AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

OBSERVATION

ABCDs of the Relative Contributions of *Pseudomonas aeruginosa* Quorum Sensing Systems to Virulence in Diverse Nonvertebrate Hosts

Alejandro Vasquez-Rifo,[a] Jamie Cook,[b] Deborah L. McEwan,[c,d]* Dania Shikara,[b] Frederick M. Ausubel,[c,d] Francesca Di Cara,[b] Zhenyu Cheng[b]

[a]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA
[b]Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada
[c]Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts, USA
[d]Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA

ABSTRACT *Pseudomonas aeruginosa* is an opportunistic bacterial pathogen that exhibits pathogenicity in an unusually broad range of plants and animals, and it is of interest to study the roles of particular virulence-related factors in diverse hosts. The production of many *P. aeruginosa* virulence factors is under the control of a quorum sensing (QS) signaling network, which has three interconnected branches that engage in intricate cross talk: Las, Rhl, and MvfR. Because there has been no systematic comparison of the roles of the three QS systems in mediating *P. aeruginosa* virulence in various hosts, we compared the virulence of wild-type (WT) *P. aeruginosa* PA14 and a set of isogenic PA14 QS in-frame deletion mutants in four selected hosts, the reference plant *Arabidopsis thaliana* (Arabidopsis), the crop plant *Brassica napus* (canola), the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*. The first letters of the selected host genera, A, B, C, and D, inspired the title of this article and indicate that this work lays the groundwork for future elucidation of the specific roles of each QS branch in mediating virulence in diverse hosts.

IMPORTANCE In this study, we performed a systematic comparison of the virulence of WT *P. aeruginosa* and QS mutants in selected hosts and conditions. This work represents an important contribution to the long-term goal of unraveling the entangled roles of different branches of the *P. aeruginosa* QS network in different hosts and will serve as a valuable resource for the field of host-pathogen interactions.

KEYWORDS *Pseudomonas aeruginosa*, quorum sensing, broad host range, opportunistic pathogen

The large genome of *Pseudomonas aeruginosa* encodes a network of regulatory systems that contribute to its metabolic versatility, adaptability to environments, and broad host range as a pathogen (1–3). The *P. aeruginosa* quorum sensing (QS) signaling network consists of three branches, each of which plays an important role in regulating virulence (4–8). To help elucidate the specific contribution of each QS branch to pathogenesis, we assembled a comprehensive set of isogenic in-frame deletion mutants in QS genes in *P. aeruginosa* PA14, including the single Δ*lasR*, Δ*lasI*, Δ*rhlR*, Δ*rhlI*, and Δ*mvfR* mutants and different combinations of higher order Δ*lasR* Δ*rhlR*, Δ*lasR* Δ*mvfR*, Δ*rhlR* Δ*mvfR*, and Δ*lasR* Δ*rhlR* Δ*mvfR* mutants. We used this set of mutants to compare their virulence to wild-type (WT) PA14 in four *P. aeruginosa* hosts. Importantly, none of the mutant strains grew at a statistically significant slower rate than wild-type PA14 as determined by growing four replicate cultures of each strain in LB medium for approximately 20 h at 25°C in an automated plate reader.

Editor Alejandro Aballay, School of Medicine, Oregon Health & Science University

Address correspondence to Zhenyu Cheng, zhenyu.cheng@dal.ca.

*Present address: Deborah L. McEwan, Foundation Medicine, Cambridge, Massachusetts, USA.

F.M.A has a financial interest in Octagon Therapeutics, Inc., a company that was previously engaged in developing antimicrobial compounds. The remaining authors declare no competing financial interests.

This article is a direct contribution from Frederick M. Ausubel, a Fellow of the American Academy of Microbiology, who arranged for and secured reviews by Andreas Vilcinskas, Institute for Insect Biotechnology, Justus Liebig University Giessen, and Marvin Whiteley, Georgia Institute of Technology School of Biological Sciences.

