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Involvement of Multiple Loci in Quorum Quenching of Autoinducer I Molecules in the Nitrogen-Fixing Symbiont *Rhizobium* (*Sinorhizobium*) sp. Strain NGR234▽†

D. Krysciak,[1] C. Schmeisser,[1] S. Preuß,[1] J. Riethausen,[1] M. Quitschau,[2] S. Grond,[2] and W. R. Streit[1]*

Abteilung für Mikrobiologie und Biotechnologie, Biozentrum Klein Flottbek, Universität Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany,[1] and Institut für Organische Chemie, Eberhard Karls Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany[2]

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***Rhizobium* sp. strain NGR234 is a unique alphaproteobacterium (order *Rhizobiales*) that forms nitrogen-fixing nodules with more legumes than any other microsymbiont. Since we have previously described the complete genome sequence of NGR234, we now report on a genome-wide functional analysis of the genes and enzymes involved in autoinducer I hydrolysis in this microbe. Altogether we identified five cosmid clones that repeatedly gave a positive result in our function-based approach for the detection of autoinducer I hydrolase genes. Of these five cosmid clones, two were located on pNGR234*b* and three were on cNGR234. Subcloning and *in vitro* mutagenesis in combination with BLAST analyses identified the corresponding open reading frames (ORFs) of all cosmid clones: *dlhR*, *qsdR1*, *qsdR2*, *aldR*, and *hitR-hydR*. Analyses of recombinant DlhR and QsdR1 proteins by using high-performance liquid chromatography-mass spectrometry (HPLC-MS) demonstrate that these enzymes function as acyl homoserine lactone (AHL) lactonases. Furthermore, we showed that these enzymes inhibited biofilm formation and other quorum-sensing-dependent processes in *Pseudomonas aeruginosa*, *Chromobacterium violaceum*, and *Agrobacterium tumefaciens*. Finally, our experimental data suggest that competitive colonization of roots in the rhizospheres of cowpea plants is affected by DlhR and QsdR1.**

Quorum sensing (QS) is a cell density-dependent system of gene regulation in prokaryotes. Through the accumulation of bacterially produced signaling molecules (autoinducers), the bacterial population is able to sense increases in cell density and alter gene expression accordingly (55). This allows coordinated expression of genes at the population level which are most effective at higher cell densities, such as those for pathogenicity, biofilm formation, production of extracellular proteins, and others. For an excellent review on interspecies signaling, see reference 41. Many quorum-sensing mechanisms involve *N*-acyl homoserine lactones (*N*-AHLs) in Gram-negative bacteria. While the general mechanisms of the synthesis of *N*-AHLs are well understood, it is quite intriguing that only a limited number of proteins that interfere with these bacterial quorum-sensing molecules via enzymatic hydrolysis are known. Three main types of enzymes act on autoinducer I (AI-I) molecules. (i) Lactonases, which hydrolyze the lactone ring moiety in a reversible way, form the major class of enzymes identified to date (11, 34, 35). More recently several novel lactonases have been reported: AidH (28), AiiM (54), and BpiB05 (3). (ii) Acylases are known to interfere with the autoinducer I-like molecules by cleaving the acyl side chain off the homoserine lactone moiety. Acylases have been identified in a variety of microorganisms, such as *Comamonas* spp. (50), *Pseudomonas aeruginosa* (20), *Pseudomonas syringae* (42), *Ralstonia* spp. (24), *Rhodococcus erythropolis* (49), *Shewanella* spp. (29), and *Streptomyces* spp. (31). (iii) Oxidoreductases are enzymes that target the acyl side chain by oxidative or reducing activities and thus catalyze the chemical modification of *N*-AHLs and not their degradation. To date only two such enzymes with oxidoreductase activity have been discovered. A P-450/NADPH-P450 reductase has been isolated from *Bacillus megaterium* and characterized in detail (6, 7). The respective AHL-oxidizing enzyme was designated CYP102A1, and it was able to hydrolyze both AHLs and fatty acids with various chain lengths. In addition, Uroz and colleagues reported the presence of another enzyme in crude cell extracts of *R. erythropolis* W2 (49). They demonstrated that the 3-oxo substituent of 3-oxo-C_{14}-homoserine lactone (3-oxo-C_{14}-HSL) was reduced to yield the corresponding derivative, 3-hydroxy-C_{14}-HSL, and this was also observed for 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL. The autoinducer was thereby inactivated.

Rhizobium sp. strain NGR234 (referred to here as NGR234) is able to nodulate more than 120 genera of legumes (33). The complete genome analysis identified two loci linked to the synthesis of autoinducer I molecules. TraI synthesizes an acyl-HSL that is probably *N*-3-oxooctanoyl-L-homoserine lactone (17), and *ngrI* probably encodes a coumestrol derivative of the autoinducer I type molecules (40).

In this study, we demonstrate that NGR234 carries a surprisingly large number of functional genes involved in the degradation of *N*-AHLs. Using a recently published function-based screening protocol (39), we identified at least five loci (three on the chromosome and two on pNGR234*b*) that are actively involved in *N*-AHL degradation or modification. Overexpression, purification, and biochemical characterization were

* Corresponding author. Mailing address: Abteilung für Mikrobiologie und Biotechnologie, Biozentrum Klein Flottbek, Universität Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany. Phone: (49) 40-42816463. Fax: (49) 40-42816459. E-mail: wolfgang.streit@uni-hamburg.de.

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TABLE 1. Bacterial strains, plasmids, and constructs used in this study

Strain or construct	Description	Reference(s) or source
EPI100-T1 phage T1-resistant *E. coli*	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	Epicentre Technologies, Madison, WI
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]	Stratagene, La Jolla, CA
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*(DE3)	Novagen, Darmstadt, Germany
Rhizobium sp. strain NGR234	Wild-type New Guinea isolate; Rif^r	47
Pseudomonas aeruginosa PAO1	Wild-type strain of *P. aeruginosa*; Amp^r	19
Agrobacterium tumefaciens NTL4(pCF218)(pCF372)	Reporter strain for AHL detection; *traI*::*lacZ* Tet^r Sp^r	14, 15, 25
Chromobacterium violaceum CV026	Reporter strain for autoinducer I; mini-Tn*5* in *cviI*	27
pWEB-TNC	Cosmid cloning vector derived from pWE15; linearized with SmaI; ColE1; *cos* site; T7 promoter; Amp^r Cm^r	Epicentre Technologies, Madison, WI
pWEB-TNC-A5	pWEB-TNC with a 40.4-kb insert from NGR234	This study
pWEB-TNC-B2	pWEB-TNC with a 34.0-kb insert from NGR234	This study
pWEB-TNC-B9	pWEB-TNC with a 42.0-kb insert from NGR234	This study
pWEB-TNC-C6	pWEB-TNC with a 37.7-kb insert from NGR234	This study
pWEB-TNC-G2	pWEB-TNC with a 33.3-kb insert from NGR234	This study
pET21a	*E. coli* His_6-tagged expression vector; Amp^r	Novagen, Darmstadt, Germany
pET21a::*dlhR*	pET21a containing *dlhR* gene cloned into NdeI and HindIII restriction sites	This study
pET21a::*qsdR1*	pET21a containing *qsdR1* gene cloned into NdeI and XhoI restriction sites	This study
pET21a::*gtfU*	pET21a containing *gtfU* gene cloned into NdeI and HindIII restriction sites; served as control	This study
pBBR1MCS	Broad-host-range vector; low copy; Cm^r	22
pBBR1MCS-5	Broad-host-range vector; low copy; Gm^r	22
pBBR1MCS::P_{taq}::His-*dlhR*	pBBR1MCS containing the His_6 tag-*dlhR* fragment amplified by T7 promoter/T7 terminator primer from pET21a::*dlhR*	This study
pBBR1MCS::P_{taq}::His-*qsdR1*	pBBR1MCS containing the His_6 tag-*qsdR1* fragment amplified by T7 promoter/T7 terminator primer from pET21a::*qsdR1*	This study

carried out for two of the five proteins and showed that one was similar to a metal-dependent β-lactamase while the other one resembled a bacterial dienelactone hydrolase. The remaining three loci, identified to be responsible for quorum-quenching (QQ) or AI-I-modifying activity, encode a metal-dependent β-lactamase, an acetaldehyde dehydrogenase, and a putative histidine triad protein linked with a predicted NUDIX hydrolase. Furthermore, we were able to show that extra copies of the *dlhR* and *qsdR1* genes strongly affect plant root colonization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Microbiological materials used in the present work are listed in Table 1. *Escherichia coli* strains and *Pseudomonas aeruginosa* PAO1 were grown at 37°C in Luria-Bertani medium (37) supplemented with appropriate antibiotics. NGR234 was cultivated in yeast extract-mannitol medium (YEMA) (52) or TY medium (37) at 30°C. *Agrobacterium tumefaciens* NTL4 (14), carrying a *traI*::*lacZ* promoter fusion, was grown in LB or AT medium (46) containing 0.5% glucose per liter at 28°C. *Chromobacterium violaceum* CV026 was cultivated in LB or TY medium at 28°C.

