Mycobacterium abscessus subsp. abscessus Is Capable of Degrading Pseudomonas aeruginosa Quinolone Signals

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Pseudomonas aeruginosa employs 2-heptyl-3-hydroxy-4(1H)-quinolone (the Pseudomonas quinolone signal, PQS) and 2-heptyl-4(1H)-quinolone (HHQ) as quorum sensing signal molecules, which contribute to a sophisticated regulatory network controlling the production of virulence factors and antimicrobials. We demonstrate that Mycobacterium abscessus1 and clinical M. abscessus isolates are capable of degrading these alkylquinolone signals. Genome sequences of 50 clinical M. abscessus isolates indicated the presence of aqdrABC genes, contributing to fast degradation of HHQ and PQS, in M. abscessus subsp. abscessus strains, but not in M. abscessus subsp. bolletii and M. abscessus subsp. massiliense isolates. A subset of 18 M. a. subsp. abscessus isolates contained the same five single nucleotide polymorphisms (SNPs) compared to the aqdr region of the type strain. Interestingly, representatives of these isolates showed faster PQS degradation kinetics than the M. abscessus type strain. One of the SNPs is located in the predicted promoter region of the aqdr gene encoding a putative transcriptional regulator, and two others lead to a variant of the AqdC protein termed AqdCII, which differs in two amino acids from AqdCI of the type strain. AqdC, the key enzyme of the degradation pathway, is a PQS dioxygenase catalyzing quinolone ring cleavage. While transcription of aqdr and aqdc is induced by PQS, transcript levels in a representative of the subset of 18 isolates were not significantly altered despite the detected SNP in the promoter region. However, purified recombinant AqdcII and AqdcI exhibit different kinetic properties, with approximate apparent K_m values for PQS of 14 µM and 37 µM, and K_cat values of 61 s^{-1} and 98 s^{-1}, respectively, which may (at least in part) account for the observed differences in PQS degradation rates of the strains. In co-culture experiments of P. aeruginosa PAO1 and M. abscessus, strains harboring the aqdc genes reduced the PQS levels, whereas mycobacteria lacking the aqdc gene cluster even boosted PQS production. The results suggest that the presence and expression of the aqdc genes in M. abscessus lead to a competitive advantage against P. aeruginosa.

Keywords: Mycobacterium abscessus, Pseudomonas aeruginosa, quorum sensing, quorum quenching, Pseudomonas quinolone signal, alkylquinolone degradation
INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen regulating its virulence via a complex quorum sensing (QS) network. Besides N-acyl homoserine lactone-mediated las and rhl systems, it possesses an alkylquinolone (AQ) dependent QS system which uses PQS [the Pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone] and its biosynthetic precursor HHQ [2-heptyl-4(1H)-quinolone] as signal molecules (reviewed in Heeb et al., 2011; Huse and Whiteley, 2011). AQ signaling is involved in the regulation of a number of virulence factors such as the siderophore pyoverdine, the redox-active phenazine pigment pyocyanin, rhamnolipid biosurfactants, and the cytotoxic lectin and adhesin LecA (Deziel et al., 2005; Heeb et al., 2011; Huse and Whiteley, 2011).

Several QS-regulated exoproducts of P. aeruginosa do not only contribute to establishing infections of the host, but also act as antimicrobials. These may affect other species coexisting with P. aeruginosa in mixed microbial communities such as those infecting the lung of cystic fibrosis (CF) patients. Studies using laboratory co-cultures of P. aeruginosa with other bacteria support the hypothesis that QS-controlled exoproducts are important for competition. For example, hydrogen cyanide, rhamnolipids, and phenazines together promoted P. aeruginosa competitiveness in co-culture with Burkholderia multivorans, with hydrogen cyanide contributing the greatest effect (Smalley et al., 2015). Pyoverdine was found to contribute to growth inhibition of B. cenocepacia by P. aeruginosa (Costello et al., 2014), and another study showed that pyoverdine-mediated iron acquisition was responsible for growth suppression of Corynebacterium glutamicum, Bacillus subtilis, and Staphylococcus aureus by cell-free culture supernatants of P. aeruginosa (Lee et al., 2016). Thus, in polymicrobial communities, bacteria capable of quenching the production of P. aeruginosa antimicrobials by interference with its QS systems should have some advantage for survival and growth.

