

## ***Drosophila* are protected from *Pseudomonas aeruginosa* lethality by transgenic expression of paraoxonase-1**

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Research Article

Infectious disease

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# *Drosophila* are protected from *Pseudomonas aeruginosa* lethality by transgenic expression of paraoxonase-1

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*Pseudomonas aeruginosa* uses quorum sensing, an interbacterial communication system, to regulate gene expression. The signaling molecule *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL) is thought to play a central role in quorum sensing. Since 3OC12-HSL can be degraded by paraoxonase (PON) family members, we hypothesized that PONs regulate *P. aeruginosa* virulence in vivo. We chose *Drosophila melanogaster* as our model organism because it has been shown to be a tractable model for investigating host-pathogen interactions and lacks PONs. By using quorum-sensing-deficient *P. aeruginosa*, synthetic acyl-HSLs, and transgenic expression of human PON1, we investigated the role of 3OC12-HSL and PON1 on *P. aeruginosa* virulence. We found that *P. aeruginosa* virulence in flies was dependent upon 3OC12-HSL. PON1 transgenic flies expressed enzymatically active PON1 and thereby exhibited arylesterase activity and resistance to organophosphate toxicity. Moreover, PON1 flies were protected from *P. aeruginosa* lethality, and protection was dependent on the lactonase activity of PON1. Our findings show that PON1 can interfere with quorum sensing in vivo and provide insight into what we believe is a novel role for PON1 in the innate immune response to quorum-sensing-dependent pathogens. These results raise intriguing possibilities about human-pathogen interactions, including potential roles for PON1 as a modifier gene and for PON1 protein as a regulator of normal bacterial flora, a link between infection/inflammation and cardiovascular disease, and a potential therapeutic modality.

## Introduction

The paraoxonase (PON) family in mammals is made up of 3 members: PON1, PON2, and PON3. PON-like proteins are evolutionarily conserved, including in *Caenorhabditis elegans*, but interestingly are absent in *Drosophila melanogaster*. PON1 is an approximately 39- to 45-kDa protein and was the first family member studied. It was originally described as an enzyme capable of degrading paraoxon and other organophosphates (1–3). Later, it was found that PONs also play an important role in lipid oxidation and atherosclerosis (4, 5). More recently, all 3 members of the PON family have been shown to also possess lactonase activity (6–10), and structure-reactivity studies suggest that PON's native enzyme activity is as a lactonase (11). Importantly, lactones are key quorum-sensing signaling molecules for a number of Gram-negative bacteria, including *Pseudomonas aeruginosa*.

*P. aeruginosa* is a major cause of clinically relevant infections in hospitalized patients, immunocompromised people, burn wound patients, and those with chronic lung disease, such as cystic fibrosis (CF). Quorum sensing is an interbacterial mode of communication accomplished through the coordinated production, secretion, and detection of chemical signals (quorum-sensing signals) that trigger expression of specific bacterial genes. The quorum-sensing

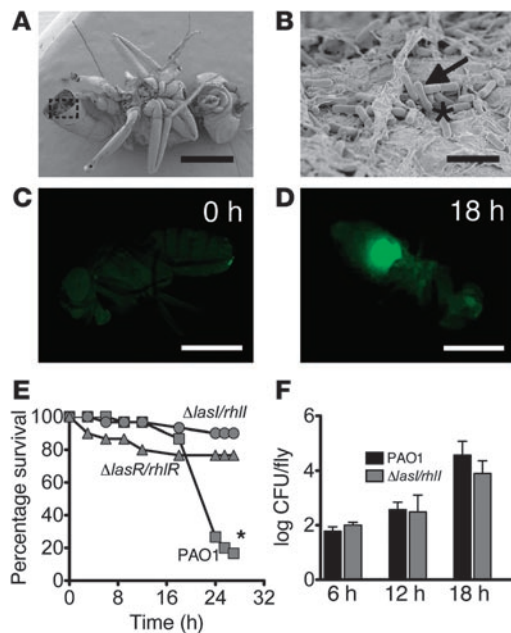
signals self produced by *P. aeruginosa* are in the form of small molecules, or autoinducers, termed acyl-homoserine lactones (acyl-HSLs) (12). *P. aeruginosa* uses acyl-HSL quorum-sensing molecules to regulate the expression of genes implicated in virulence and biofilm formation (13–15). Two hierarchically regulated acyl-HSL systems are present in *P. aeruginosa*: the *las* and *rhl* systems. LasI synthesizes *N*-3-oxododecanoyl HSL (3OC12-HSL), and LasR is the transcription factor that responds to 3OC12-HSL. RhlI synthesizes *N*-butanoyl HSL (C4-HSL), and RhlR is the transcription factor that responds to C4-HSL (12). Quorum-sensing-deficient *P. aeruginosa* is less virulent in animal models of burn wound infection, neonatal pneumonia, and acute/chronic pulmonary infections, suggesting an important role for quorum sensing in *P. aeruginosa* virulence (16–21). We (6, 9) and others (7, 8) have shown that all of the PONs can inactivate 3OC12-HSL. In addition, PON1 inhibits *P. aeruginosa* biofilm growth in an in vitro biofilm model (6).

These observations suggest the hypothesis that quorum-sensing molecules are important for *P. aeruginosa* virulence and that lactonases, specifically PONs, can protect the host from lethal *P. aeruginosa* infection. In order to test this hypothesis, an in vivo model of infection is required. However, this has been difficult to study in mammals since all 3 PONs are present on the same locus on chromosome 7, each PON can degrade 3OC12-HSL, and deletion of individual PON family members leads to compensation by the remaining members (6, 9). Therefore, we turned to other organisms and found that *D. melanogaster* does not express PON or a PON homolog. The fruit fly immune response has been extensively studied, and findings from this model organism have led to exciting discoveries of mammalian counterparts of *D. melanogaster*'s innate immune response (22, 23). We took what

**Nonstandard abbreviations used:** CF, cystic fibrosis; 3OC12-HSL, *N*-3-oxododecanoyl HSL; C4-HSL, *N*-butanoyl HSL; *da*, daughterless; DSM, demeton-S-methyl; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; HSL, homoserine lactone; PON, paraoxonase; ppm, parts per million; TBBL, 5-thiobutyl butyrolactone; UAS, upstream activation sequence.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

Quorum-sensing-dependent *P. aeruginosa* infection of *Drosophila*. (A and B) Scanning electron micrographs at low and high magnification of *Drosophila* at 24 hours following infection with wild-type *P. aeruginosa* (PAO1). (A) Low-magnification image shows a whole fly with an opening in the distal abdomen. Scale bar: 0.5 mm. (B) High-magnification image of boxed area on whole fly. Arrow indicates bacteria and asterisk denotes extracellular matrix. Scale bar: 2.5  $\mu$ m. (C and D) GFP signal (quorum-sensing activity) after infection of *Drosophila* with a quorum-sensing reporter strain of *P. aeruginosa* (under control of the *lasB* promoter). GFP signal at 0 hours (C) and 18 hours (D) following abdominal inoculation with PAO1. Scale bars: 0.5 mm. (E) Fly survival after infection with PAO1 and quorum-sensing-deficient strains of *P. aeruginosa*. *da-GAL4/+* flies were infected with PAO1 (squares),  $\Delta lasI/rhlI$  (circles), or  $\Delta lasR/rhlR$  (triangles) strains.  $n = 30$  flies per group for each experiment. \* $P < 0.001$ , comparing PAO1 versus  $\Delta lasI/rhlI$  and PAO1 versus  $\Delta lasR/rhlR$  fly survival; log-rank test. (F) *P. aeruginosa* bacterial counts after infection with PAO1 or  $\Delta lasI/rhlI$ . At 6, 12, and 18 hours following infection, flies were anesthetized, surface sterilized, and homogenized for performance of quantitative bacterial counts. Data are displayed as log<sub>10</sub> CFU/fly and represent the mean  $\pm$  SEM.  $n = 3$ –5 per time point with 10–20 flies per group per experiment.

