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Conversion of the *Pseudomonas aeruginosa* Quinolone Signal and Related Alkylhydroxyquinolines by *Rhodococcus* sp. Strain BG43

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A bacterial strain, which based on the sequences of its 16S rRNA, *gyrB*, *catA*, and *qsdA* genes, was identified as a *Rhodococcus* sp. closely related to *Rhodococcus erythropolis*, was isolated from soil by enrichment on the *Pseudomonas* quinolone signal [PQS; 2-heptyl-3-hydroxy-4(1*H*)-quinolone], a quorum sensing signal employed by the opportunistic pathogen *Pseudomonas aeruginosa*. The isolate, termed *Rhodococcus* sp. strain BG43, cometabolically degraded PQS and its biosynthetic precursor 2-heptyl-4(1*H*)-quinolone (HHQ) to anthranilic acid. HHQ degradation was accompanied by transient formation of PQS, and HHQ hydroxylation by cell extracts required NADH, indicating that strain BG43 has a HHQ monooxygenase isofunctional to the biosynthetic enzyme PqsH of *P. aeruginosa*. The enzymes catalyzing HHQ hydroxylation and PQS degradation were inducible by PQS, suggesting a specific pathway. Remarkably, *Rhodococcus* sp. BG43 is also capable of transforming 2-heptyl-4-hydroxyquinoline-*N*-oxide to PQS. It thus converts an antibacterial secondary metabolite of *P. aeruginosa* to a quorum sensing signal molecule.

Bacteria use cell-to-cell communication systems based on chemical signal molecules to coordinate their behavior within the population. These quorum sensing (QS) systems regulate a variety of physiological processes, such as bioluminescence, sporulation, competence for DNA uptake, biofilm maturation, production of secondary metabolites, and expression of virulence factors (1). The QS network of the opportunistic pathogen *Pseudomonas aeruginosa* involves the two acylhomoserine lactone (AHL)-based Las and Rhl systems, producing and responding to *N*-3-oxo-dodecanoyl homoserine lactone and *N*-butanoyl homoserine lactone, respectively, 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 in *P. aeruginosa*, but its biosynthetic precursor 2-heptyl-4(1*H*)-quinolone (HHQ) also acts as a QS signal molecule. PQS signaling is involved in the control of virulence factor production, including the formation of elastase, pyocyanin, and lectin LecA, and it influences biofilm maturation. PQS additionally has iron-chelating and membrane-altering properties (reviewed in references 2, 3, and 4).

Whereas PQS appears to be unique to *P. aeruginosa*, other *Pseudomonas* as well as *Alteromonas* spp. seem to rely on nonhydroxylated 2-alkyl-4(1*H*)-quinolones, and *Burkholderia* spp. use mainly 3-methylated AQs for signaling (5–8). However, *P. aeruginosa* produces more than 50 AQs and related compounds (9). Among these are the 2-alkyl-4-hydroxyquinoline *N*-oxides, which are close analogs of the quinones/semiquinones involved in membrane-associated electron transport chains and thus act as inhibitors of respiratory cytochromes (10, 11).

There is considerable interest in agents that selectively interfere with the QS systems of pathogenic bacteria for targeting bacterial virulence and developing new anti-infective therapies (12). Quorum sensing interference is generally believed to be less likely to select for resistance than antibiotic therapy, because in contrast to antibiotic therapy, it does not directly affect growth; however, from recent studies a more varied picture emerges (13, 14). Strategies to interfere with quorum sensing involve inhibition of QS signal biosynthesis, inhibition of signal perception or transduction, or inactivation of the signal molecules themselves. With regard to the AHLs, signal inactivation by enzymatic modification or degradation actually seems to be widespread in nature. Some oxidoreductases catalyze the reduction of the 3-oxo group of AHLs or the ω-hydroxylation of the side chain. A wide range of Gram-negative as well as Gram-positive bacteria belonging to diverse taxa, e.g., strains of *Anabaena*, *Agrobacterium*, *Pseudomonas*, *Variovorax*, *Bacillus*, *Arthrobacter*, and *Rhodococcus* spp., produce lactonases or acylases that hydrolyze AHL signaling molecules (for recent reviews, see references 15 and 16).

Rhodococci are virtually ubiquitous bacteria residing in soil and water environments. They show high resistance to harsh environmental conditions, such as desiccation (17, 18), and are well known for their catabolic versatility. The hydrophobic cell surface containing mycolic acids as well as the ability of many rhodococci to produce biosurfactants is thought to support the assimilation of hydrophobic substrates by increasing their bioavailability (19, 20). Interestingly, a number of *Rhodococcus* isolates can utilize AHL signal molecules as carbon sources. In *Rhodococcus erythropolis* W2, *R. erythropolis* R138, and related strains, the ability to efficiently degrade AHLs appears to be correlated with a conserved γ-lactone degradation pathway, with the lactonase QsdA as the key enzyme (21–23). *R. erythropolis* strains possessing this pathway significantly reduced tissue maceration of potato tubers by the soft-rot pathogens *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium atrosepticum* (23–25).

Whereas numerous reports can be found in the literature on the biodegradation of AHLs, bacteria that degrade AQ-type sig-

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naling molecules have not been described so far. The only enzyme known to be able to inactivate an AQ-type QS signal is the dioxygenase Hod (1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase) from *Arthrobacter* sp. Rue61a, which catalyzes the cleavage of PQS to form *N*-octanoylanthranilate and carbon monoxide (26). However, Hod is an enzyme involved in the 2-methylquinoline degradation pathway of *Arthrobacter* sp. Rue61a, with 3-hydroxy-2-methyl-4(1*H*)-quinolone (MHOQ) as its physiological substrate (27, 28), and its comparatively low activity toward PQS is considered fortuitous.

