# A Rhodococcus qsdA-Encoded Enzyme Defines a Novel Class of Large-Spectrum Quorum-Quenching Lactonases

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# A *Rhodococcus qsdA*-Encoded Enzyme Defines a Novel Class of Large-Spectrum Quorum-Quenching Lactonases▽†

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**A gene involved in *N*-acyl homoserine lactone (N-AHSL) degradation was identified by screening a genomic library of *Rhodococcus erythropolis* strain W2. This gene, named *qsdA* (for *q*uorum-sensing *s*ignal *d*egradation), encodes an N-AHSL lactonase unrelated to the two previously characterized N-AHSL-degrading enzymes, i.e., the lactonase AiiA and the amidohydrolase AiiD. QsdA is related to phosphotriesterases and constitutes the reference of a novel class of N-AHSL degradation enzymes. It confers the ability to inactivate N-AHSLs with an acyl chain ranging from $C_6$ to $C_{14}$, with or without substitution at carbon 3. Screening of a collection of 15 *Rhodococcus* strains and strains closely related to this genus clearly highlighted the relationship between the ability to degrade N-AHSLs and the presence of the *qsdA* gene in *Rhodococcus*. Bacteria harboring the *qsdA* gene interfere very efficiently with quorum-sensing-regulated functions, demonstrating that *qsdA* is a valuable tool for developing quorum-quenching procedures.**

Gram-negative bacteria couple gene expression to population density by a regulatory mechanism named quorum sensing (QS). QS relies upon the production and the perception of one or more signal molecules by the bacterial population. An important class of these signals is the *N*-acyl homoserine lactone (N-AHSL) class (9). Molecules belonging to this class exhibit a conserved structure, with a backbone composed of a homoserine lactone (HSL) N-linked to an acyl chain via an amide bond. Variation in *N*-acyl chain length and the oxidation status of N-AHSLs provide for specificity of the signal. Of particular interest is the finding that QS regulates pathogenicity, or pathogenicity-related functions, in bacteria of medical or environmental importance (15, 32, 50).

If QS and N-AHSLs are important components of the strategy of adaptation by bacteria to their biotic environment, especially a plant surface, one might suspect that the eukaryotic hosts and competing bacteria might have developed strategies to interfere with this communication system. Indeed, QS inhibition was reported through the production of antagonists (10) or the production of N-AHSL degradation enzymes by plants (5), animals (2, 34), and a wide range of bacterial genera (14, 16, 18–20, 30, 31, 44, 49). In spite of the large diversity of N-AHSL-degrading bacteria, only two families of N-AHSL-inactivating enzymes (N-AHSLases) have been described to date: the AiiA-like N-AHSL lactonases (6, 19, 49) and the AiiD-like N-AHSL amidohydrolases (14, 20, 30). Whatever their physiological role, N-AHSLases have been used efficiently to interfere with the expression of QS-regulated functions in bacteria (6, 19, 20, 35, 42). This strategy has been termed quorum quenching (QQ). It proved to be a valuable trail toward definition of novel biocontrol agents such as natural isolates degrading N-AHSLs (8, 23, 44). QQ occurs in natural environments as indicated by the coexistence of N-AHSL-producing and -degrading strains in biofilms (14) or in the rhizosphere (4). Among the species harboring an N-AHSLase activity, *Rhodococcus erythropolis* is remarkable because it is the only bacterium in which three enzymatic activities directed at N-AHSLs have been characterized: an oxidoreductase activity, which reduces 3-oxo-N-AHSLs to their hydroxylated equivalents (43); an amidohydrolase (43); and a lactonase (29). The marked *R. erythropolis* QQ capabilities suggest that it might be used in biocontrol protocols, especially since it is a natural inhabitant of soils worldwide (44).

In this study we report the isolation of one of the three genes encoding N-AHSLase activities from *R. erythropolis* strain W2. We show that this gene encodes a phosphotriesterase (PTE)-like broad-spectrum N-AHSL lactonase, which is found only in the *Rhodococcus* genus and solely in strains capable of degrading N-AHSLs.

## MATERIALS AND METHODS

**Strains, media, growth conditions, and chemicals.** Bacterial strains and plasmids are listed in Table 1. All strains were grown at 25°C, with the exception of the *Chromobacterium violaceum* biosensor and *Escherichia coli*, which were grown at 30°C and 37°C, respectively. Unless otherwise stated, LBm medium was buffered at pH 6.5 to prevent spontaneous lactonolysis of N-AHSLs and used as the rich medium (44). All media were solidified with 16 g/liter of agar. Antibiotics were used at the following final concentrations (in μg/ml; see Table 1 for specific strain requirements): ampicillin, 100; gentamicin, 25; tetracycline, 10; rifampin, 100; and streptomycin, 250. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were included in me-

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Characteristics or synonym | Source or reference |
|---|---|---|
| Strains tested for N-AHSL degradation and presence of the *qsdA* gene | | |
| *Actinoplanes utahensis* NRRL 12052 | Source of aculeacin A deacylase | 39 |
| *Corynebacterium glutamicum* ATCC 14752 | Glutamine-producing strain | 38 |
| *Escherichia coli* K-12 | | Lab collection |
| *Flavobacterium* sp. strain ATCC 27551 | Parathion-degrading strain | 24 |
| *Gordonia alkalivorans* strain 98 | Petroleum-degrading strain | Lab collection |
| *Microbacterium* sp. strain POLB142 | Mannopine-degrading rhizospheric strain; soil isolate | 27 |
| *Mycobacterium* sp. strain Tn20 | Drought-resistant Sahara desert isolate | This study |
| *Nocardia asteroides* CECT 3051 | ATCC 19247; type strain | CECT[a] |
| *Pseudonocardia autotrophica* CECT 3044 | ATCC 13181; type strain | 47 |
| *Ralstonia solanacearum* GMI 1000 | Type strain | Lab collection |
| *Rhodococcus erythropolis* | | |
| W2 | N-AHSL-degrading isolate from soil | 44 |
| DCL14 | Limonene-degrading strain | 46 |
| SQ1 | ATCC 4277-1; *R. erythropolis* type strain | 33 |
| Mic1 | Mycorhizosphere isolate | Lab collection |
| MP50 | Aromatic-nitrile-degrading strain | 41 |
| CECT 3008 | ATCC 11048 | CECT |
| *Rhodococcus fascians* D188 | Fasciation-causing agent on dicotyledons | 3 |
| *Rhodococcus opacus* HL PM-1 | 2,4,6-Trinitrophenol-degrading strain | 25 |
| *Rhodococcus rhodochrous* CECT 3042 | ATCC 6846 | CECT |
| *Rhodococcus corynebacteroides* CECT 420 | ATCC 14898; soil isolate | CECT |
| *Rhodococcus* sp. strain RHA1 | Polychlorinated biphenyl-degrading strain | Lab collection |
| N-AHSL biosensors and N-AHSL-producing strains used for QQ assays | | |
| *Agrobacterium tumefaciens* NTL4(pZLR4) | Hydroxy, oxo, and long-chain N-AHSL sensor strain | 21 |
| *Chromobacterium violaceum* CV026 | Reduced N-AHSL sensor strain | 22 |
| *Pectobacterium carotovorum* Pcc797 | Soft-rot-causing agent | Lab collection |
| *Pseudomonas fluorescens* 1855-344 | Rhizospheric strain | Lab collection |
| *Agrobacterium tumefaciens* | | |
| 15955tra[c] | Octopine-type strain, constitutive for Ti plasmid conjugal transfer | Lab collection |
| C58C1RS | Ti plasmid-cured C58 derivative resistant to rifampin and streptomycin | Lab collection |
| *Escherichia coli* strains used for cloning | | |
| DH5α | | Lab collection |
| VCS257 | | Stratagene |
| Plasmids | | |
| pUC19 | $Amp^r$ | Lab collection |
| pGEM-T | $Amp^r$ | Promega |
| pUC1318::Gm | $Amp^r$ $Gm^r$ | 40 |
| pME6032 | $Tc^r$ | 12 |
| pCP13/B | $Tc^r$; cosmid vector | Lab collection |
| pSU16 | $Tc^r$; cosmid clone conferring N-AHSL degradation capability | This study |
| pSU16-11 | pUC19 with a 3.2-kb EcoRI fragment from pSU16 conferring N-AHSL degradation | This study |
| pSU16-12 | pUC19 with a 3.5-kb BamHI fragment from pSU16 conferring N-AHSL degradation | This study |
| pSU40 | pME6032 harboring the 3.2-kb EcoRI fragment from pSU16-11 | This study |
| pSU8-1 | pGEM-T with a 1.8-kb PCR fragment containing *orf1* and *qsdA* | This study |
| pSU8-1 ΔSalI | pSU8-1 with a SalI deletion in *orf1* | This study |
| pSU8-1::Gm | pSU8-1 with insertion of a $Gm^r$ cassette at the AgeI site of *qsdA* | This study |
| pSU8-1 ΔSalI::Gm | pSU8-1 ΔSalI with insertion of a $Gm^r$ cassette at the AgeI site of *qsdA* | This study |
| pSU-$qsdA_{W2}$ | *qsdA* ORF amplified and cloned into pET16b | This study |
| pSUreg1 | Upstream regulatory region of *qsdA* amplified and cloned into pGEM-T | This study |

