Acyl-Homoserine Lactone Quorum Signal Degradation by Soil and Clinical *Pseudomonas sp.*

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Abstract

Acyl-homoserine lactones (AHLs) are signaling molecules that are used by several species of *Proteobacteria* in a process of cell-to-cell communication known as quorum sensing. The production, secretion, and detection of these signaling molecules are used to regulate a variety of microbial group behaviors, such as motility, the production of extracellular enzymes, antibiotics, and virulence factors. This thesis describes the ability for two *Pseudomonas sp.*, a soil - isolate strain PAI-A and a clinical - isolate *Pseudomonas aeruginosa* strain PAO1, to degrade long chain acyl-homoserine lactone quorum signaling molecules, and explores the implications for this degradation activity. *P. aeruginosa* is an opportunistic pathogen that engages in quorum sensing with a long and a short chain AHL: 3OC12HSL and C4HSL and regulates the production of its virulence genes in this way. The soil isolate does not accumulate AHLs, and there is no evidence for its engagement in quorum sensing. Both species degrade long chain AHL via an acylase mechanism in which the molecule is cleaved at the amide bond. Two enzymes, PvdQ and QuiP, encoded by the genes PA2385 and PA1032 of *P. aeruginosa,* were found sufficient for the degradation of long chain AHL, but only the PA1032 gene is necessary for this process. PA1032 is transcribed and its protein product is present during degradation of long chain AHL. Studies of $PAO1_{lagless}$, a variant of *P. aeruginosa* that always degrades long chain AHL, indicate that this strain is broken in the regulation of PA1032. $PAO1_{lagless}$ was found to express the

PA1032 gene throughout planktonic and biofilm growth states, but wild type PAO1 expressed PA1032 locally in the center of biofilm microcolonies. This finding suggests PAO1 may use its ability to degrade one of its two AHLs during this dynamic growth state. Degenerate primers designed from PA1032 of PAO1 enabled the determination of a 2.5 kb putative AHL acylase of the soil isolate. Collectively, these studies of how *Pseudomonas* soil and clinical isolates degrade AHL suggest the diverse ways in which the degradation of acyl-homoserine lactone molecules may be used.

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1. INTRODUCTION

Communication in biological systems is dependent on signals. Whether signals are chemical, auditory, or visual, signal synthesis and presence are as important as signal decay and absence in the initiation and cessation of communication. Some bacteria engage in a process of cell-to-cell communication, known as quorum sensing, where they use chemical signaling molecules to couple their gene transcription to changes in their cell population density. The signaling molecules used by some gram negative *Proteobacteria* are acyl-homoserine lactones (AHLs), and over the last few decades these signaling molecules have been found to coordinate a variety of bacterial group behaviors important for societal, environmental, and medical reasons. Quorum-sensing bacteria produce signaling molecules, such as AHLs, which increase in concentration as bacteria grow in a given area. The bacteria then monitor the concentration of signaling molecules in the local environment as a proxy for cell population density and couple this information to the expression of genes advantageous to express at high cell population densities. In certain bacteria species, AHLs mediate the production of virulence factors in disease of humans and plants (79, 120), production of antibiotics and antifungal agents (5, 128, 159), pigment production (100, 160), motility (45, 58) and biofilm formation (31).

The presence and concentration of signaling molecules in the local environment of quorum-sensing bacteria are important to the regulation of genes that underlie

bacterial group behaviors. For example, the coupling of gene transcription to cell population density is effective only if signaling molecules accurately reflect cell numbers when the environmental conditions are right for quorum sensing. In recent years, there has been interest in the biotic degradation of AHL and the implications of this process for quorum sensing bacteria. In 2000, it was discovered that AHLs are subject to microbial degradation by two bacterial species: *Variovorax paradoxus* (92) and *Bacillus sp.* (40). The bacteria degraded signaling molecules using different mechanisms: via an acylase enzyme that cleaves the molecule at the amide bond, or via a lactonase mechanism in which the homoserine lactone ring is hydrolyzed (40, 92). Since these studies, several microbial species, some of which have known quorum-sensing systems, have been identified to degrade AHL-signaling molecules (Appendix Table 1). *Agrobacterium tumefaciens*, which uses AHL-mediated quorum sensing to regulate conjugal plasmid transfer genes (129), was shown to exit the quorum sensing state using an AHL lactonase in the degradation of its AHL 3OC8HSL at stationary phase (189).

Other bacteria that do not have known AHL quorum-sensing systems also have been found to degrade AHL (40, 70, 97, 117). Interestingly, and relevant to conditions in nature, signal degradation has been found to be carried out by bacterial consortia that degrade AHL synergistically (49) and by natural soil microbial communities that degrade physiological amounts of AHL rapidly (172). In the latter paper, signal degradation is estimated to pose a formidable

challenge to AHL signal accumulation in the soil environment. Certain mammalian cells that are in contact with bacteria, such as airway epithelial cells and colon cells, have also been found to degrade AHL signaling molecules (23), indicating the diverse occurrence of this process.

This thesis describes the degradation of acyl-homoserine lactone signaling molecules by two *Pseudomonas sp*.: a soil - isolate, strain PAI-A, which does not accumulate AHL signaling molecules, and a clinical - isolate and opportunistic pathogen, *Pseudomonas aeruginosa* PAO1, which accumulates AHLs and engages in AHL mediated quorum sensing. These studies were done because relatively little was known about which bacteria have the ability to degrade AHL, the range of signal degrading enzymes, how signal degradation is used by microbes that have this ability, and if and how *Pseudomonas sp.* in particular are able to carry out this process. The mechanism by which *Pseudomonas sp.* PAI-A and PAO1 degrade long-chain AHL signaling molecules is the subject of chapter 2. This chapter, published in *Applied and Environmental Microbiology (AEM)*, includes the identification and study of an AHL-degrading enzyme encoded by the gene PA2385 in *P. aeruginosa* PAO1. PA2385 is sufficient, but not necessary, for the initial cleavage step that takes place at the amide bond of long-chain AHLs. Chapter 3, also published in *AEM,* describes the identification and study of a second enzyme in PAO1, encoded by the gene PA1032 (*quiP)*, which is sufficient and also necessary for the degradation of long-chain AHL. Unlike the PA2385 gene, *quiP* mRNA and its protein product are present when

cells are degrading AHL, and the gene is necessary for AHL degradation to take place. Chapter 4 examines the expression of *quiP* in wild type PAO1, and in $PAO1_{lagless}$, a PAO1 variant that degrades long-chain AHL all of the time. This chapter provides an initial study of the regulation of the *quiP* gene in PAO1. In chapter 5, the full sequence of a putative AHL acylase from the soil isolate strain PAI-A is determined using degenerate primers made from an alignment of the QuiP AHL acylase of PAO1 and other *Pseudomonas sp.* homologs.

BACKGROUND

AHL-MEDIATED QUORUM SENSING

Quorum sensing was first described in the 1970s by Nealson and colleagues as the process used by the marine bacterium *Vibrio fischeri* to regulate light production (110). The components of AHL mediated quorum sensing, which include a signaling molecule producng synthase, a protein that responds to the signaling molecule, and a set of genes with promoters that are regulated directly by signal-bound response regulator proteins and indirectly by signal concentration, were defined in *V. fischeri.* The structure of the *V. fischeri* signaling molecule, 3OC6HSL, was determined by Eberhard et al. (44). This was the first of a family of signaling molecules known as acyl-homoserine lactones, whose members all contain a homoserine lactone moiety, but differ in the length

of and substitutions to the acyl chain. The current range of AHL molecules produced by bacteria have acyl chains ranging from four to 18 carbons (143, 168). The AHL synthase of *V. fischeri* is encoded by the gene *luxI* (46) and the response regulator protein that interacts with the AHL signal is encoded by a gene *luxR* (46). The signal-bound LuxR acts as a transcription factor that binds to conserved promoter sequences of quorum-regulated genes.

In the time since quorum sensing was first described, many microorganisms have been found to produce AHLs and contain gene homologs to *luxI* and *luxR*, and several species of *Proteobacteria* have been described to engage in AHL mediated quorum sensing. Some examples are, the use of 3OC6HSL in the regulation of plant cell degrading enzymes that cause soft rot of vegetables by *Erwinia sp.* (79), the use of 3OC8HSL in the regulation of Ti plasmid transfer in the formation of crown galls by *Agrobacterium tumefaciens* (129, 190), and the use of C6HSL in the regulation of the antifungal agent phenazine production by *P. aureofaciens* (128).

AHL QUORUM SENSING IN *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is a common bacterium present in numerous environments in nature. It can grow virtually anywhere at temperatures up to 42 °C and can respond and adapt well to its environment. *P. aeruginosa* is an

opportunistic pathogen of humans that causes serious chronic infections in the lungs of people with cystic fibrosis (CF) and in immunocompromised people and burn victims. *P. aeruginosa* is dangerous in hospital settings where it can colonize catheters and other devices that come in contact with people (26, 59). Its versatile metabolism, ability to secrete numerous extracellular toxins and form biofilms resistant to antibiotics and traditional treatments makes infections with this bacterium formidable.

P. aeruginosa has two AHL-mediated quorum sensing systems, the *las* and *rhl* systems that consist of synthesis and response to a long chain 3OC12HSL signal and a short chain C4HSL signal. The long chain AHL 3OC12HSL is synthesized and sensed by the LuxI/R homologs LasI/R respectively (120, 122, 123). The 3OC12HSL signal bound to LasR controls expression of *lasI,* the signal synthase via positive feedback (147) and also controls a host of genes that produce virulence factors such as *toxA, lasB* and *lasA,* for elastase synthesis (127). The *las* system also regulates the short chain AHL quorum sensing system, and for this reason the two systems are described to behave in a hierarchy. Signal-bound LasR controls the transcription of RhlR, the response regulator for the short chain signal, and this links the quorum sensing systems (89). Cells do not respond to, or produce more of the C4HSL signaling molecule via positive feedback, until a critical concentration of the long chain signal has accumulated. 3OC12HSL can also bind RlhR and control the *rhl* system post translationally

(125), though C4HSL will not block 3OC12HSL and 3OC12HSL does not activate RhlR (121).

The *rhl* system synthesizes and responds to a short chain AHL C4HSL using the *rhlI/R* gene pair (89, 113, 123, 180). C4HSL-bound RhlR regulates a host of genes including autoinduction of the *rhlI* synthase as well as virulence factor encoding genes *rhlAB* for rhamnolipids, *lasB* for elastase, pyocyanin and proteases (14, 88, 127) and is also a major regulator of genes through induction of the stationary phase sigma factor *rpoS* (88). The AHL-mediated QS systems of PAO1 collectively control a program of genes that include genes for virulence factor production and biofilm formation. Regulation of virulence genes in this way is thought to enable the timing of virulence factor production until cell numbers are high enough to overwhelm host defenses. The complexity of the two interlaced AHL systems most likely enables the bacterium to respond to multiple cues in the environment and express certain genes when conditions are favorable.

The AHL quorum sensing system of *P. aeruginosa* is related to virulence and pathogenicity. Its biofilm state of growth in particular result in long-term lung infections in CF patients (25, 67). The synthesis and presence of the AHL 3OC12HSL have been found in the sputum of CF patients (56, 102, 148), suggesting the involvement of AHL quorum sensing in chronic infection by *P. aeruginosa*. The 3OC12HSL-signaling molecule itself has also been considered

a virulence factor for its ability to stimulate respiratory epithelial cells to produce an inflammatory response (37, 158). AHL synthase or response regulator mutants have been found defective in pathogenicity in several animal models (124, 137, 156), which further implicates the role of AHL-mediated quorum sensing in disease by *P. aeruginosa*.

Numerous studies of gene expression in signal synthase and response mutants and transcriptome studies of PAO1 have estimated approximately 6% of the genome is controlled by AHL signals (146, 169, 176). The influence of AHL on gene expression is complex as both the concentration and temporal presence of the two signaling molecules activates and represses genes (146). Studies show that genes known to be quorum regulated are not necessarily activated or repressed when signal synthase mutants are exposed to AHL signal; some genes are responsive to long-chain signal whereas others are responsive to both long- and short-chain signal and the growth stage of cells also influences regulation (146, 169). The amount of signal also matters for gene regulation, as the elastase gene *lasB* is activated by 10 times more 3OC12HSL than *lasI,* and the 3OC12HSL signal synthase (147) and secretory pathway genes *xcpP* and *xcpR* may be expressed only when virulence factors are induced by AHL (19). *Pseudomonas* is also known to produce other signals such as *Pseudomonas* quinolone signal (126) which interacts with the AHL system (77). The numerous global regulators that have been identified to influence AHL quorum sensing in *P. aeruginosa* also suggest how complicated this process is (1, 134, 165). These

regulators may ensure the proper timing of expression of quorum regulated genes and of integration of environmental cues to regulate quorum sensing.

The study of AHL signal degradation in the soil and clinical *Pseudomonas sp.* shows how this process might function and be relevant to the physiologies of these bacteria that have different life histories. How might this process be used in the contexts of the different environments in which these microbes live?

2. UTILIZATION OF ACYL-HOMOSERINE LACTONE QUORUM SIGNALS FOR GROWTH BY A SOIL PSEUDOMONAD AND *PSEUDOMONAS AERUGINOSA* PAO1

ABSTRACT

Acyl-homoserine lactones (AHLs) are employed by several Proteobacteria as quorum sensing signals. Past studies have established that these compounds are subject to biochemical decay and can be used as growth nutrients. Here we describe the isolation of a soil bacterium, strain PAI-A, that degrades 3-oxododecanoyl-HSL (3OC12HSL) and other long acyl, but not short acyl AHLs as sole energy sources for growth. The SSU rRNA gene from strain PAI-A was 98.4% identical to that of *Pseudomonas aeruginosa*, but the soil isolate did not produce obvious pigments or AHLs or grow under denitrifying conditions or at 42°C. The quorum sensing bacterium *P. aeruginosa*, which produces both 3OC12HSL and C4HSL, was examined for the ability to utilize AHLs for growth. It did so with similar specificity for the degradation of long acyl, but not short acyl AHLs. In contrast to strain PAI-A, *P. aeruginosa* PAO1 growth on AHLs commenced only after extremely long lag phases. LC/APCI+ MS analyses

indicate that PAO1 degrades long acyl AHLs via an AHL acylase and a homoserine-generating HSL lactonase. A *P. aeruginosa* gene, *pvdQ* (PA2385), has previously been identified as being a homologue of the AHL acylase described from a *Ralstonia* species. *E. coli* that expressed *pvdQ* catalyzed the rapid inactivation of long acyl AHLs and the release of HSL. *P. aeruginosa* engineered to constitutively express *pvdQ* did not accumulate its 3OC12HSL quorum signal when grown in rich media. However, *pvdQ* knockout mutants of *P. aeruginosa* were still able to grow utilizing 3OC12HSL. To our knowledge, this is the first report of the degradation of AHLs by pseudomonads or other γ-Proteobacteria, of AHL acylase activity in a quorum sensing bacterium, of HSL lactonase activity in any bacteria, and of AHL degradation with specificity only toward AHLs with long side chains.

INTRODUCTION

Many bacterial species control and modulate their physiology in response to increases in their population densities in a process known as quorum sensing [reviewed in (51, 105)]. Several dozen species of *Proteobacteria* use acyl-homoserine lactones (AHLs) as dedicated signal molecules in this process. A diversity of AHL structures and the enzymes and proteins involved in their synthesis and recognition have been elucidated (60, 107, 119, 144). One of the best-studied quorum sensing species is the opportunistic pathogen *Pseudomonas aeruginosa*, which makes and responds to two distinct acyl-

homoserine lactones: 3-oxododecanoyl-HSL (3OC12HSL, also known as PAI, autoinducer of the *las* QS system), and butanoyl-HSL (C4HSL, also known as PAI-2, the autoinducer of the *rhl* QS system). The two quorum circuits are known to control several physiologies and virulence factors associated with the infection of immunocompromised individuals, such as those with cystic fibrosis (164). Recently, the influence of AHLs on the global regulation of gene expression by *P. aeruginosa* has been examined and found to be vast (146, 169). Clearly, AHL-mediated signaling and signal dynamics are very important to the biology of this species, and it is important to understand issues related to signal stability.

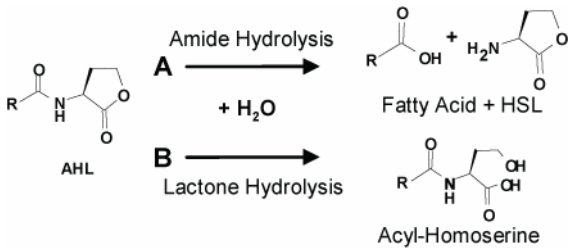


Figure 2.1. Two mechanisms by which AHLs can be inactivated. A, Cleavage of the amide bond by bacterial AHL acylase yields HSL and the corresponding fatty acid (92, 97). The AHL is chemically stable under conditions of non extreme temperature and pH. B, Cleavage of the lactone ring by bacterial AHL lactonase yields the corresponding acyl-homoserine (41, 189). The lactone ring is also subject to chemical hydrolysis; the chemical half life of the ring is ca. $10^{[7\text{-pH}]}$ days. The acyl-side chain diversity of known, naturally occurring AHLs has been reviewed (51).

AHLs are known to be chemically inactivated via alkaline hydrolysis yielding the cognate acyl-homoserine (166), but are stable for weeks or months at pH values of 5 to 6 (142). AHLs are also subject to biological inactivation (Figure 2.1). Similar to abiotic ring hydrolysis, acyl-homoserine can be generated by AHL lactonases encoded by *Bacillus cereus* (and its close relatives) and by *Agrobacterium tumefaciens* (39, 40, 94, 135, 189). None of these strains have been found to further degrade the molecule, and no net oxidation occurs during this inactivation reaction. More complete degradation can occur as shown by an *Arthrobacter* soil isolate that utilizes the acyl-homoserine degradation products of AHL ring hydrolysis reactions (49). In another mechanism of AHL inactivation, the amide bond of AHL is cleaved by AHL acylases during the utilization of quorum signals as growth nutrients by *Variovorax* and *Ralstonia* species (92, 97). HSL is released as a product of these reactions and the acyl-moiety is further metabolized as an energy substrate (90, 92). When a gene encoding an AHL acylase from *Ralstonia*, *aiiD*, was expressed in both *E. coli* and *P. aeruginosa*, it effectively inactivated endogenously produced AHL quorum signals and quenched quorum sensing in the latter strain (97).

A close homologue of the *Ralstonia* AHL acylase has been identified in *P. aeruginosa* PAO1 and in the genomes of several other sequenced pseudomonads (97). This finding raised the possibility that members of this genus, which often produce their own AHLs and engage in quorum sensing, may also be capable of degrading these signals. In this study we detail our isolation

of a soil pseudomonad capable of utilizing 3-oxododecanoyl-HSL as sole energy source, our subsequent discovery that *P. aeruginosa* also exhibits growth on long acyl AHLs, and our examination of *pvdQ*, an AiiD homologue of *P. aeruginosa*, for AHL acylase activity.

MATERIALS AND METHODS

BACTERIAL STRAINS

The bacterial strains used were: *Pseudomonas* strain PAI-A (isolation described below); *Pseudomonas aeruginosa* PA14 obtained from Dianne Newman of Caltech; *Pseudomonas aeruginosa* PAO1 and QSC112a obtained from E. Peter Greenberg of the University of Iowa (176); *Pseudomonas aeruginosa* PAO1 and an in-frame *pvdQ*-deletion/Gm-cassette-replacement mutant of this PAO1 Denver strain, both obtained from Michael Vasil of the University of Colorado Health Sciences Center (114); *Pseudomonas aeruginosa* PAO1 containing pPvdQ-Nde (see below), a constitutive PvdQ expression vector derived from pUCP-Nde (28); *E. coli* DH5α, carrying pUCP18-Nde obtained from Ciaran Cronin of the University of California, San Francisco; *E. coli* BL21PRO containing pPvdQ-PROTet, a pPROTet.E133-derived tet-inducible PvdQ expression vector encoding tetracycline and spectinomycin resistance (see below); and *E. coli*

BL21PRO containing the autonomously replicating plasmid, pPROTet.E133 (Clontech, Mountain View, CA).

MEDIA AND GROWTH CONDITIONS

LB (lysogen broth), amended with antibiotics when appropriate, was used for growth and maintenance of all strains unless otherwise stated. For the 3OC12HSL-dependent enrichment of strain PAI-A and other growth experiments performed on this strain, we modified MES 5.5, the defined medium used to enrich and study *Variovorax* paradoxus VAI-C (92). These modifications included buffering with 5 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) at pH 7.2 and the addition of sodium sulfate (14 g/L) and magnesium chloride (4 g/L). For growth experiments with *P. aeruginosa*, defined medium with MES buffer at pH 5.5 was used as described previously (92) with the exception that it contained sodium sulfate, not sulfite, as S-source, a typographical error in the reported recipe. Unless otherwise noted, the medium was buffered to a pH of 5.5 with 5mM 2-(n-morpholino)-ethanesulfonic acid (MES). Ammonium-free MES 5.5 basal medium was used to examine the utilization of AHLs and HSL as potential nitrogen sources. 100 mM stock solutions of AHLs were prepared by dissolving AHLs in ethyl acetate that had been acidified with glacial acetic acid (0.01% v/v), and stocks were stored at -20 °C. AHLs used in these studies were: n-3-oxododecanoyl-l-homoserine lactone (3OC12HSL; Quorum Sciences, Iowa City

IA), n-3-oxohexanoyl-l-homoserine lactone (3OC6HSL; Sigma), and from Fluka: n-3-butanoyl-DL-homoserine lactone (C4HSL), n-3-hexanoyl-DL-homoserine lactone (C6HSL), n-3-heptanoyl-DL-homoserine lactone (C7HSL), n-3-octanoyl-DL-homoserine lactone (C8HSL), n-3-dodecanoyl-DL-homoserine lactone (C10HSL), n-3-dodecanoyl-DL-homoserine lactone (C12HSL), n-3-tetradecanoyl-DL-homoserine lactone (C14HSL). For growth experiments, the AHL was dispensed into sterile tubes, the ethyl acetate was removed by evaporation under a stream of sterile air, and sterile medium was added to the dried AHL that remained. Stocks of L-HSL (Sigma) were prepared just prior to their use from well-desiccated reagent stored at -20 °C. Thin layer chromatography and ninhydrin staining was used to confirm the purity of HSL stock solutions (75). Cells were grown in 5 ml of medium in 18 mm tubes with shaking at 37 °C unless otherwise noted. AHL molecules are stable for 30 days under the conditions of low pH in our defined medium (142). Unless noted, all other reagents were of reagent grade.

ENRICHMENT AND ISOLATION PROCEDURES

Turf soil was collected in May of 2000 at the University of Iowa. The soil was disrupted and dispersed with a metal spatula, and remaining large particles were removed. One hundred milligrams of the soil were added to 5 ml of the vitamins replete, ammonium-replete enrichment medium containing 1 mM 3OC12HSL as

a sole energy source (see above). After two days of incubation with shaking at 37 °C, a 1% (vol/vol) transfer was made into like medium. This culture was incubated without agitation at room temperature for 3 months after which the culture was transferred once more into 3OC12HSL-containing medium before being streaked for isolation on rich media. Because 3OC12HSL is not soluble at the concentrations employed for growth, isolation was on LB agar with subsequent verification of the AHL-degradation phenotype in the defined liquid medium.

GROWTH STUDIES

Optical density measurements were performed at 600 nm using a Spectronic 20 spectrophotometer. AHLs with side chains of greater than six carbons in length were poorly soluble, so the ethyl acetate carrier was evaporated in the glass tube such that a uniform coating of AHL was beneath the spectrophotometer's light path. When care was taken to vortex tubes gently, the changes in optical density reflecting growth could be monitored accurately. Molar growth yields were determined in the defined media containing the indicated substrate at a final concentration of 1 or 2 mM. For both *Pseudomonas* PAI-A and *P. aeruginosa* PAO1, factors for converting optical density to cell dry mass were determined by growing cells in media containing succinate as the energy source and NH_4Cl as the nitrogen source, washing the cells with 50 mM ammonium acetate buffer (pH

5.5), and drying cell samples to a constant weight. Such determinations were made in quadruplicate.

OTHER ANALYSES

Strain PAI-A was examined for several traits exhibited by *P. aeruginosa*, which was used as a positive control. Fluorescent pigment production was examined using Wood lamp illumination of colonies grown on LB agar. Pyocyanin production was examined in glycerol-alanine medium (50). Production of AHLs in both LB and defined media was examined using previously described radioassay methods (141). A Beckman System Gold HPLC running a methanol gradient was used in the chromatographic analysis of ethyl acetate extracts as previously described (142). Radioactivity was monitored via on line, solid scintillation counting using an in-line HPLC β-particle detector (IN/US Model 3, Tampa, FL). Microscopic examinations were performed using a Zeiss Stemmi 2000 stereomicroscope (low magnification), and a Zeiss Axioplan research microscope (higher magnification, phase contrast and dark field). Nitrate-dependent anaerobic growth was tested using both MOPS-buffered defined media or LB amended to contain 10 mM potassium nitrate dispensed under a 100% N2 headspace in Bellco (Vineland, NJ) 18 mm butyl, serum-stoppered Balch tubes.

ANALYSIS OF CELL-FREE CULTURE AND REACTION FLUIDS

For the initial characterization of the intermediates in AHL degradation, a TLC method was used (75). For a refined analysis, a liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC/APCI+ MS) technique was developed to monitor and quantify the disappearance of AHL and appearance of a number of AHL degradation products. For this analysis, 50 μl samples of culture fluids were taken in triplicate from AHL-grown cultures and were centrifuged at 15,800 · g for 10 minutes. The cell-free culture fluids were stored at -20 ºC until all samples had been collected for analysis. For LC/MS analysis, samples were mixed 1:1 with acetic acid-acidified methanol (1% vol/vol). Dilutions were made using MES 5.5 medium. A C18 ultra aqueous reverse phase column (5 μm bead size, 50 mm x 3.2 mm; Restek no. 9178553) was used. The initial mobile phase was 50:50:1 methanol:water:acetic acid running at 0.5 ml/min isocratically over the first minute after injection and increased (via a linear gradient) to 80:20:1 methanol:water:acetic acid over the following two minutes (Figure 2.3). Using this method, a diversity of AHLs, their corresponding acyl-homoserines, HSL, and homoserine in samples could be quantified from cultures growing in the defined medium buffered at pH 5.5 with MES buffer. These analyses were performed at Caltech's Environmental Analysis Center using a Hewlett Packard 1100 Series LC/APCI+ MS.

