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Laura M
 Faure, Maria A Llamas, Karlijn C. Bastiaansen, Sophie de Bentzmann, Sarah Bigot. Phosphate starvation relayed by PhoB activates the expression of the Pseudomonas aeruginos
a $\sigma vreI$ ECF factor and its target genes. Microbiology, 2013, 159 (Pt_7), pp.1315-1327. 10.1099/mic.0.067645-0 . hal-03012634

HAL Id: hal-03012634 https://hal.science/hal-03012634

Submitted on 18 Nov 2020

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Received 11 March 2013 Accepted 2 May 2013

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

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^{VreI} extracytoplasmic function sigma factor and the VreR antisigma 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.

> 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

[†]These authors contributed equally to this work.

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 phosphatespecific ABC transport (Pst) system. Upon phosphate limitation, the Pst system mediates Pi transport and activates expression of the Pho regulon via the twocomponent 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^{Vrel} (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 1^{-1} proteose peptone (Difco Laboratories), 100 mM HEPES, 20 mM NH₄Cl, 20 mM KCl, 3.2 mM MgCl₂] contained 0.4% glucose and was adjusted to pH 7.2. For high Pi medium, 10 mM of KH₂PO₄ 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) φ80lacZΔM15 ΔlacX74 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
CC1182pir	araD139 Δ(ara leu)7697 ΔlacX74 phoA20 galE galK thi rpsE rpoB argE(Am) recA1, λpir lysogen	Herrero et al. (1990)
P. aeruginosa		
PAO1	Wild-type, prototroph	Lab collection
pPAO690::lacZ	PAO1 with the plasmid pPAO690:: <i>lacZ</i> transcriptional fusion inserted at <i>attB</i> site	This study
pphdA::lacZ	PAO1 with the plasmid pphdA:: lacZ transcriptional fusion inserted at attB site	e This study
pPAO692::lacZ	PAO1 with the plasmid pPAO692:: <i>lacZ</i> transcriptional fusion inserted at <i>attB</i> site	This study
$\Delta vreI$	Chromosomal vreI deletion in PAO1	This study
$\Delta phoB$	Chromosomal <i>phoB</i> 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, <i>lacZa</i> ColE1 f1 <i>ori</i> , Ap ^R Km ^R	Invitrogen
miniCTX-lacZ	Tcr <i>lacZ</i> +; self-proficient integration vector with <i>tet</i> , V- <i>FRT-att</i> PMCS, <i>ori, int</i> and <i>oriT</i> ; Tc ^R	This study
miniCTX-pPAO690::lacZ	PAO690 promoter fragment cloned upstream of <i>lacZ</i> gene in miniCTX <i>-lacZ</i> ; Tc ^R	This study
miniCTX-pphdA::lacZ	phdA promoter fragment cloned upstream of lacZ gene in miniCTX-lacZ; Tc ¹	This study
miniCTX-pPAO692::lacZ	PAO692 promoter fragment cloned upstream of <i>lacZ</i> gene in miniCTX- <i>lacZ</i> ; Tc ^R	This study
pMP220	<i>IncP</i> broad-host-range <i>lacZ</i> fusion vector; Tc ^R	Canter Cremers et al. (1989)
pMPR3	vreA promoter fragment cloned upstream of the lacZ gene in pMP220	This study
pMPR3 <i>mut</i> Pho	<i>vreA</i> promoter fragment with a mutated <i>pho</i> box cloned upstream of the <i>lacZ</i> gene in pMP220	This study
pKNG101	Suicide vector in P. aeruginosa; sacB, Sm ^R	Kaniga <i>et al.</i> (1991)
pKNG101-ΔvreI	vreI deletion construct	This study
pKNG101-Δ <i>phoB</i>	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 <i>Bam</i> HI/ <i>Spe</i> I the 1.3 kb PCR fragment containing the <i>vreI</i> gene	This study
pJN105-vreI	<i>vreI</i> 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.

