Regulatory interactions between the Pho and σ^B -dependent general stress regulons of *Bacillus subtilis*

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When *Bacillus subtilis* is subjected to phosphate starvation, the Pho and σ^B -dependent general stress regulons are activated to elicit, respectively, specific and non-specific responses to this nutrient-limitation stress. A set of isogenic mutants, with a β -galactosidase reporter gene transcriptionally fused to the inactivated target gene, was used to identify genes of unknown function that are induced or repressed under phosphate limitation. Nine phosphate-starvation-induced (*psi*) genes were identified: *yhaX*, *yhbH*, *ykoL* and *yttP* were regulated by the PhoP–PhoR two-component system responsible for controlling the expression of genes in the Pho regulon, while *ywmG* (renamed *csbD*), *yheK*, *ykzA*, *ysnF* and *yvgO* were dependent on the alternative sigma factor σ^B , which controls the expression of the general stress genes. Genes *yhaX* and *yhbH* are unique members of the Pho regulon, since they are phosphate-starvation induced via PhoP–PhoR from a sporulation-specific σ^E promoter or a promoter that requires the product of a σ^E -dependent gene. Null mutations in key regulatory genes *phoR* and *sigB* showed that the Pho and σ^B -dependent general stress regulons of *Bacillus subtilis* interact to modulate the levels at which each are activated.

Keywords: stress response, *psi* genes, phosphate starvation, regulatory networks

INTRODUCTION

During phosphate-starvation stress, *Bacillus subtilis* responds by activating the Pho and σ^B -dependent general stress (σ^B -GS) regulons (Hulett, 1996; Hecker & Völker, 1998; Antelmann *et al.*, 2000; Lahooti *et al.*, 2000; Prágai *et al.*, 2001). Induction or repression of genes of the Pho regulon enables the cell either to transport and use limiting phosphate resources more efficiently or to increase the accessibility of alternative phosphate sources. Genes of the Pho regulon that are induced in response to phosphate starvation include the following: *phoA* and *phoB*, encoding alkaline phosphatases (APases) (Bookstein *et al.*, 1990; Hulett *et al.*, 1990); *phoD*, encoding a phosphodiesterase/APase, which has a putative role in cell-wall teichoic acid turnover (Eder *et al.*, 1996); the *pstSACBABB* operon, encoding a high-affinity phosphate transporter (Qi *et al.*, 1997); *glpQ*, encoding a glycerophosphoryl diester phosphodiesterase

involved in the hydrolysis of deacylated phospholipids (Antelmann *et al.*, 2000); the *phoPR* and *resABCDE* operons, encoding two-component signal-transduction systems PhoP–PhoR and ResD–ResE (Hulett *et al.*, 1994; Hulett, 1996; Nakano *et al.*, 2000); and two genes, *ydhF* and *ykoL*, of unknown function (Antelmann *et al.*, 2000; Robichon *et al.*, 2000). In addition, the expression of the *tagAB* and *tagDEF* operons, involved in the synthesis of a phosphate-containing cell-wall polymer, polyglycerolteichoic acid, is repressed (Müller *et al.*, 1997; Liu *et al.*, 1998), while the expression of the *tuaABCDEFGH* operon, responsible for the synthesis of teichuronic acid, a non-phosphate-containing polymer that replaces teichoic acid in the wall, is induced during phosphate starvation (Müller *et al.*, 1997; Liu & Hulett, 1998; Lahooti & Harwood, 1999).

Genes of the Pho regulon are controlled by the interaction of at least three two-component signal-transduction systems (Hulett, 1996). The centre of this regulatory network is the PhoP–PhoR sensor–regulator system (Hulett *et al.*, 1994). During phosphate starvation, the PhoP response regulator is activated by its cognate sensor-kinase, PhoR. Phosphorylated PhoP

Abbreviations: APase, alkaline phosphatase; BFA, *Bacillus subtilis* functional analysis; HPM, high-phosphate medium; LPM, low-phosphate medium; σ^B -GS, σ^B -dependent general stress.

(PhoP~P) is required for the activation or repression of genes in the Pho regulon and to enhance the transcription of the *phoPR* and *resABCDE* operons. The second signal-transduction system, ResD–ResE, involved in the activation of genes required for aerobic and anaerobic respiration, is required for the full induction of the Pho regulon, while the third response regulator, Spo0A, terminates the phosphate response via AbrB and ResD–ResE, and initiates sporulation if phosphate-starvation conditions persist. Additionally, in the presence of the NhaC Na⁺/H⁺ antiporter, Na⁺ has a repressive effect on the expression of the *phoPR* operon, while in the absence of this antiporter the *phoPR* operon is hyper-induced (Prágai *et al.*, 2001).