we believe is a novel approach of expressing PON1 in *D. melanogaster* and testing for protection from *P. aeruginosa*-induced lethality. We chose to study PON1 as opposed to PON2 or PON3, since PON1 can degrade organophosphates and protection from organophosphate killing would confirm in vivo PON1 activity in transgenic *D. melanogaster*. By using quorum-sensing-deficient strains of *P. aeruginosa*, synthetic acyl-HSLs, and transgenic lactonase (PON1) expression in flies, we have found that 3OC12-HSL is required for *P. aeruginosa* virulence and PON1 protects flies from *P. aeruginosa*-induced lethality by disrupting quorum-sensing pathways.

**Results**

*P. aeruginosa* lethality in *D. melanogaster* is quorum-sensing dependent. For these studies, we infected *D. melanogaster* by pricking the fly’s abdomen with a needle previously dipped in a *P. aeruginosa* suspension (24, 25). In this model, *P. aeruginosa* is lethal to flies. Figure 1, A and B, shows low- and high-magnification scanning electron micrographs of a whole fly and abdomen, respectively, at approximately 24 hours following inoculation. *P. aeruginosa* infection is evidenced by the presence of rod-shaped bacteria encased in an extracellular matrix.

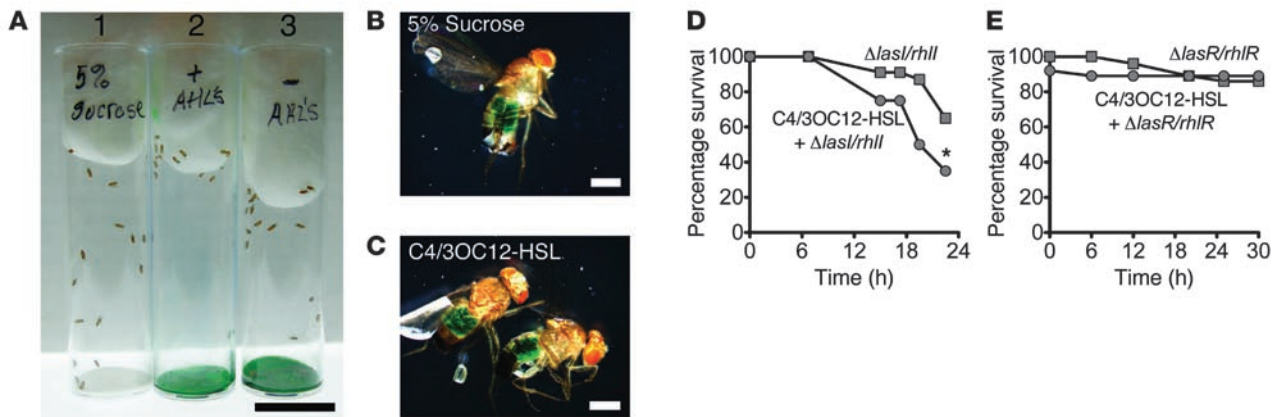
Our initial experiments were designed to test the hypothesis that quorum sensing occurs following *P. aeruginosa* infection of flies and that killing is dependent upon an intact quorum-sensing system. To test this hypothesis, we first inoculated flies with a quorum-sensing reporter strain of *P. aeruginosa* that expresses GFP under control of the *lasB* promoter (a quorum-sensing-regulated gene). Figure 1C shows that immediately following inoculation (time = 0 hours), there was no quorum-sensing activity. However, at 18 hours following infection, the fly abdomen was intensely green, confirming increased quorum-sensing activity (Figure 1D). To directly test whether quorum sensing was required for *P. aeruginosa* virulence in flies, we inoculated flies with (a) wild-type *P. aeruginosa* (PAO1); (b) strains deficient in 3OC12-HSL and C4-HSL production ( $\Delta lasI/rhlI$ ); or (c) strains deficient in the receptors required to respond to 3OC12-HSL and C4-HSL ( $\Delta lasR/rhlR$ ). Flies inoculated with medium alone had less than 10% mortality (data not shown). When flies were infected with PAO1, greater than 80% mortality occurred by 24 hours following infection

(Figure 1E). In contrast, flies infected with the  $\Delta lasI/rhlI$  or  $\Delta lasR/rhlR$  mutants showed increased survival. These results are consistent with our hypothesis and previous reports from others showing that quorum sensing is important for *P. aeruginosa* virulence in *D. melanogaster* (26–29). When flies were inoculated with a very high dose of  $\Delta lasI/rhlI$  ( $10^{11}$  CFU/ml), enhanced fly killing was observed, suggesting that this high bacterial load overwhelmed the fly’s host defense response (Supplemental Figure 1B; supplemental material available online with this article; doi:10.1172/JCI35147DS1).

One potential explanation for decreased killing by mutant strains could be that bacterial dissemination or growth is altered. We compared bacterial counts in flies at 6, 12, and 18 hours following infection with either PAO1 or  $\Delta lasI/rhlI$ . Bacterial counts were similar at all time points examined in flies infected with either PAO1 or the quorum-sensing-deficient strain  $\Delta lasI/rhlI$  (Figure 1F), suggesting that differences in bacterial replication are not responsible for the less virulent phenotype.

We next hypothesized that C4- and 3OC12-HSL feeding would restore virulence to  $\Delta lasI/rhlI$ -infected flies but not to  $\Delta lasR/rhlR$ -infected flies. Flies were fed 5% sucrose alone or containing C4- and 3OC12-HSL for 48 hours (Figure 2A), inoculated with the  $\Delta lasI/rhlI$  or  $\Delta lasR/rhlR$  mutants, and then maintained in the feeding assay for the remainder of the experiment. Acyl-HSLs did not alter food consumption rates in *D. melanogaster* (Figure 2, B and C). We found that, compared with flies fed 5% sucrose alone, flies fed C4- and 3OC12-HSL had decreased survival following  $\Delta lasI/rhlI$  infection (Figure 2D). In contrast, C4- and 3OC12-HSL feeding had no effect on fly survival following  $\Delta lasR/rhlR$  infection (Figure 2E). These data show that *P. aeruginosa* is virulent in *D. melanogaster*, virulence is dependent upon an intact quorum-sensing system, and exogenous acyl-HSLs can restore virulence to *P. aeruginosa* strains deficient in C4- and 3OC12-HSL production.