Besides acting as QS signals contributing to the regulation of P. aeruginosa exoproducts, PQS and HHQ have antagonistic effects on other microorganisms. PQS acts as an iron-trap (Bredenbruch et al., 2006; Diggle et al., 2007), HHQ exhibits bacteriostatic activity against several Gram-negative bacteria, and both PQS and HHQ repress motility in a range of bacteria (Reen et al., 2011). Moreover, the AQ biosynthetic pathway of P. aeruginosa besides 2-alkyl-4(1H)-quinolones and their 3-hydroxylated congeners also yields 2-alkyl-4-hydroxyquinoline-N-oxides (AQNOs), which act as antibiotics, inhibiting respiratory electron transfer at the cytochrome bc1 complex (Lightbown and Jackson, 1956; Cooley et al., 2005). 2-Heptyl-4-hydroxyquinoline-N-oxide (HQNO), along with siderophores produced by P. aeruginosa, drives S. aureus from aerobic respiration to fermentative metabolism. This is not only detrimental to S. aureus growth rates and fitness, but also results in the production of lactate that is preferentially utilized by P. aeruginosa (Filkins et al., 2015).

Rhodococcus erythropolis BG43, an isolate from soil, is the first bacterium described to be able to degrade HHQ and PQS (Müller et al., 2014). Two gene clusters aqdaB1C1 and aqdraB2C2, both inducible by PQS or a metabolite thereof, were shown to be involved in PQS and HHQ degradation (Müller et al., 2015). Considering their multiple biological functions, degradation of HHQ and PQS could serve for QS interference, or detoxification, or both. Interestingly, homologs to the entire aqdaRAB2C2 cluster from R. erythropolis BG43 (locus tags XU06_RS29725 to XU06_RS29740; NZ_CP011296.1) are conserved in the genomes of some other actinobacteria, especially in representatives of the rapidly growing mycobacteria (RGM) such as strains of M. fortuitum, M. mageritense, and M. abscessus (locus tags MAB_0300c to MAB_0303; NC_010397.1; Müller et al., 2015). M. abscessus, one of the most pathogenic and antibiotic-resistant RGM, is considered an emerging pathogen, causing a pseudotuberculosis lung disease to which patients with CF are particularly susceptible (Griffith et al., 2007; Roux et al., 2009). A recent study revealed that dominant clones of M. abscessus, which emerged a few decades ago and show increased virulence, have spread globally, emerging as a major threat to individuals with CF (Bryant et al., 2016).

The identification of genes potentially coding for an AQ conversion pathway in the genomes of M. abscessus raises the question of whether the strains are indeed able to transform AQ compounds. In this study, we analyzed the degradation of HHQ and PQS by M. abscessus DSM 44196 and by M. abscessus isolates from CF patients. Having identified three groups of strains that differ in their kinetics of PQS degradation and in the presence and type of aqg genes, we determined their effect on PQS levels in co-cultures with P. aeruginosa. To find out whether the differences in PQS degradation kinetics observed for the two groups of clinical isolates harboring aqg genes are due to distinct catalytic properties of their PQS dioxygenases, or due to differences in gene expression, we compared the kinetic properties of the purified enzymes and determined aqg transcript levels.

MATERIALS AND METHODS

Mycobacterium abscessus Strains

The nomenclature of Mycobacterium abscessus is complicated by the non-uniform use in the literature of species and subspecies designations. Currently, two subspecies are recognized: M. abscessus subsp. abscessus, and M. abscessus subsp. bolletii which unites the previous subspecies massiliense and bolletii (Leao et al., 2011). However, for the sake of clarity and because recent publications support the previous classification (Cho et al., 2013; Tan et al., 2015; Bryant et al., 2016; Tortoli et al., 2016), we use the three-subspecies designations. The M. abscessus isolates from CF patients used in this study were characterized previously (Rüger et al., 2014; Kehrmann et al., 2016). The type strain of M. abscessus subsp. abscessus (DSM 44196) was obtained from DSMZ, Braunschweig, Germany.