Standards over a range of concentrations (125 nM to 1 mM) were prepared using either water or MES 5.5 basal media depending on the origin of the sample, and were diluted 1:1 with acetic acid-acidified methanol (1% vol/vol). For a 20 μl sample injection, the detection limits for standards prepared in minimal medium buffered with MES buffer at pH 5.5 were: 2.5 pmoles for 3OC12HSL and its corresponding acyl-homoserine, 10 pmoles for C10HSL and its corresponding acyl-homoserine, and 100 pmoles for HSL. The limit of detection for standards prepared in water was lower than for those prepared in medium, but the former were only used to quantify AHLs recovered from evaporated ethyl acetate extracts. Ethyl acetate extraction did not recover HSL or homoserine. The accurate quantization of homoserine in standards and samples was complicated by its partial lactonization into HSL, a chemical reaction that occurred after injection into the LC/APCI+ MS instrument. Thus, while homoserine plus HSL pool sizes could accurately be quantified, homoserine itself was determined with less precision using the LC/APCI+ MS method and was usually a slight underestimate.

Nucleotide sequence analysis of the SSU rDNA Strain PAI-A. The nucleotide sequence of a PCR-amplified fragment of the 16S rDNA of strain PAI-A was determined and analyzed using previously described procedures (91, 92). Sequence reads were assembled and edited using Sequencher (Genecodes, Ann Arbor). Multiple sequence alignments, translations, and phylogenetic analyses were performed using the Linux ARB freeware package (www.arb-

home.de/). Phylograms were constructed via Puzzle-Map 5.0 maximum likelihood analyses (145). Tree layout was performed using Treeview 1.6.6 for Windows (116). The 1401 base-pair sequence for strain PAI-A has been submitted to GenBank (AY288072). The GenBank accession numbers for the other sequences presented in Figure 2.2 are as follows: *Pseudomonas* strain CRE 11, U37338 (108); *P. aeruginosa* PAO1, AE004949 (154); *Pseudomonas* strain BD1-3, AB015516; *P. anquilliseptica*, X99540 (38); *P. balearica* U26418 (8); *P. resinovorans*, AB021373 (2); *P. oleovorans*, D84018 (3); *P. citronellolis*, Z76659 (106); *Pseudomonas* strain 273, AF039488 (181); *Pseudomonas* strain B13, AJ272544 (103); *P. nitroreducens*, D84021 (3); and *Pseudomonas* strain CRE 12, U37339 (108).

Cloning and expression of *pvdQ* (PA2385) encoding a putative *P. aeruginosa* AHL acylase. Genomic DNA was isolated from *P. aeruginosa* PAO1 using the DNeasy tissue kit (Qiagen) and used as a template for PCR. The deduced coding region for PvdQ (Gene PA2385; www.*Pseudomonas*.com) was amplified from the genomic DNA using the following primers: 5′-AGGCCAAGCTTATGGG GGATGCGTACCGTACTG-3′ and 5′-GTTATATAGCGGCCGCTAGGCATTGCTT ATCATTCG-3′ (HindIII and NotI restriction sites underlined, respectively), cloned into the appropriately digested expression vector, pPROTet.E133 (Clontech), and transformed into *E. coli* BL21PRO. Recombinant AHL acylase activity was examined as follows. After growth in LB medium containing spectinomycin (50 μg/ml) and chloramphenicol (34 μg/ml), and after gene induction by the addition

of anhydrotetracycline (aTc, 100 ng/ml) at 18 ºC, cells were pelleted and resuspended to a final optical density of 1.2 in MOPS buffered media (pH 7.2) containing 10 μM 3OC12HSL. Recombinant cells that had not been induced with aTc were used as a negative control. Reaction mixtures were incubated at 18 ºC. 150 μl samples were removed at 0, 15, 30, and 60 min and analyzed for AHL disappearance and product appearance via LC/APCI+ MS (see above).

CONSTITUTIVE EXPRESSION OF *PVDQ* IN *P. AERUGINOSA*

For the constitutive expression of *pvdQ* in strain PAO1, the coding sequence was PCR-amplified with the following primers: 5′-AAGAGGACATATGGGGGATGCGT ACCGTACTG-3′ and 5′-CTAAAGCTTGGCTGTGGGCCGCCTCTATGG-3′ (underlines mark NdeI and HindIII restriction sites, respectively). The PCR product was cloned into the *E. coli-Pseudomonas* shuttle expression vector pUCP-Nde digested with NdeI and HindIII (28). The resulting construct, pPvdQ-Nde, was transformed into *P. aeruginosa* PAO1 via electroporation. Since the repression of gene expression from this vector requires LacI, and since wild-type *P. aeruginosa* PAO1 does not encode this repressor, the probable acylase was expected to be constitutively expressed, a prediction borne out after the examination of total cell proteins via PAGE.

RESULTS

ENRICHMENT AND ISOLATION OF A BACTERIUM THAT UTILIZES 3-OXODODECANOYL-HSL AS A SOLE ENERGY SOURCE

An enrichment culture using a 3OC12HSL-containing minerals and vitamins medium became turbid within 48 hours after inoculation with turf soil. No growth was evident in a control lacking energy nutrient. The cells were rods of uniform morphology and were well dispersed in the medium. They did not form clumps, a pellicle, or attach to the glass at the air-medium interface. When the culture was streaked on LB agar medium for isolation, a single, uniform colony morphotype was observed. Pure cultures were obtained after several successive streaks from single colony picks. Growth of a representative isolate, designated strain PAI-A, was confirmed in the 3OC12HSL-containing liquid medium.

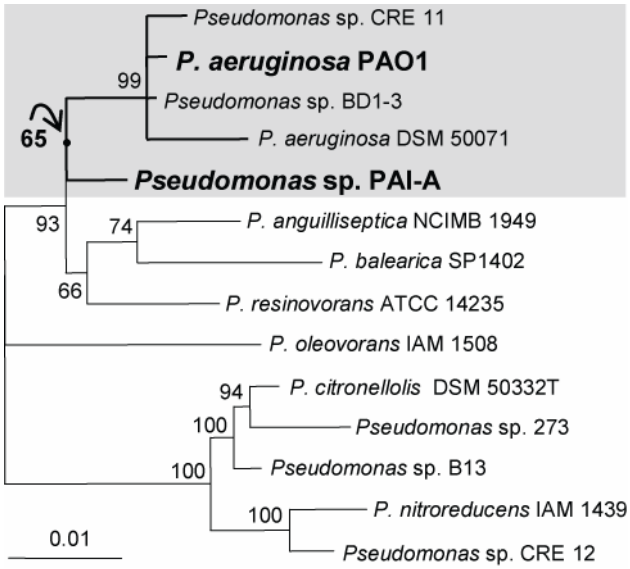


Figure 2.2. Ribosomal RNA-based phylogeny of strain PAI-A. Construction of the phylogram used 1,120 unambiguously aligned nucleotide positions in a 10,000-step Tree-Puzzle 5.0 maximum likelihood analysis (145, 155). The bar represents evolutionary distance as 0.01 changes per nucleotide position, determined by measuring the lengths of the horizontal lines connecting the species. The numbers provide support for the robustness of the adjacent nodes. The arrow points to the short node from which the 5 strains within the shaded box radiate. See methods for GenBank accession numbers.

PHYLOGENETIC ANALYSIS OF STRAIN PAI-A

A nearly complete sequence of the SSU rDNA was obtained. Web-based similarity searches against rDNA in the RDP-II and GenBank databases suggested that strain PAI-A was most closely related to *P. aeruginosa* and several other pseudomonads. The SSU rDNA shared 98.4% and 98.1% sequence identity with *P. aeruginosa* PAO1 and *P. resinovorans*, respectively. By any of the distance (not shown), parsimony (not shown) and maximum likelihood methods employed (Figure 2.1), the SSU rDNA from str. PAI-A clustered most closely with those from *P. aeruginosa* and its close relatives.

PROPERTIES OF *PSEUDOMONAS* PAI-A

Strain PAI-A grew aerobically in both defined media and LB at 30 and 37 °C, but not at 42 °C. Cultures doubled every 35 minutes in defined medium with succinate as the sole carbon source at 37 °C. The isolate grew on a number of tested substrates at both pH 7.2 and pH 5.5; however, cultures did not grow in AHL-containing media at the latter pH. Cells did not grow anaerobically in either succinate-defined or LB media amended with nitrate. Exponentially growing cells sampled from AHL-containing media were vigorously motile rods, 2.5 x 0.8 μm in dimension. The isolate formed creamy-white colonies with spreading edges.

After several days, colonies become smooth, non sticky, leathery, and extremely recalcitrant to disruption with an inoculating loop.

Strain PAI-A did not produce colored or fluorescent pigments in or on LB or glycerol-alanine (pyocyanin-production) media. Cultures did have any aroma of note. Cells did not grow in media containing 30 μg nalidixate/ml. To examine *Pseudomonas* PAI-A for the production of AHL quorum signals, cultures grown in both defined and LB media were incubated with ^{14}C-carboxyl methionine. Since no radioactive peaks were evident after chromatography of the ethyl acetate extract fraction, this isolate does not appear to accumulate AHLs under the conditions examined.

EXAMINATION OF *PSEUDOMONAS AERUGINOSA* STRAINS FOR THE ABILITY TO UTILIZE 3OC12HSL

Two clinical strains of *P. aeruginosa*, PAO1 and PA14, were examined for the ability to utilize 3OC12HSL in defined, ammonia-replete media at both pH 5.5 and 7.2. Both strains grew rapidly at both pHs using succinate as a sole energy source. Although initially it appeared as if neither would utilize the quorum signal as an energy nutrient, the strains began to grow exponentially with a doubling time ranging from 11 to 25 days after several weeks' incubation. The length of the initial lag phase in cultures inoculated using naïve cells (those not previously

grown on AHL) was highly variable, ranging from 10 to 30 days. Curiously, AHL-grown cells that were transferred directly into AHL-containing media did not show significant lags in growth, but those transferred and grown in media containing a different energy substrate followed by re-introduction into AHL broth re-exhibited long lag phases. The issues underlying the long lags exhibited by naïve cells and their subsequent adaptation to growth on AHLs have not been further clarified.

PSEUDOMONAS PAI-A AND *P. AERUGINOSA* PAO1 DEGRADE AND UTILIZE LONG ACYL AHLS

Strains PAI-A and PAO1 grew on a number of AHLs, but no growth was observed with AHLs with acyl side chains shorter than 8 carbons (Table 2.1). When provided with 1 mM C4HSL as a co-substrate in 3OC12HSL-containing media, PAI-A and PAO1 did not degrade detectable amounts of the short chain AHL or exhibit any C4HSL-dependent stimulation of their growth yields. Optical density to dry weight biomass conversion factors were determined for an OD_{600nm} of 1.0 as: 346 + 7 μg/ml for strain PAO1, and 337 + 8 μg/ml for strain PAI-A. The doubling times of strains PAI-A and PAO1 were comparable for many substrates (Table 2.1). *P. aeruginosa* PAO1 utilized both the D and L forms of AHLs, as determined by substrate disappearance and comparison of the molar yields on L and DL forms. Curiously, no increase in molar growth yield was observed as a function of AHL acyl lengths, i.e., when comparing growth on C10HSL, C12HSL,

and C14HSL (for contrast, see Figure 5 of (92)). The AHL molar growth yields for str. PAO1 were poor, e.g., only 49%-74% of that achieved during its growth on the corresponding fatty acids (Table 2.1). Growth on fatty acids revealed the expected, incremental increase in molar yield as a function of increased acyl length. Neither str. PAI-A nor str. PAO1 used HSL as sole or supplementary energy source.

Table 2.1. Growth of *P. aeruginosa* PAO1 and *Pseudomonas* PAI-A on acyl-homoserine lactones and other energy sources[1]

Substrate	*P. aeruginosa* PAO1		*Pseudomonas* str. PAI-A	
	Yield ($g \cdot mol^{-1}$)	**Doubling Time** (hours)	**Yield** ($g \cdot mol^{-1}$)	**Doubling Time** (hours)
C8-DL-HSL	95 ± 4	15.0 ± 2	+, ND[2]	ND[2]
C10-DL-HSL	97 ± 3	14.0 ± 3.8	+, ND[2]	ND[2]
3OC12-L-HSL	76 ± 10	25.0 ± 3	84 ± .2	25.0 ± 3.5
C12-DL-HSL	84 ± 7	14.9 ± 7.5	80 ± 18	16.5 ± 3
C14-DL-HSL	84 ± 12	21.0 ± 5.3	+, ND[2]	ND[2]
Succinate	43 ± 3	0.6 ± .09	49 ± 3.8	0.6 ± .02
Decanoate	130 ± 3	ND[2]	126 ± .5	ND[2]
Dodecanoate	141 ± 4	ND[2]	155 ± 8.4	ND[2]
Tetradecanoate	177 ± 16	ND[2]	198 ± 3	ND[2]

[1]Values represent the averages from at least duplicate cultures. Studies were performed in a minimal salts media buffered at pH 5.5 for strain PAO1 and pH 7.2 for strain PAI-A. Neither strain utilized HSL, homoserine, C4HSL, 3OC6HSL, C6HSL, or C7HSL as an energy source.

[2]ND, not determined.

STRAINS PAI-A AND PAO1 RELEASE HSL AS AN INITIAL PRODUCT OF AHL DEGRADATION

Thin layer chromatography of clarified reaction fluids, harvested from dense cell suspensions of strains PAI-A and PAO1 incubated with 25 mM C12HSL, revealed the AHL-dependent release of ninhydrin-reactive materials. These had the same yellow and purple staining characteristics and migration characteristics as authentic HSL and homoserine, respectively (data not presented). In contrast, cell-free, AHL-free, and cell-and AHL-free controls did not produce ninhydrin reactive materials after similar incubation periods. The TLC data suggest that both strains catalyze the initial step of AHL degradation via an HSL-releasing acylase. Because analyses of biological AHL degradations are less ambiguous at pH 5.5 than they are at pH 7.2, and because strain PAI-A does not grow on AHLs at pH 5.5, *P. aeruginosa* was chosen for further experiments.

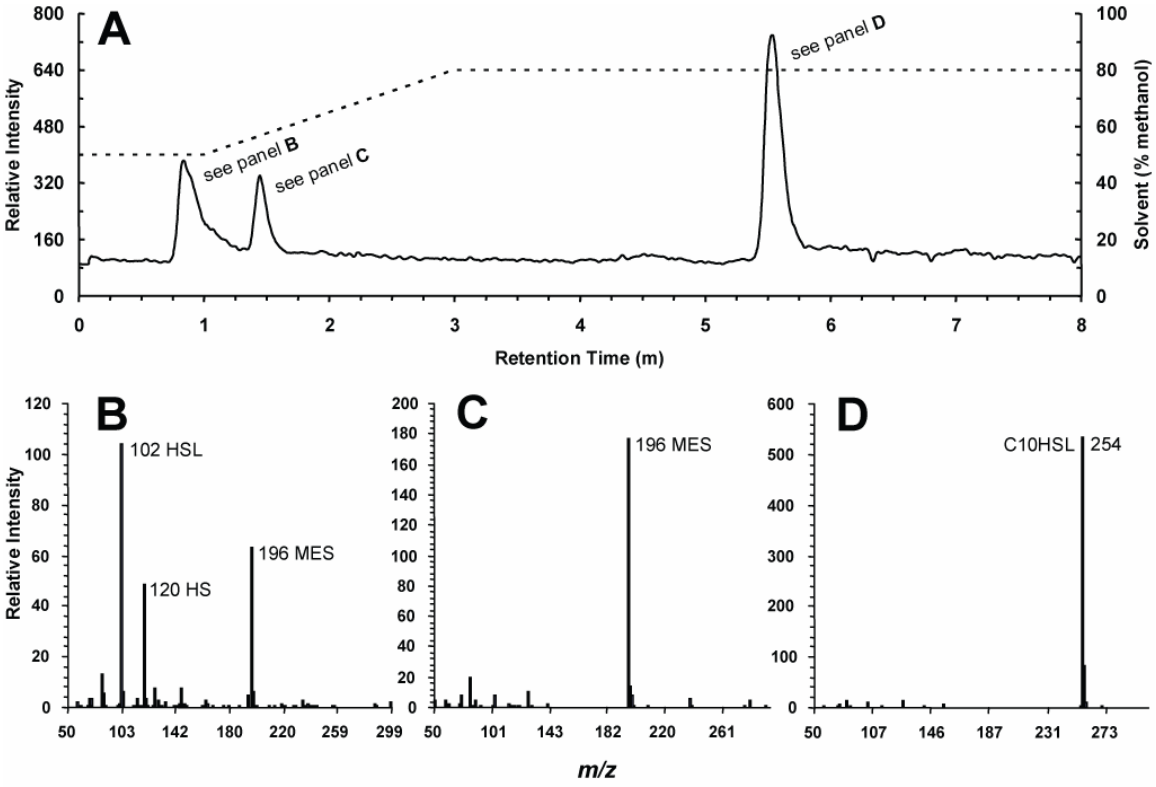


Figure 2.3. LC/APCI+ MS analysis of a cell-free fluid sampled from a *P. aeruginosa* culture utilizing C10HSL as sole energy source in MES 5.5 minimal medium. The details for resolving AHLs from their degradation products and other media components are described in methods. A, Chromatogram showing the separation of homoserine/HSL, MES buffer, and decanoyl-HSL (left axis). The hash marks correspond to changes in methanol-water solvent ratios during the course of the run (right axis). B, The mass spectrum of the first peak resolves homoserine from homoserine lactone; note that the peak tail can overlap with, but can be resolved from the component in the second peak. C, Mass spectrum of the second peak, morpholinoethane sulfonic acid (MES buffer). D, Mass spectrum of the third

peak, decanoyl-HSL. This method can be applied to separate and determine the concentrations of a number of other AHLs and any acyl-homoserine degradation products (not shown).

LC/APCI+ MS analyses confirmed that *P. aeruginosa* PAO1 releases HSL and homoserine as AHL degradation products. A representative chromatogram of cell-free fluid from a C10HSL-grown culture is shown in part A of Figure2.3. Although HSL and HS elute at similar times, both compounds were resolved by extracting the M+1 molecular ions 102 and 120, respectively, from the raw chromatogram (a standard MS practice).

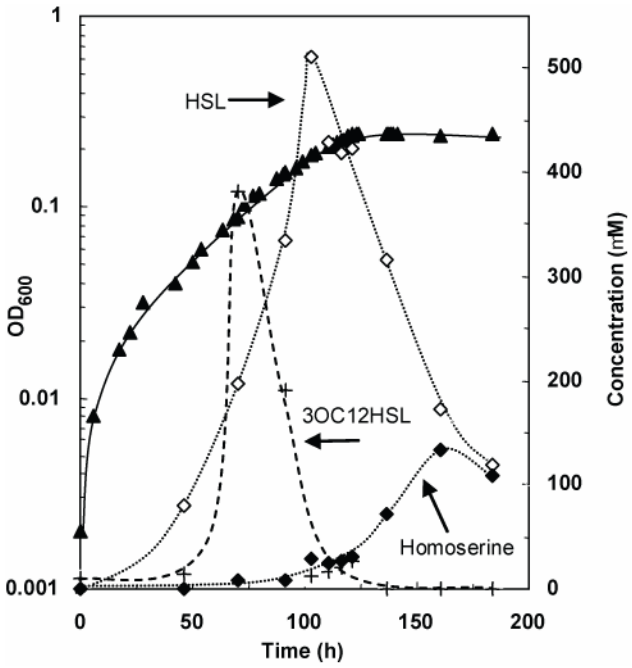


Figure 2.4. Growth of *P. aeruginosa* PAO1 in ammonia-replete MES 5.5 media containing 1 mM 3OC12HSL as sole energy source. Substrate consumption and product accumulation were determined via LC/APCI+ MS. Note that since the 3OC12HSL substrate was poorly soluble at the initial concentrations employed, virtually no AHL was observed in the culture fluid at the time of inoculation. As growth progressed, a transient spike in AHL in solution was observed. HSL accumulated throughout the growth phase but was degraded upon entry into stationary phase yielding a transient intermediate, homoserine. 3OC12-homoserine concentrations remained static throughout the course of the experiment and never exceeded 0.1% of the initial AHL concentration (not plotted). Culture pH was examined and found to be well controlled throughout.

P. aeruginosa PAO1 growth and metabolism of 3OC12HSL as the sole energy source in minimal medium buffered at pH 5.5 with MES is shown in Figure 2.4. By early stationary phase, all of the white, nearly insoluble 3OC12HSL-substrate was consumed such that concentrations of less than 125 nM remained. HSL accumulated throughout the growth phase and reached a maximum of ca. 500 μM just before the onset of stationary phase, after which it decreased to less than 80 μM by 100 hours into stationary phase. Concomitant with the disappearance of HSL, the amino acid homoserine accumulated and then decreased to concentrations below 80 μM by 100 hours into stationary phase (Figure 2.4). Since the culture pH was well-controlled at pH 5.5, and since the half life decay of HSL into homoserine at this pH is on the order of weeks, an enzymatic HSL lactonase, not abiotic alkaline hydrolysis, is most likely responsible for the evolution of homoserine. *P. aeruginosa* did not grow using either HSL or homoserine as a sole energy source in MES 5.5 media. When provided with long chain AHLs as sole sources of carbon and nitrogen, strain PAO1 grew at rates about twice as slowly as cultures utilizing AHL plus ammonium (not shown). Cells did not use either homoserine or HSL as sole sources of energy or nitrogen.

ANALYSIS AND EXPRESSION OF *P. AERUGINOSA PVDQ*, WHICH ENCODES A CANDIDATE AHL ACYLASE

We explored whether the *P. aeruginosa* gene PA2385 (recently named *pvdQ* (87)), which was identified as a close homologue to a gene encoding an HSL-releasing AHL acylase from *Ralstonia* XJ12B (97), might encode a protein with AHL acylase activity and confer the AHL-dependent growth of *P. aeruginosa*. The coding region of this gene was amplified from the genomic DNA, cloned into an expression vector, and expressed in *E. coli*. The polypeptide encoded by the gene was predicted to be post-translationally cleaved into two distinct subunits. PAGE analysis of the total proteins fraction from *E. coli* cells expressing recombinant *pvdQ* revealed small amounts of the two expected subunits (data not shown). The majority of the recombinant protein was recovered as the unprocessed 80 kDa pro-polypeptide. This observation is similar to that noted by Zhang and co-workers for recombinant *aiiD*, the *Ralstonia* AHL acylase (97).

Resting *E. coli* cell suspensions expressing *pvdQ* were incubated with 10 μM 3OC12HSL, which is a concentration relevant to the quorum sensing physiology of *P. aeruginosa*. AHL disappearance and the appearance of HSL and 3OC12-homoserine were evaluated using the LC/APCI+ MS analysis of cleared reaction fluids (Figure 2.5). Within an hour, the AHL disappeared concurrent with the accumulation of stoichiometric amounts of HSL as product. No 3OC12-homoserine accumulation was observed. Cell-free and un-induced cell controls did not catalyze HSL release or the degradation of the AHL over the same time

period. In a pattern similar to the AHL utilization data (Table 2.1), cells of *E. coli* expressing the recombinant acylase catalyzed the HSL-releasing degradation of C14HSL, C12HSL, C10HSL, and C8HSL, but not 3OC6HSL or C6HSL. The effects of the constitutive expression of *pvdQ* in *P. aeruginosa* PAO1 were also examined. In comparison to wild type, which accumulated 3OC12HSL to concentrations in excess of 6 μM during growth in LB at 30 °C (Figure 2.6), cultures expressing the acylase did not accumulate any of this quorum signal above the threshold of detection.

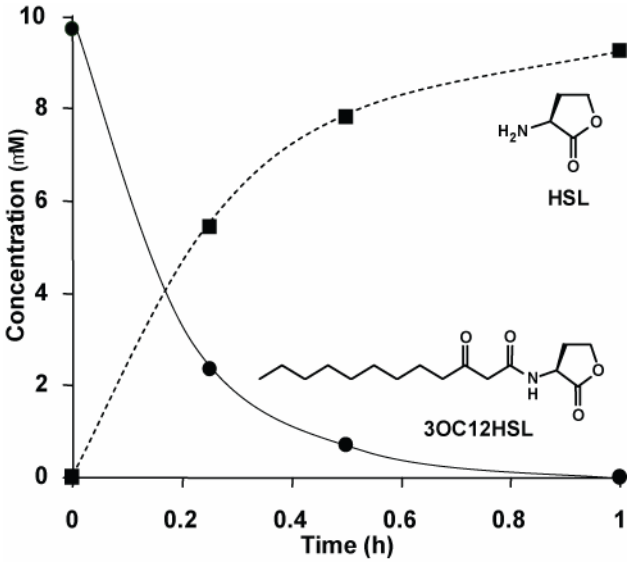


Figure 2.5. *E. coli* cells expressing recombinant *pvdQ* degrade 3OC12HSL and generate stoichiometric amounts of HSL. Substrate disappearance and product accumulation was determined using the LC/APCI+ MS method (Figure 2.3). Induced cells containing plasmid pPvdQ-PROTet were washed and suspended in MOPS (pH 7.2) buffered medium to a final OD_{600nm} of 1.2. AHL degradation and HSL accumulation were not observed over the duration of the experiment in either heat killed suspensions of the same cells, or in no cell controls (not shown).

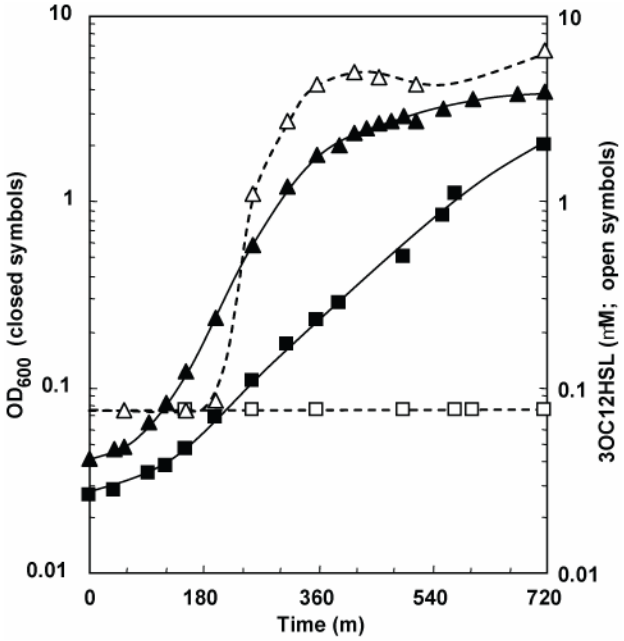


Figure 2.6. Growth and accumulation of endogenous 3OC12HSL by *P. aeruginosa* PAO1 wild type (▲, △), and a recombinant derivative constitutively expressing *pvdQ* (■, □). Because of the organic complexities of LB, sampled cell-free culture fluids were extracted with ethyl acetate before LC/APCI+ MS analysis; the limit of detection for 3OC12HSL was 75 nM and was plotted in place of zero. Cultures were grown at 30 ºC in LB. Under similar culture conditions, a *pvdQ* knockout mutant grew and accumulated 3OC12HSL in parallel with wild type (not shown).

