**The Alkylquinolone Repertoire of *Pseudomonas aeruginosa* is Linked to Structural Flexibility of the FabH-like 2-Heptyl-3-hydroxy-4(1H)-quinolone (PQS) Biosynthesis Enzyme PqsBC**

Florian Witzgall[a], Tobias Depke[b], Michael Hoffmann,[c, e] Martin Empting,[d, e] Mark Brönstrup,[b] Rolf Müller,[c, e] and Wulf Blankenfeldt*[a, f]

*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(1H)-quinolones (AQs) and AQ N-oxides (AQNOs) that serve as signaling molecules to control the production of virulence factors and that are involved in membrane vesicle formation and iron chelation; furthermore, they also have, for example, antibiotic properties. It has been shown that the β-ketoacyl-acyl-carrier protein synthase III (FabH)-like heterodimeric enzyme PqsBC catalyzes the last step in the biosynthesis of the most abundant AQ congener, 2-heptyl-4(1H)-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.

**Introduction**

The Gram-negative bacterium *Pseudomonas aeruginosa* is a serious burden for public health. It is a major causative agent of hospital-acquired lung infections that often lead to death in cystic fibrosis patients, catheter-associated urinary tract infections, bacterial keratitis owing to contaminated contact lenses, and 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 therefore, this network is 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 secretion of small signaling molecules (autoinducers, AIs) that are sensed by specific transcriptional receptors that 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...
that are also interlinked with each other.\(^{9,11}\) Two of these pathways, the \(\text{lqs}\)\(^{12–14}\) and \(\text{rhf}\)\(^{15–17}\) systems, rely on \(N\)-acyl-L-homoserine lactones, which are common autoinducers in Gram-negative bacteria.\(^{7,8}\) The third QS circuit, the \(\text{pqs}\) 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 virulence 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 \(\text{pqsABCDE}\) 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-aminobenzoylacetate (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-aminobenzoylacetyl-CoA (2-ABA), which is the building block for the 4-quinolone ring structure of HHQ.\(^{26,27}\) 2-ABA is a branch-point metabolite in the biosynthesis of HHQ, as it is shuttled into different reaction pathways. Owing to its instability, it can spontaneously decompose into 2-aminoacetophenone (2-AA) by the loss of \(\text{CO}_2\) or by undergoing intramolecular cyclization into 2,4-dihydroxyquinoline (DHQ).\(^{22,28}\) 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 is formed.\(^{28,29}\) In a final step, HHQ is hydroxylated at the \(\text{C3}\) position by the monooxygenase PqsH.\(^{30}\) Additionally, a very recent study demonstrated that PqsBC could also use 2-hydroxylaminobenzoylacetyl (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 \(\beta\)-ketoacyl-acyl-carrier protein synthase III (FabH) of the thiolase superfamily, but unlike other FabH homologues 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 was recently shown by Drees et al.\(^{29}\) that the missing asparagine side chain in PqsC was mimicked by the NH\(_2\) 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 and 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-

