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

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The *Pseudomonas aeruginosa* PAO1 genome has at least two genes, *pvdQ* and *quiP*, encoding acylhomoserine lactone (AHL) acylases. Two additional genes, *pa1893* and *pa0305*, have been predicted to encode penicillin acylase proteins, but have not been characterized. Initial studies on a *pa0305* transposon insertion mutant suggested that the gene is not related to the AHL growth phenotype of *P. aeruginosa*. The close similarity (67 %) of *pa0305* to HacB, an AHL acylase of *Pseudomonas syringae*, prompted us to investigate whether the PA0305 protein might also function as an AHL acylase. The *pa0305* gene has been cloned and the protein (PA0305) has been overproduced, purified and subjected to functional characterization. Analysis of the purified protein showed that, like β -lactam acylases, PA0305 undergoes post-translational processing resulting in α - and β -subunits, with the catalytic serine as the first amino acid of the β -subunit, strongly suggesting that PA0305 is a member of the N-terminal nucleophile hydrolase superfamily. Using a biosensor assay, PA0305 was shown to degrade AHLs with acyl side chains ranging in length from 6 to 14 carbons. Kinetics studies using *N*-octanoyl-L-homoserine lactone (C₈-HSL) and *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL) as substrates showed that the enzyme has a robust activity towards these two AHLs, with apparent K_{cat}/K_m values of $0.14 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ towards C₈-HSL and $7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ towards 3-oxo-C₁₂-HSL. Overexpression of the *pa0305* gene in *P. aeruginosa* showed significant reductions in both accumulation of 3-oxo-C₁₂-HSL and expression of virulence factors. A mutant *P. aeruginosa* strain with a deleted *pa0305* gene showed a slightly increased capacity to kill *Caenorhabditis elegans* compared with the *P. aeruginosa* PAO1 wild-type strain and the PAO1 strain carrying a plasmid overexpressing *pa0305*. The harmful effects of the $\Delta pa0305$ strain on the animals were most visible at 5 days post-exposure and the mortality rate of the animals fed on the $\Delta pa0305$ strain was faster than for the animals fed on either the wild-type strain or the strain overexpressing *pa0305*. In conclusion, the *pa0305* gene encodes an efficient acylase with activity towards long-chain homoserine lactones, including 3-oxo-C₁₂-HSL, the natural quorum sensing signal molecule in *P. aeruginosa*, and we propose to name this acylase HacB.

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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

[†]These authors contributed equally to this work.

Abbreviations: AHL, acylhomoserine lactone; BrMMC, 4-bromomethoxy-7-methyl coumarin; HSL, homoserine lactone; QS, quorum sensing; RLU, relative light units.

A supplementary figure, illustrating detection of AHLs with TLC, is available with the online version of this paper.

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- C_{12} -HSL and C_4 -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- C_{10} -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- C_{12} 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- C_{12} acyl chain, which is consistent with the observed quenching of the 3-oxo- C_{12} -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 & Lindow, 2009). The function of the *pa0305* gene and its product has not been experimentally tested to date, to our knowledge. Initial studies on a *pa0305* transposon insertion

mutant strain did not demonstrate any defect in growth on C_{10} -HSL compared with the wild-type strain, thus suggesting that the gene is not involved in the metabolism of AHLs (Huang *et al.*, 2006).

Based on the high sequence similarity between PA0305 and HacB, we reasoned that the PA0305 protein might also function as an AHL acylase. The aim of this study was to investigate whether PA0305 is able to degrade acylhomoserine lactones and whether it functions as a quorum quenching 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 ($10 \mu\text{g ml}^{-1}$ for *E. coli* and $100 \mu\text{g ml}^{-1}$ for *P. aeruginosa*) and ampicillin ($100 \mu\text{g ml}^{-1}$ for *E. coli* and $300 \mu\text{g ml}^{-1}$ 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 *NdeI* 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 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 PAO1 Δ *pa0305* 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 $2 \times$ tryptone yeast extract (TY) medium (0.5 % sodium chloride, 0.6 % tryptone and 1 % yeast extract) supplemented with $100 \mu\text{g ampicillin ml}^{-1}$ and 0.1 % glycerol, for 40 h at 37°C . The culture was induced with 0.4 mM IPTG at OD_{600} 0.8–1.0. Cells were harvested by centrifugation, sonicated in lysis buffer (50 mM Tris/HCl pH 8.0, 2 mM EDTA, 0.1 % Triton X-100) and centrifuged (30 min, $20\,000 \text{ g}$, 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/HCl (pH 8.0) on a PD10 desalting column (GE Healthcare) (Otten

