A Quorum Sensing Regulated Small Volatile Molecule Reduces Acute Virulence and Promotes Chronic Infection Phenotypes

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Abstract

A significant number of environmental microorganisms can cause serious, even fatal, acute and chronic infections in humans. The severity and outcome of each type of infection depends on the expression of specific bacterial phenotypes controlled by complex regulatory networks that sense and respond to the host environment. Although bacterial signals that contribute to a successful acute infection have been identified in a number of pathogens, the signals that mediate the onset and establishment of chronic infections have yet to be discovered. We identified a volatile, low molecular weight molecule, 2-amino acetophenone (2-AA), produced by the opportunistic human pathogen Pseudomonas aeruginosa that reduces bacterial virulence in vivo in flies and in an acute mouse infection model. 2-AA modulates the activity of the virulence regulator MvfR (multiple virulence factor regulator) via a negative feedback loop and it promotes the emergence of P. aeruginosa phenotypes that likely promote chronic lung infections, including accumulation of lasR mutants, long-term survival at stationary phase, and persistence in a Drosophila infection model. We report for the first time the existence of a quorum sensing (QS) regulated volatile molecule that induces bistability phenotype by stochastically silencing acute virulence functions in P. aeruginosa. We propose that 2-AA mediates changes in a subpopulation of cells that facilitate the exploitation of dynamic host environments and promote gene expression changes that favor chronic infections.

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Introduction

Bacteria excrete small molecules that act as specific chemical signals to positively regulate specialized processes [1], including the production of virulence factors important for pathogenic infection, host colonization, and interspecies microbial interactions [2]. Using interconnected multi-layered regulatory networks, such as quorum sensing (QS) networks, bacteria sense and respond to external and internal bacterial cell signals as well as environmental cues, thereby adapting to exploit target hosts. Adaptation and coordination of gene expression is particularly important for pathogenic microorganisms that need to colonize changing host environments since their ability to sense and respond to host environmental cues is crucial for their survival.

Pseudomonas aeruginosa is an opportunistic human pathogen that causes chronic and acute infections, and is a major agent of morbidity and mortality in cystic fibrosis (CF) patients. Establishment of chronic P. aeruginosa respiratory or wound infections requires a complex adaptive process that mediates essential physiological changes allowing bacterial cells to survive and persist in a dynamic host environment. Although insights into chronic infection pathways have been reported [3–7], the specific bacterial signals that may promote the transition and/or adaptation of known pathogens from an acute to a chronic infection remain unknown.

Many P. aeruginosa virulence factors associated with acute infections are controlled by QS [8]. This pathogen has an extensively studied complex QS-communication network that facilitates cross-talk between organisms and impacts many P. aeruginosa group-related behaviors including virulence [8]. There are at least three known QS systems in P. aeruginosa: two are dependent on the acyl-homoserine-lactone (AHL) QS transcription factors LasR and RhlR [9], and a third is dependent on the 4-hydroxy-2-alkylquinolines (HAQs) LysR-type transcription factor MvfR (multiple virulence factor regulator) [10]. MvfR is critical for P. aeruginosa acute infection through its control of genes involved in the production of secreted virulence factors and in iron assimilation.
**Author Summary**

*P. aeruginosa* causes acute as well as chronic infections in humans. In this paper we report the identification of a *P. aeruginosa* small molecule, 2-AA, that modulates this pathogen’s virulence to promote chronic infections. We show that the synthesis of 2-AA, responsible for the grape-like odor of *P. aeruginosa* cultures and of wound infections, is controlled by the multiple virulence factor regulator (Mvfr) important for virulence in acute infections. 2-AA reduces the production of Mvfr-regulated acute virulence factors, and attenuates acute virulence by negatively fine-tuning the Mvfr regulon activity. Moreover, we show that 2-AA adapts *P. aeruginosa* for chronic infections by promoting mutations in a key acute virulence gene (lasR) and by prolonging bacterial survival. The findings presented here reveal the function of a new Mvfr-regulated molecule, and highlight Mvfr’s importance as a highly promising target for the development of inhibitors that can simultaneously halt acute and chronic infections caused by *P. aeruginosa*, and possibly by other pathogenic bacteria. This study uncovers insights that paradigmatically pave the way for the search of 2-AA-like small volatile molecules that promote pathogen adaptation and establishment of chronic infections caused by foreboding human pathogens.

[10–13]. This regulator controls HAQ signaling and its own activity by positively regulating the expression of genes in the pqsABCDE [14] and phnAB [10] operons. These operons encode enzymes that catalyze the biosynthesis of at least 59 distinct low molecular weight compounds, most of which are structurally related to HAQs. While two of the most abundant HAQs, 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (Pseudomonas Quinolone Signal-PQS) [14–16], function in vivo as ligands that bind and activate Mvfr [14,17], the biological functions of other PqsABCD/PhnAB biosynthetic products remain elusive.

In this study, we show that one of these abundant Mvfr-regulated non-HAQ low molecular weight molecules, 2-aminoacetophenone (2-AA), that is used to diagnose *P. aeruginosa* infections in humans [18], reduces acute virulence by negatively fine-tuning the transcription and synthesis of the Mvfr ligand HHQ, and promotes changes that are critical for pathogen adaptation and important for chronic infection.

