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ORIGINAL PAPER

Chromobacterium violaceum adaptation to low-phosphate conditions

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Abstract *Chromobacterium violaceum* is a free-living bacterium that inhabits low-nutrient environments such as the Amazon basin. Bacteria respond to phosphate (Pi) shortage by expressing a range of genes involved in Pi uptake and assimilation, known as the PHO regulon. Several PHO regulon genes have been annotated in the genome of *C. violaceum*. Here we show that *C. violaceum* is extremely well adapted to low-Pi conditions. Remarkably, this bacterium is able to grow in media containing only traces of Pi. The PHO regulon genes are induced upon Pi depletion, but the bacteria continued to grow under these conditions. Unlike other Proteobacteria hitherto analyzed, neither PstS nor PhoU play a role in the repression of the PHO regulon under Pi excess.

Keywords *Chromobacterium violaceum* · PHO regulon · *pst* operon · Phosphate · Nutritional stress

Introduction

Free-living organisms frequently face shortage of macronutrients in natural environments, which are often poor in basic resources, such as carbon, nitrogen and phosphorus (Smil 2000). Phosphorus occurs in nature in its maximal oxidized state (PO_4^{3-} or Pi), which is also the preferred phosphorus source. Once Pi is taken up by bacteria, it is directly incorporated into phosphorylated monomers, primarily as ATP (Wanner and Wilmes-Riesenberg 1992). Given the importance of Pi and the irregular availability of this nutrient in natural habitats, bacteria must be able to sense changes in Pi concentration in the environment and to modify the pattern of gene expression accordingly. When the external concentration of Pi is below a minimum threshold that supports bacterial growth (ca. 4 μM), a set of genes involved in scavenging alternative sources of this nutrient, the PHO regulon, is activated (Wanner F1996). The most well-studied PHO genes are *phoA*, the *pst* operon and the *phoBR* operon that, respectively, encode the periplasmic enzyme alkaline phosphatase, the high-affinity Pi-transport system Pst and the two-component system that controls PHO expression.

The histidine kinase PhoR autophosphorylates in response to low Pi in the medium and transfers the Pi moiety to PhoB, which in turn binds to the PHO-boxes, that are specific sequences in the regulatory regions of all PHO genes in place of the standard −35 sequence. PhoB then interacts with the RNA polymerase associated with σ^{70}, thus initiating transcription (Makino et al. 1989). Under Pi excess, PhoR dephosphorylates PhoB, thereby repressing the expression of the PHO genes (Carmany et al. 2003).

The Pst transporter is a typical ATP-binding cassette system (ABC) comprised of four proteins: PstS or PiBP (Pi-binding protein), a periplasmic protein whose function is to scavenge Pi molecules in the periplasm and to present them to the channel formed by the integral proteins PstC and PstA. PstB is an ATPase that energizes the transport process (Surin et al. 1985). The Pst system of most Proteobacteria is

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Table 1 Bacterial strains, plasmids and DNA oligomers used in this study

Strains	Genotype	Source
C. violaceum strains		
C. violaceum ATCC 12472	Wild-type strain	Consortium (2003)
FN2	*pstS*::pKNOCK-km	This work
FN3	*phoU*::pKNOCK-km	This work
E. coli strains		
DH5α	F- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* φ80d*lacZ* δM15 δ(*lacZYA-argF*) U169, *hsdR*17 λ	
S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::*Tn*7	Simon et al. (1983)
Plasmids	***Relevant features***	
pFN13	P*phoU-lacZ* fusion	This work
pFN15	P*phoB-lacZ* fusion	This work
pKNOCK-km	Suicide vector, KmR	Alexeyev (1999)
pRK*lacZ*290	Low copy vector with a promoterless *lacZ*	Gober and Shapiro (1992)
pTZ57R/T	Cloning vector	Fermentas
Oligomers	**Sequence (5'->3')**	
lacZ1391(R)	CCTCTTCGCTATTACGCCAG	
phoB-prom(F)	CAAGATTGGTTGCGGATGAG	
phoB-prom(R)	GTTGAAGGCGATCAGCTCC	
phoU-prom(F)	GAAACCATCAACCACATCGCC	
phoU-prom(R)	GTGTTCTGCCATGATTAGCCTC	
phoU-int(F)	GTGGAGCAACAGATCCTGTC	
phoU-int(F)	GGGTCTTCCATCATGAAGGT	
phoU-ext(F)	TGGAGGAAACCATCAACCAC	
phoU-ext(R)	GGTAACCGCGAGATGTTACC	
pstS-int(F)	TGCCGCTGCAGCCGGAAG	
pstS-int(R)	CTGGTTCTGCAGCAGGCCG	
pstS-ext(F)	ACCACGTTCAGATAATCGCC	
pstS-ext(R)	GCCGATTCTTGATGGTTCAG	