For the activation or repression of Pho-regulon genes, PhoP~P binds to Pho-box sequences, direct repeats of TT(A/T/C)ACA with a 5±2 bp spacer (Eder *et al.*, 1999). For efficient PhoP~P binding, four TT(A/T/C)ACA-like sequences with an 11 bp periodicity are required. In the case of genes induced by PhoP~P, the PhoP-binding sites are on the coding strand of the promoter region, while in the case of the *tagAD* promoter, which is repressed by PhoP~P, these sites are on the non-coding strand (Liu *et al.*, 1998).

In addition to the phosphate-starvation-specific Pho regulon, phosphate limitation also induces genes of the σ^B -GS regulon (Antelmann *et al.*, 2000). The σ^B -GS regulon provides a non-specific resistance to stress. Proteins encoded by members of the σ^B -GS regulon appear to protect DNA, membranes and proteins from the damage caused by oxidative stress and contribute to survival in extreme environments such as those involving heat, salt and ethanol stress (Gaidenko & Price, 1998; Hecker & Völker, 1998). The σ^B -mediated general stress response is one of the earliest responses to growth arrest and is induced by two independent classes of stress: (1) energy limitation arising from, for example, carbon or phosphate starvation; and (2) environmental stress such as osmotic stress and heat, salt, acid or alkaline shock (Akbar *et al.*, 1997; Hecker & Völker, 1998). The activity of σ^B is controlled by anti-sigma-factor RsbW. In unstressed cells, when the antagonist protein RsbV is phosphorylated, RsbW can bind to, and inactivate, σ^B (Benson & Haldenwang, 1993). In response to energy stress or environmental stress, RsbP or RsbU PP2C phosphatases, respectively, remove the serine phosphate from RsbV~P (Voelker *et al.*, 1996; Yang *et al.*, 1996; Vijay *et al.*, 2000), allowing it to sequester RsbW. As a result, σ^B is free to activate genes in the σ^B -GS regulon (Dufour & Haldenwang, 1994). In the case of the RsbP phosphatase, its PAS domain and RsbQ, an α/β -hydrolase, appears to be required to sense energy stress (Vijay *et al.*, 2000; Brody *et al.*, 2001). In contrast, in the environmental stress pathway, the RsbU phosphatase is activated by elements higher up in the regulatory cascade and which include the RsbS antagonist, the RsbT kinase, the RsbX phosphatase, the RsbR regulator and four RsbR paralogues, namely YkoB, YojH, YqhA and YtvA (Yang *et al.*, 1996; Akbar *et al.*, 1997; Voelker *et al.*, 1997; Akbar *et al.*, 2001).

In this paper, we report the results of a systematic search for genes of unknown function that respond to phosphate starvation. The identified genes were assigned to either the Pho or the σ^B -GS regulon by monitoring their expression in *phoR*-null or *sigB*-null mutants, respectively. We also investigated the effects of σ^B on the expression of the Pho regulon and of PhoP–PhoR on the σ^B -GS regulon. The data indicate that the Pho and σ^B -GS regulons are interacting members of the phosphate stimulon.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids are listed in Table 1. Strains were grown in low-phosphate medium (LPM) or high-phosphate medium (HPM) (Prágai & Harwood, 2000a). The starting concentration of phosphate was 0.42 mM in LPM and 5.0 mM in HPM. LPA and HPA are LPM and HPM, respectively, solidified with 1.5% agar. When required, the concentrations of antibiotics were as follows: ampicillin, 50 µg ml⁻¹ (for *Escherichia coli*) and chloramphenicol, 6 µg ml⁻¹; erythromycin 0.3 µg ml⁻¹; kanamycin 10 µg ml⁻¹; lincomycin 25 µg ml⁻¹; neomycin 7 µg ml⁻¹; tetracycline 12.5 µg ml⁻¹ (for *B. subtilis*). X-Gal was used at 100 µg ml⁻¹ and IPTG at 1 mM.

DNA manipulation and general methods. Plasmid and chromosomal DNA extraction, restriction endonuclease digestion, agarose gel electrophoresis, transformation of competent *B. subtilis* cells, PCR reactions and bioinformatical analyses were carried out as described previously (Prágai *et al.*, 1997, 2001). Enzymes, molecular size markers and deoxynucleotides were purchased from Roche Diagnostics or Amersham Pharmacia Biotech.

Construction of plasmids. Primers YDCE-FOR and SIGB-REV (Table 2) were used for PCR amplification of a 4067 bp fragment of the *ycdE*–*sigB* region, and primers RSBU-FOR and SIGB-REV were used to amplify a 1677 bp fragment of the *rsbU*–*sigB* region. Primers YVFR-FOR and RSBP-REV were used for PCR amplification of a 1673 bp fragment of the *yvfR*–*rsbP* region, and primers PRS-FOR and CTC-REV were used to amplify a 1205 bp fragment of the *prs*–*ctc* region. The PCR reactions were carried out with *Pfu* DNA polymerase using chromosomal DNA of *B. subtilis* 168 as template. After *Bam*HI and *Eco*RI digestion, the PCR fragments were ligated into a *Bam*HI- and *Eco*RI-digested pBgaB integrational vector (Mogk *et al.*, 1996) and transformed into electrocompetent cells of *E. coli* XL-1 Blue (Stratagene). Transformants were selected on Luria–Bertani agar medium supplemented with ampicillin. The resulting plasmids, pZP131, pZP132, pZP133 and pZP139 (see Fig. 5), were confirmed by restriction digestion and PCR using the insert-specific primers (Table 2). pZP134 was constructed by releasing a fragment containing the 3'-end of *rsbR* through to the 5'-end of *sigB* (see Fig. 5b) from pZP131 with *Eco*RI digestion.

