Nayak et al., 2010). Many VSR rather sequester dsRNAs, thus preventing their cleavage by Dicer-2.

b) Other anti-viral mechanisms

The JAK/STAT pathway has been shown to be involved in the control of the viral load especially in the case of a DCV (*Drosophila* C Virus) infection (Galiana-Arnoux et al., 2007; Kemp et al., 2013). Upon DCV infection, some JAK/STAT controlled genes are up-regulated. Moreover, *Drosophila* flies deficient for the JAK kinase Hopscotch are more susceptible to a DCV infection and present a higher viral load. However, the JAK/STAT pathway is required but not sufficient to induce the expression of all DCV-induced genes (Dostert et al., 2005). In this way, it has been suggested that the expression of some of these genes is regulated by Dicer-2 (Deddouche et al., 2008). Moreover, the mechanism through which viruses are detected and JAK/STAT pathway activated remains to be discovered.

Autophagy is an anti-viral mechanism that, so far, was only demonstrated to be efficient against Vesicular Stomatitis Virus (VSV) (Shelly et al., 2009).

Finally, one group has shown that some Imd pathway members and hemocytes are required for an efficient antiviral response against CrPV infection (Costa et al., 2009).

II. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium. It is a Proteobacteria that belongs to the family of *Pseudomobadaceae*.

P. aeruginosa is rod-shaped and monoflagellated. With a size of 1-5 µm in length and 0.5-1 µm in breadth, these bacteria are quite small as compared to *E. coli*. Thanks to its incredible nutritional versatility, *P. aeruginosa* is a ubiquitous microorganism that can be found in water or soil and infects organisms like plants, nematodes, insects and mammals.

A. An opportunistic pathogen

Pseudomonas aeruginosa is an opportunistic human pathogen and in some rare cases infects also healthy persons. However, it much more often colonizes immunocompromised patients like individuals with AIDS or cancer, or patient with cystic fibrosis. *P. aeruginosa* is also the cause of a high number of nosocomial infection especially after a surgery, in long-term intensive care units and for burnt patients (Kerr and Snelling, 2009). Interestingly, *P. aeruginosa* is also found in the gastro-intestinal tract and can be a reservoir for infections of the host. By crossing the intestinal barrier, it can affect lung tissues after transport in the circulatory system (Marshall et al., 1993; Zaborina et al., 2006). The fight against *P. aeruginosa* is particularly important for cystic fibrosis patients for which these bacteria complicate the diseases in 90% cases.

These bacteria can induce either an acute or a chronic type of infection. *P. aeruginosa* can notably infect chronically the lung of cystitic fibrosis (CF) patient. There, the infection provokes the activation of the immune system that leads to inflammation and the progressive destruction of the lung tissue.

A second important characteristic is that *P. aeruginosa* is its intrinsic multiresistance to multiple classes of antibiotics associated with acquired resistance. Moreover, when adhering to a surface, *P. aeruginosa* grows in a biofilm. These surfaces (*e.g.* catheter, tubes…) are significantly more difficult to sterilize. This needs to be taken in account in the sterilization/cleaning procedures in hospitals.

P. aeruginosa is also able to form a biofilm in the host, thereby inducing a switch to a chronic infection. At this stage, the infection is particularly hard to treat as the biofilm

protects bacteria localized in its center from the action of the host immune system and from antibiotics.

B. *Pseudomonas aeruginosa* virulence system

An infection involves multiple steps that require each distinct set of virulence factors. A first step entails the adhesion of the bacterium to a host tissue. This can be followed or preceded by the secretion of virulence factors in the environment or the direct injection of other factors directly into the host cells. *P. aeruginosa* is also able to invade epithelial cells, for instance those of the cornea (Fleiszig and Evans, 2002). Infection is a coordinated process that requires synchronization of the bacteria and that is achieved by perceiving the local concentration of bacteria in a tissue by a process known as quorum sensing. The breadth of knowledge accumulated on this organism is vast. Here, I shall focus mostly on secretion systems and quorum sensing in *P. aeruginosa* since they are most relevant to my work.

1. *Pseudomonas aeruginosa* protein secretion systems

Protein secretion systems are nano-apparatuses localized in the envelope of the bacteria that allow the transport of specific proteins like protease or ion chelators from inside the bacteria to outside of the bacteria. Proteins can either be injected directly inside a target cell (eukaryote or prokaryote) or secreted in the extracellular space. Through this secretion process, proteins cross the inner bacterial membrane (hydrophobic), the periplasm (a hydrophilic space) and then the outer bacterial membrane (hydrophobic). Sometimes these proteins need also to cross the target cell membrane. All protein transport through bacterial secretion systems requires energy.

Bacterial protein secretion systems share remarkable resemblances with other bacterial existing structures like efflux pumps, flagella or type IV pili. Surprisingly, strong similarities have been identified between the type six secretion system and the bacteriophage tail. All together, these observations suggest that bacterial protein secretion systems arose from a progressive evolution of these different bacterial or phage structures.

So far, six secretion systems have been identified in bacteria. They are numbered from one to six, as secretion system types. Most bacteria do not possess all six secretion systems.

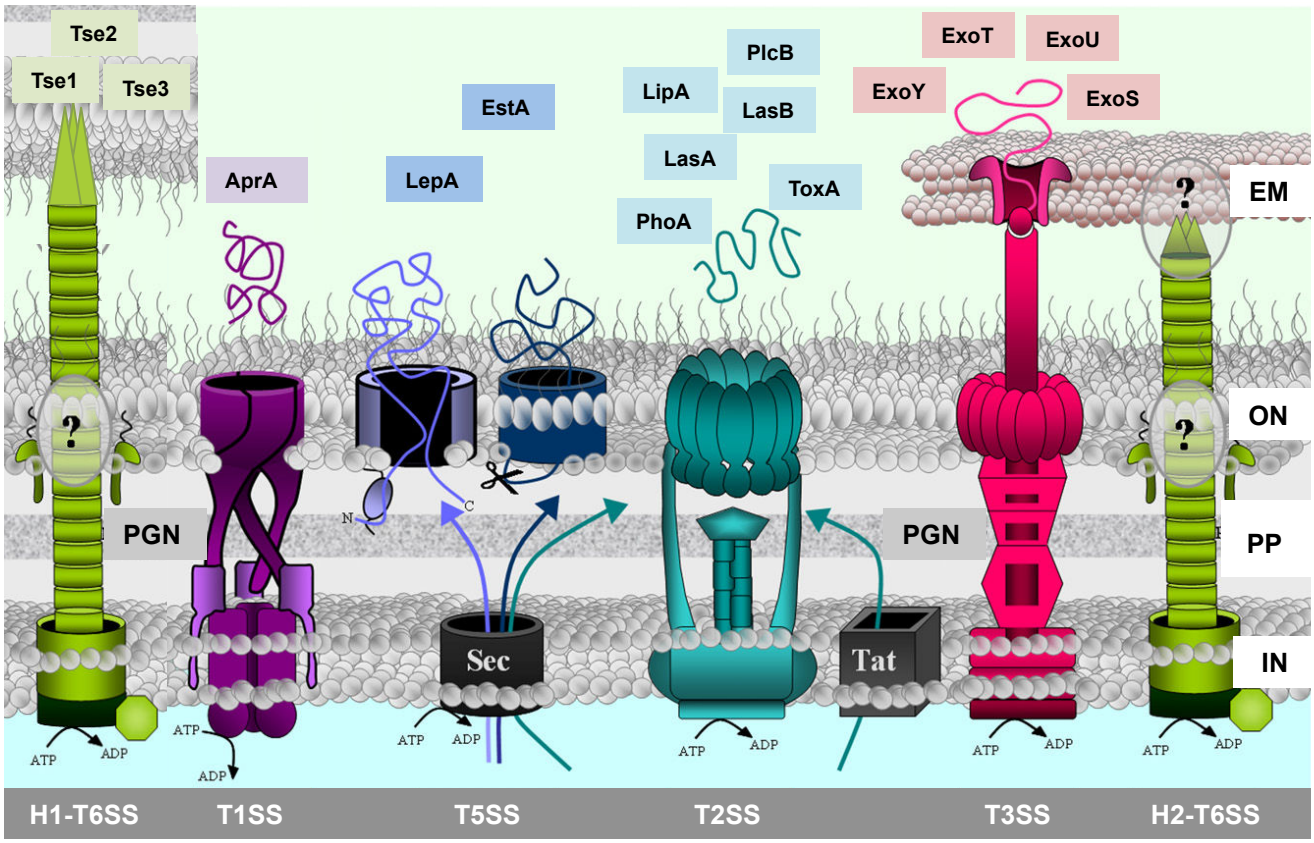


Figure 7. The *Pseudomonas aeruginosa* secretion systems.
P. aeruginosa possesses five secretion systems (T1SS, T2SS, T3SS, T5SS and T6SS).To transport proteins through the bacterial envelop, all systems need energy provided by ATP. Proteins can either cross the envelope in a one-step (T1SS, T3SS and T6SS) or in two-step (T2SS and T5SS) mechanism. In the latter case, proteins first cross the inner membrane (IN) via the Sec or the Tat machinery and once in the periplasm (PP), they are recognized by their specific secretion system type and translocate across the outer membrane (OM). Proteins that cross the bacterial envelope in one-step are either secreted in the extracellular medium (T1SS) or directly injected injected inside a host cell that can be from an eukaryotic (T3SS and H2-T6SS) or a prokaryotic (H1-T6SS) organism. All these secretion systems transport various proteins, including virulence factors. In the periplasm, there is a thin peptidoglycan layer (PGN).
(Scheme adapted from Bleves et al., 2010.)

Pseudomonas aeruginosa has the particularity to lack the type IV secretion system but acquired all five other secretion system types. They can be clustered in two groups depending on whether the proteins they secrete cross the bacterial envelop in one stage or two. In the latter case, proteins stay for a short time in the periplasm before secretion through the outer membrane. These two classes of secretion systems are referred here as one-stage and two-stage secretion systems (Fig. 7).

a) Two-stage secretion systems

Proteins transported via this class of secretion systems do not cross directly the bacterial envelope but after passing the inner membrane, they stop shortly in the periplasm. This pause is due to the fact that the proteins are transported through the inner and the outer membrane using different mechanisms. The secretion system type itself is only required to cross the outer membrane while proteins commute via general export machineries routinely used for the exchange of periplasmic and outer membrane proteins like the Sec and the Tat machineries.

The general secretory pathway (Sec) is the more common secretion system for protein transport across cytoplasmic membrane in all living organisms. It essentially transports unfolded propteins (Papanikou et al., 2007). The Twin Arginine Translocation pathway (Tat) transports through the inner membrane proteins with a double-arginine motif. It is involved in the transport of folded proteins (Sargent, 2007).

The T2SS is comparable to a pump and transports proteins from the periplasm to the extracellular space (Fig. 7). The T2SS can be divided in three parts: first, a pore in the outer membrane formed by secretin, second, a complex of proteins localized on the surface, in the inner membrane and a transperiplasmic protein, and third, a pseudopilus. The ATPase (XcpR) is located basally of the secretion system, in the cytoplasm. Proteins recognized as T2SS substrates enter the vestibule of the secretin, then contact the tip of the pseudopilus which ultimately translocates the protein (Douzi et al., 2012).

Among the T2SS substrates are elastases (LasA and LasB), lipases (LipA and LipC), phospholipases (PhoA, PlcB and PlcH), an alkaline phosphatase (PhoA) and an exotoxin (ToxA). Besides the Xcp T2SS two other systems homologous to Xcp have been identified: the Hxc system and more recently the Txc system (Ball et al., 2002; Cadoret et al., 2014).

The T5SS is classically composed by a simple channel localized in the outer membrane and transport large proteins (Sauri et al., 2009) (Fig. 7). These proteins either stayed associated to the outer membrane or are released in the extracellular space after a proteolytic cleavage. T5SS transports divers proteases and lipases. Of note, in *Serratia marcescens*, the pore forming toxin hemolysin is secreted in the medium through a T5SS.

b) One-stage secretion systems

This class of secretion systems directly transports proteins from the cytoplasm to the extracellular space or to the host cytoplasm.

The T1SS is a classical ABC transporter that release transported proteins in the extracellular medium (Fig. 7). This secretion system transports notably the alkaline protease (AprA) (Baumann et al., 1993; Guzzo et al., 1990).

The T3SS is a needle-like machinery that allows the injection of specific toxins inside eukaryotic cells (Fig. 7). This needle-like structure (constituted by Psc, Pop and Pcr proteins) assembles upon an eukaryotic cell contact and directly translocates the T3SS effectors in the cytoplasm of the host cell (Yahr et al., 1996).

There are four known T3SS effectors: ExoT, ExoU, ExoY, and ExoS. However, all *P. aeruginosa* strains possess only three effectors among these four. For example, the strain PA14 possesses ExoT, ExoY, and ExoU. These effectors are injected under an inactive form and need eukaryotic co-factors for activation (Phillips et al., 2003; Sato et al., 2003). They carry various functions. ExoT and ExoS have both a GTPase-activating function (N-terminal domain), that can interfere with the host cytoskeleton (Avet-Rochex et al., 2005), and an ADP-ribosyltransferase activity (C-terminal) domain. ExoY has an adenylate cyclase function, which provokes an increase of cAMP concentration inside the host cell. ExoU is a potent phospholipase that induces a strong cytotoxicity by inducing necrotic host cell death (Finck-Barbançon et al., 1997). Moreover, ExoU and ExoS are incompatible within one strain and the ones carrying ExoU/ExoT are called cytotoxic strains (which induce necrosis) while these carrying ExoS/ExoT are called invasive strains (promotes bacterial internalization followed by an apoptosis-like process) (Shafikhani et al., 2008).

The T3SS has been shown to act as a defense mechanism against phagocytic cells (Avet-Rochex et al., 2007; Shafikhani et al., 2008). This secretion system is a major virulence mechanism that is involved in *P. aeruginosa* acute infections.

The T6SS is a bacteriophage-like structure that like the T3SS allows the direct injection of effectors inside of "enemy" cells that can either be prokaryotic or eukaryotic (Fig. 7). Three clusters of genes have been identified in *P. aeruginosa* called H1-T6SS, H2-T6SS and H3-T6SS. So far, little is known about the H2-T6SS and the H3-T6SS. However, recent studies highlighted the function of H1-T6SS.

H1-T6SS is specifically activated in bacteria-bacteria interaction and involves the Tse1, Tse2 and Tse3 toxin effectors that are injected in the periplasm (Tse1 and Tse3 and degrade the PGN) or in the cytoplasm (Tse2) of Gram-negative bacteria. On the other side, the *P. aeruginosa* genome encodes three cognate immunity proteins, Tsi1, Tsi2 and Tsi3 which are closely linked to the toxin effectors allowing their simultaneous expression. This co-expression of the Tse1-3 and the Tsi1-3 provides a protection to *P. aeruginosa* against its own toxic effectors (Li et al., 2012; Russell et al., 2011).

In contrast, the H2-T6SS has been shown to be specifically activated against eukaryotic cell (Fig. 7). The H2-T6SS would promote *P. aeruginosa* internalization from eukaryotic cells and has been shown to be involved in the virulence of the bacteria in a *C. elegans* infection model (Sana et al., 2012).

Recently, PldB, a H3-T6SS-dependent phospholipase D effector has been identified in *P. aeruginosa* and has been shown to be involved in bacteria-bacteria interaction and to promote *P. aeruginosa* internalization inside of eukaryotic epithelial cells (Jiang et al., 2014).

2. *Pseudomonas aeruginosa* quorum sensing

Most bacteria do not only act as single bacteria but are also able to interact with each other to form communities that allow performing efficiently common tasks. Bacteria communications are achieved through their **quorum sensing**[2] systems.

Pseudomonas aeruginosa uses its quorum sensing especially to synchronize the expression of genes involved in the synthesis of virulence factors. This quorum sensing is

[2] **Quorum sensing:** a mechanism of chemical cell-cell communication that permits coordination of gene expression as a function of the local population density. (Schuster et al., 2013)

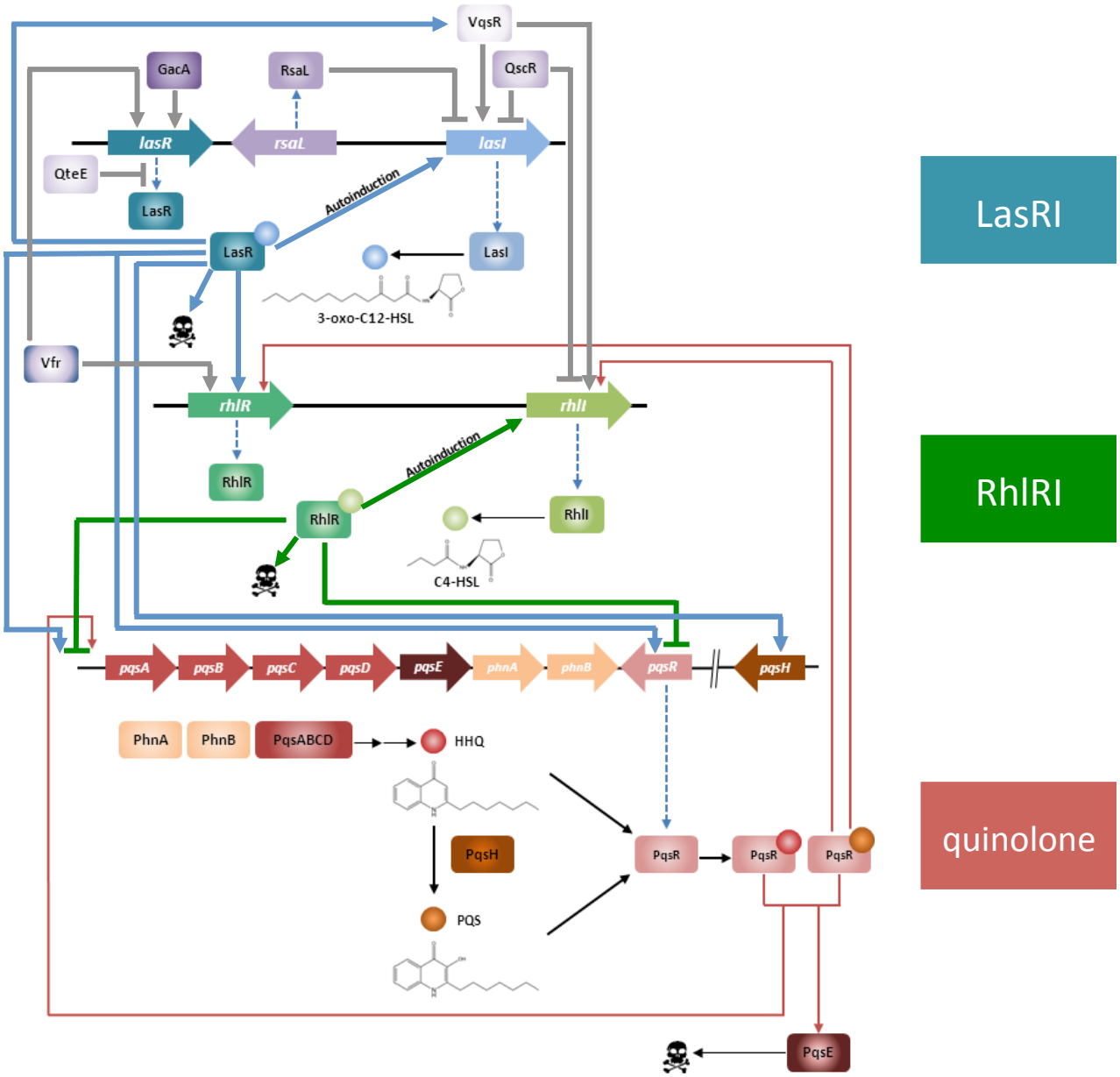


Figure8. The *Pseudomonas aeruginosa* Quorum sensing network.

P. aeruginosa bacteria communicate either through the AHL (LasRI and RhlRI) or the quinolone signaling system. Both systems are interconnected via repression and activation of specific genes for each system. Basically, LasRI circuit involves 3-OC12-HSL synthetized by LasI and that binds LasR which then activates its target genes. The RhlRI circuit is organized in a similar way, with the C4-HSL synthetized by RhlI and that binds RhlR which in turn regulates its target genes. The quinolone signaling requires the PQS or its precursor HHQ, produced by the proteins originating from the *pqsABCD* operon, PhnA and PhnB proteins. These molecules binds PqsR which than activates the expression of its target genes. Upstream of these Quorum sensing system, other proteins are required to tightly regulate qurorum sensing threshold, like Vfr, QteE, RsaL and GacA. Skulls symbolize virulence factors.

(Scheme adapted from Papaioannou et al., 2013.)

well studied and so far, depending on the molecules used for communication, two main classes have been identified: the acyl-homoserine lactone (AHL) signaling system and the quinolone signaling system. These two classes are known to interact between each other (Jimenez et al., 2012; Papaioannou et al., 2013; Schuster and Greenberg, 2006).

Basically, each bacterium produces these communication molecules that will be released in the medium and binds the transcriptional activator receptor of another bacteria from the same species. Depending on the bacterial density, and thus on the concentration of the signal, a varying fraction of receptors will bind to the signal molecule; when the fraction of occupied receptor reaches a certain threshold, the receptors will activate the quorum sensing target genes including those coding for virulence factors

a) The AHL signaling system: LasRI and RhlRI

P. aeruginosa AHL system is composed by the LasRI (elastase) and the RhlRI (rhamnolipids) circuits. Both of them communicate via acyl-homoserine lactone molecule autoinducers and are organized in a similar way (Schuster et al., 2013) (Fig. 8).

The LasRI circuit is composed by the LasI enzyme responsible for the synthesis of 3-oxo-dodecanoyl-homoserine lactone (3-OC12-HSL), the HSL autoinducer that is sensed by the LasR transcriptional activator receptor. This binding allows the expression of LasRI-dependent genes among which are *lasI*, *elastases*, *exotoxin A*, *alkaline phosphatase* and elements from the T2SS (Gambello and Iglewski, 1991).

These 3-OC12-HSL can also be sensed by QscR, an HSL-responsive orphan receptor, which will then modify the expression of another set of genes (independent of LasR) and also represses or delay the expression of many genes usually under the control of LasRI or RhlRI (Chugani et al., 2001; Lequette et al., 2006).

Similarly, the RhlRI circuit is composed by the RhlI enzyme producing butanoyl-homoserine lactone (C4-HSL). These C4-HSL autoinducers will be recognized by RhlR, the transcriptional activator receptor and induce its dimerization (Pearson et al., 1995). This last step will then allow the activation of RhlRI-dependent genes like *rhlI*, *rhlA* and *rhlB*, *rpoS* (sigma factor of stationary phases), *pyocyanin* and *hcn*.

Together, the RhlRI and LasRI circuits control more than 300 genes including virulence factor genes (Schuster et al., 2003). The RhlRI and LasRI circuits do not function in

parallel but interact together. Many genes are controlled jointly by both RhlRI and LasRI, for instance *elastases* (*lasA* and *lasB*), components of the T2SS (*xcpR* and *xcpP*) or an alkaline protease (*aprA*). In addition, there is high fidelity recognition between the autoinducer and the receptor in each circuit as RhlR presents only a very low affinity for 3-OC12-HSL and LasR does not seem to be activated by C4-HSL.

Classically, the LasRI circuit is hierarchically positioned upstream of RhlRI circuit as LasR induce the expression of *rhlR* (Pesci et al., 1997) and QscR represses *lasI* and *rhlI* (Latifi et al., 1996). However, RhlRI circuit is not exclusively dependent on the LasRI, as it has bind shown that the expression of some RhlRI-dependent genes like *pyocyanin* or *rhamnolipids* is not abolished in a lasR mutant background but only delayed (Dekimpe and Déziel, 2009).

Upstream of the AHL signaling systems, other known virulence factors control the activation of the LasRI and RhlRI circuits. For instance the virulence factor receptor (Vfr), activated by cAMP, promotes the expression of both *rhlR* and *lasR* while GacA induce only *lasR* expression (Croda-Garcia et al., 2011; Reimmann et al., 1997). Beside it, three major regulators permit a sharp control of the threshold activation. The first of them, RsaL, is a major regulator of the LasRI circuit by repressing the transcription of *lasI* (de Kievit et al., 1999). The second, the recently identified QteE, was shown to reduce the stability of LasR thus preventing its accumulation. QteE reduces also the amount of RhlR but through an yet unidentified process (Siehnel et al., 2010). The last of them, VqsR (virulence and quorum sensing regulator) positively controls the quantity of autoinducers through the induction of *lasI* and *rhlI*, and is itself activated by LasR (Juhas et al., 2004) (Fig.8).

b) The quinolone signaling system

The quinolone signaling system adds a second level of complexity to *P. aeruginosa* quorum sensing network. LasRI and RhlRI circuits control the expression of the *pqsABCD* operon responsible for the production of 4-hydroxy-2-heptylquinoline (HHQ) (Fig. 8). RhlR represses the operon while LasR activates its expression (McGrath et al., 2004; Xiao et al., 2006a). However, one study reported that neither LasR nor RhlR bound the promotor region of *pqsA* (Wade et al., 2005). These HHQ molecules are further transformed in 3,4-dihydroxy-2-heptylquinoline (PQS) by the PqsH enzyme, the expression of which is itself activated by LasR (Whiteley et al., 1999).

Both HHQ and PQS can binds to their sensor PqsR (also known as MvfR) to promote the expression of the *pqsA-E* genes or the *rhlR* and *rhlI* genes for PQS-PqsR only (Xiao et al., 2006b). In addition, *pqsR* expression is promoted by LasR and repressed by RhlR (Wade et al., 2005).

PqsE is not involved the synthesis of HHQ and PQS, but this protein possesses a metallo-β-lactamase fold and is a important virulence factor involved in the production of pyocyanine, HCN or rhamnolipids (Diggle et al., 2003; Gallagher et al., 2002). One study also suggested that PqsE activity requires RhlR, at least partially (Farrow et al., 2008).

C. *Pseudomonas aeruginosa* infection models

1. Different host models

According to its strong versatility, *P. aeruginosa* is able to proliferate in very diverse environments and therefore to infect a large range of organisms. This property of *P. aeruginosa* allowed the development of infection models in a variety of hosts from plants to mouse (Papaioannou et al., 2013).

P. aeruginosa has been reported to share similar virulence systems in plants (A. thaliana) and mouse (Rahme et al., 1995). Moreover, in nearly all host models, *P. aeruginosa* QS mutants displayed a decrease of virulence. However, specific adaptations to some hosts have been observed. For instance, phenazines and rhamnolipids, which are required in a mammalian infection model, are not involved in the virulence of the bacteria in *Dictyostelium discoideum* and *Drosophila melanogaster* infection models (Limmer et al., 2011a; Pukatzki et al., 2002).

C. elegans is an interesting host model as genetic tools are available and it is a quite prolific and easy to maintain animal with a short generation time and lifespan. Indeed, it can be grown easily in multiple well plates. Recently, a whole library of *P. aeruginosa* (PA14 strain) transposon insertion mutants (80% of PA14 genome covered) was screened using this host model (Feinbaum et al., 2012). Five different infection model have been developed in *C. elegans*: the slow killing assay (slow intestinal infection with biofilm formation), the fast killing assay (phenazine toxicity under acidic conditions), the lethal paralysis assay (cyanogenesis), the red death assay (phosphate depleted media that induces QS) and the liquid

Infection model	Procedure	Complementary informations	Pathogenicity	Reference
"Polymicrobial infection model"	-PAO1 strain -40 CF oropharyngeal flora (OF) -5% sucrose only	-Male flies only -3 hours starvation	-Microorganisms diverted to the crop -Crop damages -3 classes of microorganisms: I. OF that kill flies II. OF that do not kill flies, no influence on PAO1 III. OF not pathogenic alone but that increase PAO1 virulence when together	Sibley et al. 2008
"Intestinal dysplasia model"	-PA14 strain -10% LB and 90% sucrose (5%)		-Important ISC proliferation upon infection -JNK pathway activation in EC upon infection -Cryptic infectious cofactor?	Apidianakis et al. 2009
		-*ras1*^{v12} mutant flies	-Tumor-like structure and cell dissemination in the hindgut of *ras1*^{v12} mutants upon infection	Bangi et al. 2012
"Biofilm model of infection"	-PAO1 strain -5% sucrose only	-Male flies only -3 hours starvation -Hemolymph collection: centrifugation of whole flies	-Bacteria predominantly localized to the crop -Biofilm formation in the crop -High bacterial titer in the hemolymph at 2 days of infection	Mulcahy et al. 2011
"Exotoxins and hemocytes interaction model"	-CHA strain -5% sucrose only	-UAS-*exoSGAP* flies	-*exoS* expressed in eyes provokes a rough phenotype -Eye phenotype partially reverted in *rac1* or *rho1 GTPase* mutants -*exoS* expressed in hemocytes induces a higher susceptibility to the infection and rescues the decreased virulence phenotype of CHA *exoS* mutants	Avet-Rochex et al. 2005
"Bacteremia model"	-PA14 strain -10% BHB and 90% sucrose (50mM)	-Female flies only -2 days sucrose-only diet prior to experiments	-Bacteria predominantly in the midgut -High bacterial titer in the hemolymph at late stages (5 days) of the infection -Cellular immune defense, Toll and IMD pathways are required for the host defense	Limmer et al. 2011

Table 1. Distinct *P. aeruginosa* intestinal infection model in *Drosophila*.
Five different intestinal infection models are described. Note the differences in the preparation of the infection suspension. The treatment of the flies prior to infection seems also important as different fly treatments and different infection solutions lead to a differing pathogenicity. Surprisingly, there are only very few variations in the total amount of bacteria flies feed on.

killing assay (hypoxic response). They all share similarities with CF patient infection or other modes of infection in Humans (Utari and Quax, 2013).

2. *Drosophila melanogaster* host models

Moreover different infection models are used in a same model organism as developed here for the *Drosophila* infection model.

First, the septic infection model corresponds to a direct introduction of bacteria inside of the body cavity of the fly (Haller et al., 2014). It can be done either by injection or by pricking. Both infections lead to similar pathogenicity, in particular, to a rapid death of infected flies as it has been shown by others and us (Lau et al., 2003; Limmer et al., 2011a). In these models, bacteria grow in the hemolymph and even though *Drosophila* develops a strong humoral immune response, it is not sufficient to clear the bacteria and flies will succumb within 48 hours.

Second, different intestinal infection model have been tested. Surprisingly, they did not always lead to similar pathogenicities (see Table 1). In this type of infection model, *Drosophila* are fed with an infection suspension containing *P. aeruginosa* (Haller et al., 2014). Flies die at a much slower rate in an intestinal infection as bacteria need to cross the intestinal barrier prior to provoking a bacteremia (Limmer et al., 2011a; Mulcahy et al., 2011). Different pathogenicities were observed likely depending on variations in the infection procedure.

Most of the infection models use a mono-bacterial infection suspension to infect flies. However, one study tested the effect of polymicrobial infection (Sibley et al., 2008). The results were highly interesting as the authors showed that some non-pathogenic microbes can influence PAO1 virulence and increase its pathogenicity. Another study demonstrated a strong interaction between the phagocytic function of plasmatocytes and one effector of the T3SS, *exoS* (Avet-Rochex et al., 2005). We have shown, in our "bacteremia infection model", that upon infection, PA14 localizes in the whole digestive tract and predominantly in the midgut (Limmer et al., 2011a). We could observe only a significant high bacterial titer in the hemolymph at the second, late stage of the infection (5 days), which only then induces Imd and Toll pathways activation. Moreover, we demonstrated that the cellular immune response of the flies is required in the defense against PA14. Phagocytosis remains functional against

other microbes or particles until late in the infection, although it is unable to control *P. aeruginosa* proliferation during the second phase of the infection.

Some studies have reported massive presence of *P. aeruginosa* in the crop that damaged it (Mulcahy et al., 2011). Prior to infection these flies were starved on water, what might explain the surprisingly high bacterial number in this storage diverticulum. In the same study, the bacterial titer in the hemolymph was unexpectedly high only two days after the infection start. These bacteria were retrieved from whole flies through a centrifugation process. A procedure that likely does not allow discriminating between hemolymph-bacteria and gut-lumen bacteria.

Aim of this work

Recently, using our intestinal infection model of *Drosophila* with the PA14 strain, we have identified the *ΔrhlR* mutant that exhibited a strong decrease of virulence in wild-type flies. However, *ΔrhlR* displayed a nearly full regain of virulence in flies unable to phagocytose and we confirmed these results in *eater* mutant flies (Limmer et al., 2011a). These results indicated that RhlR is required in PA14 to circumvent the cellular immune response of *Drosophila*. However, how RhlR exerts this function remains unknown and the elucidation of its mechanism of action was one of the major aims of this work.

The major aim of my PhD was to characterize in depth our *Drosophila vs. P. aeruginosa* intestinal infection model. As described in Chapter 1, I tried to understand how RhlR could allow the bacteria to elude phagocytosis by screening a small subset of 384 PA14 mutants. In parallel of it, I tested PA14 mutant bacteria for known virulence factor in different infection models.

In Chapter 2, I present some unexpected differences in PA14 virulence depending on whether flies are infected as single individuals or as communities. These observations are likely linked to RhlR function in the bacterial quorum sensing.

Previously, we showed that upon PA14 infection both Imd and Toll pathways are induced and involved in the defense against PA14, a result that has been also observed by others but not explained (Lau et al., 2003; Limmer et al., 2011a). Using different Toll pathway PRR mutants, I tried to dissect Toll pathway activation upon PA14 infection. These results are presented in Chapter 3.

During my PhD, we observed that different fly stocks, with presumably the same genotype, exhibited different susceptibilities to an intestinal infection with PA14, as well as to other infections. A thorough analysis of these fly stocks revealed the presence of an enteric virus in the most susceptible stock. This study is detailed in Chapter 4.

For the time of my Master2 and first year of PhD, a part of my work, on *Drosophila* and *P. aeruginosa*, contributed to the paper added in Annex 1. Moreover, I also contributed to a Method chapter in which we presented the different technics we use to study bacterial virulence. This method chapter corresponds to Annex 2.

Chapter 1

A quorum-sensing independent function of *P. aeruginosa* RhlR in circumventing Thioester protein mediated phagocytosis in an intestinal infection model of *Drosophila*

In this work, I studied the role of RhlR in *P. aeruginosa* (PA14) virulence and how it could exert its function to circumvent the cellular immune response of *Drosophila*. I also tried to identify novel PA14 virulence factors.

It is written as a scientific paper in preparation. However, a few additional experiments needs to be performed before a future submission.

My contribution to this work is major as I performed all the experiments except for the screen that was done in collaboration with an intership student. I generated the PA14 deletion mutants at the ESBS in Illkirch with the help of Olivier Cunrath.

A quorum sensing independent function of *P. aeruginosa* RhlR in circumventing Thioester protein mediated phagocytosis in an intestinal infection model of *Drosophila*

Samantha Haller[a], Samuel Liegeois[a], Olivier Cunrath[b], Stefanie Limmer[a1], Sebastian Niehus[a], Eliana Drenkard[c,d], Isabelle Schalk[b], Frederick M. Ausubel[c,d], and Dominique Ferrandon[a*].

[a] Equipe Fondation Recherche Médicale, Unité Propre de Recherche 9022 du Centre National de la Recherche Scientifique, Institut de Biologie Moleculaire et Cellulaire, Université de Strasbourg, F67084 Strasbourg Cedex, France; [b] UMR 7242, Université de Strasbourg-CNRS, ESBS, Blvd Sébastien Brant, Strasbourg, Illkirch F-67413, France; [c] Department of Genetics, Harvard Medical School, Boston, MA 02115; and [d] Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

[1] Present address: Institut für Neuro- und Verhaltensbiologie, Badestrasse 9, 48149 Münster, Germany.

*To whom correspondence may be addressed. E-mail: d.ferrandon@ibmc.u-strasbg.fr.

INTRODUCTION

Drosophila melanogaster is a powerful genetic model organism for the study of innate immunity and host-pathogen interactions that has been intensely investigated in the past 20-25 years [1-3]. Genetic analysis has allowed the detailed dissection of its systemic immune response to microbial infections [4]. Indeed, two major NF-kappaB pathways regulate the induction of the expression of genes that encode potent antimicrobial peptides, which will attack most bacteria and fungi [5,6]. This response is so effective, especially in the case of Gram-negative bacterial infections, that another arm of host defense, the cellular immune

response, has remained in comparison less well understood [7]. Indeed, blocking it through saturation of the phagocytic apparatus with inert particles does not yield a strong susceptibility phenotype when infected by *Escherichia coli*, unless the systemic immune response is partially impaired [8]. Nevertheless, we have found in two intestinal infection models with the opportunistic pathogens *Serratia marcescens* and *Pseudomonas aeruginosa* that the cellular immune response plays a key role in controlling the bacteria that have escaped from the digestive tract [9,10]. In both cases, the phagocytic receptor Eater plays a cardinal role and prevents the development of a rapid bacteremia [10,11]. Of note, it is unknown whether opsonization plays a role *in vivo*, even though it has been shown to occur in cultured cells [12]. In contrast to *S. marcescens*, *P. aeruginosa* ultimately manages to establish an exponential infection in the hemocoel four to five days after its ingestion. In a previous study, we have shown that a member of the LuxR family of signal receptor-transcriptional regulators, RhlR, is required to circumvent the cellular immune response [10]. Indeed, *rhlR* mutants are almost avirulent since they remain at very low levels in the hemolymph and kill the infected flies at a much reduced rate. Interestingly, the cellular immune response remained functional until late stages of the infection.

RhlR is the major regulator of one of the three known quorum-sensing systems in *P. aeruginosa*. Quorum-sensing systems play a major role in coordinating the expression of virulence genes in several infection models [13-16]. However, we have failed to uncover a strong role of the Las and quinolone quorum sensing system in our infection model [10]. This observation was somewhat unexpected since the Las system appears to function upstream of the Rhl quorum sensing system . RhlR is activated by binding to an auto-inducer molecule, butanoyl-homoserine lactone (C4-HSL), which is synthesized by the RhlI enzyme. This activation takes place when a threshold concentration of C4-HSL is reached.

Here, by studying the phenotype of *rhlI* mutants, we show that RhlR functions both in a quorum sensing-dependent and –independent manner. We further identify other virulence factors that potentially function together with RhlR. We also demonstrate that the cellular immune response plays a critical role only during the first phase of the infection and that it requires the function of the Thioester-containing Protein Tep4, a likely opsonin.

RESULTS

A quorum-sensing independent function of RhlR?

We had previously reported that RhlR plays a key role in the virulence of *P. aeruginosa* PA14 in our intestinal infection model. As RhlR is supposedly activated by C4-HSL synthetized by RhlI, we therefore checked whether the inducer was also required for virulence. As compared to *rhlR*, *rhlI* null deletion mutants displayed a modest, yet significantly, impaired virulence (Fig. 1A). We had also checked three independent transposon insertion mutants in the *rhlI* locus and had failed to detect a consistent phenotype with these strains, although some of them sometimes displayed a slightly attenuated virulence phenotype. Of note, of all of the mutants we have tested, *rhlI* was the strain that displayed the highest variability from experiment to experiment (Fig.1B), whereas *rhlR* consistently exhibited a strongly impaired virulence phenotype.

Next, we asked whether *rhlI* regains its virulence when ingested by flies immunodeficient for the cellular immune response. An easy procedure is to saturate the hemocytes by the prior injection of latex beads into the hemocoel. The beads will be rapidly ingested. However, as they cannot be degraded, they ultimately saturate the phagocytic apparatus, thus effectively

ablating the cellular immune response [8,17]. As shown in Fig. 1C, *rhlI* display an enhanced virulence when ingested by latex beads-injected flies as compared when ingested by uninjected control flies. The virulence displayed by *rhlI* mutants in phagocytosis-deficient flies is similar to that of *rhlR* mutants, but nevertheless is not as strong as that of wild-type PA14 (Fig. 1D). Latex beads-injected flies are more sensitive to the infection, as mirrored by a difference of 2.2 days in LT50s. A similar difference of 2.8 days was observed with *rhlI*, which contrasts with the strong virulence gain observed with *rhlR*: 4.7 days.

As noted previously [10], *rhlR* mutants displayed a shallow survival curve, even when their virulence is restored in phagocytosis-impaired flies, as measured by the Hill coefficient (Fig. 1E). Interestingly, *rhlI* mutants also exhibited a significantly shallower curve than wild-type bacteria under the same conditions.

Taken together, these data indicate that RhlR presents a dual function, a quorum sensing-independent function and a quorum-sensing function that becomes apparent when the cellular immune defense is deficient.

A mini-screen to identify mutants with a **rhlR-***like phenotype*

We had previously tested several mutants that affect known effectors of the Rhl quorum-sensing pathway and failed to detect any with an altered virulence. As the *Drosophila* model is too cumbersome to screen a whole bank of bacterial mutants, we decided to follow a strategy we had employed to identify virulence factors of the entomopathogenic bacterium *Serratia marcescens* in a systemic infection model [18]. Namely, we screened virulence mutants selected in a large-scale screen in the nematode worm *Caenorhabditis elegans*, in which 180 mutant strains had been isolated on the basis of a decreased virulence [19]. In the present study, this strategy is better-suited since the PA14 transposon insertion library generated by the Ausubel laboratory was screened also in an intestinal infection model that

recapitulates at least the early stages of the infection in *Drosophila* [20]. For instance, the bacteria ingested by *C. elegans* are also exposed to AMPs and a Dual-oxidase mediated ROS-burst.

We chose to test all of the mutants that had been isolated following the first-round of screening in *C. elegans* [19]. We have therefore monitored the survival of flies after ingestion of *P. aeruginosa* for 326 PA14 mutant strains, a screen that was independently performed twice by two investigators. The results are shown in Supplementary Table 1. Thirteen strains displayed a significantly enhanced virulence phenotype and actually had not been retained in the final list in the *C. elegans* screen after multiple round of screening. In contrast, twelfe strains that had been retained in the *C. elegans* study exhibited an impaired virulence phenotype. A further 60 strains displayed a somewhat altered virulence in our mini-screen and were therefore included for a second round of screening, together with the already retained 25 mutants. Fig. S1 displays the results expressed in terms of LT50s (lethal time 50: time required to kill 50% of the infected flies) obtained for these 85 mutants in three independent experiments. As expected, *rhlR* displayed the strongest avirulence phenotype. We kept a further 11 strains that consistently displayed a decreased virulence phenotype, although it was sometimes modest. We also identified ten mutant strains that displayed a consistently increased virulence phenotype, again often with a mild phenotype (Fig. S1).

We focused on the hits with a decreased virulence and further tested other independent transposon insertion mutants whenever available, a strategy that did not allow us to confirm any candidate genes (Fig. S2). Next, we asked whether the original transposon insertion mutant strains would be able to regain their virulence in flies with a defective cellular immune response. As shown in Fig. 2, *sltB1*, *vfR*, *xcpR*, *gidA*, and *rho* mutant strains displayed a virulence similar to that of wild-type PA14 when infecting latex-beads injected flies, except that the difference in LT50s observed for *gidA* between wild-type and latex beads-injected

flies was not significant. We therefore constructed and obtained one deletion mutant for each of these genes, *rho* excepted, as it may be an essential gene. We confirmed for *vfR, xcpR,* and *sltB1* our findings obtained with the transposon insertion mutants, both in wild-type and phagocytosis-impaired flies (Fig. 3). Of note, the null mutant phenotype was still weaker than the *rhlR* null mutant phenotype.

Because the phenotypes of these three novel virulence genes was milder than that of *rhlR*, especially as regards *vfR* and *sltB1*, we asked whether compound mutants would display a virulence as strongly attenuated as that of *rhlR*. We found that this was not the case for any of the three possible double-mutants (Fig. S3)

We conclude that *vfR*, *sltB1*, and *xcpR* are likely involved in the same process, which would be related to that regulated by RhlR.

Phagocytosis is required in host defense against ingested* P. aeruginosa *during the first hours of the infection

A striking feature of our intestinal infection model is that even though PA14 bacteria are able to cross the digestive tract within a day, and likely in a shorter period, its titer in the hemolymph remains very low for at least three days. There is an exponential increase in the titer thereafter [10]. We therefore asked at which phase of the infection phagocytosis was required. To this end, we decided to block phagocytosis by injecting latex beads at different time points of the infection, namely before the infection as is our usual procedure, or a few hours or days after the beginning of the infection. Phagocytosis is likely saturated rapidly after the injection of latex-beads. Our expectation is that an increased virulence would be detected in latex beads-injected flies as long as phagocytosis was required for host defense. Once phagocytosis becomes irrelevant, then the latex beads treatment should become ineffective and no significant survival difference should be observed with PBS-injected

control flies. We first tested wild-type PA14 in this assay. As shown in Fig.4A, blocking phagocytosis one day before or four hours after infection led to a strongly increased virulence of PA14, as observed in survival experiments in which we monitored the LT50s. When phagocytosis was blocked one or two days after the ingestion of PA14, latex-beads injected flies succumbed as early as flies in which the cellular response had been inhibited four hours after ingestion, and faster than control flies to PA14 infection. However, the difference was not significant, likely because of too few data collected at these time points. In contrast, blocking phagocytosis at days four or six no longer had an influence on the survival rate of infected flies as compared to controls. We conclude that phagocytosis plays a critical role in host defense during at least the first four hours, and likely the first three days, of the infection, that is, during the initial phase of the infection.

We next tested in the same assay *rhlR* mutant bacteria and found that phagocytosis was constantly required, at least up to day four, to control *rhlR* infection (Fig. 4B). Flies would then succumb to infection in the seven-eight days following the inactivation of phagocytosis. These data indicate that phagocytosis is the only efficient defense against *rhlR* bacteria, as they become again virulent when phagocytosis is impaired, at any time of the infection. Unexpectedly, *rhlI* appeared to behave as *rhlR* in this assay (Fig. 4C). However, one should note in the case of *rhlI* that the absolute difference of LT50s at day 4 of latex-bead injection, the latest time point examined, is much smaller (2,3 days) than for injections at days -1, 1, or 4h (mean of 5.1 days). The difference for *rhlR* at day 4 is 4.2 days.

RhlR is required to circumvent an early step of phagocytosis

We have previously shown that the Eater phagocytic receptor is required to limit PA14 infections. Indeed, *rhlR* bacteria regain their virulence when ingested by *eater* flies [10]. The family of TEP proteins has been reported to act as opsonins for bacterial uptake by

macrophages in mosquitoes [21] and also in *Drosophila* cultured Schneider-2 cells [12]. Even though we had failed to detect an immune phenotype in *Tep* mutants with a variety of pathogens, we tested them in the PA14 intestinal infection model [22]. As no Tep1 mutant is currently available, we first knocked down *Tep1* expression in hemocytes or in the fat body by RNAi and did not detect an altered sensitivity of these flies to PA14 intestinal infections (Fig. 5A-B). We found that *Tep4* and *Tep2,3,4* triple mutants, but not *Tep2, Tep3,* and *Tep2,3* mutants were markedly more sensitive to the ingestion of PA14 (Fig. 5C). However, further blocking phagocytosis by injecting latex beads in this mutant background still enhanced further the sensitivity to infection of *Tep4* mutant flies (Fig. S4), which suggests that *Tep4* is only partially required for the uptake of *P. aeruginosa*. Importantly, *rhlR* regained a wild-type virulence when ingested by *Tep4* mutants (Fig. 5D).

We conclude that *Tep4* function is required in the host defense against ingested PA14.

DISCUSSION

In this study, we have analyzed the interactions of *P. aeruginosa* with *Drosophila* from the dual perspective of both pathogen and host. Our data lead us to propose a model in which RhlR plays a primordial, quorum sensing-independent, role in virulence by lessening its detection by the cellular immune arm of the host defense once it has reached the internal body cavity of the insect.

A rhlI-*independent function of* rhlR

P. aeruginosa is a pathogen that uses complex signaling mechanisms to adapt to its environment. In particular, its three quorum-sensing system appear to be involved in its virulence properties [13-15]. *In vitro* studies, sometimes complemented by *in vivo*

experiments, have revealed that these quorum-sensing systems are intricately intertwined. It is thus somewhat unusual that only the Rhl system appears to play a critical role for virulence in our infection model. Here, we report that rhlR null mutants consistently display virulence levels that are much weaker than those observed with *rhlI* mutants (Fig. 1B). This observation implies that RhlR functions at least partially independently from RhlI and presumably from C4-HSL activation. Remarkably, *rhlR* but not *rhlI*, had been picked up in a screen for reduced virulence in a *C. elegans* intestinal infection model, suggesting that this property is not specific to the *Drosophila* model [19].

One possibility is that RhlR nevertheless gets activated, possibly in a different manner, by an as yet unidentified compound. Of note, RhlR is only poorly activated by 3-oxo-C12-HSL. In addition, *lasI* mutant bacteria display only a modestly decreased virulence phenotype (SH, unpublished data). The diketopiperazines (DKPs) represent a candidate family of RhlR-activating compounds [23]; however, at least one study failed to detect any interaction of these compounds with LuxR proteins [24]. The resolution of this issue will require testing mutants that affect the synthesis of DKPs.

Another hypothesis to consider is that RhlR may function independently of auto-inducer molecules. RhlR forms dimers in the presence or absence of C4-HSL [25]. Further studies reported that RhlR functions as a repressor when unbound to C4-HSL [26,27]. Interestingly, RhlR dimers seem to bind its target DNA sequence with an altered conformation [26]. Finally, transcriptomics studies also revealed several target genes that appear to be repressed by either LasR or RhlR [28,29]. Of note, a limitation of all these studies is that they were performed *in vitro* and not *in vivo*.

Finally, our studies on the inactivation of the cellular immune response at different time points of the infection further support a quorum sensing-independent role of RhlR. Our study

revealed that phagocytosis, and thus C4-HSL-independent RhlR function, is required only when very few bacteria are present in the hemolymph, that is, during the first days of the infection. Of note, we cannot exclude that C4-HSL might be produced by the bacteria present in large numbers in the gut compartment. However, if C4-HSL were produced in the intestinal lumen and able to cross the digestive barrier, it would then be difficult to understand why it does not immediately activate RhlR: the observed lag between ingestion and full-blown bacteremia should hardly be detected. This hypothesis also does not account for why the *rhlI* virulence phenotype is much weaker than that of *rhlR*.

A RhlR-like role of other virulence factors

In this work, we have identified at least three further virulence factors required for pathogenesis in our intestinal infection model, VfR, XcpR, SltB1, and possibly Rho. We unfortunately were not able to confirm the phenotype of *rho* by generating a null mutant, as the transcription termination factor it encodes may well be essential. Of note, *gidA* has been reported to be required for RhlR function [30].

VfR is a cAMP-activated factor that is involved in *P. aeruginosa* virulence [31] and positively regulates the expression of genes of the type 2&3 secretion systems, as well as that of *rhlR* and *lasR* [32-38]. Of note, most of these studies were performed using the PAO1 strain, and not PA14. We have found that these two strains behave differently in our intestinal infection model (SH, unpublished data). Nevertheless, we have found that mutants for two genes that positively or negatively regulate VfR activation, *pilG* and *chpB,* respectively display a reduced or enhanced virulence in our infection model, further reinforcing the notion that *vfR* is important for the virulence of PA14 (SH, unpublished data).

At this stage of the genetic analysis, we do not know whether *vfR* functions upstream or downstream of *rhlR in vivo*. Our attempts to measure *vfr* transcript levels in bacteria infecting

flies in a septic injury model revealed a strong attenuation of *vfR* expression in *rhlR* mutants (SH, unpublished data), which suggests a potentially reversed regulatory link between *rhlR* and *vfR*. However, a more relevant set of *in vivo* data should be gained in our intestinal infection model, which is technically-challenging as only a few bacteria can be retrieved from the hemolymph at the relevant stage of the infection.

XcpR is a core component of the type 2 secretion (T2SS) system through which virulence factors such as proteases or lipases are secreted [39]. In keeping with this broad role, it is the mutant with the largest attenuation of virulence, *rhlR* excepted. Thus, the T2SS, in contrast to the T3SS, is required for virulence in our infection model.

SltB1 encodes a lytic transglycosylase, which is involved in peptidoglycan metabolism and thus plays a critical role in the structure of the cell wall. In keeping with this function, sltB1 mutant has been shown to exhibit an enhanced resistance to ß-lactamase [40,41]. A role of *sltB1*in virulence has not been reported, at least as regards *P. aeruginosa*. In *Escherichia coli*, a compound mutant that deletes all six lytic transglycosidases, but not single mutants, is unable to secrete the immune elicitor tracheal cytotoxin [42]. Thus, because of the redundancy of lytic transglycosylases with respect to peptidoglycan remodeling, it is implausible that SltB1 acts through the systemic Immune deficiency immune response. SltB1 is thus likely to alter the structure of the cell wall, and possibly to modify indirectly the configuration of outer membrane proteins.

All of these null mutants display a weaker phenotype than *rhlR* deletions. Yet, they regain their virulence when ingested by phagocytosis-deficient flies, a phenotype they share with *rhlR*. This opens the possibility that these genes are somehow connected with *rhlR*. As RhlR is a master regulator, it might influence in parallel several downstream processes in which SltB1, XcpR, and VfR are separately involved. If this were the case, the expectation would be

that compound mutants would exhibit a stronger avirulence phenotype, which we did not observe. Thus, it remains an open possibility that the product of these genes are involved in a common process.

A quorum sensing-dependent function of RhlR

The finding that *rhlI* mutants display a somewhat reduced virulence suggests that C4-HSL quorum sensing is nevertheless playing a role in our infection model. Interestingly, we note that in flies deprived of a cellular immune response, *rhlR* and *rhlI* mutants are still less virulent than wild-type PA14 by about two days (Fig. 1D). Both mutants display survival curves with shallow slopes (Fig. 1E) when fed to phagocytosis-impaired flies. This suggests that the switch to systemic aggressive bacteremia occurs in a less coordinated manner, consistent with a lack of quorum sensing.

At this stage, it is difficult to assess whether *xcpR*, *sltB1*, or *vfR* are involved in a quorum sensing-dependent or –independent process.

RhlR and the escape from phagocytosis

A characteristic of the infection is the ability of a few bacteria to escape into the hemocoel, where they encounter hemocytes. *rhlR* bacteria appear to be more vulnerable to the cellular immune response, which is mediated by the phagocytic receptor *Eater*. Here, we show that the thioester-containing protein Tep4 displays an *eater*-like phenotype. The simplest explanation is that it functions as an opsonin, as some other Teps. It had been reported that Tep2 was required for the uptake of *E. coli* by S2 cultured Drosophila cells and that *Tep3*for that of *Staphylococcus aureus* [12]. Here, we note that *Tep3* mutants exhibit a rather increased host defense to the ingestion of *P. aeruginosa* and strikingly that only *Tep4* displays an enhanced sensitivity to this infection in our model. Further experiments will be required to determine whether Tep4 binds with an enhanced affinity to *rhlR* bacteria as compared to wild-

type PA14, and whether the ingestion of *P. aeruginosa* by hemocytes depends on *Tep4*. Another open question is whether Eater directly binds to bacteria, *e.g.*, to peptidoglycan as shown earlier [43], or whether it also recognizes bacteria opsonized by Tep4.

Because few bacteria appear to cross the digestive tract at any time point of the infection, we have been so far unable to detect any PA14 ingested by hemocytes *in vivo*. As had been shown for *S. marcescens*, we have shown that *P. aeruginosa* continuously escape from the digestive tract (SH, unpublished data). Our data with the ablation of phagocytic function at different time points of the infection suggest that the continuous patrol by hemocytes effectively prevents PA14 in the hemocoel from developing then into a systemic infection.

Because *SltB1* likely affects the cell wall and peptidoglycan remodeling, because Eater directly binds to peptidoglycan, we propose that the absence of *SltB1* results in cell wall alterations that render *P. aeruginosa* more prone to detection by the Eater, and potentially Tep4, sensing system. As *SltB1-vfR* compound mutants do not display an enhanced reduction of virulence, which suggests that they function in a common process, and as *vfR* expression may be regulated by RhlR, we propose as a working model that the *rhlI*-independent function of RhlR is to regulate the cell wall so that the bacterium conceals its presence from the cellular immune system.

MATERIALS AND METHODS

Drosophila stock and Culture

The following strains were used: wild-type, w^{A5001} and the mutants *Tep3* (d03976) [44], *Tep4* (EY04656) [45], *Tep2,3* double mutant, *Tep2,3,4* triple mutant and UAS-*Tep1* RNAi (ML2D) were described before [22]. C564 *Gal* $Gal80^{ts}$ and hml *Gal4* $Gal80^{ts}$ fly lines were used as drivers for UAS-*Tep1* RNAi [46]. These crosses were done at 18°C. After hatching, F1 flies were transferred to 29°C for 6 days to allow a strong expression of the *Tep1* RNAi construct. To block the phagocytosis ability of *Drosophila*, latex beads were injected in these flies one day before infection or upon the infection (Fig. 4) as described before [47], except that flies were allowed to recover for four hours after injection prior to being exposed to the bacteria.

Bacteria strains and growth conditions

Pseudomonas aeruginosa strain PA14 wild-type [48], ΔrhlR and ΔrhlI mutants [10] and all 326 transposon insertion mutants [19] were grown in Brain-Heart-Infusion Broth (BHB), overnight, at 37°C with agitation.

Drosophila intestinal infections

Infections were performed as described previously with PA14 [47]. At least 20 flies were assessed per infection tube. Infected and control flies were kept at 25°C. For survival assays, the number of surviving flies was computed daily.

Generation of PA14 clean deletion mutants

In-frame deletions in PA14 vfR, xcpR and sltB1 were constructed by replacing the PA14 wild-type coding sequences of these genes with a 1.400 kb PCR-amplified sequence from PA14 in the ORF of these genes as described before for the PAO1 strain [49]. The 1.400 kb

represented around 700 bases upstream, the 20 first, the 20 last and the 700 bases downstream of the coding sequence of the gene. PCR-amplified fragments that contained the in-frame deletion of these genes were subcloned into the EcoRI and HindIII sites of plasmid pME3088. The resulting plasmids were introduced into PA14 wild-type strains to allow exchange between wild-type sequence and deleted constructs by homologous recombination resulting in the ΔvfR, ΔxcpR and ΔsltB1. Double mutants were generated in two steps by successive conjugation with the two relevant plasmids.

Newly generated mutants were identified by PCR with appropriate primers for each deleted gene and confirmed by sequencing deleted constructs in their genome.

Statistical analysis

All statistical analysis were performed on Graphpad Prism version 5 (Graphpad software Inc., San Diego, CA). Details are indicated in the legend of each figure.

REFERENCES

1. Limmer S, Quintin J, Hetru C, Ferrandon D (2011) Virulence on the fly: Drosophila melanogaster as a model genetic organism to decipher host-pathogen interactions. Current Drug Targets 12: 978-999.
2. Bier E, Guichard A (2012) Deconstructing host-pathogen interactions in Drosophila. Dis Model Mech 5: 48-61.
3. Igboin CO, Griffen AL, Leys EJ (2012) The Drosophila melanogaster host model. J Oral Microbiol 4.
4. Lemaitre B, Hoffmann J (2007) The Host Defense of Drosophila melanogaster. Annu Rev Immunol 25: 697-743.
5. Ganesan S, Aggarwal K, Paquette N, Silverman N (2010) NF-kappaB/Rel Proteins and the Humoral Immune Responses of Drosophila melanogaster. Curr Top Microbiol Immunol.
6. Ferrandon D, Imler JL, Hetru C, Hoffmann JA (2007) The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol 7: 862-874.
7. Pean CB, Dionne MS (2014) Intracellular infections in Drosophila melanogaster: host defense and mechanisms of pathogenesis. Dev Comp Immunol 42: 57-66.
8. Elrod-Erickson M, Mishra S, Schneider D (2000) Interactions between the cellular and humoral immune responses in *Drosophila*. Curr Biol 10: 781-784.
9. Nehme NT, Liegeois S, Kele B, Giammarinaro P, Pradel E, et al. (2007) A Model of Bacterial Intestinal Infections in Drosophila melanogaster. PLoS Pathog 3: e173.
10. Limmer S, Haller S, Drenkard E, Lee J, Yu S, et al. (2011) Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. Proc Natl Acad Sci U S A 108: 17378-17383.
11. Kocks C, Cho JH, Nehme N, Ulvila J, Pearson AM, et al. (2005) Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell 123: 335-346.
12. Stroschein-Stevenson SL, Foley E, O'Farrell PH, Johnson AD (2006) Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS Biol 4: e4.
13. Schuster M, Sexton DJ, Diggle SP, Greenberg EP (2013) Acyl-homoserine lactone quorum sensing: from evolution to application. Annu Rev Microbiol 67: 43-63.
14. Coggan KA, Wolfgang MC (2012) Global regulatory pathways and cross-talk control pseudomonas aeruginosa environmental lifestyle and virulence phenotype. Curr Issues Mol Biol 14: 47-70.
15. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, et al. (2012) The multiple signaling systems regulating virulence in Pseudomonas aeruginosa. Microbiol Mol Biol Rev 76: 46-65.
16. Williams P, Camara M (2009) Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol 12: 182-191.
17. Hoffmann D (1976) [Role of phagocytosis and soluble antibacterial factors in experimental immunization of Locusta migratoria]. C R Acad Sci Hebd Seances Acad Sci D 282: 1021-1024.
18. Kurz CL, Chauvet S, Andres E, Aurouze M, Vallet I, et al. (2003) Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. Embo J 22: 1451-1460.

19. Feinbaum RL, Urbach JM, Liberati NT, Djonovic S, Adonizio A, et al. (2012) Genome-wide identification of Pseudomonas aeruginosa virulence-related genes using a Caenorhabditis elegans infection model. PLoS Pathog 8: e1002813.
20. Irazoqui JE, Urbach JM, Ausubel FM (2010) Evolution of host innate defence: insights from Caenorhabditis elegans and primitive invertebrates. Nat Rev Immunol 10: 47-58.
21. Levashina EA, Moita LF, Blandin S, Vriend G, Lagueux M, et al. (2001) Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae. Cell 104: 709-718.
22. Bou Aoun R, Hetru C, Troxler L, Doucet D, Ferrandon D, et al. (2011) Analysis of thioester-containing proteins during the innate immune response of Drosophila melanogaster. J Innate Immun 3: 52-64.
23. Holden MT, Ram Chhabra S, de Nys R, Stead P, Bainton NJ, et al. (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from Pseudomonas aeruginosa and other gram-negative bacteria. Mol Microbiol 33: 1254-1266.
24. Campbell J, Lin Q, Geske GD, Blackwell HE (2009) New and unexpected insights into the modulation of LuxR-type quorum sensing by cyclic dipeptides. ACS Chem Biol 4: 1051-1059.
25. Ledgham F, Ventre I, Soscia C, Foglino M, Sturgis JN, et al. (2003) Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of Pseudomonas aeruginosa LasR and RhlR. Mol Microbiol 48: 199-210.
26. Medina G, Juarez K, Valderrama B, Soberon-Chavez G (2003) Mechanism of Pseudomonas aeruginosa RhlR transcriptional regulation of the rhlAB promoter. J Bacteriol 185: 5976-5983.
27. Anderson RM, Zimprich CA, Rust L (1999) A second operator is involved in Pseudomonas aeruginosa elastase (lasB) activation. J Bacteriol 181: 6264-6270.
28. Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J Bacteriol 185: 2066-2079.
29. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003) Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185: 2080-2095.
30. Gupta R, Gobble TR, Schuster M (2009) GidA posttranscriptionally regulates rhl quorum sensing in Pseudomonas aeruginosa. J Bacteriol 191: 5785-5792.
31. Smith RS, Wolfgang MC, Lory S (2004) An adenylate cyclase-controlled signaling network regulates Pseudomonas aeruginosa virulence in a mouse model of acute pneumonia. Infect Immun 72: 1677-1684.
32. Albus AM, Pesci EC, Runyen-Janecky LJ, West SE, Iglewski BH (1997) Vfr controls quorum sensing in Pseudomonas aeruginosa. J Bacteriol 179: 3928-3935.
33. Beatson SA, Whitchurch CB, Sargent JL, Levesque RC, Mattick JS (2002) Differential regulation of twitching motility and elastase production by Vfr in Pseudomonas aeruginosa. J Bacteriol 184: 3605-3613.
34. Whitchurch CB, Beatson SA, Comolli JC, Jakobsen T, Sargent JL, et al. (2005) Pseudomonas aeruginosa fimL regulates multiple virulence functions by intersecting with Vfr-modulated pathways. Mol Microbiol 55: 1357-1378.
35. Wolfgang MC, Lee VT, Gilmore ME, Lory S (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev Cell 4: 253-263.

36. Fox A, Haas D, Reimmann C, Heeb S, Filloux A, et al. (2008) Emergence of secretion-defective sublines of Pseudomonas aeruginosa PAO1 resulting from spontaneous mutations in the vfr global regulatory gene. Appl Environ Microbiol 74: 1902-1908.
37. Fuchs EL, Brutinel ED, Jones AK, Fulcher NB, Urbanowski ML, et al. (2010) The Pseudomonas aeruginosa Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. J Bacteriol 192: 3553-3564.
38. Croda-Garcia G, Grosso-Becerra V, Gonzalez-Valdez A, Servin-Gonzalez L, Soberon-Chavez G (2011) Transcriptional regulation of Pseudomonas aeruginosa rhlR: role of the CRP orthologue Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhlR. Microbiology 157: 2545-2555.
39. Filloux A (2011) Protein Secretion Systems in Pseudomonas aeruginosa: An Essay on Diversity, Evolution, and Function. Front Microbiol 2: 155.
40. Cavallari JF, Lamers RP, Scheurwater EM, Matos AL, Burrows LL (2013) Changes to its peptidoglycan-remodeling enzyme repertoire modulate beta-lactam resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother 57: 3078-3084.
41. Nikolaidis I, Izore T, Job V, Thielens N, Breukink E, et al. (2012) Calcium-dependent complex formation between PBP2 and lytic transglycosylase SltB1 of Pseudomonas aeruginosa. Microb Drug Resist 18: 298-305.
42. Bouskra D, Brezillon C, Berard M, Werts C, Varona R, et al. (2008) Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456: 507-510.
43. Chung YS, Kocks C (2011) Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor eater. J Biol Chem 286: 26524-26532.
44. Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, et al. (2004) A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet 36: 283-287.
45. Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, et al. (2004) The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. Genetics 167: 761-781.
46. McGuire SE, Mao Z, Davis RL (2004) Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci STKE 2004: pl6.
47. Haller S, Limmer S, Ferrandon D (2014) Assessing Pseudomonas virulence with a nonmammalian host: Drosophila melanogaster. Methods Mol Biol 1149: 723-740.
48. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, et al. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. Science 268: 1899-1902.
49. Cunrath O, Gasser V, Hoegy F, Reimmann C, Guillon L, et al. (2014) A cell biological view of the siderophore pyochelin iron uptake pathway in Pseudomonas aeruginosa. Environ Microbiol.

FIGURE LEGENDS

Figure 1. RhlR's function in circumventing the cellular immune response of *Drosophila* is at least partially independent from RhlI.

Survival experiment of wild-type flies (w^{A5001}) that are fed on wild-type PA14 bacteria or Δ*rhlR* and Δ*rhlI* mutants. (A) Survival curves of infected and uninfected (NI) flies. Flies died faster from the infection with PA14 WT than Δ*rhlR* and Δ*rhlI*. Flies infected with Δ*rhlI* exhibited an intermediate survival phenotype. One representative experiment out of seven is shown. (B) Pooled LT50 of wild-type flies (w^{A5001}) survival test in intestinal infections with PA14 WT, Δ*rhlR* or Δ*rhlI*. LT50 of flies after infection with PA14 WT was significantly lower than with Δ*rhlR* (***p=0.0003) and ΔrhlI (*p=0.0385). Flies were significantly more susceptible to infection with Δ*rhlI* than with Δ*rhlR* (**p=0.0047). The LT50 data from seven survival experiments are displayed (biological duplicates are also shown as there was as much variability between experiments as within experiments), black bars indicates medians.

(C) Survival curves of wild-type and latex bead-injected flies after intestinal infection with PA14 bacteria. In latex bead-injected flies Δ*rhlI* exhibited a regained virulence. Note however that the shift in virulence is of the same magnitude as that observed for wt PA14 and contrasts with the large shift observed with Δ*rhlR*. (D) Pooled LT50 of latex bead-injected flies (w^{A5001}-LxB) survival experiments. w^{A5001}-LxB flies died significantly slower after Δ*rhlR* infection than PA14 WT (**p=0.0065). A slight decrease of virulence, but at the border of significance, was observed between PA14 WT and Δ*rhlI* (p=0.0726). No difference in virulence was detected between Δ*rhlR* and Δ*rhlI* (p=0.3056). Data represent the LT50s from five experiments (biological duplicates are also shown as there was as much variability between experiments as within experiments), black bars indicates medians. (E) Hill coefficient of latex bead-injected flies in PA14 infection. Hill coefficient gives an indication on the slope of the

survival curves. Survival curves of w^{A5001}-LxB flies infected with PA14 wild-type (PA14 WT) had a significant higher Hill coefficient than survival curves of flies infected with Δ*rhlR* (**p=0.0092) or Δ*rhlI* (*p=0.0405). No significant difference in Hill coefficient was detected between survival curves of flies infected with Δ*rhlR* or Δ*rhlI* (p=0.6243). The results from three experiments are shown; black bars indicate medians. For all, Mann Whitney tests were used for all statistical analyses.

Figure 2. Selected mutants present a phenotype similar to that of Δ*rhlR*.

LT50s measured from survival experiments of untreated wild-type (w^{A5001}) *Drosophila* or of flies in which phagocytosis had been blocked (w^{A5001}-LxB) after intestinal infection with PA14 wild-type (PA14), the clean deletion mutant of *rhlR* (Δ*rhlR*), or transposon insertions affecting candidate virulence genes. *vfR*, *xcpR*, *rho* and *sltB1*, but not *sltB1 (N)* [N: another transposon insertion near the *sltB1* locus] nor *gidA*, exhibited a significantly decreased virulence in w^{A5001} and regained virulence in w^{A5001}-LxB. The LT50s of at least two experiments are presented; ***p<0.001, **p<0.01, *p<0.05, unpaired t test.

Figure 3. Null deletion mutants of *vfR*, *sltB1*, and *xcpR* exhibit a Δ*rhlR*-like phenotype.

Newly generated deletion mutants of *vfR*, *sltB1* and *xcpR* were tested in intestinal infection of wild-type flies (w^{A5001}) and flies in which phagocytosis had been blocked by injection of latex beads (w^{A5001}-LxB). As previously described, w^{A5001} flies infected with Δ*rhlR* (red curves) exhibited a strong increase of survival compared to flies infected with PA14 wilt-type (PA14, blue curves) but Δ*rhlR* regained a nearly full virulence in w^{A5001}-LxB (A), (B), and (C). Similar phenotypes were observed with Δ*vfR* (A), Δ*xcpR* (B), and Δ*sltB1* (C). However, none of these mutants presented a virulence as strongly decreased as that displayed by Δ*rhlR*, in keeping with data obtained with the transposon insertion mutants. Among the three mutants, Δ*xcpR* exhibited the strongest decrease of virulence in w^{A5001} but also the weakest regain of

virulence in w^{A5001}-LxB. (D) Survival curves of uninfected (NI) control flies. Note the unusual susceptibility of uninfected flies to latex beads injections in that series of experiments. Results from one representative experiment out of three are presented.

Figure 4. The *Drosophila* cellular immune response is critical only at the very beginning of PA14 intestinal infections.

Measured LT50s from survival experiments of flies after intestinal infection with wild-type PA14 (A), Δ*rhlR* (B) or Δ*rhlI* (C) mutants and injection of either latex beads (LxB) or PBS at different time points of the infection. Latex beads or PBS were injected either one day before the infection started (-1d) or four hours (+4h), one day (+1d), two days (+2d), four days (+4d) or six days (+6d) after the infection started. Grey dots correspond to the survival of infected, uninjected flies. (A) LT50s of w^{A5001}-LxB are significantly lower than w^{A5001} only at -1d (**p=0.0086) and +4h (*p=0.0154). (B) LT50 of w^{A5001}-LxB are significantly lower than w^{A5001} nearly all along the infection (-1d: ***p=0.0002, +4h: ***p=0.0002 and +4d: **p=0.0069). (C) A similar phenotype is observed with flies infected with Δ*rhlI* (-1d: *p=0.0395, +4h: **p=0.0085, +1d: ***p=0.0002 and +4d: *p=0.0400). Note however that for injections of latex beads at day4 the difference is reduced, as compared to earlier time points of injection of latex-beads. The cumulative LT50 data from at least three experiments (only two experiments for Δ*rhlI*) are shown, except for +d2 and +d6; black bars indicates medians. Statistical analyses were done with an unpaired t-test.

Figure 5. PA14 RhlR is required to circumvent *Drosophila* Tep4 function in phagocytosis.

(A) and (B) survival assay with flies overexpressing in the fat body (driver C563 Gal4 $Gal80^{ts}$) (A) or in hemocytes (driver hml Gal4 $Gal80^{ts}$) (B) an RNAi construct against *tep1* (UAS-RNAi *Tep1*) or against GFP (UAS-RNAi *GFP*) as a control. No significant difference

was observed in the survival of *tep1* knock down flies in the fat body or hemocytes and control flies. The mean ±SD of one experiment with triplicates is shown. (C), (D) and (E) *Drosophila* wild-type flies (w^{A5001}), single mutants *tep3* and *tep4*, double mutant *tep2,3* and triple mutant *tep2,3,4* were orally infected with PA14 wild-type (C) or Δ*rhlR* mutant (D) in parallel experiments. (C) *tep4* and *tep2,3,4* mutant flies are significantly more susceptible to PA14 infection compared to w^{A5001}. No difference in survival was detected between *tep2,3* mutant and w^{A5001}. Surprisingly *tep3* mutants seemed to be more resistant to the infection. (D) A strong regain of Δ*rhlR* virulence is observed with *tep4* and *tep2,3,4* mutants compared to w^{A5001} flies. *tep2,3* and w^{A5001} presented nearly the same rate of death when challenged with Δ*rhlR* . *tep3* seemed again to be more resistant to the infection. (E) Survival of uninfected flies on a sucrose solution. Unexpectedly, *tep3* mutant flies seemed to survive much longer on a sucrose-only diet. In (C), (D) and (E) one representative experiment out of three (each with biological triplicates, except for uninfected flies) is shown.

Table S1. List of the 326 PA14 mutants from the first round of test.

All mutants were tested twice in an intestinal infection of wild-type *Drosophila* (w^{A5001}). Mutants selected for the second round of test are highlighted in grey. Selected mutants exhibited a stronger virulence phenotype (highlighted in grey and written in red), a weaker virulence phenotype (highlighted in grey and written in blue) or a conflicting phenotype (altered virulence in one experiment out of two) between the first and second survival test (highlighted in grey and written in black).

Figure S1. Results of the second round of virulence tests.

60 PA14 mutants were tested a third time in an intestinal infection with wild-type *Drosophila* (w^{A5001}). The mean ±SD of the difference of the LT50s of flies infected with the mutant *vs*. the

wild-type PA14 in three experiments (primary screen and retest) are shown. *rhlR* mutants presented the least virulent phenotype. In addition, 11 other mutant strains presented a decreased virulence compared to PA14 wild-type and 10 mutants strains exhibited an increased virulence phenotype.

Figure S2. The third round of test on other transposon insertion mutants in the candidate loci did not confirm most of the decreased virulence phenotypes.

Newly ordered mutants (other transposon insertions in the candidate loci) rarely exhibited the same decreased virulence phenotype than the original mutants, except for the two *rho* mutants, which presented a similar decreased virulence. One *xcpR* mutant out of three showed a decreased virulence. Only one *vfR* mutant was available. The means ±SD of three experiments are shown (some mutants were tested only once: *pnp* (31610) and *ddlB* (55626). ***p<0.001, **p<0.01, *p<0.05, unpaired t test.

Figure S3. Double mutants did not present an enhanced avirulence phenotype as compared to single mutants.

Survival assays of wild-type flies in intestinal infection with PA14 wild-type (PA14 WT), single mutants Δ*rhlR*, Δ*vfR* or Δ*xcpR*, or double mutants Δ*vfR*-Δ*xcpR* (A), Δ*xcpR*-Δ*sltB1* (B) or Δ*vfR*-Δ*sltB1* (C). (A) The double mutant Δ*vfR*-Δ*xcpR* presented a phenotype intermediate between those of Δ*vfR* and Δ*xcpR*. Flies infected with Δ*vfR*-Δ*xcpR* exhibited a survival curve significantly different from flies infected with PA14 WT (***p<0.0001), Δ*rhlR* (***p<0.0001), Δ*vfR* (***p<0.0001) and Δ*xcpR* (**p=0.0013). (B) Δ*xcpR*-Δ*sltB1* double mutant are more virulent than Δ*xcpR*. Survival curve of flies infected with Δ*xcpR*-Δ*sltB1* is significantly different from survival curves of flies infected with PA14 WT (***p<0.0001), Δ*rhlR* (***p<0.0001) and Δ*xcpR* (***p<0.0001). (C) The double mutant Δ*vfR*-Δ*sltB1*

presented a virulence phenotype similar to that of Δ*vfR*. Survival curve of flies infected with this double mutant was significantly different from survival curves of flies infected with PA14 WT (***p<0.0001) or Δ*rhlR* (***p<0.0001) but not with Δ*vfR* (p=0.2671). In (A), (B) and (C) results of one experiment with triplicates are presented. Statistical analyses were done using the LogRank test.

Figure S4. *Drosophila* Tep4 mediates only a part of the cellular immune response against PA14.

Survival assay of wild-type (w^{A5001}) and *tep4* mutants *Drosophila* after injection of latex beads (LxB) or no injection in an intestinal infection with Δ*rhlR* PA14 mutants (A). w^{A5001}-LxB, *tep4* and *tep4*-LxB presented a strong decrease of survival compared to wA5001 in infection with Δ*rhlR* (for all ***p<0.0001). A slight, yet significant, difference was observed in survival curves between w^{A5001}-LxB and *tep4* (**p=0.0018) or *tep4*-LxB (***p=0.0003) as well as between *tep4* and *tep4*-LxB (**p=0.0083). This later comparison suggests that all phagocytic function is not blocked in the *tep4* mutants since Δ*rhlR* bacteria display an even higher virulence when phagocytosis is further blocked by the injection of latex beads, which totally ablates phagocytic function. The means ±SD of one experiment with biological triplicates is presented. (B) Survival curves of uninfected control flies from (A); one control experiment was performed.

Figure S5. w^{A5001}-LxB flies infected with Δ*rhlR* or Δ*rhlI* exhibited similar curve shapes, different than PA14 wild-type.

Hill coefficient gives an indication of the slop of curves. Survival curves of w^{A5001}-LxB flies infected with PA14 wild-type (PA14 WT) had a significant higher Hill coefficient than survival curves of flies infected with Δ*rhlR* (**p=0.0092) or Δ*rhlI* (*p=0.0405). No

significant difference in Hill coefficient was detected between survival curves of flies infected with Δ*rhlR* or Δ*rhlI* (p=0.6243). Data represented results from three experiments, black bars indicate medians, statistical analyses were done with Mann Whitney test.