A *pvdQ* deletion-replacement mutant and the strain QSC112a, which carries a Tn5-insertion into *pvdQ*, were also examined for growth in defined media with 3OC12HSL as sole energy source. Surprisingly, both mutants remained capable of growing on long acyl AHL signals. Accumulations of endogenously produced 3OC12HSL by LB-grown cultures of the *pvdQ*-deletion mutant were identical to that of the wild type grown under the same conditions. Evidently, although *pvdQ* encodes an enzyme with an HSL releasing AHL acylase activity specific toward long-chain AHLs, another enzyme must be a significant contributor to the growth phenotype on AHL.

DISCUSSION

We have enriched and isolated a novel soil pseudomonad, strain PAI-A, based on its ability to degrade 3-oxododecanoyl-HSL, a known virulence factor produced and used as a dedicated signal in the quorum sensing physiology of the opportunistic pathogen *P. aeruginosa*. Subsequently, we found that two clinical strains of *P. aeruginosa* were capable of degrading and growing on 3OC12HSL and on other long-acyl AHLs. None of the pseudomonads examined degraded either butanoyl-HSL, the other distinct AHL quorum signal produced by *P. aeruginosa*, or other short acyl AHLs tested. Although closely related to *P. aeruginosa*, str. PAI-A is not a member of that species as it did not produce

pigments, AHLs, grow at 42 °C or anaerobically with nitrate as terminal electron acceptor.

Using a newly refined LC/APCI+ MS technique, the soil and clinical pseudomonads were shown to degrade AHLs via an HSL-releasing activity. These results suggest that these pseudomonads use an AHL acylase in the initial step of AHL degradation, a mechanism previously described in *Variovorax* and *Ralstonia* isolates (92, 97). *P. aeruginosa* accumulated HSL as a transient intermediate during degradation of long acyl AHLs. The HSL was subsequently de-lactonized to form homoserine, which was then consumed (Figure 2.4). Since the culture pH was well controlled in order to preclude chemical hydrolysis of lactone ring, the observed HSL degradation was due to a biological, and not an abiotic, hydrolysis event. Enzymes with HSL lactonase activity have been found in fungal and mammalian biota (74, 86). To our knowledge, this is the first reported demonstration of an HSL lactonase activity in bacteria. Curiously, neither HSL nor homoserine were used as energy or nitrogen sources by *P. aeruginosa*, as they are by *Variovorax* and *Arthrobacter* species (49, 92). It is possible that HSL lactonase and homoserine degrading activities serve as detoxification mechanisms since both of these compounds are known to be toxic to diverse biota (49, 73, 74, 187).

There are notable differences between AHL utilization by the pseudomonads and *Variovorax paradoxus* and *Ralstonia* str. XJ12B. *Ralstonia* was reported to

degrade and grow equally rapidly with short and long acyl AHLs with doubling times of 8.5-10.5 hours on 3OC12HSL and C4HSL respectively (97). *V. paradoxus* was reported to utilize the entire spectrum of short and long acyl AHLs tested, growing most rapidly on 3OC6HSL with a doubling time of 3.5 hours with molar growth yields that corresponded well with the acyl length of a given AHL (92). The pseudomonads examined in this study, however, did not degrade AHLs with acyl side chains shorter than 8 carbons, and no correspondence between molar growth yields and AHL acyl side chain lengths was observed (Table 2.1). Curiously, such a correspondence was observed when the cells were grown with long chain fatty acids.

AHL utilization by *P. aeruginosa* exhibited another key difference from *Variovorax*, *Ralstonia*, and even *Pseudomonas* PAI-A. When cultures not previously grown on AHL were inoculated into media containing long acyl AHL, it generally took one to three weeks before logarithmic growth commenced. No such lag was observed when AHL grown cells were sub-cultured into such medium. This adaptation does not appear to reflect a stable mutation, as long lags returned if the sub-culturing process was punctuated with a transfer into or onto media containing a different energy substrate. The long initial lag time suggests that AHL degradation by *P. aeruginosa* is not immediately induced by the quorum signal, and is not controlled as a function of the catabolic needs of the cell or by cell starvation.

Recently, an acylase from *Ralstonia sp.*, AiiD, which inactivates both long and short chain AHLs was described (97). Heterologous expression in *E. coli* of *pvdQ*, the closest homologue of the *Ralstonia* acylase encoded by *P. aeruginosa*, conferred to *E. coli* AHL-acylase activity specific towards long acyl, but not short acyl chain, AHLs (Figure 2.5). Expression of *pvdQ* in *P. aeruginosa* is known to be well-regulated. Under the monikers of QSC 112a and QSC 112b, *pvdQ* was identified as being a late responder to the 3OC12HSL quorum sensing circuit (176), although gene microarray studies have not provided further support for this observation (146, 169). *pvdQ* has also been found to be iron-regulated (Fur-repressed) and appears to be involved in pyoverdine biosynthesis based on evidence from both microarray and mutagenesis studies (87, 114). Because of its complicated control, we wished to examine the effects of the constitutive expression of plasmid-encoded *pvdQ* in strain PAO1. Remarkably, in striking contrast to wild type, which produced μmolar amounts of this quorum signal 3OC12HSL when grown in rich medium, *P. aeruginosa* constitutively expressing *pvdQ* did not accumulate 3OC12HSL (Figure 2.6).

Surprisingly, two *pvdQ* knockout mutants were able to grow with 3OC12HSL as a sole energy source. This suggests that another enzyme must confer the AHL growth phenotype in str. PAO1. Although some contribution of *pvdQ* to AHL-utilization cannot be ruled out, it seems more likely that this protein is, as has previously been suggested, involved in a yet to be fully described editing reaction during the maturation of the pyoverdine siderophore (87, 114). It remains

possible, however, that 3OC12HSL is subject to inadvertent biochemical degradation by *pvdQ* during times of pyoverdine expression. In addition to PvdQ, the genome of *P. aeruginosa* encodes three other Ntn hydrolase homologues. The AHL growth phenotype may be conferred by one of these or, alternatively, another protein that is not homologous with them.

That *P. aeruginosa* can degrade one, but not the other, of its two AHL quorum signals has revealed a novel AHL degradation apparatus. Signal decay, in addition to providing utilizable nutrients, may play a role in the regulation of the LasR/LasI/3OC12HSL-controlled quorum sensing regulon. It has been noted that the two principal AHL quorum signals of *P. aeruginosa*, C4HSL and 3OC12HSL, are present in the sputum of cystic fibrosis patients and in laboratory biofilms at ratios quite different from those encountered in planktonic, liquid-grown cultures. Sputum and biofilm samples were found to contain significantly higher levels of C4HSL with respect to 3OC12HSL, and this could be a reflection of the biochemical turnover of the latter. In addition to examining this possibility, we hope to identify the loci encoding the AHL acylase and HSL lactonase enzymes involved during AHL-dependent growth and to reveal the regulatory details controlling signal decay by *Pseudomonas* PAI-A and *P. aeruginosa* PAO1 under diverse cultivation conditions.

3. IDENTIFICATION AND STUDY OF QUIP, A SECOND AHL ACYLASE OF PAO1 THAT IS SUFFICIENT TO DEGRADE LONG CHAIN AHLS

ABSTRACT

The relevance of the acyl-homoserine lactone (AHL) quorum signals 3OC12HSL and C4HSL to the biology and virulence of *Pseudomonas aeruginosa* is well investigated. Previously, *P. aeruginosa* was shown to degrade long chain, but not short chain AHLs as sole carbon and energy sources (70). A gene encoding an enzyme with AHL acylase activity, *pvdQ* (PA2385) was identified, but was not required for AHL utilization. This indicated that *P. aeruginosa* encodes another AHL acylase, which we identify here. Comparison of total cell proteins of cultures grown with long acyl AHLs versus other substrates implicated the involvement of a homolog of PvdQ, the product of gene PA1032 for which we propose the name QuiP. Transposon mutants of *quiP* were defective for growth when *P. aeruginosa* was cultured in media containing decanoyl-HSL as sole carbon and energy source. Complementation with a functional copy of *quiP*

rescued this growth defect. When grown in buffered LB, constitutive expression of QuiP in *P. aeruginosa* lead to decreased accumulations of the quorum signal 3OC12HSL relative to the wild type. Heterologous expression of QuiP was sufficient to confer long-chain AHL acylase activity upon *E. coli*. Examination of gene expression patterns during AHL-dependent growth of *P. aeruginosa* further supported the involvement of *quiP* in signal decay, and revealed other genes also possibly involved. It is not yet known under which "natural" conditions *quiP* is expressed or how *P. aeruginosa* balances the expression of its quorum sensing systems with the expression of its AHL acylase activities.

INTRODUCTION

Acyl-homoserine lactone (AHL) mediated quorum sensing is employed by numerous *Proteobacteria* in the control diverse and significant biological activities such as the production of antibiotics (179), the formation of differentiated biofilms (31, 98), cell motility (4, 45) and virulence factor production (43, 120, 138, 150). In recent years, a diversity of isolated *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (39, 40, 70, 92, 97, 118) as well as naturally occurring microbial communities from the environment (49, 172) have been shown capable of degrading AHL signaling molecules. A number of mechanisms and genes involved in signal decay have been identified (40, 70, 92, 97, 117, 118, 189), and

this knowledge has been utilized to exploit or interfere with quorum sensing (41, 188).

The opportunistic pathogen *Pseudomonas aeruginosa* causes infections in immunocompromised individuals and forms persistent lung infections in individuals with the genetic disease cystic fibrosis (132). Factors relevant to the success of *P. aeruginosa* in a diversity of habitats are known to be regulated by its quorum sensing systems, which consist of at least three interconnected signaling systems (for review see (80)). The acyl-HSL-mediated quorum sensing (QS) systems of *P. aeruginosa* are among the best studied of these signaling circuits. These systems employ two distinct AHL signaling molecules, 3-oxo-dodecanoyl-homoserine lactone (3OC12HSL) and butanoyl-homoserine lactone (C4HSL), which are produced and sensed by the products of the lasI/R and rhlI/R gene pairs, respectively (55, 113, 120, 122). The las and rhl quorum sensing systems operate in a hierarchy wherein the las system regulates the rhl system at the transcriptional and posttranslational levels (88, 125). 3OC12HSL, the signaling molecule of the las system, is thus believed to be a “gatekeeping” signal for its role in the *P. aeruginosa* quorum sensing hierarchy (80).

Previously, we demonstrated that *P. aeruginosa* PAO1 and a closely related pseudomonad isolated from soil are able to degrade and utilize long chain (≥ 8 carbons in the acyl chain), but not short acyl chain AHL molecules as sole sources of carbon and energy (70). Quorum signals were degraded via the

acylase mechanism (70, 92). Studies of the gene *pvdQ* from strain PAO1 determined that it was sufficient, but not necessary for long acyl chain AHL degradation as a diversity of *pvdQ* mutants retained the ability to degrade and utilize AHLs (70). This established that *P. aeruginosa* encodes at least one additional AHL degrading acylase enzyme. In the current study, we pursued the identification of other genes and enzymes that form the basis for the AHL degradation and utilization phenotype of *P. aeruginosa* PAO1.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITIONS

The bacterial strains used in this study are listed in Table 3.1. LB (lysogen broth) (9), amended with antibiotics when appropriate, was used for growth and maintenance of strains unless otherwise noted. A defined, freshwater medium buffered at pH 5.5 with 5 mM 2-(n-morpholino)-ethanesulfonic acid (MES) was used when AHL signal was provided as the sole carbon source, as previously described (92). AHLs used in these studies were: n-3-oxohexanoyl-l-homoserine lactone (3OC6HSL, Sigma), n-butanoyl-DL-homoserine lactone (C4HSL, Fluka), n-hexanoyl-DL-homoserine lactone (C6HSL, Fluka), n-heptanoyl-DL-homoserine lactone (C7HSL, Fluka), n-octanoyl-DL-homoserine lactone (C8HSL, Fluka), n-decanoyl-DL-homoserine lactone (C10HSL, Fluka), n-3-oxododecanoyl-L-

homoserine lactone (3OC12HSL, Quorum Sciences, Iowa City, Iowa), and n-dodecanoyl-DL-homoserine lactone (C12HSL, Fluka). 100 mM stock solutions of each AHL were prepared, aliquoted, stored as described previously (92). Decanoyl-HSL (C10HSL) was utilized for most experiments as a less expensive, representative long-chain AHL. Cells were grown in 5 ml of medium in 18 mm test tubes with shaking at 250 rpm at 37 °C unless otherwise noted. Antibiotics were used at the following concentrations: 60µg/ml tetracycline, 34 µg/ml chloramphenicol, 50 µg/ml spectinomycin, 100 µg/ml ampicillin (*E. coli*) and 250 µg/ml carbenicillin (*P. aeruginosa*).

Table 3.1 Bacterial strains used in this study

Species and strain	Characteristics	Source
Pseudomonas aeruginosa		
PAO1	WT	Lab stock (originally from B. Iglewski)
$PAO1_{UW}$	WT	(72)
PAO1 pPA1032-Nde		this study
PAO1 pPA1893-Nde		this study
PAO1 pPA305-Nde		this study
PAO1 pPA2385-Nde		(70)
$PAO1_{UW}$ mutant ID: 33050	PA1032::Tn'phoA/in	(72)
$PAO1_{UW}$ mutant ID: 8713	PA3922::Tn'lacZ/in	(72)
$PAO1_{UW}$ mutant ID: 6619	PA2385::Tn'lacZ/in	(72)
$PAO1_{UW}$ mutant ID: 7831	PA1893::Tn'lacZ/in	(72)
$PAO1_{UW}$ mutant ID: 32876	PA0305::Tn'phoA/in	(72)
$PAO1_{UW}$ PA1032 mutant	ID: 33050 with pPA1032-Nde	this study
$PAO1_{UW}$ PA1032 mutant	ID: 33050 with empty pucp-Nde	this study
Escherichia coli		
BL21Pro		Clontech
BL21Pro pPA1032	Shuttle vector	this study
BL21PRO PRO-Tet PA1032		this study
BL21PRO PRO-Tet PA1893		this study
BL21PRO PRO-Tet PA305		this study

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND PEPTIDE MASS FINGERPRINTING

Cultures were grown in MES pH 5.5 minimal medium with either 1 mM C10HSL or 1 mM sodium decanoate as a sole carbon and energy source. Cell cultures from the different growth conditions were harvested during the exponential phase at approximately OD_{600nm} of 0.2 and the volume of culture that would equate to 1 ml cell suspension at an OD_{600nm} of 1.0 was centrifuged at 5,220 x g for 10 minutes. The cell pellets were resuspended in 150 µl of 1X LDS sample buffer (Invitrogen) with 10% reducing agent (Invitrogen) and heated at 90 ºC for 10 minutes. Samples were centrifuged as above for 10 minutes and 7 µl of the clarified supernatant loaded onto a 10% Bis-Tris MOPS buffered acrylamide gel (Invitrogen). Pre-stained SDS-PAGE standards (BioRad) were used as molecular mass markers. Gels were run with MOPS SDS running buffer in an XCell Surelock system (Invitrogen) according to the protocol of the manufacturer, with the exception that the gel was run at 100 V. Gels were stained with Coomassie G-250 and de-stained with water at 18 ºC with orbital shaking over night. Protein bands of interest were carefully excised under a dissecting microscope using sterile razor blades and transferred to sterile microcentrifuge tubes. The in-gel proteins were analyzed at the Peptide Facility at Caltech using methods as previously described (62, 76, 191), with several modifications. Proteins were extracted from the gel, trypsin digested, and analyzed using both LC-MS/MS with a QSTAR pulsar mass spectrometer (Applied Biosystems) with a

C18 reverse phase 3.5 µm Vydac, 0.1 mm ID x 60 mm L column as well as with MALDI-TOFMS techniques. Protein identification from mass spectra was performed by peptide mass fingerprinting with Mascot (Matrix Science) software. Results from both LC-MS/MS and MALDI-TOFMS analyses were compared.

TRANSPOSON INSERTION MUTANT STUDIES AND CONSTITUTIVE EXPRESSION OF PA1032 IN MUTANT AND WILD-TYPE BACKGROUNDS

P. aeruginosa PAO1 mutants carrying transposon insertions into the PA1032 gene and several other loci of interest were obtained from the Manoil Laboratory (72) (Table 3.1). These mutants were chosen on the basis of the results of the protein analysis performed above, or because they were obvious homologs of those genes. The transposon location within mutants was confirmed using PCR. Presence of a transposon within each gene of interest was confirmed using PCR with flanking primers and transposon orientation determined using transposon internal primers and flanking primers. The mutants were tested for decanoyl-HSL utilization. For starter culture inocula, strains were grown overnight in MES pH 5.5 defined medium with tetracycline and 5 mM sodium succinate as the carbon source. Overnight cultures were inoculated (2% v/v) into medium of the same composition except that 1 mM C10HSL was the sole carbon source. Cultures were incubated for up to 5 weeks at 37 ºC in a shaking water bath and OD_{600nm} measured using a Spectronic 20 spectrophotometer. For insertion

mutants exhibiting no growth or strongly defective growth on AHLs, the impact of complementing the mutation with a functional copy of the gene expressed on a plasmid was examined. The gene PA1032 was amplified from the *P. aeruginosa* PAO1 genome using the primers: 5′-TAGGATACATATGGCCTCGCCA GCCTTCATG-3′ and 5′- AATCTAGATCAGCGAGCGGGAGTGAGCGT-3′ (NdeI and XbaI restriction sites are underlined, respectively) and ligated into the NdeI/XbaI digested *E. coli-Pseudomonas* shuttle expression plasmid pUCP-Nde using T4 ligase (Invitrogen) according to manufacturer's protocol. The resulting construct, pPA1032-Nde, was transformed into *E. coli* and sequenced to ensure that no mutations occurred during PCR amplification. Plasmid pPA1032-Nde was then transformed into wild-type *P. aeruginosa* PAO1 and *P. aeruginosa* PA1032 transposon mutants via electroporation using a Bio-Rad Gene Pulser II electroporator.

Wild type *P. aeruginosa* PAO1 and transposon insertion mutants transformed with the plasmid pPA1032-Nde were tested for their ability to grow utilizing AHL signal molecules by inoculating succinate-grown, overnight cultures of these strains into MES pH 5.5 medium containing 1 mM C10HSL and carbenicillin. Cultures were incubated at 37 ºC and shaken at 250 rpm. Changes in OD_{600nm} were monitored and culture fluids were analyzed using LC/APCI+ MS essentially as described previously (70). Cells were separated from culture medium by centrifugation. Clarified culture fluids were diluted ten-fold to decrease the concentration of salts and buffer in the injected samples. Samples contained a

final concentration of 50% (v/v) methanol and 50% (v/v) deionized water acidified with 0.1% (v/v) acetic acid. 50 µl of sample was injected and analyzed using a Hewlett Packard 1100 Series LC/APCI+ MS outfitted with a 150 mm x 4.6 mm mixed mode reverse phase C8 column (Alltech). The mobile phase was 1 ml/min of 75% (v/v) methanol and 25% (v/v) deionized water acidified with 0.1% (v/v) acetic acid. These conditions enabled improved separation of homoserine lactone (HSL), homoserine (HS), and a variety of chain length AHL from culture fluid over the method described previously (70). Concentrations of HSL, HS and AHL from samples could be determined by comparison to standard curves of the compounds.

Accumulation of naturally produced 3OC12HSL over time by wild type *P. aeruginosa* expressing pPA1032-Nde or the empty control plasmid was examined. 100 ml cultures were grown in LB buffered with 50 mM MOPS (pH 7.0) and incubated at 30 ºC with shaking. 4 ml culture samples were removed over the course of growth, and extracted twice with ethyl acetate acidified with 0.01% (v/v) acetic acid (44, 122). The extract was evaporated to dryness, and the dried residue re-constituted in methanol and subsequently mixed with an equal volume of de-ionized water. Triplicate samples at each time point were analyzed using LC/APCI+ MS as described above.

EXPRESSION OF GENE PA1032 AND TWO OTHER *P. AERUGINOSA* ACYLASE HOMOLOGS IN *E. COLI*

The genes PA1032, PA0305 and PA1893, which were predicted to encode N-terminal nucleophile aminohydrolases (Ntn hydrolase) and are homologs of the known AHL acylase of *P. aeruginosa* encoded by the gene *pvdQ* (PA2385), were amplified from strain PAO1 genomic DNA and ligated into pPROTet.E133 (Clontech) using primers: 1032F: ATTAGAAGCTTATGGCCTCGCCAGCCTTC, 1032R: ATTACTCTAGATCAGCGAGCGGGAGTG, 305F: ACTACAAGCTTATGAAACGCACCCTGAC, 305R: ATTACTCTAGATCAGCGCTTCGGCTCCAG, 1893F: ACTACAAGCTTATGTCGAAGAACGCACG, 1893R: ATCACTCTAGATCATGGTCGTGGCTCGC (HindIII and XbaI restriction sites are underlined for forward and reverse primers, respectively). After ligation, the resultant plasmids were electroporated into *E. coli* BL21Pro (Clontech). *E. coli* BL21Pro cultures containing the plasmid constructs were induced to express the inserted genes with 10 nM anhydrotetracyline overnight at 16 ºC upon reaching an OD600 of 0.5. SDS-PAGE analysis of the protein profiles of induced versus non-induced cultures revealed the over-expression of polypeptides of approximately 90 kDa. This mass corresponds to the pre-modified propolypeptide predicted to be generated by each of these three Ntn-hydrolase homolog encoding genes; however, the presence of the two subunits that would be predicted from the proper processing of the pro-polypeptide was not readily apparent in SDS-PAGE analysis. To assay for AHL acylase activity, induced and non-induced cultures

were concentrated to an OD_{600nm} of 1.2 in 1 ml of MOPS pH 7 medium amended with each of a various chain length AHLs ranging from C4HSL to C14HSL. Reaction mixtures were incubated at 30 ºC with shaking at 250 rpm. 50 µl samples were removed over time, clarified by centrifugation, and examined for AHL, HSL, acyl-homoserine, and HS content using LC/APCI+ MS as described above.

TRANSCRIPTOME ANALYSIS USING AFFYMETRIX GENECHIP MICROARRAYS

Wild type *P. aeruginosa* PAO1 was grown from freezer stock on LB agar and single colonies that developed were picked and grown overnight in MES pH 5.5 medium with 5 mM succinate at 37 ºC. 1 ml of overnight cell culture was inoculated into flasks containing 15 ml of sterile MES pH 5.5 medium that contained either, 3 mM C10HSL, 8 mM sodium succinate, or 3 mM sodium decanoate as carbon sources. Cultures were grown at 37 ºC and shaken at 250 rpm. Cell cultures incubated with AHL signal as the sole carbon source began growing on AHL signal after a long lag period of approximately three weeks, as previously described (70). Once cultures began growing exponentially, they were transferred once into fresh medium of the same composition and allowed to continue growing under the same conditions. Upon reaching exponential phase with an OD_{600nm} of 0.4, 10 ml samples of culture from all substrate conditions were processed for RNA. RNA in all culture samples was stabilized immediately

with RNA Protect Bacterial Reagent (Qiagen) and processed according to manufacturer's protocol. RNA was extracted from cell cultures using the RNeasy kit (Qiagen) and prepared for hybridization to GeneChips as previously described (133, 146). Hybridization, washing and scanning of Affymetrix GeneChips was performed at the University of Iowa Genome Center using an Affymetric fluidics station. Genechips were performed in duplicate and data analyzed using Microarray Suite software. We report, as arbitrarily significant, gene activity changes showing greater than five fold up-or down regulation.

RESULTS

IDENTIFICATION OF *P. AERUGINOSA* PROTEINS PUTATIVELY INVOLVED IN AHL-DEPENDENT GROWTH

SDS-PAGE analysis and subsequent peptide mass fingerprinting enabled the identification of three proteins of 60, 70, and 90 kDa in mass that were readily apparent and consistently present in cultures growing on decanoyl-HSL, but not in cultures growing on the corresponding fatty acid, decanoate, as sole source of carbon and energy (Figure 3.1). These three protein bands are not obvious in either 1. cultures grown with decanoate that have been inoculated with AHL-grown cells, 2. cultures grown with decanoate in the presence of 1 mM C10HSL, or 3. cultures grown with decanoate in the presence of 10 μM 3OC12HSL, or 10

μM C4HSL, or a combination of both of these *P. aeruginosa* quorum signals (data not shown). Peptide mass fingerprinting identified the proteins with 60 and 70 kDa in mass as the products of *P. aeruginosa* PAO1 genes PA3922 and PA4022, respectively. Gene PA3922 encodes a "hypothetical protein" that is highly conserved among several pseudomonads but that is found in few other bacteria. It has no known function and no known obvious structural motifs except for a 25-residue secretion signal sequence (http://www.cbs.dtu.dk/services/SignalP/). Within the *P. aeruginosa* genome, PA3922 is a close homolog of PA3421 (sharing 69% AA identity), and the flanking genes in their respective gene clusters are quite similar to each other as well. The second of the three, the product of gene PA4022, is predicted to be an alcohol dehydrogenase based on homology. The PA4022 protein is most likely localized to the periplasm as indicated by the expression of alkaline phosphatase from translational fusions at this locus described in a previous study (72). The identification of the third, 90 kDa protein became especially intriguing. With LC-MS/MS analyses, 17 peptides generated from this excised 90 kDa protein band were strongly matched, with 21% total coverage, to the predicted protein product of gene PA1032. PA1032 was identified from independent MALDI-TOF/MS and LC-MS/MS analysis of the in-gel protein over-expressed by *P. aeruginosa* degrading decanoyl-HSL. This locus had previously been annotated as a "putative penicillin amidase" (154). To our knowledge, PA1032 has not been previously studied by others. PA1032 is the final gene of a putative gene cluster that consists of genes PA1033, which is a distant homolog of glutathione S-

transferases, and PA1034 and PA1035, which both encode hypothetical proteins of unknown function (www.pseudomonas.com). The product of PA1032 was particularly intriguing as it is one of four Ntn-hydrolase homologs encoded by the *P. aeruginosa* genome, one of which is PvdQ (PA2385), a previously identified AHL acylase (70). PA1032 shares 21% amino acid identity with PvdQ and 23% amino acid identity with AiiD, the AHL acylase from a strain belonging to the genus *Ralstonia* (97), with the conserved regions predominantly in the signal sequence, α and linker regions but less in the β-subunit (GeneDoc: http://www.psc.edu/biomed/genedoc/). PA1032 is predicted to be secreted into the periplasm based on its signal sequence (http://www.psort.org/psortb/), and for its similarity to the Ntn-hydrolase family of proteins, is predicted to be cleaved into 4 peptides: a signal sequence, two peptides corresponding to the α and β subunits of the natural enzyme, and a spacer peptide. In our experience with the over-expression of this gene, much unprocessed propolypeptide remains. Its expression during the AHL-dependent growth of strain PAO1, coupled with its homology to previously identified, functional AHL acylases, lead us to pursue the study of PA1032 with the hypothesis that this gene encodes the second AHL acylase of *P. aeruginosa* that we had previously postulated to exist (70).