**Results/Discussion**

**2-AA Synthesis Is Controlled by Mvfr but Is Not Required for the Activation of Its Regulon**

To determine the functions of the abundant Mvfr-regulated small molecules, we first compared the liquid chromatography/mass spectrometry (LC/MS) total ion chromatograms of culture-free supernatants from highly pathogenic wild-type *P. aeruginosa* (PA14) cells versus those of isogenic mvfr mutant cells. As shown in Figure 1A, the mvfr versus PA14 supernatant lacked HHQ and PQS, as well as three other abundant low molecular weight compounds: the HAQ molecule 4-hydroxy-2-heptylquinoline N-oxide (HQNO) [19], 2,4-dihydroxyquinoline (DHQ) [20], and the non-HAQ molecule 2-AA, 2-AA is a relatively simple, non-HAQ volatile molecule responsible for the grape-like odor of *P. aeruginosa* cultures as well as burn wounds infected with *P. aeruginosa* [18,21]. Along with DHQ and HQNO, 2-AA is also produced and excreted by the HAQs-producing bacterium *Burkholderia thailandensis* [23] (Figure S1). Because HHQ and PQS both induce pqsABCDE expression [14], we investigated whether these three additional abundant molecules also induce expression of these genes. Maximum levels of 2-AA in the cell supernatant vary from micromolar to millimolar range depending on the growth medium used [18,22]. We used Luria-Bertani (LB) broth media in experiments examining the effects of exogenous 2-AA supplementation on pqs operon gene transcription. We chose to use LB broth because it supports lower levels of 2-AA production (37.5 μM = 5 μg/ml Figure 1B) than other media [18]. Using a pqsA green fluorescence protein (GFP)/ASV—transcriptional reporter fusion in a pqsR::H double mutant background that does not produce any of these molecules (Figure S2), we exogenously added to LB media the above molecules. HQNO only modestly induced pqsABCDE expression, while DHQ and 2-AA did not induce pqsABCDE expression (Figure 1C). This finding indicates that unlike HHQ and PQS, 2-AA and DHQ molecules may have biological roles other than activating pqs operon transcription.

**2-AA Down-regulates the Expression of Mvfr-regulated Loci**

The kinetics and dose-dependency effects of exogenously added 2-AA and DHQ on pqsA regulation were examined in greater detail using WT cells carrying the pqsA-GFP/ASV transcriptional reporter fusion. Figure 2A shows that 2-AA, but not DHQ (Figure S3A), greatly reduced pqsA expression, in a dose-dependent manner, with 2-AA achieving the strongest inhibition at 200 μg/ml (1.5 mM). Bacterial growth was unaffected by either 2-AA or DHQ (Figure S3B and C). In light of these results, we focused our subsequent experimental efforts on 2-AA.

We visualized pqsA-GFP expression under a fluorescent microscope in the presence of a range of 2-AA concentrations. Surprisingly, but in accordance with the established dose-dependent effects of 2-AA, only a subpopulation of the cells showed a shut-down of pqsA-GFP activity in the presence of 50 μg/ml (0.375 mM) 2-AA (Figure 2B and C); a statistically significant number of cells had turned off the pqsA expression though some still fluoresced strongly. No fluorescing cells were seen at 200 μg/ml 2-AA (Figure 2B and C). Thus 2-AA appears to promote phenotypic heterogeneity in a genetically ‘homogenous’ population and silence pqs operon expression in a fraction of the cells, suggesting that Mvfr active and inactive cells co-exist to achieve a bistable phenotype [24]. The size of the 2-AA responsive population increased with higher 2-AA concentrations. The need for higher concentrations of 2-AA to produce significant detectable transcriptional changes may be due to the compound affecting only a subpopulation of cells.

2-AA inhibition of Mvfr is likely mediated via negative feedback regulation given that Mvfr controls 2-AA synthesis (Figure 1A). To corroborate our pqsA expression data, we measured levels of HHQ in 2-AA treated cells and found that HHQ production was also inhibited by 2-AA in a dose-dependent manner (Figure 2D). Consistent with the view that 2-AA downregulates the Mvfr regulon, we observed that 2-AA decreased production of pyocyanin (Figure 2E) and pyoverdine (Figure 2F), virulence factors whose synthesis depends on Mvfr. A time course study of 2-AA production (Figure 1B) showed that 2-AA levels did not significantly decrease as occurs for the Mvfr activators HHQ and PQS (Figure 1B), indicating that the production kinetics and stability of 2-AA are distinct from those of the Mvfr activators. Together, this convergence of data strongly suggests that 2-AA has the novel biological activity of silencing the Mvfr regulon.

**2-AA Down-regulates pqsABCDE Operon Expression via Mvfr, and by Targeting HAQ Biosynthesis Enzymes**

To elucidate the mechanism by which 2-AA inhibits the Mvfr regulon, we administered 2-AA together with HHQ to PA14
Figure 1. 2-AA synthesis is controlled by MvfR. (A) LC/MS total ion chromatograms show the relative percentage of the most abundant small molecules in *P. aeruginosa* (PA14) (upper panel) and isogenic mvfR mutant (lower panel) supernatants after 9 h of growth. The most abundant molecules in PA14 cultures that were absent in mvfR mutant cultures were: DHQ (1); 2-AA (2); HQNO (3); and HHQ+PQS (4+5). The abundant peaks shown in mvfR are negligible compared to the amount of HHQ or PQS produced by PA14 since the percentage value on the Y axis is drawn with respect to the most abundant molecule detected. (B) Production kinetics of 2-AA in PA14 supernatants in LB. PA14 growth is shown as OD$\text{_{600 nm}}$ on the secondary Y axis versus time. The chemical structure of 2-AA is shown in the inset. (C) 2-AA is not a MvfR co-inducer. MvfR-dependent *pqsA-GFP(ASV)* expression in the *pqsA:pqsH* mutant with or without exogenous molecules (10 $\mu$g/ml), reported as relative fluorescence versus time, averaged for six replicates.

