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Study of virulence regulatory mechanisms of *Pseudomonas aeruginosa* in *Drosophila melanogaster*: an investigation into RhlR quorum sensing system

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Abbreviations

Abbreviation	Full name
AHL /Acyl-HSL	Acylated homoserine lactones
AI	Autoinducer
AMP	Antimicrobial peptide
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
C. elegans	*Caenorhabditis elegans*
CF	Cystitis fibrosis
CFU	Colony forming unit
C4-HSL	N-Butanoyl-L-homoserine lactone
DSF	Diffusible signal factors
DAP-type PGN	meso-diaminopymelic acid type Peptidoglycan
Duox	Dual oxidase
DMSO	Dimethyl sulfoxide
EBs	Enteroblasts
ECs	Enterocytes
EEs	Enteroendocrine cells
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
Exo	Exoenzyme
GAL4	Galactose4 gene
GNBP	Gram-negative binding protein
HSL	Homoserine lactone
LPS	Lipopolysaccharides
IMD	Immune deficiency
ISCs	Intestinal stem cells
IQS	2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde
Key	Kenny
Lys-type PGN	Lysine-type peptidoglycan
MP1	Melanization protease1
MyD88	Myeloid differentiation primary-response gene 88
MAMPs	Microbial-associated molecular patterns
NADPH	Nicotinamide adenine dinucleotide phosphate
NIM	Nimrod
Nox	NADPH oxidase
3OC12-HSL	N-(3-oxododecanoyl)-L-homoserine lactone
OMPs	Outer membrane proteins
OMVs	Outer membrane vesicles
P. aeruginosa	*Pseudomonas aeruginosa*
PAMPs	Pathogen-associated molecular patterns
P. entomophilia	*Pseudomonas entomophilia*
Psh	Persephone

PM	Peritrophic matrix
PGRP	Peptidoglycan recognition protein
PO	Phenoloxidase
PPO/proPO	Prophenoloxidase
PQS	3,4-dihydroxy-2-heptylquinoline
PRRs	Pattern recognition receptors
QS	Quorum sensing
ROS	Reactive oxygen species
SAM	S-Adenosyl-Lmethionine
S. aureus	*Staphylococcus aureus*
SPs	Serine proteases
SPE	Spätzle-processing enzyme
S. marcescens	*Serratia marcescens*
T4P/TFP	Type IV pili
TLR	Toll-like receptor
TNFR	Tumor necrosis factor-receptor
UAS	Upstream Activating Sequence
wt	Wild type
Teps	Thioester-containing proteins

General Abstract

Background:

Opportunistic pathogens such as *Pseudomonas aeruginosa* cause infection more frequently and severely in human individuals with an impaired immune system. *P. aeruginosa* infections are not easy to be eradicated due to this bacterium being resistant to multiple antibiotics, and to its capacity for forming biofilm communities, which results in persistent or chronic infection. To develop novel therapies, it is fundamental to understand how *P. aeruginosa* controls its virulence and adapts to the changing environment in the context of hosts. *P. aeruginosa* uses various regulatory mechanisms to regulate its virulence in its host. One well-studied mechanism, quorum sensing (QS), is a cell-to-cell communication process mediated by autoinducers, signaling molecules produced by bacteria, that in turn collectively control a large set of gene expression programs. In this way, the bacteria populations synchronously change their behaviors and modulate the production of virulence factors or biofilm formation. The Rhl quorum sensing system in *P. aeruginosa* uses C4-HSL as the autoinducer, RhlI as the autoinducer synthase, and RhlR as the autoinducer receptor and transcriptional regulator. PqsE protein has been proposed to be involved in the Rhl QS circuit.

Our team has studied host-pathogen interaction in *P. aeruginosa* infection models in *Drosophila* for over one decade. Previously, we have investigated the *P. aeruginosa* pathogenicity in an intestinal infection (oral infection) model of immunocompetent and immunodeficient *Drosophila*. We found that the quorum sensing regulator RhlR of *P. aeruginosa* is necessary for bacterial virulence and enables bacteria to evade the cellular immune response in the intestinal model. Meanwhile, an interesting phenomenon is that a few ingested *P. aeruginosa* bacteria manage to cross the intestinal barrier into the fly hemocoel but kill the flies in more than one week, much slower than the bacteria directly injected into the fly hemocoel, which kills the flies within a couple of days by bacteremia. This change in the pathogenicity of *P. aeruginosa* according to the infection routes led us to further investigate the underlying mechanisms. This will hopefully lead to a better understanding as to how *P. aeruginosa* regulates its virulence in the host, particularly during chronic infections.

Objective:

The investigation presented here aims to characterize bacterial behavior and pathogenicity of the ingested *P. aeruginosa*, especially of the bacteria that have crossed the intestinal barrier. Then, the further goal was to confirm whether RhlR signaling is playing a key role in the virulence regulation in different infection *Drosophila* models and to figure out how the RhlR/RhlI/PqsE signaling circuit works in the context of the *Drosophila* host in the presence or absence of its immune defense.

Chapter abstract:

In the first chapter, we developed and characterized a novel *P. aeruginosa* model in *Drosophila* based on transient intestinal infection, which we called the latent infection model. In this model, flies were challenged with oral infection with bacteria, and later the gut bacteria were eliminated by antibiotic feeding. We found that the infected flies can survive over one month, with the bacteria colonizing the tissue and remaining dormant in the tissue, without eliciting a strong systemic immune response. The cellular immune response and the humoral immune response play ancillary roles in preventing the bacteria from activating their virulence program. Melanization however is the major defense for the establishment of latency.

In the second chapter, we made a comparative analysis among four *P. aeruginosa* infection models in *Drosophila* with different pathogenic characteristics: septic injury infection, continuous oral infection, latent infection, and latent-reactivated infection, in the perspective of fly survival, bacterial load, host immune response. We revealed that there is a virulence-switching program of the tissue-colonizing bacteria in the continuous oral infection and latent-reactivated infection. Furthermore, we analyzed the phenotypes of wild-type *P. aeruginosa* and mutants affecting the Rhl QS circuit in several *Drosophila* models of immunocompetence or immunodeficiency and also *in vitro*. We identified that the Rhl quorum sensing system is necessary for virulence switching and bacterial lifestyle transition, in a way partially dependent on the signaling components, RhlI and PqsE.

Finally, my last chapter was carried out to find out why the mutant bacteria Δ*rhlI* Δ*pqsE* has a higher virulence phenotype than the mutant Δ*rhlR* in the *Drosophila* oral infection models, which might lead to a discovery of an alternative autoinducer or alternative activation mechanism of RhlR regulator independent on the function of RhlI and PqsE. For that, we performed a latent-secondary infection, C4-HSL feeding, and analyzed

biofilm formation in the intestine. Up to now, we failed to identify the existence of the alternative autoinducer but confirm a link that the different phenotypes are associated with the gut environment. Thus, we propose it may be external factors that are responsible for the alternative RhlI-independent activation of the RhlR regulator.

Keywords: quorum sensing; virulence; *P. aeruginosa; Drosophila*; host-pathogen interactions; innate immunity

General Introduction

1. *Drosophila melanogaster*

1.1 Advantages of a powerful model organism

A model organism is non-human species that allows us to study experimentally and understand a variety of biological phenomena in a practical way, with the hope that the theories we derive from it will be universal. Possibly, the identified molecular mechanisms might not be entirely conserved throughout evolution, but the underlying biological principles may apply generally, such as innate immunity. The reality is many model organisms just work within limitations to a specific research area or are eliminated as time passes. There is one standing for over a century in various fields of biology study and persisting in its contribution to our knowledge: *Drosophila melanogaster.*

Drosophila melanogaster (hereafter called *Drosophila* or fly) a species of Fly taxonomically belongs to *Dipterans*, also known as the fruit fly, because of its attraction to overripe fruits. *Drosophila* has been widely used in different research areas, including genetics, developmental biology, life evolution, physiology, neurobiology, immunity, microbial pathogenicity, and so on (>60178 published citations in PubMed), thanks to its remarkable attributes (pointed out in the following), and practically, its powerful genetics.

Up to now, many advantages for genetic manipulation have been accumulated on this small organism and make it an indispensable workhorse in biological research. For example, an important genetic tool called balancer chromosomes are modified chromosomes that have multiple rearrangements and inversions to prevent meiotic recombination from the recovery of engineering sequence. They carry recessive sterile or lethal mutations allowing their maintenance without selection. Besides, the dominant markers in the balancers make the selection easily visible [1]. Then, there are multiple mutation techniques, like transposon mutagenesis, which randomly inactivates and tags genes by insertion of transposable elements, which is also a tool widely used in microbial genetics [2]. A specific and powerful tool is RNA interference via introducing

the expression of transgenically-expressed hairpin homologous RNAs designed to target genes of interest. The tool is often applicated with the GAL4-UAS system and enables binary control of gene expression in a spatiotemporal manner [3]. Furthermore, within a decade, CRISPR-Cas9 as the most rapid technique for engineering specific genes has been widely employed in *Drosophila* [4, 5]. There are vast available resources for *Drosophila* study, for example, comprehensive information on the Flybase website (http://flybase.org), insertion mutants stock covering over 65% of known genes, RNAi lines libraries, CRISPR-Cas9 stock [6], and the ability to silence or overexpress a given gene in a spatially and temporally-controlled manner [3].

Attributes of *Drosophila* as a model organism

- Ease of breeding and low maintenance cost.
- Small size and large offspring (normally, hundreds of eggs per single female)
- Short developmental cycle and relatively long life (live up to 80 days), with distinct developmental stages for study (fig.1).
- Sophisticate genetic tools.
- Nearly 75% of human disease genes have orthologs matched in flies [7]. Flies have functional anatomy roughly analogous to that of mammals, conserved signaling systems employed during development or innate immune system.

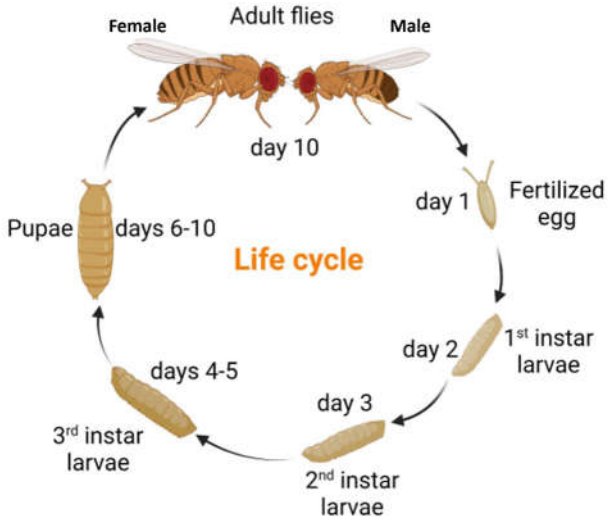

Figure 1. The life cycle of *Drosophila melanogaster*. It takes about ten days for the life cycle of flies at 25 ℃, with embryonic, larval, pupal, and adult stages. (A scheme adapted from an unpublished figure of our colleague Huang,2022.)

1.2 The host defenses of *Drosophila melanogaster*

In the natural environment, the fruit fly has been evolving a sophisticated innate immune system that allows it to fight off infections.

The discovery in the *Drosophila* of the conserved immune function in the Toll pathway and its connection to antimicrobial peptides (AMPs) [8, 9], makes *Drosophila* one of the most favorable models to study the host-pathogen interaction, together with its powerful genetic tool, allowing us to look at mechanisms from both sides that of the pathogens and host fly, in a more relevant manner and practical manner (e.g., large-scale screening).

Fruit flies do not possess an adaptive immune response, but this makes it more favorable for dissecting the hidden functions of innate immune systems and pathogen pathogenicity without compensatory interference from the adaptive immune system. *Drosophila* has multiple innate immune strategies that limit pathogenic infections, including physical barriers (like cuticle, and epithelial barriers), coagulation, melanization, phagocytosis, humoral response (like antimicrobial peptides), as well as local responsese [10].

Here I will introduce the primary immune responses in *Drosophila* that are relevant to this work. An overview of the primary defenses of the fruit flies triggered by a septic injury or intestinal infection with bacteria is shown in Figure 2. Briefly, When the bacteria were inoculated into the fly body cavity by pricking or injection (septic injury), the wound of the cuticle and epithelium triggers a rapid activation of immune defenses, including coagulation and melanization, which relies on enzymes called pro-phenoloxidases (PPOs), produced by crystal cells. This process will restrain some microorganisms around the wound, possibly through the bactericidal effect of PPO-activation byproducts, such as ROS. Subsequently, hemocytes kill the bacteria by phagocytosis, followed by a potent systemic humoral response mediated by immune deficiency (IMD) or Toll pathway, involving the production of AMPs secreted from the fat body. As for local responses in the intestinal tract, barrier epithelia deal with the invasions by producing AMPs, ROS, and acids. After the bacteria have escaped from the gut, the bacteria might be controlled by phagocytosis and AMPs.

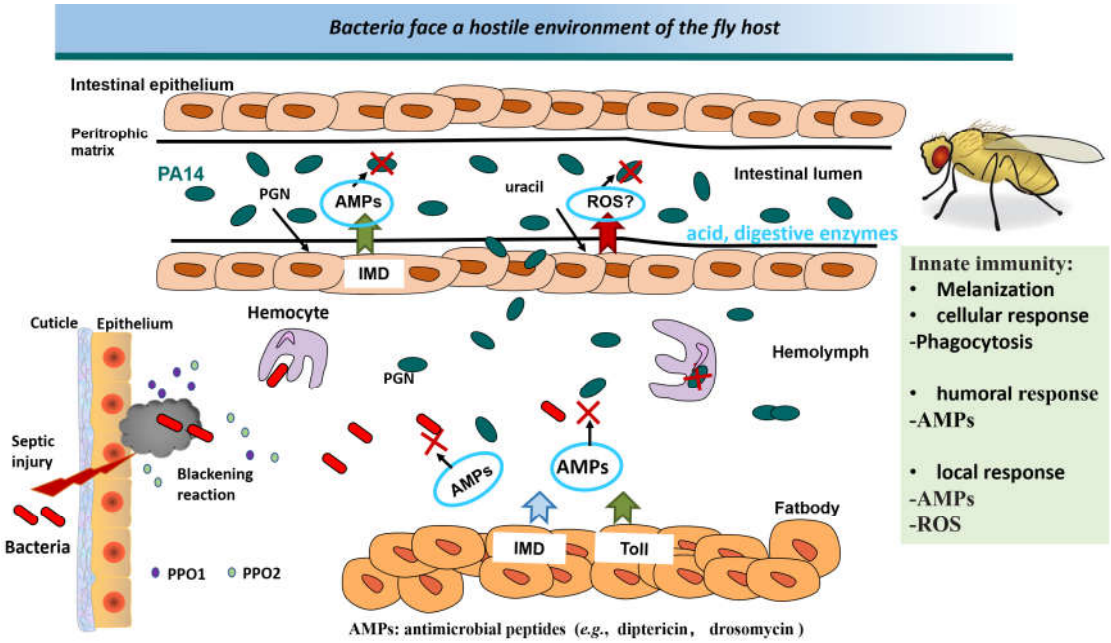

Figure 2. An overview of the primary defenses of the fruit flies triggered by a septic injury or an intestinal infection with bacteria. An infected wound quickly triggers the coagulation, and melanization cascade (PPOs activation) for wound closure and traps the invading microbes. Subsequently, microbes can be killed by the phagocytosis of hemocytes, or antimicrobial peptides (AMPs) secreted from the fat body, which is regulated by IMD or Toll pathway. In the gut, AMPs, acids, and ROS produced from barrier epithelia. After the bacteria cross the gut, the bacteria might be killed by phagocytosis and AMPs.

1.2.1 Coagulation and Melanization

Coagulation

In the laboratory setting, we often inoculate bacteria directly into the fly thoracic cavity via pricking or injection, which bypasses the physical barrier of the chitinous exoskeleton. This hydrophobic, tough cuticle has three layers and induces several responses around the wound site. Firstly, a quick reaction called coagulation (hemolymph clotting) is triggered to limit the dissemination of intruders and promote wound healing. The coagulation compounds have been well-studied in fly larvae. The clot is assembled from initially soluble coagulogens in the hemolymph, including the fat body-derived proteins, such as fondue, hexamerin (larval serum proteins), hexamerin receptor (fat body protein1), lipophorin , as well as the hemocyte-derived

coagulogens, like phenoloxidase (PO), hemolectin (Hml) and tiggrin [11, 12]. The crosslinking of the clot fibers is mediated by a conserved enzyme called transglutaminase (TG), a homolog of clotting factor XIIIa in vertebrates, which catalyzes the cross-linking of its substates in the hemolymph, such as hexamerin, Fondue. Further crosslinking and hardening of the clot matrix depend on a phenoloxidase cascade, which is also required for melanization (reviewed in the next part) [13, 14]. Deficiency of TG in the larvae increases the susceptibility to the natural invasion of entomopathogenic nematodes and nematode's commensal bacteria, as well as the human pathogen *S. aureus* [13]. But it seems that the increased sensitivity is only caused by some microbes. Nevertheless, coagulation contributes to a quick and early defense for *Drosophila* larvae against infection. In adult flies, coagulation appears to happen, but its functional importance in the immune response still needs to be confirmed.

Melanization

Another immediate immune response is the melanization reaction, resulting in a visible blackening clot at the wound site, which is an important immune defense in arthropods. Melanization can be induced by clean injury, septic injury of microorganisms, and natural infection of fungi or parasites [15]. Systemic melanization can also be induced by signaling mediated by the Pattern Recognition Receptor (PRR) after recognition of the bacterial peptidoglycan (PGN) [16]. The blackened injury site contains a clot and the deposition of melanin, contributing to wound closure as well as trapping the invading organisms at the wound site [15]. Besides, quinones, the intermediates of melanin maybe have direct toxicity to microorganisms and parasites: the melanization reaction is likely linked to the production of cytotoxic molecules, like reactive oxygen species (ROS). Thus, it is also possibly contributing to the killing of invading organisms, like fungi, and bacteria. In *Drosophila* larvae, melanization also plays a role in the encapsulation response against parasitic wasp eggs. Of note, no encapsulation occurs in adult flies [17, 18].

The melanin synthesis requires the key enzymes named pro-phenoloxidases (proPOs/PPOs), which lead to melanin polymerization by catalyzing the oxidation of Phenols to the monomer of melanin Quinones. Three PPOs are encoded in the fly genome: *PPO1*, *PPO2*, and *PPO3*. PPO3 is produced by the inducible adhesive cells in larvae called lamellocyte, and it is restricted to the encapsulation response of larvae

against parasitoid wasps. In addition to PPO3, PPO2 also contributes to capsule melanization. Interestingly, a single PPO mutant is not sufficient to abolish melanization no matter in larvae or adult flies. Only when two PPOs out of three are knocked out, can the blackening reaction be fully suppressed. The partially overlapping function of the three PPOs suggests there is a double-security strategy to ensure the function of melanization or it may need at least two trigger -signaling [19-21].

PPO1 and PPO2 are secreted by crystal cells and require proteolytic cleavage for activation in the hemolymph, while PPO3 is made in an active form in lamellocyte [19, 22]. Three serine proteases (SPs) have been identified to activate PPOs: melanization protease1 (MP1), Sp7, and Hayan [23-25]. Early studies have indicated PPOs have a systemic activation, which may be mediated by bacterial compounds. Spontaneous melanization occurs in the mutant flies with a gain of function in Toll receptor or loss of function in the serine protease inhibitor Serpin 27A or the NF-κB inhibitor Cactus [26-28]. Consistently, our group also showed that the Pattern Recognition Receptors (PRRs) responsible for the recognition of Gram-positive bacteria in upstream of the Toll pathway, namely GNBP1 and PGRP-SA, also contribute to the full activation of PPOs [29]. Besides, overexpression of PGRP-LE, the Pattern Recognition Receptor (PRR) for peptidoglycan (PGN) of Gram-negative bacteria, can also activate the PPO cascade in fly larvae [16]. Curiously, when looking at the upstream of PPOs, Hayan and Sp7 do not lead to the same ending, as loss of Hayan can completely abolish the blackening reaction, whereas an Sp7 null mutation causes a mild reduction [21]. It has been demonstrated that the blackening reaction at the wound site is owing to the Hayan and its substrates PPO1 and PPO2, but not Sp7. Upon the systemic infection with a little inoculation of the Gram-positive bacteria *Staphylococcus aureus*, there is an alternate melanization reaction mediated by SP7 and its downstream target PPO1, which is linked to the upstream part of Toll-PRR pathway, contributing to microbe killing [30]. It is in line with the previous studies, that bacterial compounds, per se, can trigger the activation signaling of PPO in systemic infection. Moreover, PPO signaling has cross-regulation with another PPR-dependent signaling, like the Toll-NF-κB pathway, and maybe more than that. A previous study of our group has shown that the GNBP3， a β-glucan receptor for sensing fungi, directly activates PPOs without the function of the Toll receptor [29]. As in the case of the injury site, it has been proposed that apoptosis of crystal cells and plasmatocyte or phosphatidylserine (PS) exposure

may release endogenous signals for activating the PPO cascade [31, 32]. Besides, melanization can be induced by epidermal DNA damage [33].

Melanization is not restricted to the injury surface cuticle or hemolymph, it has been also observed in other organs. For example, tumorous-like stress can cause damage as well as melanization in salivary glands [34]. Melanization can also be induced in the trachea when microorganisms invade the respiratory system [35]. Besides, melanization is activated on the gut surface with the oral infection of *Pseudomonas entomophila* (Pe), an entomopathogenic bacteria, which is capable of making severe destruction of the gut barrier [36]. Stress responses are also associated with melanization reaction since *Drosophila* larvae of p38b & p38a double mutant have visible melanization in the hindgut when it is challenged with *P. aeruginosa* on food [37].

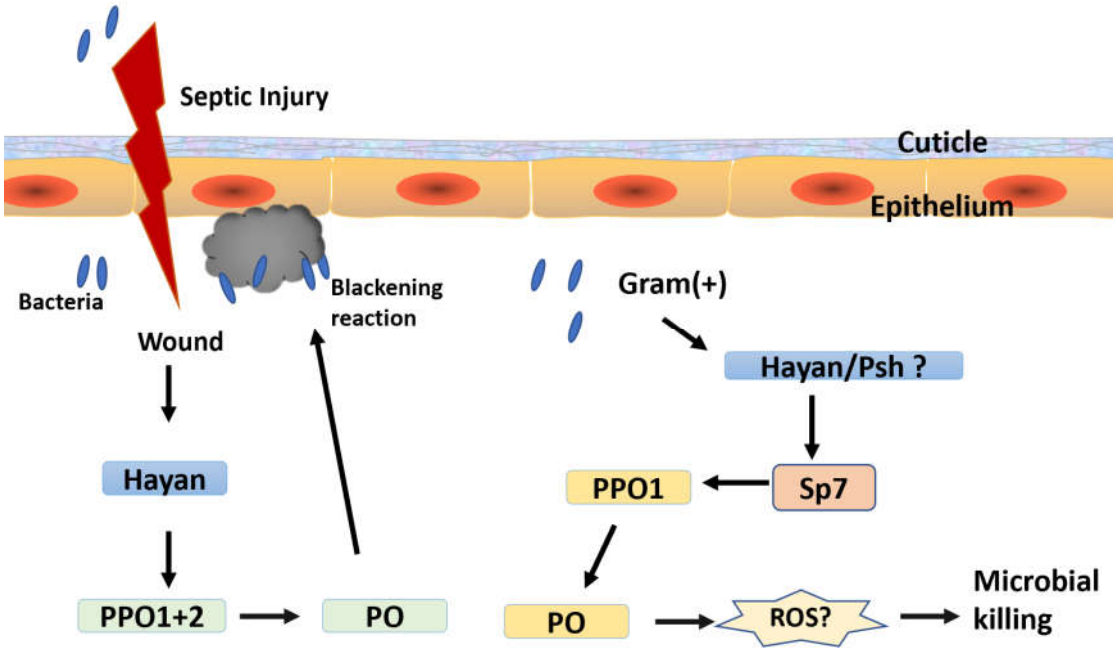

Figure 3. A model of melanization reaction triggered by a septic injury.

A wound of the cuticle and epithelium activates Hayan through an unknown mechanism, leading to the activation of PPO1 and PPO2, which are secreted from crystal cells. The process results in melanin deposition around the wound side, that is, the blackening reaction. Hayan and Psh in the Toll pathway are also proposed to activate Sp7, resulting in microbial killing mediated by PPO1.

1.2.2 The Cellular response: phagocytosis

In *Drosophila* larvae, there are three types of blood cells (hemocytes): plasmatocytes (90% ~95%), crystal cells (5%), and lamellocytes (very few, inducible upon certain infection) [38]. Adult flies possess a majority of plasmatocytes and crystal cells as the remainder. As mentioned above, lamellocytes and "crystal cells" are responsible for encapsulation response and melanization reaction, respectively. It is not clear whether "real crystal cells" are present in adult flies as no crystals are visible but 8% of adult hemocytes express PPOs [39]. Plasmatocytes, also known as macrophages, are the effector cells of the cellular response in adult *Drosophila* through phagocytosis, which is an evolutionarily conserved defense.

Phagocytosis is the engulfment process of self or foreign materials, involving particle bounding, internalization, phagosome formation, and particle degradation, thus leading to the disposal of invading microorganisms or apoptotic cell debris. Particle binding by receptors on the macrophages is the initiation of phagocytosis. The non-self-ligands can be microorganism components, known as microbial-associated molecular patterns (MAMPs), for example, lipopolysaccharides (LPS), peptidoglycan (PGN), and fungal Polymeric β-(1,3)-glucans, whereas the self-ligands commonly are the components from the abnormal cells or apoptotic cells (with exposure of phosphatidylserine) [40, 41]. Some phagocytic receptors in *Drosophila* are conserved and homologous to the mammalian ones, and some seem to only occur in insects. The common receptors of macrophages are summarized as follows:

Scavenger receptors (SRs) were initially characterized as receptors for modified low-density lipoprotein (mLDL) [42], and also serve as Pattern Recognition Receptors (PRRs) in many species with the capability to bind to multiple ligands, like polyanionic ligands. Class C Scavenger receptor I (dSrCI) is the first SRs identified in fruit flies, with four members (dSr-CI-IV). It has been shown under *in vitro* conditions that dSrC is important for the phagocytosis of bacteria but not for yeast [43, 44]. However, there is still no proper confirmation of the phagocytotic function of dSr-CI while using the loss-of-function mutants. It may be complemented by other phagocytic receptors. Besides, there are twelve class B SRs encoded in the fly genome, presenting an expansion, whereas there are only three homologs in humans (CD36) [45]. Most of the class B SRs are found expressed in the *Drosophila* gut with unknown functions [46].

Nimrod is a family of PRRs that utilize the Nimrod (NIM) repeats, also known as EGF-like repeats (EGF, epidermal growth factor), to bind with diverse ligands, contributing to adhesion, interactions, and coagulation [47]. Among the twelve members of Nimrods in *Drosophila*, Eater and Nimrod C1 are best characterized for phagocytosis. Eater is a transmembrane protein that contains 32 extracellular EGF-like repeats and is a marker of macrophage expressed both in adult flies and larvae, as well as in S2 cells. Eater has been considered a bona fide phagocytic receptor of macrophages since Kocks and colleagues identified its function in bacterial phagocytosis [48]. Deficiency of Eater by using RNAi or other mutants impaired the phagocytosis of Gram-positive bacteria, such as *E. faecalis* and *S. aureus*, as well as Gram-negative bacteria, like *E. coli.* Besides, Eater also plays an essential role in eliminating the Gram-negative bacterium *Serratia marcescens*, which crossed the gut barrier during the intestinal infection [48, 49]. However, a recent study reported that an eater null mutant lost the function in phagocytosis of Gram-positive but not Gram-negative bacteria [50, 51]. NimC1 used to be considered to contribute to phagocytosis of the Gram-positive bacterium *S. aureus*. However, a recent report showed that *NimC1* null mutant is not sufficient to abolish bacterial phagocytosis but is essential for the engulfment of latex beads or yeast particles. Double mutants of *eater* and *NimC1* severely affect the phagocytosis of both Gram-negative and Gram-positive bacteria, suggesting a synergistic effect of these two receptors [51].

Opsonins are extracellular molecules that facilitate phagocytosis by binding to the particles on the one hand, and likely to the macrophage phagocytosis receptors on the other hand. In insects, complement proteins are a family of conserved proteins named thioester-containing proteins (TEPs). *Drosophila* Teps consists of six genes called Tep1–Tep6, which share sequence similarities with the complement factor C3 family in mammals. These Teps genes are constitutively expressed in hemocytes, in some barrier epithelia, and are inducible in the fat body [52]. The functions of these Teps are still poorly understood. TEP5 is a non-expressed pseudogene, whereas TEP6 (also named MCR), which lacks a functional thioester binding domain, plays roles in septate junctions (SJs) in the gut epithelial barrier [52, 53]. Tep1-Tep4, presumably act as Opsonins, as they can be secreted out of cells. A study in *Drosophila* S2 cells showed RNAi silencing of TEP2, TEP3, and TEP6, impaired the phagocytosis of *E. coli*, *S. aureus,* and *Candida albicans*, respectively [54]. However, the role of Teps (TEP2,3, 4) in *Drosophila* immunity was not observed in a septic injury infection or natural fungal

infection [52]. A recent report using Teps mutants lacking Tep1-4 supports their roles in facilitating phagocytosis and the activation of the Toll signaling pathway, notably for Gram-positive bacteria [55]. Our colleges Haller, et al also characterized the opsonization function of Tep4 in infection models of *P. aeruginosa* wt or the QS mutants depending on the infection routes [56]

1.2.3 The systemic immune response: from detection to effectors

The *Drosophila* systemic immune response, also known as humoral immune response, is a potent defense against invading microorganisms, with the hallmark of antimicrobial peptides the conserved effectors secreted in the hemolymph, directly acting against bacteria and fungi. AMPs are expressed inducibly and released primarily from the fat body, a functional analog equivalent to the mammalian liver and adipose tissue. The Toll pathway and IMD pathways are two classical nuclear factor κB (NF-κB) pathways that can be activated in systemic infection, responsible for controlling the expression of numerous genes, including AMPs. The intracytoplasmic signaling cascades of the Toll pathway are similar to the signaling cascades downstream of Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) in mammals, whereas the IMD pathway resembles the mammalian tumor necrosis factor-receptor (TNFR) pathway [10, 57].

PRRs. The activation of these two pathways relies on the recognition of microbial-associated molecular patterns (MAMPs) by the Pattern Recognition Receptors (PRRs), or the detection of microbial proteases. Peptidoglycan-recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) are the two main receptor families involved in triggering the systemic response. PGRP-LC is a transmembrane receptor, whereas PGRP-LE, PGRP-SA, PGRP-SD, GNBP1 and GNBP3 are soluble receptors.

Toll pathway. Unlike TLR in mammals, Toll itself is not a PRR but binds with the maturation form of Spätzle cytokine [8, 58]. The Toll pathway can be activated by MAMPs from Gram-positive bacteria, fungi or some microbial proteases. The lysine-type peptidoglycan (Lys-type PGN), a cell wall component of Gram-positive bacteria, is recognized by PGRP-SA, and by GNBP1 [59-61]. GNBP1 cooperates with PGRP-SA for sensing Gram-positive bacteria [40, 61]. The fungal components β-(1,3)-glucans are specifically recognized by GNBP3. The above recognitions activate the

modSP (modular serine protease）, and successively Grass, Persephone /Hayan, and the SPE (Spätzle processing enzyme), which lead to cleavage of the Spätzle cytokine [8, 30]. Spätzle can also be activated by microbial proteases through the Persephone (Psh) pathways [62, 63]. Subsequently, the extracytoplasmic domain of Toll binds to the cleaved cytokine Spätzle, triggering the cascade signaling in the cells, leading to the degradation of the NF-κB inhibitor Cactus [64, 65]. The NF-κB transcription factor DIF (or Dorsal) is released and translocated into the nucleus, ultimately inducing multiple genes expression, for example, the AMP Drosomycin [40, 66].

IMD pathway. Lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria cannot trigger either the Toll or IMD pathway [67]. However, the DAP-type peptidoglycan (PGN) beneath the LPS from Gram-negative bacteria can be recognized by transmembrane PGRP-LC or the intracellular PGRP-LE. The activation signal of PGRP-LC/LE is mediated by the IMD adaptor, a signaling platform for activating the IKK complex or FADD-DREDD complex, leading to the phosphorylation and cleavage of the NF-κB transcriptional factor Relish and genes expression of AMPs, like *Diptericin* [68-70].

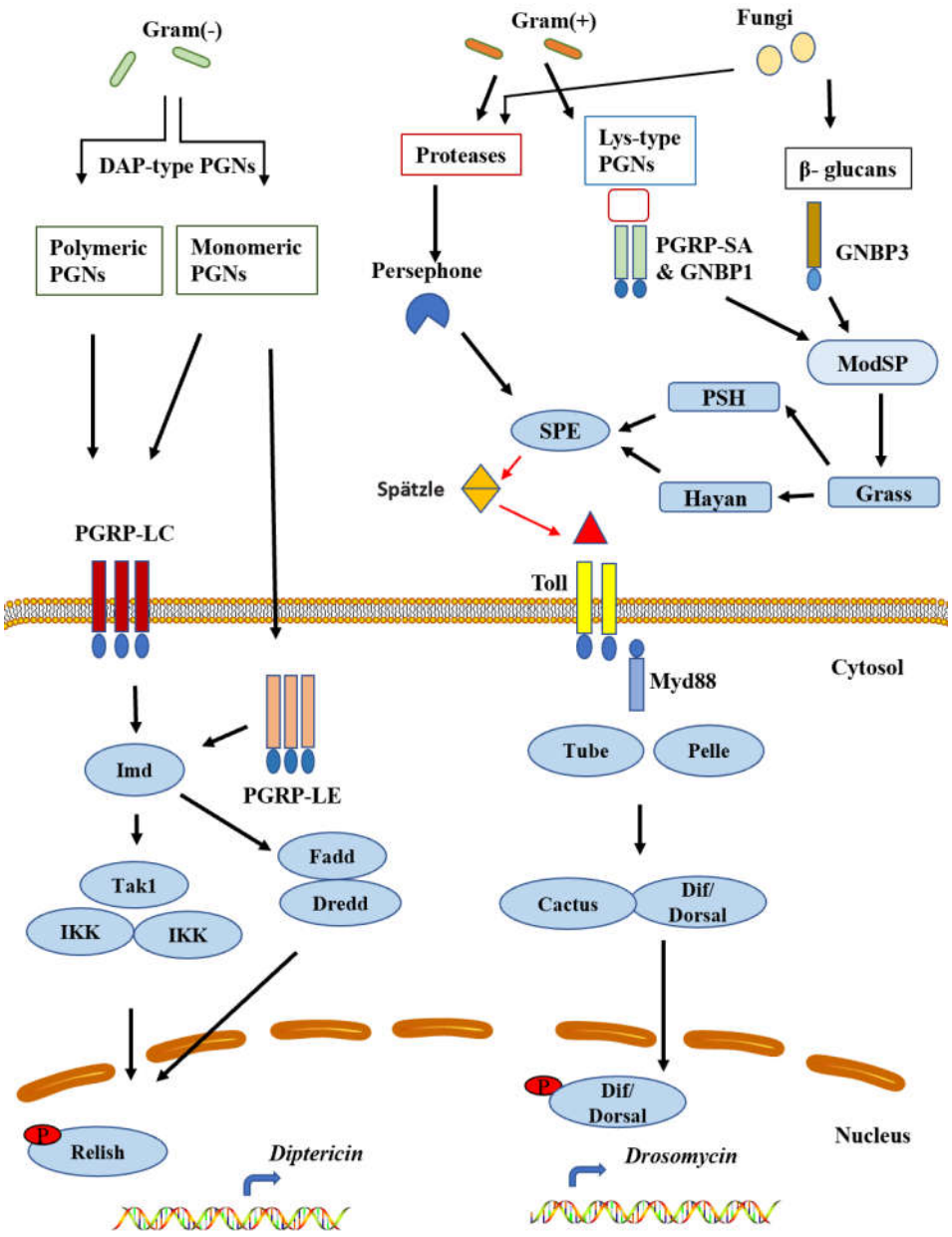

Figure 4. The Toll and IMD signaling pathway in *Drosophila*.

The Toll pathway is activated by components of the microbial cell wall from Gram-positive bacteria and fungi or some microbial proteases, triggering proteolytic cascades of Psh/Hayan-SPE-Spz. Spätzle binds to Toll, leading to the activation of the NF-κB transcription factor DIF (or Dorsal), ultimately inducing multiple genes expression, including the AMP genes. Microbial cell wall component peptidoglycans (PGNs) from Gram (-) bacteria were recognized by the PGRP-LC/LE sensor, triggering the signaling mediated by the IMD adaptor, leading to the activation of NF-κB transcription factor Relish and subsequent expression of AMPs. (The scheme was drawn partially according to a reference [71].

1.2.4 Local immune responses in the intestinal epithelium barrier

Evolutionarily conserved, the local epithelial barrier in *Drosophila* is not a simple physical barrier, which also possesses local immune responses. It was initially revealed by using the transgene fluorescent reporters of AMPs in *Drosophila*, that the AMPs were not only expressed in the fat body but also various epithelial tissues exposed to the environment, including gut, trachea, reproductive tracts, surface barrier [72-74]. Subsequently, intestinal infection models for studying the host-pathogen interaction were developed in *Drosophila* [75, 76]. Of note, before that, the intestinal infection model of *Drosophila* was just used to look at the bacterial side without taking the host defenses into account. Here, I will focus on the digestive tract and its immune defense in *Drosophila*.

Structure and function

The intestine in *Drosophila* is a digestive tube divided into three connecting regions: foregut, midgut, and hindgut. The foregut is formed of the oesophagus, proventriculus, and crop. The crop is a diverticulated structure in the insects that functions mainly as an extensible reservoir for food storage. The proventriculus (also known as cardia), is a bulbiform structure at the anterior midgut consisting of three epithelial layers. The proventriculus cells continuously produce the peritrophic matrix (PM), which distributes posteriorly on the midgut lumen and form a semipermeable membrane consisting of glycoproteins and chitin, similar to the secreted mucous of the digestive tract in vertebrate [77]. The proventriculus is also a major source of gut antimicrobial peptides [73]. Beneath the peritrophic membrane, there are multiple mucin-like proteins produced from the proventriculus, also salivary glands, and Malpighian tubules [78]. The Malpighian tubules are excretory organs branching from the junction between the midgut and hindgut, with a function equivalent to kidneys [79]. Posterior to the proventriculus, the midgut, the main digestive, and absorptive region, is roughly subdivided into the anterior, middle, and posterior parts. The midgut epithelium is composed of four kinds of epithelial cells in adult flies, namely secretory enteroendocrine cells (EEs), absorptive Enterocytes (ECs), enteroblasts (EBs), and intestinal stem cells (ISCs) [80]. ISCs contribute to the constant renewal and rapid regeneration after barrier damage [81-83]. In the middle region (equivalent to the stomach), there are also a group of specific secretory cells called copper cells

responsible for the production of gastric acid [84, 85].

Defense strategies

The foregut and hindgut are protected by an impermeable cuticle, whereas the midgut epithelium is covered by the semipermeable peritrophic matrix (PM) and mucin-like proteins, preventing direct contact of the intestinal epithelium with food particles, ingested toxins, as well as ingested microbes [86, 87]. It has been demonstrated that the reduction of PM exacerbates the susceptibility of flies to oral infection with entomopathogenic *P. entomophila* [88]. Very few bacteria can cross the gut barrier covered with PM and reach into the hemocoel, except some entomopathogenic bacteria, like *S. marcescens*, *P. entomophila, and P. aeruginosa* [75, 76, 89]. *P. entomophila* can release proteases that degrade the PM, thus promoting the damage of pore-forming toxins [87]. Upon intestinal infection of *S. marcescens*, this bacterium can secrete a pore-forming toxin named hemolysin, triggering a fast and conserved response of intestinal epithelia, thinning-purge-recovery of epithelial thickness within several hours. During this process, the apical cytoplasm (including damaged mitochondria) of the thinning enterocytes was extruded away [90]. Interestingly, *P. aeruginosa* was reported to be capable of crossing the gut without causing visible damage to the gut barrier through an unknown mechanism [89]. Besides, the gut epithelium can eliminate the Gram-negative bacteria ECC15 (opportunistic to flies) through epithelial shedding (epithelial renewal), depending on the Imd-NF-κB pathway [91]. In addition, fly digestive enzymes, presumably are capable of attacking some microbes (at least the cell wall components), like lysozymes (acting on PGNs), chitinases, and glucanases [80]. And the strong acid (pH 2–4 in the copper region) has shown a bacteriostatic or bactericidal effect. Flies with increased pH in the copper region are more susceptible to *Pseudomonas* pathogens (including *P. aeruginosa)*, and burden increased microbiota [84, 92]. The crop is also proposed to have roles in microbial control, and detoxification in other insects. Nonetheless, it still needs to be investigated in fruit flies [93].

Furthermore, *Drosophila* has two complementary local defenses against intestinal infection: the production of AMPs and ROS. Ingested Gram-negative bacteria can induce the expression of some AMP genes, such as *Diptericin* and *Attacin*, primarily in the proventriculus. The induction relies on the IMD pathway or JAK-STAT pathways, but not the Toll pathway [94]. Peptidoglycans (PGNs) of Gram-negative bacteria are

recognized by the transmembrane receptor PGRP-LC, or the intracellular receptor PGRP-LE of the epithelial cells [95, 96]. Moreover, PGN fragments can penetrate the gut barrier and trigger the AMPs production of the fat body [96]. Of note, the melanization reaction and Toll pathway are only activated in the cuticular foregut and hindgut [97]. The IMD pathway is under multiple negative feedback regulations, such as PGRPs, that avoid damage caused by an excessive immune response and thereby maintain gut homeostasis [98-100].

Second, Reactive Oxygen Species (ROSs) are also thought to be an effective antimicrobial way to control intestinal infection as well as gut microbiota [101, 102]. ROS can be produced by the NADPH oxidase (Nox) and dual oxidase (Duox), the latter is supposed to be triggered by the recognition of the pathogen-derived uracil [102, 103]. Reactive HOCl produced by the uracil-Duox pathway can promote gut defecation to eliminate food-borne pathogens, via the activation of the HOCl sensor TrpA1 in EECs. Flies lacking TrpA1 have a higher mortality rate resulting from the increased bacterial persistence in the gut lumen [104]. ROS may also cause damage to gut epithelium because of its cytotoxic side effect [80].

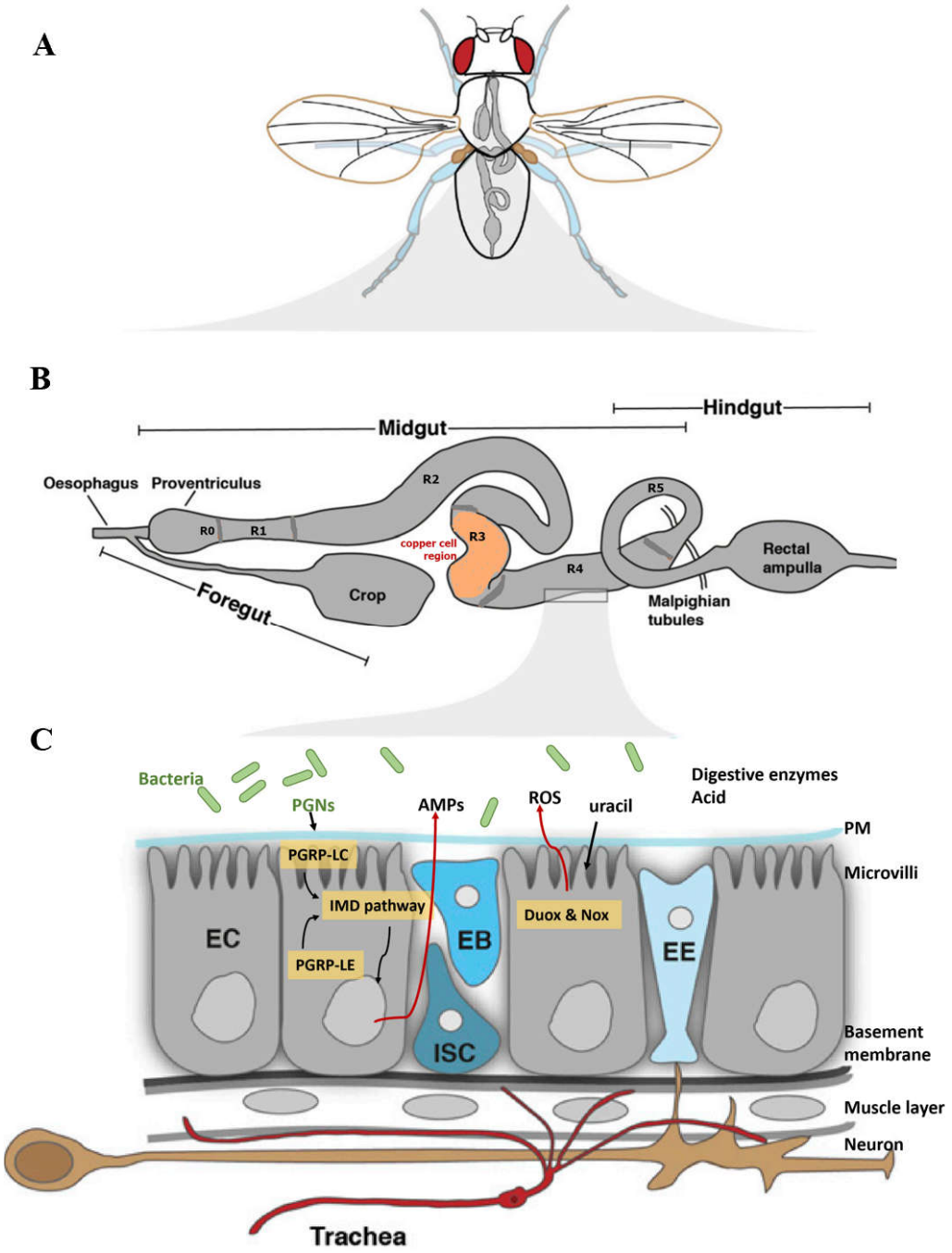

Figure 5. Anatomical structure and local immune response of the *Drosophila* intestinal tract. (A) The intestinal tract is highlighted within the fly body cavity. (B) The anatomical structure of the adult digestive tract consists of the foregut, midgut, and hindgut. The midgut is separated anatomically into six regions (R0 to R5) corresponding to distinct digestive functions. (C) Main cellular composition and local immune response of the midgut. EEs, secretory enteroendocrine cells; ECs, absorptive Enterocytes; EBs, enteroblasts; ISCs, intestinal stem cells; PM, peritrophic membrane. (An adaptation modified from a published figure [80])

2. *Pseudomonas aeruginosa*

Team history: why we chose *Pseudomonas aeruginosa* for studying host-microbe interaction in *Drosophila*?

Almost twenty years ago, we had noticed that as few as five *S. marcescens* bacteria cause a fast killing of its host, the *Drosophila*, in less than 24 hours during septic injury infection performed by injection or pricking. On the contrary, flies can survive for more than ten days when these bacteria are ingested, although some bacteria manage to cross the intestinal barrier in less than two hours, but do not cause bacteremia [75]. Intriguingly, why the virulence of these bacteria is so distinct in these two infection models of the same host? Our team in France investigated thoroughly intestinal infection with *S. marcescens*, primarily from the host perspective [105]. However, it was difficult to look from the side of bacteria because the genetic engineering of *S. marcescens* was tricky, with only a small-scale genetic screen reported this year [106]. A decade ago, we introduced the study of intestinal infections in *Drosophila* by another Gram-negative bacterium, *P. aeruginosa*, which showed similar phenotypes as *S. marcescens* infections in *Drosophila. P. aeruginosa* is one of the most well-known bacteria, under extensive study, with a vast library of knowledge (>77000 articles), advanced genetics as well as many available resources (*e.g.*, an ordered transposon insertion mutant library of PA14 [107]).

2.1 *Pseudomonas aeruginosa*, a ubiquitous and opportunistic pathogen

Pseudomonas aeruginosa (*P. aeruginosa)* is a Gram-negative, monoflagellated bacterium with a rod shape of about 1-5 μm long and 0.5-1.0 μm wide. *P. aeruginosa* had been considered as an obligate aerobe previously. But now it is categorized as a facultative aerobe since it can adapt to oxygen-free conditions by using nitrogen or other alternative electron acceptors [108, 109]. Besides, *P. aeruginosa* possesses remarkable nutritional versatility and metabolic flexibility, which makes it a ubiquitous microorganism [110]. It is detected in a variety of environments such as humans, animals, plants, soil, pools, tap water, community settings, and hospital settings including therapy equipment [111-115]. Given its strong adaptability, it is not surprising that *P. aeruginosa* infects a wide range of hosts, including humans, mice, *Drosophila*, *Caenorhabditis elegans, and* plants [116-118].

Pseudomonas aeruginosa **causes nosocomial opportunistic infections**

P. aeruginosa is one of the leading causes of nosocomial opportunistic infections, on the critical list of antibiotics-resistant bacterium [119, 120]. It is responsible for high mortality in patients with cystic fibrosis (CF), traumas, burns, cancer, chronic obstructive pulmonary disease (COPD), and ventilator-associated pneumonia (VAP), particularly in combination with the immunocompromised situation. The infection most commonly occurs at local sites such as the respiratory tract, urinary tract, skin, and soft tissue, but can also develop into bacteremia [115, 121-127]. On the one hand, *P. aeruginosa* can cause acute infection leading to acute tissue injury, acute respiratory distress syndrome, and sepsis. On the other hand, it can be persistent as chronic colonization in the biofilm niche [125, 128-130]. The most classical example is cystic fibrosis, a genetic disorder of local mucosal immunity, commonly combined with chronic pulmonary infection by *P. aeruginosa.* In the CF airway, inhaled *P. aeruginosa* adapts to the viscid mucus environments and forms biofilms that allow it to evade the immune response and be resistant to antibiotics [121, 131]. Furthermore, the coinfection of *P. aeruginosa* with other microorganisms is a common and challenging phenomenon that intensifies the infection severity and difficulty of treatment. For example, *P. aeruginosa* in the CF airways, present together with *Staphylococcus aureus*

or *Aspergillus fumigatus* results in a worse disease phenotype [132, 133]. Another recent example is hospitalized COVID-19 patients, wherein *P. aeruginosa* as one of the common bacteria causes coinfections and makes poorer outcomes, and higher mortality [134, 135].

Gastrointestinal *Pseudomonas aeruginosa*, an underestimated source of various infections in human

P. aeruginosa is a commensal bacteria in the human gastrointestinal tract [136]. But its intestinal colonization can increase in hospitalized patients with different diseases, including immunosuppression, cancer, and Crohn's disease, as well as patients with surgical injury [137-139]. Intestinal *P. aeruginosa* exhibits enhanced virulence in the vulnerable or stressful host environment of patients [124]. Besides, gastrointestinal *P. aeruginosa* can cause enterocolitis and Shanghai fever (enteric infection and sepsis) in healthy children [140]. Intestinal *P. aeruginosa* has been considered to be an important source of gut-derived sepsis and is associated with higher mortality in intensive care units [141-143]. A considerable number of lung infection of *P. aeruginosa* is caused by the translocated bacteria from the intestine to the lungs [144]. Collectively, intestinal *P. aeruginosa* not only can cause local infection in the digestive tract but also lead to systemic or remote infection by translocating.

Various infections with *Pseudomonas aeruginosa* are a significant concern, especially in immunocompromised patients. However, the therapy of *P. aeruginosa* is a major challenge due to its powerful ability to develop resistance to currently available antibiotics. Moreover, the excessive use of antibiotics promotes the evolution and adaptation of this stubborn bacterium [145, 146]. The discovery and development of alternative novel therapeutic approaches are highly desirable and in increasing demand in the past decade. Anyhow, a deeper understanding of the pathogenicity and regulatory mechanism of *P. aeruginosa* particularly in the host context is the foundation for novel therapeutic strategies.

2.2 *Pseudomonas aeruginosa* isolates

P. aeruginosa strain PAO1 and *P. aeruginosa* strain PA14 are respectively the first and the second clinical isolates that have been analyzed by full genome sequencing [147, 148]. Thus, they are the two most frequently used for the investigation of *Pseudomonas aeruginosa* in labs. Detailed information is shown in Table 1.

Table 1. Comparisons of PAO1 and PA14

Strains	Collection source	Genome Size (Mbp)	Total number of genes	Year of sequencing	Reference
PAO1	Human wound	6.3	5651	2000	[147]
UCBPP-PA14	Human burn wound	6.5	5973	2006	[148]

2.3 Pathogenicity of *Pseudomonas aeruginosa*

2.3.1 Virulence, an outcome of host-microbe interaction

Virulence is a microbial capacity that is formulated to describe the degree of pathogenicity of a microbe. It is not a precise and independent definition but a relative concept because virulence is only a specific outcome resulting from host-microbe interaction [149-151]. Historically, some classic outcomes of the host-microbe interaction were described, namely, commensalism, colonization, latency (colonization/persistence), and disease [149, 152]. The term, latency (latent infection), is often used to describe infections caused by hidden or dormant organisms which cannot be eliminated by the host immune response, leading to an asymptomatic state for a long time. Dormant bacteria have decreased activity and no growth; thus, they are avirulent, and often tolerant to many antibiotics because of their low metabolism and growth arrest. Such a state depends on the delicate balance of the interaction between the host and the microbe. Latency can evolve into a symptomatic chronic infection, where bacteria may have a heterogenous proliferating population and cause continuous damage over time. It also converts to acute infections, where organisms are actively

proliferating and potentially causing symptoms [150, 152, 153].

2.3.2 Virulence factors as effectors /contributors of pathogenicity

Bacterial virulence factors are described as components of a bacterium to cause infection by achieving at least one of the following: colonization including movement and attachment, immunoevasion, immunosuppression, subverting the host defenses, and extracellular or intracellular survival [150]. They usually contribute to a situation of survival battle at the cellular level and cause host damage. It is worth noting that most of the virulence factors contribute to pathogenicity in a combinatorial manner; very few of them can function as all-or-none determinants of pathogenicity [148, 149]. Nevertheless, as far as *P. aeruginosa,* it can be never ignored that the successful of a wide range of infections caused by *P. aeruginosa* is partly owing to its potential expression of various virulence factors.

Flagellum and pili

P. aeruginosa possesses one polar flagellum and pili, which play roles in motility and attachment, and are important for the spread and colonization of bacteria. On the one hand, the flagellum is an important apparatus that enables the bacterium to swim in liquid media and swarm upon semi-solid surfaces [154]. Chemotaxis swimming mediated by flagella is a behavioral response to the attraction of nutrients, such as sucrose, and fatty acids, or evasion from a harmful condition, such as extreme pH [155, 156]. It has been illustrated that chemotactic flagellar motility is necessary for the fitness of acute burn wounds [125]. In contrast, the lack of flagellar motility or loss of flagella of *P. aeruginosa* is considered to be an adaption feature responsible for the resistance to phagocytosis and the persistence of chronic colonization in CF patients [157, 158]. On the other hand, the flagellum can function as an adhesin binding on different surfaces including lipids that mediate the initial attachment to host cells. The flagella rotation provides force promoting the membrane interaction. Meanwhile, the flagellum length serves as an anchor inserted into the cell membrane. Besides, *P. aeruginosa* flagellum not only can bind to the apical surface of respiratory epithelial cells through interaction with glycosphingolipids but can also bind heparan sulfate

proteoglycans on the basolateral surface [159, 160]. Moreover, flagellin has been identified as a powerful immunogen and a ligand of TLR-5 that activate the innate immune response in human, animals, and plants [159, 161, 162]. Another bacterial appendage of *P. aeruginosa* related to motility and attachment is the pili. Cell adhesion is the crucial function of pili contributing to the initial step of colonization. The nanoscale protein fibers, namely, type IV pili (T4P/TFP), can strongly bind to hydrophobic surfaces but weakly to hydrophilic surfaces [163]. Similar to the binding of flagella, pili can bind to glycosphingolipids and N-glycans of epithelial cell surface [160]. When a pilus attaches to a solid surface, it can retract, then pulls, like a grappling hook, making the bacterial cells move forward. This is the twitching motility mediated by type IV pili, driving the phototaxis and chemotaxis as a response to the environment stimuli [164, 165]. Besides, Both type IV pili and flagella of *P. aeruginosa* are required for swarming [166]. Further, along with the attachment and movement on the surface, TFP can be a sensor and a mechanochemical transducer regulating surface-induced gene expression of virulence factors [167]. Type IV pili as well as flagella of *P. aeruginosa* are necessary for microcolonies organization and biofilm formation [165, 168].

LPS

Lipopolysaccharide (LPS), also known as endotoxin, is an important structural structure of the outer membrane in most Gram-negative bacteria, consisting of a hydrophobic domain named lipid A and hydrophobic oligosaccharide. Lipid A is inserted into the outer leaflet of the outer membrane, linked with the core oligosaccharide on the surface, and the outmost oligosaccharide repeats called O antigen, which is specific and highly variable [169]. Lipid A is a major toxic component of LPS, independent of the polysaccharide part, and is heat-stable and responsible for potent innate immune response and pathophysiological effects. Excess LPS in the host has long been recognized as an incentive factor for septic shock [170, 171]. Lipid A of extracellular LPS is recognized by the host Toll-like receptor 4 (TLR4) in a mammal, which subsequently mediates NF-κB activation by the MyD88 pathway to induce the expression of proinflammatory cytokines including interleukin-1beta (IL-1β), and tumor necrosis factor (TNF) [169, 172]. It is a trigger signaling event that primes the immune cells for inflammasome activation at the transcriptional level of the

inflammasome components. The activation of the inflammasome leads to the maturation of caspase-1 into an active form that cleaves pro-IL-1β into mature IL-1β and also cleaves gasdermin-D (GSDMD) into an active endogenous pore-forming toxin. GSDMD activation leads to the formation of pores on the membrane of the immune cell allowing the secretion of IL-1β; if the activation is strong enough, it causes an inflammatory cell death lysis called pyroptosis [173]. As for the intracellular LPS, lipid A as a ligand is directly recognized by caspase-4 and -5 in humans or caspase-11 in mice, finally eliciting GSDMD-mediated pyroptosis of host cells [173]. Despite the above inflammation and self-damage, a series of activated immune responses such as ROS NETs and antibodies, to a large extent, eliminate the bacteria and control the bacterial dissemination [174, 175]. To evade the host immune response and survive for a long time in the chronic infection, *P. aeruginosa* in the respiratory tract of CF patients adapts to the hostile environment by introducing lipid A modification and expressing a low level or loss of O-antigen [176-179].

Interestingly, in the *Drosophila melanogaster* host, LPS from the Gram-negative bacteria is not recognized by the Toll (the homolog of TLR) and does not activate the IMD pathway. The PGN beneath the LPS or the released PGN fragments are recognized by some PGRP receptors, and these, in turn, activate the IMD pathway and the production of antimicrobial peptides [67, 180, 181]. Interestingly, *Drosophila* neurons can sense LPS and achieve LPS avoidance depending on a gustatory chemosensor, TRPA1 (Transient receptor potential A1) [182, 183]. In an evolutionarily conserved process, the TRP orthologue in the human sensory neurons and epithelial cells functions as an irritant sensor to recognize LPS prior to the initiation of TLR4 signaling during inflammation [184].

OMVs

Outer membrane vesicles (OMVs) are extracellular vesicles released by gram-negative bacteria, ranging in size from 20 to 300 nm *in vitro* or *in vivo* of a host. OMVs are formed from the outer membrane of bacteria, consisting of phospholipids, LPS, outer membrane proteins (OMPs), periplasmic proteins such as peptidoglycan, and other enriched content including virulence factors and nucleic acid [185]. OMVs have been considered an important secretion platform that can deliver multiple bacterial lipids and proteins to reach host cells avoiding direct cell contact and achieving a remote-distance impact [185, 186]. Thus, it is directly linked to pathogenicity during infection. It has

been found that OMVs secreted by *P. aeruginosa* package multiple virulence factors, including CFTR-inhibitory factor (Cif), alkaline phosphatase, hemolytic, phospholipase C, and β-lactamase, and across a mucus layer, directly deliver into the host cytoplasm, leading to the cytotoxicity of the airway epithelial cells [187]. The OMVs of *P. aeruginosa* containing the bacterial toxin Cif, or sRNA also help bacteria cells impair mucociliary clearance and evade the immune response of airway epithelial cells [188-190]. Vesicle levels, sizes, and release are modulated by various factors, such as temperature, oxidation, quorum sensing, flagellar motion, and nutrient availability [191-195]. Lower temperature increases the vesicle production of *Serratia marcescens* whereas higher temperature results in increased versicles abundance of *E. coli* [191, 192]. However, a temperature shift from low to higher temperature does not affect the OMVs production of *P. aeruginosa,* but it is increased by hydrogen peroxide (H_2O_2) treatment and antimicrobial peptide polymyxin B treatment [195]. Interestingly, it was found that in *Vibrio fischeri*, flagellum rotation may promote OMVs release, as the number of vesicles increased in the hyperflagellated stain, whereas it was decreased in the flagella-loss mutant [194]. Furthermore, OMVs also have a role in controlling quorum sensing. The OMVs of *P. aeruginosa.* packages a considerable amount of the molecule PQS (*Pseudomonas* quinolone signal), which is an autoinducer to bind the PqsR receptor. Reduced OMV production was obviously observed in PQS-deficient mutants [196]. Besides, OMV production is modulated by extracellular PQS concentrations [197]. It was observed that the condition of bacterial culture affects PQS distribution and OMVs production. The strain PAO1 grown in brain heart infusion broth (BHI) produces more vesicles and extracellular PQS than those grown in Luria-Bertani broth (LB). In the same culture condition, no matter whether LB or BHI, the strain PA14 is a better OMV producer and better PQS exporter than PAO1[197]. Interestingly, culture supernatants from *E. coli* and *K. pneumoniae* stimulate Δ*pqsA* (the PQS-deficient *P. aeruginosa* strains) to produce OMVs that are comparable to the stimulation of PQS [198]. It suggests that the regulatory mechanism of OMV production through quorum sensing may be common and that OMVs deliver cross-signal with other species.

