FEMS Microbiology Letters, 362, 2015, fnv069

doi: 10.1093/femsle/fnv069
Advance Access Publication Date: 29 April 2015
Research Letter

RESEARCH LETTER – Physiology & Biochemistry

The *Pseudomonas aeruginosa* RhlR-controlled aegerolysin RahU is a low-affinity rhamnolipid-binding protein

Špela Miklavič[1], Polona Kogovšek[2], Vesna Hodnik[1], Jernej Korošec[1], Aleš Kladnik[1], Gregor Anderluh[1,3], Ion Gutierrez-Aguirre[2], Peter Maček[1] and Matej Butala[1,*]

[1]Department of Biology, Biotechnical Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia, [2]Department of Biotechnology and Systems Biology, National Institute of Biology, 1000 Ljubljana, Slovenia and [3]National Institute of Chemistry, 1000 Ljubljana, Slovenia

*Corresponding author: Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia. Tel: +386-1-3203397; E-mail: matej.butala@bf.uni-lj.si

One sentence summary: We provide evidence that 3OC12-HSL 'LasR is insufficient, and that C4-HSL' RhlR promotes expression of *Pseudomonas aeruginosa rahU* gene product, which is a low-affinity monorhamnolipid-binding protein.

Editor: Craig Winstanley

ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* uses quorum-sensing systems to regulate collective behaviour in response to the environment, by linking the expression of particular genes to population density. The quorum-sensing transcription factors LasR and RhlR and their cognate *N*-acyl-homoserine lactone (HSL) signals *N*-3-oxo-dodecanoyl-L-HSL ($3OC_{12}$-HSL) and *N*-butanoyl-L-HSL (C_4-HSL) control the expression of several hundred genes, which include those involved in virulence and biofilm formation. Here, we have focused on regulation of the expression of the putative virulence factor gene, *rahU*. We show that the intact *las-rhl* box immediately upstream of the −35 promoter element is needed for *rahU* expression in *P. aeruginosa*. Using β-galactosidase assays and quantification of the mRNA levels for *rahU*, *lasR* and *rhlR*, we provide evidence that for *rahU* promoter activity, $3OC_{12}$-HSL-LasR is not sufficient, and instead C_4-HSL-RhlR is the trigger. Furthermore, surface plasmon resonance analysis revealed that RahU binds the biosurfactant rhamnolipids. Thus, this is the first report of a bacterial molecule that interacts with RahU.

Keywords: induction of gene expression; transcription responses; biosurfactant; rhamnolipids; quorum-sensing

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that coordinates gene expression of an array of virulence factors and secondary metabolites through the quorum-sensing response (Wagner *et al.* 2003). The diffusible signals involved at high cell densities are *N*-3-oxo-dodecanoyl-L-homoserine lactone ($3OC_{12}$-HSL), which is produced by the synthetase LasI, and *N*-butanoyl-L-homoserine lactone (C_4-HSL), which is produced by the synthetase activity of RhlI (Passador *et al.* 1993; Ochsner and Reiser 1995). The global transcriptional regulators LasR and RhlR serve as the signal receptors for $3OC_{12}$-HSL and C_4-HSL, respectively (Fuqua, Parsek and Greenberg 2001). LasR can bind to specific DNA sequences only when coupled to $3OC_{12}$-HSL, and

Received: 2 March 2015; **Accepted:** 20 April 2015

depending on C_4-HSL binding to RhlR, this complex can activate or repress the expression of target genes (Medina *et al.* 2003; Schuster, Urbanowski and Greenberg 2004). These two networks are hierarchically organized, as the *lasRI* system can control the expression of *rhlRI*, and these are interconnected with other regulatory pathways (Williams and Cámara 2009). Furthermore, the $3OC_{12}$-HSL signal shows post-translational control of RhlR through its competition with C_4-HSL for binding to RhlR, which provokes dissociation and inactivation of the homodimer (Pesci *et al.* 1997; Ventre *et al.* 2003).

Overall, more than 300 *P. aeruginosa* promoters are responsive to *las*, *rhl* or *las/rhl* (Wagner *et al.* 2003; Schuster and Greenberg 2007). The promoter-specific determinants (i.e. *las-rhl* boxes) recognized by $3OC_{12}$-HSL-LasR and C_4-HSL-RhlR are not clearly understood (González-Valdez *et al.* 2014). For instance, *las/rhl*-responsive promoters frequently harbour only one target site, which indicates that both of these transcription factors can bind to the same promoter nucleotide sequence (Gilbert *et al.* 2009). Among quorum-sensing-regulated genes, there is also the conserved putative virulence factor gene *rahU* (PA0122) (Wagner *et al.* 2003). RahU belongs to the aegerolysin protein family (Pfam 06355, InterPro IPR009413), which contains over 300 members of small (~15 kDa) β-structured proteins that are found in several eukaryotic and bacterial taxa (Berne, Lah and Sepčić 2009; Novak *et al.* 2015). Aegerolysin from oyster mushroom has been shown to target sphingomyelin/cholesterol membrane domains, and when combined with a membrane attack complex/perforin (MACPF)-like protein, the resulting pore-forming complex can damage cell membranes (Ota *et al.* 2013). The putative MACPF-like partner of RahU has not been identified. It has been suggested that the RahU protein modulates *P. aeruginosa* biofilm formation, and that by binding oxidized low-density lipoprotein or lysophosphatidylcholine (lysoPC), this 15-kDa protein can sense the host innate immunity (Rao *et al.* 2011a,b). Transcriptome analysis has indicated that *rahU* is substantially activated by $3OC_{12}$-HSL alone, while for full promoter activation, the C_4-HSL signal is also required (Schuster *et al.* 2003). Using the chromatin immunoprecipitation in conjunction with DNA microarray analysis, LasR was shown to interact at position −52 to −37 relative to the *rahU* transcription start site (Gilbert *et al.* 2009; Wurtzel *et al.* 2012), which makes it likely that LasR makes contact with RNA polymerase (Browning and Busby 2004). However, independent evidence for direct *rahU* promoter (p*rahU*) regulation by LasR is not available.

