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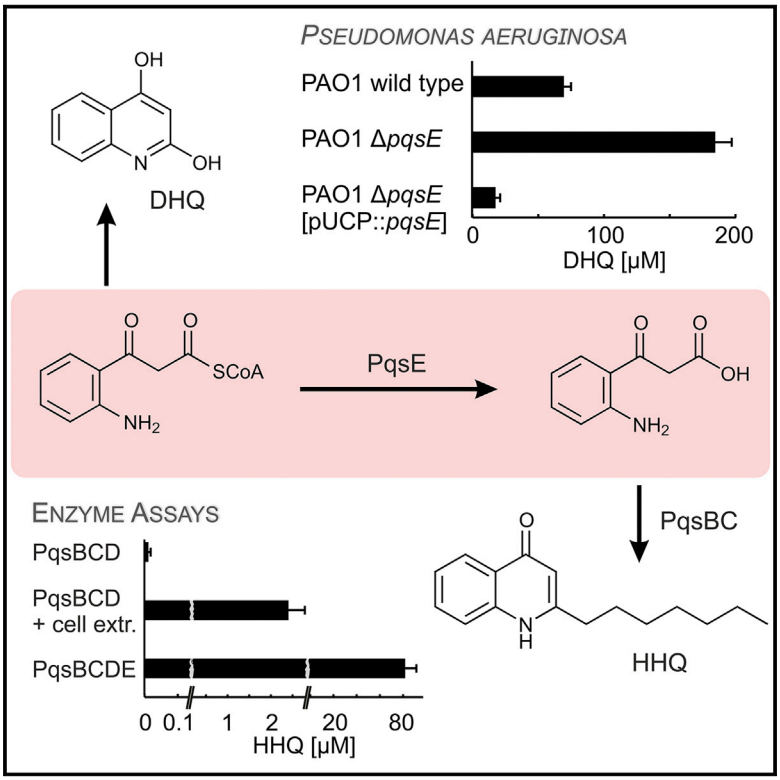
PqsE of *Pseudomonas aeruginosa* Acts as Pathway-Specific Thioesterase in the Biosynthesis of Alkylquinolone Signaling Molecules

Graphical Abstract



Highlights

- The biosynthesis of 2-alkyl-4(1*H*)-quinolones (AQs) was reconstituted in vitro
- Contrary to the current notion, PqsE contributes to AQ synthesis besides PqsABCD
- PqsE hydrolyzes the biosynthetic intermediate 2-aminobenzoylacetyl-coenzyme A
- PqsE balances the levels of metabolites deriving from the AQ biosynthetic pathway

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In Brief

Drees and Fetzner demonstrate that PqsE of *Pseudomonas aeruginosa* acts as thioesterase in alkylquinolone biosynthesis. By hydrolyzing the intermediate 2-aminobenzoylacetyl-CoA, which tends to decompose to 2,4-dihydroxyquinoline, PqsE balances the levels of quorum-sensing signal molecules and secondary metabolites deriving from this pathway.

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Chemistry & Biology
Article

PqsE of *Pseudomonas aeruginosa* Acts as Pathway-Specific Thioesterase in the Biosynthesis of Alkylquinolone Signaling Molecules

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SUMMARY

Pseudomonas aeruginosa **uses the alkylquinolones PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone) and HHQ (2-heptyl-4(1*H*)-quinolone) as quorum-sensing signal molecules, controlling the expression of many virulence genes as a function of cell population density. The biosynthesis of HHQ is generally accepted to require the *pqsABCD* gene products. We now reconstitute the biosynthetic pathway in vitro, and demonstrate that in addition to PqsABCD, PqsE has a role in HHQ synthesis. PqsE acts as thioesterase, hydrolyzing the biosynthetic intermediate 2-aminobenzoylacetyl-coenzyme A to form 2-aminobenzoylacetate, the precursor of HHQ and 2-aminoacetophenone. The role of PqsE can be taken over to some extent by the broad-specificity thioesterase TesB, explaining why the *pqsE* deletion mutant of *P. aeruginosa* still synthesizes HHQ. Interestingly, the *pqsE* mutant produces increased levels of 2,4-dihydroxyquinoline, resulting from intramolecular cyclization of 2-aminobenzoylacetyl-coenzyme A. Overall, our data suggest that PqsE promotes the efficiency of alkylquinolone signal molecule biosynthesis in *P. aeruginosa* and balances the levels of secondary metabolites deriving from the alkylquinolone biosynthetic pathway.**

INTRODUCTION

The γ-proteobacterium *Pseudomonas aeruginosa* is a ubiquitous opportunistic pathogen frequently associated with nosocomial infections, especially among immunocompromised individuals. In patients with cystic fibrosis, it is the leading cause of morbidity and mortality (Emerson et al., 2002). Once established in the host, it produces a large array of virulence factors and forms biofilms that are difficult to combat (Gellatly and Hancock, 2013; Taylor et al., 2014). Synthesis of numerous virulence factors is controlled by a process called quorum sensing (QS), whereby the bacteria communicate via diffusible chemical signal molecules to coordinate their behavior within the population. The sophisticated QS network of *P. aeruginosa* involves the two *N*-acylhomoserine lactone-based Las and Rhl systems, and the Pqs system that is based on specific 2-*n*-alkyl-4(1*H*)-quinolones (AQs). 2-Heptyl-3-hydroxy-4(1*H*)-quinolone, termed the *Pseudomonas* quinolone signal (PQS), is the major AQ signal, but its biosynthetic precursor 2-heptyl-4(1*H*)-quinolone (HHQ) also acts as signal molecule (Déziel et al., 2004; Pesci et al., 1999; Xiao et al., 2006). AQ signaling is involved in the control of virulence factor production and influences biofilm maturation (reviewed in Heeb et al., 2011; Huse and Whiteley, 2011; Nadal Jimenez et al., 2012).