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isogenic \textit{pqsA::pqsH} double mutant cells, which do not produce any HAQs [14] or 2-AA (Figure S2) but have functional MvfR. The expression of the \textit{pqsA} reporter in \textit{pqsA::pqsH} cells requires activation of MvfR. While exogenous addition of HHQ to these cells induced \textit{pqsA} reporter expression via activation of MvfR, co-addition of 2-AA, at 100 \textmu g/ml (0.75 mM) and above, attenuated this expression in a dose-dependent manner, suggesting that 2-AA may negatively impact the MvfR-regulated operon \textit{pqsABCDE} at the transcriptional level (Figure 3A) via MvfR. Unlike other anthranilic acid analogs, which act on PqsA activity and inhibit the synthesis of MvfR ligands [25], the apparent 2-AA inhibition described here can be regarded as independent of PqsA since the \textit{pqsA::pqsH} mutant was used in these experiments. Moreover, 2-AA did not perturb MvfR protein levels or MvfR ligand stability (data not shown).

To assess whether down-regulation of MvfR activity by 2-AA is due to reduced ligand levels, we engineered \textit{mvfR} mutant cells to...
synthesize HHQ independently of MvfR by constitutively expressing \( \text{pqsABCD} \). These cells produced HHQ in the absence of 2-AA whereas HHQ production decreased in the presence of 2-AA in a dose-dependent manner (Figure 3B), indicating that inhibition of the MvfR regulon can also occur independently of MvfR. This post-transcriptional inhibition could be mediated through interference with ligand biosynthesis.

The \( \text{pqsABBCDE} \) operon gene \( \text{pqsA} \) is required for the synthesis of 2-AA whereas HHQ production decreased in the presence of 2-AA in a dose-dependent manner (Figure 3B), indicating that inhibition of the MvfR regulon can also occur independently of MvfR. This post-transcriptional inhibition could be mediated through interference with ligand biosynthesis.

The \( \text{pqsABC} \) operon gene \( \text{pqsA} \) is required for the synthesis of HHQ (data not shown).

We used \( \text{pqsBC} \) mutant cells with an additional, more sensitive reporter system to further assess the effects of endogenous 2-AA produced by the \( \text{pqsBC} \) mutant on transcription of the \( \text{pqs} \) operon. We fused the \( \text{pqsA} \) promoter to the \( \text{Bacillus subtilis sacB} \) gene that codes for the levansucrase product, which is toxic when cells are grown in the presence of sucrose. The \( \text{sacB} \) gene has previously been incorporated into allelic exchange vectors as a means of counter-selection [23]. As shown in Figures 3C and S4, PA14 cells with the \( \text{pqsA} \) promoter of \( \text{pqsA-SacB} \) in the \( \text{pqsBC} \) mutant was induced by exogenous addition of 10 \( \mu \text{g/ml} \) HHQ where indicated. (D) Concentration of pyocyanin in \( \text{mvfr} \) mutant cells (OD 3.0) over-expressing MvfR or PqsE under a constitutive promoter in the presence or absence of 2-AA (200 \( \mu \text{g/ml} \)). Error bars represent standard deviations of triplicate samples. These experiments were repeated at least three times with similar results.
addition of HHQ, which promotes further induction of the pqs operon (Figure 3C and S4). Importantly, the endogenous level of 2-AA in the pqsABCDE mutant cells in presence of the inducer, had a suppression efficacy comparable to 100 μg/ml of exogenously added 2-AA Figure 3C. These results are physiologically important and consistent with the putative role of 2-AA in down-regulation of MvrR regulon. These results also suggest there may be limited uptake of exogenously added 2-AA thereby requiring addition of higher concentrations of 2-AA to generate a physiological response by exogenously added 2-AA.

The last gene in the pqs operon, pqsE, is essential for pyocyanin production [16,26–27], but dispensable for HAQ synthesis [12,15]. Importantly, 2-AA did not reduce pyocyanin in an mvrR mutant that constitutively expressed pqsE, even though it efficiently inhibited pyocyanin production when MvrR was constitutively expressed (Figure 3D). Hence we can deduce that 2-AA negative regulation of the MvrR regulon is a result of the down-regulation of pqsABCDE operon expression and interference with MvrR activity upstream of PqsE, via inhibition of HHQ biosynthesis.

2-AA Treatment Reduces the Mortality of PA14-infected Flies and Mice

We have shown previously that P. aeruginosa pathogenesis can be studied in Drosophila melanogaster [28–30]. Drosophila shares striking similarities with mammals in terms of its overall physiology and innate immunity signal transduction pathway components [28,31]. P. aeruginosa strain PA14 is highly virulent in flies, causing high mortality; meanwhile, mvrR mutants exhibit reduced virulence, causing reduced mortality [30]. Therefore, since 2-AA negatively regulates MvrR, we first tested whether it reduces P. aeruginosa virulence in Drosophila. As shown in the Figure 4A, flies co-injected with P. aeruginosa and 2-AA succumb to infection significantly later than flies injected with only P. aeruginosa.

