PA0305 of *Pseudomonas aeruginosa* is a quorum quenching acylhomoserine lactone acylase belonging to the Ntn hydrolase superfamily

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†These authors contributed equally to this work.

**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic pathogen, often detected in immune-compromised patients and hospital-acquired infections. A high percentage of cystic fibrosis patients acquire chronic *P. aeruginosa* infections leading to high mortality rates within this group (Lyczak *et al.*, 2000; Tatterson *et al.*, 2001). *P. aeruginosa* employs a complex network of quorum sensing (QS) systems necessary to control expression of density-dependent genes, including genes encoding virulence factors. Induction of these genes depends on production, secretion and detection of the 3-oxo-C12-HSL and the C4-HSL signal molecules [full abbreviations for all the acylhomoserine lactones (AHLs) used in this study are given in Table 1]. The high
resistance of this bacterium to common antibiotics has been linked to biofilm formation and to the regulation of the QS system (de Kievit, 2009). Inhibition of QS, a process referred to as quorum quenching, is thought to provide means of fighting P. aeruginosa infections (Papaioannou et al., 2009).

One of the ways quorum quenching in P. aeruginosa can be achieved is by degrading the 3-oxo-C12-HSL and C6-HSL signal molecules, which can occur via two different enzymes: acylases or lactonases. A number of studies reported the identification of acylases capable of degrading AHLs produced by Gram-negative bacteria. These acylases all belong to the Ntn-hydrolase superfamily. Ralstonia sp. strain XJ12B produces the AiiD acylase, degrading 3-oxo-C10-HSL (Lin et al., 2003). P. aeruginosa PAO1 produces at least two AHL acylases, PA2385 (PvdQ) (Huang et al., 2003; Sio et al., 2006) and PA1032 (QuiP) (Huang et al., 2006), both of which have been experimentally proven to degrade long-chain AHLs (Huang et al., 2006; Sio et al., 2006). The recently determined 3D crystal structure of PvdQ in complex with 3-oxo-C12 has confirmed the Ntn-hydrolysis mechanism typical of this class of acylases (Bokhove et al., 2010). Interestingly, this enzyme has a large hydrophobic binding pocket that can accommodate the 3-oxo-C12 acyl chain, which is consistent with the observed quenching of the 3-oxo-C12-HSL endogenous to P. aeruginosa.

In the P. aeruginosa PAO1 genome, besides pvdQ and quiP, two additional genes, pa1893 and pa0305, have been identified as genes encoding hypothetical penicillin acylase proteins belonging to the Ntn-hydrolase superfamily. The pa0305 gene is predicted to encode a 795 amino acid polypeptide that shows 26% sequence similarity to PvdQ and 29% similarity to QuiP. PA0305 also has 67% sequence similarity to HacB, an AHL acylase of Pseudomonas syringae that was initially annotated as penicillin acylase (Shepherd & Iglewski, 1989). Based on the high sequence similarity between PA0305 and HacB, we reasoned that the PA0305 protein might also function as an AHL acylase. Herein, we report that the PA0305 protein of P. aeruginosa PAO1 degrades medium- to long-chain AHLs with high efficiency resulting in a reduction of production of quorum-sensing-dependent virulence factors in P. aeruginosa. We also, for the first time to our knowledge, report kinetic data for an AHL acylase.

### METHODS

#### Bacterial strains and growth media. Escherichia coli and P. aeruginosa strains were routinely grown and maintained in Luria–Bertani (LB) broth (0.5 % sodium chloride, 1 % tryptone and 0.5 % yeast extract, buffered with 50 mM MOPS, pH 7.0), unless otherwise indicated. Where necessary, gentamicin (10 μg ml⁻¹ for E. coli and 100 μg ml⁻¹ for P. aeruginosa) and ampicillin (100 μg ml⁻¹ for E. coli and 300 μg ml⁻¹ for P. aeruginosa) were added to maintain the plasmids. AHLs (Table 1) used in this study were purchased from Sigma and Fluka.

#### Cloning of the pa0305 gene and expression in E. coli and P. aeruginosa. Chromosomal DNA was isolated from an overnight culture of P. aeruginosa PAO1 using a GenElute bacterial Genome DNA kit (Sigma). The ORF encoding the acylase gene (bases 346,690–344,303) (Stover et al., 2000) was amplified from chromosomal DNA by PCR using the primer pair ForA and RevA (Table 2). The PCR product was digested with Ndel and HindIII restriction enzymes and ligated to the similarly digested pET-20b plasmid, containing a 6-His tag (Novagen). The resulting pET-pa0305his plasmid was used for the construction of the pUCP-pa0305his by reamplifying (using primers ForB and RevB) and subcloning the pa0305 fragment containing a C-terminal 6-His tag (Table 2). For purification and enzyme activity assays the pUCP-pa0305his construct was transformed into E. coli Origami. pUCP-pa0305 was constructed in a similar fashion, but now omitting the 6-His tag. For pa0305 overexpression in PAO1 and pa0305 complementation in PA01 ΔpvdQ strains, the ORF was amplified by PCR from P. aeruginosa PAO1 chromosomal DNA using the ForB and RevC primers (Table 2). After digestion of the PCR product with SacI and XbaI restriction enzymes, the product was ligated to the pMMB67EH vector in which gene expression is under the control of a tac promoter. The resulting construct was then transformed into P. aeruginosa cells using the protocol described by Smith & Iglewski (1989).

#### Purification of PA0305his protein. E. coli Origami cells containing the pUCP-pa0305his plasmid were grown in 2 x tryptone yeast extract (TY) medium (0.5% sodium chloride, 0.6% tryptone and 1% yeast extract) supplemented with 100 μg ampicillin ml⁻¹ and 0.1% glycerol, for 40 h at 37 °C. The culture was induced with 0.4 mM IPTG at OD600 0.8–1.0. Cells were harvested by centrifugation, sonicated in lysis buffer (50 mM Tris/HisCl pH 8.0, 2 mM EDTA, 0.1% Triton X-100) and centrifuged (30 min, 20,000 g, 4 °C). The cell-free extract was then used for purification of PA0305his on an Ni-NTA agarose gravity column (Qiagen). The protein was eluted with 250 mM imidazole in 50 mM Tris/HCl pH 8.0 and afterwards desalted using 50 mM Tris/HisCl (pH 8.0) on a PD10 desalting column (GE Healthcare) (Otten et al., 2003).