Construction of strains. To analyse gene expression in a *phoR*-null strain, *B. subtilis* 168-PR (Prágai *et al.*, 2001) was transformed with chromosomal DNA from the appropriate *B. subtilis* functional analysis (BFA) mutants, selecting for Em^r, Lm^r and Tc^r. In these double mutants, *phoR* was inactivated by the replacement of an internal *Bal*I fragment with a Tc^r gene (Hulett *et al.*, 1994). For monitoring gene expression in a *sigB*-deletion strain, ML6 (Igo *et al.*, 1987) was transformed with chromosomal DNA from the appropriate BFA mutants, selecting for Em^r, Lm^r and Cm^r. Transcriptional studies of *phoA*, *phoB* and *phoP* were performed in strains ML6K and

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source* or reference
<i>Bacillus subtilis</i> strains		
BFA214	<i>trpC2 csbD</i> ::pEC16 Em ^r	MICADO
BFA1036	<i>trpC2 yttP</i> ::pYR1036 Em ^r	MICADO
BFA1631	<i>trpC2 yhaX</i> ::pYHAX1 Em ^r	MICADO
BFA1743	<i>trpC2 yhbH</i> ::pYZDC1 Em ^r	MICADO
BFA1818	<i>trpC2 ykzA</i> ::pDU1818 Em ^r	MICADO
BFA1843	<i>trpC2 ykoL</i> ::pDU1843 Em ^r	MICADO
BFA2414	<i>trpC2 yscC</i> ::pYSXCF Em ^r	Prágai & Harwood (2000b)
BFA2438	<i>trpC2 ysnF</i> ::pYSNFK Em ^r	MICADO
BFA2490	<i>trpC2 yvgO</i> ::pYVGOK Em ^r	MICADO
YHEKF	<i>trpC2 yheK</i> ::pYHEKF Em ^r	Prágai <i>et al.</i> (2001)
168-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r	Prágai <i>et al.</i> (2001)
BFA214-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>csbD</i> ::pEC16 Em ^r	BFA214→168-PR
BFA1036-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>yttP</i> ::pYR1036 Em ^r	BFA1036→168-PR
BFA1631-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>yhaX</i> ::pYHAX1 Em ^r	BFA1631→168-PR
BFA1743-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>yhbH</i> ::pYZDC1 Em ^r	BFA1743→168-PR
BFA1818-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>ykzA</i> ::pDU1818 Em ^r	BFA1818→168-PR
BFA1843-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>ykoL</i> ::pDU1843 Em ^r	BFA1843→168-PR
BFA2414-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>yscC</i> ::pYSXCF Em ^r	BFA2414→168-PR
BFA2438-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>ysnF</i> ::pYSNFK Em ^r	BFA2438→168-PR
BFA2490-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>yvgO</i> ::pYVGOK Em ^r	BFA2490→168-PR
YHEKF-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>yheK</i> ::pYHEKF Em ^r	YHEKF→168-PR
ML6	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r	Igo <i>et al.</i> (1987)
BFA214-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>csbD</i> ::pEC16 Em ^r	BFA214→ML6
BFA1036-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>yttP</i> ::pYR1036 Em ^r	BFA1036→ML6
BFA1631-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>yhaX</i> ::pYHAX1 Em ^r	BFA1631→ML6
BFA1743-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>yhbH</i> ::pYZDC1 Em ^r	BFA1743→ML6
BFA1818-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>ykzA</i> ::pDU1818 Em ^r	BFA1818→ML6
BFA1843-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>ykoL</i> ::pDU1843 Em ^r	BFA1843→ML6
BFA2414-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>yscC</i> ::pYSXCF Em ^r	BFA2414→ML6
BFA2438-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>ysnF</i> ::pYSNFK Em ^r	BFA2438→ML6
BFA2490-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>yvgO</i> ::pYVGOK Em ^r	BFA2490→ML6
YHEKF-SB	<i>trpC2 sigBΔHindIII–EcoRV</i> ::Cm ^r <i>yheK</i> ::pYHEKF Em ^r	YHEKF→ML6
ML6K	<i>trpC2 sigBΔHindIII–EcoRV</i> ::cat::Km ^r	pCm::Nm→ML6
168-A	<i>trpC2 amyE</i> ::pNK45(<i>phoA–lacZ</i>) Cm ^r	Prágai <i>et al.</i> (2001)
168-P	<i>trpC2 amyE</i> ::pNK55(<i>phoP–lacZ</i>) Cm ^r	Prágai <i>et al.</i> (2001)
168-B	<i>trpC2 amyE</i> ::pCB619(<i>phoB–lacZ</i>) Cm ^r	Prágai <i>et al.</i> (2001)
ML6K-A	<i>trpC2 amyE</i> ::pNK45(<i>phoA–lacZ</i>) Cm ^r <i>sigBΔHindIII–EcoRV</i> ::cat::Km ^r	168-A→ML6K
ML6K-P	<i>trpC2 amyE</i> ::pNK55(<i>phoP–lacZ</i>) Cm ^r <i>sigBΔHindIII–EcoRV</i> ::cat::Km ^r	168-P→ML6K
ML6K-B	<i>trpC2 amyE</i> ::pCB619(<i>phoB–lacZ</i>) Cm ^r <i>sigBΔHindIII–EcoRV</i> ::cat::Km ^r	168-B→ML6K
PZ131	<i>trpC2 amyE</i> ::pZP131(<i>sigB–bgaB</i>) Nm ^r	This study
PZ132	<i>trpC2 amyE</i> ::pZP132(<i>sigB–bgaB</i>) Nm ^r	This study
PZ133	<i>trpC2 amyE</i> ::pZP133(<i>rsbP–bgaB</i>) Nm ^r	This study
PZ134	<i>trpC2 amyE</i> ::pZP134(<i>rsbR–bgaB</i>) Nm ^r	This study
PZ139	<i>trpC2 amyE</i> ::pZP139(<i>ctc–bgaB</i>) Nm ^r	This study
PZ131-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>amyE</i> ::pZP131(<i>sigB–bgaB</i>) Nm ^r	PZ131→168-PR
PZ132-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>amyE</i> ::pZP132(<i>sigB–bgaB</i>) Nm ^r	PZ132→168-PR
PZ133-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>amyE</i> ::pZP133(<i>rsbP–bgaB</i>) Nm ^r	PZ133→168-PR
PZ134-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>amyE</i> ::pZP134(<i>rsbR–bgaB</i>) Nm ^r	PZ134→168-PR
PZ139-PR	<i>trpC2 phoRΔBall</i> ::Tc ^r <i>amyE</i> ::pZP139(<i>ctc–bgaB</i>) Nm ^r	PZ139→168-PR