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

Full name of AHL	Abbreviation
N-Butanoyl-DL-homoserine lactone	C_4 -HSL
N-Hexanoyl-DL-homoserine lactone	C_6 -HSL
N- β -Ketocaproyl-DL-homoserine lactone	3-oxo- C_6 -HSL
N-Heptanoyl-DL-homoserine lactone	C_7 -HSL
N-Octanoyl-DL-homoserine lactone	C_8 -HSL
N-3-Oxo-octanoyl-L-homoserine lactone	3-oxo- C_8 -HSL
N-Decanoyl-DL-homoserine lactone	C_{10} -HSL
N-3-Oxo-decanoyl-L-homoserine lactone	3-oxo- C_{10} -HSL
N-Dodecanoyl-DL-homoserine lactone	C_{12} -HSL
N-3-Oxo-dodecanoyl-L-homoserine lactone	3-oxo- C_{12} -HSL
N-Tetradecanoyl-DL-homoserine lactone	C_{14} -HSL
N-3-Oxo-tetradecanoyl-L-homoserine lactone	3-oxo- C_{14} -HSL

Table 2. Strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant characteristic or sequence (5' → 3')	Source or reference
Strains		
<i>P. aeruginosa</i> PAO1	Wild-type	Holloway collection
<i>P. aeruginosa</i> PAO1 $\Delta pa0305$	Deleted <i>pa0305</i> , PAO1 derivative	This study
<i>E. coli</i> S17-1 λ pir	$\Delta ara-leu7697 \Delta lacX74 \Delta phoAPvuII$ <i>phoR araD139 ahpC galE galK rpsL</i> (Sm ^r) F'[<i>lac</i> ⁺ (<i>lac</i> ^q) <i>pro</i>] <i>gor522::Tn10</i> (Tc ^r) <i>trxB::kan</i>	Simon <i>et al.</i> (1989)
<i>E. coli</i> Origami	$\Delta ara-leu7697 \Delta lacX74 \Delta phoAPvuII$ <i>phoR araD139 ahpC galE galK rpsL</i> F'[<i>lac</i> ⁺ (<i>lac</i> ^q) <i>pro</i>] <i>gor522::Tn10</i> (Tc ^r) <i>trxB::kan</i>	Novagen
<i>E. coli</i> JM109::pSB401	Biosensor for detecting C ₆ -, C ₇ - and C ₈ -HSL and its 3-O-form	Swift <i>et al.</i> (1997)
<i>E. coli</i> JM109::pSB536	Biosensor for detecting C ₄ -HSL	Swift <i>et al.</i> (1997)
<i>E. coli</i> JM109::pSB1075	Biosensor for detecting C ₁₀ - to C ₁₄ -HSL and their 3-O-forms	Winson <i>et al.</i> (1998)
Plasmids		
pET-20b	Expression vector	Novagen
pET-20b- <i>pa0305his</i>	pET20b containing <i>pa0305</i> with a six-His tag at the C-terminus	This study
pUCP18	<i>E. coli</i> - <i>Pseudomonas</i> shuttle vectors derived from pUC18, pMB1, pRO1600 replicon, <i>lacZx</i> , <i>bla</i> , Ap ^r Cb ^r	West <i>et al.</i> (1994)
pUCP- <i>pa0305his</i>	pUCP18 containing <i>pa0305</i> with a six-His tag at the C-terminus	This study
pUCP- <i>pa0305</i>	pUCP18 containing <i>pa0305</i>	This study
pMMB67EH	Amp ^r IncQ, <i>tac</i> promoter	Fürste <i>et al.</i> (1986)
pMMB- <i>pa0305</i>	pMMB67EH containing <i>pa0305</i>	This study
pEX18Gm	Gm ^r , <i>oriT</i> ⁺ <i>sacB</i> ⁺ , gene replacement vector	Hoang <i>et al.</i> (1998)
pEX- $\Delta pa0305$	pEX18Gm containing flanking regions of <i>pa0305</i>	This study
Oligonucleotides		
ForA	CGCGCAATATACATATGAAACGCACCCTGACCGTGATCG	
RevA	CATTATATTAAGCTTGCGCTTCGGCTCCAGCACCAGCAC	
ForB	GGGGAAGAGCTCATGAAACGCACCCTGACCGTGATCG	
RevB	CCGTTGCACCTCTAGAAGTTATTGCTCAGCGGTGGCAGCAG	
RevC	TTAATTCTAGATCAGCGCTTCGGCTCCAGCACCA	
ForD	CAGAACCAGACCGACCTCTA	
RevD	CGGAACAGACGGTCGGTATC	
ForE	GGTAAGCTTCAAGCGGTTCCTCGTGAGTTC	
RevE	GCTCCAGCACACGCGTATGCATCGATCCTTCTCCTTGTGTGA	
ForF	GCATACGCGTGTGCTGGAGCCGAAGCGCTGAG	
RevF	GCCGAATTCAACGCCCTCAAGCTGGTGACG	
ForG	CGTCGAGCTGGATCTCGATCTG	
RevG	GGCCGATCTACAATACCTACCG	
rpoD-For	GGGCGAAGAAGGAAATGGTC	
rpoD-Rev	CAGGTGGCGTAGGTGGAGAA	

et al., 2007). 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 β -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. After separation, proteins were transferred onto a PVDF membrane (Sequi-Blot PVDF membrane, Bio-Rad) by semi-dry electroblotting. The membrane was then stained with Coomassie Brilliant Blue (CBB) R-250 and the bands corresponding to the α -subunit and the β -subunit-his of PA0305his were excised. The first six residues of the subunits were determined by ABI Procise 494 sequencing (Alphalyse).