Pyocyanin

P. aeruginosa produces various redox-active phenazine compounds, the most well-known being pyocyanin (PCN), which is responsible for the blue/green figment as an identification feature of *Pseudomonas* spp. Pyocyanin has a complex synthesis process

mediated by gene products encoded by two *phzABCDEFG* operons, and *phzH, phzM*, and *phzS* genes [199, 200]. Production of pyocyanin is regulated by the quorum sensing system, PqsR- PqsABCDH, and RhlR–RhlI, with additional regulation of the LasR–LasI, VfR, and GacA–GacS regulatory systems [200, 201]. Multiple studies have revealed that pyocyanin has a bona fide toxicity for *in vitro* cell culture systems, *C. elegans*, *Drosophila*, plants, and mice [116, 202-205]. The concentration of pyocyanin from the sputum of CF patients can induce apoptosis of neutrophils but not of macrophages or airway epithelial cells [202]. Pyocyanin-induced neutrophil apoptosis also has been validated in a murine model of acute pneumonia, which is considered to be a strategy of bacteria to suppress the acute inflammatory response for self-survival [203, 204]. In a conserved way, pyocyanin cause a fast killing of *C. elegans*. However, *C. elegans* mutants with higher resistance to oxidative stress are not sensitive to PCN-mediated killing [116]. Besides, it was found Δ*phzB*, the *P. aeruginosa* mutant of pyocyanin was less virulent in the *Drosophila*, which was infected by pricking on the thorax [206]. However, interestingly, our colleagues Limmer S. *et al*. found the phenazine mutants (*Δphz1/2*, *phzH, phzM*, and *phzS*,) were as virulent as wild-type PA14 in both wild-type or immunodeficient flies infected by bacterial feeding [89].

Rhamnolipid

Rhamnolipids (RLs) are a class of glycolipids containing L-rhamnose and β-hydroxy fatty acids chains, produced by *P. aeruginosa,* among other microorganisms. The three key enzymes, RhlA, RhlB, and RhlC are responsible for rhamnolipid biosynthesis; their expression are modulated by various regulatory factors, such as the RhlR/I QS and PQS quorum sensing systems, global regulator RpoS, posttranscriptional regulator GacS-GacA [207]. Rhamnolipids are known as bacterial surfactants, with the property of reducing surface tension, thus, primarily play a role in adhesion, surface-associated motility (swarming) and biofilm structure development, solubilization of bacterial molecules, like quinolone signal (PQS) [208]. Rhamnolipids can cause hemolysis and necrotic killing of leukocytes, thus it may be a way to evade innate immune defense, although it is applied as a green and nontoxic biosurfactant [209-211]. Our colleagues Limmer S, *et al*. found the *rhlA* and *rhlB* mutants had the same phenotype as wild- type PA14, suggesting that rhamnolipids were not responsible for the virulence of *P. aeruginosa* in the oral infection model of *Drosophila* [89].

Alginate, Psl, and Pel

P. aeruginosa is capable to produce at least three extracellular polysaccharides, namely alginate, Psl, and Pel, which provide bacterial cells capsule-like matrix material to enhance its tolerance to the environmental conditions, such as oxidative environment, desiccation, as well as host defense [212, 213]. It has been identified that these three exopolysaccharides are involved in the formation, development, and architecture of biofilm [214]. Alginate is responsible for mucoid overproduction of *P. aeruginosa* in the CF airway during chronic infection [215]. *P. aeruginosa* adapts well in CF lung during chronic infection and turns out to be mucoid, nonmotile, with rough LPS in texture [216]. Note that apart from genes coding alginate, the *P. aeruginosa* PA14 strain only has *pel* operon, whereas the PAO1 strain has two loci, *pel* and *psl* [217, 218].

Other secreted virulence factors & secretion systems

Last but not least, *P. aeruginosa* can produce and release numerous and multiple toxin proteins like exoenzymes and proteases. Four kinds of exoenzyme have been identified in *P. aeruginosa,* including exoenzyme U (ExoU), ExoS, ExoT, and ExoY. These exoenzymes make diverse and pleiotropic effects on host cell function by disrupting the actin cytoskeleton, and tight junctions, inducing apoptosis and inhibiting cell migration and immune response [219]. Proteases represent one crucial category of virulence factors resulting in tissue damage to the host. So far, it is known that *P. aeruginosa* possesses four types: LasA elastase (staphylolysin), LasB elastase, alkaline protease (*e.g.*, AprA), and protease IV [220]. Besides, exotoxin A has been long identified to cause severe cytotoxicity in mice and contribute to the infection pathogenicity in the CF lung [221, 222].

TSSs. Bacteria possess sophisticated protein secretion apparatuses to export various virulence factors out of the bacterial cell envelope. Up to date, *P. aeruginosa* has six types of secretion systems, which are classified into the one-step system, namely type I secretion system (T1SS), T3SS, T4SS and T6SS, and two-step system, the T2SS and T5SS [223]. One-step secretion systems directly transport the substrates from the bacterial cytoplasm into a target cell or extracellular space, whereas two-step secretion systems require translocating the substrate first into the periplasmic space through inner-membrane-spanning transporters and then secreting them outside the bacterium

[224]. T2SS is evolutionarily related to Type IV pili and comprises a similar structure. In contrast to T4P, T2SS pseudopili do not extend out of the outer membrane, thus they lack motility and adhesion function [225]. T3SS is also evolutionarily associated with flagella, consisting of almost the same structure proteins as those located in the flagellar basal body, which secretes the proteins that form the rod, the hook, and the filament, the hook [226]. Roles of secretion systems in bacterial pathogenicity are closely linked to the dedicated substrates/effectors which they export (scheme figure). For example, T2SS substrates are elastases (LasA and LasB), lipases (LipA and LipC), phospholipases (PhoA, PlcB, and PlcH), an alkaline phosphatase (PhoA) and an exotoxin (ToxA). T3SS and T6SS are molecular nano-syringes mediating the direct delivery of multiple toxins to the targeted cells, including the prokaryotic competitors in the ecological niches and the eukaryotic cells in the host environments [227, 228]. T3SS effectors are exoenzymes as mentioned above, and the PA14 strain encodes ExoU, ExoT, and ExoY. *P. aeruginosa* has three T6SSs, H1-to H3-T6SS [229] H1-T6SS is committed to bacterial killing by transporting at least eight toxins (Tse1-Tse8) [230-233] .H2-T6SS and H3-T6SS have been reported to not only target prokaryotic cells and but also to mediate invasion in eukaryotic cells, such as epithelial cells [231, 234-236]. It was reported by our team that a flagellar T3SS contributes to the damage of gut epithelial cells in the *S. marcescens* intestinal infection model of *Drosophila* [106]. However, we found *P. aeruginosa* mutants of T3SS present a similar virulence as wild-type PA14 in the oral infection of *Drosophila* [89], while other reports showed that T3SS mutants cause a slightly delayed mortality in the fast killing of *Drosophila* infected by pricking with *P. aeruginosa* [237].

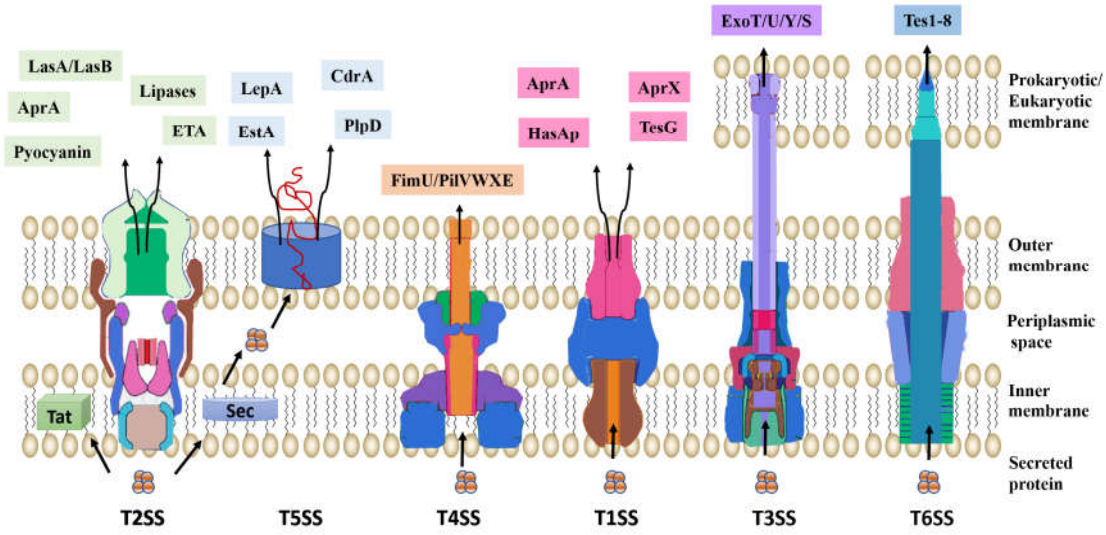

Figure 6. The secretion systems *in P. aeruginosa*. T1SS, T3SS, T4SS, and T6SS are one-step secretion systems. Proteins are transported directly out of the bacterial outer membrane through T1SS, or directly injected into a target cell through T3SS and T6SS. T2SS, and T5SS are two-step secretion systems, by which proteins are translocated to the extracellular space after first reaching periplasm via the Sec/Tat machinery. (Images of TSSs were adapted from a published figure [223])

2.4 Quorum sensing

P. aeruginosa has multiple sophisticated signaling systems to regulate its virulence. Here I will focus on the quorum sensing of *P. aeruginosa.* Quorum sensing (QS) is a bacterial cell-to-cell communication process that enables bacteria population response to their cell density, collectively controls gene expression, and thus synchronizes group behaviors, such as bioluminescence, virulence factors production, biofilm formation, and motility.

2.4.1 Discovery history of quorum sensing

Since Antonie van Leeuwenhoek revealed the existence of microorganisms 300 years ago, for a long time bacteria used to be considered as single-cell life forms endowed with only simple processes. A bacterial communication system, termed quorum sensing was discovered 300 years later, in the 1960s and 1970s. People then became thinking

that bacteria are capable to behave collectively and coordinately like a multicellular organism [238-240]. The conception of cellular communication in a bacterial population originates from a description by Tomasz in 1965 that there is a " hormone-like activator " with the capability of imposing physiological homogeneity among the bacterial population, based on the study of genetic competence in *Pneumococcus* [241]. In 1970, Nealson & Hastings found that two bioluminescent marine bacteria, *Vibrio fischeri,* and *Vibrio harveyi*, produced light when they reached a threshold of high cell density, and the bioluminescence could be induced by the cell-free culture fluids, wherein the responsible component is termed autoinducer [242, 243]. Subsequently, the autoinducer was identified as an acyl-homoserine lactone (AHL) [244]. These discoveries suggest that certain bacteria possess chemical communication systems involving the production, release, detection, and response of the signaling molecule autoinducers, which enable the bacteria to sense their population density, and behave collectively and coordinately. Thereafter, people found the communication is common in the bacterial world, and given its features, the process was named quorum sensing [239]. Furthermore, such communication mode exists in a wide range of species and extends to communication between species, and even between kingdoms [245, 246].

2.4.2 Quorum sensing system, an intricate, partially redundant regulatory system

Bacterial quorum sensing works relying on the networks of signaling molecules called autoinducers, autoinducer synthases, and regulators/ receptors. The activation of the circuit triggers the downstream signal transduction and gene expression, and in turn, changes the behavior in the population as a response to the cell density related to the variations of the environment. LuxR/LuxI type quorum sensing in *Vibrio fischeri* was first characterized based on combined findings in the 1980s and is considered a typical paradigm for QS of most Gram-negative bacteria. There are four common features of QS systems found in nearly all known Gram-negative bacteria [247, 248] : (Figure: quorum sensing in *Vibrio fischeri* as a paradigm)

- Autoinducers bind to specific receptors located either in the cytoplasm or in the inner membrane. Typically, Gram-negative bacteria use the LuxR-type receptors, which function in the cytoplasm, as transcriptional factors after binding the

autoinducer AHLs produced by partner LuxI-type synthases.

- The autoinducers are acyl-homoserine lactones (AHLs/HSLs) or other S-adenosylmethionine (SAM)- derived molecules. They can easily diffuse through the bacterial membrane.
- Quorum sensing typically controls a large subset of genes that underpin various biological processes.
- Autoinduction. Quorum sensing signaling activated by autoinducers, in turn, upregulates the autoinducer synthases in the downstream operon, forming a feed-forward loop.

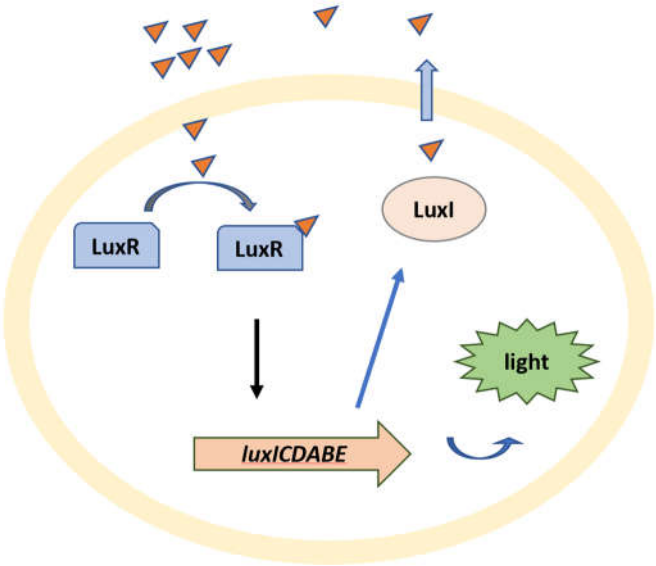

Figure 7. LuxR-LuxI quorum sensing system in *Vibrio fischeri*. The diffusible HSL autoinducer (red triangles) is synthesized by LuxI protein. When the autoinducer reaches a concentration threshold, it is bound by the LuxR. The LuxR-autoinducer complex activates the promoter of *luxICDABE*, resulting in bioluminescence. (Scheme adapted from a published figure [240].)

2.4.3 Quorum sensing system in *Pseudomonas aeruginosa*

2.4.3.1 Four interwoven quorum-sensing circuits

P. aeruginosa employs four interwoven quorum-sensing circuits currently known: two LuxR-LuxI type systems termed LasR-lasI and RhlR-RhlI, PqsR-PqsABCDH, IQS

system. The synthases, LasI, RhlI, PqsABCDH, and AmbBCDE, respectively synthesize the autoinducers, N-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL), N-butyryl-L-homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (PQS), and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS). The receptors LasR, RhlR, and PqsR, also function as cytoplasmic transcription factors that are bound by the corresponding autoinducers. The receptor for IQS has not been found yet [248].

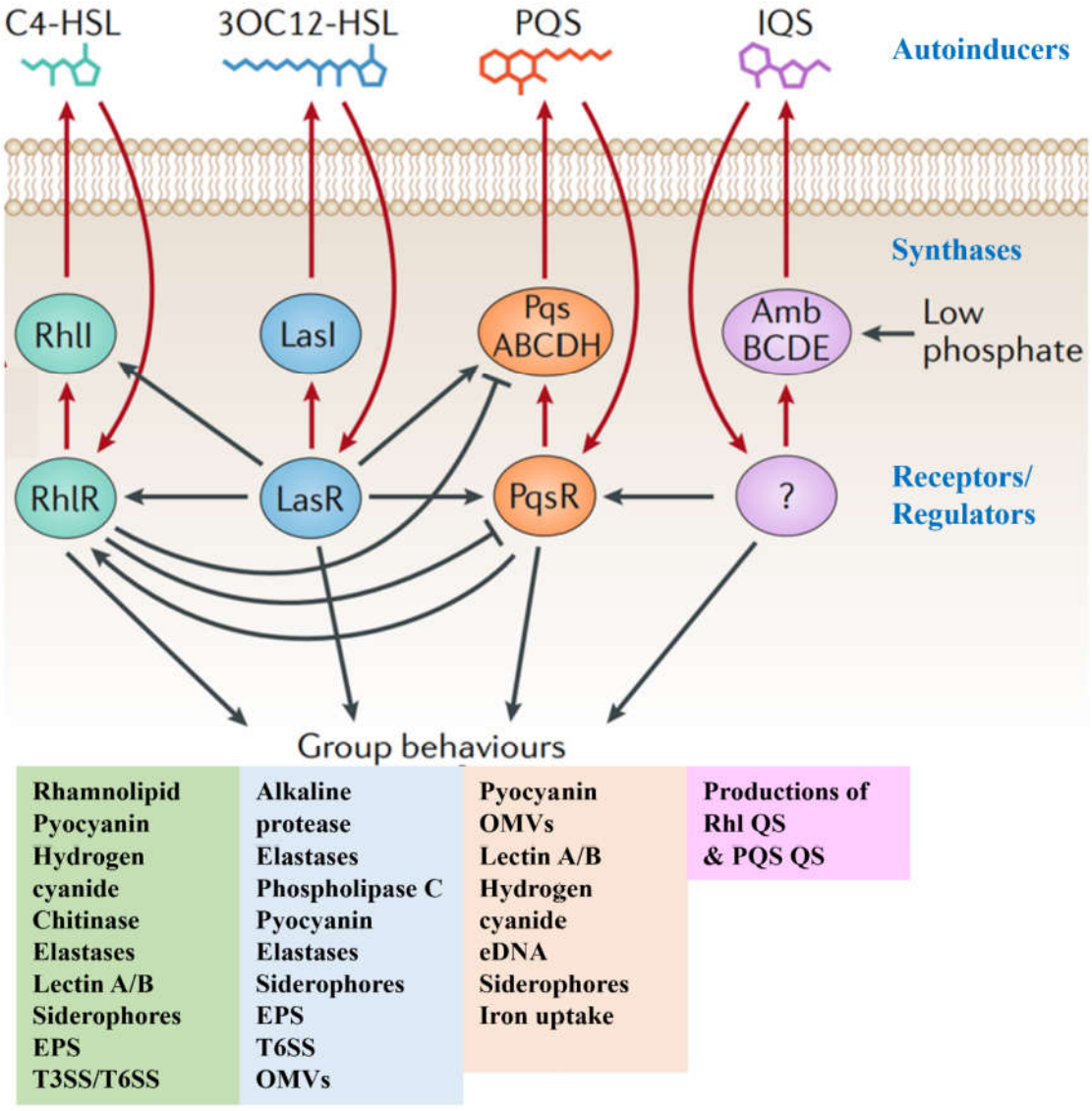

Figure 8. QS circuits in *P. aeruginosa*. *P. aeruginosa* has four interwoven QS circuits: LasR/lasI and RhlR/RhlI, PqsR/PqsABCDH, IQS system, with their respective autoinducers, 3OC12-HSL, C4-HSL, PQS, and IQS. The corresponding virulence products are also shown at the bottom of each QS loop. (An adaptation modified from published figures [249, 250])

The Las and Rhl quorum sensing systems

RhlR/RhlI and LasR/LasI circuits are two LuxR/LuxI type systems. As a homology to the protein LuxR, LasR in *P. aeruginosa* was discovered as a key transcriptional activator of the metalloprotease elastase LasB [251]. Whereafter, within two years, LasR was found to have a global regulation in the transcription of many virulence factors, such as AprA, LasA, and ToxA [252-254]. One after the other, its partner LasI and the first autoinducer were identified in *P. aeruginosa* [255, 256]. Subsequently, *rhlR,* located in the rhamnolipid synthase gene cluster *rhlABR*, was characterized as a regulator gene responsible for the synthesis of rhamnolipids, and shares sequence homology with *lasR* [257]. The second autoinducer N-butyryl homoserine lactone (now known as C4-HSL) and its synthesis gene *rhlI* at downstream of the *rhlABR* gene cluster were separately identified in the same year [258, 259]. It has been revealed that in *in vitro* culture, the RhlR/RhlI and LasR/LasI circuits of *P. aeruginosa* control more than 300 genes, representing over 10 % of the genome [260, 261]. They are involved in a large proportion of virulence phenotypes and physiological processes, including the production of secreted virulence factors and secretion apparatus, metabolism, attachment, and motility, as well as biofilm formation. LasR and RhlR have extensively overlapping regulons, but also have their distinct regulons [260-263].

PQS system and IQS system

P. aeruginosa utilizes additionally two non-LuxR/LuxI QS systems: *P. aeruginosa* quinolone signal (PQS) system and IQS system. When the bacteria were grown under iron-limited conditions, the PQS signaling was triggered by PQS, 2-heptyl-3-hydroxi-4-quinolone, an autoinducer synthesized by PqsA, PqsB, PqsC, PqsD, and PqsH. PQS molecule is recognized by the regulator/receptor PqsR (also called MvfR), which in turn, activates gene expression for virulence [264-266]. The integrated QS (IQS) system can be activated by phosphate starvation. The signaling molecule is produced by AmbBCDE and is a byproduct in the synthesis of siderophore pyochelin, yet the corresponding receptor is not known [267, 268].

Interwoven regulation among QS in *P. aeruginosa*

LasR/LasI and RhlR/RhlI QS systems are arranged in a hierarchical manner, as the activation of the LasR/Las system can upregulate the transcription not only of its synthase gene *lasI* but also that of *rhlR* and *rhlI* [269-272]. RhlR/RhlI system also forms

a positive feedback loop by activating its own regulon *rhlR* and *rhlI* [256, 273]. Expression of *pqsR* and *pqsH* is positively regulated by LasR/LasI, whereas RhlR/RhlI inhibits the expression of *pqsR* and *pqsABCD* [201, 265, 274]. In turn, the transcription of *rhlI* and *rhlR* could be enhanced by the PQS system [275, 276]. Besides, the production of IQS is completely blocked when *lasR* or *lasI* is absent under the rich medium condition. And blocking IQS biosynthesis impaired the Rhl QS and PQS system by regulating the biosynthesis of PQS and C4-HSL with an unknown mechanism [267]. As its positive regulation of the other three QS, the Las system is often considered to be at the top of the signaling hierarchy and contributes to the cascade activation of QS in *P. aeruginosa in vitro* condition. However, the situation appears to be more complex than the simple hierarchy model proposed, an earlier report showed that the deletion of lasR only delays the Rhl system and PQS system, as many virulence factors such as pyocyanin still can be produced under certain conditions [277, 278]. The activation of RhlR in the absence of LasR has already been reported to be partly due to the IQS system as mentioned before [267, 278]. Yet, most of the above regulation relationships have not been proved in the more complex host environments.

QscR. In addition, it is worth mentioning that there is another LuxR homolog in *P. aeruginosa,* called QscR, known as an orphan or solo receptor because of lacking its cognate synthase LuxI [279]. It was found that in the *Drosophila* feeding infection model, QscR dampens the virulence possibly by repressing lasI. Deletion of QscR leads to an earlier yield of C4-HSL and 3OC12-HSL; in turn, expression of genes regulated by RhlR and LasR is induced in advance, such as phenazine [280]. It has been revealed that QscR can sense and respond to 3OC-12-HSL, resulting in a functional folding for activating a single linked operon, which in turn dampens the gene transcription under the regulation of LasR and RhlR. The outcome is considered as a “brake” on the QS autoinduction in high cell density, based on combined findings [273, 281-284]. Besides, QscR can also bind to other HSLs, thus preferentially responds to non-*P. aeruginosa* signals from other species, such as *Burkholeria vietnamiensis*, that may be used to detect the cohabitating species [285].

2.4.3.2 Contribution of QS for virulence, in the perspective of Rhl and Las system

For acute and/ or chronic?

Given the powerful regulation of numerous genes, it is not surprising that quorum sensing of *P. aeruginosa* contributes to virulence during the infection, which has been proved in the different infection models, such as *C. elegans*, *Drosophila*, plants, and rodents [107, 267, 286-291]. According to earlier studies, Δ*lasI*Δ*rhlI* double mutant of PAO1 stain was nearly avirulent in acute pulmonary infection of neonatal mice, and it caused milder lung pathology and had less colonization in the chronic lung infection of adult rats [290, 291]. In the ulcer infection of rats, *ΔlasRΔrhlR* double mutant and Δ*lasI*Δ*rhlI* double mutant of PAO1 induce less tissue destruction, but the same inflammation level in comparison to wild-type PAO1. *ΔlasIΔrhlI* forms an immature biofilm in the wounding site, while *ΔlasRΔrhlR* cannot form a biofilm and displays a planktonic state, exhibiting a larger wound size [288]. It implies that the role of QS was more important for the chronic period than the acute phase of infection.

RhlR acts in its own scenarios.

A clinical phenomenon is that *lasR* and *lasI* loss of function mutants have been frequently isolated from cystic fibrosis patients with chronic infection of *P. aeruginosa* [292-296]. Correlatively, a previous study by our colleagues Limmer S *et al.* showed that it is RhlR, but not LasR, that contributes to the virulence in immunocompetent *Drosophila* with the oral infection of PA14 [89], although *lasR* mutant of PAO1 strain has less virulence in the sepsis infection of *Drosophila* [267]. Consistently, Bassler's team also found that RhlR plays a key role in virulence, whereas *lasR* mutant is as virulent as wt PA14 in the infection model of *C. elegans* [289]. The key contribution to the virulence of RhlR is also proved in the murine model of acute lung infection. But unlike in the *Drosophila* and *C. elegans*, mutants of *lasR* and *lasI* displayed attenuated virulence [289].

An alternative autoinducer?

Surprisingly, such kind of virulence regulation of RhlR, no matter in *C. elegans*, or mice, is not dependent on the canonical homoserine lactone autoinducer C4-HSL, as

the mutant of *rhlI* was as virulent as the wt bacteria. *In vitro*, colony biofilm phenotypes of Δ*rhlR* and Δ*rhlI* are totally different, and cell-free culture fluids can activate RhlR-dependent gene expression [289]. A similar finding was also made in the *Drosophila* model by our team [56] that RhlR was only partially dependent on RhlI for virulence. Moreover, RhlR may help the bacteria evade the host defense of phagocytosis and opsonization, independently of RhlI [56, 89]. The above findings imply there may be another autoinducer synthesized by an alternative ligand.

An enigmatic character, PqsE.

Subsequently, in a successive work, the protein PqsE was screened out by colony biofilm phenotype *in vitro*, as its mutant displays a hyper-rugose phenotype that is highly similar to that of Δ*rhlR*. In addition, Δ*rhlI*Δ*pqsE* has a phenotype indistinguishable from Δ*rhlR*, whereas Δ*rhlI* is smooth and wt PA14 presents a colony biofilm phenotype with a rugose center and smooth periphery [297]. In culture conditions, RhlR, RhlI, and PqsE regulate distinct but overlapping regulons. Importantly, the *pqsE* mutant as well as the double mutant of *rhlI* and *pqsE*, are avirulent in the infection of the *C. elegans* model, a Δ *rhlR*-like phenotype. In acute murine lung infection models, Δ*pqsE*, and Δ*rhlI* Δ*pqsE* strains are highly attenuated, even more than the Δ*rhlR* strain. Based on the findings, it was proposed that PqsE serves as an alternative paired synthase, producing an unknown autoinducer that also binds to the receptor RhlR [297]. As it is located in the *pqsABCDE* locus, PqsE was initially thought to be responsible for the synthesis of the PQS autoinducer, and now it has been identified that PqsE is dispensable for PQS production [264, 298, 299]. Earlier, it has been reported that PqsE functions independently of the PQS system and enhances the RhlR expression for the response to RhlI-derived C4-HSL [298]. It seems reasonable that there is an alternative autoinducer for RhlR. An example is that apart from the preferential cognate autoinducer, 3OC12-HSL, LasR is also sensitive to other homoserine lactones, such as 3OC10-HSL and 3OC14-HSL [300].

However, another possibility was proposed earlier that the effect of PqsE on RhlR might result from a protein-protein or protein-DNA/RNA interaction [301]. Later, the hypothesis was confirmed in *in vitro* experiments; that is, it is not the PqsE catalytic activity that is responsible for the full regulation function of RhlR, but a direct interaction between PqsE and RhlR, increases the affinity of RhlR for DNA, and in turn,

enhances transcriptionally the expression of virulence genes. The production of pyocyanin in liquid culture showed that RhlR cannot be activated in the absence of the ligand C4-HSL, even when *pqsE* is overexpressed. Collectively, the Rhl system can work in two putative modes in *in vitro* condition, RhlR: C4-HSL or PqsE-RhlR: C4-HSL [299, 302]. Given the above, the classic autoinducer C4-HSL is still required for the full activation of RhlR *in vitro*. It has been pointed out earlier that the cognate homoserine lactone (HSL) autoinducers are required for LuxR-type receptors as transcriptional factors to achieve dimerization, protein folding, and protease resistance [303, 304]. However, it was also reported that RhlR does not require C4-HSL for dimerization, although it needs C4-HSL for transcription activation [305]. However, RhlR is insoluble in the absence of its ligand, and thus it cannot be active [306]. It is worth mentioning that specific points mutant of *rhlR* enable the ligand-independent activity of RhlR *in vitro* and *in vivo* [307].

To sum up, there is no doubt that the Rhl system plays a key role in pathogenicity in specific scenarios, distinctly and independently from the Las system. Yet, we do not know when and why they are required and why not in a specific host environment. RhlR functions as a complex formed by the interaction with *pqsE* and C4-HSL, displaying a unique regulatory feature of QS. However, the *in vitro* working model of the PqsE-RhlR: C4-HSL complex still cannot provide a proper explanation that why a mutant of RhlI is as virulent as wt bacteria in the infection model of *C. elegans* and the murine acute lung infection model. In conclusion, it thus appears that we do not yet fully understand the complexity of quorum sensing signaling in the context of infections.

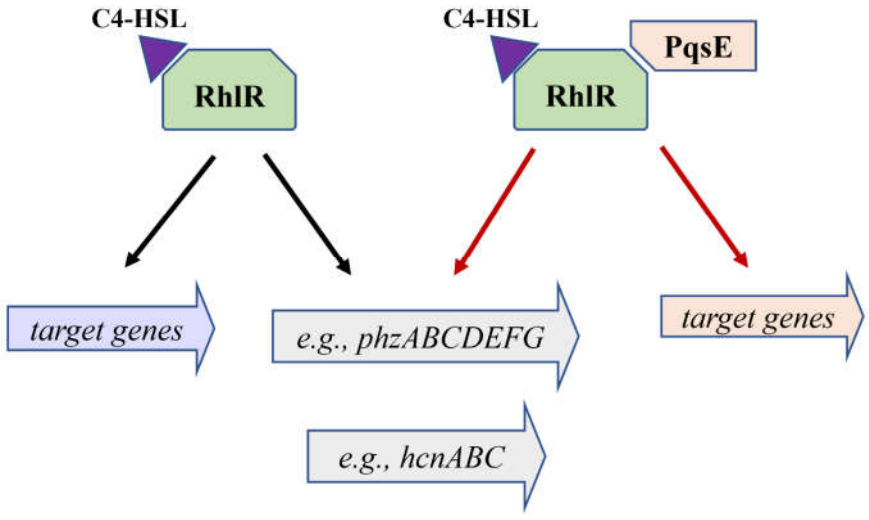

Figure 9. A scheme showing two putative modes for RhlR activation in *P. aeruginosa*. Autoinducer C4-HSL is bound by RhlR to induce the basal expression of some genes (*e.g.*, grey loci), which are enhanced when RhlR interacts directly with PqsE. The RhlR-C4-HSL and PqsE-RhlR: C4-HSL complexes also regulate distinct regulons. (The scheme was drawn partially according to a reference [299].)

2.4.4 Lifestyles transition of *P. aeruginosa* & quorum sensing

Biofilm formation and development in *P. aeruginosa*

Biofilm is a common lifestyle of *P. aeruginosa* in the environment, since it provides a physically stable and protective shed for the bacteria, with higher tolerance against environmental stresses, like high or low temperature or pH, antibiotics, as well as host defense. Thus, clinically, biofilms are most often related to chronic infections (long-term infections). It is also the major cause of chronic infection in cystic fibrosis patients, typically with the overexpression of alginate.

Biofilms are bacterial communities, which are sessile and enwrapped by a self-produced extracellular matrix. The matrix comprises extracellular polysaccharides (also called exopolysaccharides, EPS), such as alginate, Psl, and Pel, and also consists of proteins and eDNA. Development of *P. aeruginosa* biofilms *in vitro* was well characterized as a five-stage process by scanning electron microscopy, gene expression, and protein analysis [308, 309]:

I. Reversible attachment: Planktonic bacteria transiently contact the surface via flagella-mediated motility, independently of quorum sensing (confirmed by mutant Δ *lasI*).

II. Irreversible attachment： Bacteria are nonmotile, aggregate, and form microcolonies, with EPS production, and Las QS activation.

III. Early maturation: Bacteria forms thicker cell cluster with *rhlA* activation (responsible for the synthesis of rhamnolipid, controlled by Rhl QS); over 500 genes are upregulated, including the genes related to the anaerobic process.

IV. Maturation: Maximum cell cluster is formed, typically with a mushroom-shaped structure, showing the greatest difference in protein pattern from planktonic cells.

V. Dispersion/Detachment: Motile and nonmotile cells coexist. Some bacteria cells actively swim away from the interior cell clusters, presumably for the colonization of new niches. Enzymes responsible for degrading the matrix may be involved during this process.

Biofilm development, a lifestyle transition model controlled by QS

The above primary characteristics tell us that biofilm formation is indeed a sophisticated programmed group behavior, that required cell-cell communication, in turn, within the biofilm, signaling molecules for cell-cell communication can maintain an optimal and sufficient level to regulate biofilm development. Earlier or later investigations in quorum sensing and biofilm progressively developed our understanding of this process.

Swarming motility (a bacterial movement on semi-solid surfaces) has been identified as a group behavior in the control of QS and related to the early step of biofilm formation. Swarming depends on flagellar motility, pili-mediated twitching, and the rhamnolipid [166, 310]. Rhamnolipids as biosurfactants promote microcolony formation in the initial phase but are also required for the biofilm architecture in the later phase, by facilitating migration-dependent development of mushroom structure [311, 312]. *rhlAB* for rhamnolipid synthesis predominantly controlled by Rhl QS is preferentially expressed in the stalks of the mushroom structure [313], but rhamnolipids also promote the bacterial twitching motility in the mushroom cap [311]. Furthermore, at the dispersion phase, the overproduction of rhamnolipids mediates biofilm detachment from making cavities within the center structures [314]. The biofilm dispersion stage of *P. aeruginosa* involves the degradation of the matrix and also the inhibition of the synthesis of the compounds that form it, such as the major exopolysaccharides, Pel, which is upregulated by LasR/lasI system at the early stage. Here, the study we emphasized before [289], points out that Pel is responsible for the hyper rugosity of biofilm colonies in Δ*rhlR*, and Pel is downregulated by the overproduction of phenazines in Δ*rhlI*, resulting in a smooth biofilm. In contrast to the *rhlR* mutant, biofilms of *lasR* and *lasI* mutants display thin and less differentiated biofilm colonies. Besides, they are easy to be eradicated by surfactants, and more sensitive to antibiotics [315].

Other regulatory signaling pathways for biofilm formation

Signaling networks for biofilm development are intricate, redundant, and possibly conflicting. Here, I will briefly introduce three other signaling pathways involved in biofilm formation.

c-di-GMP signaling. c-di-GMP signaling is one of the important signaling pathways modulating the lifestyle transition from motility and biofilm formation. Cyclic dimeric GMP (c-di-GMP) is a diffusible second messenger extensively utilized in bacteria. The intracellular concentration of c-di-GMP fluctuates in response to changes in the environment or as a result of cellular life cycles. It has been observed that c-di-GMP controls bacterial virulence with a dichotomy model: high-level c-di-GMP corresponding to chronic infection (slow growing, biofilm formation) versus low-level c-di-GMP corresponding to acute infection (fast proliferation, motility) [316]. LasR/LasI indirectly influences the level of c-di-GMP by upregulation of tyrosine phosphatase TpbA, which not only decreases the c-di-GMP level but also inhibits the expression of *pel* [317].

Gac/Rsm cascade. Bacterial lifestyle is also under the modulation of the Gac/Rsm cascade which culminates in switching ON/OFF RsmA, a posttranscriptional regulator responsible for the translation repression of multiple target genes [318]. The Gac/Rsm network consists of the GacS/GacA two-component system, which regulates the expression of small RNAs that are encoded by the *rsmY* and *rsmZ* genes. The RsmY/Z sRNAs competitively bind to RsmA; in turn, the target mRNAs are relieved from the restraint of RsmA [319]. When the planktonic bacteria reach a high cell density, the RsmY/Z sRNAs are upregulated, leading to surface attachment [320]. During biofilm development, the sRNAs are reduced in the irreversible attachment phase, then upregulated to a higher level compared to the planktonic state during biofilm maturation [321, 322]. Finally, RsmY/Z sRNAs are reduced again when the bacteria detach from the biofilm [323].

cAMP/Vfr signaling. Virulence factor regulator (Vfr) is activated by binding to cAMP, a cyclic second messenger synthesized by adenylate cyclase (AC) enzymes, resulting in the production of a variety of virulence factors, mainly contributing to acute infections [324]. When *P. aeruginosa* cells attach to a solid surface, the flagellar load is increased to tether the surface [325]. Meanwhile, the flagellum senses the surface and triggers the activation of the cAMP/Vfr pathway, which in turn negatively regulates its synthesis, and also induces the expression of virulence factors that promote biofilm

formation [325-327]. When the biofilm becomes thicker, cAMP levels are reduced [328].

P. aeruginosa displays higher virulence during the lifestyle transition

The process of biofilm development provides a basal landscape for investigators to study a planktonic-to-sessile switch model of bacterial lifestyle, corresponding to the transition from acute infection to chronic infection. One interesting study report I would like to highlight here as it demonstrates how planktonic-sessile transition switches on bacterial virulence. Siryaporn *et al*. performed a killing assay of amoeba challenged with *P. aeruginosa* grown in liquid versus those attached to a surface from an identical culture and showed that only the bacteria attached to a surface can kill amoebas [329]. Bacterial virulence was induced by surface attachment, which triggers mechanotransduction mediated by a mechanosensory pilum PilY1. The quorum sensing Las system but not the Rhl system is required for surface-activated virulence. Planktonic bacteria in high density supplemented with autoinducers (3OC12-HSL, C4-HSL) were not virulent, suggesting that quorum sensing cannot activate virulence without surface attachment [329]. In a reverse vein, another study showed that *P. aeruginosa* cells detached from biofilms are more virulent to *C. elegans* and macrophages than the corresponding planktonic cells. Compared to the planktonic cells, the dispersed cells possess distinctive gene expression profiles, such as higher expression levels of T2SS, and lower induction of RsmY/Z sRNAs [323]. It would be interesting to investigate whether quorum sensing systems are also involved in this reverse process.

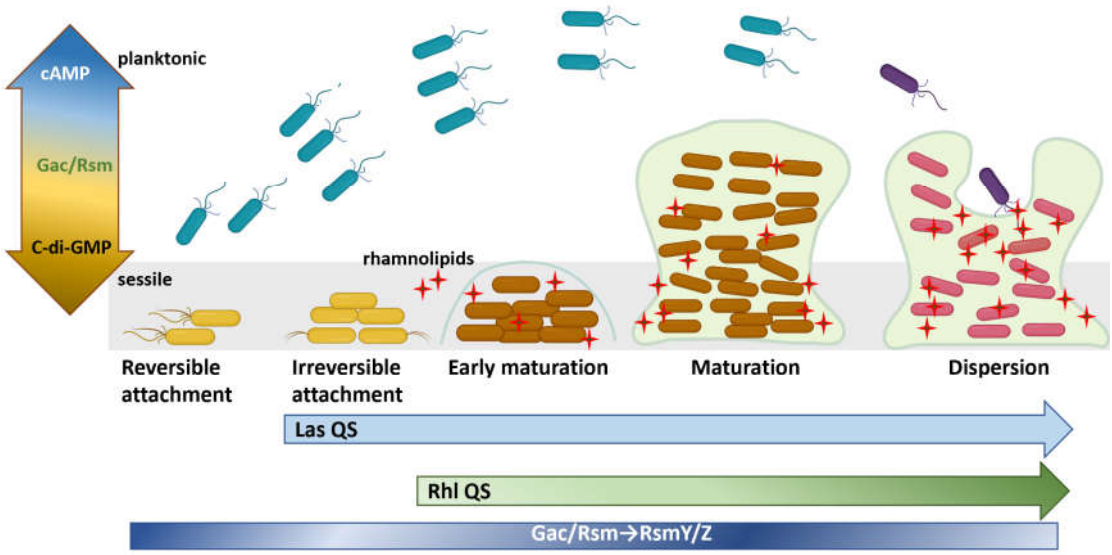

Figure 10. Biofilm development of *P. aeruginosa* is characterized by a five-stage process. I) Reversible attachment: planktonic bacteria weakly attach to a surface. II) Irreversible attachment: bacteria are nonmotile, and form microcolonies, with Las QS activation. III) Early maturation: bacteria form microcolonies with activation of Rhl QS and ensuing increased production of rhamnolipids. IV) Maturation: bacteria form a mushroom-shaped cell cluster. V) Dispersion: some bacteria cells swim away from the central cavities. Quorum sensing and cAMP, c-di-GMP pathway， and the Gac/Rsm cascade for *rsmY/Z* induction are all involved in the lifestyle switching process. (Color shade in the scheme represents the gene expression levels regulated by different pathways).

2.4.5 Communication/interaction between species and kingdoms

Coexistence and interactions between a variety of microorganisms are the norms in the natural environment, for example, plaque in the human oral cavity, microbiota in the gut, and polymicrobial infections. Quorum sensing is not limited to its bacterial population； it also allows interspecies and inter-kingdom communication. Here, I will show several examples of the interaction relating quorum sensing between *P. aeruginosa* with other organisms, including its eukaryotic hosts.

DSF-mediated interspecies communication

Diffusible signal factors (DSF) are cis-2-unsaturated fatty acids which act as QS signal molecules regulating diverse biological functions in a range of Gram-negative bacterial pathogens [330]. Over the last decade, many studies revealed that DSF-based QS systems can mediate not only intraspecies communication but also interspecies communication and inter-kingdom communication. *P. aeruginosa* , *Stenotrophomonas maltophilia*, and *Burkholderia cenocepacia* are all multidrug-resistant pathogens that often coinfect CF patients, and their distinct DSFs, respectively PDSF, XcDSF, and BDSF, were detected in sputum samples from cystic fibrosis patients [331]. In coculture conditions, DSF secreted from *S. maltophilia* influences the biofilm architecture of *P. aeruginosa,* polymyxin antibiotic tolerance, through a sensor kinase of *P. aeruginosa* PA1396 [332]. *B. cenocepacia* also influences the virulence of *P. aeruginosa,* independently of the PA1396 sensor. Exogenous addition of BDSF from *B. cenocepacia* not only decreases the transcriptional level of the QS regulator genes *lasR*, *rhlR*, and *pqsR* but also simultaneously reduces the production of the corresponding autoinducers, including 3OC12-HSL, C4-HSL, and PQS, resulting in impairment of biofilm formation and production of virulence factors. In addition, BDSF is capable of suppressing the expression of T3SS genes in *P. aeruginosa* at micromolar concentrations. *P. aeruginosa* pathogenicity was obviously reduced with the treatment of BDSF in both HeLa cell and zebrafish infection models [333].

Does *P. aeruginosa* crosstalk with gut microbiota?

As reviewed at the beginning, *P. aeruginosa* has been found within the microbial communities of the human intestines and can cause local infection and remote infection in hospitalized patients. But whether quorum sensing of *P. aeruginosa* is activated in the gastrointestinal tract remains questionable, since autoinducers like AHL, are pH sensitive and easily inactivated by the host lactonases and bacterial enzymes [334]. A few recent studies provide clues that some types of AHLs were detected in the rumen of cattle and human intestines in the context of inflammatory bowel disease [335, 336]. It is now clear that gut microorganisms do use QS communication to keep homeostasis and also crosstalk with mammalian hosts. It has been reported that gut microbiota use AI-2 (autoinducer-2) -mediated QS system in the mammalian gut and AI-2 from engineered *Escherichia coli* modulate gut microbiota dysbiosis and composition in the mouse intestine [337]. AI-2 has been recognized as a potential interspecies signaling

molecule. Besides, mammalian epithelial cells can respond to AI-2 with the upregulation of the inflammatory cytokine interleukin-8 [338]. Although *P. aeruginosa* lacks the LuxS genes responsible for AI-2 production, it does have the capability to sense and respond to AI-2, thus altering its biofilm formation and production of virulence factors [339, 340]. *In vitro*, AI-2 can change the biofilm architecture and increase the production of virulence factors in PAO1, but not in the double mutant Δ*lasR* Δ*rhlR*. In the *P. aeruginosa* lung infection model of mice, AI-2 treatment facilitates the acute infection through the IL-17A pathway in the host, depending on the QS system of *P. aeruginosa* [341, 342]. Moreover, it has also been demonstrated that AI-2 is required for biofilm formation within dental plaque [343]. Interestingly, Sibley CD *et al.* used the oropharyngeal flora isolated from CF patients to perform coinfection with *P. aeruginosa* in *Drosophila* by feeding and characterized a class of oropharyngeal flora organisms that was not pathogenic alone but could have synergistic effect with PAO1 for pathogenicity [344].

Host factors- *P. aeruginosa - S. aureus* interaction

Host factors influence QS signaling and thereby change the outcome of pathogen competition. In the clinical setting, *P. aeruginosa* is often coinfected with *S. aureus* in the chronic wound. Intriguingly, when co-cultured under standard microbiological conditions, *S. aureus* is readily eradicated by *P. aeruginosa,* which releases QS-mediated exoproducts, such as LasA protease, and redox-active phenazines that can kill *S. aureus*. However, when cocultured in the wound-like model with plasma and red blood cells, the two species persist to survive for up to seven days, close to the coinfection observed in mouse wounds. It is the host factor serum albumin that sequesters the autoinducer 3OC12-HSL, and in turn, inhibits LasR/LasI quorum system. The outcome is that *P. aeruginosa and S. aureus* coexist [345].

Host-Microbe Interactions

The autoinducers of *P. aeruginosa per se* can affect the host's innate immune system. It has been reported that treatment with 3OC12-HSL but not C4-HSL can disrupt the tight junction integrity of intestinal epithelial Caco-2 cells cultured *ex vivo*, via inducing matrix metalloprotease activation [346]. Besides, 3OC12-HSL, but not C4-HSL, has been identified as a chemoattractant to neutrophils in a dose-dependent fashion and can promote apoptosis of neutrophils by interfering with the calcium

balance of mitochondria [347, 348]. Moreover, 3OC12-HSL reduces inflammatory responses in macrophages by modulating the NF-κB pathway resulting in a reduction of TNF-α and an increase in IL-10 production [349, 350].

Host can monitor quorum sensing of *P. aeruginosa.* Many bacteria can secrete pigmented products, such as phenazines, a downstream virulence factor from *P. aeruginosa.* Pedro *et al.* demonstrated that the pigmented virulence factors phenazines as ligands can be recognized by the aryl hydrocarbon receptor (AhR), which is known as a ligand-dependent transcription factor capable of sensing environmental toxins. They also found that AhR can recognize another bacterial-pigmented virulence factor, namely the naphthoquinone phthiocol from Mycobacterium tuberculosis [351]. This team also characterized that AhR can detect and quantify *P. aeruginosa* QS molecules and the relative abundances of bacterial cells, in turn, eliciting and coordinating diverse immune defenses against the infection by *P. aeruginosa,* in human cells, zebrafish, and mice. It was proposed that AhR is a master regulator of host defense responses with a crucial role in tuning immunity according to the infection stage [352].

2.5 *Pseudomonas aeruginosa* infection models

As summarized above, *P. aeruginosa* owns remarkable nutritional versatility and metabolic flexibility, numerous virulence factors, and sophisticated regulation systems, and these properties allow *P. aeruginosa* to infect a wide range of hosts, including humans, murine, insects, nematodes, and plants.

Murine models

Murine models are the most developed animal models, given they are small animals and have similarities to humans in their genetic makeup, anatomical structures, and physiological functions, which establishes murine models as a good surrogate host to study bacterial diseases. There are several murine infection models of *P. aeruginosa*, including acute lung infection [353], chronic lung infection [354], cystic fibrosis lung infection (CFTR mutation mice) [355], ischemic wound [288], acute burn wound [125], chronic surgical wound infection [125].

The nematode *Caenorhabditis elegans*

Caenorhabditis elegans (C. *elegans*) known as a powerful genetic tool, with a short generation and easy to maintain, has been used to build convenient infection models to have a quick look at the pathogenicity of *P. aeruginosa.* Five different *P. aeruginosa* infection models of *C. elegans* have been established: the fast/slow killing assay, the lethal paralysis assay, the liquid killing assay, and the red death assay [356]. It was reported that *C. elegans* infection models were utilized for large-scale screening of the transposon mutant library of PA14, covering 80% of the genome, and 170 unique genes of PA14 including both known and novel virulence factors identified required for maintaining normal levels of virulence [107]. The immune responses of *C. elegans* to *P. aeruginosa* in the intestine and its toxins have been extensively studied [357]. However, *P. aeruginosa* pathogenicity is mostly limited to the gut [358].

***Drosophila melanogaster* infection models**

Drosophila was often used as a living test tube by microbiologists without taking the host and its defenses into account until this organism was found to be endowed with an essentially evolutionarily conserved innate immune system. It was originally confirmed by the discovery of the Toll pathway in *Drosophila* in *the* 1990s, which was a guide for the characterization of Toll-like Receptors as key Pattern Recognition Receptors in mammals. At present, *Drosophila melanogaster* is one of the best-understood organisms, given its general advantages as a model organism and the genomics approaches. Compared to *C. elegans, Drosophila* has a well-known and conserved innate immune system, especially at the level of its two NF-κB signaling pathways that are similar to those of mammals. Compared to mice, flies are more practical, especially for directed or random mutagenesis at a genome-wide scale. Thus, as for studying host-pathogen interaction, *Drosophila* possesses the advantages of *C. elegans* and murine, as well as its own properties, and this makes it a relevant model organism. Typically, the infection *Drosophila* models can be classified into two categories by the infection routes:

Septic injury infection. The septic injury model is performed by direct inoculation of bacteria into the body cavity using injection or needle pricking with the bacteria normally in PBS solution. Several studies have shown the consistency and stabilization

of this model. Flies injected with *P. aeruginosa* cells of less than 100 CFU died of bacteremia within 2-4 days [56, 118]. Upon injection, bacteria directly introduced into the body cavity bypass the physical barriers and local immune defenses, the initial steps of a natural route of infection (*e.g.*, through the intestinal or tracheal barrier) which may influence the subsequent behavior of the bacteria within the organism [118, 359].

Oral infection/intestinal infection. In this model, flies are inoculated with bacteria by feeding. The infection course and the outcome are distinct according to the fluid ingredients, feeding duration, pre-treatment, treatment during infection, and temperature. Bacteria concentration and quantity, in a wide range (from OD of 0.1 to OD of 10), seems not to make a big difference in the pathogenicity. In contrast to the fast pathogenicity kinetics of the injection model, flies normally succumb in a week to oral infection with *P. aeruginosa* that needs to cross the intestinal barrier before provoking bacteremia [359]. Some intestinal infections of *P. aeruginosa* in *Drosophila* are summarized in the following table.

Table 2. *P. aeruginosa* intestinal infection models in *Drosophila*

Infection model	Procedure	Pathogenicity & Host response
Intestinal infection (Chugani,2001) [280]	-PAO1 wt, Δ*qscR* -8X10^9 CFU in 170 µl 5% sucrose - filter disk on the agar surface -pr-starvation for 5 h, 25 ℃ -male (Canton S)	Δ*qscR* was more virulence than wt PAO1
Intestinal infection (Erickson,2004) [360]	-PAO1 wt, Δ*relA* -culture in LB -pre-starvation for 5 -bacteria in 5% sucrose - filter disk on 5% sucrose agar -28 ℃ -male (Oregon R)	Δ*relA* displayed reduced virulence. (Δ*relA*, mutant that cannot production of ppGpp and pppGpp under starvation of amino acid)
Polymicrobial Infection (Sibley，2008） [344]	-PAO1 strain, OF (oropharyngeal flora) from CF patient -5% sucrose -3 hours starvation -Male flies only	-Crop damages -3 classes of OF: I. OF only can kill flies. II. OF only is avirulent, not affect the infection of PA. III. OF alone is avirulent but combined with PA promotes the killing.
Intestinal dysplasia Model (Apidianakis,2009) [361]	- PA14 -10% LB and 90% sucrose (5%)	-JNK pathway & apoptosis of enterocytes -Proliferation of intestinal stem cells (SCs)
Intestinal Model with intestinal malignancies [362]	- PA14 in 4 % sucrose, - 29℃ - Ras1V12 oncogene mutant flies	-basal invasion and dissemination of hindgut cells to distant sites upon infection
Biofilm model of infection (Mulcahy,2011) [363]	-PAO1 in 5% sucrose. -OD of 25,0.12ml, -filter on the 5% sucrose agar -pre-starvation for 3 hr,	- most bacteria located in the crop - Biofilm formation in the crop -higher bacterial load at D2 than

	- male flies, 26℃ -Collect hemolymph by centrifugation of whole flies	it at D5 -non-biofilm forming strain ΔpelB is more virulent.
Exotoxins-hemocytes-interaction Model (Avet-Rochex,2005) [364]	-clinical cystic fibrosis isolate CHA wt； ΔexsA -OD of 0.2 in 5% sucrose, 2ml, -refresh every 3 days -absorbent paper -UAS-exoSGAP flies	-ExoS secreted from PA blocks phagocytose by inhibiting Rho GTPase -ΔexoS PA shows less virulent -virulence of ΔexoS is restored in the flies with transgenic exoS
Chronic oral infection (Limmer,2011; Haller,2018) [56, 89]	-PA14 wt； mutants (eg. Δ*rhlR*； Δl*asR*; Δ*rhlI)* - OD of 0.25 in sucrose (50mM) with 10% BHB; 2ml -absorbent pads; 25 ℃	- Bacteria predominantly in the midgut; *rhlR* is key for virulence -systemic bacterial load in the late infection phase, corresponded with the humoral immune response (5 days) -cellular & humoral immune response required for defense

PhD Objectives

In the 1990s, the discovery of the Toll pathway and its connection to the production of antimicrobial peptides (AMPs) in *Drosophila* revealed the evolutionarily conserved innate immune system and was the prelude of host-pathogen-interaction studies in this small organism [8, 9]. Subsequently, local immune responses in epithelial tissues such as in the digestive tract, trachea, and surface barrier were confirmed by transgenic fluorescent reporters of AMP gene expression [72, 73]. Thereafter intestinal infection models were introduced into the studies of the host-pathogen interactions in *Drosophila*, for example, the *S. marcescens* intestinal infection model, which was initially developed by our team in France [75]. An interesting observation is that as few as five *S. marcescens* directly injected (septic injury) in the fly hemocoel cause a rapid killing within 24 hours, whereas the ingested bacteria in the gut kill the host at a much slower speed (more than ten days), even though some bacteria have managed to cross the gut barrier in a couple of hours. The phenomenon of distinct bacterial virulence according to the infection routes was also observed in the *P. aeruginosa* intestinal infection model. In this model, ingested *P. aeruginosa* bacteria were detected in the fly hemocoel but did not induce a strong systemic immune response; they caused a slow killing over a week, while septic injury infection caused a fast killing within a couple of days. Previously, our colleagues Limmer S, Haller S, et al. identified the RhlR regulator as playing a key role in the pathogenicity in the *P. aeruginosa* (PA14) in the intestinal infection model and endowing the bacteria to elude host phagocytosis, in a way independent on the signaling circuit component RhlI [89, 289]. They also did a small screening of 384 PA14 mutants in the intestinal infection models and found most of the well-known virulence factors are not necessary for the virulence. In this context, our team studies further how the ingested *P. aeruginosa* bacterium adapts itself to the hostile host environment and how it regulates its virulence in the chronic intestinal infection model. About four years ago, I worked as a research assistant with our colleague Dr. Jing Chen, and she at that time was developing a latent infection with *P. aeruginosa* (PAO1) based on transient intestinal infection; she found that the ingested bacteria that have crossed the gut, were possibly dormant in the tissues, which was further characterized in Chapter I.

Standing on the shoulder of the contributors for the above work, my PhD study aims at

exploring whether there is a virulence switching program of the ingested *P. aeruginosa* (PA14) associated with tissues during the continuous oral/intestinal infection and to confirm whether RhlR signaling is playing a critical role in the virulence regulation in different infection *Drosophila* models with distinct pathogenic characteristics. Furthermore, I aimed to figure out whether RhlR relies on its signaling components RhlI/PqsE in modulating the virulence program in the context of the *Drosophila* host in the presence or absence of its immune defenses.

Chapter I

Melanization plays a key role in the establishment of a latent infection by *Pseudomonas aeruginosa* in the *Drosophila melanogaster* host

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Statement: Most of the data of this chapter were contributed by Dr. Jing Chen. I contributed to the data of figure S2B, figure S2C, and figure 3 F. We put this manuscript as Chapter I, since it is fundamental part for the Chapter II and Chapter III, and is important to understand the study of other two chapters.

Author Contributions: JC, GL, KM, SL, ZL, and DF designed, performed and analyzed experiments. JC and DF wrote the manuscript with inputs from all authors.

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Abstract

The pathogenicity of pathogens often depends on the route of infection. For instance, *P. aeruginosa* or *S. marcescens* cause acute systemic infections when low numbers of bacteria are injected into *D. melanogaster* flies whereas flies succumb much slower to the continuous ingestion of these pathogens, even though both manage to escape from the gut compartment and reach the hemocoel. Here, we have developed a latent *P. aeruginosa* infection model by feeding flies on the bacteria for a short period. The bacteria stably colonize internal tissues and, in most cases, do not cause any symptoms; thus, latently-infected flies live almost as long as noninfected control flies. Interestingly, the dormant bacteria display particular characteristics in terms of bacterial and bacterial colony morphology, the composition of the outer cell wall, and motility. We show that melanization but not the cellular or the systemic humoral response are required to establish latency, in contrast to the situation in acute infection or continuous ingestion models. The activation of the melanization responses in latently-infected flies likely accounts for an added degree of protection of the host against a variety of supernumerary injected bacterial or fungal pathogens. This model will be useful to investigate the host-pathogen interactions that regulate the expression of virulence programs in pathogens and the specific host defenses relevant to contain pathogens depending on their route of infection.

Keywords: *Pseudomonas aeruginosa*; *Drosophila* melanogaster; latent infection; humoral immune response; melanization

Chapter Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that can cause acute or chronic infections in immune-compromised individuals and patients suffering from AIDS, burn wounds, cystic fibrosis, or chronic obstructive pulmonary disease [365]. Most acute *P. aeruginosa* infections can be controlled by antibiotic drugs and immune responses but persister cells may tolerate the treatment. Moreover, these resilient bacteria cells can lead to the relapse of the bacterial infection when the host becomes weak, or when the antibiotic treatment is discontinued [145]. Many studies attempted to identify the mechanisms of persister cell formation focusing on bacteria cells per se, such as toxins liberated from toxin-antitoxin systems resulting in growth arrest, or metabolic regulation and protein homeostasis implicated in diauxic shift, reduced production of ATP. Also, the extracellular environment plays a vital role to trigger persister cell formation due to ineffective clearance by the host or drugs, as bacteria cells have evolved to elude host immunity in different ways [153]. Mostly, bacterial persister cell formation is the result of host immunity restricting bacteria proliferation but not powerful to eliminate them. Whereas many leads are being investigated, the definitive *in vivo* mechanism remains rather poorly understood [153]. Thus, it is important to know how host immunity works to recognize and control bacteria, not only by killing them but also by influencing their physiology and the implementation of virulence programs.

Drosophila melanogaster is an ideal animal model to study the innate immune response since it lacks a mammalian-like adaptive immune system. It has been used to study the pathogenicity of *P. aeruginosa* for more than 50 years [366]. In flies, immunity against Gram-negative bacteria relies on three arms: i) the systemic humoral immune response regulated by the NF-κB-type Immune deficiency (IMD) pathway producing antimicrobial peptides [40], phagocytosis mediated by mammalian macrophages-like plasmatocytes [367], and melanization that results from the activation of dedicated serine proteases cascades [30]. Melanization involves a series of enzymatic reactions that are catalyzed by phenol oxidases, usually found as pro-proteins, prophenoloxidases (PPOs). PPOs are cleaved into active POs by proteases such as Hayan, supposedly required for the activation of the three *Drosophila* PPOs, or Sp7. In larvae, PPOs are

stored in crystal cells and are released in the hemolymph by a JNK-dependent rupture of their cytoplasmic membranes. In adults, the situation is less clear. Whereas PPO2 is expressed in some 8% of adult hemocytes, it is not clear whether PPO2 forms crystals as observed in larvae. Furthermore, PPO1 and PPO2 are found circulating in the hemolymph. They can mediate melanization at the site of wounding upon proteolytic cleavage of their Pro-domains and are also required for a microbial killing activity that remains poorly understood. *P. aeruginosa* is strongly virulent in acute systemic infection models and a few bacteria injected into the fly body cavity and its open circulatory system kills host flies within a few days despite the induction of a strong humoral systemic immune response mediated by the IMD pathway and a second NF-κB pathway, the Toll pathway [206].

Flies in their natural environment feed on rotted fruits, a microbe-rich environment in which *P. aeruginosa* usually survive in well. A continuous feeding infection model was established in *Drosophila* [359]. *P. aeruginosa* was retrieved very early on from the hemolymph, implying that some bacteria can cross rapidly the gut barrier. Interestingly, *P. aeruginosa* displayed impaired pathogenicity and flies succumbed in about a week [89]. More importantly, the bacterial titer in the hemolymph remained at a steady low level until mid-infection and then started increasing exponentially, leading to the rapid demise of the flies that succumbed to the bacterium [56]. Several immune responses contribute to controlling ingested *P. aeruginosa* pathogenicity, such as IMD pathway activation in the gut, hemocytes, and fat body a composite analogue of the fat liver and adipose tissue. The cellular immune response limits the proliferation of bacteria that have crossed the gut barrier by elimination through phagocytosis [89]. Based on this, we do not definitely know whether the steady bacterial load in the hemolymph at the beginning is due to a balance between bacteria entrance and killing, although this is highly likely. Moreover, a role for intestinal bacteria in participating in the ultimate killing of the host cannot be fully discounted, for instance, if the gut barrier were disrupted at a late stage of infection.

