Microbiology (2009), **155**, 2265–2273 **DOI** 10.1099/mic.0.028373-0

Role of Vfr in regulating exotoxin A production by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa exotoxin A (ETA) production depends on the virulence-factor regulator Vfr. Recent evidence indicates that the *P. aeruginosa* iron-starvation sigma factor PvdS also enhances ETA production through the ETA-regulatory gene *regA*. Mutants defective in *vfr*, *regA* and *pvdS*, plasmids that overexpress these genes individually and *lacZ* transcriptional/translational fusion plasmids were utilized to examine the relationship between *vfr*, *regA* and *pvdS* in regulating *P. aeruginosa* ETA production. ETA concentration and *regA* expression were reduced significantly in PAOΔ*vfr*, but *pvdS* expression was not affected. Overexpression of Vfr produced a limited increase in ETA production in PAOΔ*pvdS*, but not PAOΔ*regA*. Additionally, overexpression of either RegA or PvdS did not enhance ETA production in PAOΔ*vfr*. RT-PCR analysis showed that iron did not affect the accumulation of *vfr* mRNA in PAO1. These results suggest that: (i) Vfr enhances *toxA* expression in PAO1 both directly and indirectly through *regA*, but not through *pvdS*; (ii) *vfr* expression is not regulated by iron; and (iii) both Vfr and PvdS cooperate in the presence of RegA to achieve a maximum level of *toxA* expression.

Received 16 February 2009
Revised 15 March 2009
Accepted 20 April 2009

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that causes acute and chronic infections in immunocompromised hosts, including severely burned patients, individuals with cystic fibrosis and cancer patients undergoing chemotherapy (Davis *et al.*, 1996; Pollack, 2000). The severity of these infections is due to the ability of *P. aeruginosa* to produce an arsenal of cell-associated and extracellular virulence factors (Frank, 1997; Govan & Deretic, 1996; Sato & Frank, 2004; Woods & Vasil, 1994). Among the extracellular virulence factors produced by *P. aeruginosa* are exotoxin A (ETA), proteins of the type III secretion system and the LasB protease (elastase) (Frank, 1997; Govan & Deretic, 1996; Pollack, 2000). ETA is an ADP-ribosyltransferase that catalyses the transfer of the NAD moiety to elongation factor 2 of eukaryotic cells, which inhibits protein synthesis and results in cell death (Iglewski & Kabat, 1975). Both clinical studies and animal models have demonstrated the importance of ETA in *P. aeruginosa* infection. For example, sputum samples obtained from cystic fibrosis patients contained *toxA* mRNA (Storey *et al.*, 1998). Additionally, *P. aeruginosa* clinical isolates obtained from different sites produced ETA (Hamood *et al.*, 1996). Furthermore, *P. aeruginosa* mutants defective in ETA production were less virulent in different animal models than their parent strains (Fogle *et al.*, 2002; Matsumoto *et al.*, 1999).

ETA production by *P. aeruginosa* is regulated by different environmental factors, including growth temperature and the level of iron in the growth medium (Hamood *et al.*, 2004; Liu, 1973). Iron represses ETA production; *P. aeruginosa* produces maximum levels of ETA when it is grown in an iron-deficient medium (Hamood *et al.*, 2004; Liu, 1973). ETA production by *P. aeruginosa* also involves several positive and negative regulators (Hamood *et al.*, 2004). The most extensively analysed of these are the positive regulators RegA and PvdS (Hamood *et al.*, 2004; Vasil & Ochsner, 1999). RegA enhances *toxA* transcription, although the mechanism by which this enhancement occurs is not completely understood (Hamood & Iglewski, 1990; Raivio *et al.*, 1996). PvdS, the iron-starvation sigma factor, enhances the expression of *toxA* and is required for expression of the pyoverdine genes (Beare *et al.*, 2003; Cunliffe *et al.*, 1995; Vasil & Ochsner, 1999). Evidence suggests that the PvdS enhancement of *toxA* occurs through *regA* (Hamood *et al.*, 2004; Ochsner *et al.*, 1996). Negative regulation of ETA production occurs

Abbreviations: ETA, exotoxin A; RNAP, RNA polymerase.

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A supplementary figure showing that overexpression of Vfr does not enhance ETA production in PAOΔ*regA* is available with the online version of this paper.

through the ferric-uptake regulator Fur (Hamood *et al.*, 2004; Vasil & Ochsner, 1999). Iron-activated Fur represses *pvdS* transcription, thereby reducing *regA* and *toxA* transcription (Hamood *et al.*, 2004; Vasil & Ochsner, 1999). Gaines *et al.* (2007) demonstrated that the PvdS–RNA polymerase (RNAP) holoenzyme complex binds specifically to the upstream region of *regA* and *toxA*.

The virulence-factor regulator Vfr, originally described as a factor that regulates ETA and protease production by *P. aeruginosa*, exerts a global regulatory effect on the production of multiple virulence factors (West *et al.*, 1994b). Studies have demonstrated that Vfr regulates different genes of the *las* quorum-sensing system (including *lasR* and *lasB*), twitching motility, flagellar biosynthesis and the stationary-phase sigma factor RpoS (Albus *et al.*, 1997; Ambrosi *et al.*, 2002; Bertani *et al.*, 2003; Dasgupta *et al.*, 2002). Using microarray analysis, Wolfgang *et al.* (2003) identified more than 200 genes, including those that encode different components of the type III secretion system including *exoS* and *exsA*, that are regulated either positively or negatively by Vfr. Vfr is a homologue of the *Escherichia coli* catabolite-repressor protein CRP, which requires cAMP for its activation (West *et al.*, 1994b). Kanack *et al.* (2006) showed that Vfr binds to a specific sequence within the upstream regions of *toxA*, *regA*, *lasR*, *prpL*, *algD* and *fimS*, although other genes, including *pvdS*, *exoS* and *exsA*, lack the Vfr-binding sequence (N. L. Carty & A. N. Hamood, unpublished results). In this study, we analysed the mechanism by which Vfr regulates ETA production in *P. aeruginosa*. Our results suggest that Vfr regulates ETA production directly by binding to the *toxA* upstream region and indirectly through *regA*. However, Vfr does not regulate *pvdS* expression; neither is *vfr* expression regulated by iron.