Chemicals

Pseudomonas quinolone signal and HHQ were purchased from Sigma Aldrich and dissolved in methanol.
Genome Sequencing
Sequencing libraries were constructed from extracted genomic DNA with the Nextera XT kit (Illumina) and sequenced on the Illumina MiSeq instrument in 2 × 300 bp paired end run or in a HiSeq 2 × 150 bp paired end Rapid Run.

Reverse Transcription PCR
For isolation of RNA, *M. abscessus*T (DSM 44196) was grown in DSM219 medium. To possibly induce the expression of *aqd* genes, 20 µM PQS was added 2 h before harvesting the cells by centrifugation. Cells were frozen in liquid nitrogen and stored at −80°C. Cells were then resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and disrupted using the Mikro-Dismembrator S (Sartorius, 3000 rpm, 2 min) after addition of glass beads (150–212 µm diameter) to the cell suspension. Subsequently, RNA purification was performed with the innuPREP RNA Mini kit (Analytik Jena) according to the manufacturer’s instructions. RNA concentration was determined using the Nanophotometer N60 (IMPLEN). Removal of DNA contamination was executed with DNase I (Thermo Scientific) at 37°C and checked via PCR (GoTaq Polymerase, Promega) after renewed RNA purification. For analysis of operon structures, cDNA synthesis was carried out using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer’s instructions. Primers were designed to amplify 500 bps of regions spanning *aqdAB* and *aqdBC*.

Table 1: Primer sequences used for RT-PCRs.

| Name       | Sequence (5′ → 3′)                                      | Application                        |
|------------|--------------------------------------------------------|------------------------------------|
| regionAB-for | TGCATTCCGGGATGAGGCGG                                   | Determination of cluster organization |
| regionAB-rev | CATACTGATGCGTCAGGCG                                     |                                    |
| regionBC-for | CGTATACGAGGCGGATG                                      |                                    |
| regionBC-rev | CCCTCATCGTCAGGCGG                                      |                                    |
| aqdc-for    | CGATCCGCATCTGGTGGG                                     | RT-qPCR of *aqdC*                  |
| aqdc-rev    | GAACTGGTCCACACCTAGGG                                    |                                    |
| aqdr-for    | TGGACCGAGAGAAACACAC                                      | RT-qPCR of *aqdR*                 |
| aqdr-rev    | ATCCGGTTCGGTGTCCAGG                                    |                                    |
| 16S-for         | CAGGCTCTTCACACATGTC                                      | RT-qPCR internal control gene      |
| 16S-rev       | AGACCCCAATCGACATG                                       |                                    |

Expression of *aqd* Genes in *E. coli* for Biotransformation Experiments
The *aqdB* and *aqdC* genes were amplified by PCR from a colony of *M. abscessus*T (DSM 44196). Both AqdB and Aqdc were produced as His6-MBP fusion proteins in recombinant *E. coli* strains, obtained by restriction-free cloning of the corresponding genes into the pET28b(+) expression vector (van den Ent and Lüwe, 2006). *E. coli* Rosetta(DE3) was transformed with pET28b(+-):his8-mbp-aqdB due to many rare codons in the *aqdB* sequence. *E. coli* BL21(DE3) was transformed with pET28b(+):his8-mbp-aqdc.

