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An *rhl*-like quorum-sensing system negatively regulates pyoluteorin production in *Pseudomonas* sp. M18

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Pseudomonas sp. M18, isolated from the watermelon rhizosphere, is antagonistic against a number of soil-borne pathogens. This strain produces an uncharacterized red pigment, pyoluteorin (Plt), and two *N*-acylhomoserine lactones (AHLs). A previously isolated red-pigment-defective mutant, M18-T510, contains an insert within a gene similar to *rhlI* in *P. aeruginosa* PAO1. The M18 *rhlI* gene product is responsible for the production of two AHL signals: *N*-butyryl-homoserine lactone and *N*-hexanoylhomoserine lactone. Mutants defective in either *rhlI* or *rhlR* showed enhanced Plt biosynthesis due to loss of transcriptional repression, which was mediated, at least in part, by suppressed expression of the activator PltR. A Plt-specific ABC transporter was also upregulated in the *rhl* mutants in a Plt-dependent manner. In comparison with the wild-type strain, the *rhl* mutants survived longer during stationary-phase growth.

INTRODUCTION

Antibiotics produced by the root-associated strains of fluorescent pseudomonads can protect plants against a range of soil-borne phytopathogens (Dowling & O'Gara, 1994; Thomashow & Weller, 1996). Various pseudomonad strains used in bio-control synthesize a battery of antibiotics, including polyketides and phenazines, as well as others (Bender *et al.*, 1999; Turner & Messenger, 1986). The biosynthesis and secretion of these antibiotics occur mostly after the cell has entered into the stationary phase of growth, when bacterial densities have reached a certain threshold; this threshold is detected by a process called quorum sensing (QS) (Fuqua *et al.*, 2001; Withers *et al.*, 2001). QS regulates gene expression via signalling molecules, such as *N*-acyl-homoserine lactones (AHLs), in Gram-negative bacteria (Taga & Bassler, 2003; Waters & Bassler, 2005). The concentration of AHL increases along with the increasing bacterial cell density. When the concentration of AHL reaches a threshold level, it binds to specific transcriptional regulators belonging to the LuxR family, and forms various activated protein–AHL complexes. These complexes regulate the transcription of specific target genes, including various antibiotic biosynthesis gene clusters in *Pseudomonas* spp. (Haas & Keel, 2003).

The QS system, and its function in the regulation of antibiotic production, have been thoroughly investigated in some phenazine-producing strains, particularly *Pseudomonas aeruginosa* PAO1. There are at least two QS systems in *P. aeruginosa* PAO1, and these are called the *las* (LasI–LasR) and *rhl* (RhlI–RhlR) systems (Gambello & Iglewski, 1991; Ochsner *et al.*, 1994). The major AHLs synthesized by LasI and RhlI are *N*-(3-oxododecanoyl)-homoserine lactone and *N*-butyrylhomoserine lactone (BHL), respectively (Pearson *et al.*, 1994, 1995). Furthermore, these two QS systems do not function independently, but rather arrange in a cascade to positively control the expression of biosynthetic genes for phenazine production (Brint & Ohman, 1995; Pesci *et al.*, 1997; Schuster *et al.*, 2003). In three other phenazine-producing *Pseudomonas* strains, i.e. *P. aureofaciens* 30-84, *P. fluorescens* 2-79 and *P. chlororaphis* PCL1391, a conserved system called the PhzI–PhzR QS system has been found to positively regulate phenazine production (Chin-A-Woeng *et al.*, 2001; Khan *et al.*, 2005; Wood *et al.*, 1997). In addition, biosynthesis of certain polyketide antibiotics depends on the QS system; for example, the biosynthesis of mupirocin (pseudomonic acid) requires the MupI–MupR QS system in *P. fluorescens* NCIMB 10586 (El-Sayed *et al.*, 2001).

Pyoluteorin (Plt) is a polyketide compound that can effectively suppress several oomycete fungi including the

Abbreviations: AHL, *N*-acylhomoserine lactone; BHL, *N*-butyrylhomoserine lactone; C_T, threshold cycle; HHL, *N*-hexanoylhomoserine lactone; PCA, phenazine-1-carboxylic acid; Plt, pyoluteorin; QS, quorum sensing.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the *rhlI* gene and the partial sequence of the *rhlR* gene of *Pseudomonas* sp. M18 is DQ345445.

seed- and root-rotting pathogen *Pythium ultimum* (Bailey *et al.*, 1973; Howell & Stipanovic, 1980; Maurhofer *et al.*, 1992). Plt is produced by several strains of *Pseudomonas* spp., including the soil-borne bacteria *P. fluorescens* Pf-5 and CHA0, and the regulation of Plt biosynthesis has been investigated in both of these strains. Studies have revealed that the regulatory circuit controlling Plt production includes positive autoregulation, with Plt acting as a signalling molecule (Brodhagen *et al.*, 2004). The extracellular concentration of Plt increases in parallel with cell density, and accumulates to detectable levels until the cells begin to enter the stationary phase (Brodhagen *et al.*, 2004). In addition, PltR, the LysR-type transcriptional activator, has been presumed to be a candidate receptor (Brodhagen *et al.*, 2004). These properties are very similar to those of AHLs; however, AHLs have not yet been found in pseudomonad strains such as *P. fluorescens* Pf-5 and CHA0, which are known to produce Plt and PltR. Except for these preliminary results that suggest the presence of AHLs, little is known about the relationship between the QS system and Plt production.

The *Pseudomonas* sp. strain M18 adopted in this study is an effective bio-control agent against soil-borne phytopathogens (Hu *et al.*, 2005). This capability is primarily due to its ability to produce two antibiotics: phenazine-1-carboxylic acid (PCA) and Plt. To the best of the authors' knowledge, this pseudomonad is the first strain that has been reported to produce these two different types of antibiotics together in a single cell (Ge *et al.*, 2004; Hu *et al.*, 2005). In previous work, we described how Plt is positively regulated, and PCA is negatively regulated, by a global regulator called GacA (Ge *et al.*, 2004); however, the reverse relationship occurs in *Pseudomonas* sp. M18 through another global regulator, RsmA (Zhang *et al.*, 2005). We have also identified a novel pathway-specific regulator of PltZ that could specifically repress Plt biosynthesis (Huang *et al.*, 2004). In a more recent study, we characterized a putative Plt-induced ABC transporter cassette required for Plt production in *Pseudomonas* sp. M18 (Huang *et al.*, 2006); however, no research has yet been done on the regulation of Plt production by potential AHL signalling molecules secreted by this strain.

This study was initiated using a red-pigment-negative transposon mutant M18-T510, which was derived from *Pseudomonas* sp. M18. We then identified an *rhl* QS system, and, to the best of our knowledge, this is the first report indicating that this system can regulate Plt production in *Pseudomonas* sp. M18. We further demonstrate that Plt production is negatively controlled by this *rhl* QS system at the transcriptional level, and that this regulation is partially mediated by PltR. In addition, we present evidence that expression of a Plt-specific ABC transporter is also negatively regulated by the *rhl* QS system in a Plt-dependent manner. Finally, we demonstrate that cell growth and red pigment production are also under the control of the *rhl* QS system.

METHODS

Bacterial strains and growth conditions. *Pseudomonas* sp. M18 is a fluorescent *Pseudomonas* strain isolated from the watermelon rhizosphere, and is an unusual strain sharing some distinct features of both *P. aeruginosa* and *P. fluorescens* (Hu *et al.*, 2005). The other bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown at 37 °C in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). *Pseudomonas* sp. M18 and its derivatives were incubated at 28 °C in King's medium B (KMB; King *et al.*, 1954). Normal growth conditions for strain M18 and its derivatives were as follows: one colony was inoculated into a 250 ml flask containing 25 ml KMB broth, and grown at 28 °C, with shaking at 220 r.p.m., in a C25KC incubator shaker (New Brunswick Scientific). After 10 h growth, 7.5 ml of each strain was transferred to a 500 ml flask containing 150 ml KMB broth, and shaken at 28 °C, as described above. Antibiotics were used at the following concentrations (μg ml^{-1}): for pseudomonads, gentamicin (Gm) 40, kanamycin (Km) 50, spectinomycin (Sp) 100, and tetracycline (Tc) 125; for *E. coli*, Km 50, Gm 15, ampicillin (Ap) 100.

