Mutation analysis of the *Pseudomonas aeruginosa* *mvfR* and *pqsABCDE* gene promoters demonstrates complex quorum-sensing circuitry

Gaoping Xiao, Jianxin He and Laurence G. Rahme

Correspondence

Laurence G. Rahme
rahme@molbio.mgh.harvard.edu

Department of Surgery, Microbiology and Molecular Genetics, Harvard Medical School, Department of Surgery, Massachusetts General Hospital, and Shriners Burns Institute, Boston, MA 02114, USA

The LysR-type transcriptional regulator MvfR (PqsR) (multiple virulence factor regulator) plays a critical role in *Pseudomonas aeruginosa* pathogenicity via the transcriptional regulation of multiple quorum-sensing (QS)-regulated virulence factors. LasR activates full *mvfR* transcription, and MvfR subsequently activates *pqsA–E* expression. This study identifies and characterizes the key *cis*-regulatory elements through which *mvfR* and *pqsA–E* transcription is regulated in the highly virulent *P. aeruginosa* strain PA14. Deletion and site-directed mutagenesis indicate that: (1) LasR activates *mvfR* transcription by binding to a *las/rhl* box, CTAACAAAAGACATAG, centred at –513 bp upstream of the MvfR translational start site; and (2) RhlR represses *pqsA* transcription by binding to a *las/rhl* box, CTGTGAGATTGGGAG, centred at –311 bp upstream of the *pqsA* transcriptional initiation site. Furthermore, it is shown that MvfR activates *pqsA–E* transcription by binding to a *LysR* box, TTCGGACTCCGAA, centred at –45 bp relative to the *pqsA* transcriptional initiation site, demonstrating that this *LysR* box has a critical role in the physical interaction between the MvfR protein and the *pqsA* promoter. These results provide new insights into the regulatory relationships between LasR and *mvfR*, and between MvfR/RhlR and the *pqs* operon, and elucidate further the complex regulation of the *P. aeruginosa* QS circuitry.

Received 18 October 2005

Revised 31 January 2006

Accepted 7 February 2006

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes deleterious infections in patients with reduced immunity and with cystic fibrosis (Lyczak *et al.*, 2000). This Gram-negative bacterium produces and responds to diffusible intercellular signals and autoinducers that mediate pathogenicity via the coordinated expression of a large array of virulence genes (Rumbaugh *et al.*, 2000; Smith & Iglewski, 2003). This cell-density-dependent regulation is termed quorum sensing (QS) (Fuqua *et al.*, 2001), as autoinducer concentrations increase, up to a threshold, with increasing cell density. QS regulation is a potential target for limiting *P. aeruginosa* pathogenicity.

The best studied QS regulatory cascade components are the LasR and RhlR transcriptional regulators that are recognized and activated by their respective autoinducers (Pesci & Iglewski, 1999). The activated LasR and RhlR proteins specifically and/or cooperatively regulate multiple genes by binding to *las/rhl* boxes located in target promoter

regions. These elements have the consensus sequence CT-[N]₁₂-AG and occur in one or more copies in QS target gene promoters (McKnight *et al.*, 2000; Whiteley & Greenberg, 2001).

The transcriptional regulator MvfR plays a critical role in *P. aeruginosa* pathogenicity. *mvfR* mutants exhibit decreased virulence in different host models, including plants, insects and mammals (Cao *et al.*, 2001; Gallagher & Manoil, 2001; Lau *et al.*, 2003; Mahajan-Miklos *et al.*, 1999; Rahme *et al.*, 1997). MvfR is required to produce virulence factors, such as pyocyanin and hydrogen cyanide (Gallagher & Manoil, 2001; Rahme *et al.*, 1997), and a large family of 4-hydroxy-2-alkylquinolines (HAQs), including the intercellular signals 3,4-dihydroxy-2-heptylquinoline (PQS) and 4-hydroxy-2-heptylquinoline (HHQ) (Déziel *et al.*, 2004; Gallagher *et al.*, 2002; Lépine *et al.*, 2004), which function in the transcriptional regulation of multiple virulence genes (Déziel *et al.*, 2004; Gallagher *et al.*, 2002; McKnight *et al.*, 2000; Pesci *et al.*, 1999).