Unless otherwise specified, media were supplemented with antibiotics, as required, at the following final concentrations: for *E. coli* containing pET21a and pWEB-TNC cosmid clones and *P. aeruginosa* PAO1 cultures, ampicillin at 100 μg/ml; for *A. tumefaciens* NTL4 cultures, spectinomycin at 50 μg/ml and tetracycline at 4.5 μg/ml; for *E. coli* and NGR234 containing pBBR1MCS and related constructs, chloramphenicol at 50 μg/ml; for *E. coli* and NGR234 containing pBBR1MCS-5, gentamicin at 10 μg/ml; and for NGR234 cultures, rifampin at 25 μg/ml.

Transformation and electroporation procedures. Plasmid or cosmid transformation in *E. coli* XL1-Blue and *E. coli* BL21(DE3) was done following standard heat shock and electroporation protocols (37). NGR234 was transformed by electroporation using a protocol for *Rhizobium leguminosarum* (16) with minor modifications.

NGR234 cosmid library construction. NGR234 was grown overnight in 30 ml of TY medium with rifampin. The genomic DNA of NGR234 was isolated with the AquaPure kit (Bio-Rad Laboratories, Hercules, CA). For the construction of the NGR234 genomic cosmid library, the pWEB-TNC cosmid cloning kit (Epicentre Biotechnologies, Madison, WI) was used, with the provided protocol modified as follows. Shearing of genomic DNA was accomplished by partial digestion with Bsp143I (Sau3AI), and the end repair reaction mixture was dialyzed against water for 2 h. The ligation products were packaged using Gigapack III Gold packaging extract (Stratagene, La Jolla, CA) as recommended by the manufacturer and recovered by transfection into EPI100-T1 phage-resistant *E. coli* cells (referred to here as EPI100). The cells were spread on LB agar medium with ampicillin and incubated overnight, and colonies were transferred into 96-well microtiter plates containing 150 μl liquid LB medium with ampicillin and allowed to grow overnight. Microtiter plates were stored at −70°C after addition of 50 μl of 86% glycerol. A total of 603 cosmid clones were generated.

Screening of the NGR234 genomic library for *N*-AHL-degrading clones and their genetic analysis. For the identification of cosmid clones capable of inactivating AHLs or of blocking AHL receptors/promoters, the biosensor strain *A. tumefaciens* NTL4 was used as previously described (3, 39, 56). This strain carries a *traI*::*lacZ* reporter and does not synthesize autoinducer, and it therefore is capable of reporting the presence of *traI*-inducing metabolites. Monitoring of a possible altered *P. aeruginosa* PAO1 motility phenotype triggered by positive tested cosmid clones and recombinant proteins was accomplished by motility assays as previously described (3, 39). The 33- to 42-kb large inserts of positive tested cosmid clones were end sequenced using M13_for and T7 promoter primers (see Table S1 in the supplemental material). For the detection of open reading frames (ORFs) involved in quorum-sensing inhibition, either subcloning, *in vitro* transposon mutagenesis, or direct cloning was employed. For subcloning, either EcoRI (Fermentas, St-Leon-Rot, Germany) fragments of cosmid clones were ligated with T4 DNA ligase (Promega, Mannheim, Germany) into EcoRI-

linearized pTZ19R vector or *Taq* polymerase-amplified genes of interest were ligated into pDrive cloning vector (Qiagen [Hilden, Germany] PCR cloning kit) using the primer pairs indicated in Table S1 in the supplemental material and subjected further analyses. For *in vitro* transposon mutagenesis, the EZ-Tn*5* <KAN-2> insertion kit (Epicentre Biotechnologies, Madison, WI) was used according to the manufacturer's protocol. ORFs were amplified using the primer pairs indicated in Table S1 in the supplemental material and directly cloned into pET21a. The amplicon and the vector were digested with NdeI and HindIII for *dllhR* or with XhoI for *qsdR1* and ligated directionally, yielding pET21a::*dllhR* and pET21a::*qsdR1*, respectively. All clones obtained by subcloning, transposon mutagenesis, or direct cloning were assayed by *A. tumefaciens* NTL4 screening and motility assays with *P. aeruginosa* PAO1 as described above. For sequencing of plasmids and constructs, the T7 promoter/T3 promoter/T7 terminator/M13_20/M13_rev primers were used. Automated sequencing was performed with an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA), based on the Sanger technique. Nucleotide and amino acid sequence comparisons were accomplished using the BLAST program (1) and the publically available databases SwissProt, GenBank, KEGG, and ProSite. Multiple-sequence alignments were performed using BioEdit.

Purification of His-tagged proteins. For preparation of purified His_6-tagged proteins, constructs pET21a::*dllhR* and pET21a::*qsdR1* were transformed into *E. coli* BL21(DE3) cells. Precultures (50 ml) were cultivated in LB medium with ampicillin overnight at 37°C. The precultures were used to inoculate 1-liter LB main cultures, where the optical density at 600 nm (OD_{600}) was adjusted to 0.1. The expression cultures were grown at 37°C to an OD_{600} of 0.6 to 0.8, expression was induced by addition of 100 μM IPTG (isopropyl-β-D-1-thiogalactopyranoside), and the main cultures were incubated overnight at 17°C with shaking at 140 rpm. Cells were harvested by centrifugation at 10,000 rpm and 4°C for 20 min and resuspended in 1× LEW buffer (Macherey-Nagel, Dueren, Germany) prior to disruption through ultrasonication (3 times for 10 min with 2-min cooling breaks). The lysate was centrifuged at 13,000 rpm and 4°C for 30 min, and the obtained supernatant was then purified using Protino Ni-TED 2000 packed columns (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol. To remove imidazole from eluted proteins, the eluted proteins were dialyzed against a 200-fold volume of 100 mM potassium phosphate buffer with pH values varied according to the protein being purified (for *dllhR*, pH 7.5, and for *qsdR1*, pH 7.0). The protein purities and molecular masses were determined by SDS-gel electrophoresis.

β-Galactosidase activity assay using reporter strain *A. tumefaciens* NTL4. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) tests were performed as previously described (39) with minor modifications; 5 μl of a 10^{-8} M solution of 3-oxo-C_8-HSL was added to 1 to 100 μl of purified protein extracts (400 μg/ml) and incubated at 30°C in 100 mM potassium phosphate buffer at pH 7.0.

HPLC-MS analysis. High-pressure liquid chromatography–mass spectrometry (HPLC-MS) tests were done as previously published (3, 39) with minor modifications as follows. For chemical analysis, a 3-oxo-C_8-HSL stock solution was prepared in dimethyl sulfoxide (DMSO) (0.2 mg 3-oxo-C_8-HSL/μl DMSO). Protein extracts were purified from *E. coli* BL21(DE3) expressing *dllhR* and *qsdR1* genes as described above. Protein amounts of 0.005 to 0.3 mg/ml were mixed with 10 μl of the 3-oxo-C_8-HSL stock solution in 100 mM potassium phosphate buffer (for *dllhR*, pH 7.5, and for *qsdR1*, pH 7.0). After incubation for 20 h at 30°C, the mixtures were extracted twice with 1 volume ethyl acetate, and the combined organic layers were concentrated *in vacuo*. For a detailed description of the subsequent HPLC-MS analysis, see references 10 and 39.

