Biosynthetic Pathway of *Pseudomonas aeruginosa* 4-Hydroxy-2-Alkylquinolines

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The role of intercellular communication in the regulation of bacterial multicellular behavior has received widespread attention, and a variety of signal molecules involved in bacterial communication have been discovered. In addition to the N-acyl-homoserine lactones, 4-hydroxy-2-alkylquinolines (HAQs), including the *Pseudomonas* quinolone signal, have been shown to function as signal molecules in *Pseudomonas aeruginosa*. In this study we unraveled the biosynthetic pathway of HAQs using feeding experiments with isotope-labeled precursors and analysis of extracted HAQs by gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. Our results show that the biosynthesis of various HAQ metabolites is directed via a common metabolic pathway involving a “head-to-head” condensation of anthranilic acid and β-keto fatty acids. Moreover, we provide evidence that the β-keto-(do)decanoic acids, crucial for the biosynthesis of the heptyl and nonyl derivatives of the 4-hydroxyquinolines in *P. aeruginosa*, are at least in part derived from a common pool of β-hydroxy(do)decanoic acids involved in rhamnolipid biosynthesis.

*Pseudomonas aeruginosa* is a ubiquitous gram-negative bacterial pathogen that causes infections not only in human hosts but also in animals and even plants. This pathogen has been identified as one of the leading causes of nosocomial infections (4, 16, 23) and is responsible for fatal chronic lung infections in patients with cystic fibrosis (CF). *P. aeruginosa* coordinates its population behavior, such as biofilm formation (6, 7, 11) and virulence factor production, by means of small extracellular signal molecules, so-called autoinducers, that are released into the environment under appropriate conditions (15, 19, 21). Since intercellular communication leads to cooperative and coordinated bacterial behavior in a cell density-dependent manner, it is referred to as quorum sensing (2, 8, 18). A common feature of intercellular communication is the transcriptional activation of quorum-sensing-controlled genes when the bacterial signal molecules reach a certain threshold. *P. aeruginosa* produces two major cell-to-cell signals that are members of the acyl-homoserine lactone signal family, N-(3-oxododecanoyl)-t-homoserine lactone (3-oxo-C12-t-homoserine lactone) and N-butyryl-t-homoserine lactone (C4-t-homoserine lactone). In addition to the two acyl-homoserine lactone-type autoinducers, signal molecules belonging to the family of 4-hydroxy-2-alkylquinolines (HAQs) (9) have been identified, which include in addition to N oxides (exhibiting antimicrobial activities) 3,4-hydroxy-2-heptylquinoline (POS) (20) and 4-hydroxy-2-heptylquinoline. The latter two molecules have been shown to be involved in intracellular communication.

There is growing global concern that multiresistant pathogenic bacteria are emerging and will gradually render antimicrobial treatment ineffective. Hence, there is an urgent need for the development of novel therapeutic approaches for the treatment of bacterial infections. An alternative to growth inhibition, which is the mode of action of most traditional antibiotics, could be the attenuation of virulence factor production. In this approach, cell-to-cell signals have been recognized as promising targets for alternative therapeutic strategies that decrease bacterial virulence (13, 14, 24) as significant amounts of signal molecules have been detected at sites of infection in vivo (5, 17). Analysis of the underlying metabolic events of intercellular bacterial communication and elucidation of the biosynthesis of the signal molecules might contribute to our understanding and provide an opportunity to interfere with the control of virulence factor production. Previous studies strongly suggested that anthranilic acid is a precursor of PQS, and it was demonstrated that inhibition of anthranilic acid resulted in a loss of PQS production (1). To produce PQS, a condensation of anthranilic acid and a β-ketodecanoic acid in a multistep reaction was proposed. More recently, anthranilic acid has been shown to be a common precursor of HAQs, including POS, 4-hydroxy-2-heptylquinoline, and N oxides (9). Moreover, a PQS biosynthetic gene cluster has been identified (10). This *pqsABCDE* operon codes for a putative coenzyme A ligase (*pqsA*), two β-keto-acyl-acyl carrier protein synthases (*pqsB, pqsC*), and a FabH1 homologous transacylase (*pqsD*), whereas *pqsE* seems to encode a response effector protein which itself is not involved in the biosynthesis of PQS. Although it has clearly been demonstrated that the *pqsABCDE* genes are essential for PQS biosynthesis, their enzymatic function remains to be elucidated. In this study we used feeding experiments with labeled isotopes, and we confirmed by gas chromatography (GC)-mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy that *P. aeruginosa* synthesizes HAQs via a common biosynthetic pathway involv-
ing the “head-to-head” condensation of anthranilic acid and β-keto fatty acids. Moreover, PQS biosynthesis seems to be dependent not only on an intact pqsABCDE operon but also on the availability of β-keto acids. Interestingly, at least some of these acids seem to be derived from a common pool of β-hydroxy-keto acids involved in rhamnolipid biosynthesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Clinical P. aeruginosa strain SCV 20265, isolated from the respiratory tract of a CF patient who attended the cystic fibrosis clinic at Hanover Medical School, Hanover, Germany (12), was used in this study. This P. aeruginosa strain produced large amounts of HAQs and therefore was especially suitable for the feeding experiments with labeled precursors. P. aeruginosa was routinely cultured at 37°C on Columbia or Luria-Bertani (LB) agar. Transposon mutants of SCV 20265 with an insertion in the carB, pyrB, or pyrD gene that was generated using the transposon construction vector EZ-TN pMOD-2 (Epiconc) were grown in LB medium supplemented with 50 µg/ml gentamicin. The PAO1 wild-type strain and the nhgT::Tc mutant strain used in this study were kindly provided by G. Soberon-Chavez (Departamento de Microbiologia Molecular, Universidad Nacional Autonoma de Mexico) (3).