In the present study, we undertook a systematic analysis of *P. aeruginosa* PA01 p*rahU* induction. We show here that the intact *las-rhl* target site centred at position −44.5 relative to the transcription start site is absolutely required for activation of p*rahU*. Our data show that $3OC_{12}$-HSL-LasR is insufficient to activate *rahU* expression. In contrast, sufficient *rhlR* expression and the presence of its cognate signal C_4-HSL can trigger p*rahU*. As C_4-HSL-RhlR also promotes the expression of genes required for the synthesis of L-rhamnose-containing glycolipids, which are also known as rhamnolipids (Medina *et al.* 2003; Aguirre-Ramírez *et al.* 2012), and as the aegerolysin family proteins target organism-specific lipids, we investigated the RahU–rhamnolipid interaction using surface plasmon resonance analysis. These data show that RahU is a rhamnolipid-binding protein.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains, plasmids and oligonucleotide primers used in this study are given in Table 1. The detailed information on the cloning protocols, expression and purification of recombinant aegerolysins, fluorescence microscopy analysis, and RNA extraction and quantitative polymerase chain reaction (PCR) is provided in the Data S1–S5 (Supporting Information).

β-Galactosidase assay and manipulation of the acyl-HSL concentrations in the growth media

To assay the activity of p*rahU*, the pLP170 plasmid harbouring either the wild-type promoter fragment or its promoter variant with two nucleotides changed in the LasR/RhlR operator were transformed into *P. aeruginosa* PAO1 wild-type and its mutant strains: PAO1 Δ*lasR*, PAO1 Δ*rhlR*, PAO1 Δ*lasR*Δ*rhlR* and PAO1 Δ*lasI*Δ*rhlI* (MW1 strain) (see Table 1), as previously described (Sinclair and Morgan 1978). To measure the promoter activity, 40 mL LB medium containing 50 mM MOPS, pH 7 (LB-MOPS) supplemented with carbenicillin (300 μg mL^{-1}), and 2 μM $3OC_{12}$-HSL and/or 10 μM C_4-HSL in the case of MW1 strain were inoculated for the overnight cultures at an optical density at 600 nm (OD_{600}) of 0.05. The cells were grown aerobically in 250-mL flasks at 37°C, with shaking at 190 rpm. To remove and substitute acyl-HSL in the growth medium, the cells were removed from the medium by centrifugation (2000 × *g*, 5 min, room temperature), washed with sterile 0.9% NaCl and resuspended in fresh growth medium with the selected acyl-HSL. The cultures were sampled (1 mL) throughout the growth phase, and then the cells were pelleted by centrifugation, frozen in liquid nitrogen and stored at −80°C. After thawing, the cells were lysed and assayed for β-galactosidase activity, as reported previously (Miller 1972). The β-galactosidase activities are means ± standard deviation (SD) of at least three independent experiments. Representative growth curves are presented.

Surface plasmon resonance

We studied the interactions of immobilized recombinant aegerolysins from *P. aeruginosa* (RahU), *Vibrio cholerae* and *Clostridium bifermentans* (cbm17.1) as ligands with mixtures of monorhamnolipids and dirhamnolipids (Sigma-Aldrich, L510025), 90% pure monorhamnolipids (Sigma-Aldrich, L511218), (C:16) 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lysoPC) (Avanti Polar Lipids, 855675), C_4–HSL (Santa Cruz Biotechnology, 205402), $3OC_{12}$–HSL (Sigma-Aldrich, O9139) and mono/di/trisaccharides as analytes. Recombinant RahU and the aegerolysins from *V. cholerae* and *C. bifermentans* (cbm17.1) were immobilized by amine coupling on CM5 sensor chips (Series S; GE Healthcare) using a Biacore T100 instrument (GE Healthcare). The surface of reference and the ligand ffiow cells were initially activated according to the coupling procedure recommended by the manufacturer, which uses a 1:1 mixture of 0.4 M 1-ethyl-3–(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.1 M N-hydroxysuccinimide. The ligands were diluted into 10 mM sodium acetate buffer, at pH 4.5, 5.0 and 5.5 for RahU, aegerolysin from *V. cholerae* and cbm17.1, respectively. The ligands were then separately immobilized to saturate the second flow cell, and each ligand was immobilized on its own flow cell. We used filtered phosphate-buffered saline (20 mM Na_2HPO_4/NaH_2PO_4, 140 mM NaCl, pH 7.2) as running buffer, at a flow rate of 10 μL min^{-1}. The ligand immobilization levels of the aegerolysins from *P. aeruginosa* (RahU), *V. cholerae* and *C. bifermentans* (cbm17.1) were 1500 resonance units (RU), 850 RU and 1150 RU, respectively. After immobilization, both of the ffiow cells were blocked with a 7-min pulse of 1.0 M ethanolamine. The interactions with rhamnolipids, (C:16) lysoPC, C_4-HSL

Table 1. Bacterial strains, plasmids and oligonucleotide primers used in this study.