[a] CECT, Coleccion Española de Cultivos Tipo.

dia at 40 μg/ml and 100 μg/ml, respectively. Hexanoyl-HSL ($C_6$-HSL) and its 3-oxo derivative were purchased from Sigma. The other N-AHSLs were generous gifts from S. R. Chhabra and P. Williams (University of Nottingham, United Kingdom).

**Detection of N-AHSLs.** N-AHSLs were separated and visualized on thin-layer chromatography (TLC) plates as described previously (37). Saturated short-chain N-AHSLs were detected by TLC using the *Chromobacterium violaceum* biosensor CV026 (22). Oxo and hydroxy derivatives and long-chain N-AHSLs were detected using the *Agrobacterium* biosensor NTL4(pZLR4) (21).

**Detection of N-AHSL degradation products.** The N-AHSL degradation assays were performed as described earlier (45), using actively growing DH5α cells expressing or not the *qsdA* gene in pH 6.5 buffered LB medium. The incubation time was set to 24 h, because this allowed work at low concentration and thus reduction of the interference of the medium with the data collected. Under these

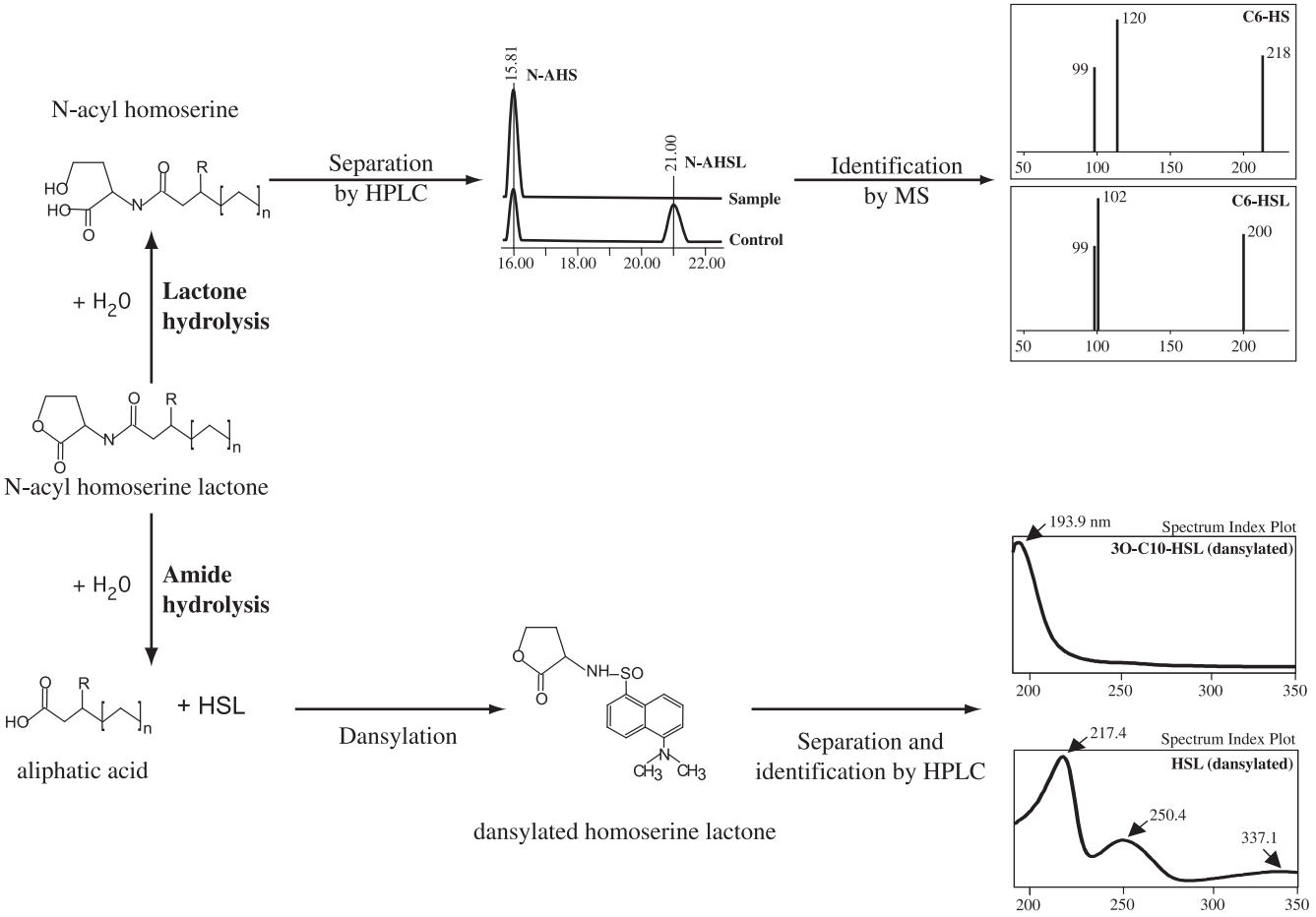


FIG. 1. Scheme for identification of N-AHSL-degradation products. N-AHSL inactivation in bacteria proceeds through two known pathways: lactonolysis (top) or amide hydrolysis (bottom). N-AHSL lactonolysis is a reversible reaction which yields the corresponding N-AHS, which can be separated by HPLC and identified by mass spectrometry (MS). Amide hydrolysis is an irreversible reaction which yields HSL and the corresponding acyl side chain. HSL can be detected by HPLC after trapping the free amine with dansyl chloride.