MvfR, LasR and RhlR are components of a complex regulatory network. While MvfR controls the *pqsABCDE* and *phnAB* operons, which encode proteins mediating HAQ biosynthesis (Déziel *et al.*, 2004; Gallagher *et al.*, 2002), the final step of PQS production, HHQ hydroxylation, appears

Abbreviations: HAQ, 4-hydroxy-2-alkylquinoline; HHQ, 4-hydroxy-2-heptylquinoline; LTTR, LysR-type transcriptional regulator; MvfR, multiple virulence factor regulator; PQS, 3,4-dihydroxy-2-heptylquinoline; QS, quorum sensing; WT, wild-type.

to require the LasR-regulated *pqsH* gene (Déziel *et al.*, 2004; Gallagher *et al.*, 2002). Furthermore, although MvfR regulates multiple *P. aeruginosa* QS-controlled genes, it is not involved in *lasRI/rhlRI* expression, or in homoserine lactone autoinducer signal production (Déziel *et al.*, 2005). Finally, while the *las* system activates *mvfR* and *pqsA–E* transcription, the *rhl* system appears to repress their expression (McGrath *et al.*, 2004; Schuster *et al.*, 2003). Recent studies indicate that the *mvfR* promoter contains a putative *las/rhl* box that is required for *mvfR* transcriptional activation (Wade *et al.*, 2005).

The MvfR protein has an N-terminal DNA-binding domain and a C-terminal ligand-binding domain, belonging to the LysR-type transcriptional regulator (LTTR) protein family. The prototypical LTTR, upon binding its corresponding small activator molecule, both represses its own gene expression, and activates the transcription of target genes whose regulatory regions carry a *LysR* box having the consensus palindromic sequence T-[N]₁₁-A (Schell, 1993). Recent studies show that PQS enhances the *in vitro* binding of MvfR to a *pqsA–E* promoter DNA fragment, suggesting that it could be the *in vivo* MvfR co-inducer (Wade *et al.*, 2005).

Here, we further interrogate and characterize the key *cis*-regulatory elements via which *mvfR* and *pqsA–E*

transcriptional regulation is mediated in the highly virulent *P. aeruginosa* strain PA14. Our results provide new insights into the molecular mechanism of MvfR regulation by characterizing the regulatory relationships between LasR and *mvfR*, and between MvfR/RhlR and the *pqs* operon.

METHODS

Bacterial strains, growth conditions and plasmids. Table 1 lists the bacterial strains and plasmids. *P. aeruginosa* and *Escherichia coli* were grown at 37 °C in Luria–Bertani (LB) broth and on LB agar. *E. coli* JM109 was used for subcloning and plasmid propagation. Ampicillin (100 µg ml^{−1}) for *E. coli*, and carbenicillin (300 µg ml^{−1}) and tetracycline (50 µg ml^{−1}) for *P. aeruginosa* were used for plasmid selection and maintenance.

Materials. Oligonucleotides were from MWG Biotech. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. Qiaquick PCR Purification kit, Qiaquick Gel Extraction kit, Qiaprep Spin Miniprep kit, RNeasy Mini kit and Rnase-free DNase I were all from Qiagen. The Expand High Fidelity PCR system was from Roche. Precast Tris/HCl PAGE gels were from Bio-Rad. [γ-³²P]ATP was from PerkinElmer.

DNA manipulations. pQF50 is the parent plasmid for all of the *lacZ* transcription fusion constructs (Farinha & Kropinski, 1990). *mvfR* and *pqsA* promoter fragments were PCR-amplified from

