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Phosphate starvation relayed by PhoB activates the expression of the *Pseudomonas aeruginosa* σ^{vrel} ECF factor and its target genes

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The cell-surface signalling (CSS) system represents an important regulatory mechanism by which Gram-negative bacteria respond to the environment. Gene regulation by CSS systems is particularly present and important in the opportunistic human pathogen *Pseudomonas aeruginosa*. In this bacterium, these mechanisms regulate mainly the uptake of iron, but also virulence functions. The latter is the case for the *P. aeruginosa* PUMA3 CSS system formed by the putative VreA receptor, the σ^{Vrel} extracytoplasmic function sigma factor and the VreR anti-sigma factor. A role for this system in *P. aeruginosa* virulence has been demonstrated previously. However, the conditions under which this system is expressed and activated have not been elucidated so far. In this work, we have identified and characterized the global regulatory cascade activating the expression of the PUMA3 system. We show that the PhoB transcriptional regulator, part of the PhoB-PhoR two-component signalling system, can sense a limitation of inorganic phosphate to turn on the expression of the *vreA*, *vreI* and *vreR* genes, which constitute an operon. Upon expression of these genes in this condition, σ^{Vrel} factor mediates transcription of most, but not all, of the previously identified σ^{Vrel}-regulated genes. Indeed, we found new σ^{Vrel}-targeted genes and we show that σ^{Vrel}-regulon genes are all located immediately downstream to the *vreAIR* gene cluster.

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INTRODUCTION

Pseudomonas aeruginosa is the third most frequent pathogen associated with nosocomial infections and the principal one for cystic fibrosis (CF) patients. Its pathogenicity depends on its unique capacity to adapt to diverse environments by switching from a planktonic (swarming motility) to sedentary lifestyle (biofilms formation) (Bordi & de Bentzmann, 2011) and on its production of an arsenal of proteins implicated in virulence. Efficient adaptation to environmental changes comes from sophisticated regulatory networks, some of which involve sigma factors that allow tight regulation of gene expression. Sigma factors are small proteins that associate with the RNA polymerase core enzyme and direct it to specific promoter sequences, thus initiating gene transcription. All bacteria contain a constitutively expressed primary sigma factor (σ^{70}), responsible for the expression of housekeeping genes, and a number of alternative sigma factors that are activated by suitable stimuli and control differential gene expression. The largest group of alternative sigma factors includes the so-called ECF (extracytoplasmic function) sigma factors (Lonetto *et al.*, 1994), which respond to signals from within the cell envelope or from outside the cell to activate genes whose products are involved in cell envelope functions, such as secretion, iron transport or stress responses (Bastiaansen *et al.*, 2012). *P. aeruginosa* contains a large number of ECF sigma factors (Potvin *et al.*, 2008), which are post-transcriptionally activated in response to an environmental signal in contrast to constitutively activated σ^{70} factor. In Gram-negative bacteria, the most common and important mechanism for the control of ECF sigma factor activity is the so-called cell-surface signalling (CSS) regulatory system (Braun *et al.*, 2006). This system is composed not only of the

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Abbreviations: CF, cystic fibrosis; CSS, cell-surface signalling; ECF, extracytoplasmic function; Fur, ferric uptake regulator; Pi, inorganic phosphate; PIA, *Pseudomonas* isolation agar; Pst, phosphate-specific ABC transport; TU, transcriptional unit.

ECF sigma factor but also by an outer membrane receptor and an anti-sigma factor, located in the inner membrane, which binds the ECF sigma factor, preventing its binding to the RNA polymerase. Receptors belong to a subfamily of TonB-dependent receptors that contain an N-terminal extension of ~80 amino acids, which interacts in the periplasm with the inner membrane anti-sigma factor to determine the specificity of the transduction pathway (Koebnik, 2005; Schalk *et al.*, 2004). The signalling cascade starts by binding of the inducing signal to its cognate TonB-dependent outer membrane receptor, which transduces the signal to the anti-sigma factor, leading to the release and activation of the sigma factor. Once activated, the ECF sigma factor binds the RNA polymerase core enzyme and directs it to the promoter of a small number of genes. Most *P. aeruginosa* surface signalling systems regulate iron uptake via siderophores, haemophores or iron-citrate (Llamas & Bitter, 2010) and their expression is usually controlled by iron via the Fur (ferric uptake regulator) repressor protein (Escolar *et al.*, 1999). These systems need both iron limitation to be synthesized and the presence of the inducing signal (i.e. ferrisiderophore) to be activated. In *P. aeruginosa*, the CSS system, termed the PUMA3 system, which is composed of the σ^{VreI} factor, the VreR anti-sigma factor and the receptor component VreA, is an unusual CSS system. It seems to be involved not in the regulation of iron uptake but in the control of *P. aeruginosa* virulence (Llamas *et al.*, 2009). The most obvious specific characteristic of this system is the receptor component VreA, which is considerably smaller than other receptors because of the lack of the 22-strand β-barrel outer membrane domain of TonB-dependent receptors and is therefore not located in the outer membrane but in the periplasm (Llamas *et al.*, 2009). However, VreA contains the typical N-terminal extension of surface signalling receptors. As a consequence, VreA seems to be involved in signalling but not in the transport of the signal (i.e. ferrisiderophore).

Microarray analysis of cells overproducing σ^{VreI} factor revealed 27 PUMA3 target genes, all positively regulated and most of them genetically associated with the PUMA3 gene cluster (Fig. 1 and Llamas *et al.*, 2009). Among those are genes encoding the type II Hxc secretion system and LapA (low-molecular mass alkaline phosphatase A), the main substrate of this secretion system. Interestingly, expression of these genes is positively regulated by inorganic phosphate (Pi) limitation (Ball *et al.*, 2002) and a link between regulation by σ^{VreI} and phosphate could exist. Pi limitation, as iron starvation, is an environmental condition that turns on the expression of genes encoding nutrient transport systems, but also genes implicated in virulence (Guerinot, 1994; Lamarche *et al.*, 2008). The level of Pi in the environment is sensed by the phosphate-specific ABC transport (Pst) system. Upon phosphate limitation, the Pst system mediates Pi transport and activates expression of the Pho regulon via the two-component PhoR-PhoB signal transduction system (Lamarche *et al.*, 2008). This regulatory system is inactive in excess of Pi through the interaction of the Pst component PhoU with PhoR. Low phosphate concentration relieves this interaction and promotes autophosphorylation of PhoR, the transmembrane histidine kinase sensor, which subsequently donates its phosphate group to its cognate response regulator PhoB. Phosphorylated PhoB controls the expression of a large set of genes by binding to a *pho* box, a 22 bp specific DNA sequence on the promoter region of the phosphate-limitation regulon genes (Blanco *et al.*, 2002).

In the present study, we have examined the link between phosphate-limiting conditions and the induction of the PUMA3 system in *P. aeruginosa*. Using reporter fusion and quantitative reverse transcriptase-PCR (RT-PCR) assays, we show that the PUMA3 genes (*vreA, vreI* and *vreR*) form an operon, whose expression is activated in low phosphate condition through the PhoB transcriptional regulator protein. We also show that, in this condition, both the PhoB and the σ^{VreI} proteins regulate the expression of 25 genes, all located immediately downstream to the PUMA3 locus. Based on our results we propose a model in which, in response to Pi limitation, PhoB enhances expression of the PUMA3 genes resulting in the production of σ^{VreI}. Free σ^{VreI} binds to the RNA polymerase and then mediates transcription of the low Pi induced σ^{VreI}-regulated genes.