conditions, a fraction of ca. 25% of the initial amount of $C_6$-HSL is spontaneously converted into $C_6$-HS by chemical lactonolysis in the presence or absence of *E. coli* cells. The enzymatic degradation of N-AHSLs can proceed through two different routes (Fig. 1), which lead to the formation of either N-AHS (lactonolysis) (Fig. 1, top) or HSL and an alkyl chain (amidolysis) (Fig. 1, bottom). The presence of HSL in the incubation medium was determined after trapping of the free amine with dansyl chloride as described earlier (48). The formation of the ring-opened derivative of N-AHSLs following lactone hydrolysis was investigated using high-pressure liquid chromatography (HPLC) on a Waters chromatograph equipped with a Waters separation module 2659 and an Atlantis reverse-phase column (4.6 by 150 mm; 5 μm) coupled to an electrospray ionization-mass spectrometry detector (Waters Micromass ZQ 200). Retention times and mass spectra were determined for individual molecules in solution as standards. Thirty microliters of the degradation assay sample was injected and eluted with water–0.1% formic acid (solvent A) and acetonitrile–0.1% formic acid (solvent B) with the following elution sequence: 100% solvent A for 5 min, a linear gradient to reach 20% solvent B in 5 min, and 80% solvent A and 20% solvent B for 10 min. Between samples, the column was rinsed by applying 100% B solvent (3 min). The column was then reequilibrated with 100% solvent A for 7 min. The specific fragments expected to appear in the mass spectra of $C_6$-HSL and $C_6$-HS are all present; fragment 200 is characteristic for $C_6$-HSL, while fragment 218 is characteristic for $C_6$-HS. Fragment 102 corresponds to HSL and therefore appears in the $C_6$-HSL spectrum, while fragment 120 is characteristic for HS and appears in the $C_6$-HS spectrum (28).

**Library construction.** As strain W2 is especially difficult to lyse by conventional methods, genomic DNA preparation was based on the method of Eulberg et al. (7) with the following modifications. Strain W2 was grown overnight in 50 ml of LB medium buffered at pH 8.0 with glycine (3%, wt/vol). Prior to DNA extraction, cells were treated for 2 hours with ampicillin and erythromycin (100 μg/ml, final concentration) to disturb cell wall synthesis and favor the subsequent lysis of the cells. A genomic library of strain W2 was constructed by partially digesting 100 μg of total genomic DNA with Bsp143I (Sau3AI) and ligating the fragments in BamHI-linearized cosmid vector pCP13/B as described by Hayman and Farrand (11). The ligation products were packaged using the Gigapack III Gold packaging kit (Stratagene, La Jolla, CA) as recommended by the manufacturer and recovered by transfection into *E. coli* VCS257. The W2 genomic library generated in this study contained over 4,000 clones, with an average insert size of 23 kb and fewer than 1% empty clones. This corresponds to a theoretical coverage of 15 times of *Rhodococcus* genome (1).

**Screening of the genomic library for N-AHSL degradation ability.** A total of 2,880 library clones were screened directly in their *E. coli* strain VCS257 host by use of a modification of the microplate N-AHSL degradation assay described by Uroz et al. (44), using *C. violaceum* CV026 as an indicator strain. Clones were first grown in presence of antibiotics and then subcultured in medium devoid of antibiotics but supplemented with 25 μM of $C_6$-HSL. Cosmid clones were considered to confer the ability to degrade N-AHSLs only if a total disappearance of the $C_6$-HSL was observed after a 24-h period. The ability of the clones to effectively degrade the N-AHSL was confirmed by separating the degradation products by TLC and detecting the presence of N-AHSL by the appropriate biosensor. This allows the detection of false-positive degradation due to the presence of compounds inhibiting N-AHSL detection or the growth of the biosensor.

**DNA sequence analysis.** Sequence analysis was performed with ORF FINDER. Nucleotide and amino acid sequence comparisons were made using

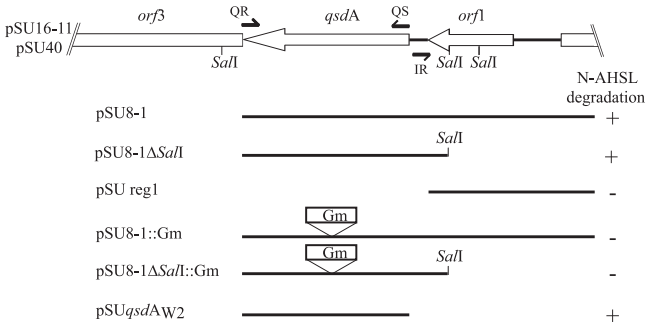


FIG. 2. Identification of the *qsdA* gene. The genetic organization of the *qsdA* locus derived from the complete sequence of the 3.2-kb EcoRI fragment conferring $C_6$-HSL degradation upon its host is shown at the top. Broken arrows symbolize the primers used for subcloning and their orientations. Restriction sites also used for subcloning are shown. See Table 1 for a description of the plasmids. Plasmid constructions used to identify the *qsdA* gene were assayed for their ability (+) or inability (−) to confer N-AHSL degradation upon their host against a set of N-AHSLs, including $C_6$-HSL, O-$C_6$-HSL, $C_7$-HSL, $C_8$-HSL, O-$C_8$-HSL, O-$C_{10}$-HSL, $C_{12}$-HSL, O-$C_{12}$-HSL, and O-$C_{14}$-HSL. Each construct gave identical degradation results regardless of the N-AHSL present in the medium.

the BLAST protocol. Multiple alignments were performed using the Pileup subroutine of the GCG package (version 10; GCG Inc., Madison, WI).

**Subcloning of the *qsdA* region.** To identify the gene coding for the N-AHSL degradation activity, the 3.2-kb EcoRI fragment was subcloned as shown in Fig. 2 using the primers QS (5′-ATGAGTTCAGTACAAACCGTTCGTG-3′), QR (5′-TCAGCTCTCGAAGTACCGACGTGGG-3′), and IR (5′-TCACCATTTT TCAACGGCCG-3′) and available restriction sites. The *qsdA* gene was disrupted by insertion of a gentamicin resistance gene from pUC1318::Gm, cloned as an XmaI fragment at the unique AgeI site of the *qsdA* gene. Southern hybridization was performed at 62°C with a *qsdA* probe amplified with the QS/QR primer pair and digoxigenin labeled according to the manufacturers' instructions (Roche/Boehringer Mannheim).