Received 24 February 2022
Accepted 28 February 2022
Published 21 March 2022

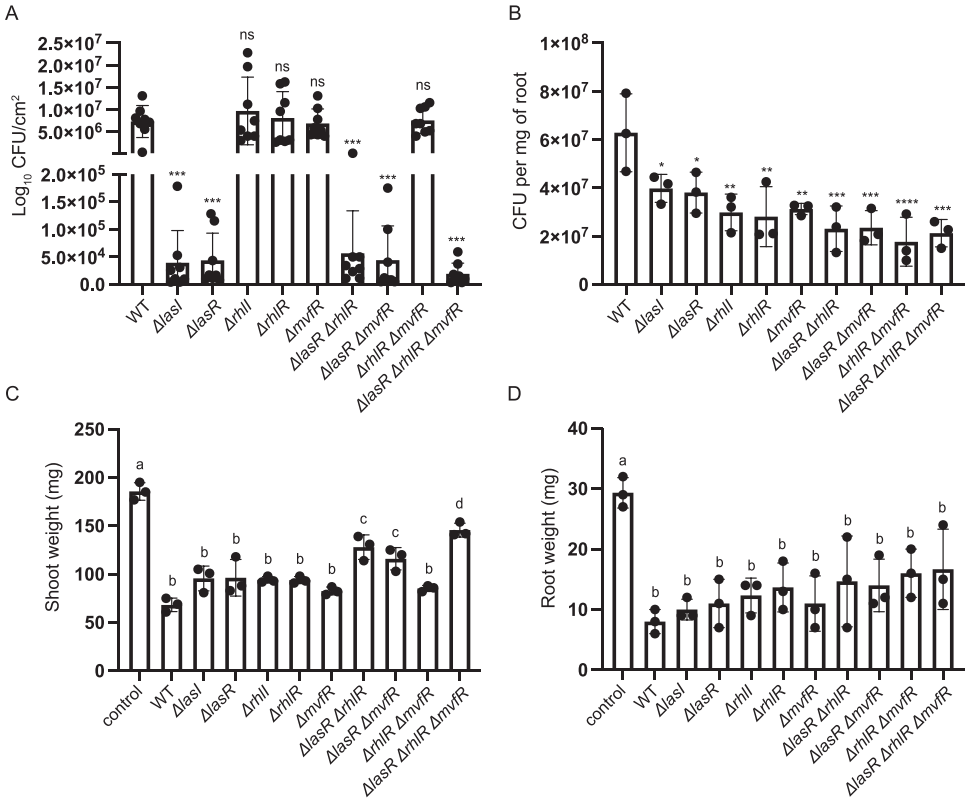

FIG 1 Plant and bacterial growth during *P. aeruginosa* infections. (A) *In planta* growth of *P. aeruginosa* PA14 WT and QS mutants 3 days after infiltration in 5-week-old *Arabidopsis* (ecotype Col-0) leaves measured by $\log_{10}$ CFU/cm^2 leaf area. All *las* mutants showed significantly decreased growth compared to WT ($P < 0.001$). (B) Colonization of canola roots by WT and QS mutants measured on day 5 postinfection. All QS mutants showed modest but highly significant reduction in root association compared to that of the WT. Canola root (C) and shoot (D) weights recorded 5 days postinfection with WT or QS mutants. In panels C and D, all strains (WT and QS mutants) showed significant shoot and root weight loss compared to that of the uninfected control. Three higher order QS mutants (Δ*lasR* Δ*rhlR*, Δ*lasR* Δ*mvfR*, and Δ*lasR* Δ*rhlR* Δ*mvfR*) had modest but significant alleviation of shoot weight loss. Error bars represent standard deviation of 10 (A) or 3 (B to D) independent biological replicates. Independent sample means were analyzed using a one-way ANOVA with a Dunnett's multiple comparison *post hoc* test to determine differences between samples. For *in planta* (A) and root-associated (B) CFU counting, significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. For plant tissue weight data (C and D), significant differences ($P < 0.05$) are reported as different letters.

Crop plants can be naturally infected by *P. aeruginosa* (9), and ornamental plants can serve as a reservoir for *P. aeruginosa* infection of hospitalized immunocompromised patients (10). We used a leaf infiltration assay in *Arabidopsis* (11) to compare the virulence of the WT and QS mutants. Plants infiltrated with a *P. aeruginosa* strain that has a functional Las QS system (WT and Δ*rhlR*, Δ*rhlI*, Δ*mvfR*, or Δ*rhlR* Δ*mvfR* mutants) showed high bacterial growth in leaves, whereas mutants lacking a functional Las QS system (Δ*lasR*, Δ*lasI*, Δ*lasR* Δ*rhlR*, Δ*lasR* Δ*mvfR*, and Δ*lasR* Δ*rhlR* Δ*mvfR* mutants) exhibited dramatically decreased growth (Fig. 1A).