Growth Conditions and Biotransformation Assays
*Mycobacterium abscessus* strains were cultivated in DSM219 medium, and recombinant *E. coli* BL21(DE3) and Rosetta(DE3) strains were grown in LB medium supplemented with 50 µg/mL kanamycin, at 37°C. For AQ biotransformation by mycobacteria, cells from pre-cultures were suspended at an optical density at 600 nm of 0.5 in fresh DSM219 medium, supplemented with 20 µM of HHQ or PQS, and incubated at 37°C. AQs were extracted at different time points as described previously (Müller et al., 2014). Samples of extracted cell suspensions were solubilized in methanol and analyzed via HPLC. For biotransformations of AQs by recombinant *E. coli* strains harboring pET28b(+-):his8-mbp-aqdB or pET28b(+-):his8-mbp-aqdc, cells were grown overnight at 30°C in the presence of 0.1 mM IPTG, harvested by centrifugation, and resuspended in fresh LB medium with 0.1 mM IPTG, adjusting an OD600nm of 3.5. Biotransformation assays were performed as described above.

Preparation of *Mycobacterium abscessus* Cell Extracts
Cell extracts were prepared from *M. abscessus*T (DSM 44196) grown in DSM219 medium. To possibly induce the expression of AQ-converting enzymes, 20 µM PQS was added 2 h before harvesting the cells by centrifugation. Cells resuspended in 50 mM potassium phosphate buffer pH 7.5 were disrupted by sonication, and cell debris was removed by centrifugation (20,000 × g, 45 min, 4°C). The supernatant (crude extract) was desalted using Zeba™ Spin Desalting columns (10 K molecular weight cut-off, Thermo Scientific). Total protein amount in cell extract supernatants was determined using the Bradford method as modified by Zor and Selinger (1996).
**Purification of AqdC Proteins**

The sequence of *aqdC* of *M. abscessus* was optimized for codon usage of *E. coli* using OPTIMIZER (Puigbó et al., 2007) and synthesized by MWG Eurofins. For the purification of AqdC proteins, codon-optimized synthetic genes were cloned in pET28b(+) using restriction-free cloning (van den Ent and Löwe, 2006). The sequence coding for TEV protease cleavage site was introduced between the coding sequences for his8-tag and AqdC protein. In the following, AqdC\(^{I}\) refers to the protein of the type strain, and the protein which differs from AqdC\(^{I}\) by the two amino acid substitutions R129P and A133T is termed AqdC\(^{II}\). *E. coli* BL21(DE3) harboring pET28b(+)::his8-aqdC\(^{I}\) or pET28b(+)::his8-aqdC\(^{II}\) were grown at 37°C in Terrific Broth. An OD\(_{600nm}\) of 1.0, cultures were supplemented with 0.2 mM IPTG and incubated at 16°C overnight (for approximately 16 h). Cells harvested by centrifugation were resuspended in washing buffer (300 mM NaCl, 20 mM Tris and 10 mM imidazole, pH 8.0), disrupted by sonication, and the AqdC proteins were purified by Ni-NTA affinity chromatography and stored in buffer containing 20 mM Tris, 10% (v/v) glycerol (pH 8.0) at −80°C.

**Enzyme Assay**

The catalytic activity of AqdC proteins was determined spectrophotometrically at 30°C by measuring PQS consumption at 337 nm. The assays contained 20 µM PQS in assay buffer (50 mM Tris, 2 mM EDTA, 10% PEG 1500, 4% (v/v) DMSO, pH 8.0). The extinction coefficient of PQS in assay buffer is 10169 M\(^{-1}\) cm\(^{-1}\) at 337 nm. Apparent steady-state kinetic constants of AqdC proteins (two biological replicates with three technical replicates each) were estimated by fitting the initial velocities measured at different substrate concentrations with the Michaelis Menten equation.

**Cocultivation of *P. aeruginosa* and *M. abscessus* Clinical Isolates**

Overnight cultures of *P. aeruginosa* PAO1 and *M. abscessus* strains were used as inocula for co-culture experiments in 10% LB medium. To account for differences in growth rates (generation times of *M. abscessus* and *P. aeruginosa* in 10% LB are 38.5 and 16.5 h, respectively), *P. aeruginosa* was adjusted to an initial OD\(_{600nm}\) of 0.05, and the mycobacterial strain to an OD\(_{600nm}\) of 0.15, as described by Costa et al. (2015). Cells were incubated at 37°C under vigorous shaking. PQS was extracted after 8 and 24 h as described previously (Müller et al., 2014) and quantified by HPLC analysis. Colony forming units (CFUs) of *P. aeruginosa* PAO1 were determined by dropping 10 µL of a diluted culture onto LB agar and counting colonies after incubation at 37°C overnight. *M. abscessus* formed colonies only after 36 to 48 h, so selective medium was not necessary.