To confirm the in vivo efficacy of 2-AA in attenuating the virulence of PA14 cells and validate the above findings in a mammalian model of infection, we used the well-studied acute mouse burn and infection model [32], in which we have shown previously that mvrR mutant cells cause attenuated virulence [10,25]. Indeed, as shown in Figure 4B, 2-AA also attenuated the virulence of PA14 cells in mice. Mice inoculated with PA14 and injected once with 2-AA survived significantly longer than control mice inoculated with PA14 alone. Importantly, this effect could not have been due to reduced PA14 proliferation since 2-AA did not affect the bacterial colony forming units (CFUs) in the muscle tissue underlying the burn injury and infection site (Figure 4C). Importantly, 2-AA greatly hampered the systemic spread of bacteria in the blood of the infected mice (Figure 4D), which is a significant problem in humans with P. aeruginosa infections. Silencing of MvrR regulon activity, and thus of acute virulence factor gene expression, is likely responsible in large part for the reduced systemic dissemination of PA14 cells and attenuated PA14 virulence observed.

2-AA Promotes the Emergence of P. aeruginosa Phenotypes that likely Promote Chronic Lung Infections

One may wonder why a pathogen such as P. aeruginosa would produce a molecule that decreases its own virulence. However, given that successful adaptation of an organism depends on its ability to regulate gene expression in response to its changing environment and thereby maximize its long-term survival, the existence of such a mechanism should not be surprising. There is ongoing debate regarding the role of QS in chronic infection due to inactivation of LasR in P. aeruginosa isolates from CF sputum, while a lack of QS has been proposed to facilitate adaptation during a chronic infection [33]. Since 2-AA down-regulates the expression of QS-related acute virulence functions, we questioned if the same molecule could promote other functions that support adaptation during chronic infections. We therefore examined a variety of phenotypes associated with chronic infection by P. aeruginosa.

A significant fraction of P. aeruginosa cells isolated from chronically infected CF patients accumulate multiple mutations in genes affecting acute virulence functions, including mucA, the regulator of alginate production, the QS regulator LasR, type III secretion system, multidrug efflux pumps, genes involved in motility, and DNA repair genes such as mutS [2,34–38]. Mutations in the lasR gene are particularly notable, not only because they accumulate in bacteria colonizing the lungs of chronically infected CF patients, but also because they have been shown to promote long-term growth and survival of CF isolates in vitro [33]. Therefore, we first examined the frequency of occurrence of lasR mutations in populations of P. aeruginosa grown in the presence of increasing concentrations of 2-AA. We found that PA14 cells exposed to increasing 2-AA concentrations for 10 d accumulated significantly more lasR mutations than untreated cells (Figure 5A). Sequence analysis showed that these lasR mutant lines harbor simple deletions or single nucleotide non-synonymous mutations in the lasR gene that produce inactive LasR protein (Table 1). One of the lasR mutant lines also carried mutations in the intergenic region of the flaQ flagellar gene regulator, which is important for motility, and five clones carried mutations in the intergenic region of mexT, a regulator of multidrug efflux. Mutations in these genes lead to loss of flagellar motility and antibiotic resistance, respectively [39–40]. However, no mutations in the DNA repair gene mutS, in type III secretion system genes (exsA, pscQ, and ppoD), or in the virulence factor gbr or rpaN, which are also associated with chronic infections [2], were identified in any of the lasR mutant lines sequenced. Additionally, 2-AA does not appear to be mutagenic, as it did not stimulate mutagenesis in a standard Ames test (data not shown). We did not identify any mutation in lasR within a day of incubation when silencing of mvrR was observed, suggesting that it’s silencing is not due to the loss of lasR. Nevertheless, the ability of 2-AA to promote lasR mutations was particularly pronounced after 10 d of incubation in isogenic mutS cells (Figure 5A), which are defective in DNA repair function. In contrast to the 2-AA dependent non-linear accumulation of lasR mutants observed in the WT background, a more linear accumulation of lasR mutants in the mutS mutant background is observed (Figure 5A). These data suggest that once a DNA repair mechanism is compromised, as in mutS mutants commonly found in P. aeruginosa cultures from CF sputum [37–38], 2-AA effect is more prominent.