Here, we have attempted to simplify the continuous ingestion infection model by allowing flies to feed on *P. aeruginosa* (PAO1) for only two days, a period during which the bacteria that have crossed the gut barrier appear to be relatively quiescent and easily

controlled by the immune system. We report that even though it is rapidly cleared from the hemolymph within three days, other escaping *P. aeruginosa* bacteria manage to colonize the host tissues and remain dormant for the lifespan of the fly causing hardly any damage unless sporadically reactivated. We study the different features of these dormant bacteria and establish that the *Drosophila* immune systems play an essential role in the establishment of quiescence in tissue-associated bacteria, with a major role played by melanization through cleaved PPO2, Hayan, and Sp7.

Results

1. Establishment of a *P. aeruginosa* latent infection model in *Drosophila*

Whereas our usual intestinal infection model relies on continuous feeding on a pad containing PAO1 in a sucrose/BHB solution, we attempted to expose the flies only for a limited period to the bacterially-laced solution at 18°C. To ensure that no bacteria remained in the gut, this initial period of feeding on the bacterial solution was followed by a 4-day period during which flies were fed on a gentamicin sucrose solution. Afterward, the flies were kept also at 18°C on a pad soaked with a sucrose solution **(Fig. 1A)**. Whereas flies feeding continuously on PAO1 usually succumb within 10 days at 18°C **(Fig. S1A)**, flies on this new regimen of a short period of ingestion of PAO1 survived much longer, with an LT50 (time taken by 50% of the flies to succumb to infection) of about 25 days. They actually survived this treatment almost as long as sucrose-only controls albeit they did succumb significantly earlier **(Fig. 1B)**. We then checked the microbial burden in the hemolymph of flies exposed for two days to PAO1 since *P. aeruginosa* is known to cross the gut barrier within hours [56, 89]. Interestingly, we detected tens of bacteria during the first three days of the protocol before the hemolymph titer progressively decreased to zero (Fig. 1C). Next, we checked whether the bacteria might have colonized the tissues by measuring the titer in whole flies or carcasses. Whereas the difference between whole flies and carcasses differed by more than 10-fold in the first two days of infection when flies were feeding on PAO1 reflecting the presence of bacteria in the gut lumen, the difference was much less

pronounced later on **(Fig. 1D)**. We checked using GFP-labeled PAO1 that the bacteria were actually cleared from the gut lumen by the gentamicin treatment. Thus, these data establish that a few thousand bacteria do colonize the fly tissues after having crossed the gut barrier during the first two days of the infection protocol. The bacterial burden appeared to remain stable in most flies throughout their lives. Of note, bacteria can also associate with the crop and gut compartments, but from the outside of the gut **(Fig. S1B)** [75]. We were actually able to detect bacteria under the fly cuticle either by DAPI staining **(Fig. 1E)** or using antibody staining **(Fig. 1F)**. We also monitored the induction of the systemic immune response by monitoring the steady-state levels of *Diptericin* mRNAs by RT-qPCR. While we detected a significant induction in the first few days of the infection that eventually subsided **(Fig. 1G)**, the level of induction was much lower than that observed during a systemic infection **(Fig. S1C)**. By monitoring transgenic flies that express a GFP protein under the control of the *Diptericin* promoter, we noted that a few flies occasionally exhibited a signal as strong as that induced during a systemic immune response induced in a septic injury model **(Fig. S1D)**. Strikingly, the p*Dipt*-GFP-positive flies were fated to die shortly after the detection, suggesting that in those flies, a full-blown bacteremia was underway. In contrast, the p*Dipt*-GFP-negative flies survived suggesting that PAO1 does not proliferate in those flies. Thus, the virulence of PAO1 can be spontaneously reactivated in a few flies. We conclude that under conditions of limited ingestion, PAO1 colonizes fly internal tissues without causing a systemic infection and therefore refers to this model of infection as a latent infection model **(Fig. 1G)**: the bacteria present in the hemocoel appear to be dormant.

2. PAO1 bacteria display distinct properties depending on the infection route

Given the strikingly different properties of virulence displayed by PAO1 depending on the infection route (injection (death within a couple of days) *vs*. ingestion) and the exact protocol (death in some 7 days upon continuous feeding on PAO1 *vs*. some 40 days in the latent infection model), we checked whether differences in bacterial morphology could be detected. To this end, we first put flies to feed on RFP-expressing PAO1 bacteria and two days thereafter injected GFP-expressing PAO1 in the body cavity (hemocoel). Under the cuticle, the injected green bacteria appeared elongated and much slenderer than the ingested red bacteria that appeared plumper **(Fig. 2A)**. These observations were confirmed by electron microscopy (insets **Fig. 2A**). We also assessed whether PAO1 bacteria displayed the O5 LPS antigen by staining bacteria in situ using

a specific antibody. Injected PAO1 bacteria found in the tissues or retrieved from the hemolymph were expressing the O5 antigen **(Fig. S2A)**. In contrast, ingested RFP-expressing PAO1 bacteria adhering to tissues did not express the O5 antigen. Interestingly, the few bacteria that are found in the hemolymph at the onset of the intestinal infection did express the LPS antigen **(Fig. S2A).**

When plated, the colonies harbored also distinct shapes. The bacterial colonies derived from injected bacteria appeared to be larger with indistinct boundaries and a tendency toward clustering with other colonies. In contrast, bacteria retrieved from the carcass of latently-infected flies appeared smaller with well-delimited contours. PAO1 grown in BHB liquid medium yielded colonies of intermediate sizes with fuzzy shapes **(Fig. 2B)**.

We next tested the motility of PAO1 bacteria in a swimming assay: whereas bacteria that had been injected and retrieved from the *Drosophila* host were motile, the ones extracted from latently-infected flies remained immobile **(Fig. 2C)**.

Dormant bacteria have been reported to be more tolerant to antibiotic treatment than metabolically active bacteria. We therefore first treated flies that had been injected a day before with PAO1 by injecting either PBS, tobramycin, or levofloxacin. The injected bacteria were clearly sensitive to tobramycin and somewhat sensitive to levofloxacin **(Fig. 2D)**. In contrast, when flies in which the latent infection had been implemented six days earlier were injected with antibiotics, the bacteria that had colonized the fly tissues appeared to tolerate well both antibiotics **(Fig. 2D').**

One possibility to account for these diverse observations would be that bacteria are selected during the establishment of the latent infection and somehow lose the ability to express their virulence programs. We therefore collected bacteria from latently-infected flies at different time points, either from hemolymph (very few bacteria) or retrieved from the crushed carcass, and then injected them directly into naive recipient flies. The flies injected with bacteria passaged in the fly using the latent infection protocol killed the naive flies very rapidly, at a pace that was almost as fast as when flies were directly injected with bacteria grown in BHB liquid culture **(Fig. 2E-E', Fig. S2B-C)**. The slightly impaired virulence observed with bacteria retrieved from hemolymph might reflect the lower number of bacteria that were collected whereas 100 liquid-culture bacteria were injected for the control.

3. The host innate immunity is required to prevent the pathogenicity of PAO1 in the latent infection model

The outcome of an infection results from the interactions between the pathogen and its host. We first monitored the survival rates of fly strains deficient for the diverse arms of the *Drosophila* immune response that ingested PAO1 under the latent infection protocol. Flies deficient either for the cellular immune response due to an absence of the Eater phagocytic receptor or flies in which the IMD pathway cannot be activated succumbed faster than wild-type flies, with a LT50 of 20 days (*vs*. about 30 days for wild-type flies) **(Fig. 3A)**. This increased virulence of PAO1 in immuno-deficient flies was mirrored in the bacterial burden measured on collected hemolymph from the second day onwards and became highly significant on the 10th day. It is likely that flies harboring a high PAO1 hemolymph titer are fated to die rapidly on the following day(s). We tested several mutant lines affecting melanization, which all displayed a greatly increased sensitivity to ingested PAO1 in the latent infection model with the exception of the Δ*PPO1* strain for which only 50% of the flies succumbed to the infection within two weeks **(Fig. 3C, FigS3A)**. The Δ*PPO2* (as well as Δ*PPO1*Δ*PPO2*) and *sp7* mutant lines exhibited the highest susceptibility with a LT50 value of respectively four and five days. Unexpectedly, *hayan* flies were somewhat more resistant and reached their LT50 by about eight days. Interestingly, the bacterial load in the hemolymph of *PPO* mutants hardly increased **(Fig. 3D)**. In contrast, the bacterial burden in the carcass was significantly higher already on the first day of infection for Δ*PPO2*. On day two, both *PPO* mutants displayed a higher PAO1 load in the carcass that was no longer observed at day five. Note that in this case, the correlation between measured bacterial load **(Fig. 3E)** and susceptibility to infection assessed in survival experiments was imperfect since Δ*PPO1* mutants do not succumb as fast as Δ*PPO2* in this two-day interval (**Fig. 3C, FigS3A)**. The systemic immune response becomes activated when the bacteria start proliferating in the hemocoel [56, 75, 89]. We observed that the expression level of *Diptericin* was increased in the *PPO* mutants from the third day onwards **(Fig. S3B)**, in keeping with the increasing bacterial load measured in the carcass **(Fig. 3E)**.

We further directly tested whether melanization is indeed activated during the latent infection. Melanization results from the activation of a proteolytic cascade that ultimately cleaves prophenoloxidases into active phenol oxidases. We therefore tested by Western blot whether the cleaved form of PPO2 was detectable in the hemolymph of flies submitted to varied immune challenges. Whereas we detected a limited partial

cleavage of PPO2 after injection of PAO1 or a cleavage of about 50% of PPO2 at day two of the ingestion of PAO1, PPO2 was nearly fully cleaved at day six of the latent infection **(Fig. 3F)**. We also monitored the expression levels of *hayan* and *sp7* transcripts by RT-qPCR. Whereas both genes were induced one or two days after a PAO1 injection **(Fig. S3C)**, such an induction was not detected in the early days (one to three) of the latent infection model **(Fig. S3D).**

In conclusion, our data suggest that the low levels of IMD pathway activation **(Fig. S1D)** prevent the proliferation of bacteria in the hemolymph whereas melanization inhibits the growth of PAO1 adhering to tissues **(Fig. 3F)**.

4. A secondary acute PAO1 infection is mitigated when occurring in latently infected flies

In the *S. marcescens* Db11 intestinal infection model, a secondary injection with Db11 leads to a fast death in which resident bacteria are reactivated and participate in the fast killing of the host [75]. We have tested whether a similar phenomenon occurs with PAO1 by injecting RFP-expressing PAO1 in flies that are at the end of the GFP-expressing PAO1 ingestion period **(Fig. 4A)**. Survival experiments showed that such flies succumbed much faster to this protocol than latently-infected flies. However, these flies died significantly slower than naive flies injected with the same PAO1 inoculum **(Fig. 4A')**. This difference was mirrored in the bacterial load measured in the hemolymph: whereas injected naive flies exhibited a higher overall PAO1 burden from day one after PAO1 injection onwards, latently-infected flies displayed a similar behavior only three days after the injection of PAO1 (that is day five of the protocol shown in Fig. 4A**) (Fig. 4B)**. In contrast, the bacterial burden in the carcass increased from day one of injection onwards for both naive and latently-infected flies, albeit the titer was always significantly higher in the naive injected flies **(Fig. 4C)**. We next asked whether the resident GFP-expressing PAO1 bacteria remained dormant when challenged by injected RFP-expressing PAO1. As shown in Fig. 4D, the contribution of the green resident bacteria to the bacterial titer in the hemolymph was higher than that of the red injected bacteria. In noninjected flies, the median hemolymph titer is of 4 bacteria **(Fig. 1C)**, which suggests that the resident bacteria are either proliferating in the hemolymph or are released from the tissues. We therefore monitored the differential burden in the carcass and found that the injected red bacteria were proliferating at a faster pace than green resident bacteria **(Fig. 4E)**. Taken together, these data establish

that the resident bacteria in latently-infected flies directly or indirectly hamper the full pathogenicity of injected PAO1. Conversely, the injection of the BHB-cultured PAO1 bacteria did reactivate to some extent the proliferation of the dormant bacteria.

5. Differential degrees of protection conferred by ingested bacteria depending on the compartment of residence

In the above experiments, the secondary injection challenge was performed on day two, that is when the PAO1 bacteria are found in the gut lumen and crop and also in the hemocoel. To assess which compartment confers protection, we first tested the gut compartment by feeding the flies with killed PAO1 that are unable to cross the gut barrier. When flies were fed heat-killed bacteria, the protection afforded by the dead bacteria was modest yet significant when compared to that provided by live bacteria, likely a few hours for the LT50 **(Fig. 5A)**. Bacteria killed using UV-treatment or chemical fixation with paraformaldehyde conferred a higher degree of protection for the former with about a day in terms of LT50s than for the latter with half a day **(Fig. S4A-B)**. Feeding the *Pectobacterium carotovorum Ecc15* strain that does not cross the intestinal barrier in adults [368] provided a degree of protection similar to that of heat-killed PAO1. We next asked whether the protection afforded by UV-killed PAO1 was mediated by the host immune defenses. **Fig. 5B** shows that the IMD pathway-mediated immune response and not Eater is required for the protection against secondarily injected PAO1. The degree of induction of the IMD pathway monitored by measuring the degree of Diptericin expression induced by both the live and heat-killed PAO1 were similar **(Fig. 5C)** yet modest **(Fig. S1D)**. It appears that this mild induction is nevertheless sufficient to protect to a significant degree the flies that have ingested PAO1 from a supernumerary acute infection.

Next, we tested the contribution of bacteria colonizing the hemocoel by injecting PAO1 secondarily at stages for which the gut lumen has been cleared from ingested PAO1, that is at days six or ten of the protocol. As apparent from Fig. 4D, the protection was much higher when flies were secondarily challenged at day ten than at day six, which was in turn higher than that observed for a day two injection. This protection was still observed in eater and key mutants **(Fig. 5E)**. Of note, the apparently higher resistance of eater mutants than key mutant flies fed on sucrose to a PAO1 injection cannot be directly compared as the genetic backgrounds of these flies differ. Thus, bacteria residing in the hemocoel may protect the host somewhat from a secondary acute

infection by stimulating another arm of the innate immune response. Alternatively, these bacteria may communicate with the incoming bacteria and modulate the expression of their virulence programs.

6. Melanization triggered during the establishment of the latent infection may protect the host from supernumerary acute infections

As the protection conferred by ingested PAO1 against a secondary PAO1 acute infection is highest when the challenge is performed at day six, we investigated whether this protection would be active also against other pathogens. We thus tested by injection a somewhat pathogenic strain of *S. marcescens*, *Enterococcus faecalis, Metarhizum robertsii, Listeria monocytogenes*, and *Candida albicans*. The prior establishment of a PAO1 latent infection conferred a significant degree of protection against all of these infections **(Fig. 6A-C, Fig. S5 A-B)**.

We have unsuccessfully previously tested the humoral and the cellular immune responses for a role in the protection afforded by PAO1 bacteria that have colonized the *Drosophila* internal tissues **(Fig. 5E)**. We therefore would need to test melanization since it is the arm of the host immune response that plays the most important role in establishing a latent infection rather than a full-blown bacteremia. However, our Δ*PPO1*Δ*PPO2* strain is currently highly sensitive to injury, likely through the uncontrolled proliferation of a member of its microbiota. Thus, we designed an experiment to test nevertheless the role of melanization in theΔ*PPO1*Δ*PPO2* mutants. After two days of feeding on PAO1, Δ*PPO1*Δ*PPO2* or wild-type flies were fed on a sucrose solution containing levofloxacin, to which PAO1 colonizing the tissues are tolerant **(Fig. 2D')**. Next, we challenged these flies by injecting *M. robertsii* conidia. This fungus is not sensitive to levofloxacin. As shown in **Fig. 6D**, wild-type flies treated with levofloxacin were partially protected from the secondary *M. robertsii* challenge. The naive Δ*PPO1*Δ*PPO2* mutant flies fed on sucrose and treated with levofloxacin (not infected with PAO1) succumbed faster to *M. robertsii*, in keeping with independent results (Wenhui Wang, Jianwen Yang, personal communication). Interestingly, the Δ*PPO1*Δ*PPO2* mutants that had initially ingested PAO1 succumbed at a rate that was very similar to that of naive Δ*PPO1*Δ*PPO2* flies. These data therefore suggest that melanization is indeed mediating the protection afforded by PAO1 colonizing the *Drosophila* hemocoel in a latent infection paradigm.

Conclusions

- Establishment of a *P. aeruginosa* latent infection model in *Drosophila*.
- PAO1 bacteria display distinct properties depending on the infection route.
- The host innate immunity is required to prevent the pathogenicity of PAO1 in the latent infection model.
- A secondary acute PAO1 infection is mitigated when occurring in latently infected flies.
- Differential degrees of protection conferred by ingested bacteria depending on the compartment of residence.
- Melanization triggered during the establishment of the latent infection may protect the host from supernumerary acute infections.

Discussion

In this work, we have established a latent infection model in which *P. aeruginosa* colonizes the tissues present within the internal cavity of its *Drosophila* host and mostly does not cause any symptoms except for a spontaneous reactivation of its virulence program in a few flies. Importantly, it relies on a passage of bacteria through the gut, a mostly hostile environment for microorganisms characterized by local chemical and immune defenses. Few bacteria have been shown to be able to escape from the adult gut compartment without significantly damaging it. This is the case of both *S. marcescens* and *P. aeruginosa* that remarkably display a much-reduced virulence in intestinal infection models [56, 75, 89]. Persistent infections are achieved in acute septic injury or injection models with microorganisms of intermediate virulence such as *Candida glabrat*a, *Enteroccocus faecalis, Providencia burhodogranariea, Providencia rettgeri* or *Lactobacillus lactis* [369-372] .However, in most cases, a significant fraction of infected flies succumb to the challenge and a persistent infection is established solely in flies that have managed to control the infection and therefore survived it. Thus, this novel intestinal infection model in *Drosophila* presents unique features. It will be interesting to determine whether *S. marcescens* is also able to silently colonize host tissues after escaping from the gut compartment [75].

The bacteria that colonize the hemocoel present phenotypes that are strikingly distinct from those in an acute infection model or in vitro growth such as the shape, exposure of LPS O-antigen, tolerance to antibiotic treatment, and adherence to tissues or motility. Importantly, they do not appear to proliferate. We however cannot formally exclude a slow growth or proliferation that would be counteracted by immune defenses such as the cellular arm of the innate immune response that would result in null net proliferation. We can however exclude a strong proliferation as the *Drosophila* immune system is tuned to sensing peptidoglycan fragments released during the cell wall remodeling that occurs during bacterial growth or division. Whereas we see a modest induction of the IMD pathway during the establishment of the latent infection, we were not able to detect a specific signal using the p*Dipt*-GFP transgenic reporter. The properties of these bacteria suggest that they may be dormant, possibly with reduced metabolic activity and growth since they are tolerant to tobramycin, which targets bacterial protein synthesis, and levofloxacin which inhibits DNA synthesis. Thus, they share many of the characteristics of bacterial persister cells, a minute fraction of a bacterial population

able to tolerate antibiotic treatments [153]. A significant difference however is that a large majority of the bacteria that have managed to reach the hemocoel appear to be associated with tissues and dormant with fewer bacteria being planktonic in the hemolymph and likely eliminated through hemocyte-mediated phagocytosis [56, 89], in contrast to the few persister cells not killed by antibiotic treatments. Although it could be argued that the bacterial burden in the hemolymph would be much higher if phagocytosis were impaired, our data show that affecting hemocyte functions, in three ways, does not cause a very rapid demise of the flies, in contrast to what happens when melanization is impaired. Thus, even though they cause a persistent infection, the bacteria colonizing the fly tissues are not bona fide persisters [153].

The study of immune-deficient flies revealed that the induction of a dormant state results from an active action of the host, most noticeably through the melanization arm of its innate immune system. In the case of persister cells in mammalian infection models, the description of an action of innate immunity in triggering the formation of persister pathogens remains mostly limited to intracellular pathogens undergoing adverse conditions in the phagosomes of macrophages [153]. In the case of *Drosophila*, a humoral immune response is here clearly required.

While an involvement of melanization and PPOs in inducing or selecting dormant bacteria has been clearly established in this work, we currently do not understand several of its features. First, we have so far been unsuccessful in determining whether melanization is required to induce dormancy and its associated reduced virulence or/and whether it is required to maintain dormancy. To address this issue, we used a RNAi transgene targeting *PPO2* and failed to observe a phenotype. We have however not yet validated this transgenic line to establish that it significantly affects the production of PPO2. We note however that when bacteria are reactivated in a few flies (Fig. S1D), the melanization machinery would presumably still be functional but this requires experimental validation. We also need to determine when and for how long we can detect cleaved PO2 in the hemolymph.

It is also not clear exactly where the melanization pathway gets activated as we did not observe any melanized tissues (a melanized hindgut had been observed in p38 *Drosophila* mutants [37]. Some PPO genes such as *PPO1* and *PPO3* are expressed in the naive gut, with a strong expression of *PPO1* in enteroendocrine cells of the gut proximal regions R1 and R2 and a limited expression of *PPO3* in enteroblasts/enterocytes as well as in visceral muscles (http://flygutseq.buchonlab.com)

[373]. It is also not clear how these PPOs would be secreted by gut epithelial cells and whether they would be released apically or basally. Nevertheless, the strongest phenotype is observed with *PPO2* mutants. Since *PPO2* is hardly expressed in the gut, it follows that melanization is unlikely to work in the gut lumen. At present, we hypothesize that bacteria that escape the gut lumen by traversing the intestinal epithelium, either through an intracellular or a paracellular route, may activate the melanization cascade when reaching the hemolymph. How melanization is triggered by Gram-negative bacteria is poorly understood. It has previously been reported that the overexpression of the PGRP-LE peptidoglycan binding-protein triggers the melanization cascade in larvae [16], its role in lack of function has not been clearly established, in as much it is now thought to act as an intracellular sensor of short peptidoglycan fragments [374]. As *P. aeruginosa* can also trigger the Toll pathway in addition to the IMD pathway [206], the proteolytic cascades that activate the Toll pathway likely also trigger the proteases that ultimately process PPOs into active POs [30]. Alternatively, the change that affects the cell wall such as the loss of the O5 antigen may also alter the way PAO1 is sensed by the immune system. In terms of effectors, our experiments document a major role for PPO2 and the Sp7 protease. Previous work with a *Staphylococcus aureus* low inoculum injection model has revealed a requirement of PPO1 and Sp7 for an uncharacterized killing activity in addition to the role of melanization in a classical blackening reaction occurring at the wound site. It is puzzling here that PPO2 and Sp7 mutants share a similar phenotype to prevent the activation of virulence programs, in the absence of a blackening reaction detectable at the macroscopic level. The activation of the PPO cascade is thought to release reactive oxygen species that may mediate their effect on the physiology of PAO1 colonizing host tissues. This possibility needs to be experimentally validated, albeit our initial attempts with vitamin C injection failed to yield a clear-cut phenotype of bacterial virulence activation.

As regards the limited role of the IMD pathway, it is not fully clear whether it plays a role at the level of the gut, of hemocytes, or of the fat body for a systemic response. We have found that this pathway is required to mediate the mild protection against a supernumerary acute PAO1 infection conferred by the prior ingestion of killed PAO1 two days earlier. As this infection by injection is systemic, it suggests that there might be a mild induction of the systemic immune response. Alternatively, hemocytes might also synthesize antimicrobial peptides or serve as a relay for IMD pathway activation

in the fat body [375, 376].

Whereas ingested PAO1 bacteria appear to be more virulent in melanization mutants, they may likely not have been induced to dormancy. In contrast, flies in which the bacterial virulence program is spontaneously or experimentally reactivated (GL, unpublished data) warrant further investigations. For instance, it will be important to determine whether there is a phenotypic switch towards the morphological and physiological forms characteristic of acute infections, in terms of bacterial morphology, O5 antigen expression, sensitivity to antibiotics, tissue adherence, and motility.

The protection induced by the ingestion of PAO1 against secondary systemic infections could in principle be mediated either by communication between the microorganisms or by the induction of immune defenses that incidentally protect against secondary infections. Whereas we cannot formally exclude the former hypothesis even though it would involve communication between *P. aeruginosa* and various bacterial and fungal species, our data with the protection against a secondary *M. robertsii* infection mediated by melanization suggests that the constitutive activation of PO2 likely mediates the protection. This will need to be experimentally confirmed either by using axenic *PPO2* lines (or re-associated with an innocuous microbiota) or by testing hayan and sp7 mutants, which may not be as sensitive to aseptic injury as our *PPO2* and Δ*PPO1*Δ*PPO2* mutant lines currently are.

In this study, we have mostly focused on the host side. It will be interesting to determine on the bacterial side which gene programs are associated with dormancy and later for the reactivation of virulence. In the case of the induction of antibiotics-treatment persister cells, a contribution from toxin-antitoxin systems has been proposed as well as a decreased production of ATP [153]. Since an atypical *P. aeruginosa* quorum-sensing system has been shown to be implicated in a continuous ingestion model in *Drosophila*, the characterization of its potential role in this latent infection model and possibly during reactivation of the virulence is warranted.

Figures

Figure 1

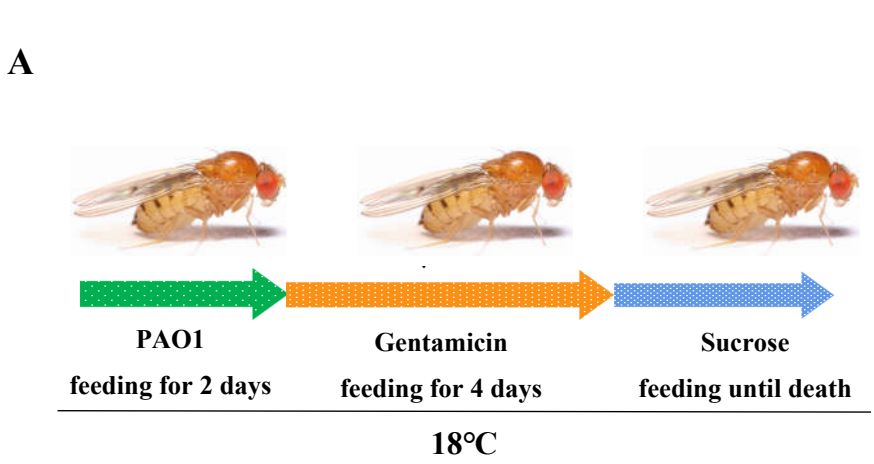


Figure 1

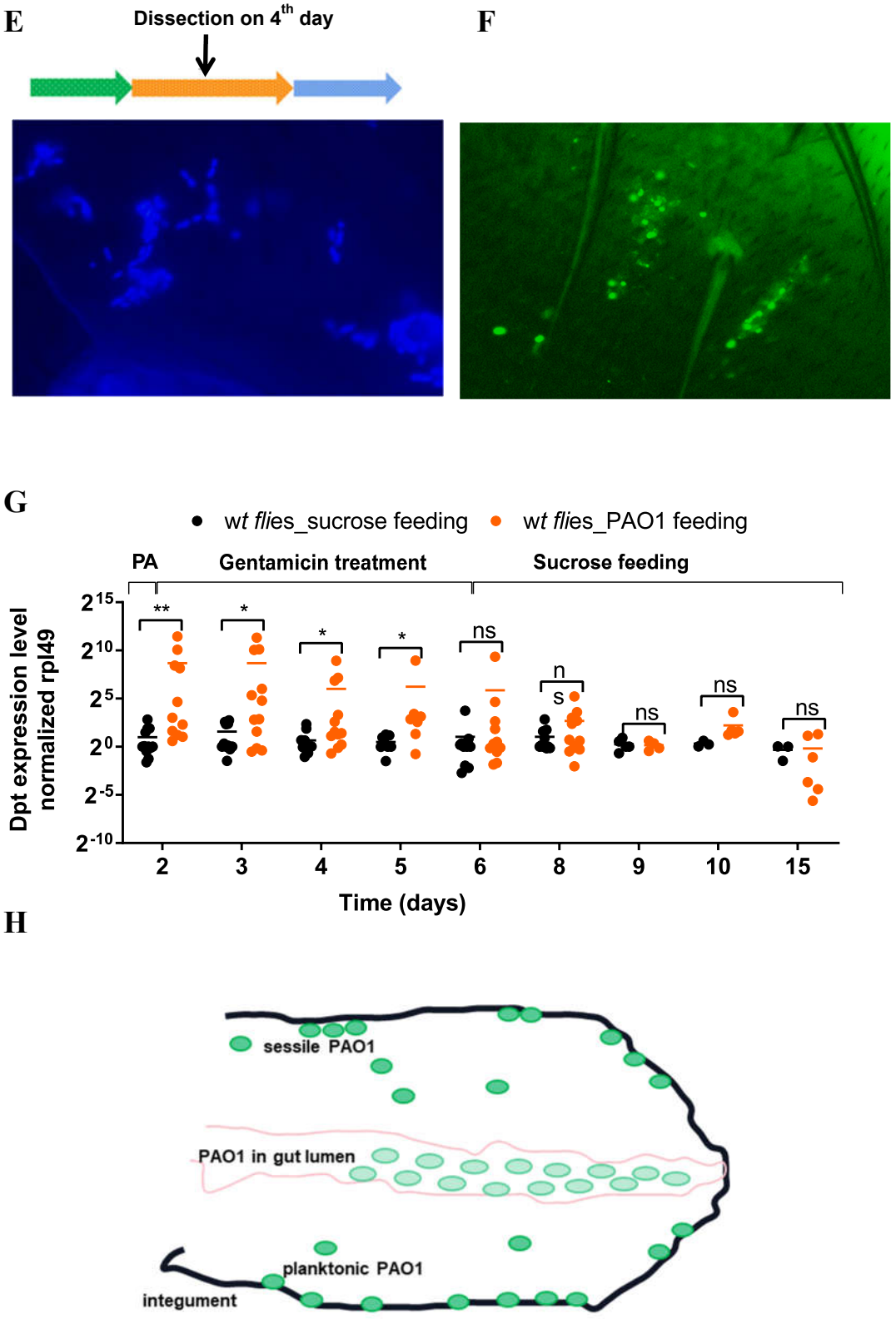

E
Dissection on 4th day
F
G
wt flies_sucrose feeding
wt flies_PAO1 feeding
PA
Gentamicin treatment
Sucrose feeding
Dpt expression level normalized rpl49
Time (days)
H
sessile PAO1
PAO1 in gut lumen
planktonic PAO1
integument

Figure S1

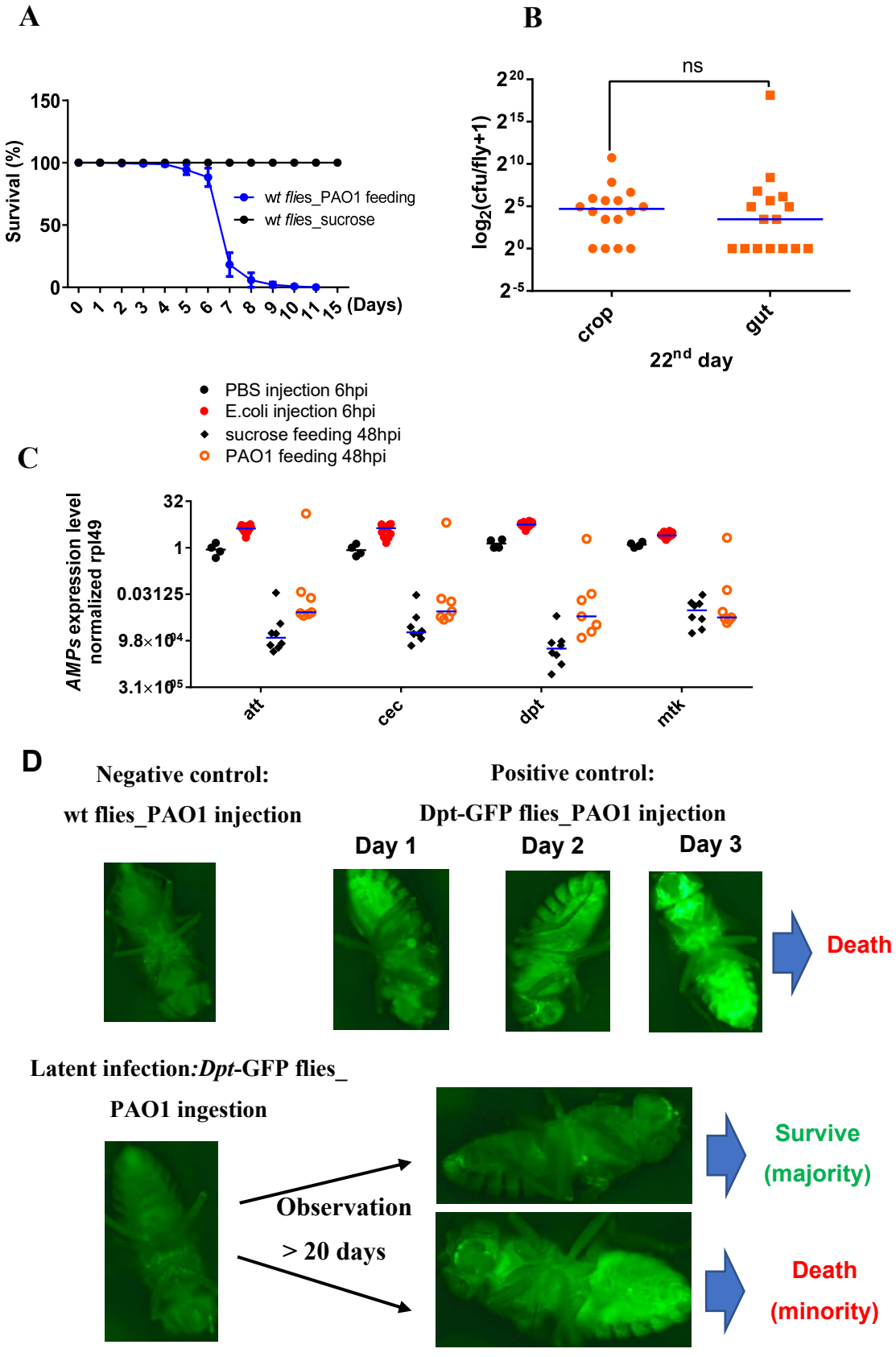

A
Survival (%)
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 15 (Days)
wt flies_PAO1 feeding
wt flies_sucrose
B
ns
log2(cfu/fly+1)
2^20
2^15
2^10
2^5
2^0
2^-5
crop
gut
22nd day
PBS injection 6hpi
E.coli injection 6hpi
sucrose feeding 48hpi
PAO1 feeding 48hpi
C
AMPs expression level normalized rpl49
32
1
0.03125
9.8×10^-4
3.1×10^-5
att
cec
dpt
mtk
D
Negative control:
wt flies_PAO1 injection
Positive control:
Dpt-GFP flies_PAO1 injection
Day 1
Day 2
Day 3
Death
Latent infection:Dpt-GFP flies_
PAO1 ingestion
Observation
> 20 days
Survive
(majority)
Death
(minority)

Figure 1. *P. aeruginosa* escaping from the gut lumen into the body cavity lose pathogenicity and may become dormant in *Drosophila.* (A). Scheme of *P. aeruginosa* latent infection model in *Drosophila*. (B). Survival curves. This experiment was done three times, pooled data are shown (C). Bacterial titer in hemolymph. (D). Bacterial titer in whole fly and carcass. Carcass refers to tissues without the head, gut, ovary and Malpighian tubules. (E-F). *P. aeruginosa* in fly tissue. (E) *P. aeruginosa* PAO1 was visualized by DAPI staining. (F) *P. aeruginosa* PA14 was stained with an antibody raised against it. (F). Activation of the IMD pathway upon ingested *P. aeruginosa* infection as monitored by RT-qPCR of *Diptericin* transcripts. (G). Scheme recapitulating the major features of ingested *P. aeruginosa* infection in *Drosophila*. (B-F). All experiments here were performed three times independently, and data were pooled together (B, C, D, F). Statistical analysis was done by Log-rank (Mantel-Cox test) in (B), by t-tests in (D and F).

Figure S1. Ingested *P. aeruginosa* exhibits impaired virulence in *Drosophila.* (A). Survival curve of *P. aeruginosa* continuous feeding flies. This experiment was done three times independently, pooled data were shown. (B). Bacterial load in tissues of latently-infected flies at late stage. Sixteen flies were dissected to grind crops and guts respectively for CFU counting. This experiment was only done once. Statistics analysis was done by t-tests.(C). IMD pathway activation measured by qRT-PCR. This experiment was only done once. (D). Visible presentation of IMD pathway activation in septic injury systemic infection and latent infection. The flies used for this experiment were *diptericin*-gfp reporter flies, the fluorescence was observed under fluorescence microscope. This experiment was done three times, and one of them was presented.

Figure 2

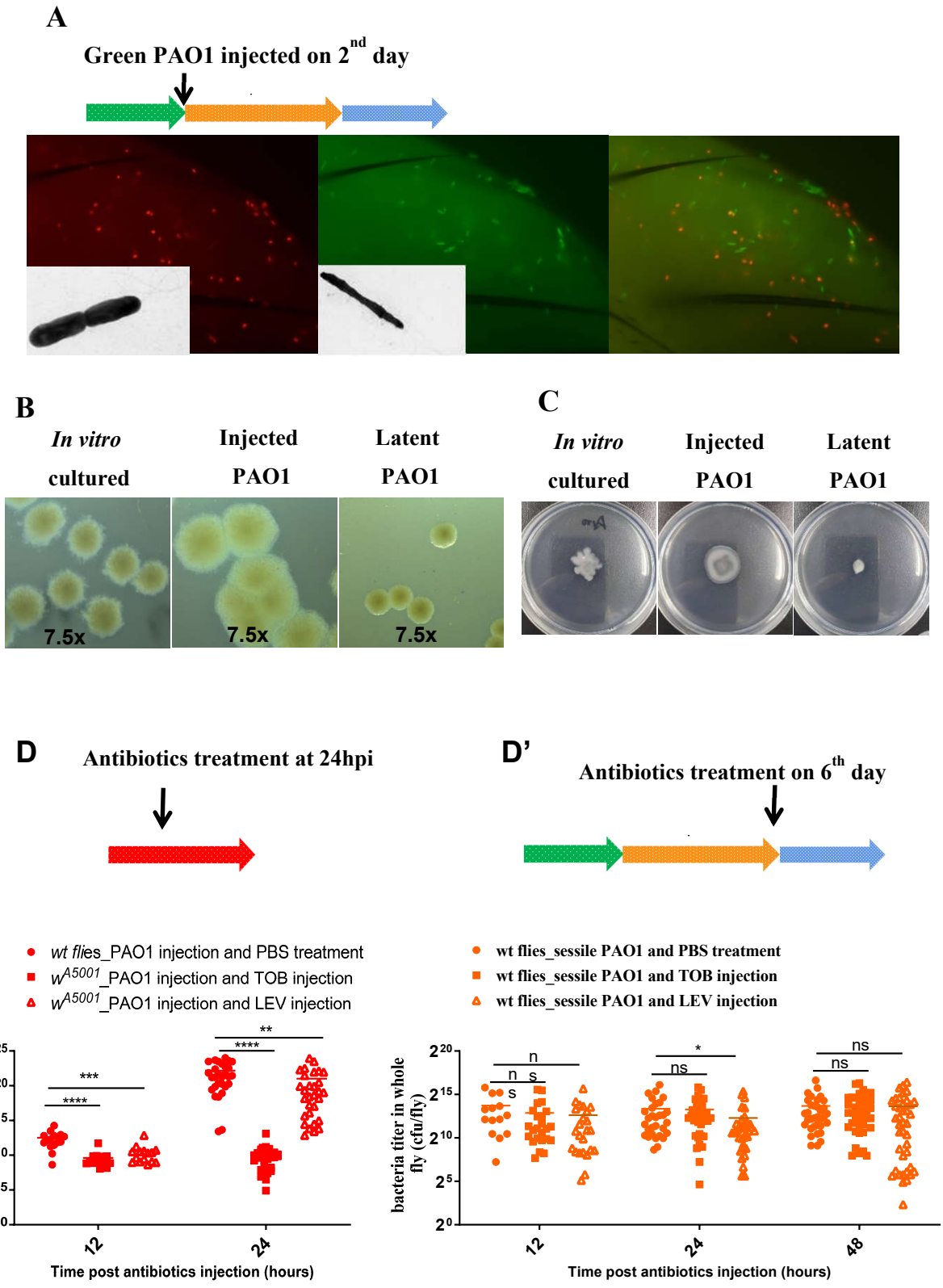

A
Green PAO1 injected on 2nd day
B
In vitro cultured
Injected PAO1
Latent PAO1
7.5x
7.5x
7.5x
C
In vitro cultured
Injected PAO1
Latent PAO1
D
Antibiotics treatment at 24hpi
D'
Antibiotics treatment on 6th day
wt flies_PAO1 injection and PBS treatment
wA5001_PAO1 injection and TOB injection
wA5001_PAO1 injection and LEV injection
wt flies_sessile PAO1 and PBS treatment
wt flies_sessile PAO1 and TOB injection
wt flies_sessile PAO1 and LEV injection
bacteria titer in whole fly (cfu/fly)
Time post antibiotics injection (hours)
ns
*
**
***
****

Figure 2

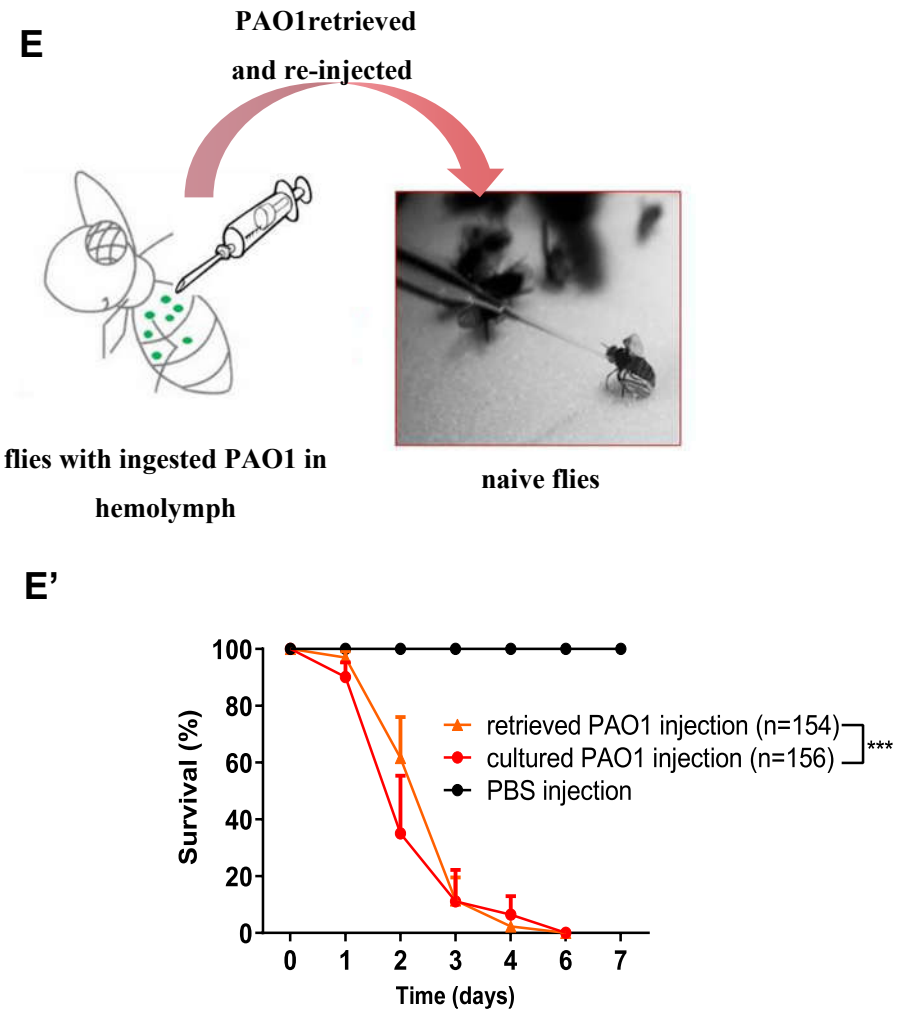


Figure 2. *P. aeruginosa* colonizing tissues in the body cavity exhibits distinct traits from injected ones. (A). Morphology of *P. aeruginosa* in latent infection and injection models. Bacteria morphology was observed by fluorescence microscopy in the same fly and also by Transmission electron microscopy in separately infected flies (Insets). (B). Colonies forming on Congo RED agar plates. (C). Motility assay on low percentage agar plates. (D and D'). Antibiotics sensitivity: bacterial titer of whole flies under the described regimens of treatment. (E). Scheme of pathogenicity experiment for retrieved *P. aeruginosa* from latently infected flies. (E'). Survival curves to injected cultured *P. aeruginosa* (red curve) or *P. aeruginosa* retrieved from latently-infected flies (orange curve). All experiments here were performed three times independently; data were pooled (D, D' and E'). Statistical analysis was done by t tests in (D and D'), by Log-rank (Mantel-Cox test) in (E').

Figure S2

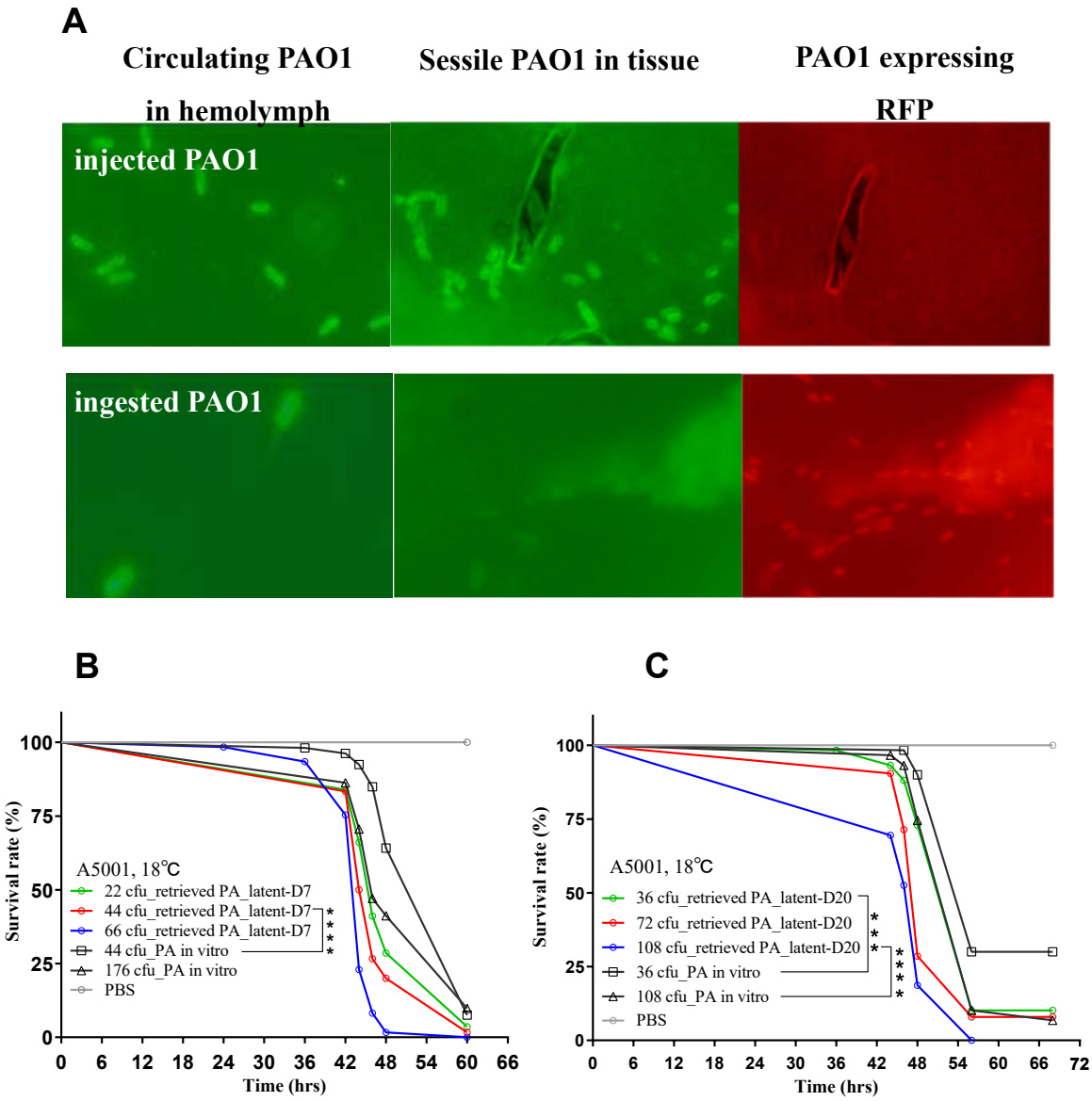


Figure S2. Features of sessile PAO1 in carcass. (A). O5 antigen staining. Hemolymph was extracted and carcass was dissected from flies with PAO1 septic injury systemic infection and latent infection respectively to observe O5 antigen using antibody staining. (B). Pathogenicity of sessile PAO1 in naive flies. *P. aeruginosa* was isolated from carcass of latently-infected flies at different time points and then injected into naive flies. This experiment was done three times and one of them was presented.

Figure 3

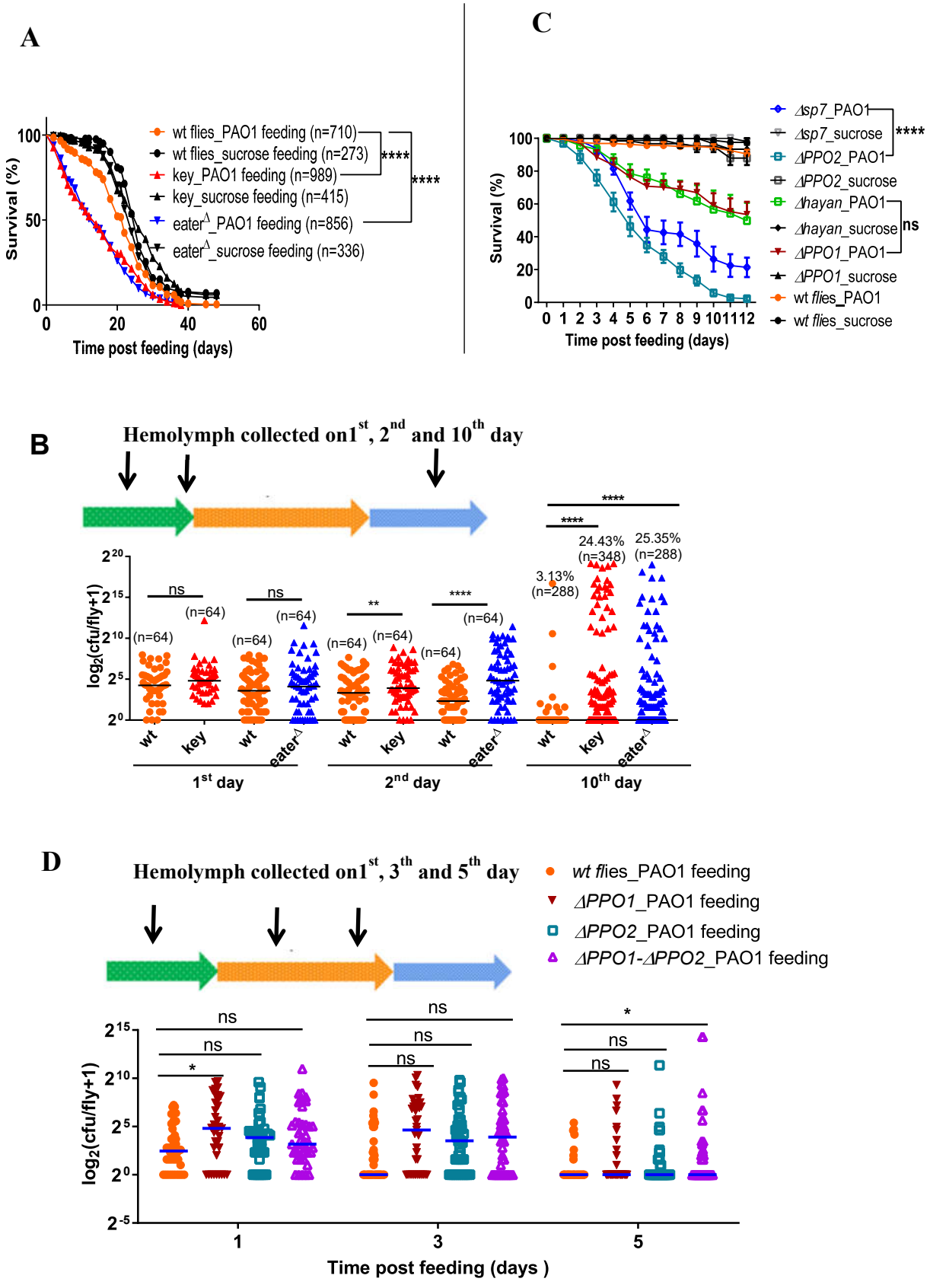

A
Survival (%)
Time post feeding (days)
wt flies_PAO1 feeding (n=710)
wt flies_sucrose feeding (n=273)
key_PAO1 feeding (n=989)
key_sucrose feeding (n=415)
eaterΔ_PAO1 feeding (n=856)
eaterΔ_sucrose feeding (n=336)
****
****
C
Survival (%)
Time post feeding (days)
Δsp7_PAO1
Δsp7_sucrose
ΔPPO2_PAO1
ΔPPO2_sucrose
Δhayan_PAO1
Δhayan_sucrose
ΔPPO1_PAO1
ΔPPO1_sucrose
wt flies_PAO1
wt flies_sucrose
****
ns
B
Hemolymph collected on1st, 2nd and 10th day
log2(cfu/fly+1)
ns
**
****
3.13% (n=288)
24.43% (n=348)
25.35% (n=288)
wt
key
eaterΔ
1st day
2nd day
10th day
D
Hemolymph collected on1st, 3th and 5th day
wt flies_PAO1 feeding
ΔPPO1_PAO1 feeding
ΔPPO2_PAO1 feeding
ΔPPO1-ΔPPO2_PAO1 feeding
log2(cfu/fly+1)
Time post feeding (days)

Figure 3

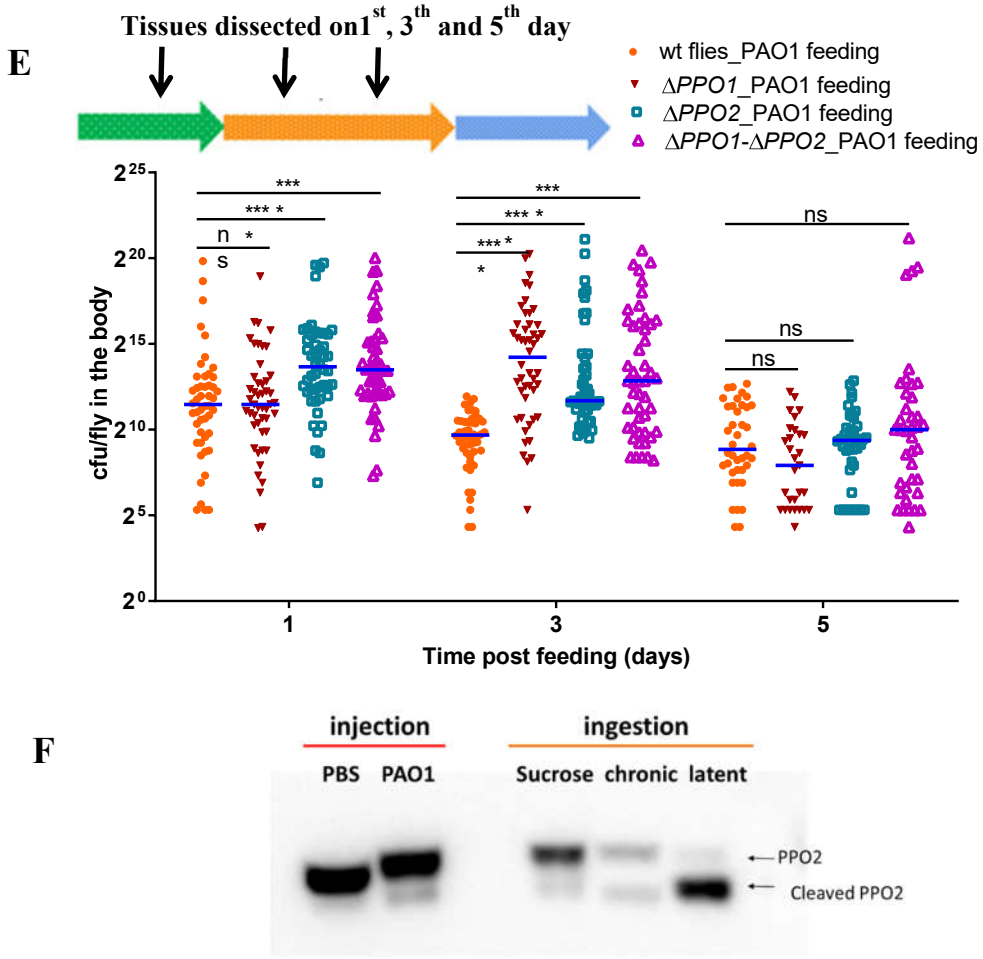
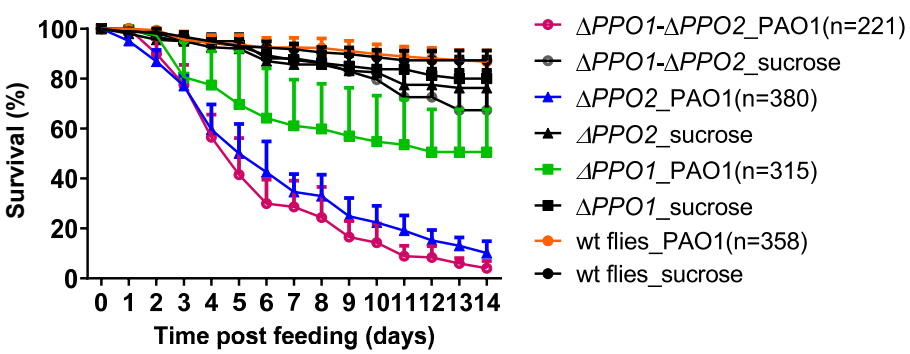


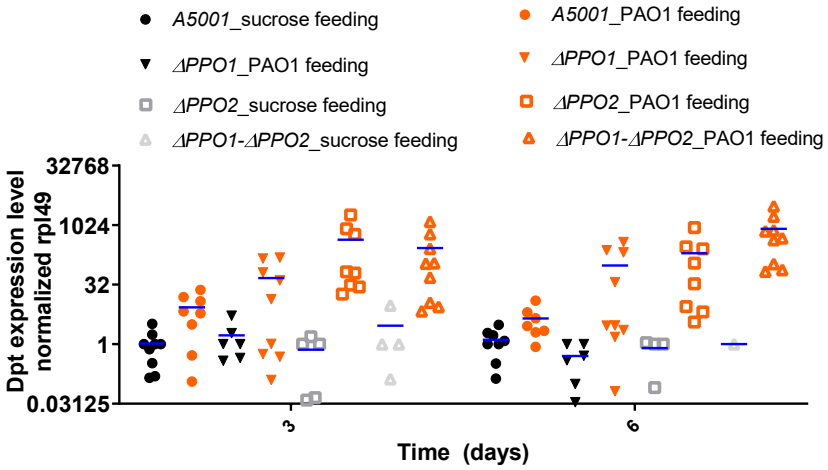
Figure 3. The *Drosophila* immune responses work together to induce into dormancy *P. aeruginosa* that have escaped from the gut into the body cavity. (A). Survival curves for *key* and *eater* deficient flies. (B). Bacterial titer in hemolymph in *key* and *eater* deficient flies. (C). Survival curves of melanization-deficient flies. (D). Bacterial titer in hemolymph in prophenoloxidase-deficient flies. (E). Bacterial titer in fly carcass of prophenoloxidases-deficient flies. (F). Scheme for roles of different immune responses in inducing *P. aeruginosa* to become dormant. *key* flies are deficient for the humoral immune response mediated by the IMD pathway; *eater* flies cannot phagocytose *P. aeruginosa* as they lack the phagocytosis receptor Eater; *sp7*, *hayan*, Δ*PPO1*, Δ*PPO2* and Δ*PPO1*Δ*PPO2* flies indicate melanization-deficient flies mediated by the serine proteases SP7 or Hayan, prophenoloxidases PPO1, PPO2 or both. All experiments here were performed three times independently and data were pooled together. Statistical analysis was done by Log-rank (Mantel-Cox test) in (A and C), by t-tests in (B, D and E).