**QQ assays.** The ability of the cloned *qsdA* genes to interfere with the expression of QS-regulated functions was tested in heterologous expression systems. Plasmid pSU40 (*qsdA*$_{W2}$ cloned into pME6032) was transferred to *E. coli* strain DH5α, *Pseudomonas fluorescens* strain 1855-344, and the octopine-type conjugal transfer constitutive strain *Agrobacterium tumefaciens* 15955tra$^c$ to yield DH5α(pSU40), 1855-344(pSU40), and 15955tra$^c$ (pSU40), respectively. The ability of these strains to interfere with QS-regulated functions was first evaluated using the streak assay described by Molina et al. (23), using *C. violaceum* CV026 as an indicator strain. The ability to interfere with virulence in *Erwinia carotovora* was tested in the potato tuber assay as described by Uroz et al. (44). Additionally, the ability of the *qsdA* locus to interfere intracellularly with QS was tested in *Agrobacterium tumefaciens*. Ti plasmid conjugation assays were performed essentially as described by Oger et al. (26), using strain 15955tra$^c$ as a transfer constitutive donor and C58C1RS as a recipient. In all experiments, strains harboring the empty vector pME6032 were used as negative control.

**Nucleotide sequence accession numbers.** The *qsdA* locus sequence has been deposited at GenBank under accession number AY541692, and the *qsdA* allele sequences have been deposited under accession numbers EF218062, EF218066, EF218065, and EF589962 (*R. erythropolis* strains SQ1, MP50 CECT 3008, and Mic1, respectively), EF218064 (*R. corynebacteroides* CECT 420), and EF218063 (*R. rhodochrous* strain CECT 3042).

## RESULTS AND DISCUSSION

**Identification of the *Rhodococcus erythropolis* gene involved in N-AHSL degradation.** Genes involved in QS signal degradation were isolated from a genomic library of *R. erythropolis* strain W2 on the basis of the ability of specific cosmids to confer $C_6$-HSL degradation capability upon the *E. coli* host cells. Degradation was clearly evidenced for 20 out of the 2,880 clones screened. Based on their restriction patterns, the 20 cosmids fell into four groups, which all shared single common EcoRI and BamHI fragments of ca. 3.2 and 3.5 kb, respectively. The cosmid pSU16, which harbors the smallest insert, was chosen for further studies. DNA fragments resulting from BamHI or EcoRI restrictions of pSU16 DNA were shotgun subcloned into pUC19. Only two sets of clones, containing either a 3.5-kb BamHI (pSU16-12) (not shown) or a 3.2-kb EcoRI (pSU16-11) (Fig. 2) fragment conferred upon their *E. coli* host the ability to degrade N-AHSLs with acyl chains ranging from $C_6$- to $C_{12}$-HSL, independently of the substitution at carbon 3. HPLC analyses of the growth media of these clones confirmed the disappearance of the N-AHSL (data not shown).

The sequence of the EcoRI fragment of pSU16-11 (3,152 bp; GenBank accession number AY541692) contains two incomplete open reading frames (ORFs) and two complete ORFs, which were named *orf1* and *qsdA* (for *Q*S *s*ignal *d*egradation) (Fig. 2). The first incomplete ORF (to the right in Fig. 2) shows similarities to alleles of the *gntR*/*fadR* family of transcriptional regulators and is most closely related to that of *Pseudomonas aeruginosa* strain PAO1 (PA1627) (identity, 40%; similarity, 58%). The first complete ORF, *orf1*, could encode a serine-rich protein of 172 amino acids. It does not show any significant homology with peptide sequences available in the databases, suggesting that it might be a pseudogene. The second complete ORF, *qsdA*, could encode a protein of 323 amino acids related to members of the PTE superfamily, which is found in a wide range of organisms from bacteria to eukarya. PTEs are zinc metalloenzymes which were initially identified as efficiently catalyzing the hydrolysis of a variety of organophosphorus compounds (13), but a growing number of PTE homologues which also show amidohydrolase or lactonase activities have been characterized (36). Finally, the protein encoded by the incomplete *orf3* shows 48% identity with proteins annotated as acyl coenzyme A synthetases (AMP-forming)/AMP-acid ligases II of *Ralstonia metallidurans* and other enzymes related to the lipid metabolism and transport. Acyl coenzyme A synthetases are involved in both the synthesis and turnover of fatty acids in bacteria.

Because the ORFs *qsdA* and *orf1* were the only uninterrupted ORFs present in pSU16-11, they were likely to determine the $C_6$-HSL degradation ability conferred by that clone. To confirm this hypothesis, various constructions were generated (Fig. 2). As expected, the constructions lacking one or both partial ORFs (e.g., pSU8-1 and pSU*qsdA*$_{W2}$) still conferred N-AHSL-degrading capabilities upon *E. coli*. Conversely, constructions lacking *qsdA* (pSU reg1) or harboring a disrupted *qsdA* gene (pSU8-1::Gm) did not confer N-AHSL degradation ability upon their host. From these results, it is clear that *qsdA* is necessary and sufficient to code for N-AHSL degradation in the original pSU16 cosmid.

***qsdA* codes for a PTE-like N-AHSL lactonase.** The identification of the degradation products of N-AHSLs was performed by a combination of HPLC and mass spectrometry analyses to detect the presence of HSL or N-AHS generated by amidolysis or lactonolysis, respectively (Fig. 1). The spontaneous degradation was evaluated in control experiments that used uninoculated LB medium or medium inoculated with *E. coli* DH5α or DH5α with the empty cloning vector. Figure 3A presents results obtained for strain DH5α(pME6032). In each

