INFECTION AND IMMUNITY, Mar. 2006, p. 1673–1682 Vol. 74, No. 3
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.3.1673–1682.2006
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Quorum Quenching by an *N*-Acyl-Homoserine Lactone Acylase from *Pseudomonas aeruginosa* PAO1

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Received 22 July 2005/Returned for modification 25 October 2005/Accepted 20 December 2005

The virulence of the opportunistic human pathogen *Pseudomonas aeruginosa* PAO1 is controlled by an *N*-acyl-homoserine lactone (AHL)-dependent quorum-sensing system. During functional analysis of putative acylase genes in the *P. aeruginosa* PAO1 genome, the PA2385 gene was found to encode an acylase that removes the fatty acid side chain from the homoserine lactone (HSL) nucleus of AHL-dependent quorum-sensing signal molecules. Analysis showed that the posttranslational processing of the acylase and the hydrolysis reaction type are similar to those of the beta-lactam acylases, strongly suggesting that the PA2385 protein is a member of the *N*-terminal nucleophile hydrolase superfamily. In a bioassay, the purified acylase was shown to degrade AHLs with side chains ranging in length from 11 to 14 carbons at physiologically relevant low concentrations. The substituent at the 3′ position of the side chain did not affect activity, indicating broad-range AHL quorum-quenching activity. Of the two main AHL signal molecules of *P. aeruginosa* PAO1, *N*-butanoyl-L-homoserine lactone (C_4-HSL) and *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), only 3-oxo-C_{12}-HSL is degraded by the enzyme. Addition of the purified protein to *P. aeruginosa* PAO1 cultures completely inhibited accumulation of 3-oxo-C_{12}-HSL and production of the signal molecule 2-heptyl-3-hydroxy-4(1*H*)-quinolone and reduced production of the virulence factors elastase and pyocyanin. Similar results were obtained when the PA2385 gene was overexpressed in *P. aeruginosa*. These results demonstrate that the protein has in situ quorum-quenching activity. The quorum-quenching AHL acylase may enable *P. aeruginosa* PAO1 to modulate its own quorum-sensing-dependent pathogenic potential and, moreover, offers possibilities for novel antipseudomonal therapies.

Pseudomonas aeruginosa PAO1 is an opportunistic pathogen which causes disease mainly in individuals who are immunocompromised, have cystic fibrosis, or suffer from serious burn wounds. It utilizes two *N*-acyl-homoserine lactone (AHL)-dependent quorum-sensing systems, termed *las* and *rhl*, which together regulate an extensive set of cell population density and growth-phase-dependent virulence factors (7, 22). The *las* and the *rhl* quorum-sensing systems employ *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), which function by activating the response regulator proteins LasR and RhlR, respectively. In diverse gram-negative bacteria, many different AHL signal molecules have been characterized. These all consist of a homoserine lactone (HSL) ring or nucleus which is connected via an amide bond to a fatty acid side chain of 4 to 14 carbon atoms in length that may contain an oxo or hydroxyl group at the 3′ position and unsaturated bonds (Fig. 1).

In *P. aeruginosa* PAO1, swarming motility, biofilm maturation, and the expression of virulence factors such as exoproteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin, cyanide, and the cytotoxic lectins PA-IL and PA-IIL, as well as the expression of the Xcp secretory apparatus, are controlled by quorum sensing, and an active quorum-sensing system is crucial for full pathogenicity (51). Interfering with the quorum-sensing systems of this pathogen may reduce virulence by preventing the release of harmful exoproducts and by inducing the development of abnormal biofilms which are more susceptible to conventional antibiotics (7, 10, 26, 36). This makes quorum-sensing components very attractive targets for the development of an anti-infective therapy (53).

So far, inhibition of quorum sensing has been achieved by destabilizing the LuxR family protein receptors for AHL signal molecules (7, 15) and by degrading AHL signal compounds by use of lactonases (26) and, more recently, acylases (16, 23, 26, 34, 56). In general, acylase enzymes remove a side chain from a ring-like molecule by hydrolyzing the connecting amide bond (Fig. 1). There are many different types of acylases, which differ in terms of substrate specificity for the side chain and nucleus. A well-known example is the β-lactam acylase class, which has been extensively investigated because of its economic value for the production of semisynthetic β-lactam antibiotics (3, 44). One of the striking features of this enzyme class is the posttranslational processing: the gene is transcribed as a single polypeptide, which autocatalytically cleaves to produce a mature active enzyme consisting of two dissimilar subunits (2, 24).

We set out to investigate acylase activity in *P. aeruginosa*. A putative acylase gene (the PA2385 gene) was located in the PAO1 genome sequence (47) by homology searching using the sequence encoding the β-lactam acylase from *Pseudomonas* SY-77 (45). This gene is also known as *qsc*112 (52) and *pvdQ*

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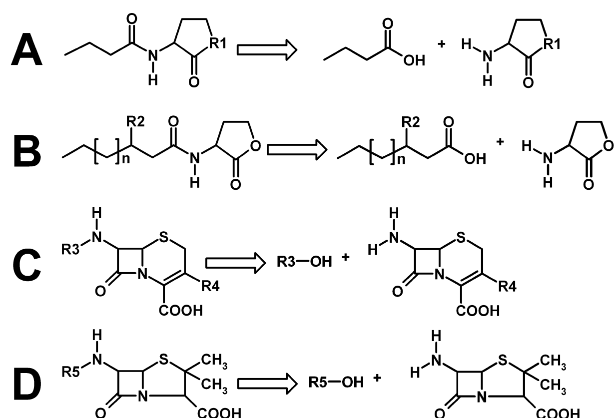

FIG. 1. Hydrolysis of AHL and β-lactam substrates by acylase enzymes. (A) Deacylation of C_4-HSL (R1 = O) and C_4-thio-HSL (R1 = S). (B) Hydrolysis of other AHL compounds (R2 = H, OH, or O; n = 1, 2, 3, 5, 7, or 9). (C) Hydrolysis of the cephalosporins glutaryl-7-aminoacetoxycephalosporanic acid (R3 = glutaric acid; R4 = methylacetoxy), adipyl-7-aminodesacetoxycephalosporanic acid (R3 = adipic acid; R4 = methylacetoxy), cephalosporin C (R3 = 5-aminoadipic acid, R4 = methylacetoxy), cephalexin (R3 = phenylglycine; R4 = methylacetoxy), and cefadroxil (R3 = 4-hydroxyphenylglycine; R4 = methylacetoxy). (D) Hydrolysis of the penicillins penicillin G (R5 = phenylacetic acid), penicillin V (R5 = phenoxyacetic acid), ampicillin (R5 = phenylglycine), and amoxicillin (R5 = 4-hydroxyphenylglycine).