Figure 1

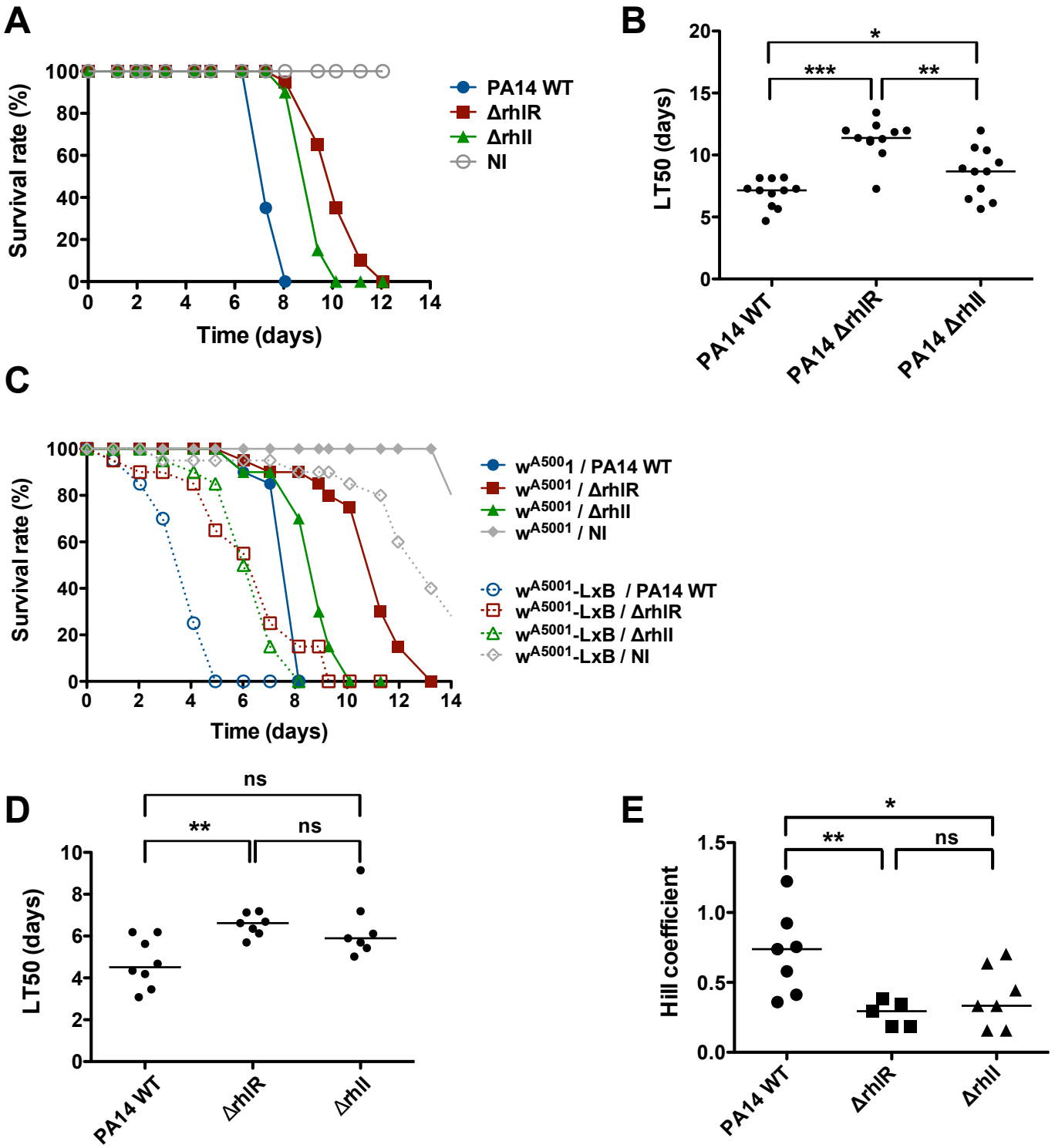

A
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔrhlI
NI
B
LT50 (days)
PA14 WT
PA14 ΔrhlR
PA14 ΔrhlI
C
Survival rate (%)
Time (days)
wA5001 / PA14 WT
wA5001 / ΔrhlR
wA5001 / ΔrhlI
wA5001 / NI
wA5001-LxB / PA14 WT
wA5001-LxB / ΔrhlR
wA5001-LxB / ΔrhlI
wA5001-LxB / NI
D
LT50 (days)
ns
E
Hill coefficient

Table S1

Mutant ID	Locus Name	Gene Name	Gene Description
23067	PA14_31260	NULL	RadC-like protein
23078	PA14_72540	NULL	ribonucleotide reductase
23182	PA14_38530	fahA	fumarylacetoacetase
23465	PA14_12030	NULL	conserved hypothetical protein
23553	PA14_57910	NULL	conserved hypothetical protein
23675	PA14_67440	NULL	putative chlorohydrolase
23709	PA14_23920	purF	amidophosphoribosyltransferase
23759	PA14_38140	NULL	putative glutamine synthetase
23737	PA14_72660	NULL	putative amidase
23757	PA14_14630	secD	protein-export membrane protein SecD
23782	PA14_23430	ORF_11	NULL
23790	PA14_09300	NULL	putative ATP-binding component of ABC transporter
23840	PA14_50070	NULL	conserved hypothetical protein
23863	PA14_36080	NULL	putative MFS transporter
23980	PA14_00550	NULL	putative membrane protein
24048	PA14_36000	PAGI-1(5)	Probable transcriptional regulator
24118	PA14_52690	aruG	arginine/ornithine succinyltransferase AII subunit
24202	PA14_21050	NULL	putative short-chain dehydrogenase
24633	PA14_60310	pilY1	type 4 fimbrial biogenesis protein PilY1
24601	PA14_59800	pvrS	kinase sensor protein
24619	PA14_60800	NULL	putative ABC transporter, ATP-binding protein
24637	PA14_02740	NULL	putative aldolase
24718	PA14_33030	sdaA	L-serine dehydratase
24724	PA14_65630	NULL	putative aspartyl protease
24904	PA14_27640	NULL	putative protein associated with synthesis and assembly of refractile inclusion bodies
24979	PA14_61150	NULL	putative oxidoreductase
24922	PA14_02750	NULL	putative transcriptional regulator
24932	PA14_43320	NULL	putative glutaminase
25054	PA14_10500	ccoN	cytochrome c oxidase subunit (cbb3-type)
25019	PA14_21110	plcN	non-hemolytic phospholipase C precursor
25035	PA14_68040	NULL	putative short-chain alcohol dehydrogenase
25062	PA14_69700	NULL	conserved hypothetical protein
25092	PA14_29720	NULL	putative lipoprotein
25168	PA14_22380	NULL	putative nuclease
25134	PA14_64270	NULL	putative branched-chain amino acid ABC transporter, periplasmic component
25153	PA14_31620	NULL	putative ABC-type periplasmic phosphate-binding protein
25351	PA14_41590	NULL	putative cytoplasmic membrane protein
25462	PA14_03210	NULL	hypothetical protein
25436	PA14_43070	hcpA	secreted protein Hcp
25529	PA14_59940	NULL	Conserved hypothetical protein
25593	PA14_24020	xcpT	general secretion pathway protein G
25530	PA14_55920	NULL	putative type II secretion system protein
25565	PA14_09730	NULL	putative dihydrodipicolinate synthase
25594	PA14_18960	NULL	hypothetical protein
25654	PA14_59310	pilR2	type IV B pilus Protein
25662	PA14_06830	norB	nitric-oxide reductase subunit B
25663	PA14_57100	NULL	putative permease
25708	PA14_42950	NULL	conserved hypothetical protein
25721	PA14_16930	NULL	putative cysteine sulfinate desulfinase
25847	PA14_25110	topA	DNA topoisomerase I
25928	PA14_41150	NULL	putative permease of ABC transporter
25882	PA14_29830	NULL	putative methyltransferase
25897	PA14_69250	NULL	putative membrane-associated protein
25935	PA14_32490	NULL	hypothetical protein
26123	PA14_08500	NULL	putative integral membrane protein
26245	PA14_54470	NULL	conserved hypothetical protein
26394	PA14_61980	NULL	conserved hypothetical protein
26553	PA14_38460	NULL	acyl-CoA carboxyltransferase beta chain

Table S1 (suite)

Mutant ID	Locus Name	Gene Name	Gene Description
26579	PA14_04890	NULL	putative zinc protease
26586	PA14_00070	NULL	putative histidinol-phosphatase
26627	PA14_38040	NULL	putative transcriptional regulator, AraC family
26664	PA14_67990	mutY	A/G-specific adenine glycosylase
26710	PA14_07170	epd	D-erythrose 4-phosphate dehydrogenase
26873	PA14_14850	pilF	type 4 fimbrial biogenesis protein PilF
27017	PA14_33690	pvdE	pyoverdine biosynthesis protein PvdE
27032	PA14_71120	NULL	putative thioesterase
27145	PA14_47350	NULL	conserved hypothetical protein
27355	PA14_49930	NULL	conserved hypothetical protein
27636	PA14_12080	sltB1	soluble lytic transglycosylase B
27757	PA14_17000	NULL	hypothetical protein
27872	PA14_43420	NULL	putative acyl-CoA dehydrogenase
27879	PA14_26910	NULL	putative MoxR protein
27934	PA14_21840	NULL	putative periplasmic binding protein
27887	PA14_30660	uvrC	excinuclease ABC subunit C
28000	PA14_36310	hcnC	NULL
28220	PA14_63710	NULL	putative glycosyl transferase
28440	PA14_13110	NULL	probable medium-chain acyl-CoA ligase
28606	PA14_19630	folE1	GTP cyclohydrolase I precursor
28622	PA14_16500	NULL	putative response regulator of the chemosensory-like operon
28646	PA14_07170	epd	D-erythrose 4-phosphate dehydrogenase
28742	PA14_07780	NULL	putative phosphotransferase
29076	PA14_58050	pmbA	PmbA protein
29310	PA14_51390	pqsD	3-oxoacyl-[acyl-carrier-protein] synthase III
29337	PA14_39730	NULL	conserved hypothetical protein
29412	NULL	NULL	NULL
29854	PA14_20960	NULL	putativ isomerase
29866	PA14_31290	pa1L	PA-I galactophilic lectin
29905	PA14_41140	NULL	putative ABC transporter, permease protein
29990	PA14_68610	hslO	putative chaperonin, 33 kDa
30058	PA14_49320	NULL	hypothetical
30196	PA14_14040	NULL	putative ATP-dependent RNA helicase RhlB
30412	PA14_57560	NULL	putative cytochrome b
30558	PA14_25880	etfA	electron transfer flavoprotein alpha-subunit
30496	PA14_20290	NULL	DNA binding-protein
30750	PA14_09820	NULL	putative acetolactate synthase large subunit
31022	PA14_08340	trpG	anthranilate synthase component II
31097	PA14_69000	pepP	aminopeptidase P
31109	PA14_50630	NULL	hypothetical protein
31176	PA14_65170	rpsR	30S ribosomal protein S18
31210	PA14_07790	NULL	putative nucleotidyltransferase
31424	PA14_52670	aruD	succinylglutamate 5-semialdehyde dehydrogenase
31480	PA14_59060	NULL	putative DNA binding protein
31610	PA14_62710	pnp	polyribonucleotide nucleotidyltransferase
31640	PA14_43350	kdpD	two-component sensor KdpD
31822	PA14_02790	pcaF	beta-ketoadipyl CoA thiolase PcaF
31824	PA14_46490	fabF2	3-oxoacyl-acyl carrier protein synthase II
31924	PA14_17880	NULL	acetyl-CoA acetyltransferase (thiolase)
32003	PA14_04410	ptsP	phosphoenolpyruvate-protein phosphotransferase
32321	PA14_41760	NULL	conserved hypothetical protein
32331	PA14_52660	aruB	succinylarginine dihydrolase
32400	PA14_73020	NULL	putative C4-type zinc finger protein, DksA/TraR family
32404	PA14_52050	purN	phosphoribosylaminoimidazole synthetase
32409	PA14_47140	NULL	putative TonB-dependent receptor
32430	PA14_52930	NULL	probable transcriptional regulator
32541	PA14_05560	NULL	putative ATP-dependent RNA helicase, DEAD box family
32561	PA14_66710	rpmE	50S ribosomal protein L31
32452	PA14_30100	NULL	conserved hypothetical protein
32578	PA14_27700	NULL	putative transcriptional regulator

Table S1 (suite)

Mutant ID	Locus Name	Gene Name	Gene Description
32602	PA14_67970	NULL	putative dehydrogenase
32801	PA14_52090	NULL	hypothetical protein
33019	PA14_25050	NULL	conserved hypothetical protein
33095	PA14_49170	phoQ	two-component sensor PhoQ
33176	PA14_08330	NULL	hypothetical protein
33387	PA14_01960	NULL	putative RND efflux membrane fusion protein precursor
33516	PA14_66120	NULL	conserved hypothetical protein
33583	PA14_42880	stk1	serine-threonine kinase Stk1
33597	PA14_35530	bkdA1	2-oxoisovalerate dehydrogenase (alpha subunit)
33623	PA14_03370	NULL	Hypothetical protein
33742	PA14_41610	NULL	conserved hypothetical protein
33817	PA14_69810	glnK	Nitrogen regulatory protein PII
34095	PA14_25690	fabF1	beta-ketoacyl-acyl carrier protein synthase II
34203	PA14_30210	NULL	putative cytoplasmic protease
34284	PA14_73370	gidA	glucose-inhibited division protein A
34708	PA14_11910	NULL	NULL
34781	PA14_30650	gacA	response regulator GacA
34827	PA14_31580	NULL	putative acyl-CoA dehydrogenase
35038	PA14_22770	NULL	conserved hypothetical protein
35005	PA14_69370	algP	alginate regulatory protein AlgP
35189	PA14_42520	NULL	conserved hypothetical protein
35639	PA14_72390	NULL	putative two-component sensor
35711	PA14_09320	NULL	putative ATP-binding component of ABC transporter
35774	PA14_04020	NULL	conserved hypothetical protein
35855	PA14_23460	wbpL	putative glycosyltransferase L
35993	PA14_64180	NULL	putative tRNA-dihydrouridine synthase
36008	PA14_12090	NULL	possible lipoprotein, rlpA family
36116	PA14_05960	NULL	putative major cold shock protein
36207	PA14_72450	dsbA	thiol:disulfide interchange protein DsbA
36226	PA14_07700	apaH	bis(5'-nucleosyl)-tetraphosphatase
36275	PA14_04410	ptsP	phosphoenolpyruvate-protein phosphotransferase
36532	PA14_51750	tolQ	TolQ protein
36598	PA14_37250	NULL	putative major facilitator family transporter
36736	PA14_43670	NULL	putative sensor/response regulator hybrid
36955	PA14_00560	exoT	exoenzyme T
37268	PA14_69010	NULL	conserved hypothetical protein
37382	PA14_69510	NULL	Hypothetical protein
37560	PA14_33610	NULL	NULL
37629	PA14_54640	NULL	probable enoyl-CoA hydratase/isomerase
37710	PA14_67560	typA	GTP-binding protein TypA/BipA
37818	PA14_69000	pepP	aminopeptidase P
37917	PA14_00120	NULL	putative 2-OH-lauroyltransferase
37913	PA14_23880	folC	folylpolyglutamate synthetase
37952	PA14_04430	NULL	conserved hypothetical protein
37943	PA14_19120	rhlR	acylhomoserine lactone dependent transcriptional regulator
38399	PA14_40010	NULL	hypothetical protein
38479	PA14_69670	lysA	diaminopimelate decarboxylase
38489	PA14_14730	iscS	cysteine desulfurase
38519	PA14_14680	NULL	inositol-1-monophosphatase
38595	PA14_05250	NULL	noncatalytic dihydroorotase-like protein noncataly
38726	PA14_73400	NULL	putative GTPase for tRNA modification and thiophene and furan oxidation
38830	PA14_27680	NULL	conserved hypothetical protein
38855	PA14_55810	NULL	putative two-component response regulator
38952	PA14_23830	fimV	pilus assembly protein
38983	PA14_38440	NULL	putative isovaleryl-CoA dehydrogenase
39053	PA14_05590	metF	5,10-methylenetetrahydrofolate reductase
39064	PA14_53940	prpB	carboxyphosphonoenolpyruvate phosphonomutase
39114	PA14_60460	rplU	ribosomal protein L21
39111	PA14_40670	metH	methionine synthase
39240	PA14_10940	NULL	putative transcriptional regulator, AraC family

Table S1 (suite)

Mutant ID	Locus Name	Gene Name	Gene Description
39292	PA14_45940	lasI	autoinducer synthesis protein LasI
39351	PA14_30230	clpA	ATP-dependent clp protease, ATP-binding subunit ClpA
39316	PA14_29980	nuoE	NADH dehydrogenase I chain E
39618	PA14_66290	aceA	pyruvate dehydrogenase, E1 component
39858	PA14_23420	ORF_10	NULL
40024	PA14_48420	NULL	putative transcriptional regulator
40221	PA14_11250	NULL	putative dTDP-4-rhamnose reductase-related protein
40356	PA14_68370	cysQ	3'(2'),5'-bisphosphate nucleotidase
40342	PA14_33650	pvdD	pyoverdine synthetase D
40396	PA14_52610	NULL	possible threonine aldolase
40436	PA14_52580	lysC	aspartate kinase alpha and beta chain
40460	PA14_41130	NULL	putative binding protein component of ABC transporter
40582	PA14_25630	rpmF	50S ribosomal protein L32
40902	PA14_65420	NULL	putative GTPase
40949	PA14_58560	NULL	Sulfite reductase
40996	PA14_43090	NULL	hypothetical protein
40982	PA14_65280	hflK	protease subunit HflK
41035	PA14_53950	prpC	citrate synthase 2
41424	PA14_22570	csaA	putative chaperone protein
41576	PA14_06510	bioF	8-amino-7-oxononanoate synthase
41602	PA14_24100	xcpZ	general secretion pathway protein M
41770	PA14_11110	cupB6	fimbrial protein cupB6
42090	PA14_06400	NULL	putative transcriptional regulator, LysR family
42176	PA14_60290	pilW	type 4 fimbrial biogenesis protein PilW
42207	PA14_05560	NULL	putative ATP-dependent RNA helicase, DEAD box family
42318	PA14_48190	NULL	putative transcriptional regulator
42489	PA14_17930	glpD	glycerol-3-phosphate dehydrogenase
42600	PA14_68730	gshA	glutamate--cysteine ligase
42695	PA14_62660	NULL	conserved hypothetical protein
42799	PA14_05310	gshB	glutathione synthetase"
42856	PA14_38530	fahA	fumarylacetoacetase
42933	PA14_66310	aceF	dihydrolipoamide acetyltransferase
43121	PA14_15360	NULL	NULL
43327	PA14_24440	NULL	putative lipoprotein
43615	PA14_09520	mexI	probable RND efflux transporter
43946	PA14_62740	rbfA	ribosome-binding factor A
44489	PA14_44120	NULL	putative 3-hydroxyisobutyrate dehydrogenase
44818	PA14_27950	NULL	putative anti-anti-sigma factor
45024	PA14_25390	sth	soluble pyridine nucleotide transhydrogenase
45143	PA14_29940	nuoG	NADH dehydrogenase I chain G
45119	PA14_00940	NULL	conserved hypothetical protein
45236	PA14_24940	NULL	putative oxidase
45399	PA14_73320	atpI	ATP synthase protein I
45410	PA14_62560	pcnB	poly(A) polymerase
45610	PA14_14470	pepA	leucine aminopeptidase
45918	PA14_64170	NULL	conserved hypothetical protein
45885	PA14_38480	NULL	putative acyl-CoA carboxylase alpha chain
45993	PA14_37490	NULL	putative TonB-dependent receptor
46221	PA14_43950	sucC	succinyl-CoA synthetase beta subunit
46283	PA14_43940	sucD	succinyl-CoA synthetase alpha chain
46250	PA14_69190	rho	transcription termination factor Rho
46254	PA14_69270	NULL	putative thioesterase
46407	PA14_45760	fliQ	flagellar biosynthetic protein FliQ
46422	PA14_62970	dnaK	putative heat shock protein
46521	PA14_04650	pfpI	protease PfpI
46670	PA14_67720	secB	secretion protein SecB
46697	PA14_65320	miaA	delta 2-isopentenylpyrophosphate transferase
46982	PA14_17900	metR	transcriptional regulator MetR
46987	PA14_00230	NULL	putative Rossmann fold nucleotide-binding protein
47013	PA14_52260	NULL	sensor/response regulator hybrid

Table S1 (suite)

Mutant ID	Locus Name	Gene Name	Gene Description
47128	PA14_19170	NULL	putative lipoprotein
47143	PA14_09100	rpsD	30S ribosomal protein S4
47923	PA14_48590	NULL	conserved hypothetical protein
47948	PA14_46840	NULL	conserved hypothetical protein
48244	PA14_51780	ruvB	Holliday junction DNA helicase RuvB
48417	PA14_34770	NULL	putative ABC transporter, periplasmic binding protein
48562	PA14_06010	NULL	conserved hypothetical protein
48544	PA14_32860	NULL	hypothetical protein
48644	PA14_67200	NULL	conserved hypothetical protein
52637	PA14_56790	NULL	putative GGDEF domain/EAL domain protein
52640	PA14_38350	galU	UTP-glucose-1-phosphate uridylyltransferase
52692	PA14_08370	vfr	cyclic AMP receptor-like protein
52787	PA14_30580	NULL	putative transcriptional regulator, LuxR family
52857	PA14_67670	ntrB	two-component sensor NtrB
52889	PA14_22020	minD	cell division inhibitor MinD
53271	PA14_62350	NULL	putative haem/haemoglobin uptake outer membrane receptor PhuR precursor
53495	PA14_08680	tufB	elongation factor Tu
53607	PA14_05190	pilU	twitching motility protein PilU
35993	PA14_64180	NULL	putative tRNA-dihydrouridine synthase
53876	PA14_41710	NULL	putative membrain protein
54153	PA14_66980	tatC	sec-independent protein translocase TatC
54161	PA14_33630	NULL	NULL
54131	PA14_18650	NULL	glutaredoxin-related protein
54272	PA14_57820	NULL	conserved hypothetical protein
54233	PA14_20730	flgM	putative negative regulator of flagellin synthesis, FlgM
54251	PA14_28490	NULL	putative membrane protein
54303	PA14_31820	NULL	putative aminotransferase
54379	PA14_20730	flgM	putative negative regulator of flagellin synthesis, FlgM
54445	PA14_40030	NULL	putative enzyme
54565	PA14_16890	NULL	putative auxiliary component of ABC transporter
54625	PA14_16270	NULL	conserved hypothetical protein
55086	PA14_63210	NULL	putative two-component response regulator
55256	PA14_62830	tpiA	triosephosphate isomerase
55834	PA14_62770	nusA	N utilization substance protein A
55443	PA14_07650	NULL	putative sporulation protein
55583	PA14_17170	NULL	putative outer membrane protein OmpH,
55833	PA14_41730	NULL	conserved hypothetical protein
55626	PA14_57320	ddlB	D-alanine--D-alanine ligase
56518	PA14_27230	NULL	putative transcriptional regulator, MarR family
56461	PA14_01100	NULL	putative ClpA/B-type chaperone
56391	PA14_08540	NULL	putative metallopeptidase
57056	PA14_48840	NULL	putative dimethylarginine dimethylaminohydrolase
56786	PA14_61500	PA4648	NULL
56790	PA14_52720	aruC	N-succinylglutamate 5-semialdehyde dehydrogenase
23102	PA14_41570	oprF	major porin and structural outer membrane porin OprF precursor
25568	PA14_61020	NULL	ankyrin-like protein
25699	PA14_67970	NULL	putative dehydrogenase
29200	PA14_41670	ppsA	phosphoenolpyruvate synthase
33692	PA14_64180	NULL	putative tRNA-dihydrouridine synthase
36369	PA14_65410	orn	oligoribonuclease
33880	PA14_41390	ppiB	peptidyl-prolyl cis-trans isomerase B
34523	PA14_27770	NULL	putative ABC transporter, ATP-binding protein
34611	PA14_29990	nuoD	NADH dehydrogenase I chain C,D
35658	PA14_12080	sltB1	soluble lytic transglycosylase B
35818	PA14_59780	rcsC	kinase sensor protein
29433	PA14_15770	NULL	conserved hypothetical protein
30251	PA14_69900	NULL	putative signal transduction protein
33890	PA14_52800	acsA	acetyl-coenzyme A synthetase
34793	PA14_62260	ppkA	cAMP-dependent protein kinase
42118	PA14_57190	NULL	putative pyrophosphohydrolase

Table S1 (suite)

Mutant ID	Locus Name	Gene Name	Gene Description
42741	PA14_52260	NULL	sensor/response regulator hybrid
41549	PA14_54390	mucD	serine protease MucD precursor
46240	PA14_61680	NULL	putative methyl transferase
54630	PA14_30650	gacA	response regulator GacA
48094	PA14_23990	xcpR	general secretion pathway protein E
53448	PA14_41020	NULL	putative Orn/Arg/Lys decarboxylase
47467	PA14_62740	rbfA	ribosome-binding factor A
57077	PA14_08490		conserved hypothetical protein
15779	PA14_50250		hypothetical protein
22339	PA14_25830		conserved hypothetical protein
22424	PA14_43950	sucC	
22525	PA14_06950		putative transcriptional regulator, LuxR family
22683	PA14_11900		hypothetical protein
22930	PA14_41710		putative membrain protein
5205	PA14_33650		pyoverdine synthetase D
5068	PA14_60990	radA	
15371	PA14_36170		putative integral membrane protein
22523	PA14_58850		conserved hypothetical protein
5691	PA14_29710		conserved hypothetical protein
6077	PA14_34010		conserved hypothetical protein
6114	PA14_50980		probable penicillin amidase
6310	PA14_47930		conserved hypothetical protein
6442	PA14_68670		putative carboxypeptidase
6472	PA14_43900		conserved hypothetical protein
6476	PA14_19670		putative transcriptional regulator, LysR family
6762	PA14_14380		putative transmembrane component of ABC transporte
6876	PA14_21990		putative aspartyl aminopeptidase
22339	PA14_25830		conserved hypothetical protein

Figure S1

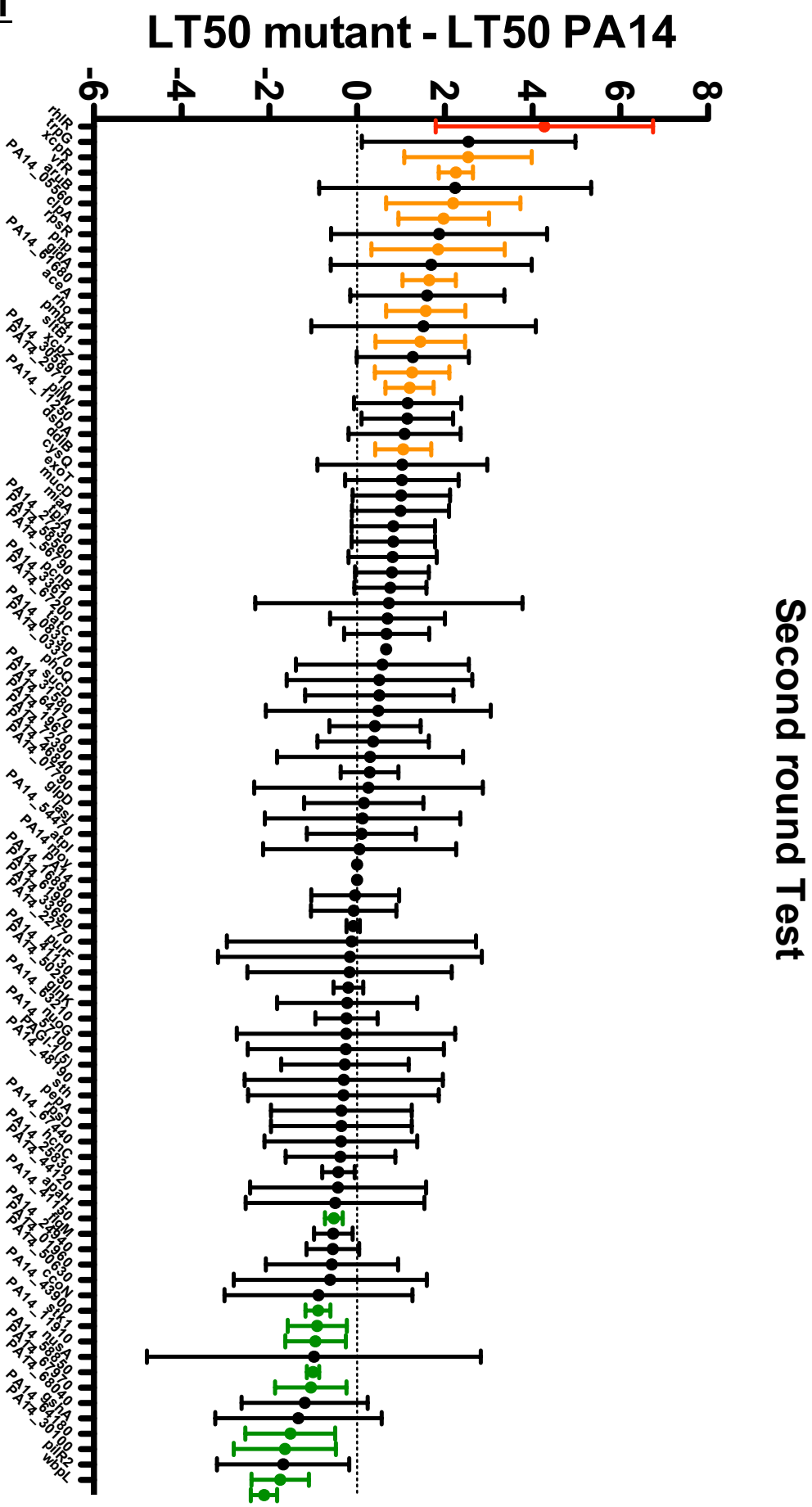


Figure S2

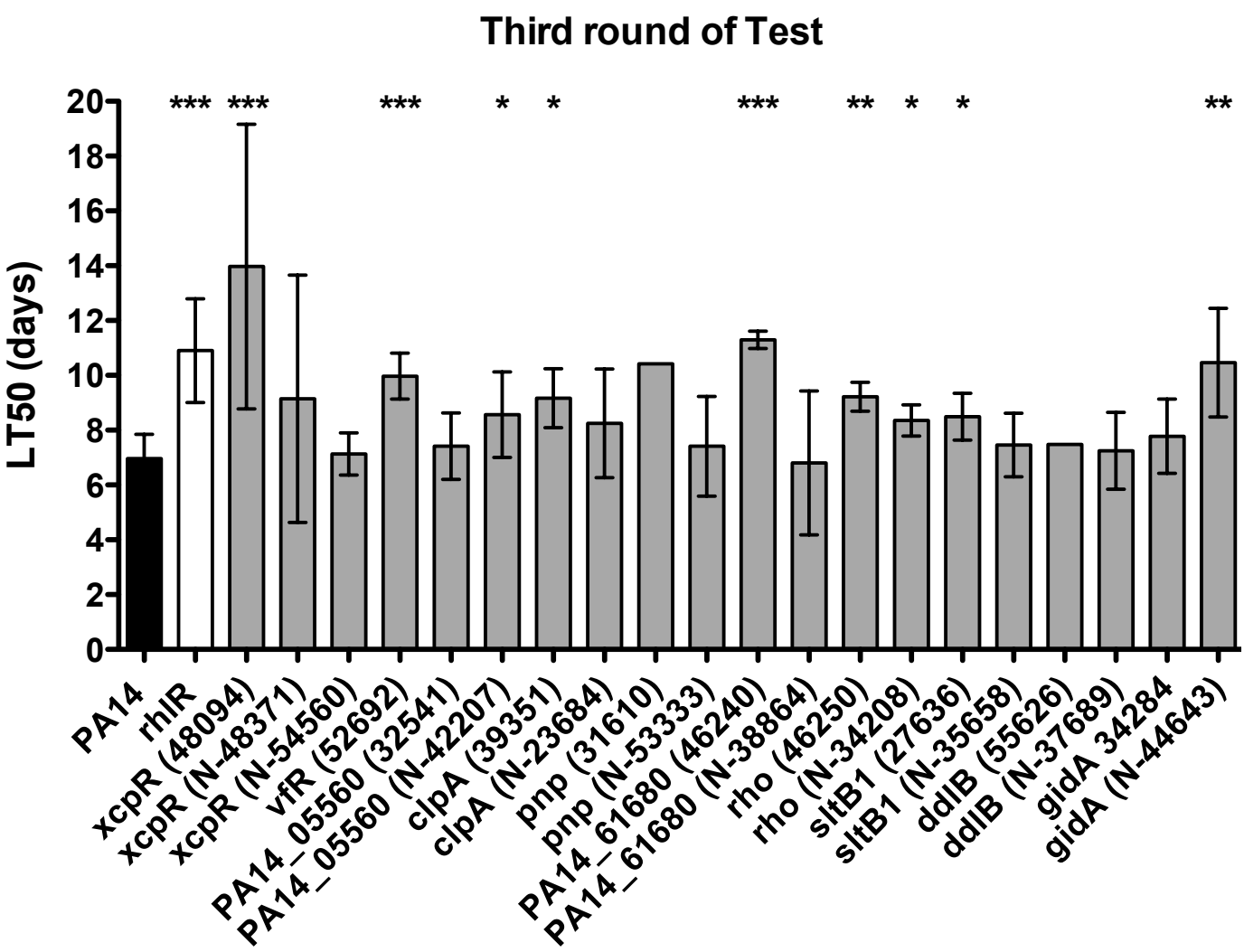

Third round of Test
LT50 (days)
0
2
4
6
8
10
12
14
16
18
20
*** *** *** * * *** ** * * **
PA14
rhlR
xcpR (48094)
xcpR (N-48371)
xcpR (N-54560)
vfR (52692)
PA14_05560 (32541)
PA14_05560 (N-42207)
clpA (39351)
clpA (N-23684)
pnp (31610)
pnp (N-53333)
PA14_61680 (46240)
PA14_61680 (N-38864)
rho (46250)
rho (N-34208)
sltB1 (27636)
sltB1 (N-35658)
ddlB (55626)
ddlB (N-37689)
gidA 34284
gidA (N-44643)

Figure 2

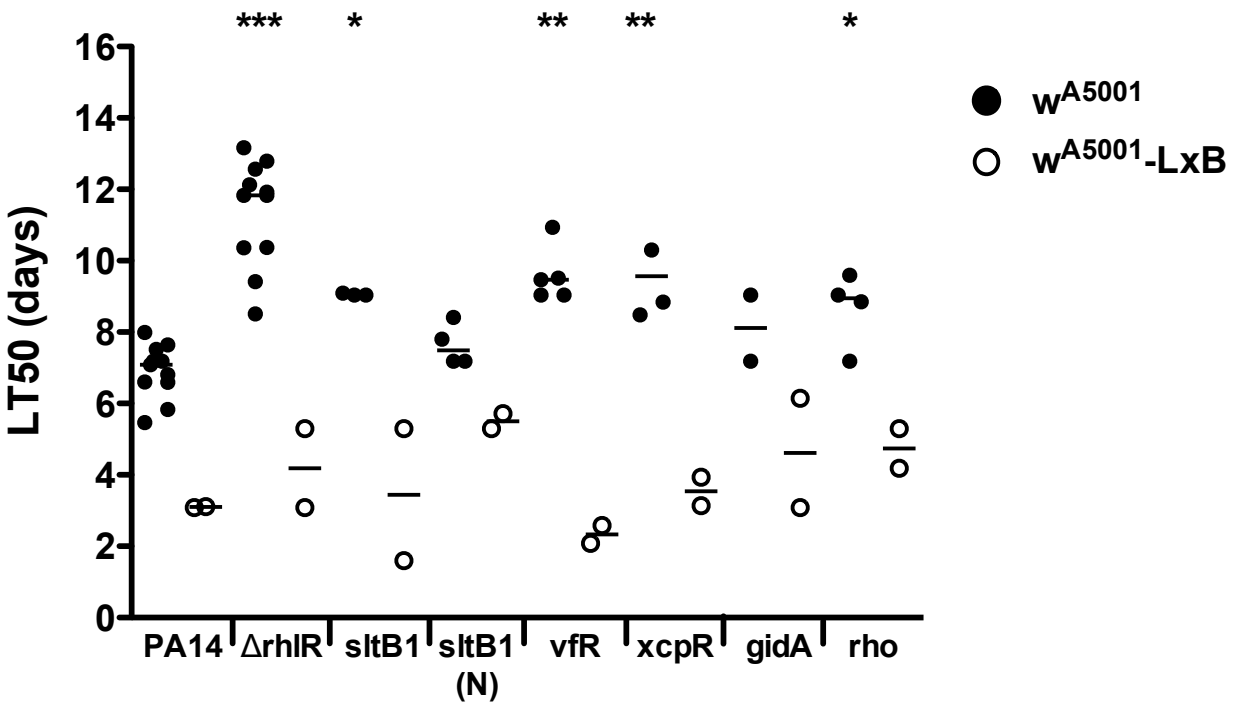

***
*
**
**
*
16
14
12
10
8
6
4
2
0
LT50 (days)
PA14
ΔrhlR
sltB1
sltB1 (N)
vfR
xcpR
gidA
rho
wA5001
wA5001-LxB

Figure 3

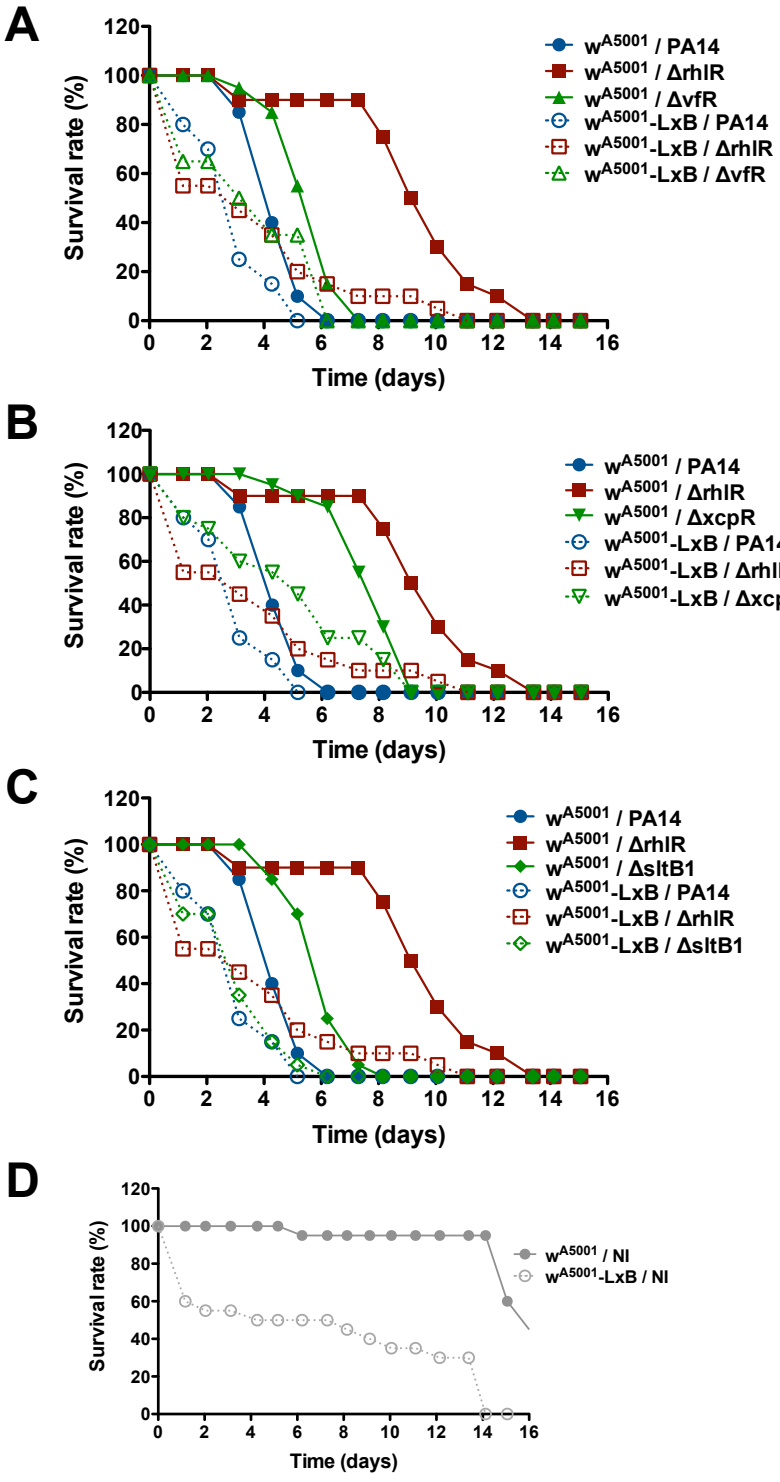
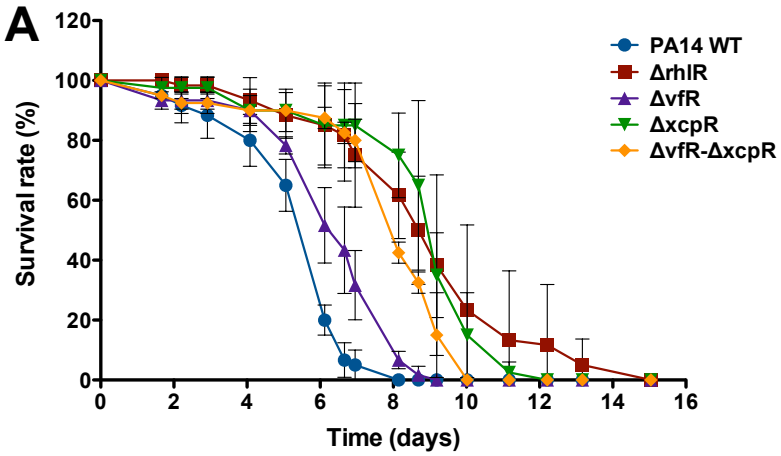
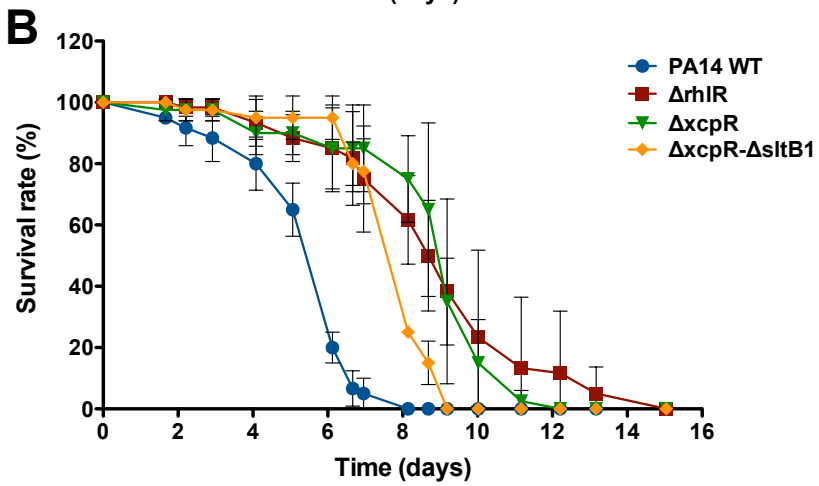

A
Survival rate (%)
Time (days)
wA5001 / PA14
wA5001 / ΔrhlR
wA5001 / ΔvfR
wA5001-LxB / PA14
wA5001-LxB / ΔrhlR
wA5001-LxB / ΔvfR
B
Survival rate (%)
Time (days)
wA5001 / PA14
wA5001 / ΔrhlR
wA5001 / ΔxcpR
wA5001-LxB / PA14
wA5001-LxB / ΔrhlR
wA5001-LxB / ΔxcpR
C
Survival rate (%)
Time (days)
wA5001 / PA14
wA5001 / ΔrhlR
wA5001 / ΔsltB1
wA5001-LxB / PA14
wA5001-LxB / ΔrhlR
wA5001-LxB / ΔsltB1
D
Survival rate (%)
Time (days)
wA5001 / NI
wA5001-LxB / NI

Figure S3


A
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔvfR
ΔxcpR
ΔvfR-ΔxcpR


B
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔxcpR
ΔxcpR-ΔsltB1

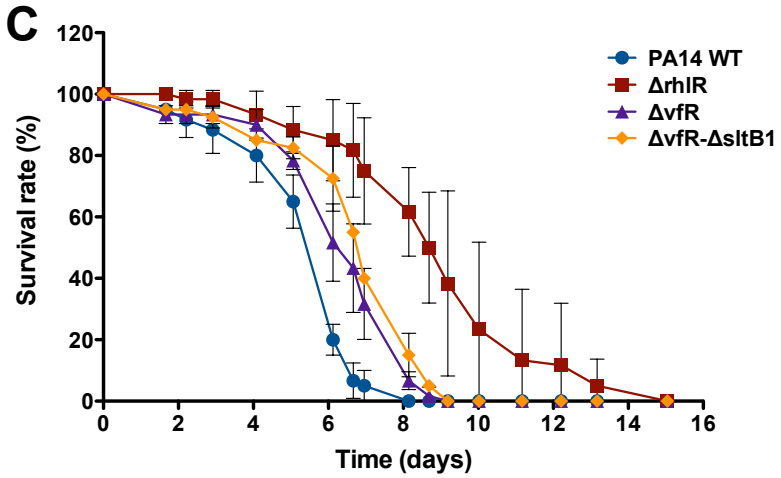

C
Survival rate (%)
Time (days)
PA14 WT
ΔrhlR
ΔvfR
ΔvfR-ΔsltB1

Figure 4

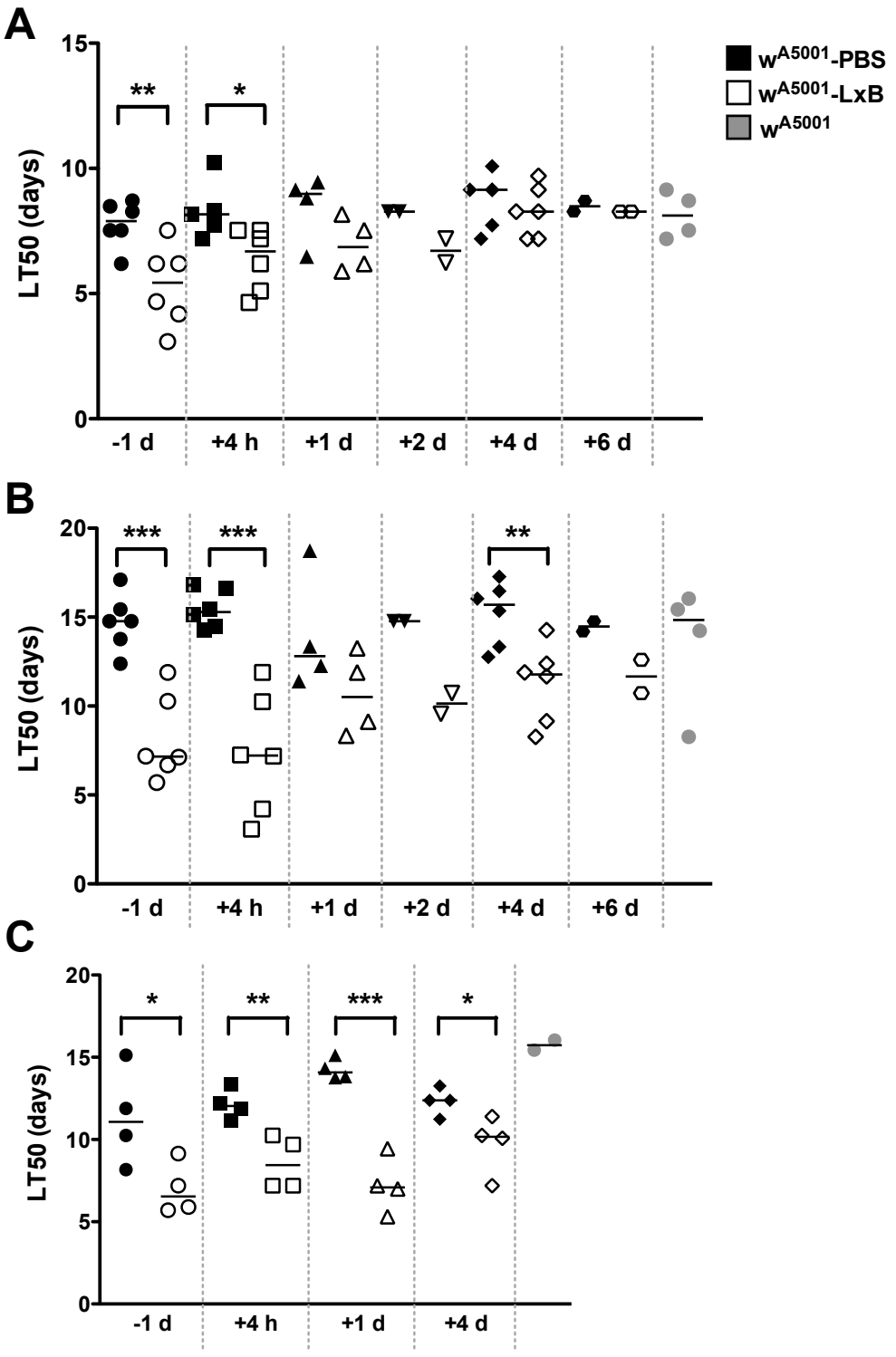


Figure 5

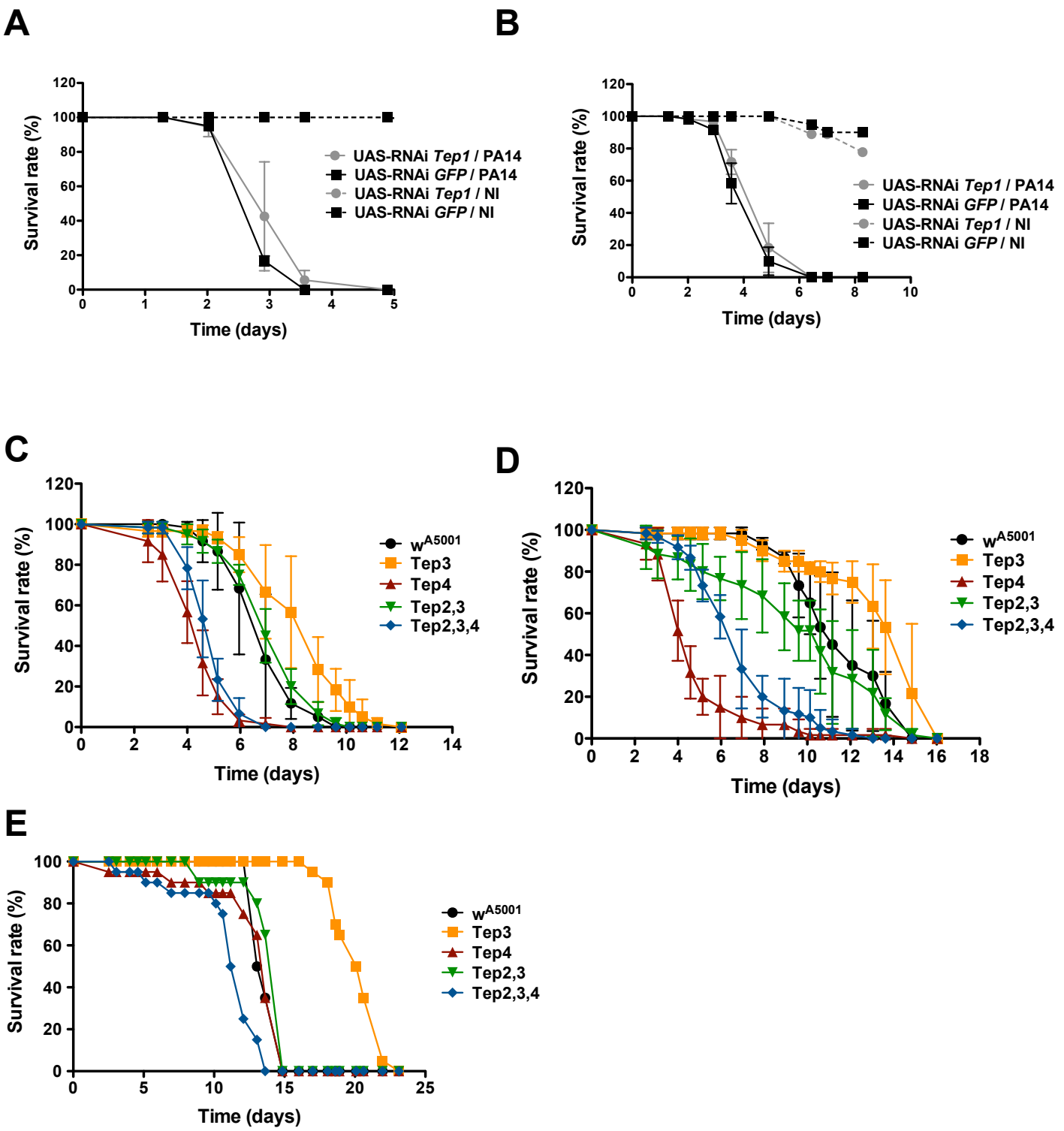
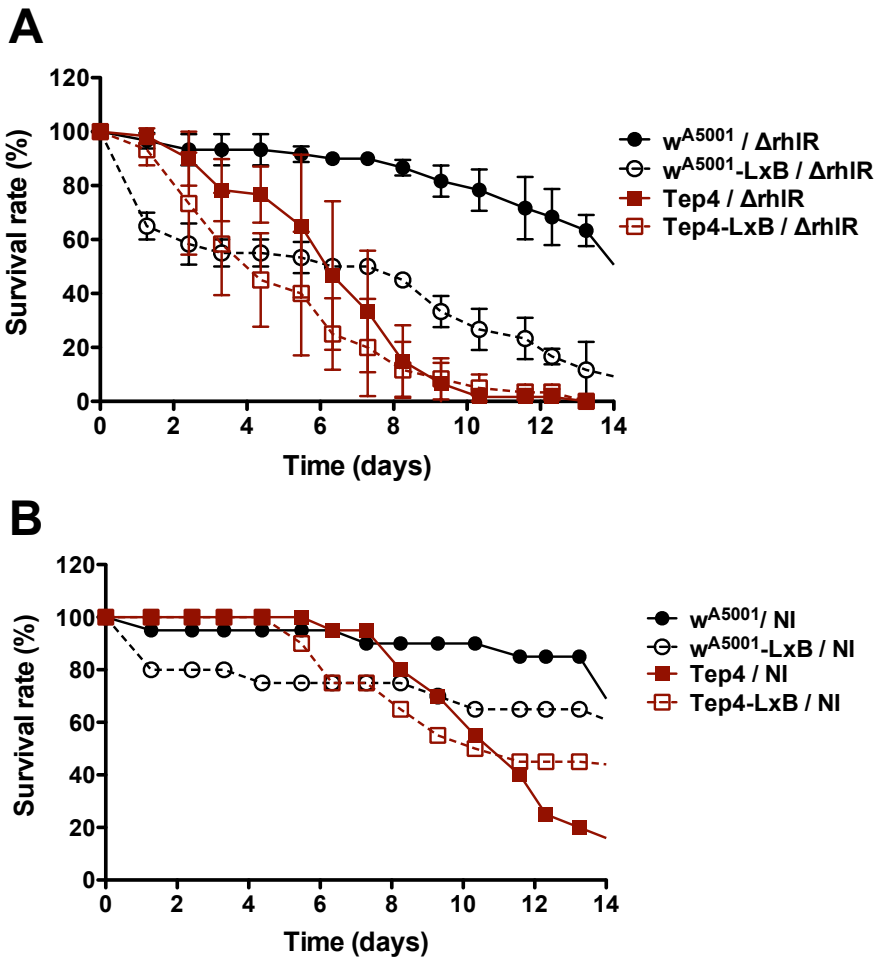

A
Survival rate (%)
Time (days)
UAS-RNAi Tep1 / PA14
UAS-RNAi GFP / PA14
UAS-RNAi Tep1 / NI
UAS-RNAi GFP / NI
B
Survival rate (%)
Time (days)
UAS-RNAi Tep1 / PA14
UAS-RNAi GFP / PA14
UAS-RNAi Tep1 / NI
UAS-RNAi GFP / NI
C
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4
D
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4
E
Survival rate (%)
Time (days)
wA5001
Tep3
Tep4
Tep2,3
Tep2,3,4

Figure S4


A
Survival rate (%)
Time (days)
wA5001 / ΔrhlR
wA5001-LxB / ΔrhlR
Tep4 / ΔrhlR
Tep4-LxB / ΔrhlR
B
Survival rate (%)
Time (days)
wA5001/ NI
wA5001-LxB / NI
Tep4 / NI
Tep4-LxB / NI

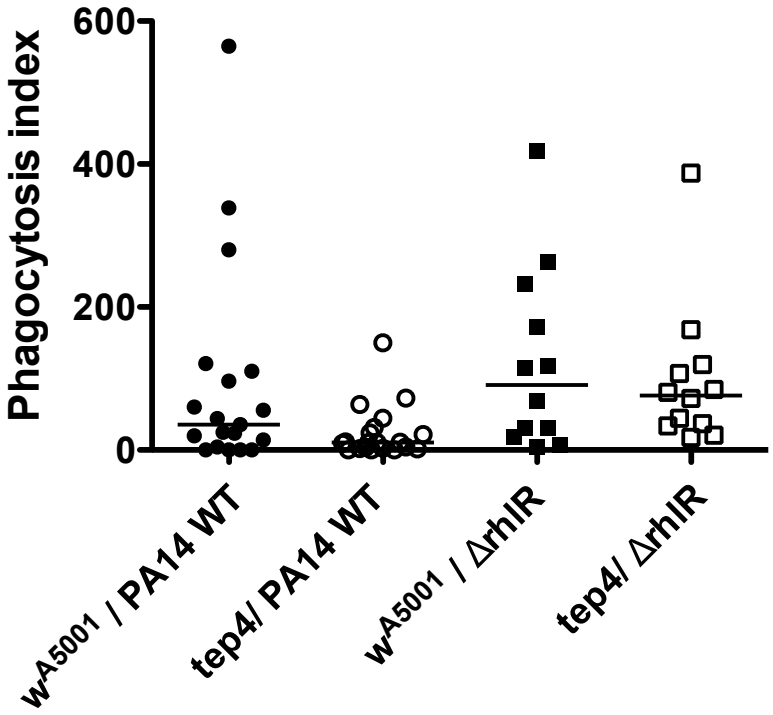


Figure C1. Phagocytosis index of PA14 WT and Δ*rhlR* mutant in *w*^{A5001} and *tep4* *Drosophila* larvae.

Even though there was not significant differences between these conditions, *w*^{A5001} larvae seemed to better phagocytose Δ*rhlR* mutants bacteria than PA14 WT. The *w*^{A5001} larval hemocytes also seemed to phagocytose PA14 WT bacteria better than *tep4* mutant hemocytes. The results of five experiments are presented. Each dot correspond to the phagocytosis index of one larva; black bars indicated medians.

Complementary results and Discussion

Measurement of phagocytic uptake of wild-type PA14 and Δ*rhlR* mutant *by Drosophila* larvae hemocytes

The cellular immune response of *Drosophila* plays an essential role in the defense against PA14 intestinal infection. We have demonstrated that PA14 RhlR, a component of the quorum sensing of the bacteria, is required in PA14 to circumvent the cellular immune response of the fly. In intestinal infection of *Drosophila*, Δ*rhlR* mutant bacteria presented a strongly decreased virulence as compared to PA14. However, this mutant regained virulence in infection with *Drosophila* for which phagocytosis had been blocked by the injection of latex beads. A similar phenotype was observed in the enteric infection of *tep4* mutant flies by Δ*rhlR*. Therefore Tep4 appears to be involved in *Drosophila* phagocytosis of PA14. Our hypothesis is that PA14 bacteria are better able to elude phagocytosis than Δ*rhlR*, which thus predicts that Δ*rhlR* bacteria should be better recognized and phagocytosed than PA14 wild-type bacteria.

To assess if there is any difference in the ability of Δ*rhlR* to be phagocytosed by wild-type *Drosophila* compared to PA14 wild-type (PA14 WT), we analyzed the phagocytosis indices (number of pH-RODO-positive signals /number of hemocytes per field of view) of these bacteria by larval hemocytes after injection into larvae of either PA14 WT or Δ*rhlR* killed pH-RODO-labeled bacteria. In contrast to adults, it is easy to collect a large number of infected hemocytes by bleeding larvae onto a slide. The pH-RODO dye has the particularity to become fluorescent only when present in an acidic environment, which is found in phagolysosomes. Then 30 minutes after injection, larvae were bled on a slide and the number of pH-RODO signals and hemocytes was counted using a fluorescent microscope. A slight increase of the phagocytosis index was observed in wild-type flies infected by Δ*rhlR* mutant bacteria compared to wild-type flies infected by PA14 wild-type (Fig. C1). Similarly, *tep4* mutant larvae tended to phagocytose PA14 WT less well than wild-type flies (w^{A5001}). However, no significant difference was detected between all these conditions because of a high variability between larvae.

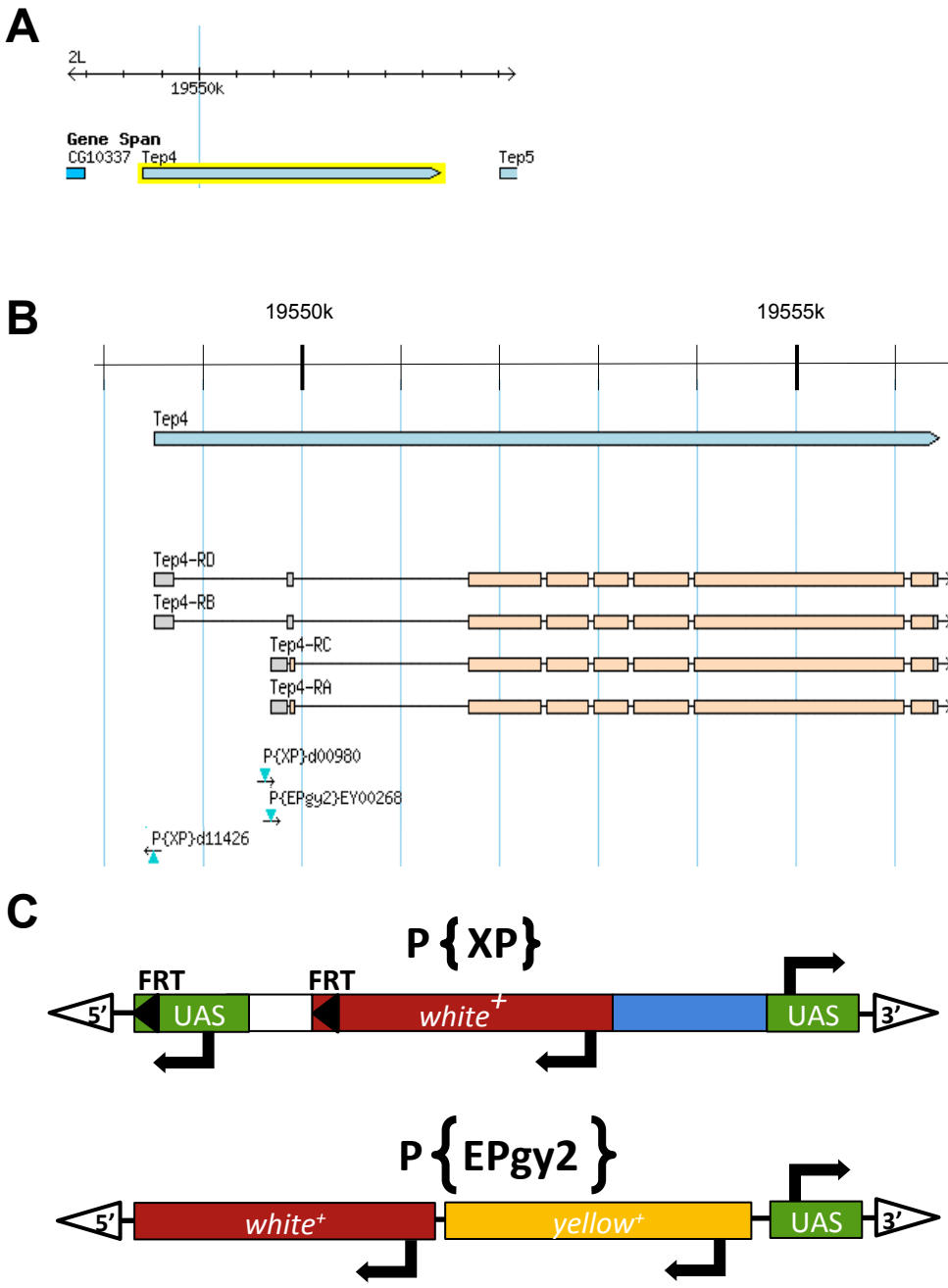


Figure C2. Tep4 transcript and transposons structure.
tep4 is located on the left arm of the second chromosome of *Drosophila* genome (A). (B) *tep4* has four predicted transcript: two short forms Tep4-RA and Tep4-RC and two long forms Tep4-RB and Tep4-RD. The functions and expression patterns of these different forms are not known. Transposons represented here were used to try to overexpress *tep4* (B). All these transposons possess a UAS sequence that might be used to overexpress either only the short form (d00980 and EY00268) or the long form (d11416) of *Tep4*. (C) Schemes of XP and *EPgy2* transposon used in this study. They both have a *miniwhite* sequence allowing to identify transgenic Drosophila by their eye color. Of note, the XP transposon possess two USA sequence, one in each direction.
(A) and (B) were adapted from flybase (http://flybase.org/reports/FBgn0041180.html). (C) was adapted from Bellen at al. 2004.

As there was too much variability, these experiments need to be optimized in another paradigm. It has been shown that pre-exposure to AMPs is crucial for recognition and phagocytosis of PA14 in a *Drosophila* cell culture model (Chung and Kocks, 2011). These labeled bacteria induce the expression of AMPs through the activation of IMD and Toll pathways. However the level of activation of these pathways may vary depending on the injection, larvae, and timing between injection and bleeding, thus potentially impacting the recognition and subsequent phagocytosis of these bacteria. To decrease this variability, one possibility could be to pre-treat the labeled bacteria with synthetic CecropinA and inject these bacteria in *key* mutant larvae, which are unable to mount an immune response. In the longer term, it would be more appropriate to measure a phagocytic index in our infection model in adults, since the bacteria would have to pass through the gut and possibly the intestinal epithelium, which is likely to alter the cell wall of the bacteria. However, this is challenging given the few bacteria that are able to cross the intestinal barrier. A *P. aeruginosa* PA14-specific antibody I raised might be useful for that purpose.

Overexpression of *tep4* did not protect flies against PA14 infection

The *tep4* gene is located on the left arm of the second chromosome, between *tep5* and CG10337 (Fig. C2A). Four transcript are predicted for the *tep4* locus: two short transcripts that are well-supported by cDNAs, *tep4*-RA and *tep4*-RC, and two long transcripts with less evidence of existence, *tep4*-RB and *tep4*-RD (Fig. C2B). These four transcripts differ from each other only at the initiation site, the middle and the end being identical.

Specific *Drosophila* lines carrying a transposon inserted at the *tep4* locus are available. Some of them contain in their sequence a UAS "promoter" sequence that can be used to induce the overexpression of genes located downstream to the UAS sequence. We chose three fly lines with an insertion of transposon upstream of *tep4*: P(XP)d11426 located upstream of all predicted *tep4* transcript, P(XP)d00980 and P(EPgy2)EY00268 both inserted in the first exons of *tep4*-RA and -RC and would thus induce an overexpression of the short transcripts only (Fig. C2B). The XP transposon possesses two UAS sequences at both extremities of the transposon, thus promoting expression from both sides of the insertion and EPgy2 only one UAS sequence (Fig.C2C).

To overexpress *tep4*, I crossed these three fly lines with two driver fly lines: p-*hml Gal4 Gal80ts* expressed in most hemocytes and p-*C564 Gal4 Gal80ts* expressed nearly in the

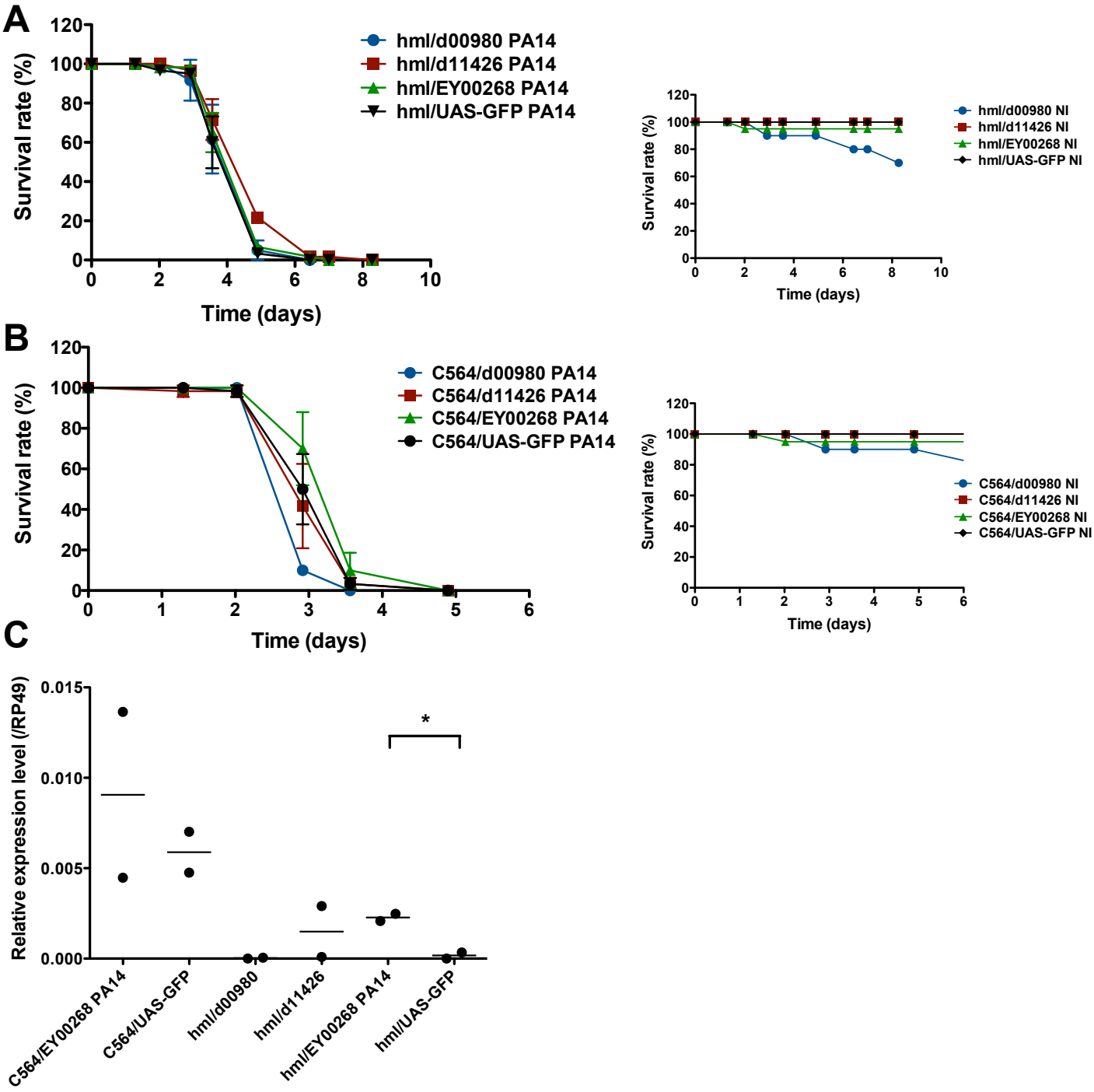


Figure C3. Trying to overexpress *Drosophila tep4* did not enhance the protection against a PA14 infection.

Fly lines carrying transposons with *UAS* enhancer sequences inserted upstream of the *tep4* sequence were crossed with either C564 *Gal4 Gal80^{ts}* driver (strong expression in the fat body) or hml *Gal4 Gal80^{ts}* driver (expressed in most hemocytes). (A) Flies overexpressing *tep4* in hemocytes were not more resistant to a PA14 intestinal infection than control flies overexpressing *GFP* in the fat body. (B) No significant difference in survival rate was observed between flies overexpressing *tep4* in the fat body or the control flies overexpression *GFP*. Right panels in (A) and (B) represent survival curves of the uninfected control flies fed on a sucrose only diet. In (A) and (B) the means ±SD of one experiment is shown. (C) RT-qPCR analysis of *tep4* expression in the flies expected to overexpress *tep4*. Only the EY00268 construct crossed with *hml Gal4 Gal80^{ts}* driver seemed to slightly, yet significantly, overexpress *tep4* (*p=0.0147, unpaired t test). Dots represent the results of one experiment with biological duplicates; black bars indicate medians.

whole fly and strongly expressed in the fat body. These crosses were performed at 18°C to prevent the overexpression during development (the Gal80 repressor is functional and inhibits the Gal4 transcription factor) and F1 progeny was transferred to 29°C for six days before intestinal infection with PA14.

No difference was observed in the survival of flies potentially overexpressing *tep4* in hemocytes and the control flies overexpressing GFP (Fig. C3A). Similarly, flies potentially overexpressing *tep4* in the fat body did not display a significant increase of survival rate compared to control flies overexpressing GFP (Fig. C3B).

We analyzed *tep4* expression in F1 progeny adults by RT-qPCR to confirm the efficiency of *tep4* overexpression. Flies possessing the p-*C564 Gal4 Gal80ts* driver presented a strong variability and no significant difference in *tep4* expression compared to control flies was detected (Fig. C3C). Only C564/EY00268 could be assessed, as crosses with this driver were not prolific enough. Among the progeny of flies crossed with the p-*hml Gal4 Gal80ts* driver only EY00268 allowed to overexpress somewhat *tep4*, presumably in hemocytes, hence the modest but nevertheless significant five-fold induction.

To draw a clear-cut conclusion, the strategy should be better validated by inducing *tep4* overexpression with a strong ubiquitous driver. However, if Tep4 is already present in saturating amount in the wild-type, in as much as very few bacteria are expected to cross at any time point, this strategy may be fruitless and hence has not been pursued.

Which step of phagocytosis is important upon PA14 infection?

One possibility is that bacteria are phagocytosed immediately upon leaving the intestine. Indeed, a population of hemocytes that migrates to the proventriculus has been described in larvae. We have attempted without success to visualize ingested PA14 in hemocytes next to the intestine. Furthermore, while we observed sometimes p-*hml-GFP* labeled cells in the proventriculus area as previously reported, this was not systematically the case. The migration of hemocytes to the proventriculus in larvae is somewhat inhibited by constitutive signaling by the phosphoinositol-3 kinase in hemocytes (Zaidman-Rémy et al., 2012). We tested a transgenic line expressing an activated form of the kinase in our infection model with either PA14 or Δ*rhlR* and did not detect any phenotypic differences with our wild-type controls (Fig. C4A).

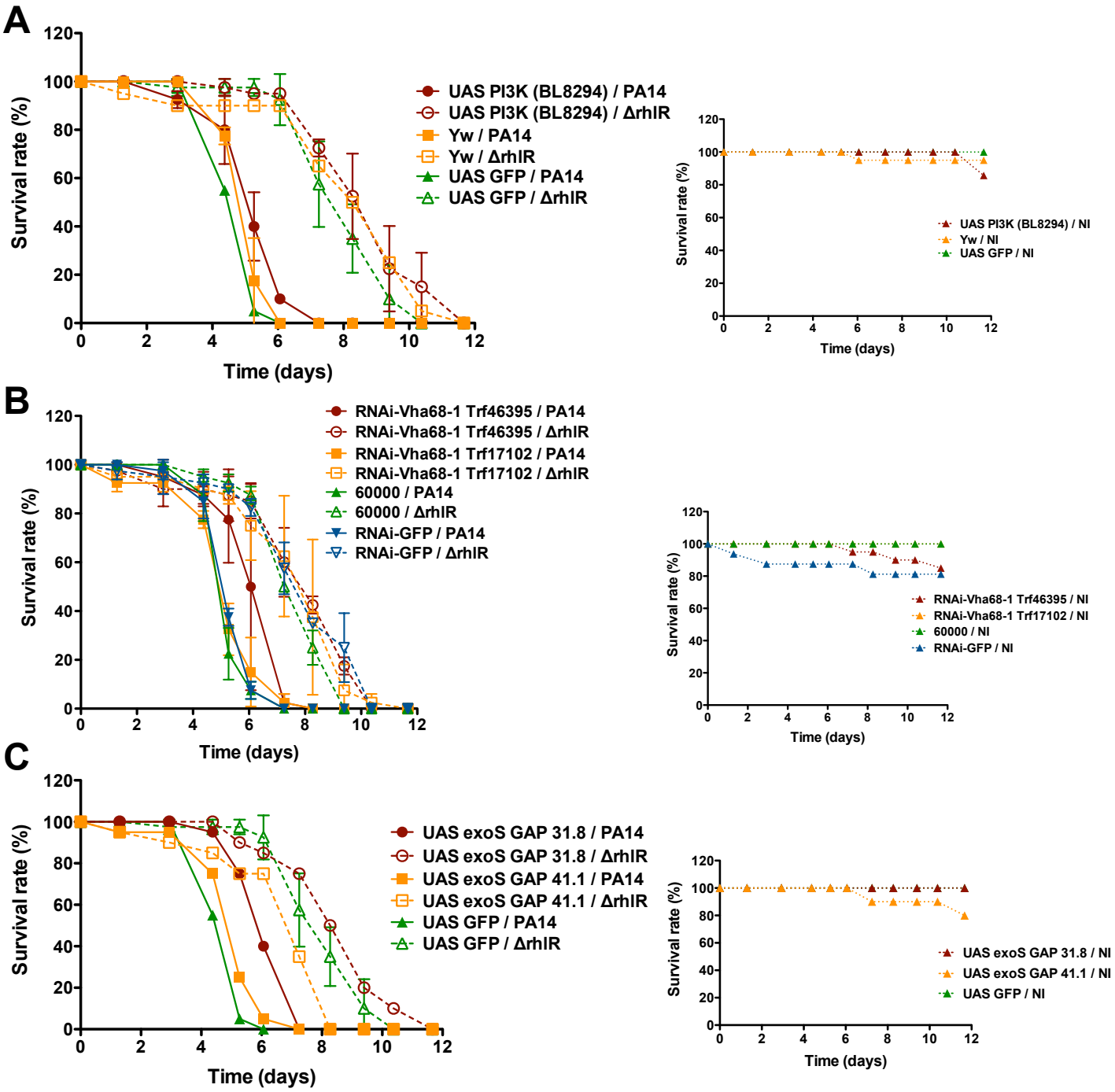


Figure C4. Δ*rhlR* mutant bacteria infecting *Drosophila* deficient for different elements of phagocytosis did not exhibit a regained virulence.

The *hml-Gal4 Gal80^{ts}* driver fly line was crossed either with two UAS-RNAi-*Vha68-1* fly lines, two UAS-exoS fly lines or one UAS-PI3K fly line. Crosses were performed at 18°C and F1 progeny transferred to 29°C for 6 days before intestinal infection with either PA14 wild-type (PA14) or *ΔrhlR* mutant bacteria. (A) Overexpression of PI3K in hemocytes did not affect *ΔrhlR* virulence compared to control flies (UAS-*GFP*). (B) As control flies (60000 and RNAi-*GFP*), flies overexpressing a UAS-RNAi-*Vha68-1* construct in hemocytes and infected with Δ*rhlR* exhibited an increased survival rate compared to flies infected with PA14. (C) Δ*rhlR* in intestinal infection of flies overexpressing a UAS-exoS GAP41.1, but not UAS exoS GAP 31.8 presented a slightly increased virulence compared to control flies infected with Δ*rhlR*. In (A), (B) and (C) right panels correspond to uninfected control flies fed on a sucrose-only diet. For all, means ±SD from one representative experiment out of three are shown.

We have previously shown that the Eater phagocytosis receptor is required to limit PA14 and to control Δ*rhlR* infections (Limmer et al., 2011a). We next asked whether other phases of phagocytosis would be important in host defense. We first tested flies in which *V-ATPase* expression was attenuated by RNA interference in hemocytes, thus potentially hampering the acidification of the phagosome that is likely required to kill ingested bacteria. We did not observe any enhanced sensitivity of these RNAi flies to PA14 or Δ*rhlR* ingestion (Fig. C4B). Rather, one of the two lines displayed a reproducible enhanced resistance to PA14 infection.

The type-three secretion system effector toxin ExoS is produced by the *P. aeruginosa* strain CHA (Avet-Rochex et al., 2005, 2007). Its GAP domain has been shown to block phagocytosis in *Drosophila* hemocytes by targeting the RhoGTPase Rac2, thus inhibiting the cytoskeletal rearrangement required for the uptake of particles. Unexpectedly, we again did not observe any impaired host defense against PA14 or Δ*rhlR* (Fig. C4C).

These experiments that yielded negative results are difficult to interpret as alternative explanations can be put forward for each of them. I did not check whether activating the PI3K had any impact on hemocyte distribution in the adult. As regards the V-ATPase, it should be checked whether RNA interference has functioned well-enough to prevent acidification, which can be tested by the injection of pH-RODO-labeled bacteria. Finally, the efficiency of the exoS-GAP transgenes in blocking phagocytosis has not been assessed using the thermos-sensitive driver system that I use to knock down the activity only at adult stages. As the results were anyway negative, I decided not to pursue further the characterization of these reagents.

PA14 wild-type bacteria are permanently crossing the intestinal barrier upon *Drosophila* intestinal infection

Upon PA14 intestinal infection of flies, only a few bacteria were detected in the hemolymph of flies at early time points of the infection but the number of bacteria dramatically increases in the late time points of infection. One hypothesis was that only a few bacteria are crossing the intestinal barrier at the beginning of the infection. Some of them are phagocytosed and some others might be able to hide from hemocytes and then proliferate in the hemolymph until the death of the fly.