Table 1 (cont.)

Strain or plasmid	Relevant characteristic(s)	Source* or reference
Plasmids		
pCm::Nm	pIC177 containing a Km ^r insertion in <i>Nco</i> I site of Cm ^r	Steinmetz & Richter (1994)
pBgaB	<i>bgaB</i> Nm ^r Ap ^r (8·811 kb)	Mogk <i>et al.</i> (1996)
pZP131	pBgaB containing a 4067 bp insert of ' <i>ycdE-sigB</i> ' region Nm ^r Ap ^r (12·872 kb)	This study
pZP132	pBgaB containing a 1677 bp insert of ' <i>rsbU-sigB</i> ' region Nm ^r Ap ^r (10·482 kb)	This study
pZP133	pBgaB containing a 1673 bp insert of ' <i>yvfR-rsbP</i> ' region Nm ^r Ap ^r (10·478 kb)	This study
pZP134	pZP131 digested with <i>Eco</i> RI and self-ligated Nm ^r Ap ^r (9·268 kb)	This study
pZP139	pBgaB containing a 1205 bp insert of ' <i>prs-ctc</i> ' region Nm ^r Ap ^r (10·01 kb)	This study

* Details of the construction and basic characterization of BFA mutants are available in the MICADO database (<http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/ACCUEIL-MUTANT.pl>). An arrow indicates transformation from donor to recipient.

Table 2. Primers

Primers	Sequence (5'→3')*	Position (range)†
YDCE-FOR	<u>CGCGGATCCTGGATGATGAAATGATGG</u>	518783–518800
RSBU-FOR	<u>CGCGGATCCACGTTGATGCCAGTATGT</u>	521165–521182
YVFR-FOR	<u>CGCGGATCCAATATCATCGAGACGACG</u>	3498166–3498183
PRS-FOR	<u>ACTGGATCCTGGAACTGCTGATTATGG</u>	57963–57980
SB-FOR	GGCTCATAAGCGATTACC	522468–522485
CAT-FOR	CTGGTTACAATAGCGACG	1465–1482‡
SIGB-REV	GAGGAATTCGGCAATCTC	522844–522827
RSBP-REV	<u>CCGGAATTCTTCCTGAGCCTCATTTCT</u>	3499832–3499815
CTC-REV	<u>CCGGAATTCAAGTGCATATAACGGCTG</u>	59161–59144
SB-REV	CTCATATCCGTCCTCCTG	522991–522974
CAT-REV	GGCCTATCTGACAATTCC	1891–1874‡

* The 5'-ends of the forward (FOR) primers included a 9 bp linker with a *Bam*HI restriction site. The 5'-ends of some of the reverse (REV) primers included a 9 bp linker with an *Eco*RI site. The linkers are underlined.