METHODS

Bacterial strains, plasmids and growth media and conditions. Strains and plasmids utilized in this study are described in Table 1. For general growth experiments, strains were grown in Luria–Bertani (LB) broth (Miller, 1972). For analysis of ETA production, *P. aeruginosa* strains were grown in the iron-deficient medium TSBDC (Chelex-treated trypticase soy broth dialysate) to which glycerol (1 %, v/v) and monosodium glutamate (0.5 M) were added (Ohman *et al.*, 1980). Iron-sufficient medium was prepared by the addition of $FeCl_3$

Table 1. Strains and plasmids used in this study

Strain/plasmid	Description*	Source (reference)
Pseudomonas aeruginosa		
PAO-SW	Prototrophic PAO1 strain	S. E. H. West (West *et al.*, 1994b)
PAOΔ*vfr*	*vfr* deletion of PAO-SW	S. E. H. West (Albus *et al.*, 1997)
PAOΔ*pvdS*	*pvdS* isogenic mutant of PAO-SW; *pvdS* disruption; Gm^r	This study
PAOΔ*regA*	*regA* isogenic mutant of PAO-SW; *regA* internal deletion; Gm^r	This study
Plasmids		
pSW205	*lacZ* translational fusion vector that replicates stably in *P. aeruginosa*; Cb^r	S. E. H. West (Storey *et al.*, 1990)
pSW228	*toxA–lacZ* translational fusion in pSW205; Cb^r	S. E. H. West (West *et al.*, 1994a)
pRL88	*regA*(*P1/P2*)–*lacZ* translational fusion in pSW205; Cb^r	D. Storey (Storey *et al.*, 1990)
pAM21-2	pUC18 recombinant plasmid in which *toxA* is expressed from the *lac* promoter carried on pKT230; Cb^r, Km^r	This study
pIN9	1.5 kb *Ava*I–*Pst*I fragment carrying intact *regA* from PA103 plus 40 bp of the *regA* upstream region expressed from the *lac* promoter in pUC18; Cb^r	This study
pIN10	pIN9 carrying the 1.8 kb stability fragment for replication of plasmid in *P. aeruginosa*; Cb^r	This study
pUCP19	*E. coli–P. aeruginosa* shuttle vector; Cb^r	H. P. Schweizer (Schweizer, 1991)
pKF917	pUCP19 carrying intact *vfr*; Cb^r	S. E. H. West (West *et al.*, 1994b)
pVLT31	Broad-host-range expression vector; Tc^r	M. Vasil (Ochsner *et al.*, 1996)
pPVD31	pVLT31 recombinant plasmid in which *pvdS* is expressed from the *tac* promoter; Tc^r	M. Vasil (Ochsner *et al.*, 1996)
pMP220	Broad-host-range *lacZ* transcriptional fusion vector; Tc^r	P. Visca (Spaink *et al.*, 1987)
pMP220::P*pvdS*	*pvdS–lacZ* transcriptional fusion in pMP220; Tc^r	P. Visca (Ambrosi *et al.*, 2002)
pMP190	Broad-host-range *lacZ* transcriptional fusion vector; Cm^r, Sm^r	I. Lamont (Spaink *et al.*, 1987)
pMP190::P*pvdE*	*pvdE–lacZ* transcriptional fusion in pMP190; Cm^r, Sm^r	P. Visca (Leoni *et al.*, 2000)
pJQ*pvdS*::Gm	*pvdS* interrupted with a Gm cassette carried in the *sacB* suicide vector pJQ200SK; Gm^r	D. Storey (Hunt *et al.*, 2002)
pSUP203-Δ*regA*::Gm	Mobilizable suicide plasmid carrying a Gm cartridge between *regA*-flanking regions; Gm^r	M. Vasil (Ochsner *et al.*, 1996)

*Cb, Carbenicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.

to TSBDC to a final concentration of 25 μg ml^{-1} (TSBDC-Fe) (Ohman *et al.*, 1980). Antibiotics were added to the growth medium as needed: carbenicillin (300 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), gentamicin (50 μg ml^{-1}), kanamycin (500 μg ml^{-1}), streptomycin (300 μg ml^{-1}) and tetracycline (80 μg ml^{-1}).

For the analysis of ETA production, as well as the expression of different genes, *P. aeruginosa* strains were first grown overnight in LB broth at 37 °C. An aliquot of the overnight culture was pelleted, washed and resuspended in TSBDC. The resuspended culture was used to inoculate fresh TSBDC or TSBDC-Fe to an OD_{540} of 0.03–0.05. The cultures were grown at 32 °C for 14–16 h with shaking at 250 r.p.m. Samples were obtained at specified time points for analysis. Each experiment was conducted in triplicate (three separate flasks), with three replicates per flask.

General DNA techniques. Plasmid DNA extraction was performed by using the Wizard *Plus* MiniPreps DNA Purification system (Promega). Restriction digestion, ligation and transformation of *E. coli* were done as described by Sambrook & Russell (2001). Plasmids were introduced into *P. aeruginosa* by electroporation (Smith & Iglewski, 1989).

