Oxylipins produced by Pseudomonas aeruginosa promote biofilm formation and virulence

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The oxygenation of unsaturated fatty acids by dioxygenases occurs in all kingdoms of life and produces physiologically important lipids called oxylipins. The biological roles of oxylipins have been extensively studied in animals, plants, algae and fungi, but remain largely unidentified in prokaryotes. The bacterium Pseudomonas aeruginosa displays a diol synthase activity that transforms several monounsaturated fatty acids into mono- and di-hydroxylated derivatives. Here we show that oxylipins derived from this activity inhibit flagellum-driven motility and upregulate type IV pilus-dependent twitching motility of P. aeruginosa. Consequently, these oxylipins promote bacterial organization in microcolonies, increasing the ability of P. aeruginosa to form biofilms in vitro and in vivo (in Drosophila flies). We also demonstrate that oxylipins produced by P. aeruginosa promote virulence in Drosophila flies and lettuce. Our study thus uncovers a role for prokaryotic oxylipins in the physiology and pathogenicity of bacteria.

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The oxygenation of fatty acids is one of the main biochemical reactions in the synthesis of lipid mediators. Oxygenated fatty acids are the starting points for the synthesis of a great variety of biologically significant metabolites known as oxylipins. The best studied oxylipins are the leukotrienes and prostanooids in mammals, which are implicated in inflammation, fever, pain and other physiological processes. The oxylipin jasmonic acid and its derivatives have been intensely studied in plants. These compounds mediate hormone-like functions and are also involved in defence responses and development. In algae and fungi, oxylipins participate in defence, reproduction and pathogenesis. However, very little is known about the role of oxylipins in prokaryotes.

Oxylipin synthesis is mainly catalysed by fatty acid dioxygenases and monoxygenases, although they can also be produced by non-enzymatic chemical oxidation of fatty acids. Fatty acid dioxygenases include lipoxygenases, cyclooxygenases, \( \alpha \)-dioxygenases and diol-synthases, which produce fatty acid-derived hydroperoxides or endoperoxides. For a long time no evidence existed for fatty acid dioxygenases in bacteria. However, in the last decade, in part due to the benefits provided by deep sequencing techniques, genes encoding putative fatty acid dioxygenases have been identified in the chromosome of many bacterial species. LoxA of Pseudomonas aeruginosa was the first prokaryotic dioxygenase to be characterized. The enzyme catalyses dioxygenation of several monounsaturated fatty acids, generating mono- and di-hydroxylated derivatives. The first prokaryotic dioxygenase to be characterized was dioxygenated at position C10 by the enzyme 10(S)-dioxygenase (10S-DOX) (ref. 8). Subsequently, the hydroperoxide derivative (10S-HPOME) could be isomerized by the enzyme (75,10S)-hydroperoxide isomerase to form 75,10S-DiHOME or be reduced to 10-HOME by an undefined mechanism.

We found that such effect was largely dependent on the dioxygenation of oleic acid, since no inhibitory effect of oleic acid on swimming was observed in ΔDS (Fig. 2a). To confirm oxylipins derived from the dioxygenase activity on oleic acid were responsible for the negative effect on swimming, we purified 10-HOME and 7,10-DiHOME from culture supernatants of PAO1 grown in the presence of oleic acid (Supplementary Fig. 3). Increasing concentrations of both 10-HOME and 7,10-DiHOME significantly reduced ΔDS’s ability to swim, although the inhibitory effect of 10-HOME was notably stronger than that observed for 7,10-DiHOME (Fig. 2b).

Swimming is strongly dependent on a functional flagellum. Thus, we investigated whether oxylipins were directly regulating flagellum motility by testing the effect of purified oxylipins on the swimming ability of ΔDS. Both 10-HOME and 7,10-DiHOME significantly reduced ΔDS swimming motility. Consistent with the stronger effect of 10-HOME on swimming, the effect of 10-HOME on swimming was higher than that observed for 7,10-DiHOME (Fig. 2c).

In addition to flagellum-dependent motilities, P. aeruginosa displays a type IV pilus-driven twitching motility. Surprisingly, we found that contrary to the negative effect of 10-HOME and 7,10-DiHOME in swimming and swimming, both oxylipins strongly promoted P. aeruginosa twitching motility (Fig. 2d). Increasing concentrations of 10-HOME or 7,10-DiHOME resulted in a direct proportional increase of ΔDS twitching motility. In addition, we directly observed the effect of oxylipins on twitching under the microscope. When supplemented with 10-HOME, ΔDS’s expanding edges consistently projected longer and thicker branches, which expanded faster (53.6 ± 10.1 pxl min⁻¹) than the controls without oxylipins (22.0 ± 11.5 pxl min⁻¹) (Supplementary Movies 1 and 2).