DNA manipulation and cloning procedures. Restriction endonucleases, DNA-modifying enzymes, *Taq* and *Pfu* DNA polymerase, DNA molecular mass markers, and other associated products, were used as recommended by the manufacturer (TaKaRa; MBI Fermentas). Small-scale preparations of plasmid DNA were performed using the MiniBEST plasmid purification kit version 2.0 (TaKaRa). Genomic DNA was extracted and purified from *Pseudomonas* sp. M18 using an EZ spin column genomic DNA isolation kit (Sangon). Restriction enzyme digestions, ligations, and agarose gel electrophoresis, were performed using standard methods (Sambrook *et al.*, 1989). Restriction fragments were purified from agarose gels using the DNA gel extraction kit (V-gene Biotech). Primers used are listed in Table 1. PCR products were recovered, and directly sequenced at the Sangon Biological Engineering Technology and Service (Shanghai, China).

Plasmid and mutant construction. Genomic DNA from strain M18-T510 was digested with the restriction enzyme *Pst*I, and the DNA fragment containing the Km^r cassette from the transposon mini-Tn*5 lacZ-tet/1* and the partial downstream flanking sequence was visualized via routine Southern hybridization, as described (Sambrook *et al.*, 1989). The probe used was a 1.7 kb *Xba*I fragment containing the Km^r cassette from plasmid pVDL24 (de Lorenzo *et al.*, 1993). The Km^r cassette was located on a 2.3 kb *Pst*I fragment from the genomic DNA of strain M18-T510. This *Pst*I fragment was cloned into pBLS, creating the plasmid pBLST510 (Table 1). The nucleotide sequence of chromosomal DNA downstream of the insertion junction was determined using the primer PSRMTN5 (Table 1).

The RhlI coding region was amplified with *Pfu* polymerase from 1 ng *Pseudomonas* sp. M18 chromosomal DNA using primers PRSMRHLI1 and PRSMRHLI2 (Table 1), under the following cycling conditions: one initial step of 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 3 min; followed by one final step of 5 min at 72 °C. The resulting 2.0 kb PCR product was purified and digested with *Xba*I and *Hin*dIII, and cloned into plasmid pBLS to create pBLSRHLI (Table 1). A 1.6 kb PCR product including *rhlR* and partial flanking sequences was obtained in a similar manner using primers PRSMRHLR1 and PRSMRHLR2 (Table 1). The 1.6 kb PCR product was purified and digested with *Eco*RI and *Pst*I, and cloned into the plasmid pEX18Tc, resulting in plasmid pEXTcRHLR (Table 1). Subsequently, a 1.9 kb *Bam*HI–*Hin*dIII fragment containing the intact *rhlI* gene, and a 1.6 kb *Eco*RI–*Pst*I fragment containing an intact *rhlR* gene, were cloned into pME6000 to produce plasmids pME6000rhlI and pME6000rhlR, respectively (Table 1).

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

Strain, plasmid or primer	Description	Reference or source
E. coli		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA–argF*) *U169 deoR* λ(ϕ80*dlacZ*ΔM15)	Sambrook *et al.* (1989)
SM10	*thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	Sambrook *et al.* (1989)
***Pseudomonas* sp.**		
M18	Wild-type, Plt$^+$ PCA$^+$ Ap^r	Hu *et al.* (2005)
M18IG	M18 Δ*rhlI*::Gm^r, Ap^r Gm^r	This study
M18RK	M18 *rhlR*::Km^r, Ap^r Km^r	This study
M18T	Plt$^-$, PCA$^+$, *pltB*::Gm^r, Ap^r Gm^r	Huang *et al.* (2006)
M18TRG	M18 *pltR*::Gm^r, Ap^r Gm^r	This study
M18TI	Plt$^-$, PCA$^+$, *pltB*::Gm^r, *rhlI*::Km, Ap^r Gm^r Km^r	This study
M18TR	Plt$^-$, PCA$^+$, *pltB*::Gm^r, *rhlR*::Km, Ap^r Gm^r Km^r	This study
M18-T510	mini-Tn*5 lacZ-tet*/1 *rhlI* mutant derived from M18; Km^r	This study
Plasmids		
pBLS	pBluescript II KS+ cloning vector, ColE1 replicon, Ap^r	Sambrook *et al.* (1989)
pBLSRHLI	pBLS with *Xba*I–*Hin*dIII insert of 2.0 kb, including gene *rhlI* and partial flanking sequence, Ap^r	This study
pBLSRHLIG	*RhlI*::Gm^r in pBLSRHLI, Ap^r Gm^r	This study
pBLST510	pBLS with *Pst*I insert of 2.3 kb from chromosome of mutant M18-T510, including Km^r cassette and partial sequence downstream, Ap^r Km^r	This study
pDSK519	Source of Km^r cassette, Km^r	Keen *et al.* (1988)
pEX18Tc	Gene replacement vector with MCS from pUC18, *oriT*$^+$ *sacB*$^+$ Tc^r	Hoang *et al.* (1998)
pEXTcIG	2.3 kb *Bam*HI–*Hin*dIII fragment from pBLSRHLIG in pEX18Tc, *oriT*$^+$ *sacB*$^+$ Tc^r Gm^r	This study
pEXTcRHLR	pEX18Tc with *Eco*RI–*Pst*I insert of 1.6 kb, including gene *rhlR* and partial flanking sequence, Tc^r	This study
pEXTcRK	*RhlR*::ΩKm in pEXTcRHLR, Tc^r Km^r	This study
pHZLF	371 bp *Eco*RI–*Pst*I PCR amplified fragment containg the first 35 codons and 243 bp DNA sequence upstream of translational start site (ATG) of *pltH* cloned into pME6015, Tc^r	Huang *et al.* (2006)
pME6000	Broad-host-range cloning vector, Tc^r	Maurhofer *et al.* (1998)
pME6015	pVS1-p15A *E.-coli–Pseudomonas* shuttle vector for translational *lacZ* fusions and promoter probing, Tc^r	Heeb *et al.* (2000)
pME6032	pVS1-p15A *E.-coli–Pseudomonas* shuttle vector, *lacI′-ptac* expression vector, Tc^r	Heeb *et al.* (2002)
pME6522	pVS1-p15A *E.-coli–Pseudomonas* shuttle vector for transcriptional *lacZ* fusions and promoter probing, Tc^r	Blumer *et al.* (1999)
pME6000rhlI	pME6000 with *Bam*HI–*Hin*dIII insert of 2.0 kb, including gene *rhlI* and partial flanking sequence, Tc^r	This study
pME6000rhlR	pME6000 with *Eco*RI–*Pst*I insert of 1.6 kb, including gene *rhlR* and partial flanking sequence, Tc^r	This study
pME6032pltR	pME6032 with *Eco*RI insert of 1.4 kb, including *pltR*, Tc	This study
pMEAZ	733 bp *Bam*HI–*Pst*I PCR-amplified fragment, containing P*pltLA–pltL–pltA′* cloned into pME6015, Tc^r	Huang *et al.* (2004)
pMEAZ-12	679 bp *Eco*RI-*Pst*I PCR-amplified fragment, containing the nucleotides −470 upstream and +209 downstream transcriptional start site of *pltL* cloned into pME6522, Tc^r	This study
pMEAZ-13	500 bp *Eco*RI–*Pst*I PCR-amplified fragment, containing the nucleotides −470 upstream and +30 downstream transcriptional start site of *pltL* cloned into pME6522, Tc^r	This study
pMERZ	681 bp *Eco*RI–*Pst*I PCR-amplified fragment, containing P*pltR′* cloned into pME6015, Tc^r	This study

Table 1. cont.