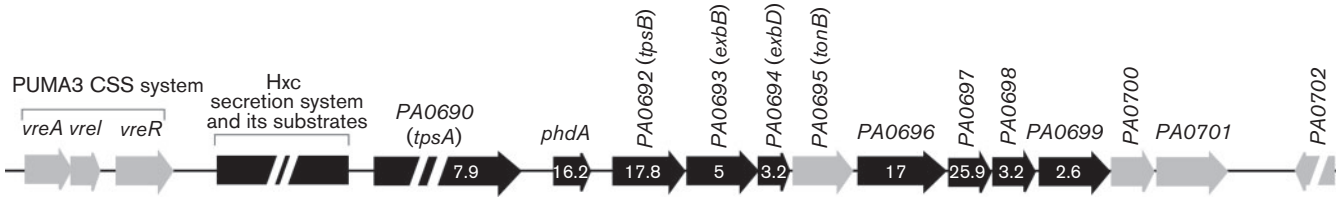


Fig. 1. Genetic organization of genes located downstream of the PUMA3 locus. Black arrows represent genes regulated by σ^{VreI} (Llamas *et al.*, 2009) with their name or their respective PA number according to the PA01 genome annotation (http://www.pseudomonas.com and Winsor *et al.*, 2011). Numbers inside the genes indicate the fold change obtained by microarray analysis (Llamas *et al.*, 2009). Grey arrows represent the neighbouring genes.

METHODS

Bacterial strains culture conditions. Strains used in this study are listed in Table 1. Strains were grown at 37 °C in Luria-Bertani (LB) medium. As required strains were cultured in low and high Pi medium. Low Pi medium [3 g l^{-1} proteose peptone (Difco Laboratories), 100 mM HEPES, 20 mM NH_4Cl, 20 mM KCl, 3.2 mM $MgCl_2$] contained 0.4 % glucose and was adjusted to pH 7.2. For high Pi medium, 10 mM of KH_2PO_4 was added to the low Pi medium. *Escherichia coli* strains were used to propagate plasmids. Plasmids were introduced into *P. aeruginosa* using the conjugative properties of pRK2013 and pRK600 plasmids (de Lorenzo & Timmis, 1994; Figurski & Helinski, 1979) or by electroporation (Choi & Schweizer, 2006). *P. aeruginosa* transconjugants were screened on *Pseudomonas* isolation agar (PIA; Difco Laboratories) supplemented with appropriate antibiotics. The following antibiotic concentrations were used. For *E. coli*: ampicillin, streptomycin, tetracycline, 15 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; gentamicin, 20 μg ml^{-1}. For *P. aeruginosa*: carbenicillin, 300 μg ml^{-1}; streptomycin, 2 mg ml^{-1}; tetracycline, 200 μg ml^{-1}; gentamicin, 75 μg ml^{-1}.

Molecular cloning and plasmids. Plasmids and primers are listed in Tables 1 and 2, respectively. PCR amplification on genomic *P. aeruginosa* PA01 DNA was performed using Phusion Hot Start

Table 1. Bacterial strains and plasmids

Ap^R, Km^R, Sm^R, Tc^R, Gm^R, resistance to ampicillin, kanamycin, streptomycin, tetracycline and gentamicin, respectively.

Strain/plasmid	Relevant characteristics	Source
Strains		
E. coli		
Top10	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC)* φ*80lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ*(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1* λ⁻	Invitrogen
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, Km^R	de Bentzmann's lab collection
CC118λpir	*araD139* Δ*(ara leu)7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argE(Am) recA1,* λ*pir* lysogen	Herrero *et al.* (1990)
P. aeruginosa		
PAO1	Wild-type, prototroph	Lab collection
p*PAO690*::*lacZ*	PAO1 with the plasmid p*PAO690*::*lacZ* transcriptional fusion inserted at *attB* site	This study
p*phdA*::*lacZ*	PAO1 with the plasmid p*phdA*::*lacZ* transcriptional fusion inserted at *attB* site	This study
p*PAO692*::*lacZ*	PAO1 with the plasmid p*PAO692*::*lacZ* transcriptional fusion inserted at *attB* site	This study
Δ*vreI*	Chromosomal *vreI* deletion in PAO1	This study
Δ*phoB*	Chromosomal *phoB* deletion in PAO1	This study
Plasmids		
pRK2013	*ori* ColE1, *tra*$^+$ *mob*$^+$, Km^R	Figurski & Helinski (1979)
pRK600	Helper plasmid, oriColE1 mobRK2 traRK2; Cm^R	de Lorenzo & Timmis (1994)
pCR2.1	TA cloning vector for PCR products, *lacZ*α ColE1 f1 *ori*, Ap^R Km^R	Invitrogen
miniCTX-l*acZ*	Tcr *lacZ*+; self-proficient integration vector with *tet*, V-*FRT*-*att*PMCS, *ori, int* and *oriT*; Tc^R	This study
miniCTX-p*PAO690*::*lacZ*	*PAO690* promoter fragment cloned upstream of *lacZ* gene in miniCTX-*lacZ*; Tc^R	This study
miniCTX-p*phdA*::*lacZ*	*phdA* promoter fragment cloned upstream of *lacZ* gene in miniCTX-*lacZ*; Tc^R	This study
miniCTX-p*PAO692*::*lacZ*	*PAO692* promoter fragment cloned upstream of *lacZ* gene in miniCTX-*lacZ*; Tc^R	This study
pMP220	*IncP* broad-host-range *lacZ* fusion vector; Tc^R	Canter Cremers *et al.* (1989)
pMPR3	*vreA* promoter fragment cloned upstream of the *lacZ* gene in pMP220	This study
pMPR3*mut*Pho	*vreA* promoter fragment with a mutated *pho* box cloned upstream of the *lacZ* gene in pMP220	This study
pKNG101	Suicide vector in *P. aeruginosa*; *sacB*, Sm^R	Kaniga *et al.* (1991)
pKNG101-Δ*vreI*	*vreI* deletion construct	This study
pKNG101-Δ*phoB*	*phoB* deletion construct	This study
pFLP2	Site-specific excision vector, Ap^R	Hoang *et al.* (1998)
pJN105	*araC-*P_{BAD} cassette cloned in pBBR1MCS-5; Gm^R	Newman & Fuqua (1999)
pUC18-miniTn7T-LAC	pUC18 vector; construction of strains with chromosomal P_{LAC} expression cassette; Ap^R Gm^R	Choi & Schweizer (2006)
pUC18-miniTn7T-LAC-*vreI*	pUC18-miniTn7T-LAC vector carrying in *Bam*HI/*Spe*I the 1.3 kb PCR fragment containing the *vreI* gene	This study
pJN105-vreI	*vreI* gene cloned into pJN105	This study

Table 2. Oligonucleotides used in this study

Fw, forward; Rv, reverse; restriction sites are underlined; complementary regions are in bold.