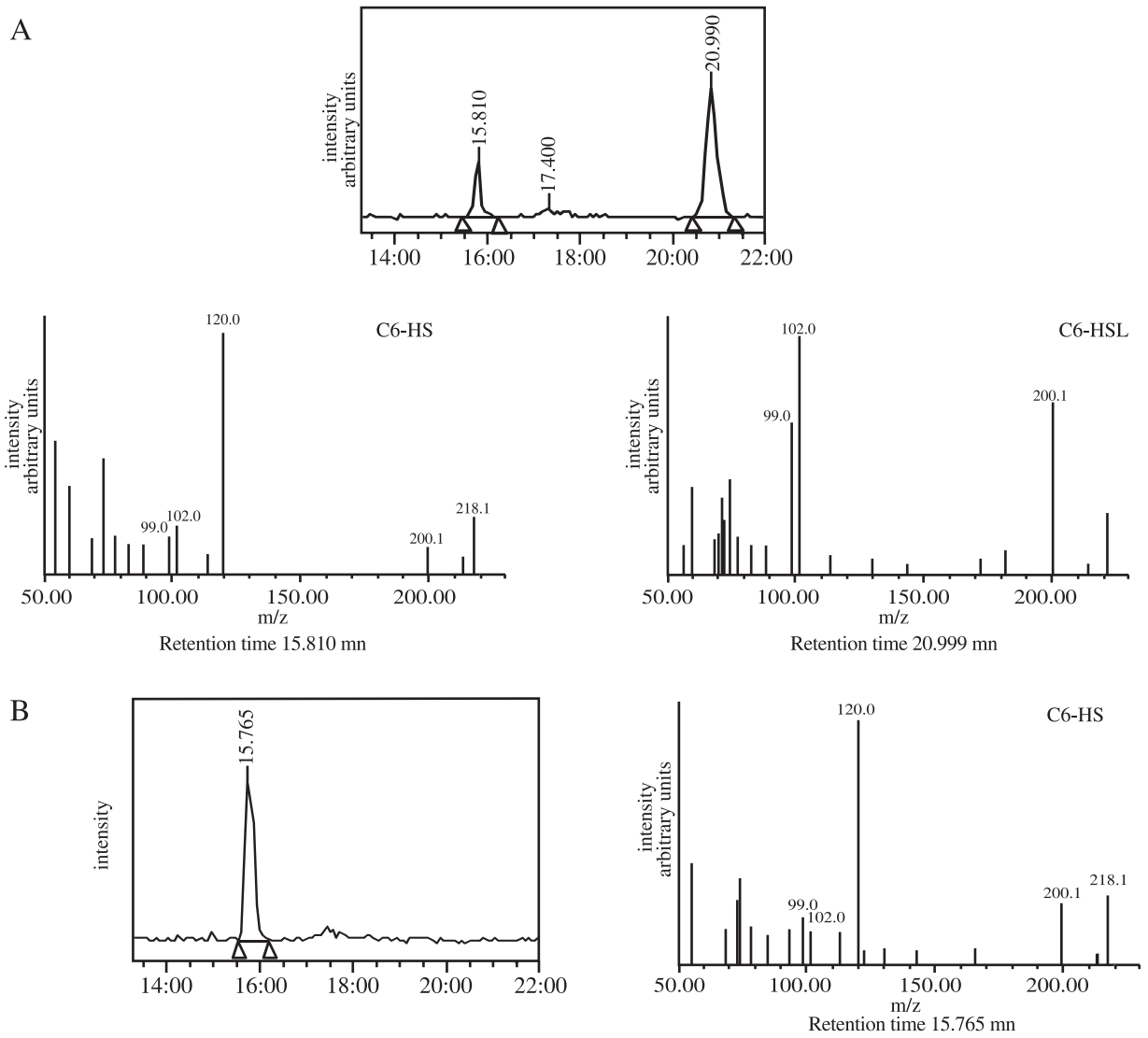


FIG. 3. QsdA lactonase activity. *E. coli* strains DH5α(pME6032) and DH5α(pSU40) were incubated in pH 6.5 buffered LBm medium with 50 μM $C_6$-HSL for 24 h. The medium was analyzed at 0 and 24 h by HPLC-mass spectrometry. Under the experimental conditions used, $C_6$-HS (molecular weight, 217) and $C_6$-HSL (molecular weight, 199) had retention times of 15.8 and 21 min, respectively, and mass spectra were composed of the following main fragments: $m/z$ = 218, 200, and 120 for $C_6$-HS, and $m/z$ = 200, 102, and 99 for $C_6$-HSL. (A) Spontaneous degradation of $C_6$-HSL in aqueous medium. (B) DH5α(pSU40) after 24 h of incubation. A single peak at a retention time of 15.8 min is visible on the HPLC spectrum, which is identified as $C_6$-HS. $C_6$-HSL has completely disappeared from the medium. The formation of $C_6$-HS, correlated with the absence of HSL in the medium, is indicative of a lactonase activity.

experiment, two peaks were visible on the HPLC spectra after a 24-h incubation. Their position at 15.8 and 21 min as well as mass spectra correspond to $C_6$-HS and $C_6$-HSL, respectively. The presence of $C_6$-HS in the control experiments results from the spontaneous lactonolysis of $C_6$-HSL in aqueous medium. Each control condition yielded the same amount of spontaneous lactonolysis (ca. 25%; data not shown), showing that DH5α does not itself facilitate the degradation of N-AHSLs. At the end of experiments performed with *E. coli* DH5α(pSU40) expressing *qsdA*, no N-AHSL could be detected (Fig. 3B), indicating that a complete conversion of the initial $C_6$-HSL occurred. Attempts to detect the presence of HSL, which would indicate a cleavage of the N-AHSL molecules by an amidohydrolase, failed (data not shown). At the same time, the presence of $C_6$-HS was clearly visible (Fig. 3). Thus, *qsdA* must code for an N-AHSL lactonase activity, the presence of which in *Rhodococcus* has been recently identified by Park and colleagues (29).

QsdA clearly belongs to the PTE family of zinc-dependent metalloproteins (Fig. 4). It is totally unrelated to the known bacterial N-AHSL lactonases, which belong to the Zn-dependent glyoxylase family, or to the known N-AHSL amidohydrolases, which belong to the cluster of β-lactam acylases. Thus, the present study extends the number of families of N-AHSL-degrading enzymes of bacterial origin to three and to include the PTE family. PTEs were first described for their ability to cleave the phosphotriester bond in phosphotriesters but were later shown to be promiscuous enzymes, harboring lactonase