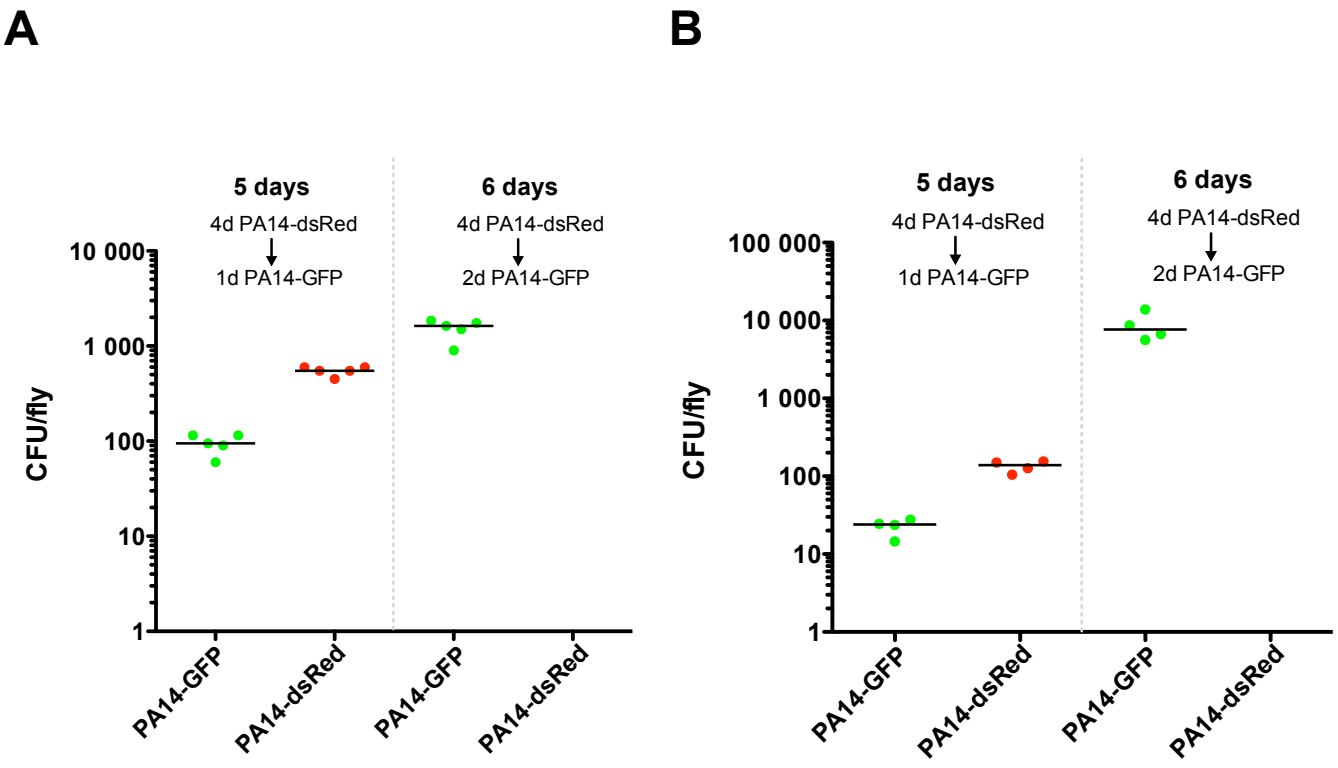


Figure C5. PA14 in intestinal infection of *Drosophila* crossed intestinal barrier all along the infection.

Wild-type *Drosophila* were orally infected with wild-type PA14 expressing dsRed (PA14-dsRed). After 4 days infected flies were transferred in tubes containing wild-type PA14 expressing GFP (PA14-GFP). At 5 days of the infection (one day after transferring flies on PA14-GFP), in *Drosophila* gut most PA14 bacteria expressed dsRed and only 10% expressed GFP (A). However, at 6 days of the infection (2 days after the transfer of these flies on PA14-GFP), only GFP positive bacteria were detected in the gut. Similarly, in the hemolymph of these flies, a majority of PA14-dsRed were detected at 5 days of the infection and only around 10% of PA14-GFP bacteria (B). In addition, 6 days after the beginning of the infection, only PA14-GFP were detected in the hemolymph of these flies. Data represent the results from one experiment.

To determine if PA14 bacteria are crossing the intestinal barrier only in the first part of the infection or if they are able to cross all along the infection, I infected wild-type flies first with PA14 expressing dsRed (PA14-dsRed). In parallel wild-type flies were infected with PA14 expressing GFP (PA14-GFP) in another tube. After four days, I exchanged both infection tubes: flies first infected with PA14-dsRed were transferred on PA14-GFP and flies first infected with PA14-GFP were transferred on PA14-dsRed. One and two days after exchanging infectious tubes, I collected hemolymph (as described before (Haller et al., 2014)) and dissected the gut of these infected flies. Guts were homogenized in sterile PBS and both hemolymph and guts were plated on LB agar with Rifampicin to select for PA14 bacteria.

Five days after the infection started (four days on PA14-dsRed and then one day on PA14-GFP), both PA14-GFP and PA14-dsRed were found in the gut (Fig. C5A) and hemolymph (Fig. C5B) of infected flies with a large majority of PA14-dsRed and around 10% of PA14-GFP. However, six days after the beginning of the infection (two days after the transfer of the flies on PA14-GFP), only PA14-GFP bacteria were found in the hemolymph and gut of infected flies.

Even though no PA14-dsRed bacteria were detected in the hemolymph at six days, it did not mean that PA14-sdRed bacteria were fully cleared from the hemolymph. The technic we use to collect hemolymph does not allow us to extract all the hemolymph of flies but only 50 to 75% of it. Similar results were obtained in the reverse experiment (four days on PA14-GFP and one or two days on PA14-dsRed): one day after transfer a majority of bacteria in the hemolymph and in the gut were PA14-GFP, and two days after transfer only PA14-dsRed were detected.

For both time point assessed, similar bacterial composition were found in infected gut and hemolymph, suggesting that bacteria composition in the hemolymph is dependent on the bacteria composition in the gut. These results clearly indicated that PA14 wild-type bacteria are able to cross the intestinal barrier all along the infection, as had been shown before in the case of *S. marcescens* oral infections (Nehme et al., 2007).

A RhlR-dependent PA14 *vfR* expression in wild-type *Drosophila*

The results from the mini-screen of PA14 mutants and subsequent infection experiments of w^{A5001} (wild-type flies) and w^{A5001}-LxB (wild-type flies pre-injected with latex beads to block phagocytosis) led to the identification of PA14 *vfR* as an interesting candidate.

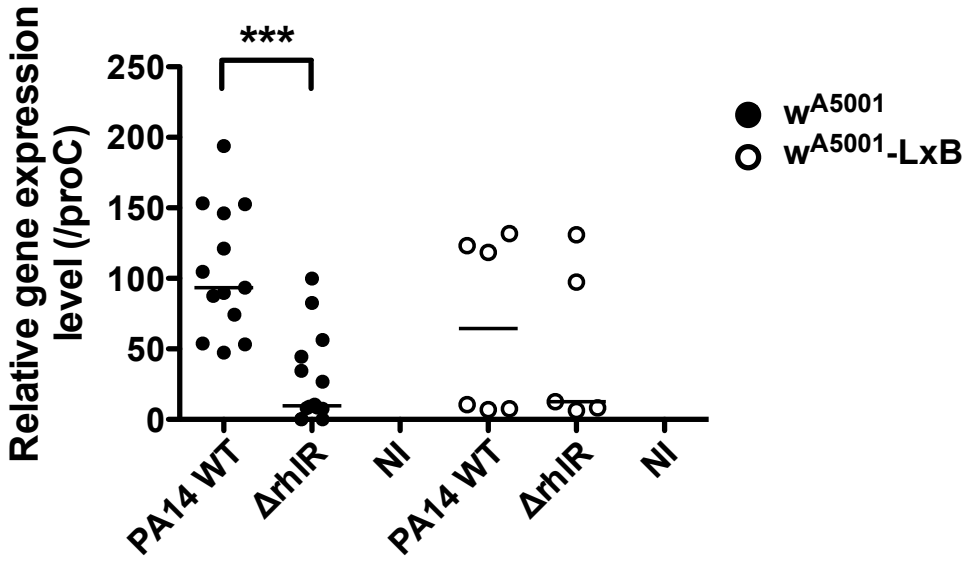


Figure C6. PA14 *vfR* expression upon *w*^{A5001} septic infection is *rhlR*-dependent.
Wild-type *Drosophila* (*w*^{A5001}) or flies for which phagocytosis had been blocked (*w*^{A5001}-LxB) were infected with PA14 wild-type or the Δ*rhlR* mutant through a septic injury infection model and expression of PA14 *vfR* was assessed by RT-qPCR after 24 hours of infection. *vfR* expression was significantly reduced in *w*^{A5001} infected with Δ*rhlR* as compared to PA14 WT (***p=0.0002, Mann Whitney test). Data represent the pooled results from four experiments (only two experiments for *w*^{A5001}-LxB) with biological triplicates. Black bars indicate medians.

We showed that undr these conditions *vfR* mutants exhibited a phenotype akin to that of *rhlR* mutants, suggesting a direct or indirect link between *vfR* and *rhlR*.

Most of our experiments were performed using an intestinal infection model. However this infection model generates a high variability in the hemolymph bacterial load. One problem is that at the relevant stages, there are very few bacteria that can be retrieved from the hemolymph. Indeed, bacterial gene expression in whole flies cannot be measured as most of the signal would originate from the bacteria present in the gut. This variability is strongly decreased in the septic infection model; in addition, many bacteria proliferate and there is no need to collect hemolymph since all of the bacteria are growing in the hemocoel compartment. Thus, the septic injury model is more convenient to assess bacterial gene expression *in vivo* upon infection. Using this septic infection model, I infected wild-type *Drosophila* (w^{A5001}) or flies pre-injected with latex beads to block phagocytosis (w^{A5001}-LxB) with PA14 wild-type (PA14 WT) or *rhlR* mutant (Δ*rhlR*) bacteria. At 24 hours of the infection, before flies started to die, I froze infected flies, extracted RNA, performed a reverse transcription and a quantitative PCR to analyze the expression of *vfR* in these different conditions.

A strong and significant decrease of *vfR* expression was detected in w^{A5001} infected with Δ*rhlR* compared to the same flies infected with PA14 WT. Even though there was a strong variability from one experiment to the other, these results suggest that at least in wild-type flies infection, *vfR* expression was dependent on *rhlR*. In contrast, vfR expression seemed to be highly variable in w^{A5001}-LxB infected either with PA14 WT or Δ*rhlR*. It is thus not clear whether phagocytosis influences *vfR* expression and further investigations would be needed to determine the phenotype. In addition, the same kind of experiments should be repeated using the intestinal infection model as these two infection routes are quite different in terms of conditions encountered by the bacteria. As already pointed out, this would be challenging as we would need to assess the expression of *vfR* only from the few bacteria inside of the general cavity of the fly and not from bacteria inside the gut lumen.

Screening other PA14 mutants for genes potentially involved in PA14 virulence

After the mini-screen presented in the previous paper, we also ordered from the PA14 NR library (and/or deletion mutants when available) of Fred Ausubel laboratory other PA14 mutants for genes that might be involved in PA14 (Liberati et al., 2006).

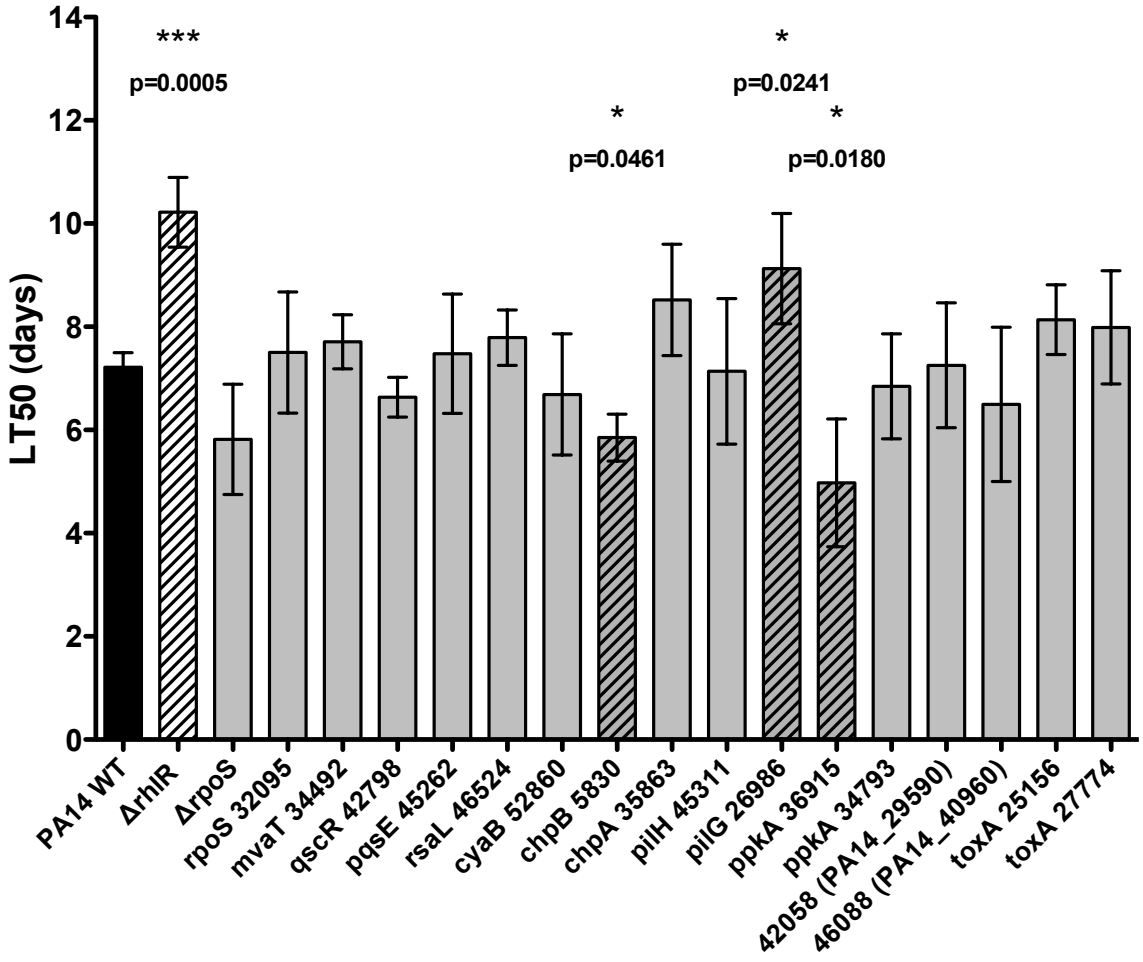


Figure C7. Other PA14 mutants tested in intestinal infection with wild-type *Drosophila*.

Wild-type (w^{A5001}) *Drosophila* were infected with PA14 wild-type (PA14 WT) and different other PA14 mutants. Two deletion mutants (Δ*rhlR* and Δ*rpoS*) and 17 transposon insertion mutants were tested in wild-type flies. Except for Δ*rhlR*, only three PA14 mutants exhibited a virulence significantly different from that of PA14 WT. Among these three mutants, two presented an increased virulence (*chpB* 5830 and *ppkA* 36915) and one mutant displayed a decreased virulence (*pilG* 26986) compared to PA14 WT. Data represent means ±SD of at least two experiments for each mutant.

Our previous results indicated that VfR is involved in PA14 virulence and that the *ΔvfR* clean deletion mutant displayed a similar phenotype than Δ*rhlR* mutant. VfR is a central component of the virulence system of PA14 by promoting acute infections (Coggan and Wolfgang, 2012). After binding to cAMP, VfR is known to interact with the QS, T2SS and T3SS among others in PA14. Therefore we wanted to assess PA14 mutants for other genes involved in the cAMP system like VfR. Among these genes I tested one mutant for *cyaB* (adenylate cyclase involved in cAMP production), *chpA*, PA14_40960 (homologue of *fimL*) and *pilG* (three positive regulators of *cyaB*) and *pilH* and *chpB* (two negative regulators of *cyaB*). In intestinal infection of wild-type *Drosophila*, only *chpB* and *pilG* mutants exhibited a slight but significant difference in virulence compared to PA14 WT (Fig. C7).

ChpB mutants were more virulent and *pilG* mutants less virulent than PA14 WT. These data fit with the hypothesis that cAMP production levels regulate the virulence of PA14, in keeping with our results with *vfR*. When VfR is more strongly activated in *chpB* mutants, an enhanced virulence in our infection model ensues. The generation of clean deletion mutants for these two genes (*chpB* and *pilG*) would confirm these results and it would then be interesting to determine the bacterial load in the hemolymph during the infection in these mutants.

In parallel, mutants for other component of PA14 quorum sensing were tested like *rsaL* (LasI repressor and global LasI -independent regulator of 130 genes), *rpoS* (stationary phase sigma factor), *mvaT* (negative regulator at protein level), *pqsE* (RhlR co-regulator of quinolone signaling) and *qscR* (block RhlR accumulation at low density). None of these mutants presented an altered virulence compared to PA14 WT (Fig. C7).

In the same way, I tested PA14 mutants for *ppkA* (essential component of H1-T6SS and involved in the expression of stress responsive genes) and *toxA* (exotoxine A that was shown to be involved in PA14 virulence with a *C. elegans* infection model (McEwan et al., 2012)). Only one of the *ppkA* mutants (36915) presented a significantly increased virulence compared to PA14 WT (Fig. C7). Given the variability we obtained with some transposon insertion mutants (like *xcpR*), the use of a clean deletion mutant of this gene would be needed to determine definitively the role of *ppkA* role in PA14 virulence.

The T3SS is not involved in PA14 virulence upon intestinal infection of *Drosophila*

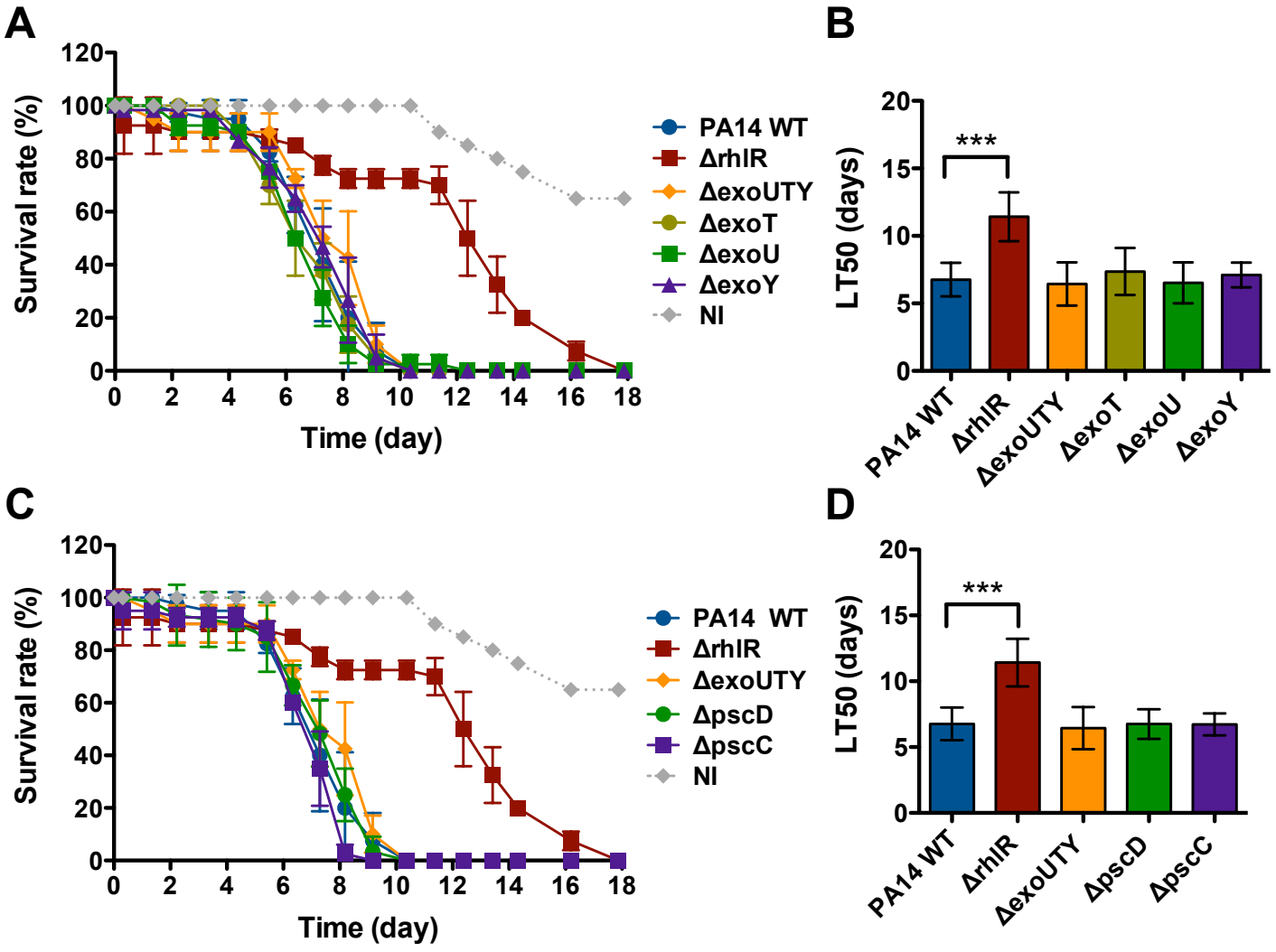


Figure C8. The PA14 T3SS is not required for virulence of the bacteria in an intestinal infection with wild-type *Drosophila*.

Wild-type *Drosophila* (w^{A5001}) were infected with PA14 wild-type (PA14 WT) or different mutants affecting the PA14 T3SS (Type 3 Secretion System). NI represent the uninfected control flies. (A) and (B) Single T3SS effector mutants (Δ*exoT*, Δ*exoU* and Δ*exoY*) and triple mutant (Δ*exoUTY*) in intestinal infection of w^{A5001} exhibited a virulence similar to that of PA14 WT. (C) and (D) PA14 mutants for core elements of the T3SS machinery (Δ*pscD* and Δ*pscC*) presented a similar virulence phenotype as PA14 WT. (A) and (C) Survival curves represent the means ±SD of one representative experiment out of four. (B) and (D) present the combined results from five experiments (means ±SD), ***p<0.0001 with Mann Whitney test.

The type three secretion system (T3SS) is an important mechanism of PA14 attack against eukaryotic cells. We have previously shown that Δ*pscD* a PA14 mutant for an element of the T3SS apparatus did not present a decreased virulence (Limmer et al., 2011a). However, ExoT, an effector of PA14 T3SS, presented a slight decrease of virulence in the mini-screen. Therefore we decided to test other T3SS mutants to determine if PA14 T3SS might nevertheless be involved in PA14 virulence in our intestinal infection model. Clean deletion mutants were kindly provided by the Frederick Ausubel (Δ*exoT*, Δ*exoU*, Δ*exoY* and Δ*exoUTY*) and Alain Filloux laboratories (Δ*pscC*).

I tested these mutants in parallel in our intestinal infection model with wild-type *Drosophila*. No significant increase of *Drosophila* survival was observed after infection with T3SS effector mutants (Fig. C8A&B) or T3SS core component of the secretion apparatus (Fig. C8C&D). These results confirmed that T3SS of PA14 is not required for a full virulence of PA14 in our intestinal infection model of wild-type flies.

The H2-T6SS is not involved in PA14 virulence upon intestinal infection of *Drosophila*

The H2-T6SS is required against eukaryotic cells (and prokaryotic cells) (Jiang et al., 2014) and seems to be regulated by the quorum sensing of the bacteria (Lesic et al., 2009; Sana et al., 2012). A PA14 deletion mutant for the whole H2-T6SS cluster of genes was kindly provided by Alain Filloux's laboratory.

I tested this T6SS mutant in our intestinal infection model using wild-type *Drosophila* (w^{A5001}), flies pre-injected with latex beads (w^{A5001}-LxB), *Myd88* mutant and key^{c02831} mutant flies. No significant increase in *Drosophila* survival was detected in any of these conditions (Fig. C9A-E). These results suggest that at least the H2-T6SS cluster of genes from the T6SS is not required for full virulence of PA14 in our infection model of *Drosophila*.

PA14 *ladS* is slightly involved in PA14 virulence when the cellular response of *Drosophila* is blocked

Recently, a mutation was detected in PA14 *ladS*, as compared to the reference strain PAO1, by sequence analysis. Other *Pseudomonas aeruginosa* strains were examined for the

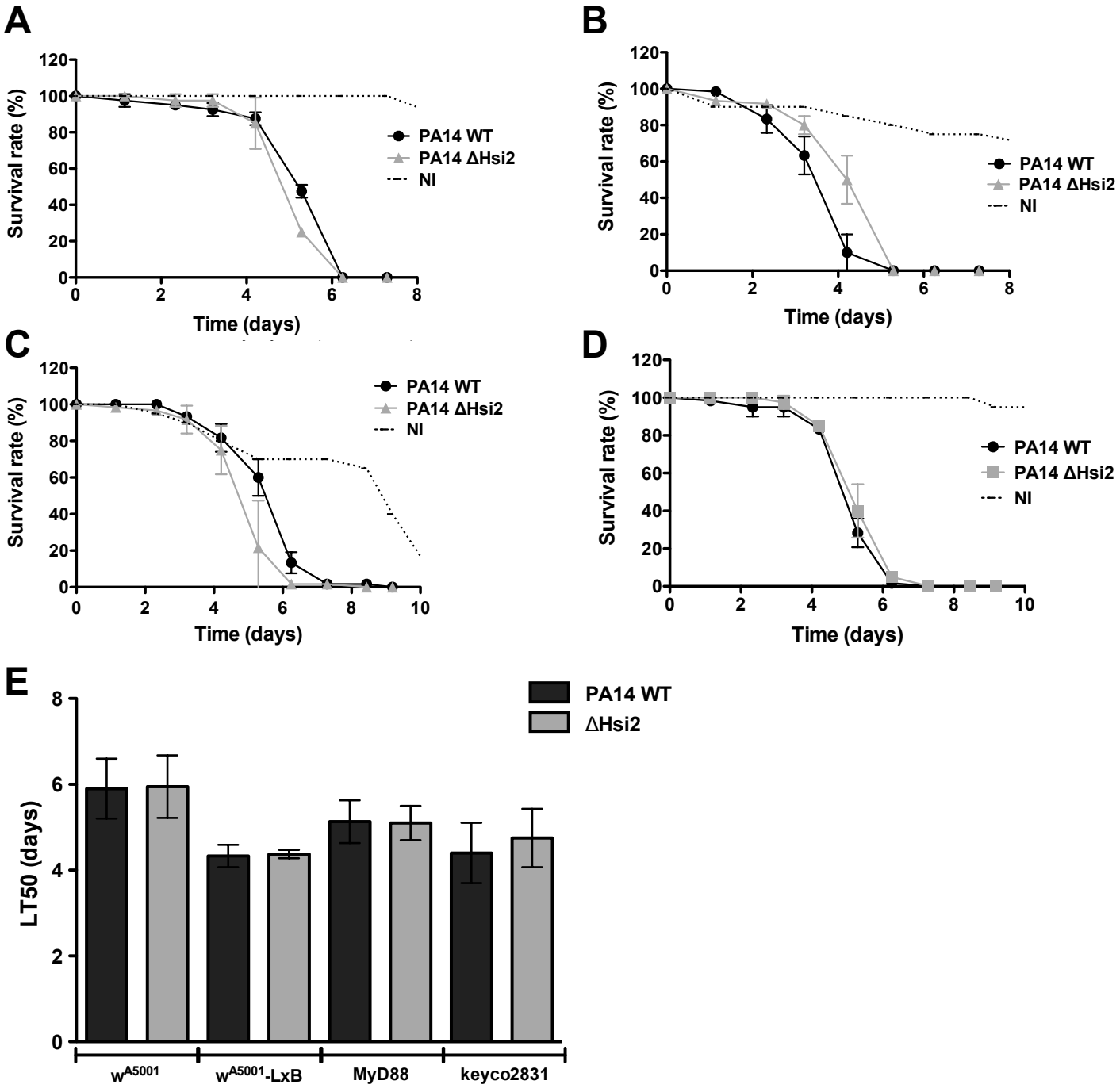


Figure C9. PA14 Hsi2 T6SS is not required for a full virulence of the bacteria in an intestinal infection with *Drosophila*.

Wild-type (*w*^{A5001}) and mutants (*MyD88* and *keyco2831*) *Drosophila* were infected with PA14 wild-type (PA14 WT) or a mutant deleted for the whole H2-T6SS cluster of genes (ΔHsi2). NI represent the uninfected control flies. Survival curves of *w*^{A5001} (A), *w*^{A5001}-LxB (B), *MyD88* (C) and *keyco2831* (D) flies after intestinal infection with PA14 WT or the *ΔHsi2* mutant bacteria. In all conditions, *ΔHsi2* mutant bacteria seemed to present a virulence similar to that of PA14 WT. A slight decrease of virulence was observed when phagocytosis was blocked by injection of latex beads (*w*^{A5001}-LxB). (E) Measured LT50s, (means ±SD)in three experiments; no significant difference in virulence was detected between PA14 WT and *ΔHsi2*.

sequence of this particular gene and no mutations were found. This gene is highly conserved in terms of sequence, even in the PA14 strain in which the *ladS* sequence differs from PAO1 by a duplication of 49 nucleotides that leads to the production of a truncated protein. By reconstructing the wild-type sequence of *ladS* in the PA14 strain, a potential role of this gene for a switch from acute to chronic infection was suggested by *in vitro* and cell culture experiments (Mikkelsen et al., 2011). Wild-type *ladS* gene was shown to up-regulate biofilm production and T6SS and to down-regulate T3SS. This reconstructed-*ladS* PA14 strain was generated in Alain Filloux laboratory and was kindly sent to us to be tested in our *Drosophila* infection model.

I orally infected wild-type *Drosophila* (w^{A5001}), flies pre-injected with latex beads (w^{A5001}-LxB), *MyD88* mutant and key^{c02831} mutant flies with wild-type PA14 (PA14 WT) or the reconstructed-*ladS* PA14 (PA14 $LadS^{r}$). PA14 $LadS^{r}$ displayed a slightly decreased virulence phenotype in intestinal infection of w^{A5001}, *Myd88* and key^{c02831} flies, which was however not significant (Fig. C10 A-E). PA14 $LadS^{r}$ exhibited a significant slight decrease of virulence only when ingested by flies in which phagocytosis was blocked (w^{A5001}-LxB), a situation in which essentially only the second phase of the infection occurs, that is, when there is a developing bacteremia. Thus, the effect of $LadS^{r}$ on virulence *in vivo* is modest at best and accounts only partially for the decreased virulence exhibited by the PAO1 strain in our model of infection (see below Fig. C11A and unpublished observations).

The results in w^{A5001}-LxB remain nevertheless difficult to interpret. The PA14 $LadS^{r}$ strain should present an increased biofilm production and T6SS activation and a decreased T3SS activation. However, I tested PA14 clean deletion mutants affecting these different mechanisms and neither the biofilm formation mutant Δ*pelA* nor the H2-T6SS mutant Δ*Hsi2* presented an increase of virulence and none of the T3SS mutants (effectors and core components of the T3SS machinery) displayed a decreased virulence, as compared to PA14 WT. Even though it has been suggested that the fly intestinal infection model is relevant to model chronic infections (Kesarwani et al., 2011), this may not be the case here.

PA14 and PAO1 strains presented different virulence mechanism in QS

PA14, which was originally isolated from a patient, is known to be one of the most virulent strains of *Pseudomonas aeruginosa*. However a majority of the *P. aeruginosa* research community is working with the PAO1 strain, which is known to be less virulent than

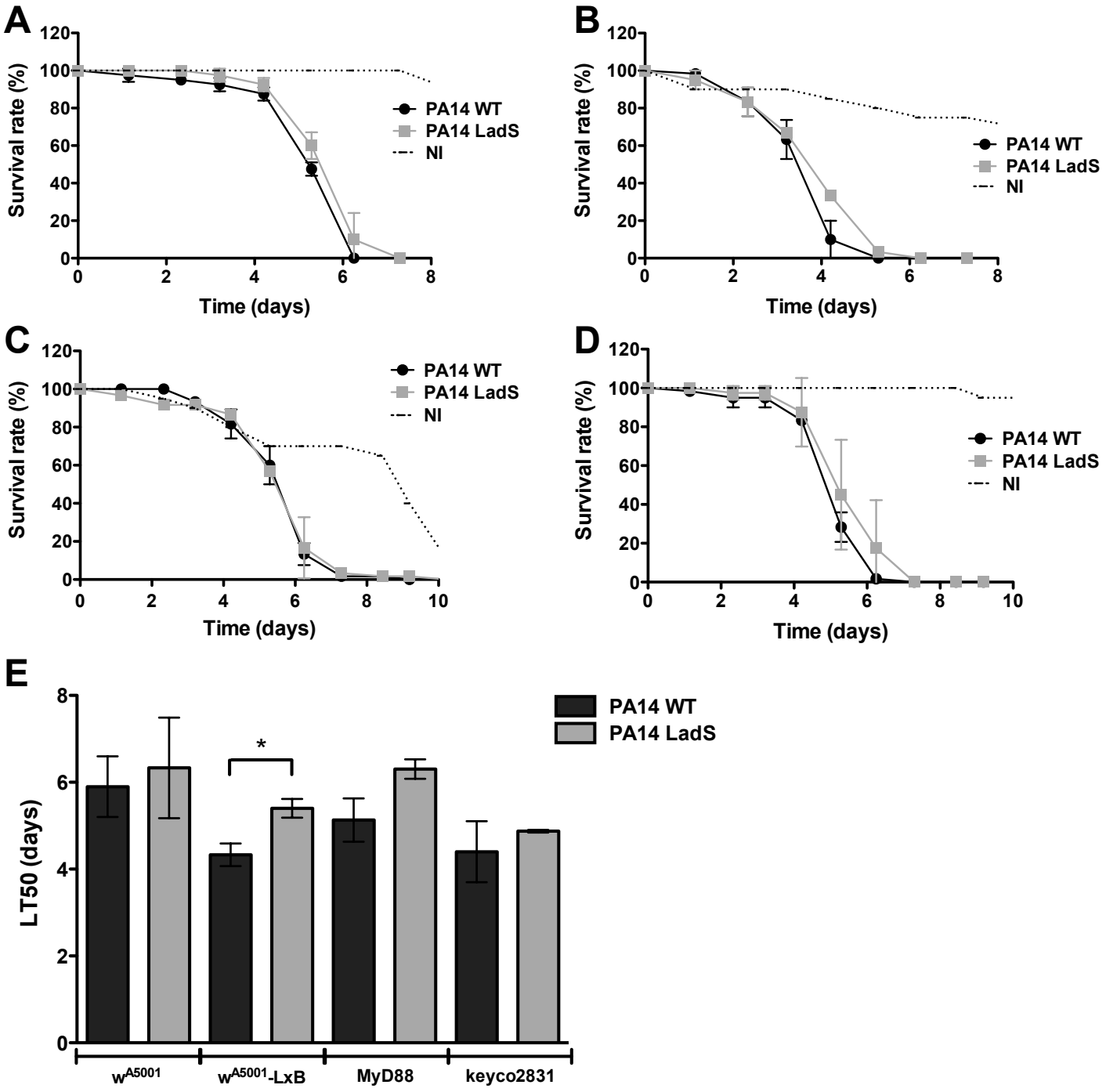


Figure C10. PA14 LadS is not required for a full virulence of the bacteria in an intestinal infection with *Drosophila*.

Wild-type (w^{A5001}) and mutants (*MyD88* and *keyco2831*) *Drosophila* were infected with PA14 wild-type (PA14 WT) or a *ladS*-"repaired" PA14 (LadS) bacteria . NI represented the uninfected control flies. Survival curves of w^{A5001} (A), w^{A5001}-LxB (B), *MyD88* (C) and *keyco2831* (D) flies after intestinal infection with PA14 WT or *LadS*. In all conditions, *LadS* bacteria seemed to present a virulence similar to that of PA14 WT. (E) The means ±SD of measured LT50S in three experiments are shown; no significant difference in virulence was detected between PA14 WT and *LadS,* except in infection of w^{A5001}-LxB where *LadS* exhibited a decreased virulence compared to PA14 WT (*p=0.0178, unpaired t test).

PA14. By testing in our intestinal infection model, PA14 wild-type (PA14) and two PAO1 strains (one strain from Nottingham: PAO1 Nott and one strain from Lausanne: PAO1 Lau) I confirmed the slight decrease of virulence in PAO1 compared to PA14. Surprisingly, I identified a slight difference of virulence between the two PAO1 strains. The PAO1 from Lausanne presented a more attenuated virulence phenotype (Fig. C11A).

In the PA14 strain, we previously described Δ*rhlR*, a quorum-sensing mutant that presented a strongly decreased virulence compared to that of PA14 WT. Moreover we noticed that wild-type *Drosophila* orally infected with Δ*rhlI* presented only a slight increase of survival rate (Fig. 1 B). Using PA14 mutant for the Las quorum-sensing system, Δ*lasR* and Δ*lasI* I observed that only Δ*lasR* displayed a slight and significant decrease of virulence and the virulence of Δ*lasI* was not significantly different from that of PA14 WT (Fig. C11 B).

In collaboration with the Miguel Camara laboratory that provided us with these strains, I assessed the same quorum-sensing mutants in the PAO1 strain background. Unexpectedly, in the PAO1 strain background (PAO1 from Lausanne), all the quorum-sensing mutants exhibited a strongly decreased virulence, including Δ*lasI* (Fig. C11D).

These results suggest that the virulence programs in the PA14 and PAO1 strains is regulated differently and that the quorum-sensing hierarchy is possibly different in PA14 and PAO1. In addition, our data indicate that RhlR has an additional function in PA14 compared to PAO1, which would be independent of the quorum-sensing system of the bacteria. A thorough understanding of this phenomenon will require an in-depth study of the infection of PAO1 in *Drosophila*, similar to the one we have achieved with the PA14 strain.

Strong discrepancies in virulence mechanisms are present from one *P. aeruginosa* strain to the other. This lead to increased difficulties to find new efficient therapeutics as these distinct strains can infect Humans and can sometimes be found together in patients undergoing mixed infections. Furthermore, the strains may evolve during chronic and possibly acute infections. These differences in virulence mechanisms need to be taken into account to develop strain-specific therapeutics.

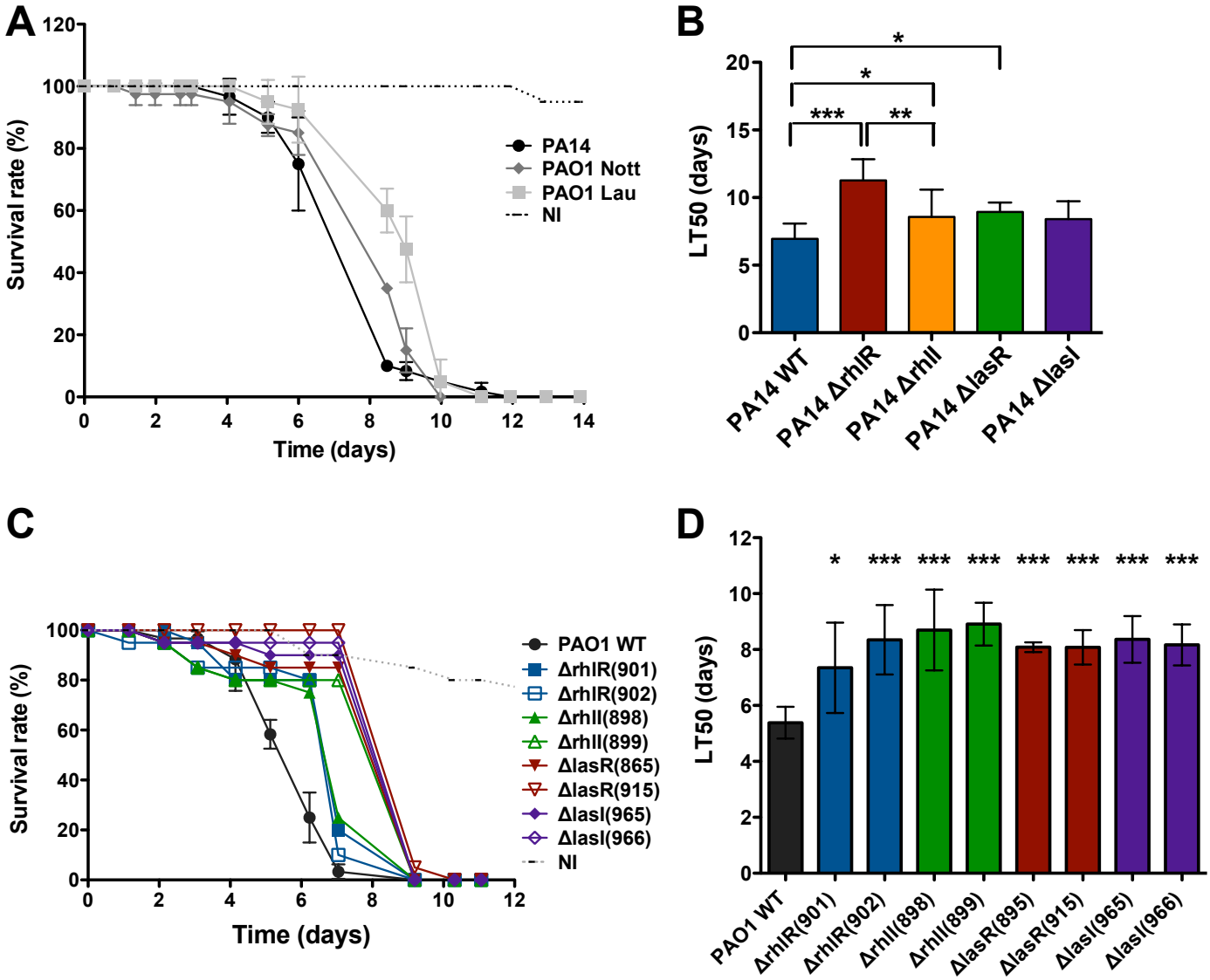


Figure C11. PA14 and PAO1 wild-type strains and QS mutants exhibit different virulence phenotype in an intestinal infection of *Drosophila*.
Wild-type (w^{A5001}) *Drosophila* were infected with PA14 wild-type (PA14), two PAO1 wild-type (PAO1 Nott and PAO1 Lau) strains and QS (quorum sensing) mutants in a PAO1 background. (A) Flies infected with PA14 presented a shorter survival rate than flies infected with PAO1 strains. Surprisingly, among the two PAO1 wild-type strains, PAO1 originating from Lausanne (PAO1 Lau) seemed to be less virulent than the PAO1 Nott (Nottingham) and PA14 strains. (B) Only PA14 Δ*rhlR* QS mutant presented a strong decrease of virulence compared to PA14 WT (Δ*rhlR*: ***p<0.0001). PA14 Δ*rhlI* and PA14 Δ*lasR* mutants displayed only a slight, yet significant decrease of virulence (Δ*rhlI*: *p=0.0313 and Δ*lasR*: *p=0.0141). The PA14 WT, Δ*rhlR* and Δ*rhlI* data are already shown in Fig. 1 B from the manuscript). (C) and (D) All QS mutants in the PAO1 strain (from Lausanne) were significantly less virulent than PAO1 wild-type (Δ*rhlR* 901: *p=0.0232 and 902: ***p=0.0008, Δ*rhlI* 898: ***p=0.0008 and 899: ***p<0.0001, Δ*lasR* 895: ***p<0.0001 and 915: ***p<0.0001, and Δ*lasI* 965: ***p<0.0001 and 966: ***p<0.0001). (A) and (C) The means ±SD of biological triplicates during a survival experiment of one experiment out of two are shown. (B) and (D) The means ±SD of LT50s measured in three (B) or two (D) experiments are shown. Statistical analysis were done with an unpaired t test.

Chapter 2

Pseudomonas aeruginosa bacteria coordinate their virulence from host to host using volatile signals

The observation of the Hill coefficient (previous chapter) from fly survival assays after PA14 wild-type or the rhlR mutants lead us to hypothesize that flies infected by PA14 wild-type have a synchronized death while flies infected by RhlR do not. This is possibly due to a synchronized PA14 virulence that is not present in the *rhlR* mutant. In this work I started to assess how this synchronized fly death/bacteria virulence occurs.

This chapter is written as scientific short paper as we think that these striking results should be published (probably as a short communication). However, some key experiments remains to be done to demonstrate the role of quorum sensing systems in the synchronization of the switch to virulence of PA14 infecting distinct hosts.

Pseudomonas aeruginosa bacteria coordinate their virulence from host to host using volatile signals

Samantha Haller[a], Samuel Liegeois[a], Eliana Drenkard[b,c], Frederick M. Ausubel[b,c], and Dominique Ferrandon[a*].

[a] Equipe Fondation Recherche Médicale, Unité Propre de Recherche 9022 du Centre National de la Recherche Scientifique, Institut de Biologie Moleculaire et Cellulaire, Université de Strasbourg, F67084 Strasbourg Cedex, France; [b] Department of Genetics, Harvard Medical School, Boston, MA 02115; and [c] Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

*To whom correspondence may be addressed. E-mail: d.ferrandon@ibmc.u-strasbg.fr.

INTRODUCTION

Living organisms often live in communities in a complex environment. Communication between members of the community allows individuals to adjust to local environmental conditions. While it had long been thought that this property was distinctive of metazoans, it has become clear that the exchange of information also plays a paramount role in prokaryotes, as manifested by quorum-sensing (Schuster et al., 2013). Communication can also take place between different species sharing the same environment, for instance in a biofilm. Quorum-sensing systems are important as they may control virulence within pathogenic microorganisms of the same species (Jimenez et al., 2012). For instance, the quinolone signaling system of *Pseudomonas aeruginosa* is required for virulence, also in a septic model

of injury in *Drosophila melanogaster* (Cao et al., 2001; Gallagher et al., 2002; Rahme et al., 1995).

It has also been reported that chemical compounds emitted by pathogenic bacteria can either serve as repulsive or attractants for their prospective hosts. For instance, geosmin is emitted by several molds and cyanobacteria that are toxic to flies (Stensmyr et al., 2012). *Drosophila* species have evolved a dedicated olfactory network that allows them to detect geosmin and to avoid the potentially contaminated food source. In contrast, the opportunistic pathogen *P. aeruginosa* emits 2-aminoacetophenone (2-AA) that lures *Drosophila* flies to feed preferentially on contaminated food (Kapsetaki et al., 2014). Conversely, bacteria are able to perceive the nature of their hosts. For instance, *P. protegens* is able to switch form a beneficial to a pathogenic mode according to the nature of the substrate, plant or insect, that it perceives through a histidine kinase receptor that evolved by shuffling sensor domains (Kupferschmied et al., 2014). Furthermore, bacteria can also kill competing microorganisms or even prospective hosts through volatile compounds (Popova et al., 2014). Finally, there are also communications between the host and pathogens. For instance, interferon-gamma binds to an outer-membrane protein of *P. aeruginosa,* which in turns triggers the expression of a quorum-sensing dependent virulence factor (Wu et al., 2005).

We are using a *P aeruginosa* intestinal infection model in *Drosophila* to decipher host-pathogen interactions (Limmer et al., 2011). We have previously shown that some ingested bacteria are able to cross the digestive tract barrier, circumvent phagocytosis by hemocytes, before ultimately causing systemic bacteremia. We have found that the rhamnolipid quorum sensing receptor RhlR is required for bacteria to elude phagocytosis (Limmer et al., 2011), a process that relies only very partially on the quorum-sensing function of RhlR (see Chapter 1). Whereas batches of 20 wild-type flies succumb in about a week to the ingestion of *P. aeruginosa* wild-type strain PA14, *rhlR* bacteria kill their hosts at a much reduced rate, except

if the cellular immune response is impaired. The survival curves have a sigmoid shape with a more or less pronounced slope during transition (Limmer et al., 2011). We have noticed that flies infected with *rhlR* or *rhlI* (Haller *et al.*, in preparation) display a shallower survival curve with a significantly altered slope, as compared to wild-type flies, even when their virulence is restored when the cellular immune response is disabled. As death is an all or none phenomenon, it may mean that bacteria somehow coordinate their virulence in distinct hosts, possibly through the Rhl quorum-sensing system. Thus, we have investigated here whether bacteria may be able to communicate and coordinate their virulence depending on whether hosts are collectively or individually infected.

RESULTS AND DISCUSSION

In a first series of experiments, we tested whether flies would succumb at the same speed when feeding on a PA14-contaminated sucrose solution by batches of 20 flies or when feeding as single flies. Thus, we infected three batches of twenty flies and infected in parallel three series of 20 single flies in vials of the same size and containing the same amount of contaminated sucrose solution. We reproducibly observed that single flies died collectively at a much slower rate than flies in batches (Fig. 1A). Namely, 50% of flies had succumbed to the infection by six days (LT50=6), in keeping with our previous results. In contrast, it took eight days for half of the single flies to die when infected. Of note, the survival curve of the individually-infected flies was much shallower than that of the collectively infected flies.

We next tested the almost avirulent bacterial mutant *rhlR* and observed a similar difference between single flies and flies feeding on batches. Because *rhlR*-fed flies hardly succumb to infection, it is likely that they die of starvation since the flies keep on feeding on the same

filter containing the sugar solution that is not replenished during the infection experiment. The batches of 20 flies fed *rhlR* died with a LT50 of 10 days (Fig. 1B). In contrast, the LT50 of individually-infected flies was about 20 days, in keeping with the larger supply of sucrose solution available to them. We conclude that the flies singly-infected with wild-type PA14 did succumb to infection, since they died much earlier than flies feeding on rhlR bacteria that ultimately starve. Of note, both wild-type PA14 and *rhlR* bacteria hardly grow on the filters, which excludes that sugar would be consumed by the *rhlR* bacteria at a slower rate. We have also checked that flies ingest the same quantity of sucrose solution (Fig. 1C). Unexpectedly, the flies drank significantly more solution after one day than after four days on the filter.

While the data shown in Fig. 1A are compatible with the hypothesis that *P. aeruginosa* is more virulent when flies are fed by batches of 20, alternative explanations can account for our observations. We therefore improved our experimental design. We placed our flies, whether single or in batches of 20, in vials with open grids on top. We then attached one vial enclosing a single fly to a vial containing 20 flies, top grid against top grid, with a spacer inserted in-between to prevent any direct physical contact between the lone fly and the flies in batches (Fig. 2A). We made 20 such coupled infection tubes. In half of them, we further separated the two vials with an impermeable Parafilm® layer so as to prevent any gaseous exchange between the two vials, whereas a bolting cloth let air flow between the two vials in the other set of ten blocks. We observed that when the vials containing single flies were connected with those containing 20 flies, all flies, whether in batch or singles, succumbed to infection at the same rapid rate (LT50=4 days) (Fig. 2C). Remarkably, when the single flies were separated from the flies in batches, they died at a significantly slower rate (LT50=6 days) than flies in batches (LT50= 4 days) (Fig. 2B).

These data indicate that there is a signal diffusing through the air that regulates either the survival of flies to infection or alternatively synchronizes the virulence of *P. aeruginosa*

infecting physically separated hosts. Thus, the signal might represent a communication between infected flies, between bacteria present in physically separated hosts, or between bacteria in one host and another infected host, which would then in response to sensing the bacterial signal indirectly modulate the virulence of the bacteria that infect it (Wu et al., 2005).

Since we used as hosts *white* flies, which are functionally blind, and since the flies are physically separated, thus ruling out the involvement of gustation, the likely cue that modulates virulence of bacteria between hosts is likely to be volatile. Any volatile signal is going to be sensed by the fly as a smell. We therefore asked whether flies with a severe olfactory defect would still be able to perceive a potential signal rendering them more vulnerable to infection. We therefore used the Orco null mutant recently employed in another study to demonstrate a requirement for olfaction in *Drosophila* larval hematopoiesis (Shim et al., 2013). We reasoned that if olfaction were involved in this differential sensitivity to infection, the flies placed in batches of 20 would behave as single flies and thus succumb later to infection. As shown in Fig. 3A, the hemizygous Orco null flies succumbed at the same rate as the heterozygous controls, thereby suggesting that olfaction may not be involved in the communication that takes place between infected flies. Of note, flies are indeed able to smell compounds of bacterial origin, *e.g.*, 2-AA, which influence their feeding behavior (Kapsetaki et al., 2014). The feeding behavior alteration induced by 2-AA was abolished in *orco* mutants (Kapsetaki et al., 2014).

Consequently, the signal that modulates the virulence of bacteria within the host is generated either by the bacteria themselves, located inside the hosts, or by the infected host, and perceived by bacteria within hosts. One alternative model would be that bacteria on the filter emit a signal that limits virulence, and that would be consumed and exhausted by the flies exposed to it in the vial. Indeed, it has been shown that PA14 emits a volatile compound, 2-AA, that reduces virulence in a septic injury model in *Drosophila* by inhibiting the mvfR

quorum-sensing regulator (Kesarwani et al., 2011). However, if this model were relevant, one would expect that in the experiments in which two vials with respectively 20 or single flies communicate, there would be a dose of 2-AA twice as important than in single vials, consumed by 21 flies instead of 20 flies for a normal dose in the single vial design. The volatile compound would then be expected to be exhausted at a slower rate by the 21 flies and thus to decrease the virulence in those 21 infected flies, as compared to the 20 flies separated from the single flies by an air-tight barrier. Our data clearly show that whether in the single or the double vial design, the batches of 20 flies succumb all at the same rapid rate, thus excluding this possibility. In addition, *orco*, which is required for sensing 2-AA, is not required for the increased virulence observed in collectivities of infected flies (Fig. 3).

Because quorum-sensing systems allow the bacteria to adapt to diverse environmental conditions, they may control the emission of volatile compound, as indeed is the case for 2-AA, which is no longer emitted in the quinolone receptor mutant *mvfR* (Kesarwani et al., 2011). We have not previously observed an altered virulence in *mvfR* mutants (Limmer et al., 2011). However, we have observed a somewhat reduced virulence in the two other quorum-sensing systems, Las and Rhl (Limmer et al., 2011). The difference in the LT50 of mutant vs. wild-type PA14 was of 1.6 days for *lasR, LasI,* and *rhlI*, vs. 4.3 for *rhlR*. This difference may be compatible with the two-day difference observed between the single fly and 20-fly experiments. To address further this question, the experimental strategy would be to feed the "emitter" flies (batches of 20) with a mutant unable to synthesize the volatile compound, *rhlI* for instance, and observe that the lone flies would die as slowly as the 20 flies feeding on *rhlI,* even though air flow would be allowed between the single-fly and the 20-flies vials. The complementary experiment would be to feed the "recipient" lone flies with a bacterium unable to perceive the signal, *rhlR* for instance, Because *rhlR* bacteria are avirulent, likely because of a quorum sensing-independent function of RhlR (Chapter 1), they cannot be tested

as a source of food for the single flies. It would however be feasible to test both component of the Las system, *lasI* and *lasR*, with this strategy. An alternative would be to expose the lone flies to quorum sensing-regulated volatile compounds and determine whether the flies would then succumb more rapidly. We do not know whether homo serine lactones are sufficiently volatile to fulfill such a function.

In conclusion, we have demonstrated that bacteria can collectively signal to each other when present (or having been present) inside distinct hosts through a volatile, as yet unidentified compound. This can be physically feasible given that flies rely on a tracheal system for respiration, in which the tracheoles directly irrigate all of the tissues. Alternatively, the signal might be emitted by infected bacteria released in the feces, as they are unlikely to be all killed by the intestinal host defenses. It will be interesting to determine whether such a communication also occurs in infected vertebrates.

REFERENCES

Cao, H., Krishnan, G., Goumnerov, B., Tsongalis, J., Tompkins, R., and Rahme, L.G. (2001). A quorum sensing-associated virulence gene of Pseudomonas aeruginosa encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. Proc Natl Acad Sci U S A *98*, 14613-14618.

Gallagher, L.A., McKnight, S.L., Kuznetsova, M.S., Pesci, E.C., and Manoil, C. (2002). Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. J Bacteriol *184*, 6472-6480.

Jimenez, P.N., Koch, G., Thompson, J.A., Xavier, K.B., Cool, R.H., and Quax, W.J. (2012). The multiple signaling systems regulating virulence in Pseudomonas aeruginosa. Microbiology and molecular biology reviews : MMBR *76*, 46-65.

Kapsetaki, S.E., Tzelepis, I., Avgousti, K., Livadaras, I., Garantonakis, N., Varikou, K., and Apidianakis, Y. (2014). The bacterial metabolite 2-aminoacetophenone promotes association of pathogenic bacteria with flies. Nature communications *5*, 4401.

Kesarwani, M., Hazan, R., He, J., Que, Y.A., Apidianakis, Y., Lesic, B., Xiao, G., Dekimpe, V., Milot, S., Deziel, E.*, et al.* (2011). A quorum sensing regulated small volatile molecule reduces acute virulence and promotes chronic infection phenotypes. PLoS Pathog *7*, e1002192.

Kupferschmied, P., Pechy-Tarr, M., Imperiali, N., Maurhofer, M., and Keel, C. (2014). Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial Pseudomonas protegens. PLoS Pathog *10*, e1003964.

Limmer, S., Haller, S., Drenkard, E., Lee, J., Yu, S., Kocks, C., Ausubel, F.M., and Ferrandon, D. (2011). Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. Proc Natl Acad Sci U S A *108*, 17378-17383.

Popova, A.A., Koksharova, O.A., Lipasova, V.A., Zaitseva, J.V., Katkova-Zhukotskaya, O.A., Eremina, S., Mironov, A.S., Chernin, L.S., and Khmel, I.A. (2014). Inhibitory and Toxic Effects of Volatiles Emitted by Strains of Pseudomonas and Serratia on Growth and Survival of Selected Microorganisms, Caenorhabditis elegans, and Drosophila melanogaster. BioMed research international *2014*, 125704.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. Science *268*, 1899-1902.

Schuster, M., Sexton, D.J., Diggle, S.P., and Greenberg, E.P. (2013). Acyl-homoserine lactone quorum sensing: from evolution to application. Annu Rev Microbiol *67*, 43-63.

Shim, J., Mukherjee, T., Mondal, B.C., Liu, T., Young, G.C., Wijewarnasuriya, D.P., and Banerjee, U. (2013). Olfactory control of blood progenitor maintenance. Cell *155*, 1141-1153.

Stensmyr, M.C., Dweck, H.K., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S*., et al.* (2012). A conserved dedicated olfactory circuit for detecting harmful microbes in Drosophila. Cell *151*, 1345-1357.

Wu, L., Estrada, O., Zaborina, O., Bains, M., Shen, L., Kohler, J.E., Patel, N., Musch, M.W., Chang, E.B., Fu, Y.X*., et al.* (2005). Recognition of host immune activation by Pseudomonas aeruginosa. Science *309*, 774-777.

EXPERIMENTAL PROCEDURES

Drosophila stock and Culture

The following strains were used: wild-type, w^{A5001} (Thibault et al., 2004), the mutant strains 7951 (w[1118]; Df(3R)Exel9029, PBac{w[+mC]=RB3.WH3}Exel9029) and 23130 (w[*]; w[+*] Orco[2]) were obtained from Bloomington Stock Center. To assess to role of *Drosophila* olfaction in flies sensitivity to PA14 infection, 7951 and 23130 fly lines were crossed together to generate an *orco* null mutant or both fly lines were crossed to w^{A5001} to generate appropriate controls (heterozygous with wild-type phenotype).

Bacteria strains and growth conditions

Pseudomonas aeruginosa strain PA14 wild-type (Rahme LG et al., 1995) and the ΔrhlR mutant (Limmer et al., 2011) were grown in Brain-Heart-Infusion Broth (BHB), overnight, at 37°C with agitation.

Drosophila intestinal infections

Infection tubes were set as described previously with PA14 (Limmer et al. 2011 or Haller et al., 2014). For classical infections, 20 flies (group of 20 flies) or one fly (single fly) were transferred to the infection tubes (or control with sucrose only). Infected and control flies were kept at 25°C. For infections allowing or not communication between grouped Drosophila and single flies, pairs of tubes were prepared. In each pair, 20 flies were transferred in one tube and a single fly in the other tube. Both tubes were closed either with Parafilm® (no communication between the two tubes) or a nylon bolting cloth (communication between the two tubes) and a separator was added between the two tubes

before fixing them tightly together. The number of fly used for each experiment is specified for each figure. For survival assays, the number of surviving flies was computed daily.

Fly ingestion assay

The amount of ingested food was measured on whole flies (10 per sample) as described before (Schneider et *al*., 2009).

Statistical analysis

All statistical analyses were performed on Graphpad Prism version 5 (Graphpad software Inc., San Diego, CA). Details are indicated in the legend of each figure.

FIGURE LEGENDS

Figure 1. Individually-infected flies are less susceptible to PA14 intestinal infection than collectively-infected flies .

A-B: The means ±SD of one tube with 20 flies in triplicates (Group of 20 flies) or 20 tubes with single flies in triplicates, from one representative survival experiment out of four are presented.

(A) Singly-infected flies survive longer to a PA14 wild-type intestinal infection than flies infected by groups of 20 flies (LT50 single flies = 8 days compared to LT50 of 20 flies in group = 6 days); ***p<0,0001, LogRank test). (B) A similar observation was made in an infection with the PA14 ΔrhlR (LT50 single flies = 20 days compared to LT50 of 20 flies in group = 10 days, ***p<0,0001, LogRank test). (C) No significant difference in the amount of ingested food was noticed between 20 flies kept together and single flies fed with a sucrose-only diet. Of note, a higher food intake at day 1 was detected in both conditions.

Figure 2. Single flies in communication with a group of 20 flies had the same rate of death than the flies in that community.

(A) Picture of the paired tubes allowing communication or not between the single flies and the group of 20 flies. Two fly tubes were fixed tightly together top to top with a spacer placed in-between the two tubes to avoid direct physical contacts between the flies placed in each tube. The tubes are closed on the top either by a bolting cloth (air flow allowed) or sealed by Parafilm® (does not allow air communication). (B) and (C) Survival test of single flies with air passage with a group of 20 flies (C) or not (B). The means ±SD of 10x 20 flies in group or 10x single flies from one representative experiment out of three are presented. (B) Single flies in tubes with no air communication was allowed with the tube of 20 flies died later from the PA14 wild-type infection than the group of 20 flies (LT50 single flies = 6 days compared to

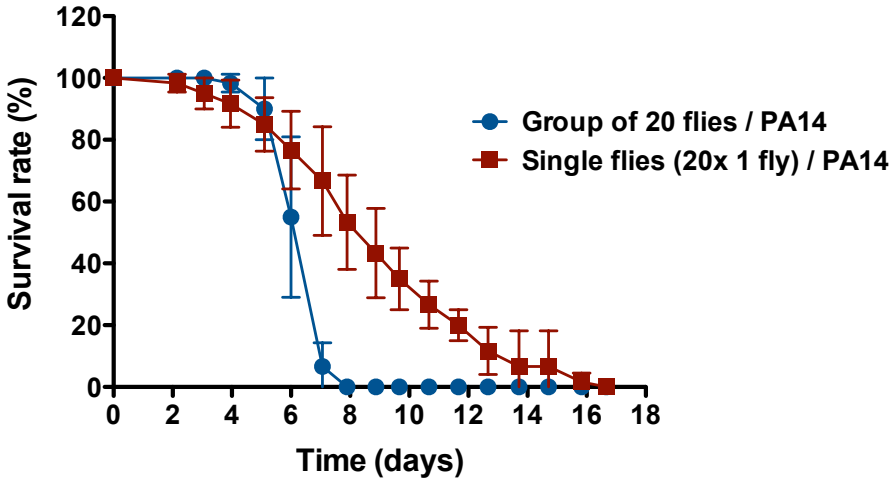
LT50 group of 20 days = 4 days, ***p<0,0001, LogRank test). (C) When air communication was allowed between the two tubes, single flies died at the same rate of death than the group of 20 flies (no significant difference, LogRank test).

Figure 3. Drosophila olfaction was not involved in the susceptibility of community flies to the PA14 infection.

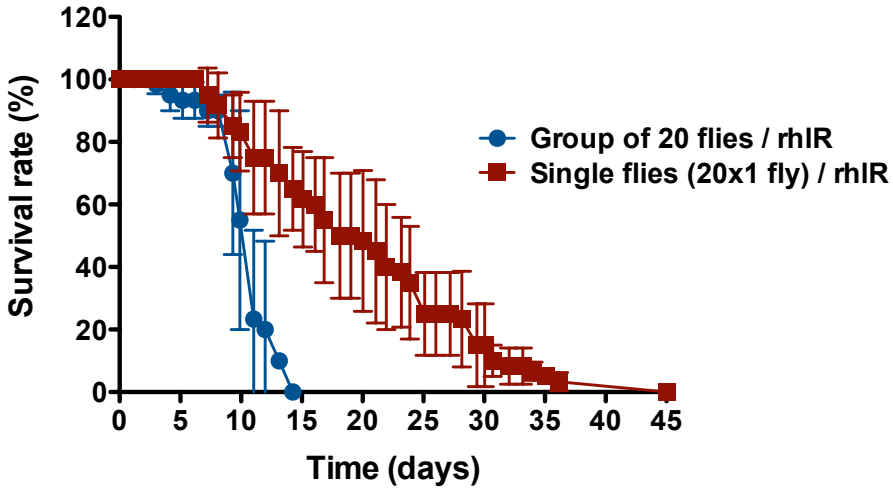
(A) Homozygous *orco* null mutant flies died at the same rate than the heterozygous control flies (null mutation/+ or *orco* deficiency/+) in a PA14 intestinal infection (no significant difference, LogRank test). The mean ±SD of triplicates (except of *orco* null mutants) from one representative experiment out of three are presented. (B) Survival of *orco* null mutants was not affected in uninfected condition (B).

Figure 1

A


Survival rate (%)
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18
Time (days)
Group of 20 flies / PA14
Single flies (20x 1 fly) / PA14

B


Survival rate (%)
120
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45
Time (days)
Group of 20 flies / rhIR
Single flies (20x1 fly) / rhIR

C

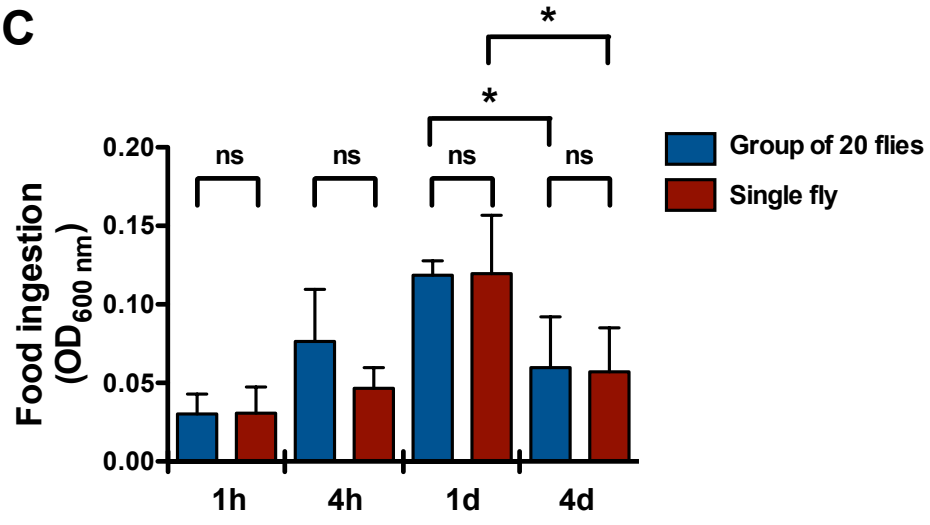
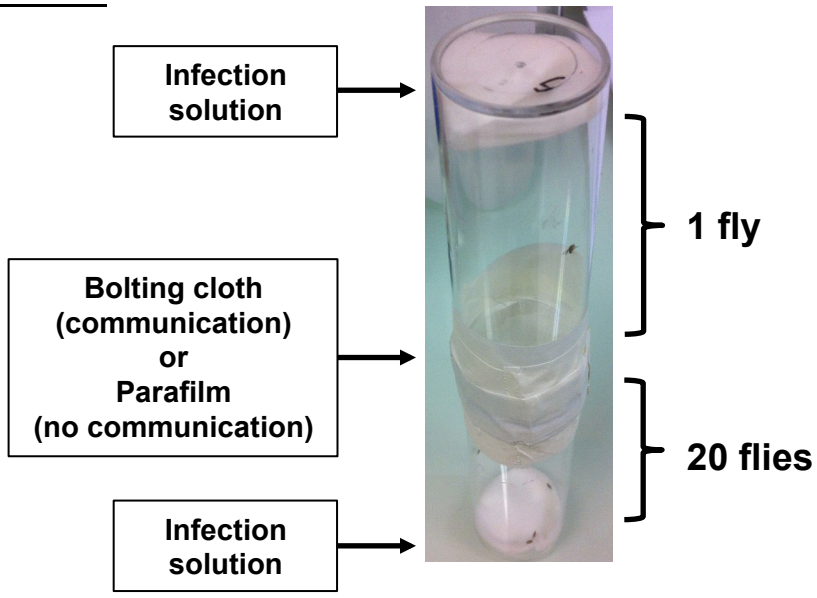
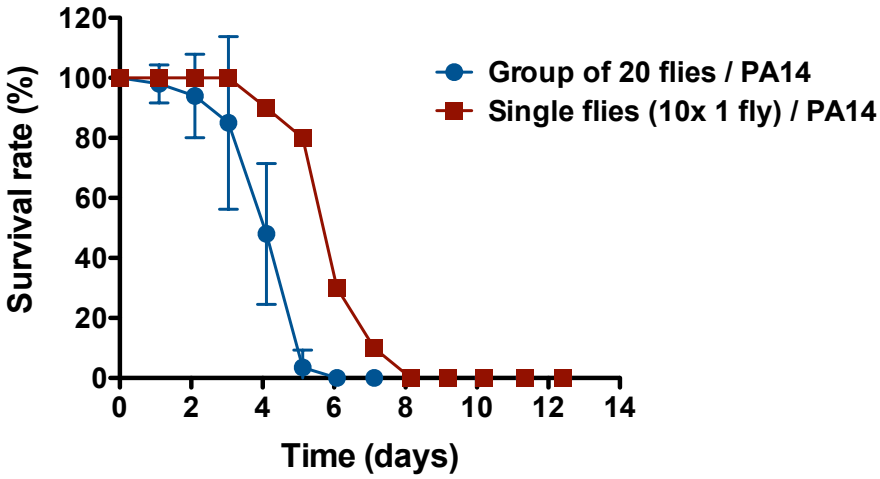

Food ingestion (OD600 nm)
0.20
0.15
0.10
0.05
0.00
ns
ns
ns
ns
*
*
1h
4h
1d
4d
Group of 20 flies
Single fly

Figure 2

A


Infection solution
Bolting cloth (communication) or Parafilm (no communication)
Infection solution
1 fly
20 flies

B


Survival rate (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
Group of 20 flies / PA14
Single flies (10x 1 fly) / PA14

C

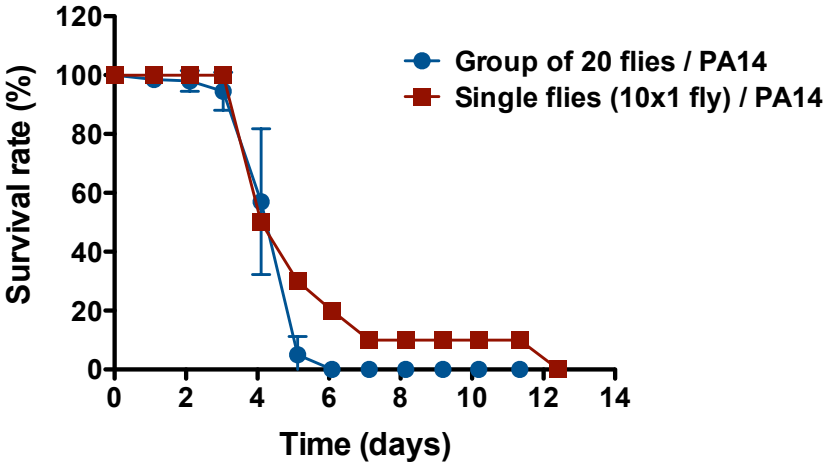
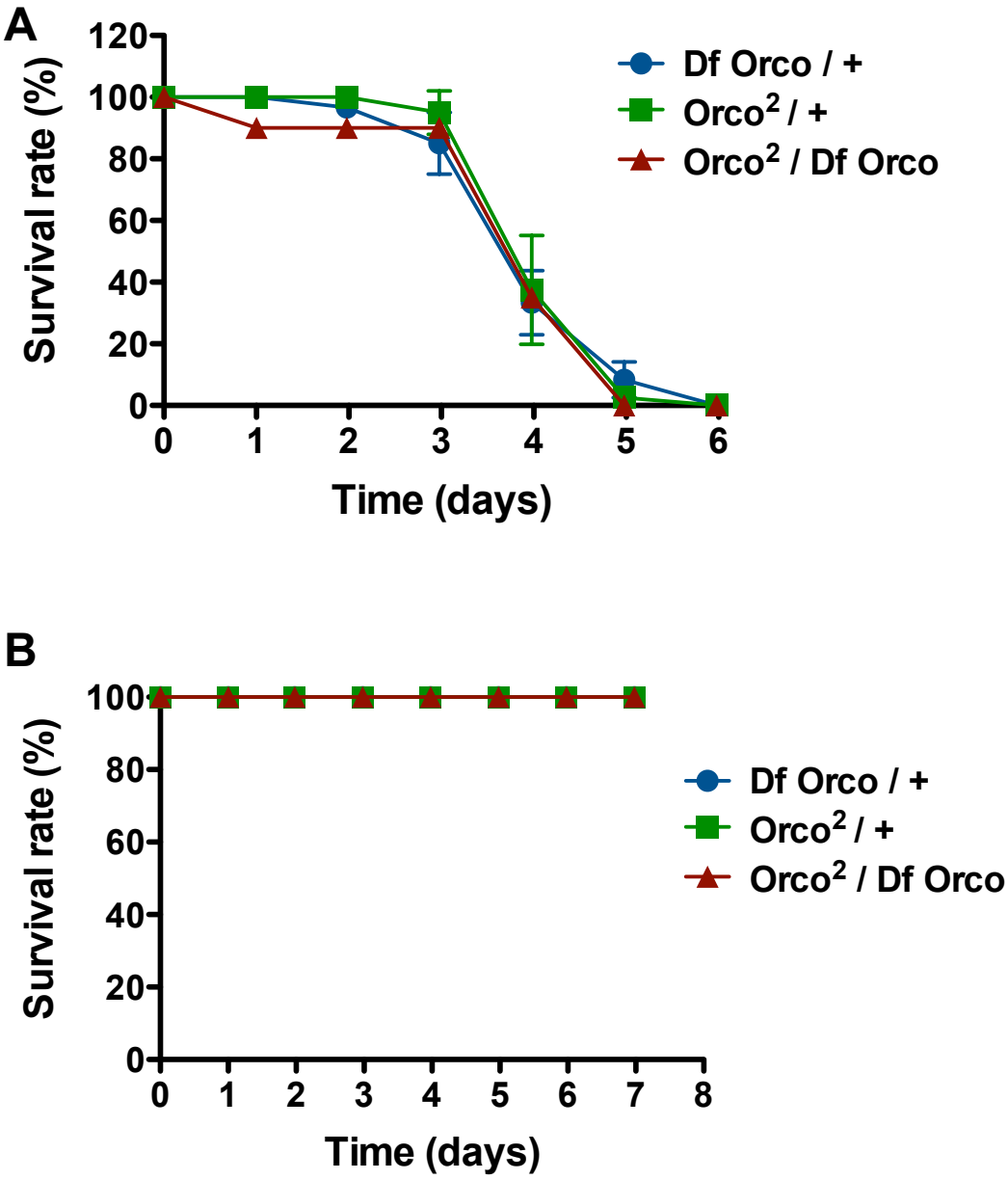

Survival rate (%)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (days)
Group of 20 flies / PA14
Single flies (10x1 fly) / PA14

Figure 3


A
Survival rate (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)
Df Orco / +
Orco2 / +
Orco2 / Df Orco
B
Survival rate (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)
Df Orco / +
Orco2 / +
Orco2 / Df Orco

Chapter 3

The Toll pathway is likely triggered by both its PRRs and virulence activity detection branches during *P. aeruginosa* infection

The aim this side project was to identify the branches of the Toll pathway that detect *P. aeruginosa* bacterial infection and lead to the activation of this pathway. My results are written as a small chapter as further experiments are required to confirm that both arm of Toll pathway activation can indeed detect PA14.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium and thus contains within its cell-wall a DAP-type peptidoglycan (PGN) layer. As described before, this type of PGN is detected by PRRs like PGRP-LC and PGRP-LE and leads to the activation of *Drosophila* IMD pathway. However, others and we have shown that besides the IMD pathway, the Toll pathway is also activated and required in the defense against wild-type PA14.

The *Drosophila* Toll pathway can be activated in different manners, either by the recognition of microbe cell-wall composition (yeast β-glucans by GNBP3 or Gram-positive bacteria Lys-PGN by GNBP1 and PGRP-SA) or the proteolytic activity of some virulence factors (fungal protease that cleaves Persephone). Moreover it has been shown that GNBP1 and PGRP-SA likely act together in a complex to activate the Toll pathway and that PGRP-SA can also bind to DAP-type PGN but with a much lower affinity than to Lys-type PGN. The analysis of the structure of PGRP-SD, another *Drosophila* PRR involved in Toll pathway activation, suggests that this PRR preferentially binds to DAP-type rather than to Lys-type PGN.

P. aeruginosa is highly virulent and secretes proteases upon infection, especially through its T2SS. Therefore *P. aeruginosa* could potentially induce Toll pathway either via the recognition of its PGN, the proteolytic activity of virulence factors, or both.

The aim of this project was to identify how PA14 activates *Drosophila* Toll pathway in our intestinal infection model of flies since usually only the IMD pathway is strongly activated by Gram-negative bacterial infections.

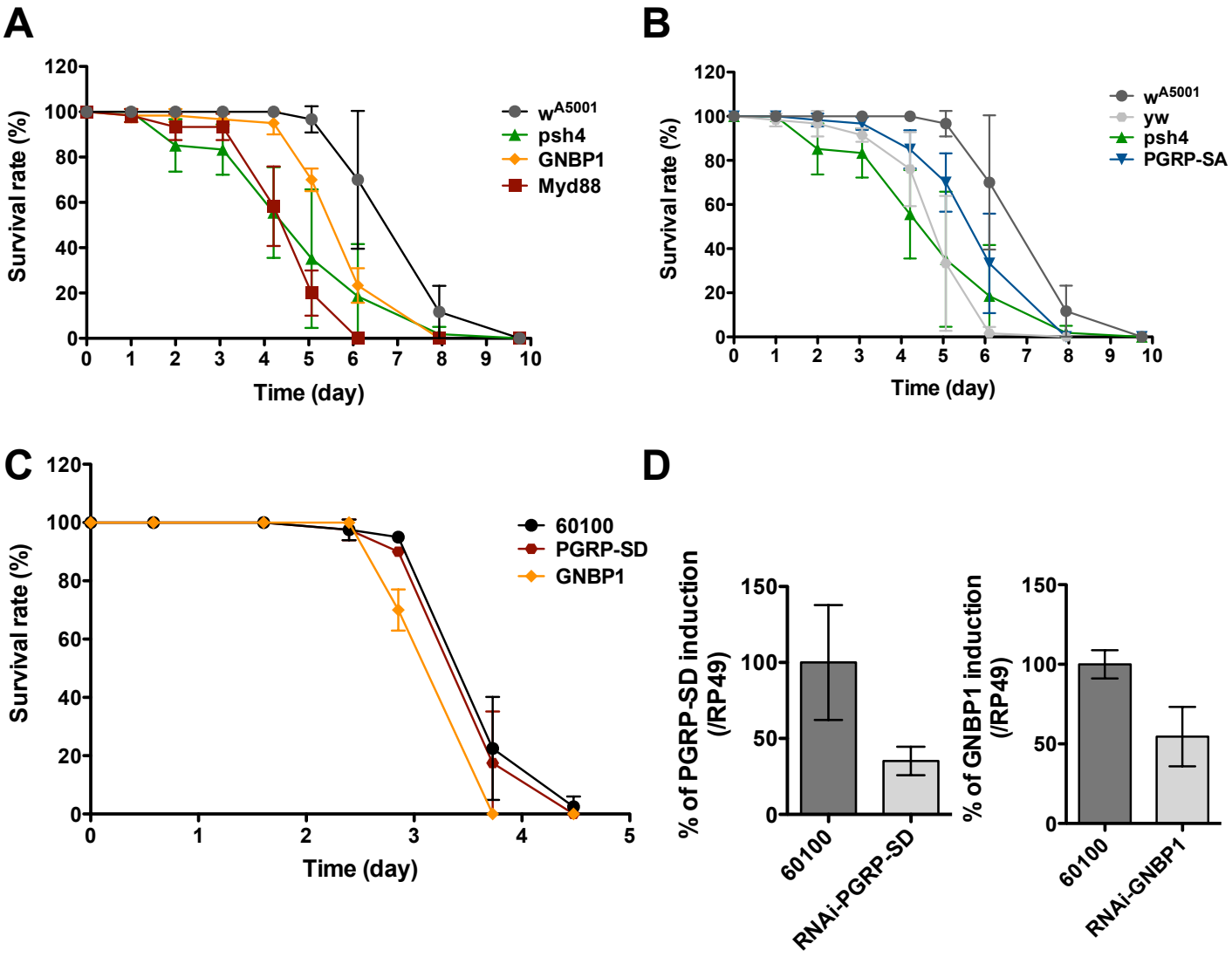


Figure 9. Both branches of Toll pathway activation are required against ingested PA14.

Different Toll pathway *Drosophila* mutants were orally infected with PA14 wild-type and the survival of flies was monitored. (A) *Myd88*, *GNBP1* and *psh4* mutants flies were more susceptible to PA14 infection than wild-type flies (w^{A5001}). Among them, *Myd88* mutants presented the strongest decrease in survival rate. (B) *PGRP-SA* mutants flies displayed an intermediate survival phenotype between w^{A5001} and *yw* control flies. Unexpectedly, *yw* flies were much more susceptible to PA14 infection than w^{A5001}. For (A) and (B) data presented means ±SD of biological triplicates from one representative experiment out of three. (C) Fly lines possessing a UAS-RNAi construct against *PGRP-SD* or *GNBP1* and the wild-type control line 60100 were crossed to the driver fly line p-*C564 Gal4 Gal80ts*. F1 progeny were infected with PA14 and the survival of flies monitored. Only flies expressing a RNAi construct against *GNBP1* (GNBP1) were more susceptible to the PA14 infection than the wild-type control (60100). Means ±SD of biological duplicates from one experiment out of two are presented. (D) Efficiency of RNAi was measured for RNAi against *PGRP-SD* and *GNBP1*. For both, a mean of around 50% reduction of transcript was observed. However, given the high variability, these difference were not significant. Means ±SD from one experiment with duplicates are presented; statistical analysis were performed with an unpaired t test.

