Regulation of *Pseudomonas aeruginosa* *ptxR* by Vfr

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Pseudomonas aeruginosa PtxR enhances the expression of the exotoxin A gene *toxA*. The expression of *ptxR* itself, which occurs from two promoters (P1 and P2), is not completely understood. We have recently demonstrated that the *ptxR* upstream region contains potential binding sites for multiple regulators, including the virulence factor regulator Vfr. In this study, we identified within the *ptxR* upstream region, a 25 bp sequence to which Vfr specifically binds. The sequence is located 20–44 (32.5) bp 5' of the *ptxR* P2 promoter, and overlaps a potential binding site for the iron-starvation sigma factor PvdS. We also show that, throughout the growth cycle, deletion of *vfr* reduces *ptxR* expression from the P2 promoter in the *P. aeruginosa* strain PAO1 by four- to eightfold, but does not affect *ptxR* expression from P1. Further, loss of Vfr eliminates the PtxR-induced enhancement in the synthesis of exotoxin A and the metalloproteinase LasB. Our results suggest that Vfr modulates *toxA* and *lasB* expression in PAO1 through PtxR. A model defining the relationships between these different genes is presented.

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INTRODUCTION

Pseudomonas aeruginosa causes serious infections in immunocompromised hosts by producing several extracellular and cell-associated virulence factors (Pollack, 2000). The production of these factors is controlled by different regulatory proteins, among which is the global regulator Vfr (virulence factor regulator) (Lory *et al.*, 2004; van Delden, 2004). Vfr belongs to the family of cAMP receptor proteins (CRPs), and has 90 % similarity to the *Escherichia coli* CRP (West *et al.*, 1994b). Vfr was originally described as a *P. aeruginosa* factor that is required for the production of exotoxin A (ETA) and protease IV (West *et al.*, 1994b). Further studies have demonstrated that Vfr activates the transcription of several other virulence genes, such as genes encoding different components of the type III secretion system; as well as the quorum sensing (QS) genes *lasR* and *rhlR*, and *rpoS*, which encodes the stationary-phase sigma factor RpoS (Albus *et al.*, 1997; Beatson, 2002; Bertani *et al.*, 2003; Wolfgang *et al.*, 2003). In addition, Vfr negatively regulates the production of the *P. aeruginosa* QS molecule PQS (*Pseudomonas* quinolone signal), and it downregulates the production of the major flagellar

regulator FleQ (Dasgupta *et al.*, 2002). Kanack *et al.* (2006) recently showed that Vfr specifically binds to the upstream regions of its target genes.

ETA is an ADP-ribosylating extracellular protein that is produced by *P. aeruginosa* through a complicated process that involves several regulators (Hamood *et al.*, 2004). One of these regulators is PtxR, which enhances the transcription of the ETA gene *toxA* by four- to fivefold (Hamood *et al.*, 1996). The 35 kDa PtxR, encoded by *ptxR*, belongs to the LysR family of transcriptional activators (Hamood *et al.*, 1996). Available evidence suggests that PtxR regulates *toxA* expression through *regA* (Hamood *et al.*, 1996, 2004). In addition to regulating *toxA*, PtxR enhances the expression of the QS gene *lasI* and its target *lasB*; however, it reduces the expression of the QS gene *rhlI* and its targets *rhlAB* and the *pqsA–E* operon, which is involved in PQS production. It also reduces expression of the pyocyanin genes, which are the target of the *pqsA–E* operon (Carty *et al.*, 2006). Colmer-Hamood *et al.* (2006) showed that the *ptxR* upstream region contains potential binding sites for several regulators, including Vfr and the iron-starvation sigma factor PvdS. In this study, we examine the relationship of Vfr to *ptxR*. We show that Vfr enhances *ptxR* expression by specifically binding to the *ptxR* upstream region near the *ptxR* P2 promoter, and that loss of Vfr impacts the ability of PtxR to enhance synthesis of ETA and LasB. These findings suggest that Vfr fine tunes or modulates ETA and LasB production through PtxR.

Abbreviations: CRP, cAMP receptor protein; EMSA, electrophoretic mobility shift assay; ETA, exotoxin A; PQS, *Pseudomonas* quinolone signal; r-Vfr, recombinant Vfr; QS, quorum sensing; Vfr-BS, Vfr-binding sequence.