*PON1 expression and enzyme activity in PON1 transgenic flies.* To test whether the lactonase function of PON1 can protect flies from *P. aeruginosa* infection, PON1 transgenic *D. melanogaster* were constructed using the GAL4-upstream activation sequence (GAL4-UAS) system, as previously described (30). Five transformed PON1-express-

**Figure 2**

acyl-HSL feeding enhances virulence of quorum-sensing-deficient *P. aeruginosa* mutants. (A) Photomicrograph of fly-feeding vials with filter paper at the base containing either 5% sucrose alone (tubes 1 and 3) or 5% sucrose with C4- and 3OC12-HSL (tube 2). Filter paper in tubes 2 and 3 contained green food dye to confirm similar rates of food consumption between feeding groups and also consumption of acyl-HSLs. Scale bar: 2.5 cm. (B and C) Representative low-power images of *Drosophila* after 24 hours feeding on either 5% sucrose alone (B) or 5% sucrose containing C4- and 3OC12-HSL (containing green dye) (C). Note similar levels of green dye in the abdomen of flies consuming sucrose alone or sucrose containing acyl-HSLs. Scale bars: 0.25 mm. (D and E) Flies were fed 5% sucrose (squares) or 5% sucrose containing C4-HSL (5  $\mu$ M) and 3OC12-HSL (60  $\mu$ M) (circles). 48 hours later, flies were infected with  $\Delta lasI/rhlI$  (D) or  $\Delta lasR/rhlR$  (E) strains of *P. aeruginosa*. Fly survival was monitored over time.  $n = 30$  flies per group for each experiment. \* $P < 0.01$ , comparing survival between  $\Delta lasI/rhlI$  versus  $\Delta lasI/rhlI$  + C4- and 3OC12-HSL groups; log-rank test.

ing fly lines were created, and PON1 protein levels were determined by immunoblotting. While *da-GAL4/+* (*da*, daughterless) flies (control flies) did not express PON1, UAS-PON1/*da-GAL4* flies (PON1 transgenic flies) expressed PON1, with variable levels of PON1 protein expression observed (PON1R9c > PON1R31 > PON1R1  $\approx$  PON1R4 > PON1R19) (Figure 3A). Multiple PON1 bands with differing electrophoretic mobility were observed and are consistent with prior reports of PON1 undergoing *N*-linked core glycosylation (8, 31).

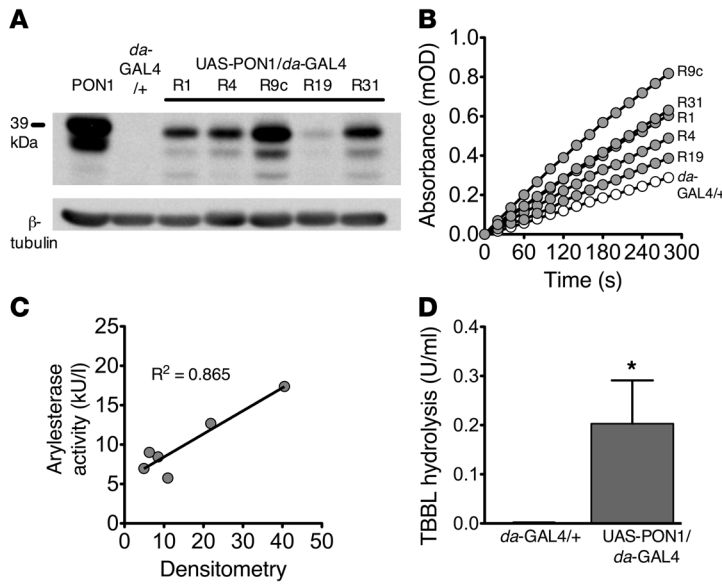
In order to confirm that PON1 was enzymatically active in transgenic flies, we measured arylesterase activity in *da-GAL4/+* and UAS-PON1/*da-GAL4* transgenic fly lysates. Consistent with presence of enzymatically active PON1, UAS-PON1/*da-GAL4* flies degraded more phenylacetate than *da-GAL4/+* flies (Figure 3B). Of interest, control flies showed low levels of arylesterase activity independent of PON1 expression. Others have reported carboxylesterase and acetyesterase activity in *D. melanogaster* but did not observe arylesterase activity (32). These contradictory findings may be due to differing approaches utilized to assay for arylesterase activity. We next compared arylesterase activity to PON1 protein expression and, as expected, found a strong correlation (Figure 3C). Finally, we also found increased arylesterase activity in hemolymph from PON1 flies compared with that in control flies (data not shown). For all subsequent *in vivo* experiments, we chose to use the PON1R9c line based upon its high level of PON1 protein expression and enzyme activity. This fly line will be referred to as UAS-PON1/*da-GAL4* in the remainder of experiments described.

We hypothesized that PON1 transgenic flies would degrade lactone molecules and, in subsequent *in vivo* experiments, disrupt *P. aeruginosa* quorum-sensing signaling pathways. Lactonase activity was quantified using a colorimetric assay that measures degradation of the synthetic lactone 5-thiobutyl butyrolactone (TBBL) (33). PON1R9c flies had significantly greater lactonase activity ( $0.203 \pm 0.088$  U/ml) compared with *da-GAL4/+* flies ( $0.001 \pm 0.001$  U/ml) (Figure 3D). Similar to what was shown in the arylesterase data, PON1 protein levels (in the 5 PON1 lines tested) correlated with lactonase activity ( $R^2$  (coefficient of determination) = 0.684, data not shown). These

*in vitro* arylesterase and lactonase studies demonstrate that PON1 is enzymatically active in transgenic flies.

*PON1 flies are protected from organophosphate toxicity.* To test for PON1 function *in vivo*, we next took advantage of PON1's promiscuous ability to hydrolyze organophosphate insecticides (1). Chlorpyrifos is a toxic organophosphate insecticide whose mechanism of action is acetylcholinesterase inhibition. Flies fed chlorpyrifos died in a dose-dependent manner (Figure 4A). At a dose of 50 parts per million (ppm), 100% of *da-GAL4/+* flies died by 4 hours after initiation of chlorpyrifos feeding. In contrast and as expected from the biochemical data, UAS-PON1/*da-GAL4* flies were markedly protected from chlorpyrifos-induced toxicity (Figure 4B). PON1 has a number of enzymatic activities, and protection from organophosphate toxicity could represent a nonspecific effect. Therefore, we asked whether PON1 transgenic flies would be protected from demeton-S-methyl (DSM) exposure. DSM is another toxic organophosphate that is not metabolized by PON1 (34). Both *da-GAL4/+* and UAS-PON1/*da-GAL4* flies died at a similar rate following DSM treatment (Figure 4C). These data show that PON1 protects *D. melanogaster* from chlorpyrifos lethality and that this protection does not represent a general resistance to toxic compounds conferred by PON1.