encoded by an operon composed of five genes—*pstS*, *pstC*, *pstA*, *pstB* and *phoU*, transcribed as a single polycistronic mRNA (Aguena et al. 2002, 2009). The most distal gene of the operon, *phoU*, encodes a protein that does not participate in Pi transport and which exact function is unknown (Steed and Wanner 1993). A complicating factor in the regulation of PHO is that the five proteins encoded by the *pst* operon act as repressors of PHO transcription when Pi is in excess. Null mutations in *phoU* or in any other *pst* gene cause the constitutive expression of the PHO regulon irrespectively of Pi concentration in the medium (Wanner 1996). The mechanism through which the Pst proteins repress PHO expression is unclear, but it has recently been shown that under Pi-excess conditions PhoU physically interacts with PstB and PhoR (Gardner et al. 2014).

Chromobacterium violaceum is a free-living β-proteobacterium that inhabits the soil and water of tropical environments and that thrives in the acidic and low-nutrient waters of the Rio Negro in the Amazon basin. A proteomic analysis revealed that 3 % of *C. violaceum* proteins are involved in inorganic ion transport and metabolism (Ciprandi et al. 2013). Several putative PHO regulon genes have been annotated in the genome of *C. violaceum* (Consortium 2003). In the present study, we investigated the growth of *C. violaceum* under Pi limitation, its ability to take up Pi and the pattern of induction of the PHO regulon. We show that this bacterium is well adapted to conditions of Pi scarcity.

Methods

Strains, plasmids and oligomers

The strains, plasmids and oligomers used in this study are described in Table 1.

Culture media and growth conditions

C. violaceum and *E. coli* cells were routinely cultured at 37 °C in lysogenic broth medium (LB) and L-agar (Miller

1992). Medium A (0.12 M Tris, 80 mM NaCl, 20 mM KCl, 20 mM NH_4Cl, 3 mM Na_2SO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2 μM $ZnCl_2$, 0.5 % glucose, 0.5 % bacto-peptone, pH 7.5) is a semi-rich Pi-limited medium (Spira et al. 2010). MM medium is modified MOPS minimal medium (Neidhardt et al. 1974) (9.52 mM NH_4Cl; 0.28 mM K_2SO_4; 0.009 mM $CaCl_2$; 0.52 mM $MgCl_2$; 50 mM NaCl; 0.01 mM $FeSO_4$; 40 mM MOPS; 3×10^{-6} mM $(NH_4)_6(Mo_7)_{24}$; 4×10^{-4} mM H_3BO_3; 3×10^{-5} mM $CoCl_2$; 10^{-5} mM $CuSO_4$; 8×10^{-5} $MnCl_2$; 10^{-5} mM $ZnSO_4$) supplemented with 0.2 % glucose and variable Pi concentrations. When required, *C. violaceum* cultures were supplemented with kanamycin (25 μg/ml), tetracycline (5 μg/ml) or ampicillin (100 μg/ml). Bacterial growth rate (μ) was calculated according to the formula: $\mu = \ln(N/N_0)/(T)$, where N and N_0, respectively, correspond to two points at the exponential growth phase and t is the time course of the growth curve.

Detection of PHO-boxes in silico

The upstream regions of putative PHO regulon genes of *C. violaceum* were scanned for PHO boxes using the consensus sequence 5′-CTGTCATAAATCTGTCAT with the Fuzznuc program from the EMBOSS software package (Rice et al. 2000); up to nine mismatches were allowed. Scores were attributed to the sequence matches based on their similarity to the PHO-box consensus as described by Yuan et al. (2006).