Transcriptional fusionsPA0609 promoter with Xla/RamH1 sites836reggggeregggereggereggereggereggeregge	Name	Sequence 5' to 3'	Description
SB3 Attcicgggccqgggccqgggccqgggccqgggccqgggccqgggccqggcqggcqggcqggcqggcqggccqggccqggcqggcqggccqggccqggqgg	Transcriptional fusions		
SH4 Taggingsoggengang SH45 studgapAGGCGCGCAGCCAATTCAACC ph/A promoter with Xhol/BanrHI sites SH46 taggingsACTCGCTGCTCACCGATTCACC PA0692 promoter with Xhol/BanrHI sites SH47 taggingsACTCGCTGCACCAATTCAC PA0692 promoter with Xhol/BanrHI sites SH48 taggingsACTCGCTGCACCAATTCAC veck promoter with Xhol/BanrHI sites PR35E AAAGAATTCAATGCAACAGCACATG veck promoter with Xhol/BanrHI sites Tormat TCAATGCACTACCAACCAGCCACGACATG weck promoter with Xhol/BanrHI sites SH57 CAACTAACCAACACACACACGCCAGGAA Fw primer verd mutant with Spel site SH58 getatgaCCGCGCACACACACGCCACGCAC Fw primer verd mutant with Spel site SH59 getatgaCAGGCGCATAACCAGCGCACCAC Fw primer verd mutant with BanrHI site SH59 getatgaCAGGCGCATAACCAGCTCACCCACCCACC Fw primer verd mutant with BanrHI site SH59 getatgaCAGGCGCATAACCAGCCACCCCCCC Fw primer verd mutant with BanrHI site SH59 getatgaCAGGCGCACACACCCCCCCC Fw primer verd mutant with BanrHI site SH59 getatgaCAGGCGCACACCCCCCCC Fw and overlapped primer vide mutant SH50 gagaacSGCAGACGCTTACCCCCTCCCCG Fw and overlapped primer vide mutant w	SB3	Att <u>ctcgag</u> gccagtgctacaaggat	PAO690 promoter with XhoI/BamHI sites
SH45att_steggACGCGCACCCATTCACCph/A promoter with Xha/Ban/HI sitesSH46tt_steggACGCGCTCACCTCCPAD692 promoter with Xha/Ban/HI sitesSH47att_steggACACCGGCACCGATTCACPAD692 promoter with Xha/Ban/HI sitesPR385AAGCATTCATTCATAGCACAAGCCvrcA promoter with EoRUXbal sitesPR395AAGCATTCATGCGACCACGACACCGWatation pho box in the vrcA promotermatPhoPCCAACTAACCACATACTAACCAGCGACCACGACACCGWatation pho box in the vrcA promotermatPhoPCCAACTAACCACACACCACCCACCCACCACFw rprimer vrcl mutant with Spl siteSB58statsgCCGCGCACCCCCCCCCCCCCCFw and overlapped primer vrcl mutantSB59statsgCCGCAGCACCACCCACCCACCFw rprimer vrcl mutant with Spl siteSB58statsgCCGCAGCACCACCCCCCCCCCCCCCCCCCCCCCCCCCC	SB4	Ta <u>ggatcc</u> gcgtaaagcgcgcggga	
SNM6 taggets_GCCCCTCATCCCAGCTCCCC PA0692 promoter with Xhal/BamHI sites SNM7 attractings_AACCGGCCCCACATTCA vre4 promoter with EoNL/Xhal sites PR35E AAACAATTCATAGGACCAAGCACCG wre4 promoter with EoNL/Xhal sites PR35E AAACAATTCATAGGACCACGCACGCACG Mutation pho box in the vre4 promoter multhof CCAACTAACCACATACTAACAGTGCCATCACGCAGT Mutation pho box in the vre4 promoter SNS7 agteringtGCGCCTACCCAGCCAGCACGCACGCACGCACGCACGCACG	SB45	att <u>ctcgag</u> AGGCGCGCAGCCAATTGACC	phdA promoter with XhoI/BamHI sites
SH37 attegggAACTCCTCTACCCCATTCCA PA0692 promoter with Xhal/BamHI sites SH38 AAAGAATTCATTCATACAGGCAAATCCA vreA promoter with Khal/BamHI sites PR335 AAAGAATTCATTCATAGGCAAAGCC vreA promoter with Khal/BamHI sites mutPho8 TAGTATGTGGTTAGTTGGTACTTCACGGGTGACATG Mutation pho box in the vreA promoter mutPho7 CCAACTAACCACATACACAGTGCCATCAGGATT Mutation pho box in the vreA promoter SB57 aglengtiGGGCGTCACACCAGCCAGGAA Fw primer vreI mutant with Spd site SB58 agtettigTCATGACCTCCCCACTCCCCT Rv and overlapped primer vreI mutant SB60 taggatesGCAAGACGCAGCCCCCCC Fw and overlapped primer vreI mutant SB83 agtettigTAGTGACTGCCTGGCTCGG Fw and overlapped primer pho8 mutant SB84 agtettigTAGTGACGCCCCCCCG Fw and overlapped primer pho8 mutant SB85 agtettigTGGCGCAGGTTCCACCATTCC Rv primer pho8 mutant SB86 taggatesGCAAGGCAGGACCCCC FW crimer pho8 mutant SB86 taggatesGCAAGGCAGGACCAGCCCCCC FW crimer pho8 mutant SB87 agtettigTGGGCGCTACACCACCCCCC FW crimer pho8 mutant SB86 taggatesGCAAGGCAGGACACACCCCCC FW crimer pho8 mutant SB86 taggatesGCAAGGCGAAGCGCACCCC FW crimer pho8 mutant SB87 agtettigTCGGCGTACACCACCCCCCC FW crimer pho8 mutant	SB46	taggatccGGCGCTGATCAAGTTCCTGC	
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mutthoRTAGTATGTGTTAGTTAGTTGGTACGTGCGGTGACATGMutation pho box in the vreA promotermutthoFCCAACTAACCACATACTAACCAGGGCAGGAGVerprimer vref mutant with Spel siteSB57agtactagCGGCGTACACCCAGCCAGGAAFw primer vref mutant with Spel siteSB58gtctagTCATGACCCCACTCCCTRv and overlapped primer vref mutantSB59gtctagTCATGACCCTCCCACTCCCFw and overlapped primer vref mutantSB60ttaggattcGCAGAGCGACACAGCCACGCFw primer vref mutant with Spel siteSB83agtcttCATGGTCTGCCGTAGTAGGFw primer phoB mutant with Spel siteSB84agtcttCATGGTCTGCCGTAGTAGGFw primer phoB mutant with Spel siteSB85uccatgAAGGGTCCACCGCTCCCGRv and overlapped primer phoB mutantSB86ttaggattcCGCAGGTCCACCACTCCRv primer phoB mutant with Spel siteSB86ttaggattcCGCAACACCACCACCACCCpUC18-mini-TnT-LACSB87agtctgTCGCGTAACACCACCACCACCACCpUC18-mini-TnT-LACSB80ttaggattcGCAACACCCACCACCACCACCACCACCpUC18-mini-TnT-LACSB129CGCACCTTCACCACTTC5' of PA0699 geneSB107CGCACATGCGCAACAACCACCACCACCACC5' of PA0699 geneSB108ACACGCACCACAACACACCACCACC5' of PA0699 geneSB109GAACACCGACCAACAACACCACCACCACC5' of PA0699 geneSB101CACGCGACTGCACAACAACACCACCACCACCACCACCACCACCACCACC	PR33X	AAA <u>TCTAGA</u> ACCGAGCAACGACACTG	
muthbric CCAACTAACCACATACCACAGCAGCAGGAG SB57 agtactgCGCGTAACACCAGCAGCAGGAA Fw primer wcl mutant with Spd site SB58 gtatgCATAGCCATCCCACCCCCCT Rv and overlapped primer wcl mutant SB59 gtatgaCATAGCCATCCCACCCCCC Fw primer wcl mutant SB60 ttaggatcCGAAGCGAGTGTGAAAACC Fw primer wcl mutant SB83 agtactgAAGGGCTTCCCCGTGTGTGG Fw and overlapped primer wcl mutant SB84 agtactgAAGAGGGCTTCCCCGTGGTGG Fw and overlapped primer phoB mutant SB85 gaccatgAAGAGGAGCGCCTCCCGG Fw and overlapped primer phoB mutant SB86 ttaggatcCGGGGAGGTTCCACCACCCC Fw primer phoB mutant with BanHI site Complementation with JNI05 T T SB7 agtactgCCGGCGAACACCACCACCCACCC pUC18-mini-Tn7T-LAC SB7 agtactgCCGCGTAACCACCACCACCCACC pUC18-mini-Tn7T-LAC SB128 GCAGCCAATTGACCGATGAA 3' of PA0690 gene SB129 CCGCAACGGTTCACCAC 5' of PA0692 gene SB160 GAACCCCAACACACACCACCACCAC 5' of PA0693 gene SB161 CAGCGGAAGCGTTCACCG 5' of PA0694 gene SB163 GTCGTGTGCATCA	mutPhoR	TAGTATGTGGTTAGTTGGTACCTGCGGTGACATG	Mutation pho box in the vreA promoter
Gene deletion constructs stagtactagt/GGCCTACACCAGCCAGGAA Fw primer vrd mutant with Spd site BB57 agtactagt/GGCCTACACCAGCCAGCAC Fw and overlapped primer vrd mutant BB59 gtatagt/AAGGACTTAAGCAAGCACAACCCC Fw and overlapped primer vrd mutant BB60 ttaggatteCCAGCGAACCCCC Fw and overlapped primer vrd mutant SB83 agtactagtAAGGGCCTTCCCGGGTGG Fw rimer vrd mutant with Spd site SB84 agtactagtGAGGCTCCCCCGGCTCCCG Fw and overlapped primer phoB mutant SB85 gaccatgAAGAGCTGACCCCCGCTCCCG Fw and overlapped primer phoB mutant SB86 ttaggatteCGCGCACCCCCCCCG Fw and overlapped primer phoB mutant SB87 gaccatgAAGAGCACACCCCCCCCCG Fw and overlapped primer phoB mutant SB86 ttaggatteCGCGCACACCACCCCCCCCCCCCCCCCCCCCCCCCCC	mutPhoF	CCAACTAACCACATACTAACAGTGCCATCAGGATG	