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 RNeasy mini kit (Qiagen), followed by DNase (Qiagen) on-column digestion according to the manufacturer's instructions. RNA was quantified by the $A_{260}:A_{280}$ ratio using a NanoDrop (ND-1000) spectrophotometer. cDNA was synthesized with 1 μ g 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 (10 min), followed by 40 cycles of 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 μ l) of 0.5 mg C₁₂-HSL ml⁻¹ in acetonitrile was placed in a 96-well microtitre plate (Lumitrac600,

GreinerBio). 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 1:100 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 1:100 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 deacylase 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 (Invitrogen). 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-C₁₂-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 A₃₄₀ 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: $y = 1.959x + 0.6966$ ($R^2 = 0.9882$). The initial rates (mM s⁻¹) were plotted versus the concentration of substrate (mM), which gives a straight line. Assuming that $[S] \ll K_m$, the slope of the line equals $(K_{cat} \times [E])/K_m$. Thus, dividing the slope by the enzyme concentration results in a value for the apparent K_{cat}/K_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 *pa0305* 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 *Hind*III and *Eco*RI 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 probes (ForG and RevG) are listed in Table 2. A double deletion ($\Delta pvdQ$, $\Delta quiP$) and a triple deletion strain ($\Delta pvdQ$, $\Delta quiP$, $\Delta pa0305$) 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 A₅₂₀ of the HCl fraction was measured. Concentrations, expressed as micrograms pyocyanin produced per millilitre culture supernatant, were determined as $(OD_{520} \times 17.072)/OD_{600}$ (Essar *et al.*, 1990).

Quantification of 3-oxo-C₁₂-HSL, C₄-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 PAO1 $\Delta pvdQ\Delta quiP$ and PAO1 $\Delta pvdQ\Delta quiP\Delta pa0305$ 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

by the biosensor was read every hour during a 24 h time-course in a multifunctional microplate reader (FLUOstar Omega, BMG Labtech). Data points obtained immediately prior to the maximum light production were used for comparisons (approximately 9 h after initiation of the bioassay). For quantification of the accumulation levels of 3-oxo-C₁₂-HSL and C₄-HSL by TLC, 2 µl of the acidified supernatants from the same amount of cells was spotted on a silica gel 60 F254 TLC plate (Merck) for 3-oxo-C₁₂-HSL and a reverse-phase TLC plate for C₄-HSL. The separation and detection were performed as described by Yates *et al.* (2002). For quantification of accumulation levels of PQS, the compound was detected as previously described (Diggle *et al.*, 2003).

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 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, PAO1 Δ pa0305, PAO1 Δ lasRlasI). 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 of the plate were excluded from the analysis. Results are presented as the percentage of living nematodes on the killing plates compared with their survival on the *E. coli* OP50 control strain. Statistical analysis was performed using the two-sample Student's *t*-test in the statistical programming language R. The 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

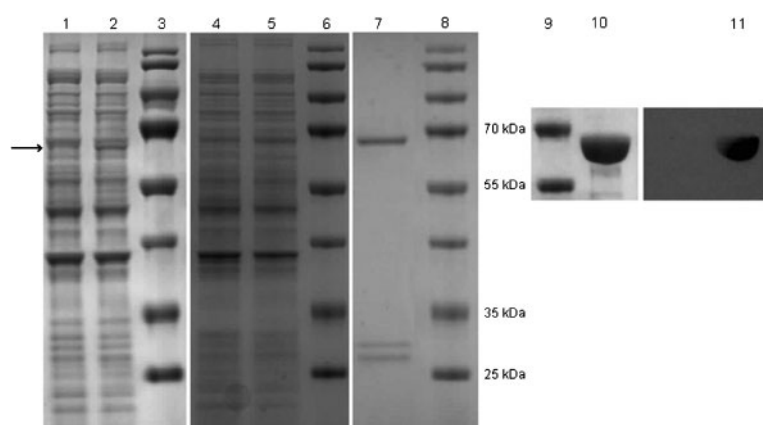


Fig. 1. SDS-PAGE of cell-free extracts of *E. coli* Origami expressing pa0305 and purified PA0305his protein. Lanes: 1 and 4, cell-free extracts of *E. coli* pUCP18; 2, cell-free extract of *E. coli* pUCP-pa0305 showing an overproduced 60 kDa protein (arrow); 5, cell-free extract of *E. coli* pUCP-pa0305his; 7 and 10, purified His-tagged PA0305; 11, purified His-tagged β -subunit of PA0305 detected on a Western blot; 3, 6 and 8, 'Page ladder' pre-stained protein.

protein band is consistent with that expected for the C-terminally processed α -subunit (i.e. with signal peptide but without spacer peptide), whereas the spectrum of the 28 kDa protein band is consistent with the α -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 α -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. C_{12} -HSL was used as the substrate since it resembles the *P. aeruginosa* signal molecule, 3-oxo- C_{12} -HSL. Incubation of C_{12} -HSL with cell-free extract prepared from *E. coli* cells overproducing PA0305his resulted in the conversion of C_{12} -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 C_{12} -HSL-degrading enzyme.