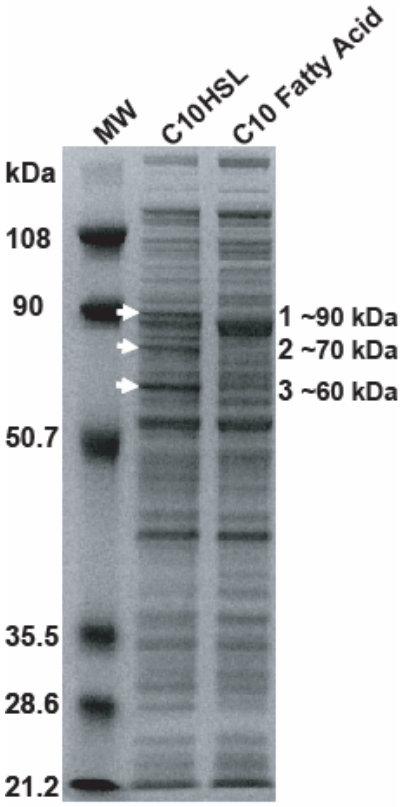


Figure 3.1. Protein profiles of cells growing on decanoyl-HSL (C10HSL) compared to cells growing on the corresponding fatty acid. Arrows indicate three readily apparent proteins specific for growth on C10HSL. The protein bands 1-3 were identified using peptide mass fingerprinting and LC-MS/MS as the products of genes PA1032, PA4022 and PA3922, respectively.

PHENOTYPIC ANALYSIS AND COMPLEMENTATION OF PA1032 TRANSPOSON INSERTION MUTANTS

Strains of *P. aeruginosa* PAO1 carrying transposon insertions into gene PA1032, into three other Ntn-hydrolase encoding genes *pvdQ*/PA2385, PA1893, and PA0305, as well as into gene PA3922, whose protein product was present during AHL degradation (see above) were examined for their ability to grow utilizing decanoyl-HSL as a growth substrate. The growth phenotype of strains carrying transposon insertions into gene PA4022, which encodes another peptide of interest (identified above), were not analyzed as we were unable to confirm the position of the insertion, i.e., after obtaining the relevant strains from UW-Seattle. Strains carrying transposon insertions into either gene PA1032 or PA3922 exhibited defective growth on decanoyl-HSL in comparison to the wild type while strains carrying insertions in the other genes exhibited no detectable phenotypic variation (Figure 3.2 A). The PA1032 mutant did not appear to grow at any rate or to any yield during the five week incubation time. The mutant PA3922 strain grew more slowly and reached at most one-fourth of the yield of the wild type. These results implicate genes PA1032 and PA3922 in the AHL dependent growth phenotype of *P. aeruginosa*. Moreover, it appears that gene PA1032, and not the other genes encoding the other three Ntn-hydrolase homologs, likely encodes the AHL acylase activity observed during the growth utilization of AHLs.

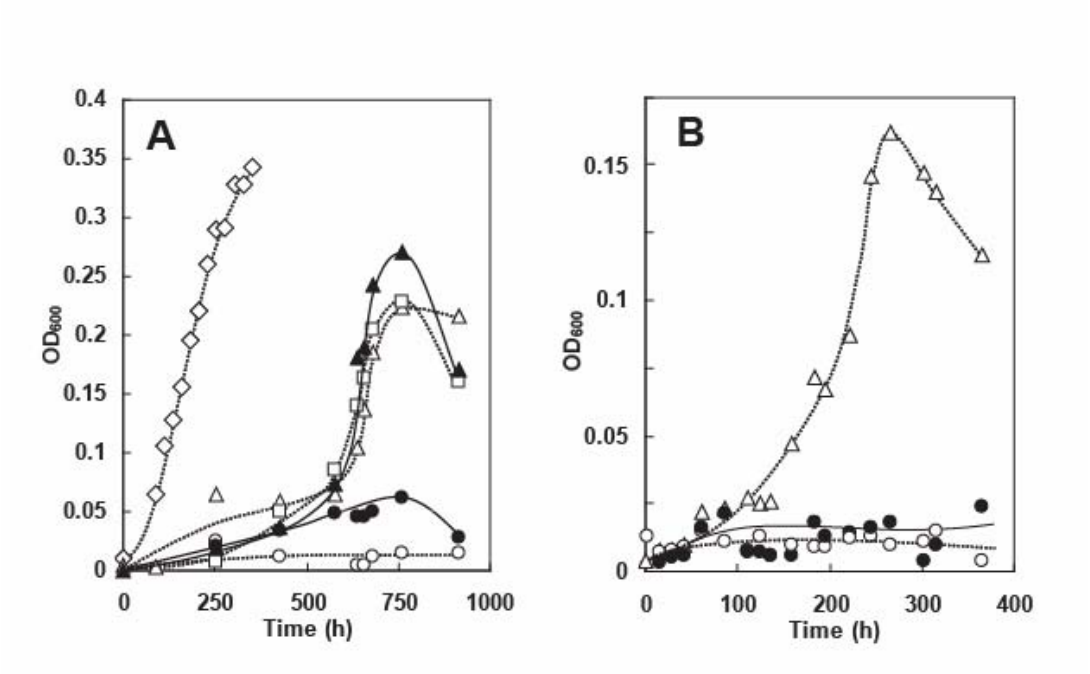


Figure 3.2. AHL growth characteristics of several transposon insertion mutants of interest. A, Comparison of growth utilization of AHLs by the UW-Seattle wild-type strain PAO1 and five insertion mutants derived from it (72). Wild type (◇), PA0305 insertion mutant (□), PA1893 insertion mutant (△), *pvdQ* (PA2385) insertion mutant (▲), PA3922 insertion mutant (●), and *quiP* (PA1032) insertion mutant (○). B, Complementation of the *quiP* (PA1032) transposon insertion mutant with a functional copy of the gene expressed from pUCP-Nde (△), versus two controls: the uncomplemented mutant (○) and the same mutant containing pUCP-Nde without the gene insert (●). Each growth curve is one representative of the results of at least 3 replicate experiments.

A plasmid that constitutively expressed a functional, wild-type copy of PA1032 was introduced into the PA1032 transposon insertion mutant. The AHL degrading capability of the mutant was complemented by the plasmid expressing PA1032, but not by the plasmid that contained no insert (Figure 3.2 B). Interestingly, complemented mutants that constitutively expressed the product of PA1032 from a plasmid degraded AHLs without lag. In this and our previous study (70), naïve *P. aeruginosa* cells (those not started with an AHL grown cell inoculum) were observed to exhibit a lag of many days to several weeks before exponential growth and AHLs utilization commenced. The introduction of the PA1032 expression construct into naïve *P. aeruginosa*, or the PA1032 transposon mutant, or the PA3922 transposon mutant conferred the ability for these strains to grow and utilize long acyl chain AHLs without any significant lag. This result suggests that the control and regulation of gene PA1032 underlies the long lag periods previously observed. Based on its involvement in the inactivation and utilization of AHLs and contribution to the catabolic and anabolic needs of the cell, we give gene PA1032 the name *quiP*, for the quorum signal utilization and inactivation protein that it encodes.

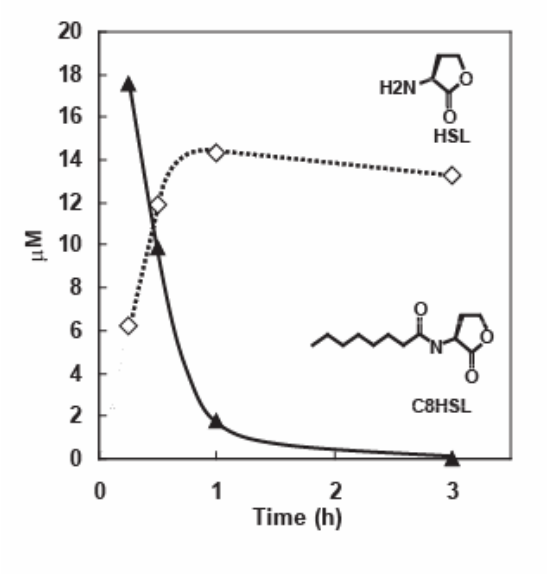


Figure 3.3. ***E. coli*** **cells expressing recombinant** ***quiP*** **(PA1032) degrade long chain AHLs and generate stoichiometric amounts of HSL. Graph shows degradation of a representative long chain AHL, C8HSL. C8HSL substrate disappearance and HSL product accumulation were determined using LC/APCI+ MS. Not plotted: recombinant** ***E. coli*** **cells expressing PA1032 degraded C7HSL, C8HSL, C10HSL, C12HSL, 3OC12HSL and C14HSL, but did not degrade C4HSL, C6HSL or 3OC6HSL.**

HETEROLOGOUS EXPRESSION OF *QUIP* CONFERS UPON *E. COLI* LONG-CHAIN AHL ACYLASE ACTIVITY

Expression of *quiP* from the inducible plasmid pPROTetE133 (Clontech) in *E. coli* resulted in the production of a 90 kDa protein corresponding to the expected mass of the unprocessed propolypeptide as determined by SDS-PAGE (data not shown). The smaller α and β subunits that would be derived from the properly processed propolypeptide (65), were not visible in SDS-PAGE analysis. Nevertheless, some fraction of the QuiP pro-polypeptide was apparently processed to maturity, as the recombinant *E. coli* cells gained the ability to catalyze the degradation of 20 µM C8HSL, generating nearly stoichiometric amounts of HSL as a product of the reaction (Figure 3.3). Such cells degraded all AHLs tested having side chains of $\geq$ C7 carbons in length (C7HSL, C8HSL, C10HSL, C12HSL, 3OC12HSL, and C14HSL), also releasing HSL as a product (data not shown). The results confirm that *quiP* encodes a protein with AHL acylase activity. The same recombinant *E. coli* cell preparations did not degrade C4HSL, C6HSL or 3OC6HSL, indicating that the enzyme has specificity for long but not short AHLs.

Constitutive expression of *quiP* in wild type *P. aeruginosa* PAO1 alters the accumulation of its long-chain AHL quorum signal, 3OC12HSL, when grown in rich media. As noted above and previously (70), signal decay by *P. aeruginosa* appears to be tightly controlled and non-trivial to upregulate experimentally. To

examine the influence of the constitutive expression of *quiP* on quorum signal accumulations, we grew *P. aeruginosa* cells that contained a QuiP expression plasmid and a variant of strain PAO1 that contained the insert-less control plasmid in the undefined, standard laboratory media, LB, buffered at pH 7. Over time, we quantified the accumulation of endogenously produced 3OC12HSL using LC-MS (Figure 3.4). For reasons that are unclear, the growth rate of the control culture containing the empty plasmid was reproducibly inferior to that of the QuiP-recombinant strain. Both cultures accumulated similar levels of 3OC12HSL during the initial seven hours of growth. However, upon reaching an optical density of OD600 of 0.2, the standing pool size of 3OC12HSL in cultures of QuiP recombinant strains decreased dramatically (Figure 3.4). Meanwhile, the pool size in the control strain continued to increase. The initial accumulation followed by the decrease of 3OC12HSL observed in QuiP recombinant cultures was reproducible, but the mechanism of regulation remains unexplained. Whether the initial accumulation phase relates to issues of inadequate expression, delays in propolypeptide maturation, or reflects other unknowns, such as a cue-mediated post translational activation of the protein, remains to be determined.

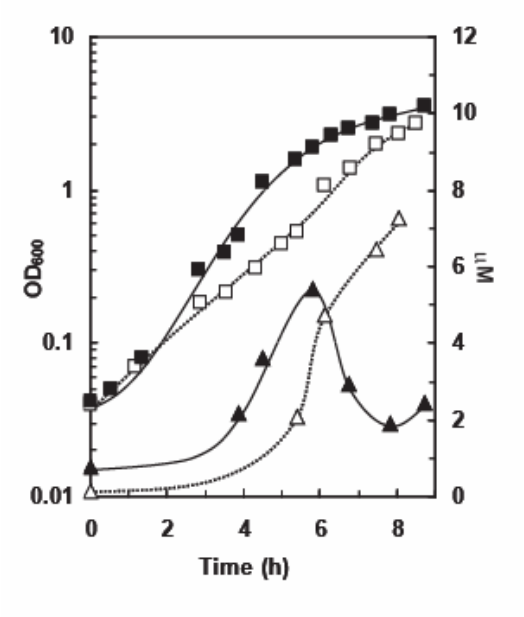


Figure 3.4. Impact of the constitutive expression of *quiP* (PA1032) on 3OC12HSL quorum signal accumulation by recombinant *P. aeruginosa* cells grown in rich medium. *P. aeruginosa* constitutively expressing *quiP* accumulate less endogenously produced 3OC12HSL than cells that contain the plasmid alone. *P. aeruginosa* strains were grown in LB buffered with 50 mM MOPS at pH 7. Optical density 600 nm (■) and AHL accumulation (▲) by wild-type strain PAO1 expressing the acylase from plasmid pPA1032. Growth (□) and signal accumulations (△) by wild-type strain PAO1 containing a control plasmid without insert.

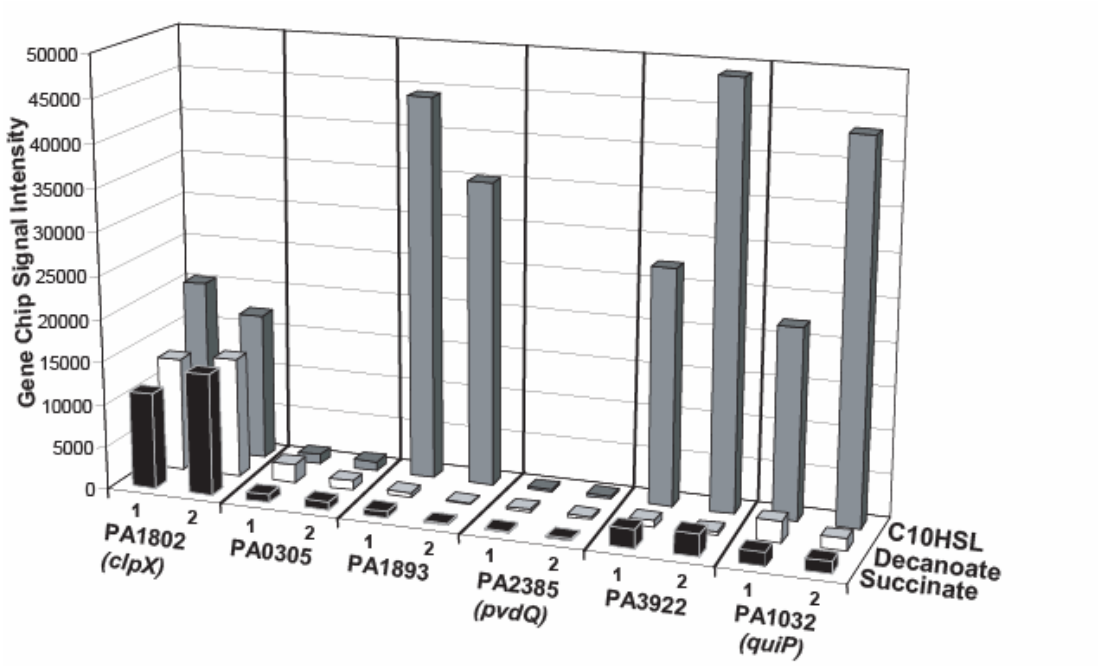


Figure 3.5. Examination of *P. aeruginosa* mRNA expression levels of four acylase-encoding genes and other genes of interest in cultures grown utilizing different carbon sources. GeneChip microarrays were employed to examine gene expression in cultures grown utilizing either decanoyl-HSL (gray bars), its corresponding fatty acid, decanoate (white bars), or succinate (black bars) as sole carbon source. The signal intensity values are quantitative measures of the relative amount of mRNA expressed for each gene under the given growth condition. The clpX protease gene was used as a control, as it is known to be expressed at similar levels under most growth conditions. A comprehensive list of genes that were up or down regulated by at least 5-fold during decanoyl-HSL utilization versus growth on decanoate is provided in Table 3.1 in the Supplemental materials.

EXAMINATION OF GENE EXPRESSION PROFILES DURING UTILIZATION OF AHLS BY *P. AERUGINOSA* SUPPORTS THE ROLE OF *QUIP* AND PA3922 IN SIGNAL DECAY

We employed a microarray approach to compare the transcriptional profiles of *P. aeruginosa* strain PAO1 cultures growing on decanoyl-HSLs versus cultures growing on decanoate or succinate. This was done to further determine whether loci we had identified using SDS-PAGE and mutant analysis and which other genes are involved in the utilization of long chain AHLs by *P. aeruginosa*. A listing of all genes significantly up or down regulated under these conditions is provided as supplemental materials. The results were in good agreement with those obtained from comparison of protein profiles under similar growth conditions (see above; and Figure 3.1). mRNA of genes *quiP* and PA3922 were strongly over-expressed in cultures actively utilizing decanoyl-HSL (Figure 3.5). Curiously, gene PA4022, identified as a locus of interest during the protein analysis (above) is not represented on the commercial *P. aeruginosa* microarrays. Thus the expression of this gene was not examined. In agreement with previous results that the AHL acylase PvdQ was not necessary for AHL utilization, there was no evidence for the expression of *pvdQ* mRNA during the growth of *P. aeruginosa* under these same conditions. Of the two remaining acylase homologs encoded by *P. aeruginosa*, PA0305 and PA1893, only the latter was expressed in great amount during decanoyl-HSL utilization relative to decanoate or succinate dependent growth. PA1893, however, was also up-regulated in cultures grown in succinate defined medium amended with 3 mM

decanoyl-HSL (data not presented) and has been shown to be quorum regulated (146, 169, 176). In agreement with protein profiles comparisons (see above) and previous transcriptome studies (146, 169), *quiP* was not expressed greatly in succinate defined medium amended with AHLs, suggesting that that *quiP* is not up regulated simply as a function of the AHL status of the cell.

DISCUSSION

P. aeruginosa has the ability to degrade AHL of eight carbons or greater, including its native long-chain AHL (70). The gene *pvdQ* of *P. aeruginosa* has been shown to be sufficient, but not necessary for AHL degradation (70). In this study we identify QuiP, the product of the *P. aeruginosa* gene PA1032, as a second AHL acylase capable of the inactivation of quorum signals in *P. aeruginosa*. *quiP* mRNA and its protein product are expressed during strain PAO1 growth on long chain AHL, (Figure 3.1 and Figure 3.5), and QuiP is sufficient to catalyze the degradation of long, but not short chain AHLs in *E. coli* (Figure 3.3). Constitutive expression of QuiP in *P. aeruginosa* resulted in decreased accumulation of 3OC12HSL signal by strain PAO1, indicating that the enzyme has activity against physiologically relevant concentrations of AHL produced by *P. aeruginosa* (Figure 3.4). QuiP appears to be the AHL acylase that underlies the ability of *P. aeruginosa* to degrade and utilize certain AHLs for growth, including one of its own quorum signals, 3OC12HSL.

QuiP has a similar specificity for the degradation of long acyl chain AHL as PvdQ (70) and another recently described AHL-degrading acylase encoded by a *Streptomyces* species (118). Proteins sharing 61%-68% amino acid identity with QuiP are encoded by other members of the family *Pseudomonadaceae*: *Azotobacter vinelandii*, *P. fluorescens*, *P. syringe*, and *P. putida*. QuiP also shared 29%-32% amino acid identity with proteins encoded by *Ralstonia metallidurans*, and *R. eutropha*, as well as several oxygenic or anoxygenic phototrophs (*Gloeobacter violaceus, Rubrivivax gelatinosus*, and *Nostoc punctiforme*). Whether these microbes also share an ability to degrade AHLs has, to out knowledge, not yet been examined.

These studies also suggest that the protein encoded by PA3922 plays an undetermined yet possibly critical role in the AHL degradation and utilization process. It was shown to be strongly up-regulated, and its protein product present in large amounts, during AHL utilization, as revealed by microarray analyses and protein profiling, respectively (Figure 3.5 and Figure 3.1). A mutant strain carrying a transposon insertion into this locus exhibited a marked growth defect during the utilization of long chain AHLs (Figure 3.2 B). However, the nature of its role in the AHL degradation process remains elusive, and its homology with other proteins in the databases provides no obvious clues. Curiously, PA3922 was previously reported to be down-regulated in *P. aeruginosa* biofilms exposed to the antibiotic tobramycin (177), but the role of this

gene in the organism's response to that perturbation is not understood either. The distribution of homologs of gene PA3922 is virtually restricted to the pseudomonads and a few other bacteria, no homologs are found in *E. coli*.

Under natural circumstances, when would these enzymes that degrade AHL be expressed, and what role could they play in the biology of *P. aeruginosa*? The expression of both QuiP, the AHL acylase described in this study, is tightly controlled. To our knowledge, up regulation of *quiP* has not previously been reported in microarray or other studies. The expression of this trait does not appear to be controlled as a function of the catabolic or anabolic needs of the cell (70). Thus, although the recycling of the carbon and energy through the degradation of quorum signals would be beneficial, the primary function(s) of QuiP seems other than the growth physiology of this bacterium.

Clearly, *P. aeruginosa* populations must carefully balance and control signal production and reception during quorum sensing with the expression of proteins exhibiting signal decay activities, either by insuring that the latter systems do not interfere with cell-to-cell communications, or at times ensuring that they do. Since *P. aeruginosa* populations maintain different ratios of their long and short chain AHL signals when grown in biofilm versus planktonic states (148), we speculate that the expression of genes such as *quiP* could potentially occur in spatially distinct subpopulations of *P. aeruginosa* growing in the biofilm state.

Supplemental Materials

Table 3.1. Genes significantly up or down regulated in wild type *P. aeruginosa* PAO1 growing with C10HSL as a carbon source compared with wild type growth on decanoate fatty acid

ORF	Gene[a]	Function or homology	Fold regulation[b]
PA0105	***coxB***	**cytochrome c oxidase, subunit II**	**6.1**
PA0106	***coxA***	**cytochrome c oxidase, subunit I**	**7.3**
PA0107		**conserved hypothetical protein**	**6.7**
PA0108	***coIII***	**cytochrome c oxidase, subunit III**	**5.7**
PA0110		**hypothetical protein**	**4.4**
PA0111		hypothetical protein	4.7
PA0112		hypothetical protein	4.3
PA0113		probable cytochrome c oxidase assembly factor	3.3
PA0122		**conserved hypothetical protein**	**2.8**
PA0141		conserved hypothetical protein	-4.4
PA0177		**probable purine-binding chemotaxis protein**	**3.3**
PA0178		**probable two-component sensor**	**3.5**
PA0179		**probable two-component response regulator**	**3.7**
PA0182		probable short-chain dehydrogenase	4.7
PA0264		hypothetical protein	3.6
PA0265	*gabD*	succinate-semialdehyde dehydrogenase	3.7
PA0266	*gabT*	4-aminobutyrate aminotransferase	3.3
PA0274		hypothetical protein	3.8
PA0296		probable glutamine synthetase	2.7
PA0297		probable glutamine amidotransferase	3.6

PA0298		probable glutamine synthetase	3.1
PA0323		probable binding protein of ABC transporter	3.8
PA0511	***nirJ***	**heme d1 biosynthesis protein NirJ**	**-3.8**
PA0513		**probable transcriptional regulator**	**-3.1**
PA0514	***nirL***	**heme d1 biosynthesis protein NirL**	**-3.7**
PA0515		**probable transcriptional regulator**	**-4.3**
PA0516	***nirF***	**heme d1 biosynthesis protein NirF**	**-4.2**
PA0517	***nirC***	**probable c-type cytochrome precursor**	**-5.3**
PA0518	***nirM***	**cytochrome c-551 precursor**	**-5.5**
PA0519	***nirS***	**nitrite reductase precursor**	**-5.4**
PA0523	***norC***	**nitric-oxide reductase subunit C**	**-3.2**
PA0524	***norB***	**nitric-oxide reductase subunit B**	**-3.6**
PA0526		hypothetical protein	-3.7
PA0527		transcriptional regulator Dnr	-3.2
PA0557		hypothetical protein	3.0
PA0565		conserved hypothetical protein	4.1
PA1032	*quip*	probable penicillin amidase	3.9
PA1123		hypothetical protein	-3.1
PA1172	***napC***	**cytochrome c-type protein NapC**	**3.2**
PA1190		conserved hypothetical protein	5.9
PA1245		**hypothetical protein**	**3.5**
PA1247	***aprE***	**alkaline protease secretion protein AprE**	**3.0**
PA1248	***aprF***	**alkaline protease secretion protein AprF**	**3.5**
PA1249	***aprA***	**alkaline metalloproteinase precursor**	**3.7**
PA1250	***aprI***	**alkaline proteinase inhibitor AprI**	**3.9**
PA1286		probable MFS transporter	3.3
PA1353		hypothetical protein	3.5
PA1354		hypothetical protein	3.5

PA1355		hypothetical protein	4.9
PA1356		hypothetical protein	2.8
PA1431	***rsaL***	**regulatory protein RsaL**	**8.2**
PA1432	***lasI***	**autoinducer synthesis protein LasI**	**7.0**
PA1546	*hemN*	oxygen-independent coproporphyrinogen III oxidase	-3.9
PA1555		probable cytochrome c	-5.0
PA1556		probable cytochrome c oxidase subunit	-6.2
PA1557		probable cytochrome oxidase subunit (cbb3-type)	-5.3
PA1596	*htpG*	heat shock protein HtpG	-2.7
PA1651		probable transporter	3.2
PA1673		hypothetical protein	-4.2
PA1746		hypothetical protein	-3.6
PA1761		hypothetical protein	2.7
PA1869		**probable acyl carrier protein**	**3.9**
PA1888		**hypothetical protein**	**2.9**
PA1891		**hypothetical protein**	**5.8**
PA1892		hypothetical protein	6.7
PA1893		**hypothetical protein**	**6.8**
PA1894		**hypothetical protein**	**6.9**
PA1895		**hypothetical protein**	**7.3**
PA1896		**hypothetical protein**	**6.9**
PA1897		**hypothetical protein**	**7.5**
PA1898		**probable transcriptional regulator**	**5.5**
Intergenic region		PA1934 and PA1935, 2068728-2069490, (-) strand	5.6
Intergenic region		PA1934 and PA1935, 2068728-2069490, (+) strand	5.3
PA1978		**probable transcriptional regulator**	**4.8**
PA1984		**probable aldehyde dehydrogenase**	**4.1**
PA1985	*pqqA*	pyrroloquinoline quinone biosynthesis protein A	3.9