† Positions of the primers specific for *B. subtilis* 168 are with respect to the entire genome as noted in the SubtiList database (<http://genolist.pasteur.fr/SubtiList>) (Kunst *et al.*, 1997).

‡ Positions of the primers specific for the chloramphenicol-resistance gene of *Staphylococcus aureus* plasmid pC194 are according to GenBank accession number NC_002013.

168. ML6K was constructed by introducing a Km^r insertion into the Cm^r gene of strain ML6 using *Sca*I-linearized plasmid pCm::Nm (Steinmetz & Richter, 1994). The insertion of the Km^r marker was verified by PCR using *sigB* (SB-FOR and SB-REV) and *cat* (CAT-FOR and CAT-REV) specific primers (Table 2). The fusions *phoA-lacZ*, *phoB-lacZ* or *phoP-lacZ* were integrated into the *amyE* locus by transforming strain ML6K with the chromosomal DNA from 168-A, 168-B and 168-P (Prágai *et al.*, 2001) and selecting for Km^r Cm^r *amyE*-null transformants. The *lacZ* fusions were verified by PCR as described previously (Prágai *et al.*, 2001). Transcriptional studies of *ctc*, *sigB*, *rsbP* and *rsbR* were performed in strains 168 and 168-PR. The fusions *sigB-bgaB*, *rsbP-bgaB*, *rsbR-bgaB* or *ctc-bgaB* were integrated into the *amyE* locus by transforming strains 168 and 168-PR with *Sca*I-linearized plasmids pZP131, pZP132, pZP133, pZP134 and pZP139,

selecting for Nm^r *amyE*-null transformants. The resulting strains were PZ131 and PZ131-PR, PZ132 and PZ132-PR, PZ133 and PZ133-PR, PZ134 and PZ134-PR, and PZ139 and PZ139-PR (Table 1).

Enzyme assays. Overnight cultures grown in HPM were diluted 200-fold in fresh LPM and HPM medium and grown at 37 °C with shaking at 200 r.p.m. Samples were collected for the determination of optical density at 600 nm, APase activity (40 µl whole culture) and β-galactosidase activity (cell pellet from 0·1–1 ml of culture). APase and β-galactosidase samples were stored at –20 °C until required.

For the APase assay, samples and a reagent blank (40 µl LPM) were thawed, 400 µl 1 M Tris/HCl (pH 8·0) containing lysozyme (200 µg ml^{–1}), DNase I (20 µg ml^{–1}), ribonuclease A (20 µg ml^{–1}), chloramphenicol (100 µg ml^{–1}) and 0·0005 % SDS were added and incubated for 10 min at 30 °C. To each

lysed sample, 300 µl pre-warmed *p*-nitrophenyl phosphate (1 mg ml⁻¹ in 1 M Tris/HCl, pH 8.0) was added and the mixture incubated at 30 °C for between 5 and 60 min. When the A_{410} value was ~0.2–0.3, the assay was stopped by the addition of 300 µl 2 M NaOH. The samples were centrifuged for 5 min and the absorbance was measured at 410 nm. Specific APase activity was calculated with the following formula (Nicholson and Setlow, 1990): $A_{410} \times 235 \times V_1 / (V_2 \times OD_{600} \times T)$ and expressed as nmol *p*-nitrophenol min⁻¹ (OD₆₀₀ unit)⁻¹. In the formula, V_1 is the final volume (in ml) of the assay (1.04 ml), V_2 is the volume (in ml) of the culture used in the assay (0.04 ml), and T is the reaction time (in min). The value 235 is a constant for the calculation of the *p*-nitrophenol concentration in nmoles.

The β -galactosidase assay, described by Miller (1972), was modified as follows: the frozen pellets were suspended in 800 µl Z buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl and 0.001 M MgSO₄) containing lysozyme (200 µg ml⁻¹), DNase I (20 µg ml⁻¹), ribonuclease A (20 µg ml⁻¹), chloramphenicol (100 µg ml⁻¹) and 0.00025% SDS and incubated for 10 min at 28 °C. Then, 200 µl pre-warmed 2-nitrophenyl β -D-galactopyranoside (4 mg ml⁻¹ in 0.1 M sodium phosphate buffer, pH 7.5) was added and the mixture incubated at 28 °C for between 5 and 60 min in the case of *lacZ* and at 55 °C in the case of *bgaB* (Mogk *et al.*, 1996). The assay was stopped by the addition of 0.5 ml 1 M Na₂CO₃. The samples were centrifuged for 5 min and the absorbance was measured at 420 nm. Specific β -galactosidase activity was calculated with the following formula: $A_{420} \times V_1 / (V_2 \times OD_{600} \times T \times 0.00486)$ and expressed as nmol 2-nitrophenol min⁻¹ (OD₆₀₀ unit)⁻¹. In the formula, V_1 is the final volume (in ml) of the assay (1.5 ml), V_2 is the volume (in ml) of the culture used in the assay (0.1–1 ml), and T is the reaction time (in min). The molar absorption coefficient of 2-nitrophenyl is 4860 M⁻¹ cm⁻¹ at pH 10.