Broth cultures of P. aeruginosa SCV 20265 and transposon mutants affected in genes of the pyrimidine metabolic pathway were grown in LB medium containing in some cases, as indicated below, either 5 mM orotic acid or 5 mM UMP.

Extraction of extracellular P. aeruginosa HAQ metabolites and thin-layer chromatography (TLC). To isolate HAQ metabolites, P. aeruginosa cultures grown on agar plates were harvested and suspended in methanol. Following a centrifugation step and evaporation of the methanol to completion, the dried residue was washed several times with distilled water. Alternatively, the secondary metabolites were extracted from P. aeruginosa broth cultures with dichloromethane. Briefly, the bacterial cultures were extracted with 1 volume of dichloromethane by vigorous shaking. After centrifugation at 2,000 × g for 15 min the lower organic layer was evaporated. Broth cultures of P. aeruginosa SCV 20265 and transposon mutants affected in genes of the pyrimidine metabolic pathway were grown in LB medium containing in some cases, as indicated below, either 5 mM orotic acid or 5 mM UMP.

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TLC was performed using a Silica Gel 60 F254 TLC plate. The extracted P. aeruginosa material was dissolved in methanol and separated by TLC using tert-butyl methyl ether–n-hexane (10:1) as the solvent. Fluorescent spots were visualized under UV light and photographed. 4-Hydroxy-2-heptylquinolines was visualized under UV light and photographed. 4-Hydroxy-2-heptylquinolines was visualized under UV light and photographed.

Analysis of isotope-labeled P. aeruginosa HAQs by GC-MS and NMR. Previous studies on the biosynthesis of HAQs revealed that anthranilic acid is a direct precursor, and it has been speculated that HAQs are produced in a multiple-step reaction by the condensation of anthranilic acid and β-keto fatty acids. However, in a previous screening for HAQ-negative P. aeruginosa transposon mutants, we isolated mutants affected in the carB, pyrB, and pyrD genes. Although these genes are not neighbors on the chromosome, they all encode enzymes successively required for the biosynthesis of orotic acid in the pyrimidine biosynthetic pathway. In these mutants HAQ biosynthesis could be complemented by addition of 5 mM orotic acid to the growth medium, as demonstrated by TLC after 3 days of growth. Thus, it may be argued that orotic acid could be a direct precursor of HAQs. In an initial step anthranilic acid and orotic acid might react to form kynurenic acid, and the pqsABCDE operon could then be responsible for the attachment of an even-number carbon chain (C6 or C8) at the C-2 carbon of the quinoline ring to produce 4-hydroxy-2-heptyl- and 4-hydroxy-2-nonylquinoline, respectively (Fig. 1). To confirm either the previously proposed biosynthetic pathway for HAQs involving the condensation of anthranilic acid and β-keto fatty acids or the possibility that anthranilic acid and orotic acid are the precursors of HAQ synthesis via the formation of kynurenic acid, we performed feeding experiments with isotope-labeled substrates. First, P. aeruginosa SCV 20265 was grown in the presence of 15N-labeled anthranilic acid on modified Vogel-Bonner swimming agar plates with β-gluconic acid as the main carbon source. After growth for 3 days the bacterial mass was extracted with methanol, and the isolated secondary metabolites were subjected to GC-MS analysis. The upper panel of Fig. 2 shows a typical gas chromatogram of the trimethylsilylated HAQ derivatives, whereas the lower panel shows the EI mass spectrum of the unlabeled heptyl derivative and the fragmentation pattern assigned in the