Strain, plasmid or primer		Relevant property	Reference or source
Strains	*Escherichia coli* DH5α	F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK⁻, mK⁺) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Novagen
	E. coli BL21(DE3) pLysS	F⁻ *dcm ompT hsdS*(rB⁻ mB⁻) *gal* λ(DE3) [pLysS Cam^R]	Novagen
	P. aeruginosa PAO1	Wild type	Holloway, Krishnapillai and Morgan (1979)
	P. aeruginosa PAO1 Δ*lasR*	PAO1 derivative; Δ*lasR*::Tc^R	Rahim *et al.* (2001)
	P. aeruginosa PAO1 Δ*rhlR*	PAO1 derivative; Δ*rhlR*::Gm^R	Rahim *et al.* (2001)
	P. aeruginosa PAO1 Δ*lasR* Δ*rhlR*	PAO1 derivative; Δ*lasR*::Tc^R Δ*rhlR*::Gm^R	Rahim *et al.* (2001)
	P. aeruginosa MW1	PAO1 derivative; a *lasI*, *rhlI* mutant that does not synthesite acyl-HSL signals	Whiteley, Lee and Greenberg (1999)
Plasmids	pCF430R	Broad host-range vector, contains the *araC*-P_{BAD} promoter and the *rhlR* open reading frame	Gupta, Gobble and Schuster (2009)
	pET-21c(+)	T7 expression vector	Novagen (Merck, USA)
	pET-21c(+).*rahU*	pET-21c(+) derivative, contains *rahU* CDS region	This study
	pET-21c(+).Vc	pET-21c(+) derivative, contains the aegerolysin CDS region from *V. cholerae*	This study
	pET-21c(+).cbm17.1	pET-21c(+) derivative, contains the cbm17.1 CDS region	This study
	pLP170	Promoterless *lacZ* transcriptional fusion vector; Cb^R; capable of replicating in *P. aeruginosa*	Preston *et al.* (1997)
	pLP170.p*rahU*	pLP170 derivative, contains the p*rahU* region	This study
	pLP170.mp*rahU*	pLP170.p*rahU* derivative with the two point mutations (p–52C to A, p–51T to C) in *las-rhl* box	This study
	pLP170.p*rahU*-R	pLP170.p*rahU* derivative, contains the intact p*rahU* region and the *araC*-P_{BAD} promoter-controlled *rhlR* open reading frame	This study
	pLP170.p*rahU*.*mCherry*	pLP170 derivative, contains the p*rahU* region linked by a 30-nt linker to the gene for the mCherry reporter protein	This study
Primers (cloning)	*rahU*promoter-f	CCCCTCGAGGCCCTGCTCGATGCCCTGGA	This study
	*rahU*promoter-r	CCCGGATCCCACCGTGCCGATGGATTTGG	This study
	rahU-f	CCCCATATGGCATACGCAGAATGGATCGC	This study
	rahU-r	CCCCTCGAGGGAGAAGCGGCCGAGGGTCA	This study
	Vc-f	CCCCATATGGCCTATGCACAATGGGTAAC	
	Vc-r	CCCCTCGAGAATTTTGGTCGTTTGAATGA	
	cbm17.1-f	CCCCATATGAATAATAATTGTGAAGTTAA	This study
	cbm17.1-r	CCCCTCGAGTGCCCATATTTTTGATACTT	This study
	egeroPAcherrP1	CCCGGATCCGGCAGCGAAGGCAAAAGCAGCGG CAGCGGCGTGAGCAAGGGCGAGGAGGATAAC	This study
	egeroCHERP2_n	GCGGGATCCTCACTTGTACAGCTCGTCCATGCC	This study
QuickChange mutagenesis	*rahU*promoter_s.	CCCCGGCCGCCGCACACCAGATCTGGCA	This study
	*rahU*promoter_antis.	TGCCAGATCTGGTGTGCGGCGGCCGGGG	This study
Primers (sequencing)	pLP170-r(1)	CCCAAAGTTAAAATGCCGCGCCC	This study
	pLP170-r(2)	CCCTGTAAAACGACGGCCAGTGA	This study
qPCR	16S rRNA-F	GGAGTACGGCCGCAAGGT	Mahnič *et al.* (2012)
	16S rRNA-probe	/56–FAM/AAAACTCAAATGAATTGACG/MGB/	Mahnič *et al.* (2012)
	16S rRNA-R	CATGCTCCACCGCTTGTG	Mahnič *et al.* (2012)
	lasR-F	ACCAGCCGGGAGAAGGAAG	Modified from Cabrol *et al.* (2003)
	lasR-probe	/56-FAM/CCGATGGCGCACCAC/36-TAMSp/	Cabrol *et al.* (2003)
	lasR-R	CCGATATCTCCCAACTGGTCTTG	Cabrol *et al.* (2003)
	rahU-F	ACCCAGGGCAGCTTCGA	This study
	rahU-probe	/56–FAM/TGCTACGACGGCAACACCAAGATGG/36-TAMSp/	This study
	rahU-R	GGGTCATCCCAGCTGAAGGT	This study
	rhlR-F	AACGCGAGATCCTGCAATG	Takaya *et al.* (2008)
	rhlR-probe	/56–FAM/TGAGCATCTCCGAGAGCACGGT/36-TAMSp/	Takaya *et al.* (2008)
	rhlR-R	GCGCGTCGAACTTCTTCTG	Takaya *et al.* (2008)
	rpoD-F	CCTGCCGGAGGATATTTCC	Modified from Belete, Lu and Wozniak (2008)
	rpoD-probe	/56–FAM/ATCCGGAACAGGTGGAAGACATCATCC/36-TAMSp/	Belete, Lu and Wozniak (2008)
	rpoD-R	GATCCCCATGTCGTTGATCAT	Belete, Lu and Wozniak (2008)