Rhizosphere colonization experiments. Rhizosphere colonization tests were done as previously described (45). Cowpea seeds (*Vigna unguiculata* subsp. *unguiculata*) were treated for 15 min in 70% ethanol and rinsed three times with sterile double-deionized water. These sterilized seeds were placed on 0.5× TY agar (lacking $CaCl_2$) and germinated at 30°C. After 24 h, germinating seeds with no visible contamination were transferred into sterile 50-ml Falcon tubes (Fisher Scientific GmbH, Schwerte, Germany) filled with ~10 g autoclaved vermiculite and 5 ml 0.25× Hoagland solution (18). NGR234 inocula consisted of NGR234 cells harboring either pBBR1MCS-5 (referred to here as pBBR-5) as a control or constructs pBBR1MCS::P_{taq}::His-*dllhR* and pBBR1MCS::P_{taq}::His-*qsdR1* (referred to here as pBBR::*dllhR*/pBBR::*qsdR1*). NGR234 inocula were grown in 250 ml YEMA medium with appropriate antibiotics, and cell densities were determined by OD_{600} and adjusted in fresh medium to either 10^3 cells/ml or 10^5 cells/ml. One hundred microliters of these bacterial suspensions was used to inoculate the 24-h-old seedlings. Bacterial solutions of the constructs and the control strain were used in combination (1:1) or as single-strain inocula. The germinated and inoculated seedlings were incubated in a light- and humidity-controlled incubator (Rumed Rubarth Apparate GmbH, Laatzen, Germany) under controlled conditions (day/night: 24/19°C, 16/8 h, and 50% relative humidity) for 4 days. Roots were harvested after this time period, shaken to remove vermiculite, cut into segments, and placed in 1.5-ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany) containing 1 ml of distilled water with 0.01% Tween 20. Appropriate dilutions were plated on YEMA agar containing Congo red and the required antibiotics.

RESULTS

Sequence-based identification of QQ genes in NGR234 that are involved in AHL degradation. In order to identify possible genes and proteins interfering with autoinducer I (AI-I) molecules, we performed a detailed analysis of the NGR234 genome data (40). The results of these searches are summarized in Table 2. A total of 18 possible QQ genes were identified by careful BLAST analyses and searches for conserved motifs. Only five of these 18 ORFs were similar to previously identified autoinducer hydrolase genes. The observed identities for the known QQ genes ranged from 24% to 34% (Table 2). Eleven of these genes were grouped as metal-dependent hydrolases, including seven proteins belonging to the metallo-β-lactamase family. The products of four ORFs were annotated as putative β-lactamase family proteins and one as a metal-dependent phosphohydrolase. Altogether, 13 of these proteins shared a conserved zinc-binding motif of previously published AHLases. This "HxHx~DH" pattern is found in several groups of metallohydrolases, including metallo-β-lactamases, glyoxalases II, and arylsulfatases, and is probably essential for AHL-degrading activity (12). Furthermore, two ORFs were similar to those for dienelactone hydrolases. In addition to the ORFs given in Table 2, we identified five other proteins encoded within the NGR234 genome sequence which may be involved in QQ processes. These proteins were annotated as putative amidohydrolases and are designated NGR_c01920, NGR_c18520, NGR_c27330, NGR_c28580, and NGR_c33720 (data not shown). Additionally, we identified 14 genes and ORFs (see Table S2 in the supplemental material) that encoded possible hydrolases with no clear function assigned.

Function-based identification of QQ loci in NGR234 involved in AHL degradation. Besides the sequence-based screening, we used a function-based approach for QQ genes to ensure identification of all potential genes involved in quenching of the bacterial autoinducer I signaling molecule. To functionally identify the genes and ORFs that were involved in QQ in NGR234 *in vivo*, we employed a previously published function-based screening technology (39). For this purpose we constructed an NGR234 cosmid clone library with insert sizes ranging from 25 to 42 kb. In the initial screen, 603 cosmid clones were tested directly in the EPI100 host using the above-mentioned screening protocol, including the *A. tumefaciens* NTL4 biosensor strain carrying a *traI*::*lacZ* reporter gene and ATsoft agar supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 3-oxo-C_8-HSL. This screening was repeated at least three times. Accumulated data from several repetitions of the function-based screening identified five clones that consistently gave a positive result. All five clones reproducibly stayed colorless in the biosensor assay and displayed a deviant phenotype compared to negative controls. At this stage the five cosmid clones were end sequenced and compared with the complete NGR234 genome sequence. All clones carried inserts ranging from 33 to 42 kb; three of the cosmid clones were mapped on cNGR234 (i.e., pWEB-

TABLE 2. Possible and verified QQ loci identified in the NGR234 genome

ORF[a]	Possible and/or annotated function	Conserved HxHx~DH motif	Size of protein (no. of amino acids)	Identity/similarity (%) to published QQ protein[b]
NGR_c10650	Metallo-β-lactamase family protein	+	556	
NGR_b16870	Putative metallo-β-lactamase family protein QsdR1	+	321	Aii2 (24/39)
NGR_b15850	Metallo-β-lactamase family protein	+	312	AhlK (34/44)
NGR_b12260	Metallo-β-lactamase family protein	+	277	
NGR_c22830	Putative β-lactamase family protein	+	214	
NGR_c27960	Metal-dependent hydrolase	−	280	
NGR_c05660	Metallo-β-lactamase family protein	+	304	
NGR_c03760	Metallo-β-lactamase family protein	+	383	Aii2 (27/44)
NGR_c05950	Putative β-lactamase family protein	+	254	AhlD (30/47)
NGR_c16020	Putative β-lactamase family protein QsdR2	+	279	
NGR_c00430	Putative metal-dependent hydrolase	−	174	
NGR_c08460	Metal-dependent phosphohydrolase	−	219	
NGR_c06480	Metal-dependent hydrolase	+	289	QlcA (27/49)
NGR_c32270	Putative β-lactamase family protein	+	336	
NGR_c17430	Metallo-β-lactamase family protein	+	306	
NGR_c10940	Metal-dependent hydrolase	+	235	
NGR_b22150	Dienelactone hydrolase-like protein DlhR	−	358	
NGR_c03800	Dienelactone hydrolase-like protein	−	292	
NGR_c23150[c]	Acetaldehyde dehydrogenase AldR	−	503	
NGR_c35560	Predicted NUDIX hydrolase (putative)	−	346	
NGR_c35570	Histidine triad protein, *hitR-hydR*	−	202	

[a] ORF identification numbers for NGR234 were derived from the GenBank entries U00090, CP000874, and CP001389. Genes/ORFs in bold were verified by functional searches in this work.

[b] Identity and similarity values were obtained using the NCBI database. Only identities and similarities to the best hit are given: Aii2 from an uncultured *Bacillus* sp. (accession no. CAD44268), AhlK from *Klebsiella pneumonia* (accession no. AAO47340), AhlD from *Arthrobacter* sp. strain IBN110 (accession no. AAP57766), and QlcA from uncultured *Acidobacteria*, cosmid p2H8 (accession no. ABV58973).

[c] The gene/ORF could be identified only by functional searches and was not found in the initial sequence-based searches.

TNC-A5, pWEB-TNC-B9, and pWEB-TNC-G2) and two on pNGR234*b* (pWEB-TNC-B2 and pWEB-TNC-C6) (Fig. 1). The insert sizes, location on the chromosome or on pNGR234*b*, and number of ORFs located on these cosmids are given in Table S3 in the supplemental material.

Crude cell extracts of cosmid clones affect motility, biofilm formation, and pyocyanin production in *P. aeruginosa* PAO1. For the further verification of the influence of the isolated cosmids on QS-dependent processes in other Gram-negative bacteria, we used *P. aeruginosa* PAO1 motility assays, as motility such as swarming/swimming or biofilm formation is QS dependent in PAO1 (21, 43, 53). Crude cell extracts were obtained from *E. coli* XL1-Blue cells harboring the cosmids pWEB-TNC-A5, -B2, -B9, -C6, and -G2. Crude cell extracts (5 to 100 μg) of cosmids were added to autoclaved and cooled swarming agar and inoculated with *P. aeruginosa* PAO1. The swarming ability of the PAO1 isolate was strongly altered after incubation for 16 h with crude cell extracts of cosmid clones. All five clones could significantly reduce the swarming motility of PAO1 and additionally alter the phenotypic appearance of colonies on the plates. The control, which was *E. coli* XL1-Blue carrying a randomly chosen negatively tested cosmid, was treated like the other five cosmids and incubated with PAO1. However, PAO1 was not affected in its swarming capabilities by the control (Fig. 2A). Further tests measuring the ability of PAO1 to form biofilms on glass surfaces suggested that the crude cell extracts of the cosmids pWEB-TNC-A5, -B2, -B9, -C6, and -G2 influenced biofilm formation. For this test, 5 to 100 μg of crude cell extracts of the cosmids and the control was added to exponentially growing cells of PAO1. In all test tubes supplemented with protein extracts from the five cosmid clones, a less developed biofilm formation was visible; no such effect was observed with the controls (Fig. 2B). Together these data further suggested that crude cell extracts of the isolated cosmid clones interfered with bacterial QS activities. Furthermore, crude cell extracts of these cosmid clones significantly reduced pyocyanin production in PAO1 (data not shown). The blue compound pyocyanin produced by *P. aeruginosa* PAO1 is also regulated by QS-dependent processes (38).