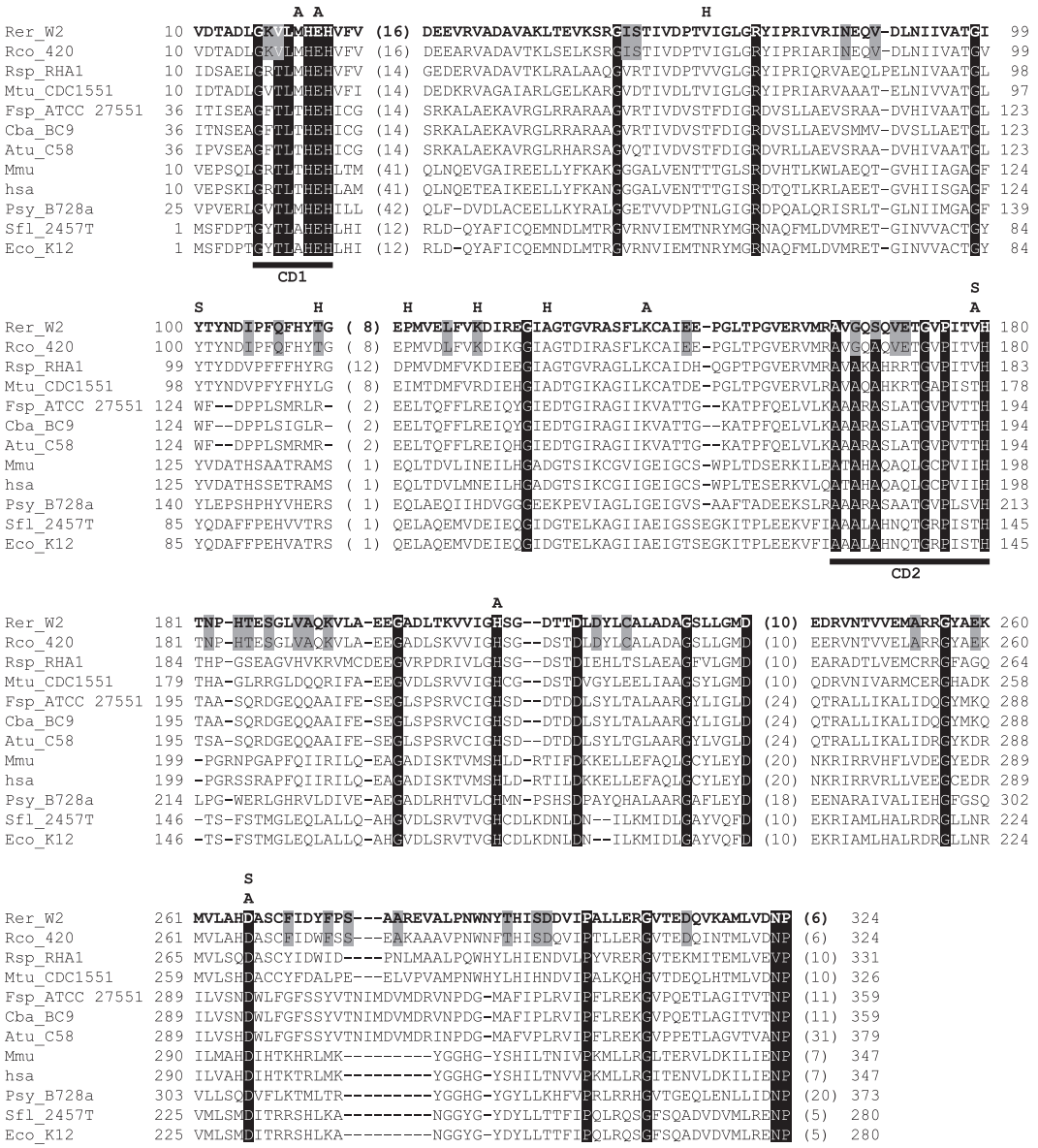


FIG. 4. Alignment of selected PTEs from bacterial and eukaryotic origins. In addition to QsdA from *R. erythropolis* strain W2 (Rer_W2), PTE homologues used in this alignment include a putative QsdA homologue from *Rhodococcus corynebacterioides* CECT 420 (Rco_420, this study); putative PTEs from *Rhodococcus* sp. strain RHA1 (Rsp_RHA1, accession no. TIG:13193), *Agrobacterium tumefaciens* strain C58 (Atu_C58, accession no. AAL43882), *Pseudomonas syringae* pv. Syringae strain B728a (Psy_B728a, accession no. YP_233869), *Shigella flexneri* strain 2457T (Sfl_2457T, accession no. AAP19319), *Mycobacterium tuberculosis* strain CDC1551 (Mtu_CDC1551, accession no. AAK44461), and *Escherichia coli* K-12 (Eco_K12, accession no. AAC76404); PTEs from *Chryseobacterium balustinum* strain BC9 (Cba_BC9, accession no. CAD19996); OPD from *Flavobacterium* sp. strain ATCC 27551 (Fsp_ATCC27551, accession no. CAD13181); and PTER from *Homo sapiens sapiens* (Hsa, accession no. Q96BW5) and from *Mus musculus* (Mmu, accession no. Q60866). The positions of the essential amino acids residues of the catalytic site (A), the substrate binding site (S), and the dimerization domains (H) are shown above the alignment. CD1 and CD2, zinc binding conserved domains 1 and 2. The 27 residues conserved among known PTE sequences are shown in reverse font. QsdA-specific amino acid substitutions which are not observed in other PTEs (including alleles not shown in the figure) are highlighted by a gray background.

or amidohydrolase activities (36). QsdA, however, does not confer the ability to degrade the prototypic phosphotriester methyl parathion, nor does the prototype PTE gene, *opd*, code for N-AHSL degradation (data not shown). Although QsdA exhibits the canonical structure of PTEs, the sequence of its metal binding site, which is central to the enzyme activity by fixing two molecules of zinc and forming the catalytic pocket in which the substrate inserts, differs from the consensus sequence of PTEs at 3 of 12 positions (CD1 and CD2 domains in Fig. 4). In contrast, the sole PTE/QsdA homologue known from *Rhodococcus*, i.e., the TIG:13193 gene from strain RHA1 (Rsp_RHA1 in Fig. 4), as well as the closest homologues of QsdA, i.e., the PTE alleles of several *Mycobacterium* strains and other actinobacteria (Mtu_CDC1551 in Fig. 4), all present the canonical signature of the PTEs, including the 12 conserved amino acids from the zinc binding domain. Interest-