SB57agtactageCGCCTACACCAGCCAGGAAFw primer vel mutant with Spel siteSB58gettageCATAAGCACTCACCTCCCRv and overlapped primer vel mutantSB60ttaggateGCAAGACGAGCACCACCCRv primer vel mutant with BamH1 siteSB83agtactagtAGGGGCTTGCCGCGTAGTAGGRv and overlapped primer vel mutant with BamH1 siteSB84agtactagtAGGGGCTTGCCGCGTAGTAGGRv and overlapped primer vhoB mutantSB85gaccatgAAGAGCTGACCCGGCTACTAGCRv and overlapped primer phoB mutantSB86ttaggatecGGCGAGGTTCCACCATCCCRv primer phoB mutant with BamH1 siteSB86ttaggatecGCGCGAGGTTCCACCAGCCAGputlemat with Spel/BamH1 sites fromSB80ttaggatecGCAGAGCAACAGCCACCCpuCl8-mini-Tn7T-LACRT-PCRstaggatecGCAAGAGCAACAGCGCACCpuCl8-mini-Tn7T-LACSB129CCGGACGCTGAATTTCTA3' of PA0690 geneSB129CCGGACGCTGAATATTCTA3' of PA0690 geneSB100GAACACCGAAACAGCACT3' of PA0690 geneSB161CAGCGATGCATGAACTTCTA3' of PA0690 geneSB162AGCTGCATGGGAAACCGACT3' of PA0690 geneSB163GTCGTCGTGGCTGAAACTTCTA3' of PA0690 geneSB164ACGGGGAGGTCACCAGAGGT3' of PA0690 geneSB165GGTGCGTGGCGTGCT3' of PA0690 geneSB164ACGGTGAGTACCCGGGTGGT3' of PA0690 geneSB165GGTGCGTGGTGCTC3' of PA0690 geneSB166GCCCCAGTGGTGTGCT3' of PA0690 geneSB167CGGCAGTGGGTGATCATCT3' of PA0690 geneSB168CTGCGGCAGTGCTCACCT3' of PA0690 geneSB169G	Gene deletion constructs		
S1558getatigtTCATGACCCTCCCACTCCCTRv and overlapped primer vrel mutantS159getatigcATAAGCATAAGCAGGTGGAAACCFv and overlapped primer vrel mutantS150ttaggatccGCAAGACGACGAGGTGGAAACCRv primer vrel mutant with BamHI siteS158agtetcGTGCTTGCCTTGCGGTGGFv and overlapped primer phoB mutantS158gaccatgAAGAGCTGACCCGGGTCCACCARv primer phoB mutant with BamHI siteS158gaccatgAAGAGCTGACCCGGCTCCCGFv and overlapped primer phoB mutantS158gaccatgAGGGGCTCACCACGCGGGAAAmplification vrel with Spel/BamHI siteS157agtacatgGCGGCTCACCCAGCCAGGAAAmplification vrel with Spel/BamHI sites fromS160ttaggatcGCGGCTGACTCACCAGCCAGCCAprimer phoB mutantS17CGGCGACGCTGAATCACCAGCCACCprimer phoBS181CGACCTCGGTACCCAGCCACCprimer phoBS181CGACCTCGGTACCCAGCGACAprimer phoBS181CGACCTCGGGTAATCTCAprimer phoBS181CGACCTCGGGAAGCGTTCACACprimer phoBS1810AACGGGACGCGAAAGCGprimer phoBS1816CGCGGTGCGCAAAGCGCprimer phoBS1816CAGCGACGCAAAAGCGCprimer phoBS1816GTGCTGCTCACCCAGGAGGGCprimer phoBS1816GCGGTGGCATGCGGAAAGCGprimer phoBS1816GCGGTGGCATGCGGCAAGGGGCGprimer phoBS1816GCGGTGGCATGCGGCAGGAGGGCprimer phoBS1816GCGGTGGCATGCGGCAGGAGGGCprimer phoBS1816GCGGTGGCATGCGCTGGGTCprimer phoBS1816GCGGTGGCATGCGCTGGGTCprimer phoBS1816<	SB57	agt <u>actagt</u> CGCGCTACACCAGCCAGGAA	Fw primer vreI mutant with SpeI site
Sh59gtagaCATAAGCATAACCAGTGGAAAACCFw and overlapped primer <i>vrel</i> mutantSh60ttaggatcgCAAGACGAACACGCACCCRv primer <i>vrel</i> mutant with Spd siteSh83agtactgAGGGGCTTGCCGGGTAGTAGGFw and overlapped primer <i>phoB</i> mutant with Spd siteSh84agtactgAGGGCTTGCCGGCTAGTAGGFw and overlapped primer <i>phoB</i> mutantSh85gacatgAAGAGCTGACCCGCGTCCGRv and overlapped primer <i>phoB</i> mutantSh86ttaggatcsCGGCGAGGTTCCACCATTCCRv and overlapped primer <i>phoB</i> mutantSh86ttaggatcsCGGCGAGGTTCCACCAGGAAAmplification <i>vrel</i> with <i>SpdIBam</i> HI sites fromSh87agtactgGCGCGAAGTGACAACGCCACCpUC18-mini-Tn7T-LACSh7agtactgGCGCGAATTGACCGATGAA3' of <i>PA0690</i> geneSh128GCAGCCAATTGACCGATGACA3' of <i>PA0690</i> geneSh129CCGACTTTCGACTACTTTC3' of <i>PA0692</i> geneSh100GAACACGGACAACACGACACC3' of <i>PA0692</i> geneSh160GAACACGGACAAACACGACT3' of <i>PA0692</i> geneSh161CAGGGATGTCACGCGTGATATCTA3' of <i>PA0693</i> geneSh162AGGTGCATGGCGGAAGC3' of <i>PA0693</i> geneSh163GCTGTCCTCATCGTCGTG5' of <i>PA0694</i> geneSh164ACGGTGCATGACCAGAAGGT5' of <i>PA0694</i> geneSh165GGGTGTGCTTGCGTG5' of <i>PA0694</i> geneSh164ACGGTGCATGCCGTACCTT5' of <i>PA0694</i> geneSh165GGTGTGCTCACTGTGGTG5' of <i>PA0694</i> geneSh166GACCAACAGAAGGTGCT5' of <i>PA0694</i> geneSh167CGCCAGCAGAAGGGTGCT5' of <i>PA0694</i> geneSh168GTCGTCGTGGTGGTGGTGG5' of <i>PA0696</i> gene <td>SB58</td> <td>gcttatgTCATGACCCTCCCACTCCCT</td> <td>Rv and overlapped primer vreI mutant</td>	SB58	gcttatgTCATGACCCTCCCACTCCCT	Rv and overlapped primer vreI mutant
SB60ttaggategCAAGAGCAACGCACCRv primer vel mutant with BamHl siteSB83agtatCagIAAGGGCTTGCCGTAGTAGGFw primer phoB mutantSB84agctttCATGGTCTGCCCGGGTCCGFw and overlapped primer phoB mutantSB85gaccatgAAGGGTGCACCCGCTCCGFw and overlapped primer phoB mutantSB86ttaggatecGGGGGGTCCACCATTCCRv primer phoB mutant with BamHl siteComplementation with pJN105TTSB57agtactgrGCGCGACGTCACCCAGCCApUC18-mini-TnT-LACSB50ttaggatecGCAAGACGACAACGCCACCpUC18-mini-TnT-LACRT-PCRTS' of PA0690 geneSB128CCGACCTTCGACTAACTTCS' of PA0690 geneSB107CGGCGCGGCTGAATATCTAS' of PA0690 geneSB108AACAGCGAACGCTTCACACS' of PA0692 geneSB109GAACCCGACAGCTCACACS' of PA0692 geneSB160GAACCCGACAGCTCACACS' of PA0692 geneSB161CAGCGCTGTGCGCGAAAGCS' of PA0693 geneSB163GTCGTCGTCATCGTCGTGS' of PA0694 geneSB164ACCGGCCGCTACGTGGCGAAGGTS' of PA0694 geneSB165GGGTGTCTGCGGTAAGGTS' of PA0694 geneSB166GACCGACCAGAAGGTGCTS' of PA0695 geneSB167CGCCGCGTTACCTTGGS' of PA0696 geneSB168GGTGTGCTGCGCTACCTTS' of PA0696 geneSB169GCCCCAGCAGGAGGTGTCS' of PA0696 geneSB169GCCCCAGACGGCCTACS' of PA0696 geneSB171CTGTCGACGAGTGGCTAATGGAGAAGGTGTTS' of PA0696 geneSB173GCATGGCGAAGTGGCTAATS' of PA0696 geneSB174	SB59	gtcatgaCATAAGCATAAGCAGGTGGAAAACC	Fw and overlapped primer vreI mutant
SIR3apticategrAAGGGCTTGCCGTAGTAGGFw rimer phoB mutant with Spd siteSIR4agctcttCATGGCTTGCCTCGGGTCGRv and overlapped primer phoB mutantSIR5gaccttgAAGAGCTGACCCGCTCCGFw and overlapped primer phoB mutantSIR5taggatc_CGGCGAGGTTCCACCATTCCRv primer phoB mutant with BamHI siteComplementation with pINTOattactagtCGCGCTACACCAGGAAAmplification vrel with SpdI/BamHI sites fromSIR5agtactagtCGCGCAACGGAACAACGCCACCpUC18-mini-Tn7T-LACRT-PCRCGGACGCGCGAATTGACCGATGAA3' of PA0690 geneSIR10GCAGCGCGCGAATATCTTC3' of phdA geneSIR10CGGCAGCGGCGGCGAAATATCTA3' of pA0692 geneSIR10GAACACCGGCGAAAACACGACT3' of PA0692 geneSIR10GAACACCGGCGAAAACACGACT3' of PA0692 geneSIR10CAGCGATGTCACGAATAAGC3' of PA0693 geneSIR10CAGCGATGCCGCGAAAGC3' of PA0694 geneSIR10CAGCGATGCCAGGAAGC3' of PA0694 geneSIR10CAGCGATGCGCGAAAGC3' of PA0694 geneSIR10CAGCGACCAGAAGGTGCT5' of PA0694 geneSIR10CGCCGCCGCAAAGGTGCT5' of PA0694 geneSIR10GCCCGCCGCAGAAGGGTGCT5' of PA0695 geneSIR10GCCCGCGCAGAAGGTGCT5' of PA0696 geneSIR10GCCCCGCGGAATTGGAAGA3' of PA0696 geneSIR10CGCGCAGAAGGCGTCTAC5' of PA0696 geneSIR11CTGGCGCAGAAGGCGTCTAC5' of PA0697 geneSIR12CGGTAACCCGGATTGGCAAGA5' of PA0697 geneSIR17CTGGCGAAGCGCGAAT5' of PA0697 geneSIR1	SB60	ttaggatccGCAAGACGAACAACGCCACC	Rv primer vreI mutant with BamHI site
SI84agtctttCATGGTCTTGCCTCGGGTCGRv and overlapped primer phoB mutantSB85gacatgAAGGTGACCCGGTCCCGGFw and overlapped primer phoB mutantSB86ttaggatcCGGCGAGGTTCCACCATTCCRv primer phoB mutant with BomHI siteComplementation with plNIb5sagtcatglCGCGCGTCACCAGCAGCAGGAAmplification vrel with Spd/BamHI sites fromSB60ttaggatcCGCGAGACGAACAACGCCACCpUC18-mini-Tn7T-LACRT-PCRsitesitesiteSB129CGGACTTTCGAGTAACTTTC5' of phdA geneSB100GAACACGGACAAACGGCAT5' of PA0699 geneSB161CAGCGATGTCAGCAAACGGCC5' of PA0692 geneSB163GTGGTGGTCATGGCGAAAGCC5' of PA0692 geneSB164CAGCGACGGTCAACGCC5' of PA0693 geneSB163GTGGTGGTCATGGTGGTG5' of PA0694 geneSB164CAGCGACCGGAAAGCG5' of PA0694 geneSB165GGGTGTGTTGGTGGTG5' of PA0694 geneSB166GACCGACCGGATCAGGT5' of PA0695 geneSB166GACCGACCGGTTCACCGTT5' of PA0695 geneSB168CTTCGACAGAGGTGCT5' of PA0695 geneSB169GCCCAGCTGGTTATGGAGAA3' of PA0695 geneSB170CGGTGAATTCGAAGGAGAT5' of PA0696 geneSB171CTGTCGACGGAAAGGTGTTG5' of PA0697 geneSB172CGGTCAACCCGTGAGA5' of PA0698 geneSB174AACAACGCTACCTGAGAGA5' of PA0709 geneSB175GCATAGCGCCAGAGA5' of PA0709 geneSB174AACAACGCGCCAGAG5' of PA0709 geneSB175GCATAGCGCGAAGAGGAGCTTGG5' of PA0709 gene	SB83	agt <u>actagt</u> AAGGGGCTTGCCGTAGTAGG	Fw primer phoB mutant with SpeI site