The AQ signaling molecules belong to a family of more than 50 compounds that share the 4-hydroxy-2-alkylquinoline structure (Lépine et al., 2004). Biosynthesis of AQs is generally accepted to require the anthranilate-coenzyme A (CoA) ligase PqsA (Coleman et al., 2008), the condensing enzyme PqsD, which has been proposed to form 2-aminobenzoylacetyl-CoA from anthraniloyl-CoA and malonyl-CoA (Zhang et al., 2008), and the PqsBC protein involved in coupling of 2-aminobenzoylacetate (2-ABA) to an octanoyl moiety to produce HHQ (Dulcey et al., 2013) (Figure 1). Two other bioactive secondary metabolites, 2-aminoacetophenone (2-AA) and 2,4-dihydroxyquinoline (DHQ), also derive from the AQ biosynthetic pathway. Formation of DHQ was shown to require PqsA and PqsD (Zhang et al., 2008), and 2-AA is thought to result from decarboxylation of 2-ABA (Dulcey et al., 2013) (Figure 1). While DHQ does not act as a signal in the AQ-based QS system and apparently has no antimicrobial effects (Lépine et al., 2007), it was observed to inhibit the viability of mouse lung epithelial cells, suggesting that it contributes to the pathogenicity of *P. aeruginosa* infection (Zhang et al., 2008). 2-AA promotes chronic infection phenotypes of *P. aeruginosa* by silencing acute virulence functions (Kesarwani et al., 2011) and by mediating persister cell accumulation (Que et al., 2013). Moreover, it modulates the innate immune response of mammalian hosts (Bandyopadhaya et al., 2012) and mediates host metabolic dysregulation that results in mitochondrial dysfunction (Tzika et al., 2013).

HHQ biosynthesis in a heterologous background requires expression of the *pqsABCD* genes (Niewerth et al., 2011; Xiao et al., 2006). However, in *P. aeruginosa* these genes form an operon together with *pqsE*. Mutants of *P. aeruginosa* deficient in *pqsE* produce wild-type levels of PQS and HHQ (Déziel et al., 2004; Gallagher et al., 2002), an observation that supported the notion that PqsE is not involved in the AQ biosynthetic pathway. PqsE was termed the "PQS response protein" because its disruption negatively affected the production of some PQS-mediated exoproducts such as pyocyanin and rhamnolipid (Déziel et al., 2005; Diggle et al., 2003; Farrow et al., 2008;

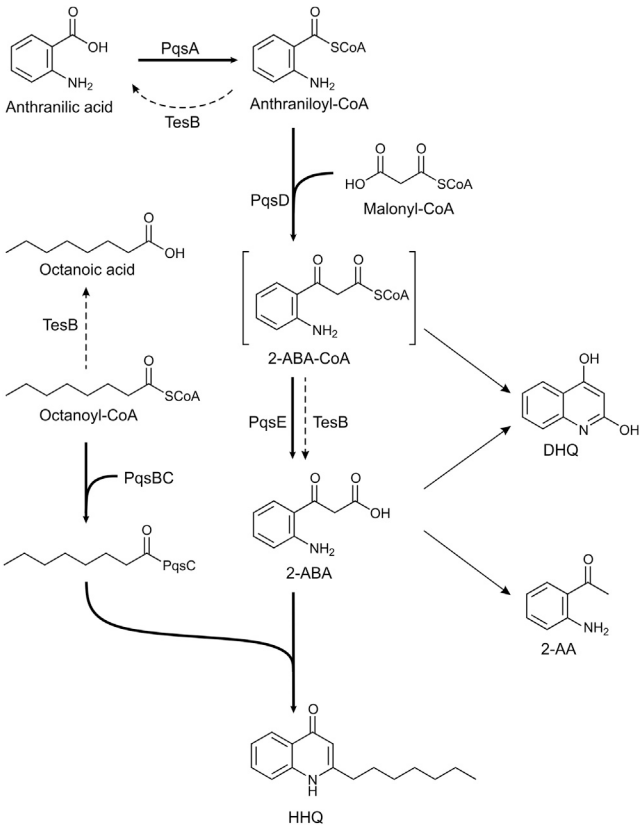


Figure 1. Proposed Pathway for the Biosynthesis of 2-Heptyl-4(1*H*)-Quinolone and Related Secondary Metabolites by *P. aeruginosa*

Biosynthesis of 2-heptyl-4(1*H*)-quinolone (HHQ) requires the PqsABCD proteins (Coleman et al., 2008; Dulcey et al., 2013; Zhang et al., 2008) and PqsE (this study). The function of PqsE can be taken over to some extent by TesB (this study) and possibly other yet unidentified thioesterases. Bold arrows indicate enzymatic reactions leading to HHQ formation. Side reactions of TesB affecting intermediates of HHQ biosynthesis, as determined in the present study, are symbolized by dashed arrows. Thin arrows indicate reactions that are thought to occur spontaneously. 2-ABA-CoA, 2-aminobenzoylacetyl-CoA; 2-ABA, 2-aminobenzoylacetate; DHQ, 2,4-dihydroxyquinoline; 2-AA, 2-aminoacetophenone.