PA2030		**hypothetical protein**	**5.2**
PA2031		**hypothetical protein**	**6.0**
PA2041		probable amino acid permease	4.0
PA2119		alcohol dehydrogenase (Zn-dependent)	-4.4
PA2127		conserved hypothetical protein	-3.0
PA2302		**probable non-ribosomal peptide synthetase**	**4.8**
PA2303		**hypothetical protein**	**7.1**
PA2304		**hypothetical protein**	**5.1**
PA2305		**probable non-ribosomal peptide synthetase**	**6.1**
PA2306		**conserved hypothetical protein**	**4.0**
PA2358		hypothetical protein	3.8
PA2365		**conserved hypothetical protein**	**4.8**
PA2366		**conserved hypothetical protein**	**4.6**
PA2367		**hypothetical protein**	**4.5**
PA2368		**hypothetical protein**	**3.5**
PA2370		**hypothetical protein**	**3.3**
PA2371		**probable ClpA/B-type protease**	**2.7**
PA2372		**hypothetical protein**	**3.3**
PA2591		**probable transcriptional regulator**	**3.4**
PA2662		conserved hypothetical protein	-2.9
PA2776		conserved hypothetical protein	3.5
PA2845		hypothetical protein	3.6
PA2862	*lipA*	lactonizing lipase precursor	5.9
PA2863	*lipH*	lipase modulator protein	4.2
PA2898		hypothetical protein	-4.3
PA2939		**probable aminopeptidase**	**5.4**
PA3032		**cytochrome c**	**2.9**
PA3038		**probable porin**	**4.2**

PA3234		**probable sodium:solute symporter**	**4.3**
PA3235		**conserved hypothetical protein**	**4.6**
PA3309		conserved hypothetical protein	-3.0
PA3329		**hypothetical protein**	**4.0**
PA3333	***fabH2***	**3-oxoacyl-[acyl-carrier-protein] synthase III**	**3.6**
PA3337	***rfaD***	**ADP-L-glycero-D-mannoheptose 6-epimerase**	**-4.1**
PA3356		conserved hypothetical protein	2.7
PA3392	***nosZ***	**nitrous-oxide reductase precursor**	**-3.0**
PA3436		hypothetical protein	-2.6
PA3446		conserved hypothetical protein	-3.3
PA3476	***rhlL***	**autoinducer synthesis protein RhlL**	**3.6**
PA3477	***rhlR***	**transcriptional regulator RhlR**	**3.8**
PA3515		hypothetical protein	5.8
PA3516		probable lyase	7.1
PA3517		probable lyase	5.4
PA3518		hypothetical protein	7.7
PA3519		**hypothetical protein**	**7.2**
PA3520		**hypothetical protein**	**3.9**
PA3521		probable outer membrane efflux protein precursor	4.7
PA3522		probable RND efflux transporter	4.5
PA3523		probable RND efflux membrane fusion precursor	6.5
PA3568		probable acetyl-coa synthetase	4.5
PA3610	*potD*	polyamine transport protein PotD	4.0
PA3724	***lasB***	**elastase LasB**	**4.8**
PA3874	***narH***	**respiratory nitrate reductase beta chain**	**-4.1**
PA3875	***narG***	**respiratory nitrate reductase alpha chain**	**-3.8**
PA3876	***narK2***	**nitrite extrusion protein 2**	**-4.5**
PA3880		conserved hypothetical protein	-3.6

PA3904		**hypothetical protein**	**5.5**
PA3906		**hypothetical protein**	**3.1**
PA3912		**conserved hypothetical protein**	**-2.8**
PA3913		**probable protease**	**-3.2**
PA3920		probable metal transporting P-type ATPase	3.4
PA3922		conserved hypothetical protein	6.0
PA3923		**hypothetical protein**	**6.0**
PA3924		probable medium-chain acyl-CoA ligase	3.9
PA4023		probable transport protein	3.5
PA4067	*oprG*	outer membrane protein OprG precursor	-4.1
PA4147	*acoR*	transcriptional regulator AcoR	2.7
PA4297		**hypothetical protein**	**3.0**
PA4306		**hypothetical protein**	**4.5**
PA4348		conserved hypothetical protein	-4.4
PA4500		probable binding protein of ABC transporter	3.5
PA4501		probable porin	2.6
PA4571		probable cytochrome c	-2.5
PA4587	***ccpR***	**cytochrome c551 peroxidase precursor**	**-5.8**
PA4601		conserved hypothetical protein	-3.8
PA4623		hypothetical protein	0.2
PA4677		**hypothetical protein**	**2.9**
PA4680		hypothetical protein	3.0
PA4688	*hitB*	iron (III)-transport system permease HitB	-2.7
PA4762	*grpE*	heat shock protein GrpE	-3.0
PA4813	*lipC*	lipase LipC	4.6
PA4828		conserved hypothetical protein	-3.6
PA4861		probable ATP-binding of ABC transporter	-3.1
PA4862		probable ATP-binding of ABC transporter	-2.7

PA4908		hypothetical protein	2.8
PA4909		probable ATP-binding of ABC transporter	3.6
PA4910		probable ATP-binding of ABC transporter	3.6
PA4911		probable permease of ABC amino acid transporter	3.7
PA4912		probable permease of ABC amino acid transporter	3.5
PA4913		probable binding protein of ABC transporter	3.4
Intergenic region		**PA4913 and PA4914, 5457716-5458499, (+) strand**	**-5.2**
PA5027		hypothetical protein	-4.6
PA5309		probable oxidoreductase	3.5
PA5312		probable aldehyde dehydrogenase	3.2
PA5313		probable pyridoxal-dependent aminotransferase	4.2
PA5314		hypothetical protein	3.9
PA5348		probable DNA-binding protein	2.9
PA5380		probable transcriptional regulator	3.8
PA5427	*adhA*	alcohol dehydrogenase	-4.7
PA5429	*aspA*	aspartate ammonia-lyase	-3.8
PA5440		probable peptidase	-3.8
PA5460		hypothetical protein	4.6
PA5475		hypothetical protein	-4.9
PA5522		probable glutamine synthetase	3.0
PA5523		probable aminotransferase	3.1
PA5530		probable MFS dicarboxylate transporter	-3.0
tRNA		tRNA_Threonine, 5809970-5810045 (+) strand	-3.0

[a] Genes in bold are previously shown to be quorum regulated (146, 169, 176)

[b] Genes with a > 2.5 –fold change in expression are shown

4. EXPRESSION STUDIES OF THE AHL ACYLASE QUIP OF *P. AERUGINOSA* PAO1 BY WILD TYPE AND BY AN AHL SIGNAL DEGRADING VARIANT OF PAO1

ABSTRACT

Pseudomonas aeruginosa PAO1 uses acyl-homoserine lactone (AHL) signaling molecules to engage in cell-to-cell communication and regulate the production of numerous genes and virulence factors. PAO1 has the ability to degrade and utilize long chain, but not short chain signaling molecules. Two genes of *P. aeruginosa*, PA2385 (*pvdQ*) and PA1032 (*quiP*), were previously found sufficient for the degradation of long chain AHL, with only the *quiP* gene necessary for AHL degradation. This study examines the expression of *quiP* in *P. aeruginosa* PAO1 and in a variant of *P. aeruginosa,* $PAO1_{lagless}$, which constitutively degrades AHL. We compare the phenotypes, transcriptomes and expression of *quiP* in PAO1 and $PAO1_{lagless}$ strains and find that while $PAO1_{lagless}$ expresses the *quiP* AHL acylase all of the time, PAO1 wild type expresses this gene for long chain AHL degradation in the center of microcolonies in the biofilm growth state.

INTRODUCTION

The acyl-homoserine lactones (AHLs) are signaling molecules used by some gram negative bacteria in cell-to-cell communication known as quorum sensing (53). In this process, bacteria couple their gene transcription to changes in cell population density. The opportunistic pathogen *Pseudomonas aeruginosa* PAO1 has two AHL mediated quorum sensing systems, the *lasI/R* and *rhlI/R* systems which involve the production and response to two main AHLs: 3OC12HSL and C4HSL respectively (96, 150, 174). The two systems are connected and behave in a hierarchy where, after a critical concentration of the long chain signal 3OC12HSL is accumulated, *rhlR,* the response regulator for C4HSL is transcribed (88, 147). The AHL mediated quorum sensing systems of PAO1 control distinct gene regulons and also interact to regulate the expression of genes for numerous virulence factors, exoenzymes, nutrient acquisition and certain aspects of biofilm formation (reviewed in (80, 146, 150, 169)). This AHL quorum sensing system is estimated to regulate approximately 6%-9% of the PAO1 genome (146, 165, 169). Multiple global regulators of the AHL quorum sensing system of PAO1 have been identified (36, 64, 134, 146) indicating that quorum regulated genes are subject to regulation and cues from the environment (185).

P. aeruginosa has the ability to degrade long chain AHL, but not short chain AHL (70). Two enzymes sufficient for AHL degradation have been identified: PvdQ, encoded by the gene PA2385 and QuiP, encoded by the gene PA1032 (69, 70,

149). PvdQ is not necessary for degradation of long chain AHL, whereas *quiP* is necessary and its transcript and protein product are present during degradation of long chain signal when AHL is the sole carbon source (69). To determine when *P. aeruginosa* may use this signal degrading ability, the expression and regulation of *quiP* in *P. aeruginosa* PAO1 and in $PAO1_{lagless}$, a variant that constitutively degrades long chain AHL, were studied.

MATERIALS AND METHODS

STRAINS AND CULTURE CONDITIONS

The bacterial strains used in this study are listed in Table 4.1. All cultures were maintained in LB (lysogen broth) amended with antibiotics when appropriate. Antibiotic concentrations used for *Pseudomonas aeruginosa* were: 60 μg/ml tetracycline, 50 μg/ml gentamycin and 250 μg/ml carbenicillin, and for *E. coli,* 100 μg/ml ampicillin, 15 μg/ml gentamycin and 10 μg/ml tetracycline. A defined freshwater medium buffered at pH 5.5 with 5mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) was used for studies with growth on defined carbon sources (92).

A variant of PAO1 was obtained from six serial transfers of PAO1 that was growing exponentially on long chain AHL signal to identical fresh medium with 1

mM C10HSL as the sole carbon source. The resulting variant strain, $PAO1_{lagless}$, constitutively degraded long chain AHL without lag. Another lagless strain, $PAO1_{lagless2}$, was obtained from a separate experiment in which selection repeated a second time starting with wild type PAO1.

Table 4.1 Bacterial strains and plasmids

Strain, plasmid or primer	Relevant characteristics	Source
P. aeruginosa		
$PAO1_{wt}$	wild type	Laboratory stock (originally from B. Iglewski)
$PAO1_{lagless}$	constitutive expression of PA1032 (*quip)* long chain AHL degrader	this study
$PAO1_{lagless2}$	long chain AHL degrader	this study
***P. aeruginosa* wild type and *P. aeruginosa* lagless mutants**		
Δ *mvfR*	PA1003::Gm	this study
Δ *vqsR*	PA2591::Gm	this study
Δ *rsaL*	PA1431::Gm	this study
Δ *lasR*	PA1430::Gm	this study
Δ *qscR*	PA1898::Gm	this study
Δ *lasR* Δ *qscR*	Δ PA1430, PA1898::Gm	this study
PAO1 YFP	Δ PA1032::YFP, Gm	this study
$PAO1_{lagless}$ YFP	Δ PA1032::YFP, Gm	this study
PAO1 YFP_att_CFP	Δ PA1032::YFP, attTn7::miniTn7T-Gm-PA1033_32, pMP4641 (eCFP)	this study
$PAO1_{lagless}$ YFP_att_CFP	ΔPA1032::YFP, attTn7::miniTn7T-Gm-PA1033_32, Gm^r, pMP4641 (eCFP), Tet^r	this study
E. coli		
TOP10	Electrocompetent cells for cloning	Invitrogen (Carlsbad, CA)

Plasmids

pCM351	allelic exchange vector, Gm^r, Amp^r, Tet^r	(99)
pCM157	Amp^r, Cre - expressing plasmid	(99)
pFUNR	plasmid containing YFP	(Sidney Cox, Caltech, unpublished)
pMW312	PQF50 plasmid, *lacZ* with *rsaL*(PA1431) promoter	(176)
pUC18TminiTN7T-Gm	Gm^r Am^r, miniTn7	(21)
pTNS1	helper plasmid for pUC18TminiTN7T-Gm	(21)
pMP4641	Tet^r, constitutive eCFP expression from P*lac*	(10)

Primers

Primer1: AAAGGTACCTGCTTTGGTGGAGCGC

Primer2: GTCGTTGGCCATCAGGCTCTT

Primer3: CCTGATGGCCAACGACAAAATAAGGAGG

Primer4: AAAAAAGCGGCCGCTTATTTATACAGTTCGTCCATA

PA1032_MCS2_F: AAAACCGGTCAAGCGGCTGACGCTCAC

PA1032_MCS2_R: AAAGAGCTCAGCAGCTCAACCTGCGCC

PA1033_32_Tn7_F: AAGGATCCACCCTTCGCACGCGCC

PA1033_32_Tn7_R. AAAAAGCTTTCAGCGAGCGGGAGTGAGCGTCA

CONSTRUCTION OF ΔPA1032 *(QUIP)*::YFP TRANSCRIPTIONAL FUSION STRAINS AND MUTANTS

Δ*quiP*::YFP reporter strains were constructed by homologous recombination. A 1 kb fragment that included 855 bp upstream of *quiP* was amplified using primer

1 (KpnI site underlined) and primer 2. Promoterless YFP, which was designed from Venus YFP (109) and Citrine YFP (61) with a monomeric A206K mutation (Sidney Cox, Caltech), was amplified from the plasmid pFUNR (Sidney Cox, Caltech, unpublished) using primers 3 and 4 (NotI site underlined). Two additional basepairs were added in primer 3 to amplify YFP, which was amplified with its own ribosome binding site from pFUNR, out of frame for a transcriptional fusion within *quiP*. The resulting deletion within *quiP* was in frame minimizing potential polar effects. The PCR product from *quiP* was fused to the YFP fragment with PCR using primers 1 and 4. This fragment was sequenced to verify the PCR sewed product. The product was cloned into pCM351 (99) by digestion with KpnI and NotI enzymes and ligation. In the second multi cloning site of pCM351, a 1 kb DNA fragment downstream of *quiP*, which included 31 bp from the end of the gene, was amplified using primers PA1032_MCS2_F (AgeI site underlined) and PA1032_MCS2_R (SacI site underlined) and cloned into pCM351 using restriction enzymes AgeI and SacI followed by ligation. The resulting plasmid was transformed into wild type PAO1, $PAO1_{lagless}$ and $PAO1_{lagless2}$ via electroporation (20). Double recombinants were selected based on resistance to gentamycin (50 μg/ml) and sensitivity to tetracycline (55 μg/ml). Putative double recombinants were confirmed by sequencing. The Gm resistance gene was removed using a cre-lox system (99). The plasmid pCM157 was transformed into strains with Δ*quiP*::YFP. Colonies that were sensitive to gentamycin, which indicated successful cre-mediated excision of the gm resistance gene, were picked. The pCM157 plasmid was lost after two rounds of

growth in LB without antibiotic selection. The resulting strains were used for *quiP* transcriptional reporting and phenotype tests. To produce cells that constitutively express CFP, the plasmid pMP4641 (10) was transformed into PAO1 cells and tetracycline selection and microscopy were used as criteria for positive transformants.

COMPLEMENTATION OF THE Δ*QUIP*::YFP MUTANTS

A fragment that contained the putative operon PA1032 and PA1033 and promoter, 122 bp upstream of PA1033 (see supplemental materials for determination), was amplified from PAO1 gDNA using primers: PA1033_32_F (BamHI site underlined) and PA1033_32_R (HindIII site underlined) primers. This fragment was inserted into a neutral site downstream of the *glmS* gene of PAO1 using a mini Tn7 transposon containing plasmid (21). The BamHI and HindIII digested PCR product was ligated into the plasmid pUC18TminiTN7T-Gm (21) and sequence fidelity verified by sequencing. The resulting plasmid, pUC18TminiTN7T-Gm_PA1033_32, was electroporated into the wild type, $PAO1_{lagless}$ and $PAO1_{lagless2}$ strains that contained Δ*quiP*::YFP reporter constructs with the aid of the helper plasmid pTNS1 (21). Putative transformants were selected based on resistance to gentamycin and insertion of the PA1033_32 genes into the correct site was verified by PCR using the primers *glmS*_Up and *glmS*_down (21). The ability for the complemented mutants to degrade signal

was tested by providing strains with AHL as the sole carbon source in minimal medium as described previously (92).

The amount of long chain AHL accumulated by the $PAO1_{wt}$ and $PAO1_{lagless}$ with Δ*quiP*::YFP and these strains complemented with PA1032 in a neutral site, was measured from cultures grown in 50 mM MOPS buffered LB. 5 ml aloquots of culture were removed at late log phase at an OD_{600nm} of approximately 2.5 and the AHL extracted using acidified ethyl acetate. The amount of signal was quantified in comparison to standards of known concentration, using a Hewlett-Packard 1100 series LC/APCI MS as described previously (70).

YFP TRANSCRIPTIONAL REPORTER ACTIVITY IN CELLS GROWN IN BATCH CULTURE

YFP reporter activity from $PAO1_{wt}$ and $PAO1_{lagless}$ strains with the YFP transcriptional fusion in the *quiP* gene was followed in cells grown in batch culture. Cells were grown in 5 ml cultures in 1/10 LB medium at 37 °C shaking at 250 rpm. Over time samples were taken for optical density at 500 nm, and fluorescence of YFP determined with excitation of 485/520nm and emission 525/540nm using a Synergy HT multi-detection microplate reader (Bio-Tek Instruments) and KC4 software. This experiment was repeated for Δ*quiP*::YFP strains complemented with *quiP* and its promoter in a neutral site downstream of the *glmS* gene.

BIOFILM EXPERIMENTS

Biofilm formation of the $PAO1_{wt}$ and $PAO1_{lagless}$ strains in a once –flow through system (153) were analyzed. 1/10 LB medium was pumped through Tygon silicone tubing with an internal diameter of 1/16", an outer diameter of 1/8" and wall of 1/32" by a Watson-Marlow peristaltic pump with a flow rate of 0.8 rpm. A biofilm flow chamber (Stovall, Greensboro, NC) was added to each line of silicone tubing. Cultures of PAO_{wt}, the $PAO1_{wt}$ strain with a Δ*quiP*::YFP reporter and expressing CFP from a plasmid, and $PAO1_{lagless}$ with the same constructs were grown in 1/10 LB. Exponentially growing cell cultures with an optical density of 600nm of approximately 0.4 were inoculated into the silicone tubing upstream of biofilm flow chambers and flow was re-started after an hour of still incubation. The biofilms that grew within the chambers were analyzed imaged using a Zeiss Axiovert 100M inverted confocal microscope with a 40X Achroplan water immersion lens and LSM5 Pascal software V 3.2 (Carl Zeiss) at the Caltech Beckman Institute Biological Imaging Center. Images were analyzed using the Imaris program (Bitplane). Additionally, from each strain, eight colonies approximately 40 μm across, imaged at ~70 hours, were selected for image analysis. The YFP and the CFP channels were separated and analyzed separately. Intensity profiles were obtained and analyzed for both channels for each image, by Tracy Teal, as previously described (157). The average fluorescence profiles for CFP and YFP for each strain was then calculated and plotted as fluorescence intensity versus distance from the center of the colony.

MICROARRAY EXPERIMENTS

The transcriptomes of $PAO1_{wt}$ and $PAO1_{lagless}$ strains were compared using microarray analysis. Strains were grown from freezer stock on LB agar and single colonies that developed were picked and grown in MES pH 5.5 minimal medium (92) with 5mM succinate as the sole carbon source overnight at 37 ºC. 1 ml of overnight cell culture was inoculated into flasks containing 15 ml of sterile MES pH 5.5 medium that contained 8 mM sodium succinate at 37º C and shaken at 250 rpm. 10 ml of cell culture were harvested at optical density 600 nm of 0.4 and RNA was stabilized immediately with RNA Protect Bacterial Reagent (Qiagen) and processed according to manufacturer's protocol. Total RNA was extracted from cultures using the RNeasy kit (Qiagen) and prepared for hybridization to *P. aeruginosa* GeneChips (Affymetrix) as previously described (133, 146). Hybridization, washing and scanning of Affymetrix GeneChips was performed at the University of Iowa Genome Center using an Affymetric fluidics station. Genechip analysis were performed in duplicate and data analyzed using Microarray Suite software. We report gene activity changes showing greater than five fold up-or down regulation. Microarray data were verified by RT PCR and comparison of expression of *rsaL,* a gene differentially expressed by $PAO1_{wt}$ and $PAO1_{lagless}$, using beta-galactosidase assays of the plasmid pMW312, which contained promoterless *lacZ* driven by the *rsaL* promoter (175).

RESULTS

CHARACTERISTICS OF $PAO1_{LAGLESS}$ STRAINS

A variant of PAO1 that, unlike the wildtype, constitutively degrades long chain AHL, was selected by successive transfers of PAO1 into minimal medium with long chain AHL as the sole carbon source. $PAO1_{lagless}$ degrades AHL even in the presence of a preferred carbon source such as succinate (data not shown). When grown at 37 °C on rich medium such as LB, the lagless strain produces significantly more blue pigment than wild type strains in plates and in liquid culture. The wild type and lagless strains have similar growth rates in LB medium, but the lagless strain accumulated about a fourth of the amount of signal as wild type (Figure 4.1). A second lagless strain, obtained through a second selection of PAO1 grown successively on signal, behaved similarly as the original $PAO1_{lagless}$ strain in that it overproduced blue pigment and constitutively degraded long chain AHL (data not shown).

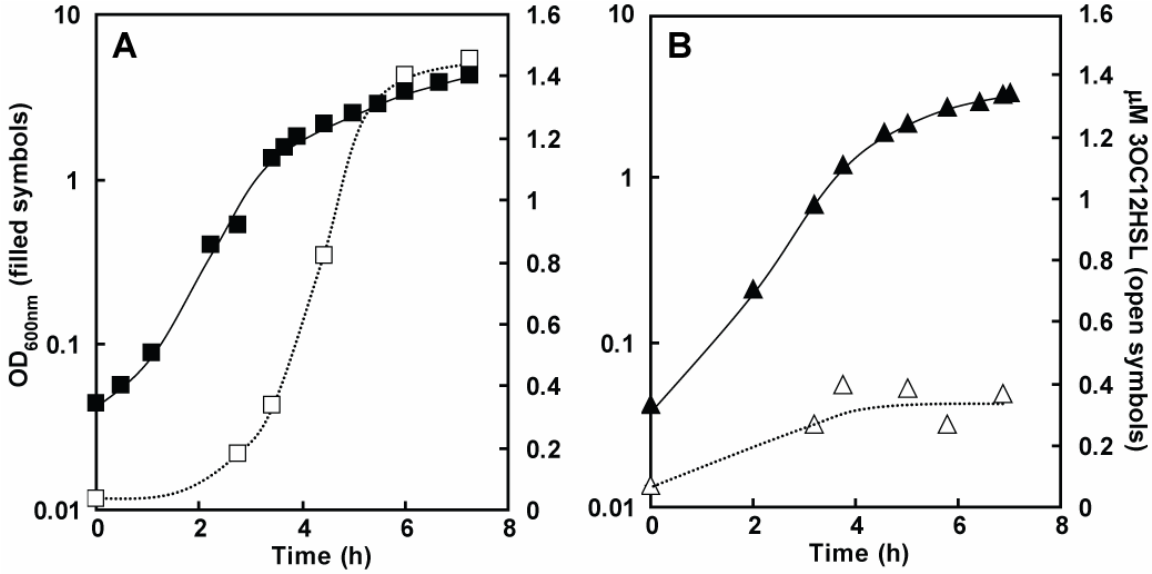


Figure 4.1. Growth and accumulation of naturally produced 3OC12HSL by $PAO1_{wt}$ and $PAO1_{lagless}$ strains. Growth of cultures in pH 7 buffered LB was measured using optical density 600 nm, and the 3OC12HSL accumulated was determined using LC/MS of culture samples extracted with acidified ethyl acetate. A, $PAO1_{wt}$, B, $PAO1_{lagless}$ strain.

DIFFERENTIAL GENE EXPRESSION OF $PAO1_{WT}$ AND $PAO1_{LAGLESS}$ GROWN IN SUCCINATE

Several genes were significantly up-regulated and a few down-regulated in $PAO1_{lagless}$ relative to $PAO1_{wt}$ when both strains were grown with succinate as the carbon source (Table 4.2). Notably, the genes differentially expressed in

$PAO1_{lagless}$ relative to $PAO1_{wt}$ were genes previously found to be quorum regulated. PA1032 (*quiP)* was significantly up-regulated in $PAO1_{lagless}$ relative to $PAO1_{wt}$. Knock-out mutants in transcriptional regulators that were significantly up-regulated in the $PAO1_{lagless}$ strain relative to wild type: PA1003 (*mvfR*), PA2591 (*vqsR*) and PA1431 (*rsaL*), did not affect the ability for the wild type or lagless strains to degrade AHL (Supplemental Material Table 4.2). Additionally, knock out mutations in PA1430 (*lasR*) and PA1898 (*qscR*) and a double mutant in the $PAO1_{wt}$ and $PAO1_{lagless}$ strain backgrounds did not affect the ability for strains to degrade AHL (data not shown).

Table 4.2. Genes significantly up or down-regulated in $PAO1_{lagless}$ compared to $PAO1_{wt}$ grown with succinate as the carbon source. Cultures were analyzed in duplicate using a *P. aeruginosa* GeneChip microarray (Affymetrix).