Table 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Relevant description	Reference/source
PAO1	Prototroph; Cm ^r	Holloway <i>et al.</i> (1979)
PAO9001	Isogenic <i>vfr</i> deletion mutant of PAO1; Gm ^r	Albus <i>et al.</i> (1997)
PA103	Prototroph; ETA hyperproducer; LasB deficient	Liu (1973)
Plasmid		
pWNP28	Expression plasmid for r-Vfr; the <i>vfr</i> gene is overexpressed from the <i>tac</i> promoter; <i>lac</i> ^q , Ap ^r	Kanack <i>et al.</i> (2006)
pSW205	<i>lacZ</i> translational fusion vector that replicates stably in <i>P. aeruginosa</i> ; Cb ^r	West <i>et al.</i> (1994a)
pBS8-4	<i>ptxS</i> :: <i>lacZ</i> translational fusion in pSW205; Cb ^r	Swanson <i>et al.</i> (1999)
pJAC24	<i>ptxR</i> :: <i>lacZ</i> translational fusion carrying the entire 567 bp <i>ptxR</i> – <i>ptxS</i> intergenic region (P1 and P2 promoters) in pSW205; Cb ^r	Colmer & Hamood (1998)
pJH2	<i>ptxR</i> P2:: <i>lacZ</i> translational fusion carrying 247 bp of the <i>ptxR</i> – <i>ptxS</i> intergenic region carrying the putative P2 promoter in pSW205; Cb ^r	Gaines <i>et al.</i> (2007)
pMP190	Broad-host-range <i>lacZ</i> transcriptional fusion vector ; Cm ^r Sm ^r Tra ⁺	Spaink <i>et al.</i> (1987)
pJAC90	<i>ptxR</i> P1- <i>lacZ</i> transcriptional fusion in pMP190; Sm ^r	Colmer-Hamood <i>et al.</i> (2006)
pJAC47	<i>ptxR</i> :: <i>lacZ</i> transcriptional fusion carrying the entire 567 bp <i>ptxR</i> – <i>ptxS</i> intergenic region (P1 and P2 promoters) in pMP190; Sm ^r	Colmer-Hamood <i>et al.</i> (2006)
p18.230	pUC18 carried on pKT230; Cb ^r Km ^r	Colmer & Hamood (1998)
pJAC7-1	The 2.1 kb <i>KpnI</i> – <i>BglII</i> fragment that carries the <i>ptxR</i> – <i>ptxS</i> intergenic region and the <i>ptxR</i> coding sequence in pUC19 cloned into pKT230; Cb ^r Km ^r	Hamood <i>et al.</i> (1996)

METHODS

Strains, plasmids, and general growth conditions. *P. aeruginosa* strains and plasmids used in this study are listed in Table 1. For routine growth and for the LasB assay, strains were grown in Luria–Bertani (LB) broth (Miller, 1972). For the ETA assay, strains were grown in TSB-DC (trypticase soy broth dialysate with 1% (v/v) glycerol and 0.5 M monosodium glutamate) (Ohman *et al.*, 1980). TSB-DC, which contains 2 µg Fe³⁺ ml⁻¹, is considered to be an iron-deficient medium; for iron-sufficient medium, FeCl₃ was added to TSB-DC to yield 25 µg Fe³⁺ ml⁻¹. If needed, antibiotics were added at the following concentrations: carbenicillin, 300 µg ml⁻¹; kanamycin, 300 µg ml⁻¹; streptomycin, 300 µg ml⁻¹; gentamicin, 40 µg ml⁻¹.

Electrophoretic mobility shift assays. Recombinant Vfr (r-Vfr) was purified from the lysate of the *P. aeruginosa* strain PA103 carrying pWNP28, in which the *vfr* gene is overexpressed from the *tac* promoter by cAMP affinity chromatography, as previously described (Kanack *et al.*, 2006). The 354, 134 and 49 bp fragments used as

probes in the electrophoretic mobility shift assay (EMSA) were synthesized by PCR (primers are listed in Table 2), and end-labelled with [γ -³²P]ATP, using T4 polynucleotide kinase (Sambrook & Russell, 2001). EMSAs were performed as described by DeVault *et al.* (1991), with minor modifications. Briefly, each 20 µl reaction contained approximately 15 pM radiolabelled DNA fragment, standardized to approximately 1000 c.p.m., and 10 ng purified r-Vfr in EMSA binding buffer [10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10 mM KCl, 5% (v/v) glycerol, 1 mM DTT and 20 µM cAMP, with 50 µg BSA ml⁻¹ and 5 µg poly(dI-dC) ml⁻¹]. Reactions were carried out for 20 min at room temperature, and the reactions were separated by 5% PAGE in 0.5 × Tris borate EDTA buffer for 16 h at 4 °C. To promote Vfr binding, 20 µM cAMP was added to the buffer in the upper chamber. Gels were dried, and exposed to film.