Oxylipins promote biofilm formation over abiotic surfaces. Bacterial motility has a direct impact on the capacity of most bacteria to grow attached to surfaces as biofilms. During biofilm formation, bacterial cells aggregate, secrete extracellular matrix and produce cell-cell communication signals. Oxylipins promote biofilm formation over abiotic surfaces, fostering bacterial aggregation and cell-cell communication. Oxylipins are also involved in the cohesion of biofilm cells, thus promoting their resistance to environmental stresses. Oxylipins also play a role in biofilm dispersion, facilitating the detachment of bacterial cells from the surface and the dispersion of bacterial cells into the surrounding environment.

**Figure 1 | Biosynthetic pathway of oxylipins derived from the diol synthase activity of P. aeruginosa over oleic acid.** Oleic acid is first dioxygenated at position C10 by the enzyme 10(S)-dioxygenase (10S-DOX) (ref. 8). Subsequently, the hydroperoxide derivative (10S-HPOME) could be isomerized by the enzyme (7S,10S)-hydroperoxide isomerase to form 7S,10S-DiHOME or be reduced to 10-HOME by an undefined mechanism.

**Results**

**Oxylipins regulate bacterial motility.** The dioxygenase activity in the model strain P. aeruginosa PAO1 relies on a pair of genes, PA2077 and PA2078, that are members of di-heme-cytochrome C peroxidase. To elucidate a possible role of these diol synthase-derived oxylipins, we introduced an in-frame deletion encompassing both PA2077 and PA2078 genes (Supplementary Fig. 1A). As expected, the resulting strain, which we called ΔDS, is a deficient 10-HOME and 7,10-DiHOME producer (Supplementary Fig. 1B). ΔDS displayed a normal growth rate in M63 complete medium supplemented with oleic acid up to 1 mg ml⁻¹, suggesting that this activity is not directly involved in the primary metabolism of the bacterium (Supplementary Fig. 2).

We then tested the effect of the dioxygenase-derived oxylipins on the different types of bacterial motility. We first observed a negative effect of oleic acid on the swimming motility of PAO1.
formation, *P. aeruginosa*, which has become a common model to study this process, transits from a free-swimming (planktonic) to a sessile phenotype. In order to know if the effect of the diol synthase-derived oxylipins on *P. aeruginosa* motility has consequences in the ability of this bacterium to form biofilms, we compared the capacity of PAO1 versus ΔDS to form biofilms in polystyrene microtitre plates. We found that increasing concentrations of oleic acid up to 1 mg ml⁻¹ proportionally increased the capacity of PAO1 to form biofilm (Fig. 3a). Interestingly, such an effect was very weak on the ΔDS mutant, suggesting that oxylipins derived from the diol synthase activity were responsible for promoting *P. aeruginosa*’s biofilm formation *in vitro* (Fig. 3a). To confirm this, we tested the effect of pure 10-HOME and 7,10-DiHOME induced biofilm formation of PAO1 or ΔDS strains (Fig. 3b,c). Consistent with the stronger effect of 10-HOME on motility compared to 7,10-DiHOME, the positive effect of 10-HOME was also visibly higher than that of 7,10-DiHOME in inducing biofilm formation (Fig. 3b,c). For example, while 10-HOME achieved saturation at 0.6 mg ml⁻¹, the effect of 7,10-DiHOME just started to show at that same concentration (Fig. 3b,c).

Inverse regulation of flagellum- and type IV pilus-based motilities was previously described to promote formation of bacterial microcolonies, a first step in the organization of a biofilm. Thus, we explored whether oxylipins were inducing initiation of a biofilm *in vitro* by upregulating the formation of microcolonies. To this end, PAO1 bacteria constitutively expressing green fluorescent protein (GFP) were directly visualized by fluorescent microscopy. After 3 h of incubation in the absence of oxylipins, attached bacteria were distributed almost homogeneously over the surface (Fig. 4a, left panels). However, when the medium was supplemented with 10-HOME or 7,10-DiHOME at 0.4 mg ml⁻¹ and 0.8 mg ml⁻¹ respectively, both oxylipins consistently promoted bacterial organization in microcolonies (Fig. 4a, panels to the right). Again, the effect of 10-HOME in inducing microcolonies formation was visibly higher than that of 7,10-DiHOME. It started to be seen at 0.2 mg ml⁻¹ (Fig. 4a, centre panel) and quantity and size of microcolonies at 0.4 mg ml⁻¹ were higher even when it was used at half the concentration of 7,10-DiHOME.