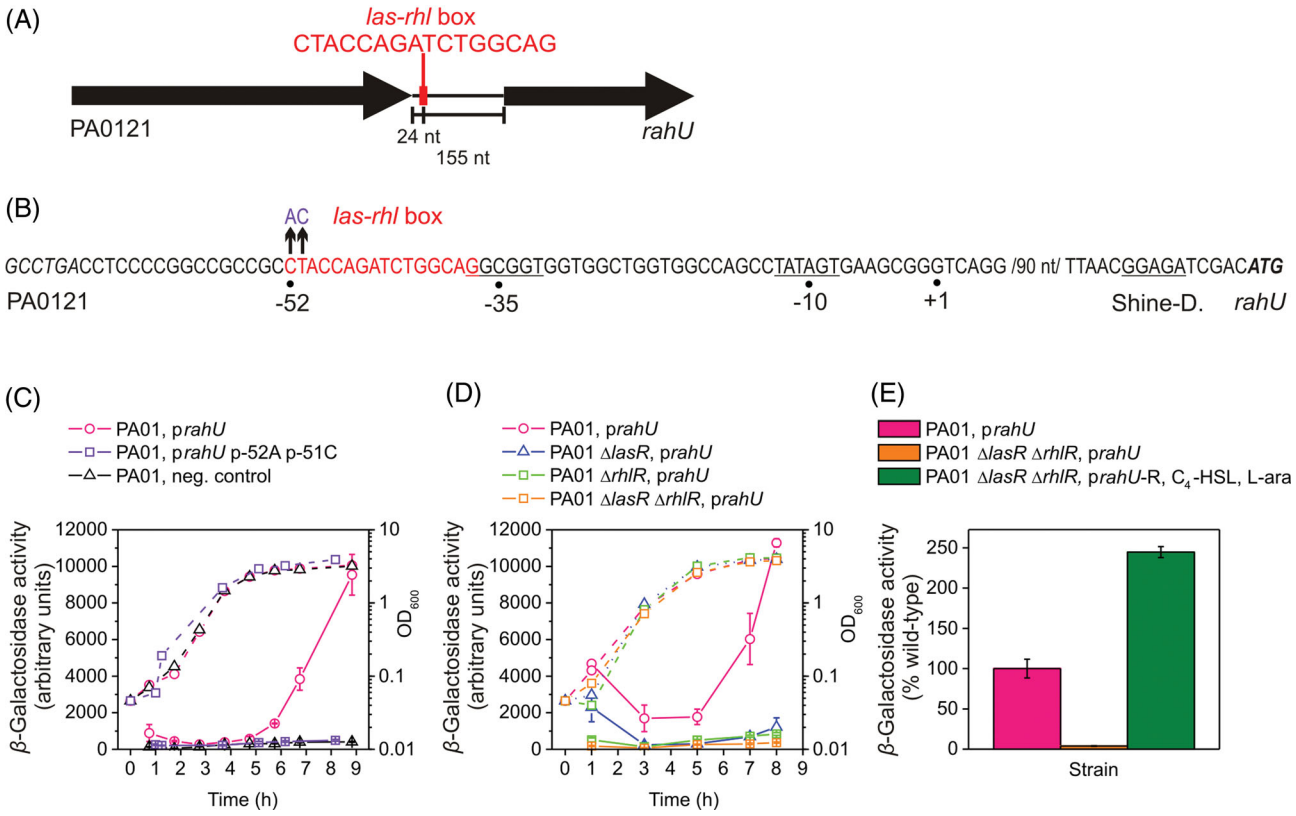

Figure 1. RhlR, and not LasR, is sufficient to trigger the *P. aeruginosa rahU* promoter. **(A)** Schematic diagram of the p*rahU* region. The previously proposed *las-rhl* box (Gilbert *et al.* 2009; Wurtzel *et al.* 2012) identified in the intergenic region (standard font) between the PA0121 and the *rahU* gene (italic font) is highlighted in red. **(B)** Sequence of the p*rahU* region from −74 to +114 relative to the transcription start site (+1) (Wurtzel *et al.* 2012). The proposed Shine–Dalgarno sequence and the −35 and −10 hexamer elements are underlined and labelled. The base substitutions described in the text are indicated by the vertical arrows and highlighted in violet. The *rahU* start codon is in bold type. **(C)** β-Galactosidase activities (solid lines) measured in *P. aeruginosa* PA01 wild-type carrying pLP170 containing either the wild-type p*rahU* region or the p*rahU* region with the −52A and −51C mutations that abrogate LasR/RhlR binding (Table 1). The empty pLP170 vector was used as the negative control. The *rahU* promoter activity measured in the *P. aeruginosa* PA01 wild-type, Δ*rhlR* mutant or in the Δ*rhlR* mutant carrying p*rahU*-R were 9540 ± 1111, 404 ± 30 and 23 460 ± 630 arbitrary units, respectively. **(D)** β-Galactosidase activities (solid lines) measured in *P. aeruginosa* PA01 wild-type or its Δ*lasR*, Δ*rhl* or Δ*rhlR* Δ*lasR* variant strains carrying pLP170 containing the wild-type p*rahU* region (Table 1). **(E)** Restoration of the RhlR phenotype in the *P. aeruginosa* PA01 Δ*rhlR* Δ*lasR* strain. The Δ*rhlR* Δ*lasR* mutant was supplied with the pLP170.p*rahU*-R vector containing the wild-type p*rahU* region derivative expressing *rhlR* from the pBAD promoter (p*rahU*-R), and was grown in the presence of 50 mM L-arabinose and 10 μM C_4-HSL. The p*rahU* activities in the wild-type or the Δ*rhlR* Δ*lasR* strain that contained either p*rahU* or p*rahU*-R were measured when the cells reached the stationary phase of growth, after 8 h of inoculation. Dashed lines in (C–E) show OD_{600}. Data are means ± SD of three biological replicates.

and $3OC_{12}$-HSL were studied at a flow rate of 30 μL min^{-1} at 25°C, using filtered 130 mM NaCl, 50 mM Tris, 50 μM EDTA, 1% dimethyl sulfoxide, pH 7.4, as the running buffer. The interactions with mono/di/trisaccharides were studied under the same conditions in running buffer without dimethyl sulfoxide. The carbohydrates were injected for 30 s, and the dissociation was monitored for 15 s, while the binding of the lipids was followed for 60 s, followed with a 30-s dissociation period. After each injection, the surface was stabilized with 60 s of buffer flow, and regeneration was not needed. The sensorgrams were double corrected for the untreated surface ffiow-cell response and the buffer response, and where indicated, the data were fitted to a steady-state affinity model to obtain the equilibrium dissociation constant (K_D), using the Biacore T100 Evaluation software (GE Healthcare). Representative sensorgrams of triplicates are shown.

RESULTS AND DISCUSSION

C_4-HSL-RhlR activates the *rahU* promoter

To investigate the growth-phase-dependent activity of the p*rahU* and the importance of the *las-rhl* box upstream of the *rahU* gene, we exploited the entire p*rahU* region, which includes 350 bp upstream and 57 bp downstream of the translation start site, and also its derivative that carried the nucleotide substitutions that disrupted two of the conserved nucleotides in the quorum-sensing operator (Fig. 1A and B; Table 1). The fragments were cloned into a broad host-range promoterless *lacZ* pLP170 plasmid (Preston *et al.* 1997). Measurements of β-galactosidase activity in *P. aeruginosa* PA01 (Table 1) containing pLP170 carrying either the wild-type p*rahU* region (pLP170.p*rahU*) or its derivatives demonstrated the importance of the *las-rhl* operon for induction of p*rahU*. In agreement with previous studies (Wagner *et al.* 2003; Schuster and Greenberg 2007; Rao *et al.* 2008), we confirmed here that p*rahU* is strongly activated at the onset of the stationary phase (Fig. 1C). Furthermore, p*rahU* was also activated in biofilm *P. aeruginosa* cells carrying the reporter p*rahU–mCherry* transcriptional fusion vector (Fig. S1, Supporting Information). Therefore, our data indicate that *rahU* expression during planktonic and biofilm growth of *P. aeruginosa* is dependent on the quorum-sensing circuitry.