\[\text{Scheme 1. Current understanding of AQ and AQNO biosynthesis in } P.\ aeruginosa, \text{ highlighting the role of PqsBC. Dashed arrows indicate breakdown reactions. TesB is a broad-specific thioesterase that can replace PqsE in this pathway.}\]
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 seven or nine 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/PQSo riginates from octanoate introduced by PqsBC.[28] However, as *P. aeruginosa* also synthesizes AQ and AQNO derivatives with shorter, longer, and mono-unsaturated alkyl chains, it seems likely that PqsBC could also utilize the respective acyl-CoA primers for condensation. We therefore investigated the acyl-CoA substrate specificity of PqsBC by using enzymatic assays and feeding experiments as well as by analyzing PqsBC crystal structures from five different crystal forms. The results show that PqsBC prefers medium-chain acyl-CoAs and that this 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. The crystal structures demonstrate that PqsBC exists in open, closed, and intermediate conformations. Such conformations were previously postulated for other FabH enzymes but were never 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 was 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 wild-type and in nonpolar *pqsB* and *pqsC* mutant strains grown in minimal medium by using LC–MS. Hydroxylated species (PQS analogues) were excluded from the analysis, as AQ/AQNO hydroxylation occurs downstream of PqsBC (Scheme 1).[20, 21, 30] Towards this, it was described previously that PQS analogues and AQNOs of the same side chain length could 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. Whereas AQs/AQNOs were completely absent in the *pqsB* and *pqsC* mutants, we observed a large variety of different AQs/AQNOs in the wild-type 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, whereas only trace amounts of AQ congeners with even-numbered alkyl chains were found (Fig-
ure 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 (2-nonyl-4(1H)-quinolone, NHQ) or C9-QNO (2-nonyl-4-hydroxyquinoline N-oxide, NQNO; Figure 1B), which is in 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 could 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 by ESI-MS. In all cases, PqsC was loaded with the respective acyl chain, whereas 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, which revealed a clear preference for the corresponding acyl-CoAs (Figure 2B). To evaluate if substrate utilization could be shifted by increasing the concentration of one acyl-CoA over the other, we incubated PqsBC with a constant amount of octanoyl-CoA and an equimolar or tenfold higher concentration of hexanoyl-, decanoyl-, dodecanoyl-, or tetradecanoyl-CoA in a 1:1 competition assay (Figure S1 in the Supporting Information). Even with a tenfold excess amount of hexanoyl- or tetradecanoyl-CoA, PqsBC was only loaded with octanoate (Figure S1A,D). In the case of a tenfold excess amount 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; this suggests that the AQ/AQNO spectrum produced by *P. aeruginosa* is also influenced by the availability of acyl-CoA.

We also investigated the acyl-CoA substrate specificity of PqsBC by endpoint measurements under turnover conditions in the presence of 2-ABA as a second substrate, as transacylation only reflects the first reaction step and the substrate specificity could also be influenced by the overall kinetics of the enzyme. The 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 λ = 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 short-chain acetyl- or butyryl-CoA or 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 six to ten carbon atoms and that the highest activity is exhibited with octanoyl-CoA.

**Structure determination of PqsBC in four different crystal forms**

To correlate the observed preference for medium-chain acyl-CoAs with structural features of PqsBC, we aimed to obtain crystal structures of octanoylated wild-type PqsBC and the
active-site mutants PqsBC\textsubscript{C129A} and PqsBC\textsubscript{C129S} to capture an enzyme–octanoyl-CoA complex, which was expected to form before the acyl group was transferred onto active-site C129 of PqsC. Despite extensive efforts in soaking and cocrystallization experiments with wild-type PqsBC and with the mutants PqsBC\textsubscript{C129A}/PqsBC\textsubscript{C129S}, however, this was not successful. Nevertheless, we determined the structures of ligand-free PqsBC wild-type and the PqsBC\textsubscript{C129A}/PqsBC\textsubscript{C129S} mutants in our different crystal forms (crystal forms 1 to 4) at 1.53 to 2.7 Å resolution. The crystals contained two, four, and 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 (Figure S5A, B), and in the wild-type 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 copurified 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)\textsuperscript{[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.\textsuperscript{[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 (Figure 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 (Figure 3B and C). The variable structural elements include helix α1 (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

| Acyl-CoA | Acyl chain length | Concentration of CoA [µM] | PqsBC | PqsBC\textsubscript{C129A} |
|---------|------------------|--------------------------|-------|--------------------------|
| acetyl-CoA | 2               | n.d.                     | –     | –                        |
| butyryl-CoA | 4               | n.d.                     | –     | –                        |
| hexanoyl-CoA | 6               | 205 ± 8                  | n.d.  | –                        |
| octanoyl-CoA | 8               | 459 ± 10                 | n.d.  | –                        |
| decanoyl-CoA | 10              | 261 ± 19                 | –     | –                        |
| dodecanoyl-CoA | 12              | n.d.                     | –     | –                        |
| tetradecanoyl-CoA | 14             | n.d.                     | –     | –                        |
| hexadecanoyl-CoA | 16             | n.d.                     | –     | –                        |

\[a\] PqsBC (1 µM) was mixed with acyl-CoA (500 µM) and 2-ABA (1 mM). The reaction was stopped after 20 min. The absorbance of the sample was measured at λ = 412 nm after the addition of DTNB (2 mM) to determine the concentration of free CoA. The conversion of octanoyl-CoA by PqsBC\textsubscript{C129A} was tested as a negative control. The errors are the standard deviations from three independent measurements. n.d.: not detectable CoA release. –: not tested.