### Table 1. Different acylhomoserine lactones (AHLs) used in this study

<table>
<thead>
<tr>
<th>Full name of AHL</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Butanoyl-DL-homoserine lactone</td>
<td>C4-HSL</td>
</tr>
<tr>
<td>N-Hexanoyl-DL-homoserine lactone</td>
<td>C6-HSL</td>
</tr>
<tr>
<td>N-β-Ketocaproyl-DL-homoserine lactone</td>
<td>3-oxo-C6-HSL</td>
</tr>
<tr>
<td>N-Heptanoyl-DL-homoserine lactone</td>
<td>C7-HSL</td>
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<tr>
<td>N-Octanoyl-DL-homoserine lactone</td>
<td>C8-HSL</td>
</tr>
<tr>
<td>N-3-Oxo-octanoyl-L-homoserine lactone</td>
<td>3-oxo-C8-HSL</td>
</tr>
<tr>
<td>N-Decanoyl-DL-homoserine lactone</td>
<td>C10-HSL</td>
</tr>
<tr>
<td>N-3-Oxo-decanoyl-L-homoserine lactone</td>
<td>3-oxo-C10-HSL</td>
</tr>
<tr>
<td>N-Dodecanoyl-DL-homoserine lactone</td>
<td>C12-HSL</td>
</tr>
<tr>
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<td>3-oxo-C12-HSL</td>
</tr>
<tr>
<td>N-Tetradecanoyl-DL-homoserine lactone</td>
<td>C14-HSL</td>
</tr>
<tr>
<td>N-3-Oxo-tetradecanoyl-L-homoserine lactone</td>
<td>3-oxo-C14-HSL</td>
</tr>
</tbody>
</table>
The purified protein was analysed on an SDS-PAGE gel. A Western blot was then carried out for immunodetection of the protein with Ni-NTA HRP conjugate (Qiagen).

**Protein identification.** To characterize the purified PA0305his protein, the α-subunit and the ςb-subunit-his were extracted from the gel. trypsin digested and analysed by using MALDI-TOF MS. Protein identification based on mass spectra was done by peptide mass fingerprinting using Mascot software (Matrix Science).

For N-terminal sequencing the purified PA0305his was loaded onto an SDS-PAGE gel, trypsin digested and analysed by using MALDI-TOF MS. Protein identification based on mass spectra was done by peptide mass fingerprinting using Mascot software (Matrix Science).

**Preparation of mRNA and real-time PCR (QRT-PCR).** Cells were harvested at the late-exponential growth phase. Total RNA was isolated and purified using the RNasy mini kit (Qiagen), followed by DNase (Qiagen) on-column digestion according to the manufacturer’s instructions. RNA was quantified by the A260/A280 ratio using a NanoDrop (ND-1000) spectrophotometer. cDNA was synthesized with 1 μg total RNA as a template using the iScript cDNA Synthesis kit (Bio-Rad). Control reactions for detection of DNA contamination contained the RNA template but lacked iScript reverse transcriptase. The expression of the pa0305 gene in *P. aeruginosa* was quantified by QRT-PCR using the primer pair ForD and RevD (Table 2) with iQ SYBR Green Supermix (Bio-Rad) in an iCycler (Bio-Rad). Amplification was performed at 95°C (15 s), 62°C (30 s), followed by melting curve analysis. The rpoD gene was chosen as an internal housekeeping gene for the baseline control in the evaluation of pa0305 expression. The relative expression of *pa0305* transcript was normalized to the *rpoD* gene and was quantified using the Pfaffl equation (Pfaffl, 2001). For each condition studied, two separate experiments were performed in triplicate.

**Bio-activity assay.** An aliquot (2 μl) of 0.5 mg C12-HSL ml⁻¹ in acetonitrile was placed in a 96-well microtitre plate (Lumitrac600,
Acetonitrile was allowed to evaporate and the AHL was reconstituted in 100 μl PBS buffer pH 7.4 (Gibco) containing 5 μl of either cell-free extract or purified enzyme. The plate was incubated at 30 °C, at 70 r.p.m. After 4 h, 100 μl of a 1:10 dilution of the lux-based E. coli pSB1075 biosensor was added to each well and the plate was further incubated for 6 h at 30 °C. Bioluminescence was detected on a FLUOstar Omega platereader (BMG labtech). As a control, reactions with only PBS mixed with the enzyme were conducted in the same way. All of the assays were performed in triplicate.

To determine the specificity of AHL-degradation activity, acetonitrile stocks of AHLs (Table 1) were transferred in a 96-well microtitre plate at a final concentration of 100, 10, 1 and 0.1 μg ml⁻¹ in the reaction mixture. The solvent was allowed to evaporate and the AHLs were reconstituted in 100 μl PBS pH 7.4 containing 2.5 μg purified enzyme. The plate was incubated at 30 °C, at 70 r.p.m. Next to this, control reactions using inactive enzyme were performed in the same way. The enzyme was inactivated by boiling a mixture of PBS with 2.5 μg enzyme at 100 °C for 15 min. After 4 h of incubation, 100 μl of a 1:10 dilution of the appropriate biosensor (E. coli pSB401, pSB536 or pSB1075) was added to each well and the plate was further incubated for 6 h at 30 °C. Bioluminescence was detected on a FLUOstar Omega platereader (BMG labtech). The experiments were performed in triplicate. The differences in relative light units (RLU) between experimental samples and controls were used to determine the degree of degradation of each AHL.