Name	Sequence 5′ to 3′	Description
Transcriptional fusions		
SB3	Attctcgaggccagtgctacaaggat	*PAO690* promoter with *Xho*I/*Bam*HI sites
SB4	Taggatccgcgtaaagcgcgcggga	
SB45	attctcgagAGGCGCGCAGCCAATTGACC	*phdA* promoter with *Xho*I/*Bam*HI sites
SB46	taggatccGGCGCTGATCAAGTTCCTGC	
SB47	attctcgagAACTGCTGTACGCGATTCCC	*PAO692* promoter with *Xho*I/*Bam*HI sites
SB48	taggatccAAACGGGTGCGCACAATTGA	
PR35E	AAAGAATTCATTCATAGGACAAAGCC	*vreA* promoter with *Eco*RI/*Xba*I sites
PR33X	AAATCTAGAACCGAGCAACGACACTG	
mutPhoR	TAGTATGTGGTTAGTTGGTACCTGCGGTGACATG	Mutation *pho* box in the *vreA* promoter
mutPhoF	CCAACTAACCACATACTAACAGTGCCATCAGGATG	
Gene deletion constructs		
SB57	agtactagtCGCGCTACACCAGCCAGGAA	Fw primer *vreI* mutant with *Spe*I site
SB58	**gcttatgTCATGAC**CCTCCCACTCCCT	Rv and overlapped primer *vreI* mutant
SB59	**gtcatgaCATAAGC**ATAAGCAGGTGGAAAACC	Fw and overlapped primer *vreI* mutant
SB60	ttaggatccGCAAGACGAACAACGCCACC	Rv primer *vreI* mutant with *Bam*HI site
SB83	agtactagtAAGGGGCTTGCCGTAGTAGG	Fw primer *phoB* mutant with *Spe*I site
SB84	**agctcttCATGGTC**TTGCCTCGGGTCG	Rv and overlapped primer *phoB* mutant
SB85	**gaccatgAAGAGCT**GACCCCGCTCCCG	Fw and overlapped primer *phoB* mutant
SB86	ttaggatccCGGCGAGGTTCCACCATTCC	Rv primer *phoB* mutant with *Bam*HI site
Complementation with pJN105		
SB57	agtactagtCGCGCTACACCAGCCAGGAA	Amplification *vreI* with *Spe*I/*Bam*HI sites from pUC18-mini-Tn7T-LAC
SB60	ttaggatccGCAAGACGAACAACGCCACC	
RT-PCR		
SB128	GCAGCCAATTGACCGATGAA	3′ of *PAO690* gene
SB129	CCGACTTTCGAGTAACTTTC	5′ of *phdA* gene
SB107	CGCGACGGCTGAATATCTA	3′ of *phdA* gene
SB108	AACAGCGAACGCTTCACAC	5′ of *PAO692* gene
SB160	GAACACCGACAAACACGACT	3′ of *PAO692* gene
SB161	CAGCGATGTCAGCAATAAGC	5′ of *PAO693* gene
SB162	AGGTGCATGGCGAAAGC	3′ of *PAO693* gene
SB163	GTCGTCGTCATCGTCGTG	5′ of *PAO694* gene
SB164	ACGGTGCAGTACCAGAAGGT	5′ of *PAO694* gene
SB165	GGGTGTGCTTGGGATCG	3′ of *PAO695* gene
SB166	GACCGACCAGAAGGTGCT	5′ of *PAO695* gene
SB167	CGCCAGTCGGTTCACTTT	3′ of *PAO696* gene
SB168	CTTCGACAAGCGCGTCTAC	5′ of *PAO696* gene
SB169	GCCCAGATCGGTAATGAGAA	3′ of *PAO697* gene
SB170	CGGGTGAAATTCGAAGAGAT	5′ of *PAO697* gene
SB171	CTGTCGACGGAAAGGTGTTC	3′ of *PAO698* gene
SB172	CGGTCAACCCGTCGAT	5′ of *PAO698* gene
SB173	GCTGGCAGATGGCTTGG	5′ of *PAO699* gene
SB174	AACAACGCTACCCTGAGCAT	3′ of *PAO699* gene
SB175	GCATAGGCGCCCCAGA	5′ of *PAO700* gene
SB176	ACTGCGGATTCGCCTCAT	3′ of *PAO700* gene
SB177	ATGCACCACCTCCAAAGAAC	5′ of *PAO701* gene
RT-vreAI-F	GTTGCTGCCGAAGACGACG	3′ of *vreA* gene
RT-vreAI-R	CCAGGTCCTCGGAGCCCAG	5′ of *vreI* gene
RT-vreIR-F	TCGCTTCGGCATTTCCACCC	3′ of *vreI* gene
RT-vreIR-R	TCGGCGGCGGAGTCTGAGGC	5′ of *vreR* gene
qRT-PCR		
SB132	AGGTCGTGCGCAGTGTC	*vreA* gene
SB133	ACCAGAACCGCCATTCC	
SB134	CCGGCAGATTCTCATCG	*vreI* gene

Table 2. cont.

Name	Sequence 5′ to 3′	Description
SB135	CCGAACCTCTGGATGACTTT	
SB137	TCTCTGGCAGGCACTCG	*vreR* gene
SB138	CCGCCGTGACATAGTCG	
SB103	GAGCAGCAGTTGTACGAGCA	*phdA* gene
SB104	TCACTTCCCCGATAATCCTG	
SB105	TACCTGCTCTCCCAGGAATG	*PAO692* gene
SB106	GCTGAAGTCGTCGTCCTGTT	
SB139	GGCCAGTCGGTGTTCG	*PAO693* gene
SB140	GCGCATTGTTGGCGTAG	
SB158	CGCCAGCTATAGCCAGTACC	*PAO695* gene
SB159	GTGATATCCCCCGAGGAAC	
SB141	GCGTGTTGAAGAAGGAACAG	*PAO696* gene
SB142	CACCTGCGGGATATAGGGTA	
SB143	GCGAAGAATCGGGAGATGTA	*PAO697* gene
SB144	TCGACCTGGGTCTTGTAGAG	
SB154	GGCCTTCCTGGAGTCG	*PAO700* gene
SB155	AACGAACGGTCCATCAGC	
SB156	GGAGTACCAGCCGCACAT	*PAO701* gene
SB157	ACCTCGATCACTCCCGACT	
SB150	CTGGACCTCAATCGCTTCC	*PA4192* gene
SB151	CCTCCGCGGGTTTCAG	
SB152	CATCCGACTAGACGTCATC	*PA5403* gene
SB153	GCTGGCAGTCCAGGAAGG	

High-Fidelity DNA Polymerase (Finnzymes) or Expand High Fidelity DNA polymerase (Roche). The pMPR3 *lacZ* transcriptional fusion was constructed by cloning the intergenic region between the *PAO673* and *vreA* genes (containing the entire *vreA* promoter) as an *Eco*RI/*Xba*I PCR fragment into the pMP220 vector (Spaink *et al.*, 1987). The pMPR3*mut*Pho plasmid was made by cloning the same insert in which the *vreA pho* box sequence CCGTCACACCACAGTCACACGA was changed to CCAACTAACCACATACTAACGA by overlapping PCR. All constructs were confirmed by DNA sequencing and transferred from *E. coli* to *P. aeruginosa* by triparental mating or electroporation. pJN105-vreI was constructed by first cloning a 1.3 kb PCR fragment containing the *vreI* gene into the *Bam*HI/*Spe*I sites of the pUC18-mini-Tn7T-LAC plasmid (Choi & Schweizer, 2006). The *Spe*I/*Pst*I DNA fragment from the pUC18-miniTn7T-LAC-vreI plasmid containing *vreI* was then subcloned into the *Nhe*I/*Pst*I sites of pJN105 plasmid (Newman & Fuqua, 1999).

Bacterial strains constructions. The p*PAO690*::*lacZ*, p*phdA*::*lacZ*, p*PAO692*::*lacZ* strains were constructed as follows. DNA fragments corresponding to *PAO690*, *phdA* and *PAO692* putative promoter regions were amplified by PCR as *Bam*HI/*Xho*I fragments and cloned into the miniCTX-*lacZ* plasmid (Hoang *et al.*, 1998, 2000) in front of a promoterless *lacZ* gene. Recombinant plasmids, checked by DNA sequencing, were then transferred from *E. coli* SM10 to *P. aeruginosa* by conjugation (Kaniga *et al.*, 1991) and inserted into the CTX phage *attB* site of the *P. aeruginosa* chromosome. An FRT tetracycline cassette-excision step was performed as described previously (Hoang *et al.*, 1998, 2000). The *vreI* and *phoB* mutant strains were obtained by first constructing the pKNG101-vreI and pKNG101-phoB suicide vectors as follows. Two DNA fragments respectively upstream and downstream of the gene of interest were PCR amplified. The resulting DNA fragments were then used as templates for an overlapping PCR run using the external pair of oligonucleotides. The PCR fragments containing the internal deletion of the gene of interest were digested and cloned into the *Spe*I/*Bam*HI sites of the suicide pKNG101 vector (Kaniga *et al.*, 1991). Once verified by DNA sequencing, pKNG101 recombinants plasmids were transferred from *E. coli* CC118*λpir* to *P. aeruginosa* PAO1 strain by triparental mating using the helper plasmid pRK2013 as previously described (Kaniga *et al.*, 1991), thus leading to the Δ*vreI* and Δ*phoB* strains.

***β*-Galactosidase activity assay.** Strains carrying the *lacZ* transcriptional fusions were grown with agitation at 37 °C in low or high Pi media supplemented with L-arabinose to induce expression from the pJN105 plasmid when indicated and 1 ml of culture was harvested after 300 min. The *β*-galactosidase activity was measured and normalized for the cell density as described previously (Miller, 1972). Each assay was run at least in triplicate and the data given are the mean.