(21). In a previous study (26), the Zhang group observed that *P. aeruginosa* PAO1 is capable of growing in minimal medium with 3-oxo-C_{12}-HSL or, alternatively, with any of several other AHL signal molecules with C_8 to C_{14} side chains, as the sole energy source. The signal molecules were cleaved to release HSL and the fatty acid side chain, indicating an acylase activity. The PA2385 gene product was identified as the possible agent for this activity based on a high level of homology with the AHL acylase from a *Ralstonia* species (26). Overexpression of this gene in *Escherichia coli* resulted in deacylation of AHLs with C_8 to C_{14} acyl side chains. Overexpression of the gene in *P. aeruginosa* PAO1 inhibited accumulation of 3-oxo-C_{12}-HSL. Unexpectedly, *pvdQ* mutants of *P. aeruginosa* PAO1 were still capable of growing in the minimal medium with 3-oxo-C_{12}-HSL as the sole energy source, and it was concluded that another enzyme must confer the growth phenotype (16). Our report describes the cloning and expression of the PA2385 gene in *E. coli*, the analysis of the substrate specificity of the purified enzyme both in vitro and in a quorum-sensing bioassay, a thorough analysis of the subunit composition, and the effect of the enzyme on virulence factor production by *P. aeruginosa* PAO1, underlining the potential of this gene's product as a novel antibacterial agent.

MATERIALS AND METHODS

Chemicals. β-Lactam compounds either were purchased from Sigma or were gifts of DSM, Delft, The Netherlands. Acyl-HSL compounds were either obtained from Fluka or synthesized as described earlier (5). 2-Heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) was synthesized as previously described (9, 38).

DNA manipulation and cloning of the PA2385 gene. All DNA-handling steps were performed following standard protocols (40). Chromosomal DNA was isolated from an overnight culture of *P. aeruginosa* PAO1 (Holloway collection) as described by Chen and Kuo (4). The open reading frame (ORF) encoding the acylase gene (bases 2638805 to 2636517, obtained from www.pseudomonas.com) (47) was amplified from chromosomal DNA by PCR and cloned into plasmid pMcTNde (45) under the control of the *tac* promoter, resulting in plasmid pMc-PA2385. For this procedure, the three bases preceding the putative start codon were altered to create an NdeI restriction site. Subsequently, the ORF was amplified from plasmid pMc-PA2385 by use of primers that added an EcoRI restriction site in front of the putative start codon and a BglII restriction site after the putative stop codon. The resulting PCR product was cloned into the *P. aeruginosa-E. coli* shuttle vector pME6032 (14) by use of these restriction sites, putting the gene under the control of a *tac* promoter, which is repressed in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside) by *lac*I^q.

Purification of the PA2385 protein. *E. coli* DH10B cells containing plasmid pMc-PA2385 were grown for 30 h at 25°C in 100-ml aliquots of 2× TY medium (40) supplemented with 50 μg/ml chloramphenicol and 0.1% glycerol. Cells (a total of 250 ml pooled from 100-ml cultures) were harvested by centrifugation, sonicated in 10 ml 50 mM Tris, 2 mM EDTA, pH 8.8, and centrifuged (30 min, 20, 000 × *g*). The PA2385 protein was purified from the resulting supernatant by column chromatography as described earlier for *Pseudomonas* SY-77 glutaryl acylase (33), by the subsequent use of Q-Sepharose for anion exchange chromatography and phenyl-Sepharose for hydrophobic interaction chromatography, and finally by a polishing step on Q-Sepharose columns (from Amersham Biosciences). Fractions were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and pooled based on the presence of the appropriate bands.

Protein identification. The N-terminal sequences of the α- and β-subunits of the acylase were determined by an automated Edman degradation performed on an Applied Biosystems 476A instrument at the Sequence Centre, Utrecht, The Netherlands. The molecular masses of the α- and β-subunits of the PA2385 protein were determined by mass spectrometry on an API 3000 triple quadrupole liquid chromatography-tandem mass spectrometry (MS) mass spectrometer (Perkin-Elmer Sciex Instruments). Peaks were analyzed by the software program MassChrom v1.2.

Activity assays. Cell extracts of *E. coli* DH10B cells containing plasmids pMcT and pMc-PA2385 were made as described above using 100 ml culture medium and 5 ml sonication buffer. The level of activity towards the β-lactam compounds shown in Fig. 1 was determined using a fluorescamine colorimetric assay (39). Deacylation of β-lactam compounds results in the formation of a primary amine group on the β-lactam ring, which can be labeled by fluorescamine. A reaction mixture consisting of 200 μl 20 mM phosphate buffer containing 5 μl cell extract and 1 or 5 mM substrate was prepared. After 1, 2, and 20 h incubation at 30°C, a 40-μl aliquot was transferred to 140 μl 0.2 M acetate buffer, pH 4.5, and 20 μl 1 mg/ml fluorescamine in acetone was added. Absorbance at 380 nm was measured after incubation at room temperature for 1 h.

Deacylation of AHL compounds will result in the formation of HSL, which can be detected following the reaction of its primary amine group with fluorescamine. Therefore, an assay similar to that used for the β-lactam compounds was performed using 0.1 mg (corresponding to 1.6 to 2.9 mM) of substrate per reaction and incubation times of 1.5, 3, and 4.5 h. Up to 5% (vol/vol) methanol was used to increase the solubility of the substrates. The tested compounds were as follows: C_4-thio-HSL, C_6-HSL, 3-oxo-C_6-HSL, C_7-HSL, C_8-HSL, C_{10}-HSL, 3-oxo-C_{10}-HSL, C_{12}-HSL, 3-oxo-C_{12}-HSL, and C_{14}-HSL. Cell extracts of *E. coli* DH10B::pMcSY77 harboring the *Pseudomonas* SY-77 glutaryl acylase gene as well as the purified enzyme (45) were also used as samples.

A gas chromatography (GC) assay was used to analyze the reagents and products of the hydrolysis reaction. A reaction mix similar to that described for the bioassay but with 1 mg (4.7 mM) substrate C_7-HSL and 25 μg (31 nM) purified PA2385 protein was incubated for 3 h, and samples were taken at 0, 1, 2, and 3 h. After evaporation was performed with a SpeedVac instrument, the residue was taken up in 300 μl ethyl acetate. GC analysis was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 injector and a Hewlett Packard 3365 Series II Chemstation system under the following conditions: column, wall-coated open tubular fused-silica J&W DB-5 column (30 m by 0.249 mm; film thickness, 0.25 μm; J&W Scientific); oven temperature program, 60° to 320°C at 10°C min^{-1}; injector temperature, 250°C; flame ionization detector temperature, 300°C; carrier gas, helium; inlet pressure, 125 kPa; linear gas velocity, 30.8 cm · s^{-1}; split ratio, 60:1; injected volume, 1.0 μl. Substrate C_{12}-HSL was incubated for 0 and 20 h, and the reaction mixture was shaken to keep the substrate resuspended. A control experiment was carried out with phosphate-buffered saline (PBS) substituting for the protein solution. Peaks in the chromatograms were identified by comparison to reference compounds and by MS, for which the samples were concentrated to 100 μl of ethyl acetate. For MS analysis, a Shimadzu QP5000 GC/MS system equipped with a 17A GC, an AOC-20i autoinjector, and the GCMSsolution software 1.10 was used. The GC conditions were as follows: column, wall-coated open tubular fused-silica CP-sil 5 CB low-bleed/MS column (15 m by