RESULTS

Screening for genes affected by phosphate starvation

Within the framework of the BFA project (Harwood & Wipat, 1996), BFA mutants were constructed by inte-

grating pMUTIN (Vagner *et al.*, 1998) into genes of unknown function. These mutants were used to identify new phosphate-starvation-induced (*psi*) and -repressed (*psr*) genes (Prágai & Harwood, 2000a), using the *spoVG-lacZ* transcriptional reporter gene of the integrated pMUTIN. BFA mutants were tested on both LPA and HPA supplemented with X-Gal. Since growth was stronger on HPA, mutants were incubated for 24 h on HPA and for 36 h on LPA before β -galactosidase activities were compared. Mutants carrying *psi-lacZ* gene fusions should give blue colonies on LPA and pale blue or white colonies on HPA. Conversely, mutants carrying *psr-lacZ* gene fusions should give blue colonies on HPA and pale blue or white colonies on LPA. Genes which are not affected by the concentration of phosphate should show similar levels of expression in both LPA and HPA. Nine of the 1147 BFA mutants exhibited high β -galactosidase activity on LPA and little or no β -galactosidase activity on HPA. These *psi* genes were *yumG* (renamed *csbD*), *yhaX*, *yhbH*, *yheK*, *ykoL*, *ykzA*, *ysnF*, *yttP* and *yvgO* (Kunst *et al.*, 1997). No *psr* genes were detected with this screening system.

Transcriptional activities of *psi* genes during phosphate starvation

To determine the influence of phosphate on the transcriptional activity of *psi* genes, β -galactosidase production by the *psi* mutants was monitored in LPM and HPM (Fig. 1). The growth kinetics of all the mutants was similar; transition from exponential to stationary phase in LPM was at an OD₆₀₀ of ~1.1, while in HPM the OD₆₀₀ was ~3.5. In both LPM and HPM, *psi* genes showed little or no expression during exponential phase (Fig. 1). In LPM, *csbD*, *ykoL*, *ykzA*, *yheK*, *ysnF*, *yttP* and *yvgO* were induced at T_{-1} – T_0 (T_{-1} is 1 h before transition from the exponential to the stationary phase) (Fig. 1a), which coincided with the point at which APase

Fig. 1. Transcriptional activities of *psi* genes in LPM (a) and HPM (b). Specific β -galactosidase activities of *yhaX* ($\blacktriangle$), *yhbH* ($\bullet$), *ykoL* ($\blacksquare$), *yttP* ($\blacklozenge$), *csbD* (∇), *yheK* ($\triangle$), *ykzA* ($\square$), *ysnF* ($\circ$), *yvgO* ($\diamond$). (a) The outer abscissa with a higher scale corresponds to the expression of *ykoL* ($\blacksquare$, dashed line). ONP, 2-nitrophenol.

Fig. 2. Transcriptional activities of *psi* genes in *phoR*-null (a) and *sigB*-null (b) mutants. The mutants were grown in LPM. The specific β -galactosidase activities of *yhaX* ($\blacktriangle$), *yhbH* ($\bullet$), *ykoL* ($\blacksquare$), *yttP* ($\blacklozenge$), *csbD* (∇), *yheK* ($\triangle$), *ykzA* ($\square$), *ysnF* ($\circ$) and *yvgO* ($\diamond$) are shown. The outer abscissa with a higher scale corresponds to the expression of *psi* genes (dashed lines). The inner abscissa corresponds to the expression of *psi* genes (continuous lines).

production was induced (data not shown). The induced specific β -galactosidase activity of *ykoL* was markedly higher than that of the other genes. *ykzA* showed constitutive low-level production of β -galactosidase in HPM, and in LPM during the exponential phase (Fig. 1). *yttP* was induced in HPM at T_{-1} – T_0 , but the specific β -galactosidase activity was at least 10-fold lower in HPM than in LPM (Fig. 1). The other *psi* genes showed no β -galactosidase production in HPM (Fig. 1b). The induction of a second group of *psi* genes, *yhaX* and *yhbH*, in LPM was delayed until T_2 – T_3 (Fig. 1a). These genes were not expressed in HPM (Fig. 1b).

