Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication

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Bacterial communities use “quorum sensing” (QS) to coordinate their population behavior through the action of extracellular signal molecules, such as the N-acyl-HSLs. The versatile and ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* is a well-studied model for AHL-mediated QS. This species also produces an intercellular signal distinct from AHLs, 3,4-dihydroxy-2-heptylquinoline (HQNO), which belongs to a family of poorly characterized 4-hydroxy-2-alkylquinolines (HAQs) previously identified for their antimicrobial activity. Here we use liquid chromatography (LC)/MS, genetics, and whole-genome expression to investigate the structure, biosynthesis, regulation, and activity of HAQs. We show that the *pqsA-E* operon encodes enzymes that catalyze the biosynthesis of five distinct classes of HAQs, and establish the sequence of synthesis of these compounds, which include potent cytochrome inhibitors and antibiotics active against human commensal and pathogenic bacteria.

We find that anthranilic acid, the product of the PhnAB synthase, is the primary precursor of HAQs and that the HAQ congener 3,4-dihydroxy-2-heptylquinoline (HHQ) is the direct precursor of the PQS signaling molecule. Significantly, whereas *phnAB* and *pqsA-E* are positively regulated by the virulence-associated transcription factor MvfR, which is also required for the expression of several QS-regulated genes, the conversion of HHQ to PQS is instead controlled by LasR. Finally, our results reveal that HHQ is itself both released from, and taken up by, bacterial cells where it is converted into PQS, suggesting that it functions as a messenger molecule in a cell-to-cell communication pathway. HAQ signaling represents a potential target for the pharmacological intervention of *P. aeruginosa*-mediated infections.

In nature, most bacteria live not as individual cells but as pseudomulticellular organisms that coordinate their population behavior by means of small extracellular signal molecules. Under appropriate conditions, these molecules are released into the environment and taken up and responded to by surrounding cells (1–3). “Quorum sensing” (QS), is the archetypal intercellular communication system used by many bacterial species to regulate their gene expression in response to cell density. This regulation allows all of the individual cells to behave coordinately and synergistically as a community, for instance, in growth dynamics and resource utilization (4). A common feature of all QS systems is the transcriptional activation and repression of a large regulon of QS-controlled genes when a minimal threshold concentration of a specific autoinducer is reached.

The well-characterized QS system used by Gram-negative bacteria is mediated by N-acyl-L-homoserine lactones (AHLs) as extracellular signaling molecules (1, 3). The versatile and ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* is one of the best-studied models of AHL-mediated QS. In this species, two separate autoinducer synthase/transcriptional regulator pairs, LasRl and RhlRI, modulate the expression of several genes, including many virulence factors, in response to increasing concentrations of the specific signaling molecules oxo-C12-HSL and C4-HSL (5, 6).

*P. aeruginosa* also produces a cell-to-cell signal distinct from AHLs: 3,4-dihydroxy-2-heptylquinoline, called PQS (7). PQS serves as a signaling molecule regulating the expression of a subset of genes belonging to the QS regulon, including the *phe* and *hcn* operons (E.D., S. Gopalani, F.L., A. N. Remick, A. P. Tampakaki, N.M.N., and L.G.R., unpublished work). PQS functions in the QS hierarchy by linking a regulatory cascade between the *las* and the *rhl* systems (8). That maximal PQS production occurs at the end of the exponential growth phase (9) supports the hypothesis that PQS acts as a secondary regulatory signal for a subset of QS-controlled genes. Although PQS has no antibiotic activity (7), it belongs to a family of poorly characterized antimicrobial *P. aeruginosa* products, the “pyo” compounds, originally described in 1945, which are derivatives of 4-hydroxy-2-alkylquinolines (HAQs) (10, 11). We have also identified a QS-associated *P. aeruginosa* transcriptional regulator, MvfR, which is required for the production of several secreted compounds, including virulence factors, and PQS (12, 13). Indeed, MvfR controls the synthesis of anthranilic acid (AA), a PQS precursor (14), by positively regulating the transcription of *phnAB*, which encodes an anthranilate synthase (12). In addition, mutations in five genes, designated *pqsA-E*, result in loss of pyocyanin and PQS production (15, 16). These genes likely mediate HAQ synthesis.