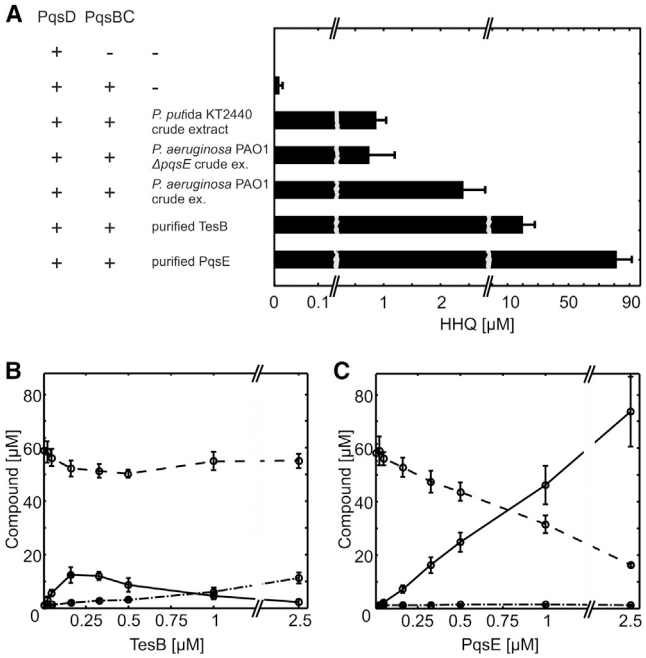


Figure 2. In Vitro Synthesis of HHQ

(A) Anthraniloyl-CoA, malonyl-CoA, and octanoyl-CoA (100 μM each) were added to PqsD (0.5 μM) and PqsBC (0.5 μM) and either 5 mg ml^{-1} of crude extract protein of *Pseudomonas putida* KT2440 or *Pseudomonas aeruginosa* PAO1 (wild-type or *pqsE* mutant), or purified thioesterase II (TesB) or purified PqsE, as indicated. Bars denote the yield of HHQ upon completion of the reaction. Error bars correspond to SE (n = 3, from independent experiments). (B and C) Reaction products as a function of TesB (B) or PqsE (C) concentrations (assay as described in A). HHQ (black continuous lines), DHQ (dashed lines), and anthranilate (dash-dotted lines) were determined with HPLC. Error bars denote SEM (n = 3). For SDS-PAGE of all purified proteins, see Figure S1.

Gallagher et al., 2002). Some (but not all) actions of PqsE are linked to the Rhl QS system, and PqsE modulates the expression of large arrays of target genes (Farrow et al., 2008; Hazan et al., 2010; Rampioni et al., 2010). However, the PqsE protein is not a transcriptional regulator, and its mode of action remains enigmatic. Its crystal structure, solved by Yu et al. (2009), revealed a metallo-β-lactamase fold with a binuclear metal center. These authors also showed that PqsE has hydrolase activity toward the artificial thioester substrate *S*-(4-nitrobenzoyl)mercaptoethane, and weak phosphodiesterase activity, although the natural substrate(s) of PqsE could not be identified.

In this study we demonstrate that PqsE acts as thioesterase of the AQ biosynthetic pathway (Figure 1). Its function can to some extent be exerted by TesB and, possibly, other yet unidentified broad-specificity thioesterases, explaining why the *P. aeruginosa* Δ*pqsE* mutant still produces HHQ and PQS. PqsE guides the biosynthetic intermediate 2-ABA-CoA toward synthesis of the HHQ QS signal molecule and thus suppresses DHQ formation. By supporting 2-ABA formation, PqsE also influences the production of 2-AA. We propose that PqsE balances the levels of the secondary metabolites that derive from the AQ biosynthetic pathway of *P. aeruginosa*.

RESULTS

PqsD Does Not Provide the Substrate for PqsBC

Genetic knockouts and complementation assays in *P. aeruginosa* and heterologous expression experiments in *Escherichia coli* and in *Pseudomonas putida* showed that the genes *pqsABCD* are sufficient to support biosynthesis of HHQ in vivo (Déziel et al., 2004; Dulcey et al., 2013; Gallagher et al., 2002; Xiao et al., 2006). To fully establish the biosynthetic pathway by reconstituting it in vitro, we tested whether enzyme assays with native PqsBC and PqsD yield HHQ from the substrates anthraniloyl-CoA, malonyl-CoA, and octanoyl-CoA (see Figure 1). However, despite varying the reaction parameters, only trace amounts of HHQ were formed (Figure 2A), while most of the anthraniloyl-CoA and malonyl-CoA were converted to DHQ. Moreover, neither 2-ABA nor 2-AA was found under any conditions tested with either PqsD or PqsBC, or both (high-performance liquid chromatography [HPLC]/electrospray ionization mass spectrometry [ESI-MS] analyses, data not shown). These

findings suggest that an additional factor is required for HHQ synthesis, which most likely is involved in providing the intermediate 2-ABA to PqsBC.