Figure S3

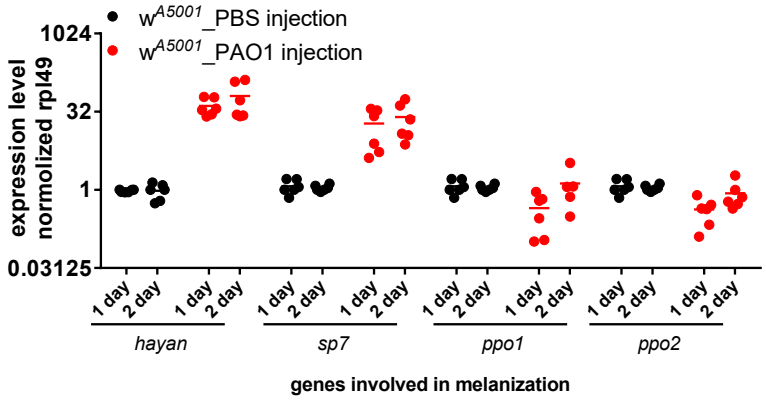
A



B



C



D

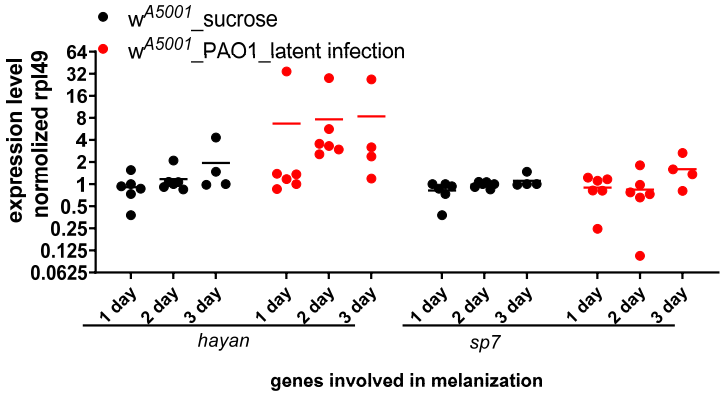
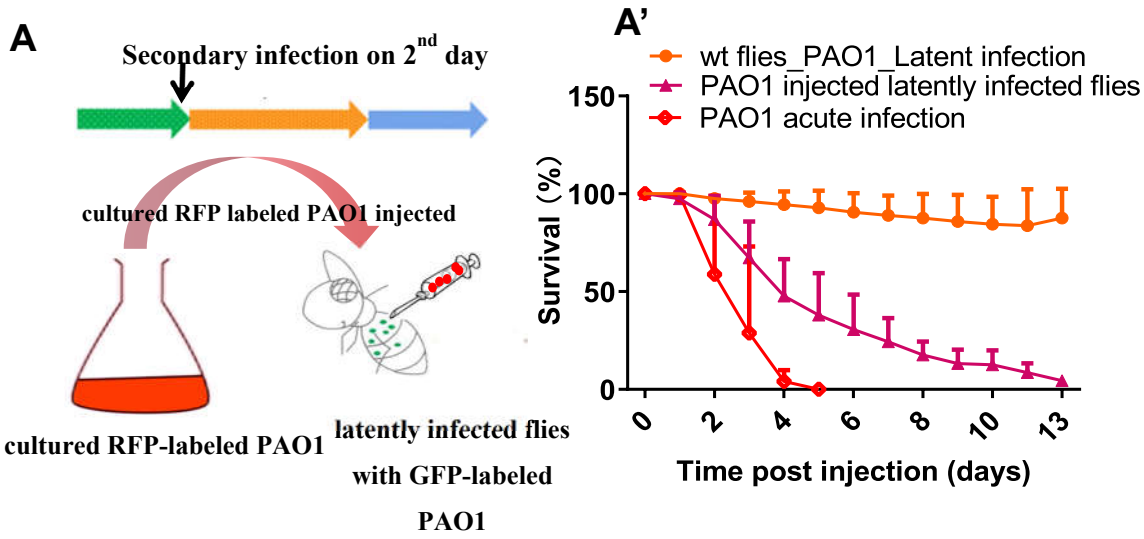
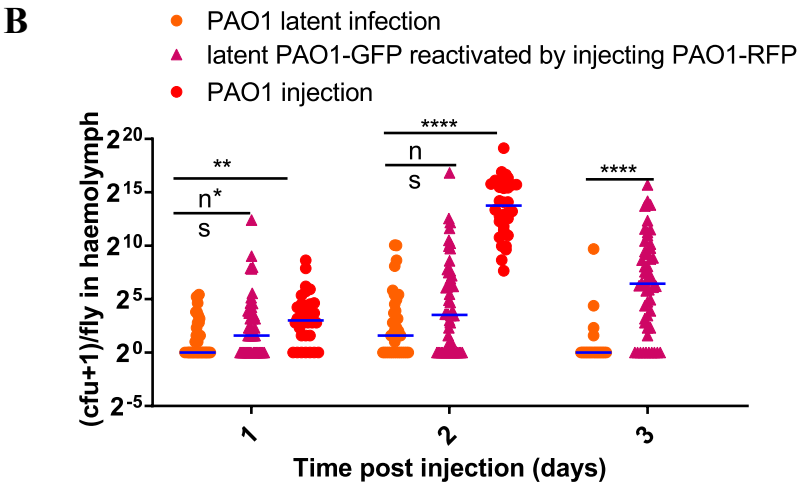


Figure S3. Melanization role against ingested *P. aeruginosa.* (A). Survival curves of prophenoloxidases flies. This experiment was done three times and pooled data were shown. (B). Measurement of IMD pathway activation in prophenoloxidases-deficiency flies. This experiment was done three times and pooled data were shown. (C). Transcription level of serine proteases and prophenoloxidases in flies with *P. aeruginosa* systemic infection. This experiment was done twice and pooled data were shown. (D). Transcription level of serine proteases in flies with *P. aeruginosa* latent infection. This experiment was done twice and pooled data were shown.

Figure 4


A
Secondary infection on 2nd day
cultured RFP labeled PAO1 injected
cultured RFP-labeled PAO1
latently infected flies with GFP-labeled PAO1
A'
wt flies_PAO1_Latent infection
PAO1 injected latently infected flies
PAO1 acute infection
Survival (%)
150
100
50
0
0
2
4
6
8
10
13
Time post injection (days)


B
PAO1 latent infection
latent PAO1-GFP reactivated by injecting PAO1-RFP
PAO1 injection
(cfu+1)/fly in haemolymph
2^20
2^15
2^10
2^5
2^0
2^-5
**
n*
s
****
n
s
****
1
2
3
Time post injection (days)

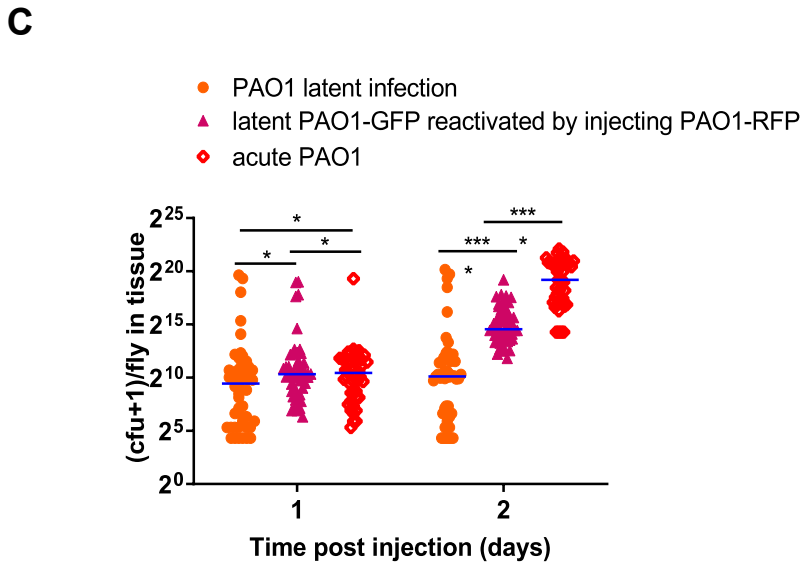

C
PAO1 latent infection
latent PAO1-GFP reactivated by injecting PAO1-RFP
acute PAO1
(cfu+1)/fly in tissue
2^25
2^20
2^15
2^10
2^5
2^0
*
*
*
***
***
*
*
1
2
Time post injection (days)

Figure 4

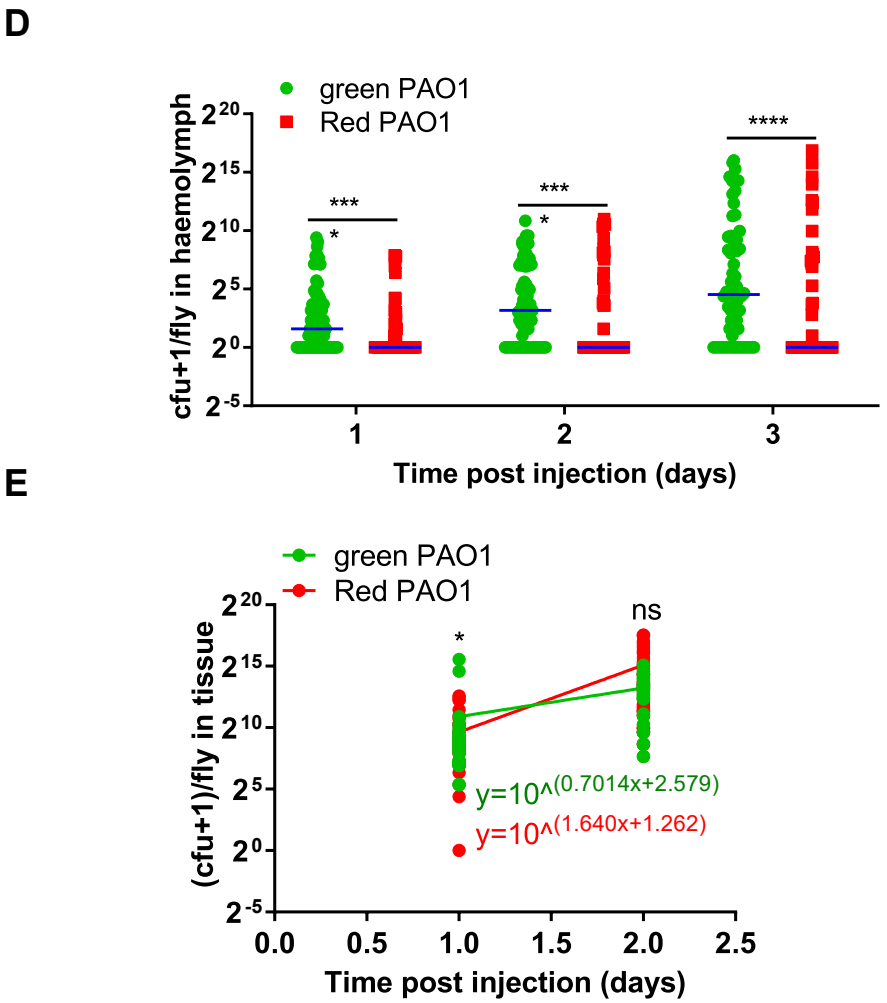


Figure 4. The immune responses elicited by ingested *P. aeruginosa* provide protection against a secondary *P. aeruginosa* injection. (A). Scheme of secondary infection by injection. (A') Survival curve of secondary infection by injecting cultured PAO1 compared to naive flies. (B). Bacterial titer in hemolymph. (C). Bacterial titer in carcass. (D). Bacterial proliferation comparison between ingested and injected PAO1 in hemolymph. (E). Bacterial proliferation comparison between ingested and injected PAO1 in carcass. All experiments here were performed three times independently and data were pooled together. Statistics analysis was done by Log-rank (Mantel-Cox test) in (A'), by t-tests in (B, C, D and E), Semi-log line (X is linear, Y is log) was plotted using Prism 6.

Figure 5

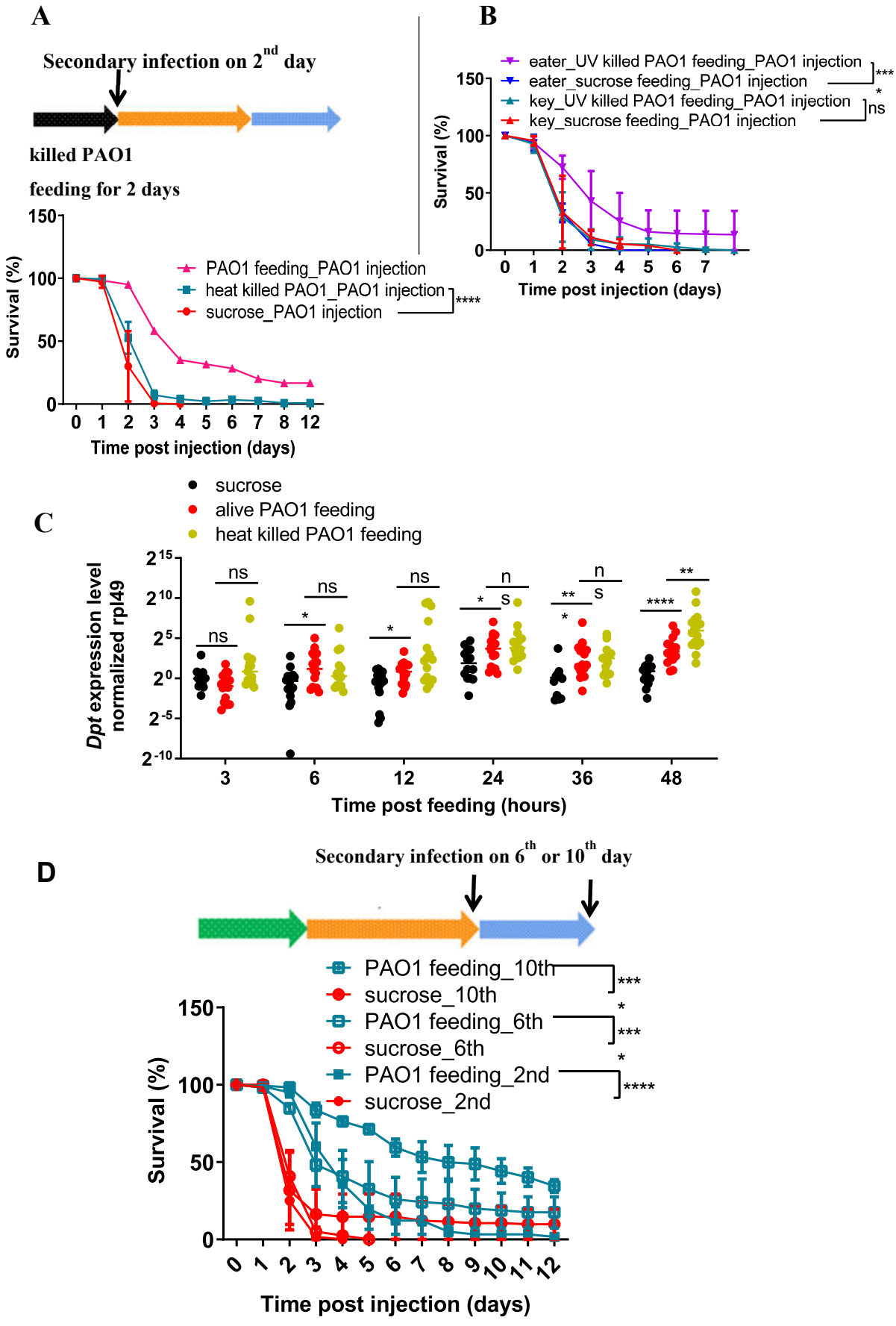

A
Secondary infection on 2nd day
killed PAO1
feeding for 2 days
Survival (%)
Time post injection (days)
PAO1 feeding_PAO1 injection
heat killed PAO1_PAO1 injection
sucrose_PAO1 injection
****
B
eater_UV killed PAO1 feeding_PAO1 injection
eater_sucrose feeding_PAO1 injection
key_UV killed PAO1 feeding_PAO1 injection
key_sucrose feeding_PAO1 injection
***
*
ns
Survival (%)
Time post injection (days)
C
sucrose
alive PAO1 feeding
heat killed PAO1 feeding
Dpt expression level normalized rpl49
Time post feeding (hours)
D
Secondary infection on 6th or 10th day
PAO1 feeding_10th
sucrose_10th
PAO1 feeding_6th
sucrose_6th
PAO1 feeding_2nd
sucrose_2nd
Survival (%)
Time post injection (days)

Figure 5

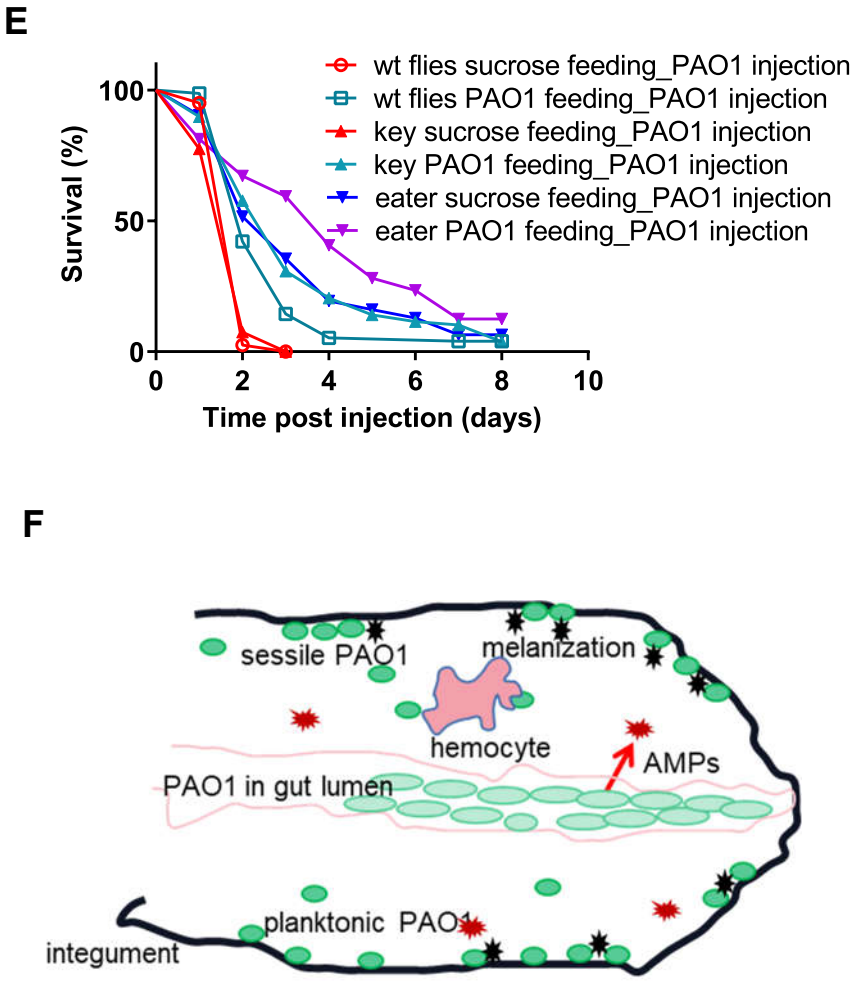


Figure 5. Both *P. aeruginosa* in the gut lumen and body cavity provide a degree of protection role against a secondary systemic infection. (A). Killed *P. aeruginosa* in gut lumen elicits partial protection against PAO1 secondary injection. (B). The partial protection role was eliminated in *key*-deficient but not *eater*-deficient flies. (C). Killed *P. aeruginosa* in the gut lumen trigger the same level of IMD pathway activation as ingested live PAO1. (D). Sessile *P. aeruginosa* in tissue elicits a strong and sustained protection against a secondary PAO1 systemic infection. (E). Sessile *P. aeruginosa* in tissue of *key*- and *eater*-deficient flies still elicits potent protection against a secondary PAOI systemic infection. (F). Scheme of *P. aeruginosa* in gut triggering the activation of the IMD pathway, possibly also indirectly. All experiments here were performed three times independently and data were pooled together. Statistical analysis was done by Log-rank (Mantel-Cox test) in (A, B, D and E), by t-tests in (C).

Figure S4

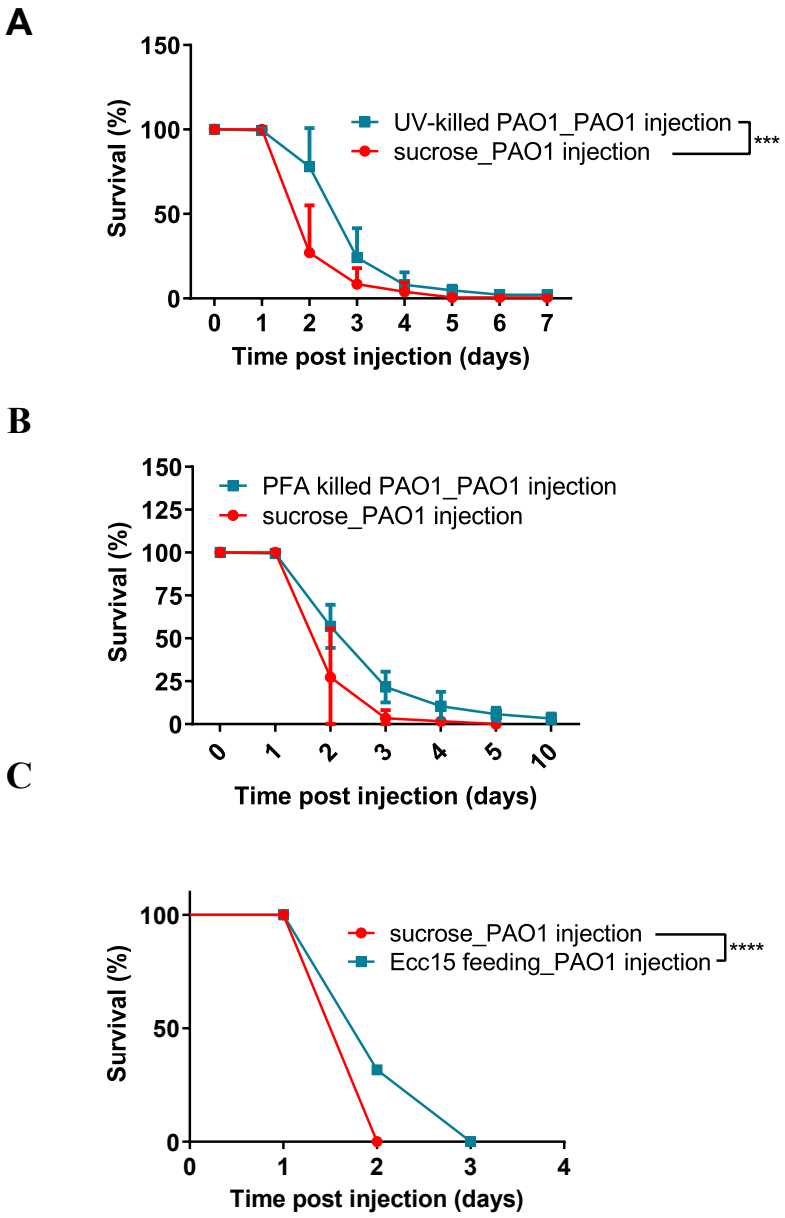


Figure S4. Protection role of killed or other alive Gram-negative bacteria in gut against *P. aeruginosa* systemic infection. (A). Protection role of UV-killed *P. aeruginosa* in gut. (B). Protection role of PFA-killed *P. aeruginosa* in gut. There experiments were done three times and pooled data were shown. (C). Protection role of *Pectobacterium carotovorum* in gut. This experiment was only done once.

Figure 6

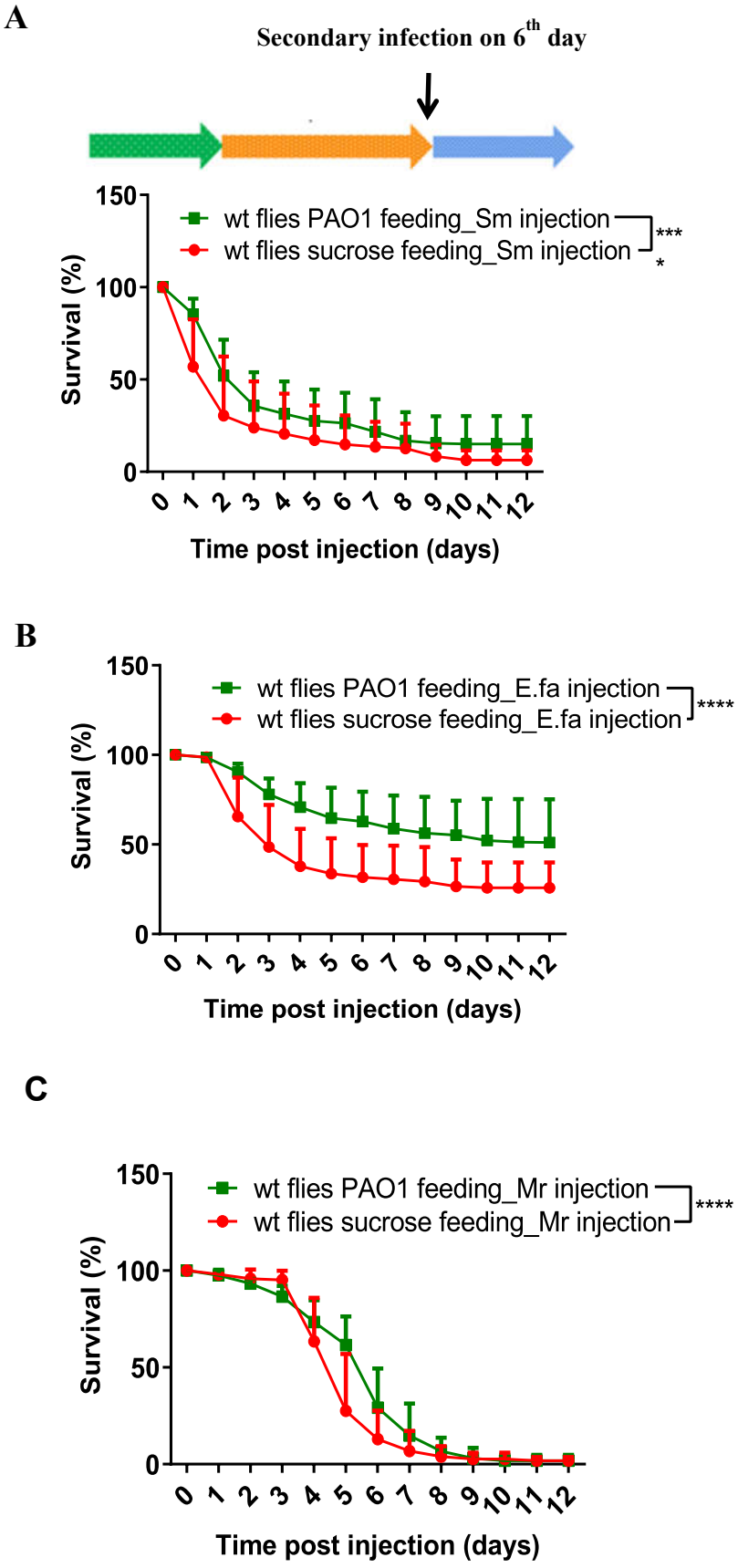

A
Secondary infection on 6th day
wt flies PAO1 feeding_Sm injection
wt flies sucrose feeding_Sm injection
***
*
Survival (%)
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time post injection (days)
B
wt flies PAO1 feeding_E.fa injection
wt flies sucrose feeding_E.fa injection
****
Survival (%)
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time post injection (days)
C
wt flies PAO1 feeding_Mr injection
wt flies sucrose feeding_Mr injection
****
Survival (%)
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time post injection (days)

Figure 6

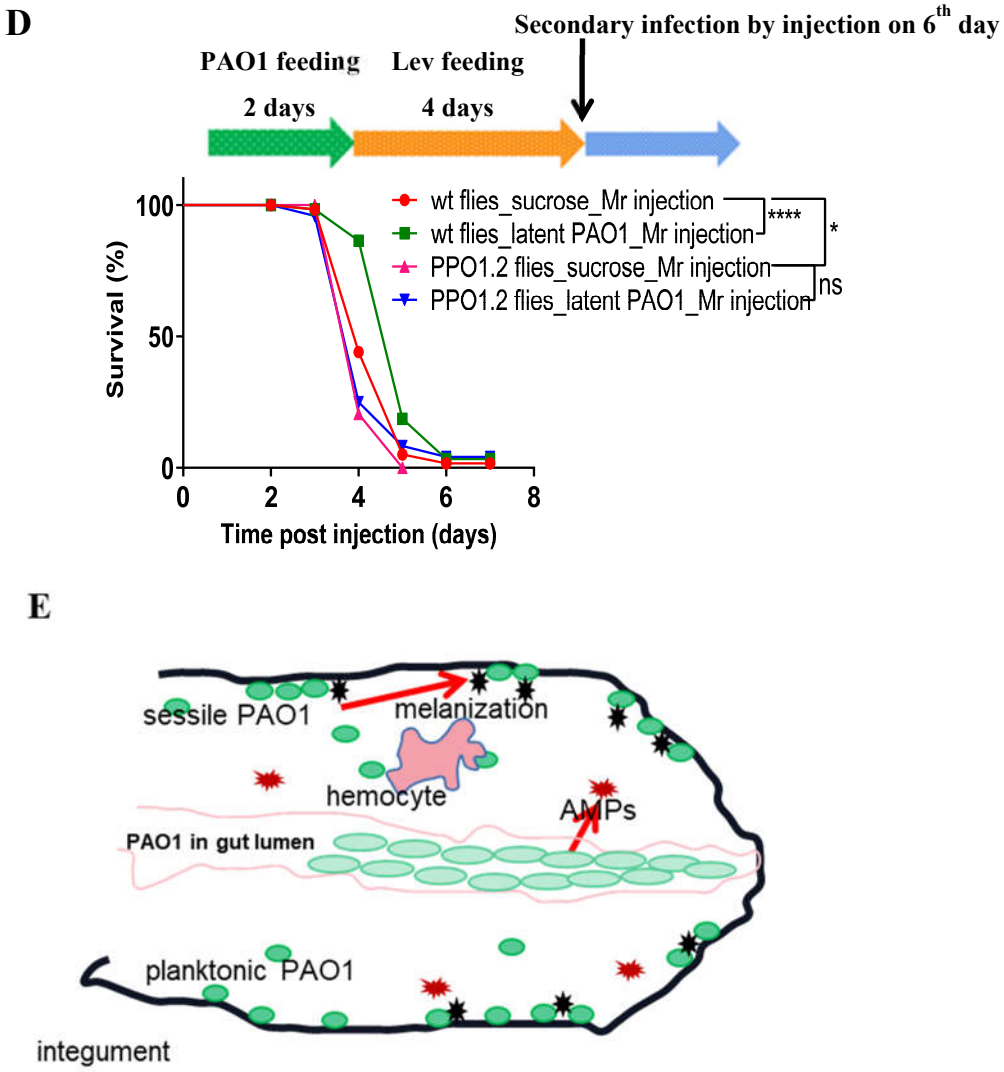


Figure 6. PAO1-latently-infected flies are protected to some degree against secondary systemic infections by fungal or bacterial pathogens, likely though the induction of the melanization response. (A). Survival curves of secondary infection by Gram-negative bacteria *Serratia marcescens* in PAO1-latently infected flies. (B). Survival curves of secondary infection by Gram-positive bacteria *Enterococcus faecalis* in PAO1-latently infected flies. (C). Survival curves of secondary infection by fungi *Metarhizium robertsii* in PAO1_latently infected flies. (D). Ingested PAO1 lose protection role against *M. robertsii* systemic infection in *ΔPPO1ΔPPO2* prophenoloxidase-deficient flies. (E). Scheme of sessile PAO1 activate prophenoloxidase PPO2 cleavage to control sessile PAO1. All experiments here were performed three times independently and data were pooled together. Statistics analysis was done by Logrank (Mantel-Cox test) in (A, B, C, D and E).

Figure S5

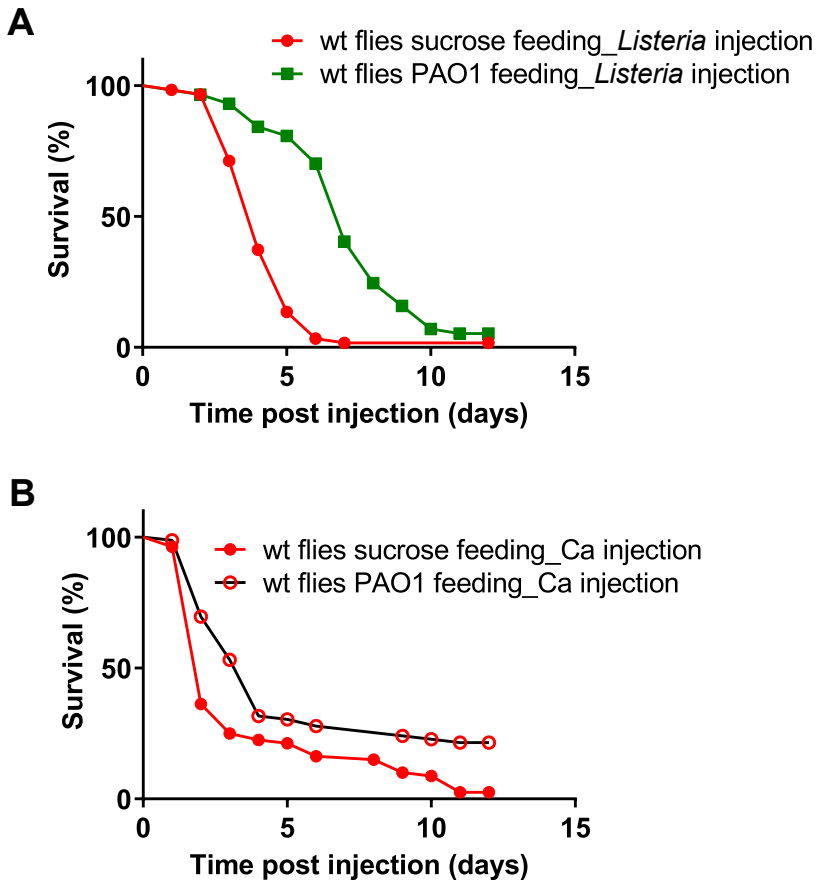


Figure S5. Protection role of *P. aeruginosa* in hemocoel against other pathogens. (A). Protection role of *P. aeruginosa* in hemocoel against *listeria monocytogenes*. This experiment was done only once. (B). Protection role of *P. aeruginosa* in hemocoel against *Candida albicans*. This experiment was done three times, and one of them was presented

Material & Methods

Flies

The wild-type fly strain used throughout the experiments is w^{A5001}, which was generated from re-isogenized w^{0118} flies [2] . Most of the fly mutant stocks used in this study have been generated in a w^{0118} flies background [377]. Δ*PPO1*, Δ *PPO2* and Δ *PPO1*Δ *PPO2* flies [20], *sp7* and *hayan* mutation flies [30] were kind gifts from Bruno Lemaitre. *key* and *eater* flies and the *Diptericin* reporter flies expressing the green fluorescence protein driven by the promoter of *Diptericin* are kept in our laboratory. Most importantly, the w^{A5001} is the isogenic control fly for the *key* and *eater* $^{\Delta}$ mutant flies.

Bacteria strains and culture conditions

The wild-type *P. aeruginosa* strain used in this study is the laboratory reference strain PAO1. The wt PAO1 labeled with GFP or RFP that was constructed to observe *P. aeruginosa* location in vivo were kind gifts from Dr. Xiaoxue Wang (Guangzhou, China). Unless mentioned otherwise, all the experiments were done using PAO1 or mutants in the PAO1 background. The other wt *P. aeruginosa* strain used here is another reference strain PA14. All the bacterial strains used in this study are noted in the Key Resource Table. Gram-negative bacteria *Serratia marcescens* and Gram-positive bacteria *Listeria monocytogenes* were kind gifts from Dr. Renjie Jiao (Guangzhou, China). Gram-positive bacteria *Enterococcus faecalis* (ATCC19433) was a kind gift from Dr. Garsin and Dr. Lorenz (Houston, USA). *Metarhizium robertsii* a kind gift from Dr. Chengshu Wang (Shanghai, China). All the bacterial stocks were kept at -80℃ refrigerator; bacteria from the frozen stocks were plated on Luria-Bertani agar plate and cultured at 37℃ incubators overnight before use. A single fresh colony was picked to inoculate the Brain-Heart Infusion broth overnight and bacteria were harvested by centrifugation. The bacteria pellet was resuspended and washed in phosphate buffered saline twice prior to its use.

Acute infection

The harvested *P. aeruginosa* pellets were suspended into PBS to measure their optical density. The optical density of bacteria suspension was adjusted to 1.0 of OD600 in PBS and then diluted to 1:1000 for injection. 3-7d old female adult flies were picked for injection, and then 13.8nl of this prepared bacteria suspension was injected into

thorax of each fly by the Nanoject III (Drummond, 3-000-032, USA). The infected flies were put in the artificial climate chamber with a temperature of 18℃ and 60% humidity and counted every 12 hours until the flies died out.

Latent infection

Before infection, 3-7d old female adult flies were picked for starvation treatment by feeding 100 mM sucrose solution on Millipore pad for 2 days under 25℃. The harvested *P. aeruginosa* pellets were suspended into PBS to measure optical density. The optical density of bacteria suspension was adjusted to 10.0 of OD600 in 100mM sucrose solution containing 10% Brain-Heart Infusion for the next infection experiment. Each tube with 20 flies was exposed to 600µl bacterial solution and put at 18℃ for 2 days. Then, the infected flies by ingestion were transferred to a new tube with 600µl of 100mM sucrose solution supplemented with 100µg per milliliter gentamicin and kept for 4 days to kill *P. aeruginosa* cells in the gut lumen. Then the flies were transferred to new tubes with 600µl of 100mM sucrose solution only. The flies were counted at regular intervals until flies died out.

Bacterial titer in hemolymph detection

The empty capillary needle was fixed into the nanoject machine (Drummond, 3-000-032, USA), then was pricked on the thorax of each fly to collect hemolymph on basis of a capillary effect. The collected hemolymph from each fly was diluted into 10ul of prepared PBS in 1.5ml Eppendorf tube. Samples collected above were diluted by a series of 2-fold dilution and then dropped on LB agar plates. These plates were put in incubator at 37℃ overnight to count the colony forming units.

Bacterial titer in carcass

The infected flies were anesthetized by carbon dioxide and then dissected under microscopy to remove heads, guts, Malpighian tubes, and ovaries; the remaining tissues of each fly were put in the Eppendorf tube containing 50ul PBS. The tissues were crushed in the Mixer Mill MM400 (Retsch, Germany) by 30Hertz for 10sec. The samples prepared above were diluted by a series of 2-fold dilutions and then dropped on LB agar plates. These plates were put in the 37℃ incubators overnight to count the colony forming units.

Bacterial titer in whole flies

Each anesthetized fly was put into 1.5ml Eppendorf tube with 50ul PBS inside and then crushed the flies using the Mixer Mill MM400 (Retsch, Germany) by 30Hertz for 10sec. The samples prepared above were diluted by a series of 2-fold dilution and then dropped on LB agar plates. These plates were incubated at 37℃ overnight to count the colony forming units.

RNA extraction and reverse transcription

Five anesthetized flies or single anesthetized fly (depending on the aims of experiments) was collected into one Eppendorf tube with 200ul Trizol for each sample, which was crushed by adding several 2mm grinding zirconium beads in the Mixer Mill MM400 (Retsch, Germany). Then, 800μl Trizol was supplemented into each tube for the next RNA extraction. For the ground samples in Trizol, 200μl chloroform was added into each tube and mixed adequately, then the layer containing RNA was separated by centrifugation with 12000g for 15min. The layer containing the RNA was transferred into a new Eppendorf tube to which 1000μl isopropanol was added to precipitate the RNA. The RNA was pelleted by centrifugation with 12000g for 10min. Then, the RNA pellet was washed with 75% ethanol once and then dissolved in DEPC water. The RNA concentration was measured on NanoDropTM One (Thermo, USA). The RNA samples were reverse transcribed into cDNA by HiScript II 1st Strand cDNA Synthesis Kit (R211-01, Vazyme, China) according to the instruction. In brief, the RNA (no more than 1μg) was mixed with the gDNA wiper and incubated at 42℃ for 2min to remove genomic DNA contamination. The reverse transcription mixture containing random primers, and reverse transcription polymerase was added into the purified RNA. The mixture was then incubated at 50℃ for 30min followed by 85℃ for 15sec in a 9600 thermocycler (Bio-rad, Germany).

qRT-PCR

The cDNA products acquired were diluted 5times and gene expression level in flies was measured by relative quantitative PCR using fluorescence dye. Primers used for quantification in this study are shown in Table S1. The samples for detection were mixed with ChamQ SYBR qPCR Master Mix (Q311-02, Vazyme, China) according to instruction and then run on CFX 96 (Biorad, USA). The expression level of target genes

was normalized by the 2-ΔΔCt.

***P. aeruginosa* visualized in vivo by expressing fluorescence proteins**

Flies were challenged with the wt PAO1labeled by expressing GFP/RFP flies and then infected flies were dissected and observed under fluorescence microscopy.

***P. aeruginosa* visualized in vivo by fluorescence**

Flies challenged with wt PAO1 were dissected and fixed with 4% paraformaldehyde for 30min. The fixed fly tissue was washed in PBS 3 times and then incubated in 1:10000 Hoechst solution diluted with PBS supplemented with 0.1% Triton X-100 for 30min. Then the fly tissue was washed 3 times in PBS to be observed under fluorescence microscopy.

***P. aeruginosa* visualized in vivo by immunofluorescence assay**

As we do not have an antibody raised against PAO1, we challenged flies with PA14 for immunofluorescence observation. Flies with PA14 infection were dissected and fixed in 4% paraformaldehyde for 30min. The fixed fly tissue was washed in PBS for 3 times and then incubated in 5% BSA suspended in PBS solution for 2 hours. Then the fly tissue was incubated with the primary antibody against PA14 for 1hour. After washed in 5% BSA solution for 3 times, the fly tissue was incubated with the 488 labeled fluorescent second antibody against rabbit for 1hour. Last, the fly tissue was washed in 5%BSA solution for 3 times to be observed under fluorescence microscopy.

Western blot for detection of PPO2 cleavage

Hemolymph was collected from infected or control flies by capillarity and then diluted into PBS with 10 micromoles per milliliter serine proteases inhibitor phenylmethyl sulfonyl fluoride. The prepared samples were added with SDS-loading buffer and boiled at 95℃ for 5min. The samples were separated by SDS-PAGE (10% acrylamide/bisacrylamide gel, 29:1; 100V; 3 h). Proteins were transferred onto a PVDF membrane (50 min, 12 V), blocked (5% BSA in 20 mM Tris/HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20 or TBST buffer, 2 h), and incubated (4℃, overnight) with antibody against PPO2/PO2 (1:5000, 5% BSA in TBST buffer, 5 mL). Membranes were washed with TBST (1x, 5 min., RT) and incubated with goat anti-mouse IgM-HRP (Southern Biotech #1021-05, 10 mL, 1:5,000, 1 h, RT). Membranes were washed with TBST (3x,

20 min., RT) and developed (SuperSignal West FEMTO Max. Sensitivity Substrate #11859290) using Amersham Imager 680 (GEHealthcare), equipped with a Peltier cooled Fujifilm Super CCD.

O-antigen staining

The hemolymph with circulating bacteria was collected by the Nanoject III with empty needles based by capillarity. The collected hemolymph was put in the wells of 8-well slides for 30min to deposit bacteria by sedimentation. The bacteria associated with the tissues were collected just by dissecting infected flies to obtain tissues. The fly tissue with adhering bacteria was fixed with 4% paraformaldehyde for 30min and then washed with PBS solution 3 times. The samples were first incubated with 5% BSA solution for 2 hours, then incubated with the O5 primary antibody (Biorbyt, orb234239) diluted in 5% BSA solution for 1 hour. The samples were washed in PBS for 3 times, and then incubated with the ALEXA488 labeled fluorescent second antibody against mice for 1hour. The samples were washed in PBS for 3 times and then observed under fluorescence microscopy.

Bacteriostatic assay in vivo

The acutely infected flies were injected with a series of doses of Tobramycin and Levofloxacin at 12 hours post *P. aeruginosa* injection to screen the most suitable dose for experimentation. The flies were injected at 12 hours post *P. aeruginosa* injection with the appropriate dose of antibiotics and then kept in a 18℃ incubator. For latently infected flies, the doses of the antibiotic for injection used were the same as for acute infections. The flies were sacrificed to measure bacteria titer by crushing the whole fly at different time points post antibiotics injection. The crushed products were diluted by a series of 10-fold and dropped on LB agar plates to count colony forming units the next day.

P. aeruginosa* cells morphology *in vivo

Fluorescence observation. The wt PAO1 expressing GFP or RFP was injected and fed to adult female flies separately. Then the infected flies were dissected and observed under a fluorescence microscope.

P. aeruginosa* colonies morphology *in vitro

The acutely and latently infected flies were sacrificed respectively on the 2nd and 6th day post-infection to be crushed. The crushed product was diluted by a series of 10-fold dilution to plate on Congo Red LB agar plates using cultured *P. aeruginosa* in vitro as control. The Congo Red LB plates were made by adding 40 μg/mL Congo-red and 20 μg/mL Coomassie brilliant blue into LB agar medium [378]. Plates were incubated at 37◦C or 25 ℃ for 1 day.

***P. aeruginosa* motility assay**

The acutely and latently infected *P. aeruginosa* were isolated for morphology observation and bacteria titer was measured by plating on LB agar plates at the same time. The crushed product was dropped on 0.3% agar plates with 1% tryptone and 0.25% NaCl to measure swimming motility [378]. Of note, the plates used in this part were adding 80μg/ml Ampicillin to inhibit microbiota to grow. These plates were then put in a 37℃ or 25℃ incubator for one day.

Secondary infection by septic injury injection

Flies were fed with live or killed bacteria in different ways and then were injected on the 2nd, 6th, and 10th-day using different kinds of pathogens, including *P. aeruginosa*, *Serratia marcescens*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Metarhizium robertsii*, *C. albicans*. Dead flies were counted each day and survival curves were plotted by Prism 5.0.

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Bacterial and fungi strains and plasmids		
Pseudomonas aeruginosa PAO1	Kind gifts from Dr. Wang	N/A
PAO1*glmS*::gfp		N/A
PAO1*glmS*::rfp		N/A
Pseudomonas aeruginosa PA14	This study	N/A
Serratia marcescens	Kind gifts from Dr. Jiao	NA
Listeria monocytogenes		N/A
Enterococcus faecalis	Kind gift from Dr. Garsin and Dr. Lorenz	N/A
Metarhizium robertsii	Kind gift from Dr. Wang	N/A
Fly stocks		
w^{A5001}	This study	N/A
key^{Δ}	This study	N/A
$eater^{\Delta}$	This study	N/A
$PPO1^{\Delta}$	Kind gift from Bruno Lemaitre	N/A
$PPO2^{\Delta}$	Kind gift from Bruno Lemaitre	N/A
$PPO1^{\Delta}$-$PPO2^{\Delta}$	Kind gift from Bruno Lemaitre	N/A
$sp7^{\Delta}$	Kind gift from Bruno Lemaitre	N/A
$hayan^{\Delta}$	Kind gift from Bruno Lemaitre	N/A
Pdpt-gfp	Kept by our lab	N/A
Antibodies and Enzymes		
Antibody against PA14, rabbit	This study	N/A
Antibody against *PPO2*, mice	This study	N/A
Antibody against O5, mice	Biorbyt	orb234239
Goat anti-mice IgG	Abcam	Ab150113
Rabbit anti-mice IgG	Abcam	N/A
Rat anti-rabbit IgG,	Abcam	N/A

Commercial Kits		N/A
HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper)	Vazyme	R211-01
ChamQ SYBR qPCR Master Mix (Low ROX Premixed)	Vazyme	Q311-02
oligonucleotides		
Rpl49 Fw	Tsingke	GACGCTTCAAGGGACAGTATCTG
Rpl49 Rv	Tsingke	AAACGCGGTTCTGCATGAG
Diptericin Fw	Tsingke	GCTGCGCAATCGCTTCTACT
Diptericin Rv	Tsingke	TGGTGGAGTGGGCTTCATG
Attacin Fw	Tsingke	GGCCCATGCCAATTTATTCA
Attacin Rv	Tsingke	AGCAAAGACCTTGGCATCCA
Cecropin Fw	Tsingke	ACGCGTTGGTCAGCACACT
Cecropin Rv	Tsingke	ACATTGGCGGCTTGTTGAG
Metchnicovin Fw	Tsingke	CGTCACCAGGGACCCATTT
Metchnicovin Rv	Tsingke	CCGGTCTTGGTTGGTTAGGA
Hayan-F	Tsingke	GCCCAATCCCAATCCCTCCC
Hayan-R	Tsingke	CCTATCCACCCGTTCGCCGT
Sp7-F	Tsingke	CGGTTTGTTTGCCTTTGGTA
Sp7-R	Tsingke	ATCGCTGCTTTATGGTGCTC
Chemicals and Antibiotics		
Trizol	Takara	9109
Trichloromethane	Guangzhou Chemical Reagent Factory	N/A
Isopropanol	Guangzhou Chemical Reagent Factory	N/A
Ethanol	Guangzhou Chemical Reagent Factory	N/A
Diethyl pyrocarbonate treated water	Sangon Biotech	B501005-0500
Congo Red	Amresco	0379
Commasie brilliant blue	Sigma	B1131
Hoechst	Thermo Fisher Scientific	H21492
4% paraformaldehyde	Biosharp	BL539A
Carbenicillin	Macklin	C805408
Gentamicin	Amresco	0304

Levofloxacin	Rhawn	R010691
Tobramycin	Rhawn	R002607
30% Polyacrylamide	Beyotime	ST003
TEMED	Beyotime	ST728
Tris-Hcl	Boster	AR1162
Phenylmethylsulfonyl fluoride PMSF	Beyotime	ST506-1
Software and algorithms		N/A
Prism	Graphpad	N/A
CE design	Vazyme	N/A
Other		N/A
LB broth	Huankai Microbial	028320
LB agar	Huankai Microbial	028330
BHB	BD	237500
Millipore pad	Merck	AP1002500
sucrose	Guangzhou Chemical Reagent Factory	N/A
Agar	Mym biological Technology	N/A
Agarose	Genesand	AG801
Calcium chloride	Tianjin Damao chemical reagent Factory	N/A

Chapter II

Rhl quorum sensing is necessary for the virulence switching during the transition from silent colonization to systemic infection

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1 Sino French Hoffmann Institute, Guangzhou Medical University, China

2 University of Strasbourg, UPR9022 du CNRS, Strasbourg France

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Abstract

P. aeruginosa is one of the leading causes of nosocomial opportunistic infections in an acute or chronic pattern, with diverse virulence programs and multiple regulatory systems. To study host-pathogen interaction between *P. aeruginosa* and *Drosophila*, we have established four infection models with different pathogenic characteristics: septic injury, continuous oral infection (intestinal infection), latent oral infection, and latent-reactivated (latent-29℃) infection. In contrast to the septic injury in which bacteria proliferate in the hemolymph, and latent infection characterized by dormant sessile bacteria, the continuous infection displays a development process with a progressive virulence switching program of *P. aeruginosa* in the tissue: dormant, transitional, and active phase. A similar virulence-switching program is also observed in the latent-29℃ reactivated infection. Compared to the latent infection, the major difference of the continuous intestinal infection is that *P. aeruginosa* bacteria are present in the gut and may continuously cross the intestinal barrier, while the main difference of the latent-29℃ reactivated infection model is that bacteria proliferate directly in the tissues. The continuous oral infection model and latent-29℃ reactivated models share the common feature that bacterial virulence is activated at a late phase, corresponding to cell density crossing a threshold in the tissue, accompanied by a transition of lifestyle from sessile to planktonic. The switching process requires the Rhl quorum sensing system but neither the Las system nor the PQS system. It is confirmed in the opposite vein, that the Rhl system is dispensable in the flies lacking the melanization response, wherein bacteria are not dormant and proliferate actively in the hemolymph. Our oral infection models also provide evidence *in vivo* that RhlR signaling modulates virulence in a way dependent on RhlI in synergy with PqsE. In addition, an alternative activation mechanism of the RhlR regulator independent of PqsE and RhlI may be involved.

Keywords: quorum sensing; virulence; *P. aeruginosa; Drosophila*; host-pathogen interactions; innate immunity

Chapter Introduction

Pseudomonas aeruginosa (*P. aeruginosa)* is a Gram-negative bacterium that is a leading cause of nosocomial opportunistic infections in immunocompromised and cystic fibrosis patients with a pattern of either acute or chronic pathogenicity [115, 127]. Moreover, it is resistant to commonly used antibiotics [120]. *P. aeruginosa* is ubiquitous in the environment, with a broad host range, affecting vertebrates, insects, nematodes, and plants through well-conserved virulence mechanisms [116-118]. An in-depth understanding of the regulatory mechanisms of virulence in *P. aeruginosa* is critical for developing alternative therapeutic interventions to control and prevent bacterial infections. Quorum sensing (QS) is a bacterial cell-to-cell communication process implemented in response to the change of cell density that enables the bacteria population to collectively control a large set of gene expression programs and thereby synchronizes group behaviors, such as virulence, biofilm formation, and motility. QS depends on networks of autoinducers, autoinducer synthases, and autoinducer receptors. *P. aeruginosa* employs four interwoven quorum-sensing loops LasR-LasI, RhlR-RhlI, PqsABCDH-PqsR, and AmbBCDE with an unknown receptor. They correspond respectively to the receptors and synthases of the autoinducers 3OC12-HSL, C4-HSL, PQS, and IQS [239, 248].

Drosophila melanogaster has been notably extensively used to study host-pathogen interactions, and to decipher the complex bacterial virulence mechanisms *in vivo*, thanks to its sophisticated genetics and its well-characterized innate immune system, which is rather similar to the mammalian one. The *Drosophila* host possesses humoral and cellular immune responses that deals with systemic infections, as well as local responses of barrier epithelia that deal with invasions in the gut tract and tracheae [10]. For example, the injection of bacteria into the fly body cavity induces the rapid activation of immune defenses, including coagulation and melanization. The melanization response is an important immune defense in arthropods, resulting in blackening clots at the wound sites, that represent melanization sites of microorganism invasion [15]. Systemic melanization can also be induced by signaling mediated by the Pattern Recognition Receptor (PRR) after the recognition of bacterial peptidoglycans (PGNs) [16]. Melanization reaction relies on enzymes called prophenoloxidases (PPOs). The Hayan and Sp7 proteases are responsible for activating PPOs into active POs, that

catalyze the deposition of melanin. Besides, the melanization reaction has an association with the production of cytotoxic molecules, such as reactive oxygen species (ROS). It is thus potentially responsible for the killing of invading organisms. It has been demonstrated that the blackening reaction at the wound site is more dependent on Hayan and its substrates PPO1 and PPO2, but Sp7 may active PPO1 and have an alternative contribution to microbe killing, possibly related to ROS production [30]. As for local microbial infection, such as that of the intestinal tract, ROS and tissue-specific AMPs are generated to combat pathogens or regulate the gut microbiota. After the bacterium crosses the epithelial barriers, it can be killed by phagocytosis of hemocytes. The bacterial component such as PGNs released during bacterial proliferation or lysis can be sensed by the fat body, triggering a potent systemic humoral response mediated by antimicrobial peptides (AMPs) such as Diptericin. The induction of multiple AMP genes is mediated by two NF-κB-type signaling pathways, the Immune deficiency (IMD) and the Toll pathways [10, 40, 71].

We have established three kinds of infection models with *P. aeruginosa* before: septic injury, continuous oral infection, and latent infection. The septic injury model corresponds to the direct inoculation of bacteria into the body cavity using injection. The very low number of injected *P. aeruginosa* cells (even as few as 1-10) can rapidly proliferate in the hemocoel and kill out the flies within four days. In contrast to the fast pathogenicity kinetics of the injection model, flies orally infected with *P. aeruginosa* at high concentration (even more than OD of 10) can survive for about a week or even longer. The ingested *P. aeruginosa* crosses the intestinal barrier before provoking bacteremia later on. This may reflect a low-virulence state of the bacteria that cross the gut barrier as described previously for *S. marcescens* [75]. Indeed, based on the above studies, our college Jing Chen developed a latent infection model by PAO1, in which the gut bacteria were cleared by antibiotic feeding after 2-days bacterial feeding (chapter I). In the latent infection model, *P. aeruginosa* crosses the gut and colonizes as dormant sessile cells in tissues, lurking over one month in the fly host without net proliferation and activation of the humoral immune response. It indicates the virulence of *P. aeruginosa* is dependent on the infection routes, and possibly the corresponding immune responses. Intriguingly, we ask how virulence is regulated in different models of *Drosophila melanogaster.*

Previously, Limmer, S. & Haller, S., *et al.* of our team found that the *P. aeruginosa* quorum-sensing regulator RhlR is required for virulence in oral infection and allows *P.*

aeruginosa PA14 to circumvent the cellular immune response, independently of its canonical synthase RhlI [56, 89]. Consistently, similar findings were made by another group using readouts of colony architecture and virulence in the infection models of *C. elegans* or murine acute lung infection [289]. Given the above findings, the possibility was that either RhlR might function independently of its QS circuit or that RhlR would be induced by an alternate autoinducer. Subsequently, an alternative synthase candidate PqsE was identified by another group as being required for RhlR activation in their *in vitro* and *in vivo* read-outs [297]. In light of these findings, it is relevant to investigate further whether RhlR signaling relies on RhlR/RhlI/PqsE circuit and whether it plays a key role in the virulence regulation in different infection *Drosophila* models, especially the chronic infection one, also in the context of immunocompromised hosts.
A work published very recently while this study was being conducted, revealed that a distinct PqsE-RhlR interaction enhances the expression of virulence genes. It proposed two working models *in vitro* of the Rhl QS circuit: the RhlR-C4HSL or PqsE-RhlR-C4HSL [299, 302]. This proposed mechanism favors the role of PqsE that has been found in our *Drosophila* models here.

Our study here makes a comparative analysis of pathogenic characteristics among four different *P. aeruginosa* infection models of *Drosophila:* septic injury, continuous oral infection (intestinal infection), latent oral infection models, and a new model, the latent-reactivated (latent-29℃) infection model. In comparison to the septic injury and latent infection with dormant sessile bacterium, the continuous infection and latent-29℃ reactivated infection models involve virulence switching programs of *P. aeruginosa*, that are activated by cell density increases of sessile bacteria in tissues, which do not induce a strong systemic immune response. The switch correlates with a passage from a sessile to a planktonic lifestyle at a late phase. The Rhl quorum sensing system but neither the Las system nor the PQS system is necessary for the switching process. It is however dispensable in the melanization mutant flies, with an immunodeficient host context associated with adhesion impairment, wherein bacteria are not dormant and easily reach the hemolymph. We also demonstrate in *Drosophila* oral infection models that RhlR signaling controls bacterial virulence in a way dependent on RhlI in combination with the function of PqsE. Additionally, we infer the existence of an atypical activation mechanism worth to be further identified.

Results

1. Distinct pathogenic characteristics of three different infection models suggest a virulence-switching program in continuous oral infection

Previously, Limmer *et al* have characterized the continuous oral infection (intestinal infection) of *P. aeruginosa* strain PA14 by monitoring fly survival and bacterial load in the hemolymph (at 25 ℃, OD of 0.25) [89]. Our colleague Jing Chen also developed and characterized a latent infection of *P. aeruginosa* strain PAO1 as described in chapter I (at 18 ℃， OD of 10). However, these studies did not determine whether PA14 colonizes tissues in the continuous infection model as PAO1 does in the latent infection model. Furthermore, the trigger for the switch of virulence has not been identified. To answer the questions and bridge the above two works, firstly, we characterized the pathogenesis of PA14 in the continuous oral infection model from more perspectives, comparing it to the pathogenesis of PA14 in the septic injury infection and latent infection models.

Three infection models were performed at 18 ℃, shown in the scheme **(Fig. 1F)**. Flies were monitored for survival and the bacterial titer was monitored at different time points in the hemolymph, carcass (dissected out of the gut, ovary, and Malpighian tubules), gut (midgut, crop), or whole body. In the septic injury model, a few injected *P. aeruginosa* bacteria PA14 proliferated rapidly in the hemolymph or the whole body and killed the flies within three days **(Fig. S1 A~C)**. In the latent infection model initially developed with PAO1(Chapter I), flies were fed with *P. aeruginosa* for 2 days, then transferred to gentamicin feeding for 4 days to kill the bacteria in the gut. Gentamicin cannot penetrate efficiently through the gastrointestinal barrier, due to its high hydrophilicity and polarity against cell membranes [379, 380]. No PA14 bacteria were detected in the hemolymph, but a few are found in the tissue and maintained at a relatively low number (about 10^2 to 10^3 c.f.u.) without proliferation for a long time **(Fig. S1 E~F)**. Correspondingly, the flies in latent infection survived for over three weeks **(Fig. S1 D)**, which is similar to the phenotype of PAO1 (in Chapter I). The septic injury and latent model represent phenotypes of acute and asymptomatic chronic infection, respectively.

In the continuous oral infection, flies were continuously fed on PA14-containing sucrose solution absorbed on a filter pad (0.6ml, OD of 4 in 50mM sucrose with 5%

BHB) at 18 ℃ until death. The infected flies survived for about a week **(Fig. 1. A)**, showing high similarity to the results reported (performed at 25 ℃) by our colleagues [89]. A novel finding is that bacteria did colonize tissues (hereafter referred to as the dissected carcass without the gut, ovary, and Malpighian tubules) and remained at a low level during the first three days, whereas PA14 cells in hemolymph were barely detectable. Subsequently, the bacterial load progressively increased in the tissues but most of the bacteria remained adherent until the late phase with planktonic bacteria detected in the hemolymph from about half of the flies, likely about to die **(Fig. 1. B & C)**. The mRNA level of *diptericin*, a readout of the systemic immune response, was just slightly induced both in the latent infection and the continuous oral infection, even at the late phase, in comparison to the septic injury infection (**Fig. S1G).** In contrast to the characteristics of the septic and latent infection, the process of continuous oral infection presents a virulence-switching program with three consecutive phases: i) a dormant phase as in the latent infection, with dormant bacteria of stable low number and low virulence; ii) a transitional phase with the sessile-bacterial load increasing in tissues; iii) a full activation phase similar to the septic infection, with sessile and planktonic bacteria of strong proliferation and possibly high virulence. Although we observed that the bacterial load was increasing in the tissues, we still do not formally know whether it is due to bacterial proliferation of sessile bacteria or the accumulation of the invading bacteria from the gut. However, since there is not a similar increase of the bacterial load in the carcass of flies infected in the latent infection protocol in which flies ingest bacteria only for two days, this increase is a consequence of flies continuously ingesting bacteria. Their influence on the carcass burden may be direct or indirect. The bacteria in the midgut and the crop were also progressively increasing from Day 4, an ambiguous time point (**Fig. 1. D & E)**. Nonetheless, there was a middle period during which the bacterial load was relatively stable. Besides, an unexpected reduction of the bacterial load in the intestine was observed from Day1 to Day2 (**Fig. 1. D & E)**. Presumably, it is due to the elimination by the local immune response in the gut, as a mass of killed *P. aeruginosa* at 18 hours was observed by PI staining, in comparison with the positive control *E. coli*, the negative control *S. marcescens* (RM66262) (**Fig. S2A&B)**. Collectively, the virulence switch in the tissues seems to depend on a certain threshold of cell density, and there may be a parallel switch happening in the gut.