SB85gacctqAAGAGCTGACCCGCTCCCGFw and overlapped primer phoB mutantSB86ttaggatccCGGCGAGGTTCCACCATTCCV primer phoB mutant with BamHI siteSB7agtactagtCGCGCTACACCACCAGCAGAAmplification vrel with Spd/BamHI sites fromSB60ttaggatccCGAAGACGAACGCCACCpullamin-TnT-LACSB60ttaggatccCGAAGACGAACGCCACCpullamin-TnT-LACSB10CGACCAATTGGACGATGAA3' of phdA geneSB128GCAGCCGATGAACATTCA3' of phdA geneSB107CGGACGGCGGAAATATCTA3' of phdA geneSB108AACAGGGACGCTCACACC5' of PAO692 geneSB160GAACACGGACAATAACCA3' of PAO692 geneSB161CAGCGACGACAACACGACCA3' of PAO692 geneSB162AGGTGCATCGCGGAAGC5' of PAO693 geneSB163GTGCTGCGCGCAAGCA5' of PAO694 geneSB164ACGGTGCATGCGGAAGC5' of PAO695 geneSB165GGCGTGCTCATCGTGTG5' of PAO695 geneSB166GACCCACGAAGGTGCT5' of PAO695 geneSB167CGCCAAGTGGGTATGAGAA3' of PAO695 geneSB168CTTCGACAGAAGGTGTC5' of PAO695 geneSB169GCCCAAGTGGTATGGAAGA3' of PAO695 geneSB170CGGGGGAAATTGGAAGGAT5' of PAO697 geneSB171CTGTGCAGGGGCTGGG5' of PAO699 geneSB173GCTGGCGAAGGCTTGG5' of PAO699 geneSB174ACAACGCGACTGCGATGG5' of PAO699 geneSB175GCTAGGCGCCAGA5' of PAO699 geneSB174ACAACGCGACTGCGATGG5' of PAO699 geneSB175GCTGGCGAAGGCCTGAG5' of	SB84	agctcttCATGGTCTTGCCTCGGGTCG	Rv and overlapped primer phoB mutant
SB86ttagggtccCGGGAGGTTCCACCATTCCRv primer phoB mutant with BamHI siteComplementation with pNUSDSB57agtactrgtCGCGCTACACCAGCAGGAAmplification vrel with Spel/BamHI sites fromSB60ttagggtccpUC18-mini-Tn7T-LACRT-PCR''''SB129CCGACGTTGCAGTGAA''SB100CGCACGCGCTGAATATCTA''SB1010CGCACGCGCTGAATATCTA''SB1010GAACACCGACACATCACCAC''SB160GAACACCGACAATAAGC''SB161CAGCGCATCTCAGCAATAAGC''SB163GTCGTCGTCATCATCGTGG''SB164ACGGTCGCTGCGTGATCGCGGATG''SB165GGCTGCGCGTGCATCGCGGATCG''SB166GACCGACCAGAAGCGTCT''SB167CGCCAGTCGCGTTACCTTT''SB168GTCGCAGTCGCGTTACCTTT''SB169GCCCAGACGGGGTCTACC''SB169GCCCAGACGGGGTCTACC''SB169GCCCAGACGGGGTCTACC''SB169GCCCAGATCGGTAATGAGAA''SB169GCCCAGATCGGTAATGAGAA''SB169GCCCAGATCGGTAATGAGAA''SB169GCCCAGATCGGTAATGAGAA''SB169GCCCAGATCGCTAATGAGAA''SB169GCCCAGATCGCTAATGAGAA''SB169GCCCAGATCGCTAATGAGAA''SB169GCCCAGATCGCTAATGAGAA''SB169GCCCAGATCGCTAATGAGAA''SB169GCCCAGATCGCTAATGAGAA''SB169GCCCAGATCGCTAATGAGAGAA''SB160GCCCAGTCGC	SB85	gaccatgAAGAGCTGACCCCGCTCCCG	Fw and overlapped primer phoB mutant
Complementation with pINU05 Statistic GCGCTACACCAGGAA Amplification ver with Spel/BamHI sites from Sp8/BamHI sites from Sp8/DamHI sites from	SB86	ttaggatccCGGCGAGGTTCCACCATTCC	Rv primer phoB mutant with BamHI site
SB57agtactagtCGGCTACACCAGCAGCAAAmplification vrl with Spdl/BamHI sites fromSB60ttagggttcGCAAGAGAACAACGCCACCpUC18-mini-Tn7T-LACSB10GCAGCCAATTGACCGATGAA3' of PAO690 geneSB128GCAGCCAATTGACCGATGAA3' of PAO690 geneSB129CCGGACTTTCAGGTAACTTTC3' of pIAd geneSB100GAACACGGACGGTTCAACCAC5' of PAO692 geneSB101CGCGACGCGGAATATACTA3' of PAO692 geneSB102AACAGCGAACGCATCACAC5' of PAO692 geneSB103AACAGCGAACAGCACATAAGC3' of PAO693 geneSB164CAGCGATGTCAGCGAAAGC3' of PAO694 geneSB165GTCGTCGTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCATGCGGATCG3' of PAO694 geneSB165GGTGTGCTTGGCATCG3' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO695 geneSB167CGCCAGCGGGTCACTTT3' of PAO696 geneSB168CTTCGACAAGCGGCTCTAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGTGAATTCGAAGAGTGTC3' of PAO698 geneSB173GCTGGCAGATGGCTTGG3' of PAO699 geneSB174AACAGCGACCCGGAT3' of PAO698 geneSB175GCATAGGCGCCCAGA5' of PAO699 geneSB176ACTGCGGATTCGCCTCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177TGCCTCGCAAGACGAGC3' of vrd geneSB176CCTGCGCAGTTCGCCCCAGA5' of VAO700 geneSB177	Complementation with pJN10	5	
SB60traggatecpUC18-mini-Tn7T-LACRT-PCR'SB128GCAGCCAATTGACCGATGAA'SB129CCGACTTTCGAGTAACTTTC'SB107CGCGACGGCTGAATATCTA'SB108AACAGCGAACGCTTCACAC'SB108AACAGCGAACGCTTCACAC'SB161CAGCGATGCTCACAC'SB162AGGTGCATGCAGCAATAAGC'SB163GTCGCGTCATCCTGG'SB164ACGGTGCATGCCATGAGGA'SB165GGGTGTCTTGGGATCG'SB166GACCACCAGAAGGTGCT'SB167CGCCACCAGAAGGTGCT'SB168GTCGCTGTCATCCAGGA'SB169GCCCACCAGAAGGTGCT'SB168CTTCGACAACGGGTCTAC'SB169GCCCACTGGTTCACTTT'SB171CTGTCGACATGGTAATGAGAA'SB172CGGTCAACTCCGTCAT'SB173GCTGCAGATGGCTTGGC'SB174ACAACGCTACCTGAAT'SB175GCTGCAGATGCCTGAGA'SB176ACTGCGGATTGCCCAAGAGG'SB177ATCGACGACCTCCAAGAAC'SB178GCTGCCGAAGACGAGAGG'SB179ATCGACGCCCCAGA'SB170CTGCTCGCGAAGACGACG'SB171ATCGACGACTGCCCAGA'SB172GCTGCCGAGACCGAGAC'SB173GCTGCCGAGACCCGAGA'SB174ACCACGCCCCCGAGA'SB175GCTGCCGCAGTCCCT'SB176ACTAGCGCCCCCGAGA'SB177ATCCACCACCTC	SB57	agt <u>actagt</u> CGCGCTACACCAGCCAGGAA	Amplification vreI with SpeI/BamHI sites from
RT-PCRSB128GCAGCCAATTGACCGATGAA3' of PA0690 geneSB129CCGACTTTTCGAGTAACTTTC5' of phdA geneSB107CGCGACGGCTGAATATCTA3' of phdA geneSB108AACAGCGAACGCTTCACAC5' of PA0692 geneSB160GAACACCGACAAACAGGACT3' of PA0692 geneSB161CAGCGATGTCAGCAATAAGC5' of PA0693 geneSB162AGGTGGCATGGCGAAAGC3' of PA0693 geneSB163GTCGTCGTCATCGTCGTG5' of PA0694 geneSB164ACGGTGCATGATCGTCGTG5' of PA0694 geneSB165GGGTGTGCTTGGGATCG3' of PA0695 geneSB166GACCGACCAGAAGGTGCT5' of PA0696 geneSB167CGCCAGTCGGTTACCATTT3' of PA0696 geneSB168CTTCGACAAGCGCGTCTAC5' of PA0696 geneSB169GCCCAGATCGGTAATGAGAA3' of PA0697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PA0697 geneSB171CTGCCACGGAAAGGTGTTC3' of PA0698 geneSB172CGGTCAACCCGTCGAT5' of PA0698 geneSB173GCTGGCAGATGGCTTGG5' of PA0699 geneSB174ACACGCTACCCTGAGCAT3' of PA0699 geneSB175GCATAGGCGCCCCAGA5' of PA0700 geneSB176ACTGCGGATTCGCCTCAT3' of PA0700 geneSB177ATGCACCACCTCCCAAGAAC5' of PA0700 geneSB176ACTGCGGATTCGCCCAGG3' of read geneRT-vreAI-FGTGCTGGCGAGAGCGACG3' of read geneRT-vreAI-FGTGCTGGCGCAGGCCCAGG5' of read geneRT-vreAI-FTGCGTCTGGCGAGTCTCACCC3' of read gene	SB60	ttaggatccGCAAGACGAACAACGCCACC	pUC18-mini-Tn7T-LAC
SB128GCAGCCAATTGACCGATGAA3' of PA0690 geneSB129CCGACTTTCGAGTAACTTTC5' of phdA geneSB107CGCGACGGCGAATATCTA3' of pA0692 geneSB108AACAGCGAACGCTTCACAC5' of PA0692 geneSB160GAACACCGACAAACACGACT3' of PA0693 geneSB161CAGCGATGTCAGCGAATAAGC5' of PA0693 geneSB162AGGTGCATGGCGAAAGC3' of PA0693 geneSB163GTCGTCGTCATCGTCGTG5' of PA0694 geneSB164ACGGTGCAGTACCAGAAGGT5' of PA0695 geneSB165GGGTGTCTTGGGATCG3' of PA0695 geneSB166GACCCACCAGAAGGTGCT5' of PA0695 geneSB167CGCCAGTCGGTTCACTTT3' of PA0695 geneSB168CTTCGACAAGCGCGTCTAC5' of PA0696 geneSB169GCCCAGATCGGTATGAGAA3' of PA0697 geneSB170CGGGTGAATTGAAGAGAT5' of PA0697 geneSB171CTGTCGCAGGAAGGTGTTC3' of PA0697 geneSB172CGGTGAAATCGATGGCTTGG5' of PA0699 geneSB173GCTGGCAAGGGCTGCGAT3' of PA0699 geneSB174AACAACGCTACCCTGAGCAT3' of PA0699 geneSB175GCATAGGCCCCCAGA5' of PA0700 geneSB176ACTGCGGGATCGCCCCAGA5' of PA0700 geneSB177ATGCACCACCTCCAAGAACG3' of vre4 geneRT-vreAL-FGTGCTGCGCAAGACGACG3' of vre4 geneRT-vreAL-FTCGCGCGCGAGTCTGAGCCC3' of vre4 geneRT-vreAL-FTCGCGCGCGAGTCTGAGGCC3' of vre4 geneRT-vreIR-FTCGCGCGCGAGTCTGAGGCC3' of vre4 geneRT-vreIR-FTCGCG	RT-PCR		
SB129CCGACTTTCGAGTAACTTTC5' of phdA geneSB107CGCGACGGCTGAATATCTA3' of phdA geneSB108AACAGCGACGCTTCACAC5' of pAO692 geneSB160GAACACCGACAGCACT3' of PAO692 geneSB161CAGCGATGTCAGCAATAAGC5' of PAO693 geneSB162AGGTGCATGGCGAAACCGGG3' of PAO693 geneSB163GTCGTCGTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCAGTACCAGAAGGT5' of PAO694 geneSB165GGGTGTGCTTGGGATCG3' of PAO695 geneSB166GACCCGACCAGAAGGTGCT5' of PAO695 geneSB167CGCCAGTCGGTTCACTTT3' of PAO696 geneSB168CTTCGACAAGCGCGTCTAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO696 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO698 geneSB171CTGCCAGCAGAAGGTGTGC3' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO698 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATGGCGAGAGGCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAGAAC5' of PAO700 geneSB177ATGCACCACCTCCAAGAACC5' of verd geneSB177ATGCACCACCTCCAAGAACC <td< td=""><td>SB128</td><td>GCAGCCAATTGACCGATGAA</td><td>3' of PAO690 gene</td></td<>	SB128	GCAGCCAATTGACCGATGAA	3' of PAO690 gene