A Thioesterase Complements PqsBCD for In Vitro HHQ Synthesis

To test whether an as yet unknown protein takes part in AQ biosynthesis, which would explain why in the in vivo situation *pqsABCD* is sufficient for HHQ formation, we supplemented the in vitro assays with cell extracts. Addition of substantial amounts of the soluble protein fraction of *P. putida* KT2440 to the PqsBCD enzyme assay indeed led to elevated levels of HHQ (Figure 2A). The yields of HHQ were proportional to the amount of protein added, with a maximum yield of 0.86 ± 0.14 μM of HHQ from 100 μM educts (Figure 2A). Since *P. putida* KT2440 does not have the AQ biosynthetic pathway, we hypothesized a widely conserved enzyme to be capable of supporting HHQ synthesis. If, as proposed by Zhang et al. (2008), 2-ABA-CoA is the direct product of the PqsD reaction (Figure 1), a thioesterase is a likely candidate for such an enzyme. 2-ABA-CoA, due to intramolecular attack of the nucleophilic 2-amino function at the thioester carbonyl, is very susceptible to intramolecular cyclization to form DHQ (Figure 1) (Dulcey et al., 2013; Yang and Drueckhammer, 2001; Zhang et al., 2008). Therefore, rapid enzyme-catalyzed hydrolysis of 2-ABA-CoA to 2-ABA, which, as tested with the chemically synthesized compound, is quite stable in slightly alkaline aqueous solution, would not only provide the substrate for PqsBC, but also reduce formation of the DHQ side product. To test the hypothesis that a ''housekeeping'' thioesterase conserved in both *P. putida* and *P. aeruginosa* is the unknown factor that in the assays with cell extracts supported HHQ synthesis, we chose to purify thioesterase II (TesB) of *P. aeruginosa* (Figure S1). The homologous TesB of *E. coli* has been demonstrated to be active toward fatty acid CoA thioesters with various chain lengths, but also toward other substrates such as hydroxyacyl-CoAs (Liu et al., 2007; Naggert et al., 1991; Spencer et al., 1978). Addition of purified TesB to the in vitro assay enhanced HHQ synthesis to a maximum yield of about 16% (Figure 2A). However, as shown in Figure 2B, excess amounts of TesB in the assays led to declining HHQ levels. This can mainly be attributed to hydrolysis of octanoyl-CoA, as suggested by the kinetic parameters of TesB for this substrate (K_M = 56.5 ± 4.1 μM, k_{cat} = 9.7 ± 0.1 s^{-1}). The observed release of some anthranilic acid in the combined PqsBCD-TesB assay (Figure 2B) also indicates minor hydrolysis of the anthraniloyl-CoA by TesB. However, the steady-state kinetic parameters of TesB for this substrate (K_M = 11.5 ± 1.9 μM, k_{cat} = 0.086 ± 1 × 10^{-4} s^{-1}) indicate that the reaction is very slow. The observation that TesB supports HHQ synthesis may explain why the *pqsABCD* genes suffice for AQ production in the *P. aeruginosa* Δ*pqsE* mutant or in heterologous hosts. It should be noted that the genome of *P. aeruginosa* contains numerous predicted thioesterases, some of which have been biochemically characterized. The PA5202 protein, for example, is highly active toward glutaryl-CoA, but also hydrolyzes a range of short- and long-chain fatty acid CoA thioesters. The PA2801 thioesterase was observed to exhibit a high level of substrate promiscuity (Gonzalez et al., 2012). It is therefore conceivable that other broad-specificity thioesterases besides TesB can also contribute to HHQ biosynthesis in *P. aeruginosa* to some extent. However, it seems very unlikely that a thioesterase that also attacks building blocks of AQ biosynthesis, as summarized in Figure 1 for TesB, is the dedicated biosynthetic enzyme of the pathway.

PqsE Acts as a Pathway-Specific Thioesterase

Interestingly, addition of crude extracts of *P. aeruginosa* PAO1 or *P. aeruginosa* PAO1 Δ*pqsE* to the in vitro assay resulted in clear differences in HHQ yields, with extracts of the wild-type strain performing significantly better (Figure 2A). This observation led us to the assumption that PqsE plays a role in AQ biosynthesis. PqsE was thus likewise heterologously expressed, purified (Figure S1), and tested for its effect on in vitro HHQ synthesis. The yields of HHQ in assays containing PqsD, PqsBC and PqsE were proportional to the amount of PqsE added (Figure 2C), in contrast to the assays with TesB (Figure 2B). Moreover, the concentration of DHQ formed in the assays decreased as a function of increasing PqsE concentrations (Figure 2C). Remarkably, a slight excess of PqsE over PqsBC and PqsD led to nearly stoichiometric conversion of the substrates to HHQ (Figures 2A and 2C). Given that the intermediate 2-ABA-CoA quickly decomposes to DHQ, it appears that in the case of near-complete conversion of substrates to HHQ, the PqsD reaction has to be the rate-limiting catalytic step of the overall reaction. The specific activities of PqsD (Zhang et al., 2008) and PqsBC (this study) are 0.22 and 0.32 μmol min^{-1} mg^{-1}, respectively; thus, the estimated specific activity of PqsE has to be in the same range. PqsE shows no activity toward anthraniloyl-CoA and octanoyl-CoA (Yu et al., 2009; this study). These data strongly indicate that PqsE is responsible for promoting the efficiency of HHQ biosynthesis versus DHQ formation.

The PqsD Reaction Produces a Short-Lived CoA Thioester

From the proposed biosynthetic pathway and our in vitro data, 2-ABA-CoA appears as the plausible substrate of PqsE. However, all attempts to identify this hypothetical direct product of the PqsD reaction, using mass spectrometry, failed. To find out whether the instability of the PqsD reaction product precludes its detection, as discussed by Zhang et al. (2008), its half-life was estimated using an indirect approach. To this end, anthraniloyl-CoA and malonyl-CoA were rapidly converted to product by a large excess of PqsD, then incubated for defined time periods before octanoyl-CoA, PqsE, and PqsBC were added in excess. The HHQ yield, which decreased as a function of the incubation time, served as readout for the decay of the PqsD product. The data (Figure S2) indicated an estimated half-life of the compound of 40 ± 5 s. Since, because of the experimental setup, the real value could be even lower, we refrained from further efforts to directly detect the PqsD product. Instead, benzoyl-CoA was used as an analog of anthraniloyl-CoA in the PqsD reaction. The anticipated product benzoylacetyl-CoA lacks the nucleophilic amino function that renders 2-ABA-CoA susceptible to spontaneous cyclization to form DHQ, and thus should be more stable. Despite very low reactivity of PqsD with benzoyl-CoA, a CoA-thioester product with the mass of benzoylacetyl-CoA was identified (Figure 3A). Addition of PqsE (Figure 3B) or TesB (data not shown) to the assay led to the release of a

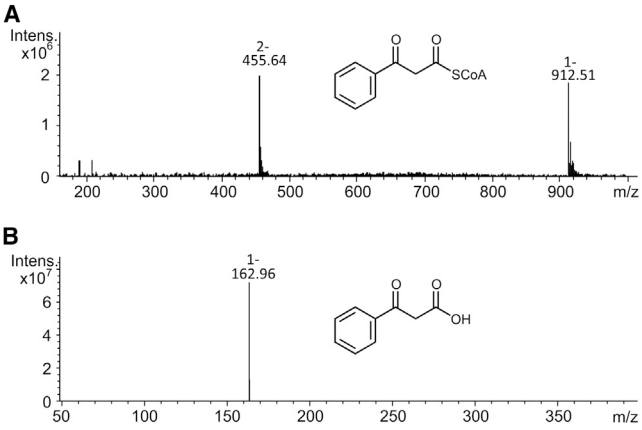


Figure 3. ESI-MS Analysis of Products from PqsD- and PqsE-Catalyzed Reactions

(A) PqsD reaction with benzoyl-CoA (as anthraniloyl-CoA analog) and malonyl-CoA. The observed masses of $[M\text{-}H]^-$ = 912.51 Da and $[M\text{-}2H]^{2-}$ = 455.64 Da correspond to benzoylacetyl-CoA with calculated monoisotopic mass of 913.15 Da.

