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Conversion of the *Pseudomonas aeruginosa* Quinolone Signal (PQS) and Related Alkylhydroxyquinolines by *Rhodococcus* sp. strain BG43

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Abstract

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 *R. erythropolis*, was isolated from soil by enrichment on PQS (the *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1*H*)-quinolone), a quorum sensing signal employed by the opportunistic pathogen *Pseudomonas aeruginosa*. The isolate, termed *Rhodococcus* sp. BG43, cometabolically degraded PQS as well as 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.

INTRODUCTION

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 (AQ). 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 refs. 2−4).

Whereas PQS appears to be unique to *P. aeruginosa*, other *Pseudomonas* as well as *Alteromonas* spp. seem to rely on non-hydroxylated 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, in order to target bacterial virulence and to develop new anti-infective therapies (12). Compared with antibiotics therapy, quorum sensing interference has been thought to less likely select for resistance, 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 wide-spread 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,*

60 *Pseudomonas*, *Variovorax*, *Bacillus, Arthrobacter,* and *Rhodococcus* spp., produce lactonases or
61 acylases that hydrolyze AHL signaling molecules (for recent reviews, see refs. 15, 16).

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

73 Whereas numerous reports can be found in the literature on the biodegradation of AHLs,
74 bacteria that degrade AQ-type signaling molecules have not been described so far. The only
75 enzyme known to be able to inactivate an AQ-type QS signal is the dioxygenase Hod (1*H*-3-
76 hydroxy-4-oxoquinaldine 2,4-dioxygenase) from *Arthrobacter* sp. Rue61a, which catalyzes the
77 cleavage of PQS to form *N*-octanoylanthranilate and carbon monoxide (26). However, Hod is an
78 enzyme involved in the 2-methylquinoline degradation pathway of *Arthrobacter* sp. Rue61a, with
79 3-hydroxy-2-methyl-4(1*H*)-quinolone (MHOQ) as its physiological substrate (27, 28), and its
80 comparatively low activity towards PQS is considered as fortuitous.

81 Quinoline and quinolone alkaloids structurally related to the AQ-type signaling molecules
82 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 *P. 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 HPLC also contained minor amounts of other AQs with C_9–C_{13} saturated and unsaturated alkyl side chains. HQNO was purified by preparative HPLC (31). For *in vitro* assays and as 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*, *Poncirus trifoliata*), as well as soil samples collected below spruce, oak and beech trees in forests in the Münster area 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/l $Na_2HPO_4 \times 2H_2O$; 3 g/l KH_2PO_4; 0.5 g/l NaCl; 1 g/l NH_4Cl; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; 15 mg/l $Na_2MoO_4 \times 7H_2O$; 1 ml/l trace element solution (34), supplemented with 50 µM PQS as sole carbon source. Culture samples were diluted into fresh medium every week for six times, even though growth or biofilm formation was hardly (if at all) detectable. Samples taken from the last transfer were spread onto Luria-Bertani agar plates (LB, 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 *E. coli* DH5α [pME6032-*pqsL*] were grown in LB medium at 30°C and 37°C, respectively. 50 µg/ml kanamycin and 50 µg/ml tetracycline were added to cultures of recombinant *P. putida* KT2440, and 12.5 µg/ml tetracycline 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 (8000 × *g*, 5 min, 4°C), washed twice with phosphate buffered saline (PBS), and used to inoculate modified KG medium to an optical density at 600 nm (OD_{600nm}) of 0.05. The modified KG medium (36) contains 1.25 g/l NaCl, 0.75 g/l KCl, 0.25 g/l Na_2SO_4, 0.25 g/l KH_2PO_4 and 1.0 g/l 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: 0.5 ml/l vitamin solution (37), NH_4Cl (0.3 g/l), $MgCl_2$ (0.5 g/l), $CaCl_2$ (0.25 g/l), $FeCl_3$ (5 mg/l), and $MnCl_2$ (2.5 mg/l). For testing the utilization of substrates as nitrogen source, NH_4Cl was omitted from the medium and 1% (wt/vol) succinate was used as 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 OD_{600nm} 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_{600nm} of 3. 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 towards 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 OD_{600nm} 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 Mini Kit 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 (nt 4687652-4688848, 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 *Eco*RI and *Sac*I, was ligated into the

appropriately digested plasmid pME6032 (40), and *E. coli* DH5α was transformed with the pME6032-*pqsL* plasmid. To generate a HQNO producing strain, *P. putida* KT2440 [pBBR-*pqsABCD*] (31) was transformed with pME6032-*pqsL* by electroporation essentially as described in ref. (39), with the following electrical settings: voltage 12.5 kV/cm; capacitor: 25 µF, 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 plating on selective media.