To understand the interplay of the quorum-sensing factors that affect *rahU* expression, we first modified the *las-rhl* box at position −44.5 relative to the *rahU* transcription start site

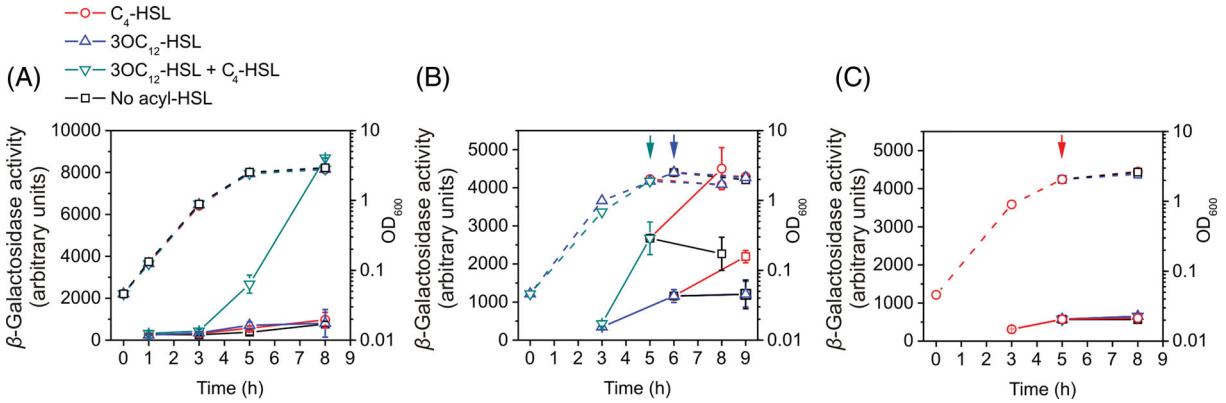

Figure 2. Signal manipulation confirms that *rahU* promoter activity is dependent on C_4-HSL-RhlR. **(A)** β-Galactosidase activities (solid lines) of the signal-generation mutant *P. aeruginosa* MW1 carrying pLP170 with a p*rahU-lacZ* transcriptional fusion vector (Table 1) grown in the absence (black squares) and presence of either 2 μM $3OC_{12}$-HSL (blue triangles) or 10 μM C_4-HSL (red circles), or in the presence of both 2 μM $3OC_{12}$-HSL and 10 μM C_4-HSL (inverted cyan triangles). Arrows in **(B)** in representative colours indicate time of centrifugation for each condition, for removal of indicated acyl-HSLs from the growth media, and suspension of cells in acyl-HSL-free or in C_4-HSL-rich or in OC_{12}-HSL medium. Arrow in **(C)** indicates the time of substitution or removal of 10 μM C_4-HSL and re-addition of HSLs, according to the representative colours. Dashed lines show OD_{600}. Data are means ± SD of three biological replicates.

(Schuster *et al.* 2003; Wurtzel *et al.* 2012). The introduction of two point mutations into the *las-rhl* box conserved region (i.e. p-52C to A; p-51T to C; Fig. 1A–C) prevented p*rahU* activation, which suggested that this region that is located immediately upstream of the −35 promoter element is essential for correct promoter function through the direct binding of either LasR or RhlR, or of both of these transcription factors.

To determine the role of the quorum-sensing transcription factors at p*rahU*, we first assayed the promoter activity from the p*rahU-lacZ* reporter plasmid in derivatives of *P. aeruginosa* PAO1 deleted for either *lasR* or *rhlR*, or for both of these genes (Table 1). Fig. 1D illustrates that negligible difference in the promoter activity was detected among the strains, in comparison to the control (i.e. the empty vector in the wild-type strain). In contrast to the transcriptome analysis (Schuster *et al.* 2003), we observed defective *rahU* expression in the *rhlR* mutant, which indicated that $3OC_{12}$-HSL-LasR and the system components under its control are not sufficient for p*rahU* induction (Fig. 1D). In addition, RhlR complementation in the *lasR*, *rhlR* mutant strain fully restored p*rahU* activity (Fig. 1E).

To confirm the observation that members of the LasR regulon cannot induce p*rahU* in the absence of the subordinate *rhl* system, we assayed p*rahU-lacZ* fusion in *P. aeruginosa* MW1, as a *lasI* and *rhlI* double mutant that does not produce diffusible acyl-HSL (Table 1). We examined a series of removal and repositioning of the extracellular signals in a similar manner to that used to study LasR activity *in vivo* (Sappington *et al.* 2011). In the $3OC_{12}$-HSL-replete medium, the *las* system was induced in *P. aeruginosa* MW1 (Schuster *et al.* 2003); however, p*rahU* activation was minimal, which supported the view that $3OC_{12}$-HSL-LasR alone cannot induce p*rahU* activation. Furthermore, C_4-HSL alone was also not sufficient to induce p*rahU*. Thus, these data indicated that both of these signals are required to obtain the full response (Fig. 2A). To obtain further insight into the regulation of *rahU* expression, we took advantage of the reversible binding of $3OC_{12}$-HSL to LasR (Sappington *et al.* 2011). *Pseudomonas aeruginosa* MW1 cells harbouring the p*rahU-lacZ* reporter were grown in either $3OC_{12}$-HSL or $3OC_{12}$-HSL plus C_4-HSL medium until the onset of the stationary phase of growth, when $3OC_{12}$-HSL was either substituted with C_4-HSL or these signals were removed from the medium (Fig. 2B). In this way, a pool of signal-free LasR was formed in the cells, which removed the LasR transcriptional activation (Sappington *et al.* 2011). In both cases, 5 or 6 h after inoculation, when the cells were resuspended in the signal-free medium, there was no increase in p*rahU* activity. This indicated that the signals were efficiently removed from the medium. In contrast, p*rahU* induction was detected as a response to the substitution of $3OC_{12}$-HSL with C_4-HSL (Fig. 2B). These data indicated that C_4-HSL-RhlR was essential for p*rahU* induction, although we cannot exclude that the p*rahU* activity is coregulated by another transcription factor that is under direct LasR/RhlR control. After the removal of the signals, p*rahU* activation stopped (Fig. 2B). This indicated that C_4-HSL binds reversibly to RhlR, which was also shown previously to be the mechanism of binding of the LasR protein and its cognate signal (Sappington *et al.* 2011). When the bacteria were growing at the onset of the stationary phase in $3OC_{12}$-HSL medium and the growth media was exchanged for fresh $3OC_{12}$-HSL medium, no induction of p*rahU* was observed (Fig. 2B). Furthermore, when MW1 bacteria were grown in media supplemented with C_4-HSL, or when the HSL was removed or exchanged for $3OC_{12}$-HSL at the onset of the stationary phase of growth, neither of the signals induced p*rahU* activity (Fig. 2C), which supports the activation of p*rahU* by C_4-HSL-RhlR.