**Analysis of C₈-HSL deacetylase activity by HPLC.** The assays were carried out using C₈-HSL as described previously (Uroz et al., 2008). The reaction mixture consisted of 1 mM C₈-HSL and 25 μg (–0.286 μM) of the enzyme in 1 ml PBS buffer pH 7.4 (In vitro gen). The mixture was incubated at 30 °C and 70 r.p.m., for 4 h. Three samples of 750 μl were taken at 0 and 4 h and processed for detection of the residual substrate, the released octanoic acid and the HSL. For detection of the substrate, residual C₈-HSL in the reaction mixture was extracted twice with 750 μl ethyl acetate. Octanoic acid in the sample was extracted three times with hexane and dried under nitrogen streaming. Derivatization of the octanoic acid was then performed by addition of 4-bromomethoxy-7-methyl coumarin (BrMMC) reagent as described previously (Wolf & Korf, 1990). The presence of free HSL was determined by addition of 750 μl dansyl chloride (2.5 mg ml⁻¹) in acetone to the reaction mixture followed by overnight incubation at 37 °C (Lin et al., 2003). The mixture was dried under nitrogen streaming and neutralized using 50 μl 0.2 M HCl. After dilution with 250 μl acetonitrile, the sample was analysed by reverse-phase HPLC separation. As a control, reference HSL (final concentration 2 mM) was dansylated in parallel with the same reagents. The presence of C₈-HSL residue and dansylated HSL was analysed by reverse-phase HPLC separation (Uroz et al., 2008; Lin et al., 2003).

HPLC analysis was performed using a Shimadzu-VP system. The column used was a LichroCART 125-4 Superspher 100 RP-18 endcapped. The mobile phase was water (solvent A) and acetonitrile (solvent B). The C₈-HSL was detected at 219 nm, whereas the dansylated HSL was detected at 267 nm and the BrMMC-derivative of octanoic acid at 328 nm.

**Kinetic studies of PA0305his on C₈-HSL and 3-oxo-C₈-HSL substrate.** Kinetic studies were performed in 0.1 M phosphate buffer pH 7.5 at 30 °C (Lin et al., 2003). For C₈-HSL kinetics, six different concentrations of the substrate (0.02–0.20 mM) were used, at which the substrate was completely soluble. For each of these concentrations, the substrate was incubated with 25 μg PA0305his (ml reaction volume)⁻¹ for 2 min. A 100 μl sample of the reaction mixture was taken and mixed directly with 100 μl o-phthalaldehyde (OPA) solution in a 96-well microtitre plate and the A₅20 was measured spectrophotometrically. To obtain a calibration curve of the OPA-derivative of the HSL reference, the same procedure was performed in an enzymic mixture containing HSL. The calibration curve is a straight line that could be fitted with the following equation: \( y = 1959.6x + 0.6966 \) \( (R^2 = 0.9882) \). The initial rates (mmol s⁻¹) were plotted versus the concentration of substrate (mM), which gives a straight line. Assuming that \( S < \leq K_m \), the slope of the line equals \( (K_m \times [E]) / K_m \). Thus, dividing the slope by the enzyme concentration results in a value for the apparent \( K_m / V_m \).

For 3-oxo-C₁₂-HSL kinetics, the substrate (at seven different concentrations, 0.01–0.12 mM, where the substrate was soluble) was incubated with 10 μg PA0305his (ml reaction volume)⁻¹ for 1 min. A 1 ml sample was mixed directly with 1 ml ethyl acetate to stop the reaction and extracted three times with the same volume of ethyl acetate. The amount of HSL product released was quantified as described above.

**Construction of precise gene deletions in PAO1.** A precise chromosomal deletion of pvd0305 was constructed as follows. The flanking regions of the target gene were amplified with PAO1 chromosomal DNA as a template, using the primer pair ForE and RevE and the primer pair ForF and RevF. The two resulting fragments were joined together in a second PCR round using ForA and RevB primers. The fusion product was then digested with HindIII and EcoRI and cloned into the similarly digested pEX18-Gm (Hoang et al., 1998). The subsequent deletion procedure was performed as described previously (Papaioannou et al., 2009). The sequences of the primers and the probes (ForG and RevG) are listed in Table 2. A double deletion (ΔpvdQΔquiP) and a triple deletion strain (ΔpvdQΔquiPΔpvd0305) were constructed with the same approach using similar primer sets.

**Elastase assay.** Elastolytic activity of extracellular supernatant was assayed in the following manner. Supernatant samples of 0.1 ml were added in Eppendorf tubes containing 20 mg Elastin Congo red (ECR; Sigma) suspended in 0.90 ml ECR buffer (100 mM Tris, 1 mM CaCl₂, pH 7.5). After 4 h incubation with agitation, insoluble ECR was removed by centrifugation (20 000 g, 5 min, 4 °C). The absorbance of the supernatants measured at 495 nm was divided by the OD₆₀₀ of the culture. LB medium was used as a negative control (Ohman et al., 1980).

**Pyocyanin assay.** Pyocyanin was extracted from the supernatants and measured by the method previously described by Essar et al. (1990). A 5 ml supernatant of culture grown in LB was extracted with 3 ml chloroform. The chloroform layer was transferred to a fresh tube and re-extracted with 1 ml 0.2 M HCl. After centrifugation, the A₅₂₈ of the HCl fraction was measured. Concentrations, expressed as micrograms pyocyanin produced per millilitre culture supernatant, were determined as \((OD₅₂₈ \times 17.072)/OD₆₀₀\) (Essar et al., 1990).

