The Alkylquinolone Repertoire of *Pseudomonas aeruginosa* is linked to Structural Flexibility of the FabH-like PQS Biosynthesis Enzyme PqsBC

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Abstract

Pseudomonas aeruginosa is a bacterial pathogen that causes life-threatening infections in immunocompromised patients. It produces a large armory of saturated and mono-unsaturated 2-alkyl-4(1*H*)-quinolones (AQs) or AQ N-oxides (AQNOs) that serve as signaling molecules to control the production of virulence factors, are involved in membrane vesicle formation and iron chelation and also have e.g. antibiotic properties. It has been shown that the FabH-like heterodimeric enzyme PqsBC catalyzes the last step in the biosynthesis of the most abundant AQ congener 2-heptyl-4(1*H*)-quinolone (HHQ) by condensing octanoyl-coenzyme A (CoA) with 2-aminobenzoylacetate (2-ABA), but the basis for the large number of other AQs/AQNOs produced by *P. aeruginosa* is not known. Here, we demonstrate that PqsBC uses different medium-chain acyl-CoAs to produce various saturated AQs/AQNOs and also biosynthesizes mono-unsaturated congeners. Further, we have determined structures of PqsBC in four different crystal forms at 1.5 Å to 2.7 Å resolution. Together with a previous report, this reveals that PqsBC adopts open, intermediate and closed conformations that alter the shape of the acyl-binding cavity and explain the promiscuity of PqsBC. The different conformations also allow us to propose a model for structural transitions that accompany the catalytic cycle of PqsBC, which may have broader implications for other FabH-enzymes, where such structural transitions have been postulated but never been observed.

Keywords: enzymes; protein structures, structure-activity relationships, transferases, enzyme catalysis, quorum sensing, FabH, conformational change

Introduction

The Gram-negative bacterium *P. aeruginosa* is a serious burden for public health. It is a major causative agent of hospital-acquired lung infections often leading to death in cystic fibrosis patients, of catheter-associated urinary tract infections, bacterial keratitis due to contaminated contact lenses and of wound infections.^[1–3] The ability of *P. aeruginosa* to adapt to diverse environments and to cause different types of infections requires a time- and habitat-dependent coordinated expression of target genes. This is achieved by a complex intercellular communication network that controls up to 10% of the *P. aeruginosa* genome and is therefore considered as a potential therapeutic target.^[4–6] Bacterial cell-to-cell communication is often referred to as “quorum sensing (QS)”, as its underlying mechanisms involve the synthesis and the secretion of small signaling molecules (autoinducers, AIs) that are sensed by specific transcriptional receptors, which are activated as soon as a certain AI level (quorum) is reached.^[7,8] Besides the production of a large arsenal of virulence factors needed for survival and persistence in the host, QS-regulated processes in *P. aeruginosa* include biofilm formation, motility and the activation of the CRISPR-Cas adaptive immune system.^[9,10] *P. aeruginosa* has three major intercellular communication circuits, which are also interlinked with each other.^[9,11] Two of these pathways, the *las*^[12–14] and *rhl*^[15–17] systems, rely on *N*-acyl-L-homoserine lactones, which are common autoinducers in Gram-negative bacteria.^[7,8] The third QS circuit, the *pqs* system, responds to 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) or its biosynthetic precursor 2-heptyl-4(1*H*)-quinolone (HHQ), which both activate the transcriptional multiple yirulence factor regulator MvfR (also known as PqsR) and belong to the class of 2-alkyl-4(1*H*)-quinolones (AQs).^[18–21] HHQ- or PQS-bound PqsR complexes induce the expression of the *pqsABCDE* operon, which encodes HHQ biosynthetic proteins (Scheme 1).^[20–23] The aryl-coenzyme A (CoA) ligase PqsA activates anthranilate to anthraniloyl-CoA, which is then condensed with malonyl-CoA to 2-aminobenzoylacetyl-CoA (2-ABA-CoA) by PqsD.^[24,25] The thioesterase PqsE and other thioesterases of *P. aeruginosa* catalyze the hydrolysis of 2-ABA-CoA to 2-aminobenzoylacetate

(2-ABA), which is the building block for the 4-quinolone ring structure of AQs.^[26,27] 2-ABA is a branch point metabolite in HHQ biosynthesis, as it is shuttled into different reaction pathways. Due to its instability, it can spontaneously decompose into 2-aminoacetophenone (2-AA) by the loss of CO₂ or by undergoing intramolecular cyclization into 2,4-dihydroxyquinoline (DHQ).^[27,28] In the main biosynthetic route to HHQ, however, the heterodimeric PqsBC complex (EC 2.3.1.180) transfers 2-ABA onto octanoate to synthesize HHQ in a decarboxylative Claisen condensation after an acyl-enzyme intermediate between the active site cysteine (C129) of PqsC and octanoyl-CoA has been formed.^[28,29] In a final step, HHQ gets hydroxylated at carbon position C3 by the monooxygenase PqsH.^[30] Additionally, a very recent study demonstrated that PqsBC can also use 2-hydroxylaminobenzoylacetate (2-HABA) as a second substrate, generated from 2-ABA by the monooxygenase PqsL, to yield 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO),^[31] which is one of the most abundant 2-alkyl-4-hydroxyquinoline *N*-oxides (AQNOs) in *P. aeruginosa*.^[21,32] PqsB and PqsC are homologous to β -ketoacyl-acyl-carrier protein synthases III (FabH) of the thiolase superfamily, but unlike other FabH homologs that have a conserved His-Asn-Cys catalytic triad, PqsB lacks all of these three residues and PqsC has only a catalytic dyad composed of His-Cys (H269-C129).^[28,29,33] It has recently been shown by Drees *et al.*^[29] that the missing asparagine side chain in PqsC is mimicked by the NH₂ group of 2-ABA in the reaction cycle.