Effect of a *phoR*-null mutation and a *sigB*-null mutation on the identified *psi* genes

To determine whether the response of *psi* genes to phosphate starvation was mediated by the PhoP–PhoR two-component signal-transduction system, *phoR*-null mutant 168-PR (Prágai *et al.*, 2001) was transformed with chromosomal DNA from the BFA *psi* mutants, selecting for Em^r , Lm^r and Tc^r . When the resulting double mutants (Table 1) were grown in LPM, three of the nine *psi* genes, *yhaX*, *yhbH* and *ykoL*, were unable to respond to phosphate starvation (Fig. 2a). In addition, the induction of *yttP* in response to phosphate starvation was reduced fourfold in this background. In contrast, the expression of the remaining five genes, *csbD*, *ykzA*, *ysnF*, *yvgO* and *yheK*, was hyper-induced five- to sevenfold in the *phoR*-null strain (Fig. 2a).

To determine whether the response of any of the *psi* genes to phosphate starvation was mediated by σ^B , *sigB*-null mutant ML6 (Igo *et al.*, 1987) was transformed with chromosomal DNA from the *psi* BFA mutants. When the double mutants were grown in LPM, five of the nine *psi* genes, *csbD*, *ykzA*, *ysnF*, *yvgO* and *yheK*, were

Fig. 3. Transcriptional activities of *ysxC* in LPM ($\blacksquare$), in HPM ($\blacklozenge$) and in *phoR*-null ($\blacktriangle$) and *sigB*-null ($\bullet$) mutants grown in LPM.

unable to respond to the growth arrest associated with phosphate starvation (Fig. 2b). These were the same genes that were hyper-induced in the absence of PhoR. Three of the four *phoR*-dependent genes (*yhaX*, *yhbH* and *yttP*) were hyper-induced two- to fourfold in the *sigB*-null strain, while the remaining gene, *ykoL*, was unaffected by this mutation (Fig. 2b).

To confirm that the σ^B - and PhoR-dependent responses to phosphate starvation were specific, we determined the influence of these regulatory proteins on the expression of a gene, *ysxC*, that is expressed in both exponential and stationary phase (Prágai & Harwood, 2000b). The data (Fig. 3) show that the pattern of expression of *ysxC* is similar in LPM and HPM, and that

Fig. 4. Influence of a *sigB*-null mutation on APase production and the transcription of *phoA*, *phoB* and the *phoPR* operon. (a) Specific β -galactosidase activities for strains 168-A (*phoA-lacZ*) (◆), 168-B (*phoB-lacZ*) (■), ML6K-A (*phoA-lacZ sigB*) (◇) and ML6K-B (*phoB-lacZ sigB*) (□) grown in LPM medium. (b) APase production (open symbols) and specific β -galactosidase activities (closed symbols) for strains 168-P (*phoP-lacZ*) (◆, ◇), ML6K-P (*phoP-lacZ, sigB*) (■, □) grown in LPM. PNP, *p*-nitrophenol.

the absence of PhoR and SigB did not lead to the hyper-induction of this gene.

Effect of a *sigB*-null mutation on transcription of *phoA*, *phoB* and *phoPR*

Our results (Fig. 2b) indicate that σ^B influences the expression of three of the four *phoR*-dependent *psi* genes. We therefore investigated whether σ^B influenced the expression of known members of the Pho regulon and, if so, whether this effect was mediated via PhoP–PhoR.

The genes encoding APaseA and APaseB, responsible for 98 % of the APase activity synthesized in response to phosphate starvation, are well-characterized members of the Pho regulon (Hulett *et al.*, 1994). To study the effect of σ^B on the transcription of these APase genes, *phoA-lacZ* or *phoB-lacZ* transcriptional fusions from strains 168-A or 168-B (Prágai *et al.*, 2001), respectively, were integrated at the *amyE* locus of strain ML6K, resulting in strains ML6K-A and ML6K-B. ML6K was ML6 in which the Cm^r gene used to inactivate *sigB* was itself disrupted by the insertion of a Km^r gene. When strains ML6K-A, ML6K-B, 168-A and 168-B were grown in LPM (Fig. 4a), *phoA* and *phoB* expression (Fig. 4a) and APase synthesis (data not shown) were induced as the cells entered stationary phase. At all subsequent time points, the transcriptional activity of *phoA* and *phoB* and APase production was approximately twofold higher in the *sigB*-null mutant, confirming the results obtained with three of the *phoR*-dependent *psi* mutants.

Since all of the tested Pho-regulon genes were hyper-induced in the *sigB*-null background, we analysed the influence of σ^B on the transcription of the *phoPR* operon. A *phoP-lacZ* transcriptional fusion, derived from strain 168-P (Prágai *et al.*, 2001), was integrated at the *amyE*

locus of strain ML6K, resulting in strain ML6K-P. When ML6K-P and 168-P were grown in LPM (Fig. 4b), both strains showed a constitutive, low level of *phoPR* expression during exponential growth phase that was induced as the cells became phosphate-starved (T_0). In the absence of σ^B (ML6K-P), the expression of *phoPR* was about threefold higher than in its presence (168-P).