Having established that PA0305 exhibits activity towards C_{12} -HSL, various AHLs with alkyl chains ranging from C_4

to C_{14} (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 $\mu\text{g ml}^{-1}$ 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 C_6 to C_{14} . The highest degradation rate (93-fold decrease in substrate concentration) was observed on C_8 -HSL at 10 $\mu\text{g ml}^{-1}$. At the lowest concentration, 0.01 $\mu\text{g ml}^{-1}$, the degradation rate was the highest on 3-oxo- C_{12} -HSL (64-fold). Activity against the 3-oxo- forms was detected from 3-oxo- C_{10} -HSL to 3-oxo- C_{14} -HSL (Table 3).

To investigate whether PA0305 functions as a lactonase or an acylase, the products of the conversion of C_8 -HSL were subjected to HPLC analysis. The reaction was performed by exposing C_8 -HSL substrate to PA0305his for 4 h. C_8 -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 C_8 -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 BrMMC was performed. HPLC analysis showed that the specific peak of the BrMMC-octanoic acid derivative was present at 12.59 min in C_8 -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 C_8 -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 C_8 -HSL and 3-oxo- C_{12} -HSL. The C_8 -HSL was chosen because it shows the highest activity with PA0305his at high concentrations and 3-oxo- C_{12} -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 C_8 -HSL and 3-oxo- C_{12} -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, $K_{\text{cat}}/K_{\text{m}}$ values were determined using seven low

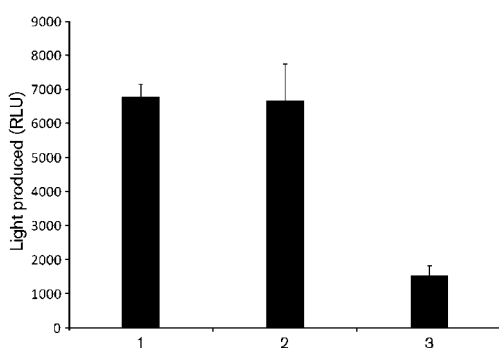


Fig. 2. Activity of cell-free extract of *E. coli* Origami producing PA0305his on C_{12} -HSL substrate. After incubation of C_{12} -HSL with extracts, the non-degraded substrate was analysed using the biosensor strain pSB1075 as described in Methods. The amount of light produced in response to C_{12} -HSL was quantified as relative light units (RLU). Lanes: 1, C_{12} -HSL incubated without cell extract; 2, C_{12} -HSL incubated with cell-free extract of *E. coli* with empty vector; 3, C_{12} -HSL incubated with cell-free extract of *E. coli* producing PA0305his. Error bars, SD.

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 $\mu\text{g ml}^{-1}$) 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.