In addition to HHQ and PQS, which are the most relevant AQs in terms of cell-to-cell communication, *P. aeruginosa* produces more than 50 distinct AQs/AQNOs with saturated or unsaturated alkyl side chains of different lengths.^[34] The repertoire of AQs in *P. aeruginosa* is highly diverse and so are their functions. Besides their role as signaling molecules in cell-to-cell communication, AQs and AQNOs are involved in iron chelation, membrane vesicle formation, show antimicrobial activities and can manipulate the immune system of an infected mammalian host.^[35] All these diverse QS-dependent and QS-independent functions of AQs and AQNOs help *P. aeruginosa* to outcompete other microorganisms in the environment or to escape cellular immune responses of the host in order to create a favorable growth habitat.^[35]

Although it is well known that *P. aeruginosa* mainly produces AQ and AQNO congeners with alkyl chain lengths of 7 or 9 carbon atoms,^[21,34,36,37] the molecular mechanisms leading to this selectivity are unknown. Until now, it has only been demonstrated that the alkyl chain of HHQ/PQS originates from octanoate introduced by PqsBC.^[28] However, since *P. aeruginosa* also synthesizes AQ and AQNO derivatives with shorter, longer and mono-unsaturated alkyl chains, it seems likely that PqsBC can also utilize the respective acyl-CoA primers for condensation. We therefore investigated the acyl-CoA substrate specificity of PqsBC, using enzymatic assays and feeding experiments as well as by analyzing PqsBC crystal structures from five different crystal forms. This shows that PqsBC prefers medium-chain acyl-CoAs and is the key factor of the *pqs* system that drives the AQ/AQNO distribution found in *P. aeruginosa*. Our data also provide evidence that PqsBC is directly involved in the biosynthesis of mono-unsaturated AQs/AQNOs. Crystal structures demonstrate that PqsBC exists in open, closed and intermediate conformations. Such conformations have previously been postulated for other FabH enzymes, but have never been observed. Our data therefore provide new insight into the acyl-CoA binding mechanism of FabH-like proteins.

Results

The diversity of AQs/AQNOs produced by *P. aeruginosa* depends strictly on PqsBC

Recently, it has been shown that the heptyl side chain of HHQ derives from octanoyl-CoA, which is incorporated by the heterodimeric FabH-like enzyme PqsBC.^[28] To investigate the importance of PqsBC for the production of the whole spectrum of AQs and AQNOs, we analyzed their production in *P. aeruginosa* PA14 wildtype and in nonpolar *pqsB*⁻ and *pqsC*⁻ mutant strains grown in minimal medium using LC-MS. Hydroxylated species (PQS analogs) were excluded from analysis, as AQ/AQNO hydroxylation occurs downstream of PqsBC (Scheme 1).^[20,21,30] Towards this, it has been described previously that PQS analogues and AQNOs of the same side chain length can be distinguished both by their MS/MS fragmentation and by their chromatographic

behavior despite identical mass-to-charge ratios.^[34,37,38] In addition, PQS congeners did not give defined peaks and had different retention times than AQs and AQNOs under the chromatographic conditions used in this study. While AQs/AQNOs were completely absent in the *pqsB*⁻ and *pqsC*⁻ mutants, we observed a large variety of different AQs/AQNOs in the wildtype strain (Figure 1A). We mainly detected saturated and mono-unsaturated (*cis* and *trans*) AQs and AQNOs with odd-numbered aliphatic side chains containing 7 to 11 carbon atoms, while only traces of AQ congeners with even-numbered alkyl chains were found (Figures 1A, B; the nomenclature for AQs/AQNOs used here was adapted from Depke *et al.*^[38]). The two most abundant saturated species produced by *P. aeruginosa* PA14 were C7-HQ (HHQ) or C7-QNO (HQNO) and C9-HQ (NHQ) or C9-QNO (NQNO) (Figure 1B), which is line with previous reports.^[21,34,36,37] These results suggest that PqsBC synthesizes not only HHQ but also other AQs/AQNOs by using shorter or longer acyl-CoAs.

PqsBC accepts a broad spectrum of acyl-CoAs

To corroborate the hypothesis that PqsBC synthesizes other AQ/AQNO derivatives directly, we performed proteomic assays to test whether PqsBC can also accept other saturated acyl-CoA substrates besides octanoyl-CoA. Towards this, PqsBC was incubated with different even-numbered saturated acyl-CoAs ranging from acetyl- to tetradecanoyl-CoA (C2- to C14-CoA) and protein acylation was analyzed with ESI-MS. In all cases, PqsC was loaded with the respective acyl chain, while PqsB remained unmodified as expected (Figure 2A). However, incubation with acetyl-CoA, butyryl-CoA and hexanoyl-CoA resulted in incomplete PqsC modification.

In a second analysis, PqsBC was incubated with a mixture containing all acyl-CoAs at equal concentrations. Notably, PqsC was only loaded with octanoate and decanoate, revealing a clear preference for the corresponding acyl-CoAs (Figure 2B). To evaluate if substrate utilization can be shifted by increasing the concentration of one acyl-CoA over the other, we incubated PqsBC with a constant amount of octanoyl-CoA and equimolar or 10-fold higher concentrations of

hexanoyl-, decanoyl-, dodecanoyl- or tetradecanoyl-CoA in 1:1 competition assays (Figure S1 in the Supporting Information). Even with 10-fold excess of hexanoyl- or tetradecanoyl-CoA, PqsBC was only loaded with octanoate (Figures S1A, D). In the case of a 10-fold excess of decanoyl-CoA, however, the loading shifted towards the decanoylated PqsBC species and only a minor fraction modified with the octanoyl moiety remained (Figure S1B). Similar observations were made with dodecanoyl-CoA (Figure S1C). Incubation of PqsBC with equimolar concentrations of octanoyl- and dodecanoyl-CoA, on the other hand, resulted in almost complete modification with octanoate. Together, these acyl-CoA loading experiments indicate that octanoyl- and decanoyl-CoA are the preferred acyl-CoA substrates for PqsBC and that transacylation of PqsC can be influenced to some extent by increasing the concentration of the acyl-CoA primers for C8- to C12-CoA, suggesting that the AQ/AQNO spectrum produced by *P. aeruginosa* is also influenced by acyl-CoA availability.

We also investigated the acyl-CoA substrate specificity of PqsBC by endpoint measurements under turnover conditions in the presence of the second substrate 2-ABA, since transacylation only reflects the first reaction step and the substrate specificity could also be influenced by the overall kinetics of the enzyme. Acyl-CoA turnover was monitored by the release of CoA upon enzyme-acyl intermediate formation, which was detected spectrophotometrically with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) at 412 nm. PqsBC displayed its highest activity with octanoyl-CoA (459 μM CoA), followed by decanoyl-CoA (261 μM CoA) and hexanoyl-CoA (205 μM CoA) (Table 1). No CoA was detected from the short-chain acetyl- or butyryl-CoA or the long-chain dodecanoyl-, tetradecanoyl- or hexadecanoyl-CoA. These data show that PqsBC has a clear preference for medium-chain acyl-CoAs with aliphatic tails of 6 to 10 carbon atoms, exhibiting the highest activity with octanoyl-CoA.