Results and discussion

Both arms of Toll pathway activation, proteolytic activity detection arm and PGN recognition may be required against PA14

Drosophila null mutants for different elements of Toll pathway were available in our laboratory. First, I decided to monitor the survival some of Toll pathway PRR mutants after an intestinal infection with PA14. I used psh^4 (*Persephone* mutants), *GNBP1*, *Myd88,* and *PGRP-SA* mutant fly lines. As control flies, I used both w^{A5001} and *yw* as *PGRP-SA* flies were generated in the *yw* background and the other mutants in the w^{A5001} background. Homozygotes female flies were sorted and orally infected with wild-type PA14. Flies were kept at 25°C.

As reported before, *Myd88* mutants flies are much more susceptible to PA14 infection than w^{A5001} (Fig. 9 A). Myd88 is a central component of the intracellular part of the Toll signaling pathway involved in the transduction of signals from both arm of Toll pathway. *GNBP1* and psh^4 mutant flies displayed also a decreased survival rate compared to the wild-type flies. These mutants presented an intermediate phenotype between *Myd88* and w^{A5001} (Fig. 9 A). Surprisingly, *yw* control flies displayed a strong susceptibility to PA14 infection as compared to w^{A5001} control flies and *PGRP-SA* mutants exhibited an intermediate survival rate between these two control flies (*yw* and w^{A5001}) (Fig. 9 B).

These results suggested that Persephone and at least GNBP1 are required in host defense against ingested PA14. However as none of them reached the susceptibility phenotype of *Myd88* (which was used here as a positive control), its lead us to hypothesize that both arm of Toll pathway activation are required in the defense against PA14. To verify this hypothesis, we should for the next step, monitor the survival of a *Drosophila psh-GNBP1* double mutant after PA14 infection. As T2SS effectors are involved in virulence, it would be interesting to test whether they display an enhanced virulence when infecting psh^4 mutant flies.

PGRP-SD involvement in the defense against PA14?

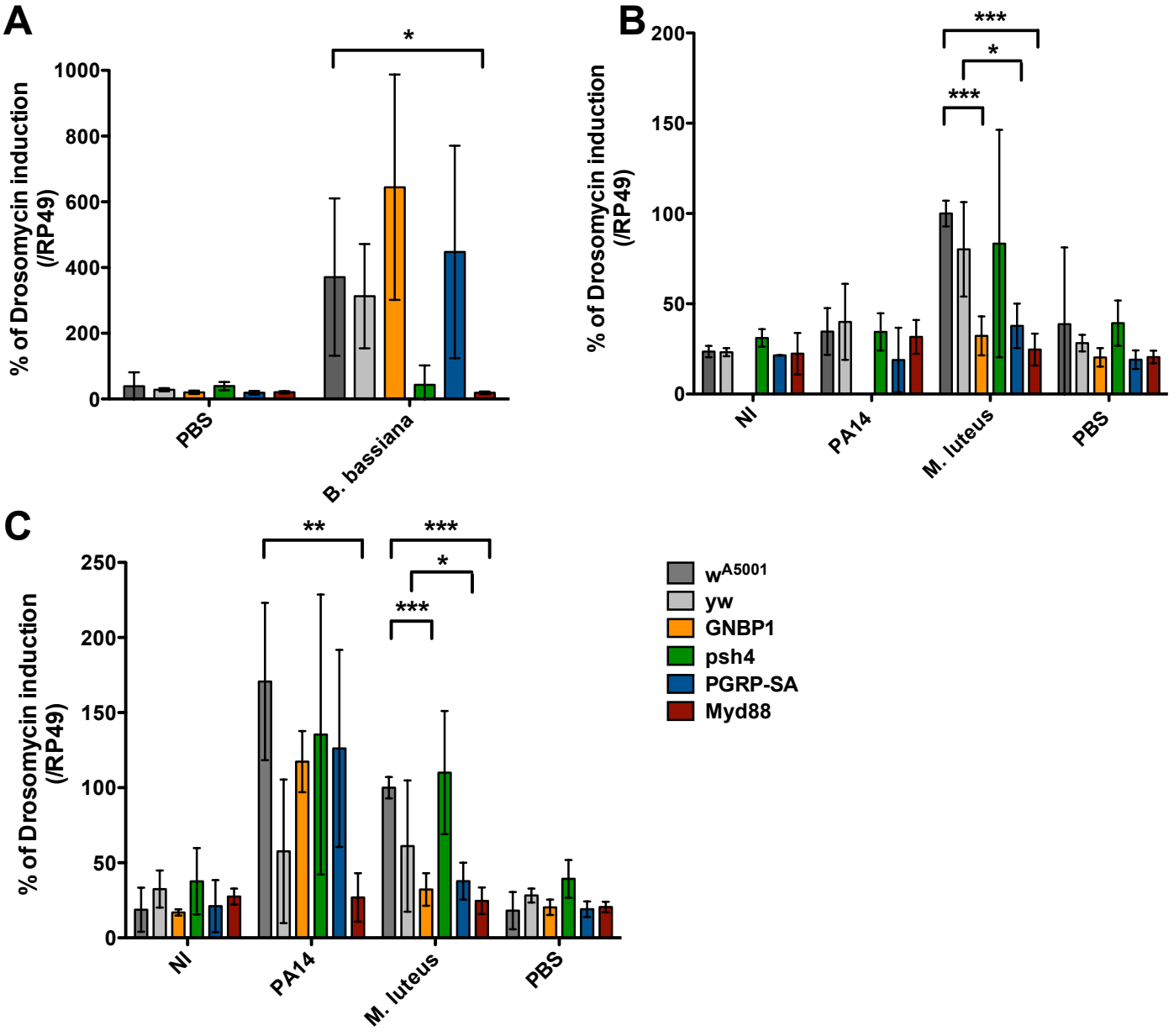


Figure 10. PA14 might activate Toll through both recognition of its PGN and through sensing of its virulence factors.

Toll pathway *Drosophila* mutants were orally infected with PA14 wild-type. At different time points of the infection flies were frozen, then RNA extracted and reverse transcribed and a quantitative PCR performed to analyze Toll pathway activation using *Drosomycin* expression as a read-out. (A) Flies infected with *B. bassiana*. A strong reduction of *Drosomycin* induction was detected in *psh[4]* and *Myd88* mutants flies when compared to control flies. However these reductions were only significant for *Myd88* flies (*p=0.0286). (B) Three days after infection with PA14 only a basal level of Toll pathway activation was detected compared to the flies infected with *M. luteus* (positive control). *Myd88*, *GNBP1* and *PGRP-SA* mutants flies presented a significant decrease of *Drosomycin* induction compared to their respective control (*Myd88*: ***p<0.0001, *GNBP1*: ***p<0.0001 and *PGRP-SA*: *p=0.0336). (C) At day five of PA14 infection, a significant decrease of *Drosomycin* induction was detected in *Myd88* mutant flies as compared to control (**p=0.0019). Moreover a reduced activation was also observed in *GNBP1* and in a smaller proportion with a high variability in *psh[4]* mutant flies. For all, means ±SD of pooled results from two experiments are presented. Statistical analyses were performed using an unpaired t test.

Our previous results suggested that the PGN sensor GNBP1 is able to detect and activate Toll pathway. PGRP-SD, has been suggested to bind to DAP-type PGN. Therefore we needed to test if this PRR is involved in the defense against PA14. No *PGRP-SD* null mutant flies were available at that time and I decided to try to use transgenic fly lines that allow the expression of an RNAi construct targeted against *PGRP-SD*. We ordered that fly line from the Vienna *Drosophila* Research Center. At the same time, I also ordered an RNAi fly line against *GNBP1* and the appropriate control flies 60100 for this type of construct.

These RNAi and control fly lines were crossed with a driver line p-*C564 Gal4 Gal80ts* at 18°C. F1 progeny were transferred to 29°C for six days and then female flies were infected with wild-type PA14 bacteria. C564/RNAi-*GNBP1* (GNBP1) presented a slight decrease of survival rate compared to the controls (60100) and no difference in survival could be observed between C564/RNAi-PGRP-SD (PGRP-SD) and control flies (60100).

I verified the efficiency of the RNAi from these two constructs by performing a quantitative RT-PCR on the cDNA. I observed a 50% decrease of expression for both genes, which however was not significant given the high variability. Because we have generated only a weak hypomorphic phenotype, we cannot exclude that *PGRP-SD* is involved in host defense against ingested PA14.

PA14 seemed to activate Drosophila Toll pathway by both recognition of its PGN and detection of virulence factors

Survival assays presented above suggested that both Persephone and GNBP1 were required in the defense against PA14 infection. GNBP1 is known to function complexed with PGRP-SA. However, survival results obtained with *PGRP-SA* mutants flies and the *yw* control flies suggested rather that PGRP-SA was not required against PA14, unless there is a problem with this control line. Indeed, *PGRP-SA* mutant flies were more susceptible to PA14 infection than another wild-type control (w^{A5001}). Given these contradictory survival results, I decided to analyze directly the level of Toll pathway activation by monitoring the level of *Drosomycin* expression.

I orally infected different Toll pathway mutant fly lines and the appropriate wild-type control flies with wild-type PA14. As a positive control for Toll pathway induction, I infected Toll pathway mutant flies either by a septic injury with *Micrococcus luteus* or by a natural infection with *Beauveria bassiana*. *M. luteus* is a Gram-positive bacterium that activates Toll

pathway through the recognition of its Lys-type PGN by GNBP1 and PGRP-SA. *B. bassiana* is a fungus that activates *Drosophila* Toll pathway via the proteolytic activity of the fungal virulence factor PR1 that cleaves and thereby activates Persephone. Flies were kept at 25°C with 60% humidity. At different time points of infection, for instance at three and five days, I froze some of these infected (PA14) and uninfected (NI) flies. Then I extracted RNA, performed a reverse transcription of these RNA and a quantitative PCR to measure the level of *Drosomycin* induction GOTTAR et al.

As reported before, *Persephone* (psh^4) and *Myd88* mutant flies exhibited a much lower *Drosomycin* induction after *B. bassiana* infection than both wild-type flies (w^{A5001} and *yw*) (Fig. 10 A). On the contrary *GNBP1* and *PGRP-SA* mutant flies presented a strong level of Toll pathway activation. In the same way, *GNBP1* and *PGRP-SA* mutants displayed a similar reduction of *Drosomycin* induction than *Myd88* mutant flies after an infection with *M. luteus* (Fig. 10 B). *Persephone* mutants (psh^4) exhibited a strong induction of *Drosomycin* expression after *M. luteus* infection that was compounded by a high variability that did not allow us to conclude in this experiment if Toll pathway activation was affected or not in this mutant as has been previously published.

After three days of PA14 oral infection, all mutant and wild-type flies presented a basal level of Toll pathway activation that is similar to the uninfected flies and the sterile PBS pricked flies (Fig. 10 B). This observation is in keeping with our previous data and correlates with the low hemolymph bacterial titer at this early stage. Of note, *GNBP1* mutant flies were not assessed for this early time point. Five days after the beginning of the PA14 infection, a strong Toll pathway activation was detected in w^{A5001} wild-type flies but not in *yw* flies (Fig. 10 C). *Myd88* mutant flies presented a significantly reduced *Drosomycin* expression compared to its wild-type control (w^{A5001}). *GNBP1* mutant flies displayed a reduced level of *Drosomycin* induction that was however not significant. Again, *psh4* presented high variability in Toll pathway activation at day five of PA14 infection, and *Drosomycin* expression seemed only slightly decreased compared to w^{A5001}. A modest reduction of *Drosomycin* inducibility in single mutants has already been observed for *GNBP3* and *Persephone* mutants after a challenge with *Candida albicans*.

These results suggested that GNBP1 is required but not sufficient to fully induce Toll pathway activation by PA14. The very low level of *Drosomycin* induction in *yw* flies compared to the other wild-type control flies (w^{A5001}) did not permit to assess the involvement of *PGRP-SA* in Toll pathway activation by PA14. The results of the analysis of *Drosomycin*

induction are compatible with the hypothesis of a dual activation of the Toll pathway through both the Persephone and the PGRP-SA/GNBP1 branches.

Further investigations would be needed to determine whether PA14 virulence factors actually cleave Persephone to induce Toll pathway activation. Of note, a Western blot on hemolymph with a Persephone-specific antibody would allow to directly determine whether Persephone gets activated during the late phase of the infection. The use of a *GNBP1-Persephone* double mutant should allow concluding on the necessity or not of both recognition of PGN and detection of virulence factors from PA14 to fully activate Toll pathway. Indeed, *GNBP3-Persephone* double mutants displayed both a strong susceptibility as well as abolished *Drosomycin* induction after a live *C. albicans* challenge. The use of *xcpR* mutants that have an impaired T2SS would also be interesting as they might prevent the secretion of the proteases that target Persephone. In this case, *Drosomycin* induction and resistance to infection would be expected to depend only on the PRR branch. This would however require an analysis in a background in which the cellular immune response is impaired to allow virulence and thus a systemic infection since no activation of Toll is possible in the absence of bacteremia, which likely does not occur or occurs only late in wild-type flies.

Moreover, we cannot formally exclude the possibility that another "virulence sensor" in *Drosophila* would act in parallel or upstream of Persephone and permit Toll pathway activation by PA14 proteases.

The *yw* strain that was used as a control for *PGRP-SA* displayed a unusual behavior. Of note, the *PGRP-SA* mutation had been isolated in this background about 15 years ago. Thus, the *yw* line may have accumulated since mutations that alter its behavior. The striking observation is that it responds like other wild-type fly lines when challenged with *B. bassiana* or *M. luteus*. However, when challenged with PA14, there is an apparent low induction of *Drosomycin*, which is in keeping with its enhanced sensitivity to this pathogen. This phenotype is difficult to account for. One formal possibility is that an upstream sensor that is activated by PA14 is inactivated in this mutant background.

In summary, the *GNBP1* and *psh* survival data point to a possible dual detection through both arms of the Toll activation pathway, which will need to be confirmed as outlined above.

Chapter 4

The enteric Nora virus affects the homeostasis of the intestinal epithelium and promotes the growth of microbiota while sensitizing *Drosophila* to bacterial infections

The aim of this project was to study the effect of an enteric virus on the fitness of Drosophila when or not associated to another infection as we have observed that Nora infected flies were more susceptible to various conditions.

This chapter is written under a scientific paper format, as we want to submit it in the near future.

My contribution to this work corresponds to all PA14 infection experiments, all the pH3, qPCR and microbiota analysis in the gut. In addition, I was in charge of the last part of the paper that corresponds to the generation of Nora-positive flies by ingestion of the Nora virus and most of the corresponding phenotype analysis.

The enteric Nora virus affects the homeostasis of the intestinal epithelium and promotes the growth of microbiota while sensitizing *Drosophila* to bacterial infections

Samantha Haller[a], Vincent Barbier[b], Stefanie Müller[b1], Laurent Daeffler[b], Kwang-Zin Lee[a], Igor Orlov[c], Danièle Spehner[d], Jean-Luc Imler[b] and Dominique Ferrandon[a*].

[a] Equipe Fondation Recherche Médicale, Unité Propre de Recherche 9022 du Centre National de la Recherche Scientifique, Institut de Biologie Moleculaire et Cellulaire, Université de Strasbourg, F67084 Strasbourg Cedex, France; [b] Unité Propre de Recherche 9022 du Centre National de la Recherche Scientifique, Institut de Biologie Moleculaire et Cellulaire, Université de Strasbourg, F67084 Strasbourg Cedex, France; [c] UMR 7242, Université de Strasbourg-CNRS, ESBS, Blvd Sébastien Brant, Strasbourg, Illkirch F-67413, France; IGBMC (Institute of Genetics and of Molecular and Cellular Biology), Department of Integrative Structural Biology, Centre National de la Recherche Scientifique, UMR 7104/Institut National de la Santé de la Recherche Médicale (INSERM) U964/Université de Strasbourg, Illkirch, France; and [d] IGBMC; CNRS, UMR 7104; Inserm U 596; Illkirch, F-67400 France; Université Louis Pasteur, Strasbourg, F-67000 France;

[1] Present address: Bernhard-Nocht-Institut for Tropical Medicine, Molecular Entomology, Hamburg, Germany.

*To whom correspondence may be addressed. E-mail: d.ferrandon@ibmc.u-strasbg.fr.

INTRODUCTION

Viruses represent a major component of biodiversity, largely through numerous bacteriophages that affect the most ubiquitous component of life on earth. With an estimated number of species reaching millions, insects represent the largest class of metazoans (Mora et al., 2011). Therefore, insects are also likely to provide the largest source of metazoan viruses and thus specific pathologies that may yield interesting insights into the biology of their hosts. *Drosophila melanogaster* is a genetic model insect. Mostly RNA viruses are known to infect

Drosophila, including the negative-stranded RNA sigma rhabdovirus, the double-stranded RNA *Drosophila* X virus, and positive-stranded RNA viruses of the *Picornavirales* order (Lamiable and Imler, 2014; Xu and Cherry, 2014). The latter comprises the *Dicistroviridae* family, which contains picorna-like viruses such as *Drosophila* C virus. Recently, an unconventional picorna-like virus has been identified, the Nora virus (Habayeb et al., 2006). Unlike other insect picorna-like viruses, its genome encodes four open reading frames corresponding to a suppressor of RNA interference, VP1 (van Mierlo et al., 2012), replicative proteins coded by ORF2, the poorly characterized product of ORF3, and capsid proteins derived from ORF4 (Ekstrom et al., 2011). This virus infests common laboratory stocks where it appears to cause a persistent infection. It is transmitted horizontally and vertically via a fecal-oral route (Habayeb et al., 2009; Habayeb et al., 2006). In keeping with this mode of transmission, the Nora virus is enteric, although it can produce an infection when injected systemically (Habayeb et al., 2009). Even though it has so far not been detected to a specific location within the digestive tract, it is likely to proliferate there as large quantities of the virus are continuously released with the feces of infected flies. The virus does not appear to have major effects on host fitness, even though some damages to the intestinal epithelium have been reported (Habayeb et al., 2009).

As for vertebrates, the *Drosophila* intestinal epithelium is simple, formed mostly by a monolayer of columnar enterocytes (Shanbhag and Tripathi, 2009). Other cell types found in the midgut epithelium include enteroendocrine cells and enterocyte progenitor cells, that is, intestinal stem cells (ISCs) and enteroblasts (Jiang and Edgar, 2011). Whereas the foregut and hindgut are protected by cuticle, the midgut is lined with a protective membrane, the peritrophic matrix, which, like mucus, prevents the direct contact of ingested food and microbes with the absorptive and secretory intestinal epithelium. The microbiota is made up of few species, at most twenty, and is usually dominated by two-three prevalent species such

as *Acetobacter pomorum*, *Lactobacillus plantarum*, or *Enterococcus faecalis* (Broderick and Lemaitre, 2012). Of note, the microbiota is relatively scarce in young flies, represented by about a thousand bacteria, but can increase considerably in older flies. The maintenance of the microbiota requires constant replenishment, as feeding flies with sterile food leads to its progressive loss (Blum et al., 2013).

Innate immunity in *Drosophila* had essentially been studied using a septic injury paradigm in which nonpathogenic or poorly pathogenic bacteria were introduced within the hemocoel through a contaminated needle or through injection (Boman et al., 1972; Lemaitre and Hoffmann, 2007). More recently, several intestinal infection models have been developed (Basset et al., 2000; Flyg et al., 1980; Nehme et al., 2007; Vodovar et al., 2005). These models and others have underlined the importance in the midgut epithelium of one of the two NF-kappaB signaling pathways that mediate systemic immunity, namely the Immune deficiency (IMD) pathway (Liehl et al., 2006; Nehme et al., 2007; Ryu et al., 2006). Interestingly, the activation level of this pathway is finely regulated through multiple loops of negative regulation and set-up by the microbiota (Guo et al., 2014; Lee and Ferrandon, 2011; Paredes et al., 2011). A dysfunction of the IMD pathway may modify the composition of the microbiota and lead to the proliferation of pathogenic bacteria (Ryu et al., 2008). A second arm of intestinal defense is provided by the Dual oxidase enzyme, which secretes Reactive Oxygen Species (ROS) when uracil is secreted by ingested microbes (Kim and Lee, 2014).

An important discovery stemming from the study of infection models as well as noxious chemicals is that of the importance of homeostasis of the midgut epithelium in the host defense (Ayyaz and Jasper, 2013; Ferrandon, 2013; Jiang and Edgar, 2011). Indeed, pathogenic microbes attack the enterocytes through secreted virulence factors. As a consequence, epithelial cells are stressed and may undergo apoptosis or anoikis. Stress and induced cell death lead to the emission of cytokines, for instance ligands that activate the

Janus kinase-Signal transducers and activators of transcription (JAK-STAT) pathway, which ultimately drive an enhanced compensatory proliferation of ISCs. Alternatively, the ROS response may be too potent and effectively kill a large proportion of enterocytes, with gut size being halved in a few hours (Buchon et al., 2010). Again, the compensatory proliferation of ISCs restores normal gut morphology and presumably function. Importantly, when ISC proliferation is hampered, flies succumb to infections as rapidly as when their innate immune response is deficient. We refer to this property of being able to endure and repair damages inflicted either by the pathogen or the host's own immune response as resilience (Ferrandon, 2013), also known as tolerance (Medzhitov et al., 2012).

We have been investigating two intestinal infection models in which flies feed on either of two opportunistic pathogens, the Gram-negative bacteria *Serratia marcescens* or *Pseudomonas aeruginosa* (Limmer et al., 2011; Nehme et al., 2007). When ingested, *S. marcescens* strain Db11 inflicts damages to the midgut epithelium during the steady-state phase of the infection. Both the IMD and the JAK-STAT pathway play important roles in host defense against the ingested entomopathogen (Cronin et al., 2009; Nehme et al., 2007). In contrast, flies that feed on *P. aeruginosa* strain PA14 undergo little apparent gut damage under our conditions (Limmer et al., 2011), a result somewhat at odds with data published by another laboratory (Apidianakis et al., 2009). The common property of both pathogens is the ability of a few bacteria to rapidly cross the intestinal barrier and to reach the hemocoel. Whereas *S. marcescens* is effectively controlled there by phagocytosis through the Eater phagocytic receptor (Kocks et al., 2005; Nehme et al., 2011), *P. aeruginosa* ultimately prevails thanks to the quorum-sensing regulator RhlR that allows PA14 to circumvent the cellular arm of host defense (Limmer et al., 2011).

Here, we demonstrate that Nora virus is a co-factor that synergizes with ingested bacteria in our intestinal infection models, thus leading to an earlier demise of infected flies. We also

report major effects of Nora infestation on the lifespan of the flies and also on the survival of flies feeding on a sucrose solution. The presence of Nora leads to a higher inflammatory response when fed pathogenic bacteria. Unexpectedly, Nora infested flies display a microbiota load that is higher than that measured in uninfested flies by four orders of magnitude. Nevertheless, the microbiota does not appear to be responsible for the increased rate of ISC proliferation we have systematically observed in our experiments.

RESULTS

Nora virus-infested stocks are shorter-lived and more susceptible to some infections

We noted that two Ore-R wild-type stocks kept by different investigators in the laboratory displayed a remarkably distinct survival pattern to systemic DCV infection (Fig. 1A). These stocks, Ore-R(SM) and Ore-R(SC), displayed the same differential sensitivity in two models of intestinal infections in which flies feed either on *Pseudomonas aeruginosa* or *Serratia marcescens* (Fig. 1B-C). We found one notable difference between the two stocks: the infection-sensitive Ore-R(SM) stock harbored the Nora enteric virus (Fig. 1D). Interestingly, flies from another reference stock, *white*, kept independently by several investigators were also found to differ in their sensitivity to enteric infections by *P. aeruginosa* and *S. marcescens*; the more sensitive flies were found to be infested with the Nora virus (data not shown). We therefore monitored the gut of the two Ore-R stocks and did not detect any major morphological differences between the two stocks. However, it is an open possibility that damaged enterocytes might be replaced by an enhanced proliferation of ISCs, thus maintaining the homeostasis of the intestinal epithelium. To measure ISC proliferation, we counted phospho-histone H3-positive cells in the intestine and found a strongly enhanced rate of ISC proliferation in the Nora-positive Ore-R (SM) stock (Fig. 1E). When we challenged

the Nora-negative Ore-R (SC) stock with *P. aeruginosa*, we found a small but nevertheless significant increase in ISC proliferation. However, this *P. aeruginosa*-induced increase was larger in the Nora-positive stock (Fig. 1E).

As we had noted in our bacterial ingestion experiments that control uninfected Nora-positive flies feeding on sucrose died more rapidly than control Nora-negative flies, we directly monitored flies feeding on a sucrose-only diet for a longer period. Whereas half the Nora-negative Ore-R (SC) flies had died by about 16 days of this regimen, 50% of Nora-positive Ore-R (SM) flies were deceased at about 10 days (Fig. 1F). We also monitored the life span of these two stocks and noted that the Ore-R(SM) flies died much earlier than the Ore-R(SC) flies (Fig. 1G).

The Nora virus is required for the enhanced susceptibility to intestinal infections but not to DCV infection

An effective procedure to eradicate Nora virus infection is to bleach eggs laid by infected females (Habayeb et al., 2009). Indeed, when we treated the Nora-infested Ore-R(SM) stock, we were not able to detect by qRT-PCR Nora virus in G0 flies and subsequent generations (Fig. 2A and data not shown). This treatment did not have a noticeable adverse impact as bleached Ore-R(SM) and Ore-R(SC) flies displayed a normal survival on regular food for at least ten days (Fig. 2B and data not shown). Remarkably, the cured Ore-R(SM) stock (now Nora-negative) succumbed slightly earlier to infection with DCV than the uncured Ore-R(SM) stock harboring Nora (Fig. 2B), thus showing that the initial presence of Nora in this stock is not causing the enhanced susceptibility of this Ore-R stock to DCV infection. Rather, a polymorphism in the gene *pastrel* was found to be the cause of the different susceptibility to DCV infection (Magwire et al., 2012).

However, we found that cured Ore-R(SM) flies fed *P. aeruginosa* succumbed later to this intestinal challenge than the uncured stock (Fig. 2C), which opens the possibility that Nora and not the distinct *pastrel* alleles harbored by the two Ore-R stocks may cause the enhanced sensitivity to intestinal infections.

In the following, we used as a Nora-negative stock the original Ore-R(SM) stock that had been cured of its Nora infection.

Reinfection of the cured stock with Nora through fecal contamination restores the sensitivity to bacterial intestinal infections

An easy way to contaminate flies with Nora virus is to place flies in a vial that had hosted infected males for several days for fecal-oral transmission (Habayeb et al., 2009). Indeed, we found that our cured flies became again Nora-positive over several generations when we submitted them to this procedure (Fig. S1A). We consistently observed over several generations that the reinfected stock was more sensitive to the ingestion of *P. aeruginosa* than the cured stock (Fig. S1B). This correlated with an enhanced ISC proliferation in the Nora reinfected flies, whether challenged with *P. aeruginosa* or not (Fig. S1C).

Reinfection of the cured stock with a purified Nora virus preparation restores the sensitivity to bacterial intestinal infections

The drawback of the fecal transmission route is that other enteric pathogens may be transferred along Nora virus and thus might account for the enhanced sensitivity to oral challenges. To exclude this possibility, we used gradient centrifugation to purify and concentrate Nora virus from an infected fly extract. This preparation was homogenous with particles of the expected size when observed by cryo-electron microscopy (Fig. 3A). The identity of the virus was confirmed by qRT-PCR (data not shown). Cured flies were fed on this pure viral preparation for 24 hours. This was sufficient to stably reinfect the stock over

several generations (Fig. 3B). As with the fecal contamination route, we observed that the flies reinfected with the pure viral preparation were more sensitive to the ingestion of *P. aeruginosa* (Fig. 3C) and displayed an enhanced rate of ISC proliferation in the gut (Fig. 3D). All together, these results demonstrate that Nora virus is responsible for the enhanced sensitivity to bacterial intestinal infections.

Nora virus causes damages to the intestinal epithelium

In the following, we further characterized the impact of Nora virus on infected flies using the Ore-R(SM) stock that had been reinfected with the pure Nora preparation, which will be referred to as Nora-positive flies. In contrast, the negative control will be the Nora-negative Ore-R(SM) cured stock.

The JAK-STAT signaling pathway is one of the major regulators of the rate of ISC compensatory proliferation occurring during infections or intestinal epithelium damage (Apidianakis et al., 2009; Buchon et al., 2009; Cronin et al., 2009; Jiang et al., 2009). We used *SOCS36E* gene expression as a read-out of JAK-STAT pathway activation. Nora-positive flies displayed an increased rate of *SOCS36E* expression, both in unchallenged and *P. aeruginosa*-challenged flies. Unexpectedly, we did not detect an enhanced induction of this gene by the ingestion of *P. aeruginosa*, whether the flies were Nora-positive or negative (Fig. 3E). As expected, SDS treatment induced the JAK-STAT pathway in both Nora –positive and –negative stocks.

As monitored by *Diptericin* expression, the IMD pathway was more induced in Nora-positive flies in the absence of *P. aeruginosa* infection (Fig. 3F). As expected, the IMD pathway was induced four-fold by the ingestion of *P. aeruginosa* by Nora-negative flies. In striking contrast, Nora-positive flies that fed on *P. aeruginosa* displayed a strong 50-fold induction of

Diptericin expression. We also noted a strong induction of the IMD pathway in Nora-positive flies fed on detergent solution (Fig. 3F), which causes enterocyte death.

We have shown previously that flies feeding on *P. aeruginosa PA14* ultimately succumb to a systemic infection resulting from the passage of the bacteria from the intestinal tract to the hemocoel. We therefore monitored the bacterial titer in the hemolymph and found a significant 20-fold increase in the number of circulating bacteria of Nora-positive flies on the third day of the infection, which confirmed the trend of a somewhat increased titer observed at day two (Fig. 3G).

Nora influences the survival of flies to septic injury

Since *P. aeruginosa* ultimately causes a systemic infection, we also tested whether Nora-positive flies would be more sensitive to other types of systemic infections. We first exposed flies to spores of the entomopathogenic fungus *Beauveria bassiana*. In this paradigm of infection, spores adhere and germinate onto the fly cuticle and secrete proteases and chitinases that allow it to pass in the body cavity. No macroscopic wounds are inflicted as the spores bore microscopic holes in the cuticle. As shown in Fig. S2A, we did not observe any difference in the survival curves between Nora-positive or -negative flies. In contrast, we noted that in septic injury models with the Gram-negative bacterial pathogen *Enterobacter cloacae* and the Gram-positive bacterial pathogen *Enterococcus faecalis*, Nora-positive flies succumbed significantly earlier than Nora-negative control flies (Fig. S2B-C). In this latter model, bacteria are introduced in the hemocoel using a sharp needle previously dipped in the bacterial suspension. We therefore asked whether Nora-positive flies were more sensitive to infection or injury *per se*. We found that Nora-positive flies pricked with a needle dipped into a sterile PBS solution succumbed at a rate that was similar to that observed in flies submitted to a septic injury, whereas control Nora-negative flies were more resistant to aseptic injury

(Fig. S3A). Unexpectedly, *Myd88* flies displayed an enhanced apparent sensitivity to a clean injury. To understand why Nora-infected flies were more susceptible to a near-sterile wound, we measured the proliferation rate of ISCs as it has been reported that injury triggers a ROS-response in the gut that leads to enterocyte apoptosis and an increased compensatory proliferation of ISCs (Takeishi et al., 2013). As reported, we observed a modest albeit significant increase of ISC proliferation in Nora-negative flies after injury (Fig. S3B). Nora-positive flies displayed a much higher rate of ISC proliferation that was however not altered by injury, suggesting that gut damage is unlikely to account for the enhanced sensitivity of Nora-positive flies to wounding. We finally compared the survival rates of flies pricked with a clean needle to that of unchallenged controls and did not find any significant difference (Fig. S3C). We conclude that the apparent sensitivity of Nora-positive flies to clean injuries is actually due to the shortened lifespan of Nora-positive flies.

Nora virus influences life span, the survival of flies feeding only on sucrose, and the microbiota

As observed earlier when comparing Nora-infested to Nora-free stocks (Fig. 1G), we found that Nora-positive flies exhibited a strongly shortened lifespan (half-life decreased by more than 15 days) with respect to Nora-negative flies (Fig. 4A). We confirmed with Nora-positive re-infected flies the results obtained with the naturally infested Ore-R(SM) Nora-positive flies with respect to nutrition on a sucrose solution (Fig. 1F): Nora-positive flies succumbed much earlier to this regimen than Nora-negative control flies (Fig. 4B). To investigate why Nora-positive flies succumb earlier to feeding on sucrose, we first measured glycogen stores (Fig. S4A) and found no significant difference between Nora-positive and –negative flies on this diet. This observation suggests that these flies are able to transport efficiently the ingested sugar across the intestinal epithelium. Next, we measured triglyceride stores and unexpectedly found a significant depletion after both five and eight days of the sucrose regimen in Nora-

positive flies (Fig. 4C). We then analyzed the composition and amount of microbiota in Nora-positive flies. To this end, gut extracts were subdivided and plated on different selective media (Guo et al., 2014). We also plated the extracts on rich medium. As shown in Fig. 4D, the Nora-positive flies harbored a dramatically-enhanced microbiotal load by four orders of magnitude when fed on sucrose solution. Similar observations were made on selective plates, suggesting that this increased amount of microbiota may not be accounted for by the overgrowth of a specific bacterial species (Fig. S4B). We also observed a similar difference in old flies feeding on regular medium: at an age at which up to 80% of Nora-positive flies were already deceased, the surviving flies also harbored four Logs more microbiotal bacteria (Fig. 4D). To investigate whether the microbiota is responsible for the enhanced sensitivity of Nora-positive flies to a sucrose regimen, we added to the sucrose solution a mixture of five different antibiotics (ampicillin, neomycin, vancomycin, metronidazole, and tetracyclin). Nora-positive flies submitted to the sucrose/antibiotics treatment succumbed at the same rate as control Nora-positive flies fed on sucrose only (Fig. 4E). We also observed that control Nora-negative flies fed the sucrose/antibiotics solution became somewhat susceptible to this regimen, as compared to Nora-negative controls fed on a sucrose-only solution. This suggests that the microbiota is required to help the flies survive this protein- and fat-depleted diet, in keeping with its role during larval development (Shin et al., 2011; Storelli et al., 2011). We also analyzed damage in the gut by monitoring ISC compensatory proliferation. Strikingly, we observed an increased rate of ISC proliferation in the antibiotics-treated flies, whereas Nora-negative flies still displayed a very modest rate of ISC proliferation (Fig. 4F).

DISCUSSION

Since the gut provides the largest host interface with the host's environment, enteric infections are likely to be common and may involve several types of pathogens. Indeed, Nora virus has been identified in laboratory stocks but also in the wild (Darren Obbard, personal communication). While this virus was deemed to have minor effects on host fitness, we show here that it has important phenotypic consequences both in the absence or presence of bacterial infections. It influences the homeostasis of the intestinal epithelium, leads to depleted triglyceride stores, increases dramatically the extent of microbiota, shortens the lifespan of infested flies, and renders them susceptible to a sucrose-only diet.

While it is experimentally easier to study one microbial infection at the time, multiple infections may occur often in the normal environment and may lead to fatal outcomes. These multiple infections are beginning to be studied. For instance, it has recently been shown that influenza-infected mice submitted to a co-infection with *Legionella pneumophila* succumb more rapidly because of a defect in the tolerance/resilience arm of host defense (Jamieson et al., 2013). Here, we have not noted an increased proliferation of Nora virus during a *P. aeruginosa* challenge, although it is difficult to make a definitive statement given the high inter-fly variability of the Nora titer (Fig. S5A) (Habayeb et al., 2009). We have however observed a strongly enhanced activation of the IMD pathway in the midgut when flies were challenged with a detergent or with *P. aeruginosa*. In the former case, it is likely that SDS also lyses the large microbiota, thus releasing peptidoglycan fragments that stimulate the IMD pathway. It will be also interesting to determine whether the microbiota is also responsible for the enhanced IMD pathway activation induced by PA14 infection. Indeed, a higher basal activation of this pathway is already observed in the Nora-infested flies. A pathogenic

bacterial challenge may activate the IMD pathway beyond the threshold that allows microbiota maintenance.

It is not clear why Nora-infested flies succumb earlier to the ingestion of *P. aeruginosa*. The increased resistance of Nora-infested flies to bacterial infections as manifested by the strong stimulation of the IMD pathway suggests a defective resilience in Nora-infested flies. There appears to be indeed a cooperation between bacterial infection and Nora infestation with respects to damages inflicted to the intestinal epithelium, as judged from the consistent synergistic increase of ISC proliferation (Fig. 1E, Fig. S1C, Fig. 3D), which may ultimately affect survival. However, increased attacks on the gut also favor the passage of the bacterial pathogen through the intestinal barrier, thus speeding the switch to a systemic infection. In favor of the first hypothesis, we note that flies fed on sucrose succumb even when the microbiota is strongly decreased by antibiotics treatment. Nevertheless, ISC proliferation was still high, demonstrating that it is likely the direct consequence of viral infestation rather than an indirect effect through the four order of magnitude higher microbiota. Indeed, the Nora-titer may increase after eight days on the sucrose regimen (Fig. S5B), although it is difficult to draw a definitive conclusion given the variability of the Nora titer (Habayeb et al., 2009).

The presence of the Nora virus influences the interpretation of survival experiments in septic injury models in which flies succumb at a slow pace. It is thus essential to include proper controls such as the absence of any infection. By the same token, the impact of Nora infestation on the homeostasis of the intestinal epithelium may represent a major confounding factor in studies that investigate the molecular mechanisms that regulate ISC proliferation. Indeed, in several instances have conflicting data been reported when investigating the specific role of pathways or tissues (Kux and Pitsouli, 2014). We have found that our *Esg-Gal4; UAS-GFP* strain that is used to monitor intestinal epithelial progenitors and to express

transgenic constructs in those cells was heavily infested by Nora virus. Therefore, it is advisable to work only with Nora-free stocks.

An unexpected finding of this study was the positive-impact of Nora virus infestation on the microbiota. Most studies have focused on the positive impact of the microbiota on the host (or negative in aged flies). Also, it has been reported that the mammalian intestinal microbiota favors enteric virus infections, including those caused by the poliovirus, a *Picornaviridae* family member (Kuss et al., 2011). However, the beneficial effects of the host to the microbiota has mostly not been documented to our knowledge, except through the perspective of the host innate immune response. It has been reported that axenic flies display elevated glucose and triglyceride levels when compared to conventionally-raised flies (Newell and Douglas, 2014; Ridley et al., 2012). Interestingly, the gnotobiotic reassociation of two microbiota species, *Lactobacillus* and *Acetobacter*, reversed the triglyceride levels to normal (Newell and Douglas, 2014). Thus, the depletion of triglyceride stores we have observed in Nora virus infected flies may be linked to the increased microbiota. It nevertheless remains striking that the presence of Nora virus in flies fed on sucrose leads to such a higher microbiotal load as compared to Nora-negative flies. We hypothesize that the content of enterocytes damaged by Nora virusis somehow released in the gut lumen and may serve as nutrient for the endogenous bacteria. Indeed, the gut epithelium must be injured: it would be difficult to account for a normal gut that is not enlarged despite the high level of ISC proliferation if enterocytes were not killed. While a previous study failed to detect signs of apoptosis, it nevertheless reported extensive damages to enterocytes and it appeared that some cytoplasm or alternatively delaminating enterocytes were indeed released in the ectoperitrophic space (Habayeb et al., 2009). Cytoplasmic extrusion may represent a novel resilience mechanism (Lee *et al*., submitted) that might also be at play in the case of viral infections.

This work underlines the connection between environmental conditions as represented by nutrition and viral and bacterial infections. A recent study has reported interactions between viral infection, a highly frequent Crohn disease susceptibility polymorphism in *ATG16L*, and the influence of the microbiota in mice (Cadwell et al., 2010). This led to an increased inflammation driven by cytokines and a loss-of-homeostasis of the intestinal epithelium when mice were fed dextran sulfate. Here, we have noted a strongly increased immune response, an augmented activation of the JAK-STAT pathway, and an enhanced susceptibility to bacterial intestinal infections when wild-type flies were exposed to a mild intestinal pathogen.

Experimental procedures

Drosophila stock and Culture

Wild-type OreR(SM) Nora(+) and Ore-R(SC) Nora(-) fly stocks were found in our laboratory. Both stocks of wild-type *Oregon-R* flies tested negative for *Wolbachia* infection. For the septic injury and natural infections the following the wild-type w^{A5001} (Thibault et al., 2004) and the mutants $MyD88^{c03881}$ (Tauszig-Delamasure et al., 2002) and key^{CO2831} (Ferrandon D., unpublished) were used in addition to Ore-R flies.

Fly stocks were kept at 25°C and nearly 60% humidity, on a standard semi-solid cormeal medium (6.4% (w/v) cornmeal (Moulin des moines, France), 4.8% (w/v) granulated sugar (Erstein, France), 1.2% (w/v) yeast brewer's dry powder (VWR, Belgium), 0.5% (w/v) agar (Sobigel, France), 0.004% (w/v) 4-hydroxybenzoate sodium salt (Merck, Germany)).

For lifespan analysis, 3 x 20 female flies were kept at 25°C with 60% humidity, on standard fly food. Flies were transferred without anesthesia on fresh food every 4 days.

For survival test on a sucrose only diet, 3x 20 female flies were fed on 2 mL on a 50 mM sucrose solution and kept at 25°C with humidity.

RT-qPCR analysis of Nora virus titer

Whole flies (6 per sample) were frozen at -80°C. RNA extraction, reverse transcription and quantitative PCR were performed as described (Haller et al., 2014). Quantification values were calculated using a standard curve. This curve was obtained with samples coming from serial dilutions of a plasmid carrying the Nora virus DNA sequence amplified by the couple of primers used for the PCR.

Primer sequence:

Nora virus: 5'-AACCTCGTAGCAATCCTCTCAAG- 3'and 5'-TTCTTGTCCGGTGTA TCCTGTATC- 3'.

Microbial strains, growth conditions and infection

The DCV viral stock was prepared in 10 mM Tris-HCl, pH 7.5.

DCV infections were performed with 4 to 8 days old adult flies by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) of 4.6 nL of DCV suspension (5×10^{10}

PFU/mL). Injection of the same volume of 10 mM Tris-HCl, pH 7.5 was used as a control. Infected and control flies were incubated at 22°C and monitored daily for survival, or frozen for RNA isolation after 3 days of infection.

Microbes were grown in these conditions: *Pseudomonas aeruginosa* PA14 in Brain-Heart-Infusion Broth (BHB), overnight, at 37°C (Rahme et al., 1995); *Serratia marcescens* Db11 (Nehme et al., 2007) in Luria Bertoni Broth (LB), overnight, at 37°C; *Enterobacter cloacae* (Lemaitre et al., 1997), in LB, overnight, at 30°C; *Enterococcus faecalis* (TX0016) in BHB overnight, at 37°C; *Beauvaria bassiana* (Lemaitre et al., 1997) on malt agar plates at 25°C.

Intestinal infections were performed as described previously with PA14 (Haller et al., 2014) and Db11 (Lestradet et al., *in press*). Infected and control flies were kept at 25°C. For PA14 infection, 3 x 20 female flies were used per experiment.

For *E. cloacae* septic injury, 50 mL of an overnight culture of bacteria was centrifuged 30 min at 3 000 x g. The supernatant was removed and pellet used to infect the flies.

For *E. feacalis* septic injury, overnight culture was diluted to 1/50 in fresh BHB and allowed to growth for 3 more hours at 37°C. The final culture was centrifuged 10 min at 3000 x g and the pellet washed one time with sterile PBS. The bacteria were resuspended to a final OD=0.5 that was used to infect female flies with a septic injury. 3 x 20 females were infected with each bacterium.

The septic injury and the PBS clean injury were performed as described (Haller et al., 2014). 20 females were injured.

For *B. bassiana* natural infection, flies were anesthetized, deposited and shaken on a sporulating plate containing the fungus. 3 x 20 females were infected.

Flies were kept at 29°C with 60% humidity and transferred, without anesthesia, to fresh food every 2-3 days.

Phospho-histone H3 immunostaining and microscopy

Fly guts were dissected, fixed, stained by immunohistochemistry with an anti-pH3 antibody (Millipore) and mounted following standard procedures (Lestradet et al., 2014, *in press*). Midguts were observed using a fluorescent microscope (Axioskop 2, ZEISS) and nuclei positive for pH3 staining were counted manually.

Dechorionation of *Drosophila* eggs

Nora(+) flies were allowed to lay eggs overnight at 25°C in a cage on apple juice agar plate with yeast paste in the center of the plate. Eggs were collected, washed with water, and dechorionated with a 50% bleach solution for 3 min with constant up and down pipetting of the solution. Eggs were abundantly rinsed with water, aligned under the microscope on a piece of agar medium and transferred by capillarity on a coverslip. One drop of mineral oil was applied to cover the eggs and the coverslip was deposited on a petri dish with normal *Drosophila* food. After 2 days, larvae were transferred to normal food vial (single bleaching) or treated again with bleach solution as previously and then transferred to normal food vial (double bleaching). Once flies emerged, they were tested for Nora virus infection. Usually, there was no difference in the results obtained with the single or double bleaching procedures.

Nora virus re-infection with feces

200 males of Nora(+) flies were allowed to deposit their feces in a food vial for 5 days at 25°C. Nora(+) flies were then replaced by 50 males and 50 females of cured Nora(+) flies. After 5 days, the vial was emptied and Nora virus infection status was monitored in those flies

(generation G0). Once the progeny emerged, 0 to 4 days old flies were transferred to a fresh vial for 4 days and then monitored for Nora virus titer or used for experiments (first generation after re-infection G1). The same procedure was repeated with the second generation after re-infection (G2).

Pure Nora virus suspension preparation

Nora(+) flies (around 5 000 flies) were crashed in ice cold PBS using a potter homogenizer. The homogenate (around 35 mL) was transferred in a 50 mL Falcon tube and centrifuged at 1 500 rpm for 10 min, at 4°C in an Eppendorf 5810R centrifuge to remove wings, legs and other fly debris. The resulting supernatant was sequentially filtrated through 0.8, 0.45 and 0.22 µm filter units and stored at -80°C. The homogenate (around 26 mL) was then layered in two 15 ml Beckmann Ultra-Clear centrifuge tubes over 1.5 mL of a 30 % (wt/wt) sucrose solution (50 mM Hepes pH 7.0, 0.1 % BSA) and centrifuged at 24,000 rpm for 6.5 hours at 11°C using a JS-24.15 rotor (Beckmann). The pellets were resuspended in a total volume of 500 µL Hepes solution (Hepes 50 mM, pH 7.0) and transferred into a 1.5 mL Eppendorf tube. The solution was then centrifuged at 10,000 rpm for 10 min in a 5424 Eppendorf centrifuge to discard insoluble material. The resulting supernatant containing virus particles was layered over a 40-10 % (wt/wt) sucrose gradient (50 mM Hepes, pH 7.0) prepared in a 15 mL Ultra-Clear centrifuge tube (Beckmann) and centrifuged at 11°C for 4h at 24,000 rpm in a JS-24.15 rotor (Beckmann). An opalescent band containing virus particles that migrated near the middle of the tube was then collected by puncturing the tube with a 25-gauge needle mounted on a 1 ml syringe. This solution was then layered onto a 30 % (wt/wt) sucrose solution contained in a 15 mL Beckmann Ultra-Clear centrifuge tubes (50 mM Hepes pH 7.0, 0.1 % BSA) and centrifuged at 24,000 rpm for 6.5 hours at 11°C using a JS-24.15 rotor

(Beckmann). The pellet was resuspended in 500 µl of Tris solution (Tris-HCl 10 mM, pH 7.5), aliquoted, and stored at -80°C.

Nora virus electronic microscopic picture

The sample was prepared by plunge-freezing of 2.5 µL of the sample on a holey carbon Quantifoil R 2/2 grid using FEI Vitrobot Mark IV machine. Images were gained on FEI Polara F30 TEM microscope with acceleration voltage 100 KV and underfocus around 2.0 microns.

Nora virus re-infection with the pure viral preparation

Nora(-) flies were fed 24 hours with a 1/100 dilution of the viral preparation in sucrose 50mM. In practice, 200 µL of the 1/100 dilution (in sucrose 50mM) of the viral suspension were deposited in an Eppendorf cup that was then placed in an empty fly tube. Flies (male and females) were then added in the tube. The flies were allowed to feed on the viral suspension for 24 hours and then transferred to a fly tube containing standard fly food.

Metabolic stores quantification

Five female flies per sample were crashed, homogenized in the proper buffer for each test (triacylglycerol or glycogen) and centrifuged 10 min at 900 x g. 10 µL of the supernatant was analyzed following the instructions of the supplier of the Triglyceride Colorimetric Assay Kit and the Glycogen Assay Kit (Cayman Chemical Company).

Microbiota quantification

Female flies were sterilized for 30 seconds in 70% ethanol and midguts were dissected in sterile PBS (10 midguts per sample) and immediately transferred in sterile PBS. Midguts were homogenized with a sterile pestle. Serial dilutions of the suspension were performed prior to plating on specific solid media and incubated at 30°C. *Acetobacteriaceae* plates, *Enterobacteriaceae* plates and MRS plates were performed as described (Guo et al., 2014). For BHB agar plates: 37 g/L BHB (Sigma), 15 g/L agar (Sigma). Media were autoclaved 15 min, at 121°C (prior to use) and stored at 4°C.

Bacterial titer in the hemolymph

PA14 presence in the hemolymph was assessed as described previously (Haller et al., 2014). Hemolymphs from 10 female flies per sample were used.

RT-qPCR analysis of gene expression in *Drosophila* midgut

Fly midguts only (without crop, hindgut and malpighian tubules) were dissected (20 per sample) and RNA were extracted as described (Lestradet, 2014, *in press*). Reverse transcription was performed using iScript™ (BIO-RAD). Quantitative PCR was performed using iQ™ SYBR® Green (BIO-RAD) and a C1000™ Thermal Cycler (BIO-RAD) device. Expression values were calculated using a standard curve (with genomic DNA) and normalized to *rp49* expression level. Results presented the average ± SD of 3 independent experiments (with biological duplicates or triplicates).

Primer sequence:

Diptericin: 5'-GCTGCGCAATCGCTTCTACT- 3'and 5'-TGGTGGAGTGGGCTTCATG-3'; Socs36e: 5'-GGGCAAACAGAACCCAGAAACCAA- 3' and 5'-TCCGAGCTGCATTC CAATAGGTGA- 3'; RP49: 5'-GACGCTTCAAGGGACAGTATCTG- 3' and 5'-AAACG CGGTTCTGCATGAG- 3'

Antibiotics treatment

3x 20 female flies (3-8 days old) were kept on a sucrose (50mM) only diet with addition of a 5 antibiotics cocktail: 100 µL/mL ampicillin, 50 µL/mL vancomycin, 100 µL/mL neomycin, 100 µL/mL metronidazole and 50 µL/mL tetracyclin. Tubes were changed every 3 days and flies transferred without anesthesia.

Statistical analysis

All statistical analyses were performed on Graphpad Prism version 5 (Graphpad software Inc., San Diego, CA). Details are given in the legend of each figure.

References

Apidianakis, Y., Pitsouli, C., Perrimon, N., and Rahme, L. (2009). Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. Proc Natl Acad Sci U S A *106*, 20883-20888.

Ayyaz, A., and Jasper, H. (2013). Intestinal inflammation and stem cell homeostasis in aging Drosophila melanogaster. Frontiers in cellular and infection microbiology *3*, 98.

Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A., and Lemaitre, B. (2000). The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc Natl Acad Sci U S A *97*, 3376-3381.

Blum, J.E., Fischer, C.N., Miles, J., and Handelsman, J. (2013). Frequent replenishment sustains the beneficial microbiome of Drosophila melanogaster. mBio *4*, e00860-00813.

Boman, H.G., Nilsson, I., and Rasmuson, B. (1972). Inducible antibacterial defence system in Drosophila. Nature *237*, 232-235.

Broderick, N.A., and Lemaitre, B. (2012). Gut-associated microbes of Drosophila melanogaster. Gut microbes *3*, 307-321.
Buchon, N., Broderick, N.A., Chakrabarti, S., and Lemaitre, B. (2009). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev *23*, 2333-2344.
Buchon, N., Broderick, N.A., Kuraishi, T., and Lemaitre, B. (2010). Drosophila EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. BMC Biol *8*, 152.
Cadwell, K., Patel, K.K., Maloney, N.S., Liu, T.C., Ng, A.C., Storer, C.E., Head, R.D., Xavier, R., Stappenbeck, T.S., and Virgin, H.W. (2010). Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell *141*, 1135-1145.
Cronin, S.J., Nehme, N.T., Limmer, S., Liegeois, S., Pospisilik, J.A., Schramek, D., Leibbrandt, A., Simoes Rde, M., Gruber, S., Puc, U.*, et al.* (2009). Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. Science *325*, 340-343.
Ekstrom, J.O., Habayeb, M.S., Srivastava, V., Kieselbach, T., Wingsle, G., and Hultmark, D. (2011). Drosophila Nora virus capsid proteins differ from those of other picorna-like viruses. Virus research *160*, 51-58.
Ferrandon, D. (2013). The complementary facets of epithelial host defenses in the genetic model organism Drosophila melanogaster: from resistance to resilience. Curr Opin Immunol *25*, 59-70.
Flyg, C., Kenne, K., and Boman, H.G. (1980). Insect pathogenic properties of Serratia marcescens: phage-resistant mutants with a decreased resistance to Cecropia immunity and a decreased virulence to Drosophila. J Gen Microbiol *120*, 173-181.
Guo, L., Karpac, J., Tran, S.L., and Jasper, H. (2014). PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell *156*, 109-122.
Habayeb, M.S., Cantera, R., Casanova, G., Ekstrom, J.O., Albright, S., and Hultmark, D. (2009). The Drosophila Nora virus is an enteric virus, transmitted via feces. J Invertebr Pathol *101*, 29-33.
Habayeb, M.S., Ekengren, S.K., and Hultmark, D. (2006). Nora virus, a persistent virus in Drosophila, defines a new picorna-like virus family. J Gen Virol *87*, 3045-3051.
Haller, S., Limmer, S., and Ferrandon, D. (2014). Assessing Pseudomonas virulence with a nonmammalian host: Drosophila melanogaster. Methods Mol Biol *1149*, 723-740.
Jamieson, A.M., Pasman, L., Yu, S., Gamradt, P., Homer, R.J., Decker, T., and Medzhitov, R. (2013). Role of tissue protection in lethal respiratory viral-bacterial coinfection. Science *340*, 1230-1234.
Jiang, H., and Edgar, B.A. (2011). Intestinal stem cells in the adult Drosophila midgut. Exp Cell Res *317*, 2780-2788.
Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343-1355.
Kim, S.H., and Lee, W.J. (2014). Role of DUOX in gut inflammation: lessons from Drosophila model of gut-microbiota interactions. Frontiers in cellular and infection microbiology *3*, 116.
Kocks, C., Cho, J.H., Nehme, N., Ulvila, J., Pearson, A.M., Meister, M., Strom, C., Conto, S.L., Hetru, C., Stuart, L.M.*, et al.* (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell *123*, 335-346.

Kuss, S.K., Best, G.T., Etheredge, C.A., Pruijssers, A.J., Frierson, J.M., Hooper, L.V., Dermody, T.S., and Pfeiffer, J.K. (2011). Intestinal microbiota promote enteric virus replication and systemic pathogenesis. Science *334*, 249-252.
Kux, K., and Pitsouli, C. (2014). Tissue communication in regenerative inflammatory signaling: lessons from the fly gut. Frontiers in cellular and infection microbiology *4*, 49.
Lamiable, O., and Imler, J.L. (2014). Induced antiviral innate immunity in Drosophila. Curr Opin Microbiol *20C*, 62-68.
Lee, K.Z., and Ferrandon, D. (2011). Negative regulation of immune responses on the fly. Embo J *30*, 988-990.
Lemaitre, B., and Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annu Rev Immunol *25*, 697-743.
Lemaitre, B., Reichhart, J.M., and Hoffmann, J.A. (1997). Drosophila host defense : differential display of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci USA *94*, 14614-14619.
Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog *2*, e56.
Limmer, S., Haller, S., Drenkard, E., Lee, J., Yu, S., Kocks, C., Ausubel, F.M., and Ferrandon, D. (2011). Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. Proc Natl Acad Sci U S A *108*, 17378-17383.
Magwire, M.M., Fabian, D.K., Schweyen, H., Cao, C., Longdon, B., Bayer, F., and Jiggins, F.M. (2012). Genome-wide association studies reveal a simple genetic basis of resistance to naturally coevolving viruses in Drosophila melanogaster. PLoS Genet *8*, e1003057.
Medzhitov, R., Schneider, D.S., and Soares, M.P. (2012). Disease tolerance as a defense strategy. Science *335*, 936-941.
Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G., and Worm, B. (2011). How many species are there on Earth and in the ocean? PLoS Biol *9*, e1001127.
Nehme, N.T., Liegeois, S., Kele, B., Giammarinaro, P., Pradel, E., Hoffmann, J.A., Ewbank, J.J., and Ferrandon, D. (2007). A Model of Bacterial Intestinal Infections in Drosophila melanogaster. PLoS Pathog *3*, e173.
Nehme, N.T., Quintin, J., Cho, J.H., Lee, J., Lafarge, M.C., Kocks, C., and Ferrandon, D. (2011). Relative roles of the cellular and humoral responses in the Drosophila host defense against three gram-positive bacterial infections. PLoS One *6*, e14743.
Newell, P.D., and Douglas, A.E. (2014). Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. Appl Environ Microbiol *80*, 788-796.
Paredes, J.C., Welchman, D.P., Poidevin, M., and Lemaitre, B. (2011). Negative regulation by amidase PGRPs shapes the Drosophila antibacterial response and protects the fly from innocuous infection. Immunity *35*, 770-779.
Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. Science *268*, 1899-1902.
Ridley, E.V., Wong, A.C., Westmiller, S., and Douglas, A.E. (2012). Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. PLoS One *7*, e36765.
Ryu, J.H., Ha, E.M., Oh, C.T., Seol, J.-H., Brey, P., Jin, I., Lee, D.L., Kim, J., Lee, D., and Lee, W.J. (2006). An essential complementary role of NF-kappaB pathway to microbicidal oxidants in Drosophila gut immunity. Embo J *25*, 3693-3701.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Shin, S.C., Ha, E.M., and Lee, W.J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science *319*, 777-782.
Shanbhag, S., and Tripathi, S. (2009). Epithelial ultrastructure and cellular mechanisms of acid and base transport in the Drosophila midgut. J Exp Biol *212*, 1731-1744.
Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., and Lee, W.J. (2011). Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science *334*, 670-674.
Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F. (2011). Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab *14*, 403-414.
Takeishi, A., Kuranaga, E., Tonoki, A., Misaki, K., Yonemura, S., Kanuka, H., and Miura, M. (2013). Homeostatic epithelial renewal in the gut is required for dampening a fatal systemic wound response in Drosophila. Cell reports *3*, 919-930.
Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J.A., and Imler, J.L. (2002). *Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections. Nat Immunology *3*, 91-97.
Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H.L*., et al.* (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat Genet *36*, 283-287.
van Mierlo, J.T., Bronkhorst, A.W., Overheul, G.J., Sadanandan, S.A., Ekstrom, J.O., Heestermans, M., Hultmark, D., Antoniewski, C., and van Rij, R.P. (2012). Convergent evolution of argonaute-2 slicer antagonism in two distinct insect RNA viruses. PLoS Pathog *8*, e1002872.
Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., Boccard, F., and Lemaitre, B. (2005). Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species. Proc Natl Acad Sci U S A *102*, 11414-11419.
Xu, J., and Cherry, S. (2014). Viruses and antiviral immunity in Drosophila. Dev Comp Immunol *42*, 67-84.

Figures Legends

Figure 1. Flies infested with Nora virus have a weaker fitness and are more sensitive to various stress conditions

Flies infested with Nora virus are noted as Nora(+) and non-infested flies as Nora(-).

(A) Ore-R (SM)[Nora(+)] and Ore-R(SC)[Nora(-)] flies were infected by injection of DCV (DCV) or Tris (NI) as an uninfected control and survivals was monitored at 22°C. Data represented the mean of 16 experiments ± SD. Nora infested flies were more susceptible to a DCV infection, ***$p < 0.0001$, LogRank test.

(B) Ore-R (SM)[Nora(+)] were more susceptible to an intestinal infection with PA14 in survival experiments at 25°C. Infected flies:PA14; noninfected control flies: NI. Data represent the mean of biological triplicates ± SD of one representative experiment out of five.

(C) Ore-R (SM)[Nora(+)] were more susceptible to an intestinal infection at 25°C with *S. marcescens* (Db11) than noninfected control flies (NI); ***p < 0.0001, LogRank test. Data correspond to one representative survival test.

(D) Nora titer of the two Ore-R stocks as measured by qRT-PCR: Ore-R(SM) [Nora(+)] and Ore-R(SC)[Nora(-)].

(E) A significant difference of the number pH3 positive nuclei in *Drosophila* midgut at 2 days after an intestinal infection with PA14 (PA14) or noninfected controls (NI) was observed between Ore-R (SM)[Nora(+)] and Ore-R(SC)[Nora(-)] flies in two experiments; ***p < 0.0001, Mann-Whitney test. One experiment shown; medians are represented.

(F) Ore-R (SM)[Nora(+)] flies were dying faster than the Ore-R(SC)[Nora(-)] flies on a sucrose diet at 25°C. Data represent the mean of biological triplicates ± SD of one representative experiment out of three.

(G) Nora infested flies presented a reduced lifespan compared to uninfested flies; ***p < 0.0001, LogRank test. Data represent the mean of biological triplicates ± SD of one representative experiment out of three.

Figure 2. Bleaching *Drosophila* eggs is sufficient to cure flies from Nora virus and to restore a normal sensibility to PA14 intestinal infection but not to DCV injection infection

Flies used for these experiments were all originally OreR(SM) *Drosophila* infested with the Nora virus. Some flies have been cured from Nora virus [Nora(-)] by bleaching the eggs and kept separately from the Nora virus infested flies [Nora(+)].

(A) Nora virus titer of the cured stocks as measured by qRT-PCR.

(B) No significant survival difference was observed between Nora cured or uncured flies after DCV injection. Data represent the mean of three experiments performed each in biological triplicates ± SD.

(C) Nora uncured flies are more susceptible to PA14 infection in survival experiments at 25°C than cured flies. Data represents the mean of biological triplicates ± SD of one representative experiment out of three.

Figure 3. Nora virus causes an enhanced susceptibility to PA14 intestinal infections that favors the passage of *P. aeruginosa* through the digestive tract

Flies used for these experiments were all coming from Ore-R(SM) stocks cured from Nora infestation by egg bleaching (Fig. 2). Some of these flies were re-infected with a pure suspension of Nora virus [re-inf pure virus - Nora(+)] or not [Nora(-)]. (PA14) were the flies infected with PA14 and (NI) the uninfected control flies.

(A) Electronic microscopic picture of the pure Nora virus preparation. Black arrows indicate Nora virus particles.

(B) A significant difference in the Nora virus titer measured by qRT-PCR was observed between Nora(+) and Nora (-) flies in the first (G1) and second (G2) generation after re-infection with virus, G1: *p=0.0127 and G2: **p=0.0061, unpaired t test. The observed difference between the initially re-infected G0 generation flies (G0-Nora(+) re-inf pure virus) or not (G0-Nora(-)) was not significant. Data correspond to biological triplicates of one experiment; medians are shown.

(C) Nora(+) flies were more susceptible than Nora(-) flies to an intestinal infection with PA14 at 25°C. The mean of biological triplicates ± SD of one representative experiment out of six are shown.

(D) A difference in the number of pHH3-positive nuclei in midguts was observed between Nora(+) and Nora(-) flies whether the flies had ingested PA14 for two days or not. A significant increase of pHH3 positive nuclei was detected after a PA14 intestinal infection in both Nora(+) and Nora(-) flies. For all conditions ***$p < 0.0001$, Mann-Whitney test. One representative experiment out of four is shown, medians are indicated.

(E) A significant increase of *SOCS36E* expression as measured by qRT-PCR was detected in the midguts of Nora(+) flies after a PA14 intestinal infection at two days. The average of three experiments (each with biological triplicates) ± SD is shown; *p=0.0288 (in PA14 infection) and *p=0.0232 (in uninfected gut), Mann-Whitney test.

(F) A marked increase of *Diptericin* expression in midguts of Nora(+) flies was detected by qRT-PCR after a PA14 intestinal infection at two days or when fed on SDS 1% for 4-6 hours. The average of 3 experiments (each with biological triplicates) ± SD is shown. This increase is less important in uninfected flies. **p=0.0091 (in Nora(-) +/- PA14 infection), ***p=0.0003 (in Nora(+) +/- infection), ***p<0.0001 (between Nora(+) and Nora(-) in PA14 infection), *p=0.0454 (between Nora(+) and Nora(-) in uninfected gut), **p=0.0022 (between Nora(+) and Nora(-) in SDS treatment), Mann-Whitney test.

(G) A significant difference of the PA14 titer in the hemolymph was measured at day three of the infection between Nora(-) (green squares) and Nora(+) flies (orange circles); **p=0.0048, Mann-Whitney test. The results of three independent experiments are shown; medians are indicated.

Figure 4. Nora virus reduces fly fitness on normal or sucrose diet

Nora(-) flies used for these experiments were from the cured Ore-R(SM) stocks obtained by egg bleaching (Fig. 2). Some of these flies were re-infected with a pure suspension of Nora virus [re-inf pure virus - Nora(+)] or not [Nora(-)].

(A) Nora(+) flies displayed a shorter lifespan than Nora(-) flies. The means of biological triplicates ± SD of one experiment out of two are shown.

(B) A marked increase of the death rate on a sucrose diet was observed in Nora(+) flies compared to Nora(-) flies.The means of biological triplicates ± SD of one representative experiment out of three are shown.

(C) No difference in triacylglycerol stores (TAG) was observed between Nora(+)(circles) and Nora(-)(squares) flies kept on standard fly food. A statistical difference was observed on a sucrose only diet at 5 and 8 days between Nora(+) and Nora(-) flies; *p=0.0246 (at 5 days) and *p=0.0182 (at 8 days), unpaired t test. Flies were kept 5 or 8 days on either a standard fly food (black) or on a sucrose only diet (grey). The results of two experiments (each with biological triplicates) are shown; medians are indicated.

(D) A strong increase of four orders of magnitude (Log scale) of the microbiotal titer was observed between Nora(+)(orange circles) and Nora(-)(green squares) flies kept for eight days on a sucrose diet. A significant increase was detected in old flies kept on standard fly food. *p=0.0180, t test. The results of one experiment out of 2 are shown; medians are indicated.

(E) No difference of survival was observed between Nora(+) flies fed or not on antibiotics (antiB). The means of biological triplicates ± SD of one experiment out of two are shown.

(F) A significant difference in the number of pHH3-positive nuclei of the midgut was observed between Nora(+) and Nora(-) flies kept on a sucrose diet for seven days, either with or without the addition of antibiotics (antiB). A significant increase of pHH3 positive nuclei was detected when antibiotics were added in Nora(-) but not Nora(+) flies; ***p<0.0001 (on sucrose diet between Nora(+) and Nora(-) flies), ***p<0.0001 (on sucrose diet plus antibiotics between Nora(+) and Nora(-) flies) **p=0.0025 (in Nora(+) on sucrose diet or with addition of antibiotics), Mann-Whitney test.

Supplementary figures

Figure S1. The re-infection with Nora virus via feces from Nora(+) flies restores the sensitivity of the Nora cured stock to a PA14 intestinal infection

Flies used for these experiments were all coming from stocks cured from Nora infestation by egg bleaching (Fig. 2). Some of these flies were re-infected via the feces originating from the initially infested Ore-R stock [re-inf feces virus - Nora(+)] or not [Nora(-)]. (PA14) were the flies infected with PA14 and (NI) the uninfected control flies.

(A) A significant difference in the Nora virus titer measured by qRT-PCR was observed between Nora(+) and Nora (-) flies in all generations (G0; G1; G2) after re-infection with the virus via feces; G1: ***p<0.0001 unpaired t test.

(B) Survival test of Nora(+) and Nora(-) flies in an intestinal infection with PA14. Data represent the mean of biological triplicates ± SD of one representative experiment out of three. Nora [re-inf feces-Nora(+)] flies were more susceptible than Nora(-) flies to the infection with PA14.

(C) pH3 positive nuclei counts in *Drosophila* midguts of Nora(+) and Nora(-) flies, at two days of an intestinal infection with PA14. Data from one experiment are shown; medians are displayed as horizontal bars. A statistical difference was observed between Nora(+) and Nora(-) flies whether they were infected by PA14 or not. A significant increase of pHH3 positive nuclei was detected after a PA14 intestinal infection in both Nora(+) and Nora(-) flies. For all conditions ***p < 0.0001, Mann-Whitney test.

Figure S2. Nora virus does not strongly increase the pathology of systemic infections

Flies used for these experiments were all coming from stocks cured from Nora infestation by egg bleaching (Fig. 2). Some of these flies were re-infected with a pure suspension of Nora

virus [re-inf pure virus - Nora(+)] or not [Nora(-)]. The initially infested flies with the Nora virus (Nora(+)) were used only in A.

(A) Survival test after a natural infection with *Beauveria bassiana* of Nora(+) and Nora(-) flies. *MyD88* mutant flies were the positive control as these flies lack a functional Toll pathway and w^{A5001} were the wild-type control of this mutant. *MyD88*, w^{A5001} and *key* flies all tested negative for the presence of the Nora virus. The means of biological triplicates ± SD of one experiment are shown. No difference was observed between Nora(+) and Nora(-) flies.

(B) Survival test after a septic injury with *Enterococcus faecalis* of Nora(+) and Nora(-) flies. *MyD88* mutant flies were the positive control and w^{A5001} were the wild-type control of this mutant. The means of biological triplicates ± SD of one experiment are shown. Both Nora (+) flies, re-infected or initially infested, exhibited a higher susceptibility to this infection, as compared to Nora(-).

(C) Survival test after a septic injury with *Enterobacter cloacae* of Nora(+) and Nora(-) flies. *key* mutant flies were the positive control, as these flies lack a functional IMD pathway and w^{A5001} were the wild-type control of this mutant. The means of biological triplicates ± SD of one experiment are shown. Both Nora (+) flies, re-infected or initially infested exhibited a higher sensitivity to this infection compared to Nora(-).

Figure S3. The apparent susceptibility of Nora-infected flies to injury is due in fact to the decreased lifespan of these flies

Nora(-) flies used for these experiments were from the cured stocks obtained by egg bleaching (Fig. 2). Some of these flies were re-infected with a pure suspension of Nora virus [re-inf pure virus - Nora(+)] or not [Nora(-)]. The flies initially infested with Nora virus [Nora(+)] were used only in A.

(A) A significant difference of survival rate at 29°C was observed between all Nora(+) flies and Nora(-) flies after a sterile PBS clean injury of flies Nora(+) and Nora (-). One experiment out of two is shown.

(B) A significant difference in the number of pHH3 positive nuclei of midguts was observed between Nora(+) and Nora(-) flies at four days at 29°C after a clean injury (CI) or no injury (NI); ***p=0.0001 (Nora(-) flies CI *vs*. NI), ***p<0.0001 (uninjured flies: Nora(+) *vs*. Nora(-)flies) ***p<0.0001 (clean injury: Nora(+) *vs*. Nora(-)flies), Mann-Whitney test. Medians measured in one experiment are shown.

(C) No difference was observed between unchallenged flies and flies submitted to a "clean" injury. Data represent the mean of biological triplicates ± SD of one experiment.

Figure S4. Nora virus does not influence glycogen stores but favors the growth of bacteria belonging to distinct genera of the microbiota

Flies used for these experiments were all coming from stocks cured from Nora infestation by egg bleaching (Fig. 2). Some of these flies were re-infected with a pure suspension of Nora virus [re-inf pure virus - Nora(+)] or not [Nora(-)].

(A) Measure of glycogen stores in whole flies between Nora(+) (circles) and Nora(-) (squares). Flies were kept 5 or 8 days on either a standard fly food (black) or on a sucrose only diet (grey). Results from two experiments (each with biological triplicates) are shown; medians are displayed as horizontal bars. No difference was observed between Nora(+) and Nora(-) flies in both conditions.

(B) Microbiota titer analysis between flies Nora(+) (circles) and Nora(-) (squares), after eight days on a sucrose diet. Results from two experiments (each with biological triplicates) are shown; medians are displayed as horizontal bars. on MRS, *Acetobacteriaceae*, and *Enterobacteriaceae* medium was observed between Nora(+) and Nora(-) fed on sucrose. A

significant increase of at least three orders of magnitude (Log scale) of bacteria growing on rich medium (BHB) were detected between Nora(+) and Nora(-) flies. *p=0.0421, t test.

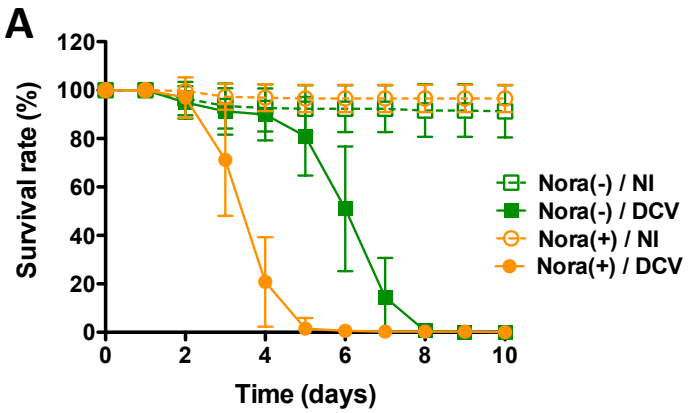
Figure S5. Nora titer in flies is not increased upon PA14 intestinal infection or a sucrose-only diet

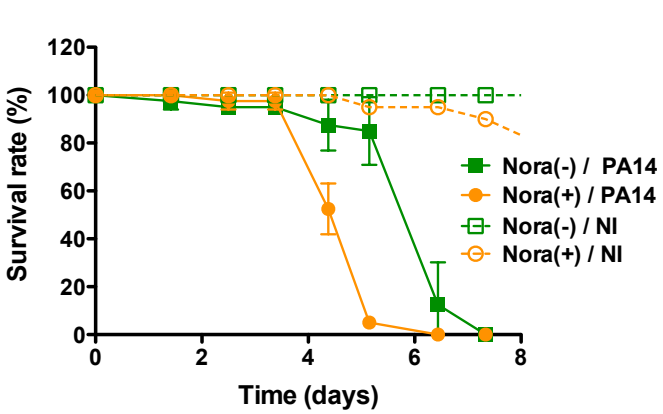
Flies used for these experiments were all coming from stocks cured from Nora infestation by egg bleaching (Fig. 2). Some of these flies were re-infected with a pure suspension of Nora virus [re-inf pure virus - Nora(+)] or not [Nora(-)].

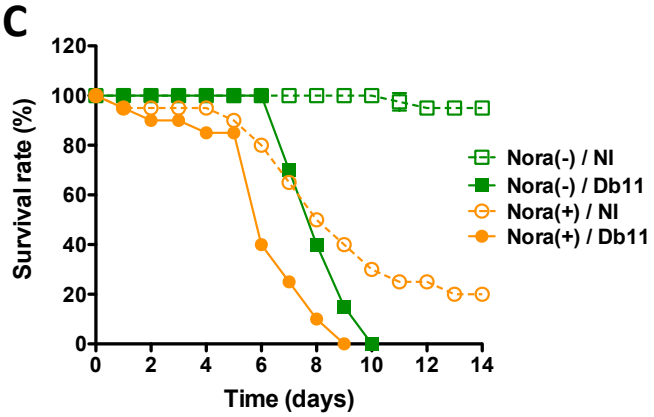
(A) Nora titer analysis of Nora(+) in an intestinal infection with PA14 (black) or uninfected (grey). Data represents biological triplicates of one experiment and medians are indicated. No difference was observed between these two conditions.

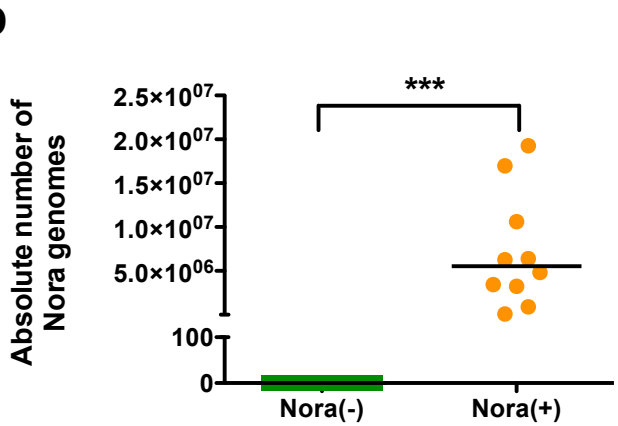
(B) Nora titer analysis of Nora(+) (circles) and Nora(-) (squares) flies kept either on a sucrose only diet (grey) or on a standard fly food (black). Results from two experiments (each with biological triplicates) are shown; medians are displayed as horizontal bars. Given the high inter-fly variability of Nora virus titer, no significant difference was observed between the two conditions and upon the time.