Construction of pIN10. A 1.5 kb *Ava*I–*Pst*I fragment was isolated from pDF18-202 (Frank *et al.*, 1989) and cloned into the *Sal*I/*Pst*I sites of pUC18. The fragment carries the intact *regA* open reading frame from *P. aeruginosa* strain PA103, plus 40 bp of the region immediately upstream of the *regA* ATG codon. In the resulting recombinant plasmid (pIN9), *regA* is expressed constitutively from the *lac* promoter. The 1.8 kb stability fragment, which allows ColEI plasmids to replicate stably in *P. aeruginosa* (Olsen *et al.*, 1982), was cloned into the *Pst*I site of pIN9, generating pIN10.

Generation of *pvdS* and *regA* knockout mutants. The mutants were constructed from PAO-SW, the strain from which the *vfr* mutant (PAOΔ*vfr*) was generated, by using the gene-replacement technique as described previously (Hunt *et al.*, 2002; Ochsner *et al.*, 1996). Plasmid pJQ*pvdS*::Gm (Hunt *et al.*, 2002) was utilized to construct PAOΔ*pvdS*, whilst plasmid pSUP203-Δ*regA*::Gm (Ochsner *et al.*, 1996) was used to generate PAOΔ*regA*. Construction of both mutants was confirmed by PCR.

RT-PCR. Bacterial RNA was extracted by using a modified hot phenol method as described previously (Carty *et al.*, 2003; Frank *et al.*, 1989). Residual chromosomal DNA was removed with DNase I in the presence of the RNase inhibitor RNasin (Promega). Enzymes and buffer were then removed from the RNA solution by using an RNeasy kit (Qiagen). To synthesize cDNA, approximately 1 μg total RNA was reverse-transcribed at 42 °C for 90 min with StrataScript reverse transcriptase (Stratagene) and random hexamers (Promega) as primers. Specific primers were used to amplify regions of *toxA* (F-5′-GCGTGCTGCACTACTCCATG-3′; R-5′-GTTACCGGCGTTCAGTTCGT-3′), *pvdS* (F-5′-ACCGTAGATCCTGGTGAAGA-3′; R-5′-CGAGTATTTCTGTTCGAGCGC-3′) and *vfr* (F-5′-GCTGCGAAACGCTGTTCTTC-3′; R-5′-GCTGCCGAGGGTGTAGAGG-3′) from the cDNA. A region of the constitutively expressed *rpsL* gene (F-5′-GCAACTATCAACCAGCTG-3′; R-5′-GCTGTGCTCTTGCAGGTTGTG-3′) was amplified as a positive control (Sobel *et al.*, 2003). PCR extension was conducted at temperatures appropriate for each primer for 30 cycles. To exclude DNA contamination, each RNA sample was subjected to PCR without reverse transcriptase. The products were examined on ethidium bromide-stained agarose gels.

Sandwich ELISA and β-galactosidase assays. The sandwich ELISA was done as described previously (Gaines *et al.*, 2005). Strains carrying various plasmids were grown in TSBDC or TSBDC-Fe for 14 h at 32 °C. ETA levels within the supernatant were determined by sandwich ELISA. Values were standardized by dividing the amount of ETA in pg μl^{-1} by the OD_{600} of the culture from which the fraction was obtained. Assays for β-galactosidase were performed as reported previously (Carty *et al.*, 2006; Miller, 1972; Stachel *et al.*, 1985).

RESULTS AND DISCUSSION

It has been suggested that both Vfr and PvdS regulate *toxA* and *regA* at the transcriptional level, although PvdS appears to regulate *toxA* expression through *regA* (Ochsner *et al.*, 1996; West *et al.*, 1994a). The *regA* upstream region contains an iron-starvation box (Hunt *et al.*, 2002; Ochsner *et al.*, 2002). Kanack *et al.* (2006) provided evidence that Vfr regulation may occur through its binding to the *toxA* and *regA* upstream region. However, Vfr does not bind to the *pvdS* upstream region (Kanack *et al.*, 2006). Thus, to understand the relationship between *vfr* and *toxA*, *regA* and *pvdS*, we addressed the following questions. (i) Does Vfr regulate *toxA* expression directly? (ii) Does Vfr regulate *toxA* expression through *regA*, *pvdS* or both? (iii) Is *vfr* regulated by iron, similarly to *toxA*, *regA* and *pvdS*? To examine the roles of *regA* and *pvdS* in the effect of Vfr on ETA production, we constructed mutants that carry specific deletions in either *regA* (PAOΔ*regA*) or *pvdS* (PAOΔ*pvdS*) as described previously (Hunt *et al.*, 2002; Ochsner *et al.*, 1996). The mutants were generated from PAO-SW, the PAO1 strain from which PAOΔ*vfr* was generated (Table 1) (Albus *et al.*, 1997).

Vfr regulates *toxA* expression at the transcriptional level only

Using sandwich ELISA, we confirmed the effect of *vfr* deletion on ETA production. Compared with its parent strain PAO-SW carrying vector pUCP19, PAOΔ*vfr*/pUCP19 produced 25-fold less ETA ($P<0.0001$) (Fig. 1a). The introduction of *vfr* into plasmid pKF917 increased the level of ETA produced by PAOΔ*vfr* significantly ($P<0.0001$), but did not restore it to wild-type level (Fig. 1a). To determine whether the effect of Vfr on ETA occurs at the transcriptional level, we examined *toxA* expression from the *toxA–lacZ* fusion plasmid pSW228 in PAO-SW and PAOΔ*vfr*. Throughout the growth cycle, *toxA* expression in PAOΔ*vfr*/pSW228 was significantly lower than that in PAO-SW/pSW228 (data not shown). As a global regulator, Vfr may regulate other cellular functions that affect ETA levels indirectly, including a general effect on protein synthesis or protein secretion. Wolfgang *et al.* (2003) suggested that a *vfr* mutation affected the type II secretion system through which ETA is secreted by *P. aeruginosa*. Therefore, we examined the level of ETA produced by PAOΔ*vfr*/pAM21-2, in which *toxA* is expressed constitutively from the *lac* promoter. ETA production in PAO1 is deregulated with respect to iron in the presence of pAM21-2 (A. N. Hamood, unpublished results). If *vfr* mutation affects the type II secretion pathway, we would expect to see very little or no ETA in the supernatant fraction of PAOΔ*vfr*/pAM21-2 grown