Up-regulated:

ORF	Gene[a]	Gene product	Fold change[b]
PA0996	***pqsA***	**probable coenzyme A ligase**	**4.3**
PA0997	***pqsB***	**homologous to acyl-carrier protein synthase**	**3.2**
PA0998	***pqsC***	**homologous to acyl-carrier protein synthase**	**4.4**
PA0999	***pqsD***	**3-oxoacyl-[acyl-carrier-protein] synthase III**	**3.1**
***PA1003**	***mvfR***	**transcriptional regulator MvfR**	**2.5**
*PA1032	*quiP*	probable penicillin amidase	4.4

PA1250	***aprI***	**alkaline proteinase inhibitor AprI**	**2.9**
***PA1431**	***rsaL***	**regulatory protein RsaL**	**7.0**
PA1432	***lasI***	**autoinducer synthesis protein LasI**	**5.9**
PA1657		**conserved hypothetical protein**	**3.4**
PA1659		**hypothetical protein**	**2.7**
PA1869		**probable acyl carrier protein**	**3.3**
PA1897		**hypothetical protein**	**3.8**
PA2065	*pcoA*	copper resistance protein A precursor	3.3
PA2193	***hcnA***	**hydrogen cyanide synthase HcnA**	**4.7**
PA2194	***hcnB***	**hydrogen cyanide synthase HcnB**	**4.1**
PA2195	***hcnC***	**hydrogen cyanide synthase HcnC**	**4.3**
PA2305		**probable non-ribosomal peptide synthetase**	**5.2**
***PA2591**	***vqsR***	**probable transcriptional regulator**	**3.1**
PA3328		**probable FAD-dependent monooxygenase**	**2.8**
PA3329		**hypothetical protein**	**3.5**
PA3330		**probable short chain dehydrogenase**	**3.5**
PA3331		**cytochrome P450**	**3.9**
PA3332		**conserved hypothetical protein**	**2.4**
PA3334		**probable acyl carrier protein**	**3.4**
PA3874	*narH*	respiratory nitrate reductase beta chain	2.8
PA3875	*narG*	respiratory nitrate reductase alpha chain	3.3
PA3876	*narK2*	nitrite extrusion protein 2	2.6
PA3877	*narK1*	nitrite extrusion protein 1	2.5
PA3904		**hypothetical protein**	**4.9**
PA3905		**hypothetical protein**	**5.5**
PA3906		**hypothetical protein**	**5.5**
PA3907		**hypothetical protein**	**3.0**
PA3908		**hypothetical protein**	**4.6**

PA3915	*moaB1*	moaB1	3.1
PA4130		**probable sulfite or nitrite reductase**	**2.8**
PA4131		**probable iron-sulfur protein**	**3.4**
PA4133		**cytochrome c oxidase subunit (cbb3-type)**	**2.7**
PA5391		hypothetical protein	3.1
Intergenic		PA1030 and PA1031, 1087095-1087843, (-) strand	2.7

Down-regulated:

ORF	Gene	Gene product	Fold change[a]
PA1320	***cyoD***	**cytochrome o ubiquinol oxidase subunit IV**	**-3.4**
PA3581	*glpF*	glycerol uptake facilitator protein	-4.3
PA3584	*glpD*	glycerol-3-phosphate dehydrogenase	-3.3

[a] Genes in bold are previously shown to be quorum regulated (146, 169, 176).

[b] Genes with a >2.5–fold change in expression are shown

* knock-out mutations in $PAO1_{wt}$ and $PAO1_{lagless}$ were made in these genes

$PAO1_{LAGLESS}$ ACCUMULATED LESS 3OC12HSL THAN WILD TYPE IN BATCH CULTURE

$PAO1_{wt}$ and the wild type strain background with Δ*quiP* accumulated similar amounts of 3OC12HSL signal when grown in batch culture in rich medium (Figure 4.2). The lagless strains accumulated significantly less 3OC12HSL long chain signal than the wild type in batch culture (Figure 4.2). The Δ*quiP* mutation in the lagless strain accumulated up to 88% of long chain AHL accumulated by the wild type strain (Figure 4.2). The signal degradation phenotype of the lagless

strain was lost in Δ*quiP* mutants, but was restored by the addition of a single copy of the *quiP* gene and its promoter at a neutral site in the chromosome (Supplemental Table 4. 2).

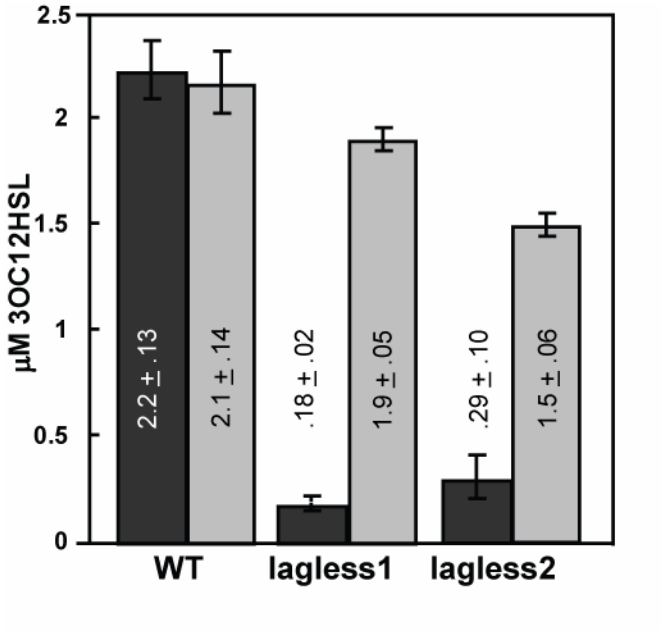


Figure 4.2. Accumulation of 3OC12HSL by $PAO1_{wt}$ and $PAO1_{lagless}$ parent strains (black bars) and strains with Δ*quiP*::YFP transcriptional fusions (grey bars). All cultures were grown in pH 7 buffered LB and 3OC12HSL was measured using LC/MS.

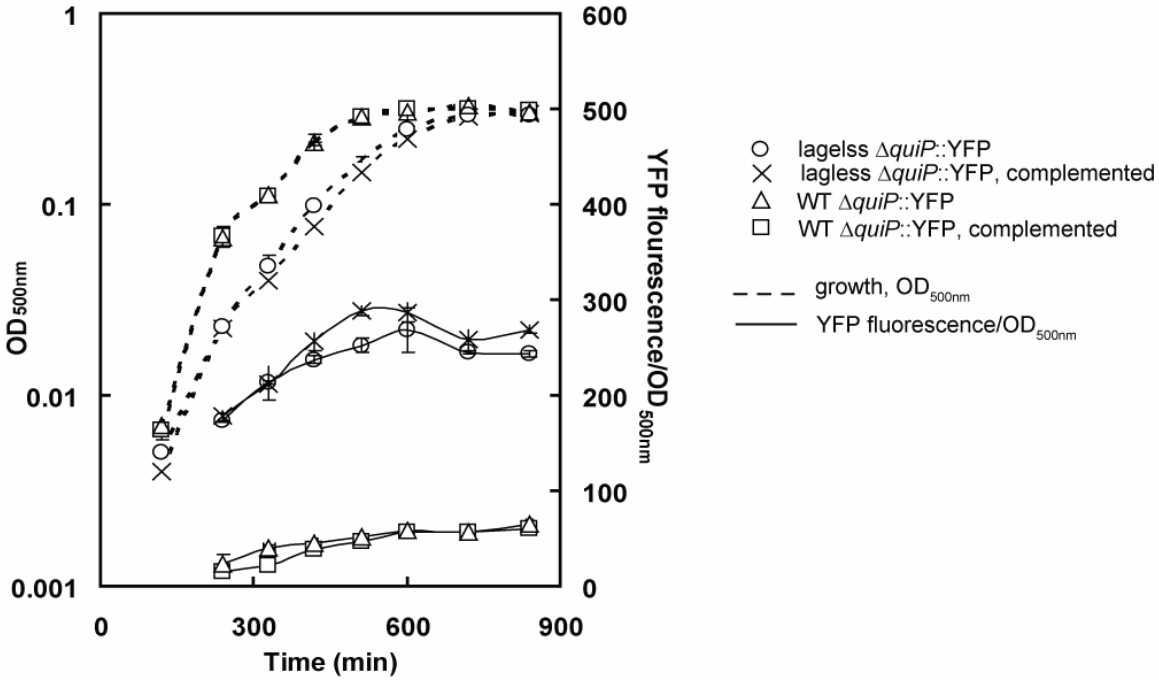


Figure 4.3. Growth and YFP expression by $PAO1_{wt}$ and $PAO1_{lagless}$ strains with Δ*quiP*::YFP transcriptional fusions and by the strains complemented with *quiP* in a neutral site (att). Cells were grown in batch culture in 1/10 LB. Growth of strains was measured by optical density at 500 nm and YFP fluorescence by excitation of 485/520 nm and emission at 525/540 nm.

STUDIES OF QUIP EXPRESSION IN BATCH CULTURE

The $PAO1_{wt}$ and $PAO1_{lagless}$ strains with Δ*quiP*::YFP transcriptional fusions and strains complemented with the *quiP* grew similarly in 1/10 LB (Figure 4.3). Both the transcriptional fusion strain and complemented strains with expressed the YFP reporter across all growth phases with similar ratios of YFP fluorescence/OD. This ratio showed two-fold variance over time of growth (Figure 4.3). The Δ*quiP*::YFP reporter gene was not expressed significantly by the wild type during any phase of growth in batch culture.

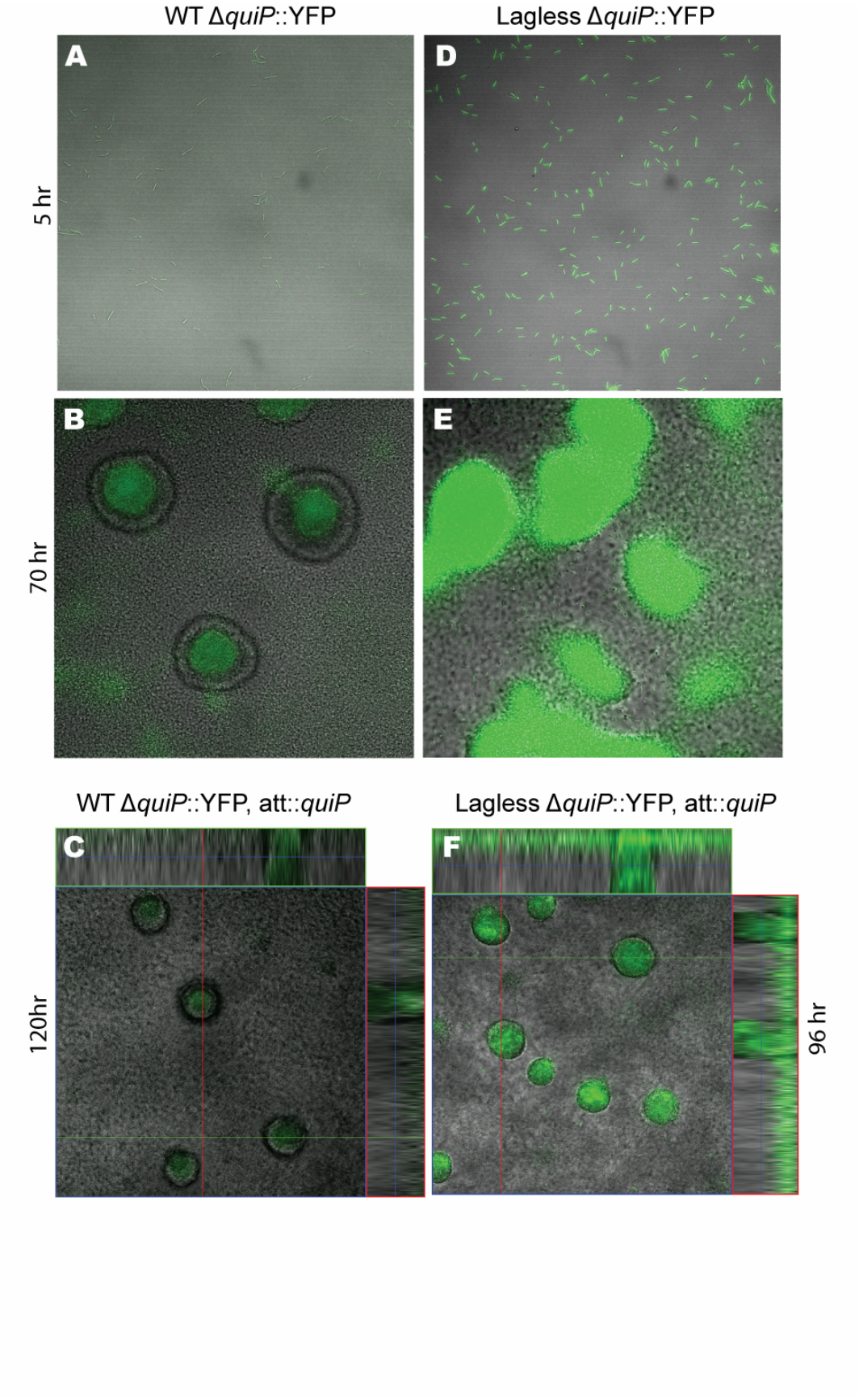

WT ΔquiP::YFP
Lagless ΔquiP::YFP
A
D
5 hr
B
E
70 hr
WT ΔquiP::YFP, att::quiP
Lagless ΔquiP::YFP, att::quiP
C
F
120hr
96 hr

Figure 4.4. Confocal microscopy of biofilms formed by $PAO1_{wt}$ and $PAO1_{lagless}$ strains containing Δ*quiP*::YFP transcriptional fusions. Strains were grown in a once flow-though biofilm system and imaged using filters for YFP and phase microscopy at 40X. A-B, $PAO1_{wt}$ Δ*quiP*::YFP. C, $PAO1_{wt}$ Δ*quiP*::YFP complemented with *quiP* at a neutral site, ortho view. D-E, $PAO1_{lagless}$ Δ*quiP*::YFP. F, $PAO1_{lagless}$ Δ*quiP*::YFP complemented with *quiP*, ortho view.

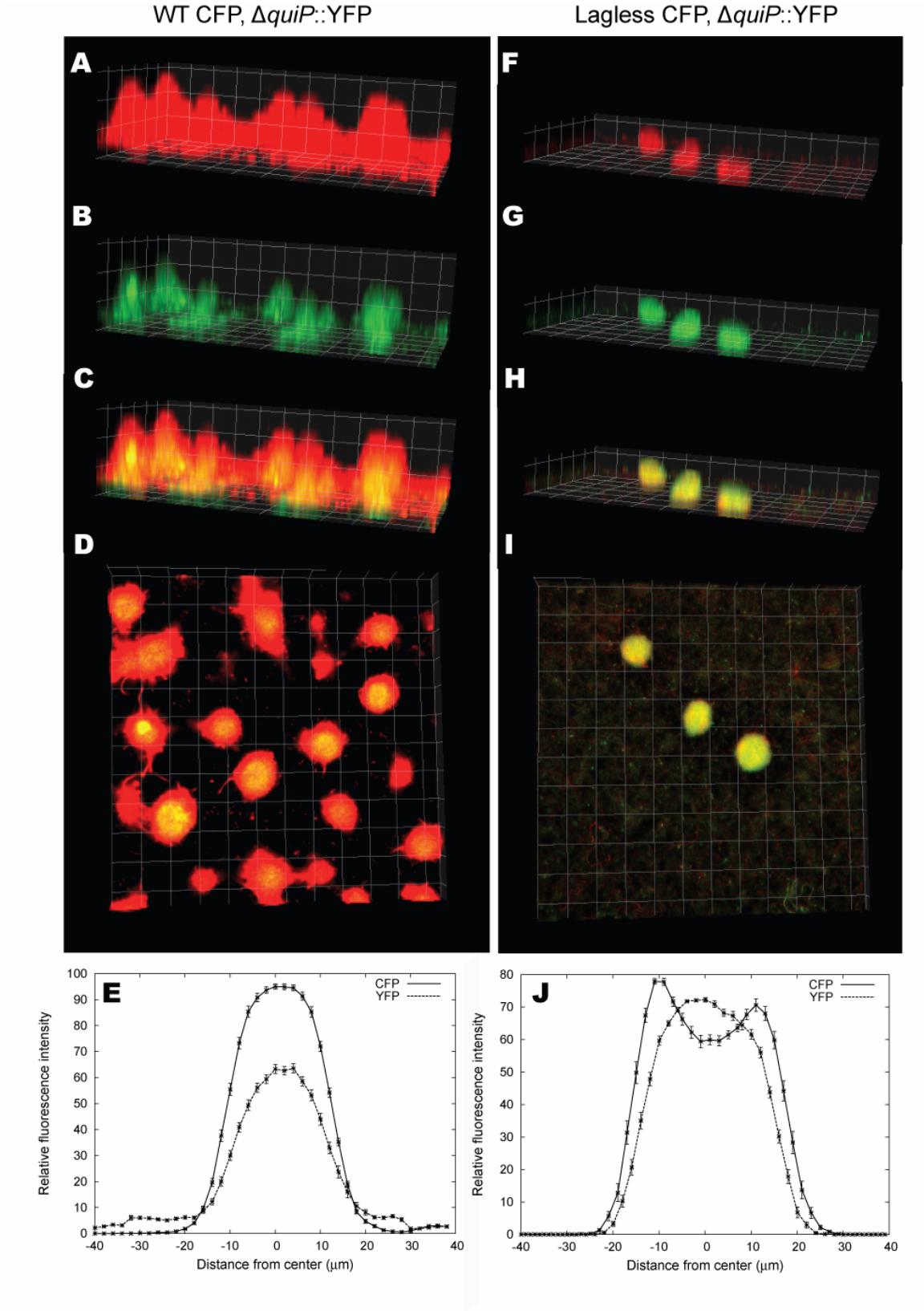

WT CFP, ΔquiP::YFP
Lagless CFP, ΔquiP::YFP
A
B
C
D
F
G
H
I
E
J
CFP
YFP
Relative fluorescence intensity
Distance from center (μm)

Figure 4.5. Confocal microscopy of biofilms formed by $PAO1_{wt}$ and $PAO1_{lagless}$ strains expressing CFP from a plasmid and with a Δ*quiP*::YFP transcriptional reporter. A-E $PAO1_{wt}$ at 96 hrs. A, CFP biofilm image; B, YFP biofilm image; C, merged fluorescence channels with overlap of CFP and YFP in yellow; D, cross section through the biofilm; E, quantification of expression profiles of CFP and YFP for $PA01_{wt}$. F-J, $PAO1_{lagless}$ strain at 120hr. F, CFP image of the biofilm; G, YFP image; H, merged fluorescence channels with overlap of CFP and YFP in yellow; I, cross section through the biofilm; J, quantification of expression profiles of CFP and YFP for $PA01_{lagless}$. Images were visualized using Imaris software. Biofilm images were quantified by averaging intensity profiles across 8 colonies ~40 μm in size for each strain. Error bars indicate the standard deviations of the binned pixel intensity values for all the images included in the plot. The double peaks in the $PAO1_{lagless}$ CFP channel are the result of increased cell numbers, and therefore increased fluorescence, at the edges of the colonies.

QUIP EXPRESSION BY $PAO1_{LAGLESS}$ AND $PAO1_{WT}$ GROWING IN BIOFILMS

$PAO1_{lagless}$ expressed the Δ*quiP*::YFP reporter gene throughout biofilm formation from initial attachment to formation of mushroom structures (Figure 4.4 D-F). The expression of the YFP reporter by the lagless strain closely followed the expression of CFP expressed constitutively by all cells from a plasmid (Figure 4.5 F-J). $PAO1_{wt}$ with the Δ*quiP*::YFP transcriptional fusion did not express YFP during the initial attachment to the surface (Figure 4.4 A), but after microcolonies formed, the center regions showed expression of the YFP gene (Figure 4.4 B, C). The wild type showed expression of CFP outside of the YFP expression in the microcolonies indicating that cells not expressing YFP were viable (Figure 4.5 C-E). The transcriptional fusion strains complemented with a functional copy of the *quiP* gene and its promoter in a neutral site showed similar patterns of YFP expression as the respective parent strains (Figure 4.4 D, H).

DISCUSSION

QuiP of *P. aeruginosa* was previously found necessary and sufficient for degradation of long chain AHL that was provided as a carbon source (69). These studies examine the role of *quiP* and its expression in *P. aeruginosa* wild type and a variant of *P. aeruginosa*, $PAO1_{lagless}$, which constitutively degrades long chain AHL. The experiments described were performed to understand how

AHL signal degradation by the *quiP* gene may be regulated by the comparison of the lagless strains with wild type PAO1, and when quorum signal degradation may occur by PAO1 by examining expression of YFP reporter fusions to *quiP*.

The $PAO1_{lagless}$ strain is defective in the regulation of the *quiP* gene. A knock out mutation in *quiP*, in which the gene was replaced with a YFP reporter gene, eliminated the ability for the lagless strain to degrade signal as a carbon source, restored the accumulation of nearly wild type levels of naturally produced 3OC12HSL (Figure 4.2), and the signal degradation phenotype could be regained by a single copy of *quiP* with its promoter present in a neutral site in the chromosome (Supplemental Table 4.2). There were no differences between the putative *quiP* promoter regions in $PAO1_{wt}$ and $PAO1_{lagless}$ (data not shown). The *quiP* gene was also necessary for long chain AHL degradation in a second lagless strain and the *quiP* mutation in this strain background restored significant accumulation of long chain AHL (Figure 4.2). These results indicate that the reduced accumulation of long chain signal in both lagless strains is not due to defects in long chain signal synthesis, but to constitutive degradation of signal produced. The *quiP* mutation in wild type *P. aeruginosa* did not influence the accumulation of long chain signal (Figure 4.2), which is consistent with lack of expression of this gene during batch growth conditions (Figure 4.3). Previous studies have shown that the ratio of C4HSL to total AHL signal is greater in batch culture grown PAO1 than in PAO1 in the biofilm state (148). A hypothesis is that AHL signal degradation within biofilms may contribute to this difference in AHL

signal ratios. Though the ratios of the C4HSL/to total AHL signal (C4HSL and 3OC12HSL) was greater in all cultures grown in biofilms compared to batch grown cultures, the absence of signal degrading ability by *quiP* mutants did not significantly affect the ratios of signal in the biofilm state (Supplemental Table 4.3). The differences in signal ratio between the two growth states were affected more by the concentration of C4HSL measured in the cultures (Supplemental Table 4.3). This experiment should be repeated as signal amounts from biofilm biomass could not be obtained with LC/MS and the method in general needs to be optimized.

The $PAO1_{lagless}$ strain differentially expressed several genes relative to the wild type strain during growth on succinate as shown by microarray analysis. The over-expression of *quiP* by lagless is consistent with its ability to degrade long chain signal without lag and accumulate less naturally produced 3OC12HSL (Figure 4.2). Several quorum regulated genes were also upregulated in the lagless strain relative to the wild type (Table 4.2). It is surprising that the lagless strain expresses quorum regulated genes (Table 4.2) when it accumulates only a fraction of the long chain signal as the wild type (Figure 4.1 and Figure 4.2). It is not known if the genes differentially expressed by the lagless strain share a common regulator that is broken in this strain or if and how *quiP* is related to them.

Knock out mutations were made in the lagless and wild type strains in transcriptional regulator encoding genes: *rsaL* (PA1431), *mvfR* (PA1003), and *vqsR* (PA1003) that were differentially expressed in the lagless strain relative to wild type (Table 4.2), and in the known response regulators for long chain AHL in PAO1: *lasR* and *qscR*. Examination of these genes cast a wide net to find out what circuits might intersect with the regulation of signal degradation. The gene *rsaL* was 7 fold up-regulated in the lagless strain and was previously reported to influence the production of 3OC12HSL by encoding a protein that is a negative regulator of *lasI* (32). The significant up-regulation of this gene in the lagless strain despite the decreased accumulation of 3OC12HSL signal suggests that there could be another regulator for the *rsaL* gene that has aberrant expression in the lagless strain. *mvfR* encodes a transcriptional regulator that affects multiple quorum regulated genes and is required for pyocyanin production and synthesis of *Pseudomonas* quinolone signal (35, 54, 167, 182). The lagless strain with the mutation in *mvfR* no longer produced blue pigment and this is consistent with studies that show *mvfR* is a key regulator of pyocyanin synthesis (16, 54). *vqsR* encodes a LuxR family regulator that that is known to control multiple quorum regulated genes (80, 165). None of the mutations in these genes affected the ability for the lagless strains to degrade signal without lag or precluded the ability for the wild type to eventually degrade signal when it was provided as a carbon source (Supplemental Table 4.2). This indicates that *quiP* is not regulated by these transcriptional regulators and the aberrant regulation in the lagless strain is potentially upstream of these genes and the *quiP* gene.

Knock-out mutations in *lasR*, which encodes the response regulator protein for 3OC12HSL, and *qscR*, a LuxR homolog that does not have a corresponding signal synthase gene, but has been shown encode a protein responsive to 3OC12HSL (22, 93, 95) as well as knock out mutations in both of these loci were made in the wild type and lagless stains. Mutations in these genes also did not influence signal degradation by the lagless or wild type strains (Supplemental Table 4.2) indicating that degradation of long chain signal is not dependent on perception of long chain signal by these known response regulators. If PAO1 has a genetic program to degrade AHL, the cue may be other than the signal itself and the lagless strain may have a mutation that is a short circuit to signal degradation.

The Δ*quiP*::YFP transcriptional reporter in the wild type background did not show significant expression during any phase of growth whereas the lagless strain expressed *quiP* throughout growth (Figure 4.3). The lagless YFP reporter strain complemented with *quiP* in a single copy in the chromosome showed similar ratios of YFP fluorescence/optical density indicating that *quiP* expression is not influenced by feedback from the gene itself. The differential expression of *quiP* by the lagless and wild type is consistent with microarray studies which show that the lagless strain over-expresses this gene in comparison to the wild type (Table 4.2) and accumulates less 3OC12HSL that the wild type due to constitutive degradation (Figure 4.2).

The stages of PAO1 biofilm formation have been well described and consist of attachment to a surface, microcolony formation, maturation to mature mushroom structures and dispersion, where sessile cells return to planktonic growth (30, 84, 139, 152). Though the lagless strain accumulated a fraction of the 3OC12HSL as the wild type (Figure 4.2), there were not significant structural differences in biofilms formed by the two strains over time. The amount of long chain signal that was accumulated by $PAO1_{lagless}$ may have been sufficient to activate genes involved in biofilm formation, or the conditions under which the biofilms were formed may not have been selective for a phenotype in the lagless strain. Previous reports have shown that the production and detection of long chain AHL signal, but not short chain AHL is important for the formation of biofilm architecture in *P. aeruginosa* (31). In other studies the differences were not as striking (33, 66, 131) and the structure of biofilms has been found to be influenced by experimental conditions such as the carbon source used for growth (85) and hydrodynamics (131). It would be interesting to compare biofilms formed by $PAO1_{lagless}$ under such different conditions.

$PAO1_{wt}$ and $PAO1_{lagless}$ differentially expressed *quiP* in biofilms. Cells of the lagless strain with the Δ*quiP*::YFP reporter fusion attached and colonized the surface similarly as the wild type strain, but $PAO1_{lagless}$ clearly expressed the YFP fusion whereas the wild type strain did not (Figure 4.4 A and D). Within 3 days, both strains formed macrocolonies that appeared similar in size and structure,

but differed in expression of the YFP reporter for *quiP* expression (Figure 4.5 E, J). $PAO1_{wt}$ showed local expression of YFP in the center regions of the biofilm (Figure 4.4 B-C, Figure 5 B-E). Cells on the outer edge of the microcolonies that did not express YFP were viable as seen by the presence of cells constitutively expressing CFP from a plasmid (Figure 4.5 A). The $PAO1_{lagless}$ strain expressed CFP and YFP in equal areas throughout the mushroom structure (Figure 4.4 E-F, Figure 4.5 G-J). These observations are consistent with constitutive expression of *quiP* by the lagless strain under all conditions tested so far (Figure 4.2, Figure 4.3, Table 4.2). The biofilms formed by wild type and lagless YFP reporter strains that were complemented with *quiP* showed similar biofilm structure and patterns of YFP expression within the microcolonies as the parent strains that contained Δ*quiP*::YFP (Figure 4.4 C and F).