AHL substrate	Purified PA0305his protein	Degradation ($10^{-3} \times \text{RLU}$)			
		0.01	0.1	1	10
C ₄ -HSL*	Inactivated	22.3 $\pm$ 1.0	70.3 $\pm$ 0.9	74.8 $\pm$ 0.9	81.2 $\pm$ 4.7
	Active	20.2 $\pm$ 2.5	68.9 $\pm$ 5.8	74.2 $\pm$ 7.1	84.2 $\pm$ 9.6
C ₆ -HSL†	Inactivated	2.3 $\pm$ 0.2	13.0 $\pm$ 2.0	40.0 $\pm$ 4.1	70.0 $\pm$ 7.5
	Active	0.94 $\pm$ 0.15	7.2 $\pm$ 0.4	31.0 $\pm$ 0.6	66.6 $\pm$ 2.8
3-oxo-C ₆ -HSL†	Inactivated	72.4 $\pm$ 0.3	99 $\pm$ 2	115 $\pm$ 6	133 $\pm$ 4
	Active	78.7 $\pm$ 8	107 $\pm$ 13	121 $\pm$ 12	136 $\pm$ 12
C ₇ -HSL†	Inactivated	5.8 $\pm$ 0.7	25.7 $\pm$ 3.0	61.8 $\pm$ 6.7	89.2 $\pm$ 3.5
	Active	0.73 $\pm$ 0.13	4.7 $\pm$ 0.6	23.9 $\pm$ 1.5	79.7 $\pm$ 1.8
C ₈ -HSL†	Inactivated	0.18 $\pm$ 0.03	2.6 $\pm$ 0.2	12.9 $\pm$ 0.4	34.4 $\pm$ 0.5
	Active	0.036 $\pm$ 0.008	0.06 $\pm$ 0.01	0.11 $\pm$ 0.01	0.37 $\pm$ 0.10
3-oxo-C ₈ -HSL†	Inactivated	41.8 $\pm$ 1.6	77.7 $\pm$ 0.5	92.8 $\pm$ 6.7	99.0 $\pm$ 1.5
	Active	32.1 $\pm$ 1.2	73.0 $\pm$ 4.9	93.1 $\pm$ 7.5	103 $\pm$ 6
C ₁₀ -HSL‡	Inactivated	0.080 $\pm$ 0.004	2.5 $\pm$ 0.3	8.6 $\pm$ 0.9	9.7 $\pm$ 1.6
	Active	0.064 $\pm$ 0.016	0.076 $\pm$ 0.012	0.105 $\pm$ 0.023	0.6 $\pm$ 0.1
3-oxo-C ₁₀ -HSL‡	Inactivated	8.3 $\pm$ 2.1	10.9 $\pm$ 0.9	11.3 $\pm$ 1.3	13.0 $\pm$ 0.4
	Active	0.240 $\pm$ 0.085	0.5 $\pm$ 0.1	2.5 $\pm$ 0.4	8.6 $\pm$ 0.4
C ₁₂ -HSL‡	Inactivated	2.41 $\pm$ 0.47	8.6 $\pm$ 0.3	9.56 $\pm$ 0.12	9.15 $\pm$ 0.13
	Active	0.110 $\pm$ 0.008	0.13 $\pm$ 0.01	0.41 $\pm$ 0.05	3.27 $\pm$ 0.25
3-oxo-C ₁₂ -HSL‡	Inactivated	10.96 $\pm$ 0.20	10.5 $\pm$ 0.1	10.0 $\pm$ 0.1	9.20 $\pm$ 0.12
	Active	0.171 $\pm$ 0.044	0.42 $\pm$ 0.08	1.57 $\pm$ 0.13	5.52 $\pm$ 0.25
C ₁₄ -HSL‡	Inactivated	4.49 $\pm$ 0.15	9.2 $\pm$ 0.6	8.9 $\pm$ 0.9	8.23 $\pm$ 0.46
	Active	0.124 $\pm$ 0.026	0.16 $\pm$ 0.03	0.46 $\pm$ 0.05	1.38 $\pm$ 0.03
3-oxo-C ₁₄ -HSL‡	Inactivated	8.0 $\pm$ 1.4	9.4 $\pm$ 0.4	9.33 $\pm$ 0.15	8.70 $\pm$ 0.25
	Active	0.19 $\pm$ 0.02	0.19 $\pm$ 0.01	0.81 $\pm$ 0.11	1.32 $\pm$ 0.13

*Biosensor *E. coli* JM109::pSB536.

†Biosensor *E. coli* JM109::pSB401.

‡Biosensor *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 ($K_{\text{cat}}/K_{\text{m}} = 7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) than C₈-HSL ($K_{\text{cat}}/K_{\text{m}} = 0.14 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$).

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 *pa0305* 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* $\Delta pa0305$ 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 $\Delta pa0305$ 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 ($\Delta pvdQ$, $\Delta quiP$, $\Delta pa0305$) was constructed and compared with the double deletion strain ($\Delta pvdQ$, $\Delta quiP$). 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).