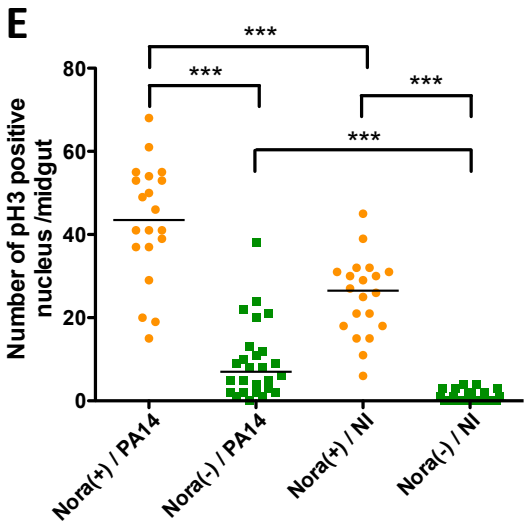
Figure 1

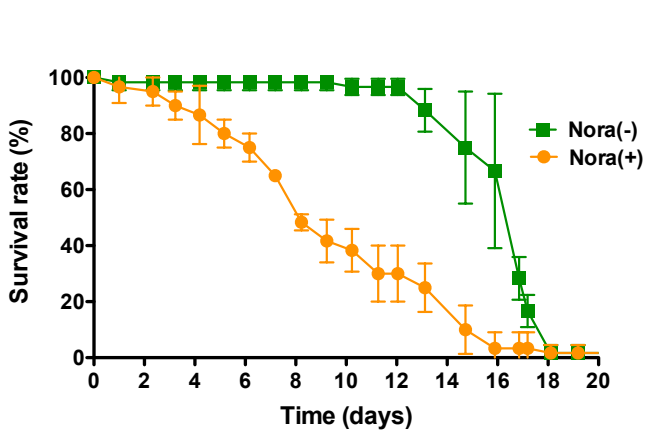

A
Survival rate (%)
Time (days)
Nora(-) / NI
Nora(-) / DCV
Nora(+) / NI
Nora(+) / DCV


B
Survival rate (%)
Time (days)
Nora(-) / PA14
Nora(+) / PA14
Nora(-) / NI
Nora(+) / NI


C
Survival rate (%)
Time (days)
Nora(-) / NI
Nora(-) / Db11
Nora(+) / NI
Nora(+) / Db11


D
Absolute number of Nora genomes
***
Nora(-)
Nora(+)


E
Number of pH3 positive nucleus /midgut
***
Nora(+) / PA14
Nora(-) / PA14
Nora(+) / NI
Nora(-) / NI


F
Survival rate (%)
Time (days)
Nora(-)
Nora(+)

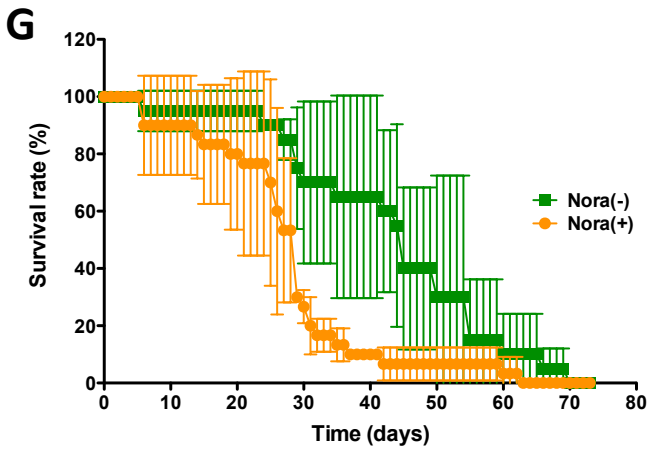

G
Survival rate (%)
Time (days)
Nora(-)
Nora(+)

Figure 2

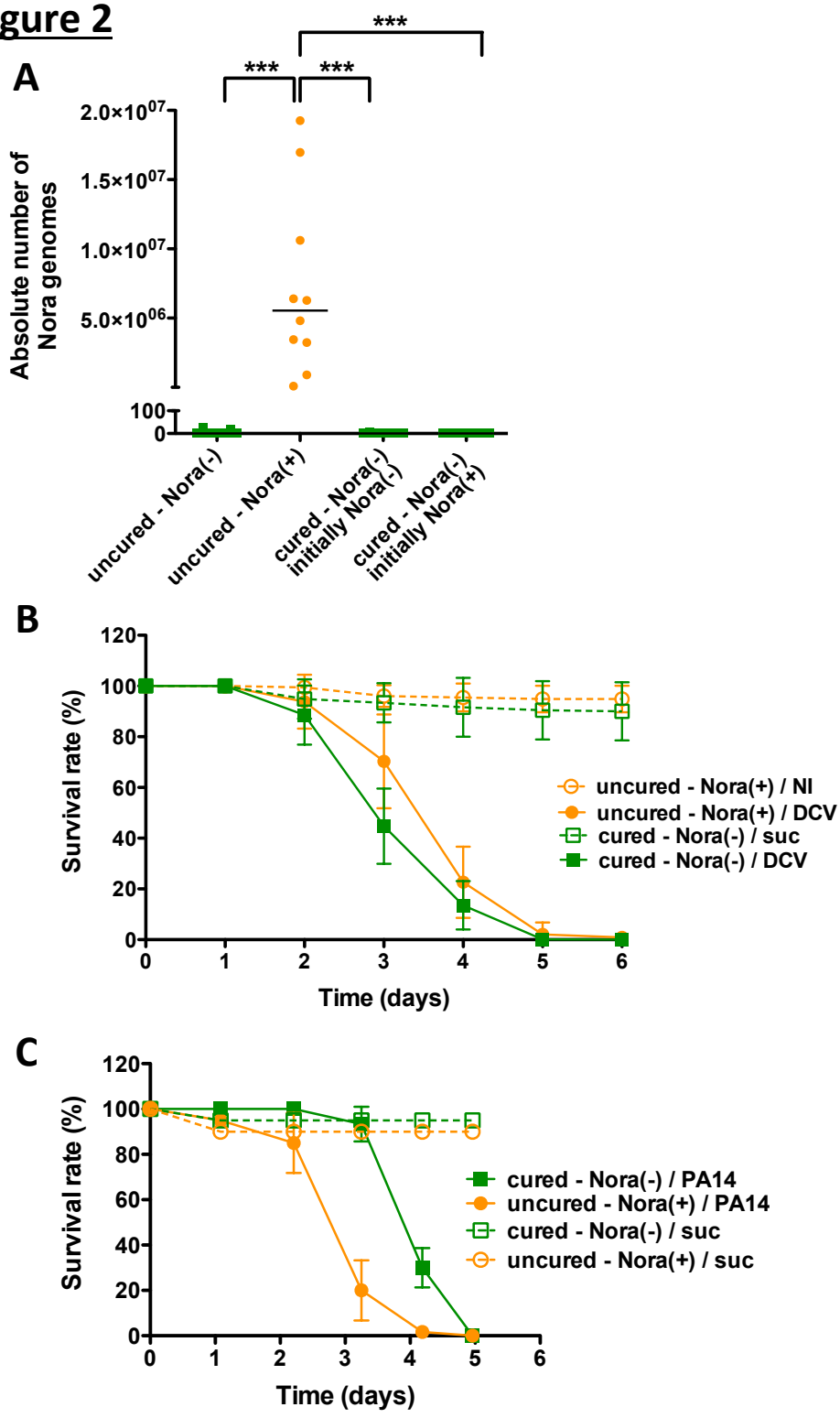

A
***
***
***
Absolute number of Nora genomes
2.0×10^07
1.5×10^07
1.0×10^07
5.0×10^06
100
0
uncured - Nora(-)
uncured - Nora(+)
cured - Nora(-) initially Nora(-)
cured - Nora(-) initially Nora(+)
B
Survival rate (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)
uncured - Nora(+) / NI
uncured - Nora(+) / DCV
cured - Nora(-) / suc
cured - Nora(-) / DCV
C
Survival rate (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)
cured - Nora(-) / PA14
uncured - Nora(+) / PA14
cured - Nora(-) / suc
uncured - Nora(+) / suc

Figure S1

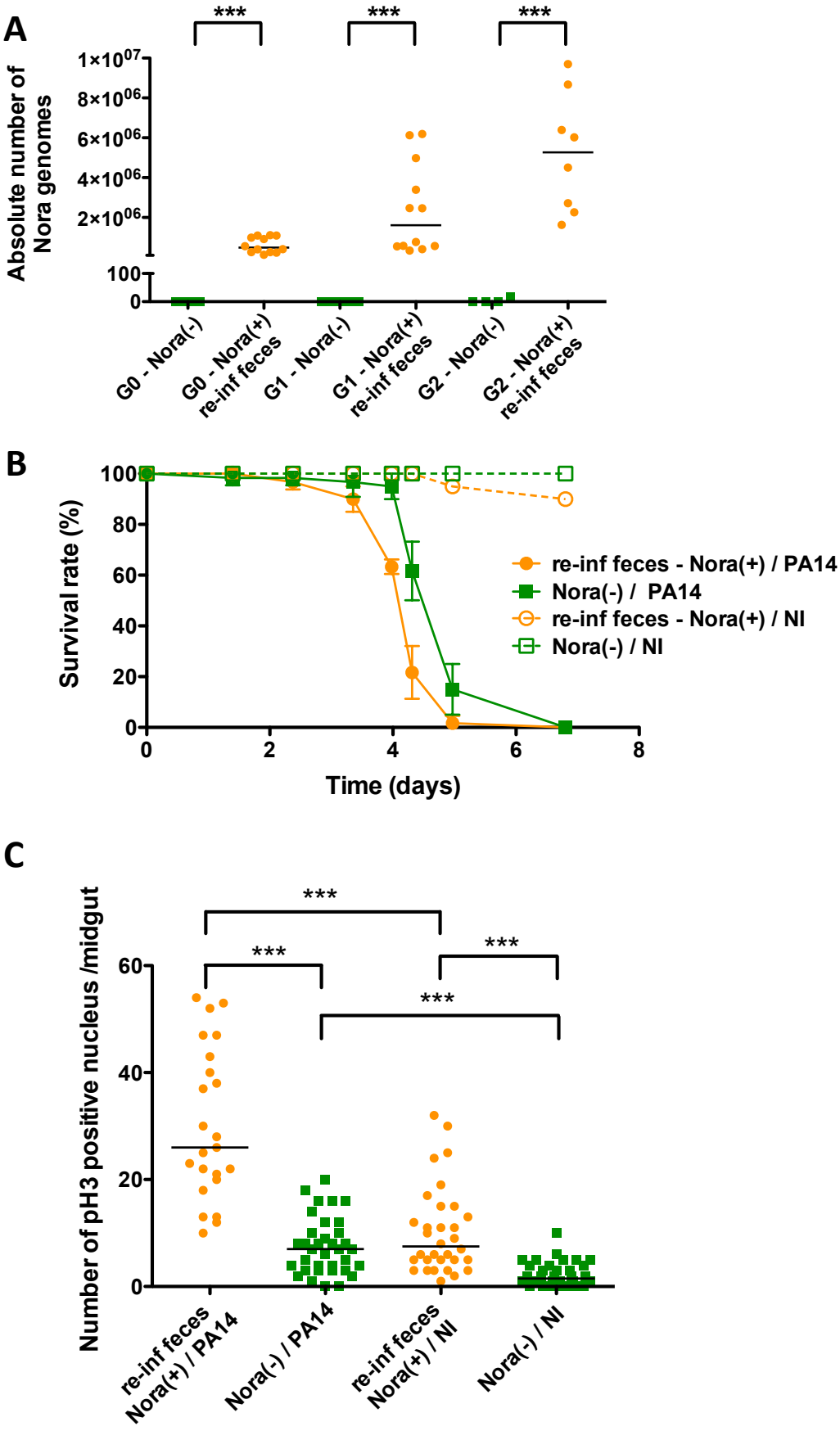

A
***
***
***
Absolute number of Nora genomes
1×10^07
8×10^06
6×10^06
4×10^06
2×10^06
100
0
G0 - Nora(-)
G0 - Nora(+) re-inf feces
G1 - Nora(-)
G1 - Nora(+) re-inf feces
G2 - Nora(-)
G2 - Nora(+) re-inf feces
B
Survival rate (%)
100
80
60
40
20
0
0
2
4
6
8
Time (days)
re-inf feces - Nora(+) / PA14
Nora(-) / PA14
re-inf feces - Nora(+) / NI
Nora(-) / NI
C
***
***
***
***
Number of pH3 positive nucleus /midgut
60
40
20
0
re-inf feces Nora(+) / PA14
Nora(-) / PA14
re-inf feces Nora(+) / NI
Nora(-) / NI

Figure 3

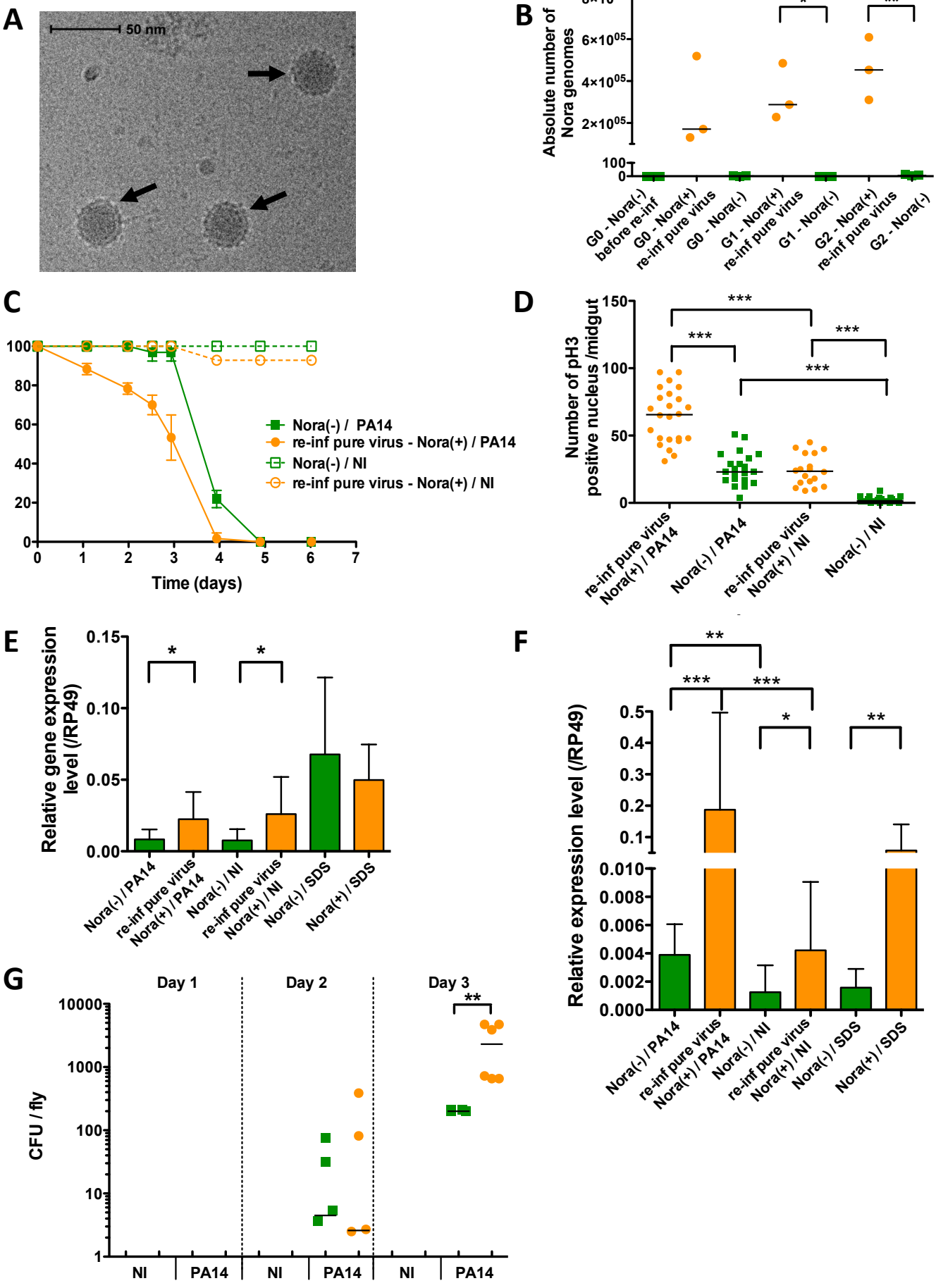

A
50 nm
B
Absolute number of Nora genomes
8×10^05
6×10^05
4×10^05
2×10^05
100
0
*
**
G0 - Nora(-) before re-inf
G0 - Nora(+) re-inf pure virus
G0 - Nora(-)
G1 - Nora(+) re-inf pure virus
G1 - Nora(-)
G2 - Nora(+) re-inf pure virus
G2 - Nora(-)
C
Survival rate (%)
Time (days)
Nora(-) / PA14
re-inf pure virus - Nora(+) / PA14
Nora(-) / NI
re-inf pure virus - Nora(+) / NI
D
Number of pH3 positive nucleus /midgut
***
re-inf pure virus Nora(+) / PA14
Nora(-) / PA14
re-inf pure virus Nora(+) / NI
Nora(-) / NI
E
Relative gene expression level (/RP49)
Nora(-) / PA14
re-inf pure virus Nora(+) / PA14
Nora(-) / NI
re-inf pure virus Nora(+) / NI
Nora(-) / SDS
Nora(+) / SDS
F
Relative expression level (/RP49)
G
CFU / fly
Day 1
Day 2
Day 3
NI
PA14

Figure S2

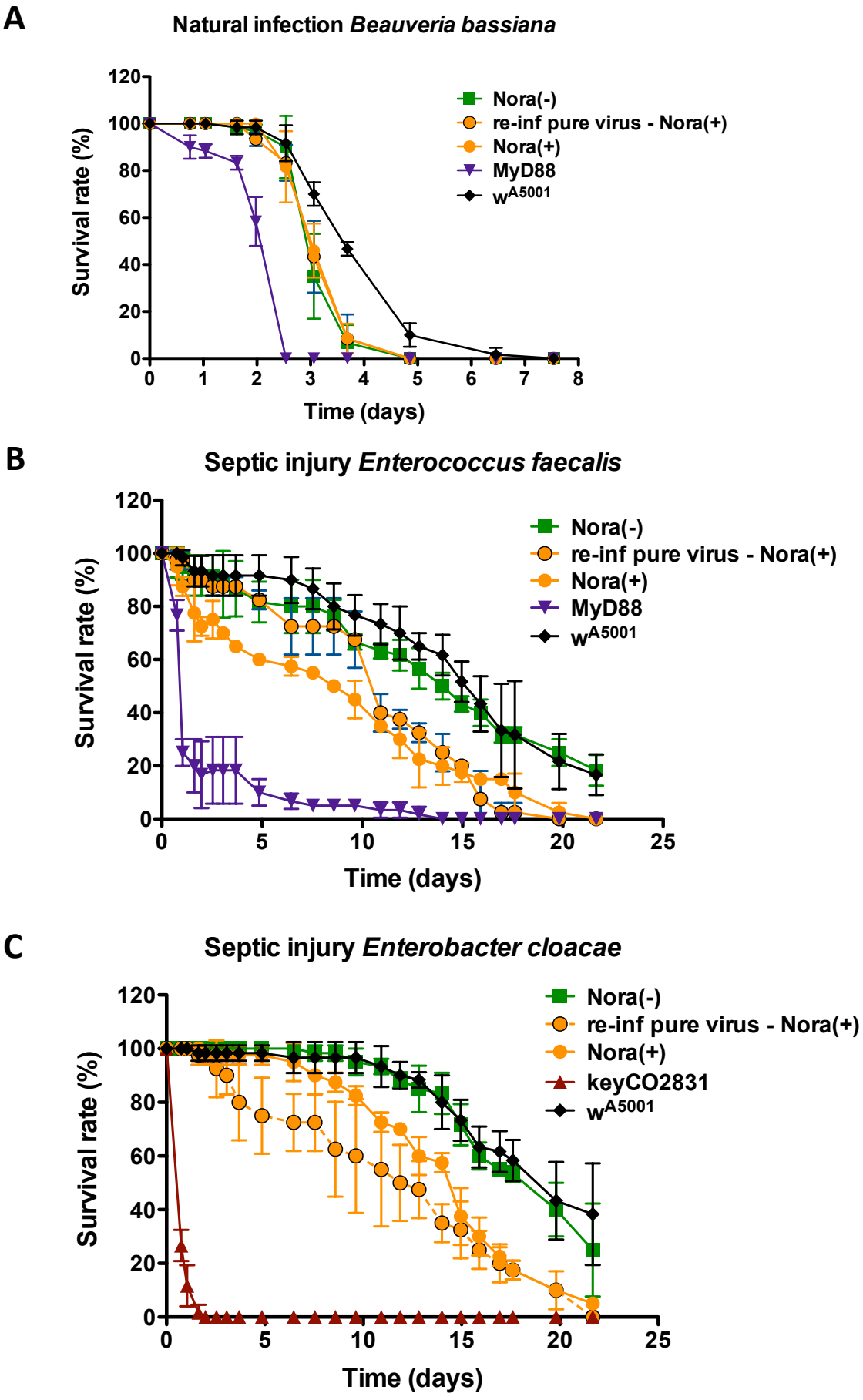

A
Natural infection Beauveria bassiana
Survival rate (%)
Time (days)
Nora(-)
re-inf pure virus - Nora(+)
Nora(+)
MyD88
wA5001
B
Septic injury Enterococcus faecalis
Survival rate (%)
Time (days)
Nora(-)
re-inf pure virus - Nora(+)
Nora(+)
MyD88
wA5001
C
Septic injury Enterobacter cloacae
Survival rate (%)
Time (days)
Nora(-)
re-inf pure virus - Nora(+)
Nora(+)
keyCO2831
wA5001

Figure 4

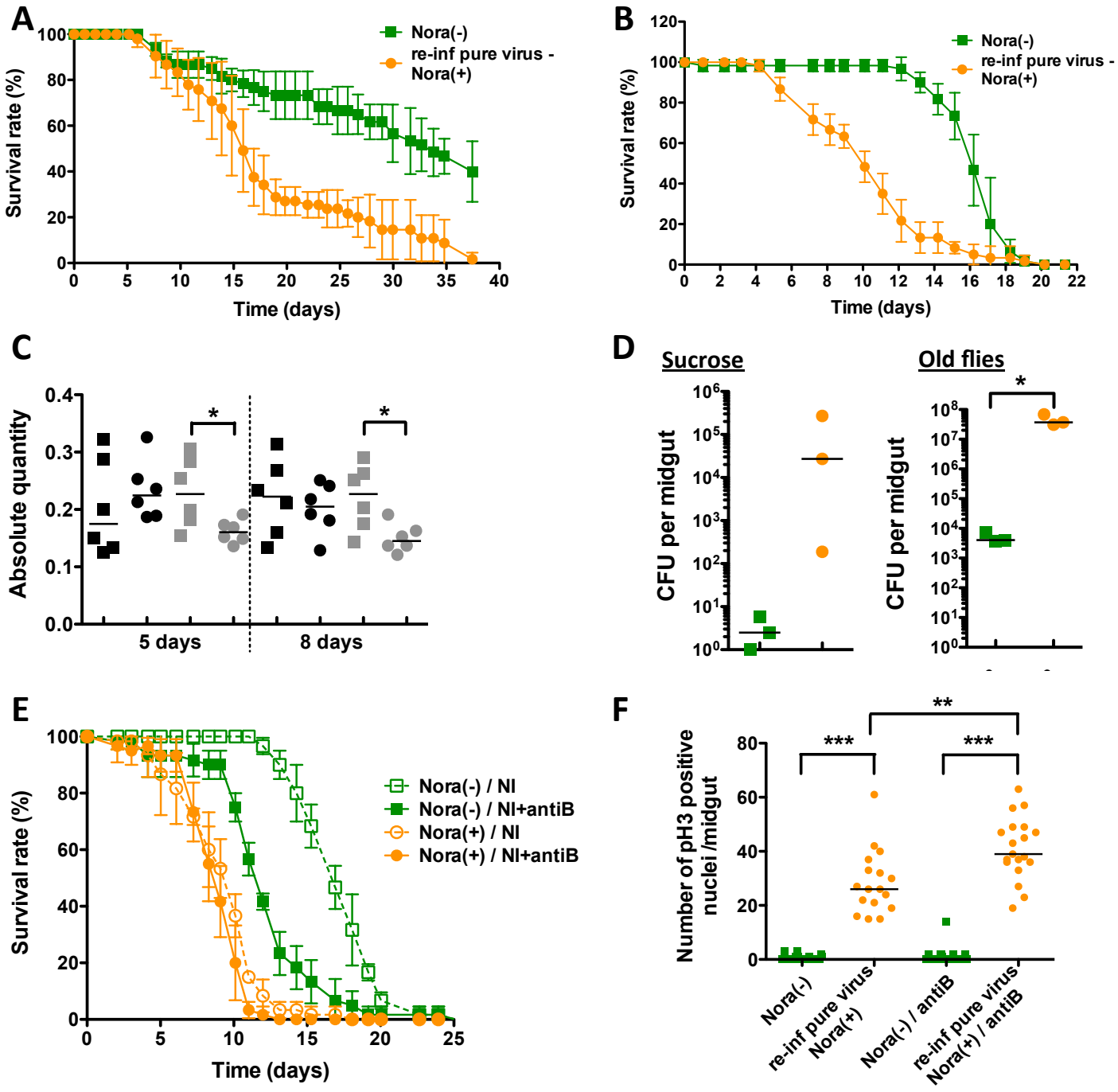
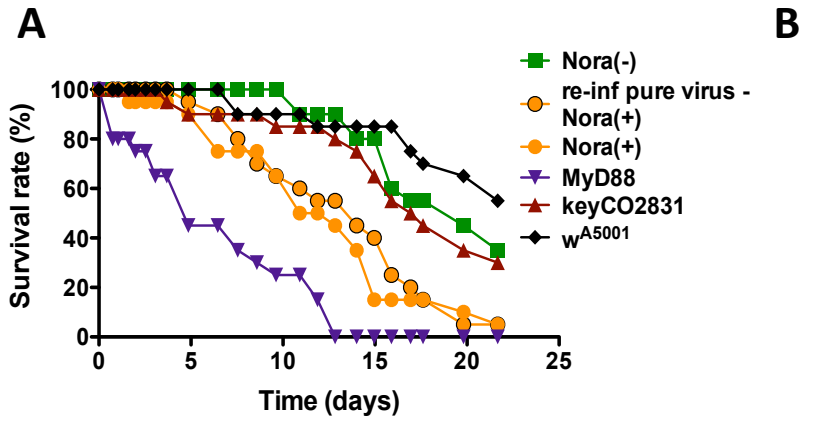
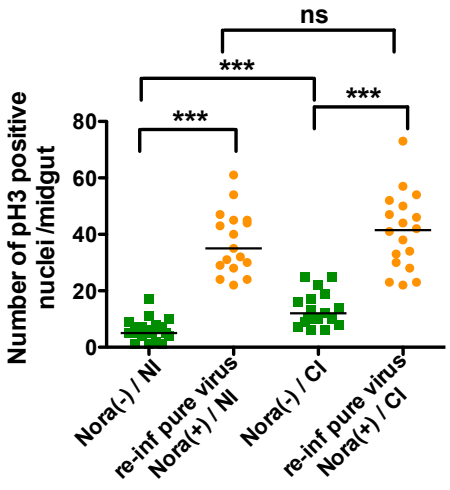

A
Survival rate (%)
Time (days)
Nora(-)
re-inf pure virus - Nora(+)
B
Survival rate (%)
Time (days)
Nora(-)
re-inf pure virus - Nora(+)
C
Absolute quantity
5 days
8 days
*
*
D
Sucrose
Old flies
CFU per midgut
CFU per midgut
*
E
Survival rate (%)
Time (days)
Nora(-) / NI
Nora(-) / NI+antiB
Nora(+) / NI
Nora(+) / NI+antiB
F
Number of pH3 positive nuclei /midgut
***
***
**
Nora(-)
re-inf pure virus Nora(+)
Nora(-) / antiB
re-inf pure virus Nora(+) / antiB

Figure S3


A
Survival rate (%)
Time (days)
Nora(-)
re-inf pure virus - Nora(+)
Nora(+)
MyD88
keyCO2831
w^A5001


B
Number of pH3 positive nuclei /midgut
ns
***
***
***
Nora(-) / NI
re-inf pure virus Nora(+) / NI
Nora(-) / CI
re-inf pure virus Nora(+) / CI

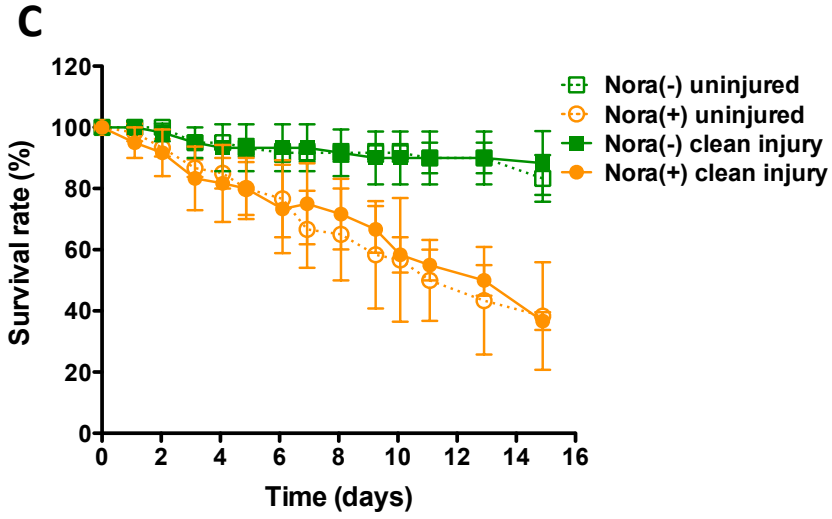
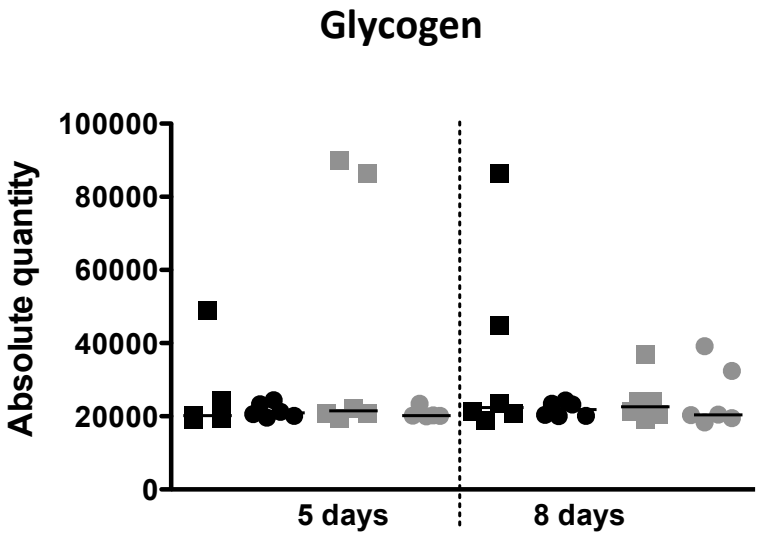

C
Survival rate (%)
Time (days)
Nora(-) uninjured
Nora(+) uninjured
Nora(-) clean injury
Nora(+) clean injury

Figure S4

A


Glycogen
Absolute quantity
100000
80000
60000
40000
20000
0
5 days
8 days

B

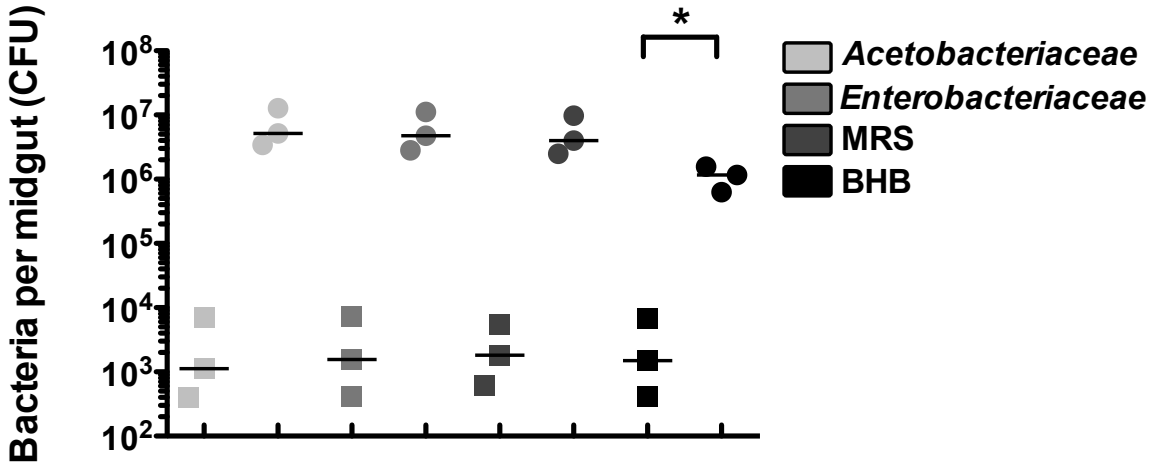
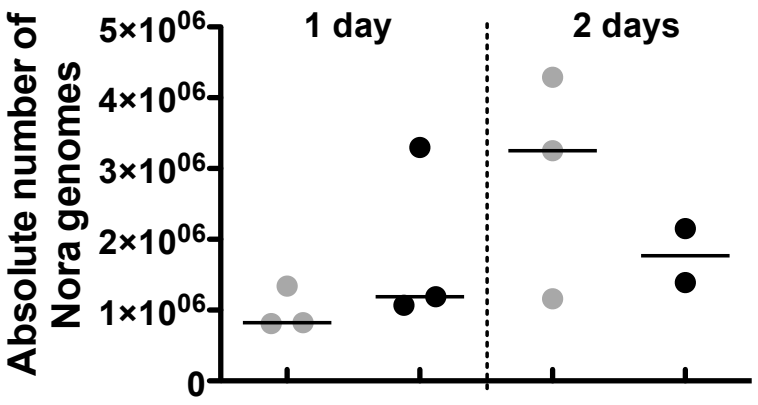
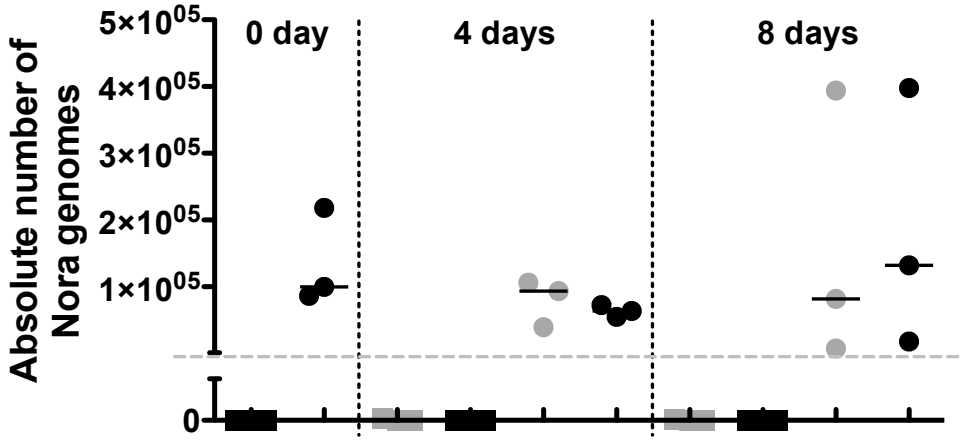

Bacteria per midgut (CFU)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
*
Acetobacteriaceae
Enterobacteriaceae
MRS
BHB

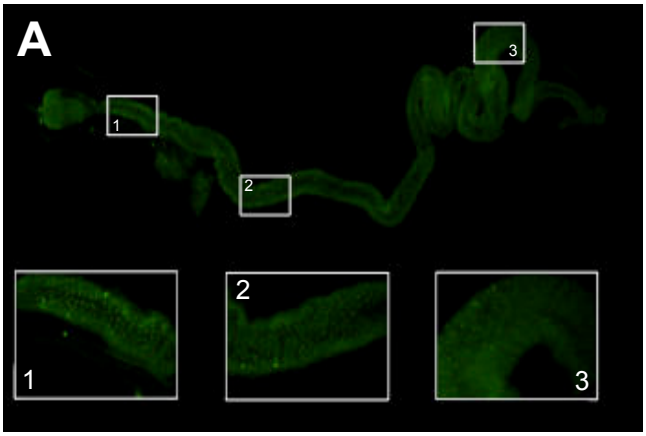
Figure S5

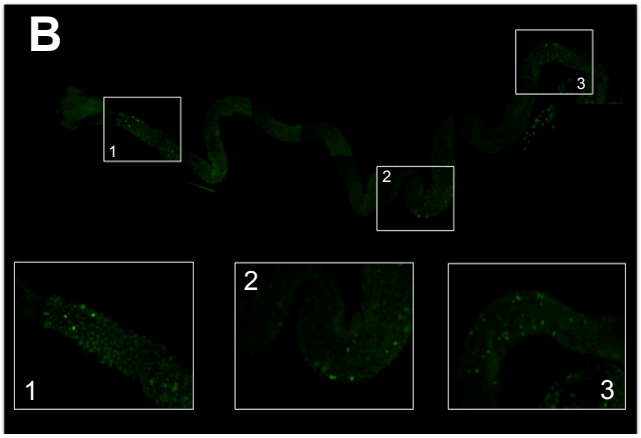
A


Absolute number of Nora genomes
5×10^06
4×10^06
3×10^06
2×10^06
1×10^06
0
1 day
2 days

B


Absolute number of Nora genomes
5×10^05
4×10^05
3×10^05
2×10^05
1×10^05
0
0 day
4 days
8 days





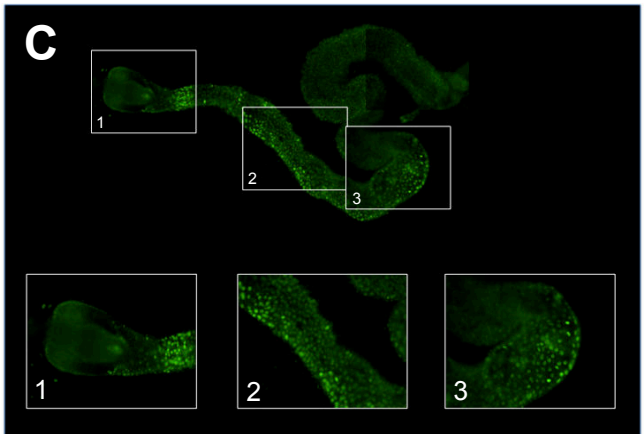


Figure C1. Nora(+) flies presented more apoptotic cell in the midgut than Nora(-) flies.

Tunel test after intestinal infection with PA14 (A & B) or SDS 1% as a positive control (C) on midguts from Nora(-) (A) or Nora(+) (B). Panels present the results of one experiment in which 10 fly midguts were observed for each condition. Nora(+) fly midguts presented slightly more apoptotic cells than Nora(-) fly midguts.

Complementary results and discussion

Nora virus may induce *Drosophila* enterocyte death through apoptosis

Nora-positive flies present a dramatic increase of phospho-histone H3 (pH3) positive nuclei in the midgut compared to Nora-negative flies indicating an increased intestinal stem cells (ISC) proliferation. In addition, the size from Nora-positive flies midguts did not seem to be increased in comparison to midguts from Nora-negative flies. The absence of increased size of the gut strongly suggests an increased rate of cell death that would be compensated for by ISC proliferation.

There are two major mechanisms of cell death: necrosis and apoptosis. We first decided to test whether the midgut cell death in Nora-positive flies could be due to apoptosis. I used a TUNEL assay to stain the cells undergoing apoptosis in Nora-positive and Nora-negative midgut in an oral infection with *P. aeruginosa*. As a positive control, I used midgut from Nora-negative flies fed with a sodium dodecyl sulfate (SDS) 1% solution for two days. Prior to the dissection, to avoid any false positive staining due to the dissection process, I injected the fixative (69 nL of 16% paraformaldehyde [PFA] in PBS) in the flies and transferred them to a 4% PFA solution in PBS. Two hours after the injection, I dissected carefully the midgut of these flies and transferred them again to a 4% PFA solution in PBT for one additional hour. I stained TUNEL positive cells using the ApopTag® kit from Millipore.

Flies fed with SDS 1% exhibited a high number of TUNEL positive cells all along the midgut (Fig. C1C). Some regions seemed to be more affected like the region R1 from the midgut (Fig. C1C, box1). In Nora-negative flies, only few cells were positive for the TUNEL staining (Fig. C1A). Nora-positive flies presented a moderate increase of TUNEL positive cells along the midgut and especially in region R1 (Fig. C1B & C1B box 1)

These results suggest that the Nora-positive ISC proliferation compensates at least partially the apoptotic cell death induced by the Nora virus infection in the midgut.

The absence of ROS does not seem to influence the death of Nora-positive flies but the proliferation rate of ISCs

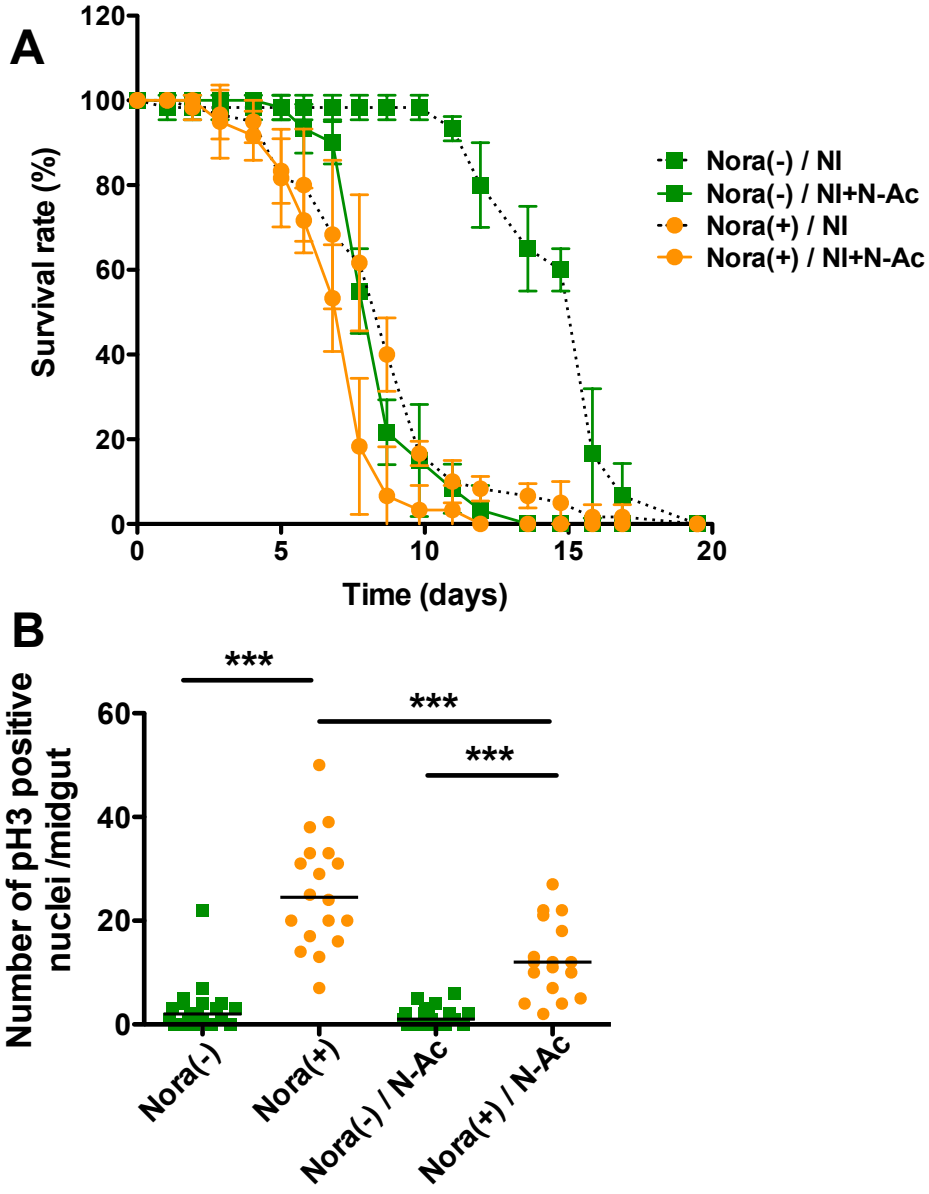


Figure C2. Decreasing ROS exposure in Nora(+) flies did not influence the survival of the flies yet decreased the proliferation rate of ISCs.

3 x 20 female Nora(-) and Nora(+) flies were fed on a sucrose-only diet either with addition of 10 mM of N-Ac or not. Tubes were changed everyday without anesthetizing the flies. In (A) the survival of these flies was assessed. No significant difference was observed in the survival of Nora(+) flies fed either sucrose/N-Ac or sucrose. Nora(-) flies fed with the addition of N-Ac exhibited a striking decrease of survival rate compared to Nora(-) flies fed only with sucrose. Data represent means ±SD of triplicates (20 female flies per tube) from one experiment. In (B) the proliferation rate of ISC was assessed by a pH3 staining. A significant decrease of pH3 positive cells was observed in Nora(+) flies with addition of N-Ac compared to the same flies fed on sucrose only (for all ***$p<0.0001$). No difference is observed in Nora(-) flies fed either with addition of N-Ac or not. Nora(+) and Nora(-) flies presented a significant difference in ISCs proliferation rate for each condition. One experiment has been performed; black bars indicate medians.

We have observed that Nora-positive flies carry an increased microbiotal load in old flies or when flies are fed only with a sucrose solution (Fig. 4D, Nora virus manuscript). This increased microbiota could be responsible for the increase of pH3 positive nuclei in these flies through triggering the activity of the Dual Oxydase (DuOx) enzyme and the production of reactive oxygen species (ROS).

To assess this hypothesis, I performed a survival experiment on a sucrose diet only with or without adding 10 mM of N-acetyl-L-cysteine (N-Ac). This antioxidant molecule is able to block ROS activity. As N-Ac is rapidly oxidized when being in solution, the fly tubes were changed every day. Nevertheless, I did not observe any striking difference in the rate of death between Nora-positive flies fed with either sucrose or sucrose with N-Ac (Fig. C2A). Unexpectedly, Nora-negative flies fed with sucrose and N-Ac died nearly at the same rate than Nora-positive flies (with and without addition of N-Ac). We do not understand this phenotype at present. It would be interesting to determine whether the microbiotal load in flies that have fed on N-Ac has increased as in Nora-positive flies. However, the enlarged microbiota is not responsible for the enhanced sensitivity of Nora-positive flies to a sucrose diet (Fig. 4E, Nora virus manuscript).

We therefore decided to perform the same experiment but monitoring this time the number of pH3 positive nuclei after 7,5 days. Surprisingly, Nora-positive flies fed with sucrose/N-Ac have fewer pH3 positive nuclei in the midgut than Nora-positive flies fed only with sucrose (Fig. C2B). However, even though the ISC proliferation rate of these Nora-positive flies (fed with sucrose/N-Ac) were sometimes reaching the low levels of Nora-negative flies, they often exhibited an intermediate rate of proliferation, between Nora-positive and Nora-negative flies. I did not detect any significant difference in the ISCs proliferation rate between Nora-negative flies with or without addition of N-Ac.

All together, these results suggest at least a partial role of ROS in the intestinal damages leading to the increased ISCs proliferation in Nora-positive flies, thus suggesting that Nora infection somehow triggers the release of ROS. This might happen for instance through the release of calcium from the endoplasmic reticulum stores, that would directly activate the Dual oxidase enzyme through its Calcium-binding domain. Conversely, when the effects of ROS production on the gut are attenuated, this does not translate into an enhanced survival. Thus, the cause of death of Nora-positive flies fed on sucrose remains to be established. In addition, the basis for the high rate of death of Nora-positive and Nora-negative flies fed on sucrose/N-Ac remains also unclear.

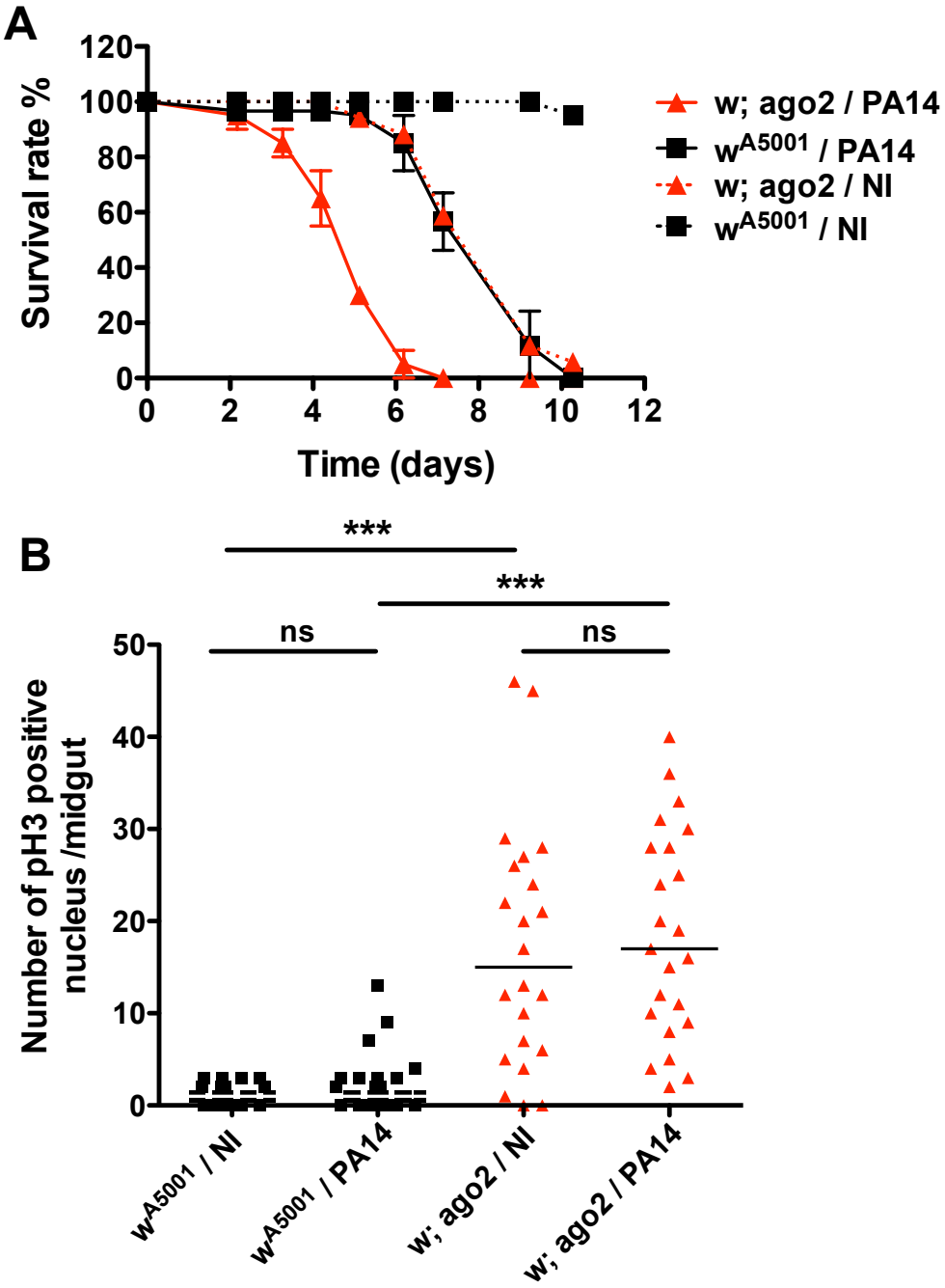


Figure C3. *Ago2* mutants flies presented a stronger pathology to a PA14 intestinal infection than wild-type flies.

3 x 20 female *ago2* mutants and wild-type flies (w^{A5001}) were orally infected with PA14. In (A) the survival of these flies was assessed. *Ago2* mutants were significantly more susceptible to the PA14 intestinal infection than w^{A5001} flies (LT50 ago2=5 days and LT50 w^{A5001}=7,5 days). Data represent the means ±SD of triplicates (20 female flies per tube) from one experiment. In (B) the proliferation rate of ISC was assessed by a pH3 staining two days after beginning of the infection. *Ago2* mutants exhibited a significantly higher ISCs proliferation rate than w^{A5001} in both infected and uninfected conditions (for both, ***p<0,0001, Mann Whitney test). One experiment has been performed; black bars indicate medians.

Nora virus does not seem to induce ISC proliferation through its interaction with Ago2

A viral suppressor of RNA interference pathway (VSR) had been identified in the viral protein one (VP1) of Nora virus (van Mierlo et al., 2012). This VSR was shown to interact with *Drosophila* Ago2. However, conflicting results about Nora virus VSR and its interaction with the RNAi pathway did not permit to establish whether this VSR effectively blocks fully the *Drosophila* RNAi pathway. On the one hand, the Nora titers are unaffected in mutants that affect the siRNA pathway (Habayeb et al., 2009a). On the other hand, transgenic expression of VP1 in the eye suppressed an RNAi-dependent phenotype (van Mierlo et al., 2012). Therefore, we first tested *ago2* mutant flies to see if they share the same kind of phenotype than Nora-positive flies in the midgut.

Ago2 flies were more susceptible to a PA14 oral infection than wild-type *Drosophila* with a LT50 of five days for *ago2* flies compared to around eight days for w^{A5001} flies (Fig. C3A). One striking observation is that *ago2* flies seemed to be highly sensitive to any kind of stress as the uninfected flies fed only with sucrose were dying at the same rate than the wild-type control flies infected with PA14.

In addition, to this survival phenotype, *ago2* mutant flies exhibited around 20 times more pH3 positive nuclei in the midgut (Fig. C3B) than the wild-type control. This later result indicated an increased ISC proliferation rate in *ago2* mutant flies. This would suggest a potential link between the siRNA pathway and the regulation of ISC proliferation. However, this result should be interpreted with caution. First, the phenotype might be caused by a second-site mutation on the *ago-2* chromosome. Second, a deficient RNAi pathway might allow the reactivation of a dormant RNA virus (Goic et al., 2013). Such infections could be detected by qRT-PCR or qPCR using primers designed against known viruses, or by deep sequencing in the case of unknown viruses.

.

All together, the increased susceptibility of *ago2* flies in an oral infection with PA14 and the increased number of pH3 positive nuclei in *ago2* midgut, on a sucrose diet or in infection with PA14, strongly correlated with the results obtained with Nora-positive flies and hinted at a weakened integrity of the intestinal epithelium. It will be important to test other mutants affecting the siRNA pathway, especially *Dcr-2*.

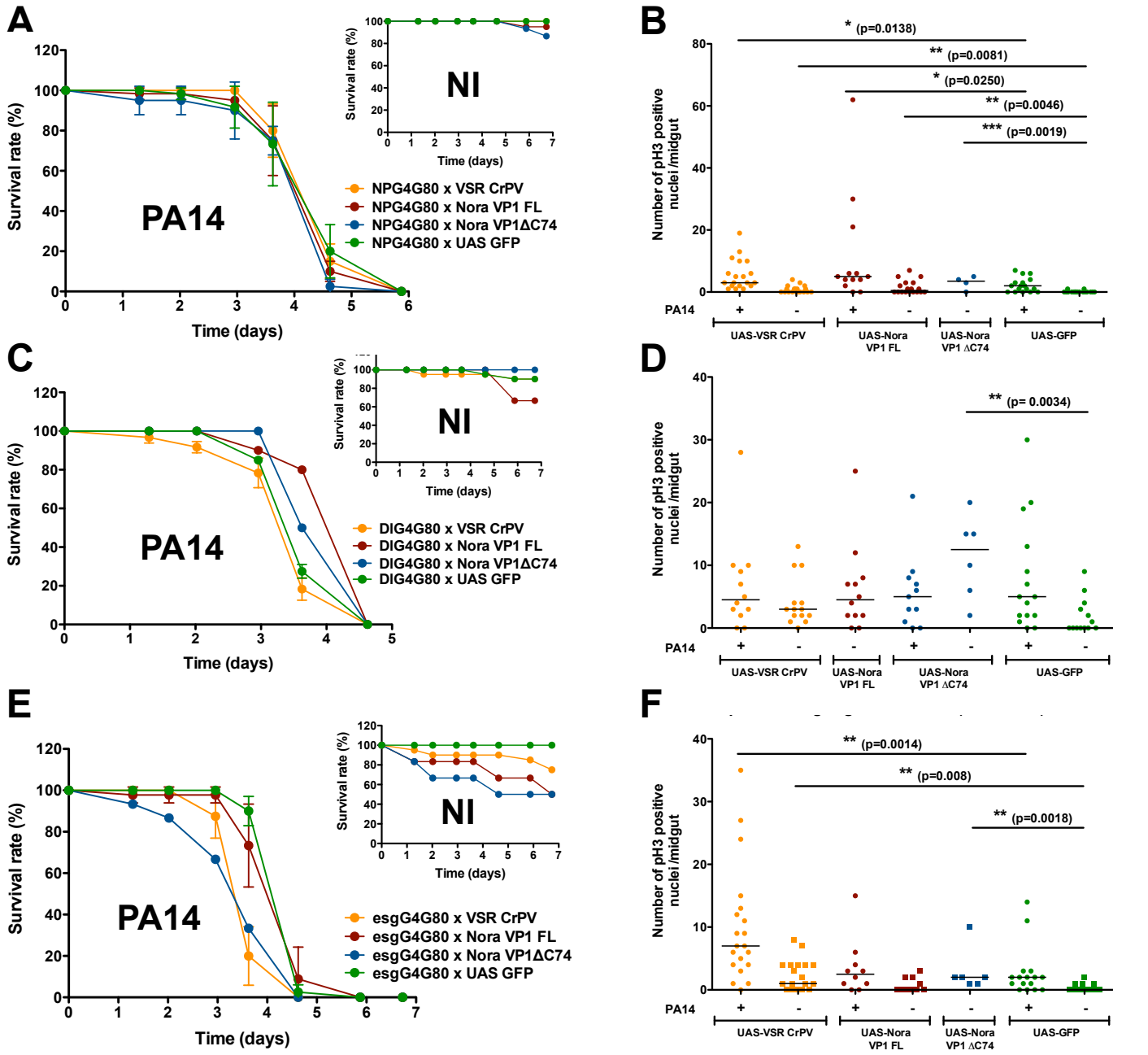


Figure C4. Overexpression of Nora virus VSR in one intestinal cell type is not sufficient to phenocopy the Nora-induced phenotype

Using different driver fly lines, transgenes allowing the overexpression of CrPV VSR, Nora virus VP1 FL (with a VSR), Nora virus VP1ΔC74 (without a VSR) or GFP (as a control) were expressed in enterocytes (*NPG4G80*), in ISCs (*dlG4G80*) or progenitor cells (*esgG4G80*) respectively. For each condition, the survival of flies and ISCs proliferation rate was assessed. (A) No difference in the survival of flies was observed but a slight increase in ISCs proliferation compared to the control was detected for all fly genotypes (B). (C) A significant increase of survival was observed with VP1 FL and VP1ΔC74 but not VSR CrPV compared to the control (***p<0,0001). (D) Only uninfected VP1ΔC74 exhibited an increased ISCs proliferation rate compared to control. (E) CrPV and VP1ΔC74 presented a significantly decreased survival (***p<0,0001) and an increased ISC proliferation rate compared to control (F). Survival results (A, C and E) represent means ±SD of duplicates or triplicates (20 female flies per tube, in some cases only one tube of 20 flies could be assessed) from one experiment. ISC proliferation assays (B, D and F) were obtained in one experiment; black bars indicate medians, statistics were done with a Mann Whitney test.

Nora virus VP1 protein contains the identified VSR. Specifically the last 74 amino acid at the C-terminal part of VP1 are important for the interaction with Ago2. Transgenic flies allowing the overexpression of either VP1 full-length (VP1 FL) protein or VP1 protein deleted from the last 74 amino acid at the C-terminal part (VP1 ΔC74) have been generated in another laboratory and kindly sent to us. Moreover, another *Drosophila* virus, the CrPV (Cricket Paralysis Virus), had been shown to possess a VSR and a transgenic fly allowing the overexpression of this CrPV VSR was available in the laboratory (van Mierlo et al., 2012).

To test whether Nora-positive flies phenotypes are due to the interaction of Nora virus with Ago2, and in the same way to localize in which cell type the Nora virus is localized, I crossed these different transgenic flies with specific GAL4 drivers allowing the expression of the transgene either in enterocytes, ISCs or progenitor cells (ISCs and enteroblasts).

By crossing these transgenic flies with an *NP3084-GAL4GAL80* driver (expressed only in enterocytes) I did not observe any difference in the survival after infection with PA14 of flies overexpression the VSR of Nora virus or CrPV and the control flies overexpression GFP (Fig. C4A). Surprisingly, flies overexpressing the VSR of Nora virus, of CrPV virus or the Nora VP1 ΔC74 presented a moderate yet significant increase of pH3 positive nuclei compared to the control flies overexpressing GFP.

The overexpression of CrPV virus VSR in ISCs using the ISC driver *dl-GAL4GAL80*, did not induce any significant changes in the survival of these flies compared to the control (Fig. C4C). However, the overexpression of VP1 FL or VP1 ΔC74 from Nora virus using the same *dl-GAL4GAL80* driver induced a slight but significant increase of the survival of the flies. These overexpressions did not affect the number of pH3 positive nuclei in the midgut of these flies compared to the control (Fig.C4C). The proliferation rate was significantly increased in VP1 ΔC74 flies compared to the control flies overexpressing GFP.

There is a slight increase of susceptibility to a PA14 oral infection for flies overexpressing the VSR of the CrPV virus in progenitor cells using the *esg-GAL4GAL80* driver (Fig. C4E). This increased susceptibility correlates also with an increased number of pH3 positive nuclei in the midgut of these flies (Fig. C4F). But no striking increase of pH3 positive number could be observed in the flies overexpressing the VSR from Nora virus, but a slight and significant increase of ISC proliferation was observed in flies overexpressing the VP1 deleted from the VSR. These two transgenic flies seemed to be more sensitive to a sucrose only diet given that both uninfected flies overexpressing VP1 full-length and VP1 ΔC74 in progenitor cells reached 50% of death at seven days (Fig. C4E).

G

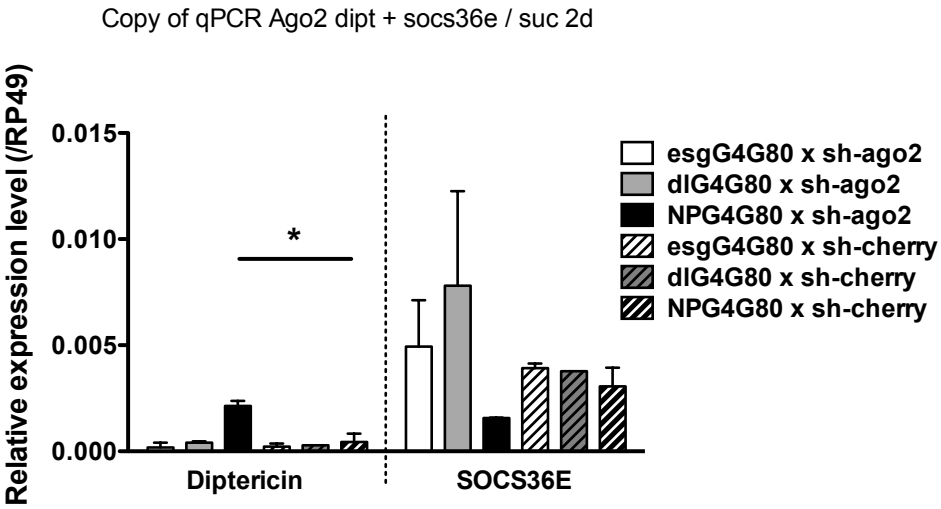


Figure C5. Down-regulation of *ago2* in *Drosophila* midgut induced an increase of *Diptericin* expression

A transgene encoding a short hairpin RNA against Ago2 mRNA was overexpressed in intestinal cells using drivers *NPG4G80* (enterocytes), *dlG4G80* (ISCs) and *esgG4G80* (progenitor cells: ISCs and enteroblasts). The overexpression of a short hairpin targeted against Cherry mRNA was used as a control. Data represent the mean ±SD of duplicates (20 midguts from female flies per sample) from one experiment. A significant increase of *Diptericin* expression was detected in midguts from flies overexpressing sh-*ago2* in enterocytes compared to the control (*p=0.0339, unpaired t-test). A slightly increased but significant expression of *SOCS36e* was observed in flies overexpressing sh-*ago2* in ISCs and progenitors compared to the control.

I observed a correlation between the survival rate and the ISC proliferation rate phenotypes only by using the *esg-GAL4GAL80* driver. In this case, flies overexpressing CrPV virus VSR were significantly more susceptible to a PA14 intestinal infection. They exhibited an increased ISCs proliferation rate compared to the control flies. The overexpression of VP1 ΔC74, but not of the full-length VP1 protein, induced a similar phenotype in survival and ISC proliferation rate. However, in contrast to full-length VP1, VP1 ΔC74 does not contain a functional VSR and should not be able to interact with Ago2. Therefore we expected to see similar results between the overexpression of CrPV virus VSR and Nora virus VP1 full-length but not with VP1 ΔC74. Even though it was very unlikely, I did not exclude the possibility of an exchange between these two transgenic lines VP1 full-length and VP1 ΔC74. The PCR test using a couple of primer with one hybridizing in the N-terminal sequence and one in the last C-terminal sequence (that correspond to the last 74 amino acid sequence), gave me clear results: VP1 full-length contains the VSR and VP1 ΔC74 does not possess the last C-terminal sequence.

These contradictory results did not permit us to clearly demonstrate a link between the possible interaction of Nora virus VSR with *Drosophila* Ago2 and the Nora-positive flies susceptibility to PA14 intestinal infection and the increase of ISCs proliferation rate in these flies.

To assess if Nora-positive fly phenotypes are dependent or not from Ago2, I overexpressed a short-hairpin against *ago2* in the midgut of flies using the same drivers as described earlier and kept these flies on a sucrose-only diet. Then I dissected their midguts and I analyzed by RT-qPCR the expression profile of *Diptericin* and *Socs36e* that were shown to be overexpressed in Nora-positive flies (Fig. 3E and F, previous paper). A slight increase of expression, but not significant, of *Socs36e* was observed in ISC and progenitor cells (Fig. C5). However I observed a significant increase of *Diptericin* expression in enterocyte when *ago2* was down regulated in this cell type (Fig. C5).

These results suggest that blockage of Ago2, and may be also of the RNAi pathway, induces an overexpression of *Diptericin* in the midgut.

All together, these results did not permit us to conclude that the Nora virus VSR interacting with Ago2 is sufficient to induce a Nora-positive fly phenotype and neither exclude an effect of this interaction in the Nora-positive fly phenotype. Moreover we could not identify which intestinal cell type Nora virus targets, if the virus targets only one. Clearly,

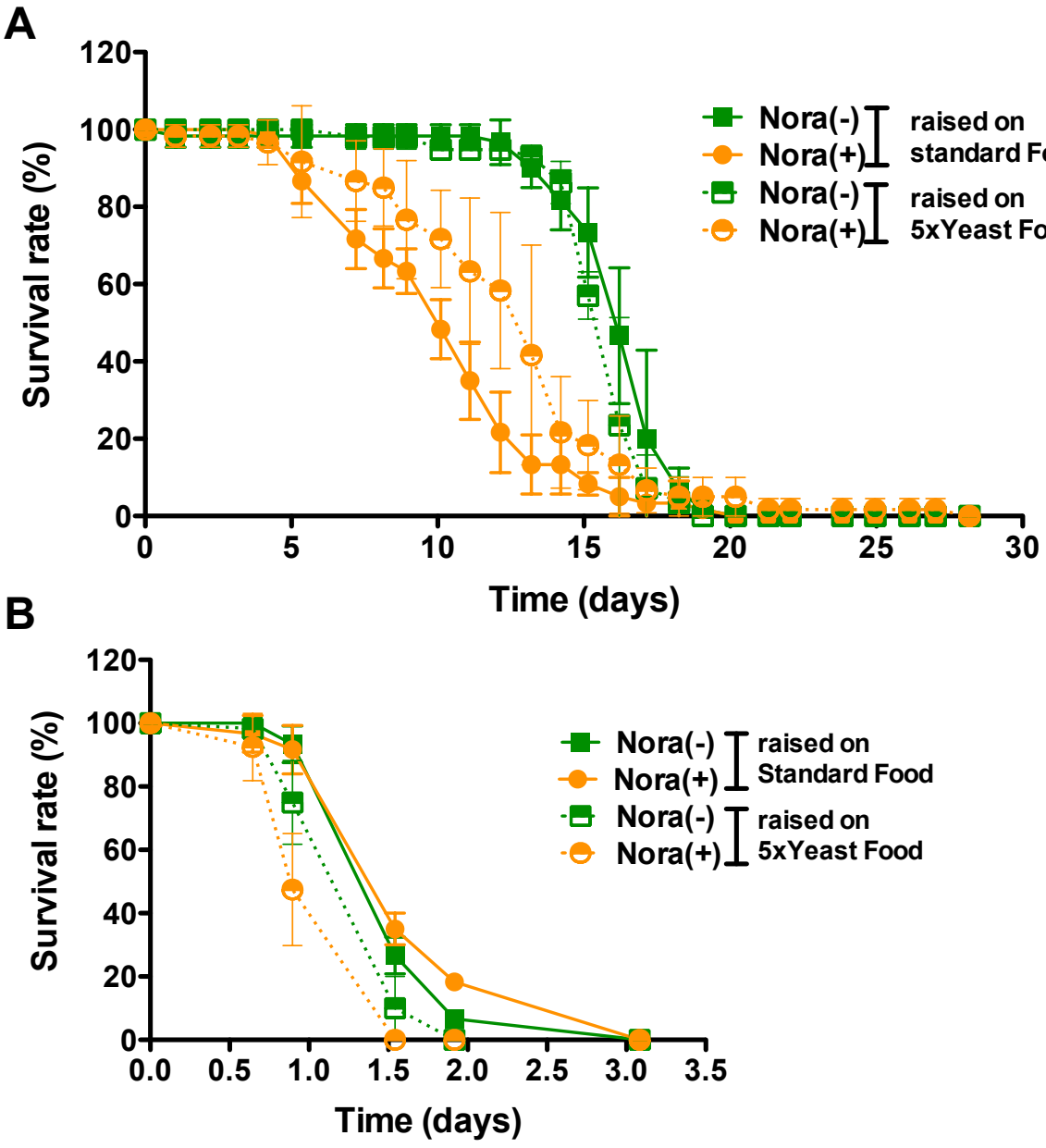


Figure C6. The quality of the fly food impacts on the survival rate of flies infected with the Nora virus.

Females flies infected with the Nora virus [Nora(+)] or not [Nora(-)] were raised either on a standard fly food (Standard Food) or on a yeast-enriched fly food (5xYeast Food). (A) Nora(+) and Nora(-) flies from each condition were kept on a sucrose-only diet. Nora(+) flies raised on a rich fly food are less sensitive to a sucrose-only diet than the same flies raised on a standard fly food. All Nora(+) flies are still more sensitive than all Nora(-) flies. Data represent the mean ±SD of triplicates (20 flies per tube) from one experiment. (B) Nora(+) and Nora(-) flies were kept on demineralized water only. Well-fed flies are slightly more sensitive in these conditions than flies raised on a standard fly food. Among the well-fed flies, Nora(+) flies are even slightly more sensitive than all the other flies. The mean ±SD of triplicates (20 flies per tube) from one experiment are shown.

overexpressing the CrPV VSR in progenitors cells but not in ISCs only induced a decreased survival of the flies after a PA14 infection and an increased number of pH3 positive nuclei in the midgut, in support of a role of the siRNA pathway in the proliferation of ISCs. In addition a down-expression of *ago2* in enterocytes induced an increase of *Diptericin* expression.

This suggests that Ago2 and may be RNAi pathway inhibition could play at least a partial role in the Nora-positive fly phenotype. Moreover we can not exclude that Nora virus targets all the intestinal cell types allowing a synergy and increased phenotype, and neither a non-cell autonomous role of Ago2 (blockage of Ago2 or RNAi pathway, for example in intestinal visceral muscles, could generate different secreted signals acting on ISCs, enteroblasts and enterocytes). However, according to our results, Nora virus interaction with Ago2 and the RNAi pathway seems to be much more complex than described and expected.

The quality of fly food influences the degree of susceptibility of Nora-positive flies to a sucrose diet

Nora-positive flies are more susceptible to a variety of intestinal infection like *P. aeruginosa* and *S. marcescens* (Fig. 1B & C, Nora virus manuscript) and to a sugar-only diet (Fig. 1F, Nora virus manuscript). Previously, we observed a significant difference in triacylglycerol stores between Nora-positive and Nora-negative flies when kept on a sucrose only diet. Therefore, we were wondering if the levels of metabolic stores could be implicated in the dramatic increase of death rate of Nora-positive flies compared to Nora-negative flies in that specific condition. I raised Nora-positive and Nora-negative flies on either our standard fly food medium (see composition in Nora virus manuscript) or on a fly food (same composition than our standard fly food) with five fold more yeast compared to our standard fly food. After a few fly generations, I transferred 3x 20 females flies of both Nora-positive and Nora-negative grown in these two conditions (standard food and rich food) on 2 mL of sucrose 50 mM. These flies were kept at 25°C with 60% humidity and their survival was monitored.

In the case of flies raised with the standard fly food, Nora-positive flies fed only on sucrose exhibited dramatic faster rate of death, with an LT50 at 10 days, compared to Nora-negative flies, that had an LT50 at around 17 days (Fig. C6A), as expected (Fig. 1F and Fig. 4B, Nora virus manuscript). No difference was observed between Nora-negative flies either kept on standard or rich fly food. Surprisingly, Nora-positive flies raised on rich fly food

survived longer than Nora-positive fed with the standard fly food. However, these well-fed Nora-positive flies were still more susceptible than Nora-negative flies to the sucrose only diet.

The difference in the survival rate of Nora-positive flies when fed either on rich or standard food, and the absence of difference with Nora-negative flies under these two same conditions, suggests at least a partial role of metabolic stores in the Nora sensitivity phenotype on a sucrose only diet.

To follow on these results, I exposed Nora-positive and Nora-negative flies to a stronger metabolic stress, full starvation, by keeping the flies on 2 mL of demineralized water only.

Under these conditions, Nora-positive and Nora-negative flies raised on standard fly food died at the same rate (Fig. C6B). Unexpectedly, both Nora-positive and Nora-negative flies grown on rich food were more sensitive to full starvation conditions than flies raised on standard food. However, Nora-positive flies grown on rich food seemed to die slightly faster than Nora-negative flies raised in the same conditions. However, in the full starvation condition, the difference in LT50 between well fed Nora-positive and Nora-positive flies raised on standard food was only 12 hours. This difference was even smaller between Nora-negative either raised on rich food or on standard food. These metabolic stress conditions were so strong that all flies died within three days and most likely too fast to see any significant difference between all Nora-positive and Nora-negative flies.

The metabolic stores did not seem to be different between Nora-positive and Nora-negative flies when flies were kept on standard food (Fig. 4C and Fig. S3A from Nora virus manuscript). Moreover, in Nora-positive flies the amount of triacylglycerol was lower than Nora-negative flies after five and eight days kept on a sucrose-only diet. This suggested that, under a sucrose diet condition, Nora-positive flies drew faster in their triacylglycerol reserves than Nora-negative flies, likely because Nora-positive flies used more energy than Nora-negative flies.

We have shown an increase of ISC proliferation in the midgut of Nora-positive flies compared to Nora-negative flies. Stem cells divisions cost energy and this increase of ISCs proliferation in Nora-positive flies could be responsible at least in part for the increase of triacylglycerol lost in these flies compared to Nora-negative flies.

All these results indicate that the levels of metabolic stores and therefore the quality of the fly food in terms of amount of metabolites plays an important role in the Nora-positive fly

sensitivity to a sucrose-only diet and most likely also in the reduced lifespan of these flies. This critical point could explain some discrepancy of Nora virus infection phenotype observed in other laboratories. For instance, Dan Hultmark, who first identified the Nora virus infection in *Drosophila* and described the virus, observed a less severe decreased lifespan in Nora-negative flies compared to our results (Habayeb et al., 2009b).

General Discussion

In this work, I have focused on a model of intestinal infections by the ubiquitous opportunistic pathogen *P. aeruginosa* using as a host the fruit fly *D. melanogaster*. This is a good model system because of the wealth of knowledge accumulated on both partners. Here, I have tried to use as much as possible the strong genetics of *Drosophila* and the advantage of its small size and rapid life cycle that allows performing many experiments in a relatively short time frame. In addition, the infection model is complex as the pathogen moves through distinct compartments within the host and encounters several types of immune defenses. I have also explored the interactions from the pathogen's perspective by performing a mini genetic screen and by generating some deletion mutants in *P. aeruginosa* PA14, in collaboration with Olivier Cunrath from the Isabelle Schalk laboratory at the ESBS in Illkirch. Before discussing more in depth the lessons I have learned from performing this work, I would like to summarize briefly my main findings that are described at length in this manuscript.

- *rhlR* but not *rhlI* displays a strongly attenuated virulence in a *Drosophila* intestinal infection model;
- *rhlI, lasI*, and *lasR* display all a similarly mildly reduced virulence in this infection model;
- *vfR, sltB1*, and *xcpR* may function in a common process together with *rhlR*, which likely affects the bacterial cell wall and thereby allows PA14 to elude at least partly detection by the immune system;
- phagocytosis is required to control PA14 infections in the hemolymph only during the first phase of the infection when few bacteria are present in the hemocoel;
- *rhlR* is required not only to circumvent Eater-mediated phagocytosis but also to avoid detection by the putative opsonin Tep4;
- the switch to virulence in the hemocoel likely requires a quorum sensing-dependent function of RhlR;

- bacteria present in distinct hosts coordinate their virulence through volatile cues that are likely perceived directly by the bacteria within their hosts;
- Toll pathway activation during bacteremia is likely mediated both by sensing PA14 DAP-type PGN and direct or indirect detection of PA14 enzymatic activity of PA14 virulence factors by the host protease Persephone;
- PA14 kills Nora virus-infected flies much quicker and only in these conditions induces strong damages to the epithelium;
- Nora-virus infected flies display an enhanced local immune response when challenged orally with PA14;
- Nora virus infection shortens the life span of the *Drosophila* host, both when flies feed on normal food or on sucrose solution;
- the presence of Nora virus promotes the growth of the microbiota in aged flies and in flies feeding on a sucrose solution;
- the Nora virus affects the homeostasis of the midgut independently from the microbiota.

A synopsis of PA14 intestinal infection in Drosophila

PA14 is placed on filters in a sucrose solution that also contains some bacterial growth medium, which is required for its virulence. There is only a very limited growth of PA14 on this filter for a day, and the titer remains more or less constant thereafter. Ingested bacteria pass through the different sections of the gut and some of them are diverted to a storage diverticulum, the crop. In the midgut, PA14 encounters AMPs, ROS, and digestive enzymes. Of note, PA14 is unable to permanently colonize the midgut, as is also the case for the microbiota (Blum et al., 2013; Limmer et al., 2011a). Throughout the infection, a few bacteria continuously manage to escape from the digestive tract into the hemocoel where they encounter plasmatocytes. As the titer remains low, it is likely that they are phagocytosed; yet, some of them may circumvent phagocytosis, largely through a quorum sensing-independent function of RhlR that likely modifies the cell wall and thus allows PA14 to escape detection by the host cellular immune system. At mid-infection, there is a switch in virulence that is likely mediated by the bacteria quorum-sensing system and that may require some coordination in-between bacteria present in distinct hosts so as to speed up the process. As a

result, PA14 proliferates within the hemocoel and thereby triggers the systemic immune response, that is however ineffective at clearing the infection.

Let me now discuss the distinct steps of the intestinal infection, as revealed by my experiments.

I have observed using a reporter transgene an induction of the IMD pathway in the proximal region of the midgut (region 1), right after the proventriculus, from seven hours onward. It is not clear why there is not an induction of the IMD pathway throughout the midgut. Possibly, PA14 might block translation in the rest of the midgut, as has been reported for *P. entomophila* in *Drosophila* and for PA14 in *C. elegans* (Chakrabarti et al., 2012; Dunbar et al., 2012).

As regards the ROS host response, I have not directly monitored whether hypochlorous acid is generated by DUOX, for instance by using the R19 dye (Lee et al., 2013). DUOX activity is induced by uracil secreted by bacteria (Lee et al., 2013). It is likely that uracil is secreted by *P. aeruginosa* as uracil has been reported to influence all three quorum sensing systems and these are affected when uracil synthesis is altered in the bacteria (Ueda et al., 2009). Indeed, it has been reported that a flavodoxin, a mobile electron shuttle, likely provides efficient protection against ROS within the *Drosophila* midgut (Moyano et al., 2014). Flavodoxin mutants display a lower overall titer and are less virulent in an intestinal infection model. It has not been tested whether this antioxidant might also protect bacteria in the phagolysosome of *Drosophila* hemocytes; however, such a role has been described in a mammalian macrophage cell line (Moyano et al., 2014).

In conclusion, the bacterium encounters in the gut rather drastic conditions that are difficult to reproduce in vitro. The work from the team on a similar infection model with *S. marcescens* suggests that these Gram-negative bacteria shed their LPS O-antigen in the midgut. Indeed, *S. marcescens* retrieved from the hemolymph hardly react with a O28-serotype-specific antibody (S. Liegeois and M. Lestradet, personal communications). Thus, it is likely that the bacteria that cross the intestinal barrier have an altered cell wall, which might facilitate their detection by phagocytes. In contrast, in the septic injury model, the cell wall has not been perturbed and is likely intact, which may partially account for the much enhanced virulence of PA14 in this infection model, in which phagocytosis appears to play at best a modest role in host defense.

We do not have a formal proof that *P. aeruginosa* penetrates the hemocoel by crossing the digestive tract. For instance the bacteria might cross weak points of the cuticle, for instance in the joints of the legs or by penetrating the tracheal system. My work provides a

hint that the passage occurs through the midgut. Indeed, I have shown that in Nora virus-infected flies the rate of transit to the hemolymph is higher than in control-uninfected flies. As Nora virus is an enteric virus that affects the homeostasis of the intestinal epithelium, it implies that the bacteria likely cross the digestive tract at the midgut. Further microscopic studies are warranted to determine whether the passage occurs through the enterocytes by transcytosis or in-between epithelial cells by a paracellular route. However, such studies are difficult to perform as few bacteria manage to cross the epithelium. Of note, it is suspected that patients in intensive care units suffer infections, e.g., lung infections inflicted by bacteria initially present in their own gastro-intestinal tract, an effect that can be somewhat recapitulated in mice models of intestinal infections (Alverdy et al., 2000; Bertrand et al., 2001; Ledingham, 1988; Ma and Kanost, 2000; Matsumoto et al., 1997, 1998; Yoshida et al., 1986). Of note, the passage of the gut barrier may involve the disruption of tight junctions, mediated by effectors of the T3SS, exoS or exoU (Okuda et al., 2010; Zaborina et al., 2006). Also, *P. aeruginosa* has been shown to be invasive, for instance in the cornea in the case of keratitis affecting contact lens wearers. Indeed, the pathogen can cross several epithelial layers. Again, the T3SS seems to be involved (Heimer et al., 2013), which is definitely not the case in our infection model with PA14, but may be the case for the strain CHA, which relies on exoS to neutralize phagocytes (Avet-Rochex et al., 2005, 2007; Fauvarque et al., 2002). One should nevertheless be aware that the studies cited above were performed at a time when the T6SS had not been discovered yet. Thus, investigators may have missed a potential link with this novel secretion system. I did not find any virulence phenotype in mutants deficient for the H2-T6SS and found a weak phenotype for one transposon inserted in the H1-T6SS whereas another insertion in the same locus did not yield any altered phenotype. This finding should be thus further validated with deletion mutants. Also, we noted a weakly decreased virulence phenotype for the reconstructed wild-type allele of *ladS* present in many strains of *P. aeruginosa* but not in PA14 when the host cellular immune response was ablated. The wild-type allele of *ladS* is known to regulate the H1-T6SS (Mikkelsen et al., 2011). Thus, we obtained somewhat contradictory results, a statement that needs to be modulated in the light of the weakness of the observed phenotypes. It actually remains to be established whether biofilms do form inside the hemocoel of infected flies. Of note, PAO1 biofilms have been reported to form in the crop and to prevent virulence in another *Drosophila* intestinal infection model (no growth medium added with the bacteria) by decreasing the rate of passage to the hemocoel (Mulcahy et al., 2011). These results should however be interpreted with great caution as the authors actually centrifuged flies to retrieve bacteria supposedly

present in the hemocoel... They found a large titer that may actually represent bacteria present in the gut. Indeed, we did not notice any phenotype when we tested similar mutants affecting biofilm formation in PA14 (Limmer et al., 2011a).