We additionally explored whether microcolonies induced by oxylipins contained extracellular DNA (eDNA) and exopolysaccharide (EPS), which are characteristic components of the matrix

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**Figure 2** | Oxylipins inversely regulate flagellum and type IV pilus associated motilities. (a) Effect of adding oleic acid on the swarming motility of PAO1 and its ΔDS-derived mutant. Oleic acid at 0.1 mg ml⁻¹ inhibited PAO1 significantly (*t*-test, *P* < 0.0001), but not ΔDS swarming. (b) Both 10-HOME and 7,10 Di-HOME oxylipins inhibit swarming motility of ΔDS significantly (one-way ANOVA, *P* < 0.0001). (c) 10-HOME oxylipin strongly inhibits flagellum-driven motility of ΔDS strain (one-way ANOVA, *P* < 0.0001), but 7,10 Di-HOME has a weak effect (*P* < 0.05). (d) 10-HOME and 7,10 Di-HOME significantly increased ΔDS twitching motility (one-way ANOVA, *P* < 0.0001). Results from three independent experiments with *N* = 3 each. Error bars represent s.d.s.

**Figure 3** | Oxylipins induce biofilm formation in *P. aeruginosa* (a) Quantitation of the oleic acid effect on biofilm formation by the WT and ΔDS strains. Oleic acid induced a threefold increase in biofilm formation by WT compared to ΔDS. (b) Quantitative effect of 10-HOME and 7,10-DiHOME on biofilm formation by PAO1. 10-HOME showed a stronger effect than 7,10-DiHOME. (c) As expected, the effect of oxylipins on ΔDS biofilm formation was similar. Means and s.d. are from at least three independent experiments.
of microcolonies deriving into biofilms. DNA- and EPS-specific fluorescence staining using 4',6-diamidino-2-phenylindole and concanavalin A-FITC conjugate, respectively, showed the presence of abundant eDNA and EPS associated to the extracellular matrix of the microcolonies (Fig. 4b).

**Non-oxylipin-producing bacteria behave as social cheaters.** Bacteria usually produce extracellular factors required for bacterial growth and persistence. These factors benefit the whole bacterial population including cell variants that exploit them by ‘cheating’, avoiding the cost of production. The diol synthase activity of *P. aeruginosa* occurs in the periplasm. However, the resulting oxylipins cross the bacterial outer membrane and accumulate in the extracellular media. This prompted us to test whether the ADS mutant can profit from the extracellular oxylipins produced by the wild type (WT) by cheating to complement its biofilm deficiency when they are co-cultured. For this, PAO1 and ADS, constitutively expressing GFP or m-Cherry were co-cultured in minimal media supplemented with oleic acid at 1 mg ml$^{-1}$. Quantitative analysis of bacteria incorporated into the biofilm revealed that, in coculture with PAO1, ADS efficiently incorporated into the biofilm. This suggested ADS cheated on oxylipins produced by the WT (Supplementary Fig. 4).

**Oxylipins induce biofilm formation over *A549* cells in vitro.** We subsequently tested whether the effect of oxylipins on biofilm formation also had an impact in the ability of *P. aeruginosa* to colonize biotic surfaces. As *P. aeruginosa* infections are frequently associated with the respiratory tract, we tested the capacity of 10-HOME and 7,10-DiHOME to promote biofilm formation over monolayers of A549 human alveolar epithelial cells. PAO1 expressing GFP was added to the cell monolayers (approximately 20 bacteria per cell) in the presence or absence of 10-HOME or 7,10-DiHOME. As observed in abiotic surfaces, both 10-HOME and 7,10-DiHOME oxylipins promoted microcolony formation of PAO1 over A549 monolayers. Once again, the effect of 10-HOME was consistently higher than that of 7,10-DiHOME when used at the same concentration (Fig. 5).