TABLE 1. Quorum quenching by the *Pseudomonas aeruginosa* AHL acylase

AHL[a]	Concentration[b]	Biosensor[d]	Response to[c]:		
			PA2385	SY77	PA2385 pure
C_4	0.1	536	−	−	−
C_6	1	401	−	−	−
OC_6	0.01	401	−	−	−
OHC_6	1	CV	−	−	−
C_7	1	401	−	−	−
OC_7	0.01	401	−	−	−
C_8	1	401	−	−	−
OC_8	0.1	401	−	−	−
OHC_8	1	CV	−	−	−
C_9	1	401	−	−	−
C_{10}	0.1	1075	−	−	−
OC_{10}	0.001	1075	−	−	−
OHC_{10}	0.1	1075	−	−	−
C_{11}	0.01	1075	+	−	+
C_{12}	0.001	1075	+	−	+
OC_{12}	0.0001	1075	−	−	+
OHC_{12}	0.001	1075	+	−	+
C_{14}	0.01	1075	−	−	+
OC_{14}	0.0001	1075	−	−	+
OHC_{14}	0.001	1075	+	−	+

[a] AHL substrates are identified by their side chains.
[b] Concentrations are given in mg substrate per ml reaction mix.
[c] −, no reduction in the response of the biosensor was detected; +, the response by the biosensor was decreased or abolished. PA2385 and SY77, cell-free extracts of *E. coli* DH10B cells containing pMc-PA2385 and pMcSY-77, respectively; PA2385 pure, purified PA2385 protein.
[d] Biosensors: 401, *E. coli* JM109::pSB401; 536, *E. coli* JM109::pSB536; 1075, *E. coli* JM109::pSB1075; CV, *Chromobacterium violaceum* CV026 (27).

0.25 mm; film thickness, 0.10 μm; Varian, The Netherlands); oven temperature program, 100°C to 320°C at 15°C min^{-1}; injector temperature, 275°C; carrier gas, helium; column inlet pressure, 75 kPa; column flow, 2.5 ml · min^{-1}; linear gas velocity, 81.4 cm · s^{-1}; split ratio, 21:1; injected volume, 5.0 μl. MS conditions were as follows: ionization energy, 70 eV; ion source temperature, 250°C; interface temperature, 250°C; scan speed, three scans · s^{-1}; mass range, 34 to 500 atomic mass units.

A bioassay to determine the activity of the putative acylases towards AHLs at low and more physiologically relevant concentrations was developed. This assay employs engineered biosensor strains in which pigment production or bioluminescence is affected by a specific range of AHLs (27, 48, 54). A reaction mixture consisting of 1 ml PBS (pH 7.4) containing 5% (vol/vol) methanol, 5 μl cell extract or 2.5 μg (3.1 nM) purified PA2385 protein, and 10 μl substrate solution was incubated at 30°C. Aliquots of 250 μl were taken after 0, 90, and 180 min, supplemented with 250 μl NaCl-saturated PBS, and extracted twice with 500 μl ethyl acetate. The combined organic layers were dried over anhydrous $MgSO_4$ and subsequently in a SpeedVac instrument (Alpha RVC, Christ, Germany). The residue was dissolved in 25 μl acetonitrile. Ten μl of this solution was added to a well of a white 96-well plate, and 100 μl of the appropriate biosensor in LB medium was added. The response of the biosensors after incubation overnight was analyzed with a ChemiGenius^2 XE imaging system (Syngene, United Kingdom). Degradation of the substrate by the sample results in a decrease in or extinction of bioluminescence. The type of biosensor used depended on the AHL being tested. The lowest concentration of AHL at which the biosensor still gave a good response was used as the assay concentration (Table 1).

Quorum-quenching assays with *P. aeruginosa* PAO1. Purified PA2385 protein was added to 50-ml fresh cultures of *P. aeruginosa* PAO1 at a concentration of 0.022 mg/ml, and aliquots were removed 6 h and 24 h postinoculation. Samples were assayed for the production of AHLs, PQS, and pyocyanin and for elastolytic activity. The expression of *lecA* was also monitored by adding 0.14 mg/ml PA2385 protein to the *lecA*::*lux* PAO1 strain (55).

Plasmid pME6032-PA2385 and the control plasmid pME6032 were transformed into *P. aeruginosa* PAO1 by electroporation (46). The resulting strains were grown in 50 ml LB medium in the presence or absence of 1 mM IPTG to analyze the effects of overexpression of PA2385. Aliquots were taken after 5, 8, and 24 h of growth and analyzed. The plasmids were also transformed into the *lecA*::*lux P. aeruginosa* PAO1 strain to determine the expression of *lecA*.

The bioluminescence of the *lecA*::*lux* PAO1 strain was determined as a function of cell population density with a combined, automated luminometer-spectrometer (Anthos Labtech LUCYI). Overnight cultures of *P. aeruginosa* were diluted 1:100 in fresh LB medium, and 0.2-ml portions were inoculated into microtiter plates. The luminescence and turbidity of the cultures (optical density at 495 nm) were automatically determined every 30 min. Luminescence is given in relative light units per unit of optical density at 495 nm. Experiments were repeated three times with similar results.

The elastolytic activity of bacterial supernatants was determined with an elastin Congo red (ECR; Sigma) assay (31). A 100-μl aliquot of bacterial supernatant was added to 900 μl of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of ECR and incubated with shaking at 37°C for 3 h. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control.

Pyocyanin produced by the cultures was extracted from the supernatants and measured by the method of Essar et al. (12). A 3-ml volume of chloroform was added to 5 ml of culture supernatant and mixed. The chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl. After centrifugation, the top (aqueous) layer was removed and its absorption at 520 nm was measured. Experiments were conducted twice with similar results.

The accumulation of AHL signal molecules was determined in aliquots of 900 μl of culture supernatant. Bacterial cells were removed by centrifugation (13,000 rpm, 5 min), and the resulting supernatant was filter sterilized through 0.2-μm-pore-size filters (Millipore). For AHL determination, culture supernatants were first acidified with 1 M HCl (100 μl) and then incubated at 37°C for 18 h. This was done to ensure that any AHLs hydrolyzed to the open-ring form during growth were recyclized. For detection of 3-oxo-C_{12}-HSL, 1 μl of acidified culture supernatant was spotted onto normal-phase thin-layer chromatography (TLC) plates (silica gel 60F254; Merck). For detection of C_4-HSL, 5 μl of acidified culture supernatant was spotted onto reverse-phase TLC plates (RP-18 F245; Merck). TLC plates were overlaid with 100 ml of soft top agar containing 1 ml either of an *E. coli* strain harboring reporter plasmid pSB1075 (to detect 3-oxo-C_{12}-HSL) or of one harboring pSB536 (to detect C_4-HSL). Plates were incubated at 37°C for 4 h. Bioluminescence was detected with a Luminograph LB 980 photon video camera (EG & G Berthold).

For the determination of PQS accumulation, 10-ml aliquots of *P. aeruginosa* cultures were extracted with 10 ml acidified ethyl acetate (9, 38), vortexed vigorously, and centrifuged at 10,000 rpm for 5 min. The organic phase was transferred to a fresh tube and dried to completion under a stream of nitrogen gas. The solute was resuspended in 50 μl methanol. A 10-μl sample was spotted onto a normal-phase silica 60F254 (Merck) TLC plate which had been previously soaked for 30 min in 5% KH_2PO_4 and activated at 100°C for 1 h. Extracts were separated by use of a dichloromethane:methanol (95:5) system (9) until the solvent front reached the top of the plate. The plate was visualized by use of a UV transilluminator and photographed. Synthetic PQS (2 μl of a 10-mM stock concentration) was used as a positive control.