Identification of possible QQ ORFs and similarities to known QQ proteins. For the identification of the genes responsible for the observed phenotypes in the *A. tumefaciens* NTL4 bioassay, a detailed analysis of the insert sequences of these cosmid clones in combination with *in vitro* transposon mutagenesis and subcloning was performed. Transposon mutagenesis was accomplished for pWEB-TNC-G2, subcloning of random EcoRI restriction fragments of cosmids into pTZ19R vector was carried out for pWEB-TNC-B2, and specific subclones of PCR-amplified genes of interest were established for pWEB-TNC-C6, -A5, and -B9 in the pDrive or pTZ19R cloning vector. In the latter cases only the genes of interest and no additional DNA was cloned. All subclones constructed from the pWEB-TNC-A5, B2, -B9, and -C6 cosmids or mutants obtained from pWEB-TNC-G2 were analyzed by functional assays using *A. tumefaciens* NTL4 and the *C. violaceum* reporter strain CV026. Together these tests identified the *dlhR* gene on cosmid pWEB-TNC-B2 on a 4.4-kb EcoRI fragment, the *qsdR1* gene on pWEB-TNC-C6, the *aldR* gene on pWEB-TNC-G2, and the *qsdR2* gene on pWEB-TNC-B9 as responsible for the observed phenotypes. On pWEB-TNC-A5, the *hitR-hydR* locus was associated with the observed phenotype. The identified genes and their flanking regions are depicted in Fig.

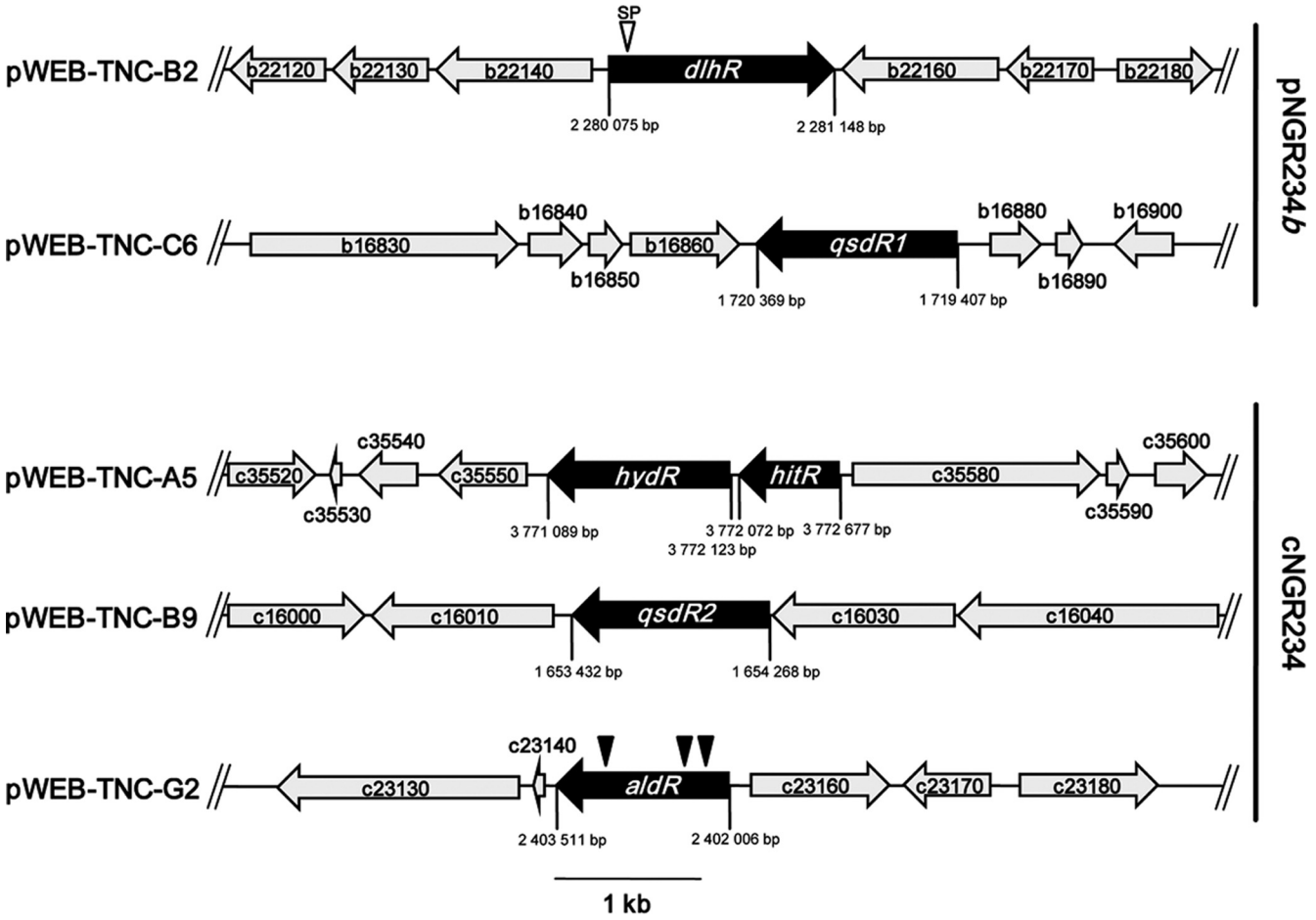

FIG. 1. Identification of NGR234 AHL-degrading cosmid clones. Partial physical maps of the identified cosmid clones pWEB-TNC-B2, -C6, -A5, -B9, and -G2 carrying the putative genes *dlhR*, *qsdR1*, *hitR-hydR*, *qsdR2*, and *aldR* are shown. Cosmid clones pWEB-TNC-B2 and -C6 were mapped on pNGR234*b*, whereas cosmids pWEB-TNC-A5, -B9, and -G2 were located on the bacterial chromosome. Black arrows indicate the ORFs that were involved in QQ. Gray-shaded arrows indicate flanking ORFs that were not linked to the QQ phenotypes. ORFs are designated using ORF identification numbers from GenBank entries CP000874 and CP001389 (prefix NGR_~). The scale bar represents 1 kb. A possible secretion signal (SP) in *dlhR* is indicated by an inverse open triangle. Positions of *in vitro* transposon insertions in the identified QQ genes are indicated by filled and inverse triangles.

1 and indicated in Table 2. These data suggested that NGR234 probably carries at least five gene loci involved in modification or possible degradation of the AI-I signaling molecules.

The NGR234 ORF NGR_b16870 (960 bp) encodes a 321-amino-acid protein designated QsdR1 (for quorum sensing signal degrading enzyme from *Rhizobium* sp. strain NGR234). The translational start codon of QsdR1 is preceded by a possible ribosome-binding site AGAGGA, and possible −10 as well as −n sequences were identified according to previously reported rhizobial consensus promoter sequences (26). QsdR1 originating from cosmid pWEB-TNC-C6 is highly similar to a hypothetical protein from *Rhizobium etli* (RetlG_22662), with an E value of 2e−124, and to a hypothetical protein of *Agrobacterium tumefaciens* (Atu4307), with an E value of 1e−121. The protein belongs to the hydrolases of the β-lactamase superfamily and is grouped in class B of these enzymes, requiring a bivalent metal ion (Zn^{2+}) for activity (8) (Table 2). Although an NCBI database search uncovered high homologies only to rather conserved hypothetical proteins with β-lactamase domains, a direct comparison with known representatives of AHLases confirmed that the *qsdR1* product is highly similar to the Aii2 hydrolase from an uncultured *Bacillus* sp. (5). Additionally, BLAST searches indicated two conserved regions among the QsdR1 sequence and several known AHLases, such as AiiA (Table 3). The first short sequence is ^{141}HMHMDHIG^{148} and the second is ^{236}TGGHTPGH^{243}.