DNase I footprinting analysis. DNase I footprinting was performed on a 151 bp fragment amplified by PCR, using the primers VfrfpF and VfrfpR (Table 2), and *Pfu* polymerase (Stratagene). *Sall* and *XhoI* restriction enzyme sites were added to the 5' and 3' ends of the fragment, respectively, to facilitate labelling of one DNA strand.

Table 2. Primers used in this study

Primer	Sequence (5'→3')	Amplicon size for each primer pair (bp)
<i>ptxR</i> 354F	AATGAATGTGCCATATCTCCATAAATAA	354
<i>ptxR</i> 354R	CCAGGGCGGCGCTCA	
<i>ptxR</i> 134F	TGAAACTTATGCGCGCCTG	
<i>ptxR</i> 134R	ATTTTTCGACAAGACCCGTCC	134
<i>ptxR</i> 49F	AAAGCACCCGCGCGA	
<i>ptxR</i> 49R	CAGTTCTCATTTATCGGAAAACGA	49
VfrfpF*	ATCGGTCGACGGTTTTGTCTCGGTAGAAAAGCGAT	151
VfrfpR†	CGATCTCGAGCGGTCAGGTCGTCGATAAA	

*Includes *Sall* site (underlined).

†Includes *XhoI* site (underlined).

Digested fragments were 5'-end-labelled with [γ - 32 P]TTP [70 μ Ci (2.59 MBq) per 25 μ l reaction] (PerkinElmer Life Science) using T7 Sequenase (GE Healthcare Bio-Sciences), according to the manufacturer's directions. Binding reactions were performed by incubating 2.9 μ M r-Vfr with the radiolabelled DNA fragments in 25 μ l EMSA binding buffer, without poly(dI-dC), for 20 min at room temperature. Reactions were incubated with 15 μ g DNase I ml^{-1} , as described by Ross *et al.* (1990), and separated on 10% acrylamide sequencing gels. Maxam–Gilbert chemical sequencing reactions (Maxam & Gilbert, 1980) were also performed.

β -Galactosidase assays. The level of β -galactosidase activity produced throughout the growth cycle of PAO1 and PAO9001 (isogenic *vfr* mutant) carrying different plasmids was determined as previously described (Colmer-Hamood *et al.*, 2006). Briefly, 2 ml aliquots from overnight cultures were pelleted, washed, and resuspended in TSB-DC medium to an OD₆₀₀ of 0.03–0.05. Cells were grown for 14 h at 32 °C, with shaking at 250 r.p.m., and 1 ml samples were obtained every 2 h beginning 4 or 6 h post-inoculation, depending on the fusion plasmid tested (pJAC24 and pJH2, respectively). Cells in the samples were lysed, and the level of β -galactosidase activity was determined (Miller, 1972; Stachel *et al.*, 1985).

Assays for ETA and LasB. For ETA analysis, overnight cultures of the *P. aeruginosa* strains were subcultured in TSB-DC, and grown for 14 h at 32 °C. Supernatant fractions were separated, and the level of ETA within each fraction was determined by sandwich ELISA, as previously described (Gaines *et al.*, 2005). Values were standardized by dividing the amount of ETA ($\mu\text{g } \mu\text{l}^{-1}$) in each supernatant by the OD₆₀₀ of the culture from which the fraction was obtained.

To determine LasB activity, overnight cultures of the *P. aeruginosa* strains were subcultured into LB broth, and grown for 14 h at 37 °C. The supernatant fractions were separated, and the level of LasB activity in each fraction was determined by the elastin Congo red assay, as previously described (Schaber *et al.*, 2004). Values were standardized by dividing the LasB activity obtained in each supernatant (A_{495}) by the OD₆₀₀ of the culture from which the fraction was obtained.

Analysis of the *pvc*-related product. PtxR regulates the production of a pigment (a coumarin derivative termed pseudoverdine)

produced by the *pvc* operon. The pigment production can be analysed by measuring the absorbance of the supernatant at 405 nm (Stintzi *et al.*, 1996). Analysis of the *pvc*-related product (PVC) was accomplished by growing the *P. aeruginosa* strains for 14 h at 32 °C in iron-deficient and iron-sufficient TSB-DC. Supernatant fractions were separated, and the A_{405} of each fraction was determined. Values for PVC were standardized as described for measurement of LasB activity.