The effect of overexpressing PA2385 on the production of the siderophore pyoverdin was determined after growth of PAO1::pME6032 and PAO1::pME6032-PA2385 in low-iron Casamino Acids medium (6) at 37°C with shaking at 200 rpm. After a 24-h incubation, aliquots of 1 ml were taken and the cells were removed by centrifugation (13,000 rpm, 5 min). The pyoverdin concentration was determined by measuring the absorbance at 405 nm of the culture supernatants. The assay was performed in triplicate in independent flasks.

RESULTS

Cloning and expression of the PA2385 gene. Initially, we set out to find and characterize homologues of the known β-lactam acylases in the (partially) finished sequences of microbial genome projects. The PA2385 gene of *P. aeruginosa* PAO1 was identified as the gene encoding a putative acylase in a BLAST search at the NCBI website by use of the protein sequence of *Pseudomonas* SY-77 glutaryl acylase (GenBank accession number AF458663) as a query. The putative ORF was amplified by PCR from *P. aeruginosa* PAO1 chromosomal DNA and cloned into plasmid pMcTNde, resulting in plasmid pMc-PA2385. The DNA sequence of the cloned gene matched the sequence reported by the *Pseudomonas* Genome Project (47).

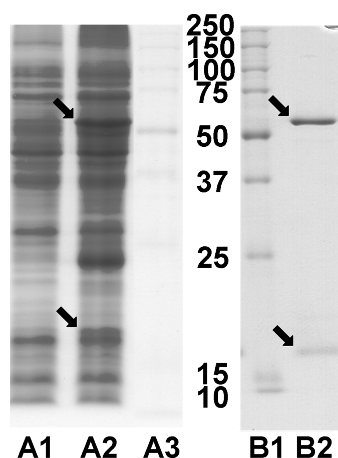

FIG. 2. The PA2385 protein in cell extracts and as purified protein. The *P. aeruginosa* AHL acylase consists of a small α-subunit and a large β-subunit. Lane A1: cell extract of *E. coli* DH10B::pMcTNde (control). Lane A2: cell extract of *E. coli* DH10B::pMc-PA2385 showing additional bands. Lanes A3 and B1: marker proteins (broad range; Bio-Rad). Lane B2: purified PA2385 protein showing two subunits.

Overexpression of the gene in *E. coli* DH10B revealed two additional protein bands on SDS-PAGE gels, which likely represented two subunits, as found with β-lactam acylases (Fig. 2, lanes A1 and A2).

Purification and subunit characterization of the PA2385 protein. The PA2385 protein was obtained at a purity of >95% and a yield of 25 mg per liter of fermentation broth by a three-step chromatography protocol, during which fractions were pooled based on the appearance of the putative β-subunit on SDS-PAGE gels. The purified enzyme consists of an α-subunit and a β-subunit of approximately 18 and 60 kDa, respectively (Fig. 2, lane B2), whereas the ORF of the PA2385 gene encodes a 726-amino-acid protein with a theoretical molecular mass of 84.0 kDa. Evidently, the precursor protein undergoes posttranslational modification, which is a characteristic of β-lactam acylases (11, 24, 25). In order to analyze the boundaries of these subunits and thereby elucidate the maturation process, the subunits were analyzed by N-terminal Edman degradation and mass spectrometry analysis.

The N-terminal sequence of the α-subunit was defined as Asp-Met-Pro-Arg-Pro, implying that the first 23 amino acids are removed from the gene translation product (Fig. 3). Indeed, this stretch of amino acids terminates with Val-Gln-Ala, a variant of the characteristic Ala-X-Ala motif found in most *Sec*-type signal sequences (49), and is in accordance with the signal sequence predicted by SignalP (28). Mass spectrometry analysis of the α-subunit showed two peaks of equal area at 18,574 and 18,645 Da. This strongly indicates that the α-subunit consists of a peptide of 170 (amino acids 24 to 193) or 171 (amino acids 24 to 194) amino acids with a theoretical mass of 18,572 or 18,643 Da, respectively. N-terminal sequencing of the β-subunit showed that it starts at amino acid 217 with the residues Ser-Asn-Ala-Ile-Ala. Mass spectrometry showed that the β-subunit has a molecular mass of 60,426 Da, corresponding with the theoretical mass of 60,424 Da. Evidently, residues 193 (or 194) to 216 bridging the α- and β-subunits are removed during the maturation process (Fig. 3).

During the last purification step, we observed a protein which migrated on SDS-PAGE gels with a band similar to that of the β-subunit and a band slightly larger than that of the α-subunit (data not shown). This could very well be the partially maturated PA2385 protein, in which the spacer peptide is still attached to the α-subunit.

Activity screening. In a preliminary screen, a cell extract of *E. coli* DH10B cells containing pMc-PA2385 was tested with the fluorescamine assay for activity towards a range of β-lactam compounds with various aromatic and aliphatic side chains (Fig. 1). No activity was detected. The extract was subsequently screened for activity towards various AHLs and was found to deacylate four of the tested compounds: C_7-HSL, C_8-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL. AHLs with shorter side chains (C_4-thio-HSL, C_6-HSL, 3-oxo-C_6-HSL) were not degraded. No hydrolysis of the compounds C_{10}-HSL, C_{12}-HSL, and C_{14}-HSL was observed, but this may reflect their poor solubilities in the aqueous reaction buffer rather than lower activity levels. A cell extract of *E. coli* cells producing *Pseudomonas* SY-77 glutaryl acylase and the purified glutaryl acylase (45) were also tested for activity towards AHLs, but no deacylation could be detected.

The PA2385 protein is an AHL acylase. A gas chromatography assay was performed to analyze the reaction catalyzed by purified PA2385 protein. From the fluorescamine assay described above, it was evident that a free primary amine is formed during the reaction with AHL substrates. The consumption of substrate during the reaction was investigated by incubating high concentrations of C_7-HSL with the purified PA2385 protein, since this AHL is soluble at high concentrations in the reaction mix and reference compounds for the substrate and reaction products are available. Aliquots were taken from the reaction mixture at different time intervals and extracted with ethyl acetate. The extracts were injected into the GC and showed a linear decrease of the substrate peak over time (data not shown). Huang et al. showed that crude extracts of *E. coli* overexpressing the gene consume 3-oxo-C_{12}-HSL and liberate HSL (16). The data obtained with the purified enzyme confirm these results. Unfortunately, the products of the reac-

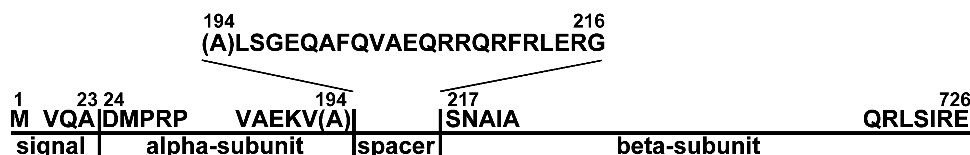

FIG. 3. Schematic diagram of the structure of the *P. aeruginosa* PAO1 PA2385 gene product. The gene product is comprised of four sections: a signal sequence, the α-subunit, a spacer peptide, and the β-subunit. The first and last residues in each section are given, and the sequence of the spacer peptide is given in full. Ala194 may be a part of the α-subunit or a part of the spacer peptide.