The ORF NGR_c16020 (834 bp) encodes QsdR2, a 279-amino-acid protein which was also assigned to the β-lactamase superfamily. Prior to the putative ATG start codon, an AGGAGA ribosome-binding site as well as possible −10 and −35 promoter sequences could be identified according to published rhizobial consensus sequences (26). QsdR2 showed a very high similarity to a hypothetical protein from *Sinorhizobium meliloti* 1021 (SMc01194), with an E value of 3e−146, and to a β-lactamase domain-containing protein of *Sinorhizobium medicae* WSM419 (E value, 4e−146). QsdR2 shares a short conserved motif within the AHLases but is otherwise not similar to AHLases. The identified pattern is ^{83}HAHADH^{89}.

A detailed amino acid analysis of ORF NGR_b22150 (1,071 bp), encoding DlhR (for dienelactone hydrolase from *Rhizo-*

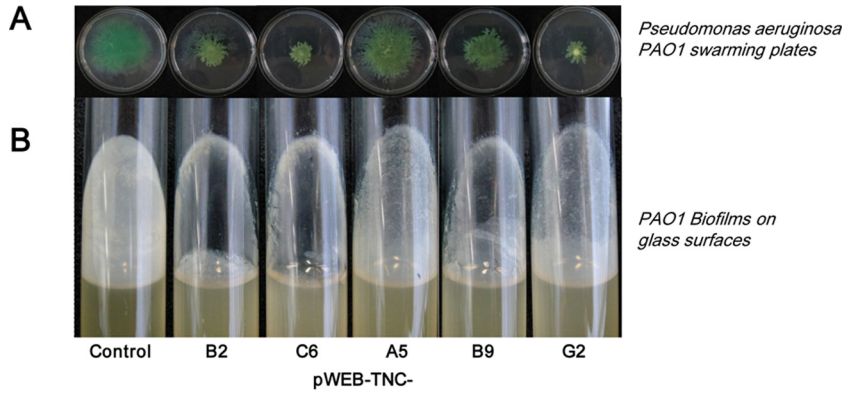

FIG. 2. *P. aeruginosa* PAO1 motility and biofilm assays. (A) Altered swarming motility of *P. aeruginosa* PAO1 on swarming plates supplemented with crude cell extracts of cosmids pWEB-TNC-B2, -C6, -A5, -B9, and -G2. Plates were supplemented with 5 to 100 μg of crude cell extracts of *E. coli* harboring the QQ cosmids. The plates were inoculated with 1×10^8 cells of *P. aeruginosa* PAO1 and incubated overnight. (B) Biofilm assays using sterile test tubes filled with LB medium and supplemented with 5 to 100 μg of crude cell extracts of cosmid clones. The tubes were inoculated with 1×10^8 cells of *P. aeruginosa* PAO1 and incubated at 37°C and 140 rpm for 6 to 8 h. Controls in panels A and B were either *E. coli* cells or *E. coli* harboring a negatively tested pWEB-TNC cosmid clone inoculated with PAO1. For overview purposes, only one control is displayed.

bium sp. strain NGR234), revealed that this gene product was most similar to a hypothetical protein (Atu0247) from *Agrobacterium tumefaciens* strain C58. The observed similarity was 75% and the identity was 58%, with an E value of 3e−89 (Table 2). The deduced amino acid sequence of DlhR contained a multidomain COG4188 spanning nearly the whole protein and a smaller Pfam03403 domain structure. COG4188 represents a predicted dienelactone hydrolase domain, whereas Pfam03403 is involved in a subfamily of phospholipases A2, which are responsible for inactivation of platelet-activating factor through cleavage of an acetyl group. In addition, a conserved GSD(L) motif containing the active-site serine residue typical of GDSL esterases (family II of lipolytic enzymes) was identified (2). Further analysis of the upstream sequence of the predicted ATG start codon of *dlhR* revealed a possible ribosome-binding site as well as both promoter (−10/−35) regions. Additionally, the prediction of the presence and location of a signal peptide (SignalP 3.0 server, http://www.cbs.dtu.dk/services/SignalP/) on *dlhR* uncovered a 99% probability for a cleavage site between amino acids 22 and 23 (beginning with methionine). The predicted signal peptide on *dlhR* is MIPSH VPAALALAVAFAAPCHAF.

ORF NGR_c23150 (1,506 bp) encodes a putative acetaldehyde dehydrogenase, a 503-amino-acid protein, which we designated AldR (for acetaldehyde dehydrogenase from *Rhizobium* sp. strain NGR234). Similarities to proteins present in *Sinorhizobium meliloti* 1021, *Sinorhizobium medicae* WSM419, *Rhizobium etli* CFN42, *Rhizobium leguminosarum* bv. *trifolii* WSM2304, and *Mesorhizobium loti* MAFF303099 could be observed, with a maximum identity up to 100% and E values of 0.0 (Table 2).

Cosmid clone pWEB-TNC-A5 carries two ORFs that appeared to be involved in QQ. ORF NGR_c35570 (606 bp), encoding HitR (for histidine triad protein from *Rhizobium* sp. strain NGR234), and NGR_c35560, encoding HydR (for NUDIX hydrolase from *Rhizobium* sp. strain NGR234), might be responsible for the QQ phenotype. Gene *hitR* is predicted to encode a protein with 202 amino acids which was highly similar to homologous proteins in *Sinorhizobium meliloti* 1021 (E value, 1e−104). The *hydR* gene (1,035 bp) is predicted to

TABLE 3. Biochemically characterized QQ hydrolases in microorganisms and metagenomes

Protein	Source or strain	Protein family	Conserved motif	Reference
AiiA	*Bacillus* sp. strain 240B1	Metallo-β-lactamase superfamily	HxHx~DH	12
AiiB/AttM	*Agrobacterium tumefaciens* strain C58	Metallo-β-lactamase superfamily	HxHx~DH	5
AhlD	*Arthrobacter* sp. strain IBN110	Metallo-β-lactamase superfamily	HxHx~DH	32
AhlK	*Klebsiella pneumoniae*	Metallo-β-lactamase superfamily	HxHx~DH	32
QlcA	Soil metagenome	Metallo-β-lactamase superfamily	HxHx~DH	36
QsdR1	*Rhizobium* sp. strain NGR234	Metallo-β-lactamase superfamily	HxHx~DH	This study
AidH	*Ochrobactrum anthropi* ATCC 49188	α/β-Hydrolase fold family	GX-Nuc-XG	28
AiiM	*Microbacterium testeceum* StLB037	α/β-Hydrolase fold family	ND[a]	54
BpiB04	Soil metagenome	Glycosyl hydrolase family	ND	39
BpiB07	Soil metagenome	Dienelactone hydrolase family	ND	39
DlhR	*Rhizobium* sp. strain NGR234	Dienelactone hydrolase family	GSD(L)	This study
BpiB01	Soil metagenome	Hypothetical protein	ND	39
BpiB05	Soil metagenome	Hypothetical protein	ND	3
QsdA	*Rhodococcus erythropolis* W2	PTE superfamily	PTE domain	51

[a] ND, none detected.