*PON1 flies are protected from P. aeruginosa-induced lethality.* Based upon the important role of 3OC12-HSL for *P. aeruginosa* virulence and the lactonase activity of PON1-expressing flies, we hypothesized that PON1 transgenic *D. melanogaster* would be protected from *P. aeruginosa*-induced lethality. As shown before (Figure 1D), wild-type *P. aeruginosa* (PAO1) killed the majority of control or *da-GAL4/+* flies. However, Figure 5A shows that UAS-PON1/*da-GAL4* flies were protected from PAO1 killing. Importantly, we also noted that this protection occurred to a degree similar to that seen when control flies were infected with  $\Delta lasI/rhlI$ . By 18 hours following infection,  $53\% \pm 13\%$  of *da-GAL4/+* flies were still alive compared with  $87\% \pm 4\%$  of UAS-PON1/*da-GAL4* flies ( $n = 5$ ;  $P < 0.05$ ). Comparable levels of bacteria were cultured from *da-GAL4/+* and UAS-PON1/*da-GAL4* flies at 6, 12, and 18 hours following infection



**Figure 3** PON1 expression and activity in PON1 transgenic *Drosophila*. **(A)** Western blot analysis for PON1 in *da-GAL4/+* (control) and UAS-PON1/*da-GAL4* flies. PON1 flies were constructed using the GAL4-UAS binary system under control of the *da* promoter driving ubiquitous PON1 expression. Fly heads were obtained from 1- to 3-day-old flies, homogenized, and used for immunoblotting with PON1 antibody. Lysates from CHO cells infected with an adenovirus-expressing human PON1 served as the positive control. All PON1 fly lines obtained were tested for PON1 protein.  $\beta$ -tubulin was used as a loading control. **(B)** Arylesterase activity was tested in fly homogenates from *da-GAL4/+* and UAS-PON1/*da-GAL4* transgenic fly lines by measuring phenylacetate degradation. mOD, optical density  $\times 10^{-3}$ . **(C)** Correlation of arylesterase activity and PON1 protein expression in control and PON1 transgenic flies. Densitometry analysis of PON1 and  $\beta$ -tubulin immunoreactive bands was performed, and data are expressed in relative units. **(D)** Lactonase activity in control and PON1 transgenic flies. Whole-fly lysates were combined with the synthetic lactone TBBL (0.25 mM), and lactonase activity (thiol moiety release) was monitored with DTNB (0.5 mM) at an absorbance of 412 nm. Data represent the mean  $\pm$  SEM with  $n = 3-4$  per group. \* $P < 0.01$ , lactonase activity between *da-GAL4/+* and UAS-PON1/*da-GAL4* flies; Student's *t* test.

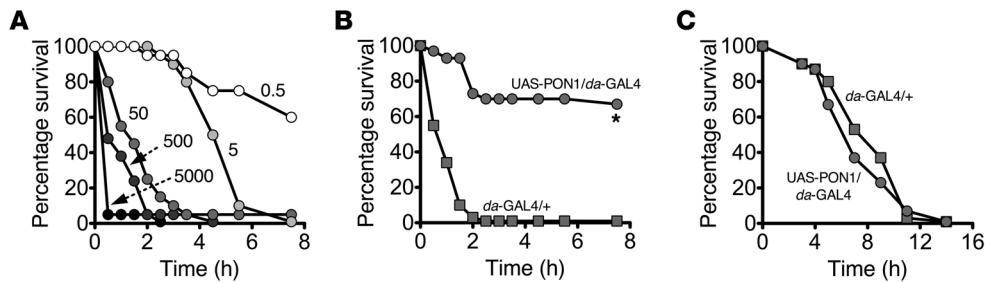
(Figure 5B). Finally, UAS-PON1/*da-GAL4* flies were also protected from high-dose ( $10^{11}$  CFU/ml) PAO1 challenge (Supplemental Figure 2A). These results indicate that PON1 expression protects flies from *P. aeruginosa* independent of bacterial growth, suggesting modulation of virulence factors. To test whether PON1 expression would select for quorum-sensing mutants, we cocultured wild-type PAO1 and  $\Delta lasI/rhlI$  in the presence or absence of PON1 (Supplemental Methods). Over 18 hours, there was no enrichment for either *P. aeruginosa* strain (Supplemental Figure 3, A and B).

When flies were infected with 2 clinical isolates of *P. aeruginosa* (PA-7JJA2 and PA-7DVM2) from CF patients, we also observed improved survival in UAS-PON1/*da-GAL4* flies (Figure 5C), demonstrating that protection is not only limited to infection with PAO1. In order to determine whether these clinical isolates were producing levels of acyl-HSLs comparable to those seen with PAO1, we used the acyl-HSL detector strain *Agrobacterium tumefaciens* NTL4. Figure 5D shows equivalent levels of acyl-HSL activity in PAO1, PA-7JJA2, and PA-7DVM2 strains, with minimal activity observed in the  $\Delta lasI/rhlI$  isolate. To determine whether PON1 could protect flies from another bacterial species that also uses acyl-HSL-dependent quorum sensing, we next infected *da-GAL4/+* and UAS-PON1/*da-GAL4*

flies with *Serratia marcescens*. Similar to our results following *P. aeruginosa* infection, we also observed that PON1 transgenic flies were protected from *S. marcescens* lethality (Figure 5E). Finally, we infected flies with the SH1000 strain of *Staphylococcus aureus* (no acyl-HSL production) and found similar fly death in *da-GAL4/+* and UAS-PON1/*da-GAL4* flies (Figure 5F). These results suggest that PON1 protection is specific and may occur against bacteria that utilize acyl-HSLs to regulate virulence factor production.

PON1 protection from *P. aeruginosa*-induced lethality is dependent upon 3OC12-HSL inactivation. As the *las* and *rhl* systems are hierarchically regulated, with increasing concentrations of 3OC12-HSL leading to virulence gene activation and C4-HSL production, we hypothesized that 3OC12-HSL alone would be sufficient for *P. aeruginosa*-induced lethality. To directly test this hypothesis, *da-GAL4/+* flies were infected with a strain of *P. aeruginosa* unable to produce 3OC12-HSL ( $\Delta lasI$  mutant). Similar to our findings with the  $\Delta lasI/rhlI$  and  $\Delta lasR/rhlR$  mutants,  $\Delta lasI$  killed fewer control flies than wild-type PAO1 (Figure 6A). Similarly, the  $\Delta lasI$  strain also failed to kill PON1-expressing flies (Figure 6B). Having established the importance of 3OC12-HSL in *P. aeruginosa* virulence, we next hypothesized that exogenous 3OC12-HSL would enhance  $\Delta lasI$  virulence in *da-GAL4/+* flies but degradation of exogenous 3OC12-HSL by UAS-PON1/*da-GAL4* flies would prevent restoration of  $\Delta lasI$  virulence. As predicted, feeding 3OC12-HSL to *da-GAL4/+* flies but not UAS-PON1/*da-GAL4* flies augmented  $\Delta lasI$ -induced lethality (Figure 6, A and B). These results further emphasize the importance of 3OC12-HSL in quorum-sensing control of *P. aeruginosa*'s virulence. Moreover, PON1 degradation of either endogenous or exogenous 3OC12-HSL can decrease mortality from *P. aeruginosa* infection.

PON1 flies disrupt *P. aeruginosa* quorum-sensing signaling. Finally, we began to investigate a possible mechanism or mechanisms for PON1's protection by studying the effects of PON1 expression on regulation of quorum-sensing-controlled genes. For these studies, we used 2 complementary approaches. First, we infected flies with a quorum-sensing reporter strain of *P. aeruginosa* (PAO1-qsc102-lacZ) that expresses  $\beta$ -galactosidase under control of qsc102, which responds primarily to 3OC12-HSL (13). Following infection, *da-GAL4/+* flies had significant X-gal staining in their abdomen, but this signal was less pronounced in UAS-PON1/*da-GAL4* flies (Figure 7A). UAS-PON1/*da-GAL4*-infected flies scored  $3.8 \pm 0.8$  compared with  $6.2 \pm 0.7$  in *da-GAL4/+*-infected flies ( $P < 0.05$ ), according to a visual analog scale for X-gal staining, and *lacZ* expression (quantified by RT-PCR) was decreased following *P. aeruginosa* infection in UAS-PON1/*da-GAL4* compared with *da-GAL4/+* flies ( $P < 0.05$ ) (Figure 7B). Second, we measured the mRNA levels of several quorum-sensing regulated genes that are important for *P. aeruginosa* virulence (*lasA*, *lasB*, *toxA*, and *aprA*). Compared with *da-GAL4/+* flies, UAS-PON1/*da-GAL4* flies had lower expression levels for 3 of the 4 genes tested (*lasA*, *lasB*, and *aprA*;  $P < 0.05$  for *lasB* and *aprA*), with greater than 50% reduction observed for *aprA* after PAO1 infection (Figure 7B). As a control, we found that the level of a non-quorum-sensing controlled gene (*polA*) was similar between *da-GAL4/+* and UAS-PON1/*da-GAL4* flies after infection (Figure 7B). Finally, since *P. aeruginosa* has other non-acyl-HSL-dependent quorum-sensing systems, PON1 could cause upregulation of these systems. As expected, we found no difference in *pqsA* expression, an upstream regulator of