Strain, plasmid or primer	Description	Reference or source
pMEZA	724 bp *Bam*HI–*Pst*I PCR-amplified fragment, containing P*phzA′* cloned into pME6015, Tc^r	Ge *et al.* (2004)
pUCGM	Source of Gm^r cassette, Gm^r	Schweizer (1993)
Oligonucleotides	**Sequence (5′–3′)***	
PRSMPLTA1	ACCGAGACCATCACCAGCATC	This study
PRSMPLTA2	CCTTCTTGAACGGACGCACC	This study
PRSMRHLI1	TGACTTTCTAGAGTGTTCGCCGTCCTGGAAAAG (*Xba*I)	This study
PRSMRHLI2	GTAATAAAGCTTGAAACGCGCTTCTTCGCTACAG (*Hin*dIII)	This study
PRSMRHLR1	GTCATCCTGCAGAGCCTTGCTGCCATCGT (*Eco*RI)	This study
PRSMRHLR2	AGCGATGAATTCGGTCGTCGGCAACAG (*Pst*I)	This study
PSRMRPOD1	GAGCGGGAGGAGCGTTTAC	This study
PSRMRPOD2	CGGGCAAAAAATAAGCAGAGG	This study
PSRMTN5	CAGATCTGATCAAGAGACAG	This study

*Specified restriction sites are underlined.

To construct the *rhlI* mutant, a 557 bp *Bgl*II–*Eco*91I fragment from pBLSRHLI, including the partial sequence of the *rhlI* gene with its promoter region, was replaced by an 850 bp *Sma*I fragment containing the *aacC1* gene from plasmid pUCGM, to create plasmid pBLSRHLIG (Table 1, Fig. 2). Next, a 2.2 kb *Bam*HI–*Hin*dIII fragment from pBLSRHLIG was cloned into pEX18Tc to generate the plasmid pEXTcRG (Table 1). After conjugation with strain M18 as the recipient, and *E. coli* SM10/pEXTcRG as the donor, we selected Tc-sensitive, Gm-resistant and sucrose-resistant transconjugants with an inactivated *rhlI* gene in the chromosome. One isolate (M18IG) was further confirmed by PCR for the additional 123 bp fragment in the *rhlI* gene using primers PRSMRHLI1and PRSMRHLI2. The *rhlI* gene in the Plt-negative strain M18T was inactivated by inserting the ΩKm Km-resistance cassette of pDSK519 into the *Pst*I site of the ORF of the *rhlI* gene (mutant M18TI). The plasmid pME6000rhlI was introduced into strain M18IG, forming the complemented *rhlI* mutant M18IG/pME6000rhlI.

To construct the *rhlR* mutant, the 1.7 kb ΩKm cassette was inserted into the *rhlR* gene at the *Sac*I site in pEXTcRHLR, resulting in pEXTcRK (Table 1). Analogous to the *rhlI* mutant construction described above, we constructed the *rhlR* mutant M18RK (Fig. 2), and the M18T derivative M18TR. Successful inactivation of *rhlR* was further verified by PCR. Plasmid pME6000rhlR was introduced into the strain M18RK, forming the complemented *rhlR* mutant M18RK/pME6000rhlR.

RNA extraction and reverse transcription. RNA was extracted from M18, M18IG, M18RK, and the complemented mutants M18IG and M18RK. Under normal growth conditions, each culture was harvested in early exponential phase, at an OD_{600} of 2.0–2.4, or in late exponential phase, at an OD_{600} of 5.0–6.0. RNA extraction and reverse transcription were performed using the EZ spin column total RNA isolation kit (Sangon), and the First Strand cDNA synthesis kit (MBI Fermentas), according to manufacturers' recommendations.

Real-time PCR. The amounts of cDNA obtained by reverse transcription were quantified utilizing the MiniOpticon Real-Time PCR System (Bio-Rad), with a SYBR green I stain. We measured the transcription of the *pltA* gene, which is the first gene of the Plt biosynthetic gene cluster in *Pseudomonas* sp. M18 (Huang *et al.*, 2004; Nowak-Thompson *et al.*, 1999). Transcription of the endogenous housekeeping gene *rpoD* (Savli *et al.*, 2003) was used as a reference. The primers used for amplifying the *pltA* gene (PRSMPLTA1 and PRSMPLTA2) and the *rpoD* gene (PSRMRPOD1 and PSRMRPOD2) were designed from their sequences in *Pseudomonas* sp. M18 (Table 1). The PCR fragments of the *rpoD* gene and *pltA* gene were 173 and 157 bp, respectively. Each 25 μl of reaction mixture contained: 2 μl of the cDNA sample diluted 1 : 5; 1 × PCR buffer; 1 × SYBR green I; 200 μM dATP, dGTP, dCTP and dTTP; 1 μM of each primer in water; and 2.5 U *Taq* DNA polymerase. Negative controls consisting of distilled water, or total RNA instead of the cDNA, were included in each test to check for DNA contamination. The target cDNA (*pltA*) and reference cDNA (*rpoD*) were amplified in separate wells. PCRs were run in an MJ Mini Personal Thermal Cycler (Bio-Rad), with the following program: one step of 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The evolution of fluorescence intensity of each reaction mixture was recorded continuously using a MiniOpticon detector (Bio-Rad). The PCR products amplified from cDNA were further confirmed by sequencing. PCR analyses for each strain in different growth phases were repeated three times.

For analysis of the data, the comparative threshold cycle (C_T) method was adopted as a mathematical model, as described by Livak & Schmittgen (2001), to calculate the differences of *pltA* gene expression among different strains grown in KMB broth. The amount of *pltA* cDNA, normalized to levels of the reference *rpoD*, and calibrated relative to the M18 strain, was expressed as a $2^{-\Delta\Delta C_T}$ value, which represents the fold change of *pltA* gene expression in a given strain relative to strain M18. The $\Delta\Delta C_T$ value was the ΔC_T value of a certain strain (M18, M18IG, M18RK, or the complemented M18IG or M18RK) subtracted from the ΔC_T value of strain M18, where ΔC_T is the C_T value of cDNA (*pltA* gene) subtracted from the ΔC_T value of the cDNA (*rpoD* gene) in each strain. Statistical significance was computed by an unpaired Student's *t* test; $P<0.05$ was considered statistically significant.

Assays for BHL, *N*-hexanoylhomoserine lactone, Plt and β-galactosidase. For extraction of AHL, a 1 ml culture of M18 strain or one of its derivatives (M18-T510, M18IG or M18IG/pME6000rhlI) was harvested at an OD_{600} of 5.0–6.0. After centrifugation (8000 ***g***), the supernatant was extracted three times with 1 ml ethyl acetate. The ethyl acetate was evaporated with a rotary evaporator, and dried extracts containing AHL were resuspended in 20 μl ethyl acetate. BHL and *N*-hexanoylhomoserine lactone (HHL) were analysed employing a TLC bioassay, as described by McClean *et al.* (1997) and Shaw *et al.* (1997). Four samples, and standard

Fig. 1. Pigments produced by the strains M18 (left), M18-T510 (middle) and the complemented mutant M18IG/pME6000rhlI (right) grown on KMB agar.

AHLs (Fluka), were spotted (2 µl) onto TLC silica Gel RP-C18 plates (Merck) for migration, and *Chromobacterium violaceum* CV026 was used as the reporter strain (McClean *et al.*, 1997; Shaw *et al.*, 1997). The quantities of BHL and HHL in each sample were estimated in comparison with standards, i.e. 0.25, 0.5, 1 and 2 nmol BHL, and 0.01, 0.1, 0.2 and 0.5 nmol HHL.