(B) Addition of PqsE leads to formation of benzoylacetate with a mass of $[M\text{-}H]^-$ = 162.96 Da (calculated monoisotopic mass 164.05 Da), verified by fragmentation releasing a 77 Da phenyl fragment and by ESI-MS analysis of chemically synthesized benzoylacetate (not shown). See also Figure S2.

compound with the mass of benzoylacetate. These results support the hypothesis that 2-ABA-CoA is the direct product of the PqsD reaction and the substrate of PqsE.

PqsE and TesB Catalyze the Release of 2-ABA

While the combination of PqsD, PqsE, and PqsBC supported in vitro HHQ synthesis, it remained to be determined which products result from combining the PqsD and PqsE reactions. From the proposed pathway (Figure 1), one should expect formation of the on-pathway intermediate 2-ABA in addition to the side products 2-AA and/or DHQ. Given the lability of 2-ABA at acidic to neutral pH (Dulcey et al., 2013), we utilized thin-layer chromatography (TLC) with an alkaline mobile phase to separate and quantify the reaction products.

Increasing the concentration of PqsE in the combined assay with PqsD not only resulted in increased yields of 2-ABA and reduced formation of DHQ, but also led to increasing release of 2-AA (Figure 4). In principle, this is also valid for the combined assay with PqsD and TesB (Figure 4B, dashed lines). Consistent with the previous findings (Figure 2), release of anthranilate was additionally observed in the assays with TesB (Figure 4B). Although less efficient than PqsE, TesB performed considerably well in this assay (31 μM 2-ABA from 100 μM substrates) compared with the combined PqsD-TesB-PqsBC assays shown in Figure 2 (16 μM HHQ formation from 100 μM substrates). This suggests that the rapid hydrolysis of octanoyl-CoA rather than a low efficiency of 2-ABA-CoA cleavage by TesB limited the yield of HHQ in the overall reaction.

When PqsE was combined in a 2- to 5-fold molar excess with PqsD, a maximum 2-ABA concentration of 60 μM was observed from conversion of 100 μM substrates, with 17–23 μM 2-AA and ~7 μM DHQ released as by-products (Figure 4). Most of the 2-AA likely can be attributed to decomposition of 2-ABA from organic solvent exposure during extraction, because samples of chemically synthesized and TLC-purified 2-ABA subjected to the same extraction protocol as the enzyme reactions likewise contained 2-AA. However, 2-ABA is stable for several hours to days when handled in aqueous, alkaline solution, as indicated by UV-spectroscopic data (not shown). We therefore assume that DHQ, the only product observed from the PqsD-catalyzed condensation of anthraniloyl-CoA and malonyl-CoA, for the most part derives from the unstable 2-ABA-CoA thioester intermediate, while 2-AA originates from decarboxylative decomposition of 2-ABA as already suggested by Dulcey et al. (2013). From the observation that the ratio of PqsD and PqsE in the in vitro assays significantly affected the proportions of the three products formed, we may hypothesize that in *P. aeruginosa*, the expression level of PqsE balances DHQ and 2-ABA formation. This would not only determine the ratio of DHQ and HHQ produced by the organism, but also affect the levels of 2-AA released.

The *P. aeruginosa* Δ*pqsE* Mutant Produces Increased Levels of DHQ

Previous publications reported that the *P. aeruginosa* Δ*pqsE* mutant produces wild-type levels of AQs (Déziel et al., 2004; Gallagher et al., 2002). However, the involvement of PqsE in AQ biosynthesis as established in our in vitro experiments raises the question of whether *P. aeruginosa* Δ*pqsE* under conditions of AQ synthesis accumulates unphysiologically high levels of 2-ABA-CoA, which (besides being converted by another thioesterase such as TesB) would decompose to DHQ. While the HHQ, PQS, and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) concentrations were virtually identical in cultures of *P. aeruginosa* PAO1 wild-type and Δ*pqsE* mutant, the DHQ levels were significantly higher in the *P. aeruginosa* Δ*pqsE* cultures (Figure 5). When complemented with the plasmid-borne *pqsE* gene, the Δ*pqsE* strain showed decreased DHQ and AQ levels (Figure 5; Table S1). However, pyocyanin was overproduced by *P. aeruginosa* PAO1 Δ*pqsE* [pUCP::*pqsE*], which is consistent with the phenotype of *pqsE* overexpression in *P. aeruginosa* described previously (Farrow et al., 2008; Rampioni et al., 2010). PqsE has an (indirect) repressive effect on *pqsA* transcription (Rampioni et al., 2010); therefore, its overproduction leads to a lower expression of *pqs* biosynthesis genes and, thus, a decrease in the total levels of HHQ and DHQ. Nevertheless, the ratio of DHQ to AQs in cultures of *P. aeruginosa* PAO1 Δ*pqsE* [pUCP::*pqsE*] corresponded to that of the wild-type strain (Figure 5; Table S1). Media composition (LB, M9, MMII [Carl et al., 2004; Sambrook et al., 1989]) and supplementation of mineral salts media (lowered/increased trace elements; with and without anthranilate and/or octanoate as precursors), despite affecting total levels of DHQ and AQs, had no influence on AQ to DHQ ratios (not shown).