Two lines of evidence support our proposal of a quorum sensing-independent function of RhlR. First, the null *rhlR* phenotype is much stronger than the *rhlI* null phenotype. Second, phagocytosis only plays a role at the early stage of the infection. Since RhlR's main function is to elude phagocytosis, it follows that RhlR is required at an early stage of the infection, when few bacteria are present, making a role of quorum sensing unlikely. Of note, we cannot formally exclude that the threshold of activation is lowered as has been described when *P. aeruginosa* is starved. However, less than 10 bacteria per fly can be retrieved from the hemolymph at these early stages. Another argument in support of my hypothesis is the discovery of the role of *sltB1* in virulence. This mutant is likely to display an altered cell wall that would make it more prone to detection by Eater or by Tep4. As *sltB1* displays a *rhlR*-like phenotype, it may be regulated by RhlR. Further studies are required to validate this point, both in terms of phagocytic uptake and in terms of expression as measured by qRT-PCR. A role of the T2SS is less easy to explain. It may be required for the secretion of virulence factors, and thus might be required for a quorum sensing-dependent function. Alternatively, it might be required for the secretion of factors that maintain the integrity and functions of the bacterial cell wall. Finally, the role of vfR in virulence is also not unexpected. My original finding is that vfR likely acts together with rhlR, and that *vfR* expression in vivo appears to require RhlR function. As this was in a septic injury model, it would be interesting to determine whether *rhlI* is also required for *vfR* expression in this septic injury model. At present, it is difficult to tell whether vfR functions in a quorum sensing-dependent or independent manner. If *sltB1* expression happens to be dependent on either *rhlR* or *vfR*, an hypothesis that remains to be tested, it will be interesting to study its promoter.

rhlI, *lasR*, and *lasI* display a mild but nevertheless significant decreased virulence phenotype in which flies succumb about two days later to infection. These data suggest that there is also a quorum sensing-dependent component in the *rhlR* mutant phenotype. The finding that the slope of the survival curves is shallower for rhlR suggests that flies succumb to wild-type PA14 infection in a coordinated manner that is best explained by the synchronization of the switch to virulence of bacteria present in distinct hosts. My experiments have demonstrated that single flies do not display such a coordinated susceptibility behavior and actually succumb at a slower rate. It is striking that this difference in death rates is also of about two days, which is also the decreased rate observed for *rhlI*

mutant infection. This observation suggests that the switch to virulence is caused by coordination between bacteria present within distinct hosts rather than within the same host. Even though I did not provide a formal demonstration, I propose that at least the Rhl quorum sensing system is involved in this phenomenon. Indeed, both the *rhlR* and the *rhlI* phenotypes display the properties expected for genes involved in sending or receiving a diffusible signal. I plan to test this hypothesis as discussed in the manuscript. Furthermore, I shall also expose single flies to 3-oxoC12-HSL and C4-HSL and determine whether they die faster. I shall also complement *rhlI* mutant flies by providing C4-HSL.

The synchronization of the virulence of bacteria infecting distinct hosts in our laboratory infection model begs the question of its relevance in nature, that is, when there is an open environment. One possibility may be that many flies are actually present on the same rotten fruit, as anyone may have experienced in his own kitchen. Another interesting possibility is that of social insects such as ants or honeybees in which many individuals reside within a closed environment. This may also concern bumblebees. Interestingly, *P. aeruginosa* has been found in beehives and bumblebee colonies but appears to be associated with larvae (Mohr and Tebbe, 2006; Wille, 1961). As discussed previously, the tracheal respiratory system of insects may allow the diffusion of chemical cues form bacteria in one host to bacteria in another host. It would be highly interesting to determine whether such a phenomenon may also occur in vertebrate hosts.

***On screening banks of bacterial mutants in* Drosophila**

My original aim was to screen the whole PA14 insertion library generated by the Ausubel laboratory (Liberati et al., 2006). I first decided to optimize the conditions of the screen by first performing a mini-screen using a selected subset of mutants that had been shown to present a reduced virulence in a *C. elegans* infection model. However, I have been confronted with the problem of phenotypic variability from one experiment to the next. To obtain reliable data, we had to perform the screen in duplicate and then to go through multiple rounds of confirmation, using also independent mutants whenever available. Because this represented already a considerable amount of work, I never was in a position to screen the whole library. Clearly, it still does represent a worthy endeavor, as the fly model is clearly distinct from the *C. elegans* model, since most of the pathology is observed when the bacteria have escaped from the digestive tract, which does not happen except possibly at the very late

stages of infection in the nematode (Irazoqui et al., 2010). We indeed found very few mutants that displayed an altered virulence from the subset displaying a decreased virulence in *C. elegans* and actually found some with an enhanced virulence, which remain to be more closely investigated. A screen of the full library will represent a major project requiring extensive means and labor. Even in *C. elegans*, in which mutants can be screened in multiple-well plates, the screen also required quite an investment. Nevertheless, this approach is still much more powerful and achievable in the *Drosophila* model than in a murine model of infection.

What is a relevant infection model to understand *P. aeruginosa* infections in patients?

We have established an intestinal infection model in *Drosophila*. As noted above, we had to deal with some variability in the phenotype. Thus, to obtain reliable data, experiments had to be repeated multiple times. There are several parameters that influence the outcome of infections in *Drosophila*. The presence of the *Wolbachia* symbiont, which affects a large number of arthropod species, has been reported to protect flies from viral infections (Hedges et al., 2008; Teixeira et al., 2008). It does not seem to influence bacterial infections though (Wong et al., 2011b). It should however be kept in mind that *Wolbachia* might affect the Nora virus. I have in any case worked with *Wolbachia*-free flies. I have demonstrated that Nora virus is a major parameter that strongly enhances the virulence of PA14, likely by causing enhanced damages to the intestinal epithelium. Another parameter that is more difficult to control is the microbiota. I have not rigorously tested axenic flies vs. conventionally-raised flies with respect to PA14 intestinal infections. The reason for this is that young flies harbor a microbiota that is not numerous, at most 1,000 bacteria per fly, and I retrieve about 3-5,000 PA14 in infected guts. In addition, the H1-T6SS is likely killing resident bacteria. The microbiota may however indirectly impact the host: our flies are grown on a food that contains relatively little yeast, thus depriving flies of a plentiful availability of proteins and fatty acids. Furthermore, the virulence of PA14 is influenced by the medium in which it is kept on the filters. PA14 kept on a pure sucrose solution do not kill wild-type flies. Thus, even though I used *Wolbachia*- and Nora virus-free flies, I observed sometimes some variability in the absolute virulence at different periods of the year. It should be kept in mind that other parasites such as viruses might be present cryptically. Indeed, deep sequencing of cell lines and fly lines always reveal the presence of some viruses and sequences of unknown origins. The genetic background of flies is also an issue. Indeed, I observed that Ore-R wild-type flies

are more susceptible to intestinal PA14 infections than another reference strain, w^{A5001} we use as a wild-type too control for innate immunity mutants. There might be loci that strongly influence host susceptibility to infection, as exemplified by the *pastrel* locus, which influences the susceptibility to DCV (Magwire et al., 2012). In conclusion, even under conditions that are as controlled as possible, variability in virulence may remain an issue, hence the requirement for always including appropriate controls in each experiment. These considerations should actually apply to any infection model, including vertebrate ones. In the future, we shall aim to work in an insectory that will be more similar to animal houses, that is that will be free of specific pathogens. This will be however a difficult endeavor requiring even stricter quarantine procedures as the major vector for microbial contamination of fly cultures is constituted by wandering mites that feed on the food, or sometimes on flies, and that are notoriously difficult to control.

Besides the obvious importance of the infection paradigm used, *e.g*., septic injury *vs*. intestinal infection (Limmer et al., 2011b), another relevant consideration is that of the bacterial strains that are used in the infection models. My work with PA14 has revealed the importance of a quorum sensing-independent function of *rhlR* in eluding phagocytosis. It therefore came as a surprise when I analyzed the phenotype of the same null mutants in a PAO1 background. *lasR, lasI, rhlI*, and *rhlR* all displayed a strongly attenuated virulence. Further studies are warranted to understand the behavior of PAO1 in our intestinal infection model. For instance, it would be important to determine whether these mutants regain their virulence when the cellular immune response is disabled. Indeed, we do not even know whether PAO1 bacteria are able to cross the intestinal barrier. The good news however is that quorum sensing is playing an important role in PAO1 virulence (Lutter et al., 2012; Stoltz et al., 2008). Thus, the fly appears to be relevant to model strategies in which quorum sensing is targeted (Papaioannou et al., 2013; Schuster et al., 2013).

Multiple *P. aeruginosa* strains have been retrieved from patients. Furthermore, these strains evolve during the infection, for instance by losing the perception to quorum sensing signals. These cheaters are thus able to profit from the common goods, that is, virulence factors, produced by the community, without paying the metabolic price of synthesizing these factors. A major asset of *P. aeruginosa* is its ability to adapt to diverse environmental host conditions. These strains may have differing genomes and only a core genome is common to all *P. aeruginosa* strains. Thus, my work as well as that of many investigators raises the question as to whether each strain may display unique characteristics that give it its own virulence patterns. A good starting point toward answering this problem will reside in the

systematic comparison of PAO1 and PA14 in our intestinal infection model. A first step was taken by investigating the *ladS* alleles, but, this clearly does not account for the observed differences. It the long term, once the difference between PAO1 and PA14 are understood, it will also be interesting to systematically test multiple clinical strains so as to determine what is the core virulome of *P. aeruginosa* and thus to determine which strategies are relevant to decrease infections *in vivo*, that is, in patients. Indeed, the ever-increasing proportion of multiple drug resistance strains is a worry that warrants the exploration of novel anti-infective strategies in which the development of microbial resistance is minimized.

One major difficulty in science today is to obtain funding. It is increasingly difficult to study biological problems for the sake of understanding and one should unfortunately more and more propose practical applications for one's research. Thus, we would like to test as a first step whether our findings in an insect model are relevant to understand infections in mammalian systems, which are supposedly closer to humans. Multiple infection models abound, from burn wound models to urinary tract infections, and of course, cystic fibrosis models of chronic infections in the lungs. Here, I am not considering cell culture models given my strong emphasis on *in vivo* infections. In practice, the ideal model for us would be intestinal infection models, which are relevant since patients often get infected by their own pathogens hosted in the gastro-intestinal tract. We note in that respect that leukopenic patients are more likely to develop such infections. Indeed, some mouse intestinal infection models rely on cyclophosphamide treatment, which will impair the host's cellular immunity. A severe limitation is to find collaborators with the relevant expertise and to convince them. Thus far, we developed contact with the group of Miguel Camara in Nottingham, that however is using a cathether colonization model that relies on PAO1, as PA14 is too virulent. Possibly, PA14 would be adequate in a mouse intestinal infection model?

Developing and understanding an infection model in *Drosophila* requires an intense effort and is a valuable project in itself. As discussed above, a thorough understanding of PA14 infection, and then of PAO1 requires still many years of work. It might only be relevant to test our findings in mice then. However, I am convinced that if we confirm a role of *rhlR* in the "invisibility" of the PA14 cell wall to the immune system, it would be highly interesting to test whether this function is relevant to elude phagocytosis by mouse macrophages once the intestinal barriers have been crossed by these bacteria.

Annex 1

Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model

Stefanie Limmer, Samantha Haller, Eliana Drenkard, Janice Leeb, Shen Yu, Christine Kocks, Frederick M. Ausubel, and Dominique Ferrandon

An in-depth mechanistic understanding of microbial infection necessitates a molecular dissection of host–pathogen relationships. Both *Drosophila melanogaster* and *Pseudomonas aeruginosa* have been intensively studied. Here, we analyze the infection of *D. melanogaster* by *P. aeruginosa* by using mutants in both host and pathogen. We show that orally ingested *P. aeruginosa* crosses the intestinal barrier and then proliferates in the hemolymph, thereby causing the infected flies to die of bacteremia. Host defenses against ingested *P. aeruginosa* included an immune deficiency (Imd) response in the intestinal epithelium, systemic Toll and Imd pathway responses, and a cellular immune response con- trolling bacteria in the hemocoel. Although the observed cellular and intestinal immune responses appeared to act throughout the course of the infection, there was a late onset of the systemic Imd and Toll responses. In this oral infection model, *P. aeruginosa* PA14 did not require its type III secretion system or other well-studied virulence factors such as the two-component response regulator GacA or the protease AprA for virulence. In contrast, the quorum sensing transcription factor RhlR, but surprisingly not LasR, played a key role in counteracting the cellular immune response against PA14, possibly at an early stage when only a few bacteria are present in the hemocoel. These results illustrate the power of studying infection from the dual perspective of host and pathogen by revealing that RhlR plays a more complex role during pathogenesis than previously appreciated.

Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model

Stefanie Limmer[a,1], Samantha Haller[a], Eliana Drenkard[b,c], Janice Lee[b,d,2], Shen Yu[b,c], Christine Kocks[b,d], Frederick M. Ausubel[b,c,3], and Dominique Ferrandon[a,3]

[a]Equipe Fondation Recherche Médicale, Unité Propre de Recherche 9022 du Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, Université de Strasbourg, F67084 Strasbourg Cedex, France; [d]Department of Pediatrics and [c]Department of Genetics, Harvard Medical School, Boston, MA 02115; and [b]Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

Contributed by Frederick M. Ausubel, September 12, 2011 (sent for review October 8, 2010)

An in-depth mechanistic understanding of microbial infection necessitates a molecular dissection of host–pathogen relationships. Both *Drosophila melanogaster* and *Pseudomonas aeruginosa* have been intensively studied. Here, we analyze the infection of *D. melanogaster* by *P. aeruginosa* by using mutants in both host and pathogen. We show that orally ingested *P. aeruginosa* crosses the intestinal barrier and then proliferates in the hemolymph, thereby causing the infected flies to die of bacteremia. Host defenses against ingested *P. aeruginosa* included an immune deficiency (IMD) response in the intestinal epithelium, systemic Toll and IMD pathway responses, and a cellular immune response controlling bacteria in the hemocoel. Although the observed cellular and intestinal immune responses appeared to act throughout the course of the infection, there was a late onset of the systemic IMD and Toll responses. In this oral infection model, *P. aeruginosa* PA14 did not require its type III secretion system or other well-studied virulence factors such as the two-component response regulator GacA or the protease AprA for virulence. In contrast, the quorum-sensing transcription factor RhlR, but surprisingly not LasR, played a key role in counteracting the cellular immune response against PA14, possibly at an early stage when only a few bacteria are present in the hemocoel. These results illustrate the power of studying infection from the dual perspective of host and pathogen by revealing that RhlR plays a more complex role during pathogenesis than previously appreciated.

innate immunity | host–pathogen interactions | systemic immunity | intestinal immunity | phagocytosis

Thanks to its powerful genetics and the lack of an adaptive immune response, the fruit fly *Drosophila melanogaster* is an ideal host in which to study many evolutionarily conserved features of host–pathogen relationships (1). The *Drosophila* host defense response in a septic injury model (in which pathogen cells are introduced directly into the body cavity) relies on the rapid activation of immune defenses, including coagulation and melanization, phagocytosis of invading microorganisms mediated by hemocytes, and a potent systemic humoral response involving the production of antimicrobial peptides by the fat body, the insect equivalent of the mammalian liver (1, 2). In the case of bacterial infections, pattern recognition receptors (PRRs) discriminate between two types of peptidoglycan (PGN). Diaminopimelic acid-type PGN triggers the immune deficiency (IMD) pathway. The antibacterial action of the IMD pathway is mediated in part by antimicrobial peptides (AMPs), including diptericin, which is active against Gram(−) bacteria. In contrast, lysine-type PGN, which is found in some Gram(+) bacteria, leads to the systemic activation of the Toll pathway that functions in parallel to the IMD pathway to activate the expression of a partially overlapping set of immune effectors, including the AMP drosomycin.

The *Drosophila* defense against infection is not limited to immunity in the body cavity (hemocoel). For example, intestinal infection models have revealed a role for the IMD pathway in barrier epithelia, including the midgut epithelium (3–6). Previously, we developed a *Drosophila* oral infection model with the potent entomopathogenic bacterium *Serratia marcescens*, which is able to cross the intestinal barrier (4). Interestingly, *S. marcescens* loses virulence in the hemocoel and is controlled by phagocytosis. We identified about 900 *Drosophila* genes that may be involved in defense against ingested *S. marcescens* (7). The well-studied human opportunistic pathogen *Pseudomonas aeruginosa* is also a potent *Drosophila* pathogen (8). Because of the extensive genetic tools available for *P. aeruginosa*, including a nonredundant transposon mutant library (9), and the multifaceted nature of *P. aeruginosa* virulence (10, 11), we and others have used *Drosophila–P. aeruginosa* oral infection models to study evolutionarily conserved mechanisms underlying infectious disease (12–16; reviewed in ref. 17). Here, we address pathogenesis from the dual perspective of host and pathogen by using mutants in both *Drosophila* and *P. aeruginosa*. We find with our infection protocol that ingested *P. aeruginosa* strain PA14 traverses the gut barrier and kills its host through a systemic infection. The *P. aeruginosa* quorum-sensing regulator RhlR is required for virulence and may allow *P. aeruginosa* to circumvent the hemocyte-mediated cellular immune response.

Results

Ingested *P. aeruginosa* Kills Flies by Bacteremia in the Hemocoel. We monitored the survival of adult flies fed on a sugar solution (supplemented, or not, with bacterial growth medium) containing *P. aeruginosa* strain PA14. The severity of infection ranged from asymptomatic (sucrose-only solution) to severe (sucrose solution supplemented with bacterial growth medium) (Fig. 1 *A* and *B* and Fig. S1*A*). Typically, flies died more slowly (after about 8 d) when *P. aeruginosa* was ingested than after direct inoculation into the hemocoel in the septic injury model (48 h; Fig. S2*A*) (8, 14, 18, 19). *P. aeruginosa* PA14 did not appear to persistently colonize the fly intestine despite the presence of a stable steady-state number of viable bacteria in the intestine when flies were continuously feeding on the pathogen (Fig. S2*B*). Indeed, flies that were fed on *P. aeruginosa* for up to 3 d did not succumb to the infection when transferred to vials containing

Author contributions: S.L., S.H., C.K., F.M.A., and D.F. designed research; S.L., S.H., J.L., and C.K. performed research; E.D. and S.Y. contributed new reagents/analytic tools; S.L., S.H., C.K., F.M.A., and D.F. analyzed data; and S.L., S.H., C.K., F.M.A., and D.F. wrote the paper.

The authors declare no conflict of interest.

[1]Present address: Institut für Neuro- und Verhaltensbiologie, Badestrasse 9, 48149 Münster, Germany.

[2]Present address: Albany Medical College, 43 New Scotland Avenue, Albany, NY 12208.

[3]To whom correspondence may be addressed. E-mail: ausubel@molbio.mgh.harvard.edu or d.ferrandon@ibmc.u-strasbg.fr.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1114907108/-/DCSupplemental.

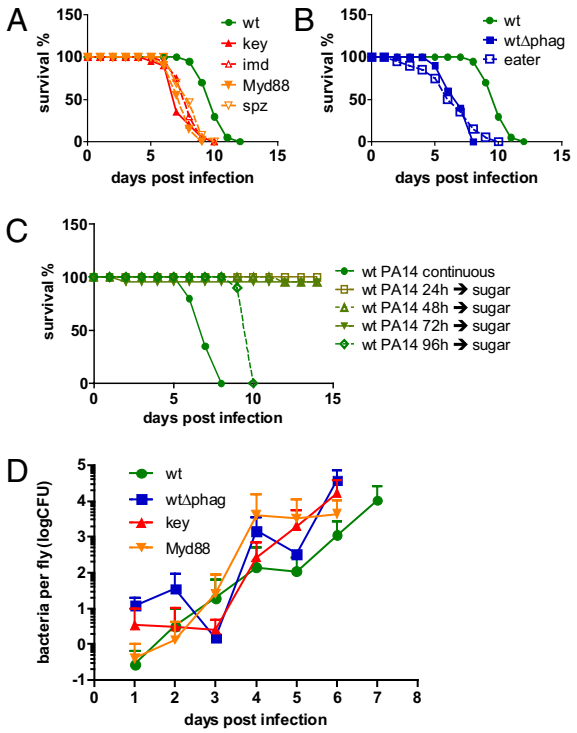


Fig. 1. Systemic and cellular immune responses contribute to host defense against orally ingested *P. aeruginosa* PA14. (*A* and *B*) Survival following PA14 oral infection. IMD pathway mutants [*imd* ($P = 0.0003$, $n = 8$), *key* ($P = 0.00005$, $n = 22$)] and Toll pathway mutants [*MyD88* ($P = 0.0001$, $n = 22$), *spätzle* (*spz*) ($P = 0.01$, $n = 4$)] succumbed faster to the infection than wild-type (wt) flies (*A*). Flies defective for phagocytosis [*eater* ($P = 0.01$, $n = 3$); latex bead-injected flies: wtΔphag ($P = 8 \times 10^{-7}$, $n = 9$)] also died faster than wild type (*B*). (*C*) Flies were either fed continuously or fed for the indicated period on the bacterial solution and then fed on a sterile sucrose solution that was changed daily; survival data are shown. At least 4 consecutive days of feeding were required to develop a lethal infection. (*D*) Bacterial titers measured in the hemolymph collected from batches of 10 flies in seven independent experiments are shown on a logarithmic scale. The values shown correspond to the bacterial titer per fly. Error bars are ±SD.

only a sterile sucrose solution (Fig. 1*C*) and actually cleared the bacteria from the gut. In contrast, flies transferred to the sterile sucrose medium after feeding on PA14 for 4 d died with similarly rapid kinetics as flies fed continuously on the pathogen, except that killing occurred about a day later, even though the pathogen was cleared from the digestive tract (Fig. 1*C* and Fig. S2*B*). In our experimental setting, we observed no significant degradation of the intestinal epithelium or an increase in intestinal stem cell proliferation, even at late stages of infection (Fig. S3 and *SI Results* and *SI Discussion*).

P. aeruginosa PA14 was able to cross the intestinal epithelium, although bacteria were barely detectable in the hemolymph after the first day of feeding on PA14 unless phagocytosis was blocked (Fig. 1*D*). Afterward, the titer of PA14 slowly increased in the hemolymph of wild-type flies or behaved somewhat erratically in flies with impaired host defense (see below) during the first 3–4 d while remaining at an absolute level of fewer than 100 bacteria per fly in the hemolymph. When we injected a similar number of bacteria in the body cavity (septic injury model), 50% of the flies succumbed within 48 h (Fig. S2*A*). In contrast, a similarly high number of bacteria were found in the hemolymph of orally infected wild-type flies around day 4, yet flies succumbed only starting from days 7–8 (Fig. 1 *A* and *D*). Thus, *P. aeruginosa* in the hemocoel appeared to be initially less virulent than in the septic injury model. Likewise, *S. marcescens*, another Gram(−) opportunistic pathogen able to orally infect *Drosophila*, is much more virulent following a septic injury. *S. marcescens* also crosses the intestinal barrier in an oral infection model, but in contrast to *P. aeruginosa*, does not proliferate in the hemocoel (4). Taken together with the lack of persistent colonization of the intestine by *P. aeruginosa* PA14, the steadily increasing bacterial titer in the hemolymph suggests that orally infected flies die from bacteremia.

Both a Systemic and a Cellular Immune Response Are Required in the Host Defense Against Ingested *P. aeruginosa*. Next, we investigated the different facets of host defense in the *P. aeruginosa* oral infection model. As expected, IMD pathway mutants succumbed significantly earlier than wild-type flies of the same genetic background (Fig. 1*A*). We observed the induction of *Diptericin* reporter transgenes in the distal proventriculus and proximal midgut from day 1 onward (the proventriculus is the valve-like structure that connects the foregut to the midgut; Fig. 2 *B–D* and Fig. S4). In contrast to its expression in the proventriculus, the expression of the *Diptericin-LacZ* reporter was induced only

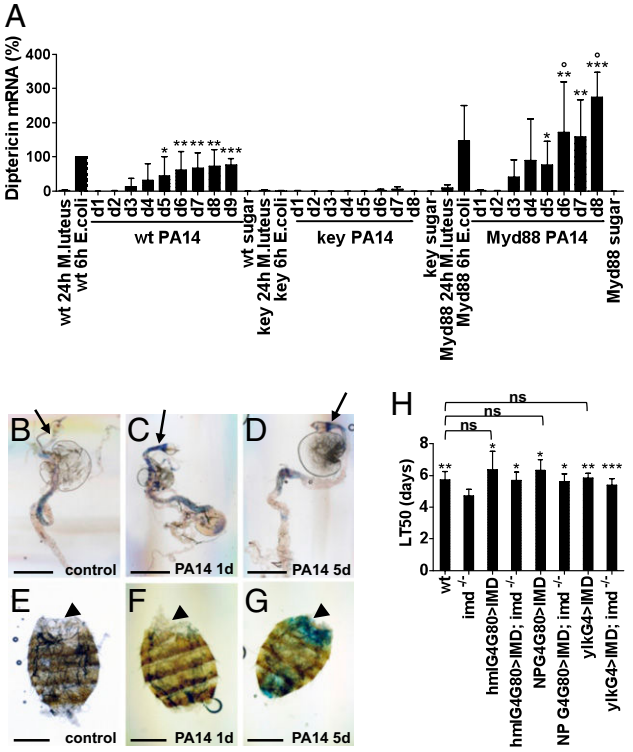


Fig. 2. An early-activated local IMD response and a late systemic IMD response both contribute to host defense against orally ingested *P. aeruginosa* PA14. (*A*) qRT-PCR analysis of the induction of *Diptericin*, a classic IMD pathway readout, in infected flies. Results are expressed as a percentage of the induction measured 6 h after a septic injury challenge with *E. coli*. *P* values (*) refer to the comparison between infected and noninfected flies of the same genotype: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 7$. Other *P* values (°) refer to the comparison between mutant and wild-type flies at the same day of infection: $°P < 0.05$; $n = 7$. (*B–G*) β-Galactosidase staining of *Diptericin-LacZ* flies. *Diptericin* is induced in the proventriculus (arrows) throughout the infection (*B–D*), whereas systemic *Diptericin* induction in the fat body (arrowheads) of the fly occurs in later stages of the infection (*E–G*). (*H*) Rescue of the *imd* PA14 susceptibility phenotype by overexpression of a UAS-*imd*$^+$ transgene (>IMD) with a gut (NPG4G80), a hemocyte (hmlG4G80), or a fat body (ylkG4)-specific driver as documented by the average time it takes to kill 50% of the flies (LT50). Note that AMPs synthesized in hemocytes and the fat body are secreted into the hemocoel. In this series of experiments, wild-type flies succumbed somewhat earlier than usual. *P* values computed by comparison with *imd* mutant flies: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 5$. Error bars are ±SD. ns: not significant. (Scale bars: 500 μm.)

from day 5 onward in fat body lobules in which the systemic immune response takes place (Fig. 2 *E*–*G*). The bulk of endogenous *Diptericin* mRNA started accumulating at days 4–5, suggesting that the majority of *Diptericin* expression occurs in the fat body (Fig. 2*A*). As expected, *Diptericin* expression was not induced in flies in which the IMD pathway gene *kenny* (*key*) is mutated, but was induced in *MyD88* mutant flies in which the Toll pathway is abrogated (Fig. 2*A*) (20, 21).

To determine which tissues are functionally relevant to the IMD defense against ingested PA14, we expressed a transgenic wild-type copy of *imd* either in the midgut, in hemocytes, or in the fat body of an *imd* mutant (rescue by overexpression) or of wild-type flies (overexpression) using the UAS-Gal4 expression system (22). To assess the degree of susceptibility to infection, for each survival experiment we computed the time required to kill 50% of the flies [median lethal time 50 (LT50)]. The overexpression of the *imd* transgene in a wild-type background did not significantly enhance protection against PA14. In contrast, the *imd* susceptibility phenotype was rescued by overexpressing the wild-type gene in the midgut, hemocytes, or fat body, suggesting that the IMD pathway can control defense responses in at least three different immune tissues (Fig. 2*H* and *SI Discussion*).

Typically, the Toll pathway is not strongly activated by Gram(−) bacteria (2), yet *P. aeruginosa* [a Gram(−) bacterium] has been shown to induce systemically the Toll pathway (18). Consistent with the latter study, Toll pathway mutant flies such as *spätzle* and *MyD88* were more sensitive to oral *P. aeruginosa* infection (Fig. 1*A*). Accordingly, a *Drosomycin-GFP* reporter transgene (Toll pathway readout) was expressed only in the fat body from day 5 onward (Fig. 3 *B* and *C*). Similarly to *Diptericin*, the expression of endogenous *Drosomycin* as measured by qRT-PCR also became significant only from day 5 onward (Fig. 3*A*). The overexpression of a wild-type copy of *MyD88* in hemocytes but not in the midgut was sufficient to rescue the *P. aeruginosa* susceptibility phenotype of a *MyD88* mutant (Fig. 3*D*). Similar to *imd* overexpression, transgene-mediated activation of the Toll pathway using UAS-*Toll*^{10b} (encoding a constitutively active form of the receptor) or UAS-*MyD88*$^{+}$ in the midgut or the hemocytes before *P. aeruginosa* ingestion did not provide enhanced protection against the infection in a wild-type background (Fig. 3*D*). Taken together, the expression data and the genetic experiments suggest that in the late stages of the infection process the Toll pathway acts through the systemic immune response to impede *P. aeruginosa* infection.

A cellular immune response constitutes an important arm of host defense in several infection models, likely through phagocytosis (13, 14, 23–25). We therefore asked whether phagocytes play an important role in our *P. aeruginosa* PA14 feeding model. We impaired the cellular response either by injecting nondegradable latex beads (26) or by using mutant flies deficient for the phagocytic receptor Eater (23). In both cases, we observed a significantly reduced resistance to ingested *P. aeruginosa* (Fig. 1*B*), even under conditions in which ingested *P. aeruginosa* does not kill wild-type flies (sucrose only, Fig. S1 *B* and *C*). Flies in which the cellular response was blocked by latex bead injection displayed a higher bacterial titer than wild-type flies (Figs. 1*D* and 4*B*). We therefore investigated the possibility that PA14 impairs the phagocytic machinery of hemocytes. Even during the final phase of the infection, however, hemocytes were still able to ingest fluorescein-labeled *Escherichia coli*, suggesting that hemocytes are present and not impaired in their ability to phagocytose bacterial particles (Fig. S5).

Taken together, our data suggest that different host defenses become relevant at distinct stages of the infection.

RhlR, but Not the LasR Acylhomoserine Lactone Quorum-Sensing Transcription Factor, Is Required for the Virulence of Orally Ingested *P. aeruginosa* PA14. To determine which bacterial factors influence the virulence of PA14 in the oral infection model, we

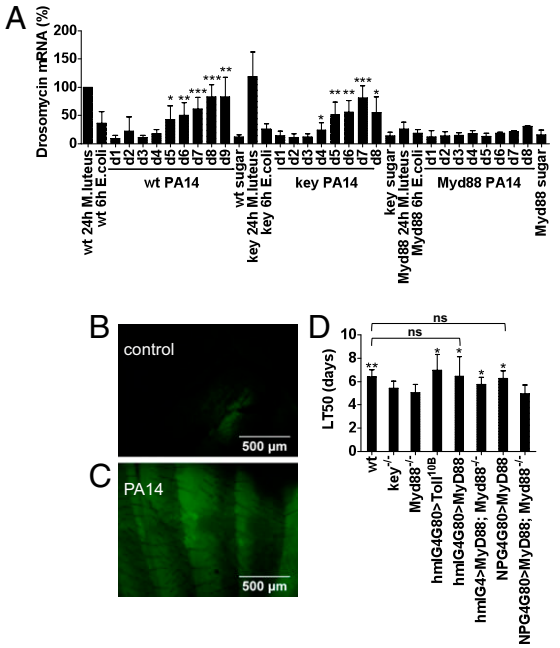


Fig. 3. Late Toll pathway activation contributes to systemic host defense against orally ingested *P. aeruginosa* PA14. (*A*) qRT-PCR analysis of the induction of *Drosomycin*, a classical readout of Toll pathway activation, in infected flies. Results are expressed as a percentage of the induction measured 24 h after a septic injury challenge with *M. luteus*. *P* values (*) refer to the comparison between infected and noninfected flies of the same genotype: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 6$. No significant difference was observed between wild-type (wt) and *key* flies with respect to *Drosomycin* expression levels. (*B* and *C*) *Drosomycin-GFP* reporter induction in the fat body upon infection. (*D*) Overactivation of the Toll pathway and rescue of the *MyD88* susceptibility phenotype by overexpression of a UAS-*MyD88*$^{+}$ transgene (>MyD88) with a gut (NPG4G80) or a hemocyte (hmlG4 or hmlG4G80)-specific driver, as documented by the average time it takes to kill 50% of the flies (LT50). Rescue was observed by overactivation of the Toll pathway in hemocytes, but not in the gut. Note that AMPs synthesized in hemocytes are secreted into the hemocoel. The UAS-*Toll*^{10B} transgene (>Toll10B) expresses a gene encoding a constitutively active form of the Toll receptor. In this series of experiments, wild-type flies succumbed somewhat earlier than usual. *P* values are compared with *MyD88* mutant flies: $*P < 0.05$; $**P < 0.01$; $n = 5$. Error bars are ±SD. ns: not significant. (Scale bar: 500 µm.)

challenged wild-type flies with bacteria defective for the type III secretion system (T3SS) (*pscD*) or one of its effectors (*exoT*); the GacA virulence regulator; the AprA alkaline protease, which is important for *Pseudomonas entomophila* virulence in an oral infection model of *Drosophila* (6); the LasR *N*-3-(oxododecanoyl)homoserine lactone quorum-sensing regulator; the LasB elastase; the MvfR quinoline quorum-sensing regulator; the PhoB low-phosphate response regulator; and PA14 deficient for the formation of biofilm (*pelA*). All of these bacterial mutants displayed normal virulence in wild-type flies (Fig. 4*A* and Fig. S6*C*). In contrast, several independent PA14 *rhlR* mutants, which are deficient for the C4-acylhomoserine lactone-dependent quorum-sensing regulator RhlR, a second acylhomoserine lactone quorum-sensing system in *P. aeruginosa* (27), were severely impaired in virulence and killed the flies 3–4 d later than wild-type PA14 (Fig. 4 *A*, *C*, and *D* and Fig. S6 *A* and *B*). A *lasR-rhlR* double mutant behaved like the single *rhlR* mutant in wild-type flies (Fig. S6*C*). Interestingly, flies infected with *rhlR* mutants did not succumb as synchronously as flies infected with wild-type PA14. The survival curve was significantly shallower as quantified by the Hill coefficient, which measures the steepness of a sigmoid curve (Fig. S7). Consistent with their reduced ability to kill flies, the titer of the *rhlR* mutants in the hemolymph was lower than

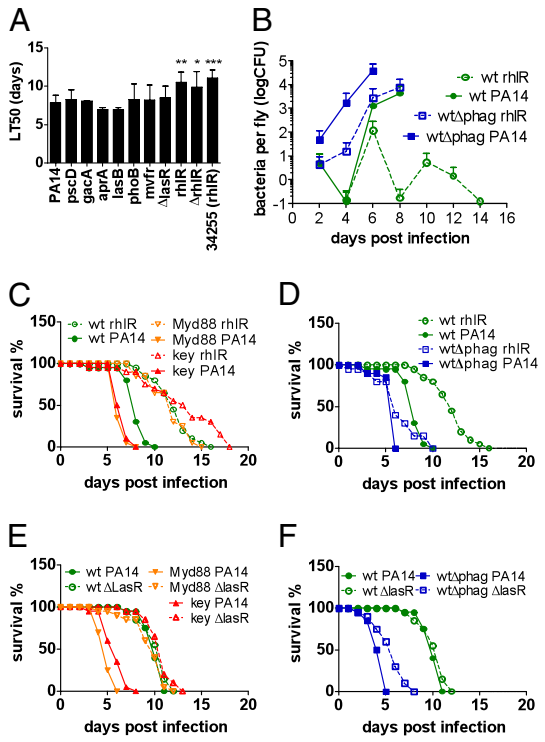


Fig. 4. RhlR, but not LasR, is required to counteract the cellular immune response against *P. aeruginosa* PA14. (*A*) Survival experiments in wild-type *Drosophila* to analyze virulence of *P. aeruginosa* mutants in known virulence factors. The average time that it takes to kill 50% of flies (LT50) is plotted. Two *rhlR* transposon insertion mutants [37943 (referred to as *rhlR*) and *34255*] and a deletion (*ΔrhlR*) displayed the same attenuated virulence phenotype, whereas other mutants were not significantly less virulent than wild-type (wt) PA14. *pscD* is a deletion mutation that affects the secretion machinery and thus prevents the secretion of all T3SS effectors, including ExoT. *exoT* mutant bacteria were tested in independent experiments using flies of a different genetic background and also did not show a phenotype ($n = 3$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n = 3$ or 4 depending on the mutant tested. (*B–F*) In the *Insets*, the genotype of the host [wild-type (wt) or mutant flies] and the genotype of the pathogen (PA14 refers to wild-type PA14) are indicated. (*B*) Bacterial counts per fly measured in the hemolymph collected from PA14 and *rhlR* infected wild-type (wt) and latex bead-injected flies (wtΔphag) expressed on a logarithmic scale ($n = 3$). (*C*) Survival experiments using wild-type PA14 and *rhlR* mutant bacteria. *rhlR* mutants are less virulent (*P* values PA14 vs. *rhlR* in wild-type flies: $P = 0.0017$, $n = 7$; *key* flies: $P = 0.0020$, $n = 6$; *MyD88* flies: $P = 0.0001$, $n = 7$). (*D*) *rhlR* mutant bacteria killed phagocytosis-deficient latex bead-injected flies as rapidly as wild-type bacteria ($P > 0.05$, $n = 6$). (*E*) Survival experiments using wild-type PA14 and *lasR* mutant bacteria. *lasR* mutants are as virulent as PA14 in wild-type flies and less virulent in *key* and *MyD88* flies (*P* values PA14 vs. *lasR* in wild-type flies: $P = 0.33$, $n = 5$; *key* flies: $P = 0.027$, $n = 2$; *MyD88* flies: $P = 0.025$, $n = 3$). (*F*) *lasR* mutant bacteria killed phagocytosis-deficient latex bead-injected flies somewhat less rapidly than wild-type bacteria ($P = 0.013$, $n = 4$). Error bars in *A* and *B* are ±SD.

the titer of PA14 and reached a maximum of around 100 bacteria per fly at day 6 of infection (Fig. 4*B*). Thus, *rhlR* mutant bacteria appear to be cleared more efficiently from the hemolymph. In keeping with these data, the systemic immune response was hardly induced, as measured by the accumulation of *Drosomycin* and *Diptericin* mRNAs (Fig. S8*A*).

RhlR Is Required to Circumvent the Cellular Immune Defense of *P. aeruginosa* Orally Infected Flies. To distinguish the possibilities that RhlR is required to counteract or elude either the systemic humoral immune response or the cellular arm of host defense, or both, we first infected wild-type, *key*, or *MyD88* mutant flies with either wild-type *P. aeruginosa* or an isogenic *rhlR* mutant. We found that *rhlR* mutant bacteria killed *key* or *MyD88* mutant flies with the same low virulence as wild-type flies (Fig. 4*C*).However, the titer of PA14 *rhlR* was somewhat higher in the *key* or *MyD88* mutants than in wild-type flies, presumably because the bacteria were not cleared as efficiently from the hemolymph (Fig. S8*B*). As expected, PA14 *rhlR* mutants did not induce *Diptericin* in *key* mutants, but *Drosomycin* was induced to somewhat lower levels by PA14 *rhlR* in the *key* mutant than in wild-type flies (Fig. S8*A*). Conversely, PA14 *rhlR* did not induce *Drosomycin* in *MyD88* mutants, and *Diptericin* was induced to significantly lower levels than those measured after an oral challenge with wild-type *P. aeruginosa* (Fig. S8*A*).

Next, we impaired the cellular immune response either by injecting latex beads before feeding the flies on *rhlR* mutant bacteria or by using *eater* mutants (sucrose-only conditions, as described above). In striking contrast to *MyD88* or *key* mutants, phagocytosis-deficient flies succumbed almost as rapidly as controls fed with wild-type *P. aeruginosa* (Fig. 4*D* and Fig. S1*B*). In other words, the *rhlR* mutant is highly virulent when the cellular immune response is impaired. Moreover, the *rhlR* bacterial titer measured in latex bead-treated flies was as high as that of PA14 in wild-type flies (Fig. 4*B*). These results suggest that RhlR's role in virulence in this infection model is to circumvent the cellular arm of immunity. Interestingly, whether the *rhlR* mutant bacteria were virulent or not in immunodeficient flies, we noted that the slopes of survival curves were shallower than with wild-type *P. aeruginosa*, indicating that the requirement for RhlR in synchronizing the rate of death among infected flies is independent of host defenses (Fig. 4 *C* and *D* and Fig. S7). We also assessed the role of RhlR in a septic injury model. Consistent with the oral infection model, *rhlR* mutants were significantly less virulent in wild-type flies, but not in phagocytosis-deficient, latex bead-injected flies (Fig. S9), further supporting the idea that RhlR is involved in counteracting hemocyte-mediated defense responses. To determine how RhlR controls virulence in our oral infection model, we tested several known direct transcriptional targets of RhlR. We found that *rhlA*, *rhlB*, and phenazine (*phzH*, *phzM*, *phzS*, and *Δphz1/2*) mutants were as virulent as wild-type PA14 in both wild-type or immunosuppressed flies (Fig. S6*C*). These data indicate that RhlR exerts its effects through other, yet unknown, effectors.

Unexpectedly, we found that LasR was required for virulence in flies defective for *key*, *MyD88*, and phagocytosis (Fig. 4 *E* and *F* and Fig. S1*C*). Thus, in contrast to RhlR, LasR contributes to virulence only in immunocompromised, but not in wild-type, flies.

Discussion

Fly Model of Generalized Bacteremia Following Gastrointestinal Infection. *Drosophila* has been widely used as a model host to study *P. aeruginosa* pathogenesis (12, 14–19, 28, 29). Here, we used an oral infection model to investigate in detail the interplay between bacterial virulence mechanisms and the host response by using both host and pathogen mutants defective in immunity or virulence, respectively. In previously described *Drosophila* oral infection models using *P. aeruginosa*, the actual cause of death has rarely been investigated (17). Unlike a previous study (15), we did not observe extensive damage to intestinal epithelial cells in our experimental setting (Fig. S3 and see *SI Results* and *SI Discussion* for further discussion). Thus, it is unlikely that flies succumb to intestinal damage. Rather, our results show that some ingested *P. aeruginosa* cross the digestive tract (a conclusion that can also be drawn from the study reported in ref. 14) and cause a systemic infection as evidenced by the high bacterial titer measured in the hemolymph before death (Fig. 1), akin to human infections caused by foodborne pathogens (30, 31). In support of this conclusion is our finding that flies succumb to

bacteremia when they are fed on *P. aeruginosa* for 4 d and are then transferred to a sterile feeding solution. The flies die, even though the bacteria are quickly cleared from the gut after being transferred (Fig. 1*C* and Fig. S2). Thus, this oral infection model in *Drosophila* provides a paradigm in which to study intestinal infections that can lead to bacteremia.

Two Phases of Infection and a Switch in the Virulence Program Controlled by the RhlR Virulence Regulator? Several studies with *Drosophila* and other insects have shown that very low numbers of *P. aeruginosa* cells (as few as 1–10) introduced into the body cavity by microinjection or pricking are able to rapidly multiply, causing a lethal bacteremia over the course of about 2 d (14, 18, 19, 32). The behavior of *P. aeruginosa* in our oral infection model is markedly different. The bacterial titer in the hemolymph remains low during the first phase of the infection (Fig. 1*D* and Fig. S8*B*). This may reflect a low-virulence state of the bacteria that cross the gut barrier as described previously for *S. marcescens* (4), or it may reflect the ability of humoral or cellular immune defenses to initially cope with the invading bacteria, or both. Indeed, a systemic immune response is significantly induced only at day 5 of feeding, when the bacterial titer in the hemolymph has increased significantly (Figs. 2 and 3). We note that a systemic immune response is induced earlier in the infection in immunodeficient flies (Figs. 2 and 3), in which case the bacterial titer also increases more rapidly (Fig. 1*D*).

Because PGN is not exposed on the surface of Gram(−) bacteria, they may not be detected by *Drosophila*'s PRRs unless the bacteria proliferate and release small PGN fragments generated during cell-wall remodeling (4). One explanation of our data showing that a systemic immune response occurs only after 5 d of feeding (Fig. 2 *A* and *E–G* and Fig. 3 *A–C*) is that the *P. aeruginosa* cells that initially cross the epithelial barrier into the hemolymph are in a relatively avirulent state but eventually switch to a high state of virulence (Figs. 1*D* and 4*B*). Alternatively, or concomitantly, the late onset of systemic immunity may reflect the gradual influx of bacteria through the gut into the hemolymph until they reach sufficiently high numbers to overcome local and phagocytic defenses. Finally, it is possible that the *P. aeruginosa* cells in the intestine or the few that translocate into the hemocoel actively suppress the systemic immune response as has been observed in a septic injury model with *P. aeruginosa* PA14 (29). This latter hypothesis may appear somewhat unlikely given the low number of bacteria retrieved from the hemolymph during the early phase of the infection. Because hemocytes are phagocytically active throughout the course of the infection and because the systemic immune response is not activated in the first phase of the infection, the cellular response may be the main active defense during the early phase in the hemocoel.

In summary, there seem to be two phases in the infection. In the early phase, bacteria cross the gut barrier and are most likely controlled efficiently in the hemocoel by phagocytes. In the late phase, *P. aeruginosa* PA14 is able to resist at least partially the cellular immune response through RhlR and then starts proliferating rapidly in the hemolymph, thus activating a systemic immune response, which in turn slows the infection process.

Genetic Analysis of Host–Pathogen Interactions Yields Insights into the in Vivo Roles of *P. aeruginosa* Quorum-Sensing Virulence Regulators. By using genetic mutants in both partners of an infectious host–pathogen relationship, we have been able to reveal unexpected in vivo roles for two global regulators of *P. aeruginosa* virulence: the transcription factors RhlR and LasR. RhlR is the major regulator of C4-homoserine lactone quorum-sensing, one of three quorum-sensing systems in *P. aeruginosa* (27, 33). Our data show that RhlR plays a key role in the oral infection model as *rhlR* mutants display strongly attenuated virulence (Fig. 4*C*). However, RhlR is unlikely to be required for passage through the intestinal barrier because *rhlR* mutants can kill phagocytosis-deficient flies as rapidly as wild-type PA14 (Fig. 4*D* and Fig. S1*B*).

It has been proposed that *P. aeruginosa* partially inhibits the systemic AMP response induced in the septic injury model (29). We do not think that RhlR is responsible for this virulence function: *rhlR* mutants also displayed an attenuated virulence phenotype in both IMD and Toll pathway mutant flies (*key* and *MyD88* mutants; Fig. 4*C*), arguing that NF-κB–independent defense mechanisms contain the infection in these cases. Furthermore, we did not detect enhanced induction of AMP gene expression when wild-type flies were infected with *rhlR* mutant bacteria (Fig. S8*A*), suggesting that RhlR is not involved in suppressing the AMP responses. Rather, the decreased induction of the AMP genes likely reflects the reduced ability of *rhlR* bacteria to proliferate in vivo (Fig. 4*B*).

In contrast to the results obtained with IMD and Toll pathway mutants, RhlR is dispensable when the cellular immune response is impaired either by the prior injection of latex beads or in *eater* mutants (Fig. 4*D* and Fig. S1*B*). These data suggest that an essential in vivo function of RhlR is to circumvent phagocytosis of *P. aeruginosa* by professional phagocytes. However, in contrast to the *P. aeruginosa* toxins secreted by the type III secretion system such as ExoS, RhlR is not required to impair the general phagocytic activity of hemocytes because killed *E. coli* appear to be ingested normally (Fig. S5). In light of the three explanations delineated above for why *P. aeruginosa* initially fails to proliferate in the hemolymph and fails to activate a systemic immune response, it is possible that RhlR is required at a critical period during the infection to protect *P. aeruginosa* bacteria from phagocytic clearance by an unknown mechanism. Interestingly, RhlR function appears to be required at a relatively early time in the infection process when the measured bacterial titer in the hemolymph is rather low (about 100/fly) (compare Fig. 4*B* to 4*D*). Quorum sensing should not be activated at this low bacterial concentration.

When either wild-type flies or flies with an impaired immune function were infected with a *P. aeruginosa rhlR* mutant, they exhibited a shallow survival curve. In contrast, flies that had ingested wild-type PA14 died in a more synchronized manner. Thus, RhlR seems to play an important role in the coordinated onset of pathology in the population of infected flies as a whole, which may be related to its classic role in quorum sensing. In the absence of RhlR, bacteria may behave in a more erratic manner because of a lack of coordination of bacterial virulence properties through quorum sensing.

The RhlR target genes—phenazines, RhlA, and RhlB—have been shown to be important for virulence in several model hosts from plants to mammals (34). Here, we find that these genes are not required for virulence in our *Drosophila* oral infection model. Thus, RhlR may circumvent the cellular immune response through other as-yet-uncharacterized target genes.

In contrast to RhlR, the transcriptional regulator LasR, which controls 3-oxo-C12-homoserine lactones, is not required for virulence in wild-type flies (Fig. 4 *A*, *E*, and *F*). *lasR* mutants display a phenotype that is the opposite of *rhlR*: *lasR* mutants, unlike *rhlR* mutants, are attenuated in phagocytosis-impaired flies (Fig. 4 *D* and *F* and Fig. S1 *B* and *C*). Classically, the two acylhomoserine lactone quorum-sensing systems of *P. aeruginosa* are thought to function in a hierarchical order, with the LasR system on top of the RhlR regulon (27). However, it was recently shown that RhlR can control the expression of LasR-specific factors independently of LasR and conversely (33). Furthermore, quorum-sensing systems themselves are under environmental control (35). Thus, our study underscores the necessity to functionally dissect the role of virulence factors in vivo in both immunocompetent and immunocompromised hosts to obtain insights into their complex regulatory roles in pathogenesis. In-

deed, quorum-sensing defective strains have been isolated from patients (36 and references therein). The finding that LasR is required for virulence in immunocompromised flies reveals a subtler LasR function that may be masked in wild-type flies. This is a reminder that bacterial screens for avirulent mutants in host-sensitized backgrounds are likely to yield insights that may not be gained using only wild-type host organisms. This and many other features of this study highlight the usefulness of model organisms in studying infectious disease.

Materials and Methods

Bacterial Strains and Growth Conditions. We used the following strains: wild-type strain—*P. aeruginosa* PA14 (37); *rhlR*—two transposon insertions, GID3229 ID#37943 (referred to as *rhlR*) and GID3229 ID#34255 [referred to as 34255 (*rhlR*)] (9), and two independent sets of deletion mutants that are further described in *SI Materials and Methods* together with *lasR*, *rhlA*, *rhlB*, *pelA* (38), and *phz1/2* (39) deletions. For deletion mutants, we used *ΔpscD*; *ΔexoT* (40), and for insertions we used *gacA* (37), *aprA* GID865 ID#23768, *lasB* GID759 ID#45691, *phoB* GID3473 ID#48234, *rhlA* GID2578 ID#23291, *rhlB1* GID1159 ID#28984, *rhlB2* GID1159 ID#27130, *pelA* GID86 ID#26187, *phzH* GID443 ID#39981, *phzM* GID2109 ID#40343, *phzS* GID1461 ID#44099 (9), and *lasR-RhlR* (33). All bacteria were grown in brain–heart infusion broth (BHB) overnight with shaking at 37 °C. We observed similar survival curves of infected flies when PA14 was grown and incubated with Luria broth (LB). *E. coli* and *Micrococcus luteus* for qRT-PCR controls were grown in LB overnight with shaking at 37 °C.

Survival Experiments. An overnight culture of bacteria was centrifuged (4,000 × *g*, 10 min, 4 °C) and diluted in fresh BHB to obtain a solution of OD_{600} = 2.5. This solution was then diluted 10 times with a sterile 50-mM sucrose solution to OD_{600} = 0.25. Two absorbant pads (Millipore AP1003700) were placed at the bottom of clean medium-size vials (3.5-cm diameter), and 2 mL of bacterial solution was added to the filters before the introduction of about 20 flies, which had been feeding on a 50-mM sucrose solution for 2 d at 25 °C. Survival experiments were performed at 25 °C and 50% humidity, and the number of surviving flies was monitored. For overexpression/rescue experiments, flies were incubated at 29 °C for 48 h (on fly food) before infection to inactivate Gal80 and allow for strong Gal4 activity (7).

For experiments using the oral infection model under conditions in which wild-type flies are not killed (Fig. S1), bacteria were centrifuged and washed in PBS. The pellet was then diluted with 5% sterile sucrose solution to an OD_{600} of 0.1, and 7-mL aliquots of this medium were pipetted onto sterile cotton balls placed at the bottom of empty fly culture vials. Further descriptions of materials and methods are found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank M. C. Lafarge and J. Bourdeaux for expert technical help. We are grateful to Dr. C. Makino and Dr. H. Fukuyama for a reporter fly line; to Dr. R. Feinbaum, Dr. D. K. Newman, Dr. R. Kolter, and Dr. E. Deziel for bacterial strains; and to Dr. S. Lory for plasmids. This work was funded by Centre National de la Recherche Scientifique; National Institutes of Health Grant P01 AI044220 (to F.M.A., C.K., and D.F.) and Grant R01 AI085581 (to F.M.A.); Fondation Recherche Médicale (Equipe Fondation pour la Recherche Médicale) (D.F.); and the French Ministry of Research (S.L. and S.H.).

1. Lemaitre B, Hoffmann J (2007) The host defense of Drosophila melanogaster. *Annu Rev Immunol* 25:697–743.
2. Ferrandon D, Imler JL, Hetru C, Hoffmann JA (2007) The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol* 7:862–874.
3. Tzou P, et al. (2000) Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. *Immunity* 13:737–748.
4. Nehme NT, et al. (2007) A model of bacterial intestinal infections in Drosophila melanogaster. *PLoS Pathog* 3:e173.
5. Ryu JH, et al. (2006) An essential complementary role of NF-kappaB pathway to microbicidal oxidants in Drosophila gut immunity. *EMBO J* 25:3693–3701.
6. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. *PLoS Pathog* 2:e56.
7. Cronin SJ, et al. (2009) Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. *Science* 325:340–343.
8. Boman HG, Nilsson I, Rasmuson B (1972) Inducible antibacterial defence system in Drosophila. *Nature* 237:232–235.
9. Liberati NT, et al. (2006) An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103: 2833–2838.
10. Rahme LG, et al. (2000) Plants and animals share functionally common bacterial virulence factors. *Proc Natl Acad Sci USA* 97:8815–8821.
11. Lee DG, et al. (2006) Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. *Genome Biol* 7:R90.
12. Sibley CD, et al. (2008) Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. *PLoS Pathog* 4:e1000184.
13. Ye YH, Chenoweth SF, McGraw EA (2009) Effective but costly, evolved mechanisms of defense against a virulent opportunistic pathogen in Drosophila melanogaster. *PLoS Pathog* 5:e1000385.
14. Avet-Rochex A, Bergeret E, Attree I, Meister M, Fauvarque MO (2005) Suppression of Drosophila cellular immunity by directed expression of the ExoS toxin GAP domain of Pseudomonas aeruginosa. *Cell Microbiol* 7:799–810.
15. Apidianakis Y, Pitsouli C, Perrimon N, Rahme L (2009) Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. *Proc Natl Acad Sci USA* 106:20883–20888.
16. Chugani SA, et al. (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa. *Proc Natl Acad Sci USA* 98:2752–2757.
17. Limmer S, Quintin J, Hetru C, Ferrandon D (2011) Virulence on the fly: Drosophila melanogaster as a model genetic organism to decipher host-pathogen interactions. *Curr Drug Targets* 12:978–999.
18. Lau GW, et al. (2003) The Drosophila melanogaster toll pathway participates in resistance to infection by the gram-negative human pathogen Pseudomonas aeruginosa. *Infect Immun* 71:4059–4066.
19. D'Argenio DA, Gallagher LA, Berg CA, Manoil C (2001) Drosophila as a model host for Pseudomonas aeruginosa infection. *J Bacteriol* 183:1466–1471.
20. Tauszig-Delamasure S, Bilak H, Capovilla M, Hoffmann JA, Imler JL (2002) Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections. *Nat Immunol* 3:91–97.
21. Rutschmann S, et al. (2000) Role of Drosophila IKK γ in a toll-independent antibacterial immune response. *Nat Immunol* 1:342–347.
22. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.
23. Kocks C, et al. (2005) Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. *Cell* 123:335–346.
24. Defaye A, et al. (2009) Genetic ablation of Drosophila phagocytes reveals their contribution to both development and resistance to bacterial infection. *J Innate Immun* 1:322–334.
25. Charroux B, Royet J (2009) Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. *Proc Natl Acad Sci USA* 106:9797–9802.
26. Elrod-Erickson M, Mishra S, Schneider D (2000) Interactions between the cellular and humoral immune responses in Drosophila. *Curr Biol* 10:781–784.
27. Williams P, Cámara M (2009) Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: A tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* 12:182–191.
28. Fauvarque MO, et al. (2002) Role and activation of type III secretion system genes in Pseudomonas aeruginosa-induced Drosophila killing. *Microb Pathog* 32:287–295.
29. Apidianakis Y, et al. (2005) Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by suppressing antimicrobial peptide gene expression. *Proc Natl Acad Sci USA* 102:2573–2578.
30. Cossart P, Toledo-Arana A (2008) Listeria monocytogenes, a unique model in infection biology: An overview. *Microbes Infect* 10:1041–1050.
31. Tsolis RM, Young GM, Solnick JV, Bäumler AJ (2008) From bench to bedside: Stealth of enteroinvasive pathogens. *Nat Rev Microbiol* 6:883–892.
32. Jander G, Rahme LG, Ausubel FM (2000) Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. *J Bacteriol* 182:3843–3845.
33. Dekimpe V, Déziel E (2009) Revisiting the quorum-sensing hierarchy in Pseudomonas aeruginosa: The transcriptional regulator RhlR regulates LasR-specific factors. *Microbiology* 155:712–723.
34. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model. *Cell* 96:47–56.
35. Hazan R, et al. (2010) Homeostatic interplay between bacterial cell-cell signaling and iron in virulence. *PLoS Pathog* 6:e1000810.
36. Karatuna O, Yagci A (2010) Analysis of quorum sensing-dependent virulence factor production and its relationship with antimicrobial susceptibility in Pseudomonas aeruginosa respiratory isolates. *Clin Microbiol Infect* 16:1770–1775.
37. Rahme LG, et al. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268:1899–1902.
38. Friedman L, Kolter R (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* 51:675–690.
39. Dietrich LE, Price-Whelan A, Petersen A, Whiteley M, Newman DK (2006) The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* 61:1308–1321.
40. Miyata S, Casey M, Frank DW, Ausubel FM, Drenkard E (2003) Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. *Infect Immun* 71:2404–2413.

Supporting Information

Limmer et al. 10.1073/pnas.1114907108

SI Results

In a recently described oral-feeding model using *Drosophila melanogaster* strain *esg-Gal4*, ingested *P. aeruginosa* PA14 was reported to cause apoptosis of enterocytes and a subsequent compensatory proliferation of intestinal stem cells (ISCs), thus maintaining the homeostasis of the posterior (*caudal*-expression region) midgut epithelium (1). We made similar observations in our oral infection model with *Serratia marcescens* (1, 2). However, under our experimental conditions using *D. melanogaster* strain A5001 and *P. aeruginosa* PA14 infection, the morphology of the midgut epithelium appeared normal throughout the infection (Fig. S3), even on the last day of the infection when most of the flies were dead. Compensatory proliferation of ISCs has been shown to be required for the homeostasis of the midgut epithelium during bacterial infection (1, 2). This repair mechanism actually masks the severe damage incurred by the epithelium, including an extensive induction of apoptosis in enterocytes. As intensive compensatory proliferation is not observed in noninfected flies, a way to indirectly monitor the damage inflicted to the gut by ingested PA14 is to measure the rate of division of ISCs. We assessed the mitotic rate by phosphohistone H3 staining and by following the incorporation of the labeled nucleotide EdU in the distal midgut region of the infected A5001 flies (Fig. S3). With both of these techniques, we failed to observe proliferation beyond normal background levels. Because the JAK-STAT pathway has been shown to be required to control ISC proliferation during infection, we also monitored the expression of Unpaired3, which stimulates the JAK-STAT pathway (Fig. S3) (2–4). We observed a weak induction of an *Unpaired3-GFP* reporter transgene in a few enterocytes. We also observed some expression in intestinal muscle cells and in some undefined epithelial cells of a *GFP* reporter transgene that is activated by STAT92E binding to 10 copies of its DNA-binding site (Fig. S3). However, we were unable to detect any expression of the 10xSTAT92E reporter in ISCs, in contrast to the previously published *S. marcescens* oral infection model from our laboratory (2). Because we failed to observe any significant degradation of the intestinal epithelium during *P. aeruginosa* PA14 oral infection, even at the end of the infection (Fig. S3), and because flies transferred to a sterile medium are eventually killed in the absence of any detectable PA14 in the gut (Fig. 1*C* and Fig. S2*B*), we conclude that death in our infection model is unlikely to be caused by gut damage.

SI Discussion

Immune Deficiency Pathways Play Multiple Roles in Host Defense Against Ingested *P. aeruginosa*. A previous study documented the effect of ingested PA14 on the intestinal epithelium and reported widespread damages to the epithelium, which caused an increased proliferation of ISCs (1). The reason for the different host pathologies in our PA14 infection model compared with that reported in Apidianakis et al. (1) is unclear. It might be due to variables in experimental protocols (for example, the starvation period before infection) and fly husbandry (such as food, microbiotia, asymptomatic viral infections) and possibly the interplay of these variables with the genetic background of the host (1, 5–7).

Apidianakis et al. (1) reported increased susceptibility to feeding on *P. aeruginosa* PA14 in fly mutants in which the integrity of the intestinal epithelium could not be maintained, a consequence perhaps of increased translocation of bacteria across intestinal epithelial cells and/or earlier activation of a virulence switch after bacteria have gained access to the hemocoel. The increased susceptibility to feeding on *P. aeruginosa* of immune deficiency (IMD) pathway mutants caused by a defective activation of the pathway may be similarly explained by a defect in the ability of the intestinal epithelium to control bacterial translocation. Indeed, we were able to rescue the susceptibility phenotype of *imd* mutant flies by overexpressing a wild-type copy of *imd* in the gut epithelium (Fig. 2*H*). Interestingly, a similar level of rescue was obtained in hemocytes and in the fat body, indicating that all three immune tissues can participate in the immune defense response (Fig. 2*H*). It was unexpected that rescue with the hemocyte-specific *hmlGal4* driver would rescue the *imd* or *MyD88* mutant phenotypes. One interpretation of this latter result is that hemocytes may secrete enough AMPs to compensate for the absence of AMP production in the fat body. An alternate hypothesis is that rescued hemocytes emit a signal that triggers the systemic immune response in the fat body, but in an IMD or Toll pathway-independent manner because fat-body cells are mutant. Finally, an expression of the *hml-Gal4* driver among intestinal cells has been reported, and therefore we cannot exclude rescue at the level of the midgut (8). Thus, the IMD pathway seems to play multiple roles in the host defense against ingested *P. aeruginosa*—locally in the gut epithelium likely by controlling at least partially the rate of passage through the gut (5) and systematically in the fat body or hemocytes, which also secrete AMPs into the hemolymph.

Models of *P. aeruginosa* Oral Infection in *Drosophila*. We used two methods to orally infect *Drosophila* in this study, in which adult unstarved flies were fed *P. aeruginosa* PA14 in the presence or absence of bacterial growth medium [brain–heart infusion broth (BHB) or Luria broth (LB)] (Fig. S1). Surprisingly, the absence of growth medium in the feeding solution has major consequences on the virulence of PA14 as they then fail to kill wild-type hosts. A possibility that remains to be tested is that RhlR is not induced in the absence of growth medium, thus explaining why wild-type flies are not killed in this setup. Indeed, *eater* mutant flies succumb in both models, consistent with the finding that RhlR is dispensable when the cellular immune response is impaired. In both cases, bacteria are found mostly in the digestive tube and to a much lesser extent in the crop, a storage diverticulum in which the bacteria accumulate when flies are fed after prior starvation. In both conditions, the bacteria are able to cross the midgut barrier where they are either controlled by the *eater*-dependent cellular immune response (sugar-only growth medium) or ultimately prevail (sugar with bacterial growth medium). The difference in the virulence behavior of the bacteria appears to be a consequence of the conditions that the bacteria experience while placed on the filter. It may be that, in the absence of bacterial growth medium, *P. aeruginosa* cells are relatively quiescent and do not secrete virulence factors in the medium. Bacteria accumulate in the crop in a third oral infection protocol (not used in our study) (5, 6). In this third oral infection model, flies were starved for food and water for 3–5 h before infection and then fed on a concentrated bacterial solution placed on a filter on top of sucrose agar (5, 6). It has not yet been determined whether bacteria cross the midgut barrier and whether flies succumb to a systemic infection in this latter feeding model. It will be interesting to measure the bacterial titer in the hemolymph and to assess the susceptibility of mutants that affect host defense in this third model of infection to determine whether flies also succumb to bacteremia or whether flies succumb to damage inflicted to the crop, as suggested by the authors (5, 6).

Distinct Virulence Patterns in Different Models of *P. aeruginosa* Infection. *P. aeruginosa* has been reported to kill several species of insects in different models of infection. For example, it was found to be a major cause of death in laboratory cultures of grasshoppers (9). Infection by feeding led to the demise of insects, which harbored a titer of 10^9 bacteria per insect. Interestingly, even though the bacteria had been rapidly cleared from the gut, they ultimately were able to proliferate to high titers in the hemolymph. Thus, this infection model displays similarities to the one that we are using in *Drosophila*.

Larvae of the Greater Wax Moth *Galleria mellonella* have been used to identify *P. aeruginosa* PA14 virulence factors (10, 11). Interestingly, it was found that the T3SS system and ExoT, which is a toxin secreted through the T3SS, were required for virulence. In contrast to these findings, we did not observe any requirement for the T3SS in our *Drosophila* oral infection model. We note, however, that another T3SS effector, ExoS, which is present in *P. aeruginosa* strain PAK but is lacking in strain PA14, blocks phagocytosis in *Drosophila* by regulating the small Rho GTPase family member Rac2 (12, 13). As regards *P. aeruginosa* PA14, the discrepancy between observations in *Galleria* and *Drosophila* may be due to the evolutionary divergence between these two species that last shared a common ancestor about 340 million years ago. Alternatively, the difference may reside in the infection route (oral vs. septic injury) or in the developmental stage (adult vs. larva).

Another difference between our *Drosophila* oral-feeding model and other insect infection models is the role of GacA in virulence. GacA belongs to a two-component system that regulates bacterial virulence in plants and animals. It regulates RhlR and LasR in vitro (14). It also controls the expression of the AprA protease in *Pseudomonas entomophila*, a major virulence factor in an oral infection model (15). GacA is also important for virulence in the *G. mellonella* infection model (10). In contrast, we observed normal virulence of *gacA* mutants in our *Drosophila* oral infection model.

Interestingly, the expression of the *P. aeruginosa lasR* gene, or the gene itself, is often lost in chronic infections that characterize cystic fibrosis patients (16). It has been proposed that this virulence factor might be required initially for establishing the infection but that, as a result of adaptation or growth in rich media, the *lasR* gene might be lost as *lasR* mutants have a growth advantage under such conditions (17). Our findings open the alternate possibility that LasR might not be required for full virulence in vivo.

These observations underscore the importance of the particular infection model used for assessing virulence factor contribution to pathogenesis and illustrate that opportunistic bacteria can resort to multiple virulence strategies depending on the context (18).

SI Materials and Methods

Fly Strains. Stocks were raised on standard cornmeal–agar medium at 25 °C. Different wild-type strains were used: Oregon R (Fig. S1), w^- A5001, and *yw P[ry$^+$, Dipt::LacZ = pDipt-LacZ], P[w$^+$mC Drom::GFP = pDrom-GFP S65T]*; *cn bw* (*yw*DD1-*cn bw*) (19, 20). The wild-type flies all behaved in the same manner when tested in parallel. In our experiments, whenever possible, we used the wild-type strain corresponding to the background in which the mutants were generated as controls. Thus, "wild-type" in main and supplementary figures (Figs. 1–4 and Figs. S1 and S7–S9) may correspond to different genetic backgrounds. Mutants in the A5001 background were *key^{c02831}* and *Myd88^{c03881}* (21), and the mutant in the *yw*DD1-*cn bw* (wild-type strain used for Fig. 2 *B–H* and Fig. 3 *B* and *C*) background was *imd^{shadok}* (22). IMD overexpression and rescue experiments used *hml-Gal4,tub-Gal80/+;UAS-imd$^+$/+*. *hml-Gal4,tub-Gal80, imd^{shadok}/imd^{shadok}; UAS-imd$^+$/+*; *NP3084-Gal4,tub-Gal80/+; UAS-imd$^+$/+*; *NP3084-Gal4,tub-Gal80, imd^{shadok}/imd^{shadok}; UAS-imd$^+$/+*; *ylk-Gal4/UAS-imd$^+$*; and *imd^{shadok}/imd^{shadok}; ylk-Gal4/UAS-imd$^+$* (23–25). Toll pathway overstimulation and rescue used *UAS-MyD88$^+$/+; hml-Gal4,tub-Gal80/+*; *UAS-MyD88$^+$/+; hml-Gal4, Myd88^{c03881}/Myd88^{c03881}*; *UAS-MyD88$^+$/+;NP3084-Gal4,tub-Gal80/+*; and *UAS-MyD88$^+$/+; NP3084-Gal4,tub-Gal80, Myd88^{c03881}/Myd88^{c03881}* (21). The *Dipt-tomato* reporter line was a kind gift from Chieko Makino and Hidehiro Fukuyama (UPR9022 Centre National de la Recherche Scientifique, Strasbourg, France). Notably, we could not use the fat body-specific driver *yolk-Gal4* for the ectopic expression of *MyD88* or *Toll^{10b}* because their overexpression was lethal, whether in a wild-type or a mutant background. JAK-STAT reporter transgenes used were *upd3-GFP* (26), *10xStat92E-binding site-GFP* (27), and *eater Df(3R)D605/Df(3R)Tl-I* (28).

Statistical Analysis of Survival Curves. Because each of the survival experiments described in this paper have been performed multiple times and because log-rank analysis can compare only two survival curves at a time in the same experiment, we decided instead to compute the median lethal time 50 (LT50) (see below) and then perform statistical analysis on the LT50s using Student's *t* test. As the slope of survival curves is reproducible from experiment to experiment (Fig. S8), it is legitimate to use this approach.

Construction of Bacterial Deletion Strains. We constructed in-frame deletions in *rhlR* and *lasR* using two independent methods. In the first method, we used plasmids carrying deletion mutant alleles for *rhlR* and *lasR* from *P. aeruginosa* strain PAK (1) kindly provided by S. Lory (Harvard Medical School, Boston, MA). These mutant alleles were then introduced into the wild-type PA14 genome by homologous recombination (2), resulting in Δ*rhlR3-10* and Δ*lasR3-5*, referred to in the main text as Δ*rhlR* and Δ*lasR*, respectively. In the second method, an *rhlR* in-frame deletion mutant was generated by replacing 1.866 kb of wild-type PA14 sequence with a 1.140-kb PCR-amplified fragment that contained a 0.726-kb deletion in the *rhlR* ORF. Similarly, an *rhlA* in-frame deletion was generated by replacing 2.100 kb of wild-type PA14 sequence with a 1.212-kb PCR-amplified fragment that contained a 0.888-kb deletion of the *rhlA* ORF. Similarly, a *rhlB* in-frame deletion was generated by replacing 2.481 kb of wild-type PA14 sequence with a 1.200-kb PCR-amplified fragment that contained a 1.281-kb deletion of the *rhlB* ORF. Similarly, a *lasR* in-frame deletion was generated by replacing 2.251 kb of wild-type PA14 sequence with a 1.603-kb PCR-amplified fragment that contained a 0.648-kb deletion in the *lasR* ORF. PCR-amplified fragments containing the deleted genes were subcloned into the KpnI and HindIII sites (*rhlR*, *rhlA*, *rhlB*) and the BamHI site (*lasR*) of pEX18Ap (3), generating plasmids pEX18rhlRΔ1, pEX18rhlAΔ1, pEX18rhlBΔ1, and pEX18lasRΔ6–1, respectively. The resulting constructs were used to introduce the *rhlR*, *rhlA*, and *rhlB* and *lasR* deleted genes into the wild-type PA14 genome by homologous recombination, resulting in the Δ*rhlR1-2*, Δ*rhlA1*, Δ*rhlB1*, Δ*lasR18-2*, and Δ*lasR18-3* mutants.

Colonies of putative mutants generated by both methods were screened by PCR using appropriate flanking primers and were further confirmed by sequencing the corresponding PCR products.

Bacterial Counts in the Hemolymph. Bacterial counts were measured as previously described (25). Hemolymph solutions were plated on LB plates containing 10 μg/mL rifampicin and incubated at 37 °C for 16 h. We checked that hemolymph counts were representative of the degree of infection of the body cavity by dissecting away the digestive tract after the fly's hemolymph had been collected and plating an extract of the carcass at different stages of the infection.

β-Galactosidase Staining and p*Drom*-GFP Observation. Flies were infected and their abdomens dissected. *pDrom*-GFP abdomens were mounted in glycerol and observed. *pDipt*-lacZ abdomens were fixed in 1% glutaraldehyde for 10 min, washed and stained for 30 min in coloration solution at room temperature [8.4 mM Na_2HPO_4, 1.6 mM Na_2HPO4, 0.15 M NaCl, 1 mM $MgCl_2$, 3.5 mM K_3FeCN_6, 3.5 mM K_4FeCN_6, 0.15% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)], washed again, mounted in glycerol, and observed using a Zeiss Axioskope 2 fluorescence microscope. Images were processed using ImageJ 1.41o.

Quantitative RT-PCR. This analysis was done as previously described (29). Samples of five flies were frozen in liquid nitrogen and crushed with a Mixer Mill 300 (Retsch) twice for 60 s at 25 Hz. Total RNA was then prepared using the RNeasy kit (Qiagen) or the nucleospin kit (Macherey Nagel), and RNAs were eluted in 100 μL of RNase-free water. A total of 2 μL was then used in a reverse transcription reaction with SuperScript II reverse transcriptase (Invitrogen) and random primers (Invitrogen) according to the supplier's instructions. The cDNAs were then diluted to a proper concentration so that the subsequent PCR reactions would not be inhibited by components of the reverse transcription preparation. PCR reactions were set up using the quantitative PCR kit (Eurogentec) and in a 1/50,000–1/75,000 final concentration of SYBRGreen. Real-time PCR was then performed in 96-well plates on an i-cycler iQ (Bio-Rad) or in 384-well plates using a CFX384 system (Bio-Rad); usual PCR conditions were preincubation at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. PCR reactions were done in duplicates; to check for specificity of the PCR, melting curves were analyzed for each data point. The levels of expression of the gene of interest was then normalized against the measured level of the RNA coding for ribosomal protein 49 determined in each sample. Primers were as follows: diptericin—forward (5′-GCTGCGCAATCGCTTCTACT-3′) and reverse (5′-TGGTGGAGTGGGCTTCATG-3′); RP49—forward (5′-GACGCTTCAAGGGACAGTATCTG-3′) and reverse (5′-AAACGCGGTTCTGCATGAG-3′); GNBP1—forward (5′-CACCAAAAGGCTGTGTATCAAGAT-3′) and reverse (5′-TCCGCCGAGATTGCAGA-3′); PGRP-SA/seml—forward (5′-CCTTCGTTGGGACTCCACTA-3′) and reverse (5′-CGTGTGATGGATGACCACAT-3′). The efficiency of the primers is usually close to 100% and must be >85% for validation of the experiment. cDNA levels are quantified against a standard ladder made with dilutions of a plasmid containing the sequence of interest.

Injection of Latex Beads. A total of 69 nL of fourfold concentrated Surfactant-Free Red CML latex beads (0.30 μm-diameter polystyrene beads, Interfacial Dynamics) were injected into recipient flies to block phagocytosis, as previously described (25). The effectiveness of the procedure was checked by testing the phagocytosis of FITC-labeled *Escherichia coli* as described below (30).

In Vivo Phagocytosis Assay and Injection of FITC-Labeled *E. coli*: A total of 69 nL of FITC-labeled *E. coli* (33 μg/μL) (Invitrogen bioparticles) were injected into the flies' thorax with PA14. Flies were kept at room temperature, and 1 h later 2 × 69 nL of Trypan blue was injected to quench the fluorescence of noningested FITC-*E. coli*. Ten minutes later, the abdomens were dissected, mounted in glycerol, and observed using a Zeiss Axioskope 2 fluorescence microscope. Images were processed using ImageJ 1.41o. This experiment was performed for each day of the infection.

Intestinal Colonization Assay. Flies were first fed on a *P. aeruginosa* PA14 solution (OD_{600} = 0.5) for different times and transferred after a given incubation period to vials containing a sterile 50-mM sucrose solution. After 1 h on sucrose solution, the flies were again transferred to a new sterile vial. After this, flies were transferred to new vials every day to avoid contamination of the sugar solution by fly feces. We were not able to detect more than 10–50 bacteria on the filters after the first two changes of sterile vials; most of the bacteria were cleared from or killed in the gut in the first hour as determined by plating gut extracts (crop included). Survival at 25 °C was monitored every day.

Immunostainings. Primary antibodies used were Rabbit α pHH3 (1:1,000; Millipore) and Mouse α GFP (1:100; Roche). Secondary antibodies conjugated to Alexa Fluor-488 (Invitrogen) were used at a 1,000× dilution. Standard immunohistochemical methods were used. Texas Red-X phalloidin [stock solution (6.6 μM) was diluted 8× (0.825 μM) (Invitrogen)] was added to the secondary antibodies or used alone after 2 h blocking at room temperature. Guts were mounted in Vectashield with DAPI (Vector Laboratories) and observed using the inverted confocal microscope Zeiss LSM 510. Images were processed using ImageJ 1.41o.

Septic Injury Assay. An overnight culture of bacteria was centrifuged (4,000 × *g*, 10 min, 4 °C) and the pellet was diluted in sterile PBS to a concentration of 10 bacteria/nL. Of this solution, 9.2 nL was injected into the thorax of the flies. Afterward, flies were put on a 50-mM sucrose solution. Survival at 25 °C was observed after 24 h (and 36 h for wild-type flies), and then surviving flies were counted each hour.

EdU Incorporation. A total of 69 nL of a 0.5-mM solution of EdU in PBS was injected into the fly thoraxes. Following incubation for 3 h at 25 °C, guts were dissected and stained following the supplier's instructions (Click-iT EdU Alexa Fluor 488, Invitrogen). Guts were mounted in Vectashield with DAPI (Vector Laboratories) and observed using a Zeiss LSM 510 inverted confocal microscope. Images were processed using ImageJ 1.41o.

Calculation of Hill Coefficient and Median Lethal-Time Analysis (LT50 Values). Hill coefficient (HillSlope) and LT50 (logEC50) were calculated with GraphPad Prism 5 software (Figs. 2*H*, 3*D*, and 4*A* and Fig. S6 *B* and *C* for LT50 and Fig. S7 for Hill coefficient). Values shown are absolute values. Statistical analysis was performed using the Student's *t* test.

1. Apidianakis Y, Pitsouli C, Perrimon N, Rahme L (2009) Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. *Proc Natl Acad Sci USA* 106:20883–20888.
2. Cronin SJ, et al. (2009) Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. *Science* 325:340–343.
3. Buchon N, Broderick NA, Chakrabarti S, Lemaitre B (2009) Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. *Genes Dev* 23:2333–2344.
4. Jiang H, et al. (2009) Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. *Cell* 137:1343–1355.
5. Sibley CD, et al. (2008) Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. *PLoS Pathog* 4:e1000184.
6. Chugani SA, et al. (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa. *Proc Natl Acad Sci USA* 98:2752–2757.
7. Cadwell K, et al. (2010) Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell* 141:1135–1145.
8. Charroux B, Royet J (2009) Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. *Proc Natl Acad Sci USA* 106:9797–9802.
9. Bucher GE, Stephens JM (1957) A disease of grasshoppers caused by the bacterium Pseudomonas aeruginosa (Schroeter) Migula. *Can J Microbiol* 3:611–625.
10. Jander G, Rahme LG, Ausubel FM (2000) Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. *J Bacteriol* 182:3843–3845.
11. Miyata S, Casey M, Frank DW, Ausubel FM, Drenkard E (2003) Use of the Galleria mellonella caterpillar as a model host to study the role of the type III secretion system in Pseudomonas aeruginosa pathogenesis. *Infect Immun* 71:2404–2413.