We also tested the virulence of the QS mutants in a *Brassica napus* root colonization model (12). Roots are in close contact with pathogens in the soil, and bacteria that colonize plant roots need to partially nullify root defense responses (13). In contrast to the *Arabidopsis* leaf infiltration model in which Las-mediated QS is a key virulence determinant, mutation of any of the QS branches resulted in a consistently modest but highly statistically significant reduction in root colonization, with the double and triple mutants exhibiting more pronounced colonization defects with higher statistical significance (Fig. 1B). Monitoring virulence by assessing the growth of the host showed that the loss of canola shoot weight caused by colonization of PA14 WT was partially abrogated in the Δ*lasR* Δ*rhlR*, Δ*lasR* Δ*mvfR*, and Δ*lasR* Δ*rhlR* Δ*mvfR* mutants (Fig. 1C). All of

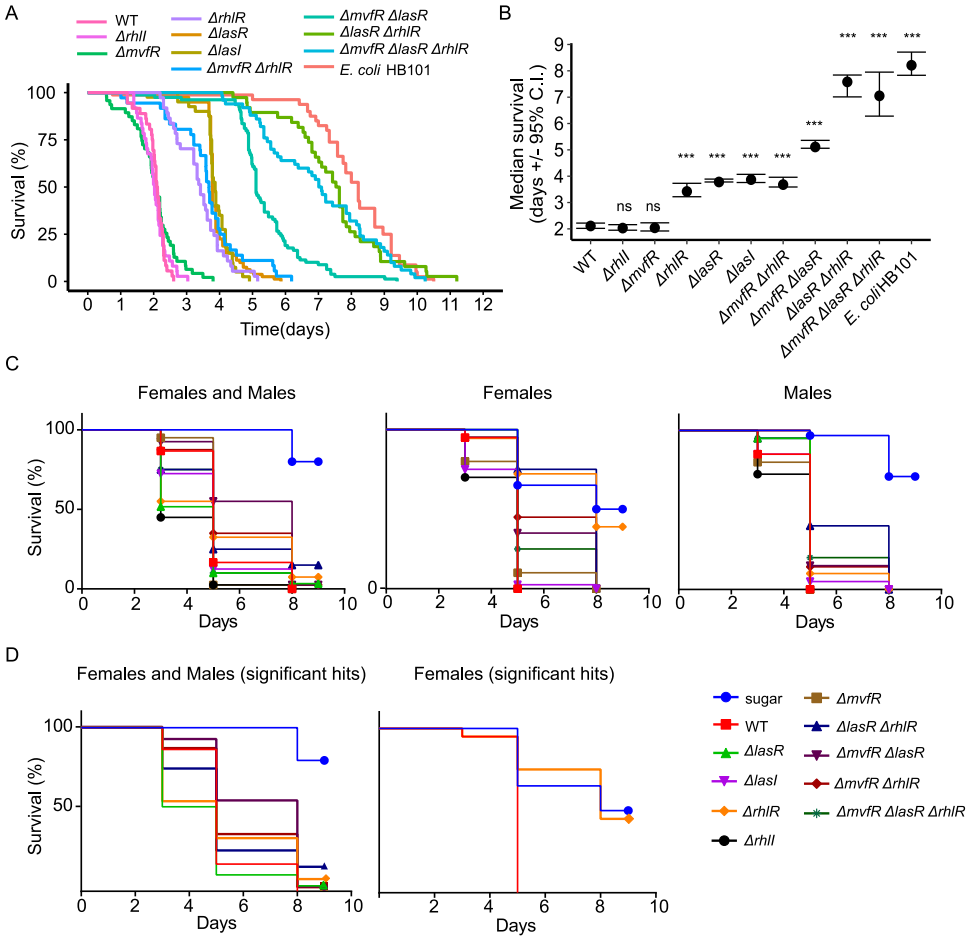

FIG 2 Survival of *C. elegans* and *D. melanogaster* exposed to *P. aeruginosa* PA14 WT and QS mutants. (A) Survival curve of adult worms (in days) following infection with the indicated *P. aeruginosa* PA14 WT and QS mutants. *E. coli* HB101 was included as a negative control. Pairwise comparisons of the survival curves between each strain and the WT were done using the log rank test. The test *P* values were all significant ($P < 0.0001$) except for Δ*mvfR* and Δ*rhlI*. (B) Median survival estimate (in days with confidence interval [CI]) for the survival curves shown in panel A. (C) Survival curves of mixed, female, and male *D. melanogaster* following infection with the *P. aeruginosa* PA14 WT and QS mutants. Uninfected sugar-fed flies were included as controls. (D) Survival differences were analyzed by Kaplan-Meier and log rank analysis. Only survival curves of mixed and female flies for WT and for statistically different ($P < 0.05$) QS mutants are shown. No QS mutants were statistically different from WT in infected male flies.

the mutants exhibited consistently modest increases in root weight compared to PA14 WT that were not statistically significant (Fig. 1D). A previous publication reported that a Δ*rhlI* mutant and a Δ*rhlI* Δ*lasI* mutant, but not a Δ*lasI* mutant, exhibited partially and completely disrupted root-associated growth in *Arabidopsis* and sweet basil, respectively (14), suggesting that different QS systems may be involved in root colonization in different hosts.

The nematode *Caenorhabditis elegans* has been used extensively as a model host to study bacterial virulence (15). Here, we monitored *P. aeruginosa*-mediated killing of *C. elegans* using a semi-automated life span device (16) (Fig. 2A and B). We found that the Δ*mvfR* or Δ*rhlI* mutations did not impair the ability of PA14 to kill nematodes, whereas all of the other mutants exhibited reduced virulence, resulting in longer nematode lifespans (Fig. 2A). We observed that both the Las and Rhl QS branches are required for full virulence. In contrast, the MvfR QS system appears to play a minor role in PA14-mediated killing. Only in one mutant (Δ*lasR* Δ*mvfR* mutant) did the loss of *mvfR* result in reduced virulence. Similar to other reports (17, 18), we observed that the Δ*rhlI* and Δ*rhlR* mutants exhibited very different phenotypes with respect to their ability to kill *C. elegans*. More specifically, the Δ*rhlI* mutant was indistinguishable from WT