Table 1. Bacterial strains and plasmids

Strain or plasmid		Relevant characteristics	Source or reference
<i>P. aeruginosa</i>			
PA14	Clinical isolate UCBPP-PA14		Rahme <i>et al.</i> (1995)
PA14 <i>rhlR</i> [−]	<i>rhlR</i> ::Tc derivative of PA14		Déziel <i>et al.</i> (2005)
PA14 <i>lasR</i> [−]	<i>lasR</i> ::Gm derivative of PA14		Déziel <i>et al.</i> (2004)
<i>E. coli</i>			
JM109	F [−] (<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ <i>M15</i>) <i>endA1 recA1 hsdR17</i> (r _K [−] m _K ⁺) <i>supE44 thi-1 gyrA96 relA1</i> Δ(<i>lac-proAB</i>)		Lab collection
Plasmids			
pQF50	<i>lacZ</i> transcriptional fusion vector; Ap ^r		Farinha & Kropinski (1990)
pGX1	pQF50 carrying <i>mvfR</i> promoter from −746 to +160 relative to the <i>mvfR</i> translational initiation site; Ap ^r		Déziel <i>et al.</i> (2005)
pGX2	pQF50 carrying <i>mvfR</i> promoter from −447 to +160 relative to the <i>mvfR</i> translational initiation site; Ap ^r		This study
pGX3	pGX1 with a C-to-T mutation at CT-[N] ₁₂ -AG position 1; Ap ^r		This study
pGX4	pGX1 with a A-to-T mutation at CT-[N] ₁₂ -AG position 11; Ap ^r		This study
pGX5	pQF50 carrying <i>pqsA</i> promoter from −486 to +231 relative to the <i>pqsA</i> transcriptional initiation site; Ap ^r		This study
pGX6	pQF50 carrying <i>pqsA</i> promoter from −246 to +231 relative to the <i>pqsA</i> transcriptional initiation site; Ap ^r		This study
pGX7	pQF50 carrying <i>pqsA</i> promoter from −89 to +231 relative to the <i>pqsA</i> transcriptional initiation site; Ap ^r		This study
pGX11	pGX5 with a C-to-T mutation at CT-[N] ₁₂ -AG position 1; Ap ^r		This study
pGX12	pGX5 with the deletion of CT-[N] ₁₂ -AG; Ap ^r		This study
pGX9	pGX7 with a T-to-C mutation at T-[N] ₁₁ -A position 1; Ap ^r		This study
pGX10	pGX7 with the deletion of a half at T-[N] ₁₁ -A; Ap ^r		This study
pH44	Cosmid clone carrying PA14 genomic DNA from PA0963 to PA1004; Ap ^r		He <i>et al.</i> (2004)

cosmid clone pH44 (He *et al.*, 2004), digested with *KpnI* and *HindIII*, and subcloned into pQF50, to generate pGX2 from primers GX30 and GX31, pGX5 from primers GX32 and GX36, pGX6 from primers GX33 and GX36, and pGX7 from primers GX34 and GX36 (Table 2). Sequential, PCR-based, site-directed mutagenesis was used to introduce mutations into the *mvfR* and *pqsA* promoter fragments, which were then digested with *KpnI* and *HindIII*, and subcloned into pQF50, to generate pGX3 from primers GX29, GX52, GX53 and GX31. Briefly, pGX3 was generated using pGX1 as a template, and GX29 and GX52, and GX53 and GX31 were used, respectively, to amplify two overlapping PCR products, which were purified and mixed with the template to amplify the final mutated PCR product, using GX29 and GX31. The mutated nucleotide was then introduced using GX52 and GX53. The final PCR products were digested with *KpnI* and *HindIII*, and subcloned into pQF50. Following the same procedure, pGX4 was generated using GX29, GX54, GX55 and GX31; pGX9 using GX34, GX56, GX57 and GX36; pGX10 using GX34, GX58, GX59 and GX36; pGX11 using GX32, GX60, GX61 and GX36; and pGX12 using GX32, GX210, GX209 and GX36 (Table 2). Constructs were confirmed by DNA sequencing, and electroporated into *E. coli* and *P. aeruginosa* PA14 cells.

β -Galactosidase activity assay. *P. aeruginosa* PA14 cultures containing the pQF50-*lacZ* derivatives were grown overnight at 37 °C, diluted to OD₆₀₀ = 0.01, and grown at 37 °C, and β -galactosidase activity was measured (Miller, 1972) at different time intervals. Results, in Miller Units (MU), are expressed as the mean $\pm$ SD from three independent experiments.

DNA mobility gel-shift assay. Lysate from *E. coli* cells carrying pDN18-*mvfR*, which overexpresses MvfR, was mixed with 2 nM ³²P-labelled wild-type or mutated *pqsA* promoter fragments in a total volume of 15 μ l, incubated at room temperature for 20 min in the presence of 40 pM PQS, and analysed by electrophoresis on 5% nondenaturated polyacrylamide gels at 200 V at 4 °C.