Alkaline phosphatase (AP) assay

Alkaline phosphatase quantitative assays were performed as described (Galbiati et al. 2014). The specific activity of AP was calculated according to the formula: A_{410} min^{-1} OD_{660}^{-1}, where A_{410} is the absorbance of enzymatic products after removal of the cells by centrifugation, *min* is the reaction time and OD_{660} is the turbidity of the bacterial culture.

β-Galactosidase assay

β-Galactosidase assays were performed as described (Miller 1992), except that the cell density was measured at 660 nm. The specific activity was calculated according to the formula: A_{420} min^{-1} OD_{660}^{-1} 1000. The variables are as described above for the AP assay.

Pi uptake

Pi-uptake assays were performed as described by de Almeida et al. with some modifications (de Almeida et al. 2015). Briefly, bacteria were cultivated in MM medium containing 10 mM Pi (KH_2PO_4) for 48 h at 37 °C, centrifuged at 5000×*g* for 10 min, washed twice, suspended in Pi-free medium at an OD_{660} of 0.1 and grown for 6 h at 37 °C to induce phosphate starvation. Pi transport was assayed by adding a mix of 0.5 mM K_2HPO_4 + 10 μCi [^{32}P] (IPEN, São Paulo, Brazil). One hundred microliter samples were withdrawn every 30 s, applied to nitrocellulose membrane disks (Millipore, MA) on a manifold and immediately washed with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, pH 7.4.). The filters were transferred to vials containing 5-ml scintillation cocktail (Perkin-Elmer, Waltham, MA) and read in a scintillation counter (Beckman Coulter, Fullerton, CA).

Microdetermination of Pi

Pi concentration in the culture medium was measured as described (de Almeida et al. 2015). The supernatants of the bacterial cultures were collected, diluted ten times and mixed with the same volume of the working reagent (1 volume of 167 mM H_2SO_4, 1 volume of 2.5 % $(NH_4)2MoO_4$, 1 volume of 10 % ascorbic acid and 2 volumes of deionized water) and incubated at 37 °C for 2 h. Following the incubation period, samples were measured in a spectrophotometer at 820 nm. Calibration solutions were prepared with the following concentrations of NaH_2PO_4: 0.16, 0.08, 0.04, 0.02 and 0.01 mM.

Construction of transcriptional fusions

lacZ transcriptional fusions were constructed in plasmid pRK*lacZ*290. DNA fragments encompassing the promoter regions of *phoB* and *phoU* were amplified by PCR using, respectively, the phoB-prom and phoU-prom pairs of primers and cloned in plasmid pTZR57 (Fermentas-Thermo) according to the manufacturer's instructions. The resulting plasmids containing P*phoU* or P*phoB* DNA sequences were digested with EcoRI or EcoRI–HindIII, respectively, and ligated to plasmid pRK*lacZ*290 digested with the same enzymes resulting in plasmids pFN13 and pFN15. Cloning was confirmed by DNA sequencing using the *lacZ* reverse primer. Plasmids pFN13 and pFN15 were transformed into *E. coli* S17-1 and mobilized to *C. violaceum*.

Construction of the *pstS* and *phoU* knockout mutants

Knockout mutants of *pstS* and *phoU* were obtained by inserting the suicide plasmid pKNOCK-Km into *C. violaceum* chromosome. DNA fragments of 461 and 470 bp corresponding, respectively, to internal regions of *phoU* and *pstS* ORFs were amplified by PCR using phoU-int or *pstS*-int sets of primers. The amplicons were cloned in vector pTZ57R/T. The *phoU* fragment was digested with XbaI and HindIII, while the *pstS* fragment was digested only

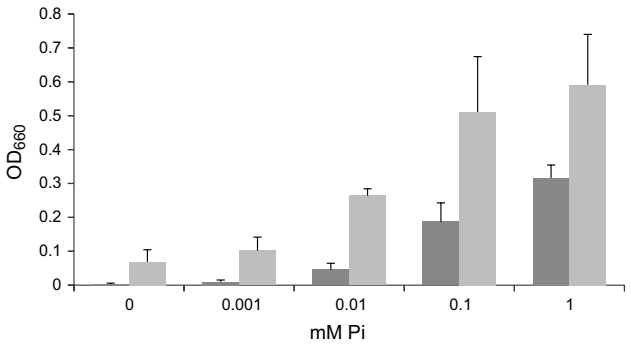

Fig. 1 Growth of *E. coli* and *C. violaceum* under different Pi concentrations. *E. coli* K-12 strain MG1655 (*dark gray bars*) and *C. violaceum* (*light gray bars*) are grown for 24 h in MOPS minimal medium supplemented with 0, 0.001, 0.01, 0.1 or 1 mM KH_2PO_4. Culture turbidity is measured at OD_{660}. *Bars* represent the mean ± SEM of three independent cultures