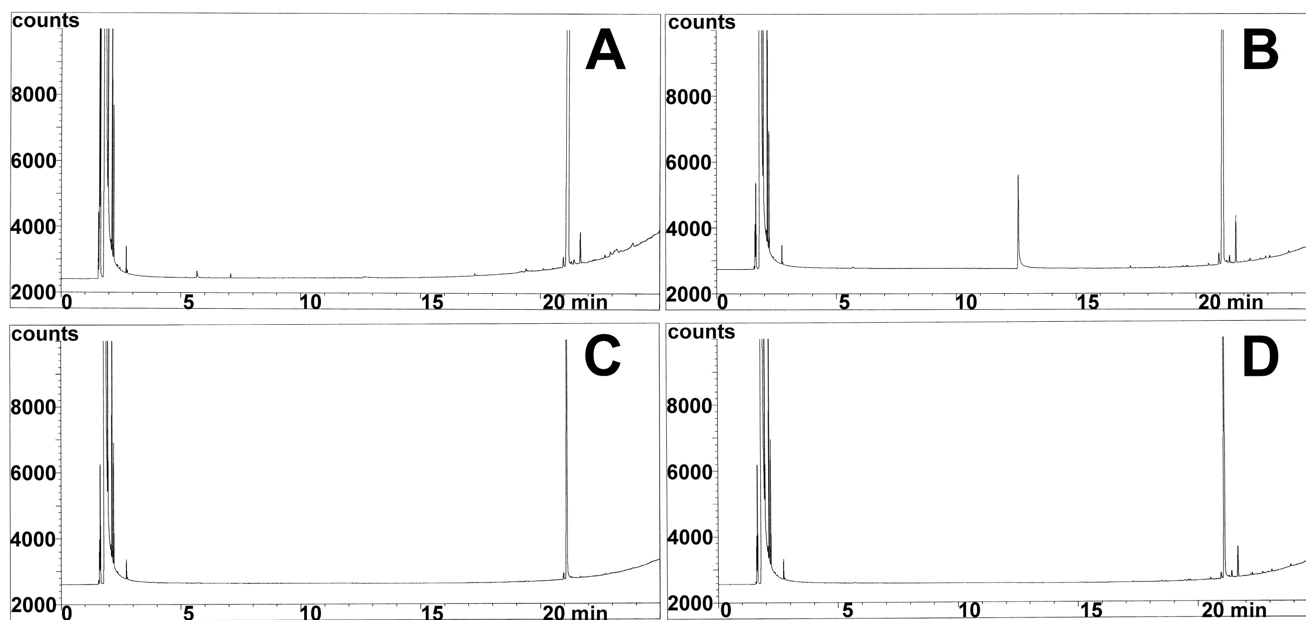

FIG. 4. GC analysis of C_{12}-HSL reaction. The incubation of the PA2385 protein with C_{12}-HSL results in the formation of lauric acid, whereas no lauric acid is formed if C_{12}-HSL is incubated without enzyme. (A) Reaction mixture at $t = 0$ h. (B) Reaction mixture at $t = 20$ h. (C) Control reaction mixture without enzyme at $t = 0$ h. (D) Control reaction mixture at $t = 20$ h. The retention time of C_{12}-HSL is 21.1 min, and that of lauric acid is 12.7 min.

tion, heptanoic acid and HSL, were not extracted with ethyl acetate due to their hydrophilic nature. In order to analyze whether the side chain is removed as a whole during the reaction or digested in several steps, a substrate with a longer, more hydrophobic side chain had to be incubated with the PA2385 protein. C_{12}-HSL was chosen, as it resembles the *P. aeruginosa* AHL 3-oxo-C_{12}-HSL and is degraded at low concentrations by the PA2385 protein (Table 1; also see the following section), whereas its side chain C_{12} acid (lauric acid) is available and stable, in contrast to the 3-oxo-C_{12} acid side chain. GC and GC/MS analysis clearly shows that lauric acid is formed during the reaction, whereas it is not formed in the control reaction (Fig. 4). Kinetic evaluation was hampered, however, by the poor solubility of C_{12}-HSL in PBS due to the hydrophobic nature of its side chain. C_{12}-HSL is present mainly as a precipitate, which makes reproducible sampling of the substrate from the reaction mixture impossible, and therefore a decrease of the substrate peak in time cannot be shown. However, combining these GC results, one can conclude that the PA2385 protein is an AHL acylase.

In vitro quorum quenching by the *P. aeruginosa* AHL acylase. The AHL-hydrolyzing capability of the PA2385 protein suggests that this enzyme may function as a quencher of quorum signaling. To determine whether the acylase is able to hydrolyze AHLs at physiologically relevant low levels, a bioassay using bacterial strains engineered to sense the presence of low concentrations of AHLs by inducing pigment production or bioluminescence was performed (27, 48, 54). Several biosensor strains, each capable of responding to the presence of different AHLs, were used to determine whether the signal molecules remained detectable after incubation with the PA2385 protein (Table 1). It can be seen that the acylase interferes with quorum sensing by cleaving AHLs with acyl side chains with lengths from 11 to 14 carbon atoms but has no activity against short-chain AHLs, such as C_4-HSL and C_6-HSL. The substituent at position 3 of the acyl side chain did not affect the susceptibility to the enzyme in this assay. The PA2385 protein can therefore be expected to act as a broad-range quorum quencher.

Activity towards C_7- and C_8-HSL was not found with this bioassay, whereas this activity was detected with the fluorescamine assay. This likely is due to the different substrate concentrations used in the two assays. The K_m of the PA2385 protein may be rather high for the AHLs with shorter side chains, with the result that activity becomes apparent only at high substrate concentrations.

In vivo quorum quenching by addition of the purified PA2385 protein to *P. aeruginosa* PAO1. To determine whether in vitro quorum quenching was also effective in vivo, the consequences of adding the purified PA2385 protein for the phenotype of *P. aeruginosa* were evaluated. The PA2385 protein was added to fresh cultures of *P. aeruginosa*, which were analyzed after 6 h of growth for the accumulation of AHL and PQS signal molecules and for the production of virulence factors. While the growth of the culture was not affected, the addition of the AHL acylase completely inhibited accumulation of 3-oxo-C_{12}-HSL. Figure 5A clearly shows that this AHL signal molecule cannot be detected in the presence of the acylase, although it is clearly present in the control cultures. Similarly, the PQS signal molecule was not detected when the acylase was added to the PAO1 cultures, in contrast to what was seen for the controls (Fig. 5B). However, the accumulation of the short-chain AHL, C_4-HSL, was similar to that of the control culture (data not shown).