**Quantification of 3-oxo-C₁₂-HSL, C₈-HSL and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) production.** The amount of 3-oxo-C₁₂-HSL produced by the PAO1 parental strain and the PAO1pvdQΔquiP and PAO1pvdQΔquiPpvd0305 mutant strains was determined using the biosensor E. coli (pSB1075), which produces light in response to long AHLs (Winson et al., 1998). 3-Oxo-C₁₂-HSL concentrations were measured 4, 15, 27 and 50 h after initiation of bacterial growth. Samples taken at the specific time points mentioned were centrifuged for 10 min at 5000 g and each of the supernatants was filtered through a 0.2 μm pore filter (Whatman). The filtered supernatants were then frozen in liquid nitrogen and stored at −20 °C until further analysis. Once all samples were collected, a bioassay was started at 37 °C by adding 180 μl of a 1:100 dilution of an overnight E. coli (pSB1075) culture and 20 μl of the supernatant sample to be analysed. The amount of light generated
Quorum quenching activity of expressed PA0305 in P. aeruginosa using a Caenorhabditis elegans model. Slow-killing kinetics of C. elegans by PAO1 and its derivatives were determined by using the procedure described previously (Papaioannou et al., 2009). Briefly, *Pseudomonas* strains were grown overnight at 37 °C in LB broth supplemented with appropriate antibiotics and then diluted 1:100-fold into fresh broth. Nematode growth medium plates (59 mm diameter) were then spread with 80 μl of the respective culture. After the plates were incubated at 37 °C for 24 h and allowed to equilibrate to room temperature for 30 min, 50 larval stage 4 (L4) nematodes from stock plates were transferred onto the *P. aeruginosa* lawn (PAO1, PA01 Δpa0305, PA01 ΔlasRlasI). The plates were then incubated at 24 °C and scored for living and dead worms every 3–4 h for 7 days. For statistical purposes, a minimum of four replicates per trial was performed. *E. coli* OP50 was used as a negative control to evaluate background levels of worm death. A worm was considered to be dead when it failed to respond to plate tapping or gentle touch with a platinum wire. Worms that died as a result of getting stuck to the wall failed to respond to plate tapping or gentle touch with a platinum wire. Worms that died as a result of getting stuck to the wall were excluded from the analysis. Results were plotted using PyLab, a python package for data analysis and visualization.

**RESULTS**

Expression and purification of PA0305

The *P. aeruginosa* pa0305 gene was amplified and cloned into the pUCP18 vector. Using this construct, expression of the gene in *E. coli* Origami resulted in the synthesis of a protein of about 60 kDa as shown by SDS-PAGE with Coomassie staining (Fig. 1, lane 2). The size of this protein matches the predicted size of the β-subunit of the putative acylase encoded by *pa0305*. Because of the relatively low-level expression of the gene, overproduction of the α-subunit was not observed. To facilitate protein detection and purification, the recombinant enzyme was also produced in *E. coli* Origami as a C-terminal His-tagged protein. In comparison with native PA0305 (without a fusion tag), however, the His-tagged PA0305 protein was produced at even lower levels and could not be detected by Coomassie staining (Fig. 1, lane 5). Nonetheless, the His-tagged protein could be efficiently purified by a Ni-based immobilized-metal-affinity chromatography protocol. SDS-PAGE analysis of the purified protein revealed three protein bands (Fig. 1, lane 7). The band of about 65 kDa represents the β-subunit, whereas the two bands of about 26 and 28 kDa represent different non-mature forms of the α-subunit (as discussed below). The identity of the 65 kDa band as the His-tagged β-subunit of the PA0305 protein was confirmed by Western blotting with anti-His antibody (Fig. 1, lane 11). Expression of the *pa0305* gene from the vector pMMB67EH in *P. aeruginosa* resulted in very low amounts of PA0305 (data not shown) and purification from *P. aeruginosa* was not further pursued.

Characterization of purified PA0305his

The purified PA0305his protein was subjected to MS and protein sequencing to establish the identity of the three protein bands observed on the SDS-PAGE gel (Fig. 1, lane 7). MALDI-TOF MS analysis of the protein band of about 65 kDa revealed that this protein is the β-subunit of PA0305. N-terminal sequencing revealed that the first six amino acid residues of the β-subunit are, as predicted, Ser-Asn-Ala-Trp-Val-Val. From these observations, it can be concluded that the β-subunit of PA0305 is correctly formed in *E. coli*, resulting in a protein with an N-terminal serine.

MALDI-TOF MS analysis of the two smaller proteins revealed similar MS spectra, indicating that both proteins correspond to the α-subunit. The spectrum of the 26 kDa...
protein band is consistent with that expected for the C-terminally processed z-subunit (i.e. with signal peptide but without spacer peptide), whereas the spectrum of the 28 kDa protein band is consistent with the z-subunit that includes both the signal peptide and the spacer peptide. N-terminal sequencing of the smallest (26 kDa) protein band revealed the sequence Met-Lys-Arg-Thr-Leu-Thr. This observation further confirms that the signal peptide indeed remains attached to the z-subunit. Apparently, the signal peptide is not efficiently removed in E. coli, which suggests that the protein is not properly secreted.

Analytical gel filtration was performed as described in Methods with thyroglobulin (670 kDa), ferritin (440 kDa) and aldolase (158 kDa) as reference proteins. PA0305his eluted with a retention time corresponding to a molecular mass of approximately 400 kDa (data not shown), a size that corresponds to a tetra-heterodimer.

**PA0305 is an AHL acylase**

The lux-based E. coli pSB1075 biosensor was used to test cell-free extracts for the presence of AHL-degrading activity. C12-HSL was used as the substrate since it resembles the P. aeruginosa signal molecule, 3-oxo-C12-HSL. Incubation of C12-HSL with cell-free extract prepared from E. coli cells overproducing PA0305his resulted in the conversion of C12-HSL, as detected by a clear reduction in bioluminescence (Fig. 2). Cell-free extracts prepared from the negative control strain (E. coli cells containing an empty plasmid) did not show this reduction in bioluminescence (Fig. 2). These data clearly indicate that PA0305 is a C12-HSL-degrading enzyme.

Having established that PA0305 exhibits activity towards C12-HSL, various AHLs with alkyl chains ranging from C4 to C14 (for abbreviations see Table 1) were tested as potential substrates for purified PA0305his. In these activity assays, purified PA0305his was mixed with four different concentrations of AHLs, ranging from 0.01 to 10 µg ml⁻¹ and the conversion of the AHLs was followed by measuring the quenching of light using a panel of three biosensor strains (Table 3). At each AHL concentration, the activity of the purified enzyme was compared with heat-inactivated protein. The PA0305his protein showed activity towards AHL with alkyl chains ranging from C6 to C14. The highest degradation rate (93-fold decrease in substrate concentration) was observed on C8-HSL at 10 µg ml⁻¹. At the lowest concentration, 0.01 µg ml⁻¹, the degradation rate was the highest on 3-oxo-C12-HSL (64-fold). Activity against the 3-oxo- forms was detected from 3-oxo-C10-HSL to 3-oxo-C14-HSL (Table 3).