**Figure 4**

PON1 flies are protected from chlorpyrifos toxicity. (A) *da-GAL4/+* flies were fed 0.5, 5, 50, 500, or 5000 ppm chlorpyrifos in 5% sucrose. Fly survival was monitored over time. (B and C) *da-GAL4/+* (squares) or UAS-PON1/*da-GAL4* (circles) flies were fed chlorpyrifos (50 ppm in 5% sucrose) (B) or DSM (150 ppm in 5% sucrose) (C), and survival was followed over time.  $n = 30$  flies per group for each experiment.  $*P < 0.001$ , comparing survival between *da-GAL4/+* and UAS-PON1/*da-GAL4* flies; log-rank test.

*Pseudomonas* quinolone signal (PQS) production, between *da-GAL4/+* and UAS-PON1/*da-GAL4* flies following *P. aeruginosa* infection (data not shown). In summary, these findings show that PON1 protection from *P. aeruginosa* infection is dependent upon 3OC12-HSL and suggest disruption of quorum-sensing-controlled signals (i.e., virulence factors) as a possible mechanism.

**Discussion**

Our results from studies with quorum-sensing-deficient strains of *P. aeruginosa* and synthetic quorum-sensing molecules, specifically 3OC12-HSL, reveal that quorum sensing is required for *P. aeruginosa* virulence in vivo. Moreover, our work is the first, to our knowledge, to demonstrate that the in vivo expression of a lactonase, PON1, is able to disrupt quorum-sensing pathways and protect from *P. aeruginosa*-induced virulence. These findings are important because they allow us to better understand a possible role for PONs in the innate immune response to quorum-sensing-dependent bacterial pathogens.

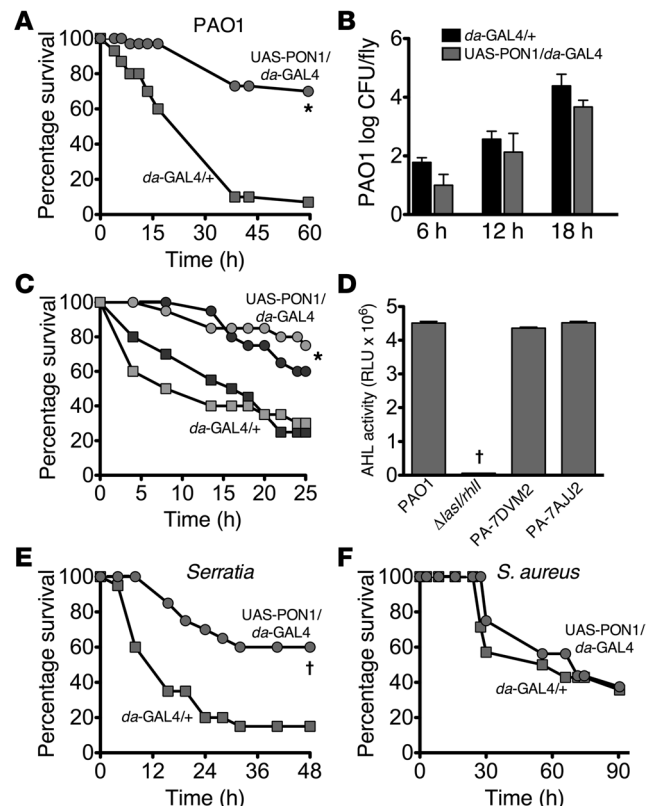
Some of the original descriptions of quorum sensing and bacterial function focused on control of bioluminescence in the marine organism *Vibrio fischeri* (35, 36). Since then, many bacterial pathogens

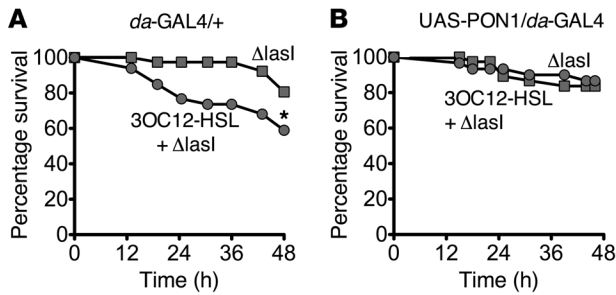
have been found to use quorum sensing, with the *P. aeruginosa las-rhl* system being one of the most widely studied. The earliest report of quorum sensing in *P. aeruginosa* focused on 3OC12-HSL's (*Pseudomonas* autoinducer or PAI-1) control of elastase production (37). Subsequent studies have shown that quorum sensing regulates a large number of *P. aeruginosa* genes, specifically those involved in virulence, pathogenesis, and biofilm formation (12).

The most convincing data demonstrating a link between quorum sensing and *P. aeruginosa* virulence come from a number of animal models of infection, including burn wound, neonatal pneumonia, acute and chronic pneumonia, and ocular keratitis (16–21, 38). These studies have primarily been performed with *P. aeruginosa* laboratory strains deficient in 1 or more of the key components of the *las* and *rhl* systems, including *lasI*, *lasR*, *rhlI*, and/or *rhlR*. In general,

**Figure 5**

PON1 flies are protected from *P. aeruginosa* lethality. (A) *da-GAL4/+* (squares) and UAS-PON1/*da-GAL4* (circles) flies were infected with *P. aeruginosa*, and fly survival was monitored over time.  $n = 30$  flies per group for each experiment.  $*P < 0.01$ , comparing *da-GAL4/+* and UAS-PON1/*da-GAL4* survival; log-rank test. (B) *P. aeruginosa* bacterial counts following infection in *da-GAL4/+* and UAS-PON1/*da-GAL4* flies. Bacterial quantification was performed as described for Figure 1. Data are displayed as log 10 CFU/fly and represent the mean  $\pm$  SEM.  $n = 3–5$  per time point with 10–20 flies per group per experiment. (C) PON1 transgenic flies are protected from lethality following infection with clinical isolates of *P. aeruginosa*. Light-shaded symbols, PA-7JJA2; and dark-shaded symbols, PA-7DVM2.  $*P < 0.01$ , comparing *da-GAL4/+* and UAS-PON1/*da-GAL4* fly survival following either PA-7JJA2 or PA-7DVM2 infection; log-rank test. (D) acyl-HSL quorum-sensing activity by *P. aeruginosa* isolates. Supernatants from overnight bacterial cultures were incubated with the acyl-HSL detector strain *A. tumefaciens* NTL4, and  $\beta$ -galactosidase activity was determined. Data are mean  $\pm$  SEM and  $n = 3$  per bacterial strain.  $\dagger P < 0.001$ , compared with PAO1. (E–F) *da-GAL4/+* and UAS-PON1/*da-GAL4* flies were infected with either (E) *S. marcescens* or (F) *S. aureus* (SH1000), and survival was followed over time.  $n = 30$  flies per group for each experiment.  $\dagger P < 0.001$ , comparing survival between *da-GAL4/+* and UAS-PON1/*da-GAL4* flies following *S. marcescens* infection; log-rank test.