Table 2. Oligonucleotides

Primer	Sequence
GX29	5'-GGATCGGTACCGACCGAACACGACATGCCG-3'
GX30	5'-GGATCGGTACCGACCGAACACGACATGCCG-3'
GX31	5'-CTAGGAAGCTTCCCGACGACAGCTCCAC-3'
GX32	5'-GGATCGGTACCGTAGGTGTCCTCTTCGGC-3'
GX33	5'-GGATCGGTACCAAGGGTTGTAACGGTTTTTG-3'
GX34	5'-GGATCGGTACCCGTGGTCTTCTCCCCG-3'
GX35	5'-GGATCGGTACCAGCGCCGCTAGTTTCCCG-3'
GX36	5'-CTAGGAAGCTTCGCCGGGCTTGAGCAGGC-3'
GX52	5'-CCGGTGGCTACGTTAACAAAAGACATAG-3'
GX53	5'-CTATGTCTTTTGTAAACGTAGCCACCGG-3'
GX54	5'-CTACGTAACAAAAGTCATAGGTTTCGG-3'
GX55	5'-CCGAAACCTATGACTTTTGTAGCGTAG-3'
GX56	5'-CCCGAACTTTTCGCTCGGACTCCGAA-3'
GX57	5'-TTCGGAGTCGCGAGCGAAAAAGTTTCGGG-3'
GX58	5'-CCCGAACTTTTCGCTCCGAAATATCGCGCG-3'
GX59	5'-GCGCGATATTTCGAGCGAAAAAGTTTCGGG-3'
GX60	5'-CGAGCGCCTCGAATTGTGAGATCTGGG-3'
GX61	5'-CCCAGATCTCACAATTCGAGGCGCTCG-3'
GX209	5'-CGAGCGCCTCGAAGCGATTTCGCGAG-3'
GX210	5'-CTCGGCAAATCGCTTCGAGGCGCTCG-3'

RESULTS AND DISCUSSION

A *las/rhl* box functions in *mvfR* transcriptional activation

Microarray and *lacZ*-fusion data suggest that LasR, and not RhlR, upregulates *mvfR* transcription (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wade *et al.*, 2005). A putative *las/rhl* box, centred at -513 bp from the MvfR translational start site (Wade *et al.*, 2005; Fig. 1A), shares 81.3% identity with the *las/rhl* box of the promoter region of the QS-regulated *rsaL* gene (Whiteley & Greenberg, 2001; Fig. 1B). The *las/rhl*-box sequence has nearly all the conserved sequence elements of the prototypical *las*-specific promoter (Schuster *et al.*, 2004). To determine if this putative element functions in *mvfR* regulation, *mvfR'*-*lacZ* transcriptional fusions were generated. The DNA fragment from the 746 bp upstream to the first 160 nucleotides of the *mvfR* gene was fused to a promoterless *lacZ* gene in pQF50, to generate pGX1 (Déziel *et al.*, 2005). As a control, we fused the *mvfR* -447 to +160 region, which lacks the putative *las/rhl* box, to *lacZ* (pGX2). These two constructs were separately introduced into strain PA14, with pGX1 also introduced into *lasR*⁻ mutant cells. Fig. 1(C) shows that the β -galactosidase activity from both fusions increased with increasing PA14 cell density. Furthermore, pGX1 gave significantly higher levels in strain PA14 versus pGX2, suggesting that its putative *las/rhl* box functions in *mvfR* regulation.

We next performed site-directed mutagenesis on pGX1 to alter highly conserved *las/rhl* nucleotides (Whiteley & Greenberg, 2001): (1) the position 1-C nucleotide was replaced with T, to give pGX3; and (2) the position 11-A nucleotide was replaced with T, to give pGX4 (Fig. 1A). Fig. 1(C) shows that both substitutions reduced LacZ activity, further demonstrating the relevance of the *las/rhl* box in *mvfR* activation. These results further elucidate the complex regulatory links between LasR and *mvfR*, and define the critical sequence elements of the prototypical *las*-specific promoter. Indeed, while the 1-C nucleotide was previously thought inconsequential for LasR recognition (Schuster *et al.*, 2004), Fig. 1(C) shows that pGX3, in which this C is replaced by T, has greatly reduced activity, indicating the 1-C nucleotide is important for LasR activation.