12. Avet-Rochex A, Bergeret E, Attree I, Meister M, Fauvarque MO (2005) Suppression of Drosophila cellular immunity by directed expression of the ExoS toxin GAP domain of Pseudomonas aeruginosa. *Cell Microbiol* 7:799–810.
13. Avet-Rochex A, Perrin J, Bergeret E, Fauvarque MO (2007) Rac2 is a major actor of Drosophila resistance to Pseudomonas aeruginosa acting in phagocytic cells. *Genes Cells* 12:1193–1204.
14. Reimmann C, et al. (1997) The global activator GacA of Pseudomonas aeruginosa PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* 24:309–319.
15. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. *PLoS Pathog* 2:e56.
16. Smith EE, et al. (2006) Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA* 103:8487–8492.
17. D'Argenio DA, et al. (2007) Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol* 64:512–533.
18. Lee DG, et al. (2006) Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. *Genome Biol* 7:R90.
19. Thibault ST, et al. (2004) A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nat Genet* 36:283–287.
20. Jung AC, Criqui MC, Rutschmann S, Hoffmann JA, Ferrandon D (2001) Microfluorometer assay to measure the expression of β-galactosidase and green fluorescent protein reporter genes in single *Drosophila* flies. *Biotechniques* 30:594–601.
21. Tauszig-Delamasure S, Bilak H, Capovilla M, Hoffmann JA, Imler JL (2002) *Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections. *Nat Immunol* 3:91–97.
22. Gottar M, et al. (2002) The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature* 416:640–644.
23. Sinenko SA, Mathey-Prevot B (2004) Increased expression of Drosophila tetraspanin, Tsp68C, suppresses the abnormal proliferation of ytr-deficient and Ras/Raf-activated hemocytes. *Oncogene* 23:9120–9128.
24. Georgel P, et al. (2001) *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Dev Cell* 1: 503–514.
25. Nehme NT, et al. (2007) A model of bacterial intestinal infections in Drosophila melanogaster. *PLoS Pathog* 3:e173.
26. Agaisse H, Petersen UM, Boutros M, Mathey-Prevot B, Perrimon N (2003) Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. *Dev Cell* 5:441–450.
27. Bach EA, et al. (2007) GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. *Gene Expr Patterns* 7:323–331.
28. Kocks C, et al. (2005) Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. *Cell* 123:335–346.
29. Gobert V, et al. (2003) Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors. *Science* 302:2126–2130.
30. Elrod-Erickson M, Mishra S, Schneider D (2000) Interactions between the cellular and humoral immune responses in *Drosophila*. *Curr Biol* 10:781–784.

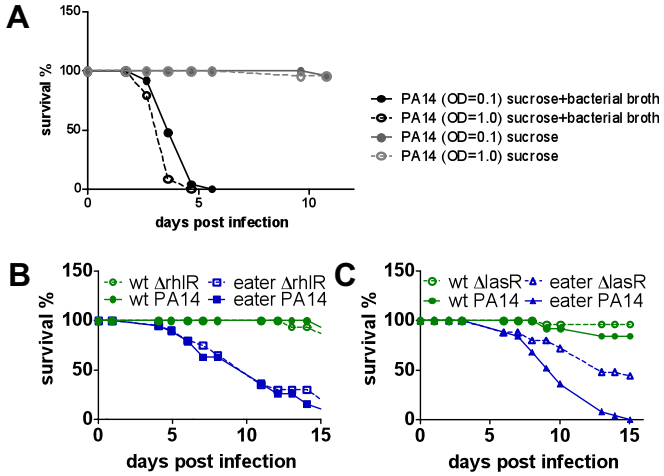


Fig. S1. Influence of the presence of bacterial broth in the medium on PA14 virulence in a *Drosophila* oral infection model. (*A*) Wild-type flies (Oregon-R) feeding on *P. aeruginosa* PA14 in 5% sucrose did not succumb to oral infection, whereas flies feeding on *P. aeruginosa* PA14 in 80% bacterial broth (LB or BHB)/ 4% sucrose succumbed rapidly to infection. Note that the bacterial concentration in the feeding medium hardly influences survival. Data are representative of five independent experiments. (*B*) *rhlR* mutants ($P > 0.05$, $n = 3$) display the same level of virulence as wild-type PA14 in phagocytosis-deficient *eater* mutant flies under conditions in which wild-type flies do not succumb to the oral infection (sucrose-only medium). (*C*) *lasR* mutants ($P < 0.001$, $n = 3$) are less virulent in *eater* mutant flies.

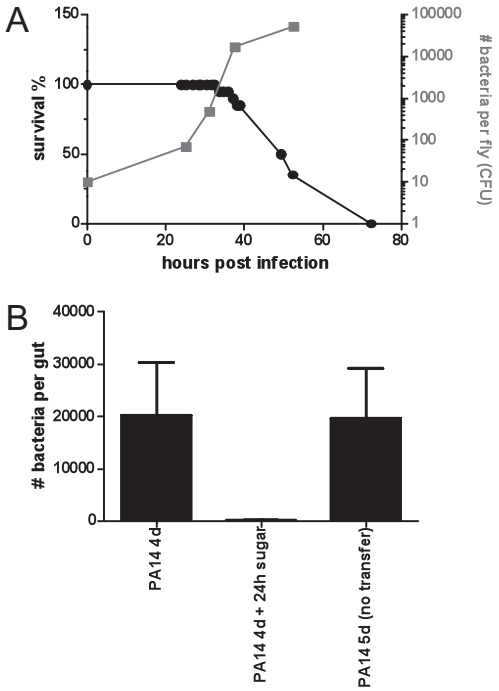


Fig. S2. Flies die from bacteremia in the septic injury model and do not show persistent intestinal colonization in the oral-feeding model. (*A*) About 100 bacteria were introduced in the hemocoel of flies by septic injury. Survival was monitored (left *y* axis: black curve, circles) and the bacterial titer in the hemolymph was measured (right logarithmic scale: gray curve, squares). This experiment is representative of three independent experiments. (*B*) *P. aeruginosa* PA14 does not persistently colonize the digestive tract of *Drosophila*: when feeding continuously on *P. aeruginosa* PA14, flies display a constant bacterial load of around 20,000 bacteria per intestine (PA14 at 4 d and PA14 at 5 d). The intestines of flies that have been transferred to a sterile sucrose solution after 4 d of feeding [PA14 4 d + 24 h sugar (PA14 at 4 d + 24 h with sugar)] have very few detectable bacteria in their intestine ($P < 0.05$); $n = 3$.

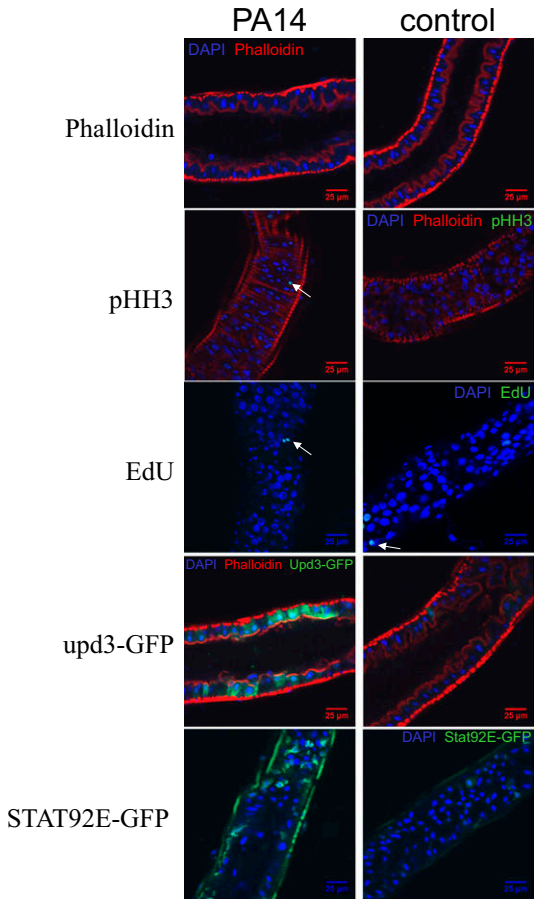


Fig. S3. Orally fed *P. aeruginosa* PA14 does not severely damage the gut epithelium. Guts of the orally infected flies (strain A5001 unless indicated otherwise) were dissected every day until 1 d before death. Control flies were fed on a sucrose solution. The data shown here are from the last time point that we examined. (First row) After fixation, some guts were stained with Texas-Red phalloidin, which binds to actin filaments and thus stains mainly the brush border and intestinal muscles. Phalloidin staining indicates no difference in gut epithelium integrity. (Second row) To monitor the proliferation of intestinal stem cells, phosphohistone H3 staining (pHH3: green) was performed (positive signal highlighted by white arrow). There was no detectable difference in proliferation. (Third row) EdU was injected into flies 3 h before fixation and staining with an EdU-specific fluorescent azide (green; positive signal highlighted by white arrow). No difference in EdU signal was detected. (Fourth and fifth rows) To investigate JAK-STAT pathway activation, *upd3-GFP* and 10xSTAT92E binding-site GFP transgenic flies were used. JAK-STAT pathway activation by *P. aeruginosa* PA14 in the latter line appeared to be restricted to intestinal muscles, whereas the *upd3* signal was weak and restricted to a few intestinal epithelial cells.

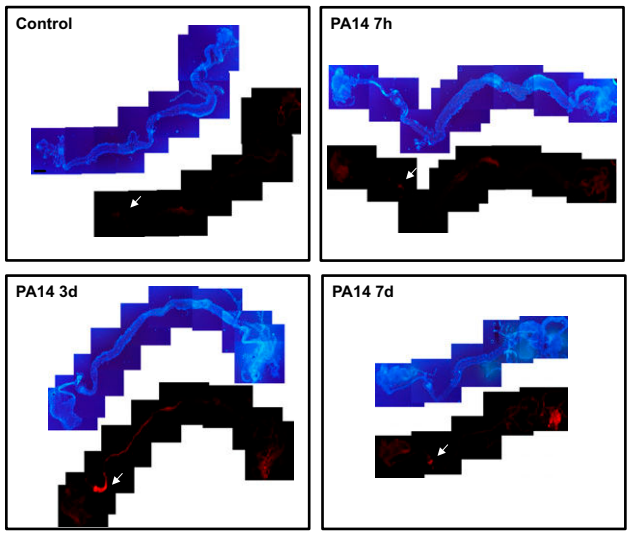


Fig. S4. *Diptericin* induction in the gut of *Diptericin-tomato* transgenic flies upon infection with wild-type PA14 bacteria. Transgenic flies carrying a *Diptericin-tomato* reporter were used to assess *Diptericin* expression in the gut of adult flies. Following oral infection with PA14, the entire guts were dissected at the indicated time points. *Diptericin* is strongly expressed in the distal proventriculus and in the proximal anterior midgut at day 3 of infection (white arrow), but not at 7 h. This pattern of expression is still present but decreased at day 7. The control is a *Diptericin-tomato* transgenic fly fed only on a sucrose solution. The visualization of the expression of the *Diptericin-tomato* reporter transgene, unlike that of the *Dipt-LacZ* transgene, is not hampered by endogenous reporter activity. The results are representative of two independent experiments. (Scale bar: 100 μm.)

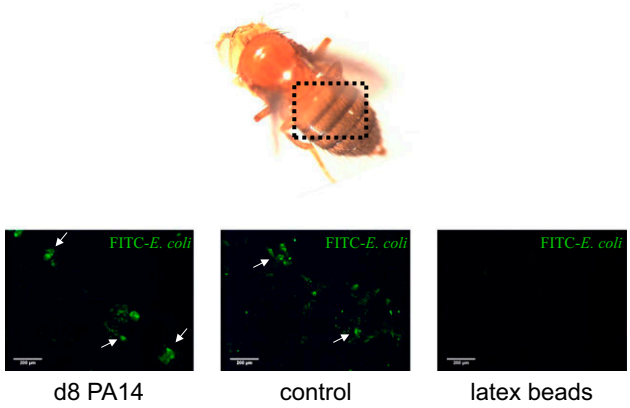


Fig. S5. Hemocytes of *P. aeruginosa* PA14-infected flies do not lose their ability to phagocytose bacterial particles. Phagocytosis of injected FITC-labeled *E. coli* particles (Invitrogen) at day 8 of infection with PA14 as observed under epifluorescent illumination. The fluorescence from free, nonphagocytosed bacteria was quenched with Trypan blue. (*Upper*) The region of the fly body corresponding to the area from which high-magnification microscopy pictures were taken is indicated by dashed lines. (*Lower* panels) The fluorescent signal corresponds to FITC-*E. coli* phagocytosed by sessile hemocytes lining the dorsal vessel (white arrows). Flies infected with *P. aeruginosa* PA14 (*Left*) and noninfected control flies (*Center*) displayed the same ability to phagocytose FITC-*E. coli*. (*Right*) No phagocytosed FITC-*E.coli* were found in latex bead-injected flies.

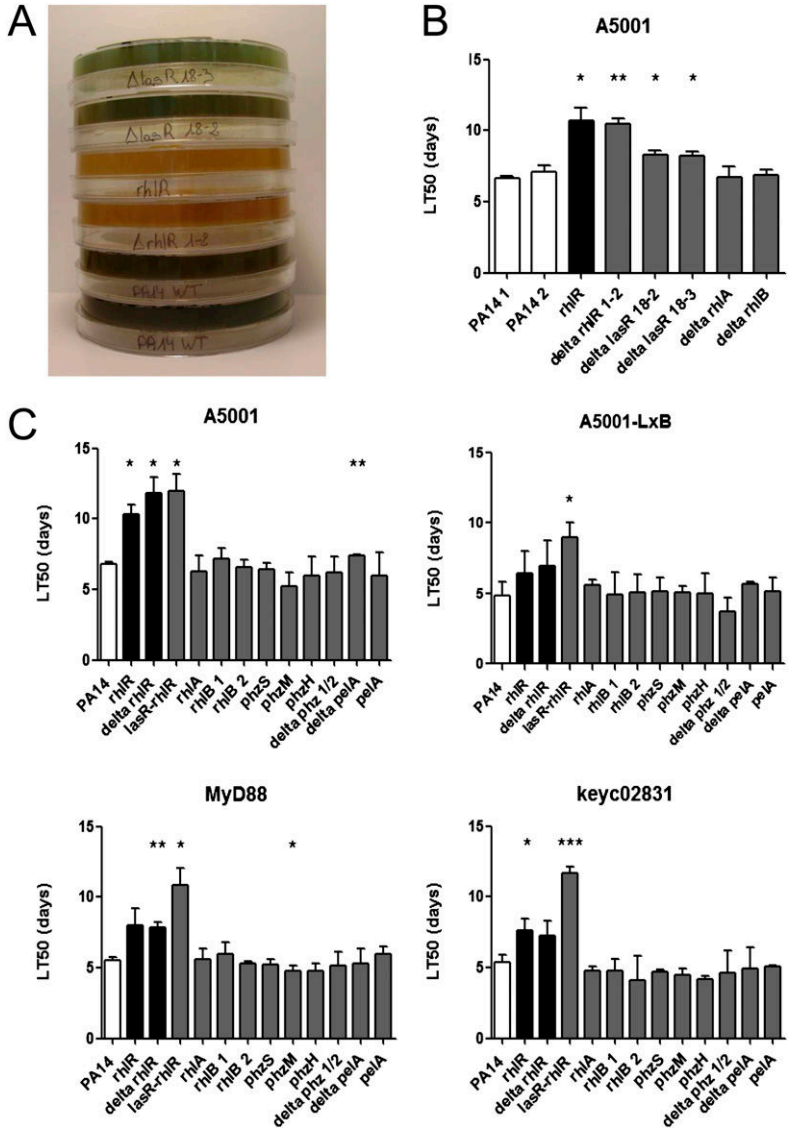


Fig. S6. Phenotypes of mutants in *lasR, rhlR,* and in some RhlR target genes. (*A*) Phenotypes due to the production of pyocyanin as observed for bacteria cultured on rifampicin LB plates of two independent colonies of wild-type PA14 (two bottom plates), *ΔrhlR 1–2* and *rhlR* (next two plates), and two *ΔlasR* deletions (two top plates). As expected, the *rhlR* mutants fail to produce any pyocyanin. Note the distinct color of *lasR* mutants. (*B*) Infection of A5001 wild-type flies with a set of PA14 in-frame deletion mutants. The results indicate the LT50. In A5001 flies, there is a statistically significant difference between wild-type PA14 and *ΔrhlR 1–2,* PA14 and the reference *rhlR* mutant (*rhlR*: GID3229 ID#37943). The two *ΔlasR* mutants showed only a minor attenuation. *ΔrhlA* and *ΔrhlB* behave as wild-type PA14. (*C*) Survival analysis of wild-type flies (A5001) or immunocompromised flies [key^{c02831} for IMD pathway, *MyD88* for the Toll pathway, and latex bead-injected flies (A5001-LxB)] infected with wild-type PA14 or PA14 mutants deficient for different targets of the RhlR quorum-sensing system, as well as biofilm-deficient mutants in *pelA*. In contrast to *rhlR* or *lasR*/*rhlR* double mutants, none of the other mutants displayed reduced virulence in any host background. The results show the LT50 of the survival. *P* values (*) refer to pairwise comparisons between flies infected with wild-type PA14 and mutant bacteria: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 3$. Error bars are ±SD.

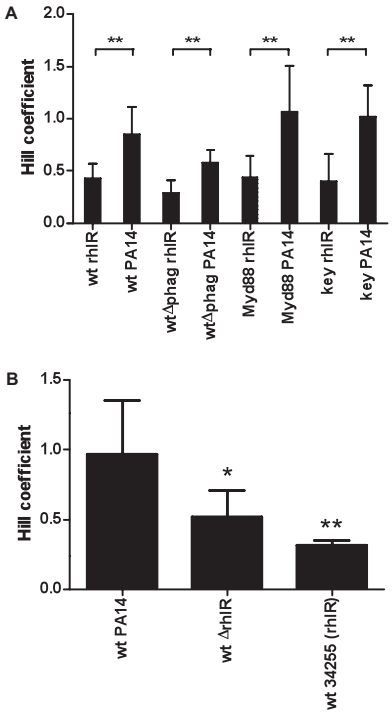


Fig. S7. RhlR is required for the synchronized killing of flies that have ingested *P. aeruginosa* PA14. (*A*) Hill coefficient (absolute value) of survival curves (some of which are shown in Fig. 4) using wild-type or immunosuppressed flies infected with wild-type PA14 or *rhlR* mutant bacteria. The Hill coefficient measures the slope of sigmoid curves. The higher absolute values of the Hill coefficient correspond to steeper curves as illustrated in Fig. 4 *C* and *D*; $^{**}P < 0.005$, $n = 3$. (*B*) Hill coefficient (absolute values) of survival curves (mean LT50 values shown in Fig. 4*A*) of wild-type flies infected either by wild-type PA14, a *rhlR* deletion mutant (*ΔrhlR*), or a *rhlR* transposon insertion mutant [*34255(rhlR)*]. These two *rhlR* mutants are distinct from the *rhlR* mutant (37943) that was used for most of the experiments (*SI Material and Methods*). $^{*}P < 0.05$; $^{**}P < 0.005$; $n = 3$. Error bars are ±SD.

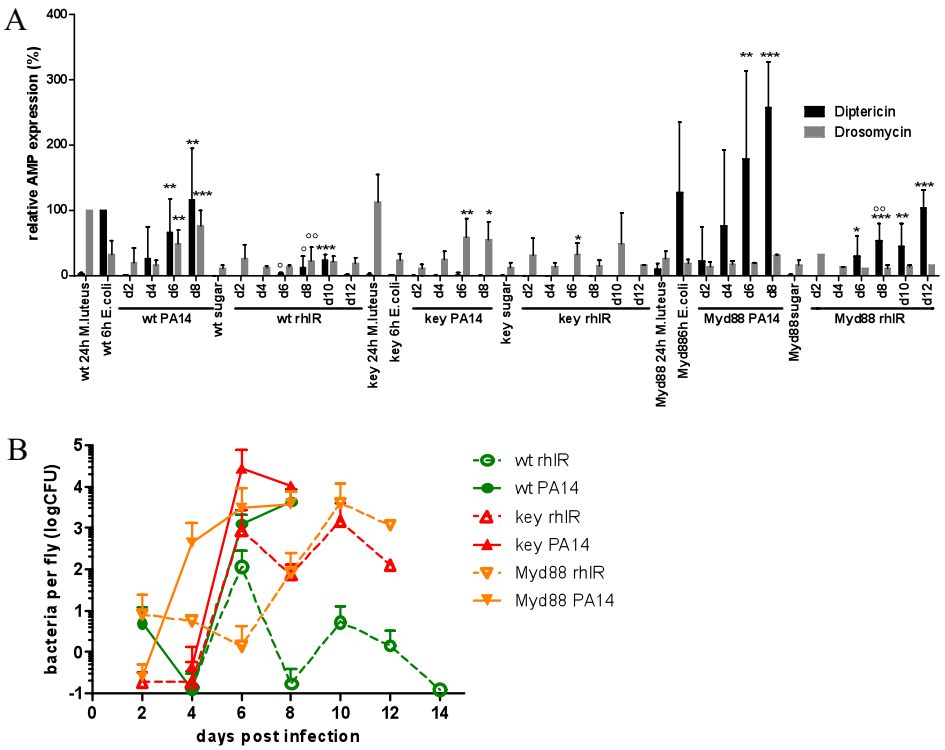


Fig. S8. *rhlR* bacteria fail to establish a strong systemic infection in wild-type or humoral immune response mutant flies. (*A*) qRT-PCR analysis of the expression of *Diptericin* and *Drosomycin* of wild-type, IMD mutant (*key*), and Toll mutant (*Myd88*) flies after infection with PA14 or *rhlR* mutant bacteria (mean of three independent experiments). Statistically significant differences between flies fed on *P. aeruginosa* and flies of the same genotype fed on sucrose solution are indicated by $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$. The signal measured in wild-type and *Myd88* flies infected with *rhlR* is significantly weaker than that induced by PA14, as indicated by $°P < 0.05$ and $°°P < 0.01$. (*B*) Bacterial numbers in the hemolymph of wild type (wt), Toll mutants, and IMD mutants (each data point is the mean of three independent experiments); this number per fly is represented with a logarithmic scale. The bacterial titer in *key* and *Myd88* mutants is higher than that measured in wild-type flies also orally challenged with *rhlR*. Note that at day 12 half of the *rhlR*-infected *key* and *Myd88* flies have succumbed (Fig. 4*C*). Thus, the lower titer measured in these flies at that time point (compared with day 10) may result from flies that have been less severely infected and as a result die more slowly, consistent with the shallow curves observed in survival experiments. Error bars are ±SD.

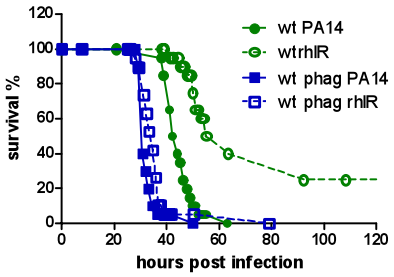


Fig. S9. *rhlR* mutant bacteria are less virulent in a septic injury model, unless the cellular immune response is impaired. Data from a septic injury survival experiment using wild-type and phagocytosis-deficient (latex bead-injected: phag) flies are shown. *rhlR* mutant bacteria kill wild-type flies significantly more slowly than *P. aeruginosa* PA14: $P = 0.012$; $n = 2$. The results are representative of two independent experiments.

Annex 2

Assessing *Pseudomonas* Virulence with a Nonmammalian Host: *Drosophila melanogaster*

Samantha Haller, Stefanie Limmer, and Dominique Ferrandon

Drosophila melanogaster flies represent an interesting model to study host-pathogen interactions as: (1) they are cheap and easy to raise rapidly and do not bring up ethical issues, (2) available genetic tools are highly sophisticated, for instance allowing tissue-specific alteration of gene expression, e.g., of immune genes, (3) they have a relatively complex organization, with distinct digestive tract and body cavity in which local or systemic infections, respectively, take place, (4) a medium throughput can be achieved in genetic screens, for instance looking for Pseudomonas aeruginosa mutants with altered virulence. We present here the techniques used to investigate host-pathogen relationships, namely the two major models of infections as well as the relevant parameters used to monitor the infection (survival, bacterial titer, induction of host immune response).

Chapter 56

Assessing *Pseudomonas* Virulence with a Nonmammalian Host: *Drosophila melanogaster*

Samantha Haller, Stefanie Limmer, and Dominique Ferrandon

Abstract

Drosophila melanogaster flies represent an interesting model to study host–pathogen interactions as: (1) they are cheap and easy to raise rapidly and do not bring up ethical issues, (2) available genetic tools are highly sophisticated, for instance allowing tissue-specific alteration of gene expression, e.g., of immune genes, (3) they have a relatively complex organization, with distinct digestive tract and body cavity in which local or systemic infections, respectively, take place, (4) a medium throughput can be achieved in genetic screens, for instance looking for *Pseudomonas aeruginosa* mutants with altered virulence. We present here the techniques used to investigate host–pathogen relationships, namely the two major models of infections as well as the relevant parameters used to monitor the infection (survival, bacterial titer, induction of host immune response).

Key words Survival assay, Bacterial titer, Host–pathogen interactions, Genetic screens for virulence factors, *Drosophila* septic injury model, *Drosophila* oral infection model

1 Introduction

A major limitation of vertebrate models is that it is difficult to screen bacterial mutant libraries for alterations in the virulence program of the pathogen. This is due to the high number of animals required to perform such screens, which are ethically questionable, costly, labor- and time-intensive. Genetic model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* provide interesting alternatives to screen for virulence factors. Indeed, while there may be "private" virulence factors required exclusively for pathogenesis in a given model, "public" virulence factors can be identified in screens involving invertebrate or plant organisms [1–4]. To be meaningful, such screens must be performed in model systems in which the immune system is well-characterized, and *Drosophila* certainly qualifies on this account [5]. Its immune system encompasses both a humoral and a cellular response that deal with systemic infections, as well as local responses that deal with invasions

Alain Filloux and Juan-Luis Ramos (eds.), *Pseudomonas Methods and Protocols*, Methods in Molecular Biology, vol. 1149, DOI 10.1007/978-1-4939-0473-0_56, © Springer Science+Business Media New York 2014

through frontier epithelia such as that of the intestinal tract [6–8]. In addition, *Drosophila* benefits from over a century of research, which results in highly sophisticated genetic tools as well as knowledge on most major biological questions, from chromosomal structure to ecology and evolution.

Several infection models have been developed using *Pseudomonas aeruginosa* as a pathogen [9, 10]. They can be regrouped into two major categories: septic injury and oral infection models [10]. Flies succumb rapidly, within 48–72 h, to the direct introduction of *Pseudomonas* into the body cavity [11–14]. This fast infection kinetics makes it difficult to screen for virulence factors having moderate effects as the difference between survival curves between flies infected with wild-type versus mutant bacteria represents usually just a few hours. In contrast, orally infected flies succumb in 5–10 days depending on the conditions [4]. While some investigators have reported damages to the intestinal epithelium, mostly in a sensitized background (Ras^{V12} mutants) [15], we have failed to detect any major impact on the gut epithelium. Rather, we have found that some PA14 bacteria are able to escape the digestive tract and ultimately launch a systemic infection that defeats both the cellular and systemic immune responses. Of note, the use of specific conditions in which the fly immune response has been inactivated (mutants, inhibition of phagocytosis) has led to the discovery that the quorum-sensing regulator RhlR is required for PA14 to initially circumvent the cellular immune defense [4].

Thus, when performing infections, several parameters can be easily monitored, including the survival rate of flies, the bacterial titer inside the fly or tissues/compartments such as gut and hemolymph, and the activation of host antimicrobial defenses such as the induction of antimicrobial peptide genes, which is the hallmark of the systemic immune response.

Here, we present the techniques we use to probe the interactions between *P. aeruginosa* and *D. melanogaster*. These include two variations of the septic injury model (pricking and injection) and an oral infection model. Wild-type and mutant flies can be used for such assays. One way to inhibit the cellular immune response is through the saturation of the phagocytic apparatus through the injection of nondegradable latex beads. Other techniques include the determination of the bacterial titer in specific tissues or in the hemolymph. To monitor the induction of the systemic immune response, the expression levels of antimicrobial peptide genes is measured using quantitative reverse-transcription PCR, a procedure that can be performed in 96-well plates or even 384-well plates. Of note, general information on *Drosophila* can be found in several books [16, 17] as well as on the Flybase Website: http://flybase.org/.

2 Materials

2.1 *Drosophila* Husbandry and Culture of Bacteria

1. *Drosophila* stocks: they are kept under standardized conditions in an air-conditioned room or incubator at 25 °C and 60 % humidity. For long-term storage, stocks are kept at 18 °C. The health status of the flies has to be checked on a regular basis: tests for the presence of microsporidia, viruses, or symbionts such as *Wolbachia* are essential, since such infections can interfere with the experimental infection (*see* **Note 1**, Table 1). Common wild-type stocks are Oregon-R and Canton-S. Often, flies carrying the *white* mutation are also used as a wild-type strain. Reporter fly lines for the expression of antimicrobial peptide genes such as *Diptericin*-Tomato, *Drosomycin*-GFP, and *Diptericin*-LacZ (*see* **Note 2**) can be used to assess the spatial and temporal expression of antimicrobial peptides in the

Table 1
Primers used for the detection of microbial contamination of *Drosophila* stocks

Pathogen	Test	Primer sequences	
Wolbachia-1	Regular PCR	Fw	TTGTAGCCTGCTATGGTATAACT
		Rv	GAATAGGTATGATTTTCATGT
Wolbachia-2	Regular PCR	Fw	AAAAATTAAACGCTACTCCA
		Rv	TGGTCCAATAAGTGATGAAGAAAC
Nora virus	Quantitative PCR	Fw	AACCTCGTAGCAATCCTCTCAAG
		Rv	TTCTTGTCCGGTGTATCCTGTATC
Drosophila C virus	Quantitative PCR	Fw	TCATCGGTATGCACATTGCT
		Rv	CGCATAACCATGCTCTTCTG
Flock house virus-1	Quantitative PCR	Fw	TTTAGAGCACATGCGTCCAG
		Rv	CGCTCACTTTCTTCGGGTTA
Flock house virus-2	Quantitative PCR	Fw	CAACGTCGAACTTGATGCAG
		Rv	GCTTTACAGGGCATTTCCAA
Vesicular stomatitis virus	Quantitative PCR	Fw	CATGATCCTGCTCTTCGTCA
		Rv	TGCAAGCCCGGTATCTTATC
Sindbis virus	Quantitative PCR	Fw	CAAATGTGCCACAGATACCG
		Rv	ATACCCTGCCCTTTCAACAA
Cricket paralysis virus-1	Quantitative PCR	Fw	GCTGAAACGTTCAACGCATA
		Rv	CCACTTGCTCCATTTGGTTT
Cricket paralysis virus-2	Quantitative PCR	Fw	GGAATTTTTGGAGACGCAAA
		Rv	GTGAAGGGGGCAACTACAAA
Microsporidia (*Tubulinosema ratisbonensis*)	Quantitative PCR	Fw	TCTCACAGTAGTGGCGAATG
		Rv	AACACCGTATTGGAATACAG

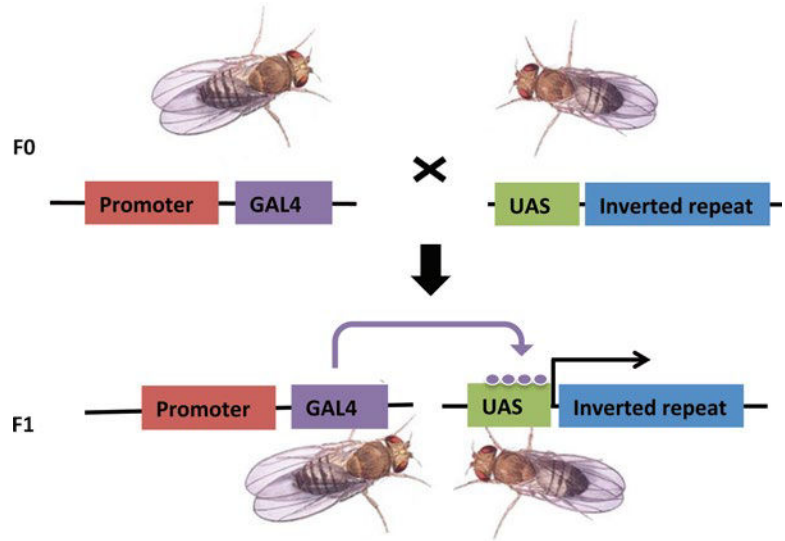


Fig. 1 The GAL4-UAS system in *Drosophila*. This system has been adapted from yeast genetics to allow the expression of any construct in a tissue-specific or cell type-specific manner. Transgenic flies containing a promoter of interest that drives the expression of the yeast transcriptional activator GAL4 are crossed to a transgenic line containing a construct that has been placed under the control of UAS enhancer sequences linked to a basal promoter. In progeny flies containing both transgenes, GAL4 will be expressed in the required tissue or cell-type and drive the expression of the construct

fly (systemic expression vs. local expression). When a gene needs to be expressed or inactivated in a given tissue, the UAS-GAL4 system is used (Fig. 1) [18]. *Drosophila* lines carrying the tissue-specific promoter of interest fused to the coding sequence of the yeast transcription activator GAL4 can either be constructed or obtained from other *Drosophila* investigators or stock centers (Vienna *Drosophila* RNAi Center: *stockcenter.vdrc.at*, Bloomington *Drosophila* Stock Center at Indiana University: *flystocks.bio.indiana.edu*, *Drosophila* Genetic Resource Center at Kyoto: *kyotofly.kit.jp*). *Drosophila* lines carrying a specific DNA sequence fused to an Upstream Activation Sequence (UAS, target sequence of GAL4) for over expression of a gene, a marker, or a hairpin construct for RNA interference mediated gene knock-down can similarly be obtained. The timing of transgene expression can be controlled by using a transgene encoding a temperature sensitive repressor of GAL4:GAL80^{ts} (*see* **Note 3**) [19].

2. *Drosophila* feed: flies are fed on a standard semi-solid cornmeal medium composed of: 6.4 % (w/v) cornmeal (Moulin des moines, France), 4.8 % (w/v) granulated sugar (Erstein, France), 1.2 % (w/v) yeast brewer's dry powder (VWR, Belgium)

(*see* **Note 4**), 0.5 % (w/v) agar (Sobigel, France), 0.004 % (w/v) 4-hydroxybenzoate sodium salt (Merck, Germany) (*see* **Note 5**). The medium is poured in plastic vials after cooking: small (Ø 25 mm), medium (Ø 32 mm), and large (Ø 50 mm). Stoppers of the adequate size are placed once the medium has cooled down. Store at 4 °C.

3. *Preparation of latex bead suspension for saturating the phagocytic apparatus*: wash the solution of beads (Invitrogen, Surfactant free red CML latex, Ø 0.30 µm, C29145) with sterile PBS 1×: centrifuge beads at 20,000 × *g* for 10 min at 4 °C. Discard the supernatant and add fresh sterile PBS 1×. Repeat wash three times. After the last centrifugation, concentrate the latex bead solution fourfold by adding only a quarter of the initial volume of sterile PBS 1×.
4. *Injections* (*latex beads suspension*): Use a Nanoinjector (Nanoject II ™, Drummond Scientific Company) and appropriate capillaries (3.5″ capillary, 3-000-203-G/X, Drummond Scientific Company). Injection capillaries are prepared using a needle puller that heats and subsequently pulls the capillary until it breaks in the middle (e.g., Model P-97, Flaming/Brown Micropipette puller, Sutter Instrument Co.).
5. *Bacterial culture medium* (*liquid*): Brain Heart Broth (BHB; Sigma-Aldrich): Beef heart, 5 g/L, calf brains 12.5 g/L, disodium hydrogen phosphate 2.5 g/L, d(+)-glucose 2 g/L, peptone 10 g/L, sodium chloride 5 g/L. Weigh 37 g of BHB powder and dissolve in 1 L distilled water. Adjust the final pH to 7.4 ± 0.2. Sterilize the solution by autoclaving at 121 °C for 15 min. Store the prepared medium below 8 °C.
6. *Bacterial plates*: Luria Bertani broth (LB, Sigma-Aldrich) with 15 % agar in Petri dish (*see* **Note 6**) with appropriate antibiotics (*see* **Note 7**). Weigh 10 g of LB powder, 7.5 g of agar powder and add 500 mL distilled water qsp. Sterilize the solution by autoclaving at 121 °C for 15 min. Once the temperature of the medium is below 60 °C, add the appropriate antibiotics and pour the medium in Petri dishes under a laminar flow cabinet. When the medium is solid, store plates below 8 °C.
7. *Dissecting microscope*: able to enlarge until 35-fold and cold light source.
8. *Material to anesthetize the flies*: Inject + Matic Sleeper (CO_2 blow gun and porous pad) linked to a CO_2 bottle or similar (*see* http://flystocks.bio.indiana.edu/Fly_Work/supplies.htm for a list of suppliers of *Drosophila* research material) (*see* **Note 8**).
9. *Tabletop centrifuge*.
10. *Pure distilled sterile water*: for instance, Advantage A10 and RiOs™ systems, Millipore, or equivalent.

2.2 Systemic Infection

1. *Fly vials* with normal fly food and appropriate stoppers.
2. *1 M Sodium hydroxide solution* (NaOH 1 M). Weigh 4 g NaOH and add pure distilled sterile water qsp 100 mL.
3. *Tungsten needle* (Ø 0.25 mm, Sigma-Aldrich) and the material to sharpen the needle (NaOH 1 M and a low voltage generator) for an infection by pricking the flies (*see* **Note 9**, Fig. 2a). For injections of bacteria, a Nanoinjector and appropriate capillaries (*see* **item 4** of Subheading 2.1) are needed.
4. *Phosphate Buffer Saline* (*PBS 1*×): 1.06 mM potassium phosphate monobasic (KH_2PO_4), 155.17 mM sodium chloride (NaCl), 2.97 mM sodium phosphate dibasic (Na_2HPO_4–$7H_2O$). Weigh 144 mg of KH_2PO_4, 9 g of NaCl, and 795 mg of Na_2HPO_4–$7H_2O$. Add pure distilled sterile water qsp 1 L.
5. *Ethanol 70%*: Measure 700 mL pure ethanol and add pure distilled sterile water qsp 1 L.

2.3 Oral Infection

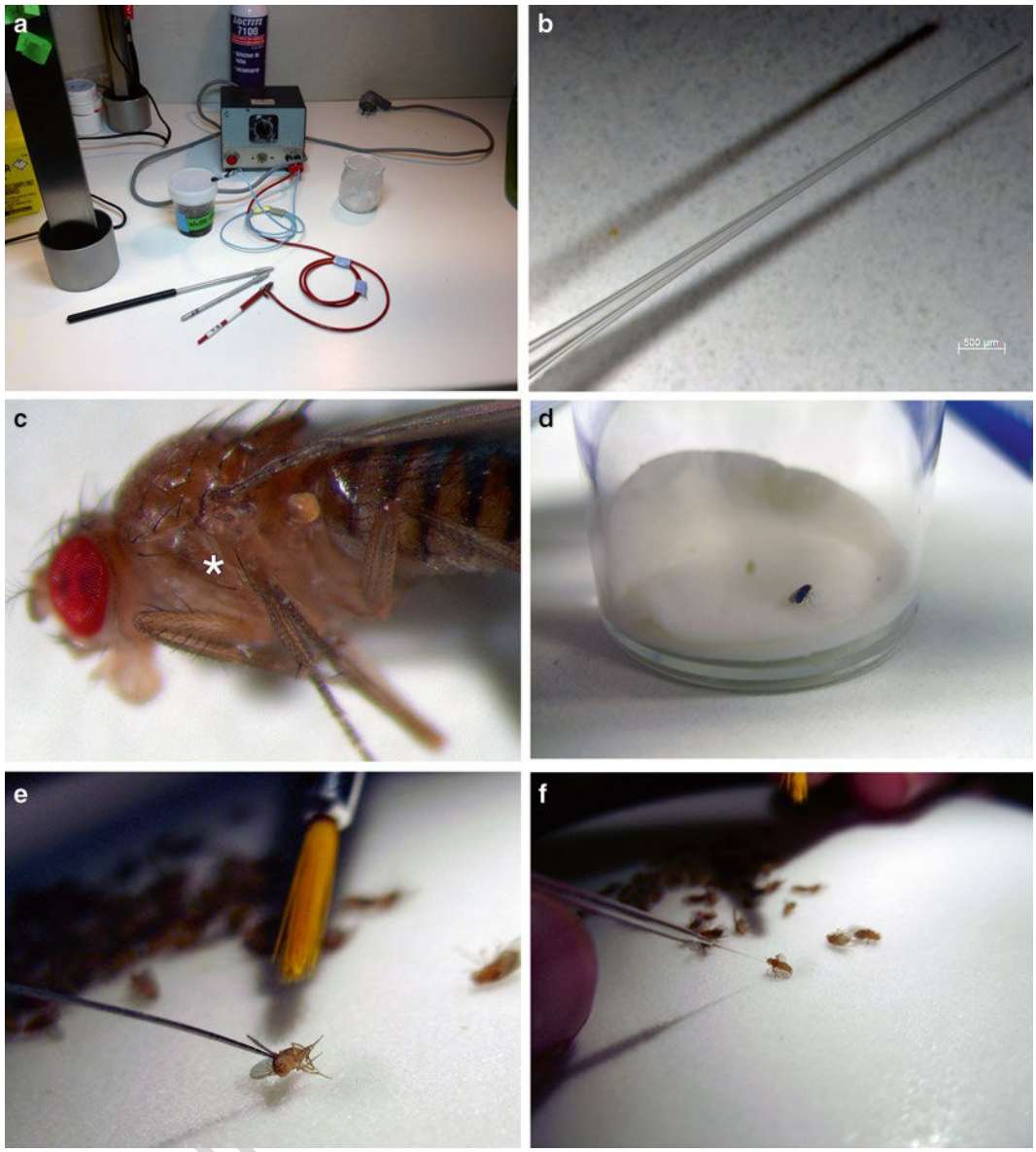
1. Medium plastic vials (Ø 32 mm, Greiner Bio-One) and appropriate stoppers.
2. *Absorbent filter*: of a size appropriate to cover the bottom of the medium plastic vial (e.g., AP1003700, Millipore).
3. *50 mM sucrose solution*: Weigh 3.42 g sucrose and add distilled water 200 mL qsp. Sterilize the solution by sterile filtration using a Ø 0.22 µm pore filter. Store at 4 °C.

2.4 Hemolymph Collection

1. *Microtubes* to collect the hemolymph: 1.5 or 900 µL volume Eppendorf or equivalent.
2. *Phosphate Buffer Saline*: *See* **item 4** of Subheading 2.2.
3. *LB agar Petri dish*: With appropriate antibiotics (*see* **Note 6**).
4. *Nanoinjector and appropriate capillaries*: *See* **item 4** of Subheading 2.1.

2.5 Analysis of Antimicrobial Peptide Gene Expression in Multiple-Well Plates

1. *Tubes to crush the flies*: specific tubes (rack with microtube strips and cap strips, Macherey-Nagel) each one containing a tungsten bead (Tungsten Carbide Beads, 3 mm diameter, Qiagen) are needed (*see* **Note 10**).
2. *Crusher* (Mixer Mill 300 MM, Retsch).
3. *Reverse transcription enzyme and reagents*: iScript™ cDNA Synthesis Kit, Bio-Rad, or equivalent.
4. *PCR tubes.*
5. *Thermocycler*: C1000™ Thermal Cycler, Bio-Rad or equivalent (*see* **Note 11**).
6. *Forward and reverse primers*: designed to detect the antimicrobial peptide mRNA sequence (*see* Table 2) at concentration of 10 µM.



this figure will be printed in b/w

Fig. 2 Septic injury of *Drosophila*. (**a**): Setup to sharpen tungsten needles. The tungsten wire is inserted in a needle-holder. Three types of needle-holders are presented on the *left foreground* of the picture. The *middle needle-holder* has been obtained by sawing off the black plastic handle of the *left-most needle-holder*, as it is too long to be convenient. The power supply is in the background. A wire from the positive pole is connected to the *right-most needle-holder*: the wire is no longer protected by its cover and is in direct contact with the metal of the holder. The graphite electrode connected to the negative pole of the generator has been placed on *top* of the NaOH solution vessel. (**b**): Elongated capillary for injecting bacterial suspensions. The tip has been broken off with sharp tweezers. (**c**) The position where the fly should be injected is shown with an *asterisk*. Inject just above the *asterisk*, at a location where the cuticle is somewhat weaker. While this is not of utmost importance for pricking or injection, it makes the collection of hemolymph easier. (**d**) Oral infection setup. Only one fly has been placed in the vial to be able to take the picture. It is strongly advised to use 20 flies so that conditions remain the same when comparing different bacterial strains. (**e**) Pricked fly. When correctly pricked, the fly is held by the needle and then dropped off either on one side or directly in a vial containing food using either a brush or tweezers (need to be disinfected when changing bacterial strains). (**f**) Injected fly. Same remark as for pricked fly

t2.1 **Table 2**
t2.2 **Primers used for the quantification of *Drosophila* antimicrobial peptide**
t2.3 **gene expressions**

t2.4 t2.5 Antimicrobial peptide	Primer	
t2.6 t2.7 Ribosomal protein L32 (RP49)	Fw Rv	GACGCTTCAAGGGACAGTATCTG AAACGCGGTTCTGCATGAG
t2.8 t2.9 Drosomycin (Drs)	Fw Rv	CGTGAGAACCTTTTCCAATATGATG TCCCAGGACCACCAGCAT
t2.10 t2.11 Diptericin (Dpt)	Fw Rv	GCTGCGCAATCGCTTCTACT TGGTGGAGTGGGCTTCATG
t2.12 t2.13 Attacin-A (Att-A)	Fw Rv	GGCCCATGCCAATTTATTCA AGCAAAGACCTTGGCATCCA
t2.14 t2.15 Defensin (Def)	Fw Rv	GCTCAGCCAGTTTCCGATGT TCCTGGTGGGCATCCTCAT
t2.16 t2.17 Cecropin (Cec)	Fw Rv	ACGCGTTGGTCAGCACACT ACATTGGCGGCTTGTTGAG
t2.18 t2.19 Metchnikowin (Mtk)	Fw Rv	CGTCACCAGGGACCCATTT CCGGTCTTGGTTGGTTAGGA
t2.20 t2.21 Drosocin (Dro)	Fw Rv	CACCCATGGCAAAAACGC TGAAGTTCACCATCGTTTTCCTG

7. *Quantitative PCR reagent and enzyme*: iQ™ Sybr® Green, Bio-Rad or equivalent.
8. *384-Well plates and cover*: specific seals to close the plate that allow fluorescence detection (*see* **Note 12**).
9. *Thermocycler with a fluorescence detector*: C1000™ Thermal Cycler with CFX384 Real-Time System, Bio-Rad and the analyzer program (CFX Manager Software, Bio-Rad) or equivalent. 96-Well plates can also be used.

3 Methods

Carry out all procedures in a P2 level laboratory (except the extraction of nucleic acids from frozen flies). As *P. aeruginosa* is an opportunistic human pathogen, the experimenter must have a functional immune system, which provides already a significant barrier against infection. Always wear a laboratory coat and gloves, and work under a microbial hood when handling the bacteria, except when injecting or retrieving hemolymph from flies. If needed, a better protection against accidentally pricking one's hand with a contaminated needle can be provided by wearing a special glove (which contains quaternary amines) on the noninjecting hand (Gant BioPro, MAP203-7, MAPA professional).

3.1 Preparation of the Flies Prior to the Infection

1. If the virulence of the bacteria needs to be tested in flies in which a gene is ectopically expressed or inactivated in a given tissue, a cross needs to be performed between two specific *Drosophila* lines (Fig. 1). Collect virgin female flies from the line carrying the regulatory sequence fused to the GAL4 sequence and male flies from the line carrying the desired coding sequence under the control of the UAS sequence (or the reverse). Deposit the males and females of the appropriate genotypes in a tube containing normal fly food, which can be supplemented with a dash of live yeast. When the cross is performed at 25 °C, transfer the parents in a new tube every 3 days and when the cross is performed at 18 °C, transfer them every 7 days.
2. Collect the emerging progeny. Anesthetize these flies with a CO_2 blow gun and sort the flies carrying the genotype of interest on a CO_2 porous pad and with the help of a dissecting microscope.
3. For an oral infection only: prepare medium size tubes with two absorbent filters and 2 mL of 50 mM sucrose solution. Sort the flies that will be infected and keep them for 48 h in these tubes at 25 °C prior starting the experiment.
4. If the phagocytic ability of the flies needs to be blocked, inject latex beads in these flies 24 h before performing the infection experiment so as to allow wound closure and sufficient time for the flies to recover from the injection procedure [20]. To this end, prepare capillaries with the puller as described in **item 4** of Subheading 2.1. Break the capillary tip with tweezers using a dissecting microscope (Fig. 2b). Fill the capillary with mineral oil and assemble it onto the Nanoinjector. Discard half of the volume of oil and fill the capillary with latex beads solution (fourfold concentrated, *see* **item 3** of Subheading 2.1). Inject each fly with 92 nL latex beads suspension in the lateral part of the thorax, slightly below and anterior to the wing hinge (*see* **Note 13**; Fig. 2c, f).

[AU1]

3.2 Culture of Bacteria

1. Grow the bacteria on an LB agar Petri dish with the appropriate antibiotics at 37 °C.
2. Inoculate the BHB culture medium with a single colony (for instance, 10 mL BHB in a 100 mL Erlenmeyer flask). Grow the bacteria overnight at 37 °C and under agitation.

3.3 Systemic Infection Model

Flies can be infected either by pricking (septic injury) or injection (*see* **Note 14**).

Flies should be 3–10 days old when the infection starts.

3.3.1 Systemic Infection by Pricking the Flies

1. Measure the OD_{600} of the bacterial culture.
2. Adjust the OD_{600} to the appropriate concentration in sterile PBS 1× (*see* **Note 15**). Centrifuge the culture at 3,000×*g*

(4,000 rpm on a A-4-62 rotor (5810R centrifuge, Eppendorf)) for 10 min at 4 °C in 50 mL Falcon tubes. Discard the supernatant and add the appropriate volume of sterile PBS 1×. Resuspend the bacteria by pipetting up and down.

3. Sharpen the tungsten needle by putting the negative graphite electrode in the 1 M NaOH solution and the positive electrode on the needle (Fig. 2a). Then plunge the needle into the 1 M NaOH solution and apply current until a sharp end is generated.
4. Dip the needle in the bacterial suspension.
5. Anesthetize the flies within the fly vial with a CO_2 blow gun and deposit them on a CO_2 porous pad.
6. Under a dissecting microscope, prick the flies one by one at the same location as for the injection (*see* **step 4** of Subheading 3.1; Fig. 2c, e) with the injection capillary. The tungsten needle has to be dipped again in the bacterial suspension after each fly has been pricked. Between each bacterial type (mutant or strain), sterilize the tungsten needle with ethanol 70 % and rinse with sterile water.
7. Transfer the infected flies in a fly vial containing normal fly food and place it at 25 °C with 60 % humidity.
8. For survival experiments, monitor the number of surviving flies every 1–2 h starting from 24 h after the infection. Do not take into account flies that have succumbed early to infection (before 18 h after infection). In addition, the bacterial titer in the hemolymph and the level of antimicrobial peptide expression can be measured (*see* below for protocols).

3.3.2 Systemic Infection by Injecting the Bacteria into the General Cavity (Hemocoel) of the Flies

1. Measure the OD_{600} of the bacterial culture.
2. Adjust the OD_{600} to the appropriate concentration in sterile PBS 1× (*see* **Note 15**). Centrifuge the culture at 3,000 × *g* for 10 min at 4 °C in 50 mL tubes (*see* section 3.3.a.1). Discard the supernatant and add the appropriate volume of sterile PBS 1×. Resuspend the bacteria by pipetting up and down. [AU2]
3. Prepare capillaries with the puller as described in **item 4** of Subheading 2.1. Break the capillary tip with tweezers using a dissecting microscope. Fill the capillary with mineral oil and assemble it onto the Nanoinjector. Discard half of the volume of oil and fill the capillary with the bacterial suspension. Use a new capillary for each bacterial strain.
4. Anesthetize the flies within a fly vial with a CO_2 blow gun and deposit them on a CO_2 porous pad.
5. Under a dissecting microscope, inject into each fly one by one the appropriate volume of bacterial suspension in order to inject the desired number of bacteria (*see* **Note 16**).

6. Transfer the infected flies in a fly vial containing normal fly food and place it at 25 °C with 60 % humidity.
7. For survival experiments, monitor the number of surviving flies every 1–2 h starting 24 h after the injection. Do not take into account flies that have succumbed early to infection (before 18 h after infection). In addition, the bacterial titer in the hemolymph and the level of antimicrobial peptide expression can be measured (*see* below for protocols).

3.4 *D. melanogaster* Oral Infection

1. Measure the OD_{600} of the bacterial culture.
2. Adjust the OD_{600} to 2.5 per mL with fresh BHB: compute the appropriate volume of bacterial culture needed for the experiment (that is, the number of tubes needed to perform the experiment, 2 mL of bacterial suspension per tube). Centrifuge the culture at 3,000×*g* for 10 min at 4 °C in 50 mL tubes. Discard the supernatant and add the appropriate volume of fresh BHB. Resuspend the bacteria by pipetting up and down.
3. Dilute the bacterial suspension 1/10 with 50 mM sucrose solution. The final OD_{600} of the infection solution is then 0.25 per mL (*see* **Note 17**).
4. Prepare the infection tubes by placing two absorbent filters at the bottom of the vials. Add 2 mL of the infection solution per tube.
5. Place 20 adult flies (3–10 days old) into each tube and put the vial at 25 °C with 60 % humidity.
6. For survival experiments, monitor the number of surviving flies at least each day. In addition, the bacterial titer in the hemolymph and the level of antimicrobial peptide expression can be measured (*see* below for protocols).

3.5 Determination of the Bacterial Titer from Collected Hemolymph

1. Prepare collecting tubes (e.g., 900 µL tubes) with 10 µL of sterile PBS 1×.
2. To manipulate the flies easily, anesthetize them with CO_2.
3. Prepare capillaries with the needle-puller as described in **item 4** of Subheading 2.1. In contrast to the injection procedure, *do not fill the capillary with oil before assembling it to the Nanoinjector*.
4. Prick the flies with the capillary into the lateral side of the thorax slightly in front and under the wing hinge, at a weak point of the cuticle (Fig. 2c). The hemolymph is drawn inside the capillary by capillarity force alone (*see* **Note 18**). Collect the hemolymph of at least ten flies, one by one (*see* **Note 19**). Use the Nanoinjector motor to force the hemolymph out of the capillary and into the collecting tube containing the sterile PBS 1× after each fly (*see* **Note 20**).

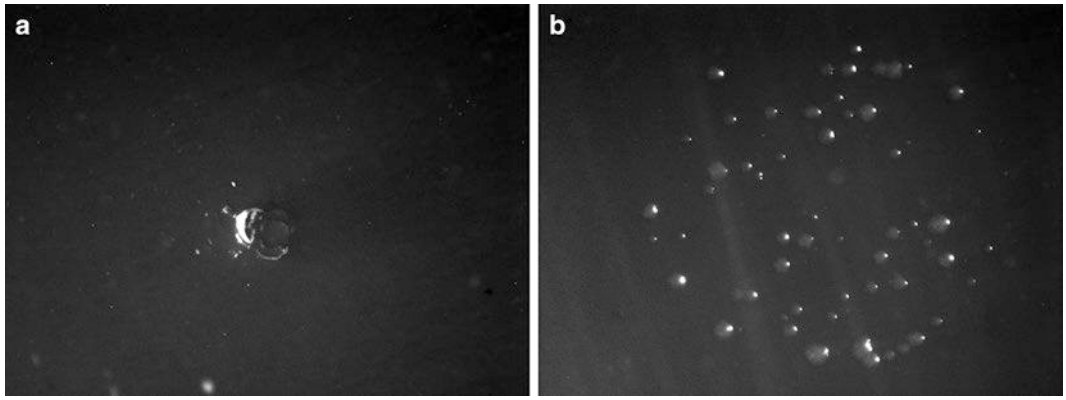


Fig. 3 Counting bacterial colonies in a drop to determine the bacterial titer. (**a**) 4 µL drop has been deposited on an agar plate containing appropriate antibiotics. Colonies are shown in (**b**), as observed under a dissecting microscope. This dilution is appropriate to count the colonies. In (**a**), no colonies have grown, and only the imprint of the tip and agar speckles can be seen. The advantage of this method is that it requires only few plates to determine the bacterial titer for many flies

5. Dilute the sample to 1/10, 1/100, 1/1,000, 1/10,000 in sterile PBS, depending for how long the flies have been fed on the bacteria (later time points tend to give higher bacterial titer).
6. Deposit two drops of 4 µL for each sample and dilutions on a single LB agar plate (*see* **Note 21**), with the appropriate antibiotics. Once the drops dried, place the plate at 37 °C for about 10 h (*see* **Note 22**) to allow bacteria to form colonies.
7. Count the number of colonies you observe for each drop using a dissecting microscope (Fig. 3). Calculate the number of colony forming units (CFU) per fly.

3.6 Analysis of Antimicrobial Peptide Gene Expression by Reverse-Transcription and Quantitative PCR (RT qPCR)

1. Freeze the flies that were used to assess the bacterial titer in the hemolymph at −80 °C (or other flies that have been infected if you are not counting CFU/fly in your experiment). Use specific tubes in which you have beforehand put a single tungsten bead in each (*see* Subheading 2).
2. Put the tubes in liquid nitrogen. Crush the flies by shaking two times for 60 s at 20 Hz using the Mixer Mill apparatus. Put the tubes in liquid nitrogen again in-between the two crushing steps. Extract total RNA with an adequate kit following the procedure recommended by the supplier (e.g., NucleoSpin 96 RNA, Macherey-Nagel). The lysis solution is added directly to the pulverized flies. Purified RNA should be kept at −80 °C.
3. Reverse transcribe the RNA into cDNA using the iScript™ cDNA Synthesis kit (BIO-RAD): 2 µL template RNA, 4 µL iScript Mix, 1 µL iScript reverse transcription enzyme, and 13 µL sterile distilled nuclease-free water (*see* **Note 23**). Use the following

program: (25 min at 65 °C, 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C).

4. Dilute the cDNA obtained in **step 3** to 1/20 with pure distilled sterile water.
5. Prepare the RT-PCR reagent solution with 5 μL of Sybr® Green Mix, 0.3 μL of each forward and reverse primer (10 μM) (against antimicrobial peptide mRNA or other mRNA of interest: Table 2), 2 μL of cDNA (diluted to 1/20 in **step 4**). Add pure distilled sterile water to the solution to obtain a final volume of 10 μL per reaction.
6. Place the sample inside a thermocycler with the following program: 15 s at 98 °C, and then 35 times: 2 s at 95 °C and 30 s at 60 °C. Ct numbers are obtained (*see* **Note 24**). The last step is to increase the temperature by 0.5 °C each 5 s from 65 to 95 °C to check that the appropriate amplicon has been amplified (*see* **Note 25**).
7. Normalize the obtained Ct with the measured Ct on the same sample of a *Drosophila* housekeeping gene like the Ribosomal Protein L32 (RP49) (Table 2).

4 Notes

1. The physiological state and genetic background of flies is an important consideration in these assays. For instance, co-infection between *P. aeruginosa* and other pathogens or unwanted symbionts (endosymbiotic bacteria such as *Wolbachia*, viruses, or others) are best avoided, since secondary infections can influence *P. aeruginosa* infections in unpredictable manners (for instance, it has been discovered that flies harboring *Wolbachia* are protected from some viral infections). On a regular basis, flies used for experiments have to be tested for known pathogens by qPCR. Flies used for experiments should always be in the same physiological state, especially the same age. Mutant and wild-type flies should as much as possible share the same genetic background to prevent irrelevant interpretations. Ideally, the flies should have the same microbiota in their intestine, a proposition difficult to implement in practice unless axenic flies or flies mono-associated with a single commensal bacterial species are used, which has not been reported so far.
2. These reporter fly lines carry the regulatory sequences of the antimicrobial peptide gene (e.g., *Diptericin* or *Drosomycin*) fused with the coding sequences of GFP or other fluorescent proteins such as Tomato (two linked RFPs) or mCherry. β-Galactosidase was used in earlier experiments [21–23].

3. The GAL80^{ts} sequence is fused to the ubiquitous promoter of *Tubulin* and will be transcribed continuously in each cell of the transgenic fly. The GAL80 protein will bind to GAL4 and prevent its binding to UAS sequences. When placed at 29 °C, the GAL80^{ts} repressor is no longer functional, thus allowing GAL4-mediated gene expression. Usually, flies are left at 29 °C for a few days to allow an optimal expression of the genes under UAS control.
4. It is preferable to use dried whole yeast, as yeast extract is not nutritive enough for the flies. Check with your supplier as we have found out that suppliers sometimes change to yeast extract without even warning their customers. Of course, use pesticide-free ingredients.
5. Flies feed and reproduce on the same medium. When larvae hatch, they will work out the medium and make it semi-liquid, if enough progeny is produced (social feeding). The adults may then get stuck in the medium and will therefore be lost. Thus, one has to transfer the adults to a new tube, every 5 days for a stock kept at 25 °C.
6. Round or square Petri dishes may be used. To test large numbers of bacteria, square Petri dishes allow the use of multichannel pipettes to increase the throughput of the procedure (especially to plate hemolymph samples).
7. Depending on the strain or mutant used the resistance against antibiotics can be different. The strain UCBPP-PA14 is naturally resistant to 100 μg/mL Rifampicin.
8. If a CO_2 setup is not available, an alternative is to anesthetize flies with ether using an etherizer. This procedure requires some practice, as it is easy to overexpose the flies to ether and kill them. It is best to use a first tester batch of flies: expose them to ether for 45 s to 1 min and monitor how long they remain asleep. With the right timing of exposure, the flies remain anesthetized up to 20 min. However, too long of an exposure kills the flies. If the flies start waking up while the experiment is still underway, flies can be reexposed, very briefly, to ether. Often, a second exposure is fatal because of overexposure.
9. We sharpen the needles using a low voltage generator, a graphite electrode (taken from a 4.5 V flat battery), and a solution of 1 M NaOH. Always use gloves when manipulating NaOH. The circuit is made by plunging the graphite electrode connected to the negative pole in the NaOH solution, connecting the positive pole to a wire, which is directly attached to the metallic part of the needle holder. By plunging the tip of the needle into the NaOH solution, the circuit is closed, and one can see metal bits falling to the bottom and the production of gas bubbles. A generator for electrophoresis may also be used,

although extreme care should be taken to make sure that the voltage is really set to 6 V, otherwise there is a death hazard with DC current.

10. Individual samples can be processed in 1.5 mL collecting tubes but the quality of these tubes has to be checked otherwise there is a risk that they break during the procedure (the tungsten bead exerts a considerable stress on the frozen tubes). We use original tubes manufactured by Eppendorf.
11. Bain-Marie or hot plates at the indicated temperatures can also be used but are less convenient.
12. Other formats can be used such as 96-well plates. The use of a robot to set up the reactions in a 384-well plate is recommended.
13. We advise to check the efficiency of this treatment. One must simply inject FITC-labeled *Escherichia coli* (or any other bacterium or fungus small enough to be phagocytosed, e.g., Molecular Probes bioparticles) in latex beads-injected flies (and noninjected controls), wait 30 min, and then inject about 300 nL of Trypan blue [20], which quenches the fluorescence emitted by extracellular bacteria but not that emitted by ingested bacteria. No fluorescence should be detected under the microscope in latex beads-injected flies. An alternative is to use pH Rhodo-labeled bacteria [24], which become fluorescent when reaching the acidified phagosome (no need for Trypan blue injection).
14. The injection system compared to the pricking system allows being more accurate with respect to the number of bacteria that will effectively be introduced into the hemocoel of the flies. However, the injection system is much more time-consuming and cumbersome than the pricking system especially if high number of bacterial strains need to be tested. In addition, there is still some variability as the injection of a small volume of liquid (2.4 nL) was found to entail a 100 % variability in the quantities effectively delivered when using a radioactive solution (Marie Gottar and DF, unpublished). In practice, the bacterial dose delivered by pricking is reproducible and accurate enough for most applications.
15. The adequate concentration of bacterial suspension should be empirically determined according to the expected phenotype, that is, the time it takes for most flies to die. If the solution is too concentrated, the difference between wild-type and mutant bacterial strains may not be easily detectable.
16. Injecting a higher volume may allow to be more precise in the effective volume injected (*see* **Note 13**). Of note, the estimated volume of *Drosophila* hemolymph is 100 nL. Thus, injections

of volumes higher than 10 nL is not advised, except for specific procedures such as the injection of latex beads, in which a significant recovery period is allowed before infections.

17. Adding new fresh BHB medium is highly important for the expression of bacterial virulence, especially for the infection of wild-type flies. Without adding fresh BHB medium, wild-type PA14 are not able to kill wild-type flies but are able to kill flies with an impaired cellular immunity [4]. Using only a sucrose solution and a tenfold higher bacterial concentration as well as a 3-h starvation period of males, Mulcahy et al. have observed the *Pel*-dependent formation of biofilm in the crop, a food-storage diverticulum [25]. Of note, the *Pel* mutants we have tested in the assay we describe here did not display an altered virulence as monitored in survival assays [4]. This observation underscores the importance of experimental conditions in these assays.
18. Do NOT use the Nanoject motor to draw hemolymph. Doing so results in collecting tissue fragments and thus alters the measure as bacteria may stick to the tissues. It is not possible to crush the flies and determine the titer, as can be done in the septic injury assay, since flies contain also bacteria in their digestive tract. An alternative is to dissect the gut and the rest of the fly (and plate both separately after homogenization). In this case, the fly must first be surface-sterilized prior to dissection by dipping it for 5 s into 70 % ethanol. When using this procedure, the number of bacterial colonies retrieved is higher than the hemolymph titer by a log.
19. The higher the number of flies that are used, the closer the number of CFU obtained is representative of the number of bacteria inside the flies. Be careful not to collect tissue fragments, which would lead to altered bacterial counts. Of note, it is much easier to collect hemolymph out of females than of males, as they are bigger.
20. For beginners, it is easier to deposit the collected hemolymph into a 10 µL sterile PBS 1× drop placed on a Parafilm strip. This drop can then be transferred to a collecting tube with a micropipette. In this way, the risk of breaking the needle is alleviated.
21. Be careful to dilute the hemolymph preparation enough because the 4 µL drops will not be spread all over the plate and CFUs have to be nonconfluent to be counted.
22. 10 h incubation should be used. If the incubation time is longer, the colonies will grow too much and reach confluence. If the incubation time is shorter, the colonies will be too small to be observed.
23. The quality of the water coming from the Millipore filtration system is pure enough for that step. The water used should not contain RNases.

24. SYBR® Green is a molecule that fluoresces strongly when bound to double-stranded DNA. The Ct number represent the number of cycles needed to reach a fluorescence signal that is higher than the background. The Ct number is inversely proportional to the initial amount of mRNA copies in the sample.
25. This last step allows the measurement of the melting curve of the amplified product. The melting curve gives an indication of the purity of the amplified product.

Acknowledgements

We thank Marie-Céline Lafarge for expert technical help, Pr. Jean-Luc Imler for the sequences of the viral PCR primers and Dr. Sebastian Niehus for critical reading of the manuscript. Work in the group is funded by CNRS, Agence Nationale de la Recherche, a National Institute of Health PO1 AI085581 grant (coordinator: Pr. F. Ausubel), and Fondation pour la Recherche Médicale (Equipe FRM).

References

1. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model. Cell 96:47–56
2. Rahme LG, Ausubel FM, Cao H, Drenkard E, Goumnerov BC, Lau GW, Mahajan-Miklos S, Plotnikova J, Tan MW, Tsongalis J, Walendziewicz CL, Tompkins RG (2000) Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci U S A 97:8815–8821
3. Kurz CL, Chauvet S, Andres E, Aurouze M, Vallet I, Michel GP, Uh M, Celli J, Filloux A, De Bentzmann S, Steinmetz I, Hoffmann JA, Finlay BB, Gorvel JP, Ferrandon D, Ewbank JJ (2003) Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. EMBO J 22:1451–1460
4. Limmer S, Haller S, Drenkard E, Lee J, Yu S, Kocks C, Ausubel FM, Ferrandon D (2011) Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. Proc Natl Acad Sci U S A 108:17378–17383
5. Lemaitre B, Hoffmann J (2007) The Host defense of Drosophila melanogaster. Annu Rev Immunol 25:697–743
6. Ferrandon D, Imler JL, Hetru C, Hoffmann JA (2007) The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol 7:862–874
7. Stuart LM, Ezekowitz RA (2008) Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol 8:131–141
8. Ferrandon D (in press) The complementary facets of epithelial host defenses in the genetic model organism Drosophila melanogaster: from resistance to resilience. Curr Opin Immunol
9. Apidianakis Y, Rahme LG (2009) Drosophila melanogaster as a model host for studying Pseudomonas aeruginosa infection. Nat Protoc 4:1285–1294
10. Limmer S, Quintin J, Hetru C, Ferrandon D (2011) Virulence on the fly: Drosophila melanogaster as a model genetic organism to decipher host-pathogen interactions. Curr Drug Targets 12:978–999
11. Lau GW, Goumnerov BC, Walendziewicz CL, Hewitson J, Xiao W, Mahajan-Miklos S, Tompkins RG, Perkins LA, Rahme LG (2003) The Drosophila melanogaster toll pathway participates in resistance to infection by the gram-negative human pathogen Pseudomonas aeruginosa. Infect Immun 71:4059–4066
12. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, Rahme LG (2005) Profiling early infection responses: Pseudomonas aeruginosa eludes host defenses by suppressing antimicrobial peptide gene expression. Proc Natl Acad Sci U S A 102:2573–2578

13. Fauvarque MO, Bergeret E, Chabert J, Dacheux D, Satre M, Attree I (2002) Role and activation of type III secretion system genes in Pseudomonas aeruginosa-induced Drosophila killing. Microb Pathog 32:287–295
14. Boman HG, Nilsson I, Rasmuson B (1972) Inducible antibacterial defence system in Drosophila. Nature 237:232–235
15. Apidianakis Y, Pitsouli C, Perrimon N, Rahme L (2009) Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. Proc Natl Acad Sci U S A 106: 20883–20888
16. Ashburner M, Golic KG, Hawley RS (2005) Drosophila. A laboratory handbook, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
17. Greenspan R (2004) Fly pushing: the theory and practice of Drosophila genetics, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
18. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415
19. McGuire SE, Roman G, Davis RL (2004) Gene expression systems in Drosophila: a synthesis of time and space. Trends Genet 20: 384–391
20. Elrod-Erickson M, Mishra S, Schneider D (2000) Interactions between the cellular and humoral immune responses in *Drosophila*. Curr Biol 10:781–784
21. Ferrandon D, Jung AC, Criqui MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, Reichhart JM, Hoffmann JA (1998) A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO J 17:1217–1227
22. Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart JM, Lemaitre B, Hoffmann JA, Imler JL (2000) Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity 13: 737–748
23. Reichhart JM, Meister M, Dimarcq JL, Zachary D, Hoffmann D, Ruiz C, Richards G, Hoffmann JA (1992) Insect immunity – developmental and inducible activity of the Drosophila diptericin promoter. EMBO J 11:1469–1477
24. Cuttell L, Vaughan A, Silva E, Escaron CJ, Lavine M, Van Goethem E, Eid JP, Quirin M, Franc NC (2008) Undertaker, a Drosophila Junctophilin, links Draper-mediated phagocytosis and calcium homeostasis. Cell 135: 524–534
25. Mulcahy H, Sibley CD, Surette MG, Lewenza S (2011) Drosophila melanogaster as an animal model for the study of Pseudomonas aeruginosa biofilm infections in vivo. PLoS Pathog 7:e1002299

Annexe 3

Résumé en français de la thèse

Introduction

Au laboratoire, nous nous intéressons aux relations hôte-pathogènes entre *Drosophila melanogaster* et différents microorganismes. La drosophile est un organisme modèle de choix pour l'étude de l'immunité innée grâce aux outils génétiques disponibles pour cet organisme et l'absence d'une immunité adaptative.

Cette immunité innée, suffisante pour se défendre contre les microorganismes, est composée de deux volets : la réponse systémique, relayée par les voies Toll et IMD (Immune Deficiency), et la réponse cellulaire (Lemaitre and Hoffmann, 2007). Les voies Toll et IMD sont des voies de signalisation déclenchant une réponse immunitaire humorale caractérisée par l'expression et la sécrétion dans l'hémolymphe de divers peptides antimicrobiens tels que la drosomycine ou la diptéricine.

Classiquement, la voie Toll est activée en réponse à une infection par certaines bactéries à Gram-positif ou des champignons, alors que la voie IMD est activée en réponse à une infection par des bactéries à Gram-négatif. La réponse cellulaire quant à elle fait intervenir le processus de phagocytose par des cellules spécialisées nommées hémocytes chez la drosophile adulte et qui baignent dans l'hémolymphe. Contrairement aux voies Toll et IMD qui sont activées par un certains types de microorganismes, les hémocytes sont capables de phagocyter une grande diversité de microstructures tels que des bactéries, des levures, des spores et même des particules inertes telle que des billes de latex.

Avant mon arrivée au laboratoire, un modèle d'infection intestinal entre la drosophile et *Pseudomonas aeruginosa* a été développé. Il s'agit d'une bactérie à Gram-négatif, capable d'infecter un large spectre d'hôtes allant des plantes aux mammifères et comprenant également des insectes et le ver *Caenorhabditis elegans*. Chez l'Homme, des infections à *P. aeruginosa* sont principalement rencontrées chez les patients en soins intensifs ou atteints de la mucoviscidose ainsi que chez les grands brulés et individus immunodéprimés. Il est à l'origine de 10 à 15% des infections nosocomiales.

Lors de mon stage de Master 2, en collaboration avec une étudiante en thèse, nous avions commencé à étudier les interactions entre la drosophile et la souche PA14 de *P. aeruginosa*. Dans ce modèle d'infection orale, la bactérie est capable de traverser la barrière intestinale et provoque une infection systémique qui emporte l'hôte. Nos premiers résultats avaient montré que dans ce modèle les deux volets de défense de la drosophile sont activés et requis pour lutter contre PA14. Cependant, la réponse cellulaire n'est pas neutralisée par PA14, même à des stades tardifs de l'infection. Par ailleurs, nous avions également identifié un mutant bactérien, affectant le gène *rhlR*, présentant une forte baisse de la virulence chez des drosophiles sauvages et un regain de virulence chez des drosophiles dont la réponse cellulaire a été bloquée (Figure 11, A & B). Nous en avions conclu que ce gène *rhlR* permet à la bactérie de circonvenir la phagocytose. Ces premiers résultats ont été publiés dans le journal *Proceedings of the National Academy of Sciences* (USA) en octobre 2011 (Limmer et al., 2011), une partie importante de ma première année de thèse ayant été consacrée à réaliser les expériences complémentaires demandées par les experts rapporteurs.

Mon projet de thèse a été de continuer l'étude de ces interactions entre la drosophile et PA14, en particulier de comprendre comment RhlR permet à la bactérie d'éluder la phagocytose d'une part, et en même temps d'identifier de nouveaux facteurs de virulence de la bactérie. Ces résultats sont décrits dans la première partie de mon manuscrit.

Dans une seconde partie, je présente les résultats obtenus en infectant les drosophiles soit de façons isolées soit en groupe de 20 individus, suggérant un rôle de RhlR et du QS dans la synchronisation de la virulence des bactéries *P. aeruginosa*.

D'autre part, dans la continuité de nos premiers résultats, nous avons voulu comprendre comment PA14 active la voie Toll alors qu'il s'agit d'une bactérie à Gram-négatif. Les résultats de l'étude des mutants de la Toll en infection intestinal par PA14 sont décrits dans une troisième partie.

Au cours de ma thèse, nous avons observé que différents stocks de drosophiles possédant a priori le même génotype, présentaient une différence significative dans la survie à une infection intestinale par PA14. Une analyse complète de ces différents stocks de drosophiles a révélé la présence d'un virus entérique dans les stocks les plus sensibles. Les résultats de cette étude sont présentés dans la quatrième partie de ce manuscrit.

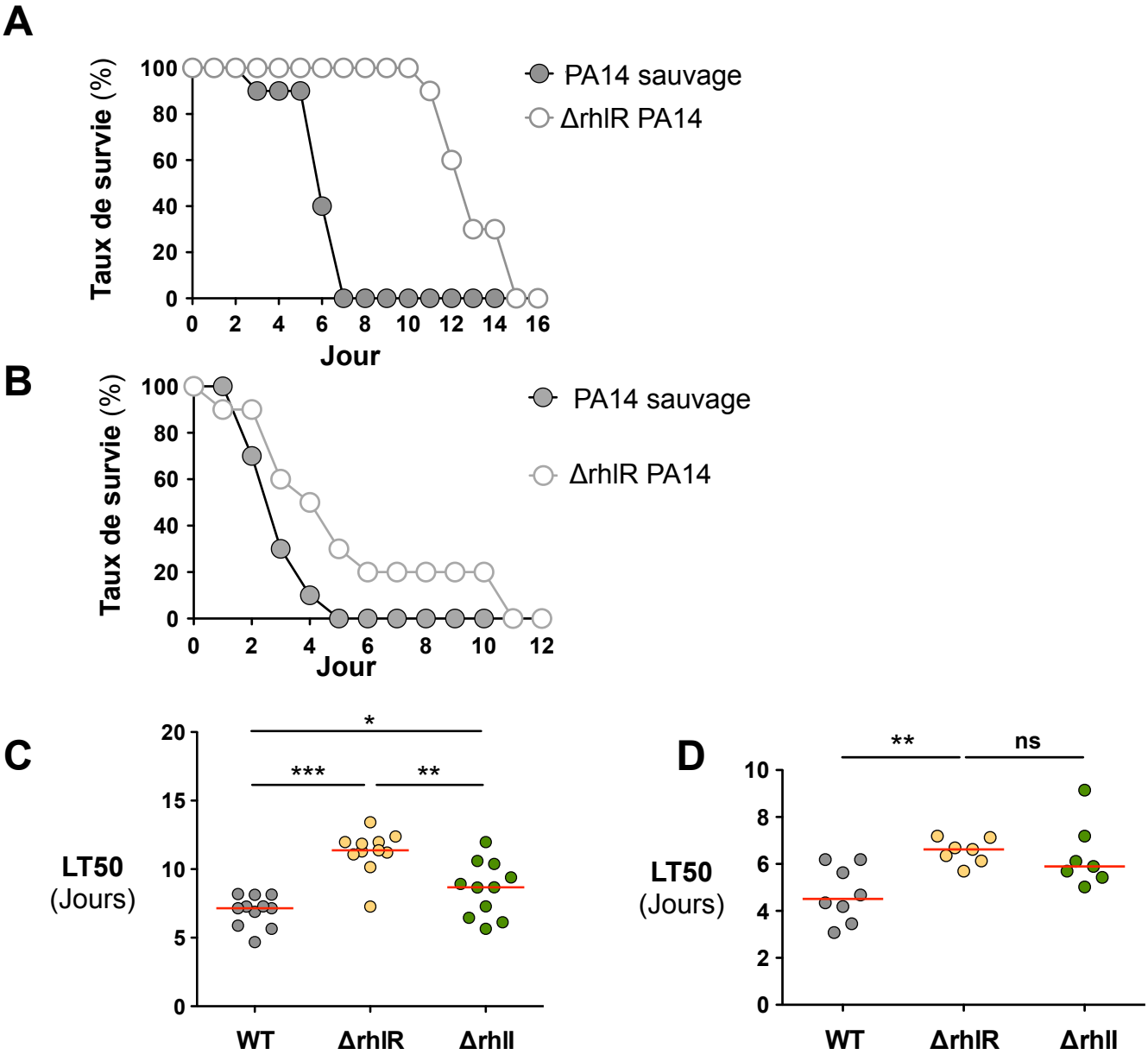


Figure 11. Une composante dépendante et indépendante du quorum sensing dans la fonction de RhlR.

Des drosophiles sauvages ou dont la phagocytose a été bloquée par une injection de billes de latex ont été infectées par voie orale avec des bactéries PA14 sauvages (PA14 sauvage ou WT) ou des mutants pour *rhlR* ou *rhlI*. (A) Des drosophiles sauvages infectées par le mutant rhlR présentent une forte augmentation de leur survie par rapport aux drosophiles infectées par la souche sauvege PA14. (B) Les mutants *rhlR* montrent un fort regain de virulence en infection de drosophiles dont la phagocytose a été bloquée. (C) Les mutants *rhlI* présentent une virulence intermédiaire entre le mutant *rhlR* et PA14 sauvage (WT) en infection de drosophiles sauvages. (D) Les mutants *rhlR* et *rhlI* présentent une virulence similaire et légèrement plus faible que PA14 sauvage (WT) en infection de drosophiles dont la phagocytose a été bloquée.

Chapitre 1 : Une fonction indépendante du *quorum sensing* pour RhlR de *P. aeruginosa* dans l'échappement à la phagocytose par l'intermédiaire de Tep4 dans un modèle d'infection intestinale des drosophiles

Chez *P. aeruginosa*, trois voies permettant la perception du *quorum* (*quorum sensing* en anglais, QS) de la bactérie ont été décrites. De plus, différentes analyses de croissance *in vitro* de *P. aeruginosa* ont montré qu'il existe une hiérarchie entre ces trois voies et que elles se régulent entre elles.