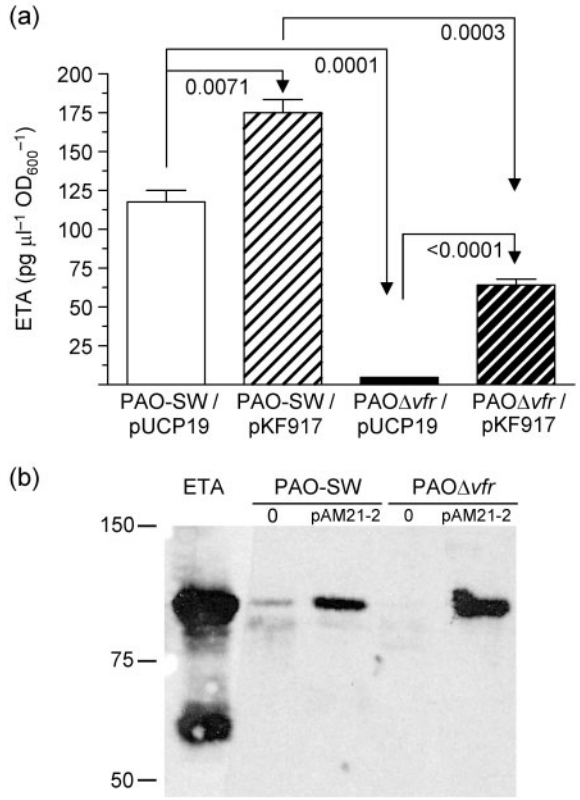

Fig. 1. (a) Vfr is required for optimum production of ETA by PAO1. PAO-SW and PAOΔ*vfr* carrying vector plasmid (pUCP19) or the *vfr* plasmid pKF917 were grown in TSBDC for 14 h at 32 °C. ETA levels within the supernatant were determined by sandwich ELISA. Values were standardized by dividing the amount of ETA in pg µl^{-1} by the OD$_{600}$ of the culture from which the fraction was obtained. Values represent the mean ± SEM of three independent experiments. Unpaired, two-tailed *t*-tests were used to determine statistical significance ($P<0.05$) between pairs. (b) Vfr does not affect ETA secretion. PAO-SW and PAOΔ*vfr* carrying pAM21-2 in which *toxA* is expressed from the *lac* promoter were grown in TSBDC-Fe for 14 h at 32 °C. Supernatants were harvested and concentrated; 10 µg protein was separated by SDS-PAGE and transferred to a membrane. ETA levels within the supernatant fractions were determined by immunoblotting with polyclonal anti-ETA. Molecular mass standards in kDa are indicated on the left; 0, no plasmid present.

under iron-sufficient conditions. However, PAO-SW/pAM21-2 and PAOΔ*vfr*/pAM21-2 produced comparable levels of ETA (Fig. 1b). Thus, Vfr affects *toxA* transcription, but does not affect ETA secretion in PAO1.

Vfr regulates ETA production indirectly through *regA* and directly through *toxA*

The DNA/protein gel-shift analyses reported by Kanack *et al.* (2006) showed that the Vfr-protected sequence within the *regA* upstream region is located 51–77 bp 5′ of the *regA* P1 transcriptional start site. To determine whether Vfr regulates *toxA* expression through *regA*, we utilized pIN10, in which *regA* is expressed from the constitutive *lac* promoter; therefore, *regA* expression is not influenced by any *P. aeruginosa* regulatory factor. The presence of pIN10 in the *regA* deletion mutant PAOΔ*regA* complemented its defect in ETA production and deregulated it with respect to iron (data not shown). As shown in Fig. 2(a), compared with PAO-SW/pUCP19, ETA production by PAO-SW/pIN10 was increased significantly ($P<0.0001$). However, pIN10 did not increase ETA production by PAOΔ*vfr* (Fig. 2a). The failure of overexpressed RegA to complement the defect of PAOΔ*vfr* in ETA production suggests that Vfr does not regulate *toxA* expression solely through *regA*. In addition, the results show that RegA requires a functional

