Phenotypic and physiological alterations by heterologous acylhomoserine lactone synthase expression in *Pseudomonas putida*

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Many bacteria harbour an incomplete quorum-sensing (QS) system, whereby they possess LuxR homologues without the QS acylhomoserine lactone (AHL) synthase, which is encoded by a luxI homologue. An artificial AHL-producing plasmid was constructed using a cviI gene encoding the C6-AHL [N-hexanoyl homoserine lactone (HHL)] synthase from *Chromobacterium violaceum*, and was introduced successfully into both the wild-type and a ppoR (luxR homologue) mutant of *Pseudomonas putida*. Our data provide evidence to suggest that the PpoR–HHL complex, but neither PpoR nor HHL alone, could attenuate growth, antibiotic resistance and biofilm formation ability. In contrast, swimming motility, siderophore production and indole degradation were enhanced by PpoR–HHL. The addition of exogenous indole increased biofilm formation and reduced swimming motility. Interestingly, indole proved ineffective in the presence of PpoR–HHL, thereby suggesting that the PpoR–HHL complex masks the effects of indole. Our data were supported by transcriptome analyses, which showed that the presence of the plasmid-encoded AHL synthase altered the expression of many genes on the chromosome in strain KT2440. Our results showed that heterologous luxI expression that occurs via horizontal gene transfer can regulate a broad range of specific target genes, resulting in alterations of the phenotype and physiology of host cells.

INTRODUCTION

The bacterial quorum-sensing (QS) system is a mechanism for coordinating gene expression in response to cell population density, and then altering the expression of those genes (Fuqua & Greenberg, 2002). The QS system appears to be involved in inter- and intra-species communication (Waters & Bassler, 2005). The altered genetic network and bacterial behaviour changes regulated by the QS system have been well characterized in *Vibrio* spp., *Pseudomonas* spp., *Escherichia coli* and several other bacteria (Ahmer, 2004; Venturi, 2006; Waters & Bassler, 2005). The QS systems have been shown to control a broad range of physiological and morphological phenotypes, including bioluminescence, virulence, biofilm formation, Ti plasmid conjugation, spore formation, competence and symbiosis, all of which confer a competitive advantage (Dunny & Winans, 1999). The QS system functions via a signalling molecule called an autoinducer, which in many proteobacteria is an N-acylhomoserine lactone (AHL). When bacteria release AHL into a confined environment where it can accumulate, the AHL can return to the interior of the cell via diffusion, and its subsequent combination with its cognate transcription factor can alter the expression of many genes.

A complete AHL-dependent QS system harbouring both transcriptional regulator (LuxR homologue) and AHL synthase (LuxI homologue) is frequently detected in many bacterial chromosomes (Fuqua & Greenberg, 2002). However, recent microbial genome data have shown that incomplete QS systems exist within many bacteria that possess the LuxR-family protein alone, and do not harbour a cognate luxI homologue. Interestingly, these luxR and luxI homologues have only been detected in proteobacteria, and only
26% of proteobacteria genomes have evidence of a complete QS system (Case et al., 2008). This unpaired luxR is occasionally referred to as an ‘orphan’ or ‘solo’ LuxR (Fuqua, 2006; Subramoni & Venturi, 2009a, b). Although the functions of many unpaired LuxR homologues have been poorly characterized thus far, they are generally assumed to be involved in sensing and responding to signals from neighbouring AHL-producing bacteria. Pseudomonas putida KT2440 has been fairly well characterized, owing to its metabolic versatility, degradative potential and mutualistic plant--microbial interactions (Ramos-Gonzalez et al., 2005). Although genome sequencing of strain KT2440 has shown that it does not possess the lux-like gene, its genome harbours several LuxR-family proteins (Nelson et al., 2002). Among the proteins, LuxR (PP6467) is highly conserved with respect to other LuxR homologues and appears to bind to exogenous 3-oxo-C6-homoserine lactone (HSL) (Subramoni & Venturi, 2009b).