With respect to the virulence factors of *P. aeruginosa* PAO1, the addition of 0.022 mg/ml PA2385 protein decreased the

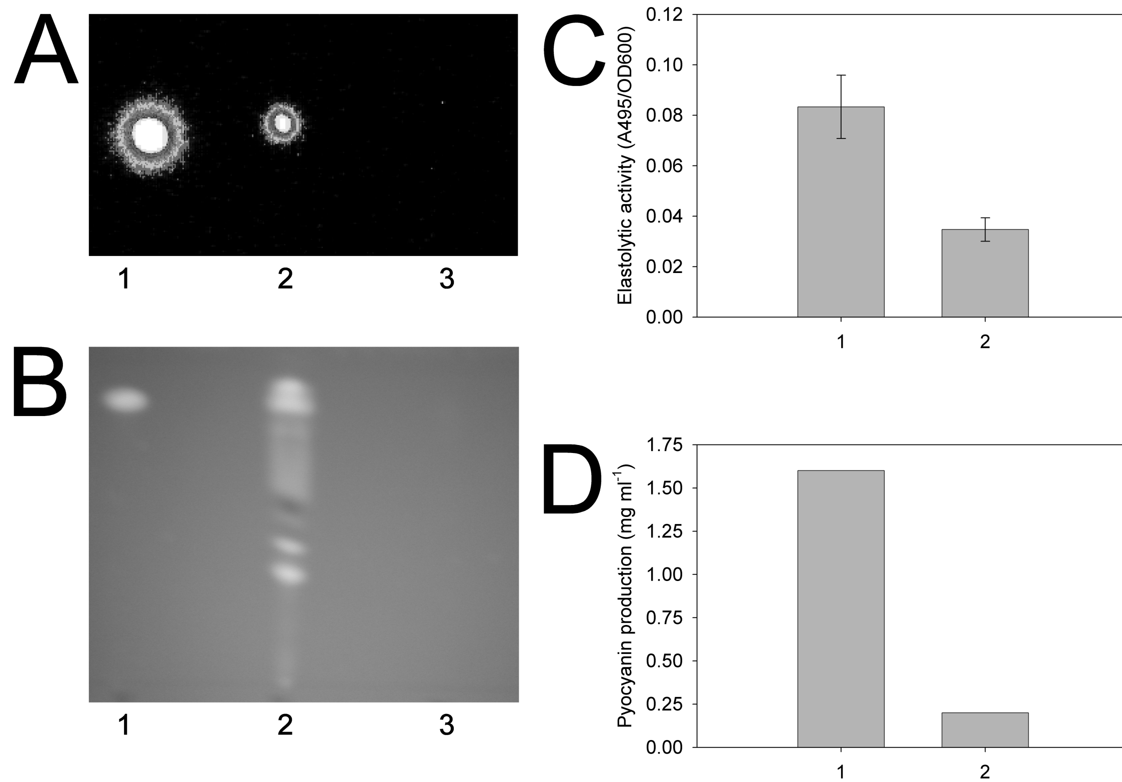

FIG. 5. Effect of exogenous addition of the AHL acylase on quorum-sensing signal molecules and virulence factors of *P. aeruginosa* PAO1. The acylase degrades 3-oxo-C_{12}-HSL (A) in *P. aeruginosa* PAO1 cultures and inhibits PQS accumulation (B), elastolytic activity (C), and pyocyanin production (D). Lanes: A1, 3-oxo-C_{12}-HSL; B1, PQS; A2 and B2, control cultures without added acylase (6 h postinoculation); A3 and B3, cultures to which acylase is added (6 h postinoculation); C1 and D1, control cultures without added acylase; C2 and D2, cultures to which acylase is added, 6 h postinoculation. OD600, optical density at 600 nm.

elastolytic activity by more than 50% (Fig. 5C). Similarly, pyocyanin production was reduced to less than 20% of the levels found in the control cultures 6 h postinoculation (Fig. 5D); this was also evident from the unusual yellow color of the cultures. However, 24 h postinoculation, all cultures produced significant levels of pyocyanin (data not shown). Finally, expression of *lecA*, which codes for the cytotoxic lectin PA-IL, was significantly inhibited by the addition of 0.14 mg/ml PA2385 protein (data not shown). Clearly, exogenous addition of the *Pseudomonas* AHL acylase to growing cells of *P. aeruginosa* PAO1 inhibits or delays the accumulation of the signal molecules 3-oxo-C_{12}-HSL and PQS and thereby the expression of several virulence factors.

In vivo quorum quenching by expression of the PA2385 gene in *P. aeruginosa* PAO1. The effects observed when the acylase protein is added to growing cultures of *P. aeruginosa* demonstrate that the enzyme can act as a quorum quencher. These data also highlight the antibacterial potential of the protein, since it is functional when supplied exogenously. The isolation of the protein from the cellular fraction of *E. coli* suggests that the PA2385 enzyme is not secreted into the medium. Therefore, the effect of overexpressing PA2385 was investigated by cloning the corresponding gene into an *E. coli-Pseudomonas* shuttle vector (pME6032) that was subsequently electroporated into *P. aeruginosa* PAO1. The resulting bacteria were grown in the absence (control) or presence of IPTG to induce expression of the gene and analyzed after 5 and 8 h of growth. SDS-PAGE gels show no additional bands without IPTG induction and additional bands at the same height as that seen for the α- and β-subunits of the purified acylase if IPTG is present (data not shown), indicating that the enzyme is produced in a mature form in *P. aeruginosa* PAO1. Additionally, SDS-PAGE analysis of the medium and cellular fractions of *P. aeruginosa* PAO1 overexpressing the AHL acylase show that the enzyme is found in the soluble cellular fraction. Overexpression of the acylase gene resulted in a small growth inhibition, probably due to production stress. For reference, *P. aeruginosa* PAO1 electroporated with the control plasmid pME6032 did not show altered phenotypes when grown in the absence or presence of IPTG.

The AHL signal molecule 3-oxo-C_{12}-HSL cannot be detected 8 h postinoculation if expression of the PA2385 gene is induced by IPTG, but it is clearly present in the culture grown in the absence of IPTG (Fig. 6A). Similarly, no PQS could be detected after the induction of PA2385 (Fig. 6B), whereas C_4-HSL levels were not affected (data not shown). Overexpression of the PA2385 gene resulted in the complete abolition of elastolytic (LasB) activity (Fig. 6C) and a significant reduction in *lecA* expression (Fig. 6D). These results clearly confirm that the intracellularly generated PA2385 acylase protein is also a functional quorum quencher. The overexpression of the PA2385 gene had no effect on pyoverdin production. For con-