Specificity of PqsE

As a thioesterase active in a pathway that starts from CoA-activated building blocks, PqsE should be highly specific for its physiological substrate. Indeed, activity of PqsE toward anthraniloyl-CoA (or benzoyl-CoA), malonyl-CoA and octanoyl-CoA was not observed (Yu et al., 2009; this study). However, PqsE can hydrolyze *S*-ethyl-acetothioacetate and acetoacetyl-CoA (Table 1), which might suggest that PqsE requires a -C_2H_4- (as

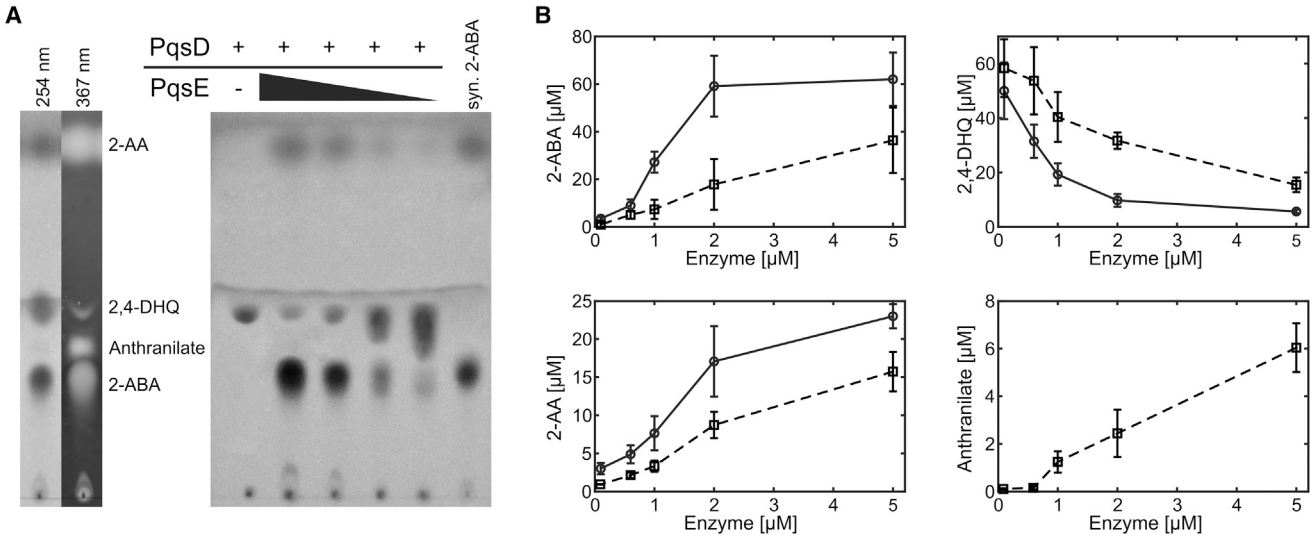


Figure 4. In Vitro Formation of 2-ABA and Side Products Formed in the PqsD-PqsE and PqsD-TesB Reactions as a Function of Thioesterase Enzyme Concentration

1 μM of PqsD and either PqsE or TesB were incubated with 100 μM substrates anthraniloyl-CoA and malonyl-CoA for 10 min. Organic molecules were prepared by solid-phase extraction (see Supplemental Experimental Procedures).

(A) Exemplary TLC plate images of 367 nm fluorescence and 254 nm fluorescence quenching (left), and a PqsE concentration series as used for densitometry analysis (right). syn., synthetic.

(B) Densitometric data on reaction products (continuous lines: PqsE; dashed lines: TesB reactions). Calibration was performed against standards of the respective commercially available compounds and chemically synthesized 2-ABA (authenticity of both was verified by mass spectrometry, data not shown). Error bars indicate SEM (n = 3) with plate-wise intensity normalization.

in CoA) or -C_2H_5 moiety adjacent to the sulfur and a β-keto-thioester group for substrate turnover. We also observed that PqsE has weak phosphorothiolate hydrolase activity toward cysteamine-*S*-phosphate (Table 1). While this compound is not a metabolite in bacteria, and phosphorothiolates are considered unphysiological, they occur as phosphocysteine in posttranslational protein modification (Deng et al., 2013; Sun et al., 2012).

DISCUSSION

The data presented in this study describe the first physiological function that can be assigned to PqsE on a molecular level. It acts as a thioesterase in the AQ biosynthetic pathway, hydrolyzing the intermediate 2-ABA-CoA to 2-ABA, which is the substrate of the PqsBC-catalyzed condensation with octanoyl-CoA to form HHQ. 2-ABA also is a precursor of 2-AA. PqsE thus counterbalances the intramolecular cyclization of the highly unstable 2-ABA-CoA to DHQ, a product not accessible to further conversion to AQ-type metabolites.

In view of the biochemistry of HHQ synthesis, a remarkable aspect is the apparent substitutability of PqsE in vivo. The fact that *pqsE*-deficient mutants of *P. aeruginosa* do not feature significantly altered levels of AQs may raise doubts about its actual relevance for the biosynthetic pathway. However, we believe that the in vivo compensation of *pqsE* is not as efficient as it seems, because PqsE, via an unknown mechanism, exerts a repressing effect on *pqsA* transcription (Rampioni et al., 2010). The resulting higher levels of PqsABCD proteins in the Δ*pqsE* mutant, due to the activity of PqsA and PqsD, should lead to overproduction of 2-ABA-CoA. Its hydrolysis by an off-pathway thioesterase (such as TesB) could still provide enough 2-ABA for PqsBC to produce approximately wild-type levels of AQs. Our observation of increased DHQ levels in the Δ*pqsE* mutant is