FIG. 1. (A) Biosynthetic pathway for P. aeruginosa HAQs involving anthranilic acid and activated β-keto fatty acids as direct precursors. Using labeled precursor substrates, the possibility of an alternative pathway via the formation of kynurenic acid as an intermediate, as shown in panel B, was excluded.

Extraction of P. aeruginosa rhamnolipids. For isolation of rhamnolipids, P. aeruginosa cultures were grown on soft agar (0.2% agarose [Difco]) in normal Vogel-Bonner medium or Vogel-Bonner medium in which the β-gluconic acid had been replaced by 25 mM sodium acetate. After incubation for 4 days at 37°C, 20-ml portions of the soft agar cultures were extracted with 10 ml dichloromethane. After centrifugation at 2,000 × g for 15 min, the lower organic layer was evaporated and subjected to GC-MS.

RESULTS AND DISCUSSION

Analysis of isotope-labeled P. aeruginosa HAQs by GC-MS and NMR. Previous studies on the biosynthesis of HAQs revealed that anthranilic acid is a direct precursor, and it has been speculated that HAQs are produced in a multiple-step reaction by the condensation of anthranilic acid and β-keto fatty acids. However, in a previous screening for HAQ-negative P. aeruginosa transposon mutants, we isolated mutants affected in the carB, pyrB, and pyrD genes. Although these genes are not neighbors on the chromosome, they all encode enzymes successively required for the biosynthesis of orotic acid in the pyrimidine biosynthetic pathway. In these mutants HAQ biosynthesis could be complemented by addition of 5 mM orotic acid to the growth medium, as demonstrated by TLC after 3 days of growth. Thus, it may be argued that orotic acid could be a direct precursor of HAQs. In an initial step anthranilic acid and orotic acid might react to form kynurenic acid, and the pqsABCDE operon could then be responsible for the attachment of an even-number carbon chain (C6 or C8) at the C-2 carbon of the quinoline ring to produce 4-hydroxy-2-heptyl- and 4-hydroxy-2-nonylquinoline, respectively (Fig. 1). To confirm either the previously proposed biosynthetic pathway for HAQs involving the condensation of anthranilic acid and β-keto fatty acids or the possibility that anthranilic acid and orotic acid are the precursors of HAQ synthesis via the formation of kynurenic acid, we performed feeding experiments with isotope-labeled substrates. First, P. aeruginosa SCV 20265 was grown in the presence of 15N-labeled anthranilic acid on modified Vogel-Bonner swimming agar plates with β-gluconic acid as the main carbon source. After growth for 3 days the bacterial mass was extracted with methanol, and the isolated secondary metabolites were subjected to GC-MS analysis. The upper panel of Fig. 2 shows a typical gas chromatogram of the trimethylsilylated HAQ derivatives, whereas the lower panel shows the EI mass spectrum of the unlabeled heptyl derivative and the fragmentation pattern assigned in the

NMR spectroscopy. NMR spectra of the purified selectively 15N-labeled 4-hydroxy-2-alkylquinolines (1D 1H and 13C) and of unlabeled synthetic material (1D 1H and 13C and two-dimensional heteronuclear multiple bond correlation) were recorded at 300 K with Bruker DPX 300 and APX 400 NMR spectrometers locked to the major deuterium resonance of the solvent, CD3OD.
inserted scheme. Compared to the unlabeled control, all extracted 4-hydroxy-2-alkylquinolines contained approximately 66% 15N, as easily deduced from the corresponding EI mass spectra of the trimethylsilylated derivatives by the characteristic shift of the molecular ions at \( m/z \) 315 (heptyl derivative) and \( m/z \) 343 (nonyl derivative) and the intense fragment ion generated by elimination of the alkyl side chain at \( m/z \) 231 by 1 Da (Fig. 2). Incorporation of the 15N from labeled anthranilic acid was also observed for all other HAQ metabolites, and this clearly indicated that anthranilic acid served as a common precursor for PQS, 4-hydroxy-2-heptylquinoline, 4-hydroxy-2-nonylquinoline, and 4-hydroxy-2-heptylquinoline-N-oxide.