Structure determination of PqsBC in four different crystal forms

In order to correlate the observed preference for medium-chain acyl-CoAs with structural features of PqsBC, we aimed at obtaining a crystal structure of octanoylated wildtype PqsBC and of the active site mutants PqsBC^{C129A} or PqsBC^{C129S} to capture an enzyme-octanoyl-CoA complex, which is expected to form before the acyl group is transferred onto active site C129 of PqsC. Despite extensive efforts in soaking and cocrystallization experiments with wildtype PqsBC and with the mutants PqsBC^{C129A}/PqsBC^{C129S}, however, this was not successful. Nevertheless, we determined the structures of ligand-free PqsBC wildtype and PqsBC^{C129A}/PqsBC^{C129S} mutants in four different crystal forms (crystal form 1 to 4) at 1.53 Å to 2.7 Å resolution. The crystals contained two, four or eight PqsBC heterodimers in their asymmetric units (Figures S2 and S3; Tables S1 and S2). Interestingly, crystals belonging to crystal forms 1, 2 and 3 could only be obtained with protein still carrying the purification tag at the N-terminus of PqsC. Crystal packing analysis revealed that the purification tag mediated crucial crystal contacts to neighboring symmetry-related PqsBC molecules in the respective crystal lattices (Figure S4).

Molecules from the precipitant were observed in the active site of PqsC in crystal forms 1 and 3 (Figures S5A, B), and in the wildtype PqsBC structure of crystal form 2, we identified additional elongated but ambiguous electron density around the active site cysteine C129 (Figure S5C). This electron density probably originated from a covalently bound co-purified ligand.

Analysis of PqsBC in five different crystal forms reveals flexibility around the active site of PqsC

We used the Protein Structural Statistics Web Server (PSSweb)^[39,40] to compare all PqsBC heterodimers in the asymmetric units of the different crystal forms. Together with the crystal structure determined by Drees *et al.*^[29] (PDB ID: 5DWZ; designated as crystal form 5), this analysis included 22 independent copies of PqsBC. PqsB shows only minor structural variations except for the C-terminus, some surface exposed residues and a loop region (residues 186-188) at the upper

area of the dimeric interface (Figures 3A, C). PqsC, in contrast, is highly variable in several regions surrounding the putative acyl-CoA binding channel near active site residues C129 and H269 (Figures 3B, C). The variable structural elements include helix α 1 (residues 33-39) with W35 and a 3_{10} helix (residues 168-171) with R168. W35 and R168 are highly conserved in FabH enzymes (Figure S6) and are known to be involved in CoA binding by intercalating the adenine moiety of CoA between the guanidine (R168) and the indole (W35) groups.^[41–44] The largest structural variations in PqsC are found in a region between β 10 and helix α 5 (residues 212-243), which is part of an area termed as “flap” in FabH from *Mycobacterium tuberculosis* (*mtFabH*), where it has been predicted to undergo large structural rearrangements during the binding of the acyl-CoA primer (residues 201-234 in PqsC correspond to the flap region in *mtFabH*; Figure S6).^[45] In contrast, amino acids in the immediate vicinity of the catalytic residues C129 and H269 are highly similar between the analyzed structures, indicating rigidity (Figures 3B, C).

PqsC adopts open, intermediate and closed conformations

Superimposition of the 22 copies of PqsBC contained in the five different crystals forms revealed that these heterodimers can be assigned to “open”, “intermediate” and “closed” states, depending on the positions of structural elements surrounding the active site (Figure 4A, Table S3). They show different conformations of the flap and helix α 1, whose flexibility is also reflected in high B-factors (Figures 4A and S7). The open form is only found in crystal form 5, while crystal forms 1 to 4 belong to the intermediate or closed states (Table S3).

In the open form, secondary structure elements containing W35 and R168 are pushed outward relative to the closed state (Figure 4B). An interesting observation is that the peptide bond between V241 and P242 adopts a *cis* conformation in open PqsBC whereas it is in the *trans* conformation in the closed and intermediate structures (Figures 4C and S8), suggesting that P242 could serve as a hinge during the catalytic cycle of PqsBC. The shape of the active site changes dramatically between the open and closed form: while residues 231 to 240 adopt a loop structure in the open

state that enlarges the catalytic cavity (termed “loop A” by Drees *et al.*^[29]; Figure S6), they form an imperfect, kinked α -helix acting as an N-terminal extension of helix $\alpha 5$ in the closed form, leading to reduction in size of the substrate-binding pocket. This transition moves residues 231 to 238 closer to the active site by up to 17.2 Å (Figure 4D). At the same time, strand $\beta 11$ of the mobile flap is raised, which also contributes to closing and shrinking of the binding pocket (Figure 4E). The distance between C α -atoms of Q234 (loop A/helix $\alpha 5$) and W35 (helix $\alpha 1$) decreases from 29 Å in the open to 11 Å in the closed conformation, highlighting the long-range structural rearrangements that accompany the interconversion of the two states.

The acyl-binding site acts as a molecular ruler that determines the acyl-CoA specificity of PqsBC

It is evident from other FabH homologs that the shape and the size of the acyl-binding channel dictate the acyl-CoA substrate specificity of these enzymes.^[44,46,47] To investigate if the geometry of the acyl-binding site of PqsBC also reflects its specificity for octanoyl-CoA (Table 1), we aligned the closed form of PqsBC^{C129A} with *mt*FabH bound to dodecanoyl-CoA (PDB ID: 1U6S^[44]) to model a complex between PqsBC^{C129A} and octanoyl-CoA (Figure 5). According to this, octanoyl-CoA occupies an L-shaped pocket in PqsC, which can be subdivided into a long pantetheinate or CoA-binding tunnel and a shorter, buried acyl-binding channel (Figures 5A, B) that is similar to those found in crystal structures of *mt*FabH-dodecanoyl-CoA^[44] or of FabH from *Micrococcus luteus* (*m*/FabH)^[48]. The substrate-binding pocket is only accessible via the pantetheinate L-arm, and the two subchannels are separated by the catalytic dyad of PqsC (C129, H269) at the bottom of the binding cleft (Figures 5A, B).

Our model suggests that the side chains of W35 and R168 sandwich the adenine ring via π -alkyl and π - π interactions, respectively, thereby anchoring the CoA portion of octanoyl-CoA like a clamp at the protein surface of PqsC (Figure 5C). This stacking recognition motif is fully conserved in FabH enzymes (Figure S6). The main chain carbonyl oxygen of R168 additionally fixes the

adenine ring by forming a hydrogen bond with the amino group. The ribose phosphate forms a salt bridge with R168 and is also hydrogen bonded to S231. Additional hydrogen bonds are formed between the diphosphate moiety and indole nitrogen atom of W39 as well as the side chain of Q234 (Figure 5C). S231 and Q234 are both located on loop A in the open state (Figure 4D), which is one of the most divergent regions between the open and the closed conformations of PqsBC. While the pantothenate moiety is stabilized by hydrophobic interactions, the β -mercaptoethylamine unit is hydrogen-bonded to the backbone carbonyl oxygen of P271 (Figure 5D). The aromatic ring of F173 mediates a sulfur- π interaction with the sulfur atom of the thioester bond and the main chain amino group of T331 forms a hydrogen bond with the thioester's carbonyl oxygen. The octanoyl chain is embedded in the hydrophobic acyl-binding pocket (Figures 5A, B, D). The distal end of this tunnel is closed by M76*/A77* of PqsB and by P87/L162/L225 of PqsC (Figure 5D).