PA14, whereas the Δ*rhlR* mutant exhibited significantly impaired killing ability. This latter observation is consistent with a report showing that in addition to C4-HSL synthesized by RhlI, RhlR can also be activated by a ligand that relies on PqsE (19).

The fruit fly *Drosophila melanogaster* has also been used to study host-bacterial interactions (18, 20, 21). We tested W^{1118} adult flies for their ability to survive infection caused by feeding flies the PA14 QS mutants. The resulting survival curves are shown in Fig. 2C. To aid data visualization, the sugar control, WT infection, and QS mutants that were significantly different from WT are plotted in Fig. 2D. When female or male flies were raised and infected separately, all of the flies died 5 days postinfection upon infection with WT PA14 or the Δ*rhlI* mutant, whereas all the other QS mutants showed a reduction in virulence (Fig. 2D). In particular, the female flies infected with the Δ*rhlR* mutant showed survival rates similar to those of the uninfected control, whereas other mutants containing the Δ*rhlR* mutant demonstrated reduced virulence that did not reach statistical significance (Fig. 2D). Conversely, when males and females were raised and infected in the same vial, they showed a slight but significant increase in survival to infections with the Δ*lasR* Δ*rhlR*, Δ*lasR*, and Δ*mvfR* mutants. The higher order mutants did not demonstrate further abrogated virulence than the Δ*rhlR* mutant in our study, which is consistent with a previous observation that in a similar feeding model, RhlR but not RhlI or LasR contributed to *P. aeruginosa* evasion of *Drosophila* immunity (19, 21). Interestingly, sexual dimorphism in infection has been reported previously and was shown to be dependent on the specific environment, as well as host and pathogen genetic backgrounds (22).

To our knowledge, this is the first study to systematically investigate the virulence of *P. aeruginosa* QS mutants representing all three branches of the *P. aeruginosa* QS network in multiple plant and invertebrate hosts. Interestingly, no consistent pattern of the role of the different QS systems in virulence emerged from these studies, suggesting that the "state of virulence" is both host and/or infection model dependent. We would argue that these results are consistent with previous results (15), which show that *P. aeruginosa* is a broad range multihost pathogen that has a variety of virulence mechanisms that allow it to physiologically adapt to particular host environments.