Isolation of total RNA and RT-PCR. Overnight cultures were subcultured and grown at 37 °C for 240 min in low or high Pi media. Total cellular RNA from 1×10^{10} bacteria was isolated using the PureYield RNA Midiprep System (Promega) or the hot phenol method using the Tri Reagent LS (Molecular Research Center) as previously described (Llamas *et al.*, 2008). Contaminating DNA was eliminated by TURBO DNase (Ambion) treatment, and samples were cleaned up and concentrated using the RNeasy kit (Qiagen). Yield and purity of RNA were further evaluated on Nanodrop and Experion devices. RT-PCR was performed on total cellular RNA using the Access RT-PCR system (Promega) or the Titan One-Tube RT-PCR system (Roche) in accordance with the manufacturer's recommendations. For each reaction, 0.5 to 1 μg of total RNA was used. DNA contamination of the RNA samples was ruled out by omission of the reverse transcriptase in the reaction or inactivation at 94 °C for 4 min prior to the RT-PCR. A positive control using *P. aeruginosa* genomic DNA was included. For quantitative reverse transcription, cDNA

synthesis was performed on 2 μg of RNA using the SuperScriptIII first strand synthesis system (Invitrogen). Cycling parameters of the real-time PCR were 98 °C for 2 min, followed by 45 cycles of 98 °C for 5 s and 60 °C for 10 s, ending with 10 min at 95 °C. To determine the amplification kinetics of each product, the fluorescence derived from the incorporation of EvaGreen into the double-stranded PCR products was measured at the end of each cycle using the SoFast EvaGreen Supermix (Bio-Rad). Experiments have been performed at least on two independent clones and the 16S gene was used as control to normalize the results.

RESULTS

PUMA3 genes are regulated by phosphate through the PhoB regulator

As PUMA3 *vreA*, *vreI* and *vreR* genes are not expressed under classic growth culture conditions (i.e. LB rich medium, data not shown), and a link between σ^{VreI} regulation and low Pi could exist (Ball *et al.*, 2002; Llamas *et al.*, 2009), we decided to determine whether the expression of PUMA3 genes depends on phosphate concentration. Therefore, we performed a quantitative RT-PCR (qRT-PCR) on each PUMA3 gene using mRNA isolated from *P. aeruginosa* grown in either high Pi (10 mM phosphate) or low Pi (0.28 mM) conditions (see Methods). This low Pi concentration allowed expression of the well-known low Pi-dependent gene *phoA*, (data not shown and Wanner, 1993) which validates the Pi-limited medium used in this work. Growing wild-type cells in low Pi led to an eight, two and fourfold induction of *vreA*, *vreI* and *vreR* gene expression respectively, compared to the expression of these genes in high Pi (Fig. 2). As phosphate regulation often requires the PhoR-PhoB two-component system, we next tested PUMA3 genes expression in a *phoB* deletion mutant. We found that expression of *vreA, vreI* and *vreR* genes was no longer induced by low Pi, indicating that PUMA3 genes are regulated by low Pi through the PhoB transcriptional regulator (Fig. 2). Finally, we showed that σ^{VreI} does not control the expression of *vreA* and *vreR* genes as expression of these genes can be detected by qRT-PCR in a *vreI* mutant (Fig. 2). As *vre* genes respond identically to a low Pi concentration and *vreA* and *vreI* genes overlap by 4 bp (Fig. 3a), we next tested if these genes form an operon. RT-PCR was performed on *P. aeruginosa* total RNA upon growth in low Pi medium using primers that amplify the *vreA-vreI* overlapping and *vreI-vreR* intergenic regions (Fig. 3a). cDNA bands of the expected sizes were obtained when the RT-PCR was performed using active reverse transcriptase but not when this enzyme was previously heat inactivated (Fig. 3b). These results show that the three components of the PUMA3 system, the *vreA, vreI* and *vreR* genes, form an operon and are likely transcribed as a polycistronic mRNA from the same promoter. To confirm this, we placed an ~300 bp DNA region upstream *vreA*, which likely contains the *vreAIR* promoter, in front of a promoterless *lacZ* gene (pMPR3 plasmid), and examined the activity of this promoter by β-galactosidase assays. As shown in Fig. 3c, activity of this promoter was ~20-fold higher in low Pi growth conditions as compared to high Pi growth conditions and no promoter activity was observed in the *phoB* mutant. This confirmed the qRT-PCR results and the induction of the PUMA3 gene expression by low Pi through the PhoB protein. Furthermore, such induction is independent on σ^{VreI} factor itself since the promoter activity did not change in a *vreI* mutant (Fig. 3c). This is in agreement with qRT-PCR results (Fig. 2) and previous results showing that σ^{VreI} does not autoregulate its own expression (Llamas *et al.*, 2009).

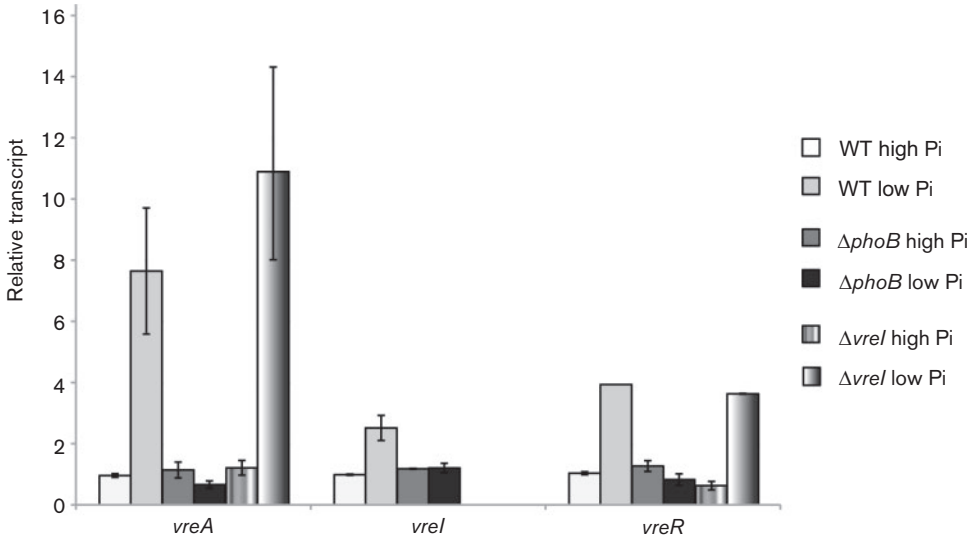


Fig. 2. Analysis of PUMA3 genes expression. *vreA*, *vreI* and *vreR* genes expression was monitored by qRT-PCR on cDNA from different strains grown in high or low Pi medium for 300 min. For each gene, bars show means and SD of the relative transcript amounts normalized to the high Pi condition obtained from at least two independent cDNA.