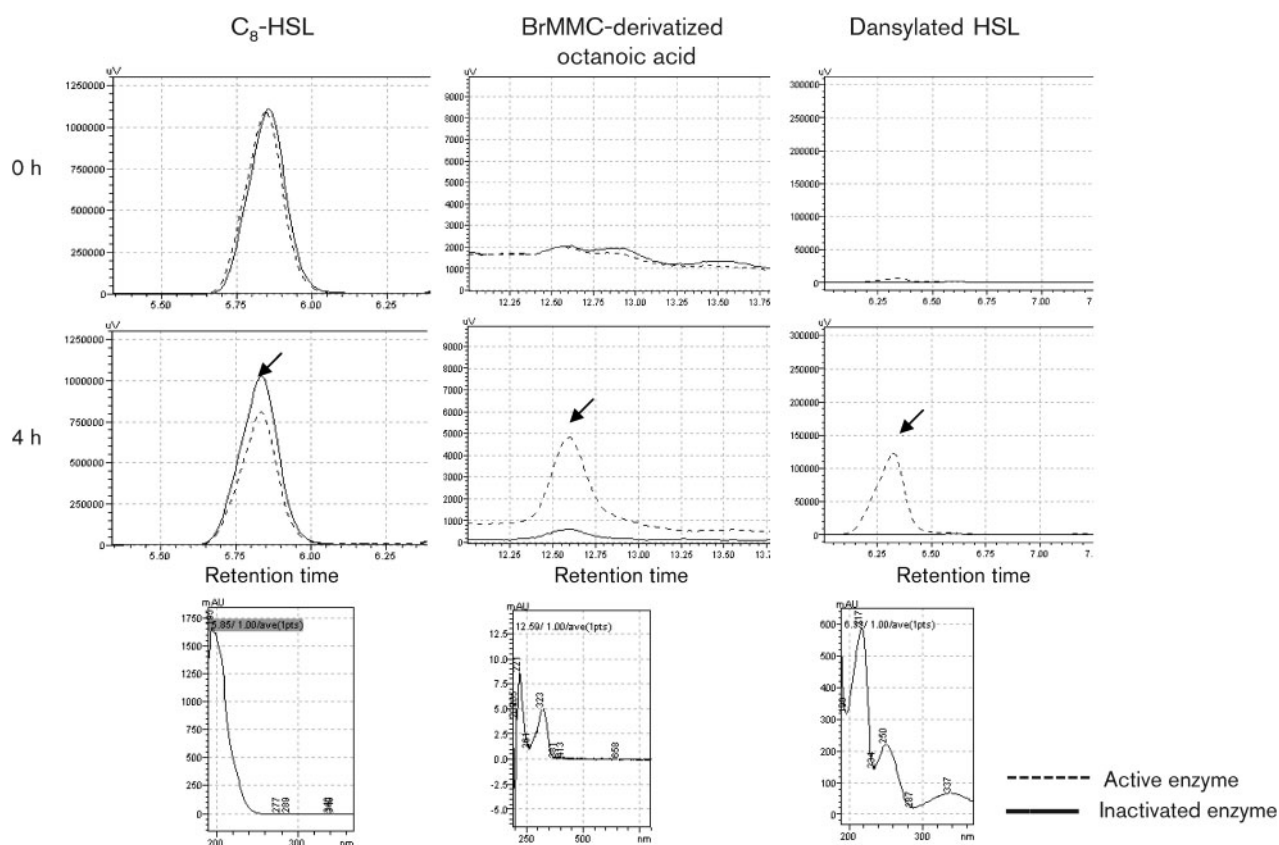


Fig. 3. Identification of the products of the PA0305his-catalysed conversion of C₈-HSL. C₈-HSL was incubated with PA0305his for 4 h. Samples were taken at time 0 (upper panels) and after 4 h (middle panels) and were then treated for quantification by HPLC analysis of the residual C₈-HSL and the octanoic acid and HSL released. Reduction of C₈-HSL levels upon exposure to active PA0305his was corroborated with the occurrence of free octanoic acid and HSL, confirming that PA0305his is an acylase that cleaves the amide bond linking the HSL ring and the acyl chain. C₈-HSL samples were extracted with ethyl acetate and injected into an RP₁₈ column as described in Methods. For efficient detection, octanoic acid was BrMMC-derivatized and HSL was dansylated, as described in Methods. Arrows indicate the specific peaks that were subjected to UV analyses (lower panels). The spectra of C₈-HSL, BrMMC-derivatized octanoic acid and dansylated HSL are identical to those described by Uroz *et al.* (2008). AU, Absorbance units.

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* $\Delta pa0305$ 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). Furthermore, 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

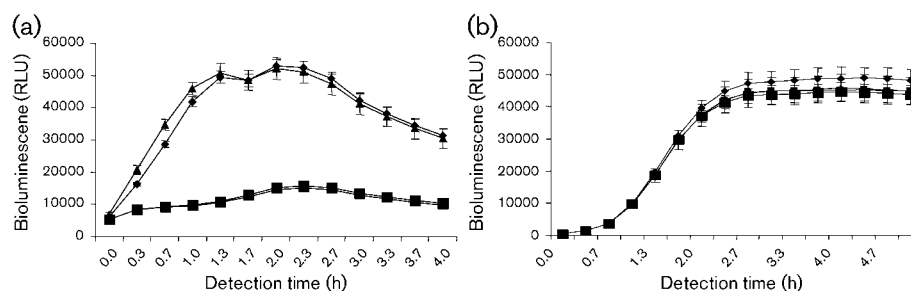


Fig. 4. Quantification of AHLs produced in a time lapse experiment. Supernatant of LB cultures of the respective strains grown for 0–5 h was extracted as described previously (Yates *et al.*, 2002) and quantified in the bioluminescence assay. (a) Results of analysis with biosensor *E. coli* JM109::pSB1075 detecting 3-oxo-C₁₂-HSL. (b) Results of analysis with biosensor *E. coli* JM109::pSB536 detecting C₄-HSL. It can be seen that the pMMB-*pa0305*-harbouring strains accumulate much less 3-oxo-C₁₂-HSL over time as compared with the wild-type PAO1 strain and the $\Delta pa0305$ strain, whereas the accumulation of C₄-HSL is similar for all strains. ♦, Wild-type empty; ■, PAO1 pMMB-*pa0305*; ▲, PAO1 pEx- $\Delta pa0305$ empty; ×, PAO1 pMMB-*pa0305*-pEx- $\Delta pa0305$.