Side views of the acyl-binding sites of PqsBC^{C129A} models bound to octanoyl- and dodecanoyl-CoA show that the 11.5 Å long alkyl-binding channel provides enough space for medium-chain acyl-CoAs (hexanoyl- to decanoyl-CoA) and has an optimal length for octanoyl-CoA (Figure 5B). Alkyl chains with more than 10 carbon atoms are expected to have to fold their aliphatic tails back to fit in the hydrophobic pocket (Figure 5B), which would lead to unfavorably strained alkyl conformations and to disadvantageous contacts with backbone atoms, whereas shorter chains will establish fewer hydrophobic interactions. The modeled PqsBC-acyl-CoA complexes thus explain the substrate specificity of PqsBC towards medium-chain acyl-CoAs.

Next, we compared the alkyl-binding site of the open and closed conformations of PqsBC (Figure 6) to obtain insight into changes at the active site that are expected to accompany substrate binding. The acyl cavity of the open state (708 Å³) is calculated to be about three times larger than that of the closed conformation (256 Å³). As mentioned above, strand β 11 of the flap region also adopts a completely different conformation between the two structures: residues F227 and L225 of PqsC are located at the side of the acyl cavity in the open state (Figure 6A) but cover

the acyl cavity from top and front in the closed conformation (Figure 6B). Other residues with significantly different orientations are M76* of PqsB and P87 of PqsC (Figures 6A, B). In the open form, M76* and P87 face each other and are oriented upwards to provide space for the alkyl chain (Figure 6A), while they are turned downwards in the PqsBC^{C129A}-octanoyl-CoA model, thereby closing the cavity at the distal end of the channel (Figure 6B).

The AQ/AQNO spectrum of *P. aeruginosa* depends on acyl-CoA availability to PqsBC

The analysis of the structure (Figure 5) and the results of the enzymatic *in vitro* assays outlined above (Figure 2 and Table 1) explain PqsBC's selectivity for medium-chain acyl-CoAs but also suggest that the enzyme should not be able to distinguish between odd- and even-numbered acyl-CoA substrates, provided they fall into the correct size regime. At equal substrate concentrations, this should lead to a bell-shaped profile for AQ/AQNO derivatives of different alkyl size in *P. aeruginosa*, suggesting that the observed deviations from this distribution in cell cultures (Figure 1B) originate from acyl-CoA availability as a consequence of the fatty acid metabolism in the bacterium. To test this, we performed feeding experiments with exogenously supplied individual fatty acid precursors from acetic acid to tetradecanoic acid in wildtype *P. aeruginosa* PA14 cultures at a fixed concentration of 2 mM and quantified the resulting relative AQ/AQNO levels (Figure 7A). In contrast to non-supplemented cultures (Figure 1B), the AQ/AQNO profile was indeed bell-shaped with a maximum at C6-HQ/C7-QNO, indicative of a preference of heptanoyl- and octanoyl-CoA. Further, the profile is characterized by a sharp increase from C3-HQ/QNO to C4-HQ/QNO and a steep drop after C9-HQ/C9-QNO. These results are in line with the preference of PqsBC for medium-chain acyl-CoAs and show that the AQ/AQNO distribution in unfed *P. aeruginosa* reflects the availability of octanoyl- and decanoyl-CoA as the C2-fragment nature of fatty acid metabolism. Unexpectedly, we observed that addition of fatty acids longer than decanoic acid did not result in an increase of the respective AQ/AQNO but in elevated levels of AQs/AQNOs with shorter alkyl chains (Figure S9). Apparently, long-chain fatty acids (> C10) run through the β -oxidation cycle

until they reach an alkyl chain length that falls into the substrate range of PqsBC, and are then channeled into the AQ/AQNO biosynthetic pathway.

PqsBC also produces mono-unsaturated Aqs/AQNOs

In addition to saturated Aqs/AQNOs, *P. aeruginosa* also produces mono-unsaturated congeners (Figure S10; C n :1-HQ/QNO, where n is the number of carbon atoms of the aliphatic side chain).^[21,34,36,38] It has been shown that the double bond is located between the α and β carbons and that the respective *cis* and *trans* isomers have different chromatographic retention times.^[34] Recently, it was found that the *trans* isomer of mono-unsaturated C9:1-QNO exhibits high bacteriostatic activity against *S. aureus* MRSA strains, while the respective *cis* isomer and its saturated AQ congener C9-HQ were inactive.^[49] Although mono-unsaturated Aqs (C9:1-HQ) have already been described by Wells in 1952,^[50] there is currently no information whether the double bond is already introduced by PqsBC-mediated incorporation of an unsaturated acyl-CoA precursor or whether it is the consequence of an unidentified desaturase that acts downstream of AQ/AQNO biosynthesis.

In the course of our feeding experiments, we found that addition of octanoic acid to the growth medium not only increased the level of saturated C7-HQ but also that of its mono-unsaturated congener C7:1-HQ (Figure S11). To shed further light onto this, we conducted an analogous experiment by adding fully deuterated octanoic-d15 acid as fatty acid precursor to the culture medium of wildtype *P. aeruginosa* PA14. We detected mass shifts of 15 Da for both C7-HQ and C7-QNO as expected, but also observed mass shifts of 13 Da for the respective mono-unsaturated C7 congeners, demonstrating that octanoic-d15 acid was also incorporated into C7:1-HQ or C7:1-QNO either prior or after unsaturation (Figure S12). To discriminate between these possibilities, cultures were supplemented with a mixture of *cis/trans*-2-octenoic acid (2 mM). As shown in Figure 7B, C7:1-HQ/QNO levels strongly increase after addition of this compound, indicating that PqsBC can also use this unsaturated precursor. It therefore seems likely that C7:1-HQ and C7:1-QNO

observed in unfed cultures derive from octenoyl-CoA that arises as an intermediate of the β -oxidation pathway.