The results of several whole-genome analyses have demonstrated that bacterial evolution has frequently proceeded via horizontal gene transfer (HTG) between species and genera, as well as kingdoms (e.g. plant--bacteria gene transfer) (Davison, 1999; Pontiroli et al., 2007). To survive and persist in a given set of conditions, bacterial cells can acquire new genes via HTG, which may result in the acquisition of novel metabolic capabilities and even promote bacterial speciation (Davison, 1999; Lawrence, 1997). Three mechanisms of gene transfer in bacteria have been identified thus far: transformation, conjugation and transduction. Among these, conjugal gene transfer is most likely to occur in environments with high bacterial density, such as the rhizosphere (Mølbak et al., 2003). A notably high frequency of HTG has been reported for strain KT2440 (Mølbak et al., 2003). This led us to investigate whether strain KT2440-like bacteria might be capable of producing AHL in the environment, which could affect the gene expression of many chromosomal genes. If this were the case, strain KT2440 might alter its phenotypic and physiological states. Our results suggest, for the first time to our knowledge, that a non--AHL-producing bacterium can be changed into an AHL-producing bacterium via the acquisition of the lux homologue via conjugation which results in pleiotropic phenotypes such as biofilm formation, antibiotic resistance, motility and siderophore production can be influenced by QS signalling.

**METHODS**

**Bacterial strains, culture media, and growth conditions.** All strains, plasmids and primers utilized in this study are listed in Supplementary Table S1. P. putida, Chromobacterium violaceum and Agrobacterium tumefaciens C58 (pZLR4) were cultivated in Luria–Bertani (LB) medium at 28 °C with vigorous agitation at 220 r.p.m. E. coli strains were cultivated in LB medium at 37 °C. P. putida strains were also grown in M9 minimal medium supplemented with 10 mM glucose and 10 mM succinate at 28 °C. When required, antibiotics were added at the following concentrations: 100 μg kanamycin ml⁻¹; 20 μg tetracycline ml⁻¹; 200 μg rifampicin ml⁻¹; and 200 μg gentamicin ml⁻¹.

**Construction of HHL-producing P. putida strains.** C. violaceum is known to generate N-hexanoyl homoserine lactone (HHL) as a QS signal. The transcriptional regulator CviR (LuxR homologue) and signal synthase CviL (LuxI homologue) are responsible for this system (McClean et al., 1997). The HHL-producing wild-type and ppoR mutant were constructed using the pHHL vector, which constitutively expresses the cvi gene of C. violaceum under the control of the lac promoter on pRK415 (Supplementary Table S1). The full-length cvi gene was amplified using the CviL-OE1 and CviL-OE2 primer pair. The 1054 bp amplified fragments were cloned into the HindIII/EcoRI cloning site of a broad-host-range vector, pRK415 (Yin et al., 2003), yielding pHHL. Either the constructed vector or empty vector (pRK415) was then conjugated from E. coli Top10 to the wild-type and ppoR mutant by triparental filter mating with E. coli HB101 (pRK2013) (Figurski & Helinski, 1979).

**Construction of the ppoR promoter and gfp transcriptional fusion in P. putida.** We fused the promoter of the ppoR gene to a promoterless gfp gene, thus creating a reporter strain. A broad-host-range promoter probe vector, pRK415gfp (Yin et al., 2003), was employed in the construction of the reporter plasmid, pRK415P_:: gfp. The 337 bp of the ppoR promoter region was amplified using the PpoR-pro1 and PpoR-pro2 primer pair. The amplicon was cloned into the EcoRI/KpnI cloning sites of the pHHL vector, thereby generating pRK415P_:: gfp.

**HHL extraction.** HHL was extracted as follows (Kang Park, 2010). The bacterial cells were removed from 100 ml M9 medium amended with 10 mM glucose and 10 mM succinate via 30 min centrifugation at 3500 r.p.m. The cell-free supernatants (CFS) were prepared by filtering a culture of each tested strain through a 0.22 μm pore-size filter (Sartorius). The filtered supernatants were then extracted twice with equal volumes of 100 % HPLC-grade ethyl acetate, and evaporated to dryness in a rotary evaporator at room temperature. The dried extracts were resuspended in 1 ml 100 % ethyl acetate, and thus the final volume of the extract was one-hundredth that of the original culture medium.

**Detection and characterization of HHL.** Individual strains were confirmed for HHL production by three different assays, as described by Elasri et al. (2001) and Shaw et al. (1997). First, each of the strains was screened on solid M9 medium containing 20 μg X-Gal ml⁻¹ in a T-streak plate assay using the QS indicator A. tumefaciens C58 (pZLR4) (Cha et al., 1998). Second, 0.5 μl of the concentrated extract was applied to Cia reverse-phase TLC plates (TLC silica gel 60 RP-18, 5 × 7.5 cm, Merck), and developed with a methanol--water solvent mixture (MeOH:water 3:2,v/v). After air-drying, the developed TLC plates were overlaid with autoinducer bioassay (AB) media (Chilton et al., 1974) containing the indicator. Third, the existence of HHL in the extracts was assessed by Miller’s β-galactosidase activity test using the same indicator. The indicator, after overnight cultivation, was inoculated in a 1 : 100 dilution in fresh M9 minimal medium containing 1.0 % glucose and 0.3 % Casamino acids. When grown exponentially (OD₆₅₀ ~ 0.3), the bacterial cells were treated with the extracts (2 μl) and incubated for 24 h until stationary phase was achieved. The presence of AHL in the extracts was quantified via β-galactosidase activity measurements using ONPG as substrate.