The extraction and quantification of Plt from the culture suspension were performed using the methods described by Huang *et al.* (2004). β-Galactosidase assays were done according to the method of Miller (Sambrook *et al.*, 1989).

RESULTS

Characterization of the chromosome region flanking the transposon in M18-T510

Pseudomonas sp. M18 produces an uncharacterized red pigment when streaked on KMB plates (Fig. 1). In previous experiments, we obtained a mini-Tn*5 lacZ-tet/1* mutant (M18-T510) that could not produce observable levels of this red pigment (Fig. 1). The fragment containing the inserted Km cassette of the mini-Tn*5 lacZ-tet/1*, along with downstream chromosomal DNA, was cloned and sequenced. For strain M18-T510, sequence analysis of plasmid pSK-T510 revealed that the partial sequence downstream of the transposon shared 98 % identity with the *rhlI* gene (accession no. PA3476) of *P. aeruginosa* PAO1. In strain PAO1, *rhlI* encodes an enzyme related to the synthesis of BHL and HHL (Jiang *et al.*, 1998). Considering such a remarkable level of identity, we gave the name *rhlI* to the gene from M18-T510. A pair of conservative primers was designed according to the nucleotide sequence flanking the *rhlI* gene in *P. aeruginosa* PAO1 (www.pseudomonas.com). A 2.0 kb PCR fragment from the chromosomal DNA of the wild-type M18 strain was purified, sequenced and cloned (see Methods). The full sequence of the 2.0 kb DNA fragment shared 99 % identity with a region of *P. aeruginosa* PAO1. The M18 *rhlI* gene was flanked upstream by sequence similar to *rhlR*, and downstream by *pheC*; this arrangement is identical to that in *P. aeruginosa* PAO1 (Ochsner & Reiser, 1995). The transposon was located at position 46 in the *rhl1* ORF (Fig. 2). The deduced product (201 aa) of the M18 strain *rhlI* gene shared 98 % similarity with the RhlI protein of *P. aeruginosa* PAO1. The intact *rhlR* gene was also sequenced and cloned (see Methods), and the predicted protein sequence of RhlR showed 100 % identity to the transcriptional regulator RhlR (PA3477) of *P. aeruginosa* PAO1.

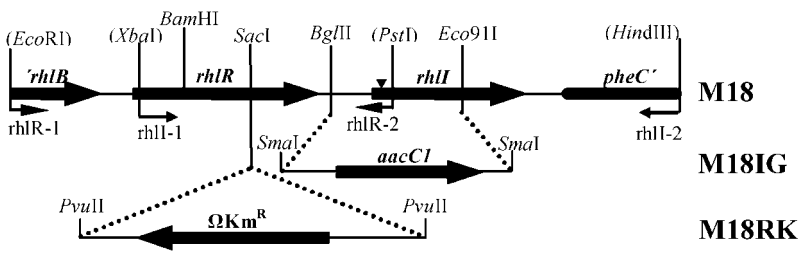

Fig. 2. Physical location of the *rhlR* and *rhlI* genes in the chromosome of *Pseudomonas* sp. M18. Artificial restriction sites on primers (rhlR-1 and rhlR-2) used for cloning *rhlR* and primers (rhlI-1 and rhlI-2) for *rhlI* are shown in parentheses. The *Bgl*II–*Eco*91I fragment containing the partial sequence of the *rhlI* gene of strain M18 was replaced by a *Sma*I fragment containing the *aacC1* gene to construct the *rhlI* mutant M18IG. A 1.7 kb ΩKm cassette was inserted into the *rhlR* gene at the *Sac*I site, resulting in the *rhlR* mutant M18RK. The filled inverted triangle indicates the insertion site of mini-Tn*5 lacZ-tet/1* in strain M18-T510.

Furthermore, the 16S rRNA gene sequence of strain M18 (AY696302) shares 99 % identity with *P. aeruginosa* PAO1 (Hu *et al.*, 2005). However, some distinct characters, such as *plt* genes, red pigment production (Fig. 1), and notable high levels of PCA production (Hu *et al.*, 2005), were markedly different from strain PAO1. Together, these data indicate that *Pseudomonas* sp. M18 is an unusual pseudomonad that is closely related to *P. aeruginosa* PAO1, but not identical to it (Hu *et al.*, 2005).

Effects of the *rhlI* null mutation on the synthesis of AHLs and red pigment

The effect of the *rhlI* mutation on AHL production in *Pseudomonas* sp. M18 was measured. A null mutant with an inactivated chromosomal *rhlI* gene was constructed by inserting a single Gm-resistance cassette by homologous recombination. The partial nucleotide sequence and promoter region of the *rhlI* gene from the wild-type strain M18 was deleted, and replaced with a fragment containing the *aacC1* gene, which is responsible for Gm resistance, to generate an *rhlI* mutant designated M18IG (Fig. 2). TLC assays indicated that strain M18IG, as compared with wild-type M18, could not produce detectable amounts of BHL or HHL in KMB broth when grown to an OD_{600} of 5.0–6.0; these results are similar to those for strain M18-T510 (Fig. 3). The inactivated *rhlI* in M18IG resulted in the disappearance of red pigment (data not shown), which is also similar to the *rhlI* mutant M18-T510 (Fig. 1). In contrast, the complemented mutant M18IG/pME6000rhlI restored the ability to produce the two AHLs (Fig. 3) and the red pigment (Fig. 1).

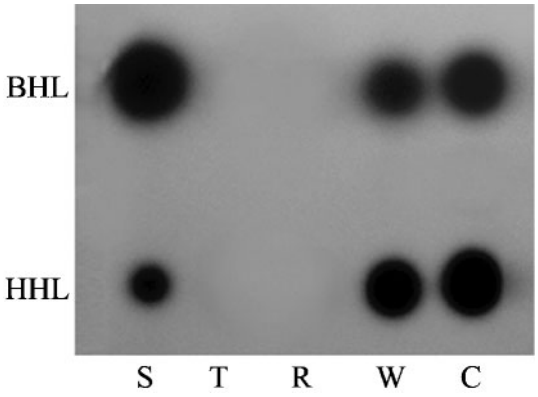

Fig. 3. TLC analysis of AHLs secreted by the wild-type *Pseudomonas* sp. M18 (W), mutant M18-T510 (T), mutant M18IG (R), and its complemented mutant M18IG/pME6000rhlI (C). The AHLs in each sample were concentrated to 50-fold more than the actual concentration in each culture after the cells had grown to an OD_{600} of 5.0–6.0. The AHL biosensor *Chromobacterium violaceum* CV026 was used to detect BHL and HHL. Lane S contains BHL (2 nmol) and HHL (0.02 nmol) standards.

Effect of the *rhl* QS system on Plt production

To investigate the regulatory function of the putative *rhl* QS system (RhlR and RhlI) on Plt production in *Pseudomonas* sp. M18, the chromosomal *rhlR* gene in strain M18 was inactivated by inserting a ΩKm cassette (Fig. 2) into the *Sac*I site of the *rhlR* gene, resulting in a mutant that was named M18RK. Inactivation of either *rhlI* or *rhlR* resulted in a dramatic increase of Plt production in strains M18IG and M18RK compared with the wild-type strain. The quantities of Plt produced by strains M18IG and M18RK were nearly identical, and were both greater than strain M18 over the same time course of growth (Fig. 4a). The expression of the Plt biosynthetic genes in the M18, M18IG and M18RK strains had to be determined in parallel. To this end, the plasmid pMEAZ harbouring a *pltA′–′lacZ* translational fusion was introduced into each of these three strains, and β-galactosidase activity was assayed in each of them. The expression of the *pltA′–′lacZ* fusion increased fivefold after 24 h growth in KMB broth for both M18IG and M18RK compared with strain M18 (Fig. 4b).