**Figure 6**  
3OC12-HSL fails to enhance *P. aeruginosa* virulence in PON1 flies. (A) *da-GAL4/+* or (B) *UAS-PON1/da-GAL4* flies were fed 5% sucrose (squares) or 5% sucrose containing 3OC12-HSL (60 μM) (circles). 48 hours later, flies were infected with the  $\Delta lasI$  mutant strain of *P. aeruginosa* (deficient in 3OC12-HSL production).  $n = 30$  flies per group for each experiment. Fly survival was monitored over time. \* $P < 0.05$ , comparing  $\Delta lasI$  and 3OC12-HSL +  $\Delta lasI$  survival in *da-GAL4/+* flies; log-rank test.

these studies have found that, compared with wild-type *P. aeruginosa*, quorum-sensing-deficient strains result in minimal inflammation and tissue destruction, decreased bacterial dissemination, and less persistent infection. In some studies, mutant strains were complemented with plasmids expressing the absent quorum-sensing components and enhanced virulence was observed (17, 18, 38). Here, we report findings similar to those described above, but in *D. melanogaster*, through the use of quorum-sensing-deficient mutant strains of *P. aeruginosa*.

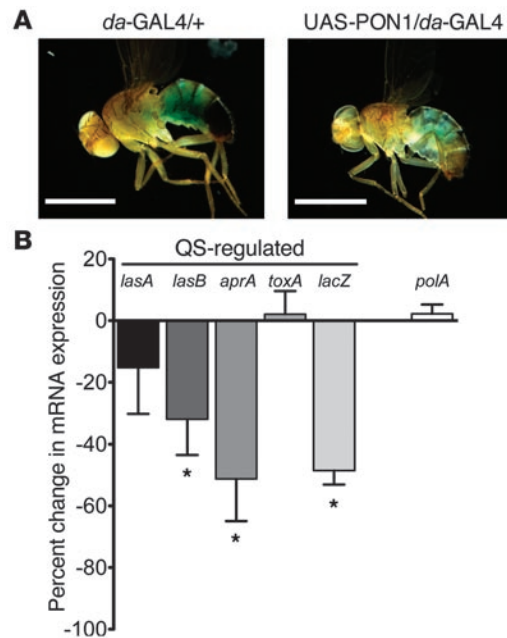
Our data show that C4- and 3OC12-HSL can complement the virulence of *P. aeruginosa* in vivo, in agreement with previous work. To the best of our knowledge, this is the first in vivo work to test the direct effect of 3OC12-HSL on bacterial virulence. Moreover, we found that the 3OC12-HSL-deficient strain of *P. aeruginosa*,  $\Delta lasI$ , resulted in decreased lethality in flies and this was complemented by 3OC12-HSL feeding. This is consistent with hierarchical control of 3OC12-HSL and C4-HSL (39, 40). These findings are important because we and others have found that C4-HSL is a poor substrate for PON (6, 7). Taken together, these data convincingly show that 3OC12-HSL is required for *P. aeruginosa* virulence in vivo.

Certain naturally occurring lactonases can degrade or inactivate quorum-sensing signals. Expression of the lactonase *aiiA*, from *Bacillus* sp. bacteria, has been shown to decrease *Erwinia carotovora*-induced soft rot in plants, a quorum-sensing-dependent process (41–43). Several recent lines of evidence from in vitro studies have led to the hypothesis that PON's lactonase activity may represent an important host defense mechanism against certain bacterial pathogens.

To test this hypothesis in vivo, we required an infection model in a whole organism and the ability to directly study PON's role in host defense. Mice have all 3 PONs, making results from single PON-knockout models difficult to interpret and a triple-knockout mouse difficult to construct. Moreover, as explained before, acyl-HSL complementation is not easily possible in murine models. Because of the absence of PON in *D. melanogaster*, well-characterized models of infection in insects, and genetic tools available for *D. melanogaster* research, we took the approach of studying *P. aeruginosa* virulence in PON1 transgenic flies. In contrast with the typical paradigm in which immune mechanisms, such as Toll, Imd, and JAK/STAT pathways (23, 44) have been first identified

in *D. melanogaster* and subsequently studied in humans, few if any studies have first used *D. melanogaster* to investigate putative components of the immune response in humans.

Our data show that PON1 transgenic flies are protected from lethality due to *P. aeruginosa* infection. In these studies, we began to investigate potential mechanisms for this effect. Although *P. aeruginosa* bacterial counts tended to be slightly lower following infection of PON1 flies compared with control flies, this small difference was neither statistically significant nor likely to be responsible for the dramatic increase in PON1 fly survival. Whether PON has a direct antibacterial effect on *P. aeruginosa* growth remains to be tested. Based upon the in vitro lactonase activity of PON1 flies and results from in vivo experiments with the  $\Delta lasI$  mutant and exogenous 3OC12-HSL, PON1 flies appear to be protected secondary to degradation of 3OC12-HSL and disruption of downstream quorum-sensing pathways. To further address this possibility, we examined regulation of quorum-sensing-controlled genes following *P. aeruginosa* infection in control and PON1 flies. Of the many genes regulated by quorum sensing in *P. aeruginosa* (13–15), 4 of the most relevant virulence genes reported to be regulated by acyl-HSLs were tested. Expression of *lasA*, *lasB*, and *aprA* was lower in PON1 flies. Interestingly, *aprA* encodes an alkaline protease, which was recently shown to be required for *P. entomophila* virulence in flies (45). While we did not test to deter-



**Figure 7**  
Quorum-sensing-dependent virulence gene activation is suppressed in PON1 flies. (A) *da-GAL4/+* and *UAS-PON1/da-GAL4* flies were infected with a quorum-sensing reporter strain of *P. aeruginosa*, PAO-1qsc102-lacZ. 18 hours later, X-gal staining was performed. Shown are representative images of *da-GAL4/+* and *UAS-PON1/da-GAL4* flies following infection. Scale bars: 0.5 mm. (B) Quorum-sensing-regulated (QS-regulated) and nonregulated genes. *da-GAL4/+* and *UAS-PON1/da-GAL4* flies were infected with *P. aeruginosa*, and 18 hours later, flies were sacrificed for mRNA analysis with RT-PCR. Data are expressed as percentage change ( $\pm$  SEM) in mRNA levels between *da-GAL4/+* and *UAS-PON1/da-GAL4* flies following *P. aeruginosa* infection. \* $P < 0.05$  for mRNA expression between *da-GAL4/+* and *UAS-PON1/da-GAL4* flies following *P. aeruginosa* infection.



mine whether 3OC12-HSL or PON1 directly affect the fly's innate immune response to *P. aeruginosa*, our data in which 3OC12-HSL feeding restored virulence to  $\Delta lasI/rhlI$  but not  $\Delta lasR/rhlR$  suggest this is not the case.