**Table 1. Acyl-CoA substrate specificity of PqsBC.**
CoA binding by intercalating the adenine moiety of CoA between the guanidine (R168) and the indole (W35) groups.\textsuperscript{[41–44]} The largest structural variations in PqsC are found in a region between β10 and helix α5 (residues 212–243), which is part of an area termed the “flap” in FabH from Mycobacterium tuberculosis (mtFabH); in this region, mtFabH is predicted to undergo large structural rearrangements during binding of the acyl-CoA primer (residues 201–234 in PqsC correspond to the flap region in mtFabH; Figure S6).\textsuperscript{[45]} In contrast, the amino acids in the immediate vicinity of the catalytic residues C129 and H269 are highly similar between the analyzed structures, indicating rigidity (Figure 3B and C).

PqsC adopts open, intermediate, and closed conformations

Superimposition of the 22 copies of PqsBC contained in the five different crystal forms revealed that these heterodimers can be assigned to “open”, “intermediate”, and “closed” states, depending on the positions of the structural elements surrounding the active site (Figure 4A and Table S3). These states have different conformations of the flap and helix α1, the flexibility of which is also reflected in high B-factors (Figure 4A and Figure S7). The open form is only found in crystal form 5, whereas 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 configuration in open PqsBC, whereas it is in the trans configuration in the closed and intermediate structures (Figures 4C and S8); this suggests 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 forms: whereas residues 231 to 240 adopt a loop structure in the open form that enlarges the catalytic cavity (termed “loop A” by Drees et al.;\textsuperscript{[29]} Figure S6), they form an imperfect, kinked α helix acting as an N-terminal extension of helix α5 in the closed form, which

Figure 4. Conformational changes in PqsBC. A) Open (red; PDB ID: 5DWZ\textsuperscript{[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 α5 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 α5 in the closed form (green). Dashed lines indicate distances between the same residue in the open and closed states. E) Amino acids of the mobile flap including β11 of the open conformation (green) move upwards relative to those in the closed conformation (red). Intermediate conformations are omitted in (B)–(E) for clarity.
leads to a reduction in the 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 β11 of the mobile flap is raised, which also contributes to closing and shrinking of the binding pocket (Figure 4E). The distance between the α carbon atoms of Q234 (loop A/helix α5) and W35 (helix α1) decreases from 29 Å in the open conformation to 11 Å in the closed conformation, which highlights 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 homologues that the shape and size of the acyl-binding channel dictate the acyl-CoA substrate specificity of these enzymes. 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 with mtFabH bound to dodecanoyl-CoA (PDB ID: 1U6S) to model a complex between PqsBC and octanoyl-CoA (Figure 5). According to this, octanoyl-CoA occupies an L-shaped pocket in PqsC that can be subdivided into a long pantetheinate or CoA-binding tunnel and a shorter, buried acyl-binding channel (Figure 5A, B) that is similar to those found in the crystal structures of mtFabH–dodecanoyl-CoA and FabH from Micrococcus luteus (mtFabH). The substrate-binding pocket is only accessible through the pantetheinate arm, and the two subchannels are separated by the catalytic dyad of PqsC (C129, H269) at the bottom of the binding cleft (Figure 5A, B).