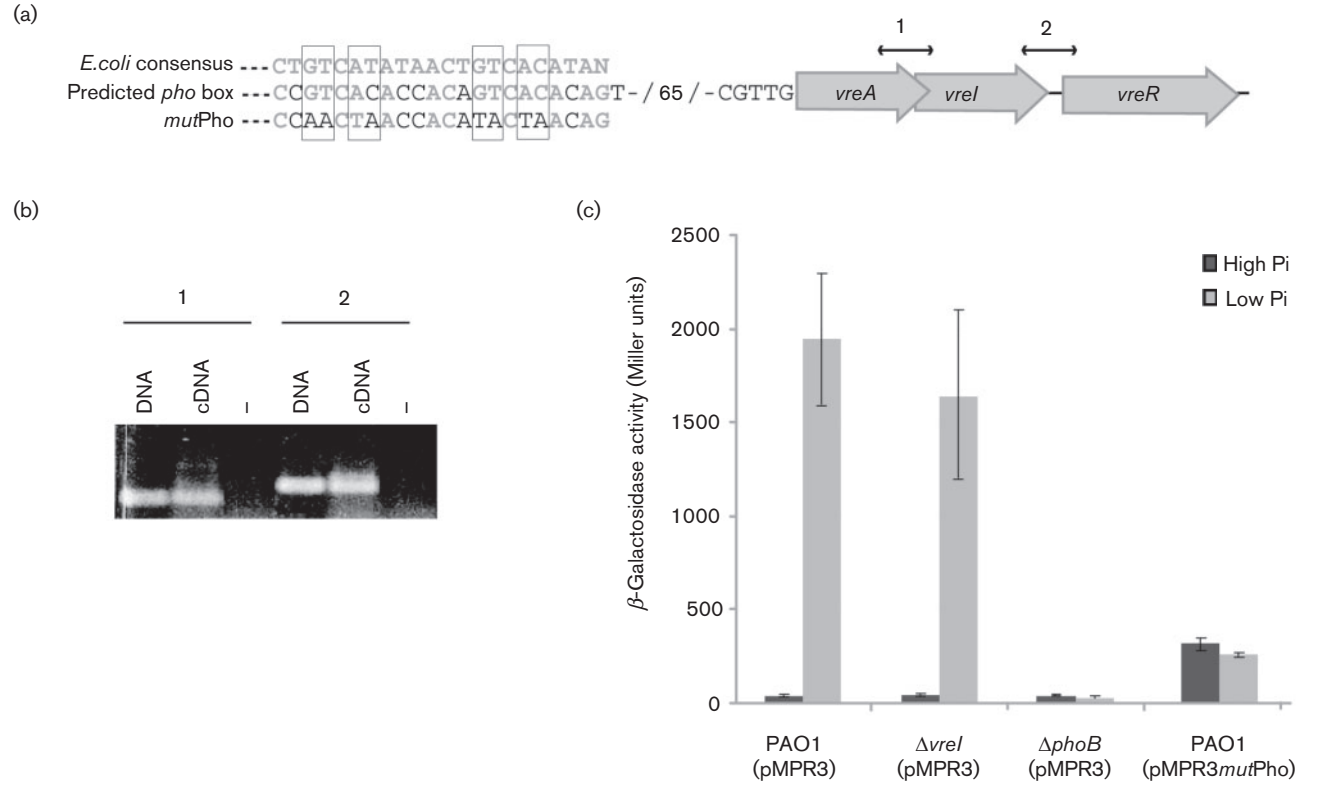


Fig. 3. Co-transcription of *vreAIR* genes and analysis of their promoter region. (a) Sequence of the putative *pho* box in the *vreAIR* regulatory region is aligned with the *E. coli pho* box consensus sequence (top). Residues matching with this consensus are indicated in grey. Bottom sequence represents the mutated *pho* box in which mutated nucleotides are boxed. (b) Amplification by RT-PCR of the cDNA corresponding to the *vreA-vreI* and *vreI-vreR* intergenic regions (arrows 1 and 2 depicted in (a) respectively). Positive controls (DNA) using *P. aeruginosa* genomic DNA and negative controls (–) containing the same amounts of RNA, primers and inactivated reverse transcriptase, are included in this assay. (c) *P. aeruginosa* PAO1 and its isogenic *phoB* mutant containing the transcriptional fusion pMPR3, in which the *vreAIR* promoter is fused to *lacZ*, or pMPR3*mut*Pho, containing the whole *vreAIR* promoter with the mutated *pho* box, were grown in high Pi (dark grey bars) or low Pi (light grey bars) and analysed for β-galactosidase activity. Bars represent the mean and SD of at least three independent experiments.

Identification of a putative *pho* box in the PUMA3 genes promoter region

No *pho* box in the *vreAIR* promoter was previously identified by a large-scale bioinformatic approach (Jensen *et al.*, 2006). However, *in silico* analysis of the PUMA3 promoter region upstream *vreA* led us to identify a putative *pho* box (Fig. 3a). This box matches 16 bp within the 22 bp consensus sequence of *E. coli* and contains two 11 bp direct repeats. In each repeat, the first 7 bp are more conserved than the last 4 bp (Fig. 3a). The contribution of the identified *pho* box to the PhoB-mediated regulation of the *vreAIR* genes was examined using a *lacZ* transcriptional fusion in which conserved *pho* box residues were changed (Fig. 3a, *mut*Pho). Mutation of the putative *pho* box abolished the induction of PUMA3 gene expression in low Pi conditions (Fig. 3c, pMPR3*mut*Pho plasmid). This result is consistent with the suggestion that the putative *pho* box is potentially a PhoB binding site. To confirm this hypothesis, we tested the ability of PhoB to bind to the *vreAIR* promoter by an electrophoretic mobility shift assay with purified PhoB that has been autophosphorylated in the presence of acetyl phosphate, a small-molecule phosphodonor (Hiratsu *et al.*, 1995). However, the *vreAIR* promoter is retarded only in the presence of a high concentration of PhoB that also shifts a *pho* box free promoter (data not shown), which made it impossible to address whether the retardation obtained with the *vreAIR* promoter was specific. As a consequence, we could not confirm that the PhoB transcriptional regulator directly binds to the promoter region of the PUMA3 genes to induce their expression.

Role of σ^{VreI} and PhoB in the activity of promoters within the PUMA3 regulon genes

We showed that the expression of *vreI*, encoding the σ^{VreI} ECF sigma factor, is induced under low Pi conditions in a PhoB-dependent manner. In order to determine whether

the genes of the PUMA3 regulon were also expressed under this condition, we first analysed the transcriptional organization of such genes. Among the 27 genes expressed upon σ^{VreI} factor overproduction, 16 are located immediately downstream of the PUMA3 locus (Llamas *et al.*, 2009) and only the expression and transcriptional organization of the *hxc* gene cluster has been characterized (Ball *et al.*, 2002). To determine the transcriptional organization of the other PUMA3-regulated genes, we performed RT-PCR on mRNA extracted from *P. aeruginosa* grown in low Pi using primers that amplified the overlapping region of the genes. Fig. 4b shows that the *PAO690* gene forms a single transcriptional unit (TU; TU-1) as no cDNA could be detected using primers that amplify the *PAO690-phdA* overlapping DNA region. Interestingly, a cDNA band could be detected with all other combinations of primers used (Fig. 4b) indicating that all these genes, from *phdA* to *PAO701*, are transcribed as a polycistronic mRNA (Fig. 4b) and thus form a second transcriptional unit (TU-2). However, based on the distance between genes (Fig. 4a) and the predicted operonic structures (http://www.pseudomonas.com; Winsor *et al.*, 2011), three intergenic regions, upstream *PAO690*, *phdA* and *PAO692* genes, may contain putative promoters of three independent TUs (Fig. 4a). Therefore, we fused these three putative promoter regions to the *lacZ* gene, encoding the *β*-galactosidase enzyme, and inserted them in the chromosome of *P. aeruginosa*. Under LB-rich growth conditions, no promoter activity was observed (data not shown). Growing the cells in low Pi conditions increased the activity of the p*PAO690*::*lacZ* and p*phdA*::*lacZ* transcriptional fusions by 2.5 and 18-fold respectively but had no effect on the p*PAO692*::*lacZ* construct (Fig. 4c). This interesting result strongly suggested that, despite the prediction, the *PAO692* and downstream genes are only expressed from the region upstream *phdA*, which contains an active promoter (Fig. 4 and Llamas *et al.*, 2009). Importantly, we showed here that the activity of TU-1 and TU-2 promoters depends on the concentration of phosphate.