If LasR regulates *mvfR* expression only via elements between -746 and -448 bp upstream of the translational start site, deletion of this region should be equivalent to a *lasR* mutation, which completely abrogates *mvfR* expression (Wade *et al.*, 2005; Fig. 1C). In contrast, complete deletion of this region, or mutagenesis of two conserved *las*-box nucleotides, results in limited *mvfR* expression in strain PA14 (Fig. 1C, pGX2-4 constructs). These results indicate that an unknown LasR-dependent transcriptional regulator(s) activate(s) *mvfR* expression via a regulatory site(s) within the -447 to +160 bp region that lacks *las/rhl* box homology, or contains a LasR cryptic site. VqsR could be a candidate for this unknown regulator, as LasR

Fig. 1. A *las/rhl* box is critical for *mvfR* transcriptional activation. (A) Sequence and localization of the putative *las/rhl* box in the *nadA*–*mvfR* intergenic region. Bold nucleotides are the most highly conserved in known and putative *las/rhl* boxes. Only the substituted nucleotides are shown in the C1T and A11T *las/rhl* box mutants. – and + indicate position relative to the MvfR translational start site. Lines are not drawn to scale. (B) Comparison between the *las/rhl* boxes of *mvfR* and *rsaL* and a proposed *las*-specific recognition sequence (Schuster *et al.*, 2004). The highly conserved consensus sequence in CT-[N]₁₂-AG is shown in bold. (C) *MvfR*–*lacZ* expression in strain PA14 is altered by deletion of the –746 to –448 region, which contains the putative *las/rhl* box, and by the C1T and A11T *las/rhl* box substitutions. β -Galactosidase activity was measured at different cell densities for strain PA14 wild-type (WT)::pGX1 (*mvfR*'–*lacZ*, –746, ■), pGX2 (*mvfR*'–*lacZ*, –447, □), pGX3 (*mvfR*'–*lacZ*, –746, C1T, ○), pGX4 (*mvfR*'–*lacZ*, –746, A11T, ●), and PA14 *lasR*[–]::pGX1 (*mvfR*'–*lacZ*, –746, ▲).

transcription initiation site, and two putative *las/rhl* boxes centred respectively at –151 and –311. While the –311 *las/rhl* box, *pqsA*–1, has nearly all the conserved sequence elements of the prototypical *rhl*-responsive promoter (Schuster *et al.*, 2004), the –151 *las/rhl* box, *pqsA*–2, is less conserved (Fig. 2B). These elements suggest that, in addition to MvfR, LasR and/or RhlR may also regulate *pqsA*–*E* transcription. To this end, we generated and assayed *pqsA*'–*lacZ* deletion constructs, starting from pGX5 (Fig. 2A), which carries the *pqsA* promoter region from –486 to +231 relative to the *pqsA* transcriptional initiation start site, fused to *lacZ*. This 717 bp fragment includes the 5' 160 nt of the *pqsA* ORF and, presumably, the entire *pqsA*–*E* regulatory region. pGX6 and pGX7 carry, respectively, the fragments –246 to +231 and –89 to +231 fused to *lacZ*.

Fig. 2(C) shows that: (1) deletion of –486 to –247 (pGX6), which carries *pqsA*–1, results in increased *pqsA* transcription, suggesting that a transcription factor(s) binds to this region to repress *pqsA*; (2) although substitution of the C-1 nucleotide of the 5' *las/rhl* box in pGX5 with T (pGX11) does not alter *pqsA* activation, deletion of the entire pGX5 *las/rhl* box (pGX12) also increases *pqsA* transcription, indicating that the repression effect is mediated via the *las/rhl* box; and (3) additional deletion of –247 to –90 (pGX7) does not further alter *pqsA* expression, versus pGX6 and pGX12, suggesting that *pqsA*–2 is unnecessary for *pqsA* regulation.

RhlR represses *pqsA* expression (McGrath *et al.*, 2004), therefore, *pqsA* transcriptional repression can be mediated via RhlR binding to the *pqsA*–1 element. To this end, we compared the LacZ activities of pGX5 and pGX12 in strain PA14 versus PA14 *rhlR*[–] mutant cells (Fig. 2D). Fig. 2(D) shows that *pqsA* expression in *rhlR*[–] mutant cells is unaffected with or without the presence of the *pqsA*–1

controls its expression, and VqsR affects *mvfR* transcription (Juhas *et al.*, 2004; Schuster *et al.*, 2003). In addition, Fig. 1(C) (and data not shown) demonstrate that the pGX1–4 constructs only display activity in *lasR*[–] mutant cells during late growth, suggesting LasR-independent transcription factors also function in *mvfR* regulation.