RESULTS AND DISCUSSION

Vfr specifically binds to a sequence within the *ptxR* upstream region

Available evidence suggests that *ptxR* is expressed from two separate promoters (P1 and P2) producing two transcripts, respectively: T1 initiating at –356 bp, and T2 initiating at –166 bp, from the *ptxR* start codon (Vasil *et al.*, 1998). In addition, computer analysis of the *ptxR*-upstream region revealed the presence of a potential Vfr-binding sequence located at 186–210 bp 5' of the PtxR ATG codon, and 20–44 bp 5' of the *ptxR* T2 transcriptional start site (Fig. 1). The sequence contains most of the conserved nucleotides within the recently described Vfr consensus sequence (Kanack *et al.*, 2006). To verify Vfr binding to the *ptxR* upstream region, we conducted EMSAs using purified r-Vfr and a 354 bp fragment that carries 340 bp 5' of *ptxR* plus the first coding sequence for the first four amino acids of PtxR. As shown in Fig. 2(a), r-Vfr produced a specific gel shift band with the 354 bp fragment. The intensity of the band was reduced in the presence of excess unlabelled probe (Fig. 2a). To further localize the binding, we divided the 354 bp fragment into a 49 bp fragment, which carries the potential Vfr-binding sequence, and a 134 bp fragment. r-Vfr specifically bound to the 49 bp fragment, but did not bind to the 134 bp fragment; this again confirmed the specificity of Vfr binding (Fig. 2b).

Fig. 1. Sequence of the *ptxR*–*ptxS* intergenic region with relevant features. The 562 bp intergenic region plus the sequences for the first three *PtxS* codons and the first 55 *PtxR* codons (interrupted) are shown (from GenBank accession no. AF012100). The deduced amino acid sequence is shown under each codon; straight arrows indicate the direction of the ORF. The region protected by Vfr is indicated by a box. The –35 and –10 regions for the P1 and P2 promoters of *ptxR* are underlined. Nucleotides for the T1 and T2 transcriptional start sites are in bold. The iron starvation box (PvdS-binding site) is overlined. Bent arrows indicate the portion of the intergenic region included in *pJAC24*, *pJAC47* and *pJH2* (continuous lines) and *pJAC90* (dashed lines). Numbering on left of the sequence is relative to the *PtxR* ATG codon.

Fig. 2. Binding of Vfr to the *ptxR*–*ptxS* intergenic region. Radiolabelled DNA fragments (~1000 c.p.m.) plus 10 ng purified r-Vfr in EMSA binding buffer were incubated for 20 min at room temperature. Samples were separated on 5% polyacrylamide gels, with 20 μ M cAMP added to the electrophoresis buffer in the upper chamber to promote Vfr binding. (a) Autoradiogram of Vfr binding to the 354 bp intergenic probe. Lanes: 1, 354 bp probe alone; 2, probe plus 10 ng Vfr; 3, probe plus 10 ng Vfr plus fivefold (5 $\times$) unlabelled probe; 4, probe plus 10 ng Vfr plus tenfold (10 $\times$) unlabelled probe. (b) Autoradiogram of Vfr binding to smaller fragments of the intergenic region. Lanes: 1, 134 bp probe alone; 2, 134 bp probe plus 10 ng Vfr; 3, 49 bp probe alone; 4, 49 bp probe plus 10 ng Vfr. Free probe size is indicated to the left of the autoradiograms. Arrows indicate DNA–protein complexes.

We then determined the exact sequence to which r-Vfr binds. We performed DNase I footprinting on a 152 bp fragment of the *ptxR*–*ptxS* intergenic region that contains the putative Vfr-binding sequence (Vfr-BS), using primers VfrfpF and VfrfpR (Table 2). As shown in Fig. 3(a), in the presence of 2.9 μ M r-Vfr, the site 5'-ACCGTCTGAAG-CAGTTCTCATTAT-3' on the *ptxR* coding strand was protected from DNase I cleavage. In addition, r-Vfr protected the corresponding sequence on the opposite strand of the DNA fragment (data not shown). Out of the 21 conserved nucleotides within the Vfr consensus sequence, 17 were matched within the protected sequence (Fig. 3b). In addition, the location of the protected sequence matched that of the potential Vfr-binding sequence that was identified by computer analysis (Fig. 1). Within this sequence, we located sites with increased hypersensitivity to DNase I treatment; the sites were located at positions 9, 10, 18 and 19 of the 25 nt protected sequence (Fig. 3a). These sites indicate a perturbation in the DNA helical structure that could include a kink or a bend (Kanack *et al.*, 2006). Similar perturbations have been detected in the Vfr-binding analyses with the upstream regions of *toxA*, *regA* and *lasR* (Albus *et al.*, 1997; Kanack *et al.*, 2006). The DNase I footprinting experiments, together with the EMSA, indicate that Vfr binds at a specific site within the *ptxR*–*ptxS* intergenic region. The proximity of this sequence to the *ptxR* T2 transcription initiation site suggests that Vfr regulates *ptxR* expression rather than *ptxS* expression.