Although the mechanisms and advantages of lasR mutations are not clearly understood, it has been proposed that lasR mutation may provide a growth advantage [33]. We further tested the effects of 2-AA on long-term survival of P. aeruginosa. When grown under the condition of limited iron and aerotaxis, PA14 cells reach stationary phase within 10–12 h and undergo significant lysis after 40 h (Figure 5B). However, addition of 2-AA inhibited entry into the lytic phase and the treated cells remained in stationary phase, promoting bacterial cell long-term survival (Figure 5B). To investigate whether 2-AA can also promote long-term survival in vivo, we developed a non-vertebrate, whole animal persistence model. When grown in the bulk of Drosophila melanogaster. Using the fly-P. aeruginosa feeding assay [28], flies were fed for 2 d with P. aeruginosa WT (PA14), 2-AA non-producer PA14 isogenic mutant strain pqsAc, and the 2-AA producer strain pqsABCDE. Flies were then transferred onto...
bacteria-free food, and the CFUs were quantified at 7 d post-feeding. Bacterial load decreased over time in the pqsA-infected flies, while flies infected with the 2-AA producing strains PA14 and pqsB::Csustained, 3 log greater CFUs (Figure 5C), indicating that 2-AA helps bacteria to survive and persist, and thus promotes better fitness in a dynamic host environment. Due to the longer incubation duration, we cannot rule out the possibility that the persistence seen here may be due to lasR mutation induced by 2-AA. Together with the above in vitro studies, these in vivo results corroborate the view that 2-AA plays a key role in switching cells to a phase that promotes concomitant adaptations that enable P. aeruginosa to persist in a chronic infection.

Synthesis of HAQs Analogs of Burkholderia are also Inhibited by 2-AA

Production of the 2-AA has been reported in plants, invertebrate animals, and vertebrate animals, including humans [41]. Moreover, several plant and human eubacterial pathogens, including Pseudomonas, Bordetella, Burkholderia, Ralstonia, Streptomyces, and Mycobacteria, as well as the Archeae Sulfolobus, encode putative PqsA and MvdR homologues, and in some cases, pqsBCD-related loci [42]. As such, these species might also produce 2-AA and use this molecule to down-regulate their respective virulence functions. Indeed, Burkholderia thailandensis, highly related and often used as a non-pathogenic surrogate for the level 3 pathogen B. pseudomallei, was confirmed to produce 2-AA (Figure S1). We assessed whether this 2-AA could inhibit HAQ biosynthesis in B. thailandensis, and found that exogenous 2-AA inhibited production of the HHQ and HNQ methylated analogs HMHQ and HMNQ (Figure S5A and B) [23], suggesting that 2-AA may perform analogous functions in B. thailandensis as observed in P. aeruginosa. 2-AA producing species may have a selective advantage in mixed microbial communities by adversely influencing the expression or activities of fitness traits of their neighbors.

Here we provide evidence that 2-AA, a small volatile molecule produced by P. aeruginosa (and B. thailandensis), is a QS regulated molecule and an important modulator of acute and chronic virulence functions. Although much work has focused on how QS cell-cell signaling leads to the activation of a plethora of virulence factors, little is known about the silencing of these factors. Degradation of AHL by an AHL acylase [43] and interference of LasR and RhlR binding to their promoter by the transcription factors QscR [44] and RsaL [45] have been shown to down-regulate AHL mediated QS. We recently demonstrated the existence of an interplay between QS systems, their components, and environmental factors that negatively impact HAQs [27]. However, no signaling molecule has been identified to date that negatively regulates acute virulence QS related functions or promotes bacterial adaptation and long-term persistence. Furthermore, no volatile compounds have been reported to be involved in

Figure 4. 2-AA reduces P. aeruginosa virulence. (A) Survival kinetics of flies injected with a PA14 bacterial suspension with (~1.6 ng/fly) and without 2-AA. Data were averaged from two experiments with n(PA14) = 55 and n(PA14+2-AA) = 56. Significance of difference of survival rate was calculated using the log-rank test of the Kaplan Meier survival estimate (p = 0.0056). (B) Survival rates of mice infected with PA14 versus PA14 + 67 µg of 2-AA/mouse. The data correspond to the averages of two independent experiments, with n(PA14) = 20 and n(PA14+2-AA) = 20. Significance of difference of survival rates was calculated using the Kaplan-Meier method (p = 0.03), with a hazard ratio of 1.8932 (95% CI, 1.0664 to 6.0718). (C and D) Bacterial loads in the local and adjacent muscle and in blood were determined for control and 2-AA treated mice, at 20 h post-burn and infection. The statistical significance of the CFU/mg muscle tissue differences between the control and experimental mice were determined using the Mann-Whitney test for independent samples, with p = 0.43 for the difference in the underlying and adjacent muscle in response to 2-AA, and p = 0.045 for the difference in the blood in response to 2-AA. CFU data are presented as log 10. Black PA14, red PA14+2-AA, green 2-AA only.

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QS regulation and virulence. Our results demonstrate that the low molecular weight molecule 2-AA silences the MvfR regulon via a negative feedback mechanism, most likely via repressed synthesis of MvfR ligands transcriptionally and post-transcriptionally, which restricts HAQ-mediated QS signaling and thus acute virulence functions, thereby enabling chronic infection. We propose the idea that there may be QS regulated aerial communication among bacteria, as 2-AA is a QS-regulated molecule and a volatile signal [46]. Such aerial communication may occur and be critical in a microenvironment such as the one found in biofilms and in infected wounds or CF lungs. It is important to note that suboptimal inhibitory concentration of 2-AA affects only a subpopulation of cells, suggesting that MvfR active and inactive cells co-exist causing a bistable phenotype. Similar bi/multi-stability phenotypes have been described as naturally occurring for several well known systems regulated by feedback mechanism, such as regulation of the Lac operon in E. coli, mucoidy in P. aeruginosa, lysogeny of the lambda phage, and transformation competency and sporulation in B. subtilis [24,47]. In our model (Figure 5D), we propose that 2-AA inhibits MvfR via feedback regulation and promotes longer cell survival in vitro and in vivo in flies, presumably by interfering with cell lysis and inducing mutations in lasR. Additionally, the accumulation of lasR mutations that allow adaptation to low oxygen conditions [48] suggest that this mechanism contributes to overall pathogen fitness in chronic infections. Whether, lasR mutations arise in WT cells, MvfR silenced cells, and/or metabolically altered cells, remains to be determined. In corroboration, the Drosophila persistence assay showed that 2-AA promotes bacterial long-term survival and as such fitness.