Here, we use liquid chromatography (LC)/MS to show that the *pqsA-E* operon encodes enzymes that direct the biosynthesis of five classes of HAQs, including molecules that function as antibiotics and cytochrome inhibitors and, significantly, as intercellular communication molecules. Furthermore, via genome-wide expression studies using the GeneChip *P. aeruginosa* oligonucleotide array, we demonstrate that the MvfR transcriptional regulator controls *pqsA-E* expression. These results reveal the HAQ biosynthesis pathway and furthermore show that one HAQ congener, 4-hydroxy-2-heptylquinoline (HHQ), is the direct precursor of PQS and itself a message molecule involved in cell-to-cell communication. This pathway represents a candidate target for the pharmacological intervention of *P. aeruginosa*-mediated infections.

Materials and Methods

**Bacterial Strains, Plasmids, and Media.** *P. aeruginosa* strains include wild-type PA14 (17); an *mvfR* mutant (12); 8C12, a TnpHαA insertsion mutant of *pqsB* (18); and an *lasR::Gm* mutant, which

Abbreviations: QS, quorum sensing; AA, anthranilic acid; HAQ, 4-hydroxy-2-alkylquinolines; PQS, *Pseudomonas* quinoline signal (3,4-dihydroxy-2-heptylquinoline); HHQ, 4-hydroxy-2-heptylquinoline; HNQ, 4-hydroxy-2-Heptylquinoline N-oxide; HNQ, 4-hydroxy-2-nonylquinoline; AHL, N-acyl-L-homoserine lactone; LC/MS, liquid chromatography/MS; CF, cystic fibrosis.

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was generated by allelic exchange using pSB219.9A as described (19). A *pqsE* deletion mutant was generated via pEX18Ap allelic replacement by using sucrose selection, resulting in a 570-bp nonpolar deletion covering 65% of the sequence (20). The *pqsA* (U479) *Tpho4* mutant was obtained from the PA14 Transposon Insertion Mutant Database. For complementation analysis, *mvfR* was cloned into pDN18 (21). The reporter fusions *phzABC-lacZ* and *hcnA-lacZ* have been described (22, 23). Plasmids were transformed into PA14 by electroporation (24). Specific β-galactosidase activity was determined as reported (25).

Bacteria were grown in LB broth or on a 1.5% Bacto-agar (Difco) LB plates. Freshly plated cells served as inoculum. For pyocyanin production, bacteria were grown in King’s A broth (26), and the pyocyanin was quantified as OD$_{600}$ after supernatant extraction (27). Tetracycline (75 mg/liter), carbenicillin (300 mg/liter), kanamycin (200 mg/liter), and gentamicin (100 mg/liter) were included as required.

**LC/MS Analysis.** Analyses were performed by using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass Canada, Pointe-Claire, Canada) in positive electrospray ionization mode, interfaced to an HP1100 HPLC equipped with a 4.5 × 150-mm reverse-phase C$_{18}$ column. Culture supernatants were twice extracted with ethyl acetate, the solvent was evaporated, and the residue was dissolved in a water/acetonitrile mixture containing the internal standard. Alternatively, culture samples were directly diluted with a methanolic solution of the internal standard, as reported (9).