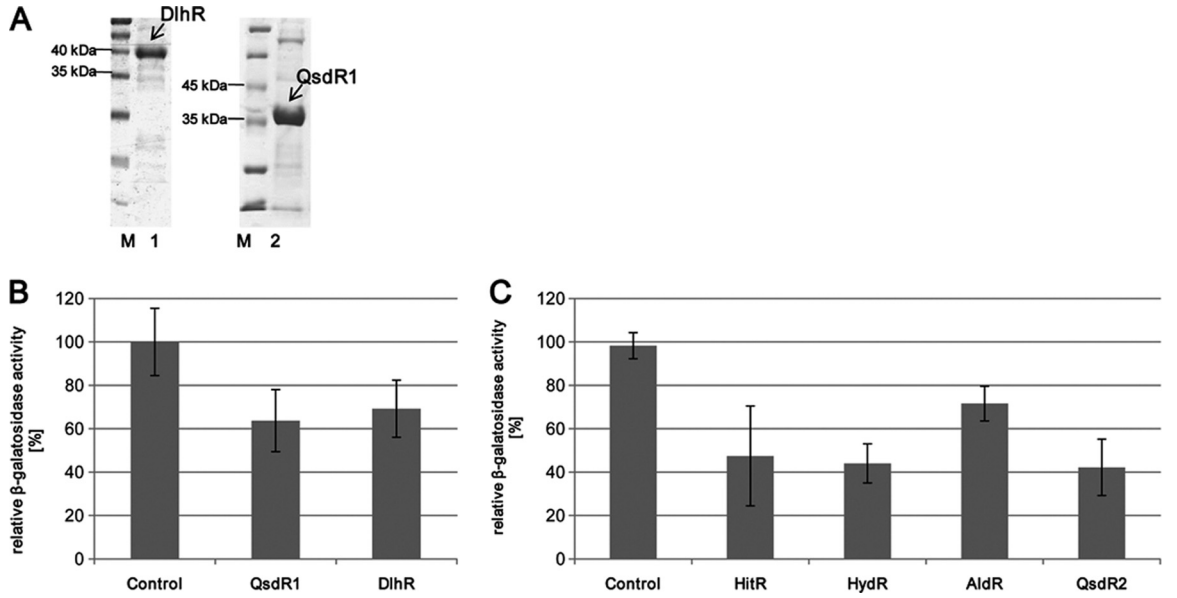

FIG. 3. Purification of recombinant DlhR and QsdR1 and β-galactosidase assay using the same QQ proteins and test with pDrive clones of *hitR*, *hydR*, *aldR*, and *qsdR2*. (A) SDS-15% PAGE of protein extracts of *E. coli* BL21(DE3) cells expressing pET21a::*dlhR* and pET21a::*qsdR1*. Lanes: M, molecular mass markers; 1, purified His_6-tagged DlhR at 39.3 kDa; 2, purified His_6-tagged QsdR1 at 36.0 kDa. (B) β-Galactosidase activity in *A. tumefaciens* NTL4 cells after addition of 3-oxo-C_8-HSL and 6 μg of either DlhR or QsdR1. (C) β-Galactosidase activity in *A. tumefaciens* NTL4 cells after addition of 3-oxo-C_8-HSL and crude cell extracts of pDrive::*hitR* (HitR), pDrive::*hydR* (HydR), pDrive::*aldR* (AldR), and pDrive::*qsdR2* (QsdR2). In panels B and C, KPO_4 buffer and crude NTL4 extracts served as controls. Data are means of at least three independent measurements. Error bars indicate simple standard deviations.

encode a protein with 346 amino acids. The highest observed similarity of HydR was to a potential NUDIX hydrolase of *Sinorhizobium medicae* WSM419 and *Sinorhizobium meliloti* 1021, with E values of 0.0. Although we have no experimental evidence, it is highly possible that the two genes form an operon (Fig. 1).

We have conclusively identified three proteins (QsdR1, QsdR2, and DlhR) that are at least weakly similar to previously known QQ proteins. Furthermore, we have identified one protein (AldR) and one locus (*hitR-hydR*) that have not been reported to be involved in QS-modifying activities. All these findings are in line with the data derived from our sequence-based analyses (Table 2), where *qsdR1*, *qsdR2*, *dlhR*, and *hitR-hydR* were already identified. Furthermore, we extend the list of potential QQ genes by the *aldR* gene, which was identified only by function-based searches.

Heterologously expressed DlhR and QsdR1 quench QS-dependent processes in *A. tumefaciens*. To verify the QQ activities of several of the identified proteins by more sophisticated methods, we focused on the enzyme candidates on pNGR234*b*. Thus, we expressed the *dlhR* and *qsdR1* genes in *E. coli* and purified the corresponding recombinant His_6-tagged proteins. For heterologous expression, the *dlhR* and *qsdR1* genes were cloned directionally into pET21a using primers with inserted restriction sites (see Table S1 in the supplemental material) and overexpressed as described above. The estimated molecular masses of the His_6-tagged and purified proteins of DlhR and QsdR1 (Fig. 3A) were in accordance with the theoretical molecular masses of 39.3 kDa (DlhR) and 36.0 kDa (QsdR1).

The purified proteins were investigated for their QQ impact using *A. tumefaciens* NTL4. The initial screening employed for detection of AHL-degrading cosmids within the NGR234 library was also used for verifying the hydrolytic activities of the recombinant and purified DlhR and QsdR1 proteins. Different concentrations of both proteins incubated with 3-oxo-C_8-HSL solution (4.1×10^{-6} M) and applied on ATsoft agar containing *A. tumefaciens* NTL4 caused a colorless appearance of the agar, indicating their effect on the QS response in *A. tumefaciens* NTL4 (data not shown).

Additionally, β-galactosidase assays combining hydrolytic cleavage of ONPG, *A. tumefaciens* NTL4, and our proteins confirmed these findings. Prior to incubation with NTL4 overnight, 5 μl of 3-oxo-C_8-HSL (4.1×10^{-8} M) was mixed with recombinant DlhR or QsdR1 protein extracts (4 to 400 μg/μl) and incubated for 1.5 h at 30°C. After this short incubation time, the levels of detected AHLs were significantly lower than those of the controls. In general, we could detect less than 70% of the added 3-oxo-C_8-HSL (Fig. 3B) after incubation with DlhR. The β-galactosidase assay with 10 to 20 μg of recombinant and purified QsdR1 revealed an even more pronounced 3-oxo-C_8-HSL degradation (Fig. 3B). On average, the β-galactosidase assays suggested that the recombinant protein degraded 50% of the added 3-oxo-C_8-HSL. Consequently, these tests confirmed the ability of recombinant DlhR and QsdR1 to degrade 3-oxo-C_8-HSL. Further tests using crude cell extracts of pDrive clones harboring the *hitR*, *hydR*, *aldR*, and *qsdR2* clones verified their hydrolytic activities in ONPG assays as well (Fig. 3C).

Uncovering the cleavage mechanism of DlhR and QsdR1 proteins by HPLC-MS analysis. To verify AHL degradation by DlhR and QsdR1 and to detect the cleavage mechanisms by which the *N*-AHLs are inactivated (lactonolysis or amidolysis),