Discussion

Alkylquinolones and their *N*-oxides (AQs/AQNOs) play an important role in *P. aeruginosa* and it has long been known that abundant amounts and a diverse spectrum of these compounds are produced by the bacterium.^[21,34–37] Here, we have used targeted metabolomics, feeding experiments, proteomics, biochemical assays and structural biology to investigate the role of the FabH-like heterodimeric PqsBC in the production of AQ/AQNO derivatives. Our study shows that PqsBC is a promiscuous enzyme that generates many of these compounds directly by utilizing acyl-CoA substrates with saturated and unsaturated alkyl chains of different length. PqsBC's preference for medium-chain acyl-CoAs provides a rationale why the most abundant AQs/AQNOs found in *P. aeruginosa* culture contain a C7- or a C9-alkyl chain, but it fails to explain why derivatives of uneven-chain acyl-CoAs and of smaller substrates with significant turnover in *in vitro* assays, such as hexanoyl-CoA, are underrepresented *in vivo*. Feeding experiments with C2- to C14-fatty acids suggest that these AQs/AQNOs are absent because the respective fatty acid/acyl-CoA precursors are not available for AQ/AQNO biosynthesis.

The level and composition of acyl-CoAs is dynamic and influenced by several enzymes involved in fatty acid degradation (Fad) and biosynthesis (Fab),^[51] some of them still uncharacterized in *P. aeruginosa*. The initial acyl-CoA pool is defined by the substrate specificity of multiple fatty acyl-CoA ligases (FadDs), which prime fatty acids with CoA for the β -oxidation cycle after uptake.^[52,53] Acyl-CoA dehydrogenases (FadE) introduce the α - β double bond to produce the respective unsaturated enoyl-CoAs in the first step of β -oxidation, before additional enzymes (FadB, FadA) catalyze their further breakdown. A recent study revealed that the transcriptional regulator PsrA counteracts the conversion of acyl-CoAs to enoyl-CoAs by repressing the transcription of the FadE-homolog PA0506, thereby increasing the level of saturated acyl-CoAs that are then

available for AQ/AQNO biosynthesis.^[54] Interestingly, octanoyl-CoA, which is the preferred substrate of PqsBC as demonstrated here, has a special role in the fatty acid metabolism of *P. aeruginosa*: in addition to degradation via β -oxidation, it can be condensed with malonyl-ACP to produce the rhamnolipid precursor β -ketodecanoyl-ACP by the enzyme PA3286, which directly links β -oxidation with *de novo* fatty acid and rhamnolipid biosynthesis.^[55,56] In addition to Fad or Fab enzymes, there are also acyl carrier proteins (ACPs) and enzymes involved in the biosynthesis of cell wall components or of rhamnolipids that also make use of the fatty acid pool. This highlights that PqsBC competes with many other fatty acid-metabolizing enzymes to branch medium-chain acyl-CoAs into AQ/AQNO biosynthesis. In this context, it may be worth noting that the *N*-acyl-L-homoserine lactone synthases RhII of the *rhl* system and LasI of the *las* circuit prefer ACP-loaded C4- and 3-oxo-C12 acyl chains, respectively.^[57,58] As PqsBC selectively uses medium-chain acyl-CoAs, it is probably ensured that the *rhl*, *las* and *pqs* quorum sensing circuits access different fatty acid subpools and do not steal acyl-substrates from each other. Further, AQs with C7- or C9-alkyl chains are the most the potent co-inducers of PqsR and the best substrates of PqsH.^[30,59,60] This shows that PqsBC, PqsR and PqsH are perfectly attuned to one another, suggesting co-evolution not only of the *rhl*, *las* and *pqs* systems but also within the *pqs* system itself.

The finding that the AQNO profile of *P. aeruginosa* cultures in both the presence and the absence of exogenous fatty acids follows the same trend as that of AQs (Figures 1B and 7A) supports the findings of a recent study^[31] that PqsBC also catalyzes the synthesis of AQNOs from 2-HABA (produced by PqsL) and from acyl-CoAs. Our study also provides evidence that in addition to saturated acyl-CoAs, PqsBC also accepts α,β -unsaturated acyl-CoAs, which are most probably formed in the first step of the β -oxidation cycle, to produce the respective mono-unsaturated AQs/AQNOs directly.

The substrate preferences of PqsBC are rooted in its three-dimensional structure, and we found that PqsBC in its closed conformation contains a hydrophobic alkyl-binding chamber that provides

sufficient space to accommodate octanoyl- and decanoyl-moieties in addition to shorter alkyl chains. These shorter substrates will establish fewer interactions with the active site whereas larger molecules will have to adopt unfavorable geometries, explaining the bell-shaped distribution of AQs/AQNOs when cultures of *P. aeruginosa* were supplied with large amounts of C2- to C14-fatty acids. Apparently, the acyl-binding tunnel of PqsBC serves as a “molecular ruler” that restricts the alkyl chain length of the substrate to 10 or fewer carbon atoms. While catalytic residues are only found in PqsC, the acyl-binding tunnel is in part also built by residues from PqsB, showing that this otherwise inactive monomer not only acts as a chaperone for PqsC but also plays a role in shaping the acyl-CoA substrate selectivity of PqsBC.

Importantly, our extensive sampling of crystallization conditions yielded four new crystal forms, of which two have large asymmetric units containing four and eight copies of the protein complex. Together with the structure recently published by Drees *et al.*^[29], this allowed us to compare 22 PqsBC heterodimers in different crystallographic environments. Structural analysis showed that PqsB is very similar in all heterodimers, at the same time revealing a structural plasticity of PqsC that manifests itself in open, intermediate and closed conformations of PqsC. With this, our study provides the first direct experimental corroboration for the existence of distinct conformational states in a FabH-like enzyme. Earlier studies have reported only closed conformations, suggesting that the closed state is in general thermodynamically favored.^[41,42,44,46–48,61–63] Evidence for the dynamic nature of these enzymes and for the existence of open conformations has been rather indirect until now.^[43,45,64,65] For example, an apo structure of *Escherichia coli* FabH (ecFabH, PDB ID: 1HNK) was found to be unstructured in regions implicated in ligand binding,^[43] and kinetic as well as crystallographic analyses with alkyl-CoA disulfide inhibitors suggested that this unstructured ecFabH resembles an open form that closes upon binding of the CoA-ligand.^[64] This view was later refined to the “open state model” by studies on *mt*FabH showing that a mutant with a blocked acyl-binding pocket could still bind an inhibitor, which is only possible if the enzyme opens by movement of the flap region and exposes its binding site.^[45,66] The open state model is

further supported by molecular dynamics (MD) simulations of the FabH-like PQS biosynthesis enzyme PqsD and of ecFabH, which demonstrated large motions of the flap region and in the areas with conserved residues for adenine stacking.^[65,67] These MD profiles are strikingly similar to the structural fluctuations observed in the ensemble of PqsC crystal structures shown in Figure 3B. We are therefore confident that the open, intermediate and closed conformations of PqsBC identified here are not crystallographic artifacts but resemble trapped “conformational snapshots” that occur during acyl-CoA binding of PqsBC.