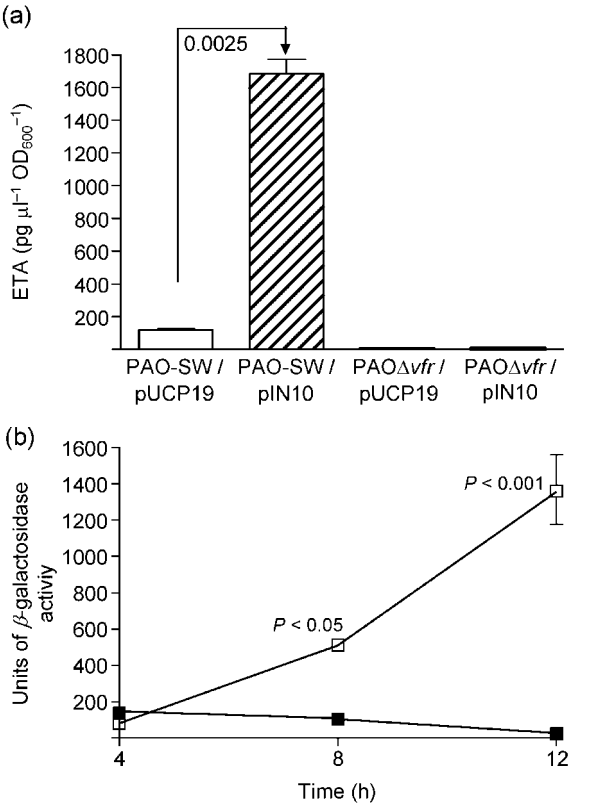

Fig. 2. (a) Constitutive expression of *regA* from the *lac* promoter in pIN10 does not bypass the defect of PAOΔ*vfr* in ETA production. Strains were grown and ETA levels determined as described in the legend to Fig. 1. Values represent the mean ± SEM of three independent experiments. (b) *vfr* mutation interferes with *regA* expression at late stages of growth. PAO-SW/pRL88 (*regA–lacZ* transcriptional fusion plasmid; □) and PAOΔ*vfr*/pRL88 (■) were grown in TSBDC for 12 h; samples were obtained at 4, 8 and 12 h post-inoculation. Cells were pelleted and the level of β-galactosidase activity was determined (Methods). Data were analysed by one-way ANOVA with a Tukey–Kramer multiple comparisons post-test. Values represent the mean ± SEM of three independent experiments.

vfr to induce *toxA* expression in *P. aeruginosa*. Next, we determined whether *vfr* deletion affects *regA* expression in PAO1. Monitoring *regA* expression from the *regA–lacZ* fusion plasmid pRL88 showed that *regA* expression in PAOΔ*vfr* was the same as that in PAO-SW at 4 h. However, at 8 and 12 h, *regA* expression in PAOΔ*vfr* was significantly ($P<0.05$ and <0.001, respectively) lower than that in PAO-SW (Fig. 2b). Therefore, Vfr may regulate *toxA* expression both directly and through *regA*. The Vfr-protected sequence within the *toxA* upstream region is located −53 to −78 bp 5′ of the *toxA* S1a start site (Kanack *et al.*, 2006), supporting the idea that Vfr regulates ETA production in PAO1 directly, bypassing *regA*. To examine this possibility, we determined the effect of the *vfr* plasmid pKF917 on ETA production by PAOΔ*regA*. In both iron-deficient and iron-sufficient medium, pKF917 produced no significant increase in ETA production by PAOΔ*regA* (Supplementary Fig. S1, available with the online version of this paper).

Results of these first three experiments revealed that Vfr regulates both *toxA* and *regA* expression at the transcriptional level, as the *vfr* deletion reduced expression of both genes significantly (Fig. 2b; data not shown). However, the failure of the *vfr* plasmid to enhance ETA production in PAOΔ*regA* indicates that RegA is essential for the enhancement in ETA production by Vfr.

Vfr does not regulate *pvdS*; neither does it regulate *toxA* through *pvdS*

Evidence indicates that ETA production in *P. aeruginosa* is regulated positively by the iron-starvation sigma factor PvdS. In the absence of functional PvdS, ETA production and *toxA* expression in *P. aeruginosa* are reduced considerably (Ochsner *et al.*, 1996). Whilst the *toxA* and *regA* upstream regions contain the Vfr-binding consensus, this sequence is not present in the *pvdS* upstream region (Kanack *et al.*, 2006), suggesting that Vfr does not regulate *pvdS* directly. Thus, we investigated whether Vfr regulates ETA production through *pvdS*.

First, we examined the effect of *vfr* mutation on *pvdS* transcription in PAO1. PAO-SW and PAOΔ*vfr* carrying the *pvdS–lacZ* transcriptional fusion plasmid pMP220::P*pvdS* were grown in iron-deficient medium and the level of *pvdS* expression was assessed at 4, 8, 12 and 16 h. As shown in Fig. 3(a), at 4 h (early stage of growth), PAO-SW/pMP220::P*pvdS* and PAOΔ*vfr*/pMP220::P*pvdS* showed low levels of *pvdS* expression. However, by 8 h, *pvdS* expression in PAO-SW increased sharply and continued unchanged until the 16 h time point (late stage of growth) (Fig. 3a). In contrast, the level of *pvdS* expression in PAOΔ*vfr*/pMP220::P*pvdS* increased until the 12 h time point and then declined to a level similar to that seen at 8 h (Fig. 3a). These results suggest that *pvdS* expression in PAO1 does not require functional Vfr. To confirm these results and to exclude the possibility that Vfr regulates *pvdS* expression post-transcriptionally, we measured the amount

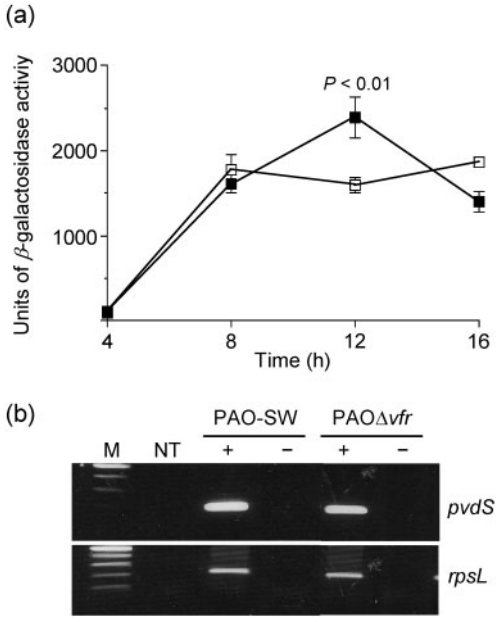

Fig. 3. *vfr* mutation does not affect *pvdS* expression. (a) PAO-SW (□) and PAOΔ*vfr* carrying the *pvdS–lacZ* transcriptional fusion plasmid pMP220::P*pvdS* (■) were grown in TSBDC for 16 h at 32 °C; samples were obtained at 4, 8, 12 and 16 h and the level of β-galactosidase was determined as described in the legend to Fig. 2. Values represent the mean ± SEM of three independent experiments. (b) Total RNA was extracted from PAO-SW and PAOΔ*vfr* grown in TSBDC for 14 h at 32 °C and the accumulation of *pvdS* mRNA was determined by RT-PCR. Transcript accumulation of *rpsL* was examined as a positive control. M, DNA size marker; NT, no-template control; −, no reverse transcriptase; +, with reverse transcriptase.

of accumulated *pvdS* mRNA in PAO-SW and PAOΔ*vfr* by using RT-PCR. As shown in Fig. 3(b), there was no apparent difference in the amount of the accumulated *pvdS* mRNA between PAO-SW and PAOΔ*vfr* under iron-deficient conditions.