Quinoline and quinolone alkaloids structurally related to the AQ-type signaling molecules of *P. aeruginosa* and *Burkholderia* spp. are produced by a variety of higher organisms, especially by plants of the family *Rutaceae* (2, 29, 30). Therefore, it is well conceivable that soil microorganisms have evolved enzymes and pathways to detoxify and/or to degrade quinolones. In this study, we isolated a PQS-degrading bacterium from soil. The isolate was identified as a *Rhodococcus* strain related to the species *R. erythropolis*. It cometabolically degrades PQS to anthranilic acid, and it is also able to convert the PQS precursor HHQ as well as related 2-alkyl-4-hydroxyquinolines of *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals. HHQ and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were produced by *Pseudomonas putida* KT2440(pBBR-*pqsABCD*) and *P. putida* KT2440(pBBR-*pqsABCD*, pME6032-*pqsL*), respectively, grown in the presence of anthranilate and octanoate, and isolated from biomass as described previously for AQs (31). HHQ extracts which were not purified further by preparative high-performance liquid chromatography (HPLC) also contained minor amounts of other AQs with C_9- to C_{13}-saturated and unsaturated alkyl side chains. HQNO was purified by preparative HPLC (31). For *in vitro* assays and as a reference compound, HQNO purchased from Enzo Life Sciences was used. 3-Hydroxy-2-methyl-4(1*H*)-quinolone (MHOQ) was synthesized from 3-formyl-2-methyl-4(1*H*)-quinolone (32, 33). PQS, *N*-acetylanthranilic acid, and anthranilic acid were from Sigma-Aldrich. Stock solutions of PQS, HHQ, and HQNO were prepared in methanol. MHOQ and *N*-acetylanthranilic acid were dissolved in ethanol and deionized water, respectively.

Isolation of a PQS-degrading bacterial strain. Soil samples, collected in the botanical garden of the University of Münster beneath plants that produce quinoline or acridone alkaloids (*Ephedra* spp., *Ruta graveolens*, *Ptelea trifoliata*, *Citrus limon*, *Citrus aurantium*, and *Poncirus trifoliata*), as well as soil samples collected below spruce, oak, and beech trees in forests in the area of Münster, Germany, and soil collected at a roadside were shaken for 1 h in 0.9% (wt/vol) NaCl solution. The suspensions were used to inoculate 24-well microtiter plates (CELLSTAR suspension culture plates; Greiner Bio-One GmbH) containing mineral salts medium (6.78 g/liter $Na_2HPO_4 \cdot 2H_2O$, 3 g/liter KH_2PO_4, 0.5 g/liter NaCl, 1 g/liter NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 15 mg/liter $Na_2MoO_4 \cdot 7H_2O$, 1 ml/liter trace element solution [34]), supplemented with 50 μM PQS as the sole carbon source. Culture samples were diluted into fresh medium every week for 6 weeks, even though growth or biofilm formation was hardly (if at all) detectable. Samples taken from the last transfer were spread onto Luria-Bertani (LB) agar plates (35). Colonies were purified by repeated alternate streaking on agar plates containing PQS mineral salts medium and LB agar. Individual isolates were tested for cometabolic PQS conversion as described below.

Bacterial strains and growth conditions. *Rhodococcus* sp. BG43 as well as *P. putida* KT2440(pBBR-*pqsABCD*), *P. putida* KT2440(pBBR-*pqsABCD*, pME6032-*pqsL*), and *Escherichia coli* DH5α(pME6032-*pqsL*) was grown in LB medium at 30°C and 37°C, respectively. Kanamycin and tetracycline, each at 50 μg/ml, were added to cultures of recombinant *P. putida* KT2440, and tetracycline at 12.5 μg/ml was added to recombinant *E. coli* DH5α cultures. To determine growth of *Rhodococcus* sp. BG43 on individual carbon or nitrogen sources, cells of overnight LB cultures were pelleted by centrifugation (8,000 × *g*, 5 min, and 4°C), washed twice with phosphate-buffered saline (PBS), and used to inoculate modified KG medium to an optical density at 600 nm (OD_{600}) of 0.05. The modified KG medium (36) contained 1.25 g/liter NaCl, 0.75 g/liter KCl, 0.25 g/liter Na_2SO_4, 0.25 g/liter KH_2PO_4, and 1.0 g/liter 2-(*N*-morpholino)ethanesulfonic acid (MES). The pH was adjusted to 6.5, and after autoclaving, the following components were added from sterile stock solutions: vitamin solution (0.5 ml/liter) (37), NH_4Cl (0.3 g/liter), $MgCl_2$ (0.5 g/liter), $CaCl_2$ (0.25 g/liter), $FeCl_3$ (5 mg/liter), and $MnCl_2$ (2.5 mg/liter). For testing the utilization of substrates as nitrogen sources, NH_4Cl was omitted from the medium and 1% (wt/vol) succinate was used as the carbon source. For all growth tests, cultures lacking the substrate to be tested were run in parallel. Strain BG43 did not grow in the MES-buffered medium in the absence of another substrate. The cultures were incubated at 30°C on a rotary shaker (160 rpm), and the OD_{600} was measured within 24 h.

The viability of cell suspensions of *Rhodococcus* sp. strain BG43 in modified KG medium with succinate, supplemented with up to 20 μM HQNO, was monitored with the BacTiter-Glo microbial cell viability assay (Promega Corporation), which quantifies ATP levels as an indicator for metabolically active cells. Cell suspensions were set up as performed for the AQ conversion assays (see below); i.e., strain BG43 was suspended in the medium to an initial OD_{600} of 3, and samples were taken at different time points within 4 h and frozen immediately. The BacTiter-Glo assay was prepared in multiwell plates as described by the manufacturer, using series of diluted samples. Antibacterial activity of HQNO toward strain BG43 was tested by growing the strain in modified KG medium with succinate in the presence of up to 500 μM HQNO. Cultures were incubated at 30°C on a rotary shaker, and the OD_{600} was determined.