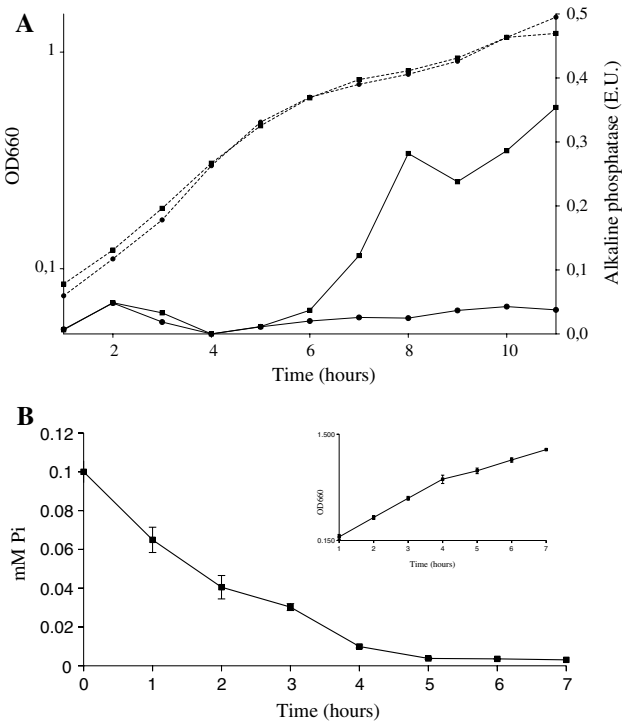

Fig. 2 Induction of AP and Pi consumption by wild-type *C. violaceum* cells. **a** Bacteria grown overnight are resuspended in non-supplemented medium A (~0.1 mM Pi) or in medium A supplemented with 1 mM Pi and grown for another 11 h. Growth (OD_{660}) (*dashed lines*) and AP activity (*solid lines*) are followed hourly. (*Square*) non-supplemented medium A; (*circle*) medium A supplemented with 1 mM Pi. **b** Bacteria suspended in non-supplemented medium A are assayed for Pi consumption. Samples are taken hourly to assay Pi concentration in the medium and cell turbidity (*inset*). *Each point* corresponds to the mean ± SEM of three independent experiments

with PstI. Both *phoU* and *pstS* fragments were subcloned in the corresponding sites of pKNOCK-Km. The resulting plasmids were transformed into *E. coli* S17-1 and mobilized by conjugation to *C. violaceum*, where they underwent insertion in the bacterial chromosome. Bacteria were selected for resistance to Kanamycin. The *phoU* and *pstS* insertions in *C. violaceum* chromosome were confirmed by PCR using *phoU*-ext or *pstS*-ext sets of primers.

Genetic transfer and molecular biology techniques

Standard bacterial genetics and molecular biology procedures, such as electroporation, conjugation, DNA restriction and cloning, were performed as described (Miller 1992; Sambrook and Russell 2001).

Results and discussion

C. violaceum growth under low-Pi conditions and induction of the PHO regulon

C. violaceum thrives in Pi-deficient habitats, such as the Rio Negro basin (McClain et al. 2001; Hungria et al. 2005). To investigate how *C. violaceum* responds to Pi availability, we monitored the growth of bacterial cultures in minimal medium supplemented with a range of Pi concentrations and compared it to the growth of the prototrophic *E. coli* strain MG1655 (Fig. 1). *C. violaceum* grew better than *E. coli* under all tested Pi concentrations. Even when no Pi was added *C. violaceum* managed to present some growth, possibly by using Pi contaminants as a P source and/or by mobilizing internal Pi stores. *C. violaceum* also formed colonies of normal size (>1.5 mm) when grown in minimal medium containing Pi concentrations ranging from 1 μM to 10 mM, while *E. coli* did not grow at all in 1 μM Pi medium (not shown). These results suggest that *C. violaceum* is well adapted to the conditions of Pi scarcity, where even very versatile bacterial species, such as *E. coli*, are unable to grow.