Results of the present study point out an important feature of the Vfr-BS within the *ptxR* upstream region. Similar to the Vfr-BS within the promoters of *lasR* and *vfr* (Albus *et al.*, 1997; Kanack *et al.*, 2006), the Vfr-BS within the *ptxR* upstream region is located 5' of the putative *ptxR* T2 initiation site (Colmer-Hamood *et al.*, 2006; Vasil *et al.*,

1998). However, the spacing between the Vfr-BS and the T2 initiation site appears to be different from the typical *E. coli* CRP-activated promoters. The Vfr-BS is centred at –32.5 bp upstream of the T2 initiation site. In *E. coli* promoters that are activated by CRP, the CRP-binding sites are usually centred at –42, –62 and –72 bp upstream of the transcription initiation sites (Kolb *et al.*, 1993). The Vfr-BS within the *lasR* promoter is centred at –47.5 and –77.5 bp upstream of the T2 and T1 transcription initiation sites (Albus *et al.*, 1997). However, Albus *et al.* (1997) have shown that increasing the spacing by 4 or 8 bp is important, but not critical, for *lasR* expression. The identified Vfr-BS within the *ptxR* upstream region overlaps with the putative binding site for the iron-regulated *P. aeruginosa* iron-starvation sigma factor PvdS (Colmer-Hamood *et al.*, 2006; Gaines *et al.*, 2007). PvdS, which is required for the expression of *toxA* and the pyoverdine genes under aerobic conditions, is synthesized in *P. aeruginosa* at a high level in iron-deficient medium (Ochsner *et al.*, 1996). Our recent EMSA has demonstrated that the PvdS–RNA-polymerase complex specifically binds to the 354 bp sequence within the *ptxR* upstream region that contains the iron-starvation box (Fig. 1) (Colmer-Hamood *et al.*, 2006; Gaines *et al.*, 2007). Moreover, in an iron-deficient medium under aerobic conditions, *ptxR* expression from P2 is significantly lower in the *pvdS* deletion strain PAO Δ *pvdS* than in its parent strain (Gaines *et al.*, 2007).

Based on the results presented in this study, the Vfr-BS within the *ptxR* upstream region may belong to the class II CRP-dependent promoters in which the site from which CRP activates transcription overlaps the DNA-binding site of the RNA polymerase (Busby and Ebright, 1999; Kanack *et al.*, 2006). However, the putative Vfr-BS within the *ptxR* upstream region differs from the typical class II

Fig. 3. DNase I footprinting analysis of Vfr. (a) The 152 bp fragment of the *ptxR*–*ptxS* intergenic region was single end-labelled with [γ - 32 P]ATP for the coding strand and incubated with (+) or without (–) 2.9 μ M purified Vfr, and then treated with DNase I as described in Methods. G + A indicates the sequencing ladder of the *ptxR* promoter. The region protected from DNase I digestion by Vfr is indicated by a bar. Arrows indicate sites with DNase I hypersensitivity. The sequence of the protected region is on the right. The DNase I hypersensitive sites are in bold. (b) Comparison of the Vfr-BS within the *ptxR*–*ptxS* intergenic region to the Vfr consensus binding sequence. Mismatches within the *ptxR*–*ptxS* Vfr-BS are in bold. The conserved half-sites within the Vfr consensus binding sequence are underlined. W represents A or T, Y represents T or C, and N indicates positions where one nucleotide or a combination of two nucleotides does not predominate (Kanack *et al.*, 2006).

CRP-dependent promoters in its location with respect to the –35 region sequences. In class II CRP-dependent promoters, the CRP-binding site partially overlaps the –35 region sequence (Busby & Ebright, 1999). However, the Vfr-BS within the *ptxR* upstream region overlaps the entire –35 region sequence (Fig. 1). Whether this represents a possible variation in class II CRP-dependent promoters is not known. At this time, we consider the T1 and T2 transcriptional initiation sites within the *ptxR* upstream region to be putative. Additional experiments (including primer extension analysis) may refine the exact position of one or both sites. This refinement may also adjust the above-discussed distance between the Vfr-BS and the T2 initiation site.