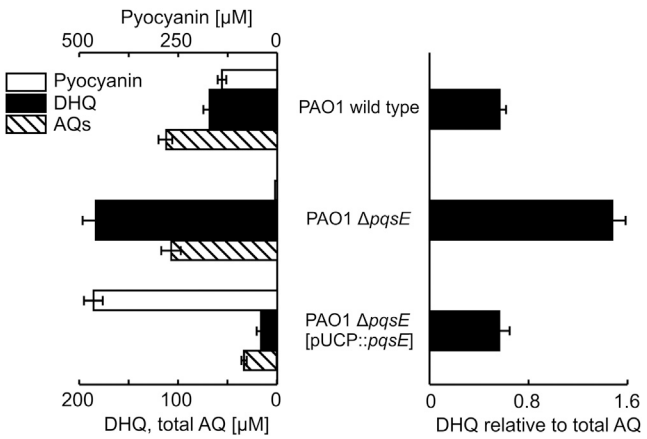
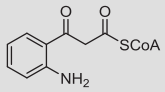
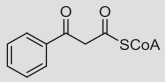
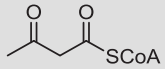
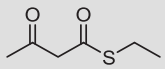
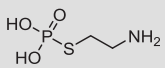


Figure 5. Increased Production of DHQ by the *P. aeruginosa* Δ*pqsE* Mutant Compared with the PAO1 Wild-Type Strain and the *pqsE*-Complemented Δ*pqsE* Mutant

Absolute levels of pyocyanin, total AQ, and DHQ, determined in cultures grown in mineral salt medium for 24 hr, are displayed on the left. AQ contents were determined from total culture extracts; pyocyanin and DHQ were quantified from acidified supernatants. On the right, DHQ levels are normalized to the total AQ content of the respective culture. Note that the DHQ observed likely is a sum of DHQ formed by *P. aeruginosa* and some DHQ resulting from intramolecular cyclization of extracellular 2-ABA. Error bars reflect SEM from at least three independent experiments. See also Table S1.

Table 1. Substrates Converted by PqsE as Identified in this Study

Compound	Turnover/Kinetic Parameters	Analysis Method
 2-Aminobenzoylacetyl-CoA (2-ABA-CoA)	turnover	TLC, LC/MS (indirectly)
 Benzoylacetyl-CoA (BA-CoA)	turnover	LC/MS
 Acetoacetyl-CoA	k_{cat} = 0.36 ± 0.08 s^{-1}, K_M = 2.5 ± 0.2 mM	spectrophotometric, thiol quantification using Ellman's reagent
 *S*-Ethyl-acetothioacetate	k_{cat} = 0.85 ± 0.12 s^{-1}, K_M = 4.4 ± 0.6 mM	spectrophotometric, thiol quantification with Ellman's reagent
 Cysteamine-*S*-phosphate	k_{cat} = 0.022 ± 0.003 s^{-1}, K_M = 0.8 ± 0.1 mM	spectrophotometric, thiol quantification with Ellman's reagent, phosphate assay according to Serrano (1978)

Data are presented as mean ± SEM (n ≥ 3).

consistent with the assumption of 2-ABA-CoA overproduction. The available transcriptomic data (Bredenbruch et al., 2006; Hazan et al., 2010; Rampioni et al., 2010) do not suggest coregulation of another thioesterase candidate gene together with *pqsABCDE* or in response to *pqsE* knockout, which argues against the presence of another pathway-specific CoA thioesterase.

The genetic organization of *pqsE*, located in the *pqsABCDE* operon together with the other AQ biosynthetic genes, also supports the notion that participation in this pathway is a primary role of PqsE. Interestingly, a recent study reported that under nutrient-limiting conditions, an alternative transcript containing *pqsDE* and the downstream *phnAB* genes is produced (Knoten et al., 2014). Upregulation of *phnAB* increases the production of anthranilate, which is required for tryptophan synthesis but also may be branched into AQ biosynthesis, provided that PqsA is not completely shut down by the indirect repressing effect of PqsE on *pqsA* transcription. In this scenario, the increased PqsDE levels will prevent accumulation of the labile 2-ABA-CoA intermediate and, thus, formation of DHQ, but will lead to accumulation of 2-ABA. In this context, it is noteworthy that 2-ABA, as observed for *pqsB*- or *pqsC*-deficient mutants of *P. aeruginosa*, can be excreted to the culture supernatant. When fed to *pqsA*- or *pqsD*-deficient mutants, it supports AQ synthesis (Dulcey et al., 2013; our unpublished data). Possibly, the extracellular 2-ABA serves as "community good" for the biosynthesis of HHQ, PQS, and other hydroxyalkylquinolines including the antibacterial 4-hydroxyalkylquinoline-*N*-oxides. On the other hand, because, depending on conditions such as solvent composition, temperature, and pH (Dulcey et al., 2013; Rosenfeld and Williams, 1991) it can decompose to either or both 2-AA and DHQ, 2-ABA may act as a kind of "environmental sensor," responding to the environment by formation of different bioactive compounds.

While the molecular basis of the regulatory effects of PqsE that are independent of the AQ pathway still remains enigmatic (Farrow et al., 2008; Hazan et al., 2010; Rampioni et al., 2010), our findings complete the understanding of AQ biosynthesis in *P. aeruginosa*, and provide a new perspective on PqsE as a key enzyme balancing the levels of AQ-type QS signal molecules and related secondary metabolites.