Moreover, since the only nitrogen of anthranilic acid was labeled, this indicates that N-1 of HAQ originates from anthranilic acid.

To shed light on the identity of the carbon substrate, especially at positions 2, 3, and 4 in the quinoline ring system, \( P. aeruginosa \) cultures were incubated for 3 days on modified Vogel-Bonner medium containing either \([1-^{13}C]\)acetate, \([2-^{13}C]\)acetate, or \([1,2-{^{13}C}]\)acetate in addition to unlabeled anthranilic acid as the main carbon source. According to the pattern of label incorporated, one can distinguish whether anthranilic acid and labeled \( \beta \)-keto acid (the pattern is dependent on the \([^{13}C]\)acetate source) condense “head to head” or whether an unusual polyketide synthesis mechanism extending activated anthranilic acid with labeled acetate moieties is responsible for PQS production. Substantial label incorporation into the HAQ metabolites was demonstrated. It is noteworthy that compared to cultures grown in minimal medium with D-gluconic acid as the main carbon source, it was 4-hydroxy-2-nonylquinoline and not 4-hydroxy-2-heptylquinoline that proved to be the main component in the HAQs. A characteristic mass shift of the molecular and fragment ions of the 4-hydroxy-2-alkylquinoline derivatives was observed for all labeling experiments by GC-MS after trimethylsilylation. Whereas the size of the molecular ion of the heptyl derivative increased by 4 Da after labeling with \([1-^{13}C]\)acetate and not \([4-^{13}C]\)acetate that proved to be the main component in the HAQs. A characteristic mass shift of the molecular and fragment ions of the 4-hydroxy-2-alkylquinoline derivatives was observed for all labeling experiments by GC-MS after trimethylsilylation.
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An identical experiment performed with [2-13C]acetate instead of [1-13C]acetate resulted in mass shifts of 5 and 6 Da for the molecular ions of the heptyl and nonyl derivatives, respectively, and a shift of 2 Da for the fragment ion at m/z 231. Therefore, the heterocyclic part of the quinoline ring system must also be synthesized from acetate units, since the labeled acetate could be clearly found not only in the alkyl side chain but also in the ring system. Furthermore, the difference between the two acetate labels can only be explained by the elimination of one CO2 molecule originating from the carboxylate moiety of acetate during biosynthesis. As expected, labeling with [1,2-13C]acetate led to a mass increase of 9 or 11 Da for the molecular ions and a shift of 3 Da of the fragment ion at 231 Da. To confirm these results, MS-MS fragmentation was performed for the molecular ions of the [1-13C]acetate- and [2-13C]acetate-labeled 4-hydroxy-2-heptylquinolines at m/z 319 and 320, respectively, as shown in Fig. 3. The alternating presence of 13C and 12C carbons in the alkyl chain of the molecules can be directly deduced from the corresponding fragments and also the fragments at m/z 232 and 233, respectively, incorporating the quinoline ring system, indirectly supporting the hypothesis that all HAQ derivatives are synthesized by “head-to-head” condensation of anthranilic acid and β-ketodecanoic or β-ketododecanoic acid with elimination of the carboxylate group of the fatty acid as CO2. A similar MS-MS experiment was performed for the molecular ion of the nonyl derivative, and analogous results were obtained (data not shown). The addition of unlabeled orotic acid in addition to anthranilic acid to the medium, which should have drastically reduced the incorporation of 13C into the ring system if the alternative biosynthetic pathway via kynurenic acid is correct, had absolutely no effect on this pattern. Addition of 3-oxo-decanoate, anthranilic acid, and [1,2-13C]acetate to the medium, however, resulted in labeling of the nonyl derivative (3 Da of the ring fragment and 11 Da of the molecular ion; compare with the data described above), whereas the labeling rate of the heptyl derivative was reduced by a factor of approximately 10, demonstrating that predominantly added unlabeled 3-oxo-decanoate is used for the biosynthesis of this derivative.