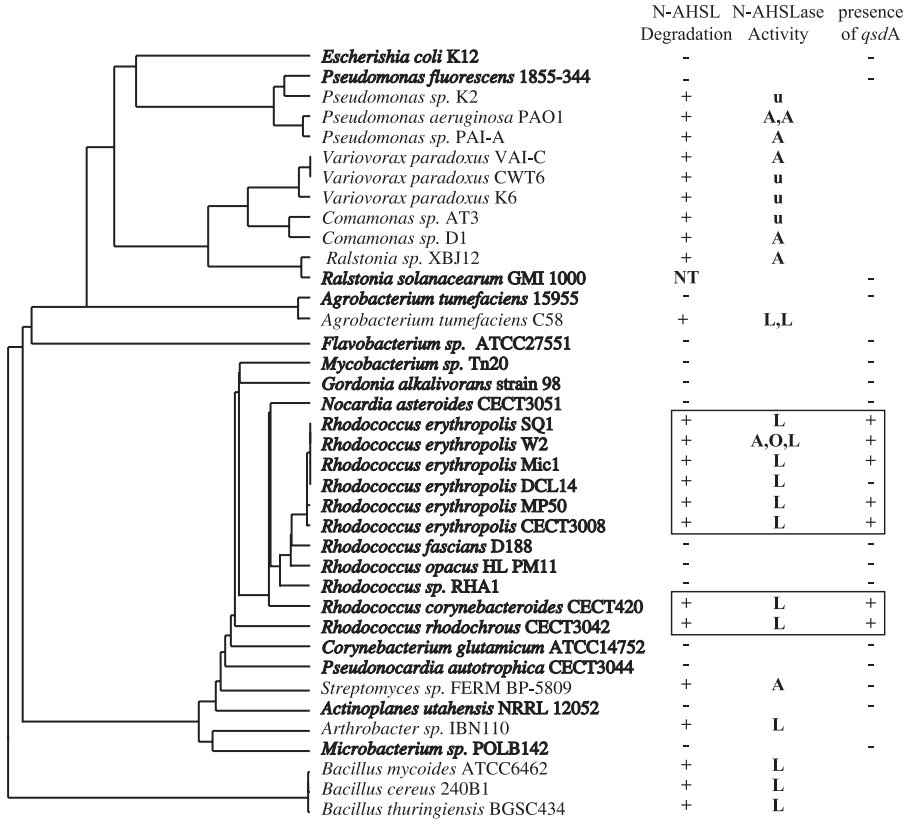


FIG. 5. Phylogenetic distribution of N-AHSL degradation in *Rhodococcus*. The ability to degrade N-AHSLs among bacteria is highlighted on a phylogenetic tree based on the 16S rRNA gene. Strains shown in bold were tested in the present study. N-AHSL degradation data from the literature are reported for all other strains. Wherever possible the nature of the enzymatic activity is noted: A, N-AHSL amidohydrolase; L, N-AHSL lactonase; O, N-AHSL oxidoreductase; u, undetermined. The number of identified activities is also reported (e.g., A, A indicates two amidohydrolases). The presence (+) or absence (−) of a *qsdA* homologue was determined by hybridization with a *qsdA* probe. The N-AHSL-degrading strains belonging to the *Rhodococcus* genus are boxed. *Agrobacterium tumefaciens* strain C58 harbors two N-AHSL lactonases (AttM and AiiB), and *R. erythropolis* strain W2 harbors at least three N-AHSL modification/degradation enzymes: an amidohydrolase (A), an oxidoreductase (O), and a lactonase (L). NT, not tested.

ingly, strain RHA1 and *Mycobacterium* sp. strain Tn20 are both unable to degrade N-AHSLs. In addition, when cloned and expressed in *E. coli*, the TIG:13193 ORF from RHA1 does not confer N-AHSL degradation capabilities to its host. Thus, the lack of N-AHSL degradation in strain RHA1 is due not to a lack of expression of the protein from its native promoter but to the lack of N-AHSL lactonase activity of the TIG:13193 protein, which will consequently be referred to not as a QsdA but as a classical PTE homologue. QsdA is therefore the first prokaryotic member of the PTE family with demonstrated N-AHSL lactonase activity.

**QsdA is a *Rhodococcus*-specific PTE-like N-AHSL lactonase.** The wide occurrence of PTE homologues in species closely related to *R. erythropolis* and the specific signature and activity of QsdA prompted us to determine the phylogenetic distribution of this gene in the actinobacterial cluster. We screened a collection of related strains belonging to the *Rhodococcus* genus, i.e., *R. corynebacteroides* (one strain), *R. erythropolis* (five strains), *R. fascians* (one strain), *R. rhodochrous* (one strain), and *R. opacus* (one strain), for their ability to inactivate N-AHSLs and the presence of the *qsdA* gene. The ability to degrade N-AHSLs appeared ubiquitous among strains of *R. erythropolis*, since all assayed isolates (5/5) degraded a range of N-AHSLs identical to that previously reported for strain W2 (acyl chain length from $C_6$ to $C_{14}$, with or without substitution on C3 [see Table S1 in the supplemental material]). N-AHSL degradation was observed in three of the five *Rhodococcus* species tested, i.e., *R. erythropolis*, *R. rhodochrous*, and *R. corynebacteroides*, but not in *R. fascians* and *R. opacus*, although these species are more closely related to *R. erythropolis* than are *R. rhodochrous* and *R. corynebacteroides* (Fig. 5), and not in the polychlorinated biphenyl-degrading *Rhodococcus* sp. strain RHA1. The degradation ability was also tested in members of genera closely related to *Rhodococcus*, such as *Pseudonocardia autotrophica* (strain CECT 3044), *Corynebacterium glutamicum* (strain ATCC14752), *Gordonia alkalivorans* (strain 98), *Microbacterium* sp. (strain POLB142), *Mycobacterium* sp. (strain Tn20), *Nocardia asteroides* (strain CECT 3051), *Actinoplanes utahensis* (strain NRRL 12052), and *Flavobacterium* sp. (strain ATCC 27551). None of these assayed strains degraded N-AHSLs.

The *qsdA* gene was detected by Southern hybridization, under low-stringency conditions using the cloned *qsdA* gene from strain W2 as a probe, in all but one of the strains able to degrade N-AHSLs, including *R. corynebacteroides*, *R. erythropolis*, and *R. rhodochrous*. Southern hybridization performed

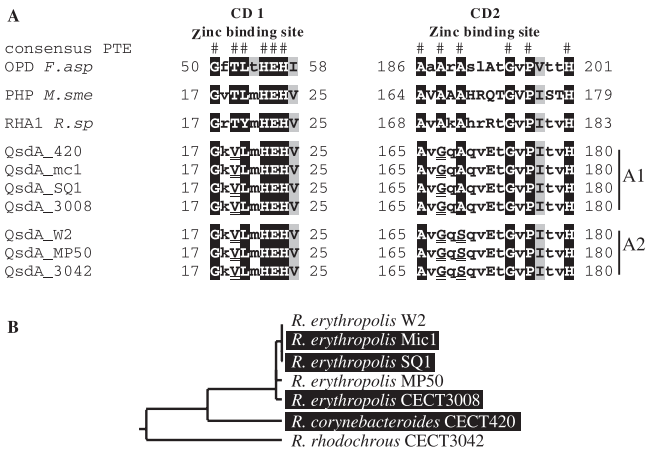


FIG. 6. Alignment of the conserved zinc biding domains of QsdA homologues. (A) OPD *F.asp*, canonical PTE sequence from *Flavobacterium* sp. strain ATCC 27551; RHA1 *R.sp*, deduced peptide sequence from the PTE homologue from *Rhodococcus* sp. strain RHA1 encoded by gene TIG:13193; PHP *M.sme*, PTE homologue from *Mycobacterium smegmatis*; consensus PTE, the 12 conserved amino acids from the PTE zinc binding domains CD1 and CD2 are indicated (#). Nonconserved positions are indicated in lowercase letters. *qsdA*-deduced protein sequences used in this alignment include those from *Rhodococcus corynebacteroides* strain CECT 420 (QsdA_420), *Rhodococcus rhodochrous* strain CECT 3042 (QsdA_3042), and *Rhodococcus erythropolis* strains SQ1 (QsdA_SQ1), CECT 3008 (QsdA_3008), W2 (QsdA_W2), Mic1 (QsdA_Mic1), and MP50 (QsdA_MP50). Divergences from the consensus PTE zinc domain sequence are underlined in the QsdA sequences. Two divergent alleles of QsdA (A1 and A2) are found in *Rhodococcus*. (B) Taxonomic relationship between strains harboring allele A1 (black background) and strains harboring allele A2 (white background) based on a 16S rRNA gene phylogeny.

on genomic DNA restricted with EcoRI, BamHI, and HindIII confirmed that the gene is present as a single copy in the genomes of these strains. The only *R. erythropolis* strain that did not hybridize with the *qsdA* probe was strain DCL14 (46). This strain is indistinguishable from strain W2 in terms of 16S rRNA gene sequence and ability to inactivate QS molecules. This result is, however, consistent with the observation by Uroz et al. (43), who demonstrated the occurrence of N-AHSLase activities other than lactonase in *R. erythropolis* strains W2 and DCL14. The *qsdA* gene could not be detected in any of the *Rhodococcus* strains unable to degrade N-AHSLs or in the strains from the related genera.