Next, to determine whether the Pi-dependent regulation of TU-1 and TU-2 promoters depends on the σ^{VreI} and PhoB transcriptional regulators, their activities were tested in a *vreI* and a *phoB* deletion mutant. *β*-Galactosidase experiments showed that the activity of both promoters was no longer induced by low Pi in the absence of σ^{VreI} or PhoB (Fig. 5a, b). Complementation of the *vreI* deletion with a plasmid overproducing σ^{VreI} from an arabinose-regulated promoter (pJN105-vreI) restored the activity of promoters (Fig. 5a, b). Indeed, these activities were related to the number of *vreI* transcripts, since a 100-fold increase of *vreI* transcripts (Fig. 5c, 0 % arabinose) led to a ~2 and 3-fold increase promoter activity of TU-1 and TU-2 respectively (Fig. 5a, b), while a 500-fold increase of *vreI* transcripts (Fig. 5c, 0.4 % arabinose) led to an ~50 and 100-fold increase in promoter activities (Fig. 5a, b). Additionally, high expression of *vreI* upon addition of 0.4 % arabinose relieved the Pi-dependent regulation of promoter activities, which confirms that an overproduction of σ^{VreI} leads to an expression of σ^{VreI}-regulated genes independently of the growth culture conditions (Llamas *et al.*, 2009). We show a contribution of PhoB in the promoter activation (Fig. 5a, b) and an absence of *vreI* expression in a *phoB* mutant (Fig. 2), which suggests that PhoB acts upstream of the σ^{VreI} factor to activate TU-1 and TU-2 promoters. To test this hypothesis, we overproduced the σ^{VreI} factor in the *phoB* mutant and measured *β*-galactosidase activities of the transcriptional fusions. TU-1 and TU-2 promoter activities are restored when *vreI* gene is introduced *in trans* (Fig. 5a, b), a result that suggests that σ^{VreI} could bypass the absence of the PhoB protein and act downstream of this determinant in the identified regulatory cascade. Surprisingly, *β*-galactosidase activities were considerably lower than in the *vreI* mutant as TU-1 and TU-2 promoters were activated only upon induction of *vreI* with 0.4 % arabinose (Fig. 5a, b). This lower activity observed in the *phoB* mutant upon addition of 0.4 % arabinose is not due to a lower expression of the *vreI* gene since equivalent levels of *vreI* transcripts were observed in the *vreI* and *phoB* mutants (Fig. 5c). This result may suggest a role for PhoB in a post-transcriptional control of σ^{VreI} activity by a remodelling of the σ^{VreI}-RNA polymerase. Alternatively, low TU-1 and TU-2 promoter activities may be a consequence of an absence of VreA and/or VreR in a *phoB* mutant (Fig. 2). Indeed, lower stability of σ^{VreI} was observed in the absence of its VreR anti-sigma factor (Llamas *et al.*, 2009). Altogether, these results clearly show that upon low Pi conditions, the σ^{VreI} factor activates TU-1 and TU-2 promoters in a PhoB-dependent manner.

Redefining the σ^{VreI} regulon

Our experiments show that the promoter of TU-2 is activated upon low Pi through the σ^{VreI} factor and PhoB regulators. This result suggested that all the genes, expressed from this promoter, undergo the same regulation. Indeed, among them, eight seem to be regulated in a σ^{VreI}-dependent manner but three (*PAO695, PAO700* and *PAO701*) seem not to be (Llamas *et al.*, 2009 and Fig. 1). To clarify this, we performed qRT-PCR to determine the level of gene expression within the TU-2. We first chose five genes (*phdA*, *PAO692*, *PAO693 PAO696* and *PAO697*), showing the highest fold expression change upon overproduction of the σ^{VreI} factor (Llamas *et al.*, 2009 and Fig. 1) and we confirmed that these genes were transcribed 6–20-fold more in low Pi than in high Pi growth conditions (Fig. 6a). Expression of these genes was significantly reduced, but not abrogated, in a *vreI* mutant (Fig. 6, Δ*vreI* low Pi). Interestingly, the absence of PhoB regulator completely turned off expression of these genes in low Pi growth conditions (Fig. 6a), suggesting that PhoB alone contributes to their expression in this condition. Finally, we show that *PAO695*, *PAO700* and *PAO701* gene expression undergoes the same pattern. Indeed, σ^{VreI} factor and PhoB protein also control their expression upon low Pi growth conditions (Fig. 6a). ECF sigma factors usually

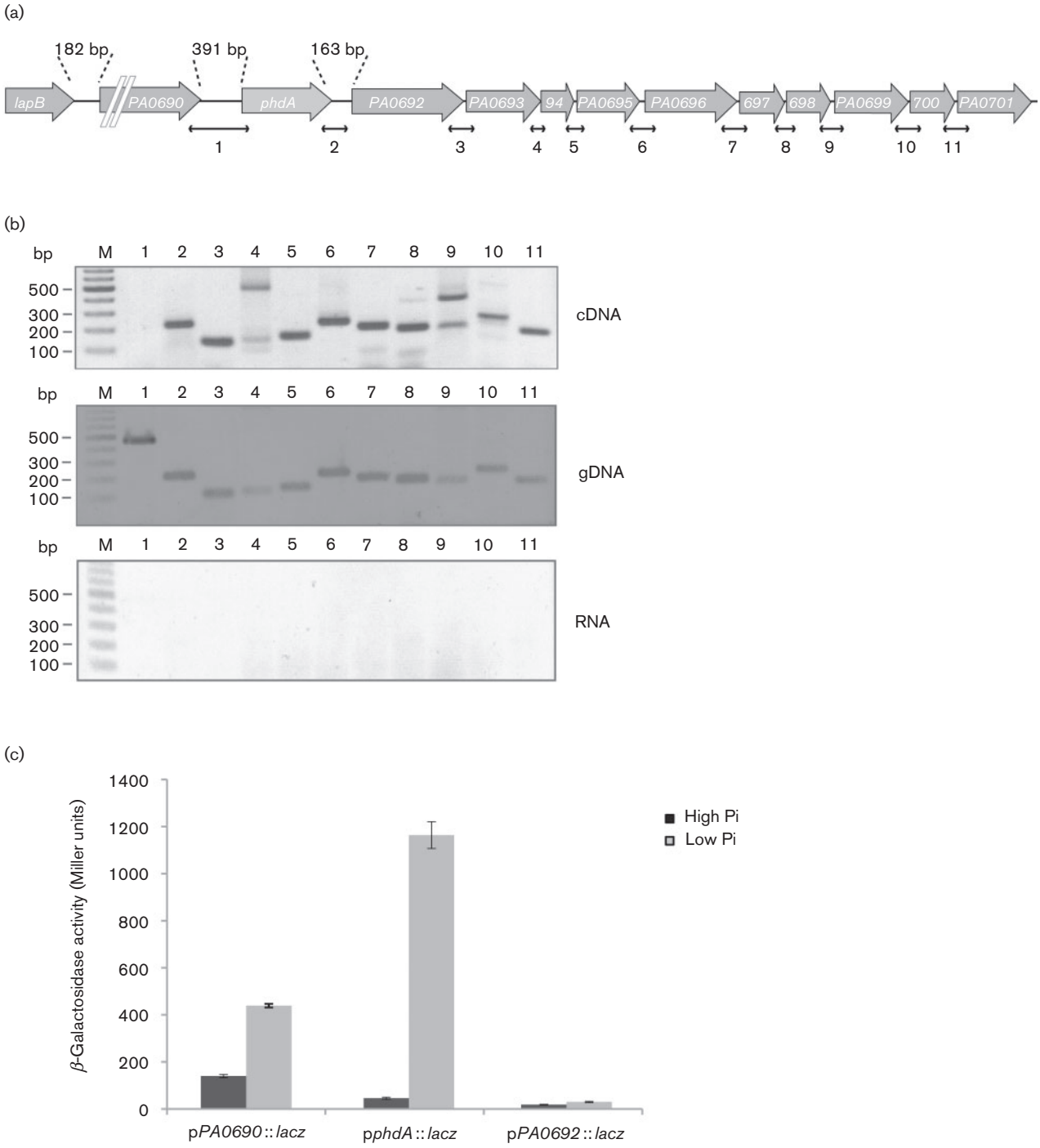
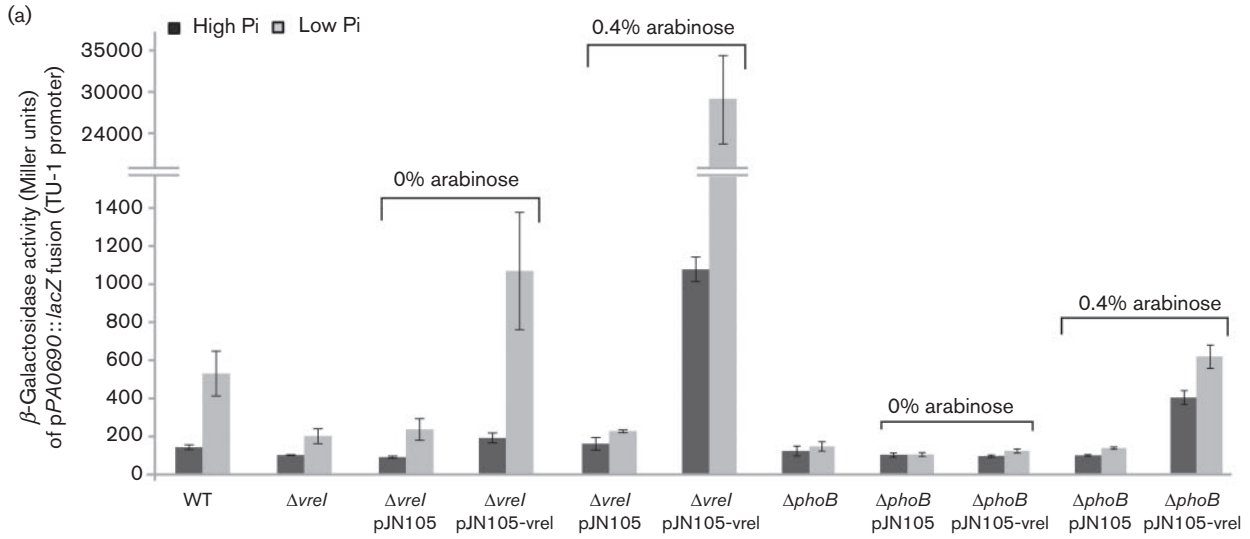
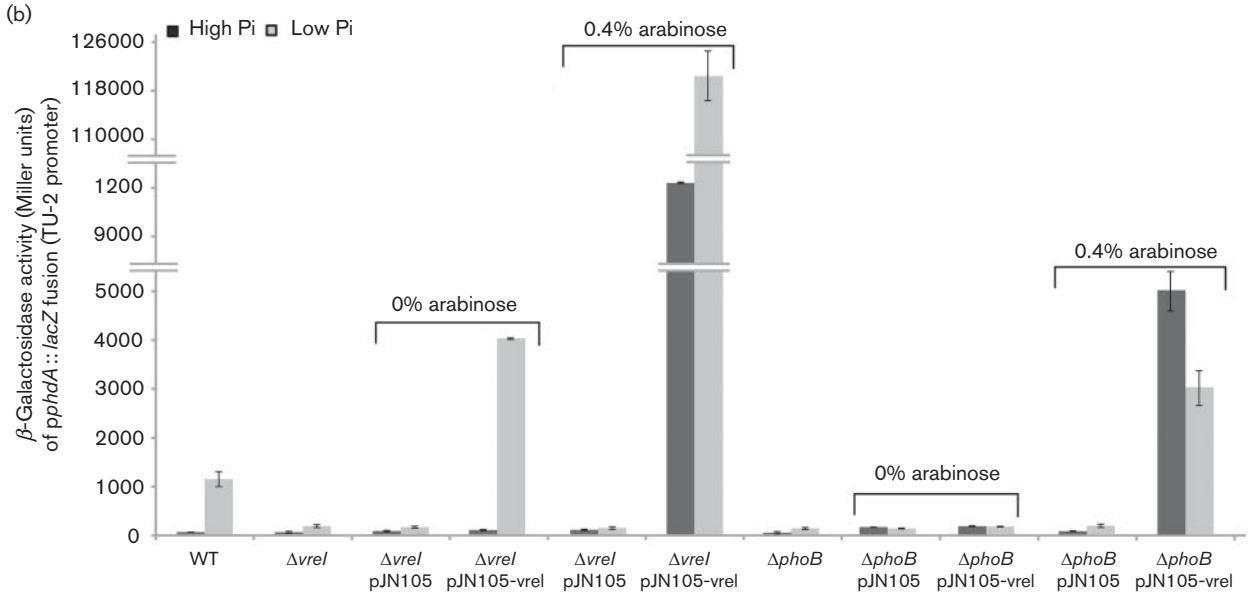