Based on 10 Vfr-BSs, Kanack *et al.* (2006) have developed a Vfr consensus binding sequence (5′-ANWWTGNGA-WNY:AGWTCACAT-3′) that contains two, more conserved, half-sites: TGNGA (left halfsite) and TCACA (right half-site) (Fig. 3b). Experimental evidence has indicated that Vfr activates the expression from the *lasR*, *regA* and *toxA* promoters, but represses the expression from the *fleQ* promoter (Albus *et al.*, 1997; Dasgupta *et al.*, 2002; West *et al.*, 1994b). Efficient regulation of *lasR* and *fleQ* by Vfr depends on specific nucleotides that exist in the conserved

half-sites of the Vfr-BSs for each gene. While *lasR* contains all the conserved nucleotides in each half-site, *fleQ* contains only three of five conserved nucleotides in the left half-site (Albus *et al.*, 1997; Dasgupta *et al.*, 2002; Kanack *et al.*, 2006). Albus *et al.* (1997) have shown that a single base pair mutation of the left half-site (TGNGA to TCNCA) and the right half-site (TCACA to TGAGA) (2 bp at a time) in the *lasR* Vfr-BS results in fivefold reduction in *lasR* expression, while changing the outermost nucleotides of each half-site has little effect. Similarly, Dasgupta *et al.* (2002) have shown that mutation of the right half-site from TCACA to TCCGC obviates Vfr binding, and that expression of *fleQ* from this mutated promoter in PAO1 is increased compared with the wild-type, and is not repressed by overexpression of *vfr* from a plasmid.

Varying numbers of nucleotides that match the ones within the conserved half-sites are found in the Vfr-BS of *ptxR* (4/5 in the left half-site, and 4/5 in the right half-site), *regA* (3/5 and 4/5), and *toxA* (4/5 and 4/5) (Fig. 3b) (Kanack *et al.*, 2006). Despite the variations in the conserved nucleotides, each half-site for these genes contains one nucleotide conserved in all the Vfr-BSs; the right halves contain a C toward the 5′ end (xCxxx), while the left halves contain an A at the 3′ end (xxxxA). These two conserved nucleotides may be

critical for Vfr binding and/or regulation of these genes by Vfr. Future *in vitro* mutagenesis analysis will be necessary to determine if changing the conserved A or C, or both, within the Vfr-BS of *ptxR*, *regA* and *toxA*, interferes with Vfr binding to the upstream regions of these genes.

Vfr enhances *ptxR* expression in PAO1

To determine if Vfr regulates *ptxR* or *ptxS* expression, we compared the levels of expression of each gene in PAO1 and its *vfr* isogenic mutant PAO9001 using *ptxR* and *ptxS* translational fusion plasmids (pJAC24 and pBS8-4, respectively; Table 1). The level of β -galactosidase activity was determined throughout the growth cycle of PAO1 and PAO9001 carrying these plasmids, as previously described (Colmer & Hamood, 1998; Swanson *et al.*, 1999). Throughout their growth cycles, PAO1/pBS8-4 and PAO9001/pBS8-4 produced comparable levels of β -galactosidase activity (data not shown), thus eliminating the possibility that Vfr regulates *ptxS* expression in PAO1. However, the level of *ptxR* expression was significantly ($P < 0.001$) higher in PAO1/pJAC24 than in PAO9001/pJAC24 throughout the growth cycle (Fig. 4a), indicating that Vfr enhances *ptxR* expression in *P. aeruginosa*. Strains containing the cloning vector pSW205 produced no detectable levels of β -galactosidase activity (data not shown). In addition, there was no variation in the growth pattern of PAO1 and PAO9001 carrying the plasmids throughout the growth cycle (data not shown).

Since the Vfr-BS is centred -32.5 bp 5' of the *ptxR* T2 initiation site, Vfr is most likely to regulate *ptxR* expression through P2. Therefore, we analysed the transcription from *ptxR* P2 using plasmid pJH2, which is identical to pJAC24, except that it carries only 247 bp of the region 5' of the PtxR start codon (P1 has been eliminated) (Gaines *et al.*, 2007). Similar to the expression from pJAC24, the level of *ptxR* expression from pJH2 was significantly ($P < 0.001$) higher in PAO1 than in PAO9001 (Fig. 4b) throughout the growth cycle. We then examined *ptxR* expression from plasmid pJAC90, which carries only the *ptxR* P1 promoter (Colmer-Hamood *et al.*, 2006), to eliminate the possibility that Vfr affects the expression from *ptxR* P1. We detected no significant difference in the level of β -galactosidase activity produced by PAO1/pJAC90 and PAO9001/pJAC90. The level of β -galactosidase activity produced by PAO1/pJAC90 was 7.6 ± 0.37 units (mean $\pm$ SEM), while that produced by PAO9001/pJAC90 was 6.3 ± 0.92 units. Plasmid pJAC90 carries a *ptxR* P1 transcriptional fusion while pJAC24 carries a *ptxR* P1/P2 translational fusion (Table 1). Thus, to eliminate any possible post-translational influence on our results shown in Fig. 4, we utilized pJAC47, which carries a *ptxR* P1/P2 transcriptional fusion (Table 1). We obtained results similar to those shown in Fig. 4(a) (data not shown). These results confirm that Vfr influences *ptxR* expression through the *ptxR* P2 promoter.