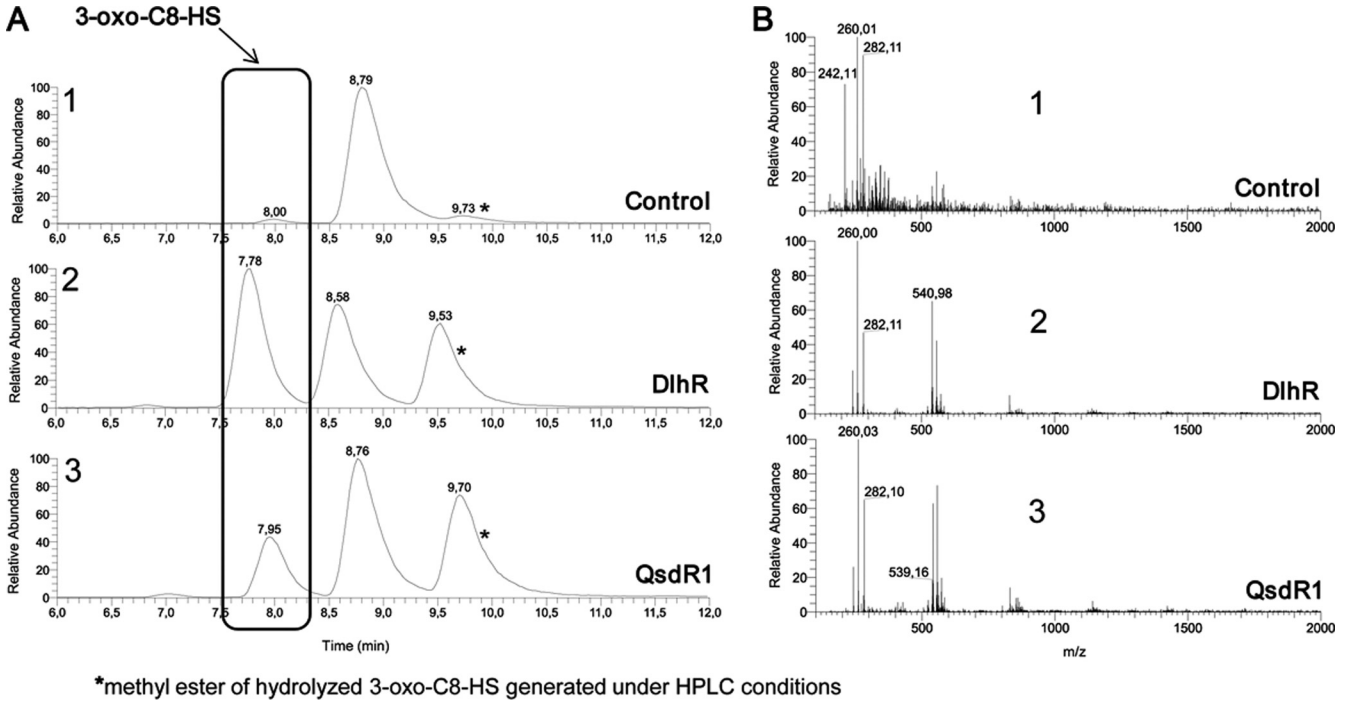

FIG. 4. HPLC-MS analysis of recombinant DlhR and QsdR1. (A) HPLC-UV spectra recorded at 252 nm for 3-oxo-C_8-HSL after incubation with GtfU as a control, DlhR, and QsdR1. (B) Respective mass spectra recorded for samples treated with either control protein GtfU, DlhR, or QsdR1. (A1) The HPLC-UV chromatogram depicts the control incubated with 3-oxo-C_8-HSL, displaying only one distinct peak at an R_t of 8.8 min representing 3-oxo-C_8-HSL. No lactone hydrolysis could be observed. (B1) The mass spectrum of the GtfU control (at an R_t of 8.0 min) shows an $(M + H)^+$ ion at an m/z of 242.1 as significant for this compound. An $(M + H)^+$ ion at an m/z of 260.01 and an $(M + Na)^+$ ion at an m/z of 282.1 were also detected owing to spontaneous degradation of 3-oxo-C_8-HSL. (A2) HPLC-UV chromatogram for 3-oxo-C_8-HSL incubated with DlhR, showing the peak at an R_t of 7.8 min for the cleavage product 3-oxo-C_8-HS (opened lactone ring). The peak at an R_t of 8.6 min indicates the unhydrolyzed 3-oxo-C_8-HSL, and an irrelevant by-product (a methyl ester), which is generated only under HPLC conditions, was detected at an R_t of 9.6 min. (B2) The corresponding mass spectrum shows three characteristic ions: an $(M + H)^+$ ion at an m/z of 260.0, an $(M + Na)^+$ ion at an m/z of 282.1, and a $(2M + Na)^+$ ion at an m/z of 540.2. (A3) HPLC-UV chromatogram of 3-oxo-C_8-HSL hydrolyzed by QsdR1. The cleavage product, an opened lactone ring form, was detected at an R_t of 8.0 min; consequently, QsdR1 was able to enzymatically degrade 3-oxo-C_8-HSL. Both peaks for the nonhydrolyzed form of 3-oxo-C_8-HSL as well as the methyl ester were recorded for QsdR1 as well. (B3) The mass spectrum as already given for DlhR showed the characteristic ions [an $(M + H)^+$ ion, an $(M + Na)^+$ ion, and a $(2M + Na)^+$ ion at the same m/z].

we performed HPLC-MS analysis. Therefore, 10 μl of 3-oxo-C_8-HSL was incubated with 0.005 to 0.3 mg/ml purified protein in 100 mM potassium phosphate buffer, pH 7.0. After incubation and extraction, reaction products were analyzed by HPLC-MS-diode array detection (DAD). Spontaneous degradation of 3-oxo-C_8-HSL was evaluated in control experiments with a purified glycosyl transferase (GtfU) subjected to the same concentrations and conditions.

Repeated measurements with both proteins indicated that the underlying mechanism of degradation was a hydrolysis of the lactone ring of 3-oxo-C_8-HSL. Determined by HPLC analysis followed by mass spectrometry, the enzymatic degradation resulted in a mixture of 3-oxo-C_8-HS with a retention time (R_t) of ~7.9 min and a methyl ester of hydrolyzed 3-oxo-C_8-HS with an R_t of ~9.6 min, generated only under given HPLC conditions. Peaks detected at an R_t of ~8.6 min display the unhydrolyzed 3-oxo-C_8-HSL form (Fig. 4).

3-Oxo-C_8-HSL incubated with control protein GtfU resulted in the detection of almost exclusively the nonhydrolyzed form of 3-oxo-C_8-HSL. Figure 4A, panel 1, shows the HPLC-UV spectrum at 252 nm for the control, displaying only one distinct peak at an R_t of 8.8 min representing 3-oxo-C_8-HSL. A peak showing the relative abundance of the hydrolyzed form was under the detection limit; consequently, no significant lactone hydrolyzation linked to the control could be detected. The mass spectrum of the control (at an R_t of 8.0 min) showed an $(M + H)^+$ ion at an m/z of 242.1 that was significant for this compound. An $(M + H)^+$ ion at an m/z of 260.01 and an $(M + Na)^+$ ion at an m/z of 282.1 were also detected owing to spontaneous degradation of 3-oxo-C_8-HSL (Fig. 4B, panel 1). Both proteins incubated with 3-oxo-C_8-HSL were able to hydrolyze the lactone ring of this *N*-AHL. HPLC-UV spectra detected at 252 nm for DlhR and QsdR1 showed almost identical retention times for the cleavage product 3-oxo-C_8-HS with the opened lactone ring. Peaks were detected at an R_t of 7.8 min for DlhR and at an R_t of 8.0 min for QsdR1 (Fig. 4A, panels 2 and 3). The relative abundance of the 3-oxo-C_8-HS compound found for both proteins was considerably higher than that for the control, thus showing a good enzymatic degradation of 3-oxo-C_8-HSL by our proteins. Mass spectra for the proteins showed a characteristic $(M + H)^+$ ion at an m/z of 260.0, an $(M + Na)^+$ ion at an m/z of 282.1, and a $(2M + Na)^+$ ion at an m/z of 540.2 (Fig. 4B, panels 2 and 3).

In summary, the control incubated under identical conditions as for DlhR and QsdR1 with 3-oxo-C_8-HSL did not produce a peak at an R_t of ~7.9 min, which is characteristic for

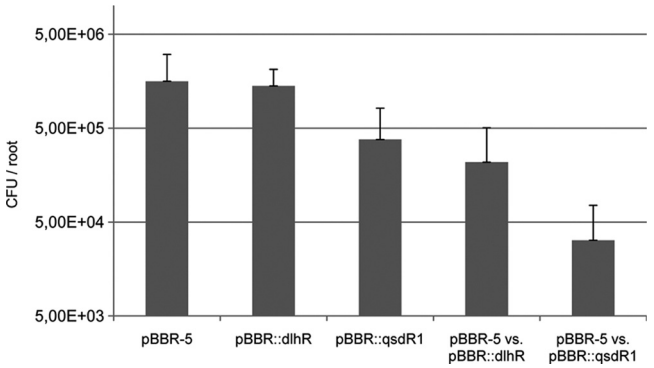

FIG. 5. Growth of NGR234 in the cowpea rhizosphere. Bacteria inoculated on day 0 were quantified in the inocula by measuring the CFU. After 4 days, bacteria were recovered from developed roots. Data represent mean numbers (CFU) of bacteria per root and standard deviations from a minimum of five replicates. From left to right: control pBBR-5 (NGR234 harboring the empty pBBR1MCS-5), pBBR::*dlhR* (NGR234 with an extra copy of *dlhR* in pBBR1MCS), pBBR::*qsdR1* (NGR234 with an extra copy of *qsdR1* in pBBR1MCS), pBBR-5 versus pBBR::*dlhR* (competition experiments with NGR234 carrying control pBBR-5 versus NGR234 carrying an extra copy of *dlhR*), and pBBR-5 versus pBBR::*qsdR1* (competition experiments with NGR234 carrying control pBBR-5 versus NGR234 carrying an extra copy of *qsdR1*). The last two bars indicate the recovered bacteria carrying the extra copies of the corresponding QQ genes (i.e., *dlhR* and *qsdR1*). Error bars indicate simple standard deviations.

the cleavage form, 3-oxo-C_8-HS. The HPLC-UV as well as mass spectral data for both recombinant proteins confirmed an enzymatic activity, giving evidence that DlhR and QsdR1 act as true lactonases in NGR234.