**Abiotic biofilm formation and motility assay.** The biofilm formation assay was conducted as previously described (Lee et al., 2009b). Poly styrene 96-well microplates were used as abiotic surfaces for biofilm formation. LB-grown bacterial cells were washed twice in PBS and inoculated at 10⁷ c.f.u. in M9 medium. After 3 days of incubation at 28 °C, biofilm formation was determined via crystal
violet (CV) staining. For the swimming assay, 2 μl of bacterial cells grown overnight were inoculated onto peptone glucose (PG)-swimming agar [0.5% proteose peptone no. 3 (Difco 212 693), 0.2% glucose and 0.25% Difco Bacto-Agar] for 24 h (Matilla et al., 2007).

**Antibiotic sensitivity assay.** Bacterial cells grown overnight were diluted 100-fold in 5 ml fresh M9 medium, to OD<sub>600</sub> ~0.7. Aliquots of each strain at early stationary phase were serially diluted and spotted onto M9 agar plates with ampicillin.

**Siderophore production.** Absorbance spectra were obtained and photography at mid-wavelength UV light (312 nm) of the CFS (Molina et al., 2006) was conducted. Siderophore production was assessed via spectrophotometric measurements of absorbance spectra (Shimadzu UV-1800 spectrophotometer).

**Northern blotting analysis and microarray experiments.** Northern blot analysis was conducted as previously described (Lee et al., 2008). The quantity of ppoR mRNA was determined by hybridizing the membrane with a ppoR-specific, 32P-labelled probe, and subsequently prepared via PCR amplification with the primer pairs PpoR-P1/-Pp2.

When grown in M9 medium to late exponential phase (OD<sub>600</sub> ~0.9), total RNA was isolated with an RNasey Mini kit (Qiagen) in accordance with the manufacturer’s recommendations. The RNA concentration and quality were photometrically assessed. The cDNA probes for cdNA microarray analysis were prepared via the reverse-transcription of total RNA (50 μg) in the presence of aminoallyl-dUTP and 6 μg random primers (Invitrogen) for 3 h. The cDNA probes were then cleaned up using a Microcon YM-30 column (Millipore) followed by coupling to Cy3 dye (for reference) or Cy5 dye (for test sample) (Amersham Pharmacia). The Cy3- or Cy5-labelled cDNA probes were purified using a QIAquick PCR Purification kit (Qiagen). The dried Cy3- or Cy5-labelled cDNA probes were then resuspended in hybridization buffer containing 30% formamide, 5 × saline sodium citrate (SSC), 0.1% SDS and 0.1 mg salmon sperm DNA ml<sup>−1</sup>. The Cy3- or Cy5-labelled cDNA probes were then mixed together and hybridized onto microarray slides. After overnight incubation at 42 °C, the slides were washed twice in washing solution 1, containing 2 × SSC, 0.1% SDS for 5 min at 42 °C, once in washing solution 2, containing 0.1 × SSC, 0.1% SDS for 10 min at room temperature, and finally four times with 0.1 × SSC for 1 min at room temperature. The slides were dried via 5 min centrifugation at 650 r.p.m. The hybridization images on the slides were scanned with an Axon 4000B microarray scanner (Axon Instruments). Hybridization images were analysed using GenePix Pro 3.0 software (Axon Instruments) to determine the gene expression ratios (control versus test sample). Clustering images were acquired via hierarchical clustering (Eisen et al., 1998), which involved computing ‘distances’ between data elements. Genes that showed changes of twofold or greater (control versus sample with respect to hybridization signal ratio) in at least two replicates were selected.