2. RhlR signaling modulates virulence in a way partially dependent on RhlI cooperating with PqsE in continuous oral infection but is dispensable in septic injury infection

The pathogenic characteristics of continuous oral infection reveal a cell-density-dependent switch of the virulence of PA14 bacteria, implying the existence of a communication system within the bacterial population. The quorum sensing systems are possibly involved in this switching process. Previously, it was identified by our team that the role of quorum sensing regulator RhlR in virulence is only partly dependent on the synthase RhlI during continuous oral infection [56, 89]. Similar findings were made by another group using the readouts of colony morphology, and virulence in the infection model of *C. elegans* and murine acute lung infection [289]. PqsE was identified as involved in the Rhl circuit *in vitro* and *in vivo* (see the introduction) [297, 299, 302]. It was therefore relevant to investigate further whether the RhlR/RhlI/PqsE is involved in the virulence switching program during continuous oral and latent infection.

We constructed an in-frame deletion mutant of the *pqsE* genes in the PA14 strain, as well as the double mutants Δ*rhlI*Δ*pqsE* and Δ*rhlR*Δ*pqsE.* Firstly, we checked the phenotypes of PA14 and mutants on the Rhl QS circuit in *vitro*. As previously reported [289, 297], none of the mutants PA14 wt and mutants (Δ *rhlR*, Δ *rhlI*, Δ*pqsE,* and Δ*rhlI* Δ *pqsE*) secreted pyocyanin neither in the liquid culture nor in the LB plate, except single mutant Δ *rhlI* produced pyocyanin in the LB plate after two-day culture (**Fig. S3A & B)**. But the colony biofilm phenotypes on Congo red agar of PA14 wt and mutants (**Fig. S3C)** didn't copy the exact architecture as what they were reported that Δ *pqsE* colony biofilm was excessively rugose, highly similar to the phenotype of Δ *rhlR* and Δ *rhlI* Δ*pqsE* [297]. In our case, the colony biofilm phenotype of Δ *pqsE* still displayed a clear difference from Δ *rhlR* in two kinds of Congo red agar recipes. Nevertheless, the colony biofilm phenotypes of Δ *rhlR* and Δ *rhlI* Δ*pqsE* were always indistinguishable **(Fig. S3C, D)**.

In the septic injury infection, both PA14 wt and all the mutants for Rhl QS killed the flies at the same kinetics within 4 days **(Fig. S4A)** and showed no difference in the bacterial load **(Fig. S4B~D)**. It means that RhlR signaling may be redundant or is not required in the septic injury infection. However, in the continuous oral infection **(Fig. 2A, B)**, Δ *rhlR* was much less virulent than wt as well as Δ *rhlI*, which is consistent

with our previous founding in the intestinal infection [56]. As expected, the double mutant Δ*rhlI*Δ*pqsE* was less virulent than Δ*rhlI* alone. Surprisingly, the single mutant Δ*pqsE* is as virulent as wt, **(Fig. 2A, B)**, which is completely different from the reported avirulent phenotypes of Δ*pqsE* in the infection model in mice or *C. elegans* [297]. We excluded the possibility of negative regulation from PqsE since the Δ*pqsE* Δ*rhlR* killed the flies as slowly as the Δ*rhlR* did **(Fig. S6A, B)**. Moreover, unexpectedly, Δ*rhlI* Δ*pqsE* was significantly more virulent than Δ*rhlR* in fly survival. **(Fig. 2A, B)**. A point that will be discussed further below. The above survival phenotypes were confirmed by another independently generated set of mutants **(Fig. S6C, D).** Additionally, we checked the phenotypes of the LasR/LasI QS system in this continuous oral infection model (at 18 ℃) **(Fig. S6E, F)**， which reconfirmed LasR/LasI QS system is not required as what had been found before [89].In general, our survival experiments favor the model according to which PqsE works synergically with RhlI in activating RhlR for pathogenicity, but PqsE on its own does not contribute to the pathogenicity in the host *Drosophila*.

Next, we extracted more information from the analysis of bacterial titers. At the late phase of infection (Day7), wt bacteria were detected in the hemolymph in more than half of the flies, and to a less extent in the hemolymph of flies that had ingested Δ*rhlI*, Δ*pqsE*, Δ*rhlI*Δ*pqsE*, whereas almost no Δ*rhlR* was detected in the hemolymph **(Fig. 2C).** Accordingly, the bacterial load of the mutants was less than PA14 wt in the carcass, midgut, and crop, in line with the survival experiments **(Fig. 2D~F)**. It implies, first, that there may be fewer bacteria deficient in Rhl QS crossing the gut. second, Rhl QS may be required for the crossing ability of bacteria, or for evading the local defense of the gut. The bacterial load in the gut confused what we primarily focus on, the behavior of the bacteria in the tissue. Perplexingly, we wondered whether the bacterial switching from sessile to planktonic state is attributed to bacterial virulence per se or is simply dependent on cell density. The first clue to the above conundrum is that the bacterial load of Δ*pqsE* in the carcass or gut is not as high as that of the wt PA14 and displayed a significant difference from that of Δ*rhlR* in the carcass **(Fig. 2D~F)**. Of note, Δ*pqsE* killed the flies as fast as the wt bacteria **(Fig. 2A, B)**. The second important clue comes from the survival phenotypes in the latent infection **(Fig. S5A, B**). There was no longer bacterium crossing from the gut, but mutants deficient for Rhl QS killed the flies at a

significantly slower speed than the wt bacteria or Δ*pqsE* did. Additionally, when the flies in the latent infection were maintained on the regular simple food instead of sucrose solution, all the infected flies survived for over one month (LT50 > 30 days), nearly as long as the non-infected flies (**Fig. S5C**)**.** It means the latent infection in the condition of sucrose feeding without nutrition supplement is not *bona fide* "latent". Somehow, the latent bacteria can be activated by the Rhl QS during a chronic lack of nutrition. Collectively, it implies that RhlR contributes to the virulence switching, possibly connecting to the lifestyle transition from a sessile to a planktonic state during oral infection. We postulate that in the continuous ingestion model, gut bacteria keep on crossing the gut barrier and silently increasingly colonize host tissues. When the cell density reaches a population threshold, RhlR signaling is triggered and may turn on a virulence switch.

We also designed experiments to find out why PqsE alone is not required for pathogenicity in the infection models of *Drosophila.* Since PqsE was considered to act as an alternative synthase, we hypothesized three possibilities: (i) PqsE-driven virulence factors are dispensable in flies; (ii) RhlI-driven virulence factors are enough to kill the flies; (iii) PqsE enhances the *rhlR* function in a way depedent on RhlI. Therefore, we performed this experiment: inject the flies with cell-free culture liquid (sterile-filtered supernatant) from PA14 wt and mutants. Firstly, we monitored the bacterial growth curve of PA14 wt and mutants Δ*rhlR*, Δ*rhlI*, Δ*pqsE,* and Δ*rhlI* Δ*pqsE*. Rhl QS system did not affect cell proliferation during the exponential phase before 16 hours; all the mutants seemed to have a delayed stationary phase **(Fig. S7A)**. Secondly, to find a proper culture time for which wt bacteria release enough virulence factors to kill flies, we injected flies with the time-course LB culture liquid from the wt PA14, and the survival showed the culture at 16 hours or 20 hours could reach the maximum virulence, in parallel with the cell quantity **(Fig. S7B)**. Of note, cell-free supernatant of BHB (Brain-Heart-Infusion Broth)-cultured wt PA14 did not kill the flies even at high cell density **(Fig. S7B)**. Thus, we injected the flies with the 20h LB-culture supernatant from the PA14 wt or mutants. The survival order of the flies injected with the culture supernatants of different *rhlR/rhlI/pqsE* mutants was roughly consistent with those following an intestinal infection **(Fig. S7C)**. More specifically, culture liquid from Δ*pqsE* is as toxic as wt to flies, which killed almost all flies within four hours. Δ*rhlI* culture liquid shows medium toxicity, whereas culture liquid from Δ*rhlI* Δ*pqsE* or Δ

rhlR is hardly poisonous. In addition, presumably, the responsible toxic factors are proteins since the boiled supernatant of wt PA14 is nontoxic (**Fig. S7D)**. Collectively, these data indicate that RhlR/RhlI-circuit-driven secreted virulence factors are sufficient for killing the flies, and that PqsE enhances the *rhlR* function dependent on RhlI both *in vitro* culture and the *Drosophila* host.

Outer membrane vesicles (OMVs) are important secreted virulence factors of *P. aeruginosa,* and their production has been shown to be under the regulation of the PQS quorum sensing system [196, 197]. We wondered whether the Rhl system also regulates OMVs production and its virulence. Thus, we performed nanoparticle tracking analysis for the vesicles produced by the PA14 wt and Δ*rhlR*, Δ*rhlI*, Δ*pqsE,* and Δ*rhlI*Δ*pqsE* mutants. The quantity and size of OMVs produced by PA14 wt and mutants showed no difference among all the strains (**Fig. S8A, B).** Then, flies were injected with 69 nl of vesicle sediment (~2.2 x10^12 particles/ml) or the corresponding supernatant simply isolated from PA14 wt or Δ*rhlR* by ultracentrifugation. We found it was the supernatant from wt bacteria but not the vesicle that was responsible for killing the flies (**Fig. S7C)**. However, when we used the concentrated vesicles (~1.7 x10^13 particles/ml), OMVs from wt bacteria but not those from Δ *rhlR* could kill the flies (**Fig. S7D)**. Collectively, it indicates, first, the Rhl QS system does not control the OMV production and size but likely affects the packaged virulence components in OMVs. Second, most of the extracellular virulence factors controlled by the RhlR axis are released directly, with a few in form of OMVs *in vitro* culture, which may endow PA14 with a potential *in vivo* of a strong and rapid local invasiveness as well as remote aggressivity when Rhl quorum sensing is activated.

3. RhlR signaling activates the virulence of tissue-colonizing *P. aeruginosa* with a transition from a sessile to planktonic form in the hemolymph that coincides with a change in pathogenicity

Since the continuous oral infection displays a complex situation with bacteria being present in the tissues, in the gut, and likely continuously crossing the gut barrier, triggering the local immune response in the gut, it is difficult to dissect the switching behavior of the sessile bacteria in the chronic infection. In contrast, there are no *P. aeruginosa* bacteria in the gut but a few *P. aeruginosa* bacteria dormant in the tissue during the latent infection. Presumably, they can be activated and start reproliferating

again. Therefore, we developed a latent-reactivated infection model by transferring the flies in latent infection from 18 ℃ to a higher temperature of 29℃ **(Fig. 3A)**. In this model, the flies infected with wt PA14 and Δ*pqsE* almost died out within 4 days post-29℃-treatment, whereas flies with Δ*rhlR*, Δ*pqsE* Δ*rhlI,* and Δ*rhlI* survived for about a week longer **(Fig. 3B, C)**. The survival phenotypes are relatively in line with those in continuous oral infection. We also measured the bacterial titer of the hemolymph and the whole body **(Fig. 3D)**. At Day 8, before transferring to 29 ℃, flies were burdened by a limited number of bacteria (around 100 CFU) in the tissue, and no bacteria were detected in the hemolymph. There was no difference in bacteria load among all bacteria strains. After transferring to 29 ℃， all the wt bacteria and mutants proliferated at a similar rate and show no difference in the load in the tissue. However, wt bacteria were detected in the hemolymph in about half of the flies at Day 11, whereas Δ*rhlR* or Δ*pqsE*Δ*rhlI* mutants are hardly detected until they reached a higher load at a later phase **(Fig. 3D)**. The data show a correlation between virulence, as measured in survival experiments with the bacterial titer in the hemolymph and not with the bacterial burden in tissues. Thus, the RhlR QS promotes virulence by favoring a switch from adhesion to tissues to a planktonic lifestyle. We used the transcriptional level of the AMP gene *diptericin* as a readout of the systemic immune response, and the septic injury infection of *E. coli* as a positive control for the induction of *diptericin.* The *diptericin* level in all the infected flies was hardly induced at different time points, except for a slight increase induced by wt PA14 in the late phase **(Fig. 3E)**., in agreement with the bacterial load in the hemolymph. Besides, we also checked the transcriptional level of *rhlA* during the latent-29 ℃ infection, a gene coding for the synthesis of rhamnolipid, directly regulated by the Rhl system [207]. The *rhlA* relative level of wt bacteria was increasing over the infection time, whereas it was not indued in the Δ*rhlR* or Δ*pqsE*Δ*rhlI* mutants. The *rhlA* expression was also induced in the single mutant Δ*rhlI,* and Δ*pqsE,* the latter with the same level as wt bacteria **(Fig. 3F).**

To ascertain the specific roles of the Rhl system in latent-reactivated infection, we checked the phenotypes of other QS systems, the LasR-LasI system, and the PQS system. None of the mutants (Δ*lasR*, Δ*lasI*, Δ*pqsR*) showed an attenuated virulence as compared to that of wt bacteria in the latent-29℃ **(Fig. S9A, B).** In light that the Rhl quorum sensing (*rhlA* transcriptional level as a readout) was already activated in the group of the sessile bacteria, and low levels of wt bacteria in hemolymph detected in

just half of the flies, at the LT50 time point, we wondered whether *Drosophila* succumbed to the colonized bacteria reactivated in the tissues before severe systemic disseminated infection. It was also possible there would be severe bacteremia before death. We examined the physiology and pathology of flies in the latent-29℃ model of *P. aeruginosa* infection, in comparison to the septic injury infection, continuous oral infection, and latent infection, through FRUMS assay and muscle staining. The FRUMS assay is used to assess the renal function of Malpighian tubules by monitoring whether flies are able to remove from the hemocoel a blue dye that gets eliminated in the feces [381]. Flies at the late phase of different infection models were injected with blue dye, then the blue color in the body was monitored as well as the number of defection blue spots on the sides of the fly vial five hours post-injection. Surprisingly, *P. aeruginosa* infection of both wt bacteria and Δ*rhlR*, in all kinds of models, even in the septic injury model, did not affect the renal function of flies, suggesting no dysfunction of internal organs. **(Fig. S10A, B).** Likewise, the phalloidin-stained flight muscle of *Drosophila* in all the infection models displayed clear sarcomeres like those in the non-infected flies and did not show a visible muscle disruption **(Fig. S10C)**. It implies the flies infected with *P. aeruginosa* do not die of substantial organ failure.

In general, firstly, the latent-29℃ infection model favors the virulence-switching program found in the continuous oral infection model. Secondly, it indicates that the sessile bacteria proliferate in the tissues independently of Rhl QS. Thirdly, virulence switching of tissue-colonizing bacteria is regulated by the Rhl system, which is necessary for the transition from silent colonization to systemic infection. Fourthly, PqsE alone is not required in the latent-reactivated infection. We postulate that in the latent-29 ℃ reactivated infection, silently colonized *P. aeruginosa* bacteria in the host tissues are activated to proliferate at a higher temperature by an unknown mechanism independent of the Rhl system with no functional impact on the tissues. When the cell density reaches a population threshold, RhlR signaling is triggered, which in turn enables the production of virulence factors and promotes bacteria to regain motility and become planktonic.

4. Rhl signaling is less required in certain *Drosophila* host contexts with immunodeficiency associated with adhesion

The opportunistic pathogens often seize the chance to be pathogenic in an immunodeficient host environment. Hence, it is important to study bacterial virulence regulatory strategies in the loss-of-function contexts of the host immune response. A previous study by our team reported that PA14 mutant Δ*rhlR* regained virulence close to the level of Δ*rhlI* and even of wild type when phagocytosis was blocked from the beginning in the continuous oral infection [56, 89]. This regained virulence phenotype of Δ*rhlR* was consistently observed in the latent-29 ℃ models when phagocytosis was blocked by latex beads injection **(Fig. S14A, B)**. Given the above studies, it may be relevant to examine further the roles of the Rhl QS system in the context of the *Drosophila* host deficiency of other immune functions. Hence, we analyzed fly mutants for another arm of host defense, namely melanization. The melanization response is an important immune defense in arthropods, which relies on enzymes for melanin deposition called prophenoloxidases (PPOs). Hayan and Sp7 enzymes are responsible for activating PPOs into POs. In chapter I, our colleague Jing Chen, revealed that ingested *P. aeruginosa* PAO1 in the latent infection model can induce systemic melanization response in flies and that phenoloxidases (POs)-deficient flies are susceptible to ingested *P. aeruginosa* PAO1 in the latent infection model.

We found there were also similar roles for POs in the continuous oral infection model with PA14. *P. aeruginosa* PA14 killed almost the POs-deficient flies faster than the wt flies (w^{1118}) **(Fig. 4E)**. More specifically, in the continuous oral infection, the POs-deficient flies exhibited different levels of susceptibility to PA14 wt, as measured by the LT50s of survival curves: Δ*PPO1* >w^{1118} > Δ*PPO2* >Δ*Sp7* > Δ*Hayan* >Δ*PPO1*Δ*PPO2* (hereafter named Δ*PPO1.2*) **(Fig. 4E)**. The double mutant Δ*PPO1.2* is the most sensitive to wt PA14 continuous ingestion, with death occurring within four days, presenting a death rate similar flies in the septic injury infection. Interestingly, PA14 mutants Δ*rhlR*, Δ*pqsE*Δ*rhlI* and Δ*rhlI* regained virulence to a large degree in Δ*PPO1.2* double mutant **(Fig. 4C, D, F)**, as well as in Δ*Hayan* **(Fig. S11G, H; Fig. 4F)**, a mild degree in Δ*Sp7* **(Fig. S11E, F; Fig. 4F)**, but neither in the single mutant Δ*PPO1* **(Fig. S11A, B; Fig. 4F)**, nor the single mutant Δ*PPO2* **(Fig. S11C, D; Fig. 4F)**. In the Δ*PPO1.2* and Δ*Hayan* mutant flies, Δ*rhlR* bacteria and Δ*rhlI*Δ*pqsE* bacteria regained virulence near to the degree of wt PA14, while Δ*rhlI* is as virulent as wt PA14 and

Δ*pqsE.* Briefly, when the melanization reaction is severely impaired, the Rhl system is no longer required for virulence in the oral infection model.

Next, we checked the bacterial load of PA14 wt and Δ*rhlR* in the POs-deficient flies, at day 2 post continuous oral infection. The bacterial burden in the hemolymph of Δ*PPO1.2* flies was high for both wt and Δ*rhlR.* In contrast, hardly any bacteria were detected in the hemolymph of w^{1118}, Δ*PPO1,* and Δ*PPO2* **(Fig. 5A)**, like for A5001 wild-type flies **(Fig. 1B)**. Δ*Sp7* is the exception with a large proportion of flies harboring a high titer of wt but not Δ*rhlR* bacteria in the hemolymph **(Fig. 5A)**. Interestingly, although bacteria increased in the hemolymph, there was no parallel increase of bacteria in the carcass, midgut, and crop of the mutants Δ*Hayan* and Δ*Sp7,* and just a slight increase of Δ*PPO1.2,* the flies that completely lack melanization (**Fig. 5B~D)**. It means the bacteria can be detected in the hemolymph, not due to the bacteria having reached a higher cell density in the tissue, which is possibly accounted for the impaired adhesion from the *Drosophila* host. Rhl signaling becomes less required when the bacteria are not trapped in the tissue, or induced to adhere to the tissues, by host melanization.

A counter-evidence that RhlR is still necessary for virulence is the mutant fly *key* (**Fig. S13A)**, which is impaired in IMD pathway-mediated immune response, including the systemic immune response as well as the local response in the gut. In *key,* although there are more wt bacteria in the tissue and intestine, bacteria are still adhering to the tissue and are not detected in the hemolymph during the early phase of the continuous oral infection. In the late phase, wt bacteria are detected in less than half of the flies, whereas Δ*rhlR* bacteria remained adherent and are much less virulent (**Fig. S13B~E)**. Besides, even though the mutant flies Δ*PPO1.2*, Δ*Hayan* and Δ*Sp7* have performed the infection following the procedure of latent infection, in which the PA14 bacteria in the gut were eliminated by gentamicin feeding, both PA14 wt and the mutant Δ*rhlR* could kill the files gradually and did not develop into a dormant state (**Fig. S12D~F)**.In contrast, in the w^{1118}, Δ*PPO1,* and Δ*PPO2*, PA14 infection can develop into a latent infection, wherein the mutant Δ*rhlR* are much less virulent than the PA14 wt (**Fig. S12A~C)**.

We also checked the involvement of other QS systems, the LasR-LasI system, and the PQS system in virulence regulation on the POs-deficient flies during continuous oral infection. In line with the phenotypes in the A5001 flies, Δ*lasR* and Δ*lasI* displayed

similar virulence as the wt bacteria in the w^{1118}, Δ*PPO1,* and Δ*PPO2* (**Fig. S15A~C)**. However, Δ*lasR*, Δ*lasI,* and Δ5 （a PA14 mutant with deletions in the *lasR, lasI, rhlR, rhlI,* and *pqsE* genes）were significantly less virulent than wt bacteria in the mutant flies Δ *PPO1.2*, Δ*Hayan* and Δ*Sp7* (**Fig. S15D~F)**. Additionally, Δ*pqsR* displayed a similar virulence as wt bacteria both in w^{1118} and POs-deficient flies. Collectively, it suggests, it is not other QS systems, but the Rhl system that is specifically required for virulence switching and lifestyle transition of the sessile bacteria population in the *Drosophila* host. In a reverse vein, the LasR-LasI system maybe contributes to the virulence of the planktonic bacteria.

In general, Rhl signaling is less required in certain *Drosophila* immunodeficiency contexts associated with impaired adhesion to tissues**.** In other words, the Rhl system is necessary for the bacteria population to be endowed with enough virulence and possibly motility to escape adhesion to the host tissues, thus achieving a lifestyle transition from a sessile to planktonic state that leads to acute systemic infection.

Conclusions

- Distinct pathogenic characteristics of three different infection models suggest a virulence-switching program in continuous oral infection, with three bacterial development phases in the tissue: dormant, transitional, and active phase.
- RhlR signaling modulates virulence in a way dependent on RhlI in combination with PqsE in oral infection, but not needed in septic injury infection.
- RhlR is involved in the virulence of OMVs without affecting the vesicle size and yield.
- Virulence activation of sessile bacteria in the host tissue depends on the cell density, which can be contributed by the bacteria continuously crossing the gut barrier or the bacteria proliferating in tissues.
- However, proliferation in the tissues is distinct from virulence, since in the latent-29 ℃ reactivated model, bacterial proliferation is independent from Rhl QS which itself is required for virulence.
- Rhl system but neither the Las system nor the PQS system is necessary for the virulence switching during the transition from silent colonization to systemic infection.
- RhlR quorum sensing is dispensable in certain *Drosophila* host contexts with immunodeficiency associated with adhesion, such as melanization.
- Does an alternative mechanism activate RhlR besides RhlI and PqsE-dependent ones: an alternative autoinducer system?

Discussion

How is the host-microbe balance broken?

To our knowledge, few studies have investigated the host-pathogen relationship through the combined analysis of more than three different models in the same host before. The flexibility and the comprehensive innate immune system of *Drosophila* allowed us to develop *P. aeruginosa* infection models with different pathogenic characteristics of acute, chronic/latent, transitional, and reactivated processes. The analysis in comparison with the septic injury and latent infection revealed that the continuous infection exhibits a virulence activation relying on the Rhl quorum sensing system, along with an increase of cell density in the carcass. It suggests a classic case of cell density-dependent activation of virulence for the sessile bacteria in the host tissue. In comparison with the latent infection, the main difference of the continuous ingestion infection model is that *P. aeruginosa* bacteria are present in the gut and might continuously cross the intestinal barrier.

However, there is a relatively stable phase of bacterial load from Day 2 to Day 4 during the continuous oral infection (Fig.1 B~E). Possibly, it is a balanced outcome of the middle phase between the ingested bacteria and the gut defense, and between the crossing bacteria and phagocytosis, possibly as well as the ROS reaction activated along with melanization [18, 30]. We infer that the gut bacteria go on crossing the gut barrier independently of their QS system in the early phase since there is no difference in the bacteria load in the tissue as well as in the gut among the PA14 wt and the mutant RhlR system at day 2 and day 5 in the intestinal infection model of A5001 flies (Fig.S13C). But at the very late phase (Day 6 and Day7), we observed an obvious increase in the load of wt bacteria (Fig.1 B~E), and a significant difference between wt bacteria and the RhlR mutant in the hemolymph, tissue, as well as midgut and crop (Fig.2 C~F).

The host-microbe balance was broken at a certain point during the late phase. We infer that the RhlR function endows *P. aeruginosa* with a higher ability to evade the *Drosophila* host immune response. We discuss this in detail in the next part. But the point is the quorum sensing should be activated based on the increase of bacterial load. In other words, classically, bacterial number increase comes first before quorum sensing activation. Two possibilities can explain this conundrum：

One possibility is that there may be crosstalk between the tissue-associated *P.*

aeruginosa and intestinal bacteria. It has been reported that the autoinducer C4-HSL is capable to cross the *Drosophila* intestinal barrier [382]. Moreover, our study showed the bacterial load of wild-type *P. aeruginosa* in the tissue at the very late phase (Day 6 & Day 7) in the continuous oral infection is obviously lower than those in the middle phase of the latent-29 ℃ infection (Fig.1C *vs*. Fig.3D). It means that for activating the Rhl QS signaling, the *P. aeruginosa* in the tissue during continuous ingestion model does not require as high density as those in the tissue in the latent-29 ℃. It further supports that the tissue bacteria and gut bacteria share the communication signaling molecule during continuous oral infection. But there is doubt that the bacteria in the gut and tissues are increasing synchronously from Day 5 onwards (Fig.1 C~ F). It is difficult to find a clear-cut relation between cell density and overall virulence. Yet, we have not excluded the outside bacteria on the feeding pad may also be involved in the communication by the secretion of its autoinducer that can be ingested. Of note, in a similar experimental setting, there was no visible damage to the gut epithelial barrier according to our previous study [89]. A study proposed the epithelial layer of the crop was severely damaged in the intestinal infection with PAO1 at the early phase [344]. We are not sure whether the crop was damaged in our experimental setting. Severe damage seems not possible during the middle period, otherwise, the bacteria load in the hemolymph and tissue should not be so confined for a long period. But the crop might be damaged at the very late phase by the virulence factors (*e.g.*, chitinase) regulated by Rhl QS. Anyway, *P. aeruginosa* starts crossing the gut barrier via an unknown route before the activation of quorum sensing. It may cross the intestinal epithelium either intracellularly through enterocytes or paracellularly in-between enterocytes which would require disrupting separate junctions that make the epithelium tight.

The second possibility is the bacteria may be proliferating both in the tissue and the gut at certain points during the continuous oral infection. We can take the latent-29 ℃ infection as an example, wherein bacteria are proliferating independently of the Rhl system (Fig.3 D). That is wt or Rhl mutants have the same proliferation ability. In addition, these proliferating sessile bacteria may have a certain ability to evade the immune response since phagocytosis cannot suppress effectively the increase of the bacteria, even though it is still functioning (Fig.S14 B, C). Besides, the expression of the *Diptericin* AMP is hardly induced (about 10% of the positive control) by the proliferating sessile bacteria (either wt or mutants) of a high load in the latent-29 ℃

infection. In contrast, in the septic injury infection, the proliferating planktonic bacteria of lower density can induce a much higher level of *Diptericin* (over 90 % of the positive control) (Fig 3 D & E *vs*. Fig S1 C & G), although it was proposed that systemic AMP induction were partially suppressed by *P. aeruginosa* in the septic injury infection [383]. Of note, Peptidoglycan (PGNs) of Gram (−) bacteria are not easily detected by *Drosophila*'s PRRs, upstream of *Diptericin* induction, since they are not exposed on the outer membrane unless the cell components are digested or small PGN fragments are released from the proliferating bacteria during cell-wall remodeling [75]. Thus, it is very interesting that sessile bacteria are proliferating without obvious AMPs induction. Yet, we have not checked whether the bacteria are forming biofilm in the tissue, which is rather unlikely given DAPI staining.

How is proliferating switched on? In the latent-29 ℃ infection model, the temperature may be a key reason to activate the bacteria to proliferate, since it is common that bacterium regulates their behavior and virulence through thermosensing [384, 385]. Besides, host metabolites may also be an incentive. Temperature stress and sucrose feeding (no nutrients) possibly change the metabolism of flies, which may activate the dormant bacteria. A possible clue is that the flies in the latent infection fed on the food instead of sucrose solution, survived for over one month (LT50 > 30 days), nearly as long as the non-infected flies (Fig.S5C), whereas the flies fed on sucrose only survive for 20 days (Fig.S5A). We also did a tentative experiment that food feeding could delay several days of the proliferation and virulence of *P. aeruginosa* during latent-29 ℃-infection. Additionally, a study of our group in Strasbourg showed that the phosphatidic acid of the *Drosophila* host is limiting for the proliferation of the intracellular parasites Microsporidia, which provides a direct link between host metabolism and the proliferation of microorganisms [381].

RhlR activation endows *P. aeruginosa* higher ability to evade the *Drosophila* host immune response.

Previously, our colleagues have found that hemocytes play a key role in controlling the bacteria crossing the gut barrier. However, at the late phase of the continuous ingestion model, phagocytosis of hemocytes no longer suppresses the bacterial increase, resulting from the activation of RhlR, which allows wild-type PA14 to evade such cellular immune response [56, 89]. Consistently, we also observed a virulence-regain phenotype

of Δ*rhlR* regain in the latent-29℃ infection model using phagocytosis-blocked flies (Fig.S14). Besides, we found an interesting relationship between the RhlR function of *P. aeruginosa* and the local immune response of the *Drosophila* intestine. Previously, we have mentioned that there is no difference in bacterial loads between *P. aeruginosa* wt and mutant Δ*rhlR* until the very late phase, in which bacterial load of Δ*rhlR* was lower in the hemolymph, tissue, midgut, and crop (Fig S13 B~E; Fig2C~F). This suggests that RhlR endows *P. aeruginosa* with higher abilities to withstand or evade the normal host immune response in the tissue as well as in the gut. Firstly, we excluded AMPs, the important effectors in the gut immune response, since the IMD-pathway mutant *key* infected with Δ*rhlR* still could survive as long as wt flies with Δ*rhlR*, although *key* mutant was killed faster than the wt flies by wt *P. aeruginosa* (Fig.S13A). We also excluded the elimination by epithelial renewal (epithelial shedding) because it is dependent on the IMD-NF-κB pathway [91] and we previously did not observe the ISCs proliferation even at the late phase of infection. A difficulty in the interpretation of the bacterial titer of the crop and midgut is that it corresponds not only to bacteria present in the lumen but also includes bacteria topologically present in the hemocoel that adhere to these organs as to other tissues. In addition, as discussed in Chapter I, melanization may not act in the lumen of the digestive tract. The simplest explanation to account for the non-increasing burden of RhlR mutant bacteria in *key* mutants is that they remain highly susceptible to the two other arms of the innate immune response, namely melanization and phagocytosis.

As noted in Chapter I, we do not really understand how melanization actually acts on *P. aeruginosa*. It would be interesting to determine whether it does not initially contribute to the adherence of bacteria escaping the gut by trapping them, that is "gluing" them to the tissue.

Rhl system and Las system regulate the virulence in an opposite lifestyle transition *in vivo* and *in vitro*

The continuous oral infection and latent-29 ℃ reactivated models both highlight that the bacterial virulence switching correlates with a lifestyle transition from a sessile to a planktonic state. The process highly relies on the Rhl QS system, but not the Las system (Fig.2 *vs*. Fig S6E; Fig.3B *vs*. Fig S9A). The opposite evidence is the phenotypes in the melanization-deficient flies or phagocytosis-blocked flies. LasR/LasI mutants turn out

to be less virulent in melanization-deficient flies (Fig.S15D-F), as well as in phagocytosis-blocked flies [89], showing opposite phenotypes to the RhlR mutant. Consistently, the opposite phenotypes of biofilm formation and architecture have been observed *in vitro*. Δ*rhlR* exhibits a hyper-rugosity biofilm phenotype, as shown in the published reports [289, 297] and our study (Fig. S3C). In contrast to the *rhlR* mutant, biofilms of *lasR* and *lasI* mutants are thin, less differentiated, easily eradicated by surfactants, and more susceptible to antibiotics [315]. Development of *P. aeruginosa* biofilms *in vitro* has been well characterized as a five-stage process [308, 309]: reversible attachment, irreversible attachment, early maturation, maturation, and dispersion. During this process, Las QS is activated from the irreversible attachment phase, while Rhl QS is activated from early maturation onwards [308]. Biofilm dispersion requires the overproduction of rhamnolipids, which are predominantly controlled by Rhl QS [314]. Given the above, the Rhl system plays a key role in biofilm architecture and biofilm dispersion, whereas lasR may be more responsible for the early stages of biofilm formation. It is worth going further to see whether there is biofilm formation in the tissue during the infection of our models.

Moreover, one interesting study was performed via a killing assay of amoeba challenged with *P. aeruginosa* and demonstrates that the planktonic-sessile transitional behavior (surface attachment) triggers the bacterial virulence relying on mechanotransduction and the Las system but not the Rhl system Also, the planktonic bacteria in high density are not virulent even though they have been supplemented with autoinducers. It means that virulence cannot be elicited without mechanotransduction [329]. Curiously, in the reverse vein, during the transition from the sessile state to the planktonic state, is there a virulence switching that also requires two such gates, mechanotransduction signaling, and quorum sensing? The latter has been identified in our *Drosophila* models which is Rhl playing a key role in the virulence switching. It was also reported that *P. aeruginosa* cells detached from biofilms show gene expression profiles distinct from the corresponding biofilm cells and from the planktonic cells. In contrast to planktonic cells, these dispersed cells have higher virulence to *C. elegans* and macrophages [323]. It may however not be a simple reverse process under the surface, particularly in the dynamic host environment. We are wondering if the Rhl system may also influence the sessile bacteria to regain their motility. It has been proven that the swarming behavior *in vitro* of PAO1 is completely abolished without RhlI or

RhlI, resulting from the lack of biosurfactant rhamnolipids [166]. However, the absence of *rhlA* or *rhlB*, the genes for rhamnolipids synthesis of *P. aeruginosa,* did not affect the virulence phenotypes in intestinal infection at least in wild-type flies [89]. Anyway, in our models, virulence switching always correlates with planktonic bacteria being present in the hemolymph. It is thus worth identifying *in vivo* whether the Rhl system allows bacteria to regain their motility and whether this motility may be coupled to the secretion of virulence factors. Of note, the flagellar synthesis apparatus is a T3SS and one of its components, FliR, has been shown to be important for the virulence of *S. marcescens* in an intestinal infection model [106]. Besides, since c-di-GMP is also an important bacterial signaling molecule that regulates the bacterial lifestyle transition [316], it may be interesting to detect the c-di-GMP level of wild-type *P. aeruginosa* and Δ*rhlR* mutant *in vitro* and in our *Drosophila* infection models.

Alternative activation mechanism of Rhl signaling in the *Drosophila* host?

Our study here shows that PqsE works synergically with RhlI for enhancing the RhlR activation and that unexpectedly PqsE alone does not contribute to the pathogenicity in the host *Drosophila.* The *Drosophila* infection models provide solid evidence *in vivo* that highly supports the PqsE-RhlR-C4HSL interaction mechanism which has been identified *in vitro* [299, 302], and was marked in the infection models of *C. elegans* and mice. We point out that the lack of a PqsE virulence phenotype excludes it as an explanation to account for the obvious difference between Δ*rhlR* and Δ*rhlI in vitro* and *in vivo*. It means there may be a separate and independent function of RhlR and RhlI or another activation mechanism. The existence of an alternative autoinducer thus remains a possibility. Moreover, we also observe the distinct phenotypes between Δ*rhlR* and Δ*rhlI* Δ*pqsE in vivo*. To our knowledge, Δ*rhlI*Δ*pqsE* should have the same phenotype as Δ*rhlR*. It is true when we look at the phenotypes *in vitro*, including the pyocyanin production, colony biofilm architecture, and supernatant injection (Fig. S3A~D; Fig. S7C), wherein the two strains shared the same phenotypes. However, there is a significant difference between Δ*rhlR* and Δ*rhlI* Δ*pqsE* in fly survival during continuous oral infection (Fig. 2A, B; Fig. S6C, D). A puzzling observation is Δ*rhlR* and Δ*rhlI* Δ*pqsE* exhibit no difference in bacterial load in hemolymph, carcass, midgut, and crop (Fig. 2C~F). Interestingly, in the latent infection model, the infection with a long-term course, and without bacteria in the gut, flies infected with Δ*rhlI* Δ*pqsE* survived for as

long as those with Δ*rhlR* (Fig. S5A, B). It suggests an alternative mechanism that activates RhlR besides RhlI and PqsE-dependent ones. Is an alternative autoinducer system or mimic signal from the host or gut microbiota? The phenomenon leads us to ask: what causes the distinguishable phenotype between Δ*rhlI* Δ*pqsE* and Δ*rhlR* in continuous oral infection? We try to ask to answer the question in Chapter III.

What are the flies dying of?

We tried to find out the actual cause of death for the infected flies in this study, from a question of whether the flies in the latent-29 ℃ reactivated infection model died of the damage inflicted by the virulence factors secreted from proliferating sessile bacteria or succumbed to bacteremia like in the septic injury model, or both. Surprisingly, we found that no significant functional renal failure of the flies was observed in all kinds of the models, including the septic injury infection, even at a time close to their death (Fig.S10A, B). Quorum sensing is activated in the septic injury infection, as we detected a high transcriptional level of *rhlA* (Fig 3F), which is regulated by the Rhl system, Las and PQS system [250]. In septic injury infection as well as other models, with a such high number of bacteria proliferating inside the fly body, severe tissue damage is supposed to be easily observed. However, we did not observe obvious flight muscle disruption by muscle staining, although it has been reported that flight muscles are degraded in the septic injury infection [206]. It is worth checking the pathology of flies by a more precise approach of cell death staining. Therefore, we are wondering if the flies might not die of physiological failure or severe tissue damage. Tissue damage by virulence factors may be very limited and not enough to inflict a deadly strike. Unlikely, in mammals, cytokine storms and endotoxic shock with severe programmed lytic cell death initiated by invading microorganisms or their cell wall components (*e.g.*, LPS) can lead to a fatal outcome [386, 387].

Another reasonable hypothesis is that although a large set of genes of virulence factors are transcriptionally activated by quorum sensing of the bacterial group, the post-translation or secretion of their virulence factors may be under a strict control. One *in vitro* evidence is that the supernatant of high cell density culture in BHB did not kill flies (Fig. S7B), although we did observe pyocyanin production indicating QS activation. In contrast, the supernatant of PA14 grown in LB killed the flies in a few hours（Fig. S7B). Another piece of evidence is the killing assay of amoeba infected

with *P. aeruginosa* that I mentioned above. Bacteria of planktonic form in high density even supplemented with autoinducers (3OC12-HSL, C4-HSL) are not virulent, unless mechanotransduction triggered by surface attachment, collaborated with the Las QS system. In the reverse vein, our models show Rhl QS is required for the virulence switching, corresponding to a bacterial transition from a sessile to a planktonic form. Also, as mentioned before, detached *P. aeruginosa* cells show higher virulence than their corresponding planktonic cells [323]. In light of the above, we infer that, in the scenario of latent-29 ℃ reactivated infection, there are four different groups of *P. aeruginosa* bacteria with different behaviors in the fly hemocoel at the late phase of infection: 1) sessile proliferating bacteria with Rhl QS activated, limited releasing of virulence factors；2) transitional bacteria from a sessile to a planktonic state, releasing virulence factors possibly for local tissue degradation, regained motility, and readiness for dispersion. 3) planktonic proliferating bacteria in the hemolymph, highly focusing on the proliferation, with QS activation, but not releasing aggressive virulence factors, and capable of evading or inhibiting the host immune response; 4) adherent bacteria, releasing invasive virulence factors dependent on the Las QS system and mechanotransduction. In the septic injury infection, planktonic proliferating bacteria and adherent bacteria possibly behave as described above. It has been proposed that systemic AMP induction is partially suppressed by *P. aeruginosa* in septic injury infection [383]. High proliferating bacteria may have strategies for evading the host's immune response. For example, our colleague Dr. Jing Chen found in the septic injury infection that *P. aeruginosa* circumvents the bactericidal or bacteriostatic effect of AMPs through the HigB-HigA toxin-antitoxin system (unpublished), which also has a function in regulating the cell proliferation in a stress environment. More specifically, antitoxin mutant Δ*higA* presents low cell density in the hemocoel and kills the flies much more slowly than wt PAO1, Δ*higB,* and Δ*higBA*, whereas Δ*higA* displays the same virulence and bacterial load in the *key* mutant or ΔAMPs flies. Moreover, I also identified the consistent phenotype of pathogenicity in the murine model of acute lung infection with the above four strains. *ΔhigA* is prone to causing less severe bacteremia (ANNEX). Besides, Dr. Jing Chen also found that the HigBA toxin-antitoxin operon is induced when bacteria are in the gut and that it possibly contributes to the establishment of persistent bacterial colonization with low virulence in the tissue. In any case, instead of being aggressive to kill the host at once, possibly, the best survival strategy for *P.*

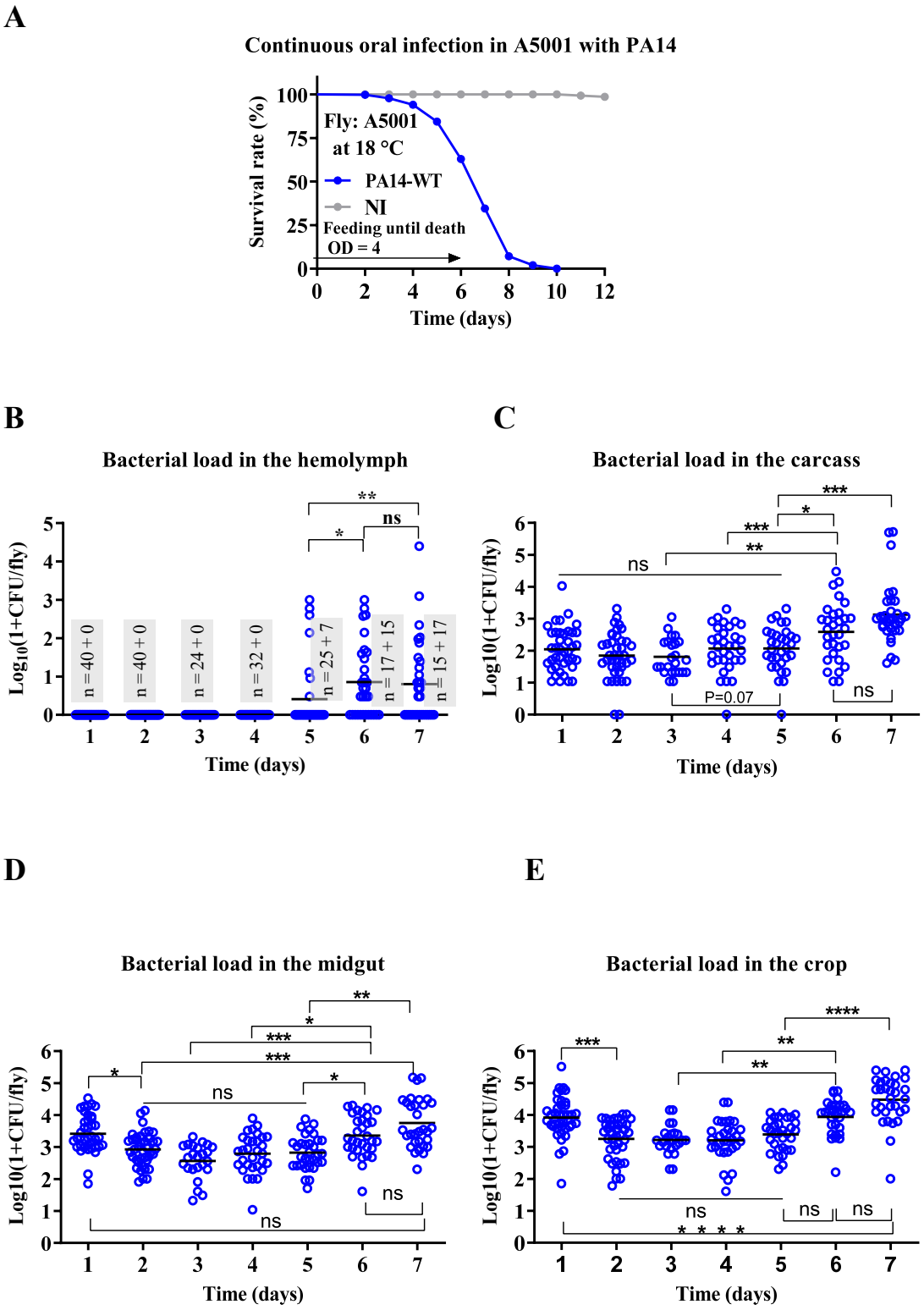
aeruginosa bacteria is to make the most of the host condition to proliferate in self-effacement, until reaching a much higher threshold of cell density, which indicates the living space and nutrient become limiting.

Our group has found that OMVs from *S. marcescens* kill flies very rapidly by acting on the nervous system through a metalloproteinase and likely by inducing apoptosis of some neurons (unpublished). It will be worth investigating whether this mechanism contributes to fly killing in *P. aeruginosa* acute infections. It would therefore be interesting to monitor the expression of the *P. aeruginosa* AprA metalloproteinase. It will also be interesting to determine whether genetically blocking apoptosis in neurons enhances the survival of flies with *P. aeruginosa* infection. Of note, *aprA* mutants display wild-type virulence when ingested [89]. It will be interesting to test the *aprA* mutants in the septic injury model.

In summary, the investigation into host-microbe interaction between *Drosophila* and the *P. aeruginosa* in different infection models with immunocompetent and immunocompromised host context, help us obtain deeper insights into the complex virulence regulatory mechanism and network of QS in pathogenicity, and the host innate immune defense.

Figures

Figure 1



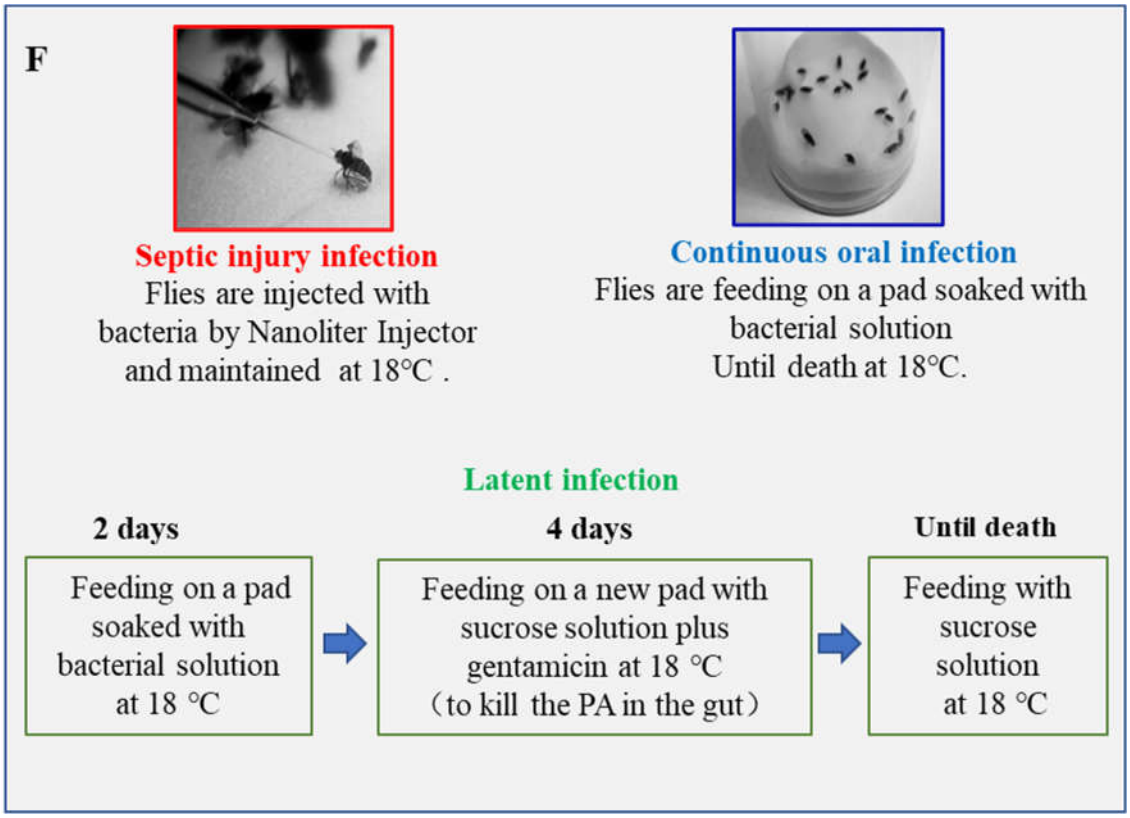


Fig. 1. Continuous oral infection presents a pathogenic process of delayed virulence. A5001 female flies were fed on a pad soaked with PA14 bacteria solution (0.6ml, OD of 4 in 50mM sucrose with 5% BHB) at 18 °C. (A) Survival curve of flies following continuous oral feeding with PA14 wt. (B ~ E) Time-course bacterial titer for the hemolymph, carcass (without the gut, ovary, and Malpighian tubules), midgut, and crop collected from a single fly post-infection. Each value point of bacterial load per fly is converted to a logarithm (CFU +1）. The geometric mean is displayed as a bar. The Fly sample number of each group is shown as total number = N (flies without bacteria in the hemolymph) + N (flies with bacteria in the hemolymph). Eight flies were analyzed for each independent experiment. Pooled data from more than three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns = not significant (F) Procedure scheme of three different infection models of *P. aeruginosa.*

Figure S1

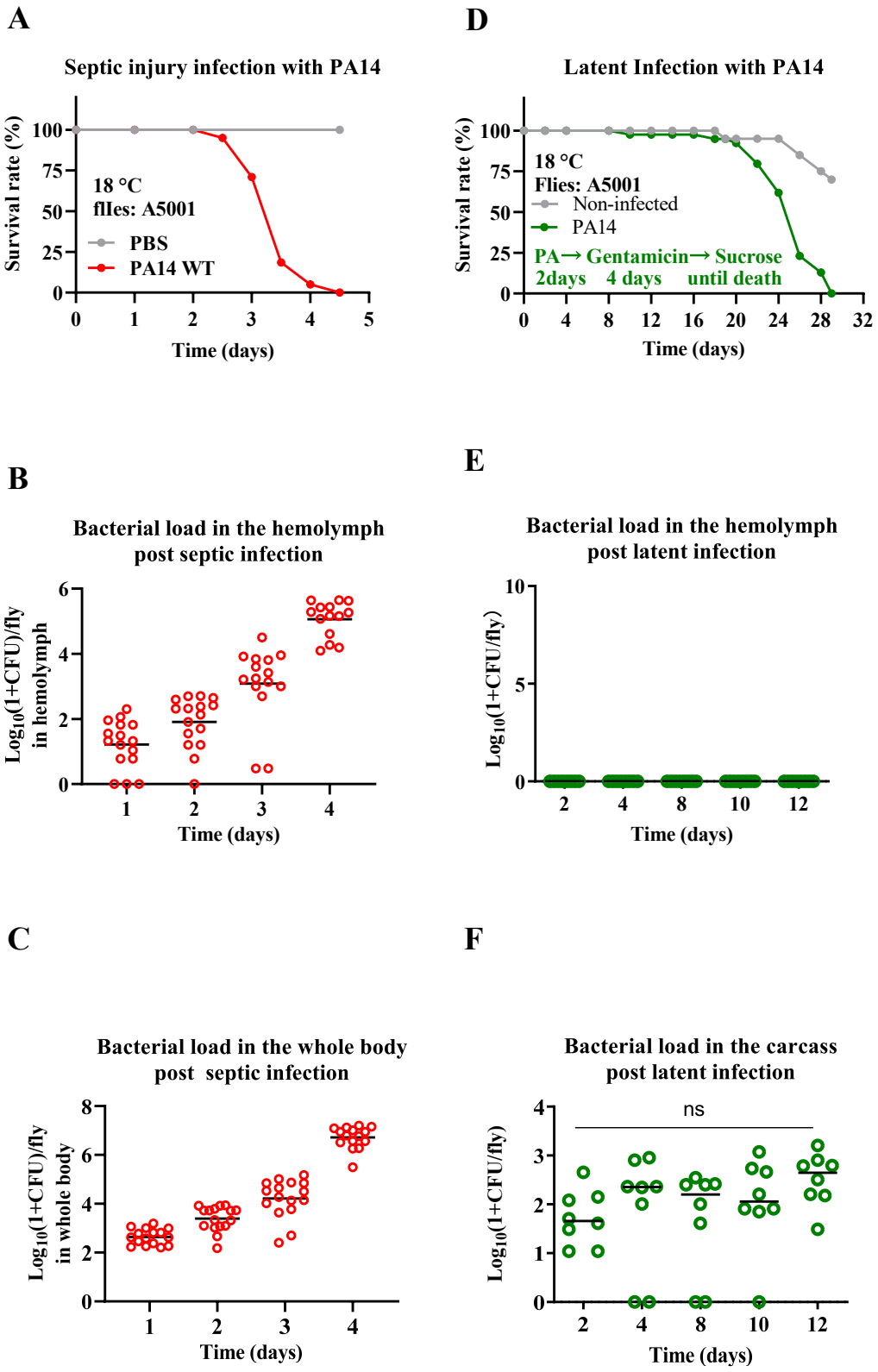

A
Septic injury infection with PA14
Survival rate (%)
100
75
50
25
0
18 °C
fIles: A5001
PBS
PA14 WT
0 1 2 3 4 5
Time (days)
D
Latent Infection with PA14
Survival rate (%)
18 °C
Flies: A5001
Non-infected
PA14
PA→ Gentamicin→ Sucrose
2days 4 days until death
0 4 8 12 16 20 24 28 32
Time (days)
B
Bacterial load in the hemolymph
post septic infection
Log10(1+CFU)/fly
in hemolymph
6
4
2
0
1 2 3 4
Time (days)
E
Bacterial load in the hemolymph
post latent infection
Log10(1+CFU/fly)
10
5
0
2 4 8 10 12
Time (days)
C
Bacterial load in the whole body
post septic infection
Log10(1+CFU)/fly
in whole body
8
6
4
2
0
1 2 3 4
Time (days)
F
Bacterial load in the carcass
post latent infection
Log10(1+CFU/fly)
ns
4
3
2
1
0
2 4 8 10 12
Time (days)

Figure S1

G

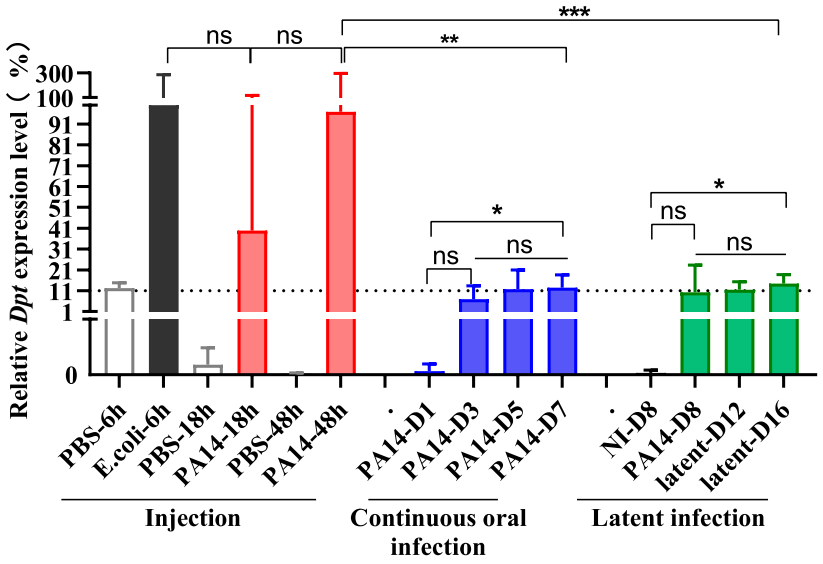
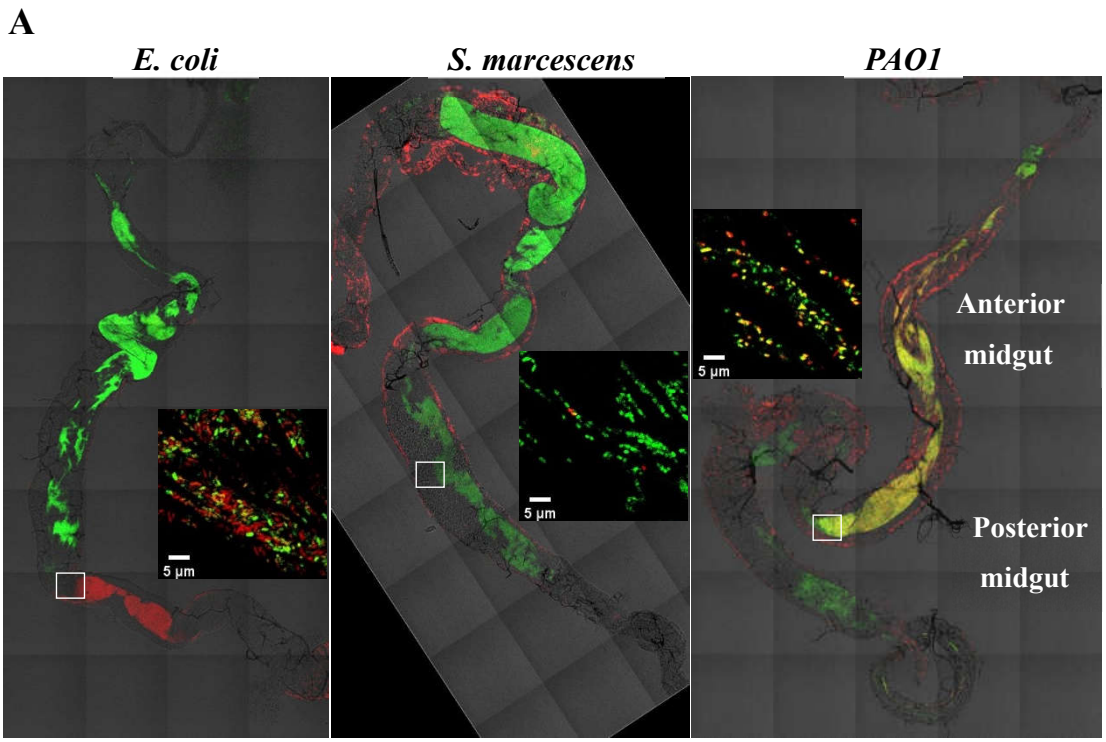


Fig. S1. Latent infection presents a pathogenic process of limited virulence versus septic injury infection of high virulence. In septic injury infection, PA14 bacteria (13.8nl, OD of 0.01 in PBS,) were directly injected into the thorax of A500 female flies at 18 ℃. In the latent infection, flies were fed on a pad soaked with PA14 solution (0.6ml, OD of 4 in 50mM sucrose with 5% BHB) for 2 days at 18 ℃. Subsequently, flies were transferred to gentamicin feeding for 4 days and thereafter fed with sucrose until death. (A, D) Survival curves of flies following septic injury infection or latent infection with PA14 wt. (B, C, E, F) Time-course bacterial titer for the hemolymph, whole body, or carcass collected from a single fly post-infection. Each value point corresponding to the bacterial load per fly is shown on a logarithmic scale. (G) Relative mRNA level of *Diptericin (Dpt)*， a classic IMD pathway readout, in the infected flies with different infections, detected by RT-qPCR. The induction level of *Diptericin* in flies after 6h post-injection with *E. coli* is used to be a positive normalization control. Data were shown as a percentage after normalization. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns = not significant

Figure. S2


A
E. coli
S. marcescens
PAO1
5 µm
5 µm
5 µm
Anterior
midgut
Posterior
midgut

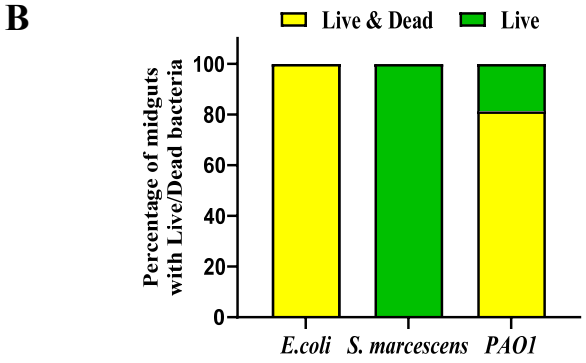

B
Live & Dead
Live
Percentage of midguts
with Live/Dead bacteria
100
80
60
40
20
0
E.coli
S. marcescens
PAO1

Fig. S2. *P. aeruginosa* faces a hostile environment in the gut before crossing the intestinal barrier to the tissue. PI staining of bacteria showed three different bacteria have distinct sensitivity to the gut antimicrobial responses. Flies were fed on pads with indicated GFP-expressing bacteria (OD600nm =10) and propidium iodide (40 μM) in 50 mM sucrose plus 10% BHB at 18℃ for 18 hours, then the gut was dissected for imaging. (A) Uninterrupted confocal images of midguts are adjacent in X and Y by Tile Scan, merged with channels of GFP-green, PI-red, and brightfield. Note that *E. coli* are alive in the anterior part of the midgut and dead in the posterior part, after the passage through an acidic region that separates the anterior from the posterior midgut. In contrast, *P. aeruginosa* appears to be killed already in the anterior midgut whereas *S. marcescens* bacteria were not killed through the gut. (B) Quantification of midguts is roughly divided into two classes: Green with live bacteria (>about 80% lumen area was green), and Yellow with a mix of live and dead bacteria (>20% lumen area was yellow or red). The number of midguts per column: 16 of PAO1, 8 of *E. coli,* and 8 of *S. marcescens* (RM66262_PBB2-GFP). Dara was pooled with two independent experiments.