To gain further evidence in support of the view that C_4-HSL is the essential environmental signal for *rahU* expression, we used quantitative PCR to quantify the relative levels of the *lasR*, *rhlR* and *rahU* transcripts in *P. aeruginosa* MW1 grown in $3OC_{12}$-HSL or C_4-HSL medium, or in medium in which the $3OC_{12}$-HSL signal was substituted for C_4-HSL. The levels of these transcripts were determined at the time of inoculation, in the exponential phase, and at the onset of the stationary phase of growth. In agreement with the β-galactosidase data, transcription of *rahU* was minimal throughout the bacterial growth in either $3OC_{12}$-HSL or C_4-HSL medium, and the *rahU* transcript level was high when $3OC_{12}$-HSL was replaced with C_4-HSL (Fig. 3B). During exponential growth and at the onset of the stationary phase of growth, the *lasR* and *rhlR* transcripts were up to 3-fold more expressed in $3OC_{12}$-HSL medium, when compared to the transcript levels for *P. aeruginosa* grown in C_4-HSL medium (Fig. 3C and D). Thus, our data suggest that $3OC_{12}$-HSL-LasR alone, and also signal-free RhlR, cannot induce p*rahU* activity (Fig. 3B and D). It has also been reported that in the logarithmic phase of growth, small amounts of *rhlR* transcript do not result in RhlR

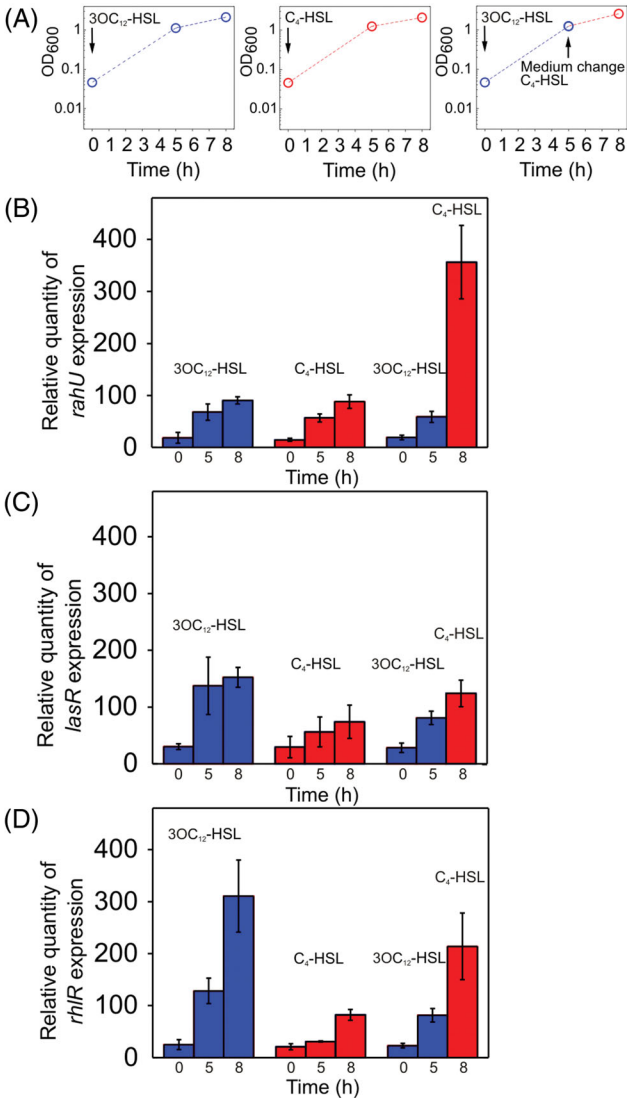

Figure 3. Quantitative PCR confirms that signal-bound RhlR, but not LasR, triggers the *rahU* promoter. **(A)** Representative growth curves of *P. aeruginosa* MW1 in media supplemented with either 2 μM $3OC_{12}$-HSL (blue) or 10 μM C_4-HSL (red), or in media where $3OC_{12}$-HSL was substituted with C_4-HSL at onset of stationary phase (arrow). **(B–D)** Relative copynumbers of *rahU*, *rhlR* and *lasR* transcripts (as indicated) in *P. aeruginosa* MW1 at time of inoculation (0 h), in exponential phase (5 h), and at onset of stationary phase of growth (8 h), as in (A). Data are means ± 95% confidence intervals of three biological replicates (Table S2, Supporting Information).

synthesis (Schuster and Greenberg 2007). Hence, this might explain why the *rahU* transcript levels were not significantly increased in the C_4-HSL-rich medium although small amounts of the *rhlR* transcripts were detected intracellularly. When the $3OC_{12}$-HSL signal was replaced with C_4-HSL in the exponential growth phase, an approximately 4-fold increase in the *rahU* transcript levels was detected 3 h later (Fig. 3B). Our data indicated that both the *rhlR* transcript and C_4-HSL at concentrations above the threshold level were prerequisites for p*rahU* induction, which indicates that C_4-HSL-RhlR is the ultimate trigger for *rahU* transcription.