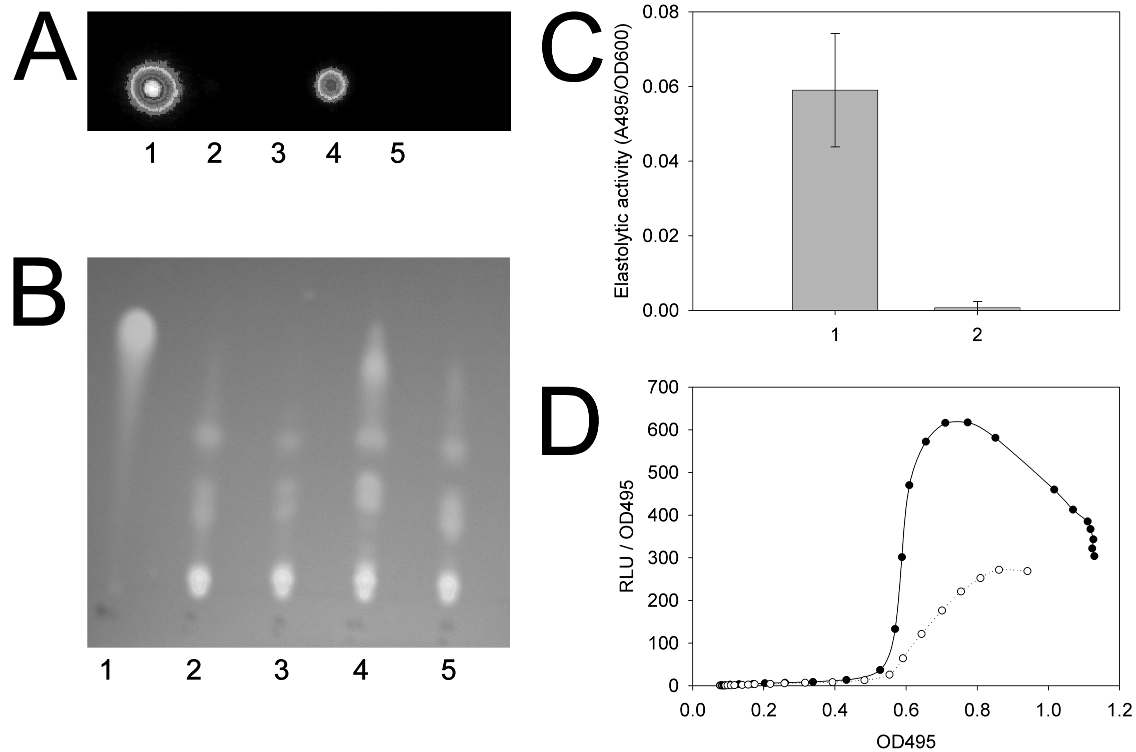

FIG. 6. Effect of overexpression of the AHL acylase gene on quorum-sensing signal molecules and virulence factors of *P. aeruginosa* PAO1. The acylase degrades 3-oxo-C_{12}-HSL (A) in *P. aeruginosa* PAO1 cultures and inhibits PQS accumulation (B), elastolytic activity (C), and *lecA* expression (D). Lanes: A1, 3-oxo-C_{12}-HSL; B1, PQS; A2 and B2, control cultures in which the gene is not induced, 5 h postinoculation; A4 and B4, as A2 and B2 but 8 h postinoculation; A3 and B3, cultures in which expression of the acylase is induced by IPTG, 5 h postinoculation; A5 and B5, as A3 and B3 but 8 h postinoculation; C1, control culture in which the gene is not induced; C2, culture in which expression of the acylase is induced by IPTG. In panel D, closed circles indicate control culture, in which the gene is not induced, and open circles indicate culture in which expression of the acylase is induced by IPTG. OD495, optical density at 495 nm; RLU, relative light units.

trol cultures, the measured relative pyoverdin production was 1.02 ± 0.08, whereas a value of 0.91 ± 0.23 was calculated for cultures overproducing the PA2385 gene.

DISCUSSION

Virulence is controlled in *P. aeruginosa* PAO1 by a number of quorum-sensing signal molecules, of which the AHLs C_4-HSL and 3-oxo-C_{12}-HSL have been the most extensively investigated. Recent publications indicate that, depending on experimental conditions, from 2.9% to over 10% of all genes in the genome of *P. aeruginosa* PAO1 are under AHL-dependent quorum-sensing control (15, 41, 50). The disruption of AHL-dependent quorum sensing has profound effects on protein production and accumulation in *P. aeruginosa* PAO1 (1, 29). Degradation of these signaling molecules results in quorum quenching and was demonstrated by AHL lactonases (10, 35, 58). In addition, hydrolysis by acylases from *Ralstonia* (26), *Pseudomonas* (16), and *Streptomyces* (34) species has recently been reported. However, many aspects of these acylases are still unknown, since most of the results were obtained with cellular extracts. In this study, we demonstrate that the *P. aeruginosa* PAO1 PA2385 gene codes for an AHL acylase that, as a purified enzyme, is capable of degrading 3-oxo-C_{12}-HSL, adding detail to the results of Huang et al., which were based on observations of *E. coli* cells expressing the gene (16). In vitro, the substrate specificity of PA2385 at physiological concentrations was shown to include AHLs with side chains ranging in length from 11 to 14 carbon atoms, irrespective of the substituent at the 3′ position of the *N*-linked acyl side chain. Because quorum sensing occurs at very low AHL concentrations (low μM range), a highly efficient enzyme is required. The results from the bioassays clearly show that the K_m of the enzyme is low enough to act as a quorum quencher.

Exogenous addition of the PA2385 acylase to a growing *P. aeruginosa* culture was shown to inhibit the accumulation of 3-oxo-C_{12}-HSL in *P. aeruginosa* PAO1. Accumulation of the PQS signal molecule was also prevented, as were elastolytic activity, the production of pyocyanin, and the expression of *lecA*; however, accumulation of the second AHL signal molecule, C_4-HSL, was not. The same phenotypes were observed when the gene was expressed under control of an inducible promoter. Previously, Huang et al. also found that overexpression of PA2385 in *P. aeruginosa* PAO1 inhibits accumulation of the 3-oxo-C_{12}-HSL signal molecule (16). All these findings corroborate the well-known phenotypes of *P. aeruginosa* PAO1 mutants in which the *las* system has been mutated (8, 9, 37). Apparently, the protein encoded by the PA2385 gene completely inhibits the *las* quorum-sensing system by degrading the cognate AHL signal molecule and thereby functions as a quorum quencher. Although a number of control mechanisms for

the quorum-sensing network are known, degradation of 3-oxo-C_{12}-HSL by an AHL acylase would represent a novel self-regulating quorum-sensing mechanism.

The loss of 3-oxo-C_{12}-HSL production in the presence of the acylase was accompanied by the loss of PQS production. Previously, it has been demonstrated that PQS is essential for the production of certain *rhl*-dependent phenotypes (9). Degradation of 3-oxo-C_{12}-HSL by the PA2385 protein delays the production of PQS and virulence factors, but at a later stage PQS will be synthesized via a LasR-independent mechanism (9). Accumulation of C_4-HSL does not seem to be strongly affected by the acylase activity. This phenotype is similar to the phenotypes of mutants defective in both PQS production (the *pqsR* mutant) and response (the *pqsE* mutant), which produce substantially reduced levels of exoproducts but retain wild-type *N*-butanoyl homoserine lactone (C_4-HSL) levels (9). It is therefore tempting to view 3-oxo-C_{12}-HSL as a signal molecule involved in controlling the timing of PQS production and thereby virulence factor production.