SIGNIFICANCE

Quorum sensing is a process in which bacteria communicate by diffusible molecules to coordinate group-related behaviors. The opportunistic pathogen *Pseudomonas aeruginosa* uses the alkylquinolones PQS (*Pseudomonas* quinolone signal) and its biosynthetic precursor HHQ as unique quorum sensing molecules. Alkylquinolone signaling is known to be involved in biofilm development and in the regulation of many virulence factors. Alkylquinolones are synthesized from CoA-activated anthranilic acid and fatty acid precursors. Notably the immunomodulatory signal 2-aminoacetophenone and the extracellular metabolite 2,4-dihydroxyquinoline, which are produced in significant amounts by *P. aeruginosa*, also derive from this biosynthetic pathway. So far, the genes *pqsABCD* have been associated with alkylquinolone biosynthesis. However, besides *pqsABCD* the *pqs* operon additionally comprises the *pqsE* gene, coding for a protein of unknown biochemical function, which has been described to act as a key regulator within the QS network

controlling *P. aeruginosa* virulence. By reconstituting HHQ synthesis in vitro, we demonstrate here that alkylquinolone synthesis requires PqsE besides PqsABCD. PqsE acts as a pathway-specific thioesterase, hydrolyzing the pathway intermediate 2-aminobenzoylacetyl-CoA. Thus, PqsE counterbalances the intramolecular cyclization of this highly unstable intermediate to the dead-end product 2,4-dihydroxyquinoline, and directs the intermediate toward HHQ and PQS biosynthesis. Because 2-aminobenzoylacetate also is the precursor of 2-aminoacetophenone, PqsE activity also affects the formation of this metabolite. Taken together, our findings not only complete our understanding of the steps of the alkylquinolone biosynthetic pathway, but also suggest that PqsE balances the levels of the signals and bioactive secondary metabolites of *P. aeruginosa* derived from this pathway. Our identification of a physiological substrate of PqsE may also inspire the design of specific inhibitors for this protein, which, due to its crucial role in *P. aeruginosa* pathogenicity, is an attractive drug target.

EXPERIMENTAL PROCEDURES

Full details are given in Supplemental Experimental Procedures.

Bacterial Strains and Growth Conditions

Heterologous overexpression of *pqs* genes was performed using *E. coli* Rosetta 2 transformed with the respective plasmids, grown in LB or TB medium. *P. putida* KT2440, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO1 *ΔpqsE* (Rampioni et al., 2010) were cultivated in LB medium or MMII mineral salt medium (Carl et al., 2004).

Protein Expression and Purification

PqsD, PqsBC, and TesB were overexpressed as His-tagged proteins from pET vectors (Merck); PqsE was expressed using the pUCP18 vector (Schweizer, 1991). Proteins were purified using Ni-NTA affinity chromatography (NiNTA Sepharose fast flow, GE Life Sciences) according to the manufacturer's instructions. Protein crude extracts were prepared by ultrasonic cell disruption, centrifugation, and buffer exchange using HiTrap desalt columns (GE Life Sciences).

Enzyme Assays

Combinations of either PqsD or both PqsD and PqsBC were incubated with TesB, PqsE, or soluble protein extracts from *P. putida* KT2440 or *P. aeruginosa* PAO1 (wild-type or *ΔpqsE*) in a buffer containing 40 mM MOPS (pH 8.2) and 150 mM NaCl. Substrates malonyl-CoA, octanoyl-CoA (Sigma-Aldrich), anthraniloyl-CoA, and benzoyl-CoA (from chemical synthesis) were added as required. Incubation was performed at 30°C and shaking at 800 rpm. PqsE activity assays with acetoacetyl-CoA and *S*-ethyl-acetothioacetate and cysteamine-*S*-phosphate (Sigma-Aldrich) were conducted under the same conditions. Reaction products were analyzed by TLC, HPLC, ESI-MS, HPLC-MS, or colorimetric quantification of thiols with Ellman's reagent (Eyer et al., 2003). For quantification of HHQ and DHQ, samples from the assay mixtures were acidified, centrifuged, and either extracted with ethyl acetate prior to HPLC analysis or analyzed directly.

Chemical Syntheses

2-ABA was synthesized from ethyl 2-nitrobenzoylacetate (Sigma-Aldrich) as reported previously (Dulcey et al., 2013). Benzoylacetate was hydrolyzed from 2-ethyl-benzoylacetate as described by Rosenfeld and Williams (1991). Benzoyl-CoA and anthraniloyl-CoA were synthesized from CoA and benzoic anhydride and isatoic anhydride, respectively, as described by Simon and Shemin (1953), and purified by preparative HPLC.

Quantification of DHQ, AQs, and Pyocyanin in Cultures

Contents of DHQ and AQs in cultures or culture supernatants of *P. aeruginosa* PAO1 or *P. aeruginosa* PAO1 Δ*pqsE* after 24 hr of growth were quantified by HPLC. Whole culture and culture supernatant samples were prepared by adding 50% methanol and either 100 mM acetic acid or 50 mM citric acid, heating to 50°C for 1 hr, and centrifugation and filtration (0.45 μm) prior to analysis. Pyocyanin was analyzed in culture supernatants by HPLC or with a photometric assay (Farrow et al., 2008).

Analytical Methods

TLC was performed on SIL-G 254 plates (Macherey-Nagel) with a mixture of methanol, ethyl acetate, heptane, and 1% ammonia solution (45:45:9:1) as mobile phase. HPLC was conducted using either Hitachi EZChrom Elite (diode array detector model 2450) or Dionex UltiMate 3000 UHPLC (for MS coupling) chromatography systems with various RP-18 columns (Knauer). Solvents were either methanol or acetonitrile (HPLC grade), in combination with citrate (pH 2.5) or ammonium acetate buffers (pH 3.8 or 7.0). ESI-MS was performed using an amaZon speed ion trap mass spectrometer (Bruker) with 20 μl min^{-1} flow, 3000 V capillary voltage, and 200°C capillary temperature. Apart from necessary adjustments, HPLC-MS was performed with the same parameters.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.04.012.

AUTHOR CONTRIBUTIONS

S.F. and S.L.D. designed research; S.L.D. performed the experiments; S.L.D. and S.F. analyzed data; and S.F. and S.L.D. wrote the paper.

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