Assessment of the potential role of 2-AA in the establishment and persistence of chronic infections has been hindered by a lack of clinically relevant chronic infection models. Nevertheless, the Drosophila persistence assay, using 2-AA producing and non-producing isogenic strains, permits us to at least determine the impact of this small molecule in a dynamic host environment that shares many immune functions with the human. Our identification of a P. aeruginosa QS regulated volatile molecule that limits...
virulence and invasive functions associated with acute infections while promoting phenotypic and genetic changes associated with chronic infection elucidates novel avenues for combating bacterial adaptation and survival in the chronic infection environment. These results also suggest that interfering with the MvfR pathway could prevent both acute and chronic infections and that 2-AA synthesis provides a potential target for the development of new anti-virulence drugs in the combateance of P. aeruginosa, and possibly of other pathogenic bacteria such as Burkholderia. This study uncovers insights that paradigmatically pave the way for the search for 2-AA-like volatile small molecules that promote pathogen adaptation and establishment of chronic infections caused by foreboding human pathogens.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Massachusetts General Hospital ( Permit Number: 2006N00093/2). All Procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Bacterial Strains and Growth Conditions

P. aeruginosa strain known as RfA1’ human clinical isolate UCBPP-PA14 (PA14) was used in the present experiments [11]. All of the PA14 mutants described in this paper are isogenic to UCBPP-PA14. The bacteria were grown at 37°C on LB broth or on plates of LB agar containing appropriate antibiotics unless otherwise indicated. The overnight PA14 cultures were grown in LB and diluted the following day in fresh media with or without 2-AA.

Bacterial Growth Curves

Bacterial cells were diluted 1/100 from overnight cultures and grown in six replicates in 20 μl in a 96-well plate (Corning, Inc., Corning, NY). Bacterial growth was measured as a function of optical density at OD600 nm using Tecan F200 automated plate reader (Infinite F200, Tecan Group Ltd, Männedorf, Switzerland). For the pqsA-mutB assay, the bacteria were diluted from overnight cultures to OD600 nm 0.1 in NaCl-free LB with tetracycline (50 μg/ml) and grown (Sigma, USA), with or without 10% sucrose (200 μl). Growth was measured in triplicate every 30 min for up to 25 h, using a Sunrise plate reader (Tecan Group Ltd, Männedorf, Switzerland).

For lysis experiments, the LB medium was treated with Chelex 100 resin 100–200 mesh, sodium glutamate (50 g/l) (BioRad, Hercules, CA) for 1 h followed by filtration with 0.2 μm filters (Corning, NY). Bacterial cells were diluted 1/100 in presence of various concentrations of 2AA. Triplicates (200 μl volumes) were inoculated in 96 wells plates. The plates were incubated and growth was recorded every 30 min for 3 d using a Sunrise plate reader (Tecan Group Ltd, Männedorf, Switzerland).

Table 1. List of clones bearing lasR, mexT, and fleQ mutations.

| Number of clones | Nucleotide position in lasR | Sequence change | Amino acid change |
|------------------|-----------------------------|-----------------|------------------|
| 1                | T452                        | Δ               | Frameshift       |
| 2                | C192G                       | C–G             | Stop             |
| 1                | C174G                       | C–G             | Ala–Gly          |
| 2*(2)            | T215                        | Δ               | Frameshift       |
| 1                | C692T                       | C–T             | Ala–Val          |
| 4                | G661T                       | G–T             | Val–Leu          |
| 1                | T235C                       | T–C             | Cys–Arg          |
| 2*(2)            | 55–59                       | Δ               | Frameshift       |
| 11*(2)           | 67–573                      | Δ               | ΔVal             |
| 2                | 67–573                      | Δ               | Δ168             |
| 17               | Full gene                   | Δ               | Δfull            |

Mutations in the mexT and fleQ intergenic regions are indicated by * and $, respectively. The number in parentheses shows the number of clones bearing the respective mutation.

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Quantification of 2-AA and HAQs in bacterial culture supernatants was performed as described previously [14,49]. The HAQs were separated on a C18 reverse-phase column connected to a triple quadrupole mass spectrometer, using a water/acetonitrile gradient [49]. Positive electrospray in MRM mode with 2×10−5 mTorr argon and 30 V as the collision gas and energy was employed to quantify 2-AA and HAQs, using the following ion transitions: 2-AA 136>91, HHQ 244>159, HHQ-D4 248>163, PQS 260>175, and PQS-D4 264>179. To quantify HAQs, Pseudomonas PA14 cells were grown in LB supplemented with different concentrations of 2-AA (Sigma, USA), with untreated LB being used as a negative control. The culture supernatant was collected at different stages of growth mixed with an equal volume of methanol, and the compounds were analyzed by LC/MS. All samples were analyzed in triplicate.