Molecular characterization and phylogenetic analysis of strain BG43. To classify the PQS-converting isolate, the gene encoding 16S ribosomal RNA 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 rDNA, *catA* and *gyrB* sequences using the Neighbor-Joining algorithm were constructed with the 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 (1000 resamplings). The *qsdA* gene encoding a "*Rhodococcus*-specific" AHL lactonase (21) was amplified with the primers qsdA-for and qsdA-rev (46) (Table 1).

Preparation of cell extracts. For preparation of crude cell extracts, *Rhodococcus* sp. BG43 was cultivated in LB medium for 24 h under 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, 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 (7000 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, 10 min) and washed twice with PBS. Subsequently the cells were resuspended in modified KG medium containing 1% (wt/vol) succinate as carbon source, and diluted to an OD_{600nm} 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 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, were 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 sacrificed 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 with 3 × 5 ml and 3 × 0.5 ml, respectively, of acidified ethyl acetate (1 ml acetic acid/l). 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, dried to completion, and the residue was re-dissolved 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 × 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/l 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 on a Dionex™ Ultimate™ 3000 UHPLC system (Thermo Scientific), coupled with an electrospray ionization ion trap mass spectrometer (amaZon Speed, Bruker Daltonics), using a scan range from 50 to 1000 *m/z* (target mass 300 *m/z*). The capillary voltage was set to 4000 V and the capillary temperature to 200°C.

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 the QS signal molecule PQS as 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 8 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 towards 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 sub-culturing 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 (1359 nt) of the 16S rRNA gene of strain BG43 revealed highest levels of sequence identity to 16S RNA 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 marker genes for *Rhodococcus* (43), were compared to those of *Rhodococcus* type species. 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* type species (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, it 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 out 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 aa) shows 99% identity to the corresponding region (aa 18-309) 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 the 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

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_{600nm} ~ 3), incubated in modified KG medium with succinate, transformed 20 μM of 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 ($OD_{600\ nm}$ of 0.5 after 24 hours) when present as 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 non-induced cells. PQS conversion to anthranilic acid occurred in the absence of added co-substrates, 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 possible mediator did neither affect the rate of NADH-dependent HHQ turnover, nor support HHQ conversion in 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 intensities 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 in 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. BG4, pre-grown in LB and incubated in modified KG medium under the same conditions as used in the PQS and HHQ biotransformation assays with 20 µM of 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 µM concentrations (up to 9 µM) in ethyl acetate extracts of the *in vitro* assays, whilst 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 pre-incubated 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 as well as AQs with C_9- and C_{11}- saturated and unsaturated alkyl chains were

342 also converted. Cell extracts of strain BG43 containing the soluble (cytoplasmic) proteins
343 hydroxylated HHQ to PQS in an NADH-dependent reaction, suggesting that strain BG43
344 produces a monooxygenase that is isofunctional to the HHQ 3-monooxygenase PqsH of *P.*
345 *aeruginosa*, which catalyzes the terminal step in PQS biosynthesis (50). PQS conversion to
346 anthranilic acid by desalted crude cell extracts was independent of added cosubstrates. The steps
347 involved remain to be biochemically characterized, however, it is conceivable that they proceed
348 analogous to the conversion of MHOQ in the 2-methylquinoline degradation pathway of
349 *Arthrobacter* sp. Rue61a. In this pathway, the intermediate MHOQ undergoes a dioxygenase-
350 catalyzed ring cleavage to carbon monoxide and *N*-acetylanthranilic acid, followed by amide
351 hydrolysis to anthranilic acid and acetate (27).

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

358 The *Rhodococcus* sp. strain BG43 was isolated from soil collected beneath plants that are
359 known to synthesize quinoline alkaloids. *Ruta graveolens*, for example, produces 2-*n*-nonyl-
360 4(1*H*)-quinolone besides other 4(1*H*)-quinolones (29). Since HHQ and PQS hardly support
361 growth of strain BG43, induction of AQ bioconversion by PQS might suggest that the reactions
362 comprise a specific pathway for the detoxification of structurally related plant alkaloids. AQ
363 transformation might even represent a natural "biocontrol pathway", enabling strain BG43 to
364 interfere with AQ-dependent quorum sensing systems. In this context, it is interesting that among
365 the isolates from the soil sample that yielded strain BG43, two were tentatively assigned to the