To correlate bacterial growth with the induction of the PHO regulon, a growth curve in medium A, a semi-rich medium with limited Pi concentration (~0.1 mM) used to induce Pi starvation and to study the expression of the PHO genes (Spira and Yagil 1999), was conducted (Fig. 2a). Interestingly, bacteria inoculated in medium A and in medium A supplemented with 1 mM Pi showed nearly identical growth curves. During the first 5 h, the growth rate of both cultures was about 0.4 h^{-1} and after that decreased to 0.15 h^{-1}. The similarity in the growth pattern of the cultures growing under Pi excess or starvation suggests that *C. violaceum* can grow equally well under high- and low-Pi concentrations and that the reduction in growth rate after 5 h was caused by factors unrelated to Pi concentration in the medium. However, only in the low-Pi medium

Table 2 *In silico* identification of PHO-box upstream to putative PHO regulon genes in *C. violaceum* genome

Gene	Mismatches	Sequence	Distance (from ATG)	Score	Hit
phoA	7	TCGTCATAAAATCTTAAT	−76	0.58	Yes
phoB	5	ATGTCATATTAGTGTCAT	−46	0.83	Yes
phoU	5	CTGTCATCTGACTGTCTT	−36	0.81	Yes
phnG	8	TTATCATGGATACGTCTA	−56	0.42	Yes
pstS	9	CGGCGAAACGGCTGACGT	−63	0.30	No
ugpB	8	ATGTCATATTATCGTAAC	−72	0.78	Yes

AP was induced, evidencing the onset of the Pi-starvation phase. This indicates that, unlike *E. coli*, where AP induction occurs concomitantly with growth arrest (Spira et al. 1995), the PHO regulon of *C. violaceum* was activated, while bacteria were still growing, albeit at a lower rate. It is worth noticing that the continued increase in OD_{660} under Pi starvation was not caused by enlargement of bacterial cells or by any other phenomenon that might have artificially increased the turbidity of the culture, but was due to bacterial replication (Fig. S1).

In another experiment, the consumption of Pi of *C. violaceum* growing in non-supplemented medium A (~0.1 mM Pi) was assessed (Fig. 2b). Bacteria growing in this medium consumed all available Pi in 5 h. During this period, the bacterial population doubled twice (from 0.16 to 0.68 OD_{660} units). After 4 h, the growth rate dropped from 0.41 to 0.21 h^{-1}, but the population kept growing. The reduction in growth rate in both Pi-starved and non-starved cultures was likely to be caused by scarcity of another macronutrient. The fact that cells in the Pi-starved culture continued to grow irrespectively of Pi concentration in the medium suggests that *C. violaceum* can mobilize internal Pi reservoirs to support growth.

The PHO regulon of *C. violaceum*

Twenty-three PHO regulon genes, such as *phoB-phoR*, the *pstSCAB* and *ugpBAEC* operons, the *phn* gene cluster, *phoA* and *phoU*, were annotated in the genome of *C. violaceum*. Sequences compatible with the PHO-box consensus (5′-CTGTCATAAATCTGTCAT) were found upstream of *phoU*, *phnG*, *ugpB*, *phoB* and *pstS* ORFs (Table 2). The sequences immediately upstream to *phoB*, *phoU* and *ugpB* showed a high degree of similarity to the PHO-box consensus. The PHO-box upstream to *pstS* displayed the lowest score.

Expression of *phoB-lacZ* and *phoU-lacZ*

Transcriptional fusions between the putative promoters of two PHO genes—*phoB* and *phoU* with *lacZ*, were constructed and used to test the pattern of expression of these genes under different growth conditions. Plasmids pFN15 (P*phoB-lacZ*) and pFN13 (P*phoU-lacZ*) were transferred by conjugation to *C. violaceum*. The exconjugants were grown in medium A and sampled hourly for bacterial growth, β-galactosidase and AP activity (Fig. 3). Both cultures entered the Pi-starvation phase after 4 h of growth in medium A, as shown by the sharp increase in AP activity. Bacteria continued growing even after AP induction, as observed previously (Fig. 2). Around this time point, β-galactosidase activity of both *phoB-lacZ* and *phoU-lacZ* also started to increase. *phoU-lacZ* and *phoB-lacZ* activities increased eightfold (from 260 to 2200 Miller units) and threefold (from 4000 to 12,000 Miller units), respectively, throughout the course of the experiment. The β-gal activity of the *phoB-lacZ* fusion was from the start considerably higher than that of *phoU-lacZ*, even though both promoters carry PHO boxes with similar scores (Table 2). Similarly, the *phoB* gene of *E. coli* also showed a higher basal level and lower level of induction than other PHO genes (Shinagawa et al. 1983). Overall, these results demonstrate that both *phoB* and *phoU* respond to Pi starvation, as expected for genes that belong to the PHO regulon.