To investigate whether PA0305 functions as a lactonase or an acylase, the products of the conversion of C8-HSL were subjected to HPLC analysis. The reaction was performed by exposing C8-HSL substrate to PA0305his for 4 h. C8-HSL was detected as a specific peak at the retention time of 5.85 min (Fig. 3). Compared with a negative control with inactivated enzyme, PA0305his caused a significant reduction of the C8-HSL concentration at 4 h. Detection of HSL liberated by acylase activity was performed by dansylating the free amine of HSL. Fig. 3 shows the presence of dansylated HSL in the 4 h sample, resulting from the cleavage of the amide bond between the HSL and the acyl chain. The specific peak was detected at a retention time of 6.33 min. This dansylated product, clearly visible in the reaction mixture with active PA0305his, was absent in the control, indicating that degradation of the substrate was caused by acylase activity. To confirm the presence of octanoic acid released by acylase activity, derivatization of the fatty acid using Brommmc was performed. HPLC analysis showed that the specific peak of the BrMmmc-octanoic acid derivative was present at 12.59 min in C8-HSL samples exposed to active PA0305 for 4 h. This peak was absent in the control samples with inactivated enzyme. These results clearly indicate that PA0305 is an AHL acylase that converts C8-HSL to HSL and octanoic acid.

**Kinetic studies of PA0305his on AHLs**

Having established that PA0305 functions as an AHL acylase, kinetic parameters were determined for the PA0305-catalysed conversion of C8-HSL and 3-oxo-C12-HSL. The C8-HSL was chosen because it shows the highest activity with PA0305his at high concentrations and 3-oxo-C12-HSL was chosen since it is one of the signal molecules produced by P. aeruginosa. The initial rates of these reactions were dependent on the concentration of the respective substrate. However, saturation with C8-HSL and 3-oxo-C12-HSL was not achieved for PA0305 because the low solubility of these AHLs in aqueous buffer does not permit rate measurements at high substrate concentrations. Hence, Kcat/Km values were determined using seven low...
Table 3. Specificity of purified PA0305his for AHL substrates

Degradation is expressed as RLU, generated by three lux-based biosensors, which produce light in response to the AHLs. The initial concentration of AHL (0.01, 0.1, 1 or 10 µg ml⁻¹) is given. The degradation assay was carried out in PBS buffer (pH 7.4) with 25 µg of either active or inactivated enzyme ml⁻¹ and incubated at 30 °C with shaking. The remaining amount of AHLs was detected by a suitable lux-based biosensor at 30°C for 6 h.

<table>
<thead>
<tr>
<th>AHL substrate</th>
<th>Purified PA0305his protein</th>
<th>Degradation (10⁻³×RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>C₄-HSL*</td>
<td>Inactivated</td>
<td>22.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>20.2 ± 2.5</td>
</tr>
<tr>
<td>C₆-HSL†</td>
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<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>0.94 ± 0.15</td>
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<tr>
<td>3-oxo-C₁₀-HSL†</td>
<td>Inactivated</td>
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<td>Active</td>
<td>78.7 ± 8</td>
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<td>5.8 ± 0.7</td>
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<tr>
<td>C₁₄-HSL‡</td>
<td>Inactivated</td>
<td>4.49 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>0.124 ± 0.026</td>
</tr>
<tr>
<td>3-oxo-C₁₄-HSL‡</td>
<td>Inactivated</td>
<td>8.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>0.19 ± 0.02</td>
</tr>
</tbody>
</table>

*BioLsensor E. coli JM109::pSB536.
†BioLsensor E. coli JM109::pSB401.
‡BioLsensor E. coli JM109::pSB1075.

Substrate concentrations where the substrate is still soluble and the detection of HSL formed was performed using the derivatization method as described above. A comparison of the values showed that PA0305 is 56-fold more efficient in catalysing the conversion of 3-oxo-C₁₂-HSL (Kₘ/Kₘ = 7.8 × 10⁻⁴ M⁻¹ s⁻¹) than C₈-HSL (Kₘ/Kₘ = 0.14 × 10⁻⁴ M⁻¹ s⁻¹).

Deletion of pa0305 in P. aeruginosa results in accumulation of 3-oxo-C₁₂-HSL

The kinetic studies with purified PA0305 have shown that the enzyme is quite efficient at degrading 3-oxo-C₁₂-HSL, which is a natural QS signal molecule in P. aeruginosa. This observation prompted us to investigate whether deletion of the pat0305 gene in P. aeruginosa would affect the ability of this strain to accumulate 3-oxo-C₁₂-HSL. In order to detect the accumulation of 3-oxo-C₁₂-HSL in cell cultures, P. aeruginosa cells grown in LB were harvested at specific time points. The supernatants were subjected to detection of 3-oxo-C₁₂-HSL using biosensor E. coli JM109::pSB1075 (Fig. 4a). The kinetics of 3-oxo-C₁₂-HSL accumulation revealed that P. aeruginosa wild-type and P. aeruginosa Δpa0305 produced similar amounts of 3-oxo-C₁₂-HSL within 7 h post-inoculation. However, from 24 to 50 h post-inoculation, 3-oxo-C₁₂-HSL was much more abundant in the Δpa0305 strain compared with the wild-type strain as seen after separation on TLC plates (see Supplementary Fig. S1, available with the online version of this paper). In order to exclude any influence from other AHL acylases encoded by P. aeruginosa, a triple deletion strain (ΔpvdQ, ΔquiP, Δpa0305) was constructed and compared with the double deletion strain (ΔpvdQ, ΔquiP). After 50 h, an approximately 250% increase in accumulation of 3-oxo-C₁₂-HSL was observed (Fig. 5). This suggests that PA0305 has a significant role in 3-oxo-C₁₂-HSL degradation in P. aeruginosa cells grown in LB medium. Notably, while deletion of the pa0305 gene affects 3-oxo-C₁₂-HSL accumulation, no influence on the accumulation of the QS signal molecules C₄-HSL and PQS was observed (Fig. 4b).
The effects of PA0305 on *P. aeruginosa* QS