A *las/rhl* and a *LysR* box are critical for *pqsABCDE* transcriptional regulation

MvfR binds to the promoter region of the *pqsA*–*E* operon (Wade *et al.*, 2005). Fig. 2(A) shows that the *pqsA* promoter region carries a putative *LysR* box, with a perfect dyad symmetry centred at –45 bp relative to the *pqsA*

Fig. 2. Analysis of *pqsA* regulatory region mutants shows that a *LysR* box and a *las/rhl* box are critical for *pqsA-E* transcriptional regulation. (A) Localization in the *ogt* and *pqsA* intergenic region of the putative *las/rhl* (–311 *pqsA*-1 and –151 *pqsA*-2) and *LysR* boxes (–45), and the putative *LysR* box sequence. The *pqsA* transcriptional start occurs 71 bp upstream of the translational start codon (McGrath *et al.*, 2004). Bold nucleotides are the most highly conserved in all known and putative *LysR* boxes. Only the substituted nucleotide is shown for the T1C *LysR* box mutant. The remaining sequence is for the half *LysR* box mutant deletion. The position –1 C of the 5' *pqsA*-1 *las/rhl* box was substituted with T to generate the C1T *las/rhl* box mutant. The whole *pqsA*-1 *las/rhl* box was deleted to create the *del* mutant. – and + indicate position relative to the *pqsA* transcriptional initiation site. Lines are not drawn to scale. (B) Comparison between the *las/rhl* boxes of *pqsA* and *rhlA* and a proposed *rhl*-responsive motif (Schuster *et al.*, 2004). The highly conserved consensus sequence in CT-[N]₁₂-AG is shown in bold. Only the substituted nucleotide is shown for the C1T *las/rhl* box mutant (pGX11). (C) Strain PA14 expression of wild-type, *LysR* box and *las/rhl* box mutants. The effect was determined by deleting the sequences –486 to –247, –246 to –90, the *pqsA*-1 *las/rhl* box and half of the *LysR* box, and substitutions of the *pqsA*-1 *las/rhl* box C1T, and the *LysR* box T1C, on *pqsA*-*lacZ* expression in PA14. β -Galactosidase activity was measured at different cell densities for strain PA14::pGX5 (*pqsA*'-*lacZ*, –486, ■), pGX6 (*pqsA*'-*lacZ*, –246, ○), pGX7 (*pqsA*'-*lacZ*, –89, ●), pGX9 (*pqsA*'-*lacZ*, –89, T1C, △), pGX10 (*pqsA*'-*lacZ*, –89, half, -x-), pGX11 (*pqsA*'-*lacZ*, –486, C1T, ▲), and pGX12 (*pqsA*'-*lacZ*, –486, del, □). (D) Expression of wild-type and a *las/rhl* box mutant in strain PA14 WT, *rhlR*[–] and *lasR*[–] mutants. The effect of deleting the *pqsA*-1 *las/rhl* box on *pqsA*-*lacZ* expression in PA14 WT, *rhlR*[–] and *lasR*[–] mutants. β -Galactosidase activity was measured at different cell densities for pGX5 (*pqsA*'-*lacZ*, –486) and pGX12 (*pqsA*'-*lacZ*, –486, del) in PA14 wild-type, *rhlR*[–] and *lasR*[–] mutants, respectively. ■, pGX5, WT; □, pGX12, WT; ▲, pGX5, *rhlR*[–]; △, pGX12, *rhlR*[–]; ●, pGX5, *lasR*[–]; ○, pGX12, *lasR*[–].

element. These results suggest that RhlR binds to this *las/rhl* box to repress *pqsA* transcription, and that the highly conserved C-1 nucleotide is unimportant for this DNA-protein interaction.