**qRT-PCR analysis.** Microarray data were confirmed by performing quantitative real-time RT-PCR (qRT-PCR) using the iCycler IQ real-time PCR detection system (Bio-Rad). The cDNA was produced with the same RNA used for microarray analysis. For real-time RT-PCR, 1 μl template cDNA, 5 pmol primers (Supplementary Table S1), 0.5 × SYBR Green and 1 U Taq polymerase (Fermentas) were used. Fluorescence was measured at the end of each 72 °C incubation and analysed with iCycler IQ software (version 3.0). Melting curve analysis (60–95 °C, 0.5 °C increments) was performed to ensure PCR specificity. For quantification, the 165 rRNA gene was used to obtain reference expression data. Four independent experiments were performed and means and standard deviations were shown.

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**RESULTS**

**Comparison of the structure of P. putida PpoR with that of E. coli SdiA**

*P. putida* KT2440 possesses a LuxR homologue (PP4647, hereinafter referred to as PpoR) (Subramoni & Venturi, 2009b) that exhibits 30.4% amino acid sequence identity with SdiA of *E. coli*, the function and structure of which have been characterized previously (Lee et al., 2007b; Yao et al., 2006). This PpoR appears to be functional and has been demonstrated to bind to 3-oxo-C6-HSL (Subramoni & Venturi, 2009b). PpoR has high amino acid sequence identity with many of the uncharacterized LuxR homologues detected in a variety of *P. putida* strains, but its amino acid sequence identity with LuxR homologues of other species is quite low (SdiA of *E. coli*, 30.4%; SdiA of *Shigella flexneri*, 28.4%; SdiA of *Salmonella enterica*, 27.5%; LuxR of *Vibrio fischeri*, 22.0%; SpLR of *Serratia plymuthica*, 21.3%; CvIR of *Chromobacterium violaceum*, 21.0%; TraR of *A. tumefaciens*, 18.4%). *E. coli* SdiA is known to be an unpaired LuxR, and is able to bind and sense a variety of AHLs, including 3-oxo-C6-HSL (Subramoni & Venturi, 2009b). Computational homology modelling shows the predicted structure of PpoR to be quite similar to the crystal structure of *E. coli* SdiA (Supplementary Fig. S1). The results of our amino acid sequence comparison showed that the AHL-binding site and the positions of the secondary structure elements are highly conserved (Fig. 1 and Supplementary Fig. S1). As with other QS LuxR homologues, PpoR comprises two functional domains: an N-terminal domain binds to the AHL and a C-terminal helix–turn–helix motif is implicated in DNA binding.

**Construction of C6-HSL (HHL)-producing P. putida KT2440**

*P. putida* KT2440 may maintain the PpoR protein via an evolutionary process in order to respond to QS signals from neighbouring bacteria. Another interesting scenario that might occur in the environment involves the acquisition of a QS signal synthase through horizontal gene transfer, whereby the *luxI* homologue is located on a broad-host-range plasmid. In an effort to gain insight into the physiological responses of *P. putida* KT2440 under both conditions, we constructed the pHHL plasmid, harbouring the *C. violaceum* *cvl* gene that encodes HHL synthase. pHHL was then introduced into wild-type *P. putida* and the ppoR mutant. The empty vector, pRK415, was also introduced into both strains as a control. To confirm the constructed strains, three different assays (T-streaking, β-galactosidase activity and TLC bioassay) were conducted using the QS indicator strain *A. tumefaciens* C58 (pZLR4), which is capable of detecting AHLs possessing side chains.
ranging from C4 to C12. The QS signal generated by the positive control \textit{C. violaceum} and the HHL-producing \textit{P. putida} wild-type (pHHL) and \textit{P. putida} \textit{ppoR} mutant (pHHL) was capable of activating the indicator strain (Fig. 2a, b). In none of the assays was any noticeable induction observed in the parental strains with negative vector controls \[\text{wild-type (pRK415) and ppoR mutant (pRK415)}.\] The TLC bioassay showed that both the wild-type (pHHL) and ppoR mutant (pHHL) generated a signal with the same \(R_F\) value as the positive control, suggesting that the signal is HHL (Fig. 2c). Interestingly, the ppoR mutant (pHHL) produced lower amounts of QS signal than the wild-type (pHHL), thereby indicating the possibility of either a QS signal secretion problem or a partial binding of the QS signal in the ppoR mutant (pHHL).