Figure 2

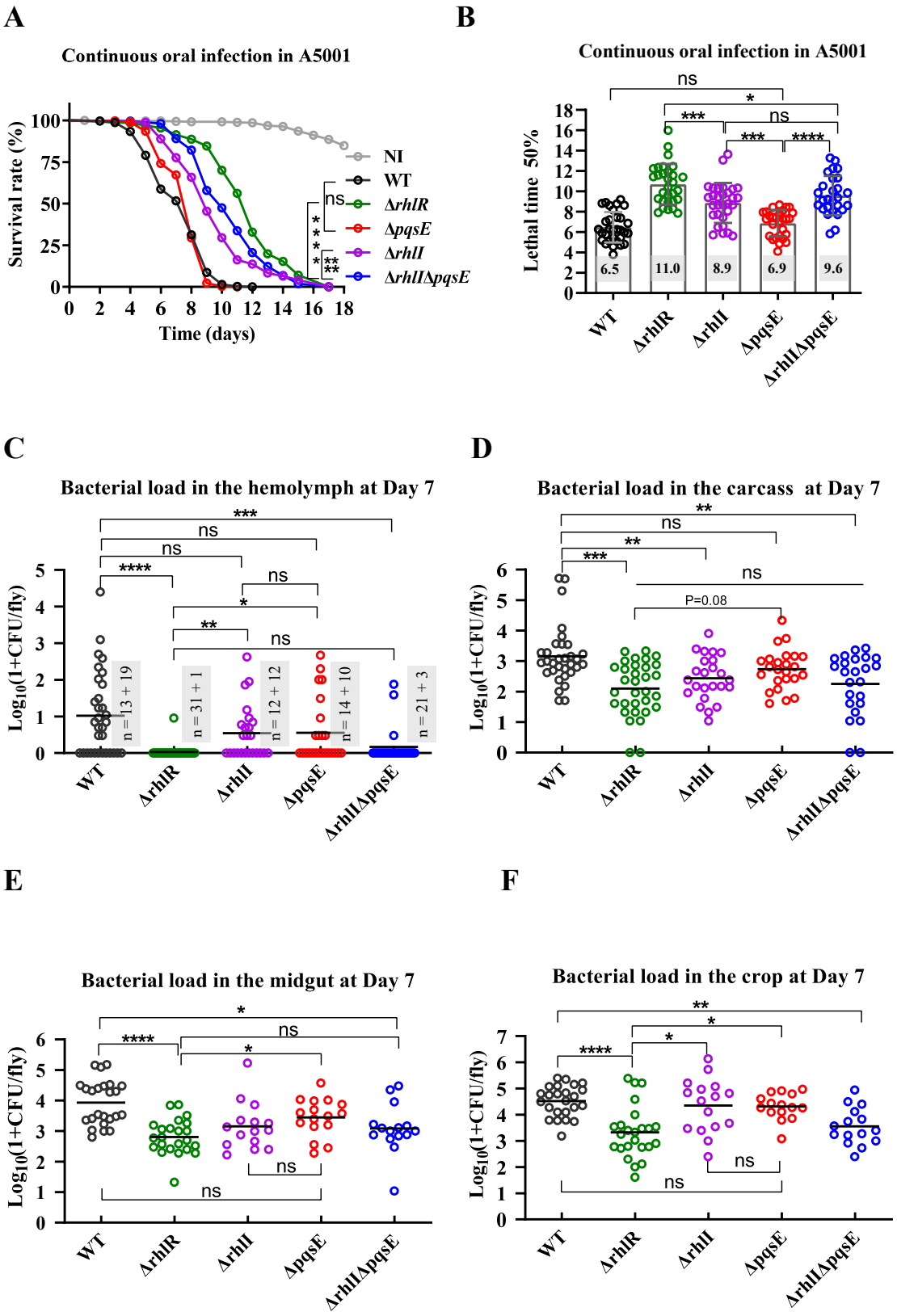
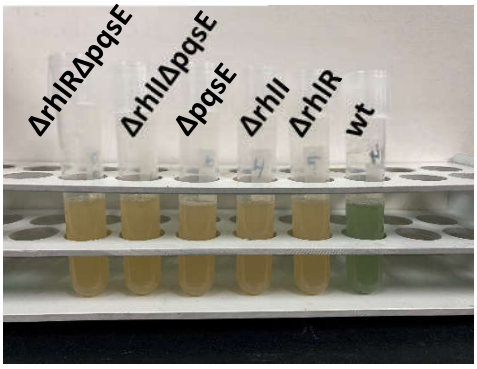

A
Continuous oral infection in A5001
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlI
ΔrhlIΔpqsE
B
Continuous oral infection in A5001
Lethal time 50%
6.5
11.0
8.9
6.9
9.6
C
Bacterial load in the hemolymph at Day 7
Log10(1+CFU/fly)
n = 13 + 19
n = 31 + 1
n = 12 + 12
n = 14 + 10
n = 21 + 3
D
Bacterial load in the carcass at Day 7
P=0.08
E
Bacterial load in the midgut at Day 7
F
Bacterial load in the crop at Day 7

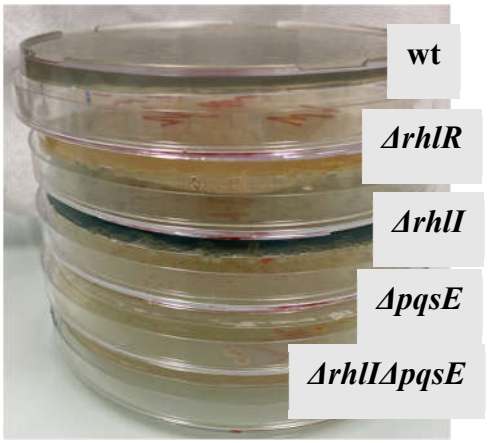
Fig. 2. Rhl quorum sensing system is required for virulence in continuous oral infection. A5001 flies were challenged with PA14 wt or mutants as indicated by continuous feeding on pads soaked with bacterial liquid at 18 ℃. (A, B) The survival of flies was monitored, and the corresponding lethal time 50% was analyzed. LT50 was shown as a scatter plot with a bar and lined at Mean with SD. One data point presented LT50 for one infected set of about 20 flies. Pool data of survival from ten times independent experiments. (C ~F) Bacterial titer separately for the hemolymph, carcass, gut, and crop collected from a single fly post 7-days infection, each value point of bacterial load per fly converted as logarithm (CFU +1). The geometric mean was lined at the bar. The fly sample number of each group is shown as total number = N (flies without bacteria in the hemolymph) + N (flies with bacteria in the hemolymph). Pool data from independent experiments two or three times.

Figure S3

A



B



C

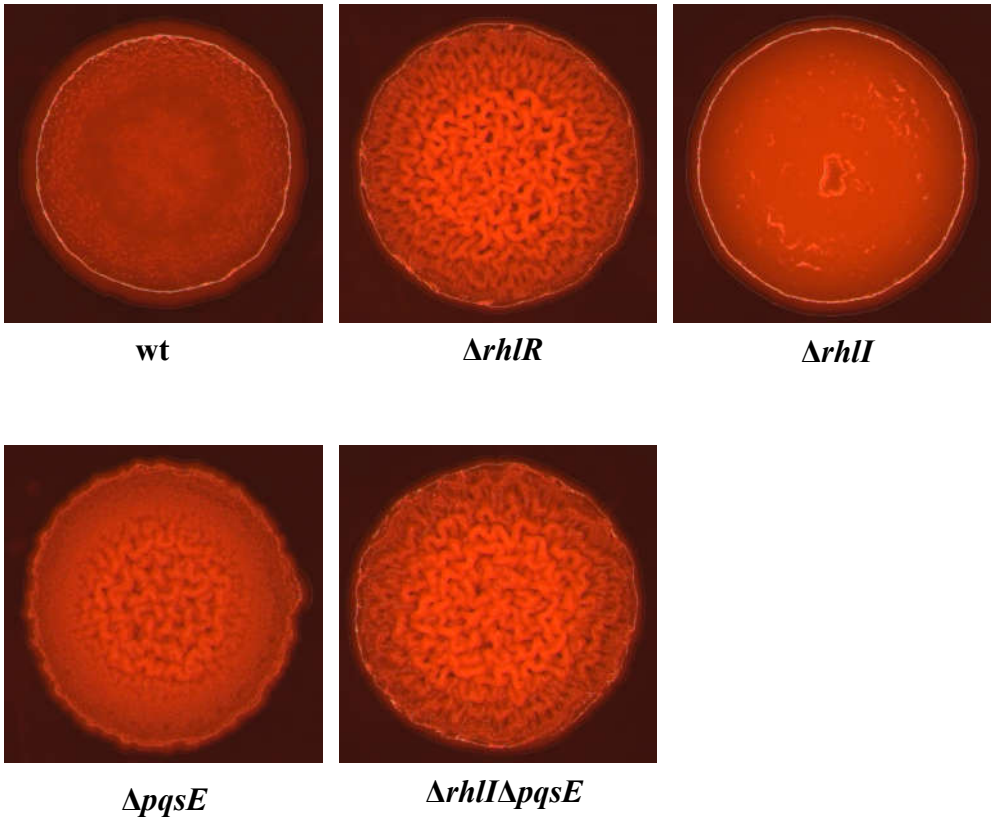


Figure S3

D

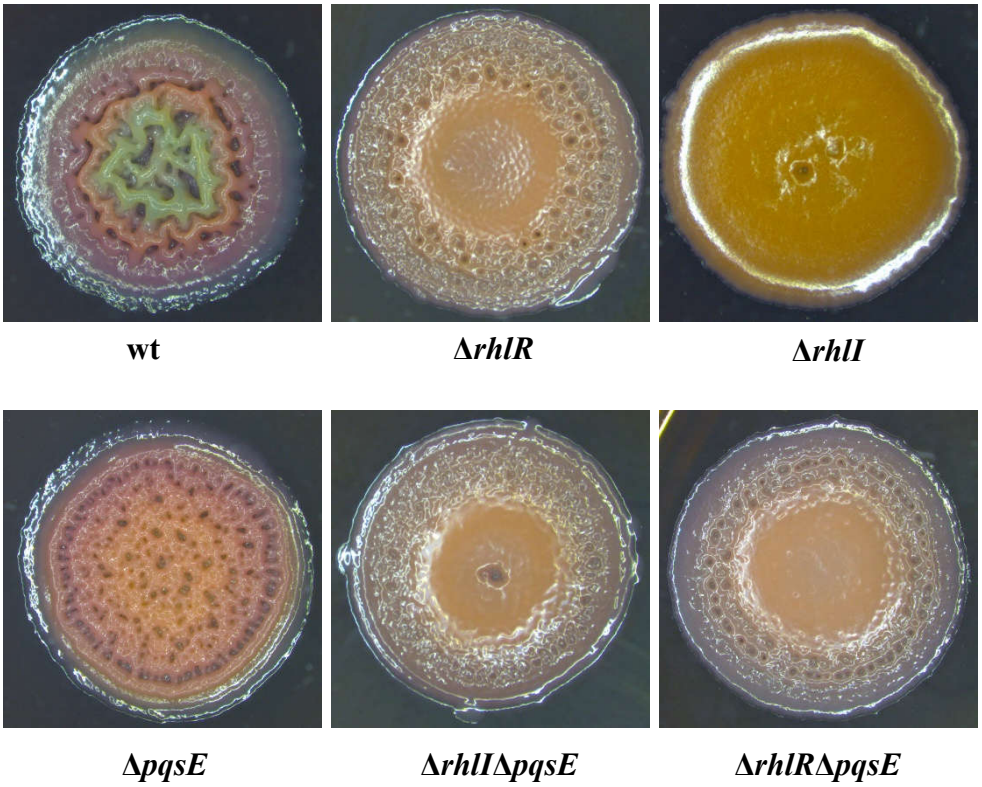


Fig. S3. Phenotypes in *vitro* culture of PA14 wt and mutants of Rhl quorum sensing system. (A) None of the PA14 mutants produced visible pyocyanin in liquid culture. Bacteria were cultured in LB liquid of 5 ml in the 37 ℃ shaker for 16 hours. （B) Pyocyanin production of bacteria cultured on the LB plate. The images were acquired after 2 days of culture at 37 ℃. (C） Colony biofilm phenotypes of wt PA14 and mutants on Congo red agar of recipe-1 after 5 days of growth at 25 ℃. (D) Colony biofilm phenotypes of wt PA14 and mutants on Congo red agar of recipe-2 after 5 days of growth at 25 ℃.

Figure S4

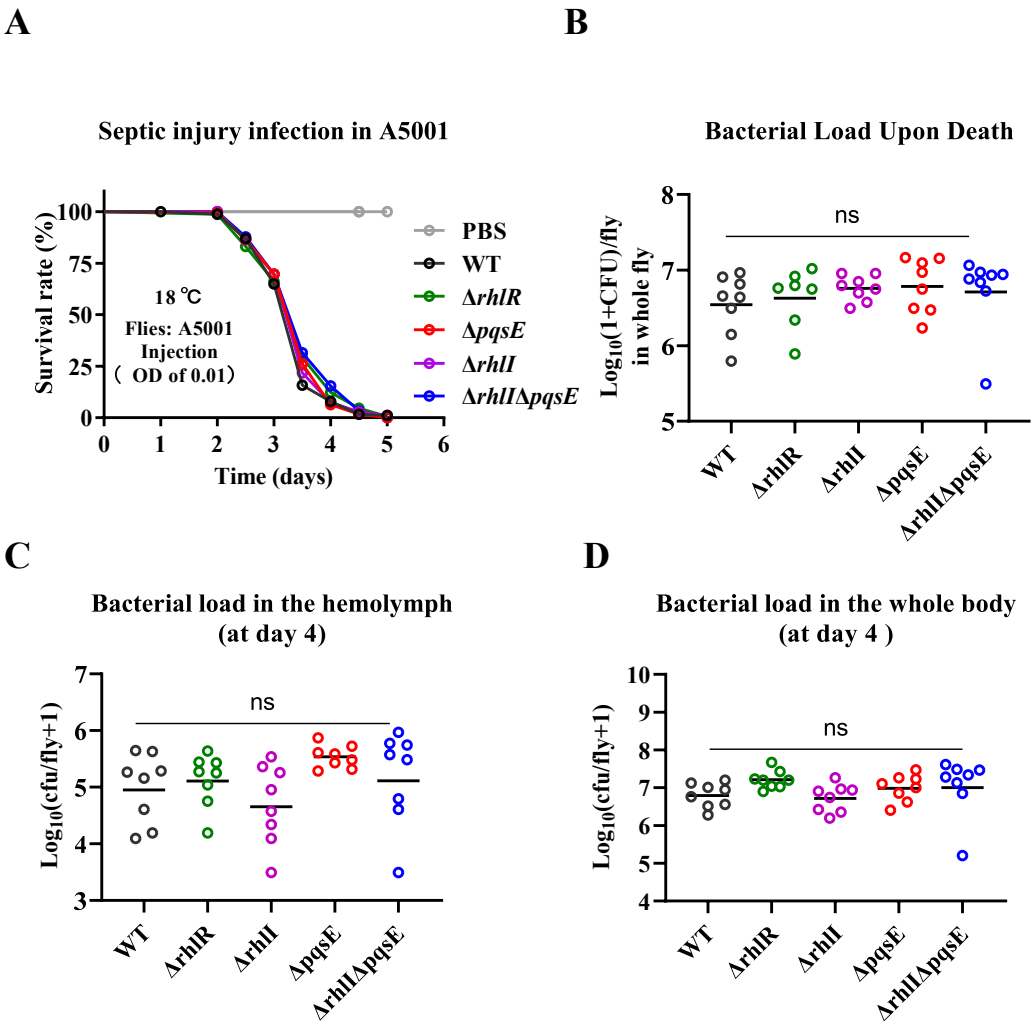
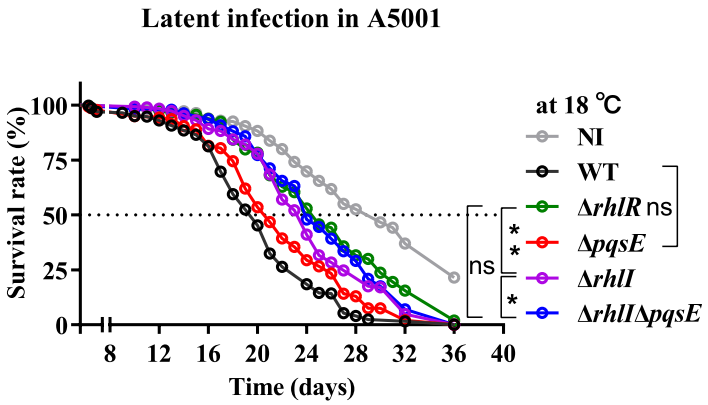


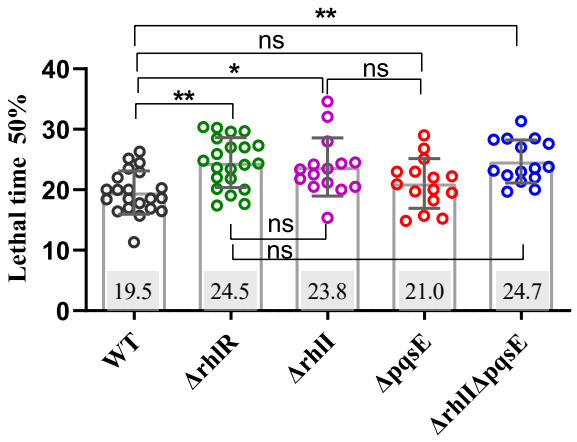
Fig. S4. Rhl quorum sensing system is not required in the septic injury infection of *Drosophila*. A5001 flies were injected with PA14 wt or mutants as indicated and monitored at 18 ℃. (A) The survival curve of flies represents pooled data from three independent experiments. (B) Bacterial load was detected upon death. (C, D) Bacterial titer in the hemolymph or the whole-body post 4 days of infection.

Figure S5

A



B



C

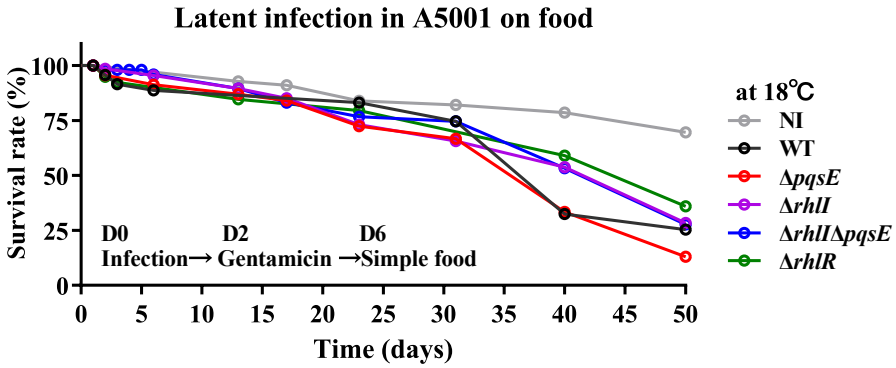


Figure S6

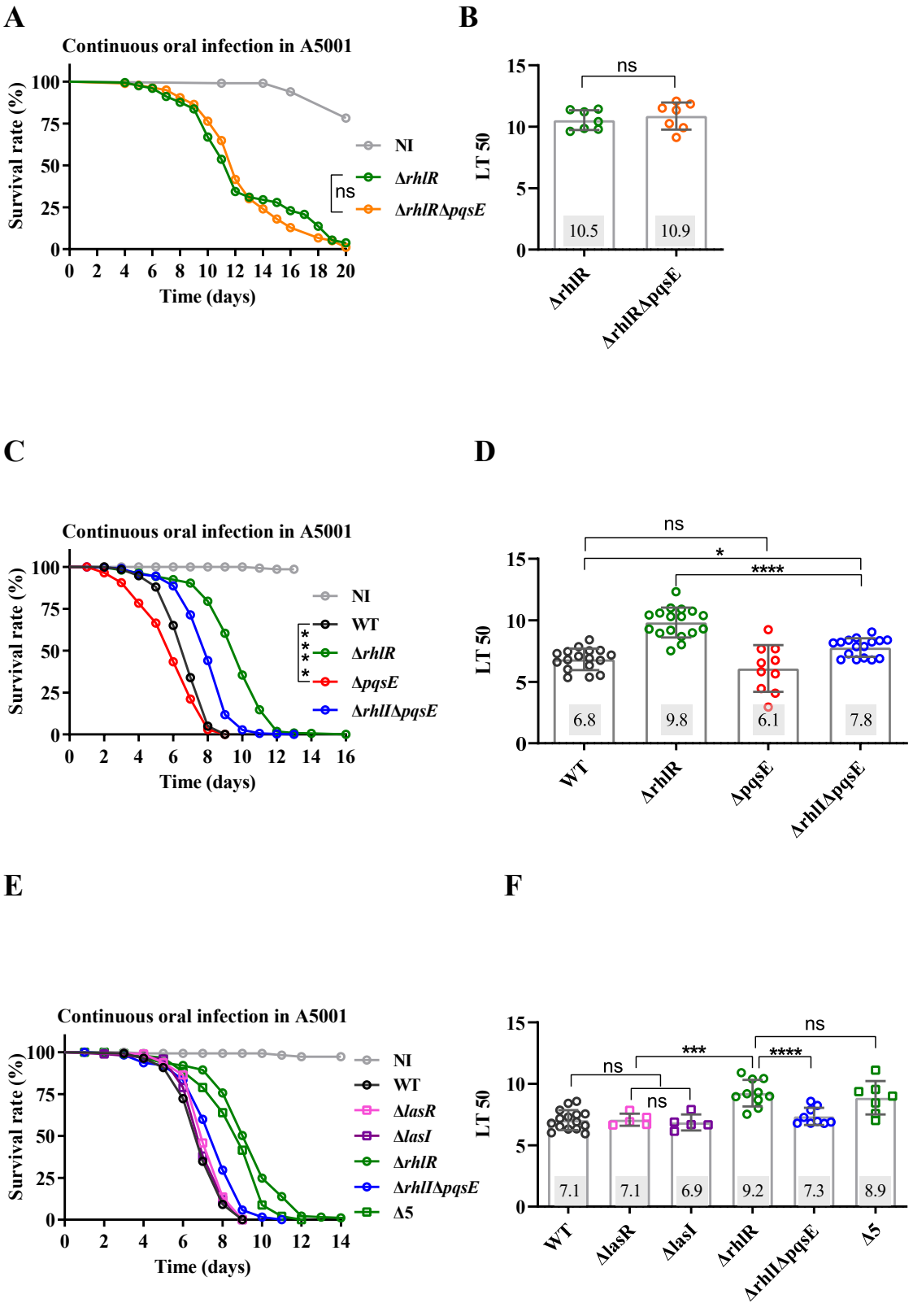

A
Continuous oral infection in A5001
Survival rate (%)
Time (days)
NI
ΔrhlR
ΔrhlRΔpqsE
ns
B
LT 50
ns
10.5
10.9
ΔrhlR
ΔrhlRΔpqsE
C
Continuous oral infection in A5001
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlIΔpqsE
D
LT 50
ns
*
****
6.8
9.8
6.1
7.8
WT
ΔrhlR
ΔpqsE
ΔrhlIΔpqsE
E
Continuous oral infection in A5001
Survival rate (%)
Time (days)
NI
WT
ΔlasR
ΔlasI
ΔrhlR
ΔrhlIΔpqsE
Δ5
F
LT 50
ns
***
****
7.1
7.1
6.9
9.2
7.3
8.9
WT
ΔlasR
ΔlasI
ΔrhlR
ΔrhlIΔpqsE
Δ5

Fig. S5. Phenotypes of PA14 wt and mutants of Rhl quorum sensing in latent infection. (A, B) The survival curve and corresponding lethal times of 50% in the latent infection with PA14 wt or mutants as indicated were analyzed. Flies underwent the procedure of latent infection at 18 ℃. Briefly, flies were fed with bacteria solution for 2 days, then transferred to gentamicin feeding for 4 days, followed by feeding on sucrose solution without antibiotics until death. (C) Survival curves of flies maintained on food in the latent infection with PA14 wt or mutants. Flies underwent the conventional procedure of latent infection until the end of gentamicin feeding and were then maintained on regular simple food.

Fig. S6. Phenotypes of other mutants in RhlR/I or LasR/I quorum sensing system are reconfirmed in the continuous oral infection. A5001 flies were fed continuously with PA14 wt or mutants as indicated at 18℃. The survival curves and corresponding lethal times of 50% were analyzed. LT50 was shown as a bar plot corresponding to the mean +/- SD. (A, B) Δ*rhlR*Δ*pqsE* is an in-frame deletion mutant in the background of Δ*rhlR.* (C, D) Bacteria strains of another independently-generated set that harbor the chromosomally encoded P*rhlA*-mNeonGreen fusion, with or without in-frame deletion. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant. (E, F) Δ*lasR* and Δ*lasI* are deletion mutants, and Δ5 is a deletion mutant of five genes including Δ*rhlR*, Δ*rhlI,* Δ*pqsE,* Δ*lasR,* and Δ*lasI*. Independent experiments two or more times were pooled for each picture.

Figure S7

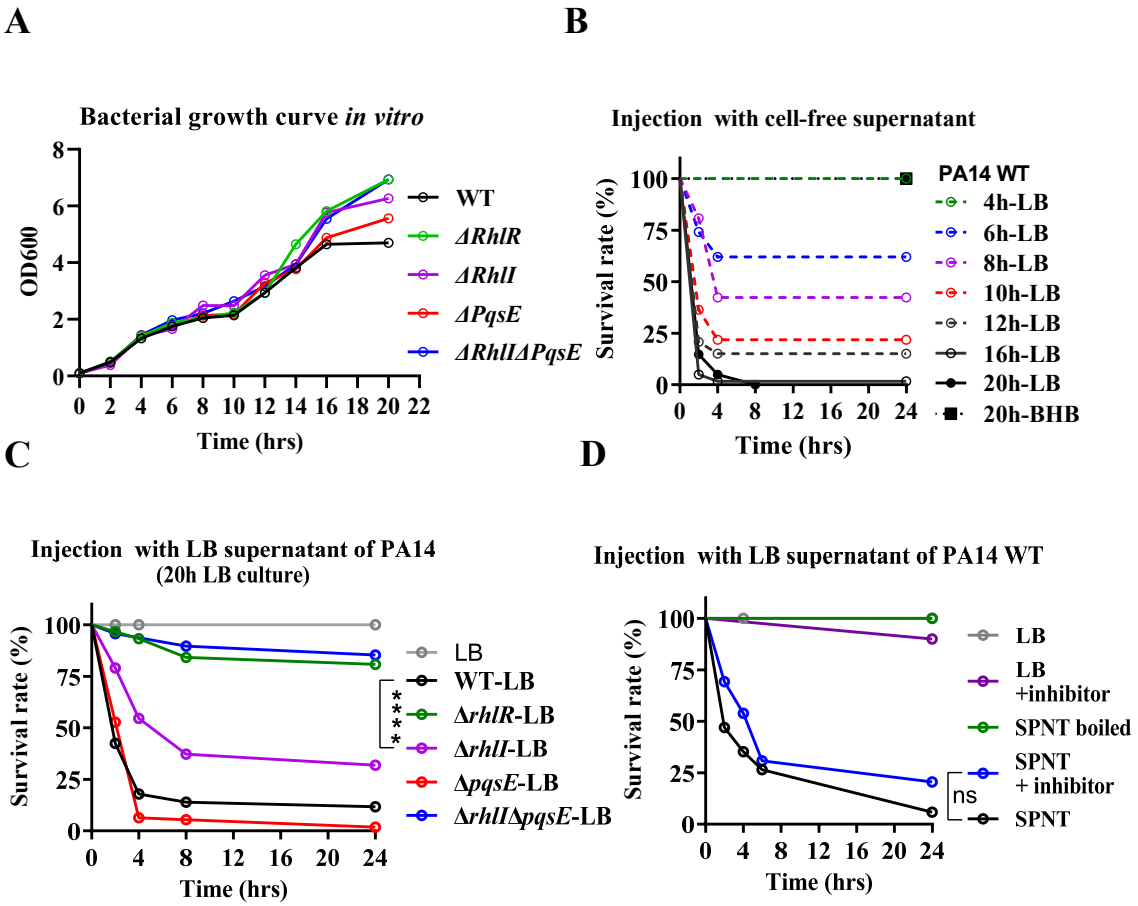


Fig. S7. Consistent sensitivity phenotypes to injection of the supernatant from bacterial cultures. All bacteria here were cultured in LB or BHB liquid at 37 ℃ -shaker. Supernatant for injection was collected from culture liquid after elimination of bacterial cells by centrifugation of 3900 RCF for 10 min, and filtration (0.2 μm). (A) Bacterial growth curves *in vitro*. PA14 wt or mutants were cultured from OD of 0.1, in 5ml LB liquid. The optical density of two separate tubes for each time point was measured. (B) A5001 flies were injected with 69 nl of PA14 wt supernatant from different culture time points. (C) A5001 flies were injected with 69 nl of 20h-culture supernatant from PA14 wt or mutants. (D) A5001 flies were injected with PA14 wt 20h culture supernatant with or without proteinase inhibitor cocktail or boiling treatment. SPNT, supernatant; LB, Lysogeny broth; BHB, Brain-Heart-Infusion Broth.

Figure S8

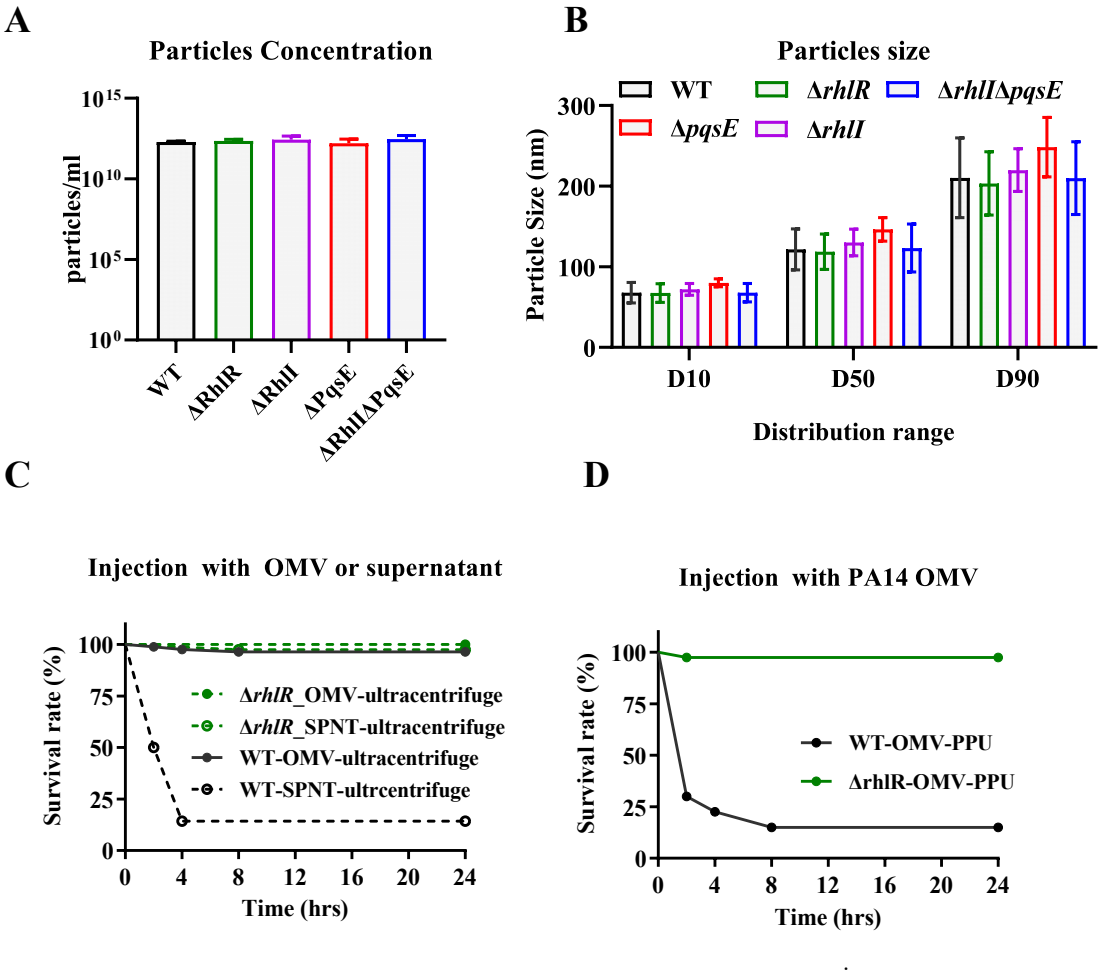
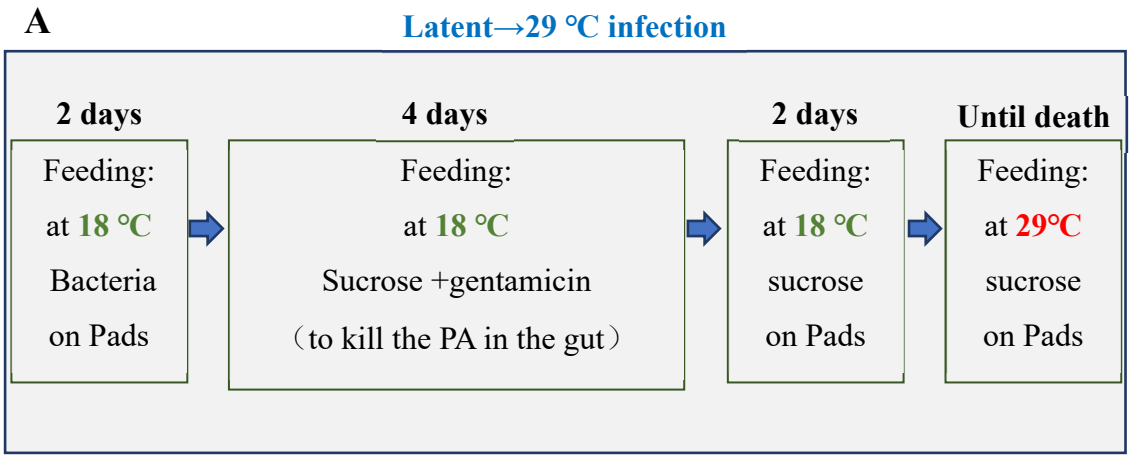
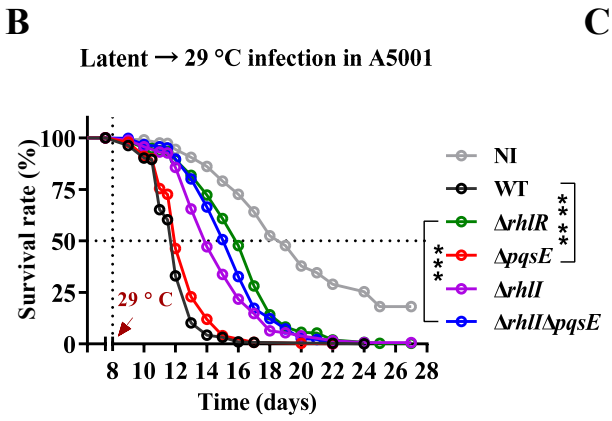
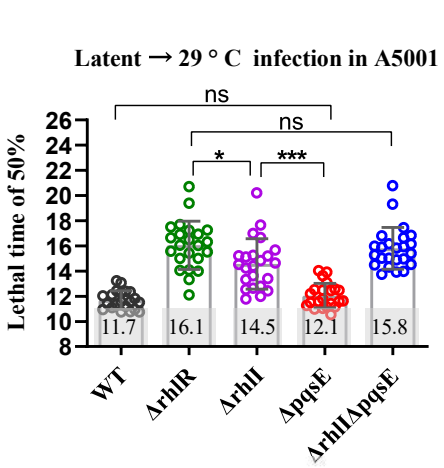


Fig. S8. RhlR is involved in the virulence of OMVs without affecting the vesicle size and yield. Bacteria were cultured in LB liquid at 37 ℃ -shaker. Supernatant for injection or subsequent isolation of OMV was collected from LB culture liquid after clearing from bacterial cells by centrifugation of 3800 x g for 15 min, and sterile filtration (0.45 μm filter). (A, B) Nanoparticle tracking analysis for the quantity and size of OMVs isolated from the culture liquid of PA14 wt or mutants by ultracentrifugation. (C) A5001 flies were injected with 69nl of supernatant (SPNT) or OMVs (~2.2 x10^12 particles/ml) simply isolated by ultracentrifugation. (D) A5001 flies were injected 69nl of high-concentrated OMVs (~1.7 x10^13 particles/ml) extracted via precipitation, purification, and ultracentrifugation.

Figure 3


A
Latent→29 ℃ infection
2 days
Feeding:
at 18 ℃
Bacteria
on Pads
4 days
Feeding:
at 18 ℃
Sucrose +gentamicin
（to kill the PA in the gut）
2 days
Feeding:
at 18 ℃
sucrose
on Pads
Until death
Feeding:
at 29℃
sucrose
on Pads


B
Latent → 29 °C infection in A5001
Survival rate (%)
100
75
50
25
0
29 ° C
8 10 12 14 16 18 20 22 24 26 28
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlI
ΔrhlIΔpqsE
****
****


C
Latent → 29 ° C infection in A5001
Lethal time of 50%
ns
ns
*
***
11.7
16.1
14.5
12.1
15.8
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE

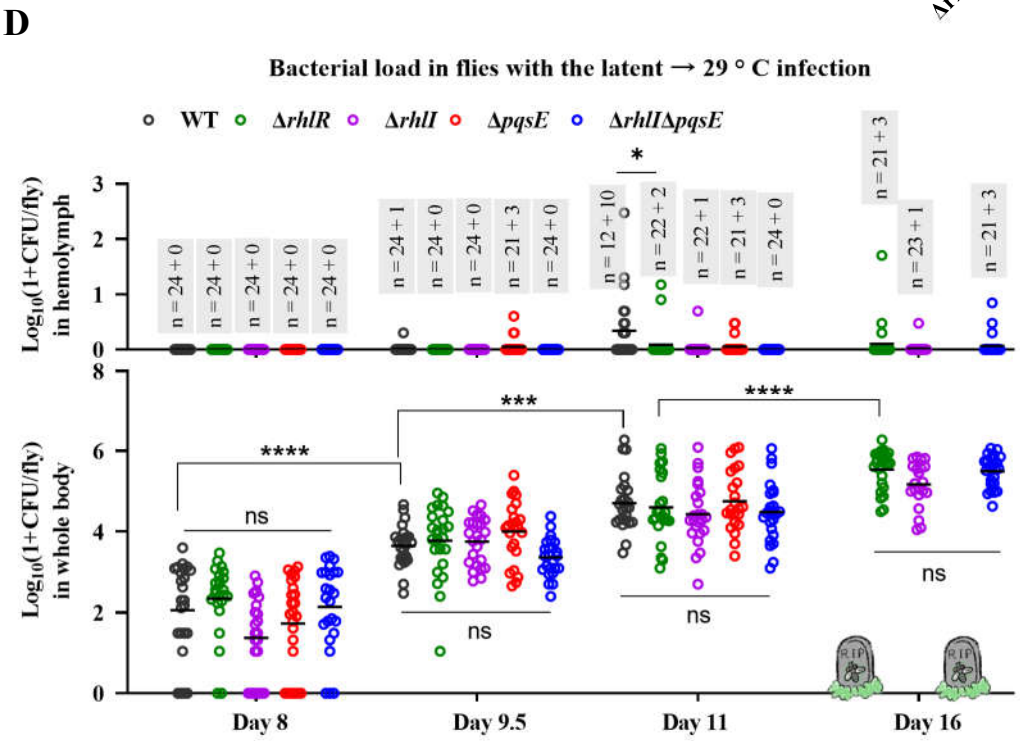
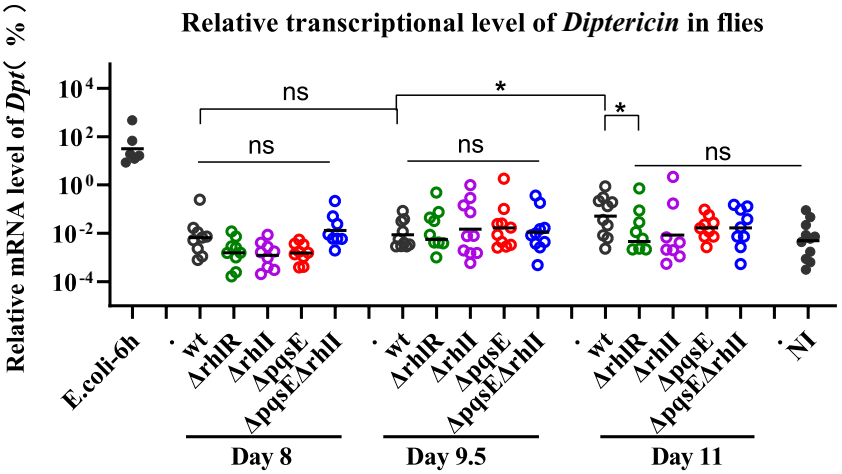

D
Bacterial load in flies with the latent → 29 ° C infection
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE
Log10(1+CFU/fly) in hemolymph
Log10(1+CFU/fly) in whole body
n = 24 + 0
n = 24 + 1
n = 21 + 3
n = 12 + 10
n = 22 + 2
n = 22 + 1
n = 23 + 1
*
****
***
ns
Day 8
Day 9.5
Day 11
Day 16

Figure 3

E


Relative transcriptional level of *Diptericin* in flies
Relative mRNA level of *Dpt*（%）
10^4
10^2
10^0
10^{-2}
10^{-4}
ns
*
*
ns
ns
ns
E.coli-6h
wt
ΔrhlR
ΔrhlI
ΔpqsE
ΔpqsEΔrhlI
NI
Day 8
Day 9.5
Day 11

F

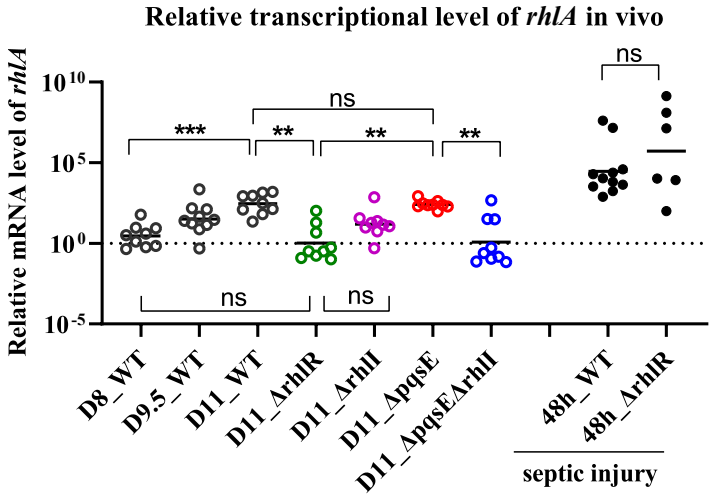

Relative transcriptional level of *rhlA* in vivo
Relative mRNA level of *rhlA*
10^{10}
10^5
10^0
10^{-5}
***
**
**
**
ns
ns
ns
ns
D8_WT
D9.5_WT
D11_WT
D11_ΔrhlR
D11_ΔrhlI
D11_ΔpqsE
D11_ΔpqsEΔrhlI
48h_WT
48h_ΔrhlR
septic injury

Fig. 3. Bacteria in the latent infection can be activated by higher temperatures, presenting a virulence-switching process that depends on RhlR signaling. (A) Scheme of operation for the latent-29 ℃ reactivated infection. (B) Survival curves of A5001 flies in the latent-29 ℃ infection with PA14 wt or the mutants as indicated. (C) Corresponding quantification of the lethal times of 50 % for the survival curve. One point presents LT50 for one infected set of about 20 flies. Bars represent the mean +/- SD. The above survival data were pooled from the data of five times independent experiments. (D) Bacterial titer in the hemolymph or the whole body in infected flies. Each value point of bacterial load per fly is converted to a logarithm (CFU +1). The geometric mean is indicated by a horizontal bar. Pooled data from three independent experiments. (E) The transcriptional level of *Diptericin*, a classic readout of the humoral immune response, was detected by RT-qPCR. 2^ (-Δ (GOI-reference)) is normalized to be a percentage of the induction level of the flies challenged with *E. coli.* for 6 hours. (F) RT- qPCR analysis for the transcriptional level of *rhlA* measured in whole fly extract, a confirmed bacterial virulence factor regulated by RhlR quorum sensing signaling. The Ct value of *rhlA is* normalized to the housekeeping gene of flies, instead of bacteria. The above two qPCR analyses are pooled data from two independent experiments. The geometric mean is indicated by a horizontal bar. One data point represents a batch level of 5 to 7 whole flies.

Figure S9

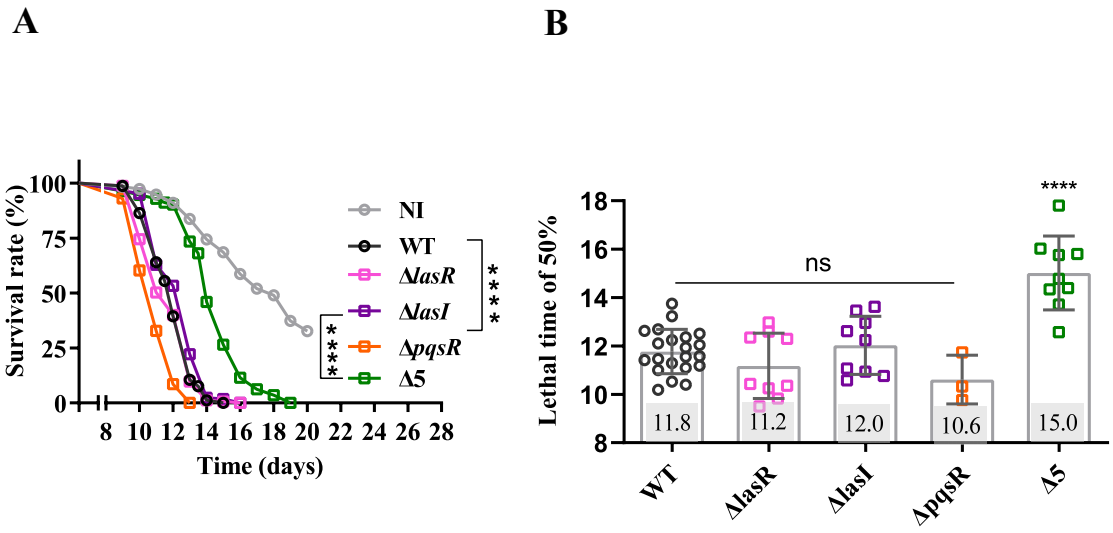


Fig. S9. LasR/I or PqsR quorum sensing system is not required in the latent reactivated infection model. (A, B) Survival curves and corresponding LT50s of A5001 flies in the latent - 29 ℃ with PA14 wt or mutants of LasR/I or PQS system. Data are pooled from three independent experiments.

Figure S10

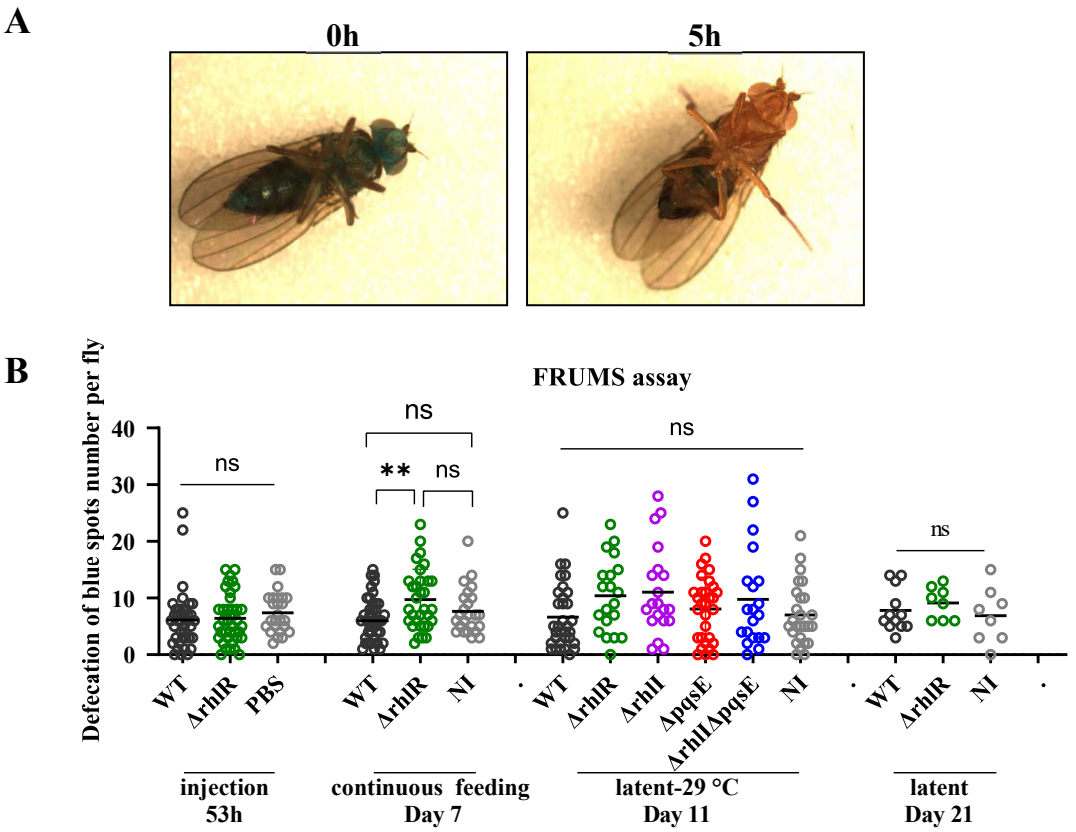

A
0h
5h
B
FRUMS assay
Defecation of blue spots number per fly
40
30
20
10
0
ns
**
ns
ns
ns
WT
ΔrhlR
PBS
WT
ΔrhlR
NI
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE
NI
WT
ΔrhlR
NI
injection
53h
continuous feeding
Day 7
latent-29 °C
Day 11
latent
Day 21
C
Non-infected
50h
Septic injury
Day 7
Continuous feeding
Day11
Latent→29 °C

Fig. S10 Physiology and pathology of flies in different infection models of *P. aeruginosa.*

(A, B) FRUMS assay was performed for A5001 flies post different infections as described before. Pictures of flies show the non-infected flies just injected with blue dye (left panel) or 5 h thereafter (right panels). The blue level of flies was observed, and the parallel defecated blue spots of each fly were counted after 5 hours. Results from two or three experiments were pooled. The mean is indicated by a horizontal bar. (C) Muscle staining of flies in different infections. Flight muscle was stained by phalloidin-FITC, whereas the nuclei were stained by DAPI. Images were captured by confocal microscopy with a 40X objective.

Figure 4

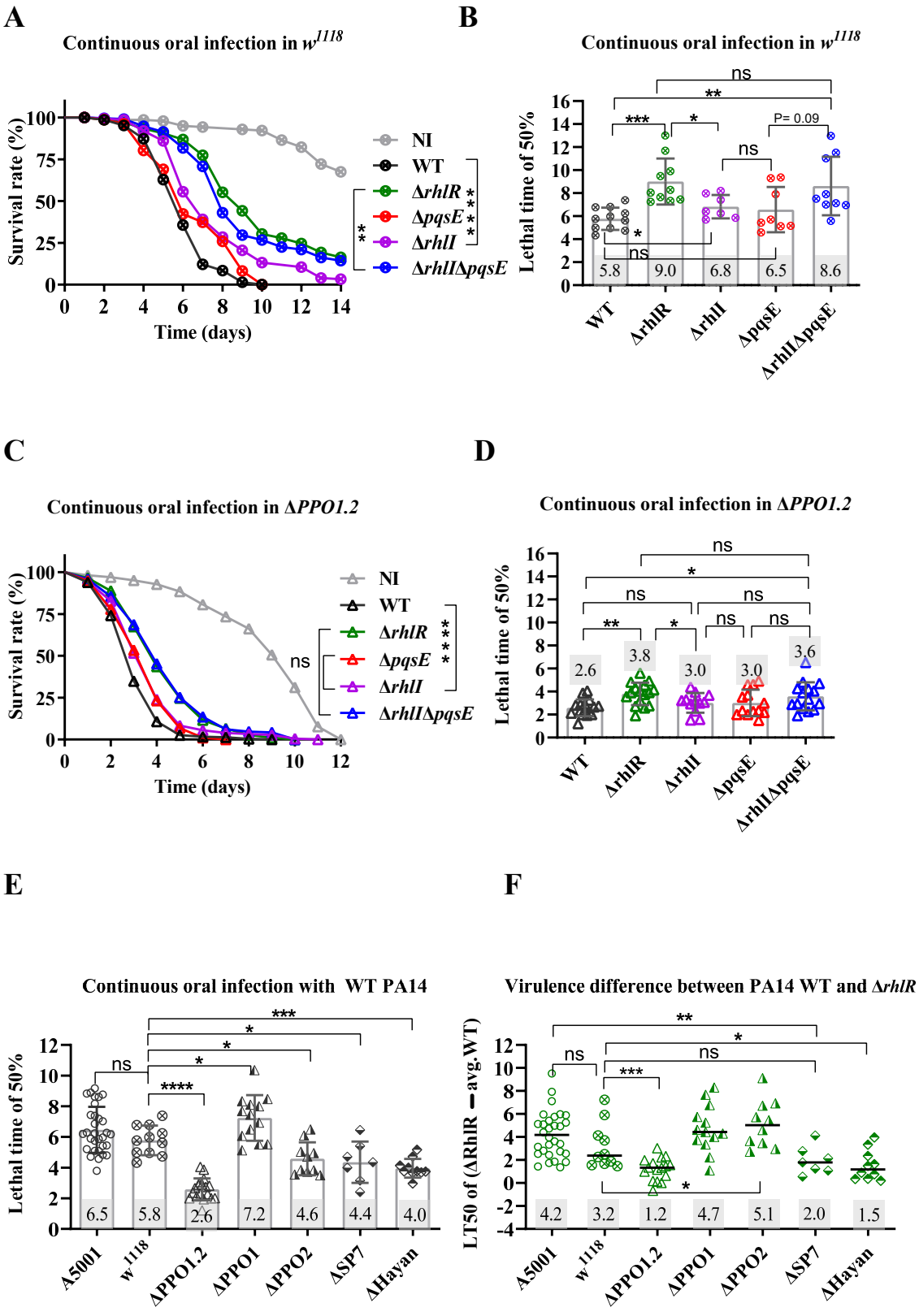
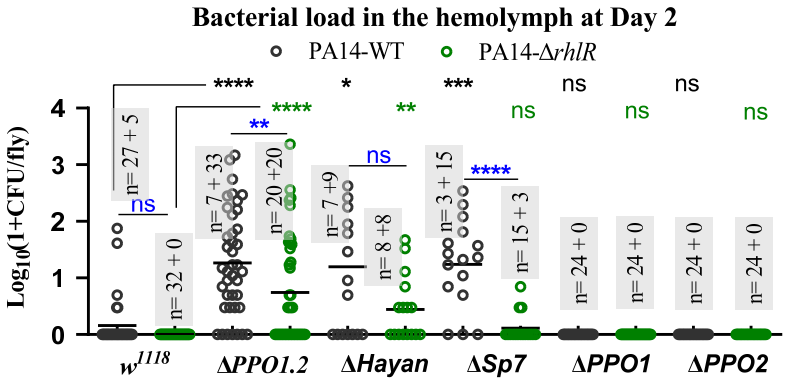
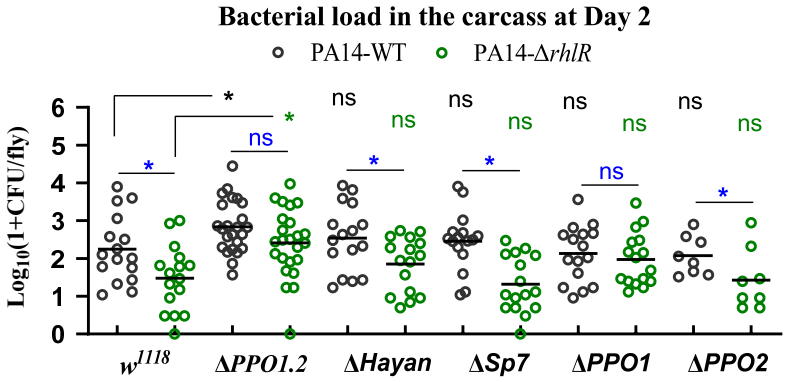

A
Continuous oral infection in w1118
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlI
ΔrhlIΔpqsE
B
Continuous oral infection in w1118
Lethal time of 50%
P= 0.09
5.8
9.0
6.8
6.5
8.6
C
Continuous oral infection in ΔPPO1.2
D
Continuous oral infection in ΔPPO1.2
2.6
3.8
3.0
3.0
3.6
E
Continuous oral infection with WT PA14
6.5
5.8
2.6
7.2
4.6
4.4
4.0
Δ5001
w1118
ΔPPO1.2
ΔPPO1
ΔPPO2
ΔSP7
ΔHayan
F
Virulence difference between PA14 WT and ΔrhlR
LT50 of (ΔRhlR – avg.WT)
4.2
3.2
1.2
4.7
5.1
2.0
1.5

Figure 5

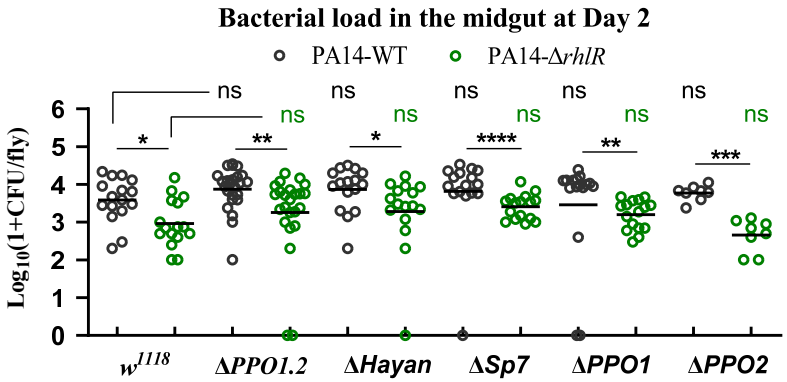
A



B



C



D

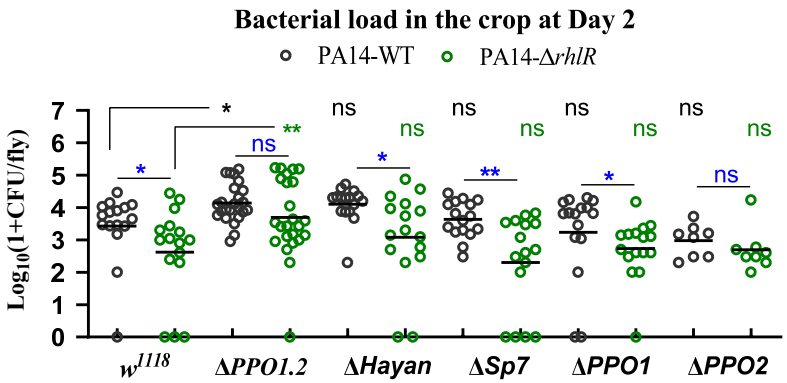


Fig. 4. Weak phenotypes of Rhl signaling for virulence of *P. aeruginosa* in the melanization-deficient *Drosophila.* Flies were infected following the classic procedure of continuous oral infection at 18 ℃ as described before. (A-B) Survival curve and the corresponding lethal time 50 % for w^{1118} flies, as wt control. The PPOs mutants have been generated in this background. LT50 was shown as a scatter plot with the bar representing mean +/- SD. Each mean value was shown inside the bar. Pooled data from four independent experiments. (C-D) Survival curve and the corresponding lethal time 50 % for the Δ*PPO1.2* flies, a double mutant of *PPO1* and *PPO2*, the prophenoloxidases contributing to melanization. Pooled data from six independent experiments. (E) Comparison analysis for the lethal time 50 % of flies in different mutants involved in the melanization pathway, in the infection with PA14 wt. (F) Virulence difference between PA14 wt and Δ*rhlR*. Each data point was a subtraction value of (LT50 of the flies infected with Δ *rhlR* - an average of those infected with PA14 wt). The mean spot is shown as a horizontal bar and the actual values were shown in the gray box. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns = not significant.