Our results were further confirmed by an *in trans* complementation experiment in KMB broth. The level of Plt produced by strain M18IG containing pME6000rhlI plasmid, and strain M18RK containing pME6000rhlR, was restored to that of the wild-type strain M18 (Fig. 4a). These complementation experiments demonstrated that the increased Plt production was indeed due to inactivation of either the *rhlI* or the *rhlR* gene in these mutant strains, and not to any other genetic event.

Effect of the *rhl* QS system on *pltA* expression occurs at the transcriptional level

Genetic evidence has revealed that RhlR regulates certain QS-controlled genes at the transcriptional level (Fuqua *et al.*, 2001). This evidence suggests that the *rhl* QS system may also regulate expression of Plt biosynthetic genes at the transcriptional level.

To confirm this hypothesis, the mRNA levels of *pltA* in M18, M18IG and M18RK were assessed by real-time PCR in two different growth phases. The housekeeping gene *rpoD* was assayed in parallel to normalize transcript levels of *pltA*. As PltA is a key component of Plt biosynthesis (Huang *et al.*, 2004; Nowak-Thompson *et al.*, 1999), the mRNA level of *pltA* can be taken as representative of the transcriptional expression of the *plt* gene cluster. The relative mRNA expression levels of the *pltA* were analysed by the $2^{-\Delta\Delta C_T}$ method, as described in Methods. According to Table 2, the level of *pltA* transcription increased in strains M18RK and M18IG, as compared with strain M18 and the complemented mutant, in both the early exponential phase ($P<0.05$) and the late exponential phase ($P<0.05$). In the early exponential phase, the normalized *pltA* levels in strains M18IG and M18RK were 1.61 and 1.92, respectively, relative to strain M18. Even greater amounts were observed when cells reached an OD_{600} of 5.0–6.0 in late exponential

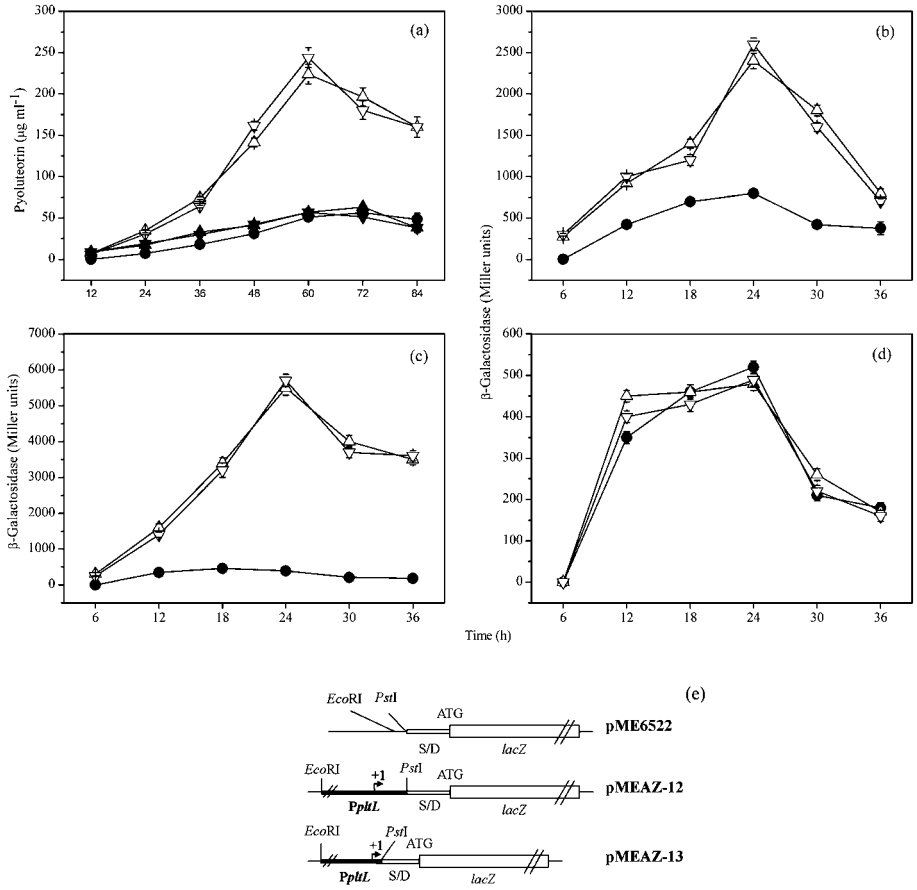

Fig. 4. Effect of *rhlR* and *rhlI* genes on Plt production and expression levels of the Plt biosynthetic gene cluster. (a) Plt was quantified in strains M18 (●), M18IG (△), M18RK (▽), the complemented M18IG (▲) and the complemented M18RK (▼), in KMB broth. β–Galactosidase expression (Miller units) of the *pltA′–′lacZ* translational fusion expression plasmid pMEAZ (b), the *plt′–lacZ* transcriptional fusion expression plasmid pMEAZ-12 (c), and the *plt–lacZ* transcriptional fusion expression plasmid pMEAZ-13 (d), was followed over time in cultures of wild-type M18 (●), M18IG (△) and M18RK (▽). Values are the means (±SD) for triplicate cultures. (e) Maps of the *plt′–lacZ* transcriptional fusion expression plasmid pMEAZ-12, and the *plt–lacZ* transcriptional fusion expression plasmid pMEAZ-13. S/D, putative Shine–Dalgarno sequence. +1, putative transcriptional start site of the *pltL*. The putative promoter region of *pltL* and its flanking sequence are shown as a thick black line.

Table 2. Analysis of relative *pltA* transcriptional levels in early exponential phase (OD_{600} between 2.0 and 2.4) and late exponential phase (OD_{600} between 5.0 and 6.0) for strains of M18, M18IG, complemented M18IG, M18RK, and complemented M18RK, using real-time quantitative PCR

Strain	Early exponential phase				Late exponential phase			
	$C_{T,rpoD}$*	$C_{T,pltA}$*	ΔC_T†	$2^{-\Delta\Delta C_T}$‡	$C_{T,rpoD}$*	$C_{T,pltA}$*	ΔC_T†	$2^{-\Delta\Delta C_T}$‡
M18	21.10±0.09	22.40±0.22	1.30±0.11	1.00 (0.93–1.08)	18.96±0.13	19.73±0.12	0.77±0.09	1.00 (0.94–1.06)
M18IG	20.94±0.17	21.55±0.10	0.61±0.08	1.61 (1.53–1.71)	18.42±0.14	16.79±0.07	−1.63±0.06	5.03 (4.82–5.24)
Complemented M18IG	21.44±0.21	22.64±0.15	1.20±0.12	1.07 (0.99–1.16)	18.72±0.06	19.27±0.19	0.55±0.10	1.16 (1.09–1.25)
M18RK	20.62±0.12	20.98±0.09	0.36±0.11	1.92 (1.78–2.07)	19.14±0.32	17.87±0.23	−1.27±0.13	4.41 (4.03–4.82)
Complemented M18RK	21.25±0.24	22.67±0.05	1.42±0.08	0.92 (0.87–0.97)	18.43±018	19.06±0.09	0.63±0.11	1.10 (1.02–1.19)

*Values are means (±SD) for three independent experiments.

†ΔC_T = mean $C_{T,pltA}$ − mean $C_{T,rpoD}$.

‡$2^{-\Delta\Delta C_T}$, Normalized amount of cDNA from the *pltA* gene in different strains relative to that in wild-type M18; $\Delta\Delta C_T$ = mean ΔC_T − mean $\Delta C_{T,M18}$.

phase, and the normalized *pltA* levels in M18IG and M18RK reached 5.03 and 4.41, respectively.