The AHL hydrolysing capability of PA0305, both in vitro and in vivo, suggests that this enzyme may function as a quorum quencher in *P. aeruginosa*. To determine the ability of overexpressed PA0305 to interfere with the expression of QS-regulated functions, the *P. aeruginosa* wild-type and *P. aeruginosa* Δpa0305 strains were transformed with the pMMB-pa0305 construct. As controls, the same strains were transformed with the empty plasmid (pMMB67EH). Gene expression was either constitutive or induced with IPTG.

Overexpression of the *pa0305* gene (LB growth, IPTG induction) in *P. aeruginosa* resulted in a significant reduction in elastolytic (LasB) activity throughout the growth phase, from 5 to 24 h post-inoculation (Fig. 6a). Pyocyanin production was also reduced after 5 h post-inoculation, but the reduction did not last until 24 h of growth (Fig. 6b). The accumulation of 3-oxo-C12-HSL was significantly reduced in all PA0305-overproducing strains and the amounts remained at low levels even after 24 h of growth (Fig. 7a). However, C4-HSL accumulation levels were not altered and this signal molecule was abundant throughout the 24 h growth period (Fig. 7b). This observation is consistent with the finding that PA0305 is not active against C4-HSL.

To prove that the reduced 3-oxo-C12-HSL accumulation was correlated with the transcription of the *pa0305* gene, mRNA levels of the *pa0305* gene in all strains were quantified using QRT-PCR. All isolated total RNA samples were of good quality as shown by analysis of the samples on a formaldehyde agarose gel (data not shown). Further- more, the RNA samples were free of chromosomal DNA, as indicated by the finding that no cDNA could be generated from the RNA samples in the absence of iScript transcriptase. The *rpoD* housekeeping gene was used as a control for comparison of the quantity and quality of all
mRNAs. The expression of *pa0305* was normalized to the *rpoD* gene which had a similar expression profile in all strains. The amount of *pa0305* transcript present in the wild-type culture at 5 h was low (Table 4). The mRNA levels of *pa0305* were significantly higher in both the wild-type strain and the Δpa0305 strain harbouring the *pa0305* expression plasmid when compared with the control strains harbouring an empty plasmid.

**Quorum quenching activity of overproduced PA0305 in *P. aeruginosa* using the *C. elegans* infection model**

To assess whether either the deletion or overexpression of the *pa0305* gene in *P. aeruginosa* have any effect on the pathogenicity of the bacterium, the killing kinetics of the wild-type PAO1 strain, the Δpa0305 strain and the pa0305 overexpressing PAO1 strain were determined using a *C. elegans* infection model and a slow killing assay as previously described by Tan & Ausubel (2000). In the slow killing assay, the LT50 value (time taken for half of the *C. elegans* to die) for PAO1 was shown to be around 68 h. We chose to screen our mutants with this method as it is highly sensitive and allows discrimination between strains that only slightly differ in their ability to kill *C. elegans*. During the first 4 days there were no differences observed between worms feeding on the three different strains. Up to the completion of the assay no significant differences could be seen between the PAO1 parental strain and the derived PA0305-overexpressing strain (*P* > 0.05). However, between days 5 and 7 after exposure, the harmful effects of the deletion of *pa0305* were more profound. At day 7 post-exposure only about 41% of the worms feeding on the deletion strain were alive compared with about 57% of the worms fed on the PAO1 wild-type or pa0305 overexpressing strain (Fig. 8). Statistical analysis showed a significant difference between the PA0305 deletion strain and the PAO1 mutant for day 7 of the assay (*P* > 0.05).

These observations give an insight into the effect of the *pa0305* gene on tempering the virulence of *P. aeruginosa* PAO1.

**DISCUSSION**

In recent years, numerous genes encoding new members of the Ntn-hydrolase superfamily have been discovered in the genomes of prokaryotes. The first characterized members of this superfamily were described as penicillin acylases and recently they have been more broadly classified as members of the family of β-lactam acylases. At present it is clear that β-lactam deacylation is unlikely to be the natural function of this class of enzymes, as this...
conversion does not inactivate penicillins or cephalosporins (Krzeslak et al., 2007; Sio & Quax, 2004) and, hence, does not give a competitive advantage. For example, the paradigm of penicillin acylases, PGA from *E. coli*, generates a molecule with even higher antibiotic activity upon conversion of penicillin-G into 6-APA (Meevootisom et al., 1983; Schumacher et al., 1986). It has been argued that this conversion is therefore not likely to be the natural function of this industrially important enzyme. The *P. aeruginosa* PAO1 complete genome sequence revealed the presence of four genes with significant sequence similarity to penicillin and cephalosporin acylases. By now it has been established that two of these genes encode products, PvdQ and QuiP, that can deacylate long-chain AHLs resulting in quorum quenching, which points to a major physiological role for these enzymes (Huang et al., 2006; Sio et al., 2006). Phenotypic analysis of a *quiP* (quorum signal utilization and inactivation protein) transposon mutant showed that a strain carrying the transposon insertion was impaired in growth on decanoyl-HSL when compared with the parental strain. QuiP complementation revealed that, when *quiP* was constitutively expressed from a plasmid, AHL degrading activity potential was restored. A remarkable decrease in 3-oxo-C$_{12}$-HSL accumulation levels was observed.

**Fig. 6.** Effects of PA0305 overexpression on virulence factor production in *P. aeruginosa* PAO1 and PAO1 Δpa0305. The production of virulence factors was tested in wild-type and Δpa0305 strains carrying either an empty vector (pMMB) or the pa0305-expressing vector (pMMB-pa0305). The elastolytic activity (a) and pyocyanin production (b) in the supernatants of the respective strains are shown. Error bars, SD.