Interestingly, extracellular addition of the protein and intracellular production of the enzyme gave the same results. As it is highly unlikely that the added acylase is taken up as a functional enzyme by the bacterial cells, this similarity indicates that 3-oxo-C_{12}-HSL diffuses through the growth medium in an acylase-susceptible form and is not disseminated by cell-to-cell contact. This highlights the potential use of the enzyme in antibacterial therapy, since expression of the gene in the bacterium as well as administration of the protein will result in quorum quenching.

The PA2385 gene product was initially selected by us as a putative β-lactam acylase on the basis of its homology with the glutaryl acylase from *Pseudomonas* SY-77. Indeed, our results point to many similarities between these two acylases. The protein encoded by the gene was shown to be a precursor consisting of four parts: a putative signal sequence, the α-subunit, a spacer peptide, and the β-subunit (Fig. 3), whereas the active enzyme consists only of the α- and β-subunits. This organization resembles the gene structure of the β-lactam acylases. The β-subunit starts with the characteristic Ser-Asn motif that is found in all β-lactam acylases. This strongly suggests that the PA2385 protein, like the β-lactam acylases, is an N-terminal nucleophile hydrolase, with the first residue of the β-subunit, serine, as the catalytically active residue (19, 25). During purification, moreover, a protein that presumably is a partially maturated precursor in which the spacer peptide has not been cleaved from the α-subunit, as has been observed in mutants of cephalosporin acylase, was obtained (18, 32). A variable cleavage site between the α-subunit and the spacer peptide, resulting in a heterogeneous enzyme sample, was observed. Variable cleavage sites have also been reported for several highly homologous glutaryl acylases, and it was postulated that this step of the maturation process may not be very specific (17). In the crude cell extracts both of *E. coli* and of *P. aeruginosa* PAO1, the mature β-subunit is visible, but no precursor can be detected. In contrast, Huang et al. saw mainly precursor protein on SDS-PAGE gels while overexpressing *pvdQ* in *P. aeruginosa* PAO1 (16). This may be due to the high sensitivity of the autocatalytic processing: we observed that production of mature protein occurs efficiently only at low temperatures (25 to 30°C) and is strongly affected at higher temperatures (37°C). Some additional bands visible in the crude cell extracts that cannot be contributed to the acylase are thought to be the result of overproduction stress in the host.

During incubation of C_{12}-HSL with the acylase, a free amine was formed and lauric acid (C_{12} carboxylic acid) was liberated. Previously, it was shown that during incubation of 3-oxo-C_{12}-HSL with the PA2385 acylase, HSL was generated as the free amine (16). These results indicate that the reaction is a deacylation in which the amide bond connecting the aliphatic side chain and the homoserine nucleus is cleaved, similar to the reaction catalyzed by β-lactam acylases (11) (Fig. 1). Despite these similarities, however, no activity towards a range of β-lactam compounds was found, and glutaryl acylase (this study) and *E. coli* penicillin acylase (26) did not degrade AHLs. Obviously, β-lactam acylases and AHL acylases have different substrate specificities.

Although AHL acylase activity has been detected in *Variovorax paradoxus* (23), in *Ralstonia* XJ12B (26), and, very recently, in *Streptomyces* sp. (34), *P. aeruginosa* PAO1 is the first human pathogen shown to possess AHL acylase activity (16). In BLAST searches with the sequence of the PA2385 gene, close homologues of this gene, including three additional genes in *P. aeruginosa*, are found in all sequenced *Pseudomonas* species and in over 100 other organisms. The fluorescamine assay described in this study would be a simple but efficient screening method to identify AHL acylases in these organisms. Huang et al. found that *P. aeruginosa* PAO1 was capable of degrading 3-oxo-C_{12}-HSL for use as a source of carbon and nitrogen. The PA2385 acylase was an obvious candidate for this activity; however, PA2385 mutants remained capable of degrading the AHLs (16). Analysis of the other three acylase homologues in *P. aeruginosa* PAO1 is currently in progress in this laboratory.

As yet, the true physiological role of the PA2385 AHL acylase in *P. aeruginosa* has not been determined. The gene has been reported to be upregulated by quorum sensing and has therefore also been identified as *qsc*112 (52). However, the observed effects range from 12- or 15-fold (52) to less than 2.5-fold (41) or to no significant effect (50), depending on the study. According to Whiteley et al., the upregulation of PA2385 is under the control of 3-oxo-C_{12}-HSL but not that of C_4-HSL (52), which could imply a function as a negative feedback loop on the *las* system. On the other hand, the PA2385 gene is part of the pyoverdin synthesis operon, and it is therefore also known as *pvdQ* (21). Although an insertion mutation of *pvdQ* results in the loss of pyoverdin production, the function of the gene in pyoverdin synthesis is yet unknown (21). Pyoverdins contain an acyl group that is coupled to the chromophore by an amide bond, implying a function of the acylase in the synthesis of pyoverdins. However, the acyl groups of the known pyoverdins are short, comprising four or five carbon atoms, and contain a charged or polar group at the end (13). These side chains would be a substrate for some β-lactam acylases but not for the AHL acylase discussed in this study. *pvdQ* mutants did not exhibit any obvious changes in the levels of accumulation of 3-oxo-C_{12}-HSL in LB medium (16) or of production of 3-oxo-C_{12}-HSL, C_4-HSL, and PQS in iron-rich LB medium (data not shown), as determined by use of a previously described *pvdQ* mutant (20). Furthermore, we found that overexpression of *pvdQ* in an iron-deficient medium had no effect on pyoverdin production. Nevertheless, the 11-fold upregulation of

pvdQ under iron starvation conditions (30) combined with the quorum-quenching function implies a role as a virulence repressor during iron deprivation. The antibacterial action of lactoferrin by its iron-chelating capability seems to be in agreement with such a mechanism. The formation of fragile biofilms by *P. aeruginosa* PAO1 was observed at concentrations of lactoferrin that did not inhibit growth (43). An upregulation of the AHL acylase due to reduced iron availability would interfere with quorum sensing and may explain these observations. This mechanism is also in accordance with the recently reported quorum quenching in *Agrobacterium tumefaciens* by upregulation of an AHL lactonase during carbon or nitrogen deprivation (57).

In conclusion, the recognition that *P. aeruginosa* PAO1 encodes a quorum-quenching enzyme may offer us a new way to treat infections with this organism. Targeting the expression of the gene or the administration of the enzyme could be used to prevent the development of disease via inhibition of the formation of biofilms and the release of proteolytic enzymes and toxins. Whereas anti-infectives have great difficulties penetrating a formed biofilm, hydrolysis of the signal molecules may dismantle a biofilm from the outside in, since signal molecules are thought to be required not only for biofilm formation but also for biofilm maintenance (42). The increased expression of the gene by iron starvation calls for an investigation of clinically applicable iron chelators, preferably to be administered topically, e.g., in the pulmonary tract. A detailed investigation of promoters and other inducers of PA2385 expression may provide additional leads.

ACKNOWLEDGMENTS

This work was sponsored by contract GBI.4707 from STW, which is part of the Dutch Organization for Science, and project QLK3-2001-02086 from the EU Commission (Vth Framework Programme). R. H. Cool was supported by the EU-community initiative Interreg IIIA. S. P. Diggle was supported by EU grant QLK3-2000-01759 (Vth Framework Programme).

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Editor: V. J. DiRita