Using NNPP (Promoter Prediction by Neural Network; Reese, 2001), we predicted the putative promoter and transcriptional start site to be 254 bp upstream of the translational start site (ATG) of *pltL* (data not shown). Thus, the plasmid pMEAZ-12, harbouring the transcriptional *plt′–lacZ* fusion, which contains the nucleotides −470 upstream and +209 downstream of the transcriptional start site (Fig. 4e), was introduced into M18, M18RK and M18IG. We then assayed β-galactosidase activity in each strain. The transcriptional expression of the *plt′–lacZ* fusion in strains M18RK and M18IG was significantly enhanced, and was 10-fold higher than strain M18 in KMB broth during some growth phases (Fig. 4c). These data, together with the results from real-time PCR, show that the expression of the Plt biosynthetic genes is negatively controlled by the *rhl* QS system at the transcriptional level. Another plasmid, pMEAZ-13, carrying a different transcriptional *plt′–lacZ* fusion, containing 470 bases upstream, but only 30 bases downstream of the transcriptional start site (Fig. 4e), was constructed, and introduced into the M18, M18RK and M18IG strains. It is interesting that the transcription of the *plt′–lacZ* fusion in these three strains was similar (Fig. 4d).

Differential contributions of BHL and HHL to the regulation of Plt production

RhlI in strain M18 was able to synthesize BHL and HHL at a ratio of approximately 10 : 1 in KMB broth; the concentrations of BHL and HHL, estimated by comparison with standards, reached maximum concentrations of 10 and 1 μM, respectively, during late exponential phase (OD_{600} between 5.0 and 6.0).

An AHL-supplementation experiment was carried out to investigate the respective contributions of BHL and HHL to Plt production in *Pseudomonas* sp. M18 in KMB broth. BHL and HHL were added to separate M18IG cultures at an OD_{600} of 2.4 before synthesis of AHL (BHL and HHL) is normally initiated in strain M18 (data not shown). The final concentrations of BHL and HHL in the M18IG cultures were 15 and 1.5 μM, respectively; both values were 1.5-fold higher than the estimated maximum values produced in M18 cultures, as described above. At the same time, another M18IG culture was treated with an equal volume of ethyl acetate vehicle to dissolve the AHLs, as a negative control. When cells entered late exponential phase (OD_{600} between 5.0 and 6.0), Plt production in the three different M18IG cultures was measured, and the results are presented in Fig. 5. In the M18IG culture supplemented with BHL, the concentration of Plt declined to 57 μg ml^{-1} compared with 230 μg ml^{-1} in the M18IG culture treated with ethyl acetate alone. Similarly, addition of HHL to the M18IG culture led to decreased Plt production (119 μg ml^{-1}), but the extent of the reversion was much less than in the BHL-treated culture. Furthermore, increasing the concentration of each AHL to threefold more than in the wild-type caused a similar trend of differential decreases in Plt production (52 μg ml^{-1} for BHL, and 108 μg ml^{-1} for HHL).

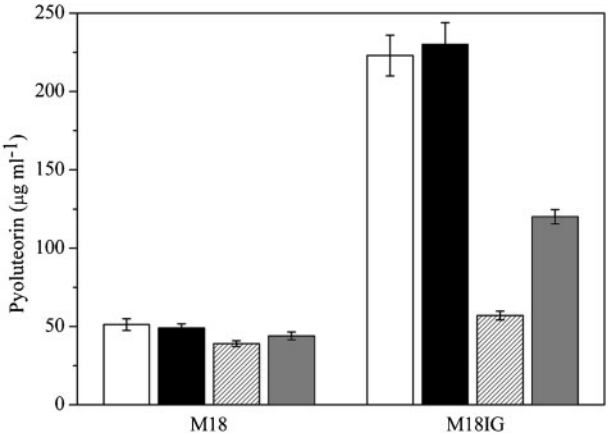

Fig. 5. Effect of BHL and HHL on Plt production. Plt production was assayed in strain M18 and the *rhlI* mutant M18IG in KMB broth, and KMB supplemented with ethyl acetate (black bars), BHL (hatched bars) and HHL (grey bars) after the culture had entered late exponential phase (OD_{600} between 5.0 and 6.0). The final concentrations of BHL and HHL added into the M18IG culture were 15 and 1.5 μM, respectively. White bars, blank.

The activator PltR mediates repression of the Plt biosynthetic gene cluster expression by the *rhl* QS system

A putative transcriptional activator, PltR, has been recently identified upstream of the Plt biosynthetic genes (Huang *et al.*, 2004), and the deduced peptide sequence (343 aa) exhibited 63 % identity over 313 aa to the homologous PltR protein in *P. fluorescens* Pf-5 (Nowak-Thompson *et al.*, 1999).

The *pltR* mutant M18TRG was constructed (data not shown), and the levels of Plt in the culture of the wild-type M18 and mutant M18TRG were quantified at an OD_{600} of 5.0–6.0 (late exponential phase). The inactive *pltR* mutant (M18TRG) showed significantly decreased Plt production (Fig. 6b). The empty plasmid pME6032, and the *pltR* overexpression plasmid pME6032pltR (Fig. 6a), were introduced into the mutant M18TRG. Following induction with IPTG, the quantity of Plt in culture of mutant M18TGR harbouring pME6032 plasmid was 9.0 μg ml^{-1}, which was similar to that of the mutant alone (12 μg ml^{-1}, Fig. 6b). In contrast, Plt production by M18TGR harbouring the plasmid pME6032pltR increased appreciably. After IPTG-induction, the Plt expression of M18TGR, harbouring the pME6032pltR plasmid, increased to 389 μg ml^{-1}, which was 13-fold more than wild-type strain M18 (Fig. 6b). In addition, disruption of *pltR* in the mutant M18TRG resulted in a notable decline in the expression of the transcriptional

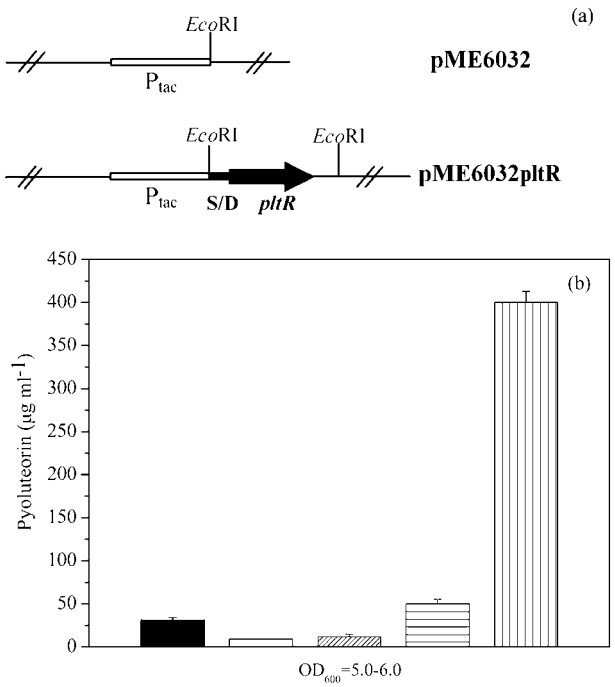

Fig. 6. (a) Construction of the *pltR* overexpression plasmid pME6032pltR. The P_{tac} promoter is shown by the white boxes, and the *pltR* gene with its putative Shine–Dalgarno sequence (S/D) is shown by the thick black arrow. (b) Plt production in strain M18 carrying plasmid pME6032, the *pltR* mutant M18TRG carrying plasmid pME6032, and M18TRG carrying the *pltR* overexpression plasmid pME6032pltR, after the cells had entered late exponential phase (OD_{600} between 5.0 and 6.0). IPTG was added to cultures as indicated, and the final concentration was adjusted to 1 mmol ml^{-1}. Black bar, M18; white bar, M18TRG; diagonal hatched bar, M18TRG/pME6032+IPTG; horizontally hatched bar, M18TRG/pME6032pltR; vertically hatched bar, M18TRG/pME6032pltR+IPTG.