To explore further the relationship between PvdS and Vfr in regulating ETA production, we determined whether increased levels of Vfr would compensate for the loss of functional PvdS in PAO1 (PAOΔ*pvdS*). As shown in Fig. 4, in both iron-deficient and iron-sufficient media, PAOΔ*pvdS*/pKF917 produced significantly higher levels of ETA than PAOΔ*pvdS*/pUCP19 ($P=0.0009$ and 0.0001, respectively). The level of ETA produced by PAOΔ*pvdS*/pKF917 in iron-deficient medium paralleled the amount produced in iron-sufficient medium (Fig. 4). Comparison of these results with those shown in Fig. 1 suggests that, in the absence of functional PvdS, the effect of Vfr on ETA production is limited. In addition, whilst iron represses ETA production in PAO-SW/pKF917, it does not in PAOΔ*pvdS*/pKF917, indicating that Vfr is not one of the factors through which iron regulates ETA production in *P. aeruginosa*.

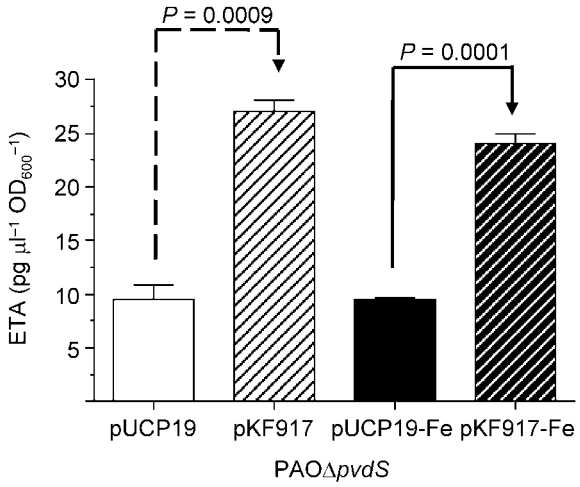

Fig. 4. Overexpression of Vfr enhances ETA production under low-iron and high-iron conditions in PAOΔ*pvdS*. Strains were grown in TSBDC and TSBDC-Fe and ETA levels were determined as described in the legend to Fig. 1. Values represent the mean ± SEM of three independent experiments.

We also excluded the possibility that Vfr regulates ETA production through *pvdS* by examining the effect of pPVD31, in which *pvdS* is expressed constitutively from the *tac* promoter, on ETA production in PAOΔ*vfr*. Similarly to *regA* expression in pIN10, *pvdS* expression in pPVD31 is not influenced by any *P. aeruginosa* regulator. Ochsner *et al.* (1996) showed that pPVD31 deregulated ETA production in PAO1 with respect to iron. Plasmid pPVD31 did not affect ETA production by PAOΔ*vfr* in iron-deficient or iron-sufficient medium, suggesting that Vfr does not regulate ETA production in PAO1 through *pvdS* (Fig. 5).

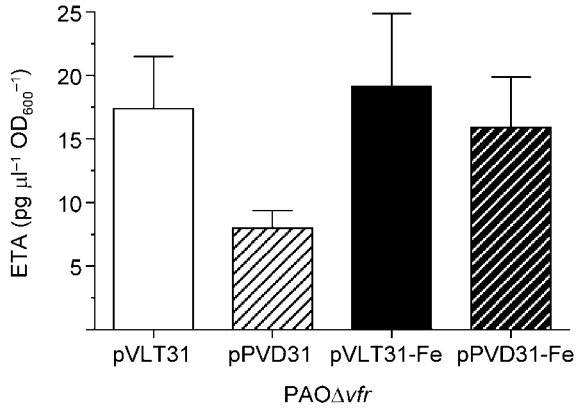

Fig. 5. Overexpression of PvdS does not increase ETA production in PAOΔ*vfr* under low-iron or high-iron conditions. Strains were grown and ETA levels determined as described in the legend to Fig. 1. Values represent the mean ± SEM of three independent experiments. There were no significant differences in the levels of ETA produced by PAOΔ*vfr* carrying either plasmid.

These experiments clarify the relationship of Vfr to *pvdS* and *toxA*. Firstly, Vfr does not regulate *pvdS*; levels of *pvdS* expression, as well as the accumulation of *pvdS* mRNA, were comparable in PAO1 and PAOΔ*vfr* (Fig. 3a, b). Secondly, whilst an increase in the level of Vfr produced a limited increase in ETA production in PAOΔ*pvdS* (Fig. 4), *pvdS* overexpression did not affect ETA production by PAOΔ*vfr* (Fig. 5). Finally, overexpression of Vfr in PAOΔ*pvdS* enhanced ETA production, irrespective of the presence of iron (Fig. 4).

These results also suggest that, unlike *toxA*, *regA* and *pvdS*, *vfr* may not be regulated by iron. To address this possibility, we examined the accumulation of *vfr* mRNA in PAO-SW that was grown in iron-deficient or iron-sufficient medium. As shown in Fig. 6(a), there was no apparent difference in the accumulation of *vfr* mRNA in the presence or absence of iron. As a control, we monitored the accumulation of *toxA* mRNA in PAO-SW under those same conditions (Fig. 6b). Thus, Vfr regulation of *regA* and *toxA* may be responsible for the low level of ETA production that occurs under high-iron conditions.