**Synthesis of Labeled HAQ.** 4-Hydroxy-2-heptylquinoline *N*-oxide (HONO) was from Sigma. 2,3,4,5-Tetradeuteroanthranilic acid (AA-d$_{4}$) was from CDN isotopes (Pointe-Claire, Canada). The internal standards, 5,6,7,8-tetra analogue-4,5-dihydroxy-2-heptylquinoline (PQS-d$_{4}$), and 5,6,7,8-tetraethoxy-4-hydroxy-2-heptylquinoline (HHQ-d$_{4}$) were synthesized as reported (9). 5,6,7,8-Tetraethoxy-4-hydroxy-2-heptylquinoline *N*-oxide (HONO-d$_{4}$) was synthesized from HHQ-d$_{4}$ (28).

**RNA Isolation and Transcriptome Analysis.** Whole genome expression profiles were produced in duplicate for PA14 and the *mvfR* mutant. Cultures were grown in 1-liter Erlenmeyer flasks with 100 ml of LB at 37°C and shaking at 200 rpm. Cells were sampled at OD$_{600}$ = 1.5, 2.5, 3.5, and 4.5, and their RNA was immediately stabilized with RNAprotect Bacteria Reagent (Qiagen, Valencia, CA) and stored at −80°C. Total RNA was isolated with the RNeasy spin column (including an on-column DNase digestion step) according to the manufacturer (Qiagen), treated with RNase-free DNase I (Promega) for 1 h at 37°C, and repurified through an RNeasy column.

Samples were labeled according to the manufacturer (Affymetrix, Santa Clara, CA) and hybridized to the Affymetrix GeneChip *P. aeruginosa* genome array for 24 h at 50°C by using the GeneChip hybridization oven at 60 rpm. Washing, staining, and scanning were performed according to Affymetrix. The original data files, obtained from the array scans hybridized with the different probes, were converted to cell intensity files (.CEL files) by using MICROARRAY SUITE 5.0. Data analysis/clustering was performed with the DNA-CHIP ANALYZER (DCHIP) software (29).

**Antimicrobial Activity Assay.** HAQ antimicrobial activity was evaluated on well-plates. An overnight culture (30 µl) was plated to produce a bacterial lawn, and 5-mm-diameter holes were punched in the agar and filled with 60 µl of a 25% methanol solution of test extract or pure HAQ. Plates were incubated overnight at 37°C and scored for growth inhibition zones around the test wells.

**Cell-to-Cell Communication Assay.** To test whether *P. aeruginosa* cells produce PQS in response to HHQ released by other cells, we compared the concentrations of PQS in cultures of an *lasR* mutant, grown in 30 ml of LB in 250-ml flasks, with cocultures containing 50% of an *lasR* mutant and an *mvfR* mutant. pDN18*mvfR* was introduced into the *lasR* mutant to compensate for the lower expression of *mvfR* in this background. The effect on gene expression of exogenous HHQ was assayed by comparing the β-galactosidase activity of PA14 vs. lasR− cells carrying the *phzABC-lacZ* or *hcnA-lacZ* fusions, grown in the absence or presence of 10 mg/liter of HHQ.

**Results**

**HAQ Identification: *mvfR* Is Required for the Production of Five Distinct Series of HAQs from the Common Precursor Anthranilic Acid.** Calfee et al. (14) recently reported that 14C-labeled AA is incorporated into PQS, but that this PQS represents only 12% of the newly synthesized compounds, indicating that the ethyl acetate extract contains additional AA-derived molecules. Because HHQ biosynthesis proceeds from the coupling of AA and an α-keto fatty acid (30), we hypothesized that these unidentified AA-derived molecules might correspond to HAQs related to PQS and HHQ. To this end, we fed AA or deuterated AA (AA-d$_{4}$) to PA14 cultures and analyzed the culture supernatants by using LC/MS (9). The resulting chromatograms exhibit several peaks in the vicinity of PQS, and the mass spectra of these compounds all show the addition of 4 Da in the cultures fed AA-d$_{4}$, demonstrating that AA is their common precursor (Fig. 8, which is published as supporting information on the PNAS web site). Because we previously reported that PQS production is abrogated in an *mvfR* mutant (12), we investigated the synthesis of these compounds in this mutant. Fig. 1 shows that all of the deuterium-labeled peaks are absent from the *mvfR* mutant culture supernatant, with the only residual peaks found in the *mvfR* mutant culture supernatant, with the only residual peaks found at HAQ retention times corresponding to two conformers of the siderophore pyochelin (31), which give M+H ions at m/z 325 and are structurally unrelated to HAQs.