pqsA-GFP(ASV) Fluorescence Measurement and pqsA-LacZ, β-galactosidase Assay

PA14 or pqsA::pqsH cells carrying pAC37 plasmids with pqsA promoter fused to short-lived GFP {pqsA-GFP(ASV)} [50] were grown overnight in LB supplemented with gentamycin (50 μg/ml). The cultures were diluted in the morning with the test compound at desired concentrations and aliquoted (200 μl) into 6 replicates in a 96-well assay plate (Corning, Inc., Corning, NY). Green fluorescent protein (GFP) fluorescence (excitation at 485 nm, emission at 535 nm) and OD600 nm for growth was measured every 30 min using a Tecan F200 automated plate reader (Infinite F200, Tecan Group Ltd, Männedorf, Switzerland). The cultures were incubated and growth was recorded every 30 min for 3 d using a Sunrise plate reader (Tecan Group Ltd, Männedorf, Switzerland).

pqsB::C

Construction of pqsB::C

The mutant was constructed as described by Leslie et al [52] such that most of the pqsB and pqsC genes were replaced with a Kanamycin resistance marker. The deletion was made from nt 151 in pqsB to nt 456 in pqsC (within the coding regions), leaving 150 bp of the 5’ end of the pqsB coding region and 149 bp of the 5’ end of the pqsC coding region. The mutants were selected on LB plates containing Kanamycin (200 μg/ml).
Visualization of 2-AA Affected Cells

PA14 cells harboring the plasmid pAC37, which contained the pqsA-GFP(ASV) reporter fusion, were grown to mid-logarithmic phase in the presence of various concentrations of 2-AA. The cells were washed in phosphate buffered saline (PBS) and their membranes were stained with FM-64 (Invitrogen) according to the manufacturer’s instructions. Five-microliter aliquots of bacterial cells were spotted onto slides and covered with Poly-L-lysine coated coverslips (Sigma Aldrich, US). The bacteria were visualized using an Eclipse E800 (Nikon) microscope with FM-64-stained membranes visualized as red and pqsA-GFP visualized as green. The pictures were processed using Spot V4.0.9 (Diagnostic Instruments) software.

Plasmid Construction

To constitutively express pqsABCD, a genomic fragment containing pqsABCD was amplified by PCR using pqsABamHI F 5’ CATGGATCCAGGTTTGTTGTTGTTG3’ and pqsDPSacB R 5’CGACTTGACTCAATGCGCCGGTTCAC3’ primers from an H44 cosmid [53] as template. The BamHI and PstI digested PCR product was ligated to a pDN18 vector digested with BamHI and PstI. The construct was introduced into E. coli Top10 (Invitrogen) cells and P. aeruginosa mcrF mutant cells by electroporation.

To construct pqsA-sacB reporter fusion, the pqsA promoter was amplified using the primers 5’GACTATGCGAGCAAGGGTTGTAAAGGTGTGATGGTTGAG3’ and 5’GAAGATCTGACAGAACATCAAAAAGTTTGCA3’ and the PA14 chromosome was used as template. The sacB gene was amplified using the primer pairs 5’GAAACTGTATGAACATCAATGAGGTTTGGG3’ and 5’AAACTGGAGTTGATGAAATAGAAAAATGCCG3’ from pKOBEG-sacB plasmid [54]. PqsA promoter (PpqsA) was then digested with SpeI/BglII and the fragment containing sacB was digested with BglII/PstI. The two fragments were ligated to the CTX (TetR) plasmid digested with SpeI/BglII. The ligated vector was electroporated into E. coli SM10 lambda pir and used to integrate the CTX-PpqsA-sacB plasmid into PA14 chromosome. Rif and TetR plates were used to select for the PA14 CTX-PqsA-sacB clones and the presence of CTX-PqsA-sacB in the PA14 chromosome was further confirmed by PCR.

Pyocyanin Quantification

 Overnight PA14 cultures were diluted to OD_600 nm = 0.05 in 5 ml LB or LB +200 µg/ml 2-AA. The bacteria were grown in triplicate at OD 3.0. Pyocyanin was extracted with chloroform from 5 ml cell culture supernatant and then extracted with an equal volume of HCl (0.2 N); optical density was measured at every 30 min by a Tecan F200 automated plate reader (OD_520 nm). Pyocyanin concentrations were calculated using a calibration curve of fluorescein for a range of pyocyanin concentrations (Sigma Aldrich, USA).

Pyoverdine Quantification

PA14 bacterial cells (200 µl) were grown in 96-well plates in D- TSB medium with a range of 2-AA concentrations. The plates were incubated and pyoverdine production and growth were measured every 30 min by a Tecan F200 automated plate reader (Infinite F200, Tecan Group Ltd., Mannedorf, Switzerland). Pyoverdine levels were measured using excitation at 400 nm and emission at 460 nm. The values were normalized to cell growth (OD_600 nm). Pyoverdine concentrations were calculated using a calibration curve of fluorescein for a range of pyoverdine concentrations (Sigma Aldrich, USA).