**Fig. 7.** Effects of PA0305 overproduction on accumulation of QS signal molecules in *P. aeruginosa* PAO1 and PAO1 Δpa0305. The same strains as described in Fig. 6 were also tested for the accumulation of 3-oxo-C$_{12}$-HSL as detected by luminescence measured with *E. coli* pSB1075 biosensor. (a) Overproduction of PA0305 reduces the accumulation of 3-oxo-C$_{12}$-HSL in wild-type and Δpa0305 supernatant. (b) *E. coli* pSB536, which is sensitive to C$_4$-HSL, was incubated with the same cell extracts. It can be concluded that overproduction of PA0305 does not reduce the accumulation of C$_4$-HSL. Error bars, SD.
Cells were harvested at the late-exponential growth phase (5 h post-inoculation). The $pa0305$ expression levels of all strains were compared with the expression of PAO1 $\Delta pa0305$ strain harbouring an empty plasmid, which is given the value 1. All of the values were normalized to the housekeeping gene $rpoD$.

### Table 4. Fold changes of $pa0305$ gene expression in $P. aeruginosa$ strains

<table>
<thead>
<tr>
<th>$P. aeruginosa$</th>
<th>Plasmid</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1 wild-type</td>
<td>pMMB67EH</td>
<td>205 ± 35</td>
</tr>
<tr>
<td>PAO1 $\Delta pa0305$</td>
<td>pMMB-pa0305</td>
<td>144488 ± 19344</td>
</tr>
<tr>
<td></td>
<td>pMMB67EH</td>
<td>1 ± 0</td>
</tr>
<tr>
<td></td>
<td>pMMB-pa0305</td>
<td>93335 ± 9506</td>
</tr>
</tbody>
</table>

detected when quiP was constitutively expressed in $P. aeruginosa$. Interestingly, even though QuiP has been proven to be involved in AHL utilization, this gene was not classified in microarray analysis as a QS-regulated gene. Similarly, PvdQ also effectively degrades AHL side chains ranging from 11 to 14 carbons and overexpression of this gene, as well as exogenous addition of purified protein in growing $P. aeruginosa$ cultures, delays the accumulation of the 3-oxo-C12-HSL molecule and as a result decreases the expression of virulence factors such as elastase and pyocyanin (Sio et al., 2006).

Different organisms were also found to produce enzymes acting on autoinducer molecules and disrupting the QS systems. Apart from the AHL acylases produced by $P. aeruginosa$, Ralstonia strain XJ12B produces the AiiD AHL acylase that when expressed in $P. aeruginosa$ results in reduction of virulence (Lin et al., 2003). In addition to the AHL acylases, a second group of enzymes that act on autoinducer molecules have been identified. These enzymes are characterized as AHL lactonases and they inactivate AHL signals by hydrolysing the lactone ring, thus yielding the corresponding acylhomoserine. Examples are the AiiA (autoinducer inactivator) AHL lactonase from the Gram-positive bacterium Bacillus sp. 240B1 (Dong et al., 2001) and the AiiB AHL lactonase produced by Agrobacterium tumefaciens (Liu et al., 2007). It has already been shown that transgenic plants expressing AHL lactonase displayed an enhanced resistance to infection by Erwinia carotovora (Dong et al., 2001).

In $P. aeruginosa$ two additional genes, $pa1893$ and $pa0305$, have been annotated as genes possibly encoding penicillin acylases (Krzeslak et al., 2007) although they have not been characterized. The results of this study clearly demonstrate that PA0305 has acyl-HSL degrading activity similar to that of PvdQ and QuiP.

$P. aeruginosa$ PA01 $pa0305$ is predicted to encode a protein of 795 amino acids in length (Stover et al., 2000) and the polypeptide shows the striking features of post-translational processing typical of the members of the N-terminal nucleophile aminohydrolases (Ntn hydrolases) superfamily. The gene is transcribed as a single polypeptide and as a result of autocatalytic cleavage a mature active enzyme consisting of two dissimilar subunits is formed. The strongest evidence for PA0305 being an Ntn hydrolase is the presence of the conserved Ser 1 residue, the first residue of the $\beta$-subunit. The N-terminal amino acids 1–25 of PA0305 are predicted to compose a signal sequence peptide (LipoP v.1.0, Phobius, SignalP v.3.0 Hidden Markov Models) (Winsor et al., 2009), which is in line with the observation that most bacterial Ntn hydrolases are secreted. However, determination of the N-terminal sequence of the $x$-subunit purified from $E. coli$ revealed that the signal peptide is not cleaved off from the $x$-subunit under the experimental conditions used, indicating that the protein may not be translocated to the periplasm in $E. coli$ or that the $E. coli$ signal peptidase is less capable of cleaving this sequence. The finding that the protein migrates as a tetra-heterodimer on a gel filtration column is unique in comparison with other characterized AHL acylases, but is not surprising considering that some cephalosporin acylases, also members of the Ntn-hydrolase superfamily,