Mutant compiling and construction. The Δ*lasR* (DH164), Δ*rhlR* (DH2742), Δ*mvfR* (DH1110), Δ*lasR* Δ*rhlR* (DH2944), and Δ*lasR* Δ*mvfR* (DH1111) mutants in the *P. aeruginosa* strain PA14 (DH123) background (the labels in the brackets represent the lab stock numbers of these strains) were constructed previously and kindly provided by D. Hogan (23). The Δ*lasI* and Δ*rhlI* mutants of PA14 have been described (18, 21), and the Δ*rhlR* Δ*mvfR* and Δ*lasR* Δ*rhlR* Δ*mvfR* mutants were constructed using previously described protocols (18, 21). All mutations were confirmed by diagnostic PCR.

Host infection assays. All infection assays using the four selected hosts were performed using previously published protocols. *Arabidopsis* (ecotype Col-0) leaf infiltration and canola root colonization experiments were carried out as described previously (13, 14). For the *C. elegans* infection, the life span and slow killing assay were performed using a *C. elegans* Lifespan Machine as previously described (16). An oral infection model of *Drosophila* (24) was used to measure fly survival. In brief, 20 flies (age-matched males and females together or separate) were cultured on filter paper soaked in 5% sucrose or 5% sucrose containing *P. aeruginosa* WT or individual mutant strain (all optical density at 600 nm [OD_{600}] = 3). Flies were transferred to fresh vials every 2 days, and the number of dead flies was determined daily.

Statistical analysis. For *in planta* and root-associated CFU counting and plant tissue weight data, independent sample means were analyzed using a one-way analysis of variance (ANOVA) with a Dunnett's multiple comparison *post hoc* test to determine differences between samples. Significance was measured at $P < 0.05$, and significant differences were reported as different letters. The curated *C. elegans* survival data obtained from the Lifespan Machine were analyzed using R 3.5 (survival and survminer packages), and the log rank test was used to evaluate differences in worm survival. *Drosophila* survival data were analyzed in Prism 9, and statistical significance between the different survival curves was determined by Kaplan-Meier and log rank analysis.

ACKNOWLEDGMENTS

We thank Deborah Hogan at the Geisel School of Medicine at Dartmouth for generously sharing *P. aeruginosa* QS mutant strains.

This project was supported by Natural Science and Engineering Research Council (NSERC) of Canada Discovery Grants (grant numbers RGPIN/04912-2016 to Z.C. and RGPIN/04083-2019 to F.D.C.), an NSERC USRA Scholarship to D.S., a PHS postdoctoral fellowship (F32 AI098307 to D.L.M.), and an NIH R01 grant (R01 AI085581 to F.M.A.).