Our model suggests that the side chains of W35 and R168 sandwich the adenine ring through 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 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 the indole nitrogen atom of W39 as well as the side chain of Q234 (Figure 5C). This stacking recognition motif is fully conserved in FabH enzymes (Figure S6). The main-chain carbonyl oxygen atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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 atom of R168 additionally fixes the adenine ring through 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).
moiety is stabilized by hydrophobic interactions, the β-mercaptoethylamine unit is hydrogen bonded to the backbone carbonyl oxygen atom 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 carbonyl oxygen atom of the thioester. The octanoyl side chain is embedded in the hydrophobic acyl-binding pocket (Figure 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 the PqsBC129A models bound to octanoyl- and dodecanoyl-CoA show that the 11.5 Å long acyl-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 ten carbon atoms are expected to have to fold their aliphatic tails back to fit into 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 acyl-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 β11 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 the top and front in the closed conformation (Figure 6B). Other residues with significantly different orientations are M76* of PqsB and P87 of PqsC (Figure 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), whereas they are turned downwards in the PqsBC129A–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 the selectivity of PqsBC 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 sizes in P. aeruginosa, thus suggesting that the observed deviations from this distribution in cell cultures (Figure 1B) originate from acyl-CoA availability as a consequence of 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 wild-type P. aeruginosa PA14 cultures at a fixed concentration of 2 mM and quantified the resulting relative AQ/AQNO levels (Figure 7A). In contrast to nonsupplemented cultures (Figure 1B), the AQ/AQNO profile is indeed bell-shaped with a maximum at C9–HQ/C7–QNO, indicative of a preference for heptanoyl- and octanoyl-CoA. Further, the profile is characterized by a sharp increase from C9–HQ/QNO to C10–HQ/QNO and a steep drop after C11–HQ/C12–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 the addition of fatty acids longer than decanoic acid did not result in an increase in the respective AQ/AQNO but in elevated levels of AQS/AQNOs with shorter alkyl chains (Figure 5B). 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; Cn−1–HQ/QNO, for which n is the number of carbon atoms of the aliphatic side chain) [21, 34, 36, 38]. It was previously shown that the double bond is located between the α and β carbon atoms 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 exhibited high bacteriostatic activity against S. aureus MRSA strains, whereas the respective cis isomer and its saturated AQ congener C9–HQ were inactive [48]. Although mono-unsaturated AQS (Cn−1–HQ) were described by Wells in 1952, 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 the addition of octanoic acid to the growth medium not only increased the level of saturated C$_7$-HQ but also that of its mono-unsaturated congener C$_7$-$\alpha$-HQ (Figure S11). To shed further light onto this, we conducted an analogous experiment by adding fully deuterated [D$_{15}$]octanoic acid as a fatty-acid precursor to the culture medium of wild-type P. aeruginosa PA14. We detected mass shifts of 15 Da for both C$_7$-HQ and C$_7$-QNO as expected but also observed mass shifts of 13 Da for the respective mono-unsaturated C$_7$ congeners, which demonstrates that [D$_{15}$]octanoic acid was also incorporated into C$_7$-$\alpha$-HQ or C$_7$-$\alpha$-QNO either prior or after unsaturation (Figure S12). To discriminate between these possibilities, cultures were supplemented with a mixture of cis/trans-oct-2-enoic acid (2 mM). As shown in Figure 7B, C$_7$-$\alpha$-HQ/QNO levels strongly increased after the addition of this compound, which indicates that PqsBC can also use this unsaturated precursor. It therefore seems likely that C$_7$-$\alpha$-HQ and C$_7$-$\alpha$-QNO observed in unfed cultures derive from octenoyl-CoA that arises as an intermediate of the $\beta$-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. Here, we 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 lengths. The preference of PqsBC for medium-chain acyl-CoAs provides a rationale as to why the most-abundant AQs/AQNOs found in P. aeruginosa culture contain a C$_7$, or C$_8$ alkyl chain, but it fails to explain why derivatives of uneven-chain acyl-CoAs and smaller substrates with significant turnover in vitro assays, such as hexanoyl-CoA, are under-represented in vivo. Feeding experiments with C$_7$ to C$_{14}$ 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 levels and compositions of acyl-CoAs are dynamic and influenced by several enzymes involved in fatty-acid degradation (Fad) and biosynthesis (Fab), 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) that prime fatty acids with CoA for the $\beta$-oxidation cycle after uptake. Acyl-CoA dehydrogenases (FadEs) introduce the $\alpha$-$\beta$ double bond to produce the respective unsaturated enoyl-CoAs in the first step of $\beta$-oxidation before additional enzymes (FadB, FadA) catalyze their further breakdown. A recent study revealed that the transcriptional regulator PsrA...