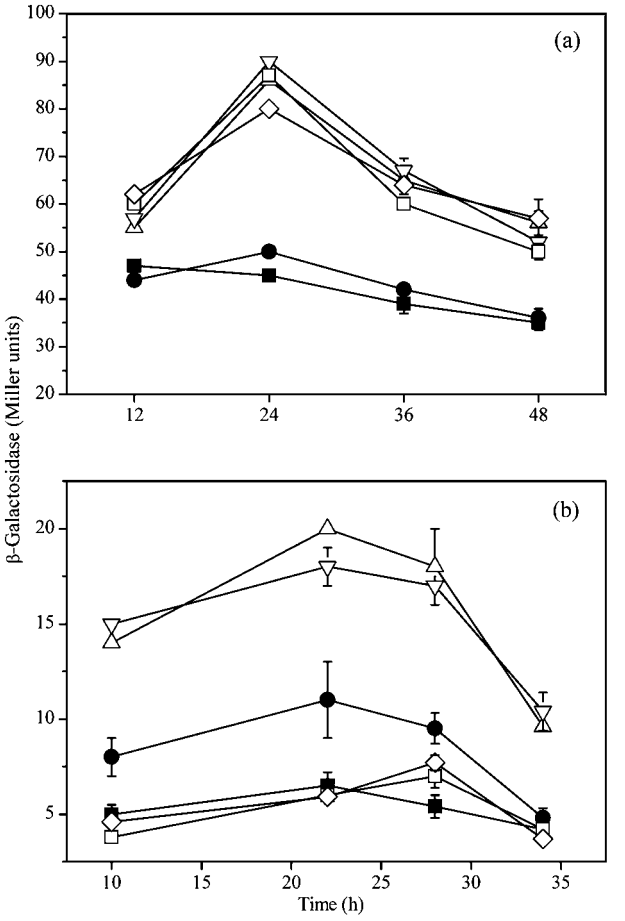

Fig. 7. Effects of the null mutation of the *rhl* QS system on the expression of *pltR* and the ABC transport gene cluster. Activity of *β*–galactosidase of a translational *pltR′–′lacZ* fusion expression plasmid pMERZ (a), and a translational *pltH′–′lacZ* fusion expression plasmid pHZLF (b), in strains M18 (●), M18T(■), M18IG (△), M18RK (▽), M18TI (□) and M18TR (◇) grown in KMB broth. Values are the means (±SD) for triplicate cultures.

plt′–lacZ fusion carried by plasmid pMEAZ-12, for which *β*-galactosidase activity was 172 Miller units, as compared with M18 (450 Miller units) after 18 h. This confirms that PltR acts as a transcriptional activator of the Plt biosynthetic gene cluster in *Pseudomonas* sp. M18, and it is consistent with results found in *P. fluorescens* Pf-5 (Nowak-Thompson *et al.*, 1999).

Such a regulatory mechanism, together with the described relationship between Plt production and the *rhl* QS system, is reminiscent of the hypothesis that PltR may mediate repression of the Plt biosynthetic gene cluster by the *rhl* QS system. To confirm this hypothesis, expression of a *pltR′–′lacZ* translational fusion (carried by pMERZ) in the M18, M18RK and M18IG strains grown in KMB broth was investigated. Mutation of either *rhlR* or *rhlI* resulted in an increase in *pltR′–′lacZ* fusion expression in the M18RK or M18IG strains, as compared with the wild-type (Fig. 7a). *β*-Galactosidase activity of the *pltR′–′lacZ* fusion expressed in either M18RK or M18IG reached a peak of about 90 Miller units after 24 h, while the peak activity in the wild-type was less than 50 Miller units. These data indicate that inactivation of the *rhl* QS system enhances expression of *pltR*.

Plt-specific ABC transporter expression by the *rhl* QS system requires Plt

To determine whether the *rhl* QS system regulates expression of the Plt-specific ABC transporter PltHIJKN, we followed expression of the *pltH′–′lacZ* translational fusion (carried by pHZLF) over time in strains M18, M18IG and M18RK. *β*-Galactosidase activity in strains M18IG and M18RK was higher than that in the wild-type during the growth process (Fig. 7b). The increase was consistent with the trend of increased Plt production in the mutant M18IG and M18RK strains, as described above. In addition, it has been reported previously that the Plt molecule itself induces

expression of the ABC transport gene cluster in *Pseudomonas* sp. M18 (Huang *et al.*, 2006). These results suggest that Plt might participate in this regulation. To examine this possibility, expression of a *pltH′–′lacZ* translational fusion was measured in the Plt-negative strain M18T, the *rhlI* Plt-negative mutant M18TI, and the *rhlR* Plt-negative mutant M18TR. β-Galactosidase activity was almost identical in each of these three mutants, and was approximately 50 % of that in the wild-type M18 (Fig. 7b). The promotional effect of the *rhlI* mutation on the *pltH′–′lacZ* reporter was clearly suppressed by the absence of Plt in the M18TI and M18TR strains.

Effect of the *rhl* QS system on cell division and death

Survival of the *rhlR* mutant M18RK was better than strain M18. The growth rates of strains M18 and M18RK were indistinguishable before the stationary phase of growth (Fig. 8a, b). However, after entering stationary phase, the cell density of strain M18 declined more quickly than that of strain M18RK after 48 h incubation, and the cell density of the former was only 50 % of the latter at 72 h during the late stationary phase (Fig. 8a). Unlike the trend of decreased cell density, the population size [mean log (c.f.u. ml^{-1})] of strain M18 declined, while it increased for strain M18RK, reaching 15.0 log (c.f.u. ml^{-1}) after 66 h incubation. This is much higher than the peak value of 13.0 log (c.f.u. ml^{-1}) attained by the wild-type M18 at 48 h (Fig. 8b). In the complementation experiment, the growth rate and population size for the complemented mutant (M18RK/pME6000rhlR) were similar to those of the wild-type M18 strain (Fig. 8a, b). We also found similar results using strain M18IG (data not shown). This suggests that *rhl* QS is involved in long-term survival in *Pseudomonas* sp. M18.

DISCUSSION

Though well-known beneficial (to plants) root-colonizing bacteria, such as *P. fluorescens* CHA0 and Pf-5, produce Plt, to the best of our knowledge, an AHL-dependent QS system has not been identified in these bacteria. The red-pigment-producing *Pseudomonas* sp. M18 adopted in this study also produces Plt, and the structural gene cluster encoding Plt biosynthesis has been identified in previous research (Huang *et al.*, 2004). In this study, we identified an *rhl* QS system (*rhlI* and *rhlR*) in *Pseudomonas* sp. M18, and this provided an excellent model to study the regulatory functions of AHL-dependent QS on Plt production.

Our results demonstrate clearly that Plt production, and expression of the Plt biosynthetic genes, are negatively controlled by the *rhl* QS system (RhlR and RhlI) in *Pseudomonas* sp. M18. In *P. aeruginosa*, RhlR can act as either an activator or a repressor for different target genes (Medina *et al.*, 2003). In this study, we demonstrated that RhlR acts as a repressor of Plt production by showing that chromosomal inactivation of *rhlR* leads to increased expression of the Plt biosynthetic genes (Fig. 4b) and, subsequently, to increased Plt production (Fig. 4a). Similar results occurred in the *rhlI* mutant M18IG. Furthermore, an *rhlI* and *rhlR* double mutant was phenotypically similar to the M18IG and M18RK mutant strains, with respect to both Plt production and *pltA′–′lacZ* fusion expression (data not shown). These data suggest that *rhlI* and *rhlR* comprise an *rhl* QS system, and that they function together in the negative regulation of Plt production in *Pseudomonas* sp. M18.