The differential expression of the *quiP* gene in the lagless and wild type strains within biofilms is the first report of *quiP* expression under standard laboratory conditions as it has only been previously identified as the gene necessary for degradation of long chain AHL when it is provided as a carbon source (69). The center of microcolonies in biofilms is also the location of multiple activities which could give clues as to how this signal degradation process is related to the rest of *P. aeruginosa* physiology. Biofilm detachment by seeding dispersal, where cells vacate the center of microcolonies, has been shown to originate from the center where cells become highly motile, increase expression of flagella and decrease expression of pillus genes and produce the surfactant rhamnolipid (11, 71, 130,

140). Cells in the biofilms are differentiated from free-living, planktonic bacteria (173) and in the dispersal process cells transition from sessile growth within biofilms to planktonic growth (83). The long term survival of bacteria that form biofilms is thought to be dependent on the ability for bacteria to leave sessile growth and re-colonize new areas (151). In some biofilms we observed wild type cells expressing the Δ*quiP*::YFP transcriptional fusion that appeared to be leaving the center of the microcolony (Figure 4.4 B). It is possible that signal degradation could be part of the switch to prepare bacteria for growth in less population dense areas, and this could potentially include a re-setting of AHL mediated quorum sensing by degrading signals in the local environment. Rhamnolipid surfactant produced in the center of microcolonies in dispersion state of growth could potentially mediate the degradation of the fatty acid hydrocarbon (7), which could potentially aid in long chain AHL degradation. The environmental cues and molecular mechanisms that trigger cells to enter planktonic growth are not well known (130). Nutrient availability has been found to promote detachment (57, 71, 140) and recently NO was identified to promote dispersal (6). Understanding biofilm dispersal has implications for treatment infections caused by biofilms (6, 11). It is not known if degradation of AHL signal is related to the cell dispersal process, but it would be interesting to investigate the timing of *quiP* expression relative to known dispersal processes such as up-regulation of flagella or rhamnolipid production.

It is not clear what environmental cues *P. aeruginosa* may be using to regulate the expression of *quiP* within biofilms. The data suggest a cue or integration of multiple cues from within the center of biofilm microcolonies for the expression of *quiP*. Biofilms are heterogeneous environments with microniches (27), and there is also diversification of cells in this growth state that is thought to be beneficial for nutrient utilization and survival within the biofilm (12). The center of microcolonies in particular is where there are the most intense gradients of oxygen (96), nutrients (24) and where signaling molecules may be concentrated according to modeling studies of biofilms (68). The sessile microbial cells in the biofilm themselves can produce gradients of microbial metabolic products (6). The cues for *quiP* expression could therefore be complex, and this may explain why the expression of this gene has not been seen under any conditions in batch culture (Figure 4.3). Expression of *quiP* has not been identified in any previous microarray studies in batch culture (146, 169) or biofilms (63, 170), but it is unclear whether localized gene expression could be captured using this method. Expression of an AHL acylase in the center of microcolonies could be a phenotype that serves to prepare bacteria for conditions outside of the biofilm and return to growth in the planktonic state.

SUPPLEMENTAL MATERIALS

Table 4.1. Plasmids and primers for knock out mutations and promoter probing experiments

Plasmids	Characteristics	Source
pQF50	contains promoterless *lacZ* for promoter probing, Amp^r	(48)
pCM351	Gm^r, Amp^r, Tet^r, allelic exchange plasmid	(99)
pCM157	Amp^r, cre enzyme-containing plasmid	(99)

Primers used for knock-out mutations:

QscR_MCS1_KpnI_F: AAAGGTACCACGAAGTAGGCGGCGAA

QscR_MCS1_NdeI_R: AAAAAAACATATGATGCATGCCAGCTTCACCAGT

QscR_MCS2_AgeI_F: AAAACCGGTAACTGAATCGACGCCTC

QscR_MCS2_SacI_R: AAAGAGCTCTATCTCCTCTTGAATTGGAATAGACAT T

LasR_MCS1_KpnI_F: AAAGGTACCAGATCCTCTGGATCAACATGG TC

LasR_MCS1_NdeI_R: AAAAAAACATATGGCCATAGCGCTACGTTCTTCTTA

LasR_MCS2_AgeI_F: AAAACCGGTCTCTGATCTTGCCTCTCAGGTCG

LasR_MCS2_SacI_R: AAAGAGCTCCGACAGGTCCCCGTCATGAAAC

PA1431_MCS1_Kpn_F: AAAGGTACCCGAAGCGGTCTATCGCA

PA1431_MCS1_Nde_R: AAAAAACATATGAGCCATTGCTCTGATCTTTTCGGA

PA1431_MCS2_SacII_F: AAAAAACCGCGGGAGTAATAAGACCCAAATTAA

PA1431_MCS2_SacI_R: AAAGAGCTCTGCTTACCCTCTAGGA

PA2591_MCS1_KpnI_F: AAAGGTACCGCATCCTTGAGCAGC

PA2591_MCS1_NdeI_R: AAAAAAACATATGGATATCCACACAACTACTCC

PA2591_MCS2_AgeI_F: AAAACCGGTCGCTAGTGCGCCGC

PA2591_MCS2_SacI_R: AAAGAGCTCAAGCCGTCGCCGTCGCC

PA1003_MCS1_KpnI_F: AAAGGTACCAGTAGTAATGCGCCACCA
PA1003_MCS1_NdeI_R: AAAAAAACATATGCAGGTTATGAATAGGCATCCC
PA1003_MCS2_AgeI_F: AAAACCGGTGAGTAGAGCGTTCTCCAGCAGA
PA1003_MCS2_SacI_R: AAAGAGCTCGGTACGCGGCAGGTGCG

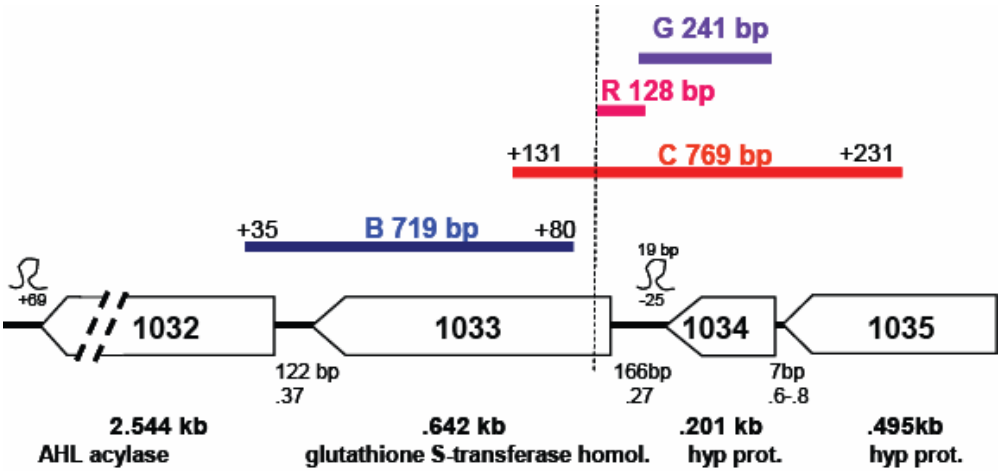
Primers used for promoter probing experiments:

PQF50C_F: AAAAAAGGATCCGTGCAACTGAACGACATCCA
PQF50C_R: AAAAAAAAGCTTAGGAAATCCGCCTGGAACT
PQF50B_F: AAAAAAGGATCCCATCCATCCGATCAACATCA
PQF50B_R: AAAAAAAAGCTTGGGGAAGAAAACGCATGAAG
PQF50D_F: AAAAAAGGATCCAACGAAGAGTTCTTCGCTCTG
PQF50D_R: AAAAAAAAGCTTAGGCGCGAATAGATGTCGTA
PQF50R_F: AAAAAAGGATCCCTTTTCGTTGGCGCG
PQF50R_R: AAAAAAAAGCTTGATCATCGGTGCGTCT
PQF50G_F: AAAAAAGGATCCTACACGGCCCGAACTGCAA
PQF50G_R: AAAAAAAAGCTTCGCGCCAACGAAAAG

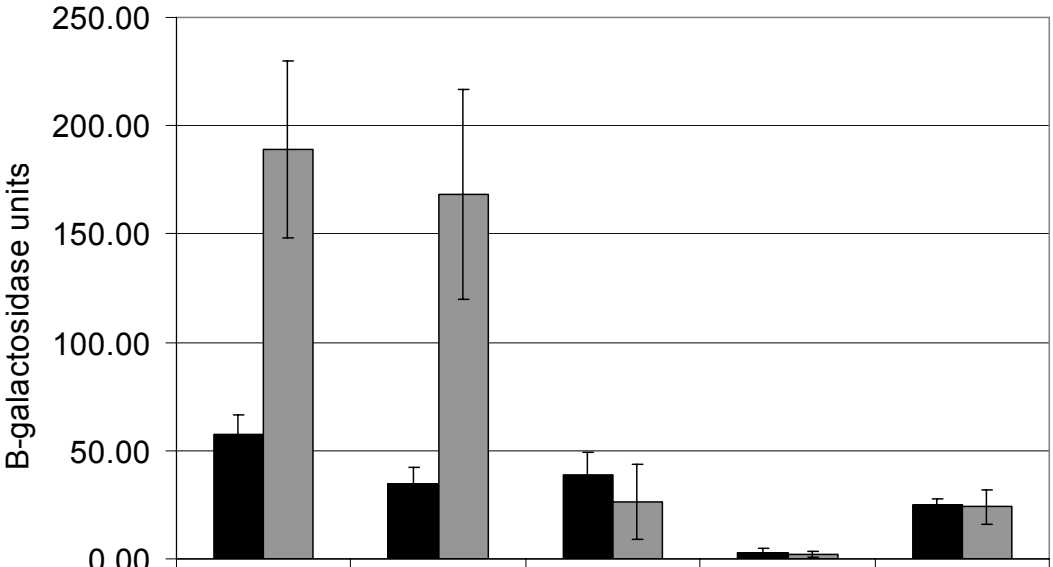
B-GAL ASSAYS AND PROMOTER PROBING

The plasmid PQF50 containing a promoterless *lacZ* was used for reporter fusion experiments (48). Putative promoter fragment C was a total of 769 bp and covered regions from 131 bp from the start of PA1033 and 638 bp upstream of PA1033, was made by amplifying the region from PAO1 genomic DNA with primers PQF50C_F and R, digested with HindIII and BamHI restriction enzymes and ligated into PQF50. Fragment B, which was 719 bp with 35 bp after the start of *quiP* (PA1032) and 684 bp upstream of *quiP*, was amplified with primers PQF50B_F and R, digested with HindIII and BamHI restriction digests and ligated into the PQF50 plasmid. Fragment C was positive for activity and fragments G and R, which were smaller regions within C, were amplified and

cloned into PQF50 to narrow down the section with promoter activity (primers provided in Supplemental Table 4.1). Fragment D, a region of DNA known not to have promoter activity, was cloned into PQF50 as a control. Constructs were confirmed by PCR and DNA sequencing. Putative promoter constructs were purified from *E. coli* and PQF50 plasmids transformed into PAO1 and PAO1lagless strains via electroporation. Beta galactosidase assays (104) were performed on cultures grown at 37 °C in a shaker at 250 rpm with minimal medium with 5 mM succinate, 1 mM C10HSL (Sigma) or 5 mM succinate with 5, 10 or 20 μM C4HSL, 3OC12HSL, or both signals.



Supplemental Figure 4.1. Map of putative promoter sequences for PA1032 (*quiP)* used in promoter probe experiments.



	C	R	B	G	D
5mM succinate	57.69	34.31	38.82	2.73	24.84
1mM C10HSL	189.04	168.19	26.46	2.18	24.03

Supplemental Figure 4.2. Promoter probing experiment with $PAO1_{lagless}$. The $PAO1_{lagless}$ strain expressing either of several PQF50 plasmids that contained different regions of DNA sequence upstream of the *quiP* gene: regions B,C,D,G, or R (Supplemental Figure 4.1) cloned in front of a promoterless *lacZ* within the plasmid, were assayed for B-galactosidase activity during growth in succinate or 1 mM C10HSL. Assays were performed at least in triplicate with averages shown under the bars.

GENERATION OF KNOCK-OUT MUTANTS

Knock-out mutants in the genes: PA1431 (*rsaL),* PA2591 (*vqsR),* PA1003 (*mvfR)*, PA1430 (*lasR),* PA1898 (*qscR),* and a *lasR* and *qscR* double mutant were made in the background of $PAO1_{wt}$ and in $PAO1_{lagless}$ by homologous recombination. One kb flanking regions of the genes were amplified from PAO1 genomic DNA using primers listed in Supplemental Table 4.1 and cloned into the multi-cloning sites of the plasmid pCM351 (99). Three bp from the start codon and three bp from the stop codon remained in the PA1431, PA1003, PA1898 and PA1430 (*lasR*) mutants and six bp from the start and three basepairs from the stop codon remained of the gene in the PA2591 knock out mutant. The Gm resistance gene was removed from the *lasR* mutant using a cre-lox system (99). The plasmid pCM157 (99) was transformed into the *lasR* mutant strain and colonies that were gentamycin sensitive, which indicated successful cre-mediated excision of the gm resistance gene, were picked. The pCM157 plasmid was lost after two rounds of growth in LB without antibiotic selection. The *qscR* double mutant was made by transforming the *lasR* mutant strain with the plasmid pCM351_*qscR* and double recombinants were selected for by resistance to gentamycin and sensitivity to tetracycline.

MEASUREMENT OF AHL SIGNAL RATIOS IN BATCH AND BIOFILM GROWN CULTURES

C4HSL and 3OC12HSL accumulated by $PAO1_{lagless}$, $PAO1_{wt}$ parent strains and Δ*quiP* knock-out mutants grown in batch culture in 1/10 LB and cultures grown in biofilms were examined. AHL from batch grown cultures were extracted with ethyl acetate as described above. Exponentially growing cells in 1/10 LB liquid culture were inoculated into silicone tubing as described above, and biofilms formed after six days were assayed for the amount of AHL signal accumulated. To assay for the amount of AHL from biofilm growth, 5 ml of effluent was collected from the biofilm lines of all strains and AHL extracted from the effluent as described above for batch grown cultures. Additionally, one foot of silicone tubing starting 1 inch from the inoculation point was removed and the biofilm washed from within the tube by rolling the tube and flushing with sterile medium. 5 ml of biofilm effluent was also collected for analysis of signal accumulation by extraction with acidified ethyl acetate and quantification with LC/ES MS (Hewlett Packard Series 1100) at the Caltech Environmental Analysis Laboratory. An C8 column (Eclipse XDB-C8, 2.1 x 50mm 3.5 μm, Agilent) was used with a gradient of 15% MeOH and for 3 min to 90% MeOH for 4 min to elute AHL. The LC/MS detection was not good for ethyl acetate extracted biofilm biomass samples; in the future less of the biofilm biomass should be extracted as the sample extracted was too complex to accurately extract AHL concentrations.

Supplemental Table 4.2. Long chain AHL signal degradation and other phenotypes of $PAO1_{lagless}$ and $PAO1_{wt}$ mutants. The parent strains and mutants were tested for the AHL degradation and growth phenotype by providing cells with 1mM C10HSL as a sole carbon source in pH 5.5 minimal medium. Degradation of and growth on long chain AHL and other phenotypes are noted.

Strains	**Parent strain**	**Δ *quiP*::YFP**	**Δ *quiP*::YFP, attTn7::miniTn7T-Gm-PA1033-32 (single copy)**	**Δ *mvfR*::Gm**	**Δ *rsaL*::Gm**	**Δ *vqsR*::Gm**	**Δ *lasR*::Gm**	**Δ *qscR*::Gm**	**Δ *lasR* Δ *qscR*::Gm**
$PAO1_{WT}$	+, 3 weeks	-	+, 3 weeks	+, several weeks, not blue	+, several weeks	+, several weeks	+, no lag not blue	+, several weeks	+, several weeks
$PAO1_{lagless}$	+, no lag	-	+, 4 days	+, no lag, not blue	+, no lag	+, no lag	+, no lag not blue	+, no lag	+, no lag
$PAO1_{lagless2}$	+, no lag	-	+, 6 days	+, no lag, not blue	+, no lag	+, no lag	+, no lag, not blue	+, no lag	ND

+ = growth on the long chain AHL C10HSL

- = no growth

ND = not determined

Supplemental Table 4.3. Ratio of C4HSL/total signal (C4HSL + 3OC12HSL) in batch and in biofilm grown cultures of $PAO1_{wt}$ and $PAO1_{lagless}$ parent strains and Δ*quiP* mutants. Strains were grown in batch culture or in a once flow-through biofilm for 6 days. Cultures were extracted with ethyl acetate and signal ratios were calculated from AHL concentrations determined by LC/MS by averaging data obtained by SIM and Scan methods.

	average of C4HSL/(C4HSL + 3OC12HSL) ratio				standard deviation			
	WT	**WT Δ*quiP***	**lagless**	**lagless Δ*quiP***	**WT**	**WT Δ*quiP***	**lagless**	**lagless Δ*quiP***
biofilm effluent	0.66	0.83	0.98	0.96	0.098	0.02	0.001	0.003
1/10 LB batch	0.44	0.64	0.89	0.69	0.02	0.02	0.02	0.021

Supplemental Table 4.4. Amounts of C4HSL, 3OC12HSL, and the ratio C4HSL/3OC12HSL measured in batch vs. biofilm grown cultures of $PAO1_{lagless}$ and $PAO1_{wt}$ parent strains and Δ*quiP* mutants. 5 ml 1/10LB batch cultures and 5 ml of biofilm effluent were extracted with acidified ethyl acetate and AHL concentration was determined by LC/MS. Scan and SIM are two methods for retrieving MS signal from C4HSL and 3OC12HSL. The values obtained from Sim and Scan data was averaged.

	SIM	SCAN	SIM	SCAN	SIM ratio	Scan ratio	SIM and SCAN	
WT	**μM C4HSL**	**μM C4HSL**	**μM 3OC12HSL**	**μM 3OC12HSL**	**C4HSL/ 3OC12HSL**	**C4HSL/ 3OC12HSL**	**Average C4HSL/ 3OC12HSL**	**std. dev.**
Batch	1.63	2.29	2.11	2.69	0.77	0.85	0.81	0.05
Biofilm	4.68	5.33	1.73	3.68	2.70	1.45	2.08	0.89
lagless								
Batch	5.22	8.18	0.73	0.79	7.18	10.26	8.72	2.18
Biofilm	8.05	12.59	0.13	0.22	61.58	58.24	59.91	2.36
WT Δ*quiP*								
Batch	5.96	8.28	3.56	4.24	1.68	1.95	1.81	0.19
Biofilm	5.82	8.77	1.09	2.03	5.31	4.32	4.81	0.70
lagless Δ*quiP*								
Batch	6.32	8.62	2.93	3.47	2.16	2.48	2.32	0.23
Biofilm	16.67	23.07	0.63	0.99	26.28	23.18	24.73	2.19

5. IDENTIFICATION OF A HOMOLOG OF THE QUIP AHL ACYLASE IN THE SOIL ISOLATE, *PSEUDOMONAS* STRAIN PAI-A

ABSTRACT

A soil bacterium, *Pseudomonas sp.* PAI-A, was previously identified to degrade long chain acyl-homoserine lactone quorum signaling molecules using an acylase mechanism (70). These studies identify a putative AHL acylase from *Pseudomonas sp.* PAI-A that is 77% identical to QuiP, an acylase that is necessary and sufficient for long chain AHL degradation in *Pseudomonas aeruginosa* PAO1. A knock-out mutation in the *quiP* homolog in PAI-A did not eliminate degradation and growth on AHL indicating that PAI-A has additional AHL acylases.

INTRODUCTION

Acyl-homoserine lactones (AHLs) are a class of signaling molecules used by several gram negative *Proteobacteria* in a process of cell-to-cell communication known as quorum sensing (QS). Quorum sensing mediated by AHLs was first discovered as the process that underlies bioluminescence by the marine

bacterium *Vibrio fischeri* (110). Numerous *Proteobacteria* have since been found to engage in cell-to-cell communication using these signaling molecules. AHLs all share a homoserine lactone moiety, but differ in the length of and substitutents in the acyl chain. AHLs are known with acyl chains from 4 to 18 carbons in length (143, 168) and are synthesized and detected in the cell by mainly homologs of the *V. fischeri* LuxI and LuxR proteins, respectively (52, 53). Quorum sensing bacteria detect the concentration of signaling molecules in the environment and use this to couple gene transcription to changes in cell population density. Quorum sensing using AHLs has been found to regulate a host of microbial group behaviors from the production of extracellular enzymes and antibiotics to motility and biofilm formation (reviewed in (174)).

In recent years, numerous microbes have been identified with the ability to degrade AHL (40, 92, 97, 118, 189). These studies have shown that AHL signal degradation could potentially take place in a range of environments and by both microbes that quorum sense as well as those that do not have known quorum sensing systems. In one mechanism of AHL degradation, bacteria (40, 117, 135, 161) and also eukaryotic cells (15, 23, 34) have been found to degrade AHL via a lactonase mechanism, by which the homoserine lactone ring is hydrolyzed to form acyl homoserine (40). In another mechanism, microbes use an acylase mechanism to degrade AHL (97, 118). The AHL acylases so far identified are part of a larger family of proteins known as Ntn (N-terminal nucleophile) hydrolases (70, 118). These proteins share characteristic processing of a

propolypeptide where a linker region is removed and alpha and beta subunits fold to form a heterodimer (13, 65, 82).

Pseudomonas sp. PAI-A is a soil - isolate that was previously found to degrade AHL signal via an acylase mechanism that is specific for long chain acyl homoserine lactones (70). *Pseudomonas sp*. PAI-A was able to degrade and grow using long chain AHL as a sole carbon source without the long lag observed in *P. aeruginosa* (70). Two genes of *P. aeruginosa*, PA2385 (*pvdQ*) and PA1032 (*quiP*), were found sufficient for the degradation of long chain AHL, but only the *quiP* gene was necessary for AHL degradation (69). These studies identify a QuiP homolog in *Pseudomonas sp.* PAI-A.

MATERIALS AND METHODS

STRAINS AND CULTURE CONDITIONS

Pseudomonas str. PAI-A was originally isolated from soil in Iowa from an enrichment with 3OC12HSL as the sole carbon source (70). All cultures were maintained in LB (lysogen broth) amended with antibiotics when appropriate. Antibiotic concentrations used *Pseudomonas* str. PAI-A were: 15 μg/ml gentamycin, 10 μg/ml tetracycline, 250 μg/ml carbenicillin, and for *E. coli,* 100 μg/ml ampicillin. A defined freshwater medium buffered at pH 5.5 with 5 mM 2-

(*N*-morpholino)-ethanesulfonic acid (MES) was used for studies with growth on defined carbon sources (92).

IDENTIFICATION OF A QUIP AHL ACYLASE HOMOLOG FROM PAI-A

Degenerate primers were designed from an amino acid alignment of the protein QuiP (PA1032) of *Pseudomonas aeruginosa* PAO1 (154) to proteins most similar from other pseudomonads: *P. mendocina* (NCBI accession # ZP_01527301), *P. fluorescens* pfo-1(NCBI accession # ABA72956), *P. syringe* (NCBI accession # YP_273648, (78)) and *P. putida* (NCBI accession # NP_743265) (111) using the GeneDoc alignment program (http://www.psc.edu/biomed/genedoc/). The degenerate primers F: CIGARTAYTGGAARCCIGARGA (16-fold degenerate) and R: GRTCIGCRTAICCRTCCC (8-fold degenerate) were designed using aligned amino acids 171-179 and 494-500 for the forward and reverse primers respectively (Figure 5.1). Genomic DNA was extracted from cells of *Pseudomonas* str. PAI-A growing exponentially in LB medium, using the DNeasy tissue kit (Qiagen) according to the manufacturer's protocol. PCR using the degenerate primers amplified a 1.1 kb fragment from PAI-A genomic DNA that was highly similar to penicillin amidases by comparisons to protein databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The complete gene and flanking regions were determined using the method of inverse PCR (iPCR) (112). The restriction enzyme XhoI, which cut once in the gene fragment obtained from

PAI-A, was used to digest PAI-A genomic DNA. The digested fragments were ligated and used in PCR with outward facing primers iPCR_F1: TGAACATCCTCTACGCCGATGACA and iPCR_R1: TTGCCGTTGGCCTG GTAGTAAAGA, which generated a 1.5 kb fragment of DNA and iPCR_F6: TCATGGGCGACAACCAGGATCTGT, iPCR_R3: CAAAGTTCAGCAGGG TGAAGACCA, which amplified approximately 1.2 kb. The PCR amplified products were cloned using the TOPO TA cloning system (Invitrogen). At least 3X coverage of the fragments were obtained and the sequencing traces checked and contigs assembled using the SeqMan program (Lasergene). The full sequence of the putative AHL acylase, 600bp upstream and approximately 400bp downstream of flanking regions was determined.

DELETION OF THE QUIP HOMOLOG OF *PSEUDOMONAS SP.* PAI-A

A 2025 bp deletion of the QuiP homolog in strain PAI-A was made by homologous recombination. A 793 bp fragment, which included 120 bp from the start of the PAI-A *quiP* gene, was amplified using primers: F: AGAATTCGAAGCCGGCCTGCCCTATC and R: AAAGGTACCCAGGCTGTCC GAGTAGC (EcoRI and KpnI sites underlined, respectively). This fragment was digested with EcoRI and KpnI restriction digests and ligated into one of two multi-cloning sites in the allelic exchange plasmid pCM351 (99).

A fragment of 829 bp, which included 430 bp downstream of the *quiP* homolog of strain PAI-A, was amplified using primers: F: AAAACCGGTAAGCTGCACACCTA TGAGTGG and R: AAAGAGCTCGAAGTTCAGTTGGTCAGGAGTGAT (AgeI and SacI restriction sites underlined). The fragment was digested with AgeI and SacI restriction enzymes and ligated into a second multi-cloning site of pCM351 (99). The resulting plasmid was transformed into str. PAI-A via electroporation and putative double recombinants were selected for by resistance to gentamycin at 15 μg/ml and sensitivity to tetracycline selection at 10 μg/ml. Mutants were confirmed by PCR.

CONSTITUTIVE EXPRESSION OF THE *QUIP* GENE HOMOLOG OF STR. PAI-A

The *quiP* gene homolog of str. PAI-A was amplified from PAI-A genomic DNA using primers: F: AAAAAAACATATGATGGCTTCGCCAGCCTTCAAG and R: AAGGATCCTGCCGATCATTTTTGCGG (NdeI and BamHI restriction sites underlined, respectively). The complete *quiP* gene sequence was digested using NdeI and BamHI restriction digests and ligated into multi cloning site of the expression plasmid pucp18Nde (28), which replicated stably in str. PAI-A. The fidelity of the gene cloned was verified by sequencing. The PAI-A *quiP* expression plasmid, $pQuiP_{PAI\text{-}A}$ was transformed into PAI-A strains via electroporation.