phoU and *pstS* are not involved in PHO repression

In most Proteobacteria hitherto analyzed *phoU* is the most distal gene of the *pst* operon. In contrast, in *C. violaceum phoU* forms a separate transcriptional unit together with *rpiA*, *ppx* and *corA*, that, respectively, encode a ribose 5-phosphate isomerase, an exopolyphosphatase and a protein involved in magnesium/cobalt transport. This genomic organization can also be found in other β-Proteobacteria. An analysis with the String program (Franceschini et al. 2013) showed that 12 out of 72 β-Proteobacteria species with sequenced genomes have the *phoU* gene separated from the *pst* operon, while only two out of 117 alpha and one out of 238 γ-Proteobacteria display this genomic arrangement. All δ and ε Proteobacteria carry *phoU* integrated in the *pst* operon. This suggests that *phoU* segregation from the *pst* operon is a relatively recent event in the evolution of *C. violaceum*, which may have been selected in response to severe low-Pi conditions.

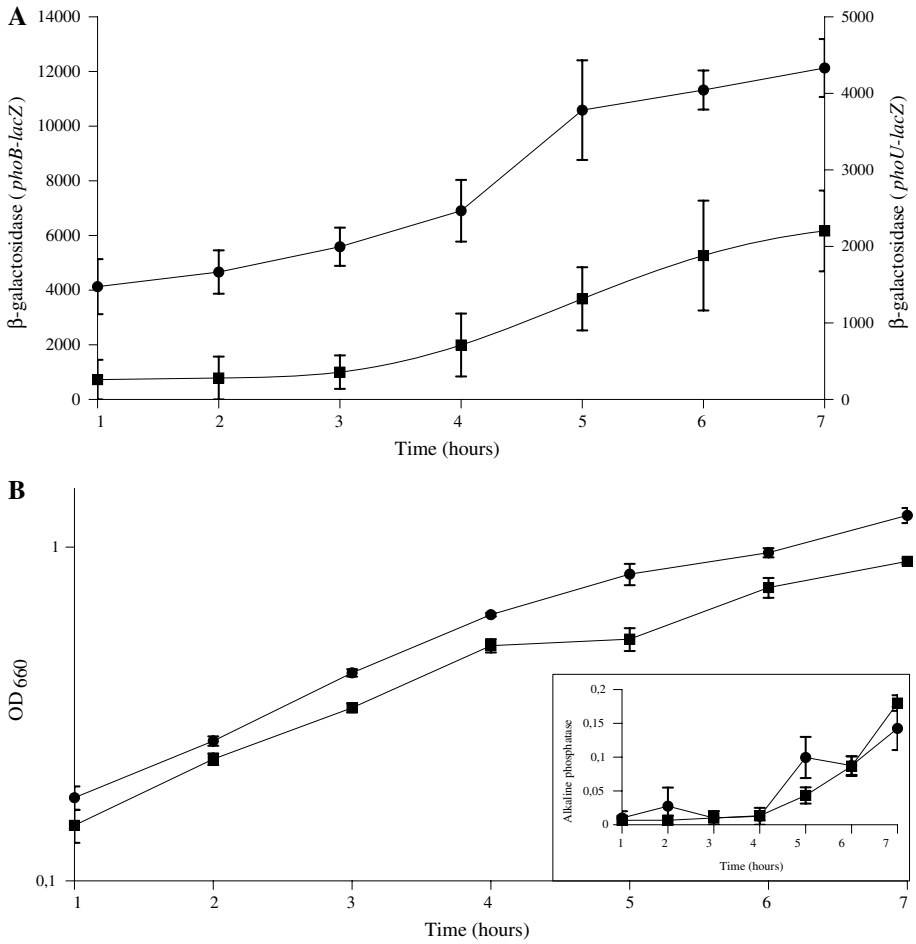

Fig. 3 Expression of *phoB* and *phoU* under Pi starvation. Wild-type *C. violaceum* cells carrying either *phoB-lacZ* (*circle*) or *phoU-lacZ* (*square*) fusions are grown in non-supplemented medium A and assayed for **a** β-gal activity and **b** growth and AP activity (*inset*). *Each point* represents the mean ± SEM of three independent experiments