Fig. 5. The high bacterial load in the hemolymph coincides with the low requirement of RhlR in the virulence of *P. aeruginosa in Drosophila.* (A~D) Bacterial titer side by side for the hemolymph, carcass, midgut, and crop form wt or mutant flies. Flies were infected by continuous oral as usual. Each value point represents the bacterial load per fly, shown as a logarithm (CFU +1). The Fly sample number of each group is shown as total number = N (flies without bacteria in the hemolymph) + N (flies with bacteria in the hemolymph). The geometric means are shown as a horizontal bar. Pooled data from at least three independent experiments

Figure S11

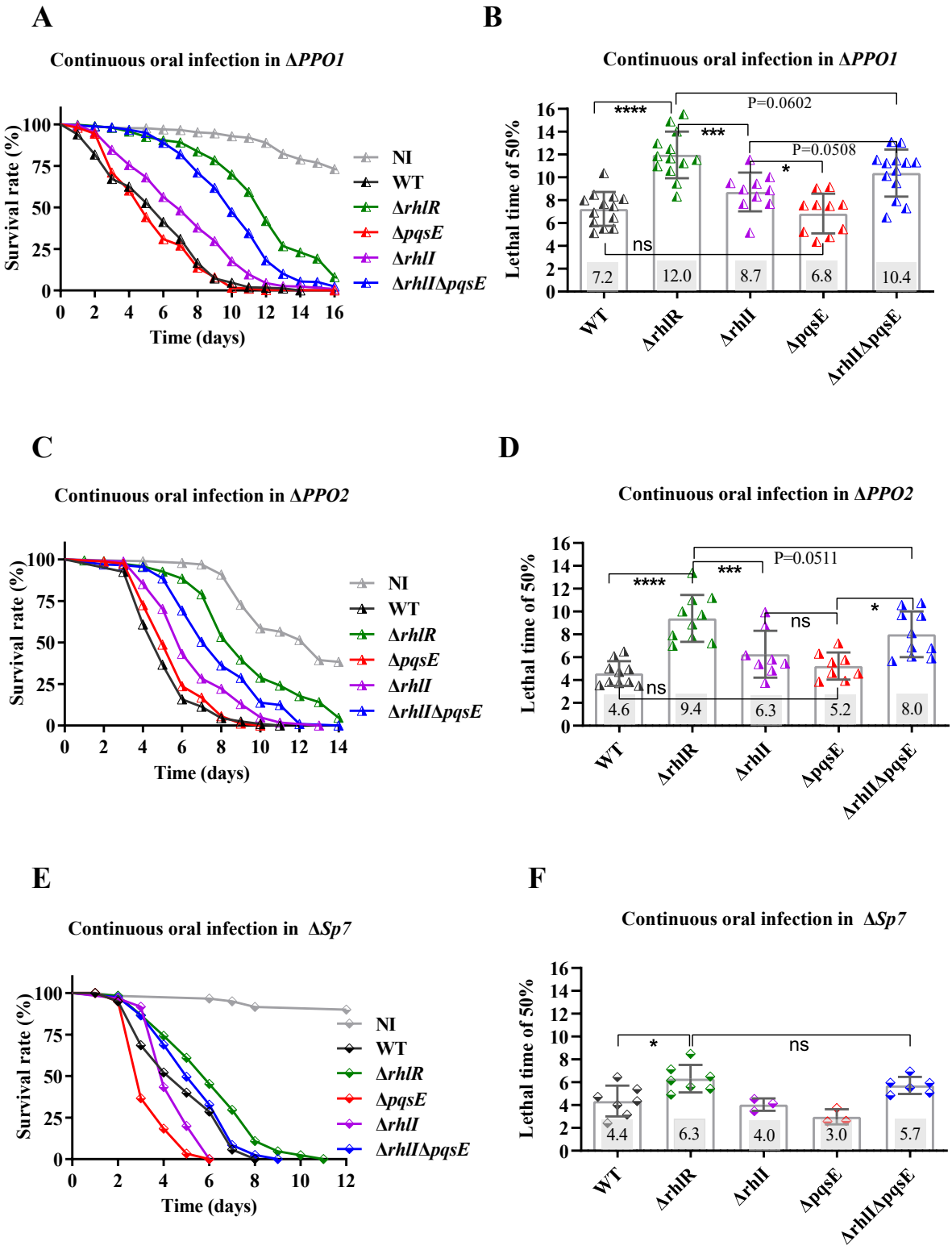

A
Continuous oral infection in ΔPPO1
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlI
ΔrhlIΔpqsE
B
Continuous oral infection in ΔPPO1
Lethal time of 50%
P=0.0602
P=0.0508
****
***
*
ns
7.2
12.0
8.7
6.8
10.4
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE
C
Continuous oral infection in ΔPPO2
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlI
ΔrhlIΔpqsE
D
Continuous oral infection in ΔPPO2
Lethal time of 50%
P=0.0511
****
***
*
ns
4.6
9.4
6.3
5.2
8.0
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE
E
Continuous oral infection in ΔSp7
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔpqsE
ΔrhlI
ΔrhlIΔpqsE
F
Continuous oral infection in ΔSp7
Lethal time of 50%
*
ns
4.4
6.3
4.0
3.0
5.7
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE

Figure S11

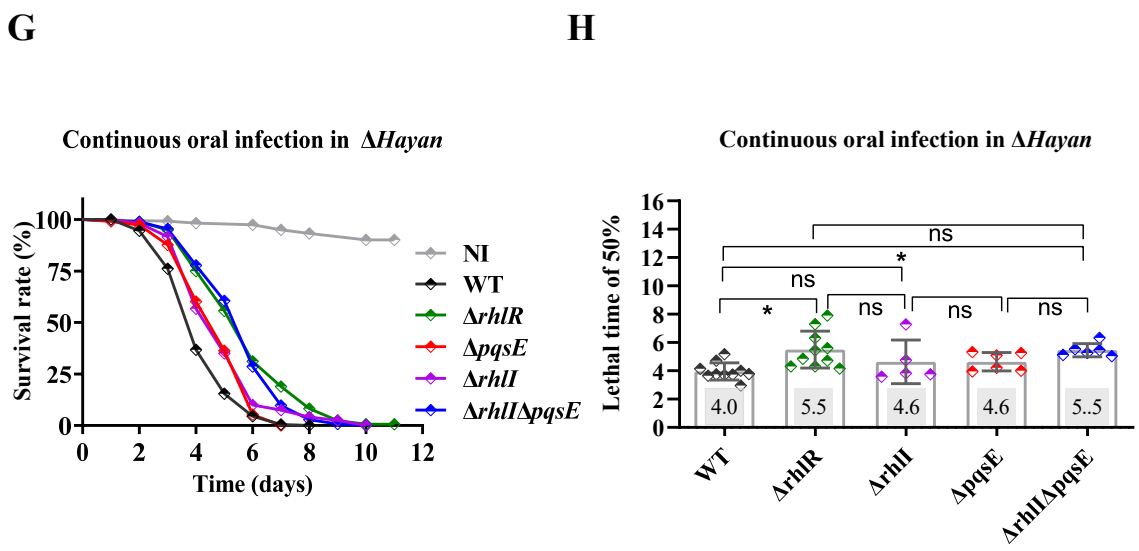


Fig. S11 Phenotypes of fly mutants in the melanization signaling challenged by continuous oral infection. All the flies underwent continuous oral feeding with PA14 wt or mutants as indicated at 18 ℃. (A, B) Survival curves and the corresponding lethal time 50 % *PPO1* mutant flies (C, D) Survival curves and the corresponding lethal time 50 % in *PPO2* mutant flies (E, F) Survival curves and the corresponding lethal time 50 % in the flies of *Sp7* mutant flies (G, H) Survival curves and the corresponding lethal time 50 % of *Hayan* mutant flies. Data were pooled from at least two independent experiments.

Figure S12

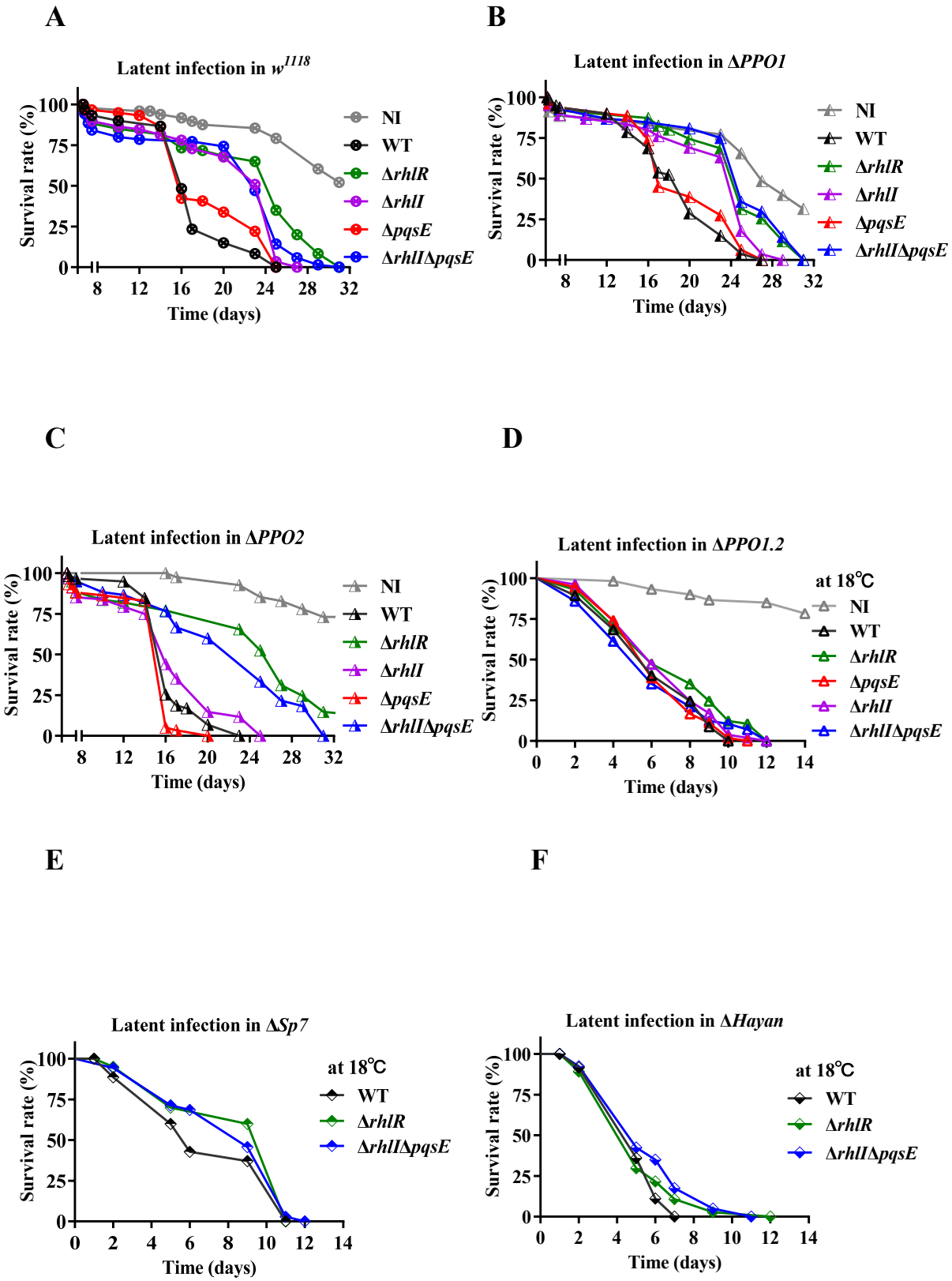

A
Latent infection in w1118
Survival rate (%)
Time (days)
NI
WT
ΔrhlR
ΔrhlI
ΔpqsE
ΔrhlIΔpqsE
B
Latent infection in ΔPPO1
C
Latent infection in ΔPPO2
D
Latent infection in ΔPPO1.2
at 18℃
E
Latent infection in ΔSp7
F
Latent infection in ΔHayan

Figure S13

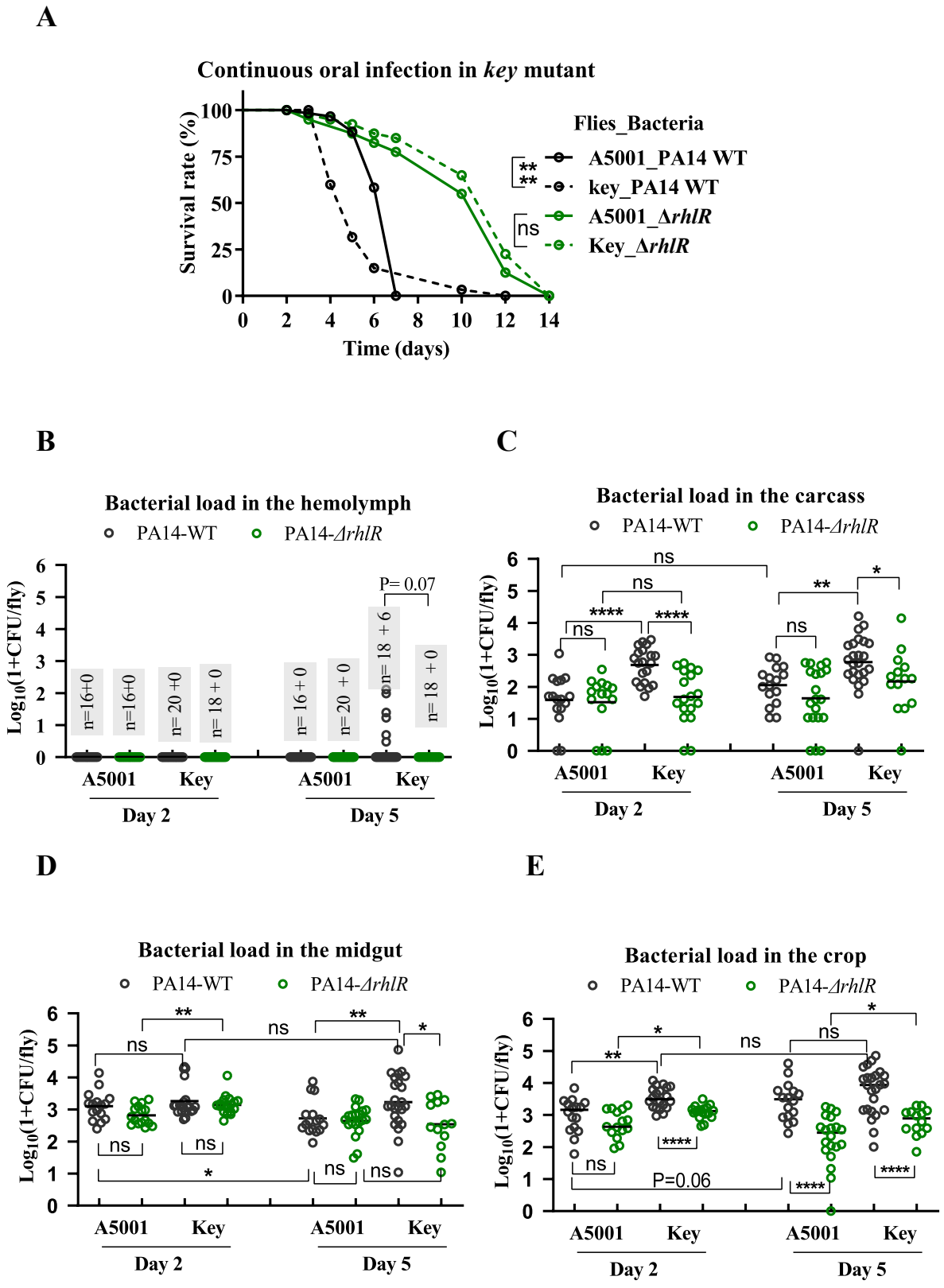
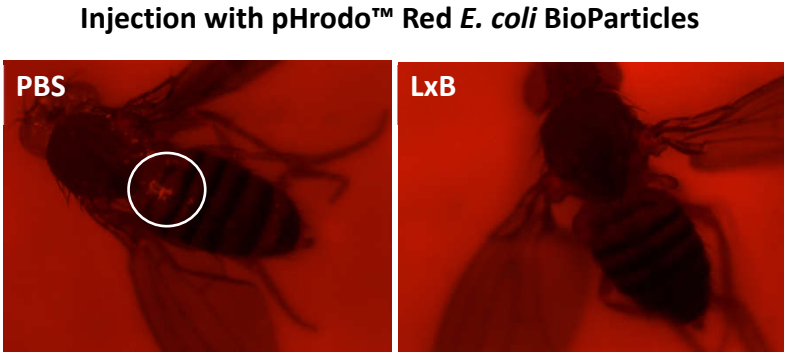
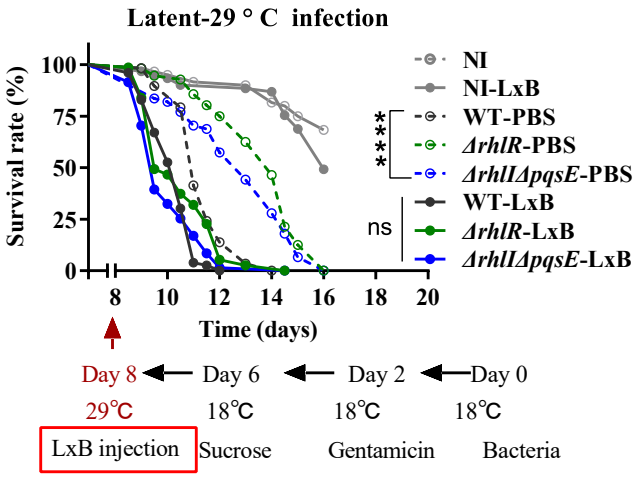

A
Continuous oral infection in key mutant
Survival rate (%)
Time (days)
Flies_Bacteria
A5001_PA14 WT
key_PA14 WT
A5001_ΔrhlR
Key_ΔrhlR
**
**
ns
B
Bacterial load in the hemolymph
PA14-WT
PA14-ΔrhlR
Log10(1+CFU/fly)
P= 0.07
A5001
Key
Day 2
Day 5
C
Bacterial load in the carcass
D
Bacterial load in the midgut
E
Bacterial load in the crop
P=0.06

Figure S14

A



B



C

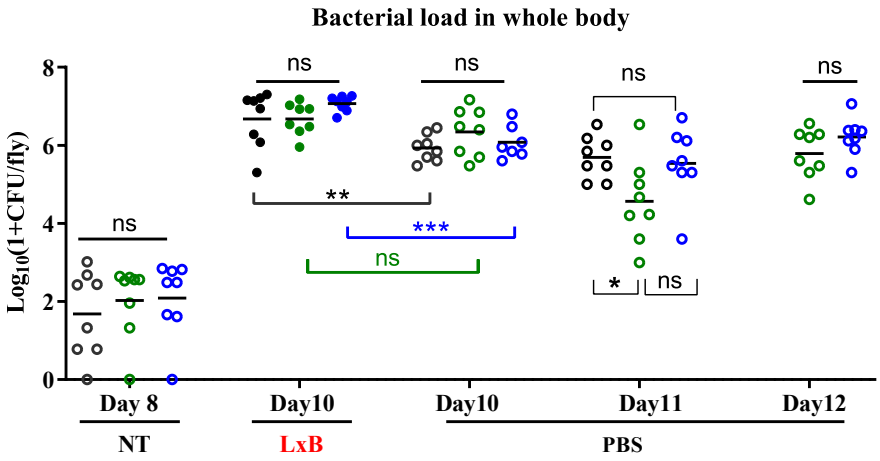


Figure S15

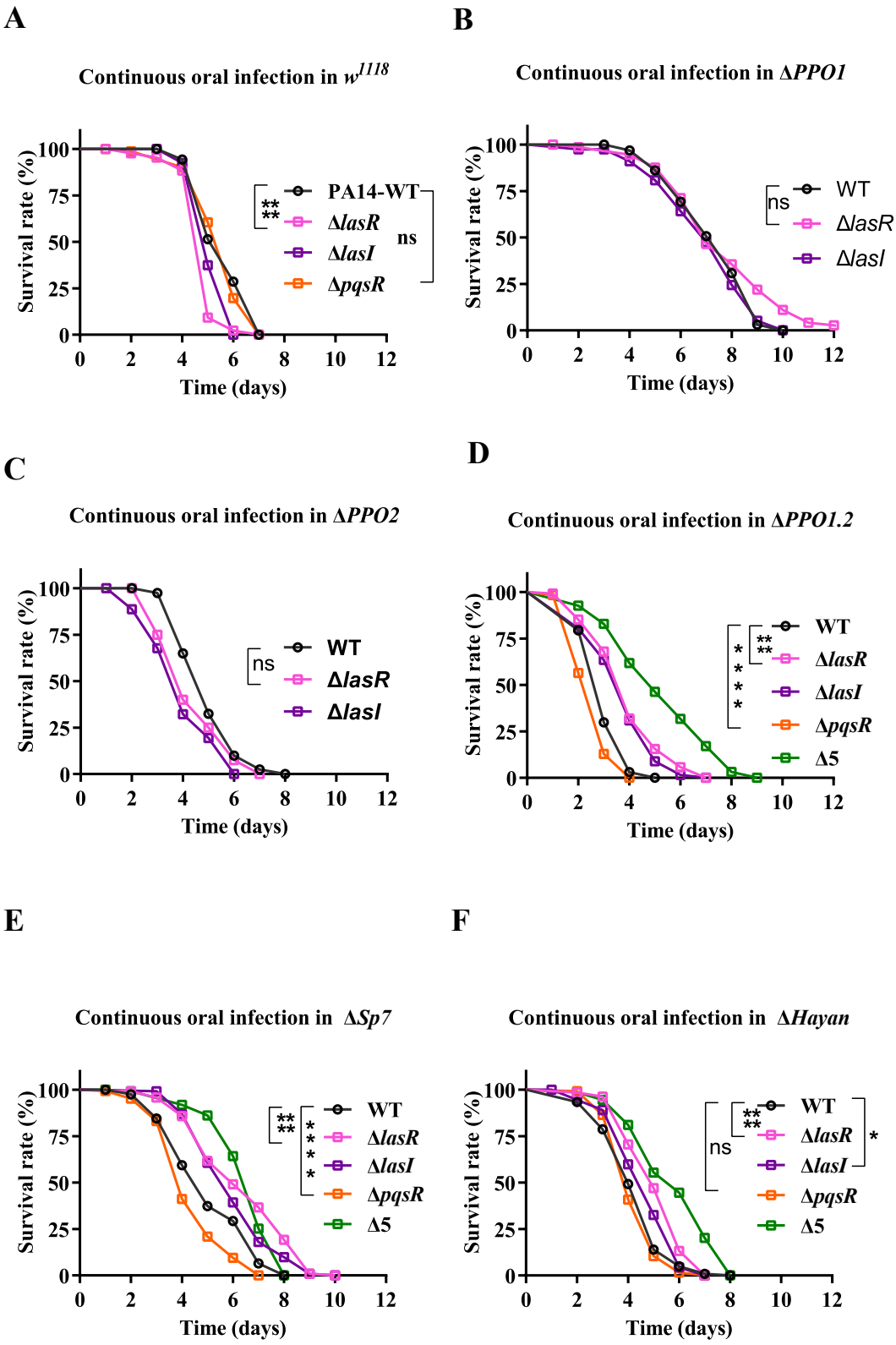

A
Continuous oral infection in w1118
Survival rate (%)
Time (days)
PA14-WT
ΔlasR
ΔlasI
ΔpqsR
**
**
ns
B
Continuous oral infection in ΔPPO1
Survival rate (%)
Time (days)
WT
ΔlasR
ΔlasI
ns
C
Continuous oral infection in ΔPPO2
Survival rate (%)
Time (days)
WT
ΔlasR
ΔlasI
ns
D
Continuous oral infection in ΔPPO1.2
Survival rate (%)
Time (days)
WT
ΔlasR
ΔlasI
ΔpqsR
Δ5
**
**
****
E
Continuous oral infection in ΔSp7
Survival rate (%)
Time (days)
WT
ΔlasR
ΔlasI
ΔpqsR
Δ5
**
**
****
F
Continuous oral infection in ΔHayan
Survival rate (%)
Time (days)
WT
ΔlasR
ΔlasI
ΔpqsR
Δ5
ns
**
**
*

Fig. S12 Phenotypes of latently infected POs-deficient flies. All the flies underwent the classic procedure of latent infection at 18 ℃ with PA14 wt or mutants as indicated. (A~ D) Survival curve of w^{1118} flies and mutants as indicated in the graph title. Each picture is representative of two independent experiments.

Fig. S13 RhlR signaling is still required for virulence in flies lacking the IMD humoral immune response during continuous oral infection. wt flies or *key* mutants were continuously fed with PA14 wt or Δ*rhlR* at 18 ℃. (A) Survival curves of flies and mutants are shown and representative of two independent experiments. (B ~ E) Bacterial titer side by side for the hemolymph, carcass, gut, and crop form flies of wt or mutants. Data were pooled from two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant.

Fig. S14. RhlR signaling is not required when phagocytosis is blocked by the injection of latex beads in the latent-29 ℃ reactivated infection. A5001 flies were submitted to the classic procedure of latent-29 ℃ reactivated infection. At day 8 before transferring to 29 ℃, flies were injected with 12% Latex beads (LxB) of 69 nl to saturate the phagocytic apparatus or injected with PBS as control. (A) The phagocytosis performance was checked by injection with pHrodo™ Red *E. coli* BioParticles. No fluorescent signal was observed when the phagocytosis was completely blocked by LxB injection. (B) Survival curves of flies with or without LxB injection in the infection were monitored. (C) The bacterial load of the whole body was detected in parallel. Each graph is representative of two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001, ns = not significant.

Fig. S15. LasR-LasI system is partially required in the melanization-impaired context of *Drosophila*. (A~F) Survival curves of wt flies or mutants. Flies were continuously fed with bacteria wild-type PA14, and the mutants as indicated at 18 ℃， following the classic procedure. Each graph is pooled from two independent experiments or representative of two independent experiments. Δ5, a PA14 mutant with deletions in the *lasR*, *lasI*, *rhlR*, *rhlI*, and *pqsE* genes.

Materials and Methods

Drosophila Strains and Culture

Fly stocks were raised at 25 °C under 60% humidity and fed with standard cornmeal–agar medium with selectively added yeast. Recipe for 10 L of fly food medium: 480g glucose, 480g cornmeal, 48 g agar, and 36 g nipagin (VWR Chemicals), was diluted into 140 ml absolute ethanol and mixed with distilled water to volume. 5 to 7-day-old adult female flies were used for experiments. A5001 flies and w^{1118} flies were used as wild-type flies. key^{c02831}, a mutant of the gene *Kenny*, which encodes the regulatory protein of the IKK complex. Mutant flies Δ*PPO1*, Δ*PPO1*, Δ*PPO1*Δ*PPO 2* (Δ*PPO1.2*) were isogenized in the w^{1118} (DrosDel) background, kindly provided by Prof. Bruno Lemaitre [20].

Bacterial strains and culture

P. aeruginosa UCBPP-PA14 was the wild-type strain and mutants used in this study were listed in the table below. Most of them were kindly provided by Prof. Bonnie Bassler and Prof Xiaoxue Wang. All the bacteria were cultured at the LB agar plates without antibiotics at 37 ℃ overnight， then maintained at 4 ℃ for no more than two weeks. For infection models, *P. aeruginosa* was cultured in liquid (25ml) of Brain-Heart-Infusion Broth (BHB) (Bacto™ Brain Heart Infusion, BD #237500) in flasks at 37 ℃ for 16 hours. For the supernatant injection experiment or OMVs isolation, *P. aeruginosa* was cultured in lysogeny broth (LB) liquid. Lysogeny broth (LB) is formulated with 5g NaCl, 5 g yeast extract (OXOID_LP0021), 10 g tryptone (OXOID_LP0042), 15g agar, ultrapure water to a total volume of 1 liter. LB agar plates included 1.5 % agar. 50 μg/mL Ampicillin was used in the LB agar for bacteria .100 μg/mL gentamicin was used for eliminating the *P. aeruginosa* in the *Drosophila* gut.

Infection models

An overnight BHB-culture of *P. aeruginosa* was centrifuged at 3,800 x g, for 15 min, 25 °C, and the pellet was washed with sterile PBS buffer twice, finally diluted in sterile PBS to an optical density at 600 nm (OD600) of 0.01, or in 50 mM sucrose solution containing 5% (v/v) BHB to OD600 of 4. For the septic injury models, flies were inoculated with 13.8nl of bacteria in PBS (OD of 0.01) by direct injection into the

thorax using the Nanoliter Injector (Drummond Nanoject II or III) and fed on sucrose solution (50mM) at 18 ℃ until death. For continuous oral infection (intestinal infection), flies were fed on an absorbent pad (AP1002500, Millipore; dia. 25 mm) soaked with 0.6ml bacterial solution (OD=4) at 18 ℃ and supplemented daily with 80 µl sucrose solution（50mM) until death. For latent infection, flies were fed on an absorbent pad (AP1002500, Millipore; dia. 25 mm) soaked with 0.6ml bacterial solution (OD of 4), at 18 ℃ for 2 days, subsequently transferred to a new vessel with a pad absorbed with 0.6 ml of sucrose solution (50mM) containing gentamicin (100 µg /ml) for 4 days at 18 ℃. Thereafter, flies were fed with sucrose solution（50mM) until death at 18 ℃. For latent-29℃ infection, flies followed the classical procedure of latent infection at 18 ℃ until day 8, when the flies were transferred to 29 ℃ until death. all vials were supplemented with a sucrose solution of 80 µl (at 18℃) or 120 µl (at 29 ℃) every day until death, except on the first day of feeding. 20 female files per vial and two or three independent replicates were performed for each infection experiment.

Supernatant injection and OMVs injection

Experiments were carried out using *P. aeruginosa* strains PA14 or mutants in PA14 background. Flies were injected with 69 nl of the cell-free supernatant or OMVs and monitored for survival at 18 ℃. Cell-free supernatant or OMVs were prepared as follows: lysogeny broth was used for culture here unless otherwise indicated. 4ml LB cultures were inoculated with a single colony and were grown for 6 h at 37°C with shaking at 250 rpm. Secondary cultures were inoculated with the first culture to get a final concentration OD600 of 0.01， then were grown in a flask at 37° C with shaking at 250 rpm for 20h unless otherwise stated. The secondary culture was used for injection or OMVs isolation after centrifugation of 3900 x g for 15 min and cleared from bacterial cells by sterile filtration of 0.22 µ M filter or 0.4 µ M filter, respectively. Two methods for the isolation of OMVs： (1) Simple isolation. Cell-free supernatant of 10ml/ tube was ultracentrifuged at 100,000 x g for 2h (Beckman SW41-Ti rotor, tube#344059) to pellet vesicles. Pellets were resuspended in PBS buffer 10ml, then went through ultracentrifugation again as above. Finally, pellets were resuspended in PBS of 200µl, for the subsequent analysis in concentration and particle size using ZetaView® Nanoparticle Tracking Analyzer (ZetaView® BASIC NTA). OMVs were adjusted to

the concentration (~2.2 x 10^12 particles / L) for fly injection. (2) Concentrated isolation: Cell-free supernatant of 300ml was used for OMVs extraction via precipitation, purification, and ultracentrifugation as described [388, 389]. OMVs solution was quantified by direct nanoparticle counting as above and normalized to 1.7 x10^13 particles/ml for fly injection.

Bacterial titer

Single-fly bacterial titer was measured in the fly hemolymph, or whole body, or in the dissected carcass, midgut, or crop. The infected flies for bacterial titer determination were rinsed into 75% ethanol for 5 seconds then washed with PBS and dried on absorbent paper. The hemolymph of each fly was extracted by a capillary with an injection needle through the thorax and collected into 20 µl sterile PBS in each well of 8-tube strips. After hemolymph collection, each fly was dissected into three parts: carcass (without the gut, ovary, and Malpighian tubules), midgut, and crop. Each dissected part or whole body without dissection was collected into 100 µl sterile PBS separately in each well of 8-tube strips and crashed by 1.4-mm ceramic beads using Tissue Homogenizer. If necessary, the samples were diluted at a 5-fold dilution ratio. 10 µl of each sample was plated on an LB plate with Ampicillin (50 µg/ml) and colonies were counted after overnight culture at 37 ℃.

Phagocytosis blocking by Latex beads

To block the phagocytosis functions of hemocytes in flies, 69 nl of 12% (w/v) latex beads were injected into the fly thorax to saturate the phagocytic apparatus. Latex beads were pretreated as follows: the initial latex beads (CML Latex Beads, 4% w/v, 0.29 µm，Invitrogen™) were washed with PBS and centrifugated twice, then resuspended with PBS to get a threefold concentrated latex bead. Before injection, the latex beads were homogenized by ultrasonication at 4 ℃. The phagocytosis performance was checked by injection with pHrodo™ Red E. coli BioParticles (P35361_Invitrogen™) with 13.8 nl and imaged by microscope after 30 mins.

Life/dead in vivo assay for intestinal *P. aeruginosa*

Flies were fed with the GFP-expressing bacteria (OD of 10) in 50 mM sucrose solution containing 5% BHB and 40 µg/mL of PI (propidium iodide). After 18h, the guts were

dissected out of the infected flies in PBS buffer, fixed with 8% paraformaldehyde solution (PFA) for 30 mins, then washed with PBS buffer three times. The stained guts were mounted with VECTASHIELD Mounting Media (Vector Laboratories) and cover slides. Images were captured by the LSM780 confocal microscope (Zeiss).

FRUMS

The FRUMS assay is used to assess the renal function of Malpighian tubules into the intestinal lumen [381]. Flies at the late phase of different infection models were injected with 69 nl of the nontoxic Blue Dye (Brilliant Blue FCF E133), then transferred into ventilated Eppendorf microtubes. Each fly was monitored for the disappearance of blue color in the fly body and the defecated blue spots (blue feces) on the tube were counted five hours post-injection**.**

Colony Biofilm Assay by Congo Red Assay

P. aeruginosa bacteria were cultured at 37 ℃ with shaking for 16 hours, then washed and resuspended with PBS. 1 μl of bacteria (OD of 0.1) was spotted onto Congo red agar (CRA) plates, grown at 25℃. Colonies were imaged after 5 days, using a Zeiss stereomicroscope in brightfield (yellow light or white light) at 8 x zoom. Recipe-1 for Congo red agar: 40 mg/L Congo red， 20 mg/L Coomassie brilliant blue dyes，1% (w/v) TB （Tryptone broth），1% (w/v) agar， deionized water [297]. Recipe-2 for Congo red agar: 40 mg/L Congo red， 20 mg/L Coomassie brilliant blue dyes，1% (w/v) Terrific Broth，1% (w/v) agar， deionized water. 200 mL of the above mixture was sterilized by autoclaving for 20 min at 121 ℃，15 psi. 10 mL medium was poured into each Petri plate (60 x15 mm) and leave it solidified at room temperature.

Detection of gene transcriptional level

RNA isolation, Reverse Transcription, and Real-time Quantitative PCR (RT-qPCR) were carried out for the detection of the relative mRNA level of genes in flies or bacteria. Specific oligonucleotide primers were designed using Primer Premier 5 and shown in the supplementary Table2. Alive flies were collected in an EP tube and washed successively in 70% ethanol with vortex for about 10 sec, in 10 % sodium hypochlorite for about 1 min, in PBS buffer with vortex twice, then dried on absorbent papers, to remove the bacteria and destroy the nucleic acids attached on the fly body outside in

the oral infection models. Subsequently, 5 to 7 flies were collected into a tube with TRIzol® Reagent and crushed by 1.4-mm ceramic beads using the Tissue Homogenizer (Bertin Technologies). Total RNA was isolated via the classical procedure of phase separation and precipitation. Reverse transcription was carried out following the manual of the iScript cDNA Synthesis Kit (Bio-Rad #1725035). cDNA samples were diluted to 1/10 in MilliQ water for the detection of fly genes, or not diluted for bacterial genes. The kit iTaq™ Universal SYBR® Green Supermix (Biorad #1725125) was used for RT-qPCR. The PCR application efficiency within a range of 90% to 110% was validated for each primer pair. Ct values of GOI (gene of interest) were first normalized to the reference gene from the same sample, then secondary normalized to the negative control or positive control as indicated.

Statistical analysis

All the data were performed on GraphPad Prism (version 8.4) or SPSS (version 23). Two methods were performed for survival data, log-rank analysis, and LT50s analysis. The log-rank analysis is performed for only two survival curves at one dimension. To make multiple comparisons and synthesis of survival data, we computed the lethal time 50% (LT50: time taken for 50% of the flies to die) through nonlinear regression, then perform statistical analysis on the LT50s as measurement data, as previously described [56, 89]. All the measurement data were first analyzed by nonparametric tests, Mann-Whitney for comparison between two independent samples, or Kruskal-Wallis for multiple comparisons. If necessary, the data of Gaussian distribution was analyzed again by parametric tests, the two-independent-sample t-test, or the Analysis of Variance (ANOVA) test with Tukey's multiple comparisons test. Significance values: ****P<0.0001; ***P< 0.001; **P<0.01; *P< 0.05; ns = not significant.

Construction of deletion mutant *P. aeruginosa*

The protocol for constructing the in-frame deletion mutant of *P. aeruginosa* was modified according to the materials that we had in our laboratory, based on the principle of the two-step allelic exchange as previously described [390]. The gene *pqsE* in the PA14 wt, Δ*rhlR*, Δ*rhlI* was deleted by the following procedure:

(1) Vector selection. To choose an allelic exchange vector/replacement vector. pEX18Ap vector was kindly provided by Prof. Xiaoxue Wang's laboratory [391]. pEX18Ap [392] is a gene replacement vector harboring antibiotic resistance of

Ampicillin /carbenicillin, conjugative machinery (oriT+), SacB gene for sucrose counter selection, a multiple cloning site (MCS) from pUC18, which is a suicide plasmid in PA containing pMB1 ori with high-copy capability in *E. coli*.

(2) Cloning methods and design of primers for synthesizing mutant alleles.

PCR was carried out for cloning deletion mutant alleles fragments. Fragments were assembled with linearized vectors using the Vazyme Clon Express MultiS One Step Cloning Kit based on homologue recombination. Primers were designed following the guidelines of the kit manual (ClonExpress MultiS). The first primer pair (Δ*pqsE*-Up-F/R) was designed to target the noncoding region upstream of the gene *pqsE*, whereas the second (Δ*pqsE*-down-F/R) on the noncoding region downstream of the gene *pqsE*. The third pair of primers (pEX_F/R) was designed to sequence the recombinant plasmid with the targeted sequence. The fourth pair of primers (Δ*pqsE*-Seq-F/R) is used for sequencing and confirmation of the deletion site on the bacterial chromosome (Primers shown in Table 2).

(3) Cloning, recombination, and transformation.

PCR was carried out to synthesize mutant alleles in vitro using the PCR kit (Phanta Max Super-Fidelity DNA Polymerase), PA14 lysis as DNA template, and the first (UP) and the second (Down) primer pairs respectively. Vectors were linearized by restriction digestion with the selected restriction enzymes (SmaI), then purified by gel DNA extraction. The target fragments and linearized vector were assembled according to the manual of Vazyme Clon Express MultiS One Step Cloning (ClonExpress MultiS). The above reaction mix was transformed into competent *E. coli* DH5α. 100 μl of culture evenly on an agar plate supplemented with 100 μg/ml carbenicillin and followed by overnight incubation at 37℃.

(4) Identification of positive colonies. Vectors were identified via colony PCR and sequencing with the pEX-F/R primer pairs

(5) Introduction of the allelic exchange vector harboring the target allele into the donor bacteria.

The recombinant plasmids were transferred into competent donor bacteria, an auxotrophic *E. coli* strain WM3064 harboring the RP4(tra) in the chromosome as conjugative machinery. *E. coli* transformants were recovered in 0.5 ml of LB medium with DAP at 37℃, with slow shaking for 45 min. 100 μl of the culture was plated evenly on an agar plate supplemented with 10mM DAP, 100 μg/ml carbenicillin followed by

overnight incubation at 37℃.

(6) Conjugation and merodiploid selection

a) Overnight cultures of the donor WM3064 and the recipient bacteria PA14 were reinoculated and regrown in LB liquid to an OD of 0.5~0.6 at 37 ℃. Donor WM3064 must be grown in the presence of DAP (0.3 mM)

b) 1.5 ml of Donor WM3064 and 1ml of recipient PA14 were gently centrifuged down (3000 g for 5 minutes) and washed with PBS 3 times and finally resuspended in 50 μL LB.

d) The donor and recipient cells (50 μL: 50 μL) were mixed in an EP tube, and 100 μL of the mixture was spotted onto prewarmed non-selective LB agar plates with DAP (0.3 mM). The solo recipient PA14 without a donor on the non-selective plates was used as a negative control.

e) Incubate conjugation plate overnight at 30℃.

f) Each conjugation culture mixture was scraped out into a tube with 5 ml PBS and washed by centrifugation 2 times.

g) Merodiploid selection. The conjugation mixture was resuspended in 1 mL of PBS. 100 μL of the mixture or the 10-fold dilutions were evenly plated onto selective plates with carbenicillin (300 μg/ml), but no DAP, on which donor WM3064 cannot grow other than the recipient PA14. Incubation overnight at 37 ℃.

(7) Counter-selection and identification.

An isolated single colony was picked after merodiploid selection and streaked onto NSLB-sucrose agar (No-salt LB agar with 15% (w/v) sucrose), incubated for 36~48 h at 30 °C. A single bacterial colony on NSLB-sucrose agar was picked and suspended in 40 μl of PBS, for PCR identification and plating. The selective bacteria were plated on LB agar with rifampicin for stock, and the LB agar containing carbenicillin (300 μg/ml), which was used for merodiploid selection and should kill the positive PA14. Overnight Incubation was at 37 °C. At least eight colonies on each NSLB-sucrose agar plate were picked for screening of the desired PA14 mutation. Sanger sequencing was performed for the positive candidate mutants after PCR identification and antibiotic plate selection.

Table. 1. Bacterial strains and plasmids

Strain	Description	Origin
UCBPP-PA14	Wild type *Pseudomonas aeruginosa*	[289]
Δ*rhlR*	A PA14 mutant with in-frame deletion in gene *rhlR*	[289]
Δ*rhlI*	A PA14 mutant with in-frame deletion in gene *rhlI*	[289]
Δ*pqsE*	A PA14 mutant with in-frame deletion in gene *pqsE*	This study
Δ*rhlI* Δ*pqsE*	A PA14 mutant with in-frame deletion in genes *rhlI* and *pqsE*	This study
Δ*rhlR* Δ*pqsE*	A PA14 mutant with in-frame deletion in genes *rhlR* and *pqsE*	This study
PA14-P*rhlA*-mNeonGreen (SM381)	wt *P. aeruginosa* strains harboring the P*rhlA*-mNeonGreen fusion in chromosome	[89]
PA14-Δ*rhlR* P*rhlA*-mNeonGreen (SM383)	PA14-Δ*rhlR* strains harboring the P*rhlA*-mNeonGreen fusion in chromosome	[89]
PA14-Δ*pqsE* P*rhlA*-mNeonGreen (SM563)	PA14-Δ*pqsE* strains harboring the P*rhlA*-mNeonGreen fusion in chromosome	[89]
PA14-Δ*rhlI* Δ*pqsE* P*rhlA*-mNeonGreen (SM568)	PA14-Δ*rhlI*Δ*pqsE* strains harboring the P*rhlA*-mNeonGreen fusion in chromosome	[89]
PA14- Δ5 (SM762)	Δ*lasR* Δ*lasI* Δ*rhlR* Δ*rhlI* Δ*pqsE*	[299]
PA14- Δ*lasR*	Δ*lasR* deletion mutant of *P. aeruginosa* strain PAK	[89]
PA14- Δ*lasI*	ΔlasR deletion mutant of *P. aeruginosa* strain PAK	
PA14- Δ*pqsR*	transposon mutant of gene *pqsR* of PA14	[107]
PAO1 glms::GFP	GFP from pCA24N-GFP with pPS856 GM promoter was inserted into *glms* down stream	[391]
*S.m*_RM66262	*S. marcescens RM66262,* chromosomally encoded *PBB2-GFP*	[393]
pEX18Ap	An allelic exchange plasmid with Amp resistence; oriT+ sacB+, with MCS from pUC18	[391]

Table .2. Primers used in this study

Primer	sequence(5'to3')
Δ*pqsE*-Up_F	cgactctagaggatccccgggCGCCGGCGAGAGTCTCGA
Δ*pqsE*-Up_R	ggagagaatCAACAGGCACAGGTCATCATCC
Δ*pqsE*-Down_F	tgtgcctgttgATTCTCTCCCGCCAGGCG
Δ*pqsE*-Down_R	aattcgagctcggtacccgggGCTGGACAGGCCATGCAG
pEX_F	TAACGCCAGGGTTTTCCCAGT
pEX_R	GGAATTGTGAGCGGATAAC
Δ*pqsE*-Seq1_F	CGCCGGCGAGAGTCTCGA
Δ*pqsE*-Seq1_R	GCTGGACAGGCCATGCAG
Δ*pqsE*-Seq2_F1	GCGGGTTGCTGTACGGCTT
Δ*pqsE*-Seq2_R1	TGGAATTCGTCGGCACACTG
*rhlA*_PA14_F	TGGACTGAACCAGGCGATGC
*rhlA*_PA14_R	GTGCTGATGGTTGCTGGCTTT
oprL-F	CGTGCGATCACCACCTTCTA
oprL-R	ACGCGCTGACCGCTGCCTTT
*16Sr*_PA_F	TACGGTAGAGGGTGGTGGAA
*16Sr*_PA_R	ACATCGTTTACGGCGTGGAC
*diptA*_F	GCTGCGCAATCGCTTCTACT
*diptA*_R	TGGTGGAGTGGGCTTCATG
*RP49*_F	GCCGCTTCAAGGGACAGTATC
*RP49*_R	AAACGCGGTTCTGCATGAG

Chapter III

What causes the difference between the Δ*rhlR* and Δ*rhlI*Δ*pqsE* virulence phenotypes in a continuous *P. aeruginosa* oral infection of *Drosophila*?

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Abstract

Pseudomonas aeruginosa, a Gram-negative bacterium with multiple antibiotic resistance, causes nosocomial opportunistic infection in patients with immunosuppression or cystic fibrosis. Multiply virulence factors are controlled by quorum sensing (QS), a process of cell-to-cell communication in response to cell density. QS involves a network that includes a transcriptional regulator, a synthase, and its product, the autoinducer as a signaling molecule that activates the regulator. RhlR/RhlI system is one of the well-known QS in *P. aeruginosa*, that relies on the autoinducer C4-homoserine lactone (C4-HSL) synthesized by RhlI. PqsE protein has recently been proposed to enhance RhlR activation via direct interaction with RhlR. Previously, we found there are differences in the strength of virulence phenotypes between the mutants Δ*rhlR*, Δ*rhlI,* and Δ*rhlI*Δ*pqsE* in the continuous oral infection models of *Drosophila*, and we speculated that an alternative mechanism activated RhlR regulator independently of both PqsE and RhlI. Here, we tried to use a latent-secondary-oral infection model to find out whether there is an alternative autoinducer that accounts for the difference in phenotypes between Δ*rhlI* Δ*pqsE* and Δ*rhlR*. We failed to identify the existence of an alternative autoinducer but confirmed a solid link that Δ *rhlI* Δ*pqsE* gains higher virulence dependent on RhlR when they are in the gut environment. Additionally, we present some evidence for different biofilm phenotypes of Δ*rhlI* Δ*pqsE* and Δ*rhlR* in the crop. We propose that it may be an external factor in the gut environment that contributes to the distinct phenotype of virulence between Δ*rhlR* and Δ*rhlI*Δ*pqsE* in continuous oral infection.

Keywords: quorum sensing; virulence; *P. aeruginosa*; *Drosophila*; host-pathogen interactions

Chapter Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen with multiple drug resistance, which can cause life-threatening infections in patients. *P. aeruginosa* possesses a variety of virulence regulatory systems contributing to its pathogenicity in its host. One well-known system, quorum sensing (QS), is a cell-to-cell communication process in response to the self-produced signaling molecules when the bacteria population reaches a cell-density threshold. This will in turn regulate the expression of numerous genes, allowing the bacteria population collectively to alter their behaviors, such as the production of virulence factors, biofilm formation, or motility [248]. QS circuits are typically composed of signaling molecules named autoinducers, of autoinducer synthases, and of partner autoinducer receptors. LuxR-LuxI type quorum sensing is the classical QS system in most Gram-negative bacteria， which relies on cytoplasmic LuxR-type receptors that function as transcriptional factors after binding the autoinducer acyl-homoserine lactones (HSLs or AHLs), produced by partner LuxI-type synthases. *P. aeruginosa* employs two LuxR-LuxI type systems, LasR-lasI, RhlR-RhlI, and another two QS circuits, PqsR-PqsABCDH and IQS system, with the respective autoinducers, namely N-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL), N-butyryl-L-homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (PQS), and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) [248]. Typically, LuxR-type receptors are insoluble and require the cognate HSL autoinducers for their dimerization, protein folding, and stability [303, 304]. It has been demonstrated that 3OC12-HSL and C4-HSL are required respectively for the solubilization of LasR and RhlR [306]. However, it was also reported that RhlR does not require C4-HSL for dimerization, although it needs C4-HSL for transcription activation [305]. It is worth mentioning that specific points mutant of *rhlR* enable the ligand-independent activity of RhlR *in vitro* and *in vivo* [300].

The four QS systems have complex cross-regulatory relationships. For example, RhlR/RhlI is positively regulated by LasR-LasI QS and PQS systems [272]. Besides, the loss of IQS attenuates bacterial virulence by dampening the biosynthesis of PQS and C4HSL via an unknown mechanism [267]. In addition, there is another LuxR homolog in *P. aeruginosa,* called QscR, known as an orphan or solo receptor since it lacks its cognate LuxI [279]. Loss of QscR induces earlier production of C4-HSL and

3OC12-HSL, and the premature transcription or overexpression of the genes regulated by RhlR and LasR, such as phenazines [280]. QscR can bind to 3OC-12-HSL with a high affinity, resulting in DNA binding activity [282, 284]. It then activates a single linked operon, which in turn decreases the gene activation of genes regulated by LasR and RhlR [273, 283]. Besides, QscR also shows sensitivity to other non-*P. aeruginosa* HSLs, such as 3OHC10, may be used to detect cohabitating species [285].

Indeed, quorum sensing is not limited to its bacterial population but is also involved in interspecies and inter-kingdom communication or interactions, including with eukaryotic hosts. In nature, it is a norm that a variety of microorganisms coexist and have cross interactions, microbiota in the gut, human oral cavity, polymicrobial coinfection, and host-microbe interaction. A well-known example of interspecies communication is the QS signal molecules diffusible signal factors (DSFs), which regulate diverse biological functions in a range of Gram-negative bacterial pathogens in humans, like *P. aeruginosa* and *S. maltophilia*, *B. cenocepacia* [330]. The exogenous addition of DSF from *B. cenocepacia* impaired *P. aeruginosa* biofilm formation and virulence factors production, which resulted from the suppressed expressions of *lasR*, *rhlR*, and *pqsR* and the reduced production of their corresponding autoinducers. In HeLa cell and zebrafish infection models, type III secretion system (T3SS) genes of *P. aeruginosa* were sensitively inhibited by DSF [333]. Besides, *P. aeruginosa* might communicate with the gut microbiota. *P. aeruginosa* has been found to exist within the microbial communities of the human intestines and is capable to cause local and remote infections in hospitalized patients [141, 143, 144]. It has been reported that gut microbiota uses AI-2 (autoinducer-2) -mediated quorum sensing system in the mammalian gut, to keep homeostasis and also crosstalk with the mammalian host [337]. *P. aeruginosa* cannot produce AI-2, but it does have the capability to sense and respond to AI-2, in turn, it changes the formation of biofilm and the production of virulence factors [340]. AI-2 treatment can alter the biofilm architecture of *P. aeruginosa* PAO1 and increase its virulence factors production *in vitro*, as well as facilitate acute infection in the murine lung infection model. It was proposed that AI-2 influences such group behavior of *P. aeruginosa* via the Las system and/or Rhl system, since phenotypes of double mutant Δ*lasR* Δ*rhlR* are not affected by AI-2 treatment [341, 342]. Moreover, AI-2 also contributes to biofilm formation within dental plaque [343]. Interestingly, Sibley et al. performed a *Drosophila* intestinal infection by coinfection with the oropharyngeal flora isolated from CF patients and *P. aeruginosa*. A class of

oropharyngeal flora bacteria was characterized that was nonpathogenic alone but could increase the total pathogenicity when coinfected with *P. aeruginosa* [344]. Quorum sensing of *P. aeruginosa* has a direct role in host-microbe Interactions. The autoinducers 3OC12-HSL of *P. aeruginosa per se* can cause damage to the integrity of the tight junctions of intestinal epithelial cells *ex vivo*, by the activation of a matrix metalloprotease [346]. Moreover, 3OC12-HSL decreases inflammatory responses of macrophages by downregulation of the NF-κB pathway [349, 350]. Besides, quorum sensing of *P. aeruginosa* can be monitored by its mammalian host. The host aryl hydrocarbon receptor (AhR) serves as a ligand-dependent transcription factor and sensor for environmental toxins, including phenazines, the secret pigmented products of *P. aeruginosa* regulated by QS [351]. In this way, *P. aeruginosa* QS molecules relevant to its cell density were detected and quantified by its host, which acts coordinately in the diverse immune responses against the infection of *P. aeruginosa* [352].

Given the above background, although QS is intrinsically in response to its autoinducer relevant to the cell density, its outcome is affected by a variety of internal and external factors in the cohabitation environment, particularly in the complex and dynamic host context. Therefore, it is essential to investigate the virulence regulatory mechanism of *P. aeruginosa* in the host context, to obtain a deeper understanding of the development of novel therapies against bacterial infection.

Up to now, as described in Chapter II, we have developed several oral infection models with different pathogenicity in *Drosophila*, including continuous oral infection (intestinal infection), latent oral infection, and latent-reactivated (latent-29℃) infection, and defined the roles of quorum sensing in the pathogenicity in the above models. Our previous study [56, 89] and investigation in Chapter 2 have demonstrated that the Rhl system plays a key role in virulence regulation in oral infection. The interesting observation is that we found there might be an atypical activation of the RhlR regulator, independently of its classical autoinducer, which is produced by RhlI. The story began from our previous finding that the regulator RhlR but not RhlI is required for bacterial virulence and allows *P. aeruginosa* PA14 to evade the cellular immune response [56]. Consistently, it was also found that mutant Δ*rhlI* displayed similar virulence as the wild-type *P. aeruginosa* in C. *elegans* and murine acute lung infection, whereas Δ*rhlR was* much less virulent [289]. *In vitro*, Δ*rhlR* and Δ*rhlI* display completely different

biofilm colonies. RhlR still can control the expression of multiple genes expression in the absence of RhlI [289]. The above findings suggest there may be an alternate autoinducer that activates RhlR. A subsequent study proposed PqsE was an alternative synthase candidate responsible for RhlR activation based on their similar phenotypes *in vitro* and *in vivo* [297]. The same group revealed in a subsequent study that PqsE does not work as a synthase, but directly binds to RhlR, which enhances RhlR binding to DNA. It was proposed that the Rhl QS circuit works as two kinds of interaction models *in vitro*: the RhlR-C4HSL or PqsE-RhlR-C4HSL [299, 302]. During the period of that study that revealed the real function of PqsE, we were also investigating the role of PqsE in our *Drosophila* models. In chapter II, we demonstrated that PqsE alone does not contribute to virulence, but that it functions synergistically with RhlI for the activation of RhlR. In theory, Δ*rhlI*Δ*pqsE* should have a phenotype identical to that of Δ*rhlR*. It is true for their phenotypes *in vitro*, including colony biofilm architecture, and virulence production. However, we always observed significant differences in the virulence phenotypes between Δ*rhlR* and Δ*rhlI*Δ*pqsE* in the continuous oral infection, although they showed no difference in the bacteria load. Interestingly, Δ*rhlI* Δ*pqsE* displayed a degree of attenuated virulence similar to that of Δ*rhlR* in the latent infection or latent-29 ℃ reactivated infection model, both of which are characterized by the absence of *P. aeruginosa* in the gut once colonization has been established.
In summary, since the proposed function of PqsE is dependent on RhlI, the existence of PqsE still cannot provide a proper explanation for the significant phenotypic difference between Δ*rhlR* and Δ*rhlI in vitro* and *in vivo*. It means an alternative autoinducer still possibly exists but is not synthesized by PqsE. Alternatively, there may exist another activation mechanism, or RhlR and RhlI respectively have independent functions. The *Drosophila* intestinal infection paradigms provide us with working models because they show distinguishable phenotypes between Δ*rhlI* Δ*pqsE* and Δ*rhlR*. We speculated that there is possibly an alternative mechanism for the activation of RhlR besides the roles of RhlI and PqsE, but it may also be due to an interaction with the host environment, such as gut microbiota.

In this study, based on the current data, we have not figured out whether there is an alternative autoinducer that is responsible for the distinct phenotypes of Δ*rhlI*Δ*pqsE* and Δ*rhlR* in the continuous oral infection model, using a latent-secondary oral

infection model or by feeding C4-HSL in the latent infection and latent-29℃ reactivated infection models. Given the different biofilm phenotypes of Δ*rhlI*Δ*pqsE* and Δ*rhlR* in the digestive tract, we propose there may be external factors from the gut environment that contribute to the alternate activation of RhlR.

Results

1. Tissue-colonizing bacteria in latent infection can be activated by a secondary oral infection of bacteria lacking the classic autoinducer, yet the activation process requires RhlR both in the resident and the newly invading bacteria

Our first step was to identify whether a possible alternative activation mechanism of the regulator RhlR would occur within the cell population of *P. aeruginosa*, that is whether *P. aeruginosa* itself could produce an alternative autoinducer to activate RhlR, independently of RhlI or PqsE. We designed a latent-secondary infection: perform a second continuous oral infection in the flies in which a latent infection with *P. aeruginosa* had been established eight days prior to the second ingestion infection. In other words, the flies harboring the dormant *P. aeruginosa* wt or mutants were challenged again by the continuous oral infection with wt or mutants *P. aeruginosa*. Our rationale was as follows: if there were a putative autoinducer that contributes to the activation of RhlR in Δ*rhlI*Δ*pqsE* bacteria, the RhlR of the dormant wt bacteria in the tissue from the primary infection should be activated by the putative autoinducer produced by the Δ*rhlI*Δ*pqsE* from the secondary infection from initially in the gut and then next in the tissues. In contrast, the dormant Δ*rhlR* bacteria in the tissue from a primary infection would not be activated because of the absence of RhlR as the specific receptor and regulator. Thus, it would show a difference in fly survival between the above two sets of infections. In short, the overall idea was to complement the missing inducer in a latent-reactivated infection model with that produced by the bacteria from the secondary infection, which are expected to be essential in the gut with some bacteria

reaching the hemocoel. For the primary infection, flies followed the classical latent infection procedure, respectively with wt, Δ*rhlR*, Δ*rhlI*Δ*pqsE P. aeruginosa*, or without bacteria. At day 8 post latent infection, the flies were secondarily challenged by continuous oral infection respectively with wt, Δ*rhlI*, Δ*rhlI*Δ*pqsE,* Δ*rhlR P. aeruginosa*, or without bacteria. For each group, the following notation was taken: "1st → 2nd". For example, flies with Δ*rhlR*→wt represent the flies primarily infected with Δ*rhlR* and secondarily infected wt. Flies with NI →wt represent the flies primarily noninfected and secondarily infected wt. The pooled survival curves and the integrated data of the computed lethal time 50% were analyzed, as follows：

- The survival experiments showed that wt→wt flies died significantly faster than NI →wt, as well as Δ*rhlR*→wt **(Fig.1A),** yet the LT50% analysis did not reveal any difference, reflecting that any difference is very modest at best **(Fig.1F)**.

- It suggests the dormant wt bacteria in the tissue can possibly be reactivated and in a way dependent on RhlR.

- The survival curve showed that Δ*rhlI*Δ*pqsE*→wt flies died as faster as wt→wt flies (**Fig.1A).**

- It suggests that RhlR in Δ*rhlI*Δ*pqsE* bacteria is functioning normally as that in wt bacteria.

- The survival curve shows that wt→ Δ*rhlI* flies died significantly faster than NI→ Δ*rhlI* flies, as well as Δ*rhlR*→ Δ*rhlI* flies **(Fig.1B).** However, the difference is too mild to be significant in the LT50% analysis **(Fig.1F).**

- It suggests the dormant wt bacteria in the tissue can be activated in an RhlR-dependent manner, without the autoinducer C4-HSL, which is synthesized by RhlI.

- The survival curve and LT50% analysis show wt→ Δ*rhlI*Δ*pqsE* flies died faster than those with NI→ Δ*rhlI*Δ*pqsE*, and there was no difference between NI→ Δ*rhlI*Δ*pqsE* and Δ*rhlR*→ Δ*rhlI*Δ*pqsE* **(Fig.1C; Fig.1F).**

- It indicates again that dormant wt bacteria in the tissue can be activated independently of the autoinducer C4-HSL but relying on the function of RhlR.

- In the wt→ Δ*rhlI*Δ*pqsE*, or Δ*rhlI*Δ*pqsE*→ Δ*rhlI*Δ*pqsE*, the primary bacteria wt or Δ*rhlI*Δ*pqsE* in the tissue are supposed to be activated, but wt→ Δ*rhlI*Δ*pqsE vs.* Δ*rhlI*Δ*pqsE*→ Δ*rhlI*Δ*pqsE* showed no difference **(Fig.1C; Fig.1F)**.

- It suggests the surface virulence is not an outcome of a simple addition mode as

“1+1=2”, since Δ*rhlI*Δ*pqsE* is less virulent than wt bacteria, but the above coinfection shows no difference.

Up to here, basically, the results are in line with the hypothesis of the existence of an alternative autoinducer. However, when the Δ*rhlR* as a secondary-inoculated bacteria was introduced into the comparison, we found there might be another possibility. According to the logic of the rationale, we raised at the beginning, if there were an alternative autoinducer, both Δ *rhlR* and Δ*rhlI*Δ*pqsE* should produce the putative autoinducer. Thus, wt bacteria in the tissue should be activated after the accumulation of Δ*rhlR* from the secondary infection. As Δ *rhlR* is much less virulent, thus there should be an obvious difference between wt→ Δ *rhlR* and Δ *rhlR* → Δ *rhlR*, as pronounced as the difference between wt→ Δ *rhlR* and NI→ Δ *rhlR*. However, the analysis showed the following: there was no difference in LT50% analysis among wt→ Δ *rhlR*, NI→ Δ *rhlR*, Δ *rhlI* Δ *pqsE*→ Δ *rhlR*, Δ *rhlR*→ Δ *rhlR*, although there was a slight difference between wt→ Δ *rhlR* and NI→ Δ *rhlR* in the survival curves **(Fig.1D; Fig.1F)**. It indicated that the activation of the wt bacteria residing in the tissues by the secondary infection required the function of RhlR in the secondary-inoculated bacteria.

We speculated two possibilities to explain the results: 1) There was an alternative autoinducer and its responsible synthase under the regulon of RhlR, like the synthase RhlI. Thus, without RhlR, the alternative autoinducer cannot be produced or is limited because of no feed-forward induction. 2) The activation of the wt bacteria or Δ *rhlI*Δ*pqsE* residing in the tissues was built on the virulence outcome of the bacteria from secondary infection. Two-step activation of the bacteria might be involved: proliferation first, then virulence activation. The dormant bacteria might not be sensitive to the signal molecules of QS and require to be equipped with enough RhlR receptors, possibly as a result of proliferation. To figure out the above, we next designed the experiments described in the following part (Figure 2).

We note that the phenotypes of flies that have been first latently infected and secondarily orally challenged were roughly similar to those of controls that had not been latently infected and submitted only to the secondary infection **(Fig.1E; G)**. The phenotypes were similar to those previously shown in the continuous oral infection in Chapter II. It indicates bacteria from the secondary infection provide the major contribution to virulence in the coinfection. As shown **(Blue *vs*. Green in Fig.1E; G),**

flies infected with 1st /NI→ Δ*rhlI*Δ*pqsE* always died faster than 1st /NI→ Δ*rhlR*. But in the latent infection **(Gray panel in Fig.1E, F)**, survival curved and LT50% analysis showed an ambiguous difference between Δ*rhlI*Δ*pqsE*→ NI and Δ*rhlR*→ NI. Similarly, in the study in Chapter I, the virulence phenotypes of Δ*rhlI*Δ*pqsE* and Δ*rhlR* had a slight difference in latent-29 ℃ infection and no difference in the latent infection. It indicates that the Δ*rhlI*Δ*pqsE* bacteria are always more virulent than Δ*rhlR* when they are in the gut but not in the tissue. We designed further experiments described next (Figure 3).