Fig. 2(A) presents two pGX7 derivatives used to assess the importance of the putative *LysR* box. pGX9 (T1C) was generated by replacing the highly conserved 1-T nucleotide (Schell, 1993) with a C, and pGX10 (half) was generated by deleting the 5' 6 nt of pGX7, to destroy its dyad symmetry. Fig. 2(C) shows that the T1C substitution severely reduced *pqsA* activation, and that this activation was completely abrogated by the *LysR* box half deletion. Since all the altered nucleotides are 5' of position –45, these mutations likely do not perturb RNA polymerase binding. These data further demonstrate that the putative *LysR* box centred at –45 is a critical *pqsA-E* regulatory element, likely via MvfR recognition and

binding (Wade *et al.*, 2005). Nonetheless, Fig. 2(D) also shows that *pqsA* expression in *lasR*[−] mutant cells (*lasR*/pGX5) increases later in growth, in agreement with the *mvfR* expression kinetics in *lasR*[−] mutant cells (Fig. 1C, *lasR*/pGX1). These data concur with previous results for late PQS and pyocyanin production in these cells (Diggle *et al.*, 2003), further suggesting that, in the absence of LasR, an unknown LasR-independent transcription factor(s) activate(s) *mvfR*, and, consequentially, *pqsA–E* during late growth.

MvfR binds to a *LysR* box to activate *pqsABCDE* transcription

PQS potentiates MvfR binding to a *pqsA–E* promoter DNA fragment (Wade *et al.*, 2005). To determine if the two putative *LysR* box mutations affect the DNA binding of MvfR, we separately mixed ³²P-labelled wild-type and mutated *pqsA* promoter fragments with *E. coli* cell lysate

expressing MvfR in the presence of PQS (Fig. 3A, B). Consistent with the Fig. 2(C) expression results, the T1C substitution was seen to reduce MvfR binding. Furthermore, binding to the half *LysR* box deletion was significantly reduced, resulting in a protein–DNA complex with higher mobility and weaker affinity. These results suggest that an MvfR monomer weakly binds the right-half *LysR* box, and that MvfR dimerization is important for the biologically productive protein–DNA interaction.

Conclusions

The data presented demonstrate that MvfR regulates *pqsA–E* transcription in a complex manner via LasR, LasR-dependent and LasR-independent transcription factors, and provide for the future identification of new factors involved in QS regulation. Dissection of the regulatory elements that mediate *mvfR* transcription further defines the essential sequences of the prototypical *las*-specific promoter

Fig. 3. The *LysR* box functions in MvfR–*pqsA* promoter DNA–protein interaction. (A) A ³²P-labelled 174 bp DNA fragment of the *pqsA* promoter region was mixed with *E. coli* cell lysate containing MvfR plus 40 pM PQS (lanes 2–6, 8–10 and 12–14). Protein added per reaction: lanes 1, 7 and 11, 0 ng μl^{−1}; lane 2, 0.6 ng μl^{−1}; lane 3, 3 ng μl^{−1}; lanes 4, 8 and 12, 6 ng μl^{−1}; lanes 5, 9 and 13, 30 ng μl^{−1}; and lanes 6, 10 and 14, 60 ng μl^{−1}. Mixtures were electrophoresed on 5% nondenaturated polyacrylamide gels. (B) Relative signal levels were determined by densitometry.

and uncovers the codependency of the *mvfR* and *las/rhl* systems in the QS circuitry. Our results indicate that the LasR protein binds to the *mvfR* promoter, *las/rhl* box, to activate *mvfR*; suggest the involvement of an unknown LasR-dependent transcription factor(s) that further stimulate(s) *mvfR* expression via interaction with an additional *mvfR* promoter element(s), located 3' of the *las/rhl* box; and that, in the absence of LasR, an unknown LasR-independent transcription factor(s) stimulate(s) expression of the MvfR pathway in late growth. Furthermore, we present strong evidence that the RhlR protein binds to the -311 *las/rhl* box in the *pqsA* promoter to repress *pqsA-E* transcription. Our genetic and biochemical results demonstrate that the MvfR protein binds to the *LysR* box in the *pqsA-E* regulatory region to activate *pqsA-E* transcription, and that *mvfR* pathway regulation is complex and *las*-codependent for both QS regulation and *P. aeruginosa* pathogenesis. To this end, molecules that specifically interfere with this regulation are potential inhibitors of bacterial virulence.