There are limited and conflicting data on the role of quorum sensing in human diseases. Most studies have focused on chronic infections such as those in CF and burn wounds. Singh et al. suggested a role of quorum sensing in biofilm formation in CF patients and found a characteristic acyl-HSL signature from sputum samples (46). 3OC12-HSL activity in CF sputum samples has subsequently been measured by others (47, 48). In contrast, genomic analysis of CF *P. aeruginosa* strains has shown that over time, quorum-sensing genes are lost (49). Similarly, several groups have found quorum-sensing mutants in isolates from mechanically ventilated patients and from respiratory tract, urinary tract, and wound infections (50, 51). These studies have ignored the host factors that may regulate acyl-HSLs. We speculate that PON may play an important role in setting up these chronic infections by degrading 3OC12-HSL and decreasing virulence, thus avoiding catastrophic outcomes. Interestingly, a recent paper showed social cheating by *P. aeruginosa* quorum-sensing mutants. Isolates deficient in *lasR* emerged over time and took advantage of members of the bacterial population, thereby decreasing the metabolic burden imposed by quorum sensing (52).

The role of PONs in the host defense response to infection in humans remains to be fully determined. Our findings may be applicable to human *P. aeruginosa* infection, and the relative contribution of different PONs may be different in sepsis, burn wound infections, pulmonary infections, and other human *P. aeruginosa* infections. Since PON1 is primarily localized in the bloodstream, we postulate that PON1 would be most important for bloodstream infections. Alternatively, PON1 may reach tissue sites of infection, similar to other serum proteins, by leakage out of the vasculature (as might occur in burn wound infections). We have previously shown that PON2 is the predominant isoform of PON in human airway epithelial cells and that loss of PON2 (in murine airway epithelial cells from PON2-knockout mice) impairs 3OC12-HSL-inactivating activity (9). PON1-knockout mice demonstrated slightly improved survival following an intraperitoneal challenge with *P. aeruginosa*; however, absence of PON1 led to increased PON2 and PON3 levels, making these data difficult to interpret (6). Several polymorphisms have been described for PON family members, and natural variation in PON activity as a genetic modifier may be responsible for differing rates of pulmonary decline in CF patients with a similar CFTR genotype, development of ventilator-associated pneumonia or burn wound infections by *P. aeruginosa*, or chronic *P. aeruginosa* colonization in idiopathic bronchiectasis.

A limitation of this work is that our studies were performed using *D. melanogaster* and an injury model of infection. Whether PON will confer protection from *P. aeruginosa* infection in other model organisms, such as mice, or is important in humans for host defense against infection remains to be determined. However, in humans, decreased PON levels and PON single-nucleotide polymorphisms have been linked to numerous diseases, including cerebrovascular disease, atherosclerosis, coronary heart disease, inflammatory bowel disease, Behçet disease, sporadic amyotrophic lateral sclerosis, and *Helicobacter pylori* infection (53–57). The basis for these linkages is thought to be disruptions in the cellular oxidant-antioxidant balance. We speculate that some of these disease associations may be secondary to PON's unique ability to disrupt quorum-sensing pathways. Yet-to-be-identified microbial pathogens or bacteria that are not culturable with traditional microbiology techniques may use lactone-based molecules

for their disease pathogenesis. Some of these microorganisms might play an unrecognized role in these clinically important diseases. For example, infectious etiologies have been proposed for a number of illnesses not originally thought to be dependent upon microbial pathogens, including a proposed link between inflammation/infection (*Chlamydia pneumoniae* and *H. pylori* infection) and coronary heart disease (58, 59). Since acyl-HSL-dependent quorum-sensing pathways are very prevalent in Gram-negative bacteria (60), we speculate that PON may be important in the pathogenesis of a number of human illnesses with an underlying infectious etiology.

Taken together, our results using genetically modified strains of *P. aeruginosa* (deficient in key quorum-sensing components), acyl-HSL complementation, and PON1 transgenic *D. melanogaster* prove the importance of 3OC12-HSL for *P. aeruginosa* virulence. We speculate that genetic variability in PONs may be linked to the pathogenesis of *P. aeruginosa* infection. Novel therapeutic interventions aimed at directly regulating quorum sensing or PON activity may show promise for treatment. Finally, these results provide insight into what we believe is a newly described function of PON and suggest how this lactonase might be important for control of *P. aeruginosa* pathogenesis.

## Methods

**Fly and bacterial strains.** All flies were maintained and cultured on standard yeast-agar-sucrose-cornmeal medium. Flies were bred and tested at 25°C. The binary GAL4-UAS system and *da* promoter were used for the ubiquitous transgenic expression of PON1 (30). Human PON1R cDNA was cloned into the pUAST plasmid and subsequently injected into *D. melanogaster* embryos ( $w^{1118}$ ) using standard techniques (Rainbow Transgenic Flies Inc.). Five transformants were used for the described studies.  $w^{1118}; da-GAL4$  virgin females were crossed to  $w^{1118}; UAS-PON1$  males to obtain the experimental lines  $w^{1118}; UAS-PON1/da-GAL4$ , which is referred to as UAS-PON1/*da-GAL4*. Control flies ( $w^{1118}; da-GAL4/+$ ) were obtained by crossing  $w^{1118}$  males with  $w^{1118}; da-GAL4$  virgin females and are denoted as *da-GAL4/+*.

For bacterial infection studies, the wild-type strain of *P. aeruginosa*, PAO1, or strains deficient in various components of the quorum-sensing system were used:  $\Delta lasI/rhlI$  mutant (no C4- or 3OC12-HSL production),  $\Delta lasI$  mutant (no 3OC12-HSL production), and  $\Delta lasR/rhlR$  mutant (lacks the transcriptional activators for C4- and 3OC12-HSL signaling). PA-7JJA2 and PA-7DVM2 are clinical isolates of *P. aeruginosa* that were obtained from 2 pediatric CF patients at the time of initial colonization by *P. aeruginosa* (kindly provided by Tim Starner, University of Iowa). All bacteria were grown overnight while being shaken at 37°C in Luria Bertani broth. Two quorum-sensing-reporter strains of *P. aeruginosa* were used, including 1 expressing lacZ under control of the *qsc102* 3OC12-HSL-responsive promoter (PAO1-*qsc102-lacZ*) (cultured in the presence of 100 µg/ml gentamicin) and 1 expressing GFP under control of *lasB*. *S. marcescens* (13880; ATCC) was grown at 26°C in Luria Bertani media. *S. aureus* (SH1000; kindly provided by Alex Horswill, University of Iowa) was grown at 37°C in brain heart infusion broth. Flies were inoculated with stationary-phase bacteria adjusted to an OD<sub>600</sub> of 3 or, for high-dose experiments with PAO1 and  $\Delta lasI/rhlI$ , an OD<sub>600</sub> of 73.

***D. melanogaster* infection assays.** Fly infection was performed according to previously described methods (25, 26, 61). Under CO<sub>2</sub> anesthesia, 1- to 3-day-old male flies were infected by pricking the abdomen with a 10-µm needle (Ernest F. Fullam Inc.) previously dipped in a bacterial suspension (OD<sub>600</sub> = 3) of the specific bacterial strain required for an experiment. Flies were returned to standard medium and maintained at 25°C. A minimum of 30 flies per experimental group (10 flies/vial × 3 vials) was used for each experiment. Using this method, we were able to reproducibly inoculate flies with a similar number of bacteria. We found a linear relationship between the CFU/ml of the





bacterial suspension and the number of bacterial organisms adherent to the needle as well as delivered into the flies (Supplemental Figure 1A). At 0 hours following fly inoculation with an  $OD_{600}$  of 3, we recovered  $2.55 \pm 0.14$  log CFU/fly, similar to others (25, 26, 61). Fly survival was determined by recording the number of flies alive at various time intervals following infection and plotted using survival curves. Reproducibility of all fly survival curves was confirmed in 3 or more independent experiments.