Physiological and phenotypic alterations by the presence of the HHL signal: growth rate, siderophore production and antibiotic sensitivity

In order to determine whether growth was influenced by the signal or PpoR, we measured the growth rates of the \textit{P. putida} derivatives. Although the lag phase of the wild-type (pHHL) was slightly shorter, the growth rate of the wild-type (pHHL) was substantially lower than those of the other tested strains \[\text{wild-type (pRK415), 1.53 \pm 0.36 h}^{-1}; \text{wild-type (pHHL), 0.97 \pm 0.18 h}^{-1}; \text{ppoR mutant (pRK415), 1.57 \pm 0.42 h}^{-1}; \text{ppoR mutant (pHHL), 1.54 \pm 0.21 h}^{-1}\] (Fig. 3a). Our data indicated that PpoR is responsible for growth defects in response to QS signalling, but that the QS signal without PpoR could not be implicated in this loss of biological fitness. In order to further investigate other phenotypic alterations that might be caused by the QS signal, we evaluated the siderophore production and antibiotic sensitivity of each strain. When grown in M9 medium, we observed that the wild-type (pHHL) generated greater quantities of siderophore than the wild-type (pRK415), while the ppoR mutant lost the ability to generate fluorescence, which was observed under mid-wavelength UV light. However, fluorescence in the ppoR mutant was increased in the presence of the HHL signal (Fig. 3b), thereby suggesting the existence of two independent siderophore production pathways (AHL-dependent and AHL-independent). Fluorescent \textit{Pseudomonas} spp. evidenced three peaks, which could be detected at 210, 260 and 400 nm. The peak observed at 400 nm is believed to be a corresponding pyoverdine, and the other peaks might also be siderophores (Molina \textit{et al.}, 2006; Xiao & Kisaalita, 1998). When the absorbance spectra of the CFS of the strains were measured via spectrophotometry, the two peaks at 260 and 400 nm were not detected in the ppoR mutant, and those peaks were restored by the QS signal (Fig. 3b). Our data indicated that siderophore production might also be regulated by at least two independent pathways.
In *E. coli*, SdiA controls multidrug resistance by regulating the multidrug resistance (MDR) pump AcrAB. The deletion of the *sdiA* gene induces a hypersensitivity to drugs and reduces AcrB protein expression (Rahmati *et al.*, 2002). In order to confirm whether or not PpoR and the HHL signal affect antibiotic resistance, the antibiotic sensitivity of the *P. putida* derivatives was measured. Bacterial cells (10⁸ c.f.u. ml⁻¹) at early stationary phase (OD₆₀₀ ~0.7) were serially diluted and spotted onto M9 plates with ampicillin (Fig. 3c). Interestingly, the wild-type was ampicillin-hypersensitive only in the presence of the QS signal, but the *ppoR* mutant remained unaffected, regardless of the presence of the HHL signal. This result implied that the PpoR–HHL complex somehow confers antibiotic sensitivity, although the QS signal alone is not sufficient to give rise to antibiotic sensitivity. Additionally, GFP expression in the reporter strain (*P. putida* *ppoR*-*gfp*) was induced by ampicillin, thereby suggesting that PpoR could respond to ampicillin (Fig. 4a). The results of a recent study suggest that ampicillin can generate oxidative stress within cells (Kohanski *et al.*, 2007). However, this PpoR–HHL complex was not involved in the response to oxidative stresses caused by paraquat, hydrogen peroxide, cumene hydroperoxide (CHP) and salicylate (data not shown).

**Phenotypic alterations due to the presence of HHL signal and indole: biofilm formation, swimming motility and indole degradation**

Indole has been reported to repress *E. coli* biofilm formation and swimming motility via SdiA, and to increase biofilm formation of *P. aeruginosa* (Lee *et al.*, 2007a). However, the involvement of PpoR of *P. putida* in the response to the indole signal and in biofilm formation has remained unclear. Interestingly, our reporter strain (*P. putida* *ppoR*-*gfp*) and Northern analysis showed a response to indole (Fig. 4a, b). *P. putida* KT2440 harbouring the pHHL plasmid evidenced significantly reduced attachment to a polystyrene surface (Fig. 4c). The abilities of the *ppoR* mutants were quite similar in the presence and absence of the HHL signal, and were reduced slightly as compared with the wild-type (*pRK415*) and control strains (Fig. 4c). For the Congo red-binding assay, biofilm formation by PpoR with the signal was not attributable to the reduced production of extracellular matrix components (data not shown). The addition of exogenous indole stimulated the attachment of all strains except for the wild type with the pHHL plasmid (Fig. 4c). Taken together, our data indicated that PpoR reduced biofilm formation in the presence of the HHL signal. Our data also indicated the possibility of an indole-signalled PpoR-independent regulation of biofilm formation in *P. putida* KT2440, which could be masked by the effects of the PpoR–HHL (or PpoR–indole) complex. In opposition to biofilm formation, the HHL signal increased the swimming activity of the wild-type, but reduced that of the *ppoR* mutant (Fig. 4d). In the absence of the signal, the *ppoR* mutant exhibited significantly increased swimming motility, as compared with the wild-type strain. We were unable to observe any swarming mobility in these *Pseudomonas*...
derivatives (data not shown). Our interpretation is made more complicated by the fact that the HHL signal alone without PpoR could induce a reduction in swimming motility, but not to the same degree as in the wild-type strain (pRK415). There is no clear explanation for this observation, but it appears likely that PpoR inhibits swimming motility and that the HHL signal could relieve this repression. Interestingly, indole repressed the swimming motilities of all the *P. putida* strains (Fig. 4d), which is consistent with the case of the *E. coli* SdiA–indole signal (Lee et al., 2007a). These data indicate that the increased biofilm formation caused by indole addition was not attributable to increments in swimming motility.