366 species *P. aeruginosa,* based on partial 16S rRNA sequences and their ability to synthesize PQS
367 (data not shown), indicating that *Rhodococcus* sp. and *P. aeruginosa* coexisted in this sample.
368 The identification of the *qsdA* gene in strain BG43, which codes for an AHL lactonase active
369 against a broad range of AHL signal molecules (21, 46, 51), suggests that strain BG43 can also
370 disrupt AHL-based communication, like other *Rhodococcus* strains that contain *qsdA* (21, 23).
371 Remarkably, *Rhodococcus* sp. BG43 was observed to slowly convert the respiratory
372 inhibitor HQNO to PQS. Thus, *Rhodococcus* sp. BG43 transforms a secondary metabolite of *P.*
373 *aeruginosa* with antibiotic activity to a *Pseudomonas* QS signal molecule (Fig. 6). Considering
374 the comparatively fast elimination of PQS added to cell suspensions of strain BG43, it is
375 interesting to note that PQS formed intracellularly from HQNO slowly accumulated to up to
376 several μM (compare Figs. 2A and 4A). The molecular basis of this effect is not yet known,
377 however, based on the kinetics of anthranilate formation from PQS by HQNO-treated cell
378 extracts, we can exclude the possibility that HQNO or hydroxy-HQNO act as inhibitor of the
379 PQS-converting enzyme.
380 The observation of transient formation of HHQ as well as the identification of PQS in the
381 HQNO bioconversion assays suggest that strain BG43 has an *N*-oxide reductase. While reduction
382 of several organic *N*-oxides by gut bacteria has been described (52, 53), we are not aware of a
383 report on reduction of 2-alkyl-4-hydroxyquinoline-*N*-oxides by axenic cultures of aerobic or
384 anaerobic bacteria. In mammalians, enzymatic reduction of aromatic *N*-oxides such as
385 quinoxaline-1,4-dioxides, which are used as drugs and animal feed additives, is catalyzed by liver
386 aldehyde oxidase and xanthine oxidoreductase (54−56). Recently, the mitochondrial amidoxime
387 reducing component 1 (mARC1), another mammalian molybdenum enzyme, was reported to
388 catalyze the reduction of nicotinamide-*N*-oxide in the presence of cytochrome b_5 and NADH-
389 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 opens up interesting new perspectives for studying bacterial inter-species 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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Figure legends

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 (1000 resamplings).

Fig. 2: Cometabolic conversion of AQs by *Rhodococcus* sp. BG43. Cell suspensions of *Rhodococcus* sp. BG43 (OD_{600nm} ~ 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, 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* isomer 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}-, C_{13}-AQ at 41.3, 43.2, 46.0 and 47.0 min) and the *cis*/*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, gray bars represent extracts from LB cultures supplemented with PQS 2 hours prior to harvesting. Data represent mean values from two independent biological replicates ± standard error. Cell extracts treated for 10 min at 99 °C did not support AQ conversion.

Fig. 4: Conversion of HQNO by *Rhodococcus* sp. BG43. **A**: Cell suspensions of *Rhodococcus* sp. BG43 (OD_{600nm} ~ 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; up-pointing triangles: HHQ; down-pointing triangles: metabolite identified as a hydroxylated form of HQNO, detected at 350 nm. Filled symbols indicate substrate added to cultures, 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 (dotted line) and after 24 hours (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 pre-incubated with 500 µM

NADH and 0, 20 or 50 µM of HQNO for 30 min, and subsequently (at t=0 min) 20 µM of 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 pre-incubated with 0, 20 µM, and 50 µM of HQNO, respectively. Data are mean values from two independent experiments.

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

626 **Table 1**: Primers used in this study.

Primer designation	**Sequence 5'→3'**	**Application**	**Reference**
pqsL-for	ATATGAGCTCTCA*G*TGGTGGT GGTGGTGGTGGCCGAGCGGC GCCGGCGACCGCACCGGCTG	Amplification of *pqsL* (nt 4687652-4688848 of *P. aeruginosa* PAO1)	This study
pqsL-rev	ATATGAATTCATGACGGACAA CCATATCGATGTACTGATC		
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		

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Table 2: Growth of *Rhodococcus* sp. BG43 and closely related type strains on selected carbon sources. Data for the type strains are from references (48, 49). Strain BG43 was cultured in modified KG medium with shaking at 30 °C. D-fructose, lactose, glycerol, D-mannose, D-xylose or *myo*-inositol: 2% (wt/vol); sucrose: 4 mM; D-sorbitol: 1% (wt/vol); catechol: 1%, 0.5% or 0.1% (wt/vol). OD_{600nm} was measured after 24 hours of incubation. +: $OD_{600nm} > 0.5$; w: weak, $OD_{600nm} < 0.5$; −: no growth observed.

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	+	+	−[a]/ +[b]	−	+
Catechol	−	−	−	+	−
Lactose	−	−	−	−	−
Glycerol	+	+	+	+	+
D-Mannose	w	−	w	+	+
D-Xylose	w	−	−	−	+
myo-Inositol	+	+	−	−	−

[a] (48); [b] (49)