DNA techniques. Genomic DNA of *Rhodococcus* sp. BG43 was extracted with the innuSPEED Bacteria/Fungi DNA kit (Analytik Jena AG). PCR was performed using Q5 Hot Start High-Fidelity DNA polymerase (New England BioLabs GmbH). Plasmids and PCR products were purified with the innuPREP Plasmid minikit and innuPREP DOUBLEpure kit (Analytik Jena AG), respectively. Agarose gel electrophoresis, restriction, and ligation were performed using standard protocols (35). Restriction enzymes were purchased from Thermo Scientific. For transformation of *E. coli* DH5α, cells were prepared according to the method of Hanahan (38). Oligonucleotides were purchased from Eurofins MWG Operon. DNA sequencing was carried out by GATC Biotech AG.

Construction of pME6032-*pqsL*. The *pqsL* gene (nucleotides [nt] 4687652 to 4688848; GenBank accession number NC_002516) of *P. aeruginosa* PAO1 (University of Nottingham strain) was amplified using the primer set pqsL-for/pqsL-rev (Table 1). The PCR product, digested with EcoRI and SacI, was ligated into the appropriately digested plasmid pME6032 (39), and *E. coli* DH5α was transformed with the pME6032-*pqsL* plasmid. To generate an HQNO-producing strain, *P. putida* KT2440(pBBR-*pqsABCD*) (31) was transformed with pME6032-*pqsL* by electroporation essentially as described in reference 40, with the following electrical settings: voltage, 12.5 kV/cm; capacitor, 25 μF; and resistor, 200 Ω. After discharge, 400 μl of LB medium was added, and the cell suspension was incubated for 1 h at 30°C with shaking before being plated on selective media.

Molecular characterization and phylogenetic analysis of strain BG43. To classify the PQS-converting isolate, the gene encoding 16S rRNA as well as genes coding for catechol 1,2-dioxygenase (*catA*) and gyrase B (*gyrB*) were amplified using the primer pairs GM3F/GM4R, catA-for/catA-rev, and gyrB-for/gyrB-rev, respectively (41–43) (Table 1). Phylogenetic trees generated from the 16S rRNA gene, *catA*, and *gyrB* sequences using the neighbor-joining algorithm were constructed with Molecular Evolution Genetics Analysis (MEGA) software version 6.0 (44). Nucleotide alignment was carried out with MUSCLE (45). The reliability of the trees was evaluated by bootstrap analysis (1,000 resamplings). The *qsdA* gene, encoding a "*Rhodococcus*-specific" AHL lactonase (21), was amplified with primers qsdA-for and qsdA-rev (46) (Table 1).

TABLE 1 Primers used in this study

Primer designation	Sequence (5′→3′)	Application	Reference(s)
pqsL-for	ATATGAGCTCTCAGTGGTGGTGGTGGTGGCCGAGCG GCGCCGGCGACCGCACCGGCTG	Amplification of *pqsL* (nt 4687652–4688848 of *P. aeruginosa* PAO1)	This study
pqsL-rev	ATATGAATTCATGACGGACAACCATATCGATGTACTGATC		
GM3F	AGAGTTTGATC(AC)TGGC	Amplification of 16S rRNA gene	41, 42
GM4R	TACCTTGTTACGACTT		
catA-for	GCCGCCACCGACAAGTT	Amplification of catechol 1,2-dioxygenase gene (*catA*)	43
catA-rev	CACCATGAGGTGCAGGTG		
gyrB-for	GGCGGCAAGTTCGACTTCGA	Amplification of gyrase B gene (*gyrB*)	43
gyrB-rev	GCCTTCTCGACGTTGATGATC		
qsdA-for	ATGAGTTCAGTACAAACCGT	Amplification of AHL lactonase gene (*qsdA*)	46
qsdA-rev	TCAGCTCTCGAAGTACCGAC		

Preparation of cell extracts. For preparation of crude cell extracts, *Rhodococcus* sp. BG43 was cultivated in LB medium for 24 h with vigorous shaking. To possibly induce the expression of genes of an AQ degradation pathway, 20 μM PQS was added 2 h before cells were harvested by centrifugation (12,000 × *g*, 4°C, and 45 min). Cells resuspended in 50 mM potassium phosphate buffer (pH 7.5) were disrupted by sonication at 4°C. Cell-free crude extracts containing soluble proteins were obtained by centrifugation for 40 min at 38,360 × *g* and 4°C. For removal of salts and small molecules, Zeba spin desalting columns (7,000-molecular-weight cutoff; Thermo Scientific) were used. The method of Bradford as modified by Zor and Selinger (47) was applied to estimate the protein concentration. Bovine serum albumin served as a standard protein.

AQ conversion by whole cells and crude cell extracts. Cells of *Rhodococcus* sp. BG43 cultures grown for 24 h in LB medium were pelleted by centrifugation (9,000 × *g*, 4°C, and 10 min) and washed twice with PBS. Subsequently, the cells were resuspended in modified KG medium containing 1% (wt/vol) succinate as the carbon source and diluted to an OD_{600} of 3. After addition of 20 μM MHOQ, HHQ, PQS, or HQNO, the cultures were incubated at 30°C with constant shaking. Cultures without added AQ were run in parallel. Samples (25 ml of cell suspension) were taken at different time points, and AQs were extracted as described below. For measuring AQ conversion by desalted crude cell extracts, the protein concentration of the extracts was set to 2 or 1 mg/ml. When indicated, NADH or NADPH, with or without additional 500 μM FAD, was added to a final concentration of 500 μM. Sets of test tubes containing 1-ml aliquots were supplemented with 20 μM PQS, HHQ, or HQNO and incubated at 30°C with shaking at 900 rpm. Test tubes were taken at different time points for extraction with ethyl acetate.