The biosynthetic pathway via β-keto-(do)decanoic acid could be independently demonstrated by 13C NMR spectrosopy. For this experiment, HAQ produced with relatively small amounts of [1,2-13C]acetate added to the medium was used, resulting in a degree of labeling of ca. 1%. Since the presence of two neighboring 13C atoms can be expected in the side chain and also in the heterocyclic ring system if our proposed biosynthetic model is correct, C-C coupling between intact acetate-derived units should be observed by 1D 13C NMR, whereas the probability of coupling between 13C atoms originating from different acetate units is too low to be observable. In this way, coupling between C-2 of the quinoline ring system and the first carbon of the side chain, C-9, with a characteristic coupling constant was indeed observed (Table 1), unambiguously demonstrating the presence of an intact acetate-derived unit at this position. The observation of doublet signals for the three carbons at the chain end confirmed the regular incorporation of acetate units in the fatty acid section of the molecule. No coupling was detected between C-3 and C-4 of the quinoline ring (Table 1), confirming the elimination of the carboxylate carbon of the final acetate unit as CO2 used to synthesize HAQ derivatives via β-keto fatty acids. Therefore, the C-4 carbons of these molecules must originate from anthranilic acid.

Since we demonstrated that β-keto acids are direct precursors of HAQs, it might be speculated that the probable coenzyme A ligase encoded by pqsA is responsible for the activation of the β-keto acids. pqsD might then be involved in a transacylation reaction, and finally, β-keto-acyl-acyl carrier protein might be condensed with anthranilic acid to form POS. These processes are probably also dependent on enzymes of the primary fatty acid metabolism. Future studies will have to elucidate the enzymatic function of the proteins encoded by the pqsABCDE operon and to identify whether and which enzymes of the primary fatty acid metabolism are also required for PQS biosynthesis.

**Table 1.** 13C NMR data for synthetic 4-hydroxy-2-heptylquinoline and the main components of the biosynthetic product after feeding with doubly labeled [13C]acetate

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Shift(s) (ppm)</th>
<th>Multiple 13C satellite</th>
<th>fC (Hz) (±0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>157.1</td>
<td>d</td>
<td>44.3</td>
</tr>
<tr>
<td>C-3</td>
<td>108.8</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>180.7</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-4a</td>
<td>125.5</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-5</td>
<td>126.0</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td>125.0</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>133.4</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-8</td>
<td>119.0</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-9</td>
<td>141.6</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-10</td>
<td>35.0</td>
<td>d</td>
<td>44.3</td>
</tr>
<tr>
<td>C-11</td>
<td>30.1</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-12</td>
<td>30.1</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-13</td>
<td>32.8</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>C-14</td>
<td>23.6</td>
<td>d</td>
<td>34.1</td>
</tr>
<tr>
<td>C-15</td>
<td>14.3</td>
<td>d</td>
<td>34.0, 34.1</td>
</tr>
</tbody>
</table>

* As determined by the MS analysis, the main components are a mixture of several compounds, the main components of which are the heptyl and nonyl derivatives. The structure of one heptyl derivative in the tautomeric keto form is

The signals were unambiguously assigned from correlations in the two-dimensional HMBC spectrum.

Overlap of signals prevented assignment of the internal chain carbons in the main components.

Doublet 13C satellite signals centered on the shift of the major signal were observed for the signals indicated by “d” but not for the signals indicated by “s”.

The assignments are interchangeable.

End units from major components of the mixture.

**Growth characteristics of P. aeruginosa mutants affected in the pyrimidine metabolic pathway.** To further verify that orotic acid is not a direct precursor of HAQ production, we tested whether HAQ production by mutants with mutations in the pyrimidine metabolic pathway could be restored by not only orotic acid but also UMP. UMP is a direct precursor of DNA
biosynthesis, and orotic acid is metabolized into UMP in the pyrimidine metabolic pathway. Indeed, both orotic acid and UMP could restore HAQ production in the carB, pyrB, and pyrD mutant strains. Since the lack of HAQ production in mutants affected in the pyrimidine metabolic pathway cannot be explained by the lack of orotic acid as a direct HAQ precursor, we analyzed the growth characteristics of the carB, pyrB, and pyrD mutant strains compared to those of the wild type. Despite comparable growth behavior in log-phase cultures, not only HAQ production but also growth in stationary-phase cultures was affected in all three mutants. The optical densities approached only about 30% of the optical densities of the wild-type cultures, unless the medium was supplemented with either orotic acid or UMP. These results imply that the absolute bacterial cell density in stationary-phase P. aeruginosa cultures and HAQ production are directly linked and that the mutants affected in the pyrimidine metabolic pathway lack HAQ production due to a general metabolic defect.