ACKNOWLEDGEMENTS

We thank Dr E. P. Greenberg (University of Iowa) for providing pQF50 and Dr S. E. Stachel (BioDiversity LLC) for comments and editing. This work was supported by the Claflin Distinguished Scholar Award, and Shriners Research grant no. 8850, to L. G. R.

REFERENCES

- Cao, H., Krishnan, G., Goumnerov, B., Tsongalis, J., Tompkins, R. & Rahme, L. G. (2001). A quorum sensing-associated virulence gene of *Pseudomonas aeruginosa* encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. *Proc Natl Acad Sci U S A* **98**, 14613–14618.
- Déziel, E., Lépine, F., Milot, S., He, J., Mindrinos, M. N., Tompkins, R. G. & Rahme, L. G. (2004). Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci U S A* **101**, 1339–1344.
- Déziel, E., Gopalan, S., Tampakaki, A. P., Lépine, F., Padfield, K. E., Saucier, M., Xiao, G. & Rahme, L. G. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of *N*-acyl-L-homoserine lactones. *Mol Microbiol* **55**, 998–1014.
- Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Camara, M. & Williams, P. (2003). The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* **50**, 29–43.
- Farinha, M. A. & Kropinski, A. M. (1990). Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J Bacteriol* **172**, 3496–3499.
- Fuqua, C., Parsek, M. R. & Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**, 439–468.
- Gallagher, L. A. & Manoil, C. (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* **183**, 6207–6214.
- Gallagher, L. A., McKnight, S. L., Kuznetsova, M. S., Pesci, E. C. & Manoil, C. (2002). Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 6472–6480.
- He, J., Baldini, R. L., Déziel, E. & 7 other authors (2004). The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci U S A* **101**, 2530–2535.
- Hentzer, M., Wu, H., Andersen, J. B. & 15 other authors (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J* **22**, 3803–3815.
- Juhas, M., Wiehlmann, L., Huber, B. & 8 other authors (2004). Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. *Microbiology* **150**, 831–841.
- Lau, G. W., Goumnerov, B. C., Walendziewicz, C. L., Hewitson, J., Xiao, W., Mahajan-Miklos, S., Tompkins, R. G., Perkins, L. A. & Rahme, L. G. (2003). The *Drosophila melanogaster* toll pathway participates in resistance to infection by the gram-negative human pathogen *Pseudomonas aeruginosa*. *Infect Immun* **71**, 4059–4066.
- Lépine, F., Milot, S., Déziel, E., He, J. & Rahme, L. G. (2004). Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas aeruginosa*. *J Am Soc Mass Spectrom* **15**, 862–869.
- Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* **2**, 1051–1060.
- Mahajan-Miklos, S., Tan, M.-W., Rahme, L. G. & Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa* – *Caenorhabditis elegans* pathogenesis model. *Cell* **96**, 47–56.
- McGrath, S., Wade, D. S. & Pesci, E. C. (2004). Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). *FEMS Microbiol Lett* **230**, 27–34.
- McKnight, S. L., Iglewski, B. H. & Pesci, E. C. (2000). The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 2702–2708.
- Miller, J. H. (1972). Assay of β -galactosidase. In *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Pesci, E. C. & Iglewski, B. (1999). Quorum sensing in *Pseudomonas aeruginosa*. In *Cell-Cell Signaling in Bacteria*, pp. 147–155. Edited by G. M. Dunny & S. C. Winans. Washington, DC: American Society for Microbiology.
- Pesci, E. C., Milbank, J. B. J., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229–11234.
- Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899–1902.
- Rahme, L. G., Tan, M.-W., Le, L., Wong, S. M., Tompkins, R. G., Calderwood, S. B. & Ausubel, F. M. (1997). Use of model plants hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proc Natl Acad Sci U S A* **94**, 13245–13250.
- Rumbaugh, K. P., Griswold, J. A. & Hamood, A. N. (2000). The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes Infect* **2**, 1721–1731.

Schell, M. A. (1993). Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* **47**, 597–626.

Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**, 2066–2079.

Schuster, M., Urbanowski, M. L. & Greenberg, E. P. (2004). Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A* **101**, 15833–15839.

Smith, R. S. & Iglewski, B. (2003). *Pseudomonas aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* **6**, 56–60.

Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P. & Pesci, E. C. (2005). Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 4372–4380.

Whiteley, M. & Greenberg, E. P. (2001). Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes. *J Bacteriol* **183**, 5529–5534.