REFERENCES

1. He J, Baldini RL, Déziel E, Saucier M, Zhang Q, Liberati NT, Lee D, Urbach J, Goodman HM, Rahme LG. 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc Natl Acad Sci U S A 101:2530–2535. https://doi.org/10.1073/pnas.0304622101.
2. Diggle SP, Whiteley M. 2020. Microbe profile: *Pseudomonas aeruginosa*: opportunistic pathogen and lab rat. Microbiology (Reading) 166:30–33. https://doi.org/10.1099/mic.0.000860.
3. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B. 2011. *Pseudomonas aeruginosa* genomic structure and diversity. Front Microbiol 2:150. https://doi.org/10.3389/fmicb.2011.00150.
4. Whiteley W, Diggle SP, Greenberg EP. 2017. Progress in and promise of bacterial quorum sensing research. Nature 551:313–320. https://doi.org/10.1038/nature24624.
5. Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science 260:1127–1130. https://doi.org/10.1126/science.8493556.
6. Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, Iglewski BH, Greenberg EP. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc Natl Acad Sci U S A 91:197–201. https://doi.org/10.1073/pnas.91.1.197.
7. Déziel E, Lepine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG. 2004. Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. Proc Natl Acad Sci U S A 101:1339–1344. https://doi.org/10.1073/pnas.0307694100.
8. Maura D, Hazan R, Kitao T, Ballok AE, Rahme LG. 2016. Evidence for direct control of virulence and defense gene circuits by the *Pseudomonas aeruginosa* quorum sensing regulator, MvfR. Sci Rep 6:34083. https://doi.org/10.1038/srep34083.
9. Clara FM. 1930. A new bacterial leaf disease of tobacco in the Philippines. Phytopathol 20:691–706.
10. Kominos SD, Copeland CE, Grosiak B, Postic B. 1972. Introduction of *Pseudomonas aeruginosa* into a hospital via vegetables. Appl Microbiol 24:567–570. https://doi.org/10.1128/am.24.4.567-570.1972.
11. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science 268:1899–1902. https://doi.org/10.1126/science.7604262.
12. Cook J, Douglas GM, Zhang J, Glick BR, Langille MGI, Liu KH, Cheng Z. 2021. Transcriptomic profiling of *Brassica napus* responses to *Pseudomonas aeruginosa*. Innate Immun 27:143–157. https://doi.org/10.1177/1753425920980512.
13. Millet YA, Danna CH, Clay NK, Songnuan W, Simon MD, Werck-Reichhart D, Ausubel FM. 2010. Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. Plant Cell 22:973–990. https://doi.org/10.1105/tpc.109.069658.
14. Walker TS, Bais HP, Déziel E, Schweizer HP, Rahme LG, Fall R, Vivanco JM. 2004. *Pseudomonas aeruginosa*-plant root interactions. pathogenicity, biofilm formation, and root exudation. Plant Physiol 134:320–331. https://doi.org/10.1104/pp.103.027888.
15. Feinbaum RL, Urbach JM, Liberati NT, Djonovic S, Adonizio A, Carvunis AR, Ausubel FM. 2012. Genome-wide identification of *Pseudomonas aeruginosa* virulence-related genes using a *Caenorhabditis elegans* infection model. PLoS Pathog 8:e1002813. https://doi.org/10.1371/journal.ppat.1002813.
16. Stroustrup N, Ulmschneider BE, Nash ZM, López-Moyado IF, Apfeld J, Fontana W. 2013. The *Caenorhabditis elegans* Lifespan Machine. Nat Methods 10:665–670. https://doi.org/10.1038/nmeth.2475.
17. Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL. 2017. The RhlR quorum-sensing receptor controls *Pseudomonas aeruginosa* pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. PLoS Pathog 13:e1006504. https://doi.org/10.1371/journal.ppat.1006504.
18. Haller S, Franchet A, Hakkim A, Chen J, Drenkard E, Yu S, Schirmeier S, Li Z, Martins N, Ausubel FM, Liégeois S, Ferrandon D. 2018. Quorum-sensing regulator RhlR but not its autoinducer RhlI enables *Pseudomonas* to evade opsonization. EMBO Rep 19:e44880. https://doi.org/10.15252/embr.201744880.
19. Mukherjee S, Moustafa DA, Stergioula V, Smith CD, Goldberg JB, Bassler BL. 2018. The PqsE and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and biofilm development in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 115:E9411–E9418. https://doi.org/10.1073/pnas.1814023115.
20. Lutter EI, Purighalla S, Duong J, Storey DG. 2012. Lethality and cooperation of *Pseudomonas aeruginosa* quorum-sensing mutants in *Drosophila melanogaster* infection models. Microbiology (Reading) 158:2125–2132. https://doi.org/10.1099/mic.0.054999-0.
21. Limmer S, Haller S, Drenkard E, Lee J, Yu S, Kocks C, Ausubel FM, Ferrandon D. 2011. *Pseudomonas aeruginosa* RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model. Proc Natl Acad Sci U S A 108:17378–17383. https://doi.org/10.1073/pnas.1114907108.
22. Belmonte RL, Corbally MK, Duneau DF, Regan JC. 2019. Sexual dimorphisms in innate immunity and responses to infection in *Drosophila melanogaster*. Front Immunol 10:3075. https://doi.org/10.3389/fimmu.2019.03075.
23. Mould DL, Botelho NJ, Hogan DA. 2020. Intraspecies signaling between common variants of *Pseudomonas aeruginosa* increases production of quorum-sensing-controlled virulence factors. mBio 11:e01865-20. https://doi.org/10.1128/mBio.01865-20.
24. Di Cara F, Sheshachalam A, Braverman NE, Rachubinski RA, Simmonds AJ. 2017. Peroxisome-mediated metabolism is required for immune response to microbial infection. Immunity 47:93–106. https://doi.org/10.1016/j.immuni.2017.06.016.