Vfr does not affect pyoverdine production in PAO1

As Vfr overrides *pvdS* to regulate ETA production, we determined whether Vfr affects the regulation of another PvdS-regulated virulence factor, pyoverdine. Compared with its parent strain, expression of the pyoverdine genes and the level of pyoverdine produced are reduced significantly in a PAO1 *pvdS* deletion mutant (Cunliffe *et al.*, 1995; Leoni *et al.*, 2000). We utilized the *pvdE–lacZ* fusion plasmid pMP190::P*pvdE* to examine the effect of Vfr on the expression of the pyoverdine genes. Transcriptional analysis revealed that the level of *pvdE* expression in PAOΔ*vfr* was similar to that by PAO-SW (data not shown). Additionally, the presence of pKF917 in PAOΔ*pvdS* failed to complement the defect in pyoverdine production (data not shown). Furthermore, whilst *pvdS* expressed constitutively from pPVD31 failed to affect ETA production in PAOΔ*vfr*, it deregulated pyoverdine production with respect to iron (data not shown). These results suggest that Vfr affects ETA production directly, but has no effect on pyoverdine production.

Conclusion

Results of this study indicate that Vfr, RegA and PvdS are required for optimum ETA production in *P. aeruginosa* (Fig. 7). Based on these results, we propose a model for *toxA* regulation by Vfr under iron-deficient (Fig. 7b) and iron-sufficient (Fig. 7c) conditions. PvdS binds to a specific sequence within the upstream region of several genes, including *toxA*, *regA* and the pyoverdine genes (Leoni *et al.*, 2000; Wilson *et al.*, 2001). Thus, binding of the PvdS-recruited core RNAP (PvdS–RNAP complex) to this sequence enhances the transcription of these genes. In

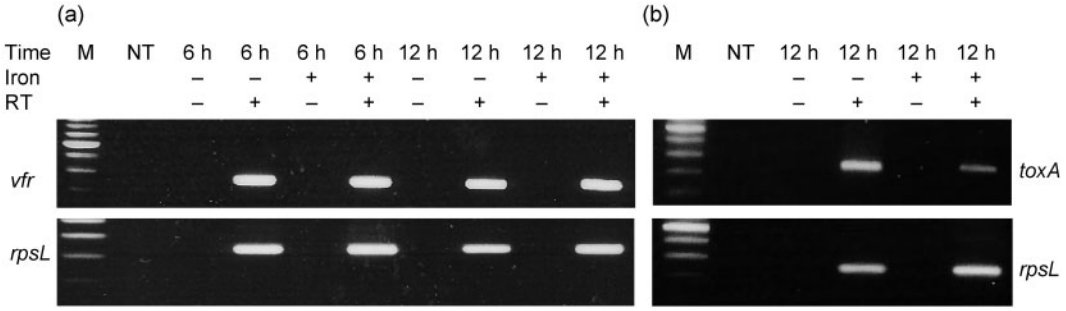

Fig. 6. (a) Accumulation of *vfr* mRNA in PAO-SW is not influenced by the level of iron in the growth medium. (b) Accumulation of *toxA* mRNA in PAO-SW is influenced by the level of iron in the growth medium (a control representing an iron-regulated gene). Total RNA was extracted from PAO-SW grown in TSBDC or TSBDC-Fe at 32 °C for 6 or 12 h (for *vfr*) or 12 h (for *toxA*) and the accumulation of mRNA was determined by RT-PCR. Transcript accumulation of *rpsL* was examined as a positive control. M, DNA size marker; NT, no-template control; RT, reverse transcriptase; −, absence of iron and/or RT; +, presence of iron and/or RT.

low-iron medium and under aerobic conditions, both the PvdS–RNAP complex and Vfr bind to the *regA* upstream region, leading to an increase in *regA* transcription and the level of RegA protein, which leads to a moderate level of ETA production (Fig. 7b). Under the same conditions, the PvdS–RNAP complex would bind efficiently to the *toxA* promoter and enhance *toxA* transcription. This binding is strengthened further by the binding of Vfr and its