**HPLC Analysis**

For the identification and quantification of PQS and other AQs, compounds were separated on a 250 × 4 mm Eurosphere II RP-18 column using a Hitachi EZChrom Elite HPLC system with diode array detector model 2450, or an Agilent 1100 series system with diode array detector model G1315B. Methanol with 0.1% (w/v) citric acid and 0.1% (w/v) citric acid in water were used as solvents. Separation of PQS and HHQ was carried out via a linear gradient from 80 to 100% methanol (v/v) over 20 min at a flow rate of 0.5 mL min\(^{-1}\).

**RESULTS**

**AQ Degradation in *M. abscessus*\(^T\)**

The presence of an *aqdRABC* gene cluster in the genome of *M. abscessus* suggested that it might degrade HHQ and PQS via reactions analogous to those identified in *R. erythropolis* BG43 (Figure 1). The *aqdB2* gene of *R. erythropolis* BG43 codes for an NADH-dependent HHQ monooxygenase, whereas the gene product of *aqdC2* is a PQS-cleaving dioxygenase which requires O\(_2\) as only co-substrate (Müller et al., 2015). AqdB and Aqdc activity was present in cell extract supernatant suggesting cytoplasmic localization. Desalted cell extract supernatants of *M. abscessus* supplemented with NADH transformed HHQ (Figure 2A), and HHQ consumption was accompanied by transient formation of 2.0 ± 0.3 µM PQS, as identified by HPLC. Interestingly, extracts from cells pre-incubated with PQS converted HHQ and especially PQS faster than extracts from non-induced cells (Figure 2). To analyze whether transformation of HHQ and PQS is indeed catalyzed by the mycobacterial AqdB and Aqdc protein, respectively, recombinant *E. coli* strains expressing *aqdB* or *aqdC* of *M. abscessus* were constructed and tested for AQ biotransformation. *E. coli* cells producing the His\(_8\)-MBP-AqdB protein converted HHQ to PQS, and *E. coli* cells expressing the *M. abscessus*\(^T\) His\(_8\)-MBP-Aqdc fusion protein were able to cleave PQS to N-octanoylanthranilic acid, as identified by HPLC and comparison of UV/Vis spectra and fluorescent properties with reference compounds (Figure 3).

Taken together, the observations strongly support the hypothesis that the mycobacterial *aqdRABC* gene cluster (locus tags MAB\_0300c to MAB\_0303 in NC_010397.1) codes for an inducible AQ degradation pathway which proceeds analogous to that identified in *R. erythropolis* BG43 (Figure 1). *N*-Octanoylanthranilic acid formed by the PQS dioxygenase Aqdc presumably is hydrolyzed by Aqda, a member of the carboxylesterase type B family, to anthranilic acid and octanoate, which both can be channeled into the central metabolism. The *aqdR* gene codes for a putative transcriptional regulator of the TetR family.

**Presence of *aqd* Genes in *M. abscessus* Strains Isolated from CF Patients**

The genomes of *M. abscessus* strains, previously isolated from respiratory samples of CF patients (Rüger et al., 2014), were analyzed for the presence and sequence of the *aqd* gene cluster with BLASTN analyses (Altschul et al., 1997). Among these 50 strains, 22 (44%) lack the *aqd* gene cluster. Interestingly, absence of the *aqd* genes correlates with the assignment of the strains to the subspecies *bolletii* (3 strains) and *massiliense* (19 strains), whereas all 28 strains of the subspecies *abscessus* harbor the *aqd* gene cluster. In all cases where two isolates were
obtained from the same patient, the nucleotide sequences of the aqd gene clusters did not differ between the first and second isolate.