counteracted the conversion of acyl-CoAs into enoyl-CoAs by repressing the transcription of the FadE homologue PA0506, which thereby increased the level of saturated acyl-CoAs that were 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 by 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 and Fab enzymes, there are also acyl carrier proteins (ACPs) and enzymes involved in the biosynthesis of cell-wall components and 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 is worth noting that the N-acyl-l-homoserine lactone synthases Rhl 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 C₆ or C₅ alkyl chains are the most potent coregulators of PqsR and the best substrates of PqsH.\[30, 36, 59, 60\] This shows that PqsBC, PqsR, and PqsH are perfectly attuned to one another and suggests coevolution not only of the rhl, las, and pqs systems but also within the pqs system itself. The finding that the AQNO profiles of *P. aeruginosa* cultures in both the presence and absence of exogenous fatty acids follow the same trend as those of the AQS profiles (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 acyl-binding chamber that provides sufficient space to accommodate octanoyl and decanoyl moieties in addition to shorter alkyl chains. These substrate interactions will establish fewer interactions with the active site, whereas larger molecules will have to adopt unfavorable geometries, and this explains the bell-shaped distribution of AQS/AQNOs upon supplying cultures of *P. aeruginosa* with large amounts of C₄ 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 ten or fewer carbon atoms. Whereas catalytic residues are only found in PqsC, the acyl-binding tunnel is in part also built by residues from PqsB, which shows 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.,\[64\] 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 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 reported only closed conformations, thus suggesting that the closed state is, in general, thermodynamically favored.\[61, 62, 44, 46–48, 61–63\] 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 resembled an open form that closed upon binding of the CoA ligand.\[32\] This view was later refined to the “open-state model” by studies on mtFabH, which showed that a mutant with a blocked acyl-binding pocket could still bind an inhibitor, but this was only possible if the enzyme opened by movement of the flap region and exposed its binding site.\[65, 66\] The open-state model is further supported by molecular dynamics (MD) simulations of the FabH-like PQS biosynthesis enzyme PqsD and 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 then binds between conserved W35 and R168 (Figure 5C) and cis/trans isomerization at V241–P242 accompanies the transition of loop A of the open state to the elongated, kinked helix α5 observed in the closed conformation (Figure 4C, D), which concomitantly pushes strand β11 upward (Figure 4E). This allows residues S231 and Q234 of the extended helix α5 to establish favorable interactions 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 closes further by movement of strand β11 from PqsC, which slides over the acyl-binding pocket like a zipper. As a result of this displacement, F227 (PqsC) then shields 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 (Figure 6A, B). As a consequence, the bound substrate is locked in the closed conformation to undergo the transacylation reaction with C129. Interestingly, the finding that PqsBC is also loaded with long-chain dodecanoyl or tetradecanoyl units (Figure 2A) that are expected not to fit into the acyl-binding cavity of PqsC seem to indicate that full closing is not required for this reaction. However, competition experiments (Figure S1) and enzymatic reactions including a 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 is likely initiated by dissociation of CoA, which does not require large structural perturbations, as 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, whereas the enzyme remains in the closed state. The AQ/AQNO product forms by decarboxylative condensation followed by intramolecular cyclization of the condensation intermediate. Although it is, at present, unclear if this final cyclization proceeds within the active site or 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 are interrupted, which allows reopening and dissociation of the product.