Fig. 4. Transcriptional fusion analysis and co-transcription of genes located downstream of the PUMA3 locus. (a) Diagram showing the putative promoter regions with distance between genes. Arrows represents the expected amplified region after RT-PCR. (b) RT-PCR products from RNA isolated from *P. aeruginosa* grown for 300 min in low Pi conditions. cDNA corresponds to the RT-PCR using mRNA as template, gDNA is a positive control using the PAO1 genomic DNA template and RNA is a negative control lacking reverse transcriptase in the RT-PCR. (c) *P. aeruginosa* strains, containing the chromosomal transcriptional fusion p*PAO690*::*lacZ*, p*phdA*::*lacZ* or p*PAO692*::*lacZ* were grown in high or low Pi conditions and β-galactosidase activity was measured after 300 min of growth. Error bars represent the mean and SD of at least three independent experiments.


(a)
High Pi
Low Pi
β-Galactosidase activity (Miller units) of pPA0690::lacZ fusion (TU-1 promoter)
35000
30000
24000
1400
1200
1000
800
600
400
200
0
0% arabinose
0.4% arabinose
0% arabinose
0.4% arabinose
WT
Δvrel
Δvrel pJN105
Δvrel pJN105-vrel
Δvrel pJN105
Δvrel pJN105-vrel
ΔphoB
ΔphoB pJN105
ΔphoB pJN105-vrel
ΔphoB pJN105
ΔphoB pJN105-vrel


(b)
High Pi
Low Pi
β-Galactosidase activity (Miller units) of pphdA::lacZ fusion (TU-2 promoter)
126000
118000
110000
1200
9000
5000
4000
3000
2000
1000
0
0% arabinose
0.4% arabinose
0% arabinose
0.4% arabinose
WT
Δvrel
Δvrel pJN105
Δvrel pJN105-vrel
Δvrel pJN105
Δvrel pJN105-vrel
ΔphoB
ΔphoB pJN105
ΔphoB pJN105-vrel
ΔphoB pJN105
ΔphoB pJN105-vrel

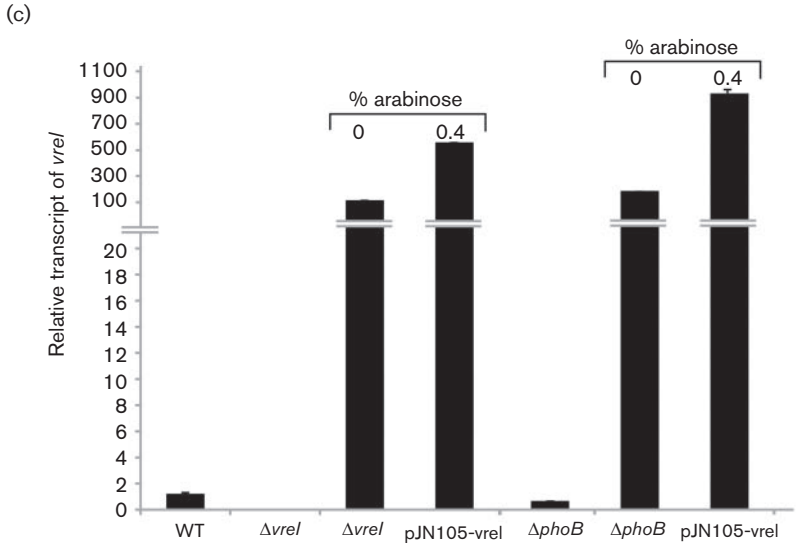

(c)
Relative transcript of vrel
1100
900
700
500
300
100
20
18
16
14
12
10
8
6
4
2
0
% arabinose
0
0.4
% arabinose
0
0.4
WT
Δvrel
Δvrel
pJN105-vrel
ΔphoB
ΔphoB
pJN105-vrel

Fig. 5. σ^{VreI} factor and PhoB regulate TU-1 and TU-2 promoter activity. *P. aeruginosa* and its isogenic *vreI, phoB* mutants containing the chromosomal transcriptional fusion p*PAO690*::*lacZ* (a) or p*phdA*::*lacZ* (b) were grown in high or low Pi conditions for 300 min. When indicated, 0.4 % of L-arabinose was added during the growth of mutant conjugated with the empty pJN105 or pJN105-vreI plasmids. Bars represent mean of β-galactosidase activity for at least three independent experiments. (c) mRNA level of *vreI* from wild-type, *vreI*, *phoB* mutants containing the pJN105-vreI plasmid grown in low Pi conditions for 300 min with or without 0.4 % L-arabinose. Level of transcript was normalized to 16S expression and shown relative to the wild-type level. Bars represent mean and SD of least two independent replicates.

regulate genes located immediately downstream on a chromosome. In the case of the PUMA3 system, microarray data revealed that σ^{VreI} factor could also control other genes located in different loci of the *P. aeruginosa* genome (Llamas *et al.*, 2009). To expand our study, we picked two genes (*PA4192* and *PA5403*), showing the highest fold change expression between wild-type and σ^{VreI} factor overproducing strains (Llamas *et al.*, 2009), and tested whether they undergo an identical cascade of regulation. Interestingly, expression of these genes is not induced upon low level of Pi and is dependent on neither σ^{VreI} factor nor PhoB protein, contrasting with TU-1 and TU-2 gene regulation (Fig. 6b).