PHENOTYPE STUDIES OF THE STR. PAI-A WITH Δ*QUIP* AND MUTANTS COMPLEMENTED WITH P$QUIP_{PAI-A}$

The ability for the PAI-A Δ*quiP* mutant to degrade AHL was tested by incubation of cultures with 1 mM C10HSL as the sole carbon source in a minimal medium buffered at pH7 with MOPS, as described previously (70). The cultures were incubated in a 37 °C water bath shaking at 220 rpm. Products of AHL degradation were measured using LC/MS.

RESULTS

IDENTIFICATION OF A QUIP HOMOLOG IN *PSEUDOMONAS* PAI-A

The AHL acylase homolog in *Psuedomonas str.* PAI-A is 77% identical to the QuiP protein encoded by the gene PA1032 of *P. aeruginosa* PAO1. The alpha and beta regions of this protein from post translational modification are predicted to begin at residues 40 and 268, respectively based on alignment to other acylases, PvdQ and AhlM, in which amino terminal sequencing was performed to determine the location of the subunits (118). The region upstream of *quiP* of PAI-A is 78% identical to PA1033, the gene upstream of *quiP* in *P. aeruginosa,* which encodes a putative glutathione S transferase. The downstream gene of *quiP* of PAI-A is 74% identical to a protein, nicotimanideribosyltransferase of

PAO1, encoded by PA4317 from the PAO1 genome (154), but this is not similar to the gene downstream of *quiP* in *P. aeruginosa*.

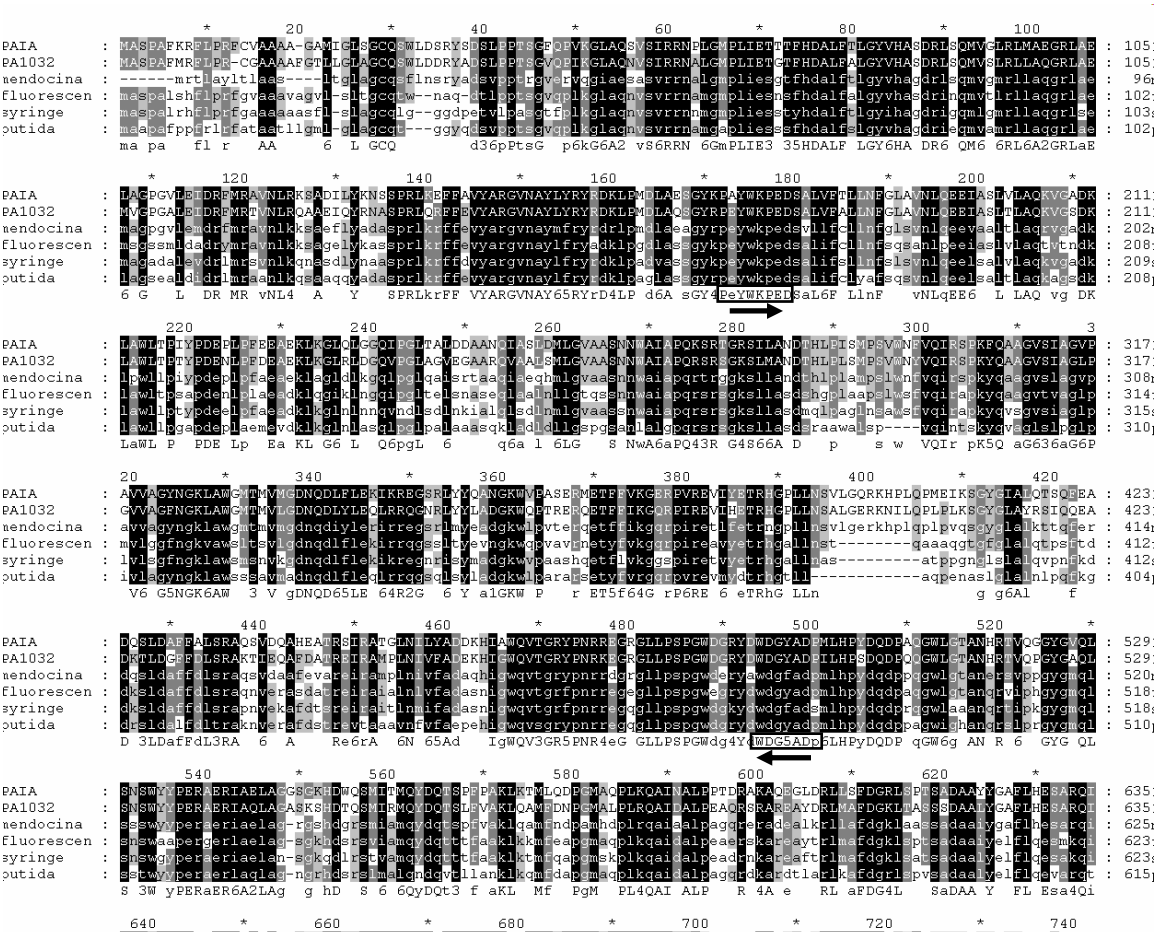


Figure 5.1. Alignment using GeneDoc (www.psc.edu/biomed/genedoc/) of *Pseudomonas sp.* protein homologs to QuiP, an AHL acylase of *P. aeruginosa* encoded by the gene PA1032. The black and grey areas indicate identical and similar amino acids, respectively. Regions used to design degenerate primers to amplify the QuiP homolog from PAI-A are indicated by boxes. The sequences used are listed as PA1032 from *P. aeruginosa,* and homologs from *Pseudomonas mendocina* ZP01527, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas syringe* phaseolicola and *Pseudomonas putida* KT2440. The full sequence of the PAI-A homolog, obtained using the degenerate primers and inverse PCR, is aligned with the rest of the sequences. The QuiP homolog of PAI-A is 77% identical to QuiP of *P. aeruginosa*.

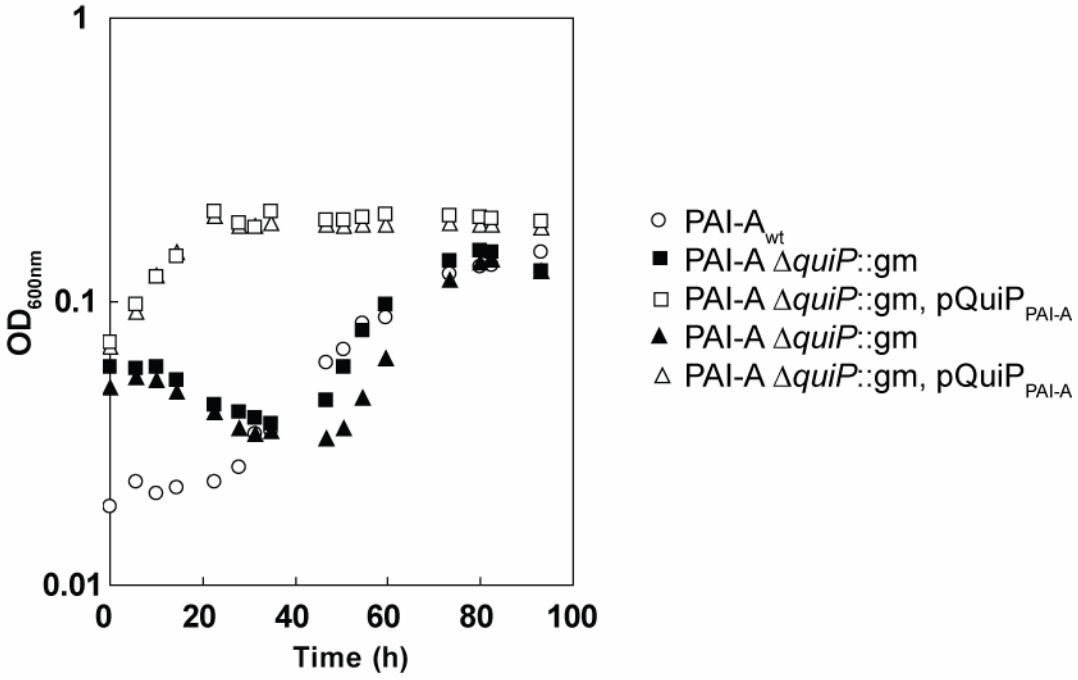


Figure 5.2. Growth of PAI-A wild type, Δ*quiP*::gm mutants, and knock-out mutants complemented with pQuiP$_{PAI-A}$. Mutant cultures were grown in duplicate in a defined, pH 7 minimal medium with 1mM C10HSL as a sole carbon source. Growth was measured using optical density at 600 nm.

The PAI-A Δ*quiP* mutant strains and wild type parent strain began to grow using long chain AHL C10HSL as a sole carbon source after approximately 30 hours. The *ΔquiP* knock out mutants that constitutively expressed *quiP* from a plasmid degraded and grew on AHL immediately. All of the strains had a similar doubling time of approximately 15 hours on C10HSL.

DISCUSSION

These studies identify a putative AHL acylase of *Pseudomonas sp.* PAI-A. The acylase is a homolog of QuiP of *P. aeruginosa* PAO1, which was found to be necessary and sufficient for the degradation of long chain AHL (69). *Pseudomonas* PAI-A is closely related to *Pseudomonas aeruginosa* PAO1 sharing 98% identity in 16srDNA, but it is not an *aeruginosa* strain as it does not grow at 42 °C, produce colored or fluorescent pigments or grow anaerobically with nitrate (70). PAI-A has the ability to degrade long chain AHL without the long lag seen prior to *P. aeruginosa* degradation of AHL as a carbon source (70).

The QuiP homolog of PAI-A is 77% identical to the QuiP AHL acylase of *Pseudomonas aeruginosa* PAO1 and other *P. aeruginosa* strains, and is highly similar (expect value = 0) to penicillin amidases homolog from *P. mendocina* (75% identity), *Azotobacter vinelandii* (69% identity), *P. fluorescens* PfO-1 (65%), *P. syringe* (63%), *P. putida* KT2440 (59%) and *Gleobacter violacens* PCC 7421 (30% expect value = 2e -88) by database comparisons (www.ncbi.nlm.nih.gov /BLAST/). *P. aeruginosa* PAO1 has two known AHL acylases, PvdQ and QuiP, but only expresses the mRNA and protein product of the QuiP acylase when degrading AHL as a sole carbon source (69). To determine if the QuiP homolog in str. PAI-A is solely responsible for degradation and growth on AHL phenotype of this strain, a knock out mutation of *quiP* in PAI-A was made. The PAI-A *quiP* mutant was still able to degrade and grow on long chain AHL as a sole carbon source (Figure 5.2). These mutants degraded and grew on C10HSL with similar

doubling times as the PAI-A wild-type parent strain and the strains constitutively expressing the PAI-A *quiP* gene from a plasmid, though the *quiP* constitutive strains initiated degradation and growth on AHL earlier (Figure 5.2). The wild-type and mutant strains produced HSL as a product of the degradation, indicating an acylase mechanism was used. The production of HSL was not stoichiometric with C10HSL degradation suggesting that PAI-A may degrade HSL (data not shown).

The QuiP homolog of PAI-A is not necessary for PAI-A degradation and growth on AHL, however, constitutive expression of this gene in str. PAI-A caused earlier degradation of AHL than the wild type parent strain (Figure 5.2) and constitutive expression in *P. aeruginosa* PAO1, which usually shows long lags before degradation of AHL as a sole carbon source, enabled the lagless degradation of AHL as a carbon source at pH 7, but not at pH 5.5 (data not shown). These initial studies suggest that the QuiP homolog of PAI-A has the ability to degrade AHL, but more stringent experiments would need to be done to determine sufficiency. None of the strains constitutively expressing *quiP* were able to grow on the short chain AHL 3OC6HSL. It is not clear if PAI-A may use its QuiP homolog in addition to another AHL acylase.

Other bacteria, such as *Bacillus sp.* (40) *Streptomyces sp.* (118), and *Arthrobacter sp.* (117), have been found to degrade AHL signal though they do not produce or utilize these signaling molecules in a quorum sensing system.

Pseudomonas str. PAI-A has the ability to degrade long chain AHL though it does not accumulate AHL or show any indications of having an AHL mediated quorum sensing system under the conditions examined (70). Microbial soil consortia were found to rapidly mineralize acyl homoserine lactones, indicating that soil microbes are primed to degrade AHL (171). *Pseudomonas* str. PAI-A could play a role in AHL degrading activities in the soil, though identifying the AHL acylase that this bacterium uses may not easy. If AHL degradation confers and advantage to str. PAI-A, or plays an important role in this bacterium's physiology in the soil, PAI-A may have redundant enzymes with AHL degrading capability. Knowledge of the AHL acylases used by str. PAI-A could enable studies of the expression of AHL degrading enzymes and examination of their role in microbial interactions in natural settings.

6. CONCLUSIONS

This thesis explores the degradation of acyl-homoserine (AHL) quorum signaling molecules by two *Pseudomonas* sp., a soil pseudomonad and a clinical isolate of *Pseudomonas aeruginosa* strain PAO1. Though closely related at the 16s rRNA level, the pseudomonads differ in physiology and life history. PAO1 is an opportunistic pathogen that produces numerous extracellular enzymes, toxins, and pigmented compounds, and this bacterium uses two well-known AHL-mediated quorum sensing systems in the regulation of virulence factor production. The soil isolate, strain PAI-A, does not accumulate AHL, does not produce pigments and there is no evidence that it has a quorum sensing system. Despite their differences, both species are able to degrade AHLs and show specificity for long chain signal degradation. Is there a place for this AHL degradation in the daily lives of both pseudomonads?

This thesis shows how the pseudomonads are able to degrade AHL, and for the clinical isolate PAO1, demonstrates where and when this signal degrading activity may occur. The QuiP AHL acylase of PAO1 is encoded by the gene PA1032 and, other than under conditions in which the AHL signal is provided as a sole carbon source, YFP reporter gene studies show that PAO1 expresses the *quiP* gene in the center of microcolonies within the biofilm growth state. For what reason(s) might PAO1 express its long chain AHL acylase in the center of microcolonies in the biofilm state? There are several possibilities:

1. PAO1 is starved in the center of biofilm microcolonies which contain microniches and steep gradients of oxygen, nutrients, and products of cellular metabolism (24, 27). PAO1, which has the ability to degrade AHL, may do so to obtain carbon and energy, and modeling studies of the amount of signal in a flow through biofilm system have predicted that the concentration of signaling molecule will be greatest in the center of microcolonies (68).

2. Similarly, degradation may be related to cell starvation, but degradation of AHL signal could occur as part of a program to exit from the energetically expensive quorum sensing state.

3. Since populations of PAO1 cells expressing the AHL acylase with the YFP reporter gene have been seen leaving the center of biofilm microcolonies (Figure 4.4 B), signal degradation could be related to a previously described dispersal state of growth (84, 139) where cells transition from sessile growth in the biofilm to the planktonic growth state. Signal degradation may prepare cells returning to the planktonic growth state for a fresh cycle of quorum sensing. The options listed are not mutually exclusive. The cues from the environment or signals for initiation of signal degradation remain to be determined.

A variant of PAO1, $PAO1_{lagless}$, constitutively degrades naturally produced AHL. Though the variant strain aberrantly degrades AHL signal all of the time, relatively few consequences of this activity were seen in traditionally quorum regulated activities, such as the formation of biofilms with complex architecture (31). If *P. aeruginosa* does naturally engage in AHL signal degradation,

degradation may occur in response to multiple factors in the environment and occur as part of a series of changes in gene expression. Signal degradation expressed on its own may not reveal the effects of a degradation program by cells any more than experiments, where AHL signal added to *P. aeruginosa* cultures did not activate genes normally regulated by AHL, would reveal about AHL quorum regulation of genes (185). The context under which both activities, initiation of quorum sensing and signal degradation occur, must make a difference.

Though QuiP is necessary and sufficient for PAO1 degradation of AHL, the QuiP homolog of the soil isolate str. PAI-A was not necessary for the degradation and growth on AHL phenotype that we observe in this strain. This suggests that, like PAO1, the soil isolate has at least one other AHL acylase in its genome. It is not known whether the soil isolate uses the QuiP acylase in addition to another AHL acylase, or to what extent an enzyme that is found active in the laboratory would be expressed in nature, where numerous cues in from environment may affect the gene expression of the bacterium.

Pseudomonas str. PAI-A is one AHL degrading bacterial species from the soil, an environment with potential for high microbial species diversity (178) and for which rapid AHL signal degradation has been found to be carried out by microbial consortia (172). Knowing the genes involved in degradation of AHL by str. PAI-A could enable studies of expression of signal degradation in the soil *in situ,* and of

the interaction between AHL producers and degraders, which to my knowledge has not been visualized. Wang and Leadbetter have shown that soil microbial communities can mineralize AHL to below what is sensed by known quorum sensing bacteria indicating that AHL degradation can challenge quorum sensing bacteria in natural communities (172). Their study shows the extent to which AHL degradation can occur in nature, and suggests the heated interactions that may be occurring between quorum sensing and quorum signal degrading microbes in the soil.

The AHL degrading enzymes known and predicted (Appendix Table 2) from three domains of life suggest the possibility for interesting interactions in diverse environments. The further studies of known microbes (136), and microbial surveys of environments from the rhizosphere to marine habitats (18, 29, 168) have identified an increasing number of AHL-producing bacteria that could participate in heated battles over AHL. For example, it would be interesting to study AHL interactions from microbes in acidic mine drainage environments, where abiotically, AHLs are predicted to persist based on the stability of the molecule at low pH (186). This extreme environment suggests where AHL degraders may have a niche, and the bacterium *Acidithiobacillus ferrooxidans,* which is now known to have two AHL producing systems (47, 136), could have interactions with AHL degraders. Additionally, synergistic interactions, which are most likely how signals are degraded in nature, can take place between bacteria that work together to degrade AHL signals as shown by co-cultures of microbes

from the soil that degrade AHL signals synergistically in the lab (49, 184). The range of microbes and eukaryotes with signal degrading activity (Appendix Table 1) suggest this process is wide-spread, and as more bacterial-bacterial and bacterial-eukaryotic interactions are studied, we may gain further insights into the intra- and inter-species interactions with acyl-homoserine lactones.

APPENDIX

Table 1. Mechanisms and proteins involved in AHL degradation by diverse bacteria and eukaryotes

(Table adapted from Y-J Wang, J.J. Huang and J.R. Leadbetter, *Adv. Appl. Microbiol.*, *in press*)

	Species	Mechanism	Protein	Reference
Bacteria				
Proteobacteria				
α-proteobacteria	*Agrobacterium tumefaciens* A6	Lactonase	AttM	(189)
	A. tumefaciens C58	Lactonase	AttM & AiiB	(17)
β-proteobacteria	*Variovorax paradoxus* VAI-C	Acylase	ND*	(92)
	Ralstonia sp. XJ12B	Acylase	AiiD	(97)
γ-proteobacteria	*Pseudomonas aeruginosa* PAO1	Acylase	PvdQ/PA2385 QuiP/PA1032	(69, 70)
	Pseudomonas sp. PAI-A	Acylase	ND	(70)
	Klebsiella pneumoniae KCTC2241	Lactonase	AhlK	(117)
	Acinetobacter sp. C1010	ND	ND	(81)
Firmicutes Bacilli (low-G+C Gram-positive)	*Bacillus* sp. 240B1	Lactonase	AiiA	(40, 41)
	B. thuringiensis	Lactonase	AiiA homologues	(39, 94)
	B. cereus	Lactonase	AiiA homologues	(39, 135)
	B. mycoides	Lactonase	AiiA homologues	(39)
	B. stearothermophillus	Lactonase	ND	(117)
	B. anthracis	Lactonase	AiiA homologues	(161)
Actinobacteria Actinobacteria (high-G+C Gram-positive)	*Rhodococcus erythropolis* W2	Acylase & oxido-reductase	ND	(162, 163)
	Arthrobacter sp. IBN110	Lactonase	AhlD	(117)
	Streptomyces sp. M664	Acylase	AhlM	(118)
Eukarya	Mammalian sera/tissues	Lactonase	Paraoxonases (PON1, PON2, & PON3)	(23, 42, 115, 183)
	Alga *Laminaria digitata*	Oxidized halogen reaction	Not identified	(15, 101)
	Legume *Lotus corniculatus*	ND	ND	(34)

*ND, Not determined.

Table 2. Homologs of AHL lactonases and acylases. Homologs to known AHL lactonase and acylase enzymes were obtained using protein BLAST database searches (http://www.ncbi.nlm.nih.gov/BLAST/)

(Table adapted from Y-J Wang, J.J. Huang and J.R. Leadbetter, *Adv. Appl. Microbiol.*, *in press*)

Organism with Homolog	**Protein** (as annotated)	**% AA Similarity**	**AHLase Homolog**	**Phylum**
AHL lactonase homologs				
Deinococcus radiodurans R1	Hypothetical protein	57%	AiiB	*Deinococcus-Thermus*
Moorella thermoacetica ATCC 39073	Beta-lactamase-like	53%	AiiB	*Firmicutes*
Thiomicrospira crunogena XCL-2	Beta-lactamase-like	51%	AiiB	*Proteobacteria*
Bradyrhizobium japonicum USDA 110	AttM/AiiB family protein	51%	AiiB	*Proteobacteria*
Natronomonas pharaonis	Hydrolase	50%	AiiB	*Euryarchaeota*
Photorhabdus luminescens subsp.	Hypothetical protein plu2238	88%	AttM	*Proteobacteria*
Yersinia intermedia ATCC 29909	Zn-dependent hydrolases	87%	AttM	*Proteobacteria*
Nocardioides sp. JS614	Beta-lactamase-like	56%	AttM	*Actinobacteria*
Clostridium beijerincki NCIMB 8052	Putative metallohydrolase	56%	AhlD	*Firmicutes*
Staphylococcus haemolyticus	Hypothetical protein	52%	AhlD	*Firmicutes*
Aspergillus oryzae	Hypothetical protein	49%	AhlD	*Fungi*
Haloarcula marismortui	Hypothetical protein	45%	AhlD	*Euryarchaeota*
Sulfolobus solfataricus	Conserved hypothetical protein	55%	AhlD	*Crenarchaeota*
Thermoplasma volcanium	Zn-dependent hydrolase	50%	AhlD	*Euryarchaeota*
Paracoccus denitrificans PD1222	Beta-lactamase-like	56%	AiiA	*Proteobacteria*
Archaeoglobus fulgidus DSM 4304	Hypothetical protein	54%	AiiA	*Euryarchaeota*
Desulfovibrio desulfuricans	Metallo-beta-lactamase family	52%	AiiA	*Proteobacteria*
Bacillus licheniformis ATTC 14580	Putative hydrolase	51%	AiiA	*Firmicutes*
Leptospira interrogans sv. Copenhageni	Metallo-beta-lactamase	51%	AiiA	*Spirochaetes*
Ralstonia eutropha JMP134	Beta-lactamase-like	51%	AiiA	*Proteobacteria*

Frankia sp. EAN1pec	Beta-lactamase-like	50%	AiiA	*Actinobacteria*
Rubrobacter xylanophilus DSM 9941	Beta-lactamase-like	50%	AiiA	*Actinobacteria*
Deinococcus geothermalis DSM 11300	Beta-lactamase-like	50%	AiiA	*Deinococcus-Thermus*
Rhizobium etli	Hypothetical protein	49%	AiiA	*Proteobacteria*
Canis familiaris (Dog)	Serum paraoxonase/arylesterase	91%	PON1	*Metazoa*
Bos taurus (Cow)	Similar to paraoxonase 1	90%	PON1	*Metazoa*
Oryctolagus cuniculus (Rabbit)	Serum paraoxonase/arylesterase 1	90%	PON1	*Metazoa*
Tetraodon nigroviridis (Pufferfish)	Unnamed protein product	78%	PON1	*Metazoa*
Xenopus laevis (African Clawed Frog)	MGC80011 protein	77%	PON1	*Metazoa*
Streptomyces coelicolor	Gluconolactonase precursor	53%	PON1	*Actinobacteria*
Trichodesmium erythraem	Senescence marker protein-30	53%	PON1	*Cyanobacterium*
Aspergillus fumigatus (Green Mold)	Hypothetical protein	50%	PON1	*Fungi*
Homo sapiens (Human)	Paraoxonase/arylesterase	94%	PON2	*Metazoa*
Pan troglodytes (Chimpanzee)	Similar to paraoxonase/arylesterase	94%	PON2	*Metazoa*
Gallus gallus (Domestic Chicken)	Similar to paraoxonase 2	84%	PON2	*Metazoa*
Pongo pygmaeus (Bornean Orangutan)	Paraoxonase 1	83%	PON2	*Metazoa*
Xenopus tropicalis (Western Clawed Frog)	Paraoxonase 1	80%	PON2	*Metazoa*
Platichthys flesus (Wild Flounder)	Paraoxonase (arylesterase)	73%	PON2	*Metazoa*
Danio rerio (Zebrafish)	Similar to paraoxonase 2	72%	PON2	*Metazoa*
Strongylocentrotus purpuratus (Urchin)	Similar to paraoxonase 2	54%	PON2	*Metazoa*
Caenorhabditis briggsae (Nematode)	Hypothetical protein CBG10483	50%	PON2	*Metazoa*
AHL acylase homologs				
Deinococcus radiodurans R1	Aculeacin A acylase	68%	AiiD	*Deinococcus-Thermus*
Hahella chejuensis KCTC 2396	Protein related to penicillin acylase	51%	AiiD	*Proteobacteria*
Nocardioides sp. JS614	Peptidase S45, penicillin amidase	52%	AiiD	*Actinobacteria*
Psychrobacter cryohalolentis K5	Peptidase S45, penicillin amidase	53%	AiiD	*Proteobacteria*
Ralstonia metallidurans	Penicillin amidase	81%	AiiD	*Proteobacteria*
Ralstonia solanacearum GMI1000	Conserved	89%	AiiD	*Proteobacteria*

	hypothetical protein			
Rubrivivax gelatinosus PM1	Protein related to penicillin acylase	48%	QuiP	*Proteobacteria*
Azotobacter vinelandii AvOP	Peptidase S45, penicillin amidase	82%	QuiP	*Proteobacteria*
Gloeobacter violaceus PCC 7421	Probable penicillin amidase	49%	QuiP	*Cyanobacteria*
Nostoc punctiforme PCC 73102	Related to penicillin acylase	48%	QuiP	*Cyanobacteria*
Pseudomonas fluorescens PfO-1	Penicillin amidase family protein	79%	QuiP	*Proteobacteria*
Pseudomonas putida	Penicillin amidase family protein	69%	PvdQ	*Proteobacteria*
Pseudomonas syringae	Penicillin amidase family protein	74%	PvdQ	*Proteobacteria*
Streptomyces lavendulae subsp.	Penicillin V acylase precursor	52%	PvdQ	*Actinobacteria*
Actinoplanes utahensis	Aculeacin A acylase	54%	AhlM	*Actinobacteria*
Shewanella baltica OS155	Peptidase S45, penicillin amidase	48%	AhlM	*Proteobacteria*

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