**Fig. 8.** The effect of overexpressing and deleting the $pa0305$ gene on the virulence of $P. aeruginosa$ in the C. elegans model. C. elegans nematodes were fed on a layer of Pseudomonas cells as described previously (Papaioannou et al., 2009). Wild-type $P. aeruginosa$ PAO1, $P. aeruginosa$ PAO1 $\Delta pa0305$ and $P. aeruginosa$ PAO1 harbouring pMMB-pa0305 were used as feeding layers. The number of surviving animals was counted for 7 consecutive days. A significant difference of the effects of the PAO1 $\Delta pa0305$ strain compared with the PAO1 parental strain is indicated by an asterisk.
have a tetra-heterodimer structure (Kwon et al., 2000). PA0305 has been purified to homogeneity from *E. coli* and it was used for further enzymic characterization revealing that it has activity towards medium- and long-chain HSLs. When compared with PvdQ and QuiP, which show 26 and 29% sequence similarity, respectively, PA0305 shows broader substrate specificity. However, in spite of the 67% sequence similarity with HacB from *P. syringae*, PA0305 is unable to degrade C4-HSL. Interestingly the ability to degrade C4-HSL, the second major autoinducer, seems to be absent from all AHL acylases of *P. aeruginosa* absent from all AHL acylases of *P. aeruginosa*, it may be concluded that C8-HSL is the best substrate for PA0305. Overproducing PA0305 in both PAO1 wild-type and PA0305 has been purified to homogeneity from the broad activity of PA0305 on different chain-length HSLs it may be concluded that C8-HSL is the best substrate for PA0305 (93-fold decrease at 10 µg ml⁻¹; Table 3). This must be considered with care, however, as the solubility of long-chain HSLs may become limiting at high concentrations. In fact this is demonstrated by comparing the conversion rates of C8-HSL at 0.01 and 10 µg ml⁻¹ with the conversion rates of 3-oxo-C12-HSL at these respective concentrations (Table 3). The deacylation rate of the enzyme at low concentrations appears to be best for 3-oxo-C12-HSL and not for C8-HSL (64-fold versus 1.3-fold). To explain these observations, one likely possibility is that at high substrate concentrations (10 µg ml⁻¹) of 3-oxo-C12-HSL, higher order assemblages (e.g. micelles) may form, limiting the enzyme activity. Nonetheless, our data suggest that at physiological concentrations the natural autoinducer of *P. aeruginosa* is the best substrate for the PA0305 enzyme. To further substantiate this conclusion kinetic studies were performed using HPLC detection of derivatized products. For the first time, to our knowledge, we report an apparent *Kcat*/*Km* value for an acyl-HSL acylase. Indeed the catalytic efficiency of PA0305 is higher for 3-oxo-C12-HSL in comparison with C8-HSL, which is consistent with 3-oxo-C12-HSL being one of the natural substrates of this enzyme. Although individual *Kcat* and *Km* values have not been assessed, the apparent *Kcat*/*Km* value of 7.8 × 10⁴ M⁻¹ s⁻¹ implies that the enzyme has a high catalytic efficiency towards 3-oxo-C12-HSL, which would require an efficient acyl binding pocket in the enzyme similar to the substrate binding pocket described for PvdQ (Bokhove et al., 2010).

The deletion of the *pa0305* gene from chromosomal DNA resulted in an increase of the 3-oxo-C12-HSL levels in the growth medium, which became most apparent after 50 h incubation. This phenotype was evident in both the wild-type and the PAO1ΔpvdQ ΔquiP background. Previous studies using transposon insertions on *pa0305* revealed that this gene is not crucial for cell growth on C10-HSL (Huang et al., 2006). In addition, microarray data published previously (Wagner et al., 2003, 2004) did not categorize *pa0305* as a QS-regulated gene. In this study, we show that the *pa0305* gene is expressed in cells grown in LB medium and is involved in the degradation of the *P. aeruginosa* las-regulon signal molecule, 3-oxo-C12-HSL. From the overexpression studies we conclude that the level of *pa0305* transcript can be 50–100-fold increased and it may be interesting to study the regulation of the *pa0305* expression under different experimental conditions, including infection models.

Unlike *pvdQ*, which is located in the pyoverdin operon (Lamont & Martin, 2003), *pa0305* does not seem to be part of an operon and there is no suggestion of a function from the genetic surroundings of *pa0305*. To verify the predicted activity of PA0305 as a penicillin acylase, we tested a number of potential substrates. Using *p*-dimethylaminobenzaldehyde for the detection of 6-amino penicillanic acid (Sio et al., 2003) and 7-aminocephalosporanic acid (Sio et al., 2002), we were able to determine that PA0305 has low activity towards penicillin V, a slight activity on penicillin-G and no activity on glutaryl 7-ACA (data not shown). However, based on the fact that the conversion of β-lactams will not provide an advantage to *P. aeruginosa*, we believe that the deacylation of β-lactams is not the natural function of PA0305 (Krzeslak et al., 2007).

The broad activity of PA0305 on different chain-length HSLs suggests a possible function of this protein under natural conditions. In addition to adding to the understanding of bacterial signal communication, the availability of purified PA0305 offers a potential agent that can be used to reduce virulence of pathogenic *P. aeruginosa* strains or other pathogens by quenching the QS system. Initial experiments in this study using the *C. elegans* infection model indicate that the effect of reducing virulence is relatively limited and detectable only after 5 days. It is possible that the stability of the protein is insufficient for its use in the nematode infection assay. Analogous to other Ntn hydrolases, the stability may be increased by protein accumulation of 3-oxo-C12-HSL (Fig. 4a). This may be due to the localization of PA0305, which is predicted to be restricted to the periplasm, whereas the substrate, 3-oxo-C12-HSL, is dispersed from the cytoplasm to the extracellular medium.
engineering or by in vitro formulation of purified enzyme in order to obtain a more stable acylase that might be used for more significant reductions in pathogenicity (Sio & Quax, 2004; Verhaert et al., 1997). The finding that at least three of the four Ntnhydroases of P. aeruginosa are capable of degrading AHLs and are thus capable of interfering with crucial gene regulatory circuits provides an explanation for the widespread occurrence of this class of enzymes in the genomes of prokaryotes. In view of the observed function of PA0305 and its homology to P. syringae HacB, we propose to rename pa0305 as hacB (AHL acylase B).

ACKNOWLEDGEMENTS

We gratefully acknowledge Miguel Câmara and Paul Williams (University of Nottingham) for providing the biosensor strains pSB1075, pSB536 and pSB401. We thank Stephan Heeb (University of Nottingham) for the gift of pEX18-Gm and Bert-Jan Baas (University of Groningen) for help with calculating enzyme kinetic parameters. In addition we thank Eric Daniel Fraenkel (University of Groningen) for the help in the statistical analysis of the C. elegans assay data. M.W. was a recipient of a Bernoulli grant from the University of Groningen. This research was partly funded by EU grant ANTIBIOTARGET MEST-CT-2005-020278.

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Edited by: D. Demuth