Comparison of the three conformational states of PqsBC enables us to speculate about a structural mechanism for the opening and closing of PqsBC during catalysis, which is summarized in Figure 8. At the outset of the catalytic cycle, substrate-free PqsBC is expected to be in the open form with P242 in the *cis*-conformation. The adenine moiety of the acyl-CoA substrate would then bind between the conserved W35 and R168 (Figure 5C) and *cis-trans* isomerization at V241-P242 would accompany the transition of loop A in the open state to the elongated, kinked helix $\alpha 5$ observed in the closed conformation (Figures 4C, D), which concomitantly pushes strand $\beta 11$ upward (Figure 4E). This allows residues S231 and Q234 of the extended helix $\alpha 5$ to establish favorable interaction with the CoA moiety of the substrate (Figure 5C), pulling the structural elements containing the W35/R168 clamp towards the active site (Figure 4B). The active site will close further by movement of strand $\beta 11$ from PqsC, which slides over the acyl-binding pocket like a zipper. Due to this displacement, F227 (PqsC) can then shield the pocket from the top like a lid and L225 (PqsC) moves to the far end of the tunnel, thereby pushing P87 (PqsC) and M76* (PqsB) downwards to establish the distal walls of the channel (Figures 6A, B). As a consequence, the bound substrate would be locked in the closed conformation to undergo the transacylation reaction with C129. Interestingly, the finding that PqsBC was also loaded with long-chain dodecanoyl or tetradecanoyl units (Figure 2A), which are expected not to fit into the acyl-binding cavity of PqsC, seems to indicate that full closing is not required for this reaction. However, competition experiments (Figure S1) and enzymatic reactions including the second substrate 2-ABA (Table 1)

suggest that transacylation in the open state of these long-chain primers is less efficient and blocks structural changes required for the second half-reaction of the catalytic cycle. This second half-reaction likely is initiated by dissociation of CoA, which would not require large structural perturbations, since CoA is bound mostly at the surface of PqsC. The release of CoA is expected to open a channel through which the second substrate 2-ABA can enter the active site while the enzyme would remain in the closed state. The AQ/AQNO product forms by decarboxylative condensation followed by intramolecular cyclization of the condensation intermediate. While it is at present unclear if this final cyclization proceeds within the active site and if it requires catalysis,^[29] the decarboxylative condensation releases the acyl group from C129. As a consequence, forces that hold the enzyme in the closed state would be interrupted, allowing re-opening and dissociation of the product.

A similar model has already been suggested for *mtFabH*.^[45,66] It has to be noted, however, that P242 of PqsC is not conserved in other FabH enzymes such as *mtFabH* (Figure S6) and therefore, due to the potential importance of its cis/trans isomerization, details of the hypothetical model purported here may not apply to all FabH proteins. This is not surprising because of the large diversity of this family and the special role that PqsBC takes within these proteins. Most FabH enzymes are homodimers involved in fatty acid biosynthesis, have a catalytic triad, and utilize acyl-CoA primers and malonyl-ACP to produce 3-ketoacyl-ACPs. In contrast, PqsBC is heterodimeric, requires only a catalytic dyad, and produces a bicyclic AQ product from acyl-CoA and 2-ABA. The flexibility of the flap region in most other FabH enzymes may therefore be encoded in other residues, and Sachdeva *et al.*^[45] have previously suggested that conserved glycine residues that flank the flap region in other FabH enzymes but not in PqsBC (Figure S6) play a role in opening and closing the active site. Although it is obvious from the crystal structures that the orientation of P242 has large structural consequences for the active site of PqsBC, further in-depth investigation will be necessary to evaluate its potential role in the catalytic mechanism of PqsBC.

Due to its importance in controlling virulence and because of its unique occurrence in *P. aeruginosa*, the *pqs* system is currently evaluated as a drug target in novel anti-virulence strategies.^[68–70] The finding that PqsBC adopts distinct conformations that have been resolved here to high resolution and could be addressed selectively may offer new opportunities for these drug discovery programs.

Experimental Section

Full experimental procedures can be found in the supporting online information.

Chemicals and bacterial strains

UCBPP-PA14 was used as the wildtype strain for all experiments. The nonpolar mutant strains ED117 (*pqsB*::*TnphoA*) and ED218 (*pqsC*::*Kan*) were kindly provided by Eric Déziel and co-workers.^[28] 2-ABA was kindly provided by Steffen L. Drees and Susanne Fetzner.^[27] All acyl-CoAs were purchased from Larodan with the exception of acetyl-CoA, which was acquired from Sigma-Aldrich. DTNB was obtained from Thermo Fisher Scientific and CoA was purchased from AppliChem.

Metabolomic analysis of *Pseudomonas aeruginosa*

AQ/AQNO analysis in *P. aeruginosa* has been performed as described elsewhere^[38]. Briefly, bacteria were grown in BM2 minimal medium with or without exogenous fatty acids (2 mM). Methanol extracts of cells and culture supernatants were analyzed by HPLC- coupled mass spectrometry, using a 150 mm Kinetex C18 reversed-phase column with 1.7 μ m particle size and 2.1 mm inner diameter (Phenomenex) and a quadrupole time-of-flight mass spectrometer (maXis HD QTOF, Bruker) with positive mode electrospray ionization. Signal quantification was based on the calculation of peak areas in extracted ion chromatograms of the respective analytes.

Acyl-CoA loading assays and LC-MS measurements of intact proteins

Acyl-CoA loading assays were performed with PqsBC (5 μ M) and acyl-CoAs (50 μ M) in Tris/HCl (50 mM, pH 7.6) at 30 °C. After incubation for 30 min, the samples (20 μ L) were directly submitted for intact protein analyses. All ESI-MS measurements were performed on a Dionex Ultimate 3000 RSLC system using a ProSwift RP-4H (monolithic PS-DVB), 250 x 1 mm column (Thermo Fisher Scientific) and a maXis 4G hr-ToF mass spectrometer (Bruker Daltonics) equipped with the standard Bruker ESI source.

Cloning and site-directed mutagenesis

The *pqsB* (PA0997) and *pqsC* (PA0998) genes from *Pseudomonas aeruginosa* PAO1 were amplified from chromosomal DNA by PCR (primers listed in Table S4). *pqsB* was cloned into pET26b (Merck Millipore) and *pqsC* was ligated into pET19m or p10\$,^[71] both based on the pET19b vector (Merck Millipore). The resulting plasmid pET19m-*pqsC* produces PqsC with an N-terminal His₆ tag followed by a recognition motif for TEV (tobacco etch virus) protease, while p10\$-*pqsC* encodes PqsC with an N-terminal His₆-tagged T7 lysozyme removable by human rhinovirus 3C protease. The active site cysteine C129 of PqsC was mutated to alanine (PqsC^{C129A}) or serine (PqsC^{C129S}) in pET19mod-*pqsC* and p10\$-*pqsC* by PCR-based mutagenesis (Table S4).