The mass spectra of these labeled peaks show that they correspond to five distinct series of HAQs (Fig. 2). All these congeners share the common basic 4-hydroxyquinoline structure of series A with an additional hydroxyl at the 3-position, as in series B, or with an --oxo group as in series C and E. Within each series, the 2-position alkyl chain varies in length. Also, the series D and E alkyl side chain is unsaturated. The most abundant congeners contain an odd carbon number alkyl chain, with seven or nine carbons preponderant.

The HAQ congeners include both previously identified and previously uncharacterized compounds. The C$_{7}$ (HHQ) and C$_{8}$ (HNO) congeners, shown in Fig. 2, were reported by Wells in 1952 (11) whereas the structures of the other series A congeners were later determined by using GC/MS (32). In contrast, the only reported series B congener is 3,4-dihydroxy-2-heptylquinol...
line, first isolated in 1959 (33), and later fully characterized and named PQS (7). For series C, the C7, C9, C8, and C11 congeners have been reported (28, 34) and are further discussed below whereas the series E and the series B PQS congeners are identified in this study. The simultaneous mass spectrometric detection of all these HAQ congeners, notably those of series C and E, which have polar N-oxide functions, could not be achieved by GC/H2O862MS (32, 35). Positive electrospray ionization MS is better suited than GC/H2O862 electron impact-MS for the detection of such relatively basic and polar compounds.

HAQ Regulation: MvfR Controls the Expression of the phnAB and pqsA-E Operons, Which Are Required for HAQ Synthesis. That MvfR regulates phnAB expression (12) suggests that it might also direct HAQ biosynthesis by regulating genes that encode anabolic pathway enzymes. As part of a project to identify MvfR-regulated genes, we carried out a transcriptome comparative analysis between PA14 and its isogenic mvfR mutant at set time points during a growth time course, by using the Affymetrix P. aeruginosa GeneChip oligonucleotide array (E.D., S. Gopalan, F.L., A. N. Remick, A. P. Tampakaki, M.N.M., and L.G.R., unpublished work). The expression profiles of the five genes just upstream from the anthranilate synthase phnAB operon tightly cluster with phnAB expression (Fig. 3 and Fig. 9, which is published as supporting information on the PNAS web site), suggesting they are coregulated. Fig. 4 shows that the expression patterns of these seven genes correlate with the kinetic rates of HAQ production, which are maximal at the end of exponential/early stationary phase (i.e., OD600 ≈ 2.5) (9). Because phnAB expression is under the control of MvfR (12), it is not surprising that the transcription of these seven genes is almost completely abolished in the mvfR mutant (Figs. 3 and 9).

Table 1 shows that knockout inactivation of pqsA or pqsB results in the complete elimination of HAQ production and the striking accumulation of AA in culture supernatants. AA likely accumulates because these mutants fail to consume AA for

<table>
<thead>
<tr>
<th>Compound</th>
<th>mvrfr−</th>
<th>pqsA</th>
<th>pqsB</th>
<th>mvrfr− compl.</th>
<th>lasR−</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHQ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1017 ± 247</td>
<td>250 ± 11</td>
</tr>
<tr>
<td>HQO N-oxide</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>200 ± 41</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>PQS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>247 ± 65</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>HQN</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>502 ± 106</td>
<td>551 ± 262</td>
</tr>
<tr>
<td>HQO N-oxide</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>286 ± 52</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>dHQQ</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>156 ± 49</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>458 ± 221</td>
<td>188 ± 102</td>
<td>0 ± 6</td>
<td>1</td>
</tr>
<tr>
<td>Pyocyanin</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>177 ± 26</td>
<td>NT</td>
</tr>
</tbody>
</table>