Extraction of AQs. Prior to extraction with ethyl acetate, samples were spiked with 1 μM *N*-acetylanthranilic acid in order to monitor the reproducibility of sample extraction. Samples of *Rhodococcus* sp. BG43 cultures incubated with AQs (25 ml each) and crude cell extract samples (1 ml each) were extracted three times with a 5-ml volume and three times with a 0.5-ml volume, respectively, of acidified ethyl acetate (1 ml acetic acid/liter). After centrifugation at 9,000 × *g* for 5 min and 20,000 × *g* for 5 min, respectively, the organic phases of each sample were combined and dried to completion, and the residue was redissolved in methanol. Using this protocol, about 86%, 79%, 65%, 21%, and 80% of HHQ, PQS, HQNO, MHOQ, and anthranilic acid, respectively, could be recovered from 20 μM solutions in modified KG medium.

Analytical methods. HPLC was performed on a 250- by 4-mm Eurospher II RP-18 column at 35°C. Extracts containing PQS or HQNO and extracts of negative controls without AQs were separated using a linear gradient (20 min) of 80% (vol/vol) methanol in water to 100% methanol, at a flow rate of 0.5 ml/min. For analysis of extracts containing HHQ or MHOQ, a linear gradient (40 min) of 15% (vol/vol) methanol in water to 100% methanol was applied at a flow rate of 0.5 ml/min. All eluents were acidified with 1 g/liter citric acid. Light absorption spectra were recorded with a diode array detector (L-2450 LaChrome Elite; Merck Hitachi). Reference compounds were used to calibrate the column for quantitative determination of AQs. Intermediates of AQ transformation were analyzed by liquid chromatography-mass spectrometry (MS) on a Dionex UltiMate 3000 ultrahigh-performance liquid chromatography system (Thermo Scientific), coupled with an electrospray ionization ion trap mass spectrometer (amaZon Speed; Bruker Daltonics), using a scan range from 50 to 1,000 *m/z* (target mass, 300 *m/z*). The capillary voltage was set to 4,000 V and the capillary temperature to 200°C.

Nucleotide sequence accession numbers. The (partial) sequences of the 16S rRNA gene and the *catA*, *qsdA*, and *gyrB* genes obtained in this study were deposited in the GenBank nucleotide sequence database under accession numbers KM093741, KM093742, KM093743, and KM093744, respectively.

RESULTS

Isolation of the PQS-converting strain BG43. Enrichment cultures were established in mineral salts medium containing PQS as a carbon source using soil samples as inocula. Purification of bacterial colonies by repeated alternate streaking on PQS mineral salts agar and LB agar plates resulted in eight isolates, all from soil samples of the botanical garden of the University of Münster. When tested for cometabolic PQS biotransformation in modified KG medium supplemented with succinate, seven out of the eight isolates showed tolerance toward PQS rather than PQS degradation. One isolate, termed strain BG43, which transforms PQS as described below, originated from soil collected below *Ruta* and *Ephedra* plants. Apparently, the protocol used for subculturing of the enrichment cultures and strain isolation predominantly selected for bacteria able to survive comparatively high concentrations of PQS and extended periods of starvation.

Phylogenetic analysis and carbon source utilization pattern of strain BG43. BLASTn analysis (standard nucleotide blast; http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) of the partial sequence (1,359 nt) of the 16S rRNA gene of strain BG43 revealed the highest levels of sequence identity to 16S rRNA genes of *R. erythropolis* strains zzx26, D7, and WZ010 (99.78%). The highest level of sequence identities to type strains occurred with *R. qingshengii* strain djl-6 (DSM 45222^T) (99.71%). The phylogenetic tree based on the 16S rRNA gene sequences of *Rhodococcus* sp. BG43 and type strains of other *Rhodococcus* species supports a close relatedness to *R. qingshengii* DSM 45222^T and *R. erythropolis* DSM 43066^T (Fig. 1A). Since the identities among the partial 16S rRNA gene sequences were very high, additionally the sequences of PCR products of the *gyrB* and *catA* genes, which have been used as

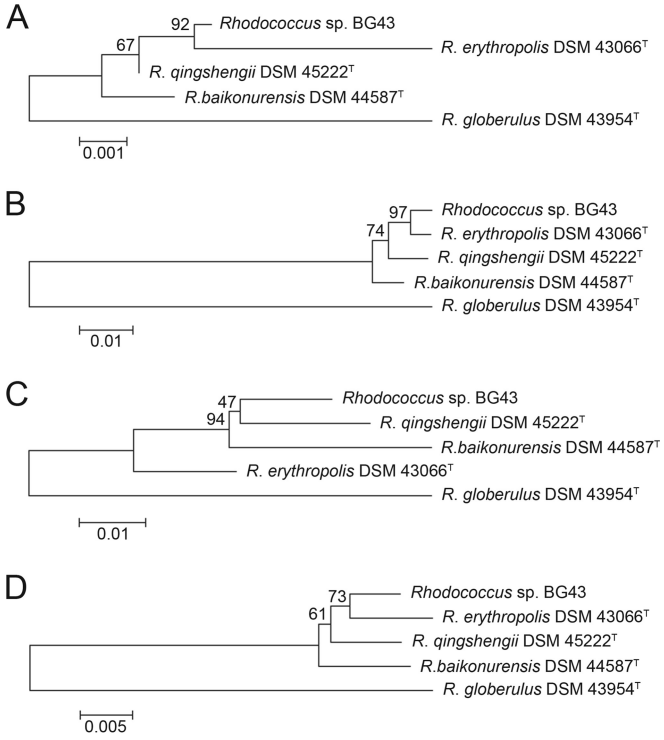

FIG 1 Phylogenetic trees based on marker genes of *Rhodococcus* sp. BG43 and closely related type strains. (A) 16S rRNA gene; (B) *gyrB*; (C) *catA*; (D) joined *catA-gyrB*-16S rRNA genes. Trees were constructed with Molecular Evolution Genetic Analysis (MEGA) software version 6.0 using the neighbor-joining algorithm (44). Nucleotide alignment was performed with MUSCLE (45). The reliability of the trees was evaluated with bootstrap analysis (1,000 resamplings).

marker genes for *Rhodococcus* (43), were compared to those of *Rhodococcus* species type strains. The phylogenetic tree based on *gyrB* sequences suggested that strain BG43 and *R. erythropolis* DSM 43066^T are closely related (Fig. 1B), whereas analysis of *catA* led to a tree clustering the isolate with *R. qingshengii* DSM 45222^T (Fig. 1C). However, a phylogenetic tree based on the concatenated sequences places strain BG43 closer to the *R. erythropolis* type strain (Fig. 1D).