RhlR est le régulateur transcriptionnel d'une des trois voies du QS de la bactérie. Classiquement, RhlR est activé en par la liaison d'une acyl-homosérine lactone, le C4-HSL qui est produit par RhlI. Suite à la liaison du C4-HSL à RhlR, RhlR se dimérise et active alors l'expression de centaines de gènes parmi lesquels se trouvent des facteurs de virulence.

Contrairement à nos attentes, aucun autre mutant affectant les trois voies du QS ne présente ce phénotype de perte de virulence chez les drosophiles sauvages, en particulier lorsque le gène nécessaire pour synthétiser le signal auquel RhlR répond, RhlI, est délété (Figure 11, C). Nous avons aussi pu établir que la fonction de RhlR pour éluder la phagocytose est requise à un stade précoce de l'infection, lorsque très peu de bactéries ont pu passer dans l'hémolymphe et pendant lequel le QS ne peut être activé en raison de la faible densité. **Il s'agit donc de la première démonstration que RhlR a une fonction indépendante de sa fonction dans le QS au cours de l'infection**. Ces résultats soulignent l'importance d'un travail *in vivo* et d'étudier à la fois l'hôte et le pathogène.

Bien que le mutant *rhlI* ne présente pas de phénotype de perte de virulence aussi important que le mutant *rhlR*, ce mutant *rhlI* est tout même moins virulent que la bactérie PA14 sauvage. De plus, nous avons pu remarquer que lorsque la phagocytose était bloquée chez la drosophile, ces deux mutants, *rhlR* et *rhlI* présentent des pertes de virulence similaire (Figure 11, D).

Ces résultats indiquent qu'il y a bien deux composantes impliqué dans la virulence dépendante de RhlR : l'échappement à la phagocytose qui serait une fonction de RhlR indépendante de son rôle dans le QS et une autre fonction qui elle serait dépendante de son rôle dans le QS, la synchronisation de la virulence des bactérie au cours de l'infection.

Par ailleurs, en testant ces même mutants bactériens *rhlR* et *rhlI* ainsi que des mutants du système de *quorum sensing* Las, *lasR* et *lasI*, dans la souche PAO1, j'ai pu observer que dans cette souche PAO1 tous ces mutants présentent une forte perte de virulence en infection intestinale de drosophiles sauvages. Cette forte perte de virulence était similaire à la perte de virulence du mutant *rhlR* d'une souche PA14 en infection intestinale de drosophiles sauvages.

Ces résultats indiquent qu'il existe des différences dans les systèmes de virulence chez *P. aeruginosa* d'une souche à l'autre.

Initialement, le mutant RhlR avait été testé dans notre modèle d'infection car il faisait partie d'un groupe de 300 mutants isolés chez *C. elegans* pour une virulence atténuée lors du crible d'une banque de mutants identifiés recouvrant 80% du génome de PA14 (Feinbaum et al., 2012). Nous avons commencé par tester tous les mutants de cette banque en infection intestinale chez la drosophile. Ce crible nous a permis d'identifier quelques gènes

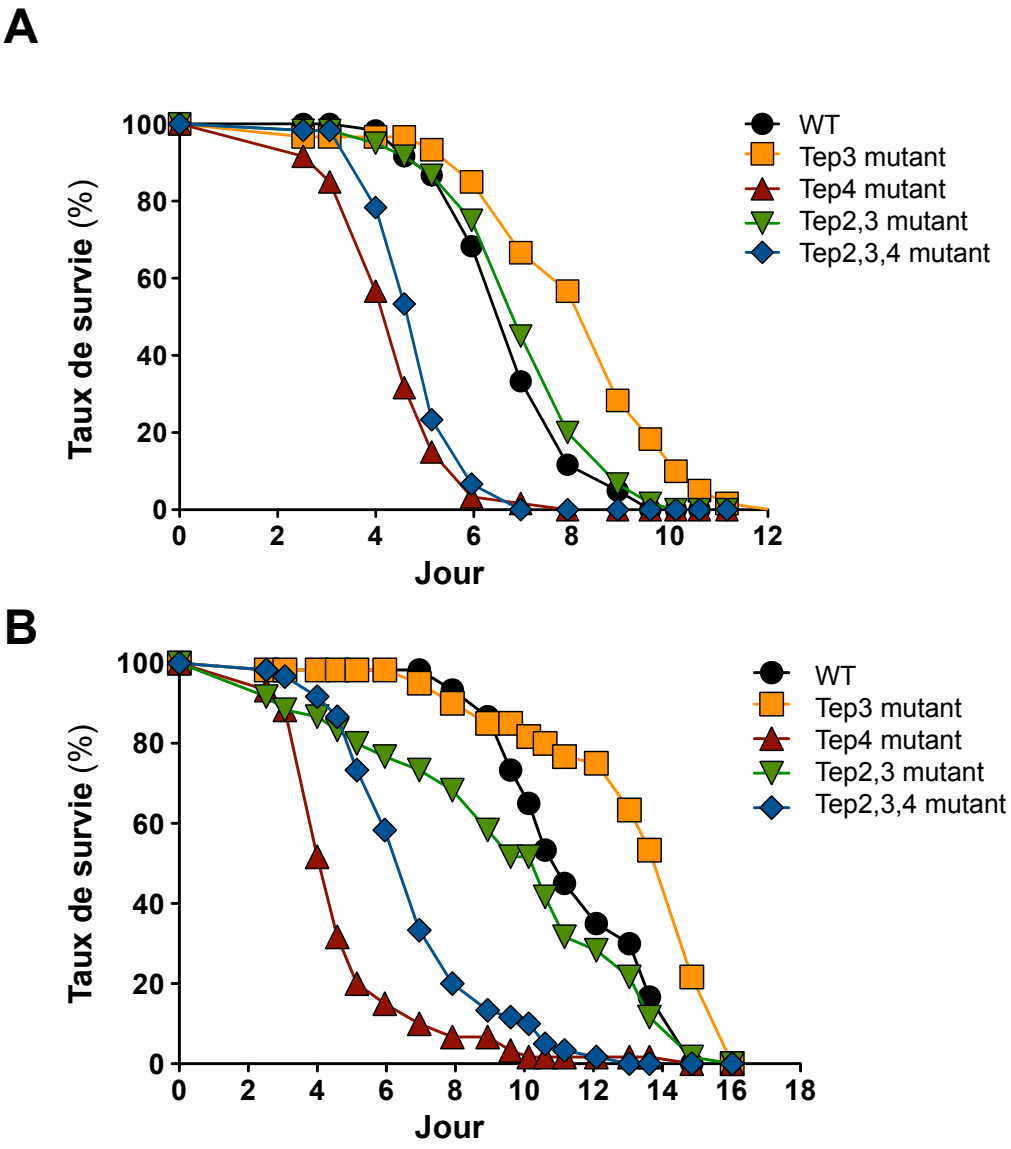


Figure 12. Chez la drosophile, Tep4 est spécifiquement requis dans la défense contre PA14, certainement en jouant un rôle d'intermédiaire essentiel pour la phagocytose.

Des drosophiles sauvages ou mutante pour un ou plusieurs gènes *tep* ont été infectées par voie orale avec des bactéries PA14 sauvages (A) ou des mutants pour *rhlR* (B). (A) Seul les drosophiles mutantes pour *tep4* et le triple mutant *tep2,3,4* présentent une plus forte sensibilité à une infection intestinale par la bactérie PA14 sauvage. (B) Les mutants *rhlR* montrent un fort regain de virulence en infection uniquement en cas d'infection de mutants *tep4* ou du triple mutant *tep2,3,4*.

bactériens qui semblent être important pour la virulence de la bactérie tels que *xcpR, vfR, gidA et sltB1* qui comme *rhlR* regagnent leur virulence lorsque la réponse cellulaire est bloquée.

En collaboration avec l'équipe d'Isabelle Schalk (ESBS), nous avons créé des mutants par délétion propre de ces gènes et ensuite confirmé le phénotype de perte de la virulence lors d'une infection intestinale chez la drosophile par ces mutants. Comme le phénotype des mutants nuls est moins fort que le mutant *rhlR,* il est possible que ces gènes agissent en aval de *rhlR*. De plus, les mutants nuls de *xcpR*, *vfR* et *sltB1* présentent tous un regain de virulence lorsque la réponse cellulaire de la drosophile est bloquée.

J'ai également pu montrer que in vivo l'expression de *vfR* semble être contrôlée par RhlR, étant donné que l'expression de *vfR* est très fortement réduite dans un mutant *rhlR* comparé à un PA14 sauvage.

En parallèle de l'étude de ces mutants bactériens, nous avons également testé des mutants de drosophile pour différents gènes liés à la phagocytose et qui pourraient intervenir dans l'interaction avec RhlR. La technique d'interférence par l'ARN, induite spécifiquement dans les hémocytes, nous a permis de déterminer que PA14 échappe à la phagocytose lors des premières étapes de celle-ci c'est-à-dire au moment de la reconnaissance de la bactérie.

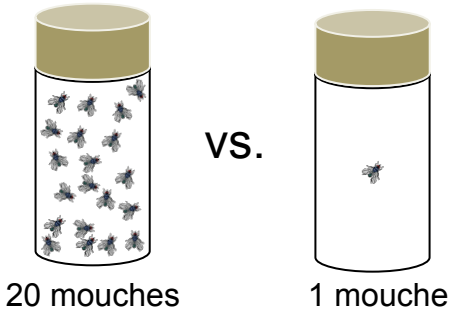
Les protéines Tep (ThioEster-containing Protein) sont structuralement très similaires aux éléments du complément chez les mammifères et pourraient jouer un rôle d'opsonine en cas d'infection par des microorganismes (Bou Aoun et al., 2011). Parmi les quatre gènes *Tep* testés, nous avons démontré que seul *Tep4* semble être impliqué dans le phénotype d'échappement à la phagocytose (Figure 12, A). J'ai également montré que les mutants *rhlR* regagnent leur virulence quand ils infectent des mutants *Tep4*. Ce phénotype est similaire à celui observé lorsque l'on bloque la phagocytose (Figure 12, B).

Notre modèle est que RhlR permet à la bactérie de modifier l'accès au peptidoglycane ce qui lui permettrait d'échapper à la reconnaissance par Tep4 et donc d'éluder la phagocytose.

Par ailleurs, j'ai également testé toute une variété de mutants chez *P. aeruginosa* dans un modèle d'infection intestinale dans l'idée d'identifier de nouveaux facteurs de virulence chez cette bactérie.

J'ai par exemple testé des mutants pour les systèmes de sécrétion de types 2, 3 et 6 chez PA14. Cependant, contrairement à ce qui avait été observé dans un modèle de culture cellulaire, le système de sécrétion de type 3 ne semble pas impliqué dans notre modèle d'infection intestinal des drosophiles. De même pour les systèmes de sécrétion de type 6 (H1-SS et H2-SS). Le système de sécrétion de type 2 quant à lui semble bien impliqué dans la virulence de cette bactérie.

A



B

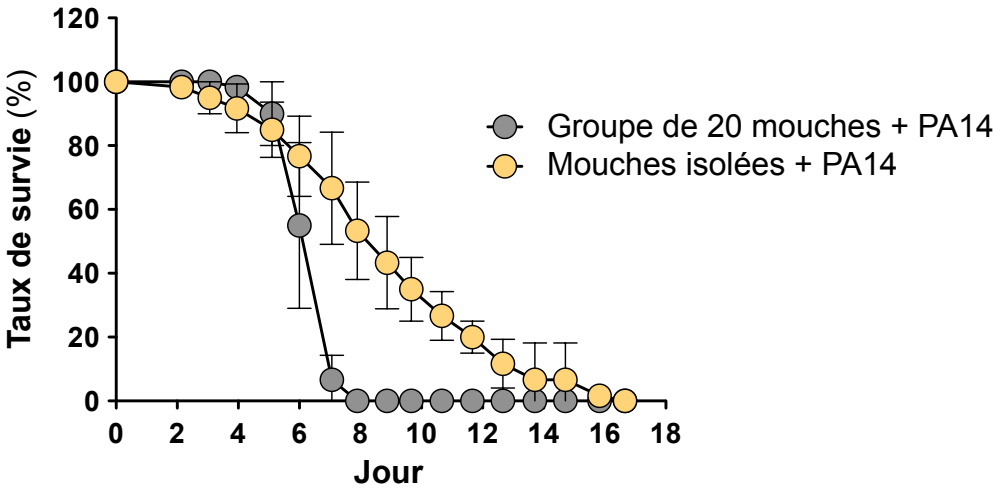


Figure 13. Des drosophiles isolées sont moins sensible à une infection intestinale par PA14 que des drosophiles en groupe de 20 individus.

Des drosophiles sauvages isolées ou en groupe de 20 individus ont été infectées par voie orale avec des bactéries PA14 sauvages. (A) Schéma représentant le système d'infection. (B) Les drosophiles isolées meurent moins vite de l'infection à PA14 que les drosophiles en groupe de 20 individus. De plus, il semble que drosophiles isolées meurent de façon désynchronisé par rapport aux drosophiles en groupe.

Chapitre 2 : Les bactéries *P. aeruginosa* coordonnent leur virulence d'un hôte à l'autre par l'intermédiaire de signaux volatiles

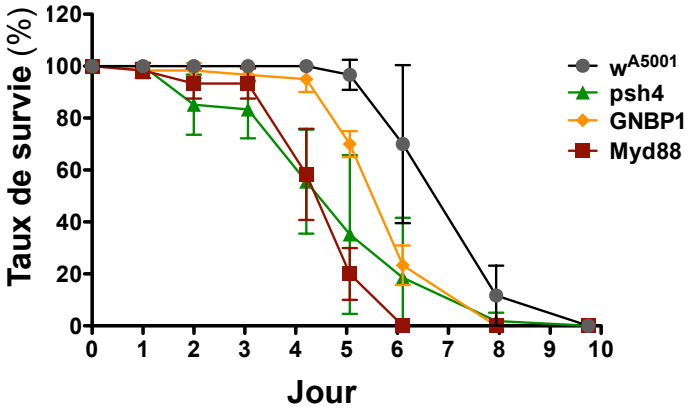
Au cours de ma thèse j'ai également observé que des drosophiles infectées et gardées isolément survivaient mieux à l'infection intestinale par PA14 que les drosophiles infectées et gardées en groupe de vingt (Figure 13, A & B). Nous avons également montré que la quantité de nourriture absorbée par les drosophiles variait au cours de l'infection mais qu'il n'y avait pas de différence d'ingestion entre les drosophiles isolées et les drosophiles en groupe.

J'ai réalisé des expériences dans lesquelles j'ai permis la communication ou non de l'air entre les drosophiles isolées et celles en groupe. Lorsque le passage de l'air est permis, les mouches isolées meurent aussi rapidement que celles en groupe. Ces résultats suggèrent donc qu'un composé volatile interviendrait pour coordonner entre les hôtes soit les défenses immunitaires, soit la virulence du pathogène. L'hôte ou la bactérie devrait ainsi pouvoir percevoir ce composé volatile.

Nous avons testé un mutant de drosophile affectant le système olfactif, lequel ne semble pas impliqué.

Ces résultats suggèrent que dans notre système d'infection, les bactéries peuvent communiquer d'un hôte à l'autre et synchroniser leur virulence.

A



B

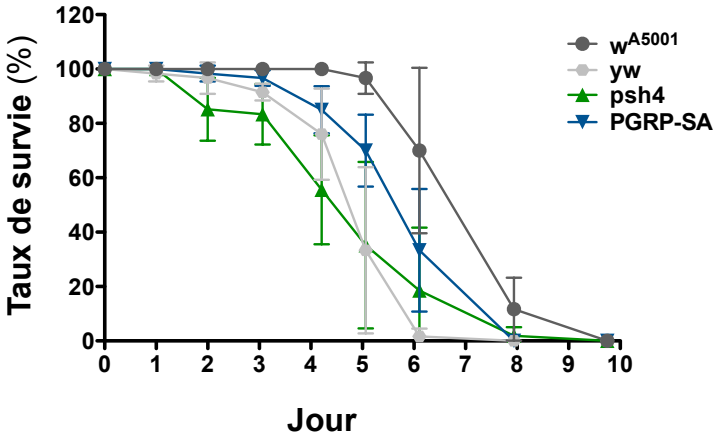


Figure 14. La détection du PGN ainsi que la détection des facteurs de virulence de PA14 sont requis pour la défense des drosophiles contre PA14.

Des drosophiles sauvages (w^{A5001}) ou mutantes pour différents éléments de la voie Toll ont été infectées par voie orale avec des bactéries PA14 sauvages. (A) Les mutants psh4 et GNBP1 sont plus sensible à une infection à PA14 que les mouche sauvage. (B) Les mutants psh4 et PGRP-SA sont plus sensible à une infection à PA14 que les mouches sauvages.

Chapitre 3 : La voie Toll de la drosophile est surement activée à la fois par la détection du PGN et par la détection des facteurs de virulence de *P. aeruginosa*

Nos premiers résultats avaient montré que les deux volets de défense de la drosophile étaient activés et requis dans la défense contre PA14. Classiquement la voie Toll peut être activée par deux voies de signalisation distinctes au niveau extracellulaire, dans l'hémolymphe.

D'une part, la reconnaissance du peptidoglycane (PGN) par des protéines telles que PGRP-SA et GNBP1 active le récepteur Toll. D'autre part, la détection de l'activité enzymatique de facteurs de virulence par Persephone active le récepteur Toll par une autre voie (Gottar et al., 2006).

PA14 possède un peptidoglycane de type diaminopimélique. Ce type de peptidoglycane est classiquement reconnu par le récepteur PGRP-LC activant ainsi de la voie IMD. Il est aussi reconnu pas PGRP-SA, mais moins efficacement. PA14 est connu pour sécréter divers facteurs de virulence qui pourraient être perçus et activer la voie Toll.

Cependant, en infectant par PA14, des drosophiles mutantes pour des éléments de ces deux voies, *Persephone* ou *PGRP-SA* et *GNBP1*, nous n'avons jamais observé d'absence totale d'activation de la voie Toll comme nous avons pu l'observer avec le mutant *MyD88*, un élément central de la voie intracellulaire de signalisation Toll (Figure 14, A & B).

Mes travaux ont montré qu'à la fois le peptidoglycane et les facteurs de virulence de PA14 sont détectés en parallèle et activent la voie Toll.

Chapitre 4 : Le virus entérique Nora affecte l'homéostasie de l'épithélium intestinale et provoque la croissance de la flore intestinale et sensibilise les drosophiles aux infections intestinales

Par ailleurs, il avait été remarqué que des isolats différents de la même souche présentait une susceptibilité différente à différentes infections dont PA14. Une étude plus approfondie a permis de montrer que les mouches plus susceptibles sont infectées par un virus entérique, le virus Nora, qui agit en synergie avec PA14.

En nettoyant les stocks de ce virus, nous perdons l'effet synergique que nous retrouvons après une réinfection avec du virus purifié. En plus de la susceptibilité à PA14, nous observons des dommages de l'intestin entrainant une prolifération compensatoire des cellules souches et une bactérémie fatale se développant plus rapidement en présence du virus (Figure 14, B & C).

De plus, les drosophiles infectées par le virus présentent une forte réduction de leur longévité lorsque les mouches sont gardées à 25°C et cette réduction de longévité et encore augmenté lorsque celles-ci sont gardées à 29°C (Figure 14, A). Nous avons aussi observé une plus grande sensibilité des drosophiles lorsque celles-ci sont nourries avec une solution de sucre uniquement.

Par ailleurs, en étudiant la flore intestinale (ou microbiota) de ces drosophiles j'ai pu constater que les drosophiles infectées par le virus possédaient en moyenne une augmentation de la quantité de bactéries de trois log par rapport aux drosophiles non-infectées par le virus. Cette forte augmentation de la flore intestinale chez les drosophiles infectées par le virus a été observé chez des mouches âgées de environ 26 jours et gardées sur de la nourriture classique

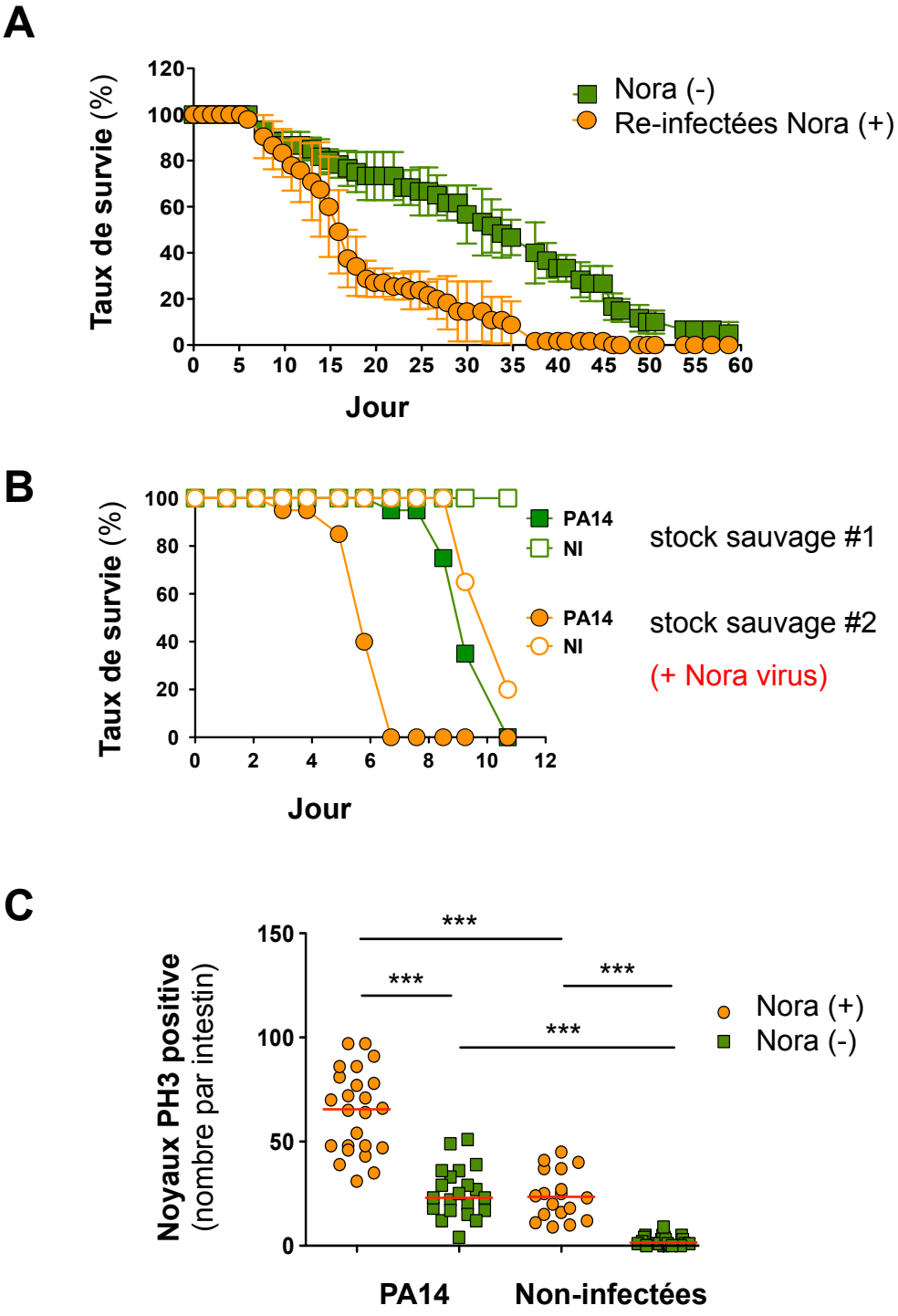


Figure 15. Les drosophiles infectées par le virus entérique Nora présentent divers pathologies.

Des drosophiles sauvages infectées ou non par le virus Nora sont étudiées sous différents aspects, ainsi que en co-infection avec PA14. (A) Les mouches infectées par le virus présente une forte diminution de la longévité part rapport aux mouche non-infectées par le virus. (B) Les mouches infectées par le virus sont plus sensibles à une infection intestinales par PA14. (C) Les mouches infectées par le virus présentent une augmentation du taux de prolifération des cellules souches intestinales et qui est dramatiquement augmenté en cas de co-infection entre le virus et PA14.

de drosophiles et chez des mouches âgées de environs 11 jours et nourri avec une solution de sucre uniquement.

Des analyses par PCR quantitative de l'expression des gènes ont montré une forte induction de l'expression de *diptéricine* chez des drosophiles possédant le virus et cette expression est fortement augmentée en cas de co-infection entre le virus Nora et *P. aeruginosa.* Des résultats similaires ont été observés dans l'étude du gène Socs36e, indiquant une augmentation de l'expression l'activité de la voie JAK/STAT dans les mouches infectées par le virus. Ces résultats sont en accord avec les résultats des counts de noyaux phopho-histone H3, indiquant une augmentation du nombre de p-hostone H3 et donc des divisions cellulaire.

Ce virus a déjà été identifié dans d'autres laboratoires mais son impact sur l'intestin moyen de la drosophile n'a pas encore été étudié (Habayeb et al., 2006). Cependant, un laboratoire a montré que ce virus pouvait bloquer la voie d'interférence par les petits ARN en bloquant la protéine Ago2 (van Mierlo et al., 2012).

Nous avons réalisé des expériences complémentaires en utilisant des mutants Ago2 et en surexprimant des protéines virales suppressives dans les différent types de cellules intestinales de la drosophile. Ces résultats montrent une augmentation de la prolifération intestinale principalement lorsque la voie d'interférence par ARN est bloquée dans les entéroblastes (cellules en voie de différentiation).

Ces résultats démontrent que ce virus à un effet très important sur les infections, voir même sur les carences en acides aminés. Ce virus est très présent dans les stocks de drosophiles dans les laboratoires à travers le monde. Depuis 2006, il y a eu une explosion des

études sur l'homéostasie de l'intestin de la drosophile avec beaucoup de résultats contradictoires. Il serait important de revisiter ces résultats pour déterminer si ce virus entérique n'est pas à l'origine des différences observées dans des laboratoires différents.

Conclusion

Au cours de ma thèse, j'ai pu montrer que chez la souche PA14 de *P. aeruginosa*, le régulateur transcriptionnel RhlR possède à la fois une fonction dépendante de ses interactions avec le *quorum sensing* de la bactérie et notamment RhlI mais en plus, RhlR exerce une fonction indépendante de ce *quorum sensing*.

D'une part, la fonction de RhlR dépendante du *quorum sensing* serait la synchronisation de la virulence de la bactérie, et d'autre part, la fonction de RhlR indépendante du *quorum sensing* serait l'échappement à la réponse cellulaire de la drosophile, qui passerait par un évitement de la détection de la bactérie par Tep4.

J'ai également observé que certains facteurs de virulence de la bactérie identifiés dans d'autres modèles d'infection ne sont pas nécessaires à la virulence de PA14 dans notre modèle d'infection intestinale des drosophiles.

En réalisant différentes expériences, nous avons observé que des drosophiles isolées meurent moins vite de l'infection à PA14 que des drosophiles en groupe. Ces résultats et d'autres suggèrent qu'il existe un composé volatile produit par PA14 et qui permettrait de synchroniser la virulence de la bactérie.

P. aeruginosa est une bactérie à Gram-négatif, cependant d'autres équipes de recherche ainsi que la notre ont montré que une infection des drosophiles par cette bactérie conduit à l'activation de la voie IMD mais aussi de la voie Toll de la drosophile. Nos résultats suggèrent que l'activation de la voie Toll serait due à la fois par la détection du PGN et de facteurs de virulence de cette bactérie.

En fin, nous avons montré que une infection des drosophiles par un virus entérique augmente la sensibilité de ces drosophiles à une infection par PA14.

De plus, les drosophiles infectées par ce virus présentent une dramatique diminution de la longévité et une forte augmentation de la prolifération compensatrice des cellules souches intestinales. Ces drosophiles sont également nettement plus sensible lorsqu'elles sont nourri avec une solution de sucre uniquement.

Mon travail de thèse a permis d'intégrer les interactions entre la drosophile et le pathogène P. aeruginosa en montrant les interactions directes entre facteurs de virulence du pathogène et défense de l'hôte et en montrant l'importance du contexte microbien, en particulier viral.

BIBLIOGRAPHY

Agaisse, H., Petersen, U.M., Boutros, M., Mathey-Prevot, B., and Perrimon, N. (2003). Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. Dev. Cell *5*, 441–450.

Aggarwal, K., Rus, F., Vriesema-Magnuson, C., Erturk-Hasdemir, D., Paquette, N., and Silverman, N. (2008). Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. PLoS Pathog *4*, e1000120.

Ahlund, M.K., Rydén, P., Sjöstedt, A., and Stöven, S. (2010). Directed screen of Francisella novicida virulence determinants using Drosophila melanogaster. Infect. Immun. *78*, 3118–3128.

Akimana, C., Al-Khodor, S., and Abu Kwaik, Y. (2010). Host factors required for modulation of phagosome biogenesis and proliferation of Francisella tularensis within the cytosol. PLoS One *5*, e11025.

Alverdy, J., Holbrook, C., Rocha, F., Seiden, L., Wu, R.L., Musch, M., Chang, E., Ohman, D., and Suh, S. (2000). Gut-derived sepsis occurs when the right pathogen with the right virulence genes meets the right host: evidence for in vivo virulence expression in Pseudomonas aeruginosa. Ann Surg *232*, 480–489.

Avet-Rochex, A., Bergeret, E., Attree, I., Meister, M., and Fauvarque, M.O. (2005). Suppression of Drosophila cellular immunity by directed expression of the ExoS toxin GAP domain of Pseudomonas aeruginosa. Cell Microbiol *7*, 799–810.

Avet-Rochex, A., Perrin, J., Bergeret, E., and Fauvarque, M.-O. (2007). Rac2 is a major actor of Drosophila resistance to Pseudomonas aeruginosa acting in phagocytic cells. Genes to Cells Devoted to Mol. Cell. Mech. *12*, 1193–1204.

Ball, G., Durand, E., Lazdunski, A., and Filloux, A. (2002). A novel type II secretion system in Pseudomonas aeruginosa. Mol. Microbiol. *43*, 475–485.

Basbous, N., Coste, F., Leone, P., Vincentelli, R., Royet, J., Kellenberger, C., and Roussel, A. (2011). The Drosophila peptidoglycan recognition protein PGRP-LF interacts with PGRP-LC to down regulate the IMD pathway. EMBO Rep. *in press*.

Bätz, T., Förster, D., and Luschnig, S. (2014). The transmembrane protein Macroglobulin complement-related is essential for septate junction formation and epithelial barrier function in Drosophila. Development *141*, 899–908.

Baumann, U., Wu, S., Flaherty, K.M., and McKay, D.B. (1993). Three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa: a two-domain protein with a calcium binding parallel beta roll motif. EMBO J. *12*, 3357–3364.

Beebe, K., Lee, W.C., and Micchelli, C.A. (2009). JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the Drosophila intestinal stem cell lineage. Dev Biol.

Belvin, M.P., Jin, Y., and Anderson, K. V (1995). Cactus protein degradation mediates Drosophila dorsal-ventral signaling. Genes Dev *9*, 783–793.

Bertrand, X., Thouverez, M., Patry, C., Balvay, P., and Talon, D. (2001). Pseudomonas aeruginosa: antibiotic susceptibility and genotypic characterization of strains isolated in the intensive care unit. Clin. Microbiol. Infect. *7*, 706–708.

Bidla, G., Hauling, T., Dushay, M.S., and Theopold, U. (2009). Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. J. Innate Immun. *1*, 301–308.

Binggeli, O., Neyen, C., Poidevin, M., and Lemaitre, B. (2014). Prophenoloxidase activation is required for survival to microbial infections in Drosophila. PLoS Pathog. *10*, e1004067.

Bischoff, V., Vignal, C., Boneca, I.G., Michel, T., Hoffmann, J. a, and Royet, J. (2004). Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat. Immunol. *5*, 1175–1180.

Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., Hoffmann, J.A., and Royet, J. (2006). Downregulation of the Drosophila Immune Response by Peptidoglycan-Recognition Proteins SC1 and SC2. PLoS Pathog *2*, e14.

Biteau, B., and Jasper, H. (2011). EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. Development *138*, 1045–1055.

Biteau, B., and Jasper, H. (2014). Slit/Robo Signaling Regulates Cell Fate Decisions in the Intestinal Stem Cell Lineage of Drosophila. Cell Rep.

Biteau, B., Hochmuth, C.E., and Jasper, H. (2008). JNK activity in somatic stem cells causes loss of tissue homeostasis in the aging Drosophila gut. Cell Stem Cell *3*, 442–455.

Blandin, S.A., Marois, E., and Levashina, E.A. (2008). Antimalarial responses in Anopheles gambiae: from a complement-like protein to a complement-like pathway. Cell Host Microbe *3*, 364–374.

Blum, J.E., Fischer, C.N., Miles, J., and Handelsman, J. (2013). Frequent replenishment sustains the beneficial microbiome of Drosophila melanogaster. MBio *4*, e00860–13.

Bosco-Drayon, V., Poidevin, M., Boneca, I.G., Narbonne-Reveau, K., Royet, J., and Charroux, B. (2012). Peptidoglycan sensing by the receptor PGRP-LE in the Drosophila gut induces immune responses to infectious bacteria and tolerance to microbiota. Cell Host Microbe *12*, 153–165.

Bou Aoun, R., Hetru, C., Troxler, L., Doucet, D., Ferrandon, D., and Matt, N. (2011). Analysis of thioester-containing proteins during the innate immune response of Drosophila melanogaster. J Innate Immun *3*, 52–64.

Broderick, N.A., and Lemaitre, B. (2012). Gut-associated microbes of Drosophila melanogaster. Gut Microbes *3*, 307–321.

Broderick, N.A., Buchon, N., and Lemaitre, B. (2014). Microbiota-Induced Changes in Drosophila melanogaster Host Gene Expression and Gut Morphology. *5*, 1–13.

Buchon, N., Poidevin, M., Kwon, H.M., Guillou, A., Sottas, V., Lee, B.L., and Lemaitre, B. (2009a). A single modular serine protease integrates signals from pattern-recognition receptors upstream of the Drosophila Toll pathway. Proc Natl Acad Sci U S A *106*, 12442–12447.

Buchon, N., Broderick, N. a, Poidevin, M., Pradervand, S., and Lemaitre, B. (2009b). Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe *5*, 200–211.

Buchon, N., Broderick, N.A., Kuraishi, T., and Lemaitre, B. (2010). Drosophila EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. BMC Biol *8*, 152.

Buchon, N., Osman, D., David, F.P., Fang, H.Y., Boquete, J.P., Deplancke, B., and Lemaitre, B. (2013). Morphological and molecular characterization of adult midgut compartmentalization in Drosophila. Cell Rep *3*, 1725–1738.

Cadoret, F., Ball, G., Douzi, B., and Voulhoux, R. (2014). Txc, a New Type II Secretion System of Pseudomonas aeruginosa Strain PA7, Is Regulated by the TtsS/TtsR Two-Component System and Directs Specific Secretion of the CbpE Chitin-Binding Protein. J. Bacteriol. *196*, 2376–2386.

Chakrabarti, S., Liehl, P., Buchon, N., and Lemaitre, B. (2012). Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the Drosophila gut. Cell Host Microbe *12*, 60–70.

El Chamy, L., Leclerc, V., Caldelari, I., and Reichhart, J.M. (2008). Sensing of "danger signals" and pathogen-associated molecular patterns defines binary signaling pathways "upstream" of Toll. Nat Immunol *9*, 1165–1170.

Chandler, J.A., Lang, J.M., Bhatnagar, S., Eisen, J.A., and Kopp, A. (2011). Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet. *7*, e1002272.

Chang, C.I., Chelliah, Y., Borek, D., Mengin-Lecreulx, D., and Deisenhofer, J. (2006). Structure of tracheal cytotoxin in complex with a heterodimeric pattern-recognition receptor. Science (80-.). *311*, 1761–1764.

Charroux, B., and Royet, J. (2009). Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. Proc. Natl. Acad. Sci. U. S. A. *106*, 9797–9802.

Cheng, L.W., and Portnoy, D.A. (2003). Drosophila S2 cells: an alternative infection model for Listeria monocytogenes. Cell Microbiol *5*, 875–885.

Choe, K.M., Werner, T., Stoven, S., Hultmark, D., and Anderson, K. V (2002). Requirement for a Peptidoglycan Recognition Protein (PGRP) in Relish Activation and Antibacterial Immune Responses in Drosophila. Science (80-.). *296*, 359–362.

Choe, K.M., Lee, H., and Anderson, K. V (2005). Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. Proc Natl Acad Sci U S A *102*, 1122–1126.

Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C., and Greenberg, E.P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. *98*, 2752 –2757.

Chung, Y.-S.A., and Kocks, C. (2011). Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor Eater. J. Biol. Chem. *286*, 26524–26532.

Claesson, M.J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., Marchesi, J.R., Falush, D., Dinan, T., Fitzgerald, G., et al. (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc. Natl. Acad. Sci. U. S. A. *108 Suppl* , 4586–4591.

Claesson, M.J., Jeffery, I.B., Conde, S., Power, S.E., O'Connor, E.M., Cusack, S., Harris, H.M.B., Coakley, M., Lakshminarayanan, B., O'Sullivan, O., et al. (2012). Gut microbiota composition correlates with diet and health in the elderly. Nature *488*, 178–184.

Coggan, K.A., and Wolfgang, M.C. (2012). Global regulatory pathways and cross-talk control pseudomonas aeruginosa environmental lifestyle and virulence phenotype. Curr Issues Mol Biol *14*, 47–70.

Combe, B.E., Defaye, A., Bozonnet, N., Puthier, D., Royet, J., and Leulier, F. (2014). Drosophila Microbiota Modulates Host Metabolic Gene Expression via IMD/NF-κB Signaling. PLoS One *9*, e94729.

Corby-Harris, V., Pontaroli, A.C., Shimkets, L.J., Bennetzen, J.L., Habel, K.E., and Promislow, D.E.L. (2007). Geographical distribution and diversity of bacteria associated with natural populations of Drosophila melanogaster. Appl. Environ. Microbiol. *73*, 3470–3479.

Costa, A., Jan, E., Sarnow, P., and Schneider, D. (2009). The Imd pathway is involved in antiviral immune responses in Drosophila. PLoS One *4*, e7436.

Croda-Garcia, G., Grosso-Becerra, V., Gonzalez-Valdez, A., Servin-Gonzalez, L., and Soberon-Chavez, G. (2011). Transcriptional regulation of Pseudomonas aeruginosa rhlR: role of the CRP orthologue Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhlR. Microbiology *157*, 2545–2555.

Cronin, S.J.F., Nehme, N.T., Limmer, S., Liegeois, S., Pospisilik, J.A., Schramek, D., Leibbrandt, A., Simoes, R.D.M., Gruber, S., Puc, U., et al. (2009). Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. Science *325*, 340–343.

Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., Galiana-Arnoux, D., Dostert, C., Antoniewski, C., Hoffmann, J.A., and Imler, J.-L. (2008). The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. Nat. Immunol. *9*, 1425–1432.

Dekimpe, V., and Déziel, E. (2009). Revisiting the quorum-sensing hierarchy in Pseudomonas aeruginosa: the transcriptional regulator RhlR regulates LasR-specific factors. Microbiology *155*, 712–723.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151–156.

Diggle, S.P., Winzer, K., Chhabra, S.R., Worrall, K.E., Cámara, M., and Williams, P. (2003). The Pseudomonas aeruginosa quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. Mol. Microbiol. *50*, 29–43.

Dionne, M.S., Ghori, N., and Schneider, D.S. (2003). Drosophila melanogaster is a genetically tractable model host for Mycobacterium marinum. Infect. Immun. *71*, 3540–3550.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A., and Imler, J.L. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nat Immunol *6*, 946–953.

Douzi, B., Filloux, A., and Voulhoux, R. (2012). On the path to uncover the bacterial type II secretion system. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *367*, 1059–1072.

Drier, E.A., Huang, L.H., and Steward, R. (1999). Nuclear import of the Drosophila Rel protein Dorsal is regulated by phosphorylation. Genes Dev *13*, 556–568.

Dunbar, T.L., Yan, Z., Balla, K.M., Smelkinson, M.G., and Troemel, E.R. (2012). C. elegans detects pathogen-induced translational inhibition to activate immune signaling. Cell Host Microbe *11*, 375–386.

Edwards, D.N., Towb, P., and Wasserman, S.A. (1997). An activity-dependent network of interactions links the Rel protein Dorsal with its cytoplasmic regulators. Development *124*, 3855–3864.

Elwell, C., and Engel, J.N. (2005). Drosophila melanogaster S2 cells: a model system to study Chlamydia interaction with host cells. Cell Microbiol *7*, 725–739.

Erturk-Hasdemir, D., Broemer, M., Leulier, F., Lane, W.S., Paquette, N., Hwang, D., Kim, C.H., Stoven, S., Meier, P., and Silverman, N. (2009). Two roles for the Drosophila IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. Proc Natl Acad Sci U S A *106*, 9779–9784.

Fairn, G.D., and Grinstein, S. (2012). How nascent phagosomes mature to become phagolysosomes. Trends Immunol. *33*, 397–405.

Farrow, J.M., Sund, Z.M., Ellison, M.L., Wade, D.S., Coleman, J.P., and Pesci, E.C. (2008). PqsE functions independently of PqsR-Pseudomonas quinolone signal and enhances the rhl quorum-sensing system. J. Bacteriol. *190*, 7043–7051.

Fauvarque, M.-O., Bergeret, E., Chabert, J., Dacheux, D., Satre, M., and Attree, I. (2002). Role and activation of type III secretion system genes in Pseudomonas aeruginosa-induced Drosophila killing. Microb. Pathog. *32*, 287–295.

Feinbaum, R.L., Urbach, J.M., Liberati, N.T., Djonovic, S., Adonizio, A., Carvunis, A.R., and Ausubel, F.M. (2012). Genome-wide identification of Pseudomonas aeruginosa virulence-related genes using a Caenorhabditis elegans infection model. PLoS Pathog *8*, e1002813.

Ferrandon, D., Imler, J.L., Hetru, C., and Hoffmann, J.A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol *7*, 862–874.

Filipe, S.R., Tomasz, A., and Ligoxygakis, P. (2005). Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway. EMBO Rep *6*, 327–333.

Finck-Barbançon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J.P., Fleiszig, S.M., Wu, C., Mende-Mueller, L., and Frank, D.W. (1997). ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. Mol. Microbiol. *25*, 547–557.

Fleiszig, S.M.J., and Evans, D.J. (2002). The pathogenesis of bacterial keratitis: studies with Pseudomonas aeruginosa. Clin. Exp. Optom. *85*, 271–278.

Galiana-Arnoux, D., Deddouche, S., and Imler, J.-L. (2007). [Antiviral immunity in drosophila]. J. Soc. Biol. *201*, 359–365.

Gallagher, L.A., McKnight, S.L., Kuznetsova, M.S., Pesci, E.C., and Manoil, C. (2002). Functions required for extracellular quinolone signaling by Pseudomonas aeruginosa. J. Bacteriol. *184*, 6472–6480.

Gambello, M.J., and Iglewski, B.H. (1991). Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. J. Bacteriol. *173*, 3000–3009.

Ganesan, S., Aggarwal, K., Paquette, N., and Silverman, N. (2010). NF-kappaB/Rel Proteins and the Humoral Immune Responses of Drosophila melanogaster. Curr Top Microbiol Immunol.

Georgel, P., Naitza, S., Kappler, C., Ferrandon, D., Zachary, D., Swimmer, C., Kopczynski, C., Duyk, G., Reichhart, J.-M., and Hoffmann, J.A. (2001). Drosophila Immune Deficiency (IMD) is a Death Domain protein that activates antibacterial defense and can promote apoptosis. Dev. Cell *1*, 503–514.

Gobert, V., Gottar, M., Matskevich, A.A., Rutschmann, S., Royet, J., Belvin, M., Hoffmann, J.A., and Ferrandon, D. (2003). Dual activation of the Drosophila toll pathway by two pattern recognition receptors. Science *302*, 2126–2130.

Goic, B., Vodovar, N., Mondotte, J.A., Monot, C., Frangeul, L., Blanc, H., Gausson, V., Vera-Otarola, J., Cristofari, G., and Saleh, M.-C. (2013). RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat. Immunol. *14*, 396–403.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., and Royet, J. (2002). The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature *416*, 640–644.

Gottar, M., Gobert, V., Matskevich, A.A., Reichhart, J.-M., Wang, C., Butt, T.M., Belvin, M., Hoffmann, J.A., and Ferrandon, D. (2006). Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. Cell *127*, 1425–1437.

Guo, L., Karpac, J., Tran, S.L., and Jasper, H. (2014). PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell *156*, 109–122.

Guzzo, J., Murgier, M., Filloux, A., and Lazdunski, A. (1990). Cloning of the Pseudomonas aeruginosa alkaline protease gene and secretion of the protease into the medium by Escherichia coli. J. Bacteriol. *172*, 942–948.

Ha, E.M., Oh, C.T., Bae, Y.S., and Lee, W.J. (2005). A direct role for dual oxidase in Drosophila gut immunity. Science (80-.). *310*, 847–850.

Ha, E.M., Lee, K.A., Seo, Y.Y., Kim, S.H., Lim, J.H., Oh, B.H., Kim, J., and Lee, W.J. (2009a). Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. Nat Immunol *10*, 949–957.

Ha, E.M., Lee, K.A., Park, S.H., Kim, S.H., Nam, H.J., Lee, H.Y., Kang, D., and Lee, W.J. (2009b). Regulation of DUOX by the Galphaq-phospholipase Cbeta-Ca2+ pathway in Drosophila gut immunity. Dev Cell *16*, 386–397.

Habayeb, M.S., Ekström, J.-O., and Hultmark, D. (2009a). Nora virus persistent infections are not affected by the RNAi machinery. PLoS One *4*, e5731.

Habayeb, M.S., Cantera, R., Casanova, G., Ekström, J.-O., Albright, S., and Hultmark, D. (2009b). The Drosophila Nora virus is an enteric virus, transmitted via feces. J. Invertebr. Pathol. *101*, 29–33.

Hall, S., Bone, C., Oshima, K., Zhang, L., McGraw, M., Lucas, B., Fehon, R.G., and Ward, R.E. (2014). Macroglobulin complement-related encodes a protein required for septate junction organization and paracellular barrier function in Drosophila. Development *141*, 889–898.

Haller, S., Limmer, S., and Ferrandon, D. (2014). Assessing Pseudomonas Virulence with a Nonmammalian Host: Drosophila melanogaster. Methods Mol. Biol. *1149*, 723–740.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2008). Wolbachia and virus protection in insects. Science *322*, 702.

Heimer, S.R., Evans, D.J., Stern, M.E., Barbieri, J.T., Yahr, T., and Fleiszig, S.M.J. (2013). Pseudomonas aeruginosa utilizes the type III secreted toxin ExoS to avoid acidified compartments within epithelial cells. PLoS One *8*, e73111.

Holz, A., Bossinger, B., Strasser, T., Janning, W., and Klapper, R. (2003). The two origins of hemocytes in Drosophila. Development *130*, 4955–4962.

Horn, L., Leips, J., and Starz-Gaiano, M. (2014). Phagocytic ability declines with age in adult Drosophila hemocytes. Aging Cell.

Hu, S., and Yang, X. (2000). dFADD, a novel death domain-containing adapter protein for the Drosophila caspase DREDD. J Biol Chem *275*, 30761–30764.

Imler, J.-L., and Bulet, P. (2005). Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem. Immunol. Allergy *86*, 1–21.

Irazoqui, J.E., Troemel, E.R., Feinbaum, R.L., Luhachack, L.G., Cezairliyan, B.O., and Ausubel, F.M. (2010). Distinct pathogenesis and host responses during infection of C. elegans by P. aeruginosa and S. aureus. PLoS Pathog. *6*, e1000982.

Ismail, N., Olano, J.P., Feng, H.-M., and Walker, D.H. (2002). Current status of immune mechanisms of killing of intracellular microorganisms. FEMS Microbiol. Lett. *207*, 111–120.

Jang, I.H., Chosa, N., Kim, S.H., Nam, H.J., Lemaitre, B., Ochiai, M., Kambris, Z., Brun, S., Hashimoto, C., Ashida, M., et al. (2006). A Spatzle-processing enzyme required for toll signaling activation in Drosophila innate immunity. Dev Cell *10*, 45–55.

Jiang, H., and Edgar, B.A. (2009). EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors. Development *136*, 483–493.

Jiang, F., Waterfield, N.R., Yang, J., Yang, G., and Jin, Q. (2014). A Pseudomonas aeruginosa Type VI Secretion Phospholipase D Effector Targets Both Prokaryotic and Eukaryotic Cells. Cell Host Microbe *15*, 600–610.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. (2009). Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell *137*, 1343–1355.

Jiang, H., Grenley, M.O., Bravo, M.J., Blumhagen, R.Z., and Edgar, B.A. (2010). EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in Drosophila. Cell Stem Cell *8*, 84–95.

Jimenez, P.N., Koch, G., Thompson, J. a, Xavier, K.B., Cool, R.H., and Quax, W.J. (2012). The multiple signaling systems regulating virulence in Pseudomonas aeruginosa. Microbiol. Mol. Biol. Rev. *76*, 46–65.

Juhas, M., Wiehlmann, L., Huber, B., Jordan, D., Lauber, J., Salunkhe, P., Limpert, A.S., von Götz, F., Steinmetz, I., Eberl, L., et al. (2004). Global regulation of quorum sensing and virulence by VqsR in Pseudomonas aeruginosa. Microbiology *150*, 831–841.

Jung, S.-H., Evans, C.J., Uemura, C., and Banerjee, U. (2005). The Drosophila lymph gland as a developmental model of hematopoiesis. Development *132*, 2521–2533.

Kambris, Z., Brun, S., Jang, I.H., Nam, H.J., Romeo, Y., Takahashi, K., Lee, W.J., Ueda, R., and Lemaitre, B. (2006). Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. Curr Biol *16*, 808–813.

Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., Harley, W., Fox, A., Golenbock, D., and Silverman, N. (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity *20*, 637–649.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.H., et al. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol *7*, 715–723.

Karlsson, C., Korayem, A.M., Scherfer, C., Loseva, O., Dushay, M.S., and Theopold, U. (2004). Proteomic analysis of the Drosophila larval hemolymph clot. J. Biol. Chem. *279*, 52033–52041.

Kemp, C., and Imler, J.-L. (2009). Antiviral immunity in drosophila. Curr. Opin. Immunol. *21*, 3–9.

Kemp, C., Mueller, S., Goto, A., Barbier, V., Paro, S., Bonnay, F., Dostert, C., Troxler, L., Hetru, C., Meignin, C., et al. (2013). Broad RNA interference-mediated antiviral immunity and virus-specific inducible responses in Drosophila. J. Immunol. *190*, 650–658.

Kerr, K.G., and Snelling, A.M. (2009). Pseudomonas aeruginosa: a formidable and ever-present adversary. J. Hosp. Infect. *73*, 338–344.

Kesarwani, M., Hazan, R., He, J., Que, Y.A., Apidianakis, Y., Lesic, B., Xiao, G., Dekimpe, V., Milot, S., Deziel, E., et al. (2011). A quorum sensing regulated small volatile molecule reduces acute virulence and promotes chronic infection phenotypes. PLoS Pathog *7*, e1002192.

De Kievit, T., Seed, P.C., Nezezon, J., Passador, L., and Iglewski, B.H. (1999). RsaL, a novel repressor of virulence gene expression in Pseudomonas aeruginosa. J. Bacteriol. *181*, 2175–2184.

Kim, Y.S., Ryu, J.H., Han, S.J., Choi, K.H., Nam, K.B., Jang, I.H., Lemaitre, B., Brey, P.T., and Lee, W.J. (2000). Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signaling for the induction of innate immune genes in Drosophila melanogaster cells. J Biol Chem *275*, 32721–32727.

Kleino, A., Myllymaki, H., Kallio, J., Vanha-aho, L.M., Oksanen, K., Ulvila, J., Hultmark, D., Valanne, S., and Ramet, M. (2008). Pirk is a negative regulator of the Drosophila Imd pathway. J Immunol *180*, 5413–5422.

Kocks, C., Cho, J.H., Nehme, N., Ulvila, J., Pearson, A.M., Meister, M., Strom, C., Conto, S.L., Hetru, C., Stuart, L.M., et al. (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell *123*, 335–346.

Lagueux, M., Perrodou, E., Levashina, E. a, Capovilla, M., and Hoffmann, J. a (2000). Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of Drosophila. Proc. Natl. Acad. Sci. U. S. A. *97*, 11427–11432.

Latifi, A., Foglino, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996). A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. *21*, 1137–1146.

Lau, G.W., Goumnerov, B.C., Walendziewicz, C.L., Hewitson, J., Xiao, W., Mahajan-miklos, S., Tompkins, R.G., Perkins, L.A., and Rahme, L.G. (2003). The Drosophila melanogaster Toll Pathway Participates in Resistance to Infection by the Gram-Negative Human Pathogen Pseudomonas aeruginosa. *71*, 4059–4066.

Ledingham, I.M. (1988). Prevention and management of infections with gram-negative bacteria in intensive care units. Br. J. Clin. Pract. Suppl. *57*, 45.

Lee, K.-Z., and Ferrandon, D. (2011). Negative regulation of immune responses on the fly. EMBO J. *30*, 988–990.

Lee, K.A., Kim, S.H., Kim, E.K., Ha, E.M., You, H., Kim, B., Kim, M.J., Kwon, Y., Ryu, J.H., and Lee, W.J. (2013). Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in Drosophila. Cell *153*, 797–811.

Lemaitre, B., and Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annu Rev Immunol *25*, 697–743.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973–983.

Lemaitre, B., Reichhart, J.M., and Hoffmann, J.A. (1997). Drosophila host defense : differential display of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. USA *94*, 14614–14619.

Leone, P., Bischoff, V., Kellenberger, C., Hetru, C., Royet, J., and Roussel, A. (2008). Crystal structure of Drosophila PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan. Mol. Immunol. *45*, 2521–2530.

Lequette, Y., Lee, J.-H., Ledgham, F., Lazdunski, A., and Greenberg, E.P. (2006). A distinct QscR regulon in the Pseudomonas aeruginosa quorum-sensing circuit. J. Bacteriol. *188*, 3365–3370.

Lesic, B., Starkey, M., He, J., Hazan, R., and Rahme, L.G. (2009). Quorum sensing differentially regulates Pseudomonas aeruginosa type VI secretion locus I and homologous loci II and III, which are required for pathogenesis. Microbiology *155*, 2845–2855.

Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., and Lemaitre, B. (2000). The Drosophila caspase Dredd is required to resist gram-negative bacterial infection. EMBO Rep *1*, 353–358.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.-H., Caroff, M., Lee, W.-J., Mengin-Lecreulx, D., and Lemaitre, B. (2003). The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat. Immunol. *4*, 478–484.

Levashina, E.A., Langley, E., Green, C., Gubb, D., Ashburner, M., Hoffmann, J.A., and Reichhart, J.M. (1999). Constitutive activation of Toll-mediated antifungal defense in serpin- deficient Drosophila. Science (80-.). *285*, 1917–1919.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., and Kafatos, F.C. (2001). Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae. Cell *104*, 709–718.

Lhocine, N., Ribeiro, P.S., Buchon, N., Wepf, A., Wilson, R., Tenev, T., Lemaitre, B., Gstaiger, M., Meier, P., and Leulier, F. (2008). PIMS modulates immune tolerance by negatively regulating Drosophila innate immune signaling. Cell Host Microbe *4*, 147–158.

Li, H., Qi, Y., and Jasper, H. (2013). Dpp signaling determines regional stem cell identity in the regenerating adult Drosophila gastrointestinal tract. Cell Rep. *4*, 10–18.

Li, M., Le Trong, I., Carl, M.A., Larson, E.T., Chou, S., De Leon, J.A., Dove, S.L., Stenkamp, R.E., and Mougous, J.D. (2012). Structural basis for type VI secretion effector recognition by a cognate immunity protein. PLoS Pathog. *8*, e1002613.

Liberati, N.T., Urbach, J.M., Miyata, S., Lee, D.G., Drenkard, E., Wu, G., Villanueva, J., Wei, T., and Ausubel, F.M. (2006). An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. U. S. A. *103*, 2833–2838.

Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog *2*, e56.

Ligoxygakis, P., Pelte, N., Hoffmann, J.A., and Reichhart, J.M. (2002a). Activation of Drosophila Toll during fungal infection by a blood serine protease. Science (80-.). *297*, 114–116.

Ligoxygakis, P., Pelte, N., Ji, C., Leclerc, V., Duvic, B., Belvin, M., Jiang, H., Hoffmann, J.A., and Reichhart, J.M. (2002b). A serpin mutant links Toll activation to melanization in the host defence of Drosophila. Embo J *21*, 6330–6337.

Lim, J.H., Kim, M.S., Kim, H.E., Yano, T., Oshima, Y., Aggarwal, K., Goldman, W.E., Silverman, N., Kurata, S., and Oh, B.H. (2006). Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. J Biol Chem *281*, 8286–8295.

Limmer, S., Haller, S., Drenkard, E., Lee, J., Yu, S., Kocks, C., Ausubel, F.M., and Ferrandon, D. (2011a). Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. Proc. Natl. Acad. Sci. U. S. A. *108*, 17378–17383.

Limmer, S., Quintin, J., Hetru, C., and Ferrandon, D. (2011b). Virulence on the fly: Drosophila melanogaster as a model genetic organism to decipher host-pathogen interactions. Curr. Drug Targets *12*, 978–999.

Lin, G., Xu, N., and Xi, R. (2008). Paracrine Wingless signalling controls self-renewal of Drosophila intestinal stem cells. Nature *455*, 1119–1123.

Lin, G., Xu, N., and Xi, R. (2010). Paracrine unpaired signaling through the JAK/STAT pathway controls self-renewal and lineage differentiation of drosophila intestinal stem cells. J. Mol. Cell Biol. *2*, 37–49.

Lindgren, M., Riazi, R., Lesch, C., Wilhelmsson, C., Theopold, U., and Dushay, M.S. (2008). Fondue and transglutaminase in the Drosophila larval clot. J. Insect Physiol. *54*, 586–592.

Lu, Y., Wu, L.P., and Anderson, K. V (2001). The antibacterial arm of the Drosophila innate immune response requires an IkappaB kinase. Genes Dev *15*, 104–10.

Lutter, E.I., Purighalla, S., Duong, J., and Storey, D.G. (2012). Lethality and cooperation of Pseudomonas aeruginosa quorum-sensing mutants in Drosophila melanogaster infection models. Microbiology *158*, 2125–2132.

Ma, C., and Kanost, M.R. (2000). A beta1,3-glucan recognition protein from an insect, Manduca sexta, agglutinates microorganisms and activates the phenoloxidase cascade. J Biol Chem *275*, 7505–7514.

Magwire, M.M., Fabian, D.K., Schweyen, H., Cao, C., Longdon, B., Bayer, F., and Jiggins, F.M. (2012). Genome-wide association studies reveal a simple genetic basis of resistance to naturally coevolving viruses in Drosophila melanogaster. PLoS Genet. *8*, e1003057.

Maillet, F., Bischoff, V., Vignal, C., Hoffmann, J., and Royet, J. (2008). The Drosophila peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. Cell Host Microbe *3*, 293–303.

Marshall, J.C., Christou, N. V, and Meakins, J.L. (1993). The gastrointestinal tract. The “undrained abscess” of multiple organ failure. Ann. Surg. *218*, 111–119.

Mathur, D., Bost, A., Driver, I., and Ohlstein, B. (2010). A transient niche regulates the specification of Drosophila intestinal stem cells. Science *327*, 210–213.

Matskevich, A.A., Quintin, J., and Ferrandon, D. (2010). The Drosophila PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function. Eur J Immunol *40*, 1244–1254.

Matsumoto, T., Tateda, K., Miyazaki, S., Furuya, N., Ohno, A., Ishii, Y., Hirakata, Y., and Yamaguchi, K. (1997). Adverse effects of tumour necrosis factor in cyclophosphamide-treated mice subjected to gut-derived Pseudomonas aeruginosa sepsis. Cytokine *9*, 763–769.

Matsumoto, T., Tateda, K., Furuya, N., Miyazaki, S., Ohno, A., Ishii, Y., Hirakata, Y., and Yamaguchi, K. (1998). Efficacies of alkaline protease, elastase and exotoxin A toxoid vaccines against gut-derived Pseudomonas aeruginosa sepsis in mice. J. Med. Microbiol. *47*, 303–308.

McEwan, D.L., Kirienko, N. V, and Ausubel, F.M. (2012). Host translational inhibition by Pseudomonas aeruginosa Exotoxin A Triggers an immune response in Caenorhabditis elegans. Cell Host Microbe *11*, 364–374.

McGrath, S., Wade, D.S., and Pesci, E.C. (2004). Dueling quorum sensing systems in Pseudomonas aeruginosa control the production of the Pseudomonas quinolone signal (PQS). FEMS Microbiol. Lett. *230*, 27–34.

Meister, M. (2004). Blood cells of Drosophila: cell lineages and role in host defence. Curr. Opin. Immunol. *16*, 10–15.

Mellroth, P., and Steiner, H. (2006). PGRP-SB1: an N-acetylmuramoyl L-alanine amidase with antibacterial activity. Biochem Biophys Res Commun *350*, 994–999.

Mellroth, P., Karlsson, J., and Steiner, H. (2003). A scavenger function for a Drosophila peptidoglycan recognition protein. J Biol Chem *278*, 7059–7064.

Micchelli, C.A., and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature *439*, 475–479.

Michel, T., Reichhart, J.M., Hoffmann, J.A., and Royet, J. (2001). Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature *414*, 756–759.

Van Mierlo, J.T., Bronkhorst, A.W., Overheul, G.J., Sadanandan, S. a, Ekström, J.-O., Heestermans, M., Hultmark, D., Antoniewski, C., and van Rij, R.P. (2012). Convergent evolution of argonaute-2 slicer antagonism in two distinct insect RNA viruses. PLoS Pathog. *8*, e1002872.

Mikkelsen, H., McMullan, R., and Filloux, A. (2011). The Pseudomonas aeruginosa Reference Strain PA14 Displays Increased Virulence Due to a Mutation in ladS. PLoS One *6*.

Mishima, Y., Quintin, J., Aimanianda, V., Kellenberger, C., Coste, F., Clavaud, C., Hetru, C., Hoffmann, J.A., Latge, J.P., Ferrandon, D., et al. (2009). The N-terminal domain of drosophila gram-negative binding protein 3 (GNBP3) defines a novel family of fungal pattern recognition receptors. J Biol Chem *284*, 28687–28697.

Mohr, K.I., and Tebbe, C.C. (2006). Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. Environ. Microbiol. *8*, 258–272.

Moule, M.G., Monack, D.M., and Schneider, D.S. (2010). Reciprocal analysis of Francisella novicida infections of a Drosophila melanogaster model reveal host-pathogen conflicts mediated by reactive oxygen and imd-regulated innate immune response. PLoS Pathog. *6*, e1001065.

Moyano, A.J., Tobares, R.A., Rizzi, Y.S., Krapp, A.R., Mondotte, J.A., Bocco, J.L., Saleh, M.-C., Carrillo, N., and Smania, A.M. (2014). A long-chain flavodoxin protects Pseudomonas aeruginosa from oxidative stress and host bacterial clearance. PLoS Genet. *10*, e1004163.

Mulcahy, H., Sibley, C.D., Surette, M.G., and Lewenza, S. (2011). Drosophila melanogaster as an Animal Model for the Study of Pseudomonas aeruginosa Biofilm Infections In Vivo. PLoS Pathog *7*, e1002299.

Nayak, A., Berry, B., Tassetto, M., Kunitomi, M., Acevedo, A., Deng, C., Krutchinsky, A., Gross, J., Antoniewski, C., and Andino, R. (2010). Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in Drosophila. Nat. Struct. Mol. Biol. *17*, 547–554.

Nehme, N.T., Liegeois, S., Kele, B., Giammarinaro, P., Pradel, E., Hoffmann, J.A., Ewbank, J.J., and Ferrandon, D. (2007). A Model of Bacterial Intestinal Infections in Drosophila melanogaster. PLoS Pathog *3*, e173.

Nehme, N.T., Quintin, J., Cho, J.H., Lee, J., Lafarge, M.C., Kocks, C., and Ferrandon, D. (2011). Relative roles of the cellular and humoral responses in the Drosophila host defense against three gram-positive bacterial infections. PLoS One *6*, e14743.

Neyen, C., Poidevin, M., Roussel, A., and Lemaitre, B. (2012). Tissue- and ligand-specific sensing of gram-negative infection in drosophila by PGRP-LC isoforms and PGRP-LE. J Immunol *189*, 1886–1897.

Nicolas, E., Reichhart, J.M., Hoffmann, J.A., and Lemaitre, B. (1998). In vivo regulation of the IkB homologue cactus during the immune response of Drosophila. J. Biol. Chem. *273*, 10463–10469.

Ohlstein, B., and Spradling, A. (2006). The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature *439*, 470–474.

Ohlstein, B., and Spradling, A. (2007). Multipotent Drosophila intestinal stem cells specify daughter cell fates by differential notch signaling. Science (80-.). *315*, 988–992.

Okuda, J., Hayashi, N., Okamoto, M., Sawada, S., Minagawa, S., Yano, Y., and Gotoh, N. (2010). Translocation of Pseudomonas aeruginosa from the intestinal tract is mediated by the binding of ExoS to an Na,K-ATPase regulator, FXYD3. Infect. Immun. *78*, 4511–4522.

Ooi, J.Y., Yagi, Y., Hu, X., and Ip, Y.T. (2002). The Drosophila Toll-9 activates a constitutive antimicrobial defense. EMBO Rep *3*, 82–7.

Papaioannou, E., Utari, P.D., and Quax, W.J. (2013). Choosing an appropriate infection model to study quorum sensing inhibition in Pseudomonas infections. Int. J. Mol. Sci. *14*, 19309–19340.

Papanikou, E., Karamanou, S., and Economou, A. (2007). Bacterial protein secretion through the translocase nanomachine. Nat. Rev. Microbiol. *5*, 839–851.

Paquette, N., Broemer, M., Aggarwal, K., Chen, L., Husson, M., Ertürk-Hasdemir, D., Reichhart, J.-M., Meier, P., and Silverman, N. (2010). Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for Drosophila NF-kappaB signaling. Mol. Cell *37*, 172–182.

Paredes, J.C., Welchman, D.P., Poidevin, M., and Lemaitre, B. (2011). Negative regulation by amidase PGRPs shapes the Drosophila antibacterial response and protects the fly from innocuous infection. Immunity *35*, 770–779.

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995). A second N-acylhomoserine lactone signal produced by Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. *92*, 1490–1494.

Peltan, A., Briggs, L., Matthews, G., Sweeney, S.T., and Smith, D.F. (2012). Identification of Drosophila gene products required for phagocytosis of Leishmania donovani. PLoS One *7*, e51831.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H. (1997). Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. J. Bacteriol. *179*, 3127–3132.

Phillips, R.M., Six, D.A., Dennis, E.A., and Ghosh, P. (2003). In vivo phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. J. Biol. Chem. *278*, 41326–41332.

Pili-Floury, S., Leulier, F., Takahashi, K., Saigo, K., Samain, E., Ueda, R., and Lemaitre, B. (2004). In vivo RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in Drosophila adults. J Biol Chem *279*, 12848–12853.

Pukatzki, S., Kessin, R.H., and Mekalanos, J.J. (2002). The human pathogen Pseudomonas aeruginosa utilizes conserved virulence pathways to infect the social amoeba Dictyostelium discoideum. Proc. Natl. Acad. Sci. U. S. A. *99*, 3159–3164.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. Science (80-.). *268*, 1899–1902.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R.A. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. Nature *416*, 644–648.

Regan, J.C., Brandão, A.S., Leitão, A.B., Mantas Dias, A.R., Sucena, E., Jacinto, A., and Zaidman-Rémy, A. (2013). Steroid hormone signaling is essential to regulate innate immune cells and fight bacterial infection in Drosophila. PLoS Pathog. *9*, e1003720.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., and Haas, D. (1997). The global activator GacA of Pseudomonas aeruginosa PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol Microbiol *24*, 309–319.

Russell, A.B., Hood, R.D., Bui, N.K., LeRoux, M., Vollmer, W., and Mougous, J.D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. Nature *475*, 343–347.

Rutschmann, S., Jung, A.C., Hetru, C., Reichhart, J.-M., Hoffmann, J.A., and Ferrandon, D. (2000a). The Rel protein DIF mediates the antifungal, but not the antibacterial, response in Drosophila. Immunity *12*, 569–580.

Rutschmann, S., Jung, A.C., Rui, Z., Silverman, N., Hoffmann, J.A., and Ferrandon, D. (2000b). Role of Drosophila IKKg in a Toll-independent antibacterial immune response. Nat Immunol. *1*, 342–347.

Ryu, J.H., Nam, K.B., Oh, C.T., Nam, H.J., Kim, S.H., Yoon, J.H., Seong, J.K., Yoo, M.A., Jang, I.H., Brey, P.T., et al. (2004). The homeobox gene Caudal regulates constitutive local expression of antimicrobial peptide genes in Drosophila epithelia. Mol Cell Biol *24*, 172–185.

Ryu, J.H., Ha, E.M., Oh, C.T., Seol, J.-H., Brey, P., Jin, I., Lee, D.L., Kim, J., Lee, D., and Lee, W.J. (2006). An essential complementary role of NF-kappaB pathway to microbicidal oxidants in Drosophila gut immunity. Embo J *25*, 3693–3701.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Shin, S.C., Ha, E.M., and Lee, W.J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science (80-.). *319*, 777–782.

Sana, T.G., Hachani, A., Bucior, I., Soscia, C., Garvis, S., Termine, E., Engel, J., Filloux, A., and Bleves, S. (2012). The second type VI secretion system of Pseudomonas aeruginosa strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. J. Biol. Chem. *287*, 27095–27105.

Sargent, F. (2007). The twin-arginine transport system: moving folded proteins across membranes. Biochem. Soc. Trans. *35*, 835–847.

Sato, H., Frank, D.W., Hillard, C.J., Feix, J.B., Pankhaniya, R.R., Moriyama, K., Finck-Barbançon, V., Buchaklian, A., Lei, M., Long, R.M., et al. (2003). The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. EMBO J. *22*, 2959–2969.

Sauri, A., Soprova, Z., Wickström, D., de Gier, J.-W., Van der Schors, R.C., Smit, A.B., Jong, W.S.P., and Luirink, J. (2009). The Bam (Omp85) complex is involved in secretion of the autotransporter haemoglobin protease. Microbiology *155*, 3982–3991.

Scherfer, C., Karlsson, C., Loseva, O., Bidla, G., Goto, A., Havemann, J., Dushay, M.S., and Theopold, U. (2004). Isolation and characterization of hemolymph clotting factors in Drosophila melanogaster by a pullout method. Curr Biol *14*, 625–629.

Schuster, M., and Greenberg, E.P. (2006). A network of networks: quorum-sensing gene regulation in Pseudomonas aeruginosa. Int. J. Med. Microbiol. *296*, 73–81.

Schuster, M., Lostroh, C.P., Ogi, T., and Greenberg, E.P. (2003). Identification, Timing, and Signal Specificity of Pseudomonas aeruginosa Quorum-Controlled Genes: a Transcriptome Analysis. J. Bacteriol. *185*, 2066–2079.

Schuster, M., Sexton, D.J., Diggle, S.P., and Greenberg, E.P. (2013). Acyl-homoserine lactone quorum sensing: from evolution to application. Annu. Rev. Microbiol. *67*, 43–63.

Scopelliti, A., Cordero, J.B., Diao, F., Strathdee, K., White, B.H., Sansom, O.J., and Vidal, M. (2014). Local Control of Intestinal Stem Cell Homeostasis by Enteroendocrine Cells in the Adult Drosophila Midgut. Curr. Biol. *24*, 1199–1211.

Shafikhani, S.H., Morales, C., and Engel, J. (2008). The Pseudomonas aeruginosa type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. Cell. Microbiol. *10*, 994–1007.

Shelly, S., Lukinova, N., Bambina, S., Berman, A., and Cherry, S. (2009). Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity *30*, 588–598.

Shia, A.K.H., Glittenberg, M., Thompson, G., Weber, A.N., Reichhart, J.-M., and Ligoxygakis, P. (2009). Toll-dependent antimicrobial responses in Drosophila larval fat body require Spätzle secreted by haemocytes. J. Cell Sci. *122*, 4505–4515.

Shin, S.C., Kim, S.-H., You, H., Kim, B., Kim, A.C., Lee, K.-A., Yoon, J.-H., Ryu, J.-H., and Lee, W.-J. (2011). Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science *334*, 670–674.

Sibley, C.D., Duan, K., Fischer, C., Parkins, M.D., Storey, D.G., Rabin, H.R., and Surette, M.G. (2008). Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. PLoS Pathog *4*, e1000184.

Siehnel, R., Traxler, B., An, D.D., Parsek, M.R., Schaefer, A.L., and Singh, P.K. (2010). A unique regulator controls the activation threshold of quorum-regulated genes in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. *107*, 7916–7921.

Soldati, T., and Neyrolles, O. (2012). Mycobacteria and the intraphagosomal environment: take it with a pinch of salt(s)! Traffic *13*, 1042–1052.

Stenbak, C.R., Ryu, J.H., Leulier, F., Pili-Floury, S., Parquet, C., Herve, M., Chaput, C., Boneca, I.G., Lee, W.J., Lemaitre, B., et al. (2004). Peptidoglycan molecular requirements allowing detection by the Drosophila immune deficiency pathway. J Immunol *173*, 7339–7348.

Stoltz, D.A., Ozer, E.A., Taft, P.J., Barry, M., Liu, L., Kiss, P.J., Moninger, T.O., Parsek, M.R., and Zabner, J. (2008). Drosophila are protected from Pseudomonas aeruginosa lethality by transgenic expression of paraoxonase-1. *118*, 3123–3131.

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F. (2011). Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab *14*, 403–414.

Stroschein-Stevenson, S.L., Foley, E., O'Farrell, P.H., and Johnson, A.D. (2006). Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS Biol *4*, e4.

Sun, H., Towb, P., Chiem, D.N., Foster, B.A., and Wasserman, S.A. (2004). Regulated assembly of the Toll signaling complex drives Drosophila dorsoventral patterning. Embo J *23*, 100–110.

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., and Kurata, S. (2004). Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. Embo J *23*, 4690–4700.

Tauszig, S., Jouanguy, E., Hoffmann, J.A., and Imler, J.L. (2000). Toll-related receptors and the control of antimicrobial peptide expression in Drosophila. Proc Natl Acad Sci U S A *97*, 10520–5.

Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J.A., and Imler, J.L. (2002). Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections. Nat Immunol. *3*, 91–97.

Teixeira, L., Ferreira, A., and Ashburner, M. (2008). The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol. *6*, e2.

Tepass, U., Fessler, L.I., Aziz, A., and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. Development *120*, 1829–1837.

Theopold, U., Schmidt, O., Soderhall, K., and Dushay, M.S. (2004). Coagulation in arthropods: defence, wound closure and healing. Trends Immunol *25*, 289–294.

Ueda, A., Attila, C., Whiteley, M., and Wood, T.K. (2009). Uracil influences quorum sensing and biofilm formation in Pseudomonas aeruginosa and fluorouracil is an antagonist. Microb. Biotechnol. *2*, 62–74.

Utari, P.D., and Quax, W.J. (2013). Caenorhabditis elegans reveals novel Pseudomonas aeruginosa virulence mechanism. Trends Microbiol. 1–2.

Wade, D.S., Calfee, M.W., Rocha, E.R., Ling, E.A., Engstrom, E., Coleman, J.P., and Pesci, E.C. (2005). Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa. J. Bacteriol. *187*, 4372–4380.

Wang, L., Weber, A.N.R., Atilano, M.L., Filipe, S.R., Gay, N.J., and Ligoxygakis, P. (2006). Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA. EMBO J. *25*, 5005–5014.

Weber, A.N., Tauszig-Delamasure, S., Hoffmann, J.A., Lelievre, E., Gascan, H., Ray, K.P., Morse, M.A., Imler, J.L., and Gay, N.J. (2003). Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. Nat Immunol *4*, 794–800.

Weber, A.N., Moncrieffe, M.C., Gangloff, M., Imler, J.L., and Gay, N.J. (2005). Ligand-receptor and receptor-receptor interactions act in concert to activate signaling in the Drosophila toll pathway. J Biol Chem *280*, 22793–22799.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., and Hultmark, D. (2000). A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster. Proc Natl Acad Sci U S A *97*, 13772–13777.

Whiteley, M., Lee, K.M., and Greenberg, E.P. (1999). Identification of genes controlled by quorum sensing in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. *96*, 13904–13909.

Wille, H. (1961). Septikämie der Honigbiene. Schweiz Bienen-Zeitung *83*, 315–325.

Wong, C.N.A., Ng, P., and Douglas, A.E. (2011a). Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. *13*, 1889–1900.

Wong, Z.S., Hedges, L.M., Brownlie, J.C., and Johnson, K.N. (2011b). Wolbachia-Mediated Antibacterial Protection and Immune Gene Regulation in Drosophila. PLoS One *6*, e25430.

Xiao, G., He, J., and Rahme, L.G. (2006a). Mutation analysis of the Pseudomonas aeruginosa mvfR and pqsABCDE gene promoters demonstrates complex quorum-sensing circuitry. Microbiology *152*, 1679 –1686.

Xiao, G., Déziel, E., He, J., Lépine, F., Lesic, B., Castonguay, M.-H., Milot, S., Tampakaki, A.P., Stachel, S.E., and Rahme, L.G. (2006b). MvfR, a key Pseudomonas aeruginosa pathogenicity LTTR-class regulatory protein, has dual ligands. Mol. Microbiol. *62*, 1689–1699.

Yahr, T.L., Goranson, J., and Frank, D.W. (1996). Exoenzyme S of Pseudomonas aeruginosa is secreted by a type III pathway. Mol. Microbiol. *22*, 991–1003.

Yang, J., and Steward, R. (1997). A multimeric complex and the nuclear targeting of the Drosophila Rel protein Dorsal. Proc Natl Acad Sci U S A *94*, 14524–14529.

Yoshida, H., Ochiai, M., and Ashida, M. (1986). Beta-1,3-glucan receptor and peptidoglycan receptor are present as separate entities within insect prophenoloxidase activating system. Biochem Biophys Res Commun *141*, 1177–1184.

Zaborina, O., Kohler, J.E., Wang, Y., Bethel, C., Shevchenko, O., Wu, L., Turner, J.R., and Alverdy, J.C. (2006). Identification of multi-drug resistant Pseudomonas aeruginosa clinical isolates that are highly disruptive to the intestinal epithelial barrier. Ann. Clin. Microbiol. Antimicrob. *5*, 14.

Zaidman-Rémy, A., Hervé, M., Poidevin, M., Pili-Floury, S., Kim, M.-S., Blanot, D., Oh, B.-H., Ueda, R., Mengin-Lecreulx, D., and Lemaitre, B. (2006). The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity *24*, 463–473.

Zaidman-Rémy, A., Regan, J.C., Brandão, A.S., and Jacinto, A. (2012). The Drosophila larva as a tool to study gut-associated macrophages: PI3K regulates a discrete hemocyte population at the proventriculus. Dev. Comp. Immunol. *36*, 638–647.

Samantha HALLER

Etude des interactions hôte-pathogène entre *Pseudomonas aeruginosa* et *Drosophila melanogaster* dans un modèle d'infection intestinale

Résumé

Au cours de ma thèse je me suis intéressée aux relations hôte-pathogène entre *Drosophila melanogaster* et *Pseudomonas aeruginosa* PA14. RhlR, un facteur de transcription bactérien permet à la bactérie d'échapper à la phagocytose. Mon projet de thèse consistait à identifier comment RhlR exerce cette fonction.

Mes résultats suggèrent que RhlR exercerait également une fonction indépendante du quorum sensing. Un crible de mutants PA14 nous a permis d'isoler trois gènes importants pour la virulence de la bactérie et possiblement reliés à RhlR: *xcpR, vfR* et *sltB1*. L'utilisation de mutants de drosophile *tep4*, m'a permis de montrer que le rôle d'échappement à la phagocytose se ferait au niveau de la détection de la bactérie.

Par ailleurs, mes résultats suggèrent aussi l'intervention d'un composé volatil qui permettrait de synchroniser la virulence de la bactérie. Dans une dernière partie, j'ai étudié les effets d'une co-infection entre un virus entérique et PA14.

Drosophile, *Pseudomonas aeruginosa*, phagocytose, virulence, relations hôte-pathogène

Résumé en anglais

During my PhD, I studied the host-pathogen interactions between *Drosophila melanogaster* and *Pseudomonas aeruginosa* PA14. We previously identified RhlR as a bacterial transcription factor that allows the bacteria to circumvent phagocytosis. My main PhD project was to study and identify how RhlR exerts this function.

My first results suggested that RhlR plays also a role independently its the quorum sensing. A screen of PA14 mutants allowed me to identify three genes involved in PA14 virulence and possibility in RhlR function: *xcpR*, *vfR* and *sltB1*. By using *tep4* fly mutants, I have shown that RhlR's role against phagocytosis is most likely required at the level of PA14 detection.

Beside this, my results indicated that possibly a volatile compound is involved to synchronize PA14 virulence. In the last part, I studied the effects of a co-infection between an enteric virus and PA14.

Drosophila, Pseudomonas aeruginosa, phagocytosis, virulence, host-pathogen interactions