**Oxylipins promote biofilm formation in vivo.** We next investigated the effect of 10-HOME or 7,10-DiHOME in biofilm formation in vivo. The crop of *Drosophila melanogaster* orally inoculated with *P. aeruginosa* has been proposed as a model to visualize biofilm formation in vivo. We orally inoculated flies with PAO1 or ADS expressing GFP in M63 complete medium. Twenty hours post infection the flies were killed and dissected crops were analysed by microscopy. Under these conditions no difference was observed between PAO1 and ADS. Both strains produced small microcolonies that were almost homogeneously distributed throughout the crops (Fig. 6a and Supplementary Fig. 5A). This result suggested that oxylipins were not produced under these conditions, probably by the lack of fatty acids availability in the crop compartment. However, when fly food was supplemented with 10-HOME or 7,10-DiHOME, both strains formed visibly larger microcolonies and biofilm covering greater area over the luminal epithelium of crops (Fig. 6b,c and Supplementary Fig. 5B,C). These observations suggest that *P. aeruginosa* can initiate biofilms in vivo over epithelial tissues, provided it has a source of fatty acids available for the synthesis of oxylipins.
Figure 5 | Oxylipins induce microcolony and biofilm formation over human epithelial cell surfaces. GFP-expressing PAO1 was added to cell monolayers of A549 cells in the absence (upper panels) or presence of 10-HOME and 7,10-DiHOME (lower panels). Oxylipins were added at 0.4 mg ml⁻¹. Approximately 20 bacteria per human cell were added and pictures were taken 3 h after the addition of bacteria. Bars represent 200 μm. The size/resolution for each panel was adjusted to 2.125 × 1.550 in/600 dpi from 7.770 × 13.333 in/72 dpi of the originals. Pictures are representative of three independent experiments with three replicates each.

Figure 6 | Oxylipins promote biofilm formation in D. melanogaster crops. Fluorescence microscopy pictures of dissected crops from Drosophila flies fed with M63 media supplemented with 10-HOME or 7,10-DiHOME, into which GFP-expressing PAO1 strain was inoculated. (a) PAO1 formed few microcolonies and the bacteria were mostly homogeneously distributed all over the crop’s lumen. However, when the media was supplemented with 10-HOME (b) or 7,10-DiHOME (c) PAO1 was able to abundantly form microcolonies and early biofilm in flies’ crops. Bars represent 200 μm. The size/resolution for each panel was adjusted to 2.125 × 1.587 in/600 dpi from 17.770 × 13.333 in/72 dpi of the originals. Pictures are representative of two independent experiments, in which five flies were dissected in each case.
Oxylipins promote *P. aeruginosa* virulence in *Drosophila* flies and lettuce. Further, we assessed whether the effect of oxylipins on motility, and consequently on biofilm formation, correlated with the capacity of *P. aeruginosa* to colonize and develop an infection process in vivo. For this, we evaluated the virulence of PAO1 versus ΔDS against *D. melanogaster* through the oral and thoracic routes of inoculation, which are established models to study *P. aeruginosa* pathogenesis\(^\text{20,21}\). After several attempts we were unable to find any significant difference between the WT and the ΔDS mutant using the feeding model of inoculation (Fig. 7a). However, using the pricking method of bacterial inoculation we found that virulence of ΔDS was significantly attenuated in *Drosophila* flies. While PAO1 caused 100% of mortality 35 h post-inoculation, ΔDS was approximately half as virulent as PAO1 at the same time point (Fig. 7b).

In investigating the reasons for the different results using the feeding or pricking model of infection we hypothesized that this was probably because *P. aeruginosa* cannot acquire free fatty acids from the intact digestive tract of the flies as it does from the wounded tissues in the pricking model. To test this hypothesis we evaluated the presence of oxylipins in homogenates of flies infected by both routes of inoculation. We performed organic extractions of homogenates of flies inoculated with PAO1 or ΔDS and analysed the extracts by thin layer chromatography (TLC). We were unable to detect 10-HOME or 7,10-diHOME in homogenates of flies orally inoculated with PAO1 but not in those inoculated with ΔDS (shown in blue). (d) Graph of an infection experiment done in lettuce. The percent of established infections over the total number of inoculation events is graphed. Means were significantly different (ordinary one-way ANOVA, ***, P < 0.01, three independent experiments, error bars are s.d.). N, total of inoculation events; L, number of independent leaves inoculated.
attenuation to mutants of the most important virulence factors of *P. aeruginosa*, such as the exotoxin A, lysozyme S, phospholipase C, flagellum motility or type IV pilus (Fig. 7c).