Mortality Studies

Fly mortality. Fly maintenance and the infection assays were performed as described previously [29]. For the survival assays, 50–60 flies were infected with a bacterial suspension of 5×10^7 cells/ml (grown in LB to OD_600 nm = 3.0) in the presence (1.6 ng of 2-AA/ml) and absence of 2AA. The bacteria were mixed with 2-AA immediately before injection, and the flies were incubated at 21°C following infection. Fly mortality was assessed over time as described previously [29]. The experiment was performed twice with qualitatively similar results between the replicates. Bacterial inoculations were accomplished via pricking the middle dorsolateral thorax of the fly with a needle previously dipped into the bacterial suspension. The number of inoculated cells was ~100 (2 nl) on average per fly. Statistical significance of survival kinetics was assessed by the Kaplan Meier method.

Mouse mortality. A thermal injury mouse model [32] was used, as described previously [11], to assess bacterial pathogenicity in 6-week-old CD1 mice (Charles River, Boston, MA, USA). The animal protocol was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Following administration of anesthesia, a full-thickness thermal burn injury involving 5–8% of the total body surface area was produced on the dorsum of the shaved mouse abdomen, and an inoculum of 2.5×10^5 PA14 cells in 100 µl of saline or 2-AA solution (67 µg of 2-AA in 100 µl saline/mouse, mixture prepared immediately before injection) was injected intradermally into the burn eschar. Mortality was recorded for 1 week. CFU counts were performed for 5–6 mice per group. Local muscle biopsies underneath the eschar, and from adjacent muscle on either side of the burn eschar, 20 h post-burn and infection were collected and homogenized in 1 ml of 1× PBS. The samples were diluted and plated on LB-agar plates containing rifampicin (50 mg/L). The systemic spread of the bacteria was assessed by counting the CFUs in the blood at 20 h post-burn and infection.

Fly Feeding Infection and Bacterial Persistence

Fly feeding on a P. aeruginosa-containing solution was performed as described previously [28]. Briefly, 5–7-day-old female Oregon- R flies (N=26 per group) were fed for 1 d with a mixture of 0.05 ml of LB bacterial culture at OD_600 nm = 1.8, 1 ml of 20% sucrose, and 4 ml of water. Thus, the feeding mix contained a final concentration of 1% LB, ~5×10^3 bacteria/ml, and 4% sucrose. A sterile cotton ball was placed at the bottom of each fly vial and was impregnated with 5 ml of the feeding mix. The flies in each treatment group were sub-divided into three fly vials (13 flies per vial), sealed with a clean cotton ball, and incubated at 25°C. A day later, the flies were transferred to 50 ml plastic screw-cap tubes (10 flies per tube). The tubes were perforated with a heated 0.9-mm needle to enable aeration, while the caps were perforated with a heated 1.2-mm needle and covered with a 2.3 cm Whatman disc (Fisher scientific, USA). A 0.2-ml volume of 4% sucrose solution was dropped onto the Whatman disc and covered with paraffin to provide food for the flies with minimal contamination. New fly tubes were prepared daily and the flies were incubated at 25°C. CFU counts per fly were measured at the indicated time points by dipping 6–9 flies per time point in 95% ethanol for 3 s and letting them dry out on paper tissues (Kimwipes). Then each fly was ground up using a 1.5-ml tube pestle in 0.1 ml of LB, and 1:10 dilutions were plated on LB plates. Statistical analysis of the CFU data was performed using the Student’s t-test.

Assessment of Mutations

PA14 and mcrS mutant were grown overnight in LB. The saturated culture was diluted 1:10 in triplicate in 4% sucrose with
varying concentrations of 2-AA, or without 2-AA. The diluted cultures were incubated statically at 25°C for 10 d. The cells were then diluted and plated to allow the numbers of total colonies to be counted the following day. After 2 d of plating, the lasR cells were identified by their excessive pyocyanin production. The percent- age of lasR mutants in each sample was calculated and statistical significance was determined by Student’s t-test, assuming equal variance. The lasR mutant colonies were further confirmed by sequencing of the complete lasR gene. In addition, the complete sequences of mexA, mexE, mexS, mexT, mexZ, wspE, βOQ, excD, accC, νF, popD, mutS, νFd, νPo, rhlR, pqsA, pqsB, pqsE, pqsH, and νfR were also determined in these lasR mutant colonies.

Supporting Information

Figure S1 Production Kinetics of 2-AA in Burkholderia thailandensis. Production kinetics of 2-AA at different stages of growth. Growth is shown as OD 600 nm. The error bars represent the standard deviation from 3 replicates.

Figure S2 Levels of 2-AA in various isogenic mutants of PA14. 2-AA levels were quantified by LC/MS and presented as relative fluorescence over time. (B and C). Growth curve of PA14 cells carrying the pqsA-GFP (ASV) in the presence of 2-AA. LC/MS data are averaged for triplicate samples.

Figure S3 DHQ does not affect the expression of the pqsA promoter, neither 2-AA nor DHQ inhibit bacterial growth. (A) Expression of pqsA-GFP (ASV) in the presence of increasing concentrations of DHQ measured as GFP fluorescence and expressed as relative fluorescence over time. (B and C). Growth curve of PA14 cells carrying the pqsA-GFP (ASV). Growth is represented as the OD 600 nm with increasing concentration of DHQ (B) and 2-AA (C) respectively. The values presented are average from 3 replicates.

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Author Contributions

Conceived and designed the experiments: MK LGR. Performed the experiments: MK RH JH YA QL GX VD SM. Analyzed the data: MK ED FL LGR. Contributed reagents/materials/analysis tools: FL LGR. Wrote the paper: MK LGR.

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