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 $\Delta pa0305$ 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

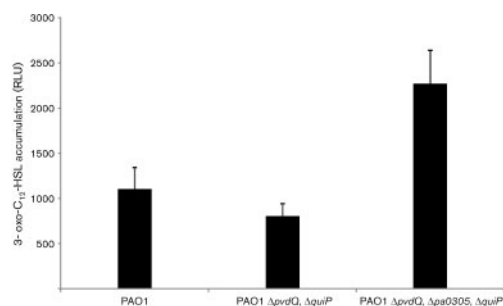


Fig. 5. Accumulation of 3-oxo-C₁₂-HSL in HSL-acylase deletion strains. The PAO1 parental strain, the *P. aeruginosa* $\Delta pvdQ$, $\Delta quiP$ double mutant and the *P. aeruginosa* $\Delta pvdQ$, $\Delta quiP$, $\Delta pa0305$ triple mutant were screened for 3-oxo-C₁₂-HSL accumulation levels after 50 h of growth in LB medium. The graph illustrates the higher 3-oxo-C₁₂-HSL levels present in the $\Delta pvdQ$, $\Delta quiP$, $\Delta pa0305$ triple mutant, in which PA0305 is absent, compared with the PAO1 and the $\Delta pvdQ$, $\Delta quiP$ double mutant background levels, where PA0305 is still present. Error bars, SD.

pathogenicity of the bacterium, the killing kinetics of the wild-type PAO1 strain, the $\Delta pa0305$ 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

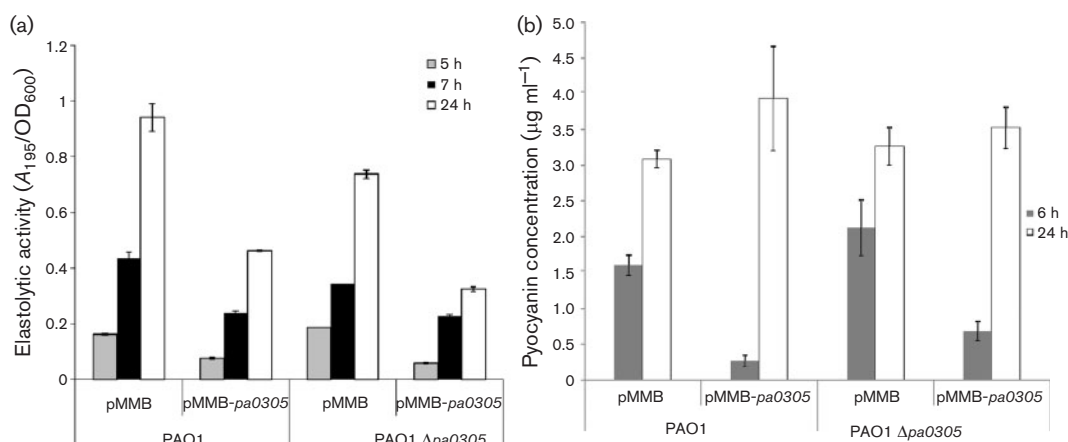


Fig. 6. Effects of PA0305 overexpression on virulence factor production in *P. aeruginosa* PAO1 and PAO1 $\Delta pa0305$. The production of virulence factors was tested in wild-type and $\Delta pa0305$ strains carrying either an empty vector (pMMB) or the *pa0305*-expressing vector (pMMB-*pa0305*). The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

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

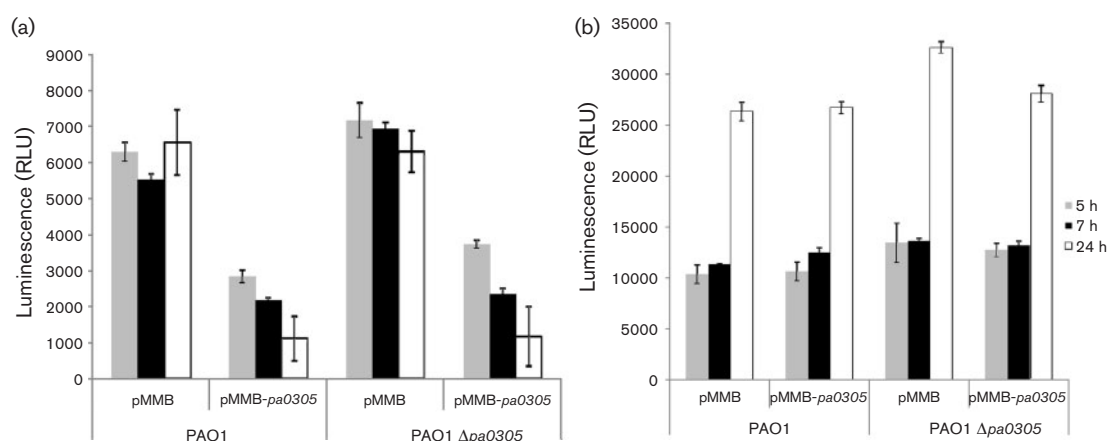


Fig. 7. Effects of PA0305 overproduction on accumulation of QS signal molecules in *P. aeruginosa* PAO1 and PAO1 $\Delta pa0305$. The same strains as described in Fig. 6 were also tested for the accumulation of 3-oxo- C_{12} -HSL as detected by luminescence measured with *E. coli* pSB1075 biosensor. (a) Overproduction of PA0305 reduces the accumulation of 3-oxo- C_{12} -HSL in wild-type and $\Delta pa0305$ 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.

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

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*.