A comparison of the carbon source utilization patterns of strain BG43 and related *Rhodococcus* species type strains (48, 49) (Table 2) shows that all strains are able to utilize glycerol but not lactose. Even though the *catA* gene, encoding catechol 1,2-dioxygenase, is present in the genome of *Rhodococcus* sp. BG43, the strain did not grow on catechol under the conditions tested, as also observed for some other *Rhodococcus* sp. strains. In contrast to *R. qingshengii* DSM 45222^T, *R. globerulus* DSM 43954^T, and *R. baikonurensis* DSM 44587^T, *Rhodococcus* sp. BG43 is able to grow on *myo*-inositol, as reported for *R. erythropolis* DSM 43066^T. In contrast to *R. qingshengii* DSM 45222^T, strain BG43 can utilize D-sorbitol. Taken together, the comparison of the marker genes tested and the carbon source utilization patterns support the hypothesis that strain BG43 clusters with *R. erythropolis*; however, more detailed analyses will be required for species allocation.

Since the AHL lactonase QsdA, a member of the phosphotriesterase (PTE) family, has been identified in all of six *R. erythropolis* strains tested (21), we speculated that strain BG43 might also contain this quorum quenching enzyme. PCR amplification indeed resulted in a specific product, whose deduced amino acid sequence (292 amino acids [aa]) shows 99% identity to the corresponding region (aa 18 to 309) of QsdA of *R. erythropolis* strain SQ1. Concordant with other QsdA enzymes from *Rhodococcus* spp., the protein sequence of $QsdA_{BG43}$ diverges from the consensus PTE zinc domain sequence. The sequence of motif 2 of the zinc binding site of $QsdA_{BG43}$ (AVGQAQVETGVPITVH; conserved residues of the zinc binding domain CD2 of PTEs are underlined) corresponds to allele A1 as defined by Uroz et al. (21), with a conserved alanine at position 5 of the motif, whereas another group of rhodococcal QsdAs (allele A2) has a serine at this position. Consistent with the role of QsdA as a key enzyme in the γ-lactone catabolic pathway, strain BG43 was capable of growing on γ-octalactone (4.5 mM) as a source of carbon and energy, but not on γ-butyrolactone (9 mM, 4.5 mM, or 1 mM), as described for *R. erythropolis* R138 (22).

Cometabolic degradation of PQS, HHQ, and MHOQ. Cell suspensions of *Rhodococcus* sp. BG43 (OD_{600} ~ 3), incubated in

TABLE 2 Growth of *Rhodococcus* sp. BG43 and closely related type strains on selected carbon sources[a]

Carbon source	Growth on carbon source				
	Rhodococcus sp. BG43	*R. erythropolis* DSM 43066^T	*R. baikonurensis* DSM 44587^T	*R. qingshengii* DSM 45222^T	*R. globerulus* DSM 43954^T
D-Fructose	+	+	+	−	+
Sucrose	+	+	−	w	+
D-Sorbitol	+	+	−[b]/+[c]	−	+
Catechol	−	−	−	+	−
Lactose	−	−	−	−	−
Glycerol	+	+	+	+	+
D-Mannose	w	−	w	+	+
D-Xylose	w	−	−	−	+
myo-Inositol	+	+	−	−	−

[a] Data for the type strains are from references 48 and 49. Strain BG43 was cultured in modified KG medium with shaking at 30°C. Carbon sources were used at the following concentrations: D-fructose, lactose, glycerol, D-mannose, D-xylose, and *myo*-inositol, 2% (wt/vol); sucrose, 4 mM; D-sorbitol, 1% (wt/vol); and catechol, 1%, 0.5%, and 0.1% (wt/vol). OD_{600} was measured after 24 h of incubation. +, $OD_{600} > 0.5$; w, weak ($OD_{600} < 0.5$); −, no growth observed.
[b] Per reference 48.
[c] Per reference 49.