When cultivated in M9 medium with indole, the other strains produced a pink pigment which was not found in the wild-type strain (pRK415) (data not shown). Indole is oxidized by a number of oxygenase enzymes, resulting in...
Fig. 4. Influence of indole on the expression of the *ppoR* gene, biofilm formation and swimming motility. (a) The response of the *ppoR* promoter to ampicillin and indole was monitored using a *ppoR*-**gfp** transcriptional fusion. The numbers below the figures show the actual fluorescence intensities. (b) Northern blot analysis of the *ppoR* gene in the presence of indole (1 mM). EtBr: images obtained after ethidium bromide staining as a loading control. (c) Effects of the HHL signal, PpoR and indole on biofilm formation. Biofilm formation ability was normalized to cell numbers measured using OD600. Light-grey bars, wild-type (pRK415); grey bars, wild-type (pHHL); dark-grey bars, *ppoR* mutant (pRK415); black bars, *ppoR* mutant (pHHL). (d) Swimming motility with and without indole addition in the *P. putida* derivatives.
the production of indigo (blue pigment) and the related indigoid pigment, indirubin (pink pigment) (McClay et al., 2005). In order to determine whether the pigment was the product of indole degradation, we measured indole degradation via HPLC analysis. For the assay, the P. putida derivatives were cultivated at 28 °C with vigorous aeration (180 r.p.m.) for 2 days in M9 medium amended with 2 mM indole. The indole residue in the supernatant was determined by the measurement of A276 using HPLC (Hirakawa et al., 2009). Consistent with the phenotype, our analysis demonstrated that the degradation of indole was stimulated by either the HHL signal or the deletion of the ppoR gene [mean residue of indole concentration (mM), wild-type (pKK415), 1.16; wild-type (pHHL), 0.10; ppoR mutant (pRK415), 0.07; ppoR mutant (pHHL), 0.65]. Our data indicated that indole degradation was promoted by either the PpoR–HHL complex or PpoR-independent pathways, but that the PpoR–HHL complex exerted a more dramatic effect on indole degradation.

Identification of HHL-signalling target genes regulated by PpoR

Microarray analysis was conducted with P. putida strains to identify genes that respond to the HHL signal (Supplementary Fig. S2, Supplementary Tables S2–S4). In order to avoid any effect of the growth state on gene expression, the total mRNAs of the bacterial cells at the early stationary phase (OD600 ~0.9) were isolated because the growths of all strains were quite similar at the entry into stationary phase (8 h in Fig. 3a). Our analysis demonstrated that a total of 71 genes (induction in red circles in Supplementary Fig. S3, 25 genes; repression in blue circles in Supplementary Fig. S3, 46 genes) in the wild-type (pHHL), 56 genes (38 up and 18 down) in the ppoR mutant (pKK415) and 36 genes (35 up and 12 down) in the ppoR mutant (pHHL) are expressed differentially by more than twofold (Supplementary Fig. S3, Supplementary Table S2), as compared with the wild-type (pRK415). Interestingly, many identified genes were exclusively expressed or downregulated under one condition, while other genes were not, which indicates that the effects of HHL addition, ppoR mutation and PpoR–HHL are quite specific (Supplementary Fig. S3). Among 157 genes that are expressed differentially by more than twofold (upregulated and downregulated), approximately 36.8% are of unknown function or are hypothetical proteins (HPs), thereby suggesting that their specific physiological function remained to be characterized (Supplementary Tables S2–S4). Because many regulatory proteins (RPs) are differentially expressed under each condition, we speculated that there might be more pleiotropic alterations in the physiology and phenotypes of strain KT2440.