PtxR and RegA regulate *toxA* expression, and their expression is positively regulated by Vfr (Fig. 4)

Fig. 4. Effect of Vfr on *ptxR* expression. PAO1 (■) and PAO9001 (▲) carrying pJAC24 (*ptxR* P1 and P2) (a) or pJH2 (*ptxR* P2 only) (b) were grown as described in Methods, and the level of β -galactosidase activity was determined. Values represent the mean of three independent experiments ($\pm$ SEM); * $P < 0.001$

(Hamood *et al.*, 1996; Hindahl *et al.*, 1987; Kanack *et al.*, 2006). Similar to *ptxR*, *regA* is expressed from two separate promoters (P1 and P2) that are differentially regulated by iron (Frank *et al.*, 1989; Storey *et al.*, 1990). While iron does not affect the expression from the P1 promoter of either gene, it negatively regulates the expression from both P2 promoters (Frank *et al.*, 1989; Storey *et al.*, 1990; Vasil *et al.*, 1998). However, the two genes differ in the location of the Vfr-BS with respect to the two promoters, and in the influence of Vfr on their P1 and P2 promoters. While the Vfr-BS is centred 32.5 bp 5' of the *ptxR* P2 promoter (Fig. 1), it is centred 63 bp 5' of the *regA* P1 promoter (Kanack *et al.*, 2006). Our expression analysis suggests that Vfr is required for *ptxR* P2 expression throughout the growth cycle in iron-deficient medium (Fig. 4b). In contrast, Vfr regulates the expression of *regA* P1 rather than P2 (West *et al.*, 1994a). The expression of *regA* P1 is iron insensitive, and occurs at early stages of growth, whereas that of *regA* P2 is iron repressible (detected in

iron-deficient medium only) and occurs during later stages of growth (Frank *et al.*, 1989). It is known that RegA regulates *toxA* expression in iron-deficient medium throughout the growth cycle (Frank *et al.*, 1989; Storey *et al.*, 1990). Vfr is required for efficient ETA production at late stages of growth, and in iron-deficient medium (Table 3) (West *et al.*, 1994a). In addition, Vfr specifically binds to the *toxA* upstream region (Kanack *et al.*, 2006). Whether Vfr regulates *toxA* expression directly through this binding at later stages of growth and in iron-deficient medium is yet to be determined.

vfr deletion obviates PtxR enhancement of ETA and LasB, but has little effect on synthesis of the PVC

Vfr positively regulates the expression of several QS-related genes, including *toxA* and *lasB* (Albus *et al.*, 1997; West *et al.*, 1994a). In comparison with its parent strain, the *vfr* mutant PAO9001 produced significantly lower levels of ETA and LasB (Table 3) (West *et al.*, 1994a). PtxR also positively affects *toxA* and *lasB* expression and production (Table 3) (Carty *et al.*, 2006; Hamood *et al.*, 1996). Therefore, PtxR may be one of the factors through which Vfr regulates the expression of these genes. Thus, we explored the possibility that pJAC7-1, a high-copy *ptxR* plasmid, would partially or completely complement the defect of PAO9001 in ETA and LasB production. Plasmid pJAC7-1 increased ETA production in PAO1 by tenfold, but did not restore ETA production in PAO9001 (Table 3). Similarly, pJAC7-1 did not restore LasB activity in PAO9001 (Table 3).

Deletion of *vfr* may impact another of the functions of PtxR. One of the targets of PtxR is the *pvc* operon, whose product is not completely defined. In PAO1, pJAC7-1 enhanced the synthesis of PVC by about 2.5-fold, regardless of the level of iron in the growth medium (Table 3). The loss of Vfr led to enhancement in synthesis of PVC; however, unlike with ETA and LasB, pJAC7-1 enhanced the levels of PVC in PAO9001 in the presence or absence of iron (Table 3).