Common biosynthetic pathway for the fatty acid moiety of rhamnolipids and P. aeruginosa HAQs. It is obvious that the majority of the HAQs of P. aeruginosa are either 4-hydroxy-2-heptylquinolines or 4-hydroxy-2-nonylquinolines. Accordingly, either β-ketodecanoic or β-ketododecanoic fatty acids are essential precursors of HAQ biosynthesis. In this context it is intriguing that rhamnolipids of P. aeruginosa are composed of rhamnose and β-hydroxy fatty acids that are the same chain length (namely, β-hydroxydecanoic and β-hydroxydodecanoic fatty acids). Recently, evidence has been provided that the rhlG gene of P. aeruginosa codes for a FabG homolog specifically involved in the synthesis of the β-hydroxy acid moiety required for rhamnolipid biosynthesis (3). While a mutation of the rhlG gene had no apparent effect on the growth rate and total lipid content of P. aeruginosa cells, the production of rhamnolipid was abrogated and the synthesis of poly-β-hydroxyalkanoate was delayed. This suggests that the biosynthetic pathway for the fatty acid moiety of rhamnolipids is separate from the general fatty acid pathway starting with a specific keto-acyl reductase step catalyzed by RhIG. In addition, the rhlG mutant strain was shown to produce significantly less pyocyanin, whose production is strongly dependent on PQS, a defect that could be complemented in the mutant strain after the parental gene was provided on a plasmid in trans (3). Since it was tempting to speculate that RhIG is required to provide a pool of β-hydroxy C10 to C12 fatty acids in P. aeruginosa that are essential precursors of not only rhamnolipid biosynthesis but also of the biosynthesis of HAQs, we tested whether the PAO1 rhlG::Tc mutant (provided by G. Soberon-Chavez) was deficient in HAQ biosynthesis. Indeed, total HAQ production was reduced in the PAO1 rhlG::Tc mutant strain after 4 h, 9 h, and 30 h of growth, as determined by TLC (data not shown). These findings suggest that RhIG plays a role not only in the biosynthesis of fatty acids used as substrates for rhamnolipid and poly-β-hydroxyalkanoate but also in HAQ production, albeit for the latter process an intact rhlG gene is not an absolute requirement. Moreover, assuming a common pathway for the fatty acid moiety of rhamnolipids and HAQs, one would expect that the addition of acetate to the growth medium would lead not only to a shift from 4-hydroxy-2-heptylquinoline to 4-hydroxy-2-nonylquinoline (as demonstrated in this study) but also to a shift in the C side chain length of P. aeruginosa rhamnolipids from C10 to C12. Indeed, acetate supplementation led to a shift in the biosynthesis of rhamnolipids with the longer C12 side chain, as demonstrated by GC-MS. Whereas before addition of acetate the short-chain variants of the rhamnolipids were synthesized predominately, the availability of large amounts of acetate caused increased synthesis of 4-hydroxy-2-nonylquinoline and rhamnolipids with the longer C12 side chain (Fig. 4). These results further strengthen the presumption that the fatty acid moiety of rhamnolipids and HAQs are derived at least in part from the same pool.

Conclusions. In this study we elucidated the biosynthetic pathway of HAQs that are signal molecules involved in the regulation of virulence factor production in P. aeruginosa. Using labeled precursors and analysis of extracted HAQs by GC-MS and NMR spectroscopy, we confirmed the previously proposed biosynthetic pathway involving a “head-to-head” condensation of anthranilic acid and β-keto fatty acids. Moreover, the results of this study imply that the P. aeruginosa rhlG gene coding for an NADPH-dependent β-ketoacyl reductase provides a pool of C10 and C12 β-hydroxy fatty acids which not only are essential for rhamnolipid biosynthesis but also serve as substrates for HAQ production.

HAQ signal molecules are produced in the stationary phase of growth, and they seem to be important when the bacterial
population is under stress. Under these conditions (for instance, during a chronic infection of a CF lung) maintenance of viability is of major importance for the bacterial population rather than cell division. The elucidation of the biosynthetic pathway and future research on the molecular mechanisms of HAQ signal transduction should provide valuable clues regarding the process of chronic infection and the pathogenesis of persistent disease. More insight could make the HAQ signal molecules a unique drug target for new therapies for treating P. aeruginosa infections.

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