To screen the genes regulated by the PpoR-dependent HHL signal, we attempted to identify only the genes that were differentially expressed by more than twofold in the wild-type strain (pHHL) (PpoR+, HHL+), but not under any other conditions (Supplementary Fig. S2a for upregulated genes, Supplementary Fig. S2b for downregulated genes, Supplementary Table S2). Our analysis revealed 15 upregulated genes (red-labelled genes in Supplementary Fig. S2a), and 30 genes were downregulated (red-labelled genes in Supplementary Fig. S2b) only by PpoR–HHL. Among the positively affected genes, PP4472 (csrcA, encoding a pleiotropic regulator of carbon source metabolism), PP5027 (dtD, encoding a D-tyrosyl-tRNA deacylase), PP4740 (hsdR, encoding a type I restriction–modification system), PP1786 (putative glycosyltransferase), PP0030 (sensor histidine kinase), PP0607 (fimT, encoding a type IV pili biogenesis protein), PP0089 (ompC, encoding an osmotically induced protein OsmC) and PP2915 (putative membrane protein) were identified with many other genes of unknown function (Supplementary Fig. S2a, Supplementary Table S2). These results indicated that PpoR–HHL signal-mediated regulation is probably involved in metabolism, as well as adaptation to environmental stimuli (Doronina & Murray, 2001; Gao & Stock, 2009; Lairson et al., 2008). In particular, in E. coli, CsrA activates motility by an increased induce in flagellar biogenesis via direct binding to the untranslated leader segment of the flhDC transcript and an increase in its half-life (Wei et al., 2001); this is consistent with our data (Fig. 4d). The fimT gene is known to be involved in type IV pili biogenesis, which is important for twitching motility (Alm & Mattick, 1996; Li et al., 2007). Downregulated genes are largely involved in the transporter system (PP4461, PP4789, PP1299, PP2592 and PP0960) or are hypothetical proteins (Supplementary Fig. S2b, Supplementary Table S2). In particular, the level of csgF mRNA was downregulated. The interaction of the CsgF protein with CsgG is required for the assembly of curli, which is relevant to both cell–cell aggregation and surface adhesion (Barnhart & Chapman, 2006). Lee et al. (2009a) have reported that SdiA, via QS signalling, represses the expression of genes associated with curli synthesis, including csgF, thereby resulting in a reduction in biofilm formation in E. coli. Consistent with that report, our data indicated that PpoR–HHL might induce a reduction in biofilm formation via the repression of the csgF gene. Additionally, PP1337, also referred to as murG, is involved directly in peptidoglycan synthesis and assembly, and is required for cell division and growth. The penicillin-binding proteins (PBPs) and FtsQ are required for the localization of MurG. Furthermore, PBPs are attractive targets for glycopeptides and β-lactam antibiotics (Chandrakala et al., 2004; Mohammadi et al., 2007). The ftsQAZ cell division genes are activated when SdiA is overexpressed in E. coli (Ahmer, 2004). It appears likely that the downregulated murG gene is linked to growth rate and amycolin sensitivity (Fig. 3a, c). In the plant pathogen Pectobacterium atrosepticum, vgrG, involved in the type VI secretion system (T6SS), is downregulated in an expI mutant defective in QS signal production, and is recovered by the exogenous QS signal (Liu et al., 2008). The vgrG-like gene identified from PpoR–HHL-dependent downregulated genes presumably contributes to bacterial expression in P. putida

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fitness and adaptation during root colonization in the rhizosphere. Unfortunately, the genes associated with swimming motility, pyoverdine production and indole degradation were not found in our microarray data, thereby suggesting that these phenotypic alterations result from other hypothetical genes. We confirmed the microarray data using qRT-PCR to analyse the 13 genes mentioned above (Supplementary Fig. S4). Our results indicate that gene expression analysis using qRT-PCR is consistent with the microarray data, except for the result for PP4740 (hsdR, encoding a type I restriction–modification system). Because the qRT-PCR experiments were repeated four times, we believe that the microarray data for the PP4740 gene represent an experimental error.

**DISCUSSION**

In this study, we constructed AHL-producing *P. putida* strains via the introduction of the HHL synthase gene (*cviI*) by conjugational gene transfer. Our data raise the possibility that a non-AHL-producing strain might be able to assemble a complete QS system via gene transfer in the natural environment. This gene transfer could take place via either cell-to-cell-dependent conjugation or transformation, taking up and incorporating naked foreign DNA from dead cells (Bertolla & Simonet, 1999; Mølbak et al., 2003; Pontiroli et al., 2007). The AHL synthase gene from dead AHL producers, either within the chromosome or on plasmids, becomes naked DNA, and can constitute good HGT material for non-AHL-producing bacteria. To date, these possibilities have not been adequately explored.