**Oxylipins promote *P. aeruginosa* virulence in lettuce.** *P. aeruginosa* is also a common pathogen of plants. Thus, we compared the ability of PAO1 versus ADS to develop an infection process in lettuce leaves, a model of *P. aeruginosa* plant infection. While PAO1 was able to establish an infection in more than 50% of the inoculation sites, ADS could establish infection only in 5% of the inoculation events. However, when ADS was inoculated with 10-HOME or 7,10-DiHOME the infection ratio increased to 48 and 25%, respectively (Fig. 7e). These results suggest that oxylipins also promote *P. aeruginosa* colonization of plant tissues.

**Discussion**

Bacterial biofilms are widely recognized to play an important role in pathogenesis during bacterial infections. The biology of biofilm has been extensively studied, little is understood about the signals governing the initiation of biofilms in vivo. Our results provide strong evidence suggesting that *P. aeruginosa* (and probably other bacteria as well) transforms fatty acids scavenged from the host into oxylipins as a way to sense the host environment and promote initiation of a biofilm lifestyle. We show that two oxylipins, 10-HOME and 7,10-DiHOME derived from a diol synthase activity of *P. aeruginosa* oleic acid are directly involved in this process. These oxylipins seem to play a critical role in the initial stages of biofilm formation by inducing a microcolony organization of attached bacteria. The mechanism by which 10-HOME and 7,10-DiHOME mediate this process involves the inverse regulation of flagellum- and type IV pilus-dependent motilities. The induced microcolonies can subsequently lead to a mature biofilm, a process involving induction of the second messenger c-di-GMP that, among other functions, inhibit both flagellum- and type IV pilus-dependent motilities (Fig. 8).

Although our results suggest both 10-HOME and 7,10-DiHOME oxylipins act on a common physiological pathway, the effect of 10-HOME on biofilm formation was consistently higher than that of 7,10-DiHOME. We currently do not know why *P. aeruginosa* produces two metabolites with a redundant function; however, the kinetic study of appearance/disappearance of 10-HOME and 7,10-DiHOME in *P. aeruginosa* culture supernatants showed that while 10-HOME appears first in time, 7,10-DiHOME remains longer in the stationary phase of the culture (Supplementary Fig. 7). Based on this observation it is tempting to speculate that 10-HOME could be responsible for producing an abrupt transient switch from a planktonic to a sessile behaviour at the very beginning phase of the biofilm formation once fatty acids are sensed. On the other hand, 7,10-DiHOME, with a more moderate activity, may prolong such effect during later stages of biofilm maturation.

We want to remark here that we found an effect of oxylipins on virulence in *Drosophila* inoculated by pricking the thoracic segment of the flies, which implies tissue damage, but not in flies inoculated by the oral route. This is consistent with our observation that PAO1 and ADS similarly colonized the fly crops in the absence of oxylipins using the oral route (Fig. 6a and Supplementary Fig. 5A). Outstandingly, exogenous oleic acid in the inocula was not required to observe an attenuated virulence of ADS compared to PAO1 in *D. melanogaster* by the pricking method. This suggests that *P. aeruginosa* inoculated by the pricking method have access to a source of host fatty acids in wounded tissues but not in the intact digestive tract of flies inoculated by the oral route (at least under the conditions tested). These fatty acids could serve as substrates for oxylipin synthesis, which this bacterium uses for its own benefit.