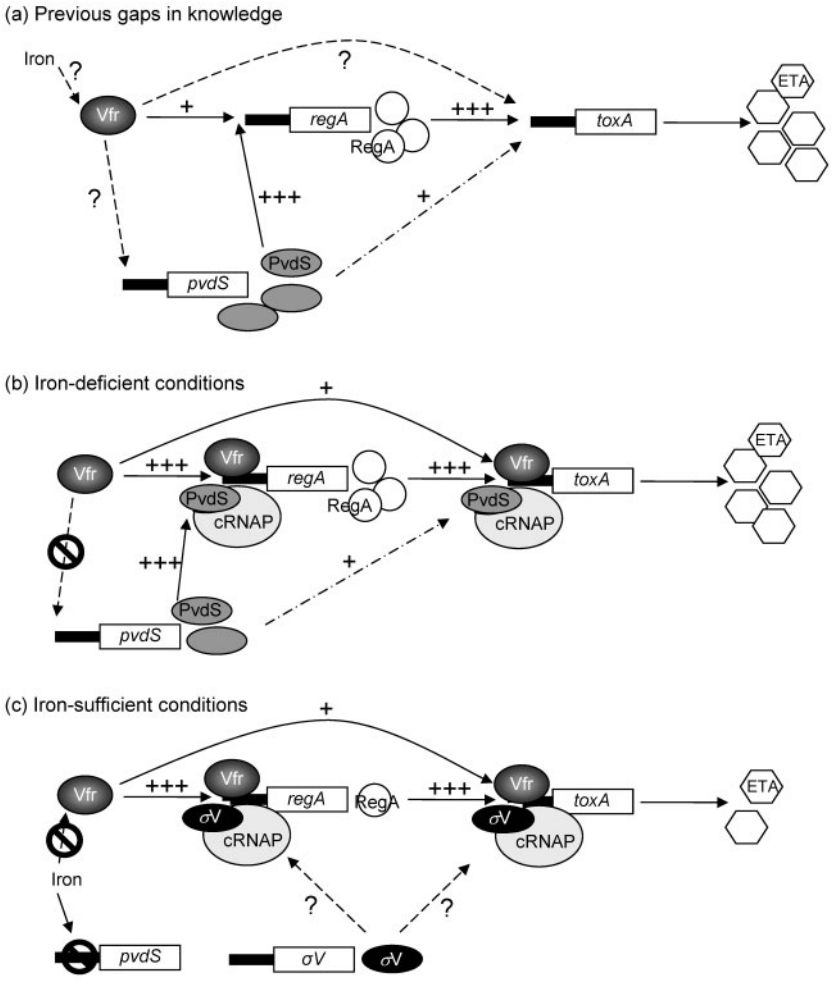

Fig. 7. (a) Diagram illustrating the current knowledge and existing gaps in our understanding of the complicated interactions between different *toxA*-regulatory genes. (b, c) Modification of (a) based on the results obtained in the present study. (b) Interactions between the *toxA*-regulatory genes under iron-deficient conditions. (c) Interactions of these genes under iron-sufficient conditions. ? indicates a previously undetermined mechanism of regulation; a dotted arrow indicates a potential regulatory pathway based only on DNA-binding studies with the PvdS–RNAP complex and the *toxA* upstream region. cRNAP, Core RNA polymerase; σV, any sigma factor other than PvdS.

interaction with the PvdS–RNAP complex, producing the maximum level of ETA. However, in the absence of RegA, binding of PvdS and Vfr to the *toxA* upstream region is not sufficient to enhance ETA production. Therefore, maximum expression of *toxA* occurs when functional Vfr, PvdS and RegA are present (Fig. 7b).

Under iron-sufficient conditions, iron–Fur represses *pvdS* expression and reduces the cellular level of PvdS severely. Consequently, PvdS will be replaced within the RNAP complex by another sigma factor that recognizes the promoters of *toxA* and *regA* less efficiently, resulting in a reduced level of *toxA* expression (Fig. 7c). However, binding of Vfr to the *toxA* and *regA* upstream region would not be altered by iron, i.e. Vfr binds at the same efficiency under iron-deficient and iron-sufficient conditions. Accordingly, an increase in the intracellular concentration of Vfr would enhance the binding of the RNAP complex to the *toxA* and *regA* promoter regions (Fig. 7c). This effect still depends on RegA. Under iron-sufficient conditions, a basal level of RegA exists in *P. aeruginosa*. The ability of sigma factors other than PvdS to transcribe the *toxA* promoter is supported by the findings of Walker *et al.* (1995), who showed that the *E. coli* RNAP holoenzyme recognizes the *toxA* promoter and transcribes *toxA* in an *in vitro* transcription system.

The effect of *vfr* and other genes on ETA production by *P. aeruginosa* has been examined in several studies. Our current understanding of the interaction between these genes and/or their products in regulating ETA production is outlined in the Introduction. Despite the wealth of knowledge in this area, several gaps still exist (Fig. 7a). For example, it is not known whether Vfr regulates ETA production directly or indirectly (through *regA*, *pvdS* or both), whether Vfr requires RegA, PvdS or both for its effect on ETA production, or whether iron interferes with the enhancement of ETA production by Vfr (Fig. 7a). As shown in Fig. 7(b, c), information provided by the present study fills these gaps. Our results provide the following new critical components. (i) Vfr does not enhance ETA production through PvdS (Fig. 7b). In the absence of PvdS, Vfr still enhances ETA production (Fig. 4). However, this effect is less dramatic than that in the presence of functional PvdS (Figs 1 and 4). Thus, maximum production of ETA requires the function of PvdS and Vfr. (ii) Vfr does not enhance ETA production through RegA (Fig. 7b). However, a minimal level of RegA is essential for the effect of Vfr on ETA production. Under conditions in which very low levels of RegA are produced, such as in iron-sufficient medium (Frank *et al.*, 1989) or when *pvdS* has been mutated, Vfr enhances ETA production (Fig. 4). On the other hand, in the absence of functional RegA (PAOΔ*regA*), Vfr does not enhance ETA production (Supplementary Fig. S1). (iii) Vfr enhances the expression of *toxA* and *regA*, possibly by binding to the upstream region of each gene, but does not enhance *pvdS* expression (Figs 2b, 7b) (West *et al.*, 1994a). (iv) Vfr enhances ETA production under iron-sufficient conditions (Fig. 4), indicating that *vfr* is not part of the regulatory network through which iron regulates ETA production (Fig. 7c). This is due to the fact that, unlike *toxA*, *regA* and *pvdS* expression, *vfr* expression is not repressed by iron (Fig. 6) (Ochsner *et al.*, 2002; Palma *et al.*, 2003). In summary, our results indicate that maximum production of ETA by *P. aeruginosa* requires the cooperation of PvdS and Vfr, as well as the presence of functional RegA.

ACKNOWLEDGEMENTS

The authors would like to thank Douglas Storey, Michael Vasil, Iain Lamont, Herbert Schweizer, Paolo Visca and Susan West for the plasmids and strains. This work was supported by grant AI33386 from the National Institutes of Health to A. N. H. The authors thank Joanna Swickard for her assistance in editing the manuscript.

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Edited by: M. A. Curtis