DlhR and QsdR1 interfere with rhizosphere colonization of cowpea roots. We speculated that DlhR and QsdR1 would be important for plant rhizosphere colonization. In order to test this hypothesis, we constructed two strains carrying extra copies of the two genes in the vector pBBR1MCS. NGR234 cells containing pBBR1MCS constructs expressed the *dlhR* and *qsdR1* genes under the control of the P_{taq} promoter. Constructs were verified by sequencing the inserts. We then carried out root colonization tests on cowpea roots by inoculating each mutant individually and in competition with the control strain (NGR234 harboring pBBR1MCS-5). The plants were harvested after 4 days and root colonization analyzed as described in Materials and Methods. Under our experimental conditions, NGR234 grew and colonized the root most rapidly at between 3 and 6 days after inoculation (data not shown), and these tests indicated that the control strain was most efficient in root colonization in all tested combinations (Fig. 5). While the strain carrying an extra copy of the *dlhR* gene was in general only slightly affected in its capability to colonize the root surface, the strain carrying the *qsdR1* gene was significantly affected in its rhizosphere colonization capabilities. Additional competition experiments confirmed these findings (Fig. 5). In these tests the strains carrying extra copies of the *dlhR* or the *qsdR1* gene were outnumbered by the control strain and colonized the rhizospheres at a statistically significantly ($P \leq 0.01$) lower level. In fact, the *qsdR1* strain was outnumbered by at least an order of magnitude by the control strain when coinoculated into the rhizosphere at equal numbers (CFU/ml), and the strain carrying extra copies of *dlhR* was outnumbered by a factor of 5 to 7. From these tests we concluded that both genes contribute to rhizosphere colonization fitness of NGR234 and are possibly involved in the degradation of plant-derived or microbial autoinducer molecules in the rhizosphere.

DISCUSSION

In this paper we describe the isolation of five NGR234-derived genes and loci that interfere with AI-I signaling molecules, and we have partially biochemically characterized two of the proteins. The NGR234-derived QQ clones were initially identified because they repeatedly interfered with quorum sensing in an *A. tumefaciens* NTL4-based bioassay using a *traI*::*lacZ* promoter fusion. The genes and loci identified and linked to the QS inhibitory phenotypes were designated *dlhR*, *qsdR1*, *qsdR2*, *aldR*, and *hitR-hydR.* All these genes, when expressed, resulted in strongly reduced motility and biofilm phenotypes in *P. aeruginosa* PAO1 (Fig. 2A and B). With respect to the reduced biofilm formation and motility induced through the expression of the NGR234-derived QQ genes, we hypothesized that these phenotypes were a result of the *N*-AHL degradation caused by the identified proteins. In fact, the results presented in Fig. 3B strongly supported that hypothesis, and further data using more sophisticated analytical technologies confirmed these findings for the recombinant and purified DlhR and QsdR1 proteins (Fig. 4). These results are all in line with the reports on enzymatic degradation of *N*-AHLs. Enzymatic degradation of the quorum-sensing signal molecules is an established method of quorum quenching and has been reviewed recently (11, 34, 35). Three main types of microbial enzymes that act on the *N*-AHLs are known: (i) oxidoreductases, (ii) acylases, and (iii) lactonases. Besides oxidoreductases, which have been uncovered in Gram-positive bacteria (6, 7, 49), oxidases have been identified in algae, acting on the 3-oxo group (4). Acylases were found in organisms such as *Comamonas* (50), *P. aeruginosa* (20), *P. syringae* (42), *Ralstonia* spp. (24), *R. erythropolis* (49), *Shewanella* (29), and *Streptomyces* (31). Aminoacylases cleave the lactone ring off the fatty acids, whereas lactonases, which constitute the largest group of AHL-degrading enzymes, hydrolyze the lactone ring in a reversible way. Today, only a small number of different functionally verified classes of lactonase proteins that efficiently hydrolyze the lactone ring in *N*-AHLs are known. The experimentally verified and biochemically characterized lactonases are summarized in Table 3. The lactonases are grouped into several clusters according to their overall similarity and the original microbe in which the first gene of the cluster was identified. The first known clusters are designated AiiA, AttM, and PON1-3 (13). Recently six other types of lactonases (i.e., QsdA [51], BpiB01, BpiB04, and BpiB07 [39]; AiiM [54], and AidH [28]) have been identified and extend the diversity of lactonase family proteins. These proteins are probably each members of novel *N*-AHL lactonase families. Zn^{2+}-dependent lactonases have been described for *Bacillus* (5, 9, 23, 48), *Agrobacterium* (5), *Rhodococcus* (30), *Streptomyces* (31), *Arthrobacter* (32), *Pseudomonas* (44), and *Klebsiella* (32). The novel lactonase derived from *Rhodococcus*, with the gene designated *qsdA*, forms a new family within the metal-dependent lactonases (51). Furthermore, we have recently identified four novel lactone-hydrolyzing enzymes isolated from the

TABLE 4. NGR234 QQ genes in related and completed rhizobial genomes

NGR234 QQ locus	Homologue/orthologue in[a]:				
	Rleg3841	SM1021	Bjap110	Retli42	ML99
dlhR (NGR_b22150)	RRZL01754	ND	ND	RRET01614	ND
qsdR1 (NGR_b16870)	RRZL02285	RSM05795	RBRJ02791	RRET00556	ND
qsdR2 (NGR_c16020)	RRZL02453	RSM02893	RBRJ04514	RRET02102	RMLO00322
aldR (NGR_c23150)	RRZL04239	RSM03645	RBRJ04784	RRET03644	RMLO05206
hitR-hydR (NGR_c35560/c35570)	RRZL00137/ RRZL00138	RSM01522/ RSM01523	RBRJ08122/ RBRJ08123	RRET00118/ RRET00119	RMLO04260/ RMLO04261

[a] Homologous/orthologous genes are indicated by the corresponding gene/ORF number. Data for the analysis were extracted from the respective genome projects and the corresponding GenBank files: NGR234, *Rhizobium* sp. strain NGR234 (CP000874 and CP001389); Rleg3841, *Rhizobium leguminosarum* bv. viciae 3841 (AM236080 to AM236086); SM1021, *Sinorhizobium meliloti* strain 1021 (AL591786); Bjap110, *Bradyrhizobium japonicum* strain USDA110 (BA000040); Retli42, *Rhizobium etli* strain CFN42 (AF311739); ML99, *Mesorhizobium loti* strain MAFF303099 (AP003017). ND, not detected.

metagenome (3, 39). Within this framework it is noteworthy that we have not only identified three lactonases (DlhR, QsdR1, and QsdR2) in NGR234, but we also identified two completely novel genes in NGR234 that had not been linked to QQ in earlier studies. These are the *aldR* gene and the *hitR-hydR* locus. Although we have not characterized all the respective proteins in detail, our data strongly suggest that they are involved in autoinducer degradation or modification in the sense that *P. aeruginosa* PAO1 responds with reduced motility and other QS-dependent phenotypes.

The richness of ORFs linked to QQ activity in NGR234 probably suggests that this is an important feature, which is possibly needed during rhizosphere colonization and during growth in free soil. This hypothesis is strongly supported by our rhizosphere colonization tests using NGR234 mutant strains carrying extra copies of the *dlhR* and the *qsdR1* genes (Fig. 5). These strains were in general less effective during root colonization than a wild-type control strain carrying an empty vector.

A detailed genome comparison of several of the sequenced rhizobial genomes suggests that rhizobial isolates appear to commonly have several QQ genes encoded in their genomes, and those appear to be present on the chromosomes or the larger megaplasmids but not on the symbiotic plasmids (Table 4). A comparison of the genomes revealed the presence of four of the NGR234 QQ genes in the *S. meliloti* SM1021 genome. SM1021, however, lacks the *dlhR* locus. Also, *dlhR* as well as the *qsdR1* locus are missing in *M. loti* MAFF303099. Furthermore, an analysis of the nearly complete USDA257 genome revealed that besides the *dlhR* gene, all NGR234 QQ genes were present in this broad-host microbe as well (unpublished data from our lab). A more detailed analysis of the presence and absence of the NGR234 QQ genes in other rhizobia is given in Table 4.

The results from this study probably have two main implications. First, NGR234 and perhaps many other rhizosphere-associated organisms appear to have a surprisingly large number of different ways to degrade or modify autoinducers present in their environment. Second, data from this study emphasize the ecological importance of QQ during root colonization.

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