An inevitable arising question is whether the diol synthase is active during infections of organisms closer to humans, like other mammals. A previous *Drosophila*-based screening to determine virulence factors of *P. aeruginosa* PA14 revealed that a transposon insertion into the PA2077 gene not only attenuated PA14 virulence in the fly, but also in a murine model of peritonitis. Another study focused on gene expression and fitness determinants during acute and chronic infections of murine models with *P. aeruginosa*, revealed that genes encoding the diol synthase activity, PA2077 and PA2078, are over-expressed in both acute and chronic infections (18.90 and 16.23 times in acute versus 36.20 and 29.47 times in chronic, respectively) compared to a non-infective well-defined *in vitro* condition. Additionally, they found that PA2077 and PA2078 genes were important for bacterial fitness. These studies, which are in agreement with our results, represent an independent corroboration of the impact caused by disrupting the diol synthase activity on *P. aeruginosa* pathogenicity and additionally reveal a possible role of oxylipins production during *P. aeruginosa* colonization of mammalian tissues.
The wide distribution of oleic acid among human tissues also supports the feasibility of oxylipins production during P. aeruginosa infections. Oleic acid is the most abundant fatty acid in human adipose tissue and second in abundance in human tissues in general including the skin, where it functions as an important component of the innate immune response. Particularly, in cystic fibrosis patients, the alveoli secretion is enriched in host-derived lipids triggered by the host immune response, which might be a source of fatty acids for the production of oxylipins. Additionally, these patients exhibit an increased bronchial cytotoxic phospholipase (cPLA) A2 activity, which hydrolyses membrane phospholipids at the sn-2 position, releasing free fatty acids. Interestingly, specific inhibition of cPLA A2 reduces mouse mortality induced by P. aeruginosa pulmonary infection, which could, at least in part, be due to the reduced availability of fatty acids for oxylipin production. While our experiments suggest that the motility phenotype of ΔSIS and its impaired ability to form biofilm directly affects its virulence in Drosophila and lettuce, an additional role of oxylipins in the context of bacterial-host interaction should not be discarded. In animals, plants and fungi, oxylipins are signalling molecules involved in cross kingdom communication. Thus, a role of prokaryotic oxylipins in mediating bacterial-host cross communication should be seriously considered. Altogether, the role of prokaryotic oxylipins in mediating bacterial-host cross kingdom communication should not be discarded. In animals, plants and fungi, oxylipins are signalling molecules involved in cross kingdom communication. Thus, a role of prokaryotic oxylipins in mediating bacterial-host cross communication should be seriously considered. Altogether, the role of prokaryotic oxylipins in mediating bacterial-host cross kingdom communication should not be discarded. In animals, plants and fungi, oxylipins are signalling molecules involved in cross kingdom communication and virulence.

**Methods**

**Strains and culture conditions.** P. aeruginosa strain PAO1 (obtained from the Manoil Lab at the University of Washington, Seattle, WA, USA) and its isogenic mutant ΔSIS (diol synthase deletion mutant) were used throughout the entire study. Deletion of the diol synthase operon was performed by allelic exchange using the suicide vector pEX100TiTn5 and a previously described method. Briefly, a fragment of *P. aeruginosa* chromosome containing the genes PA2077 and PA2077 plus ~300 bp of chromosomal flanking regions were amplified by PCR using the primers JC1G1 (5′-ggcggagagcttcgccttcgccg-3′) and JC2G2 (5′-ggcgg gaatttcggttacacctc-3′). These primers introduced sites EcoRI and HindIII at the ends of the amplified fragment that were used for cloning into the suicide vector pEX100TiTn5. The plasmid pEX100TiTn5 was constructed from this plasmid pMD19-T (TaKaRa) and the Stu fragment internal to the diol synthase operon was deleted to obtain the final construction pEXADS. This plasmid was used to in-frame deletion of PA2077-78, through allelic exchange in the chromosome of PAO1 (Supplementary Fig. 1A). The diol-synthase-activity-lacking strain obtained was named ΔSIS. The mutation was confirmed by PCR and sequencing in an independent mutant phenotype was confirmed by TLC (see below). Complementation of the mutant was performed by replacing the mutated allele with the original copy from the parental strain PAO1, also through allelic exchange. Green and red fluorescent *P. aeruginosa* were obtained by transformation with the plasmids pMF230 or pMF440, which constitutively express the GFP mut2 and mCherry fluorescent proteins, respectively. Plasmids pMF230 and pMF440 (Addgene plasmids # 62546 and 62550) were a gift from Michael Franklin (Montana State University). Other *P. aeruginosa* strains used were the transposon mutants of genes encoding exotoxin A (exoA, strain PW3709), phospholipase C (phiV, PW6586), exoenzyme S (exoS, PW7479), a cAMP-dependent transcriptional regulator (lasR, Preventa type 4 fimbrial precursor (pla, PW8622) and the flagellar motor switch protein (flaM, PW3621), which were acquired from the transposon library collection of University of Washington. Escherichia coli DH5α (Invitrogen) was the host for plasmid constructions and E. coli S17-1 λpir (a gift from Jorge Benitez—Morehouse School of Medicine) was used as a donor strain for bacterial conjugation when required.