phoU encodes a protein that in *E. coli* and in other Proteobacteria acts as a repressor of the PHO regulon under Pi-excess conditions (Wanner 1996). In these bacteria, null mutations in *phoU* result in the constitutive expression of the PHO regulon. To analyze the role of PhoU in PHO repression in *C. violaceum*, a *phoU* knockout was constructed. The *phoU* mutant and the wild-type strain were grown in medium A and assayed for AP activity (Fig. 4). Under Pi-limiting conditions, AP was induced in both wild-type and *phoU* mutant strains, while under Pi excess the level of AP was significantly lower (repressed) in both strains. This suggests that *C. violaceum*'s PhoU is not involved in PHO repression. In contrast, the *phoU* mutant of *E. coli* (Muda et al. 1992; Steed and Wanner 1993) and *P. aeruginosa* (de Almeida et al. 2015) express AP constitutively even under Pi-excess conditions. The exact function of PhoU in *E. coli* and in other bacteria is unknown, but it is well established that PhoU plays a role in PHO repression (Wanner 1996; Gardner et al. 2014). Interestingly, the firmicute *B. subtilis* does not carry a copy of *phoU* and its Pst system is not involved in PHO regulation (Qi et al. 1997). In contrast, *C. violaceum* does carry *phoU*, which is inducible by Pi starvation, but it is not located in the *pst* operon.

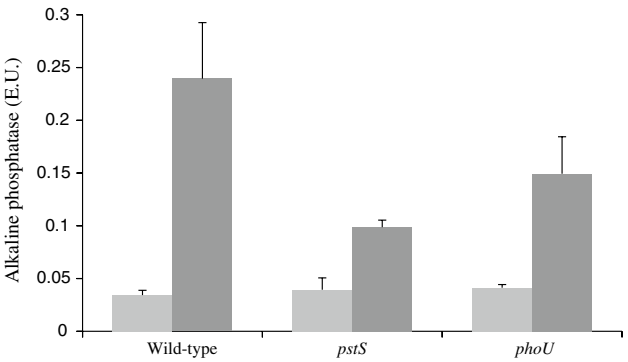

Fig. 4 AP activity of the wild-type, *pstS* and *phoU* mutants. Bacteria are grown overnight in medium A supplemented with 1 mM Pi (*light gray bars*) or in non-supplemented medium A (*dark gray bars*) and assayed for AP activity. *Each bar* represents the mean ± SEM of three independent experiments

This different genomic arrangement suggests that PhoU plays a different and still unknown role in this bacterium.

The role of *pstS*, the most proximal gene of the *pst* operon, on PHO repression was also analyzed. Similarly to what has been observed with the *phoU* mutant, the AP

activity of the *pstS* mutant was repressed under Pi-excess conditions, suggesting that *pstS* also does not participate in PHO regulation. It is interesting to note that induction of AP by Pi starvation (the ratio of AP activity in the Pi-starved and Pi-excess cultures) was significantly lower ($p = 0.045$) in the *pstS* mutant (AP induction = 2.5) than in the wild-type strain (AP induction = 7.0). On the other hand, the AP activities of the Pi-starved wild-type strain and *phoU* mutant were not statistically different ($p = 0.20$), implying that AP induction in the *phoU* mutant was not significantly lower than in the wild-type strain.