SB107CGCGACGGCTGAATATCTA3' of phdA geneSB108AACAGCGAACGCTTCACAC5' of PAO692 geneSB160GAACACGGACAACACGACT3' of PAO692 geneSB161CAGCGATGTCAGCAATAAGC5' of PAO693 geneSB162AGGTGCATGGCGAAAGC3' of PAO693 geneSB163GTCGTCCTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCAGTACCAGAAGGT5' of PAO694 geneSB165GGCTGTCCTTGGGATCG3' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO696 geneSB167CGCCAGTCGGTTCACTT3' of PAO696 geneSB168CTTCGACAAGCGCGTCTAC5' of PAO696 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO697 geneSB171CTGTCGACGACGTGTGG3' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO698 geneSB174AACAACGCTACCCTGAGAT3' of PAO709 geneSB175GCATAGGCCCCAGA5' of PAO709 geneSB177ATGCACCACCTCCAAGAAC5' of PAO700 geneSB177ATGCACCACCTCCAAGAAC5' of PAO700 geneSB177ATGCACCACCTCCAAGAACC5' of PAO700 geneSB177ATGCACCACCTCCAAGAACC5' of rAO700 geneSB177ATGCACCACCTCCAAGAACC5' of real geneSB177ATGCACCACCTCCAAGAACC5' of real geneSB177ATGCACCACCTCCAAGAACC5' of real geneSB177ATGCACCACCTCCAAGAACC5' of real geneSB177ATGCACCACCTCCAAGAACCC5' of real geneSB177ATGCACCACCTCCAAGAACCC5'	SB129	CCGACTTTCGAGTAACTTTC	5' of <i>phdA</i> gene
SB108AACAGCGAACGCTTCACAC5' of PAO692 geneSB160GAACACCGACAAACACGACT3' of PAO692 geneSB161CAGCGATGTCAGCAATAAGC5' of PAO693 geneSB162AGGTGCATGGCGAAAGC3' of PAO693 geneSB163GTCGTCGTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCAGTACCAGAAGGT5' of PAO695 geneSB165GGGTGTGCTGGGAATGGCT5' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO695 geneSB167CGCCAGTCGGTTCACTTT3' of PAO696 geneSB168CTTCGACAAGCGCGTCTAC5' of PAO696 geneSB169GCCCAGATCGGTATGAGAAT3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGGTTC3' of PAO697 geneSB171CTGTCGACAGCGCTGCAT3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO699 geneSB173GCTGCCAGATGGCTTGG5' of PAO699 geneSB174AACAAGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGCCGCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACTCCCAAAGAAC5' of PAO701 geneSB177ATGCACCACTCCCAAAGAAC5' of rea geneSB177ATGCACCACTCCCAAGAACC5' of rea geneRT-vreAI-FCTGCTGCGGAAGCACG3' of rea geneRT-vreAI-FTCGCTCGCGCAGGCCCAG5' of rea geneRT-vreAI-FTCGCTCGCGCAGGCCCAGC5' of rea geneRT-vreIR-FTCGCTCGCGCAGTCTCACCC3' of rea geneRT-vreIR-FTCGCTCGCGCAGGCTCGAGGC5' of rea geneRT-vreIR-FTCGCGCGCGGAG	SB107	CGCGACGGCTGAATATCTA	3' of <i>phdA</i> gene
SB160GAACACCGACAAACACGACT3' of PAO692 geneSB161CAGCGATGTCAGCAATAAGC5' of PAO693 geneSB162AGGTGCATGGCAGCGGAAAGC3' of PAO693 geneSB163GTCGTCGTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCATACCAGAAGGT5' of PAO694 geneSB165GGGTGTGCTTGGGATCG3' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO696 geneSB167CGCCAGTCGGTCACCTT3' of PAO696 geneSB168CTTCGACAAGCGGCTCAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO698 geneSB171CTGTCGACGGAAAGCTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO699 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of vra geneSB177ATGCACCACCTCCAAGAAC5' of vra geneRT-vreAI-FTCGCTTCGGCAAGACGACG5' of vra geneRT-vreAI-FTCGCTTCGGCAAGGCC5' of vra geneRT-vreRI-FTCGCTTCGGCAGAGTCTGAGGC5' of vra geneRT-vreRI-FTCGCTTCGGCCAAGGCC5' of vra geneRT-vreRI-RTCGCGCGCGGAGTCTGAGGC5' of vra geneRT-vreRI-RTCGCGCGCGCGAGTCTGAGGC5' of vra geneRT-vreRI-RTCGCGTCGCGC	SB108	AACAGCGAACGCTTCACAC	5' of PAO692 gene
SB161CAGCGATGTCAGCAATAAGC5' of PAO693 geneSB162AGGTGCATGGCGAAAGC3' of PAO694 geneSB163GTCGTCGTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCATCACCAGAAGGT5' of PAO694 geneSB165GGGTGTGCTTGGGATCG3' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO695 geneSB167CGCCAGTCGGTTCACITT3' of PAO696 geneSB168CTTCGACAGCGGCGTCTAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO697 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO699 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTGCGCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO700 geneSB178CCAGGTCCTCGGAAGACGACG3' of vred geneRT-vreAI-FTCGCTCGGAAGCACAGA5' of vred geneRT-vreAI-FTCGCTTCGGCATTTCCACCC3' of vred geneRT-vreR-FTCGCTTCGGCATTCCACCC3' of vred geneRT-vreR-FTCGCCTGGCGAGTCTGAGGC5' of vred geneRT-vreR-FTCGCTGCGCAGTCTCAGGCC5' of vred geneRT-vreR-RTCGGCGGCGGAGTCTGAGGC5' of vred geneRT-vreR-RTCGGCGCGCAGTCTCAGGCC5' of vred geneRT-vreR-RTCGGCGCGCAGTCTCAGGCC5' of vred geneRT-vreR-R <t< td=""><td>SB160</td><td>GAACACCGACAAACACGACT</td><td>3' of PAO692 gene</td></t<>	SB160	GAACACCGACAAACACGACT	3' of PAO692 gene
SB162AGGTGCATGGCGAAAGC3' of PAO693 geneSB163GTCGTCGTCATCGTCGTG5' of PAO694 geneSB164ACGGTGCAGTACCAGAAGGT5' of PAO695 geneSB165GGGTGTGGTTGGGATCG3' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO696 geneSB167CGCCAGTCGGTTCACTTT3' of PAO696 geneSB168CTTCGACAAGCGCGTCTAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGAGT5' of PAO698 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB173GCTGGCAAGCCGTCGAT5' of PAO698 geneSB174AACAACGCTACCTTGG5' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO699 geneSB176ACTACGCGATGCCTCAT3' of PAO700 geneSB177ATGCACCACTCCAAAGAAC5' of PAO700 geneSB176ACTAGGCGCCCCAGA5' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO700 geneSB176ACTGCGGAGATCGCCCAG3' of vred geneRT-vreAI-FGTGCTGCGAAGACGACG5' of vred geneRT-vreAI-FTCGCTTCGGAGTCTGAGGCC3' of vred geneRT-vreIR-RTCGCCGCGGAGTCTGAGGC5' of vred geneRT-vreIR-RTCGCCGCGGAGTCTGAGGC5' of vred geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vred geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vred geneRT-vreIR-RTCGGCGGCGCGAGTCTGAGGC5' of vred geneRT-vreIR-RTCGGCGGCGCGAGTCTGAGGC5' of vred geneRT-vreIR-R <t< td=""><td>SB161</td><td>CAGCGATGTCAGCAATAAGC</td><td>5' of PAO693 gene</td></t<>	SB161	CAGCGATGTCAGCAATAAGC	5' of PAO693 gene
SB163GTCGTCGTCGTCGTCGTG5' of PA0694 geneSB164ACGGTGCAGTACCAGAAGGT5' of PA0695 geneSB165GGGTGTGCTTGGGATCG3' of PA0695 geneSB166GACCGACCAGAAGGTGCT5' of PA0695 geneSB167CGCCAGTCGGTCACTTT3' of PA0696 geneSB168CTTCGACAGAGGGGTCTAC5' of PA0697 geneSB169GCCCAGATCGGTAATGAGAA3' of PA0697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PA0698 geneSB171CTGTCGACGGAAAGGTGTC3' of PA0698 geneSB172CGGTCAACCGTCGAT5' of PA0698 geneSB173GCTGGCAGATGGCTTGG5' of PA0699 geneSB174AACAACGCTACCCTGAGCAT5' of PA0699 geneSB175GCATAGCGCCCCAGA5' of PA0700 geneSB176ACTGCGGATCGCTCAT3' of PA0700 geneSB177ATGCACCACCTCCAAAGAAC5' of PA0700 geneSB177ATGCACCACCTCCAAAGAAC5' of PA0700 geneSB177ATGCACCACCTCCAAAGAAC5' of PA0701 geneRT-vreAI-FGTTGCTGCGCAAGACGACG3' of vreA geneRT-vreAI-FTCGCTTCGGCAGATTCCACCC3' of vreA geneRT-vreIR-FTCGCTCGGCGCAGTGTC5' of vreA geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreA geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreA geneRT-vreIR-RTCGGCGCGCGCGAGTGTCseneRT-vreIR-RTCGGCGCGCGCAGTGTCseneRT-vreIR-RTCGGCGGCGCGCAGTGTCseneSB133ACCAGAACCGCCATTCCsene	SB162	AGGTGCATGGCGAAAGC	3' of PAO693 gene