DISCUSSION

In the present study, we have elucidated the regulatory pathway allowing the activation of genes controlled by the PUMA3 system. We show that the PUMA3 system expression is activated under phosphate limitation through the PhoB transcriptional regulator allowing expression of the *vreI* gene, which codes for the σ^{VreI} factor. Once produced, σ^{VreI} mediates transcription of specific target genes. Microarray data identified a number of genes positively controlled by the σ^{VreI} factor (Llamas *et al.*, 2009), among which ~two-third are located immediately downstream of the *vreI* gene and ~one third dispersed onto the *P. aeruginosa* genome. Based on our findings, expression of σ^{VreI}-regulated genes should be induced in response to phosphate depletion. *hxc* gene expression occurs upon low Pi conditions (Ball *et al.*, 2002), and we found here that the other genes downstream of the PUMA3 gene cluster follow an identical regulation and are regulated by the σ^{VreI} factor and PhoB. However, we clearly showed that *PA4192* and *PA5403* genes, belonging to the one-third of genes located somewhere else in the chromosome, are not expressed under scattered limitation conditions. These results confirmed a previous microarray study that observed, under phosphate-limited conditions, a clear upregulation of only PUMA3 genes and all genes located downstream this locus, from *hxcW* to *PAO701* (Bains *et al.*, 2012). All together, these results strongly suggest that only genes from *hxcW* (*PAO677*) to *PAO701*, located downstream of the PUMA3 locus, constitute the σ^{VreI} regulon.

Expression of PUMA3 genes has been for long thought to be controlled by iron and Fur (Ochsner & Vasil, 1996), and therefore originally named *pigCDE* (from *Pseudomonas* iron-regulated genes). However, a deeper analysis of these studies revealed that the identified Fur-box is actually located in the promoter region of the *pigA* (*PAO672* or *hemO*) gene, located upstream of the *vreAIR* (*pigCDE*) genes (Ochsner & Vasil, 1996; Ochsner *et al.*, 2002). In this work, we demonstrated that PUMA3 genes, namely *vreA*, *vreI* and *vreR*, form an operon that has its own promoter

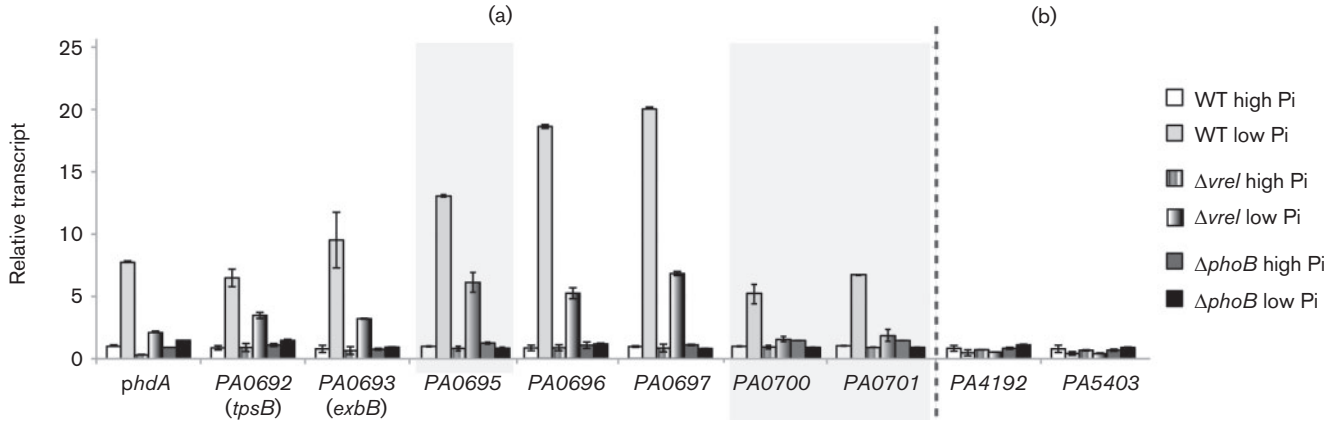


Fig. 6. Redefining the σ^{VreI} regulon. Results of qRT-PCR analysis of selected genes are shown. RNA was collected from *P. aeruginosa* strains grown at 37 °C in high or low Pi conditions for 300 min. For each gene, expression was normalized to 16S expression and shown relative to the WT high Pi condition level. Mean and standard deviation from two independent replicates are shown. Grey blocks highlight genes not previously identified as σ^{VreI}-regulated genes (Llamas *et al.*, 2009).

region upstream of *vreA* with a putative *pho* box but no Fur box (Fig. 3a). In agreement with this observation, none of the transcriptional fusions used in this work responds to iron limitation (data not shown), which further supports the suggestion that iron does not play a role in PUMA3 expression and its regulatory role.

As illustrated here, low phosphate growth conditions are required to trigger the PUMA3 pathway, including expression of PUMA3 gene and further activation of genes from the σ^{VreI} regulon. This is an intriguing observation since the σ^{VreI} factor is coproduced in this condition both with its cognate VreR anti-sigma factor, which inhibits the function of σ^{VreI} and is required for its activity (Llamas *et al.*, 2009), and the VreA protein which could sense an activating signal. This raises the question of how the VreA-dependent release of σ^{VreI} from its anti-sigma VreR occurs. Two possibilities emerge from our observations. A potential inducing signal either (i) is produced and integrated by VreA and VreR under low Pi condition to release the σ^{VreI} factor which further activates target gene transcription, or (ii) is absent under the conditions in which PUMA3 genes are expressed but the amount of free σ^{VreI} is sufficient to bind the RNA polymerase core and initiate transcription of σ^{VreI}-dependent genes. We observed that increasing the quantity of free σ^{VreI} *in trans* from a plasmid activates expression of σ^{VreI}-dependent target genes to a level considerably higher that of the level reached in low Pi (Fig. 5). Moreover, σ^{VreI}-regulated genes are also induced upon contact of *P. aeruginosa* with human airway epithelial cells (Chugani & Greenberg, 2007; Frisk *et al.*, 2004). All together, these data support the second hypothesis and suggest that an additional signal, absent in phosphate-limited conditions, is needed to completely unbind σ^{VreI} from the VreR anti-sigma factor and obtain a large σ^{VreI}-dependent gene expression.

From our study, it appears that expression of the genes belonging to the σ^{VreI} regulon is partially reduced in a *vreI* mutant and abrogated in a *phoB* mutant (Fig. 6a). This suggests that PhoB can also activate these genes by a σ^{VreI}-independent mechanism that remains to be elucidated. Putative *pho* boxes are present in both TU-1 and TU-2 promoters (our observation and Jensen *et al.*, 2006). However, the identity level of each box to the *E. coli* consensus is low and the efficiency of PhoB binding to a *pho* box correlates with this level (Diniz *et al.*, 2011; Kim *et al.*, 2000). Since we also encountered an unspecific shift when testing binding of PhoB to the TU-1 and TU-2 promoter regions (data not shown), we cannot state whether this PhoB-dependent control is direct by co-acting with the σ^{70}-RNAP holoenzyme. As σ^{VreI} is able to mediate gene transcription without PhoB (Fig. 5 and Llamas *et al.*, 2009) and PhoB complexed to the *pho* box complex interacts with a σ^{70} subunit of the RNA polymerase to control initiation of gene transcription (Blanco *et al.*, 2011; Makino *et al.*, 1993), the presence of putative *pho* boxes in the TU-1 and TU-2 promoter regions would only allow PhoB to enhance transcription initiation by remodelling the σ^{VreI}-RNA polymerase. One of our goals now is to perform further experiments to clarify the recognition and cooperation between the σ^{VreI} factor and the PhoB protein in the transcriptional regulation of these genes.

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