Cells were cultivated in LB medium at 37°C to OD600 = 4.0–4.5. Data are averages of triplicate experiments ± SD. diHQQ, 3,4-Dihydroxy-2-nonylquino- line; NT, not tested. mvrfr compl., the mutant strain complemented with pDN18/mvfR.
HAQ synthesis, further supporting AA as the HAQ precursor. In contrast, a pqsE mutant produces wild-type levels of HAQ and AA (Fig. 4 and data not shown), in agreement with the observation that pqsE inactivation does not affect PQS production (15). Genetic complementation suggests that pqsABCDE is a single operon (15). Our expression profiling data corroborate the LC/MS results and further indicate that MvfR controls the transcription of the coregulated pqsABCDE and pnsAB operons.

**HAQ Activity: MvfR Regulates HQNO Antimicrobial Activity Against Gram-Positive Bacteria.** Fluorescent pseudomonads, and perhaps just *P. aeruginosa*, are the only microorganisms identified to produce HAQs (9, 36). Although their biological functions are unknown, many HAQs were initially isolated as antibiotics (10, 11). We assayed the antimicrobial activity of total organic extracts from *P. aeruginosa* and *PqsA-D* mutants, and purified PQS, HHQ, and HNQ, against the Gram-positive species *Staphylococcus aureus* and *Bacillus subtilis*. PA14 and pqsE− extracts clearly inhibit both species whereas the pqsE− extracts have low or no antibacterial activity (Fig. 10, which is published as supporting information on the PNAS web site). Thus, MvfR regulates the production of antibiotics that can function in niche competition via a common biosynthetic pathway. To this end, we added various compounds whose synthesis is under PQS control.

Collectively, our results show that the series A compounds are the end-product of the mvaR-controlled biosynthetic pathway and are subsequently converted into the series B PQS analogues via a lasR-dependent pathway, likely via the PqsH enzyme, which is not under MvrR regulation. These results suggest that the final synthesis of the active PQS signal is highly regulated and under additional controls beyond those of the primary HAQ pathway.

**HHQ, the PQS Precursor, Functions in Cell-to-Cell Communication.** PQS is an extracellular signal that participates in the QS circuitry. Several observations suggest that HHQ also functions as an intercellular messenger: (i) it is released by bacteria; (ii) its concentration rises during exponential growth phase and then decreases during PQS production (Fig. 4); (iii) it is taken up by bacterial cells, converted into PQS, and then released into the extracellular milieu, as shown in the labeling experiments; and (iv) its synthetic pathway, by means of PsaA-D, is distinct and under different regulation than that of HHQ-to-PQS conversion, by means of PqsH. These results collectively suggest the model depicted in Fig. 6, in which HHQ is released by cells and acts as a messenger that is subsequently converted into
the PQS signal by the cells that take it up. To test this hypothesis, we compared PQS production in a lasR mutant culture vs. that of a mixed culture of lasR and mvfR mutant cells. Table 1 shows that the lasR mutant produces low levels of PQS and accumulates high concentrations of HHQ due to its low PqsH activity and the mvfR mutant produces no PQS because it is unable to synthesize the HHQ precursor. Thus, using these two mutants, one able to produce HHQ but unable to process it into PQS and the other unable to produce HHQ but able to convert it into PQS, should allow us to verify whether a P. aeruginosa cell can produce PQS by using HHQ produced by another cell. Indeed, Table 2 shows that, when the two mutants are grown together, PQS concentration is up to five times higher than if the cells fail to exchange the signaling information.