The nucleotide sequences of the aqdRABC genes of strains P5a, P5n, P23a, P23n, P28a, and P28n are identical to those of M. abscessus subsp. abscessus (DSM 44196, ATCC 19977). Compared to the aqd gene region of the type strain, those of strains P13, P24 and another 16 isolates contain the same five single nucleotide variations (SNPs). Two of these SNPs within the protein coding regions of AqdA (triplet encoding L173) and AqdC (triplet encoding I187) are silent, and one is a transition within the predicted promoter region of aqdR. The two other SNPs lead to differences in the amino acids at position 129 and 133 of the predicted PQS dioxygenase AqdC (Table 2 and Supplementary Figure 1). Two other aqd gene modifications observed in the genome of individual strains lead to single amino acid deviations in AqdA (G394S) and AqdB (G15S), respectively (Table 2 and Supplementary Figure 1). However, with respect to AqdC, the key enzyme in PQS degradation, the strains can be divided into two groups: Nine isolates produce the same protein as the type strain (AqdC\(^1\)), and 18 strains form a protein (AqdC\(^2\)) which differs in two amino acids (Table 2). Supplementary Figure 1 shows the translated nucleotide sequence obtained from the same patient, the nucleotide sequences of the aqd gene clusters did not differ between the first and second isolate.
of the aqdB region and indicates the changes in nucleotide and amino acid sequences.

**AQ Degradation by Clinical *M. abscessus* Isolates**

Ten of the clinical *M. abscessus* isolates (Table 2) were analyzed for their ability to degrade PQS and HHQ. Interestingly, members of the subset of 18 strains carrying the aqdB gene cluster with the five SNPs (as compared to that of *M. abscessus*) appeared to be the more potent PQS degraders. Surprisingly, even those strains that do not harbor aqdB genes reduced the amount of PQS to different extents within 24 h (Figure 4).

We selected a representative of each of the groups of strains – strain P5a with an aqdB gene cluster identical to that of *M. a.* subsp. *abscessus*, strain P13 with the five SNPs in the aqdB region, and strain P4a lacking aqdB genes – to determine the time course of HHQ and PQS conversion by cell suspensions. HHQ consumption by strains P5a and P13 followed similar kinetics. However, as already suggested by the preliminary data shown in Figure 4, strain P13 converted PQS faster than strain P5a, and strain P4a lacking the gene cluster very slowly converted HHQ as well as PQS (Figures 5A, B).

Strains without the aqdB-cluster which slowly consume PQS and HHQ must use alternative enzymes to modify or even degrade these AQs. HPLC analyses of culture extracts revealed transient formation of 4.9 ± 3.9 µM PQS after 2 h of incubation with 20 µM HHQ, besides an arsenal of other metabolites. Thus, it appears that other monoxygenases besides AqdB can mediate HHQ hydroxylatation to PQS. Due to the complexity of metabolites formed, incomplete peak separations, and the low concentrations of intermediates (expected to be produced by the cultures at the nM range), elucidation of their structures will require the establishment of improved extraction and separation protocols, as well as considerable upscaling, to enable NMR analyses for structural identification. However, because the UV spectra of many of the peaks of the HPLC elution profile resemble that of PQS, we assume that the corresponding metabolites are quinolones, with modifications introduced mainly to the alkyl chain.

**Transcription of aqdB Genes in Strains P13 and P5a**

To analyze whether the aqdB ABC genes are co-transcribed, RT-PCR was performed with primer pairs addressing adjacent gene transcripts (for primer sequences see Table 1). Formation of PCR products, as verified by gel electrophoresis (not shown), indicate that aqdBAB and aqdBBC are co-transcribed, suggesting organization of the aqdBABC genes in an operon.