The *rhlI* gene was shown to be essential for the production of BHL and HHL in *Pseudomonas* sp. M18, based on TLC analysis results (Fig. 3). The AHL-supplementation experiment confirmed that both BHL and HHL can take part in the negative regulation of Plt production; however, it was obvious that BHL was more effective than HHL in this

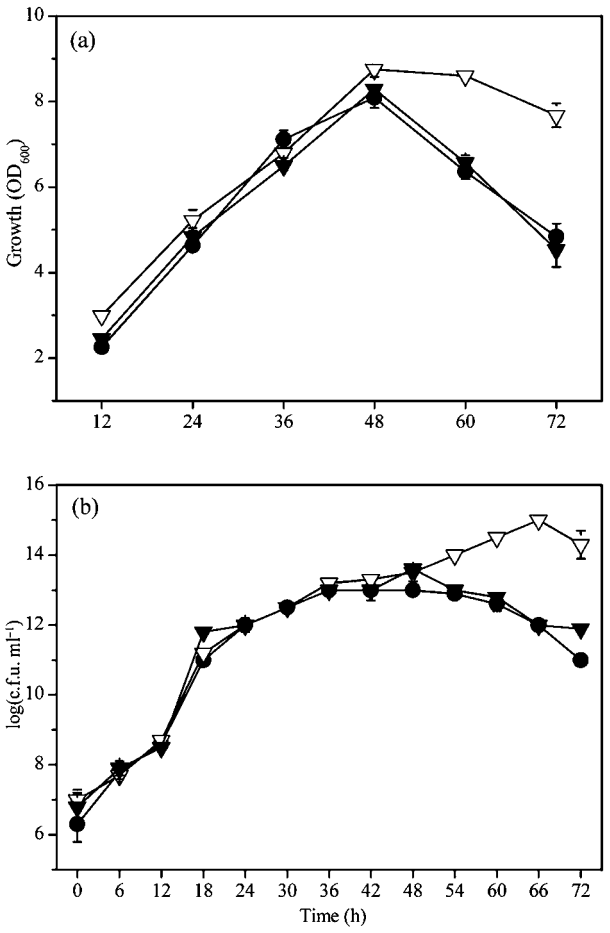

Fig. 8. Growth curves of M18, mutant M18RK, and its complemented mutant M18RK/pME6000rhlR, in KMB broth. (a) Cell densities of M18 (●), mutant M18RK (▽), and its complemented mutant M18RK/pME6000rhlR (▼), were estimated by OD_{600} measurements. Each point represents the mean (± SD) of three independent measurements. (b) The population densities [log (c.f.u. ml^{-1})] of these three strains in three parallel cultures were estimated by plating three selected dilutions on KMB plate.

regulation (Fig. 5). Perhaps the explanation for this might be that RhlR interacts specifically with the BHL molecule. It seems possible that the RhlR(HHL) complex may be a redundant component in strain M18 because there is no compelling mechanism to explain its presence when another more effective RhlR(BHL) system exists. Whether the RhlR(HHL) complex controls additional genes is unknown. Furthermore, enhancement of exogenous AHL in the M18IG culture, from 1.5- to 3.0–fold more than that produced by the wild-type, failed to result in a proportional decrease in Plt production. This disproportionate effect might be explained by the degradation of exogenous AHL during the process of culturing (Chen *et al.*, 2005), and/or the hypothesis that the relatively limiting amounts of RhlR protein were already saturated by excessive AHLs.

Our findings provide evidence that an *rhl* QS system negatively controls the expression of the Plt biosynthetic genes at the transcriptional level. This conclusion was drawn from two different sets of experiments. First, the transcriptional levels of Plt biosynthetic genes in strains M18IG and M18RK were enhanced as compared with the wild-type M18 strain or the complemented mutants (Table 2). Second, the expression of a transcriptional *plt′–lacZ* fusion in strains M18IG and M18RK increased as compared with the wild-type strain (Fig. 4c). More intriguingly, the *β*-galactosidase activity displayed a marked difference between the different transcriptional fusions (the *plt′–lacZ* carried by pMEAZ-12, and the *plt–lacZ* fusion carried by pMEAZ-13) when they were expressed in the same *rhl*-disrupted strain, such as M18IG or M18RK. The difference between the *plt–lacZ* and *plt′–lacZ* fusions is that the latter has an additional 176 bp sequence (Fig. 4e), indicating that the additional 176 bp region may be essential for regulation of the *rhl* QS system on Plt production, though further investigation will be required to detail the function of this region.

A pathway-specific transcriptional activator, PltR, was also identified upstream of the Plt biosynthesis genes. An inactive mutant of this protein significantly decreased both Plt production and *pltA′–′lacZ* fusion expression, while overexpression of *pltR* led to increased Plt production. Furthermore, the expression of *pltR* in the M18RK and M18IG strains was increased over the wild-type strain grown in KMB broth (Fig. 7a). We encountered preliminary evidence suggesting that the *rhl* QS system may function as a repressor of *pltR*, through which the *rhl* QS system negatively regulates Plt production indirectly.

In previously published work, we identified a putative ABC (ATP-binding cassette) transport gene cluster (*pltHIJKN*) required for Plt production, and this was characterized within a 7.5 kb genomic region downstream of the antibiotic Plt biosynthetic gene cluster in *Pseudomonas* sp. M18 strain. Overexpression of *pltHIJKN* led to increased Plt production (Huang *et al.*, 2006), suggesting that the ABC transport system may be involved in the regulatory function of the *rhl* QS system on Plt production. Using a translational *pltH′–′lacZ* fusion, we obtained preliminary evidence that mutation of either *rhlI* or *rhlR* enhanced *pltH′–′lacZ* fusion expression (Fig. 7b). However, this enhancement disappeared when another *pltB* mutation was introduced. *β*-Galactosidase activity of the *pltH′–′lacZ* fusion was similar in the Plt-negative strains M18T, M18TI and M18TR (Fig. 7b), and was approximately 50 % of that of the wild-type strain M18. Consistent with studies reporting that Plt could act as a signalling molecule (Brodhagen *et al.*, 2004) to induce expression of ABC transport gene cluster (Brodhagen *et al.*, 2005; Huang *et al.*, 2006), we also found that the *rhl* QS system could directly regulate this Plt-specific ABC transporter alone, but did so in a Plt-dependent manner. However, this must be distinct from the regulation of the QS system on *pltR* expression, because our evidence showed that expression of *pltR′–′lacZ* fusion in Plt null mutant strains M18TI and M18TK was maintained at a high level (Fig. 7a).

Mutants defective in the *rhl* QS system survived longer than wild-type cells, suggesting retard cell death (Fig. 8a, b), which might confer a selective advantage on *Pseudomonas* sp. M18 in KMB broth. The mechanism behind this phenomenon may be related to a similar result that was recently discovered in a *lasR* mutant of *P. aeruginosa* (Heurlier *et al.*, 2005). Whether the *las* system exists in strain M18 will need to be further investigated. The fact that the defective *rhl* QS system led to enhanced cell viability, along with increased Plt production, may provide some hints for understanding the ecological roles of QS (Manefield & Turner, 2002). However, the effect of a defective *rhl* QS system on root colonization and bio-control capacity should be investigated further.

In summary, we have presented preliminary results, and posited several suggestions, that may explain how the *rhl* QS system functions on several significant cell activities, including antibiotic biosynthesis, cell growth and pigment production. Future studies will determine the detailed mechanism of this RhlR–AHL complex on the regulation of these processes, and broaden the search for the putative regulators and pathways at work in strain M18.

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