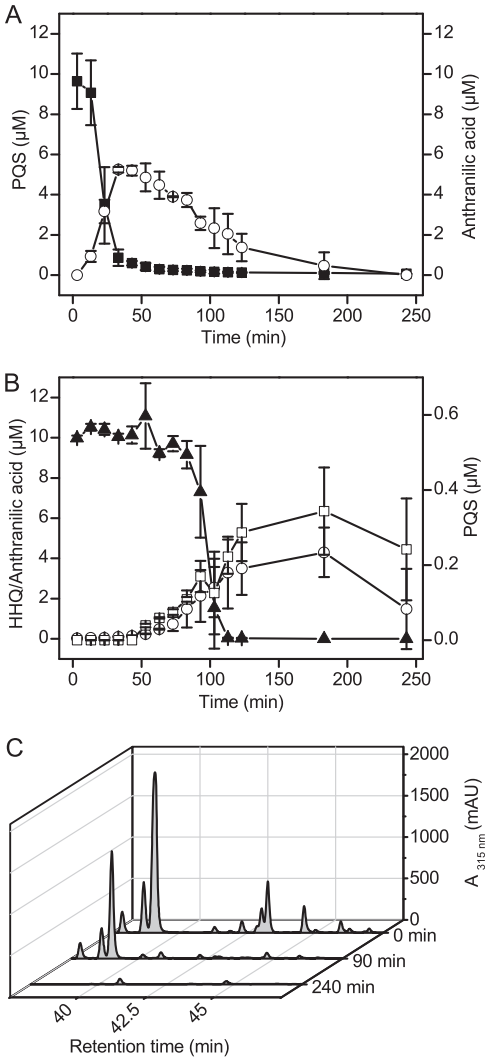

FIG 2 Cometabolic conversion of AQs by *Rhodococcus* sp. BG43. (A and B) Cell suspensions of *Rhodococcus* sp. BG43 (OD_{600} ~ 3) were incubated in modified KG medium with succinate and 20 μM PQS (A) or HHQ (B). The first culture sample was withdrawn and mixed with acidified ethyl acetate 3 min after AQ addition to the cells. The culture samples were extracted with ethyl acetate, and AQs and anthranilic acid in the extracts were quantified by HPLC. Squares, PQS; circles, anthranilic acid; triangles, HHQ. Filled symbols indicate substrates added to cultures, and open symbols indicate intermediates or products formed. Data represent mean values from three independent biological replicates ± standard deviations. (C) HPLC elution profiles of the conversion of an AQ preparation that besides HHQ (major peak at retention time, 39.1 min) additionally contains the *trans* and *cis* isomers of unsaturated HHQ ($C_{7:1}$; at 37.9 min and 38.7 min, respectively), as well as long-chain AQs (C_8-, C_9-, C_{11}-, and C_{13}-AQ at 41.3, 43.2, 46.0, and 47.0 min) and the *cis* and *trans* isomers of their unsaturated congeners ($C_{9:1}$, $C_{11:1}$, $C_{13:1}$; *trans* isomers have shorter retention times than the corresponding *cis* isomers [31]). PQS elutes at 40.2 min (90-min trace).

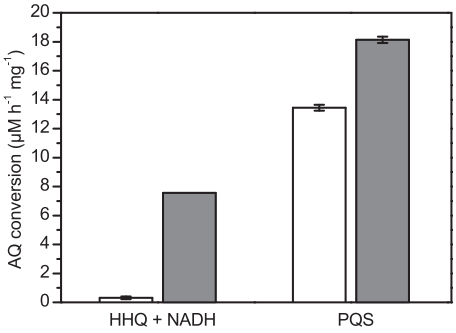

FIG 3 Rates of AQ conversion by cell extracts of *Rhodococcus* sp. BG43 (μM AQ converted per hour and mg total protein). Desalted crude cell extracts (2 mg protein/ml) were incubated with 20 μM HHQ and 500 μM NADH or with 20 μM PQS. White bars represent extracts from cells grown in LB medium, and gray bars represent extracts from LB cultures supplemented with PQS 2 h prior to harvesting. Data represent mean values from two independent biological replicates ± standard errors. Cell extracts treated for 10 min at 99°C did not support AQ conversion.

modified KG medium with succinate, transformed 20 μM PQS within 30 min. PQS conversion was accompanied by formation of an intermediate which showed the same HPLC elution behavior, UV spectrum, and fluorescent properties as authentic anthranilic acid. It accumulated in the culture and was only slowly degraded further (Fig. 2A). When anthranilic acid (0.5 mM) was the only carbon source in modified KG medium, growth of *Rhodococcus* sp. BG43 was not observed. However, it supported growth (OD_{600} of 0.5 after 24 h) when present as the sole source of nitrogen.

MHOQ, the substrate of 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (Hod) of *Arthrobacter* sp. Rue61a (26–28), is very slowly converted by cell suspensions of strain BG43. After 2 and 3 h of incubation, about 50% and 90% of the MHOQ were consumed. Only trace amounts (below 0.1 μM) of anthranilic acid were detected in the cultures during MHOQ conversion.

Cell suspensions of strain BG43 were also able to cometabolically degrade the HHQ signaling molecule. Besides anthranilic acid, PQS was formed at low concentrations during HHQ conversion (Fig. 2B). In cultures without any AQ addition, anthranilic acid was not detected (data not shown). Interestingly, other AQ congeners which were present in the HHQ extracted from biomass of *P. putida* KT2440(pBBR-*pqsABCD*) were also consumed by cell suspensions of strain BG43 (Fig. 2C).

Conversion of PQS and HHQ by crude cell extracts. To get an indication of whether the AQ degradation pathway is inducible, we compared the rates of AQ conversion by crude extracts from *Rhodococcus* sp. BG43 cells grown in LB and extracts from LB-grown cells that were incubated with PQS for 2 h prior to harvesting. As illustrated in Fig. 3, desalted extracts of PQS-induced cells converted HHQ as well as PQS faster than extracts from noninduced cells. PQS conversion to anthranilic acid occurred in the absence of added cosubstrates, whereas HHQ conversion required the addition of NADH. As also observed in the *in vivo* assays (Fig. 2B), HHQ turnover by cell extracts was accompanied by transient formation of PQS (data not shown). When NADH was replaced by NADPH, about 85% of the initial HHQ was still present in the assays after 7 h of incubation, suggesting that the HHQ monooxygenase has a high specificity for NADH. The additional presence of FAD as a possible mediator neither affected the rate of NADH-dependent HHQ turnover nor supported HHQ conversion in the presence of NADPH.