Cells induced with 20 µM PQS have higher transcript levels of aqdB (fold change between 4.0 and 6.8), indicating autoregulation of aqdB transcription. Transcript levels of aqdBII were also increased when the cells were induced with PQS (fold change between 5.4 and 5.7) (Figure 6A). In strains harboring the aqdB genes with five SNPs compared to the aqdB region of the type strain, the transition within the predicted promoter region of aqdB changes the inverted repeat sequence TTGTCGCATCGCA to TCCTGCATCGCACAA. To determine whether the SNP results in different expression levels, transcript levels of aqdB and aqdBII were compared for strains P5a and P13. RT-qPCR analyses revealed only slight reduction of transcription amounts in strain P13 (Figure 6B). Thus, it seems unlikely that differential expression of the aqdB gene coding for the key enzyme, a PQS dioxygenase, accounts for the observed differences in PQS degradation rates (Figure 5).

**Catalytic Activity of AqdB Proteins**

To analyze the possibility that the AqdBI and AqdBII proteins differ in their catalytic efficiency, recombinant proteins were purified to electrophoretic homogeneity, and their steady-state kinetic parameters were determined. Under the conditions of...
the assay, a specific activity of 53.2 U mg\(^{-1}\), an apparent \(k_{\text{cat}}\) of 97.8 ± 19.1 s\(^{-1}\) and an apparent \(K_m\) value for PQS of 37.2 ± 9.8 \(\mu\)M were observed for AqdC\(^I\), the PQS dioxygenase form of the \(M. a.\) subsp \(abscessus\) type strain and the group of strains represented by the isolate P5a. For AqdC\(^II\), the enzyme of the group of isolates represented by strain P13, a specific activity of 50.5 U mg\(^{-1}\), an apparent \(k_{\text{cat}}\) of 61.0 ± 4.3 s\(^{-1}\) and apparent \(K_m\) value of 13.6 ± 1.6 \(\mu\)M were determined.

PQS Concentrations in Co-cultures of \(P. aeruginosa\) and \(M. abscessus\)

Representatives of each \(M. abscessus\) group were co-cultivated with \(P. aeruginosa\) PAO1 to test whether PQS produced by \(P. aeruginosa\), which is packaged into membrane vesicles for trafficking between cells (Mashburn-Warren et al., 2008), is amenable to degradation by the mycobacteria. Indeed, the clinical isolates P5a and P13, which degraded synthetic PQS, also were able to reduce the PQS concentration in co-culture with \(P. aeruginosa\) PAO1 (Figure 7). Most interestingly, however, the PQS concentration increased significantly when \(P. aeruginosa\) PAO1 was cultivated with strain P4a, a representative of the group lacking the \(aqd\) genes. Compared with the \(P. aeruginosa\) PAO1 cultures, the amount of PQS in \(P. aeruginosa\) PAO1 – \(M. abscessus\) P4a co-cultures was fivefold higher after 24 h of cultivation. \(P. aeruginosa\) PAO1 cultivated with dead (autoclaved) \(M. abscessus\) strain P13 cells produced increased amounts of PQS as well. After 24 h of incubation, the PQS concentration was almost eightfold higher than in the PAO1 solo cultures. CFUs of \(P. aeruginosa\) PAO1 cultures were similar to those of PAO1 in co-culture with \(M. abscessus\) strain P13, or PAO1 cultivated in the presence of autoclaved \(M. abscessus\) P13 cells. Therefore, the increase in PQS production especially in presence of autoclaved cells is not due to increased population density of \(P. aeruginosa\).

The boosting of PQS production may rely on the recognition of cell components by PAO1. It makes the reduced levels of PQS in PAO1-P5a and PAO1-P13 co-cultures even more remarkable.

DISCUSSION

\(Pseudomonas aeruginosa\) often dominates the microbiome of the lungs of adult CF patients, however, the CF lung is usually colonized with multiple pathogens. CF-related lung disease also is a risk factor for chronic pulmonary infection with RGM, and RGM are actually detected with increasing prevalence in the CF population. Especially \(M. abscessus\) is considered an emerging threat to individuals with CF (Bar-On et al., 2015; Bryant et al., 2016).