The strains were routinely grown in lysogeny broth (LB) medium at 30°C, to which agar was added when solid medium was required. LB agar without NaCl 15% sucrose was used to segment suicide plasmid from merodiploids during construction of ΔSIS by allelic exchange. Biofilm formation was performed in M63 media supplemented with 2% glucose, 5% caseinomacid and MgSO₄ 1 mM (M63 complete). Antibiotics were added, when necessary, at the following concentrations: Ampicillin (Amp), 100 µg ml⁻¹; Carbencillicin (Cb), 300 µg ml⁻¹. Oleic acid 90% (Sigma 364525) was added to cultures for oxylipin production and purification. M63 complete media was supplemented with oleic acid 99% (Sigma, O1008) or purified oxylipins when required for the study of biofilm formation over polystyrene surface of microtiter plates or over biotic surfaces.

**HPLC/MS analysis.** Purified 7,10-DHOME and 10-HOME were prepared at 1 mg ml⁻¹ in methanol (stock solution), from which samples to be analysed were prepared by diluting in ddH₂O with 0.1% formic acid. For each sample, a 20 µl injection was loaded onto a Synergi Hydro-RP 80A 2.5 x 2 mm C18 column (Phenomenex) using a Shimadzu Prominance System Binary Pump (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) at a flow rate of 350 µl min⁻¹ using ddH₂O with 0.1% formic acid and acetonitrile with 0.1% formic acid for mobile phase A/B respectively. The gradient proceeded from 10 to 80%B over 11 min, then to 100%B at 14 min, then re-equilibrated back at initial conditions for 6 min for a total of 20 min per analysis using the SCIEX 4000 Triple Quadrupole Mass Spectrometer (Concord, Ontario, Canada) in the ESI negative ion mode. Nitrogen was used as a nebulizer and curtain gas (TMG – 20). The collision gas, collision energy and temperature were set at 10°C (–30eV for 10-HOME, –34eV for 7,10-DHOME) and 600°C, respectively. GS1 and GS2 were heated at 40°C and 60°C respectively. Analyst 1.6.2 software controlled the LC/MS/MS system.

**Motility assays.** Swimming, swarming and twitching motilities were studied using the methods described by Bashall and Kornberg. To capture videos of twitching motility, 100 µl aliquots of the twitching medium were deposited over microplate cover slips. Once the medium solidified, it was punctured in the centre up to the bottom with an extended inoculation loop embedded with a bacterial suspension (OD₆₀₀ = 1) of the strain to be filmed. The cover slips were incubated for 6 h at 37°C, then placed inverted on the microscope stage (Olympus BX53 microscope) and the twitching motility filmed using an XM10 incorporated camera (Olympus) controlled by the software cellSens Standard 1.6 (Olympus).

**Quantification of biofilm formation.** Biofilm assays were performed following the O’Toole protocol. Briefly, *P. aeruginosa* strains were cultured overnight in LB agar plates at 37°C. Bacterial suspensions were prepared in M63 medium to an OD₆₀₀ of 0.5. 10 µl of microtiter bacterial suspension was inoculated into each well of a 96-well microtiter plate containing 200 µl of M63 complete media. Oleic acid or pure oxylipins were added to the medium at desired concentrations when required. Biofilms were allowed to form at 30°C overnight. For biofilm quantification the wells were washed two times with 1 × PBS and 200 µl of 0.1% crystal violet was added to the wells and incubated for 10 min. Wells were washed three times with 1 × PBS, then crystal violet-stained biofilm was solubilized with 250 µl of 30% acetic acid and the absorbance was measured at 550 nm.

**Bacterial attachment to microtiter plates.** To study bacterial attachment and microcolony formation simulation conditions to the previous section were used, but using 10³ bacteria per well constitutively expressing GFP from the plasmid pMF230. Subsequently, the plates were incubated for 3 h at 37°C, observed under the fluorescence microscope and pictures were taken.

**Detection of eDNA and EPS in the extracellular matrix.** Microcolonies induced by the addition of 10-HOME or 7,10-DHOME, obtained as described in the previous section, were stained with 4,6-diamidino-2-phenylindole and concanavalin A-FTTC conjugate at 0.5 µg ml⁻¹ and 50 µg ml⁻¹ respectively. In this case *P. aeruginosa* constitutively expressing mCherry RFP from the plasmid pME440, was used to distinguish green fluorescence (TETC) from the FITC signal. 30 min of incubation with the fluorescent compounds the wells were washed with PBS 1 × and observed under the fluorescence microscope.