Conversion of HQNO. The viability of strain BG43 in the presence of the quinone oxidoreductase inhibitor HQNO was assessed by monitoring ATP levels in the cultures by measuring luminescence in the BacTiter-Glo assay. When cell suspensions of strain BG43 were cultured in modified KG medium with succinate and in the presence of 20 μM HQNO for 4 h, the luminescence inten-

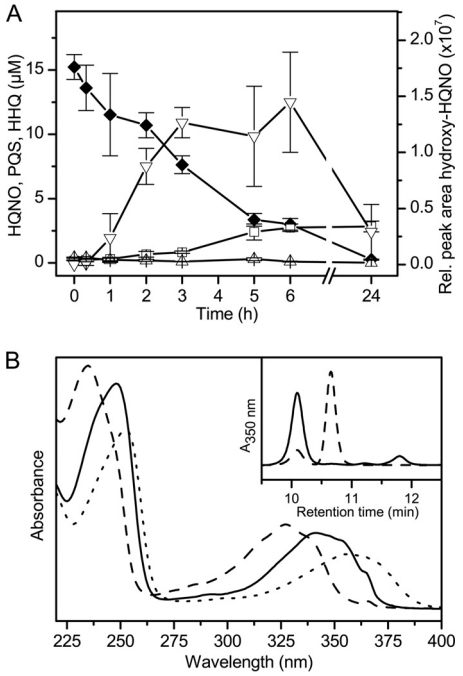

FIG 4 Conversion of HQNO by *Rhodococcus* sp. BG43. (A) Cell suspensions of *Rhodococcus* sp. BG43 ($OD_{600} \sim 3$) were incubated in modified KG medium with succinate and 20 μM HQNO. AQs in ethyl acetate extracts of culture samples were quantified by HPLC. Diamonds, HQNO; squares, PQS; triangles, HHQ; inverted triangles, metabolite identified as a hydroxylated form of HQNO, detected at 350 nm. Filled symbols indicate substrates added to cultures, and open symbols indicate intermediates or products formed. Data represent mean values from three independent biological replicates ± standard deviations. (B) UV absorption spectra (HPLC-diode array detection) of HQNO and the metabolites formed. Spectra of HQNO, PQS, and hydroxy-HQNO are represented by dashed, continuous, and dotted lines, respectively. The inset shows the corresponding HPLC elution profiles of ethyl acetate extracts of culture samples, extracted after 5 min (dashed line) and after 24 h (continuous line). Peaks represent PQS (retention time, 10.1 min), HQNO (at 10.8 min), and hydroxy-HQNO (at 11.8 min).

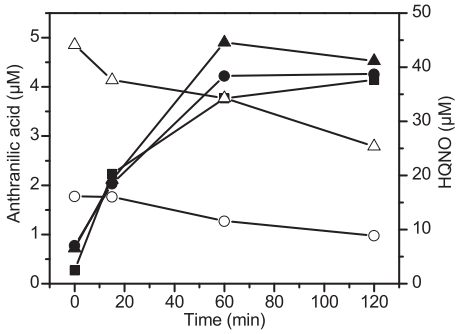

FIG 5 Anthranilic acid formation from PQS by HQNO-treated crude cell extracts. Desalted crude cell extracts (1 mg protein/ml) of PQS-induced cells were preincubated with 500 μM NADH and 0, 20, or 50 μM HQNO for 30 min, and subsequently ($t = 0$ min), 20 μM PQS was added. Samples were extracted with ethyl acetate, and HQNO (open symbols) and anthranilic acid (filled symbols) were quantified by HPLC. Squares, circles, and triangles represent samples from crude extracts preincubated with 0, 20 μM, and 50 μM HQNO, respectively. Data are mean values from two independent experiments.

sities of culture samples were in the same range as those of control cultures without HQNO, suggesting that HQNO at the concentration tested does not affect cell viability. Growth assays performed with modified KG medium with succinate indicated that 20 μM HQNO led to slight growth retardation, which was more pronounced in the presence of 100 μM HQNO. However, after cultivation for 24 h, similar optical densities were reached in cultures supplemented with up to 100 μM HQNO and cultures without HQNO. Growth of strain BG43 was fully inhibited by 300 μM HQNO.

Remarkably, cell suspensions of *Rhodococcus* sp. BG43, pregrown in LB and incubated in modified KG medium under the same conditions as used in the PQS and HHQ biotransformation assays with 20 μM HQNO, were capable of cometabolically converting the *N*-oxide. HQNO was very slowly transformed to PQS (Fig. 4A), identified by HPLC-MS which revealed an *m/z* of 260.14 (for $[C_{16}H_{21}NO_2 + H^+]$). The HPLC retention time and UV spectrum also were identical to those of the authentic PQS reference compound. Minor amounts of a compound which based on its *m/z* of 276.16 (for $[C_{16}H_{21}NO_3 + H^+]$) was identified as a hydroxylated form of HQNO were also detected in the culture extracts (Fig. 4); the UV spectrum of the compound (Fig. 4B) supports the assignment as an AQ congener. However, anthranilic acid was not detected in the extracts.

When desalted crude cell extracts of LB-grown, PQS-induced cells were incubated with 20 μM HQNO and 500 μM NADH, trace amounts of HHQ were detected already after 5 min. After 2 h of incubation, PQS was found in micromolar concentrations (up to 9 μM) in ethyl acetate extracts of the *in vitro* assays, while HHQ was no longer present. Again, anthranilic acid was not detected, and the HPLC elution profiles showed a minor peak of hydroxy-HQNO. NADPH did not support HQNO conversion by the cell extracts. The data indicate that *Rhodococcus* sp. strain BG43 detoxifies HQNO by *N*-oxide reduction and hydroxylation.

To assess whether the apparent accumulation of PQS from HQNO was due to direct inhibition or inactivation of the PQS-converting enzyme by HQNO or a metabolite thereof, desalted crude cell extracts of PQS-induced cells were preincubated with NADH and 0, 20, or 50 μM HQNO, and subsequently PQS was added to each sample. Since the kinetics of anthranilate formation from PQS were not affected by the presence of HQNO (Fig. 5), there is no indication of enzyme inhibition.