Expression and purification of (His₆-)PqsBC, (His₆-)PqsBC^{C129A} and PqsBC^{C129S}

Recombinant proteins were produced in *E. coli* BL21(DE3)pLysS (Promega) or BL21-CodonPlus(DE3)-RIL (Agilent Technologies) co-transformed with pET26b-*pqsB* and pET19m-*pqsC* or p10\$-*pqsC*. Purification involved nickel affinity and size exclusion chromatography with or without an intermittent protease cleavage and chromatography step to remove the His₆-affinity tag (Table S5). The purified proteins were concentrated to 20 – 35 mg mL⁻¹, flash-cooled in liquid nitrogen and stored at -80 °C.

PqsBC activity assay with 2-ABA and acyl-CoAs of different carbon chain lengths

Enzymatic activities were measured in a spectrophotometric assay by using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) to monitor the formation of free CoA at 412 nm.^[72,73] Acyl-CoA or DTNB stock solutions were freshly prepared in MilliQ water or DMSO, respectively. The concentrations of acyl-CoA stocks were measured at 259 nm^[74] and the concentration of free CoA already present in the respective acyl-CoA stock was calculated by adding DTNB and comparing to a standard curve.

For the enzyme activity assay, PqsBC (1 μ M) was mixed with acyl-CoA (500 μ M) and 2-ABA (1 mM) and the samples were incubated at room temperature for 20 min. The reaction was stopped with SDS before measuring released CoA with DTNB.

Crystallization, data-collection, phasing, refinement and structural analysis

Initial crystallization conditions were identified with commercial screens using the sitting drop vapor diffusion method. After optimization by random and grid screening, crystals were harvested in cryo-protectant (Table S1, Figure S2) and diffraction data were collected on beamlines X06DA/X10SA at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) and on PETRA III beamline P11 at DESY (Hamburg, Germany). Diffraction data were indexed and integrated with XDS^[75] or XDSAPP^[76], and scaled with Aimless^[77]. Since no other PqsBC structure was available at the outset of these studies, initial phases were derived by a combination of molecular replacement and heavy atom derivatization, using data from crystal form 2. The model was finalized by alternating steps of manual model building in COOT^[78] and maximum-likelihood refinement in phenix.refine.^[79] The final model was used to determine the structures of PqsBC in other space groups by molecular replacement using Phaser.^[80] Model qualities were evaluated with MolProbity.^[81] Data collection and refinement statistics are listed in Table S2. Atomic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (www.rscb.org)^[82] with accession codes 6ESZ, 6ET0, 6ET1, 6ET2 and 6ET3.

Structural representation were rendered with PyMOL,^[83] secondary structure elements were assigned with DSSP^[84] and protein cavities were calculated with KVFinder.^[85]

Modeling of PqsBC^{C129A}-acyl-CoA complexes

PqsBC^{C129A}-acyl-CoA complexes were modeled with the structure of His₆-PqsBC^{C129A} determined in this study (chains IJ of crystal form 3, PDB ID: 6ET2) and the X-ray coordinates of *mt*FabH in complex with dodecanoyl-CoA (PDB ID: 1U6S^[44]) using Molecular Operating Environment (MOE, Chemical Computing Group).^[86]

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Abbreviations

2-AA, 2-aminoacetophenone; 2-ABA, 2-aminobenzoylacetate; 2-ABA-CoA, 2-aminobenzoyl-acetyl-CoA; 2-HABA, 2-hydroxylaminobenzoylacetate; AI, autoinducer; AQ, 2-alkyl-4(1*H*)-quinolone; AQNO, 2-alkyl-4-hydroxyquinoline *N*-oxide; ASU, asymmetric unit; DHQ, 2,4-dihydroxyquinoline; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; *ec*FabH, *Escherichia coli* FabH; Fab, fatty acid biosynthesis; Fad, fatty acid degradation; HHQ, 2-heptyl-4(1*H*)-quinolone; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; MD, molecular dynamics; *m*/FabH, *Micrococcus luteus* FabH;

mtFabH, *Mycobacterium tuberculosis* FabH; MvfR, multiple virulence factor regulator; PDB, Protein Data Bank; PQS, 2-heptyl-3-hydroxy-4(1*H*)-quinolone; PSSweb, Protein Structural Statistics Web Server; QS, quorum sensing; TEV, tobacco etch virus.

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Schemes

Scheme 1. Current understanding of AQ and AQNO biosynthesis in *Pseudomonas aeruginosa*, highlighting the role of PqsBC. Dashed arrows indicate breakdown reactions. TesB is a broad-specific thioesterase that can replace PqsE in this pathway.

Figures

Figure 1. AQ/AQNO profile of *Pseudomonas aeruginosa* PA14. A) Total ion chromatograms of bacterial cell extracts of wildtype *P. aeruginosa* PA14 (black), ED117 (*pqsB*⁻, blue) and ED218 (*pqsC*⁻, magenta). The magnified insert shows the relevant AQ/AQNO section. Chromatograms from three replicates are shown for each strain. CX:n-HQ are AQs (HHQ series) and CX:n-QNO are AQNOs (HQNO series) with X and n representing the number of carbon atoms and the number of double bonds in the aliphatic chain, respectively (e.g. C7-HQ is equivalent to HHQ). The most prominent AQ/AQNO peaks in the chromatograms of wildtype *P. aeruginosa* PA14 are annotated with the *m/z* of their [M+H]⁺ ions. None of the annotated AQs/AQNOs is present in the mutant strains. B) Mean peak areas of saturated AQs (white bars) or AQNOs (gray bars) in combined cell and supernatant extracts of exponentially growing wildtype *P. aeruginosa* PA14 in arbitrary units (A.U.). Differences in AQ/AQNO levels shown in panels A) and B) are due to the fact that A) shows only cellular AQs/AQNOs, while both cellular and extracellular AQs/AQNOs were quantified for B). Since the annotation of AQs and AQNOs is based on their MS² spectra containing the characteristic radical cation with *m/z*= 159, which cannot be formed from C1-HQ/C1-QNO, the identity of C1-HQ/C1-QNO cannot be verified. The error bars are the standard deviations from three independent measurements. For comparability, the scaling is identical to Figure 7A.

Figure 3. Structural comparison of 22 individual PqsBC heterodimers in crystal forms 1 to 5 with the Protein Structural Statistics Web Server (PSSweb).^[39,40] A/B) Average standard deviations of backbone coordinates (Å) of all PqsB (A) and PqsC (B) chains by residue. The catalytic dyad (C129, H269) and conserved residues for acyl-CoA binding (W35, R168) as well as the potential hinge residue P242 are indicated as triangles and dots, respectively. C) Average PqsBC heterodimer in cartoon representation colored by the standard deviation (Å) for the backbone coordinates. Backbone atoms of PqsB or PqsC with an average standard deviation smaller than 0.6 Å are colored in dark and light gray, respectively. Important residues and secondary structure elements of PqsC discussed in the main text are highlighted.