SB164ACGGTGCAGTACCAGAAGGT5' of PA0694 geneSB165GGGTGTGCTTGGGATCG3' of PA0695 geneSB166GACCACCAGAAGGTGCT5' of PA0695 geneSB167CGCCAGTCGGTCACTTT3' of PA0696 geneSB168CTTCGACAAGCGCGTCAC5' of PA0697 geneSB169GCCCAGATCGGTAATGAGAA3' of PA0697 geneSB170CGGGTGAAATTCGAAAGAGTGTTC3' of PA0698 geneSB171CTGTCGACGGAAAGGTGTTC3' of PA0698 geneSB172CGGTCAACCCGTCGAT5' of PA0699 geneSB173GCTGGCAGATGGCTTGG5' of PA0699 geneSB174AACAACGCTACCTGAGCAT3' of PA0699 geneSB175GCATAGGCGCCCCAGA5' of PA0700 geneSB176ACTGCGGATTCGCCTCAT3' of PA0700 geneSB177ATGCACCACCTCCAAAGAAC5' of PA0700 geneSB178GCTGGCGAAGACGACG3' of vreA geneRT-vreAI-FTGCGTCGCGAGACCAGA5' of vreA geneRT-vreAI-FTCGCTTCGGCAGTTCGAGCC3' of vreA geneRT-vreIR-RTCGCTTCGGCAGTCTCAGGCC5' of vreA geneRT-vreIR-RTCGCTTCGGCAGTCTCAGGCC5' of vreA geneRT-vreIR-RTCGCTTCGGCAGTCTGAGGC5' of vreA geneRT-VreIR-RTCGCTGCGCAGTCTCGAGGCCAG5' of vreA geneRT-VreIR-RTCGCTGCGCAGTCTGAGGC5' of vreA geneRT-VreIR-RTCGCTGCGCAGTCTCAGGCC5' of vreA geneRT-VRER-RTCGCTGCGCAGTCTCAGGCC5' of vreA geneRT-VRER-RTCGCTGCGCAGTCTCAGGCC5' of vreA geneRT-VRER-RTCGCTGCGCAGTGTCC5' of vreA geneRT	SB163	GTCGTCGTCATCGTCGTG	5' of PAO694 gene
SB165GGGTGTGCTTGGGATCG3' of PAO695 geneSB166GACCGACCAGAAGGTGCT5' of PAO695 geneSB167CGCCAGTCGGTTCACTTT3' of PAO696 geneSB168CTTCGACAAGCGCGCTCTAC5' of PAO697 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATCGAAGGTGTTC3' of PAO698 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO699 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneSB177ATGCACCACCTCCGAGCCCAG3' of vre4 geneRT-vreAI-FCGGCGCGGAGTCTGAGGCC3' of vre4 geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGCC5' of vre1 geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vre4 geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vre4 geneRT-vreIR-RTCGGCGCGGAGTCTGAGGC5' of vre4 geneRT-VreIR-RTCGGCGCGCGAGTCTCAGGCC5' of vre4 geneRT-VreIR-RTCGGCGCGCGCGCGTGTCvre4 geneSB133ACCAGAACCGCCATTCC5' of vre4 gene	SB164	ACGGTGCAGTACCAGAAGGT	5' of PAO694 gene
SB166GACCGACAGAAGGTGCT5' of PA0695 geneSB167CGCCAGTCGGTTCACTTT3' of PA0696 geneSB168CTTCGACAAGCGCGTCTAC5' of PA0696 geneSB169GCCCAGATCGGTAATGAGAA3' of PA0697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PA0697 geneSB171CTGTCGACGGCAGTGTC3' of PA0698 geneSB172CGGTCAACCGTGAT5' of PA0698 geneSB173GCTGGCAGATGGCTTGG5' of PA0699 geneSB174AACAACGCTACCCTGAGCAT3' of PA0699 geneSB175GCATAGCGCGCCCAGA5' of PA0700 geneSB176ACTGCGGATTCGCCTCAT3' of PA0700 geneSB177ATGCACCACCTCCAAAGAAC5' of PA0700 geneSB177ATGCACCACCTCCAAAGAAC5' of PA0701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreIR-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-RTCGGCGGCGGAGTTCGCGCAGTGTC3' of vreA geneRT-PCRTCGGCGGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCCVreA gene	SB165	GGGTGTGCTTGGGATCG	3' of PAO695 gene
SB167CGCCAGTCGGTTCACTTT3' of PAO696 geneSB168CTTCGACAAGCGCGTCTAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO697 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACTCCAAGAAC5' of PAO701 geneSB177ATGCACCACTCCAAGAACA5' of vreA geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-FTCGCTTCGGCATTTCCACCC3' of vreA geneRT-vreIR-RTCGGCGGCGCAGTGTC5' of vreA geneqRT-PCRKKKSB133ACCAGAACCGCCATTCCK	SB166	GACCGACCAGAAGGTGCT	5' of PAO695 gene
SB168CTTCGACAAGCGCGTCTAC5' of PAO696 geneSB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO697 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneSB177ATGCACCACCTCCAAAGAAC5' of vreA geneRT-vreAI-FGCGTCGGGGGGGAGTCTGAGGC3' of vreA geneRT-vreIR-FTCGCTTCGGCAATTCCACCC3' of vreA geneRT-vreIR-RCCGGTCGGGGAGTCTGAGGC5' of vreA geneqRT-PCRKTSGCTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCC3' of vreA gene	SB167	CGCCAGTCGGTTCACTTT	3' of PAO696 gene
SB169GCCCAGATCGGTAATGAGAA3' of PAO697 geneSB170CGGGTGAAATTCGAAGAGAT5' of PAO697 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-FTCGCTTCGGCAATTTCCACCC3' of vreA geneRT-vreIR-FTCGGCTGCGCAGAGTCTGAGGC5' of vreR geneRT-vreIR-RCCAGGTCCTCGGCAGTCTGAGGCC5' of vreA geneRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCC10 vreA gene	SB168	CTTCGACAAGCGCGTCTAC	5' of PAO696 gene
SB170CGGGTGAAATTCGAAGAGAT5' of PAO697 geneSB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCGATTCCACCC3' of vreI geneRT-vreIR-RCGGCGGCGGAGTCTGAGGC5' of vreI geneRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCC1' of vreA gene	SB169	GCCCAGATCGGTAATGAGAA	3' of PAO697 gene
SB171CTGTCGACGGAAAGGTGTTC3' of PAO698 geneSB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGCCGGCGGAGTCTGAGGC5' of vreR geneqRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCCVreA gene	SB170	CGGGTGAAATTCGAAGAGAT	5' of PAO697 gene
SB172CGGTCAACCCGTCGAT5' of PAO698 geneSB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreIR-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-RTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreI geneRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCC3' of vreA gene	SB171	CTGTCGACGGAAAGGTGTTC	3' of PAO698 gene
SB173GCTGGCAGATGGCTTGG5' of PAO699 geneSB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreI geneRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCC3' of vreA gene	SB172	CGGTCAACCCGTCGAT	5' of PAO698 gene
SB174AACAACGCTACCCTGAGCAT3' of PAO699 geneSB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreI geneRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCC5' of vreA gene	SB173	GCTGGCAGATGGCTTGG	5' of PAO699 gene
SB175GCATAGGCGCCCCAGA5' of PAO700 geneSB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreI geneQRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCCVreA gene	SB174	AACAACGCTACCCTGAGCAT	3' of PAO699 gene
SB176ACTGCGGATTCGCCTCAT3' of PAO700 geneSB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreI geneqRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCCVreA gene	SB175	GCATAGGCGCCCCAGA	5' of PAO700 gene
SB177ATGCACCACCTCCAAAGAAC5' of PAO701 geneRT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreA geneqRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCCVreA gene	SB176	ACTGCGGATTCGCCTCAT	3' of PAO700 gene
RT-vreAI-FGTTGCTGCCGAAGACGACG3' of vreA geneRT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of vreI geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of vreI geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of vreR geneqRT-PCRSB132AGGTCGTGCGCAGTGTCvreA geneSB133ACCAGAACCGCCATTCCVreA gene	SB177	ATGCACCACCTCCAAAGAAC	5' of PAO701 gene
RT-vreAI-RCCAGGTCCTCGGAGCCCAG5' of <i>vreI</i> geneRT-vreIR-FTCGCTTCGGCATTTCCACCC3' of <i>vreI</i> geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of <i>vreR</i> geneqRT-PCRsB132AGGTCGTGCGCAGTGTC <i>vreA</i> geneSB133ACCAGAACCGCCATTCCvreA gene	RT-vreAI-F	GTTGCTGCCGAAGACGACG	3' of <i>vreA</i> gene
RT-vreIR-FTCGCTTCGGCATTTCCACCC3' of <i>vreI</i> geneRT-vreIR-RTCGGCGGCGGAGTCTGAGGC5' of <i>vreR</i> geneqRT-PCRsB132AGGTCGTGCGCAGTGTC <i>vreA</i> geneSB133ACCAGAACCGCCATTCCvreA gene	RT-vreAI-R	CCAGGTCCTCGGAGCCCAG	5' of <i>vreI</i> gene
RT-vreIR-R TCGGCGGCGGAGTCTGAGGC 5' of vreR gene qRT-PCR sB132 AGGTCGTGCGCAGTGTC vreA gene SB133 ACCAGAACCGCCATTCC vreA gene	RT-vreIR-F	TCGCTTCGGCATTTCCACCC	3' of <i>vreI</i> gene
qRT-PCR AGGTCGTGCGCAGTGTC vreA gene SB132 ACCAGAACCGCCATTCC VreA gene	RT-vreIR-R	TCGGCGGCGGAGTCTGAGGC	5' of <i>vreR</i> gene
SB132 AGGTCGTGCGCAGTGTC vreA gene SB133 ACCAGAACCGCCATTCC	aRT-PCR		0
SB133 ACCAGAACCGCCATTCC	SB132	AGGTCGTGCGCAGTGTC	<i>vreA</i> gene
	SB133	ACCAGAACCGCCATTCC	
SB134 CCGGCAGATTCTCATCG <i>vreI</i> gene	SB134	CCGGCAGATTCTCATCG	vreI gene