phz1 operon expression, which is required for the synthesis of pyocyanin, depends on both PQS signaling and pqsE expression (E.D., S. Gopalan, F.L., A. N. Remick, A. P. Tampakaki, M.N.M., and L.G.R., unpublished work). Therefore, to determine whether the PQS that is produced and released by mvfR cells, when they are cocultured with the lasR– cells, is biologically active in adjacent cells, we introduced a phzABC-lacZ reporter fusion into the lasR– mutant, where pqsE is expressed, and compared the β-galactosidase activity with and without co-cultivation with mvfR mutant cells. Table 2 shows that phz1 operon expression is indeed up-regulated in the presence of mvfR– cells, indicating that the PQS produced by mvfR mutant cells is taken up by the lasR– bacteria where it activates the phz1 operon. Accordingly, the mixed culture, but not the cultures of either mutant alone, also generates pyocyanin (Fig. 7). The lasR mutant is responsible for this pyocyanin production because, whereas the mvfR mutant “sees” PQS, it fails to express pqsE. Indeed, no β-galactosidase induction is obtained in a cocultivation experiment where the phzABC-lacZ reporter is carried by the mvfR– vs. the lasR– cells (data not shown). We note that, although mutants were used for this demonstration, we believe that the “conversational” pathway presented in Fig. 6 occurs in wild-type cell cultures because PA14 cells similarly perform HHQ release, uptake, and PQS conversion.

Table 2. Concentration of PQS (μg/ml) and phz1 operon expression in an lasR mutant culture and a 1:1 lasR mutant: mvfR mutant culture

<table>
<thead>
<tr>
<th></th>
<th>PQS, μg/ml</th>
<th>β-gal activity, MU*</th>
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<tbody>
<tr>
<td>lasR–</td>
<td>2.1 ± 0.1</td>
<td>63.6 ± 2.6</td>
</tr>
<tr>
<td>lasR– + mvfR–</td>
<td>5.2 ± 1.8</td>
<td>110.5 ± 4.3</td>
</tr>
<tr>
<td>Ratio†</td>
<td>5.0 ± 1.8</td>
<td>5.0 ± 0.2</td>
</tr>
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 Cultures were assayed at 8-hr sampling time, corresponding to OD600 4.3 to 4.5.

*The lasR mutant carries a phzABC-lacZ fusion: Data correspond to averages of triplicates ± SD. MU, Miller units.

†Ratios have been corrected by taking into account that the mixed culture contains 50% fewer lasR mutant cells than the lasR culture. mvfR mutant cells do not produce PQS.

Discussion:

Using LC/MS and DNA microarray analyses, we have determined that the transcriptional regulator MvfR, originally identified via its requirement for full P. aeruginosa broad-host virulence, regulates the expression of pqsABCDE and phnAB, which encode enzymes involved in the synthesis of five distinct families of structurally related HAQ congeners. By adding labeled HAQ precursors to bacterial cultures, we find that AA, mostly the product of the PhnAB synthase, is the precursor of all HAQs, and establish the sequence of their synthesis. Significantly, the last step in PQS

Table 3. Effect of HHQ addition on phz1 operon expression in PA14 and lasR– cultures

<table>
<thead>
<tr>
<th></th>
<th>PA14</th>
<th>lasR–</th>
</tr>
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<tbody>
<tr>
<td>−HHQ</td>
<td>371 ± 8</td>
<td>78.7 ± 1.5</td>
</tr>
<tr>
<td>+HHQ</td>
<td>473 ± 23</td>
<td>83.7 ± 0.2</td>
</tr>
</tbody>
</table>

 Cultures were assayed at 8 hr, corresponding to OD600 4.3–4.5. The bacteria carry a phzABC-lacZ fusion. Data correspond to averages of triplicates ± SD. MU, Miller units.
The PqsH monoxygenase, which probably mediates the hydroxylation of HHQ used by cells in a population is taken up by bacteria, suggesting that it functions as an intercellular communication pathway.