**Bacterial quantification.** At various time points following infection, flies were anesthetized with  $CO_2$  and then placed on ice. To remove surface-adherent bacteria, flies were submerged in 70% ethanol for 5 seconds and then rinsed 4 times in PBS. Subsequently, flies (5 flies in 100  $\mu$ l of PBS) were homogenized with a pestle. Quantitative bacterial count studies using serial dilutions of homogenized flies were performed with standard plating methods.

**acyl-HSL reporter assay.** acyl-HSL activity was determined in different *P. aeruginosa* isolates with the acyl-HSL detector strain *A. tumefaciens* NTL4 (pZLR4) (62). Supernatants from overnight cultures of test strains were incubated with an overnight culture of *A. tumefaciens* NTL4, and 4 hours later,  $\beta$ -galactosidase was determined.

**TBBL assay.** Activity measurements were performed in a 96-well plate using an automated microplate reader (Bio-Tek; optical length  $\sim 0.5$  cm) as previously described (33). Lactonase activity was measured with 5  $\mu$ l of cleared fly lysate in activity buffer (50 mM Tris, pH 8.0, 1 mM  $CaCl_2$ ) containing 0.25 mM TBBL and 0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) by monitoring the absorbance at 412 nm in a final volume of 200  $\mu$ l ( $\epsilon_{412}$  (extinction coefficient at 412 nm) = 7,000 OD/M). DTNB was diluted from a 100-mM stock in DMSO. TBBL was diluted from a 250-mM stock in acetonitrile. All reaction mixtures contained a final 1% acetonitrile concentration. Rates of spontaneous hydrolysis of TBBL in buffer were subtracted from all measurements. Activities were expressed as U/ml (1 unit = 1  $\mu$ mol of TBBL hydrolyzed per minute per 1 ml of undiluted serum).

**Western immunoblotting.** PON1 expression was confirmed by immunoblotting for PON1 in fly heads. Flies were anesthetized with  $CO_2$  anesthesia and then frozen in liquid nitrogen. Fly heads were separated from the thorax/abdomen after briefly vortexing frozen flies. Ten fly heads were placed in 100  $\mu$ l of lysis buffer (50 mM Tris-HCl, pH 7.5, 138 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin) with 1% Triton X-100 and homogenized with a pestle. Sonication was performed for 10 seconds, and debris was cleared by centrifugation at 4,500 g for 30 seconds at 4°C. The fly lysates were then combined with loading buffer and separated by SDS-PAGE. Protein was then transferred to PVDF membranes (Immobilon-P; Millipore). PVDF membranes were blocked for 1 hour at room temperature in 5% instant nonfat milk. Membranes were then incubated with monoclonal, anti-human PON1 at 1:1000 (Abcam) for 2 hours at room temperature and then washed 3 times in 1 $\times$  TTBS (137 mM NaCl, 2.7 mM KCl, 2.5 mM Tris, 0.05% Tween 20). Secondary antibody, conjugated to horseradish peroxidase (Amersham), was then incubated with the membrane at 1:10,000 for 1 hour. Following 3 washes with 1 $\times$  TTBS, immunoreactive bands were detected with SuperSignal solution (Thermo Scientific) and exposed to film.

**Organophosphate toxicity assays.** Chlorpyrifos (Sigma-Aldrich) and DSM (Chem Service) toxicity assays were adapted from the protocol described by Phillips et al. (63). Thirty 1- to 3-day-old males were placed in standard fly vials that had a Whatman 1 filter disc (GE Healthcare) (saturated with either chlorpyrifos or DSM, all in 5% sucrose) on the bottom surface. Flies were maintained at room temperature, and fly survival was monitored over time. To ensure that similar levels of chemicals were consumed between control and PON1 flies, initial experiments were performed using green food dye in the chemical solutions. Fly abdomens from *da-GAL4/+* and *UAS-PON1/da-GAL4* flies displayed similar levels of green coloring following ingestion of all toxins (data not shown).

**Arylesterase assay.** Arylesterase activity was determined by measuring the breakdown of phenylacetate into the colorimetric substrate phenol.

*D. melanogaster* lysates were prepared by placing 10 flies in 200  $\mu$ l of lysate buffer (20 mM Tris-HCl, pH 8.0; 1 mM  $CaCl_2$ ), homogenizing, sonicating for 20 seconds, and then clearing the lysates with a centrifugation step (4500 g  $\times$  5 m, 4°C). Supernatants were combined with phenylacetate (4 mM final concentration), and phenol formation was measured at 270 nm (25°C) using a 96-well plate reader. Similar measurements were made on hemolymph that was isolated by poking fly larvae with glass micropipettes.

***D. melanogaster*-feeding assay.** In some experiments, flies were first fed acyl-HSLs prior to bacterial infection. 5% sucrose alone or containing 3OC12-HSL (60  $\mu$ M) (RTI International) and C4-HSL (5  $\mu$ M) (Sigma-Aldrich) was added to filter paper (as described above). Flies were allowed to feed on these mixtures for 48 hours and then subsequently inoculated with various strains of *P. aeruginosa*.

**$\beta$ -galactosidase staining for PAO1-*qsc102-lacZ*.** Following bacterial infection, whole flies were placed in 30% Ficoll and freeze-thawed in liquid nitrogen twice, followed by washing in PBS.  $\beta$ -galactosidase-staining solution was added to the flies and incubated for 6 hours at 37°C. Flies were washed twice with PBS and then mounted on slides in 50% glycerol (in PBS). Quantitative analysis of X-gal staining was performed using a visual analog scale by having 5 blinded reviewers rate staining intensity per fly on a scale of 1 to 10 (1 = no staining and 10 = maximal staining).

**Quantitative real-time RT-PCR.** Total RNA was isolated with RNAprotect Bacteria Reagent (QIAGEN) and with the RNeasy Mini Kit (QIAGEN), DNase I treated, and further purified using the RNeasy Micro Kit (QIAGEN). Subsequently, 0.6  $\mu$ g of total RNA was reverse transcribed with random hexamers (Applied Biosystems) by SuperScript III (Invitrogen). Real-time PCR was performed with primers specific for the gene of interest and the control 16S transcripts (primers are listed in Supplemental Table 1). The amounts of PCR products were measured using SYBR Green and the ABI PRISM 7500 Sequence Detection System (Applied Biosystems).

**Scanning electron microscopy.** Samples were prepared for scanning electron microscopy imaging using routine methods. In brief, flies were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, post fixed in 1% osmium tetroxide, and dehydrated in a graded series of ethanol. The flies were transitioned to hexamethyldisilazane for 30 minutes and then air dried overnight. The samples were then mounted on aluminum stubs, sputter coated with gold/palladium, and imaged using an Hitachi S-4800.

**Statistics.** All experiments were performed in duplicate or triplicate, and data are presented as a representative survival curve or mean  $\pm$  SEM. Comparisons between 2 groups were made with 2-tailed Student's *t* test. The log-rank test was used for statistical analysis of survival curves. Significance was defined as  $P < 0.05$ .

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