Table 2. cont.

Name	Sequence 5' to 3'	Description
SB135	CCGAACCTCTGGATGACTTT	
SB137	TCTCTGGCAGGCACTCG	<i>vreR</i> 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 EcoRI/ XbaI PCR fragment into the pMP220 vector (Spaink et al., 1987). The pMPR3mutPho 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 BamHI/SpeI sites of the pUC18-mini-Tn7T-LAC plasmid (Choi & Schweizer, 2006). The Spel/PstI DNA fragment from the pUC18-miniTn7T-LAC-vreI plasmid containing vreI was then subcloned into the NheI/PstI sites of pJN105 plasmid (Newman & Fuqua, 1999).

Bacterial strains constructions. The pPAO690::lacZ, pphdA::lacZ, pPAO692::lacZ strains were constructed as follows. DNA fragments corresponding to PAO690, phdA and PAO692 putative promoter regions were amplified by PCR as BamHI/XhoI 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 *SpeI/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 $\Delta vreI$ and $\Delta phoB$ 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 realtime 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 Pilimited 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 sp 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, mutPho). Mutation of the putative pho box abolished the induction of PUMA3 gene expression in low Pi conditions (Fig. 3c, pMPR3mutPho 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 $\sigma^{\rm Vrel}$ 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 pPAO690:: lacZ and pphdA:: lacZ transcriptional fusions by 2.5 and 18-fold respectively but had no effect on the pPAO692:: 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 $\sigma^{\rm VreI}$ 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 $\sigma^{\rm Vrel}$ regulon