In the majority of bacterial species hitherto analyses—*E. coli* (Wanner 1996), *P. aeruginosa* (Nikata et al. 1996), *S. meliloti* (Geiger et al. 1999), *P. mirabilis* (Jacobsen et al. 2008), *P. putida* (Wu et al. 1999) and *Synechocystis sp.* (Burut-Archanai et al. 2009), the five *pst* genes play a repressive role in the expression of the PHO regulon under Pi-excess conditions. The Pst system of *C. violaceum* might have lost the ability to repress the PHO regulon concomitantly with the rearrangement of the *phoU* locus as a result of bacterial adaptation to poor Pi environments, where the lack of a PHO repressor might have conferred a selective advantage. In *E. coli*, the signal of Pi availability passes through the Pst system to PhoR (Gardner et al. 2014), as the PhoR protein of *E. coli* does not present a large periplasmic domain that senses the concentration of Pi (Scholten and Tommassen 1993). In contrast, according to an in silico analysis with the program Cello (Yu et al. 2006) *C. violaceum*'s PhoR appears to have a larger periplasmic domain and might, therefore, receive the signal of Pi availability directly from the environment. In bacteria such as *E. coli* that colonize habitats with different levels of Pi concentration, such as the intestines of mammals and birds (relatively high Pi) and water bodies and soil (low Pi), repression by the Pi-transport system is required to avoid overconsumption of Pi that can be toxic. On the other hand, in *C. violaceum* and other bacterial species that usually inhabits Pi-deficient places and rarely face abundance of Pi, an extra level of PHO repression by Pst would be unnecessary. In fact, it has recently been suggested that PhoU does not repress the PHO regulon of the oligotrophic α-Proteobacterium *Caulobacter crescentus* that similarly to *C. violaceum* dwells in low-nutrient environments (Lubin et al. 2015). More broadly, bacteria may adapt to low-Pi conditions in at least three different ways: (1) by selection of a very efficient high-affinity Pi-uptake system; or (2) an efficient Pi storage system, which could be readily used to promote growth under severe Pi starvation; or (3) by incorporating genetic modifications in the regulatory system that controls the PHO regulon. The latter being more common as evolutionary jumps often involve altered patterns of gene regulation (Wang et al. 2010).

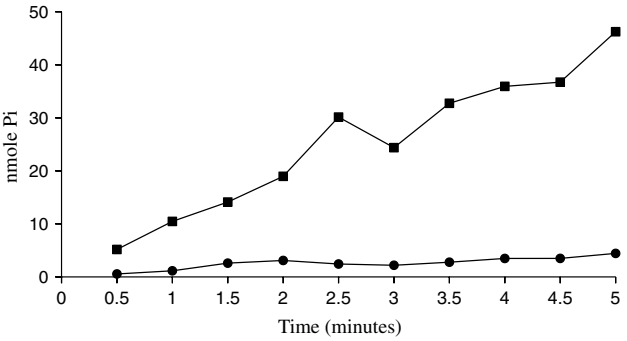

Fig. 5 Pi uptake in the wild-type strain and *pstS* mutant. Pi-starved bacteria are resuspended in MOPS minimal medium containing 0.5 mM ^{32}P-KH_2PO_4 and grown for five minutes. Samples are taken every 30 s, and the radioactivity content is measured. (*Square*) wild-type strain; (*circle*) *pstS* mutant

pstS is essential for Pi uptake

PstS is an integral part of the Pst transport system acting as a periplasmic Pi-binding protein. Null mutations in *pstS* abolish Pi transport altogether (Wanner 1996; Luz et al. 2012; Qi et al. 1997; Braibant et al. 1996; Poole and Hancock 1984; Diaz et al. 2005). To test whether *C. violaceum*'s *pstS* participates in Pi transport, a Pi-uptake assay was conducted (Fig. 5). Pi starvation was induced in the wild-type strain and in the *pstS* mutant by growing the bacteria in MOPS minimal medium lacking Pi. The bacteria were then exposed to the addition of 0.5 mM ^{32}Pi, and samples were withdrawn every 30 s. The wild-type bacteria took up Pi at a *Vmax* of 9.1 nmol Pi OD_{660}^{-1} min^{-1}, while the *pstS* mutant rate of Pi uptake was ten times lower (0.89 nmole Pi OD_{660}^{-1} min^{-1}). It can be concluded that *pstS* plays a fundamental role in Pi uptake in *C. violaceum*.

In summary, *C. violaceum* is well adapted to low-Pi conditions. It is able to grow with traces of Pi in the medium and does not stop growing upon Pi depletion. The PHO genes, *phoA*, *pstS* and *phoB*, were found to respond to Pi limitation, but unlike most Proteobacteria, *pstS* and *phoU* do not play a role in PHO repression under Pi excess.

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