In the rhizosphere, biofilm formation is an important determinant for attachment to and interaction with plant tissues. Many factors, including the QS system, are important for successful biofilm formation (Danhorn & Fuqua, 2007). Interestingly, many plant-associated *P. putida* strains have been identified as non-AHL-producing bacteria (Case et al., 2008; Elasri et al., 2001; Subramoni & Venturi, 2009b).

PpoR appears to be a functional LuxR homologue and has a folding structure similar to that of SdiA of *E. coli*. The positions of secondary structure elements are very similar between the two proteins (Fig. 1 and Supplementary Fig. S1). The folding of SdiA is stabilized by a hydrophobic interaction between two α-helices (α2 and α5). These two helices could be found in PpoR (α2 and α6). The two proteins also have similar binding sites for the AHL ligand. It is known that hydrogen bonds form between SdiA residues Trp67 and Asp80 and the lactone ring of the AHL ligand (Yao et al., 2006). Both residues (Trp106 and Asp119) are conserved in PpoR (Fig. 1). Other conserved residues interacting with the AHL ligand are located at the same position in the two active sites (Tyr63, Tyr71 and Trp95 for SdiA, and Tyr102, Tyr110 and Trp134 for PpoR, highlighted in black in Fig. 1). Analysis of other LuxR homologues (LuxR, TraR, LasR and SdiA) shows that these five residues interacting with the ligand are strictly conserved (Soulère et al., 2007). Other residues involved in the hydrophobic interaction between AHL and the SdiA residues are either conserved or substituted (Fig. 1). Taken together, these analyses strongly suggest that PpoR is capable of interacting with HHL.

Binding of AHL to PpoR has previously been shown in *P. putida* KT2440 (Subramoni & Venturi, 2009b). 3-oxo-C6-HSL appears to be a cognate signal of PpoR, and to a lower extent other AHLs could bind to PpoR (Subramoni & Venturi, 2009b). We constructed an HHL (C6-HSL)-producing *P. putida* strain. Given the fact that LuxR could sense many different types of AHL with different specificity, it seems likely that HHL can bind to PpoR. This assumption may be supported by the fact that C6-HSL produced by our recombinant strains (both wild-type (pHHL) and the ppoR mutant (pHHL)) could bind to the TraR of the Agrobacterium indicator (Fig. 2), although TraR of *A. tumefaciens* preferentially binds 3-oxo-C8 HSL (Cha et al., 1998).

We evaluated the physiological and phenotypic alterations of *P. putida* KT2440 in the presence of the QS signal. With a complete PpoR/HHL pair, its growth rate and biofilm formation were lower, and its ampicillin sensitivity was increased. Interestingly, the results of our experiments suggested that *P. putida* PpoR could sense and respond to indole. The ppoR gene was upregulated in the presence of indole (Fig. 4). Biofilm formation was reduced by PpoR–HHL and was induced significantly by the addition of indole. However, the effect of indole was cancelled out by PpoR–HHL. The deletion of *ppoR* profoundly induced swimming motility. Our previous data suggested that motility is not directly or indirectly involved in biofilm formation in *P. putida* (Lee et al., 2009b), which is inconsistent with the results thus far reported in *E. coli* (Wood et al., 2006). We speculated that the HHL signal could repress PpoR activity, which might reverse swimming motility. Indole strongly repressed swimming activity, but could not affect it in the presence of both HHL and PpoR. Collectively, these data revealed that the mediation by PpoR of biofilm formation and motility might be modulated by HHL signalling, rather than by indole. PpoR also appears likely to repress indole degradation, which could be released in the presence of the HHL signal. It is worth investigating whether the pink pigment observed is indirubin because of its anti-cancer effects (McClay et al., 2005).

Understanding the manner in which heterologous AHL synthase expression affects the lifestyles of soil microorganisms may suggest new ways to manipulate bacterial growth in the environment. Collectively, the transition from a non-AHL producer to an AHL producer after acquiring the *luxI* gene from the environment might lead to a loss of biological fitness, affecting growth rate and antibiotic resistance, which might result in an increase in motility and siderophore production to avoid any competition with other bacteria. It appears worthwhile to...
determine how this consequence (loss of function versus gain of function) of the horizontal transfer of the luxI gene alters microbial populations and evolution in the environment.

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