Figure 4. Conformational changes in PqsBC. A) Open (red; PDB ID: 5DWZ^[29]), intermediate (yellow; PDB ID: 6ET1, this study) and closed conformations (green; PDB ID: 6ET2, this study) of PqsC. PqsB is shown in dark gray and residues with only small or no changes in PqsC are shown in light gray. B) R168 and W35, which clamp the adenine ring of the acyl-CoA substrate in other FabH enzymes, move inwards upon closing of the active site. C) Residue P242 in helix $\alpha 5$ adopts a *cis* configuration in the open form (red) and a *trans* configuration in the closed state (green). D) Residues S231-A238 of loop A in the open conformation of PqsC (red) are part of helix $\alpha 5$ in the closed form (green). Dashed lines indicate distances between the same residue in the open and closed state. E) Amino acids of the mobile flap including $\beta 11$ of the open

conformation (green) move upwards relative to those in the closed conformation (red). Intermediate conformations are omitted in panels B-E) for clarity.

Figure 5. Modeling of PqsBC-acyl-CoA complexes. A) Schematic representation of the L-shaped acyl-CoA binding site of PqsBC, which can be subdivided into a channel for the CoA moiety (CoA-binding channel) and a tunnel for the hydrophobic fatty acid part (acyl-binding channel). Octanoyl-CoA (blue) and important residues (dark gray for PqsB, indicated by asterisks; black for PqsC) lining the binding pocket are indicated. B) Surface cutaway side view of the L-shaped acyl-CoA binding pocket of PqsBC in complex with octanoyl-CoA (white) superposed with dodecanoyl-CoA (black). C) Predicted interactions between residues of the CoA-binding channel of PqsC (green) and octanoyl-CoA (white). Hydrogen bonds and salt bridges are indicated with dashed black and light blue lines, respectively. The adenine moiety of octanoyl-CoA is sandwiched by the side chains of R168 and W35. D) Alkyl-binding channel of PqsBC in complex with octanoyl-CoA (white). Hydrogen bonds (black dashed lines) and sulfur- π interactions (magenta dashed lines) between PqsC and the cysteamine moiety or the thioester of octanoyl-CoA are shown. The octanoyl chain is embedded in a hydrophobic pocket formed by residues of both PqsB (dark gray) and PqsC (green).

Figure 6. Transition from the open A) to the closed state B) reduces the size of the acyl-binding pocket from 708 to 256 Å³ (white surfaces, calculated with KVFinder^[85]) as a consequence of changes in the indicated residues. The model of octanoyl-CoA (white, ball-and-stick) is shown to highlight the tight fit to the acyl-CoA binding site in the closed state. PqsB and PqsC are shown in dark gray and red/green, respectively. Residues of PqsB are indicated by an asterisk.

Figure 7. The AQ/AQNO spectrum of *P. aeruginosa* depends on the availability of fatty acids and 2-octenoic acid is the precursor of C7:1-HQ and C7:1-QNO. A) Mean peak area in A.U. of AQs (white bars) and AQNOs (gray bars) in combined cell and supernatant extracts generated from cultures of exponentially growing wildtype *P. aeruginosa* PA14 fed with 2 mM of the respective precursor fatty acid (e.g. octanoic acid for C7-HQ). Error bars show the standard deviation from three independent measurements. Note that the AQ/AQNO levels of C4-HQ/C4-QNO to C9-HQ/C9-QNO after precursor feeding are higher than those of unfed cultures (Figure 1B) and show a bell-shaped profile, indicative of incorporation of the supplied fatty acids into AQs/AQNOs. B) Mean peak area of *cis/trans* C7:1-HQ and C7:1-QNO in cell extracts of exponentially growing wildtype *P. aeruginosa* PA14 fed with EtOH (control, black bars), with octanoic acid (2 mM, white cross-striped bars) or with 2-octenoic acid (2 mM, grey cross-striped bars). The error bars represent the standard deviation from three independent measurements. The large increase in C7:1-HQ/C7:1-QNO upon the addition of 2-octenoic acid suggests that the mono-unsaturated C7-alkyl chain originates from 2-octenoic acid.

Figure 8. A structural, hypothetical model of the catalytic cycle of PqsBC. (1) After octanoyl-CoA binds to the enzyme, PqsBC (PqsB: dark gray; PqsC: light gray) transitions from the open (red) into the closed state (green). (2) The octanoyl chain is transferred to active site C129 of PqsC, followed by the release of CoA. (3) 2-ABA enters the active site and undergoes decarboxylative condensation with the octanoyl-PqsC intermediate to produce HHQ. (4) The re-opening of PqsBC leads to the release of HHQ (or of the Claisen condensation intermediate that finally undergoes cyclization to HHQ). A surface cutaway side view of the L-shaped acyl-CoA binding channel is shown at each reaction step

Tables

Table 1. Acyl-CoA substrate specificity of PqsBC

Acyl-CoA	Acyl chain length	[CoA] (μ M) (PqsBC)	[CoA] (μ M) (PqsBC ^{C129A})
Acetyl-CoA	2	n. d.	-
Butyryl-CoA	4	n. d.	-
Hexanoyl-CoA	6	205 $\pm$ 8	-
Octanoyl-CoA	8	459 $\pm$ 10	n. d.
Decanoyl-CoA	10	261 $\pm$ 19	-
Dodecanoyl-CoA	12	n. d.	-
Tetradecanoyl-CoA	14	n. d.	-
Hexadecanoyl-CoA	16	n. d.	-

PqsBC (1 μ M) was mixed with acyl-CoA (500 μ M) and 2-ABA (1 mM). The reaction was stopped after 20 min. The absorbance of the sample was measured at 412 nm after the addition of DTNB (2 mM) to determine the concentration of free coenzyme A (CoA). The conversion of octanoyl-CoA by PqsBC^{C129A} was tested as a negative control. The errors are the standard deviations from three independent measurements. n. d.: no detectable CoA release. -: not tested.

Table of Contents

The dynamic and fuzzy FabH-like PqsBC heterodimer: We investigated the acyl-CoA specificity of *Pseudomonas* Quinolone Signal biosynthesis enzyme PqsBC, finding selectivity for medium-chain acyl-CoAs that determines the alkylquinolone repertoire of *Pseudomonas aeruginosa*. Comparisons of several crystal structures unveil that PqsC is highly flexible, while PqsB is rather rigid.