Aegerolysin RahU interacts with rhamnolipids

As the expression of genes involved in rhamnolipid biosynthesis can also be activated by C_4-HSL-RhlR (Medina *et al.* 2003; Aguirre-Ramírez *et al.* 2012), and as RahU bound amphiphilic lysoPC (Rao *et al.* 2008), we determined whether RahU was able to bind rhamnolipids. Thus, surface plasmon resonance analysis was carried out to determine the specific binding and the interaction affinity between recombinant RahU and rhamnolipids. The used recombinant RahU was more than 95% pure (Fig. S2, Supporting Information). We injected different concentrations of either monorhamnolipids or a mixture of monorhamnolipids and dirhamnolipids, either with ß-hydroxyalkanoic acid tails ranging from C:8 to C:14 that is also representative for rhamnolipids synthesized by *P. aeruginosa* (Abdel-Mawgoud, Lépine and Déziel 2010), over RahU immobilized on the CM5 sensor chip. These data showed that RahU interacts with monorhamnolipids, with a K_D of 33.9 ± 1.4 μM (Fig. 4). Monorhamnolipids were also applied over the *V. cholerae* and *C. bifermentans* (Cbm17.1) chip- immobilized RahU orthologues, but no interactions were detected (Fig. S3A, Supporting Information). Among these two orthologues, the biological function was only determined for the Cbm17.1 that is a part of the protein complex toxic to mosquito larvae (Qureshi *et al.* 2014). Furthermore, (C:16) lysoPC was examined for binding to immobilized aegerolysins. LysoPC interacted with chip-immobilized RahU with a K_D of 33.5 ± 4.7 μM, which is in agreement with a previous study (Rao *et al.* 2008) in which (C:6) lysoPC was used (Fig. S3B, Supporting Information). In contrast, no lysoPC binding was detected for the other two orthologues. In addition, RahU did not interact with C_4-HSL, $3OC_{12}$-HSL (Fig. S4A, Supporting Information), rhamnose and several other carbohydrates, which were tested up to 1 mM (Fig. S4B, Supporting Information). Here, these data show that RahU interacts in μM range with *P. aeruginosa*-specific rhamnolipid biosurfactant molecules. Although our data show that this is a low-affinity interaction, we believe that it is biologically significant, for instance due to the observation that RahU protein synthesis is induced at the onset of the stationary phase of growth (Rao *et al.* 2008), when *P. aeruginosa* culture can produce approximately 100 μM of rhamnolipids (Mulligan, Mahmourides and Gibbs 1989). In addition, the *rahU* mutant was shown to generate more biofilm in comparison to its isogenic variant (Rao *et al.* 2008) and as the *P. aeruginosa* surface attachment and the development of biofilm structures is affected by rhamnolipids (Davey, Caiazza and OÀTolle 2003), we predict that this process could be modulated by RahU.

CONCLUSIONS

Our data demonstrate that RhlR coupled to C_4-HSL activates p*rahU*. In addition, these data suggest that the signal binds reversibly to RhlR. Furthermore, we show that RahU is a novel rhamnolipid-interacting protein. Thus, this study uncovers a small molecule from *P. aeruginosa* that binds to aegerolysin RahU, and provides a further contribution to the better understanding of the basic biology of these molecules, which can also be used in biotechnology applications (Lang and Wullbrandt 1999). The implications of this interaction relate to modulation of the uptake of hydrophobic substrates, antimicrobial activity, motility or decreased biofilm formation by the opportunistic pathogen *P. aeruginosa* (Lang and Wullbrandt 1999; Haba *et al.* 2003), thus lowering its virulence potential. In

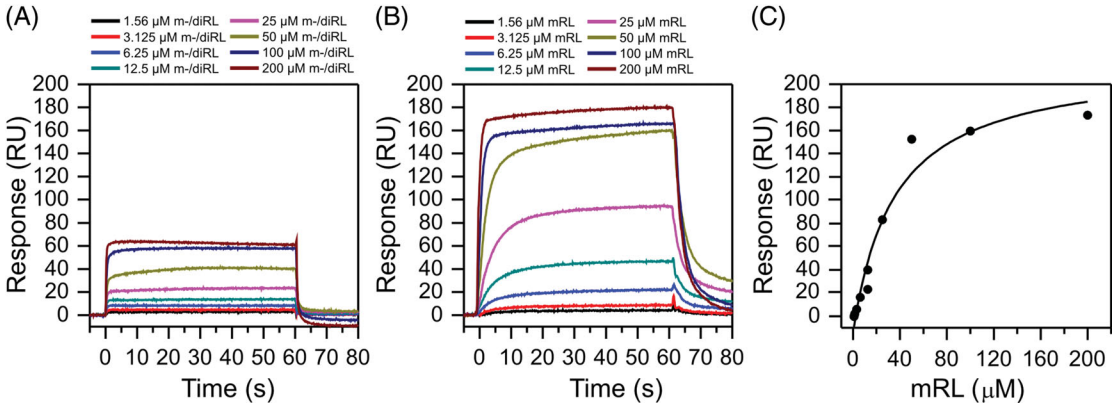

Figure 4. Surface plasmon resonance sensorgrams measuring the binding between rhamnolipids and aegerolysin RahU. The monorhamnolipid/ dirhamnolipid (m-/diRL) mixture **(A)** or the 90% pure monorhamnolipids (mRL) **(B)** (concentration range, 1.56–200 μM) were injected over the chip- immobilized 1500 RU of RahU for 60 s at 30 μL min^{-1}. Dissociation was followed for 30 s. **(C)** Determination of the equilibrium dissociation constant (K_D) from the plot of the response as a function of the monorhamnolipid concentration injected over RahU. The K_D determined for the mRL–RhlR interaction was 33.9 $\pm$ 1.4 μM. Data are means of three replicates.

addition, this study adds to the number of organism-specific lipids that bind to aegerolysins, indicating interesting structural characteristics among the aegerolysins and their cognate lipids.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

ACKNOWLEDGEMENTS

The authors thank Sudha Chugani and Peter E. Greenberg for generously providing the *P. aeruginosa* PA01 (wilde-type), PA01 Δ*lasR*, PA01 Δ*rhlR*, PA01 Δ*rhlR* Δ*las* and *P. aeruginosa* MW1 strains, Cassie Kruczek and Abdul N. Hamood for the pLP170 plasmid, and Martin Schuster and Brett Mellbye for the pCF430R plasmid. We also thank Andrej Blejec for advice on the statistical analysis of the data.

FUNDING

This study was funded by grant P1-0207 from the Slovenian Research Agency (ARRS). Špela Miklavič is a recipient of a PhD grant from the ARRS.

Conflict of interest. None declared.

REFERENCES

Abdel-Mawgoud AM, Lépine F, Déziel E. Rhamnolipids: diversity of structures, microbial origins and roles. *Appl Microbiol Biot* 2010;**86**:1323–36.

Aguirre-Ramírez M, Medina G, González-Valdez A, *et al*. The *Pseudomonas aeruginosa rmlBDAC* operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by the quorum-sensing transcriptional regulator RhlR and the alternative sigma factor σS. *Microbiology* 2012;**158**:908–16.