A similar model was 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, owing to the potential importance of its cis/trans isomerization, details of the hypothetical model purported here might 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 might therefore be encoded in other residues, and Sachdeva et al. previously suggested that conserved glycine residues that flank the flap region in other FabH enzymes but not in PqsBC (Figure S6) might 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 investigations will be necessary to evaluate its potential role in the catalytic mechanism of PqsBC.

Conclusions

As a result of its importance in controlling virulence and because of its unique occurrence in P. aeruginosa, the pqs system is currently under evaluation as a drug target in novel antivirulence strategies.[68–70] The finding that PqsBC adopts distinct conformations that were resolved here to high resolution and could be addressed selectively could offer new opportunities for these drug-discovery programs.
Experimental Section

Detailed experimental procedures can be found in the Supporting Information.

Chemicals and bacterial strains: UCBPP-PA14 was used as the wild-type strain for all experiments. The nonpolar mutant strains ED117 (pqsB::TnphoA) and ED218 (pqsC::Kan) were kindly provided by Eric Déziel and co-workers.[24] 2-ABA was kindly provided by Steffen L. Drees and Susanne Fetzner.[25] All acyl-CoAs were purchased from Larodan (Solna, Sweden) with the exception of acetyl-CoA, which was acquired from Sigma–Aldrich. DTNB was obtained from Thermo Fisher Scientific, and CoA was purchased from Appli-Chem.

Metabolomic analysis of P. aeruginosa: AO/AQNO analysis in P. aeruginosa was performed as described elsewhere.[19] 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 by using a 150 mm Kinetex C18 reversed-phase column with 1.7 μm 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 μM) and acyl-CoAs (50 μM) in Tris-HCl (50 mM, pH 7.6) at 30 °C. After incubation for 30 min, the samples (20 μL) were directly submitted for intact protein analyses. All ESI-MS measurements were performed with a Dionex Ultimate 3000 RSLC system by using a ProSwift RP-4H (monolithic PS-DVB), 250 × 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 (PA0097) and pqsC (PA0098) genes from P. aeruginosa PA01 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$-6-tagged T7 lysozyme removable by human rhinovirus 3C protease. The active-site cysteine C129 of PqsC was mutated to alanine (PqsC6129A) or serine (PqsC6129S) in pET19mod-pqsC and p10$-pqsC by PCR-based mutagenesis (Table S4).

Expression and purification of (His6)pqsBC, (His6)pqsBC6129A, and PqsBC6129S: Recombinant proteins were produced in E. coli BL21(DE3)pLyS5 (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, 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 λ = 412 nm.[72,73] The acyl-CoA or DTNB stock solution was freshly prepared in Milli-Q water or DMSO, respectively. The concentration of the acyl-CoA stock was measured at λ = 259 nm.[84] and the concentration of free CoA already present in the acyl-CoA stock was calculated by adding DTNB and comparing to a standard curve.

For the enzyme activity assay, PqsBC (1 μM) was mixed with acyl-CoA (500 μM) 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 by using the sitting drop vapor diffusion method. After optimization by random and grid screening, crystals were harvested in cryoprotectant (Table S1, Figure S2) and diffraction data were collected on beamlines X06DA/X10SA at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) and PETRA III beamline P11 at DESY (Hamburg, Germany). Diffraction data were indexed and integrated with XDS[74] or XDSAPP[75] and were scaled with Aimless.[76] Data collection and refinement statistics are listed in Table S2. Atomic coordinates and structure factor amplitudes were deposited in the Protein Data Bank (www.rcsb.org)[72] with accession codes 6ESZ, 6ET0, 6ET1, 6ET2, and 6ET3.

Structural representation were rendered with PyMOL,[85] secondary structure elements were assigned with DSSP,[86] and protein cavities were calculated with KVFinder.[87]

Modeling of PqsBC6129A–acyl-CoA complexes: PqsBC6129A–acyl-CoA complexes were modeled with the structure of His6-pqsBC6129A determined in this study (chains I/J of crystal form 3, PDB ID: 6ET2) and the X-ray coordinates of mFabH in complex with dodecanoyl-CoA (PDB ID: 1U65[88]) by using Molecular Operating Environment (MOE, Chemical Computing Group).[87]

Acknowledgements

We thank the Swiss Light Source (SLS, Paul Scherrer Institute, Villigen, Switzerland) and PETRA III (DESY, Hamburg, Germany) for beamline access and the beamline staff for technical assistance. We are grateful to the X-ray community at the Helmholtz Centre for Infection Research (H2, Braunschweig, Germany) for their help with data collection. We also thank Steffen L. Drees and Susanne Fetzner (University of Münster, Germany) for providing 2-ABA for PqsBC activity experiments and Eric Déziel for the kind provision of the Pseudomonas aeruginosa mutant strains used in this study. F.W. and T.D. are supported by the HZI Graduate School for Infection Research. T.D. acknowledges financial and nonmaterial support from the Studienstiftung des deutschen Volkes.

Conflict of Interest

The authors declare no conflict of interest.
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Manuscript received: March 19, 2018
Accepted manuscript online: May 3, 2018
Version of record online: June 22, 2018