A total of six *qsdA* alleles (in addition to *qsdA* from strain W2) were cloned into pGEM-T Easy following PCR amplification from the genomic DNAs of the four *R. erythropolis* clones, as well as the *R. rhodochrous* and *R. corynebacteroides* strains showing a positive hybridization signal in Southern analysis. All alleles conferred the ability to degrade N-AHSLs upon *E. coli* DH5α. The deduced QsdA peptides fell into two homology groups, which were termed A1 and A2 (Fig. 6A). Sequences (DNA and protein) were almost identical within each group and were ca. 88% identical and 93% similar at the protein level between groups A1 and A2. The conserved zinc binding domains CD1 and CD2 of the QsdA alleles of clusters A1 and A2 diverge at one position (residue 167) (Fig. 6A). Meanwhile, domains CD1 and CD2 of allele clusters A1 and A2 also diverge from the consensus PTE domains at two or three positions (QsdA residues 19, 167, 169), suggesting that the six alleles might derive from the same PTE ancestor. However, the *qsdA* allele phylogeny is not congruent with the *Rhodococcus* 16S rRNA gene phylogeny (Fig. 6B). Furthermore, the *Rhodococcus* species harboring these alleles do not appear to form a clade within the *Rhodococcus* genus (Fig. 5). This suggests that *qsdA* is a *Rhodococcus*-specific N-AHSL lactonase that evolved in this genus and was transferred horizontally at several points during the speciation of *Rhodococcus*.

***qsdA* confers QS quenching abilities in heterologous systems.** We tested the ability of all the cloned *qsdA* alleles, including $qsdA_{W2}$ as well as $qsdA_{420}$, $qsdA_{SQ1}$, $qsdA_{3008}$, $qsdA_{MP50}$, $qsdA_{Mic1}$ and $qsdA_{3042}$, to interfere with the expression of QS-regulated functions, using three different quenching assays in which *qsdA* is expressed in heterologous systems.

All alleles conferred upon their host the ability to inactivate the same range of N-AHSLs as the wild-type strain W2 (i.e., N-AHSLs with or without substitution on carbon 3 and with an acyl chain ranging from 6 to 14 carbons [data not shown]), regardless of the plasmid vector used to express the gene. They were able to quench the synthesis of violacein by *C. violaceum* CV026 grown in the presence of $C_6$-HSL with the same efficiency as the wild-type *R. erythropolis* strain W2 (data not shown).

When expressed in *P. fluorescens* strain 1855-344, $qsdA_{W2}$ conferred quenching capabilities closely matching that of the wild-type *R. erythropolis* strain W2 (Table 2). Strains 1855-344(pSU40) and 1855-344(pME6032) were inoculated independently at various ratios with the potato soft rot pathogen *P. carotovorum* strain PCC797 on potato tubers. In the absence of the quencher, the maceration zones averaged 2.8 cm on the potato tubers. Conversely, in the presence of the quenching strain expressing $qsdA_{W2}$, a clear reduction of symptom severity was visible, since no maceration occurred at quencher/pathogen ratios of 10:1 and 1:1 whatever the concentration of pathogen used for the assay ($10^5$ or $10^6$ cells $ml^{-1}$).

When expressed in the *Agrobacterium tumefaciens* conjugal transfer constitutive strain 15955tra$^c$, $qsdA_{W2}$ prevented the accumulation in the medium of the *N*-AHSLs normally produced by this strain (data not shown). In addition, the $qsdA_{W2}$ gene conferred upon this strain the ability to degrade N-AHSLs provided exogenously (data not shown). Furthermore, the expression of the $qsdA_{W2}$ gene totally abolished Ti plasmid con-

TABLE 2. QQ capabilities of *P. fluorescens* strain 1855-344 harboring *qsdA*

| *P. carotovorum* PCC 797 concn (cells $ml^{-1}$)[a] | Pathogen/quencher ratio | Avg extent of maceration zone (cm) ± SE with *P. fluorescens* 1855-344 carrying[b]: | |
|---|---|---|---|
| | | pME6032 | pSU40 |
| $10^5$ | 1:10 | 2.6 ± 0.5 | NM |
| | 1:1 | 2.8 ± 0.5 | NM |
| | 10:1 | 2.5 ± 0.6 | 2.2 ± 0.8 |
| $10^6$ | 1:10 | 2.8 ± 0.4 | NM |
| | 1:1 | 2.8 ± 0.5 | NM |
| | 10:1 | 2.8 ± 0.5 | 2.8 ± 0.8 |

[a] Concentration of the *P. carotovorum* cell suspension used for inoculation of potato tubers.
[b] Taken from three repeats. NM, no maceration observed.

jugal transfer by this strain. Indeed, 15955tra$^c$(pME6032) transferred its Ti plasmid to the recipient strain C58C1RS at high frequency ($1.7\ 10^{-2}$ transconjugants per donor cells), while 15955tra$^c$(pSU40) no longer conjugated its Ti plasmid to detectable levels; i.e., transfer frequencies were below the detection level of $5 \times 10^{-8}$ transconjugants per donor cell. Therefore, the *qsdA*-based N-AHSL-turnover was sufficient to prevent the accumulation of the typical *A. tumefaciens* QS signal molecules in the growth medium and the QS regulation of Ti plasmid conjugal transfer.

Due to the worldwide distribution of this genus in the soil and to the marked quenching abilities of the wild-type strains and cloned *qsdA* genes, *Rhodococcus* isolates and derivatives harboring this gene could become interesting natural biocontrol agents directed toward QS-regulated traits in plant pathogens.

**Role of *qsdA* in *Rhodococcus*.** The above observations raise questions regarding the role of *qsdA* in *Rhodococcus*. The analysis of the neighboring sequences (encoding an acyl coenzyme A synthetase and a FadR peptide analogous to a fatty acid biosynthesis regulatory protein) suggests a possible involvement in fatty acid metabolism. The observation that even closely related clones, such as *R. erythropolis* strain DCL14, or species, such as *R. opacus* and *R. fascians*, do not possess the *qsdA* gene or a N-AHSLase activity supports the view that the function encoded by *qsdA* may be either nonessential or redundant. This view is also supported by the observation that a W2 *qsdA* mutant (harboring a disrupted *qsdA* gene) has growth properties and N-AHSL degradation ability similar to those of the wild-type parent (data not shown). In such a light, the hypothesis suggested by Kaufmann et al. (17) that 3-oxo-dodecanoyl-HSL and spontaneous reorganization products could play a role as antibiotics targeting specifically gram-positive bacteria is a very tempting alternative explanation accounting for the presence of *qsdA* within the *Rhodococcus* genus. Strain W2 and related N-AHSL degraders therefore appear very well equipped to resist N-AHSLs produced by gram-negative soil competitors, with a complete degradative arsenal composed of at least two N-AHSL degradation activities, including a lactonase and an amidohydrolase, and an additional N-AHSL modification activity (29, 43). The putative toxicity of N-AHSLs on *Rhodococcus* remains to be demonstrated, since it has not been observed in preliminary experiments performed with the wild-type strains used in the present work.

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