Results of this and previous studies suggest that Vfr enhances *toxA* expression either directly through Vfr binding to the *toxA* upstream region, or indirectly through

Vfr binding to the upstream regions of *regA* and *ptxR*, and enhancing their expression (Figs 2, 4 and 5) (West *et al.*, 1994a; Kanack *et al.*, 2006). Therefore, instead of being the primary factor through which Vfr regulates *toxA* expression, Vfr may fine tune or modulate *toxA* expression through *ptxR*. This suggestion is based on the following observations. (1) Our results indicate that Vfr enhances the expression from the *ptxR* P2 promoter, but not the P1 promoter (Figs 1 and 4). Thus, an increase in the expression of the Vfr-independent *ptxR* P1 promoter (due to its presence on the multicopy plasmid pJAC7-1) should increase ETA production. However, as shown in Table 3, the level of ETA produced by PAO9001/pJAC7-1 was similar to that produced by PAO9001 carrying a vector control. (2) Recently, we have shown that *ptxR* expression from an exogenous *lac* promoter (plasmid pJAC5-1) does not bypass the defect of PAO9001 in ETA production (data not shown). (3) ETA production in PAOΔ*ptxR*, which carries an intact copy of *vfr*, is reduced but not eliminated (Hamood *et al.*, 1996).

Unlike *toxA*, Vfr enhances *lasB* expression in PAO1 indirectly by binding to the upstream region of the *lasB* transcriptional activator *lasR* and enhancing *lasR* expression (Fig. 5) (Albus *et al.*, 1997; Kanack *et al.*, 2006). Yet, similar to *toxA*, Vfr may utilize PtxR to modulate *lasB* expression. Results of our recent analysis suggest that PtxR enhances the expression of *lasI* rather than *lasR* (Carty *et al.*, 2006). LasI is involved in the synthesis of the homoserine lactone molecule 3OC₁₂-HSL, which activates LasR (de Kievit & Iglewski, 2000) (Fig. 5). Thus, a modest increase in PtxR synthesis by Vfr would increase the amount of activated LasR, and enhance *lasB* expression in PAO1, but not PAO9001 or PAOΔ*lasR* (Fig. 5, Table 3; data not shown).

With respect to PVC production, Vfr appears to have a unique effect. As shown in Table 3, *vfr* deletion enhanced PVC production by PAO1 in iron-deficient medium only (A_{405} 0.08 for PAO1/18.230 versus A_{405} 0.14 for PAO9001/p18.230). This effect is not related to PtxR, since pJAC7-1 enhanced PVC production in PAO1 and PAO9001 under both iron-deficient and iron-sufficient conditions (Table 3). Whether a possible relationship exists between Vfr, iron and PVC production is not known at this time. Several aspects of the *pvc* operon, including the functions of the

Table 3. Effect of *ptxR* on *P. aeruginosa* secreted virulence factors in the presence or absence of Vfr

Values were normalized by dividing the absorbance obtained by the growth index (OD_{600}) of the culture from which the supernatant was harvested. Values represent the means of three independent experiments ($\pm$ SD).

Strain	LasB (A_{495})	ETA ($\mu\text{g } \mu\text{l}^{-1}$)	PVC (A_{405}), low iron	PVC (A_{405}), high iron
PAO1/p18.230	0.393 ± 0.02	30.34 ± 14.5	0.40 ± 0.05	0.08 ± 0.003
PAO1/pJAC7-1	4.12 ± 0.6	341.43 ± 18.4	1.11 ± 0.06	0.19 ± 0.01
PAO9001/p18.230	0.06 ± 0.06	3.59 ± 0.09	0.51 ± 0.03	0.14 ± 0.01
PAO9001/pJAC7-1	0.07 ± 0.07	3.50 ± 0.04	0.91 ± 0.06	0.25 ± 0.01

Fig. 5. Schematic diagram illustrating the regulation of *toxA* and *lasB* by Vfr, and the role of *ptxR* in this regulation. Continuous lines indicate direct regulation of their target genes by Vfr and activated LasR. Dashed lines indicate possible indirect regulation of their target genes by PtxR and RegA. Inverted triangles represent the homoserine lactone molecule 3OC₁₂-HSL. Horizontal arrows indicate the direction of transcription of each gene.

pvc-encoded proteins and the mechanism of *pvc* regulation by PtxR, are yet to be determined.

In conclusion, our results show that Vfr specifically binds to the *ptxR* upstream region, and enhances the expression from the *ptxR* P2 promoter. In addition, our results suggest that Vfr modulates *toxA* and *lasB* expression in PAO1 through PtxR.

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