**Biofilm assay over A549 monolayers.** A549 human alveolar epithelial cells (ATCC CCL-185) were plated into black clear bottom 96-well-tissue culture plates
at 20,000 cells per well in Dulbecco's modified Eagle medium supplemented with bovine fetal serum and glutamine and allowed to grow up to confluence. Cells were washed with PBS 1× and stored at –80°C until use. Bacteria were resuspended in LB to OD600 = 1. Then 100 μl of the suspension was spotted onto a sterile filter (Whatman) that was placed on the surface of 5 ml of LB agar containing 5% sucrose. The medium was supplemented with 10-HOME or 7,10-DHOME when required. Plates were allowed to grow under this condition for 20 h and then killed. Crops were plated on a drop of PBS on a microscope slide, sealed with a coverslip and observed using an EVOS FL Cell Imaging System. Pictures were captured using the same settings for each picture.

Virulence assay in orally inoculated Drosophila. Drosophila flies were inoculated with the tested strains as described in the previous section, but instead of killing the flies they were incubated at room temperature (~25°C) and fly survival was followed and recorded for 14 days.

Virulence assay in Drosophila inoculated by prickling. Flies were anaesthetized with FlyNap (Carolina) and pricked in the thorax using a needle (BD Ultra-Fine 32 g 5/32 inch) that was loaded with the tested strain of bacteria and homogenates were centrifuged to eliminate fly and bacterial debris and total fatty acids were extracted as described above (see section Purification of diol synthase-derived oxylipins). Extracted samples were analysed first by TLC and then by HPLC/MS (see above sections for TLC and HPLC/MS analyses) to identify the presence of 10-HOME and 7,10-DHOME. To determine the presence of the oxylipins, samples were analysed by the MRM method using mass transitions m/z 297.3/155.1 for 10-HOME and 313.3/141.1 for 7,10-DHOME.

Detection of oxylipins in P. aeruginosa infected flies. Groups of 20 infected flies were homogenized using an Omni THQ homogenizer with disposable Omni Tips. The homogenates were centrifuged to eliminate fly and bacterial debris and total fatty acids were extracted as described above (see section Purification of diol synthase-derived oxylipins). Extracted samples were analysed first by TLC and then by HPLC/MS (see above sections for TLC and HPLC/MS analyses) to identify the presence of 10-HOME and 7,10-DHOME. To determine the presence of the oxylipins, samples were analysed by the MRM method using mass transitions m/z 297.3/155.1 for 10-HOME and 313.3/141.1 for 7,10-DHOME.

Lettuce infection. Individual lettuce leaves taken from the external foliage of romaine hearts (Lactuca sativa L. var. longifolia) were inoculated at 2–3 cm apart intervals into the central nerve of each leaf with the P. aeruginosa strain to be tested (3 μl of a bacterial suspension at an ODH0 = 1.0 in M63 medium) using a 25/5/8 G needle connected to a pipette. When required 10-HOME or 7,10-DHOME, or both were added to the inocula or the control vehicle at a concentration of 0.5 mg ml⁻¹. Inoculated leaves were placed in plastic beakers with the inferior part of the central nervure submerged in a solution of 10 mM MgSO₄ in water. The leaves were incubated for 5 days at room temperature. At the end of the experiment the leaves were evaluated for necrosis around the inoculation points. A dark brownish necrotic area of more than 5 mm of diameter was recorded as a successful infective event.

Statistical analysis. Kaplan-Meier plots of Drosophila flies survival experiments were compared using the log-rank (Mantel-Cox) test. We used 15 flies per condition to be able to detect an effect size proportion of surviving subjects = 0.5 with 99% power using a type I error of 0.01 (calculated using GraphPad StatMate 2). For randomization of Drosophila the flies were anesthetized and distributed to the final experimental container disregarding sex or size. In the case of the lettuce leaves, we were careful to take only external leaves of romaine hearts (up to 2–3 layers of leaves were taken per lettuce plant). Leaves coming from different plants were mixed and randomized, then the leaves were taken and distributed to the final containers. One lab member distributed and inoculated the flies for 10-HOME or 7,10-DHOME, or both were added to the inocula or the control vehicle at a concentration of 0.5 mg ml⁻¹. Inoculated leaves were placed in plastic beakers with the inferior part of the central nervure submerged in a solution of 10 mM MgSO₄ in water. The leaves were incubated for 5 days at room temperature. At the end of the experiment the leaves were evaluated for necrosis around the inoculation points. A dark brownish necrotic area of more than 5 mm of diameter was recorded as a successful infective event.

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**Author contributions**

E.M. and J.C.-G. designed, performed experiments and wrote the manuscript.

**Additional information**

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