At least two branch pathways seem to have evolved from an ancestral synthetic HHQ pathway: one leading to the production of an extracellular vs. intracellular origin and engenders the question of whether the HHQ that is converted to PQS typically has an extracellular vs. intracellular origin and engenders the question of whether the HHQ is destined to serve as the PQS precursor.

Thus, HHQ and PQS analogues are largely cell-to-cell communication pathway molecules that could mean different things to cells in a population, even if their ultimate signaling mechanisms are the same. For instance, their concentrations peak at different growth stages; their production is under different regulation (MvfR for HHQ, LasR for PQS); and they are likely produced in different cellular compartments. Indeed, the PsORT program (psort.nibb.ac.jp) predicts that the PqsH monooxygenase, which probably mediates the hydroxylation of HHQ into PQS, is localized to the periplasmic space. This result suggests that the HHQ that is converted to PQS typically has an extracellular vs. intracellular origin and engenders the question of whether the HHQ is destined to serve as the PQS precursor.

We thank the Massachusetts General Hospital-ParabiobSys/National Heart, Lung, and Blood Institute (Mgh-ParabiobSys/NHLBI) Program for Genomic applications, Massachusetts General Hospital and Harvard Medical School, Boston, MA (http://pga.mgh.harvard.edu/cgi-bin/pal4/ mutants/retrieve.cgi) for the pqc7 mutant; S. Beaton for pSB219.9A, and P. Greenberg and D. Haas for the fusion reporters. We are grateful to Andrea Remick and Guillerme Coello for technical assistance and to Scott Stachel for comments and editing. We thank Cystic Fibrosis Foundation Therapeutics for subsidizing the P. aeruginosa microarrays. This work was supported by Cystic Fibrosis Foundation Award 02G0 and Shiners Hospitals Award 8800 (to L.G.R.). E.D. is supported by a postdoctoral fellowship from the Canadian Institutes of Health Research.

synthesis requires an activity whose regulation depends on LasR vs. MvfR. Our results also reveal that one HHQ, PQS, is the precursor of the PQS-signaling molecule and is itself both released from, and taken up by, bacteria, suggesting that it functions as an intercellular message molecule. These results provide insights into the structure, biosynthesis, regulation, and function of HHQs and have allowed us to uncover a “cellular” cell-to-cell communication pathway used by P. aeruginosa.

Interestingly, this activity has been associated with the clearance of S. aureus lung colonization by P. aeruginosa (35). HHQ is found in cystic fibrosis (CF) lung exudates (35), and PQS occurs in the sputum and bronchoalveolar lavage fluid of CF lungs (44), indicating that HHQs are produced in vivo. Guina et al. (45) have reported that P. aeruginosa isolates from CF patients produce more PQS than isolates from other diseases, suggesting that the PQS pathway could be up-regulated in CF strains. Thus, P. aeruginosa, when colonizing novel infectious niches, might increase HHQ production to inhibit the growth of competing microorganisms.

This study provides insights into PQS signaling, which functions in the expression of QS-regulated genes. Our results demonstrate that this system acts via at least two distinct extracellular molecules: PQS and its precursor HHQ. As illustrated in Fig. 6, this pathway can be viewed as a conversational cell-to-cell communication because an HHQ molecule released by a cell in a population is taken up by another cell and converted into PQS where it is then released into the extracellular milieu to signal cells in the population. Several observations indicate that this signaling is not artifactual and that HHQ and PQS could mean different things to cells in a population, even if their ultimate signaling mechanisms are the same. For instance, their concentrations peak at different growth stages; their production is under different regulation (MvfR for HHQ, LasR for PQS); and they are likely produced in different cellular compartments. Indeed, the PsORT program (psort.nibb.ac.jp) predicts that the PqsH monooxygenase, which probably mediates the hydroxylation of HHQ into PQS, is localized to the periplasmic space. This

result suggests that the HHQ that is converted to PQS typically has an extracellular vs. intracellular origin and engenders the question of whether the HHQ is destined to serve as the PQS precursor.