Belete B, Lu H, Wozniak DJ. *Pseudomonas aeruginosa* AlgR regulates type IV pilus biosynthesis by activating transcription of the *fimU-pilVWXY1Y2E* operon. *J Bacteriol* 2008;**190**:2023–30.

Berne S, Lah L, Sepčić K. Aegerolysins: structure, function, and putative biological role. *Protein Sci* 2009;**18**:694–706.

Browning DF, Busby SJ. The regulation of bacterial transcription initiation. *Nat Rev Microbiol* 2004;**2**:57–65.

Cabrol S, Olliver A, Pier GB, *et al*. Transcription of quorum-sensing system genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *J Bacteriol* 2003;**185**:7222–30.

Davey ME, Caiazza NC, O'Tolle GA. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PA01. *J Bacteriol* 2003;**185**:1027–36.

Fuqua C, Parsek MR, Greenberg EP. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* 2001;**35**:439–68.

Gilbert KB, Kim TH, Gupta R, *et al*. Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. *Mol Microbiol* 2009;**73**:1072–85.

González-Valdez A, Servín-González L, Juárez K, *et al*. The effect of specific *rhlA*-las-box mutations on DNA binding and gene activation by *Pseudomonas aeruginosa* quorum-sensing transcriptional regulators RhlR and LasR. *FEMS Microbiol Lett* 2014;**356**:217–25

Gupta R, Gobble TR, Schuster M. GidA posttranscriptionally regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 2009;**191**:5785–92.

Haba E, Pinazo A, Jauregui O, *et al*. Physicochemical characterisation and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. *Biotechnol Bioeng* 2003;**81**:316–22.

Holloway BW, Krishnapillai V, Morgan AF. Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* 1979;**43**:73–102.

Lang S, Wullbrandt D. Rhamnose lipids—biosynthesis, microbial production and application potential. *Appl Microbiol Biot* 1999;**51**:22–32.

Mahnič M, Baebler S, Blejec A, *et al*. Gene expression profiling of recombinant protein producing *E. coli* at suboptimal growth temperature. *Acta Chim Slov* 2012;**59**:59–69.

Medina G, Juárez K, Valderrama B, *et al*. Mechanism of *Pseudomonas aeruginosa* RhlR transcriptional regulation of the *rhlAB* promoter. *J Bacteriol* 2003;**185**:5976–83.

Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1972.

Mulligan CN, Mahmourides G, Gibbs BF. The influence of phosphate metabolism on biosurfactant production by *Pseudomonas aeruginosa*. *J Biotechnol* 1989;**12**:199–210.

Novak M, Kraševec N, Skočaj M, *et al*. Fungal aegerolysin-like proteins: distribution, activities, and applications. *Appl Microbiol Biot* 2015;**99**:601–10.

Ochsner UA, Reiser J. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *P Natl Acad Sci USA* 1995;**92**:6424–8.

Ota K, Leonardi A, Mikelj M, *et al.* Membrane cholesterol and sphingomyelin, and ostreolysin A are obligatory for pore-formation by a MACPF/CDC-like pore-forming protein, pleurotolysin B. *Biochimie* 2013;**95**:1855–64.

Passador L, Cook JM, Gambello MJ, *et al.* Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 1993;**260**:1127–30.

Pesci EC, Pearson JP, Seed PC, *et al.* Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 1997;**179**:3127–32.

Preston MJ, Seed PC, Toder DS, *et al.* Contribution of proteases and LasR to the virulence of *Pseudomonas aeruginosa* during corneal infections. *Infect Immun* 1997;**65**:3086–90.

Qureshi N, Chawla S, Likitvuvatanavong S, *et al.* The Cry toxin operon of *Clostridium bifermentas* subs. *malaysia* is highly toxic to aedes larval mosquitoes. *Appl Environ Microb* 2014;**80**:5689–97.

Rahim R, Ochsner UA, Olvera C, *et al.* Cloning and functional characterization of the *Pseudomonas aeruginosa rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. *Mol Microbiol* 2001;**40**:708–18.

Rao J, DiGiandomenico A, Artamonov M, *et al.* Host derived inflammatory phospholipids regulate *rahU* (PA0122) gene, protein, and biofilm formation in *Pseudomonas aeruginosa*. *Cell Immunol* 2011a;**270**:95–102.

Rao J, DiGiandomenico A, Unger J, *et al.* A novel oxidized low-density lipoprotein-binding protein from *Pseudomonas aeruginosa*. *Microbiology* 2008;**154**:654–65.

Rao J, Elliott MR, Leitinger N, *et al.* RahU: an inducible and functionally pleiotropic protein in *Pseudomonas aeruginosa* modulates innate immunity and inflammation in host cells. *Cell Immunol* 2011b;**270**:103–13.

Sappington KJ, Dandekar AA, Oinuma K-I, *et al.* Reversible signal binding by the *Pseudomonas aeruginosa* quorum-sensing signal receptor LasR. *MBio* 2011;**2**:e00011.

Schuster M, Greenberg EP. Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon. *BMC Genomics* 2007;**8**:287.

Schuster M, Lostroh CP, Ogi T, *et al.* Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* 2003;**185**:2066–79.

Schuster M, Urbanowski ML, Greenberg EP. Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *P Natl Acad Sci USA* 2004;**101**:15833–9.

Sinclair MI, Morgan AF. Transformation of *Pseudomonas aeruginosa* strain PAO with bacteriophage and plasmid DNA. *Aust J Biol Sci* 1978;**31**:679–88.

Takaya A, Tabuchi F, Tsuchiya H, *et al.* Negative regulation of quorum-sensing systems in *Pseudomonas aeruginosa* by ATP-dependent Lon protease. *J Bacteriol* 2008;**190**:4181–8.

Ventre I, Ledgham F, Prima V, *et al.* Dimerization of the quorum sensing regulator RhlR: development of a method using EGFP fluorescence anisotropy. *Mol Microbiol* 2003;**48**:187–98.

Wagner VE, Bushnell D, Passador L, *et al.* Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* 2003;**185**:2080–95.

Whiteley M, Lee KM, Greenberg EP. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *P Natl Acad Sci USA* 1999;**96**:13904–9.

Williams P, Cámara M. Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr Opin Microbiol* 2009;**12**:182–91.

Wurtzel O, Yoder-Himes DR, Han K, *et al.* The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. *PLoS Pathog* 2012;**8**:e1002945.