Our experiments show that the promoter of TU-2 is activated upon low Pi through the σ^{Vrel} 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 $\sigma^{\rm VreI}$ -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, $\Delta vreI$ 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*, pphdA::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.



Fig. 5. σ^{Vrel} factor and PhoB regulate TU-1 and TU-2 promoter activity. *P. aeruginosa* and its isogenic *vrel, phoB* mutants containing the chromosomal transcriptional fusion pPAO690::*lacZ* (a) or pphdA::*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-vrel plasmids. Bars represent mean of β -galactosidase activity for at least three independent experiments. (c) mRNA level of *vrel* from wild-type, *vrel, phoB* mutants containing the pJN105-vrel 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^{Vrel} 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^{Vrel} 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^{Vrel} 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 phosphatelimited 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^{Vrel} 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^{Vrel} -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 $\sigma^{\rm VreI}$ 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 antisigma factor and obtain a large σ^{VreI} -dependent gene expression.

From our study, it appears that expression of the genes belonging to the σ^{Vrel} regulon is partially reduced in a *vrel* 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.

ACKNOWLEDGEMENTS

We are grateful to Wilbert Bitter for helpful discussions, Yann Denis for technical assistance on qRT-PCR, Eric Cascales for the electrophoretic mobility shift assay and Bérengère Ize for discussions, helpful comments and encouragement. We thank Marie-Anne Lafaulace for encouragement. This work is supported by Vaincre la Mucoviscidose (the French cystic fibrosis foundation) through a grant (FR20110600567/1/1/69). Research in S. deB.'s laboratory is supported by Bettencourt-Schueller Foundation, CNRS Institutional and ANR grants (ERA-NET ADHRES 27481, PCV-ANR 27628, ANR-09-JCJC-0047 and GDR3171). The Medical Research Foundation supports S. B. Research in M. A. L.'s lab is supported by the EU through a Marie Curie CIG grant (3038130) and the Spanish Ministry of Economy through a Ramon&Cajal grant (RYC-2011-08874).

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Edited by: M. Whiteley