DISCUSSION

The soil isolate *Rhodococcus* sp. strain BG43 is capable of degrading the *P. aeruginosa* quorum sensing signaling molecules HHQ and PQS to anthranilic acid (Fig. 6). The $C_{7:1}$ unsaturated congener of HHQ and AQs with C_9- and C_{11}-saturated and unsaturated alkyl chains were also converted. Cell extracts of strain BG43 containing the soluble (cytoplasmic) proteins hydroxylated HHQ to PQS in an NADH-dependent reaction, suggesting that strain BG43 produces a monooxygenase that is isofunctional to the HHQ 3-monooxygenase PqsH of *P. aeruginosa*, which catalyzes the terminal step in PQS biosynthesis (50). PQS conversion to anthranilic acid by desalted crude cell extracts was independent of added cosubstrates. The steps involved remain to be biochemically characterized; however, it is conceivable that they proceed analogously to the conversion of MHOQ in the 2-methylquinoline degradation pathway of *Arthrobacter* sp. Rue61a. In this pathway, the intermediate MHOQ undergoes a dioxygenase-catalyzed

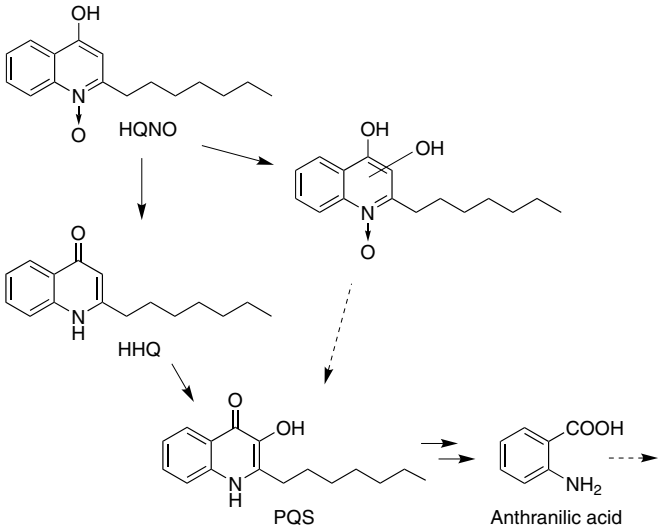

FIG 6 Proposed pathways of HHQ, PQS, and HQNO conversion by *Rhodococcus* sp. BG43.

ring cleavage to carbon monoxide and *N*-acetylanthranilic acid, followed by amide hydrolysis to anthranilic acid and acetate (27).

MHOQ, the physiological substrate of the dioxygenase Hod, which has weak activity toward PQS (26), was also transformed by cell suspensions of *Rhodococcus* sp. BG43, but significantly more slowly than PQS. Assuming that the same enzymes of strain BG43 catalyze the conversion of MHOQ and PQS, they are more specific for PQS. Moreover, in *Rhodococcus* sp. BG43, the pathway of HHQ degradation via PQS appears to be PQS inducible, supporting the hypothesis of AQ-specific rather than fortuitous reactions.

Rhodococcus sp. strain BG43 was isolated from soil collected beneath plants that are known to synthesize quinoline alkaloids. *Ruta graveolens*, for example, produces 2-*n*-nonyl-4(1*H*)-quinolone besides other 4(1*H*)-quinolones (29). Since HHQ and PQS hardly support growth of strain BG43, induction of AQ bioconversion by PQS might suggest that the reactions comprise a specific pathway for the detoxification of structurally related plant alkaloids. AQ transformation might even represent a natural "biocontrol pathway," enabling strain BG43 to interfere with AQ-dependent quorum sensing systems. In this context, it is interesting that among the isolates from the soil sample that yielded strain BG43, two were tentatively assigned to the species *P. aeruginosa*, based on partial 16S rRNA sequences and their ability to synthesize PQS (data not shown), indicating that the *Rhodococcus* sp. and *P. aeruginosa* coexisted in this sample. The identification of the *qsdA* gene in strain BG43, which codes for an AHL lactonase active against a broad range of AHL signal molecules (21, 46, 51), suggests that strain BG43 can also disrupt AHL-based communication, like other *Rhodococcus* strains that contain *qsdA* (21, 23).

Remarkably, *Rhodococcus* sp. BG43 was observed to slowly convert the respiratory inhibitor HQNO to PQS. Thus, *Rhodococcus* sp. BG43 transforms a secondary metabolite of *P. aeruginosa* with antibiotic activity to a *Pseudomonas* QS signal molecule (Fig. 6). Considering the comparatively fast elimination of PQS added to cell suspensions of strain BG43, it is interesting that PQS formed intracellularly from HQNO slowly accumulated to a concentration up to several μM (compare Fig. 2A and 4A). The molecular basis of this effect is not yet known; however, based on the kinetics of anthranilate formation from PQS by HQNO-treated cell extracts, we can exclude the possibility that HQNO or hydroxy-HQNO acts as an inhibitor of the PQS-converting enzyme.

The observation of transient formation of HHQ as well as the identification of PQS in the HQNO bioconversion assays suggests that strain BG43 has an *N*-oxide reductase. While reduction of several organic *N*-oxides by gut bacteria has been described previously (52, 53), we are not aware of a report on reduction of 2-alkyl-4-hydroxyquinoline-*N*-oxides by axenic cultures of aerobic or anaerobic bacteria. In mammals, enzymatic reduction of aromatic *N*-oxides such as quinoxaline-1,4-dioxides, which are used as drugs and animal feed additives, is catalyzed by liver aldehyde oxidase and xanthine oxidoreductase (54–56). Recently, the mitochondrial amidoxime reducing component 1 (mARC1), another mammalian molybdenum enzyme, was reported to catalyze the reduction of nicotinamide-*N*-oxide in the presence of cytochrome b_5 and NADH-cytochrome b_5 reductase (57).

The isolation of *Rhodococcus* sp. BG43 and the identification of reactions for the degradation of AQ-type quorum sensing signaling molecules and for *N*-oxide reduction of the antibacterial compound HQNO open up interesting new perspectives for studying bacterial interspecies interactions, for the biochemical characterization of novel quorum quenching and detoxification enzymes, and for the development of therapeutic agents that target AQ signaling.

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