<i>P. aeruginosa</i>	Plasmid	Fold change
PAO1 wild-type	pMMB67EH	205 $\pm$ 35
	pMMB- <i>pa0305</i>	144488 $\pm$ 19344
PAO1 $\Delta pa0305$	pMMB67EH	1 $\pm$ 0
	pMMB- <i>pa0305</i>	93335 $\pm$ 9506

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 PAO1 *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 β -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 α -subunit purified from *E. coli* revealed that the signal peptide is not cleaved off from the α -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,

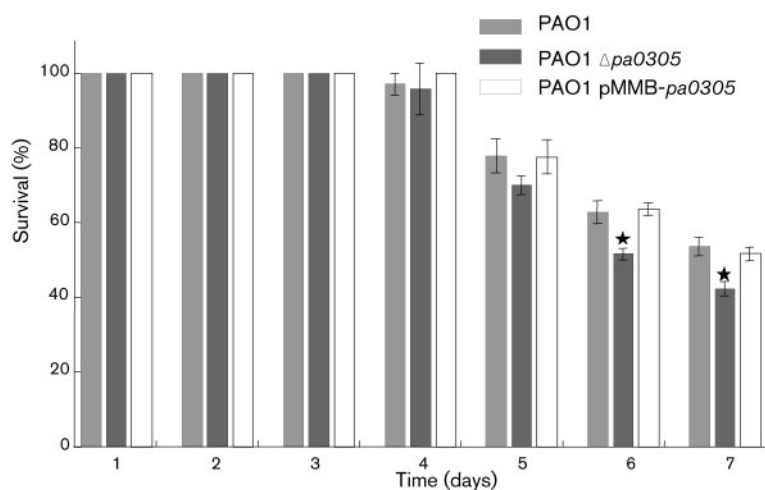


Fig. 8. The effect of overexpressing and deleting the *pa0305* gene on the virulence of *P. aeruginosa* in the *C. elegans* model. *C. elegans* nematodes were fed on a layer of *Pseudomonas* cells as described previously (Papaioannou *et al.*, 2009). Wild-type *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 $\Delta pa0305$ 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 C_4 -HSL. Interestingly the ability to degrade C_4 -HSL, the second major autoinducer, seems to be absent from all AHL acylases of *P. aeruginosa* PAO1 described to date (Huang *et al.*, 2006). Judging from conversion rates at high concentrations of different acyl-HSLs it may be concluded that C_8 -HSL is the best substrate for PA0305 (93-fold decrease at $10 \mu\text{g ml}^{-1}$; 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 C_8 -HSL at 0.01 and $10 \mu\text{g ml}^{-1}$ with the conversion rates of 3-oxo- C_{12} -HSL at these respective concentrations (Table 3). The deacylation rate of the enzyme at low concentrations appears to be best for 3-oxo- C_{12} -HSL and not for C_8 -HSL (64-fold versus 1.3-fold). To explain these observations, one likely possibility is that at high substrate concentrations ($10 \mu\text{g ml}^{-1}$) of 3-oxo- C_{12} -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 $K_{\text{cat}}/K_{\text{m}}$ value for an acyl-HSL acylase. Indeed the catalytic efficiency of PA0305 is higher for 3-oxo- C_{12} -HSL in comparison with C_8 -HSL, which is consistent with 3-oxo- C_{12} -HSL being one of the natural substrates of this enzyme. Although individual K_{cat} and K_{m} values have not been assessed, the apparent $K_{\text{cat}}/K_{\text{m}}$ value of $7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ implies that the enzyme has a high catalytic efficiency towards 3-oxo- C_{12} -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- C_{12} -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 PAO1 Δ pvdQ Δ quiP background. The increase in 3-oxo- C_{12} -HSL did not result in alterations of the C_4 -HSL accumulation levels, the growth rate (data not shown) nor the morphology of the mutant. This is all in agreement with previous observations with HacB mutants of *P. syringae* (Shepherd & Lindow, 2009). It seems that the apparent 3-oxo- C_{12} -HSL concentration is close to saturation level for regulating the AHL-responsive system in both the wild-type and Δ pa0305 mutant.

Overproducing PA0305 in both PAO1 wild-type and Δ pa0305 did reduce, but not completely abolish, the

accumulation of 3-oxo- C_{12} -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- C_{12} -HSL, is dispersed from the cytoplasm to the extra-cellular medium.

Interestingly the reduction of the 3-oxo- C_{12} -HSL levels showed the highest effect on pyocyanin production after 24 h, whereas the reduction in elastase was most pronounced after 6 h and the effect on reducing C_4 -HSL was absent. This difference in response probably reflects the difference in response regulators involved and their interaction with the regulatory elements of the respective genes.

According to mRNA quantification by RT-PCR, in rich media such as LB without exogenous addition of signal molecules, *pa0305* is expressed in low amounts at the late-exponential phase, at about 5 h post-inoculation. Previous studies using transposon insertions on *pa0305* revealed that this gene is not crucial for cell growth on C_{10} -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- C_{12} -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 pyoverdinin 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*-dimethylamino-benzaldehyde for the detection of 6-aminopenicillanic 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 AHLs 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

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 Ntnhydrolases 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).

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