2. Tissue-colonizing bacteria dormant in latent infection cannot be aroused by the classic autoinducer C4-HSL

Given the last part, we hypothesized that the dormant bacteria in the latent infection could not sense the autoinducers until they were proliferating. Therefore, we designed this experiment： flies were submitted to the infection with the PA14 bacteria or mutants and supplemented by continuous feeding with C4-HSL from Day8 post-infection. The infections followed the protocols of two models: 1) the latent-29℃ reactivated infection model in which the bacteria are proliferating in the tissues (as described in Chapter II); 2) the latent infection model in which the bacteria are dormant. The survival curves in the latent-29 ℃ infection **(Fig.2A)** showed that treatment of C4-HSL accelerated the killing of flies by the PA14 wt, Δ*rhlI*, Δ*rhlI* Δ*pqsE*, except the Δ*rhlR*. The difference of LT50% analysis **(Fig.2B)** was too mild to show the significance, except that Δ*rhlI* Δ*pqsE* with C4-HSL killed the flies much faster than those without C4-HSL did. So far, we just performed the latent infection with wt bacteria. In the latent infection with a much slower killing kinetic, we did not observe that the treatment of C4-HSL did accelerate the killing of flies by the PA14 wt **(Fig.2C, D)**. Conversely, flies feeding with C4-HSL seemed to survive a litter longer. The above suggests the latent-colonizing bacteria could not respond to the orally provided autoinducers, possibly unless they are proliferating.

3. Rhl quorum sensing is involved in the biofilm formation of *P. aeruginosa* in the crop during the continuous oral infection in *Drosophila*

As mentioned in the part1, Δ*rhlI*Δ*pqsE* and Δ*rhlR* have always displayed obvious

differences in the continuous oral infection, whereas there was only mild or no difference in the latent infection or latent-29 ℃ infection. It suggests the gut environment may influence the RhlR activation of *P. aeruginosa* bacteria. We considered whether extrinsic signaling was responsible for activating RhlR of *P. aeruginosa* Δ*rhlI* Δ*pqsE*, for example, host metabolites or signaling molecules from the gut microbiota, which is a reasonable candidate. Here, we first tried to identify whether the Rhl quorum sensing is activated in the gut, including the midgut and the crop.

A5001 flies were continuously fed with PA14 wt or mutants harboring the P*rhlA*-mNeonGreen fusion reporter in the chromosome. *rhlA* is a gene regulated by the Rhl system and responsible for the synthesis of rhamnolipids. On Day6 after infection, the guts were dissected for imaging. Because of a technical problem, we just show the results of the crops here. Consistent with a published report [363], we observed the biofilm of wild-type *P. aeruginosa* was formed in the crop. Besides, the biofilm of wt bacteria showed an obvious fluorescent signal from the *rhlA* reporter **(Fig.3A)**, whereas there was a clean background, no specific signal of *rhlA* reporter, and no visible biofilm colonies in the crops infected with Δ*rhlR*. Interestingly, we also observed cloud-like substances with a weak DAPI signal, formed in the crops infected with Δ*rhlI*Δ*pqsE*. The cloud-like colonies were not condensed as biofilm colonies as wt bacteria, and the specific green signal of *rhlA* report was sparse. Nonetheless, we did find a suspected signal of *rhlA* expressing in the Δ*rhlI* Δ*pqsE* (shown in the zoom insets). We counted the crops with cloud-like substances in each group and found there was a significant difference among the wt bacteria, Δ*rhlR,* and Δ*rhlI*Δ*pqsE* **(Fig.3B)**.

Collectively, Rhl quorum sensing is involved in the biofilm formation of *P. aeruginosa* in the crop during the continuous oral infection in *Drosophila*. Different from the Δ*rhlR*, Δ*rhlI* Δ*pqsE* displayed a visible biofilm phenotype, possibly with a mild activation of the Rhl QS.

Conclusions

Tissue-colonizing dormant *P. aeruginosa* in latent infection can be activated by the accumulation of the *P. aeruginosa* (Δ*rhlI*Δ*pqsE*) unable to synthesize the classic autoinducer, and the activation process requires RhlR function both in the colonizing and the bacteria from the secondary challenge. Tissue-colonizing dormant *P. aeruginosa* cannot respond to the exogenous autoinducer C4-HSL supplemented by feeding unless the bacteria are proliferating. Taken together, these experiments suggest that it is not an alternative autoinducer that contributes to the activation of tissue-colonizing dormant *P. aeruginosa* during the accumulation of the *P. aeruginosa* deleted for *rhlI* and *pqsE*. The activation possibly takes place dependent on the virulence outcome of the secondary inoculated bacteria. Thus, we still cannot exclude the existence of an alternative autoinducer responsible for the difference in the virulence phenotypes between Δ*rhlI* Δ*pqsE* and Δ*rhlR*. Biofilm formation in the crops differed among *P. aeruginosa* wt, Δ*rhlI* Δ*pqsE,* and Δ*rhlR*. RhlR quorum sensing is activated in the biofilm of wild-type *P. aeruginosa* and likely the Δ*rhlI*Δ*pqsE* when more experiments are performed. It suggests that Δ*rhlI*Δ*pqsE* may gain more virulence when they are in the gut environment. We propose it may be an external host-provided but not an internal alternative activation mechanism accounting for the different virulence phenotypes of Δ*rhlI*Δ*pqsE* and Δ*rhlR*.

Discussion

Our goal in this study was to find out the reason that is responsible for the virulence difference between the Δ*rhlI*Δ*pqsE* bacteria and Δ*rhlR*, which may lead to a discovery of an alternative activation mechanism of RhlR-mediated quorum sensing. The data we obtained so far drives us to consider it might be the external factors but not the internal factors that contribute to the different phenotypes of virulence in the *Drosophila* models. The biofilm phenotypes in the crop provide possible support for this hypothesis. Therefore, we are wondering whether *P. aeruginosa* communicates with the gut microbiota. The gut microflora of the laboratory-reared *Drosophila* is composed of 1 to 30 species, which are most commonly of *Acetobacteraceae* and *Lactobacillaceae*, for example, *Acetobacter* and *Lactobacillus*, Sometimes *Enterobacteriaceae* and yeasts are also found [394-396]. The gut microbiota of laboratory flies has low diversity and often be shaped by the food content or the feeding condition. For example, *Acetobacter* can be a dominant bacterium in the food medium containing high sucrose, since it is good at sucrose processing [397]. *Lactobacillus* is also a bacterial species in human gut microflora and has been identified to use the AI-2 QS system *in vitro* and *in vivo* [398, 399]. It has been reported that *P. aeruginosa* is capable to respond to the AI-2 [340], and the process is associated with the Rhl system or Las system [341, 342]. In further study, we will confirm this hypothesis by performing the *P. aeruginosa* intestinal infection in germ-free flies.

The study we showed is just focused on the possibility of an alternative activation mechanism for Rhl quorum sensing, but we infer another possibility on the opposite side here. The binding interaction of the autoinducer and its receptor is bidirectional. Although the recognition is relatively specific, it is not rare that one receptor can recognize several signaling molecules and one autoinducer can be sensed by more than one receptor. For instance, LasR recognizes 3OC12-HSL and also has different binding affinities to other exogenous HSL autoinducers, such as 3OC10-HSL, and 3OC6-HSL [300]. And 3OC12-HSL can also be recognized by the QSCR, the orphan receptor, which acts as a transcriptional factor and negatively regulates the Las system and Rhl system [273, 282]. This phenomenon demonstrates that the functions of the autoinducer and its receptor are actually not strongly coupled, that is, they have independent roles leading to separate effects. To our knowledge, it has been not reported that RhlR can

respond to other ligands, or that the C4-HSL can directly activate other receptors. Nonetheless, RhlR was shown to have the ability to interact with 3OC12-HSL, resulting in RhlR dimer dissociation but the interaction did not induce gene expression [305]. It has been also identified that 3OC12-HSL is not accountable for the different virulence activity of the Δ*rhlI* mutant and Δ*rhlR* mutant [289]. On the side of the autoinducer, C4-HSL cannot substitute for 3OC12-HSL to directly activate the transcriptional function of QscR, but it did promote the QscR oligomer dissociation [273, 282]. Moreover, interestingly, when acyl-HSLs are absent, QscR can bind to LasR or RhlR and form inactive heterodimers [273]. Based on that, we infer that in the Δ*rhlI* mutant, on the one hand, there is no C4-HSL to bind to QscR. On the other hand, QscR may bind to RhlR to form an inactive complex. The above two possibilities may dampen the negative regulatory function of QscR, although it may be compensated by 3OC12-HSL. In the case of our *Drosophila* intestinal model, the Las system is not required shown in chapter II and our previous report [89]. Besides, an earlier study showed that QscR mutant displays higher virulence than wild-type PAO1 in the *Drosophila* intestinal infection model [280]. Another two important clues provide a possible link: 1) QscR negatively regulates the expression of phenazines (*phz2*, *phz1*), which are greatly overproduced in the QscR mutant [273]; 2) phenazines are overproduced in Δ*rhlI* mutant resulting in a smooth colony biofilm phenotype, whereas phenazines are less expressed in Δ*rhlR*, leading to a hyper-rugose biofilm phenotype [289]. Of note, phenazines of *P. aeruginosa* are not necessary virulence factors in the *Drosophila* intestinal model [89], as well as in the murine acute lung infection model [289]. But not just one or two virulence factors may be under such negative control of QscR. Given the about, the negative function of QscR may provide a reasonable explanation for the different phenotypes of Δ*rhlR* and Δ*rhlI in vitro*, and to a certain extent, for the virulence phenotypes in the infection models of *Drosophila*, mice, and *C. elegans*. However, the arguments above are not enough to figure out why the phenotypes of Δ*rhlI*Δ*pqsE* and Δ*rhlR* are nearly identical *in vitro* but are significantly different in the continuous oral infection models. Further studies are required to figure out the relationship among QscR, RhlI, PqsE, and RhlR in the context of *Drosophila* intestinal infection models, which may lead to an answer to the question we are asking.

Figures

Figure 1

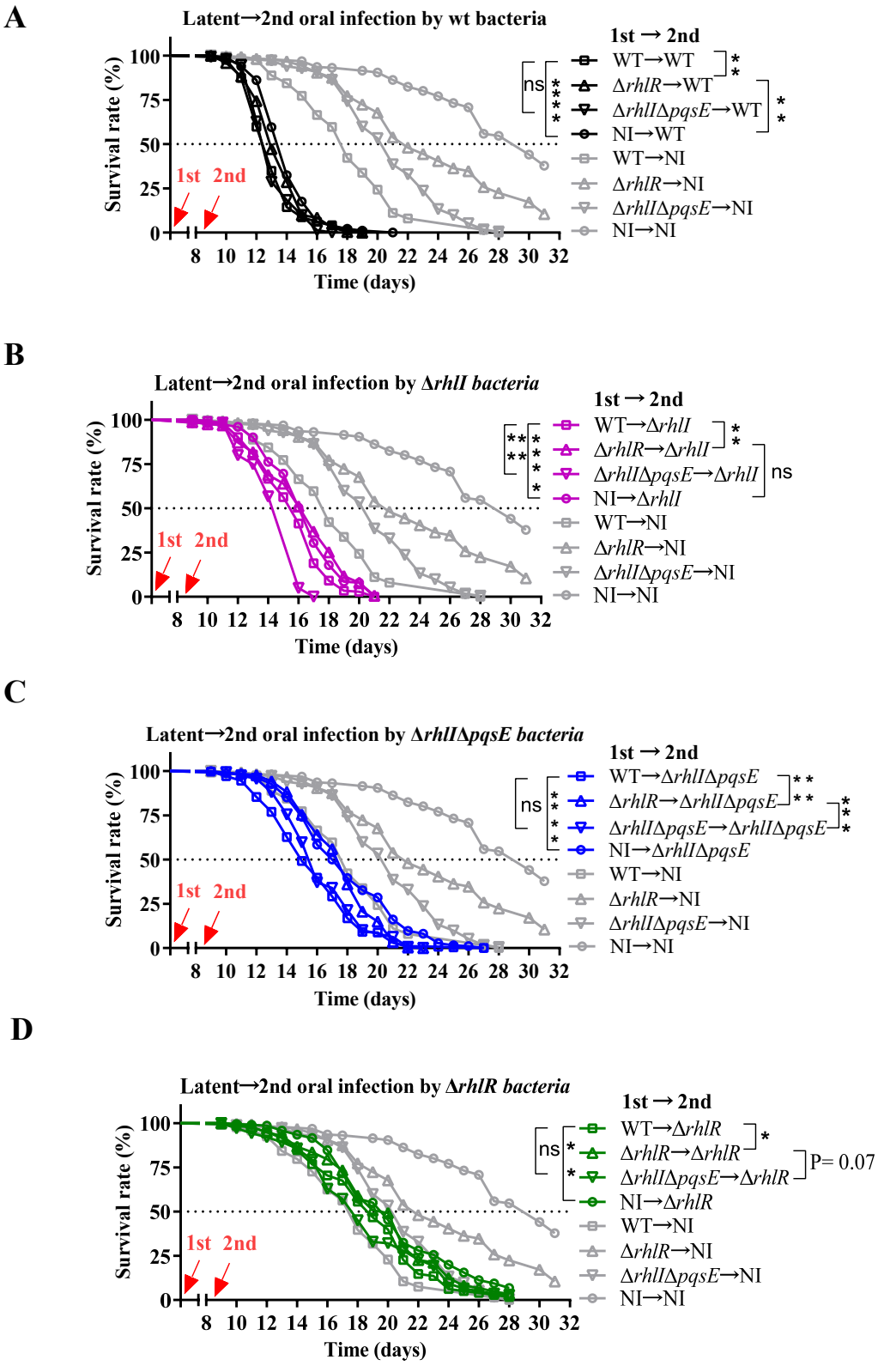
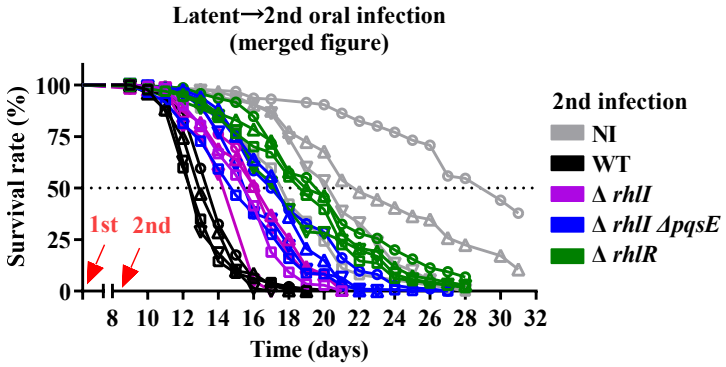
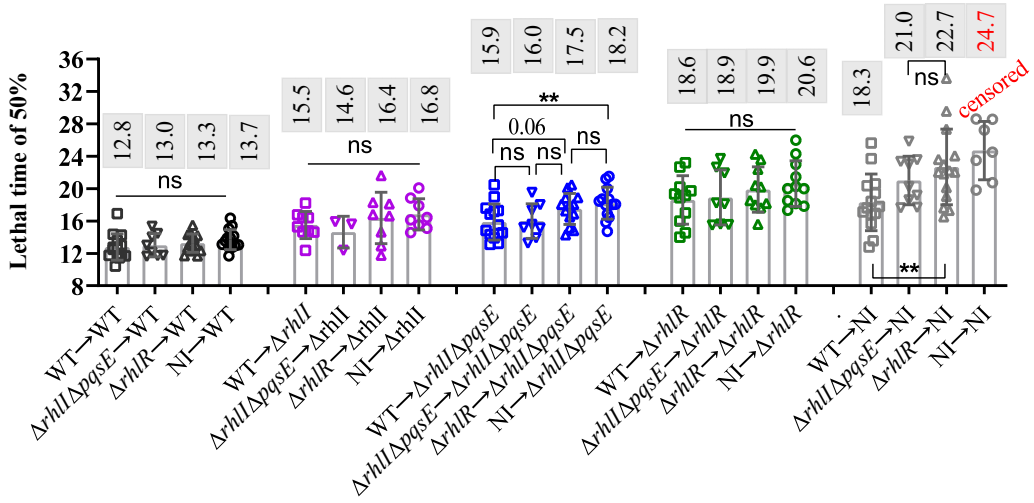


Figure 1

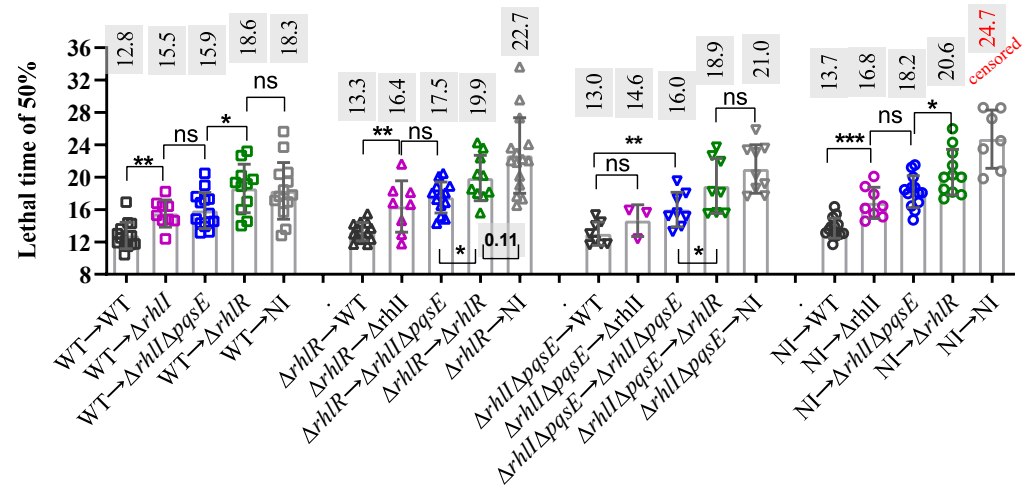
E


Latent→2nd oral infection
(merged figure)
Survival rate (%)
100
75
50
25
0
1st
2nd
8 10 12 14 16 18 20 22 24 26 28 30 32
Time (days)
2nd infection
NI
WT
Δ rhlI
Δ rhlI ΔpqsE
Δ rhlR

F


Lethal time of 50%
36
32
28
24
20
16
12
8
12.8
13.0
13.3
13.7
15.5
14.6
16.4
16.8
15.9
16.0
17.5
18.2
18.6
18.9
19.9
20.6
18.3
21.0
22.7
24.7
censored
ns
0.06
**
WT→WT
ΔrhlIΔpqsE→WT
ΔrhlR→WT
NI→WT
WT→ΔrhlI
ΔrhlIΔpqsE→ΔrhlI
ΔrhlR→ΔrhlI
NI→ΔrhlI
WT→ΔrhlIΔpqsE
ΔrhlIΔpqsE→ΔrhlIΔpqsE
ΔrhlR→ΔrhlIΔpqsE
NI→ΔrhlIΔpqsE
WT→ΔrhlR
ΔrhlIΔpqsE→ΔrhlR
ΔrhlR→ΔrhlR
NI→ΔrhlR
WT→NI
ΔrhlIΔpqsE→NI
ΔrhlR→NI
NI→NI

G


Lethal time of 50%
36
32
28
24
20
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12.8
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15.9
18.6
18.3
13.3
16.4
17.5
19.9
22.7
13.0
14.6
16.0
18.9
21.0
13.7
16.8
18.2
20.6
24.7
censored
ns
0.11
WT→WT
WT→ΔrhlI
WT→ΔrhlIΔpqsE
WT→ΔrhlR
WT→NI
ΔrhlR→WT
ΔrhlR→ΔrhlI
ΔrhlR→ΔrhlIΔpqsE
ΔrhlR→ΔrhlR
ΔrhlR→NI
ΔrhlIΔpqsE→WT
ΔrhlIΔpqsE→ΔrhlI
ΔrhlIΔpqsE→ΔrhlIΔpqsE
ΔrhlIΔpqsE→ΔrhlR
ΔrhlIΔpqsE→NI
NI→WT
NI→ΔrhlI
NI→ΔrhlIΔpqsE
NI→ΔrhlR
NI→NI

Figure 2

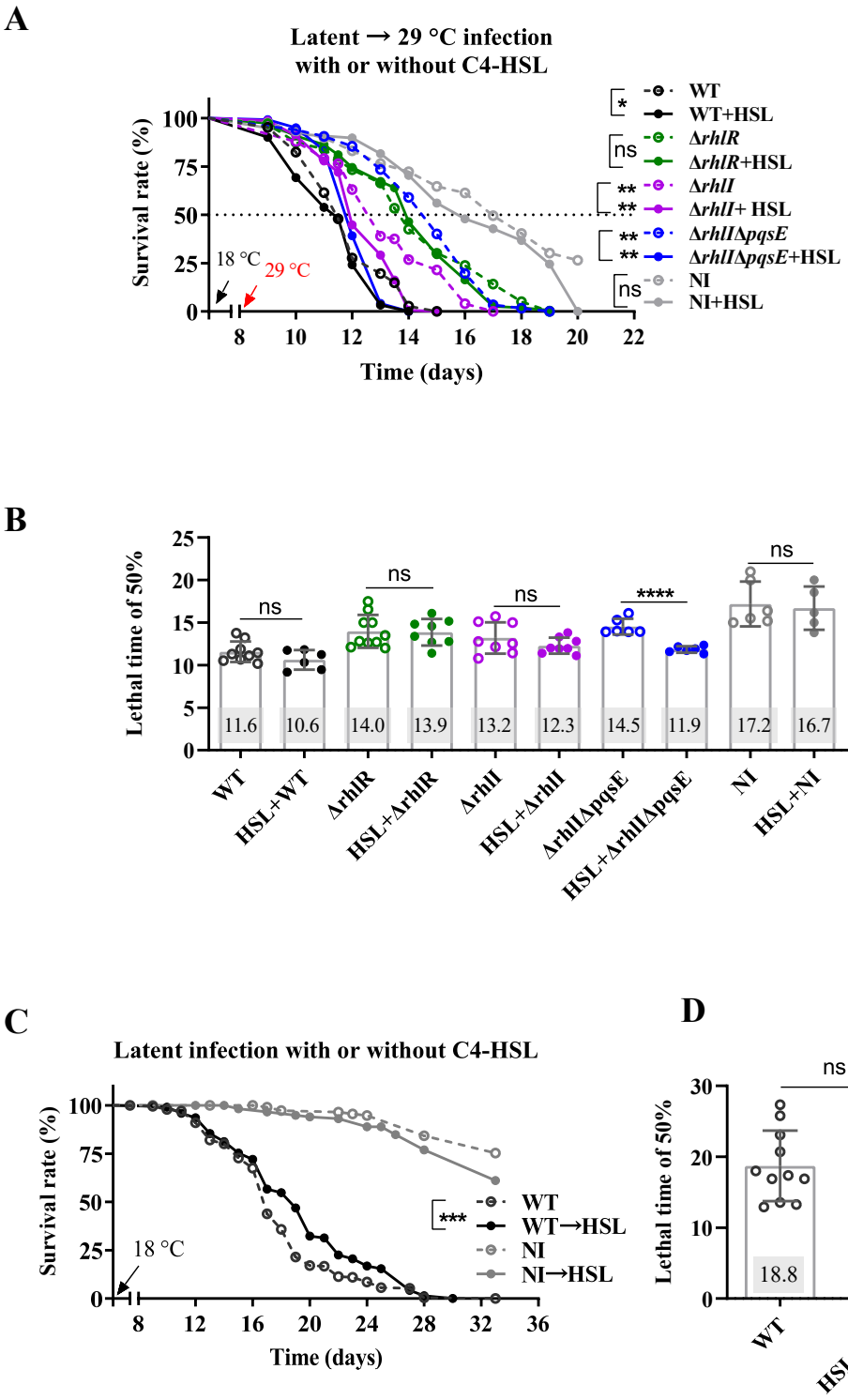
A
Latent → 29 °C infection
with or without C4-HSL
Survival rate (%)
Time (days)
18 °C
29 °C
WT
WT+HSL
ΔrhlR
ΔrhlR+HSL
ΔrhlI
ΔrhlI+ HSL
ΔrhlIΔpqsE
ΔrhlIΔpqsE+HSL
NI
NI+HSL
B
Lethal time of 50%
11.6
10.6
14.0
13.9
13.2
12.3
14.5
11.9
17.2
16.7
WT
HSL+WT
ΔrhlR
HSL+ΔrhlR
ΔrhlI
HSL+ΔrhlI
ΔrhlIΔpqsE
HSL+ΔrhlIΔpqsE
NI
HSL+NI
C
Latent infection with or without C4-HSL
Survival rate (%)
Time (days)
18 °C
WT
WT→HSL
NI
NI→HSL
D
Lethal time of 50%
18.8
19.1
WT
HSL+WT

Fig. 1. Bacteria dormant in the tissue with latent infection can be activated, depending on RhlR, by the secondary infection with Δ*rhlI*Δ*pqsE* but not with Δ*rhlR*. A5001 flies previously followed the classic procedure of latent infection with PA14 wt or mutants. At day 8, two days after the end of the gentamicin feeding, flies were challenged with secondary infection by continuous oral feeding with the PA14 wt or mutants as indicated at 18 ℃. (A~D) survival curve of flies in the latent → secondary oral infection. The primarily inoculated bacteria and secondarily inoculated bacteria were shown as 1st →2nd in the graph legend. (E) Merged survival curves of graphs A, B, C, and D. (F, G) Corresponding lethal time 50% for the survival curves in two arrangement orders. Each point is a set of about 20 flies. Pooled data from more than three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001； ****P < 0.001, ns = non-significant.

Fig. 2. Tissue-colonizing bacteria dormant in latent infection cannot be aroused by feeding on the classic autoinducer C4-HSL. (A, B) Survival curve and corresponding LT50 of flies in the latent → 29 ℃ reactivated infection with or without the autoinducer C4-HSL treatment. A5001 flies had previously followed the classic procedure of latent infection with PA14 wt or mutants at 18 ℃. At day 8, flies were transferred to 29 ℃ and fed meanwhile with or without C4-HSL of 30 μM in sucrose until death at 18℃. Pooled data from two independent experiments. (C, D) Survival curve and corresponding LT50 of flies in the latent infection with or without the autoinducer C4-HSL treatment. A5001 flies had previously followed the classic procedure of latent infection with PA14 wt or mutants. At day 8, flies were fed with or without C4-HSL of 30 μM in sucrose until death at 18 ℃. Pooled data from three independent experiments.

Figure 3

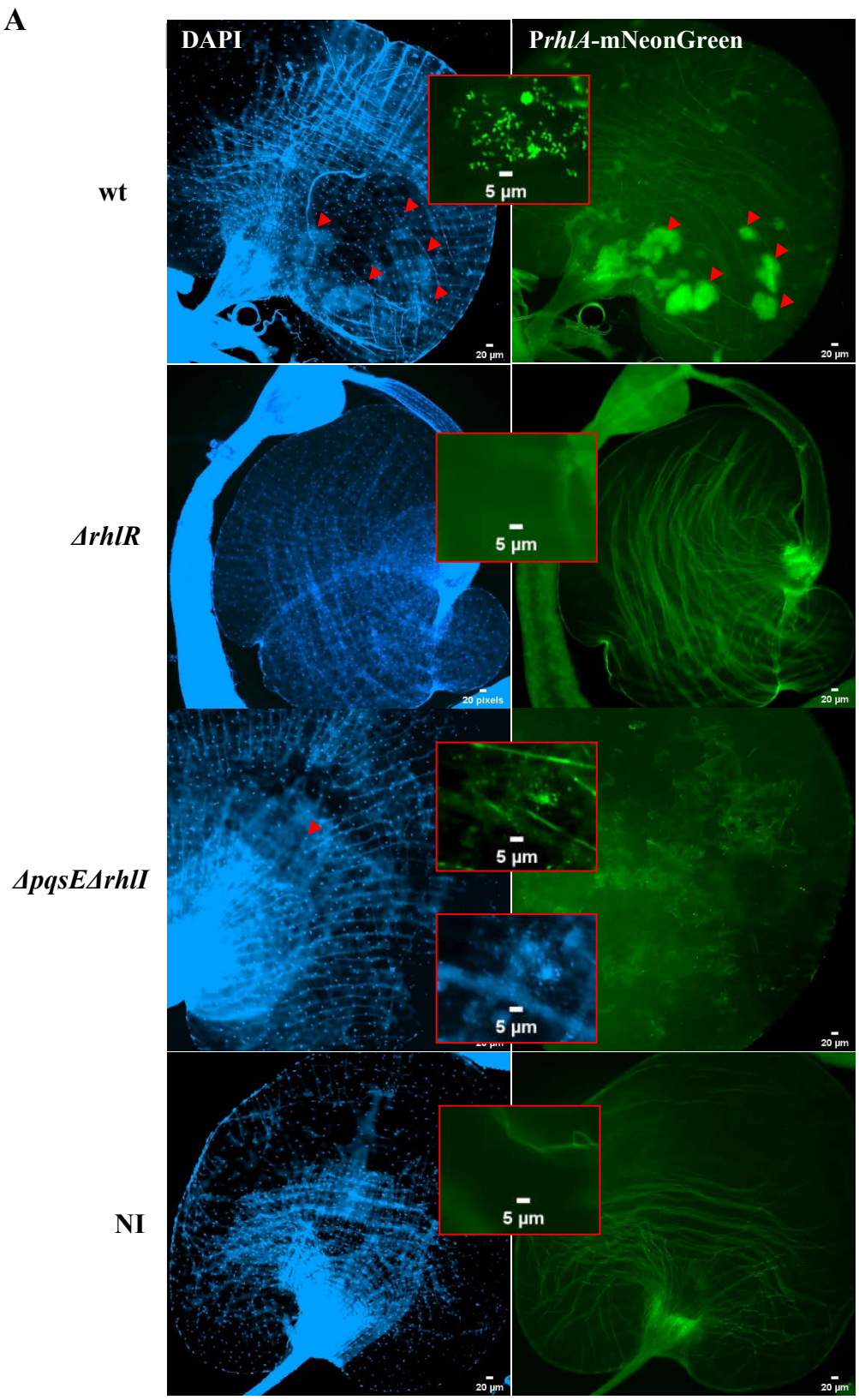
A
DAPI
PrhlA-mNeonGreen
wt
ΔrhlR
ΔpqsEΔrhlI
NI
5 µm
20 µm
20 pixels

Figure 3

B

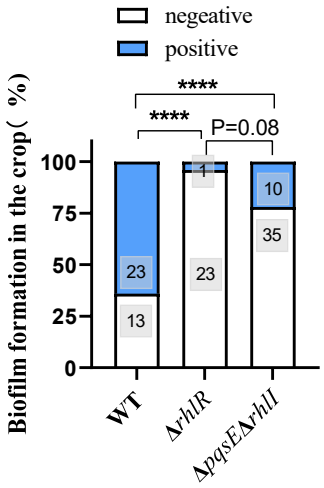

Fig. 3. Biofilm formation of *P. aeruginosa* in the crop relies on the RhlR quorum sensing during the continuous oral infection in *Drosophila*. A5001 flies were continuously fed with PA14 wt or mutants harboring the P*rhlA*-mNeonGreen fusion reporter inserted on the chromosome. At day 6, the crops of flies were dissected for DAPI staining and fluorescent imaging. (A) The images of crops from infected files. Cloud-like signals of biofilm are indicated by red arrowheads. The magnified image of the PA14 wt with mNeonGreen or DAPI shows in the inset. (B) Quantitative analysis of biofilm formation in the infected crops. The crops with cloud-like signals of DAPI or mNeonGreen were counted. The counted numbers of positive or negative were shown in the bar. The data were pooled from three independent experiments. The Chi-square test was performed for the statistical analysis. ****P < 0.0001; ns= non-significant.

Materials and Methods

Drosophila Strains and Culture

Fly stocks were raised at 25 °C under 60% humidity and fed with standard cornmeal–agar medium with selectively added yeast. 5 to 7-day-old A5001 females were used for experiments.

Bacterial strains and culture

P. aeruginosa UCBPP-PA14 was used as wild type strain and three in-frame deletion mutant strains Δ*rhlR*, Δ*rhlI*, Δ*rhlI*Δ*pqsE* were used in this study. Bacteria strains with PrhlA-mNeonGreen fusion reporter were kindly provided by Prof. Bonnie Bassler (shown in the Table 1 of Chapter I). All the bacteria were cultured on LB agar plates without antibiotics at 37 ℃ overnight， then maintained at 4 ℃ for at most two weeks. To infect flies, a single colony of *P. aeruginosa* was inoculated in the liquid (25ml) of Brain-Heart-Infusion Broth (BHB) (Bacto™ Brain Heart Infusion, BD #237500), and grown in flasks at 37 ℃ for 16 hours.

Infection procedure

An overnight BHB-culture of *P. aeruginosa* wt or mutants was transferred to a 50ml tube and spun down by centrifugation at 3,800 x g, for 15 min, 25 °C. The pellet was washed with sterile PBS buffer twice, finally diluted in 50 mM sucrose solution containing 5% (v/v) BHB to an optical density at 600 nm (OD600) of 4. For continuous oral infection, flies were fed on an absorbent pad (AP1002500, Millipore; dia. 25 mm) soaked with 0.6ml bacterial solution (OD=4) at 18 ℃ until death, and daily supplemented with 80 µl sucrose solution（50mM) from the second day onwards. For latent infection, flies were continuously fed with bacteria as above for two days, then were transferred to a new tube with a pad soaked with 0.6 ml of sucrose solution (50 mM) supplemented with gentamicin (100 µg /ml) at 18 ℃ for 4 days. Thereafter, flies were fed on sucrose solution（50mM) without gentamicin, with daily supplementation of 100 µl sucrose solution（50mM) until death. For latent-secondary infections, flies underwent the latent infection protocol as above from Day 0 to Day 8. On Day 8, flies were challenged again by continuous feeding on a pad with wt or mutant *P. aeruginosa*

until death, in the same condition as the treatment of Day 0. For latent-29℃ reactivated infection, flies followed the classical procedure of latent infection at 18 ℃ until Day 8, at which the flies were transferred to 29 ℃ until death, with daily supplementation of 120 µl sucrose solution (at 29 ℃). For C4-HSL feeding, flies were fed with the sucrose solution containing C4-HSL (30 µM) from Day 8 until death, with daily supplementation of 80 µl (at 18 ℃) or 120 µl (at 29 ℃) of this solution. C4-HSL (Sigma, 09945-25MG) was dissolved in 1.46 ml DMSO to obtain a stock solution of 100 mM and diluted in sucrose solution （50mM) to a final concentration of 30 µM. 20 female files for each vial, and two or three replicates were performed for the above infection for each independent experiment.

Statistical analysis

All the data were performed on GraphPad Prism (version 8.4). Two analyses were performed for survival data, log-rank analysis, and LT50 analysis. The lethal time 50% (LT50) was computed through nonlinear regression analysis. Statistical analysis used Mann-Whitney for pairwise comparisons or Kruskal-Wallis tests for multiple comparisons. Data of frequency distribution was analyzed by Pearson's chi-square. Significance values: ****P<0.0001; ***P< 0.001; **P<0.01; *P< 0.05, ns; non-significant.

General Discussion

Our work here aims to understand how *P. aeruginosa* changes its virulence in the *Drosophila* infection model, particularly in chronic infections. In this study, we have established a novel model named latent infection, with the distinct characteristics that *P. aeruginosa* solely colonizes the tissues, possibly with arrested growth that may underlie antibiotic tolerance; importantly, it does not cause any symptoms in most flies and does not elicit a strong systemic humoral immune response. We propose that the tissue-associated bacteria are dormant, but not *bona fide* persisters because in contrast to the few persister cells that tolerate antibiotic treatments, most colonizing bacteria appear to be dormant (it is not clear whether the few bacteria detected in the hemolymph during the initial period display features of the colonizing bacteria such as the expression of the O5-antigen, which will need to be tested). We still do not know how the *P. aeruginosa* bacterium adapts itself and crosses the intestinal barrier without causing visible damage to the gut epithelium. The humoral immune response and phagocytosis by hemocytes make an ancillary contribution to killing the bacteria that occasionally circulate in the hemolymph, while melanization plays a critical role in trapping the ingested bacteria that have crossed the intestinal barrier. Although we did not observe blackened spots in the latently-infected flies, we did detect the fully-cleaved, mature active form of *PPO2*, which is required for the melanization reaction. Besides, the melanization triggered by Gram (-) bacteria is currently unknown. Further work is however needed to identify when, where, and how the melanization gets activated, and whether its effect is required for maintaining bacterial dormancy. Indeed, a key issue is to understand in which compartment the bacteria change their phenotypes to the one found in tissues: is it occurring already in the gut? This would change the surface properties of the bacteria that may then be sensed by the immune system. For instance, it has been reported that the phagocytic receptor Eater is not required for the phagocytosis of Gram-negative bacteria [50]. Yet, it had been found that *S. marcescens* that had escaped from the gut lumen were actually controlled by Eater [48, 75]. Thus, the PO proteolytic activation cascade might be triggered upon the detection of *P. aeruginosa* with altered surface properties. Alternatively, the bacteria just leaving the gut epithelium might trigger melanization and be induced by this host defense to become dormant. To discriminate between these two possibilities, it will be important

to determine whether bacteria retrieved from the carcass and those captured in the hemolymph display or not the O5 LPS-antigen. Thus, melanization might be either required to initiate dormancy or alternatively, to actively maintain it in bacteria that would change their surface phenotype and physiology upon crossing the intestinal barrier. In addition, it will be interesting to investigate the induction of dormancy from the perspective of the bacterium, that is, how the bacterium adapts itself to the tissues by becoming dormant. From an evolutionary perspective, it may favor the dissemination of the bacterium by its flying host and *P. aeruginosa* would be released into the environment upon the death of its host.

The establishment of the latent infection model may help us obtain a deeper understanding of the behavior of *P. aeruginosa* in intestinal infections, which might be relevant to its behavior in mammalian hosts, including human patients. It is imperative to figure out how this opportunistic pathogen regulates its virulence, corresponding to the transition from a chronic to an acute infection or conversely to become dormant. In chapter II, the comparative analysis among *Drosophila* models with pathogenic characteristics, reveals a virulence-switching program of tissue-colonizing *P. aeruginosa*. Rhl quorum sensing is necessary for this virulence-switching process with the bacterial lifestyle switching from a sessile to a planktonic form whereas proliferation in the tissues is independent of Rhl QS. This specific function of Rhl QS was confirmed by the dispensability of Las and PQS systems in the *Drosophila* oral infection models. In the reverse vein, Rhl quorum sensing is no longer necessary in the melanization-impaired flies wherein the dormancy is not initiated or maintained; bacteria are present in the hemolymph already at the early phase of infection. Puzzlingly, Las QS mutants were found to be less virulent in melanization-impaired flies. It has been identified that Las QS combined with mechanotransduction switches on virulence when the bacteria are attaching to a surface [329]. Besides, the Rhl and LasR QS systems have overlapping and reverse roles in biofilm development [289, 308, 315]. Our *Drosophila* models here confirm *in vivo* that the Rhl system and Las system control the virulence switching on in opposite ways. It will be interesting to investigate further whether Rhl system activation directly regulates bacteria to regain their motility and whether this motility is coupled to the release of the virulence factors that are upregulated by Rhl QS. This can be achieved by investigating flagella, which are required for swimming and swarming, and Type IV-pili, which are required for swarming and twitching motility [164].

The bacterium in the latent infection with autoinducer C4-HSL feeding appear not to be activable, but the virulence of ΔrhlIΔpqsE mutant was enhanced when C4-HSL was fed to flies in the latent-29°C reactivated infection model. It would mean the dormant bacteria cannot sense the signaling molecules for communication, that is, they are not communicating when they rest until they are proliferating. Besides, its virulence switching is always conjugated with the increased bacterial load in the continuous oral infection and latent-29℃ infection. In addition, bacterial proliferation is not dependent on Rhl quorum sensing, which is confirmed in the latent-29℃ infection. Therefore, we propose that the passage to full virulence activation of the dormant bacteria in our *Drosophila* models requires two canonical steps in sequential order: first proliferation and then Rhl quorum sensing activation.

Typically, Rhl QS relies on the network of RhlR regulator/receptor, autoinducer C4-HSL, and autoinducer synthase, with the synergy of PqsE, which was recently proposed [299, 302]. In the case of our *Drosophila* oral infection model, PqsE alone is not required for virulence, which is different from the situation in the murine lung infection and *C. elegans* infection models [297]. Moreover, the *ΔrhlIΔpqsE* double mutant is less virulent than either single mutant but still significantly different from *ΔrhlR* in the continuous oral infection model, although these two strains have nearly identical phenotypes *in vitro*. It suggests that in the continuous oral infection model, there is possibly an alternative mechanism of RhlR activation. We considered whether it is an alternative autoinducer, but we failed to identify its existence. However, the survival difference between *ΔrhlIΔpqsE* and *ΔrhlR* is only detected in the continuous oral infection model but neither in the latent infection nor latent-reactivated infection model. Besides, different biofilm formation in the crops of *ΔrhlIΔpqsE* and *ΔrhlR* implies that *ΔrhlIΔpqsE* may be more virulent in the intestinal tract. It would be interesting to assess whether pqsE alone is required for biofilm formation in the crop. Thus, we proposed it may be an external but not an internal alternative activation mechanism accounting for the different virulence phenotypes of *ΔrhlIΔpqsE* and *ΔrhlR*. It will be worth studying further whether RhlR can nevertheless be activated in *ΔrhlIΔpqsE* double mutants and identifying which factor (from hosts or microbiota) is responsible for RhlR activation independent of the function of RhlI and PqsE. It is surprising that the RhlR regulator and its signaling components RhlI and PqsE have distinct regulation outcomes, although they are working in the same circuit in theory. One first step would

be to monitor the expression of genes uniquely regulated by the RhlI-RhlR-PqsE axis *vs*. those regulated by the classical RhlI-RhlR axis in bacteria colonizing tissues. As there are too few bacteria in the tissue that can be retrieved for transcription analysis, specific transgenic reporters might need to be designed, although the risk is that translation is inhibited in dormant bacteria. In the case of the gut, transcription analysis may be practical since there are more bacteria in the continuous oral infection.

In summary, the bacterial virulence regulatory mechanism is more complex than we thought in the dynamic host environment. The study of host-microbe interaction in *Drosophila* models allows us to finely dissect the bacterial pathogenicity and the regulatory mechanism of *P. aeruginosa* in this small but fully-equipped. host platform which complements “higher animal” models. In this study, what we learned from the *Drosophila* models revealed the important relationship of bacterial dormancy to the host's innate immune response and allowed us to investigate with high resolution the function of Rhl quorum sensing in virulence switching during chronic infection. Our work will keep going on for deeper insights into how opportunist pathogens activate or inhibit their virulence, as well as how hosts defend against their infection via their innate immune systems. In the long term, it would be important to be able to induce dormancy with a drug in patients undergoing acute *P. aeruginosa* infections or prevent the virulence activation of bacteria in the chronic infections. Such a strategy might decrease the rate of selection for bacteria able to avoid such an action.

ANNEX

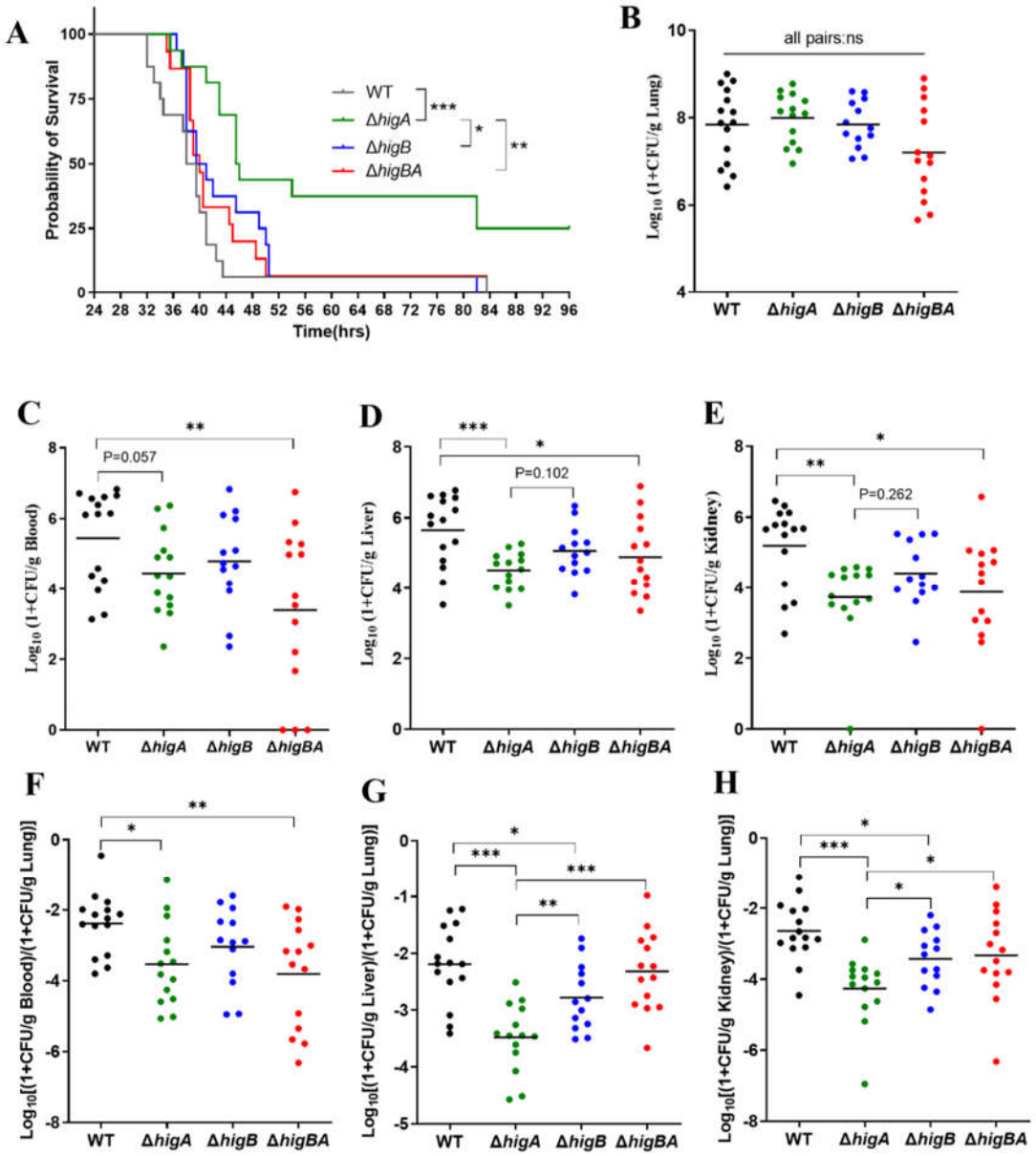

Figure. Deletion of HigA alleviated the pathogenicity of PAO1 in a murine model of acute lung infection. C57BL/6J mice (7-to-8-week-old, females) were infected with PAO1 wt or the mutants as indicated by direct intratracheal inoculation. (A) Mice survivals were monitored for up to 4 days post-infection and are represented on Kaplan-Meier curves. Significant differences were analyzed by Log Rank. Inoculation dose = 1 × 10^7 CFU/mouse, n = 16. Time (hrs.) of each mouse =Time of death –Time of inoculation. (B) Bacterial load in the lung was detected at 36 h post-infection.

Inoculation dose = 0.85 × 10^7 CFU/mouse, n = 13~15. Each dot represents one mouse. (C-E) Bacterial load in the blood, liver, and kidney was detected in parallel with the lung, and (F-H) data was normalized with the bacterial load in the lung correspondingly. Data of (B-E) was analyzed by Kruskal-Wallis one-way ANOVA. Data of F-H was analyzed by one-way ANOVA and LSD. ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant. Images are representative of one set from two independent replicates with similar results. (Ethics statement: mice experiment procedures were performed according to the guidelines of the Laboratory Animal Center at South China Agricultural University.)

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Résumé de la thèse de doctorat

Les agents pathogènes opportunistes tels que *Pseudomonas aeruginosa* provoquent des infections plus fréquentes et plus graves chez les personnes humaines dont le système immunitaire est affaibli. Les infections à P. aeruginosa ne sont pas faciles à éradiquer en raison de la résistance de cette bactérie à de multiples antibiotiques et de sa capacité à former des communautés de biofilm, ce qui entraîne une infection persistante ou chronique. Une compréhension approfondie des mécanismes de régulation de la virulence chez P. aeruginosa est essentielle pour développer des interventions thérapeutiques alternatives pour contrôler et prévenir les infections bactériennes. Un mécanisme bien étudié, la détection du quorum (QS), est un processus de communication bactérien de cellule à cellule mis en œuvre en réponse au changement de densité cellulaire qui permet à la population bactérienne de contrôler collectivement un grand nombre de programmes d'expression génique et synchronise ainsi les comportements de groupe, tels que la virulence, la formation de biofilm et la motilité. QS dépend des réseaux d'auto-inducteurs, d'auto-inducteurs synthases et de récepteurs auto-inducteurs. P. aeruginosa utilise quatre boucles de détection de quorum entrelacées LasR-LasI, RhlR-RhlI, PqsABCDH-PqsR et AmbBCDE avec un récepteur inconnu. Ils correspondent respectivement aux récepteurs et synthases des auto-inducteurs 3OC12-HSL, C4-HSL, PQS et IQS.

Drosophila melanogaster a été particulièrement largement utilisé pour étudier les interactions hôte-pathogène et pour déchiffrer les mécanismes complexes de virulence bactérienne in vivo, grâce à sa génétique sophistiquée et à son système immunitaire inné bien caractérisé, assez similaire à celui des mammifères. L'hôte drosophile possède des réponses immunitaires humorales et cellulaires qui traitent des infections systémiques, ainsi que des réponses locales de barrière épithélienne qui traitent des invasions dans le tractus intestinal et les trachées. Par exemple, l'injection de bactéries dans la cavité corporelle de la mouche induit l'activation rapide des défenses immunitaires, y compris la coagulation et la mélanisation. La réponse de mélanisation est une défense immunitaire importante chez les arthropodes, entraînant un noircissement des caillots sur les sites de la plaie, qui représentent les sites de mélanisation de l'invasion des micro-organismes. La mélanisation systémique peut également être induite par la signalisation médiée par le récepteur de reconnaissance de

formes (PRR) après la reconnaissance des peptidoglycanes bactériens (PGN). La réaction de mélanisation repose sur des enzymes appelées prophénoloxydases (PPO). Les protéases Hayan et Sp7 sont responsables de l'activation des PPO en PO actifs, qui catalysent le dépôt de mélanine. En outre, la réaction de mélanisation a une association avec la production de molécules cytotoxiques, telles que les espèces réactives de l'oxygène (ROS). Il est donc potentiellement responsable de la mort des organismes envahisseurs. Il a été démontré que la réaction de noircissement au site de la plaie dépend davantage de Hayan et de ses substrats PPO1 et PPO2, mais Sp7 peut activer PPO1 et avoir une contribution alternative à la destruction des microbes, peut-être liée à la production de ROS. Comme pour les infections microbiennes locales, telles que celles du tractus intestinal, les ROS et les AMP spécifiques aux tissus sont générés pour lutter contre les agents pathogènes ou réguler le microbiote intestinal. Une fois que la bactérie a traversé les barrières épithéliales, elle peut être tuée par phagocytose des hémocytes. Le composant bactérien tel que les PGN libérés lors de la prolifération bactérienne ou de la lyse peut être détecté par le corps adipeux, déclenchant une puissante réponse humorale systémique médiée par des peptides antimicrobiens (AMP) tels que la diptéricine. L'induction de plusieurs gènes de l'AMP est médiée par deux voies de signalisation de type NF-κB, la voie de déficit immunitaire (MI) et la voie de Toll.

Notre équipe étudie l'interaction hôte-pathogène dans des modèles d'infection à *P. aeruginosa* chez la drosophile depuis plus d'une décennie. Auparavant, nous avons étudié la pathogénicité de *P. aeruginosa* dans un modèle d'infection intestinale (infection buccale) de drosophile immunocompétente et immunodéficiente. Nous avons constaté que le régulateur de détection de quorum RhlR de *P. aeruginosa* est nécessaire à la virulence bactérienne et permet aux bactéries d'échapper à la réponse immunitaire cellulaire dans le modèle intestinal. Pendant ce temps, un phénomène intéressant est que quelques bactéries P. aeruginosa ingérées parviennent à traverser la barrière intestinale dans l'hémocèle de mouche mais tuent les mouches en plus d'une semaine, beaucoup plus lentement que la bactérie directement injectée dans l'hémocèle de la mouche, qui tue les mouches en quelques jours par bactériémie. Ce changement dans la pathogénicité de *P. aeruginosa* selon les voies d'infection nous a amenés à approfondir les mécanismes sous-jacents. Nous espérons que cela permettra de mieux comprendre comment *P. aeruginosa* régule sa virulence chez l'hôte, en particulier lors d'infections chroniques.

L'étude présentée ici vise à caractériser le comportement bactérien et la pathogénicité de *P. aeruginosa* ingéré, en particulier des bactéries qui ont traversé la barrière intestinale. Ensuite, l'objectif supplémentaire était de confirmer si la signalisation RhlR joue un rôle clé dans la régulation de la virulence dans différents modèles d'infection par la drosophile et de comprendre comment le circuit de signalisation RhlR/RhlI/PqsE fonctionne dans le contexte de l'hôte drosophile en présence ou en l'absence de sa défense immunitaire.

Dans le chapitre I, basé sur le modèle d'infection buccale transitoire, nous avons développé un modèle d'infection latente à *P. aeruginosa* en nourrissant les mouches de la bactérie pendant une courte période. Les bactéries colonisent de manière stable les tissus internes et, dans la plupart des cas, ne causent aucun symptôme; Ainsi, les mouches infectées de manière latente vivent presque aussi longtemps que les mouches témoins non infectées. Fait intéressant, les bactéries dormantes présentent des caractéristiques particulières en termes de morphologie bactérienne et bactérienne des colonies, de composition de la paroi cellulaire externe et de motilité. Nous montrons que la mélanisation mais pas la réponse humorale cellulaire ou systémique sont nécessaires pour établir la latence, contrairement à la situation dans les modèles d'infection aiguë ou d'ingestion continue. L'activation des réponses de mélanisation chez les mouches infectées de façon latente explique probablement un degré supplémentaire de protection de l'hôte contre une variété d'agents pathogènes bactériens ou fongiques injectés surnuméraires. Ce modèle sera utile pour étudier les interactions hôte-pathogène qui régulent l'expression des programmes de virulence chez les agents pathogènes et les défenses spécifiques de l'hôte pertinentes pour contenir les agents pathogènes en fonction de leur voie d'infection.

Dans le chapitre II, pour étudier l'interaction hôte-pathogène entre *P. aeruginosa* et la *Drosophile*, nous avons établi quatre modèles d'infection avec des caractéristiques pathogènes différentes: lésion septique, infection buccale continue (infection intestinale), infection buccale latente et infection latente réactivée (latente à 29 ° C). Contrairement à la lésion septique dans laquelle les bactéries prolifèrent dans l'hémolymphe et à l'infection latente caractérisée par des bactéries sessiles dormantes, l' infection continue présente un processus de développement avec un programme de commutation progressive de la virulence de *P. aeruginosa* dans le tissu: phase dormante, transitoire et active. Un programme similaire de changement de virulence est également

observé dans l'infection réactivée latente à 29 °C. Par rapport à l'infection latente, la principale différence de l'infection intestinale continue est que la bactérie *P. aeruginosa* est présente dans l'intestin et peut traverser continuellement la barrière intestinale, tandis que la principale différence du modèle d'infection réactivée latente à 29 °C est que les bactéries prolifèrent directement dans les tissus. Le modèle d'infection buccale continue et les modèles réactivés latents à 29°C partagent la caractéristique commune que la virulence bactérienne est activée à une phase tardive, correspondant à une densité cellulaire franchissant un seuil dans le tissu, accompagnée d'une transition du mode de vie sessile au planctonique. Le processus de commutation nécessite le système de détection du quorum de la Rhl, mais ni le système Las ni le système PQS. Il est confirmé dans la veine opposée, que le système Rhl est dispensable chez les mouches dépourvues de la réponse de mélanisation, dans laquelle les bactéries ne sont pas dormantes et prolifèrent activement dans l'hémolymphe. Nos modèles d'infection orale fournissent également des preuves in vivo que la signalisation RhlR module la virulence d'une manière dépendante de RhlI en synergie avec PqsE. En outre, un mécanisme d'activation alternatif du régulateur RhlR indépendant de PqsE et RhlI peut être impliqué.

Dans le chapitre III, précédemment, nous avons constaté qu'il existe des différences dans la force des phénotypes de virulence entre les mutants Δ*rhlR*, Δ*rhlI* et Δ*rhlI*Δ*pqsE* dans les modèles d'infection orale continue de la drosophile, et nous avons spéculé qu'un mécanisme alternatif activait le régulateur RhlR indépendamment de PqsE et RhlI. Ici, nous avons essayé d'utiliser un modèle d'infection latente-secondaire-orale pour savoir s'il existe un auto-inducteur alternatif qui explique la différence de phénotypes entre Δ*rhlI* Δ*pqsE* et Δ*rhlR*. À ce jour, nous n'avons pas réussi à identifier l'existence d'un auto-inducteur alternatif mais avons confirmé un lien solide selon lequel Δ*rhlI* Δ*pqsE* gagne une virulence plus élevée en fonction de RhlR lorsqu'ils sont dans l'environnement intestinal. De plus, nous présentons des preuves pour différents phénotypes de biofilm de Δ*rhlI* Δ*pqsE* et ΔrhlR dans la culture. Nous proposons qu'il puisse s'agir d'un facteur externe dans l'environnement intestinal qui contribue au phénotype distinct de virulence entre Δ*rhlR* et Δ*rhlI*Δ*pqsE* dans l'infection buccale continue.

En résumé, le mécanisme de régulation de la virulence bactérienne est plus complexe que nous ne le pensions dans l'environnement dynamique de l'hôte. L'étude de

l'interaction hôte-microbe dans des modèles de drosophiles nous permet de disséquer finement la pathogénicité bactérienne et le mécanisme de régulation de *P. aeruginosa* dans ce petit mais entièrement équipé. plate-forme hôte qui complète les modèles « animaux supérieurs ». Dans cette étude, ce que nous avons appris des modèles de drosophile a révélé la relation importante de la dormance bactérienne avec la réponse immunitaire innée de l'hôte et nous a permis d'étudier avec une haute résolution la fonction de détection du quorum de la Rhl dans la commutation de virulence au cours d'une infection chronique. Notre travail se poursuivra pour mieux comprendre comment les agents pathogènes opportunistes activent ou inhibent leur virulence, ainsi que comment les hôtes se défendent contre leur infection via leur système immunitaire inné. À long terme, il serait important de pouvoir induire la dormance avec un médicament chez les patients subissant des infections aiguës à P. aeruginosa ou d'empêcher l'activation de la virulence des bactéries dans les infections chroniques. Une telle stratégie pourrait diminuer le taux de sélection des bactéries capables d'éviter une telle action.

Guiying LIN

Study of virulence regulatory mechanisms of *Pseudomonas aeruginosa in Drosophila melanogaster*: an investigation into RhlR quorum sensing system

Résumé

Ce travail vise à établir comment la bactérie *P. aeruginosa* s'adapte à l'environnement hostile de l'hôte et comment elle contrôle ses programmes de virulence dans un modèle d'infection orale continue de la drosophile.

1) Nous avons mis au point un modèle d'infection latente chez la drosophile et observé que les bactéries ayant franchi la barrière intestinale s'associent aux tissus. Elles y sont dormantes et ne déclenchent pas une forte réponse immunitaire systémique. La mélanisation joue un rôle essentiel dans l'établissement de l'état de latence tandis que les réponses cellulaires et humorales ne contribuent que partiellement à l'inhibition de l'activation de la virulence bactérienne.

2) Nous avons réalisé une analyse comparative de différents modèles d'infection de la drosophile et caractérisé une activation de la virulence des bactéries associées aux tissus dans le modèle d'ingestion chronique. De plus, nous avons confirmé que le système de détection du quorum Rhl est nécessaire pour l'activation de cette virulence et le changement de comportement lié à la perte de dormance. L'activation du senseur RhlR ne dépend cependant que partiellement des autres composantes de ce système, RhlI et PqsE.

3) Finalement, nous avons posé la question de l'existence d'une autre molécule auto-inductrice ou d'un mécanisme alternatif d'activation de RhlR pouvant expliquer la différence de phénotypes observées entre les mutants Δ*rhlR* and Δ*rhlI*Δ*pqsE* de *P. aeruginosa* dans le modèle d'ingestion chronique. Nous n'y sommes pas arrivés jusqu'à présent. Toutefois, nous avons identifié un lien potentiel pouvant expliquer la différence de ces phénotypes par une interaction avec des facteurs de l'hôte dans l'environnement du tractus digestif.

Mots-clés: détection de quorum : virulence ; *Pseudomonas aeruginosa*, *Drosophila*, interactions hôtes-pathogènes ; immunité innée

- Résumé en anglais

The work here aims to figure out how *P. aeruginosa* adapts to the hostile host environment and how it controls virulence programs in chronic oral infection models of *Drosophila*.

1) We established a *P. aeruginosa* latent infection model in *Drosophila* and characterized that the bacteria that have crossed the intestinal barrier associate with host tissues. They are dormant and do not trigger a strong systemic immune response. Melanization plays a key role in the establishment of latency, while the cellular and the humoral immune responses make an ancillary contribution to preventing the virulence activation of the bacteria.

2) We made a comparative analysis of different infection models of *Drosophila* and characterized a virulence-switching program of the tissue-colonizing bacteria in the chronic infection model. Furthermore, we confirmed that the Rhl quorum sensing of *P. aeruginosa* is necessary for the virulence switching and lifestyle transition, in a way only partially dependent on the signaling components, RhlI and PqsE.

3) Finally, we asked whether there is an alternative autoinducer or alternate activation mechanism of the RhlR sensor that is responsible for the difference of virulence phenotypes between the Δ*rhlR* and Δ*rhlI*Δ*pqsE* mutants in the continuous oral infection. So far, we have not figured out the existence of an alternative autoinducer but identified a potential link between the distinct phenotypes and the gut environment, suggesting further interactions between *P. aeruginosa* and host-provided factors.

Keywords: quorum sensing; virulence; *Pseudomonas aeruginosa*; *Drosophila*; host-pathogen interactions; innate immunity