Alkylquinolones produced by \(P. aeruginosa\), acting as QS signals and antimicrobials, have been detected in the sputum
of CF patients (Collier et al., 2002; Barr et al., 2015). Thus, M. abscessus, when co-colonizing the CF lung, may well encounter these secondary metabolites. In this study, we demonstrate that M. abscessus is capable of degrading the P. aeruginosa signal molecules HHQ and PQS. Interestingly, clinical M. abscessus strains isolated from patients with CF showed different kinetics in PQS degradation, correlating with the presence and type of aqdBABC genes coding for an inducible HHQ and PQS degradation pathway. Differences in transcript levels, possibly related with the SNP in the promoter of the aqdB gene, differences in the kinetic properties of the PQS dioxygenases AqdB and AqDC, and additional factors such as mRNA or protein stability might influence the degradation rates. While RT-qPCRs showed no significant differences in expression levels of the aqdB and aqDC genes of strains P13 and P5a, the catalytic efficiency \((k_{cat}/K_m)\) of the PQS dioxygenase AqDC was found to be about 1.7-fold higher than that of AqDC. Strain P13 and the majority of the isolates harboring the aqdB gene cluster produce the AqDC variant. Considering that PQS levels in P. aeruginosa cultures are about 16 and 2 \(\mu M\) when cultivated in LB and artificial sputum medium, respectively (Collier et al., 2002; Lépine et al., 2003), and those in CF sputum can reach high \(nM\) ranges (Barr et al., 2015), the AqDC protein with its lower \(K_m\) value should perform better under physiological conditions.

Co-cultivation of M. abscessus isolates P13 and P5a with P. aeruginosa PAO1 reduced PQS concentrations in these cultures, whereas the presence of strain P4a (which lacks the aqdB genes), or presence of dead mycobacterial cells significantly enhanced PQS production by P. aeruginosa PAO1. Thus, P. aeruginosa seems to recognize and respond to the presence of M. abscessus cells, cellular components, or exoproducts. Upregulation of PQS production by a mycobacterial effector should have broad implications on virulence as well as competitiveness of P. aeruginosa, because the pqs system controls a diverse array of virulence factors, such as the redox-active pigment pyocyanin, rhamnolipid surfactants, the siderophore pyoverdine, and the antimicrobial 2-alkyl-4-hydroxyquinoline N-oxides (Heeb et al., 2011). Especially the latter compounds not only affect competing microorganisms but also the fitness of P. aeruginosa itself, even promoting cell autolysis and DNA release (Hazar et al., 2016). As regards a possible mycobacterial effector, it is interesting that P. aeruginosa has been reported to enhance production of PQS and phenazine antimicrobials in response to N-acetylglucosamine, a peptidoglycan turnover product shed by Gram-positive bacteria (Korgaonkar and Whiteley, 2011; Korgaonkar et al., 2013). However, since mycobacteria have a complex cell envelope dominated by arabinogalactan and mycolic acids besides peptidoglycan, P. aeruginosa may sense additional or other components.

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Remarkably, among the M. abscessus isolates tested, the presence of aqdB genes strictly correlated with the subspecies abscessus, which is the subspecies most frequently isolated from CF patients worldwide (Bryant et al., 2016). It will be interesting to analyze whether the ability to rapidly inactivate P. aeruginosa QS signals, which not only control the production of virulence factors and antimicrobials but act as antimicrobials themselves, contributes to co-colonization competitiveness of the subspecies abscessus. However, AQ degradation by M. abscessus likely is only one aspect in a multi-faceted interaction between the two pathogens and our observation of increased PQS production in response to mycobacterial cell material opens up new questions of how P. aeruginosa monitors and responds to its biotic environment.

**AUTHOR CONTRIBUTIONS**

SF and FSB conceived the experiments. TK performed genome sequencing, TW performed and analyzed RT-qPCRs, and FSB performed all other experiments. JK analyzed genome sequences. KR and FB provided mycobacterial strains. SF and FSB analyzed data and wrote the paper. All authors contributed to the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00339/full#supplementary-material
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