Effect of a *phoR*-null mutation on transcription of *ctc*, *sigB*, *rsbP* and *rsbR*

Since σ^B modulates the activity of the Pho regulon, we investigated whether PhoP–PhoR could influence members of the σ^B -GS regulon and the expression of *sigB* itself. Transcriptional fusions were constructed for *ctc*, a well-characterized σ^B -dependent gene, *sigB*, *rsbP* and *rsbR*.

Plasmids pZP131, pZP132, pZP133, pZP134 and pZP139 (Fig. 5, Table 1) were integrated independently into the chromosomes of strains 168 and 168-PR to generate isogenic pairs PZ131/PZ131-PR, PZ132/PZ132-PR, PZ133/PZ133-PR, PZ134/PZ134-PR and PZ139/PZ139-PR, respectively (Table 1).

PZ139 and PZ139-PR, encoding the *ctc-bgaB* reporter fusion, were used to confirm the influence of *phoR* on the activity of the σ^B -GS regulon. The data show that *ctc* is induced at T_0 in response to phosphate starvation (Fig. 6). In the *phoR*-null mutant (PZ139-PR), the induction level was approximately fourfold higher than that of the wild-type, in agreement with the data from the newly identified members of the σ^B -GS regulon (Fig. 2a).

PZ131 and PZ131-PR encode a *sigB-bgaB* reporter fusion located downstream of both the σ^A promoter and the σ^B promoter. *sigB* was induced at T_0 in response to

Fig. 5. Integrated plasmids used for monitoring the expression of the *ctc* and *rsbR* genes, and the *sigB* and *rsbP* operons. Schematic representation of inserts cloned into plasmid pBgaB, which carries a *bgaB* transcriptional reporter gene encoding a heat-stable β -galactosidase from *Bacillus stearothermophilus* (Mogk *et al.*, 1996). Filled arrows indicate structural genes, promoters are marked with fine arrows, and putative Rho-independent terminators are shown as stem-loop structures. Inserts are shown with thick lines under the chromosomal regions. Only relevant restriction enzyme sites are indicated. (b) 'P_A' is the σ^A promoter and 'P_B' is the σ^B promoter of the *sigB* operon. pZP139 (a), pZP131, pZP132, pZP134 (b), and pZP133 (c) were integrated into the *amyE* locus of *B. subtilis* strains 168 and 168-PR.

Fig. 6. Influence of a *phoR*-null mutation on the transcription of *ctc*, *sigB* and *rsbR*. Specific β -galactosidase activities are shown for strains PZ131 (*sigB-bgaB*) (◆), PZ131-PR (*sigB-bgaB phoR*) (◇), PZ132 (*sigB-bgaB*) (■), PZ132-PR (*sigB-bgaB phoR*) (□), PZ134 (*rsbR-bgaB*) (●), PZ134-PR (*rsbR-bgaB phoR*) (○), PZ139 (*ctc-bgaB*) (▲) and PZ139-PR (*ctc-bgaB phoR*) (△) grown in LPM medium. The outer abscissa corresponds to the expression of the *ctc* gene (dashed lines).

phosphate starvation, and at T_5 the induction level in the *phoR*-null mutant was more than twice that of the wild-type, and the initial rate of induction was approximately 10-fold higher (Fig. 6). To determine whether the hyper-induction of *sigB* was due to σ^A - or σ^B -directed transcription, β -galactosidase activities were determined in fusion strains in which the activities of these promoters could be determined independently. PZ134 and PZ134-PR encode an *rsbR-bgaB* reporter fusion downstream of the σ^A promoter. When grown in LPM, both mutants showed an identical, very low, constitutive level of expression, confirming that the σ^A promoter at the 5'-end of the *rsbR-rsbX* operon is not involved in the induction of *sigB* in response to phosphate starvation. PZ132 and PZ132-PR, encoding a *sigB-bgaB* reporter fusion downstream of the internal σ^B promoter, exhibited a similar response to that of PZ131/PZ131-PR, except that the levels of induction were enhanced about twofold (Fig. 6). These data confirm that the σ^B promoter upstream of *sigB* is responsible for the induction of this gene in response to phosphate starvation, and that this response was enhanced in the *phoR*-null background. The enhanced transcription of *sigB* in PZ132/PZ132-PR as compared with PZ131/PZ131-PR presumably reflects the fact that the former has only single copies of the *rsbR*, *rsbS*, *rsbT* and *rsbU* genes.

Finally, the expression of *rsbP*, a phosphatase responsible for sensing and signalling energy stress, was studied in PZ133 and PZ133-PR. The expression of σ^A -dependent *rsbP* was very low and was unaffected by the presence of the *phoR*-null mutation (data not shown).

DISCUSSION

In this paper, we have identified nine *B. subtilis* genes of unknown function that are induced in response to phosphate starvation. These genes are therefore members of the phosphate-stress stimulon. We used a combination of reporter-gene technology and *phoR*- and *sigB*-null mutants to assign these genes into specific regulons; *yhaX*, *yhbH*, *ykoL* and *ytpP* are members of the Pho regulon, while *yheK*, *ykzA*, *ysnF*, *yvgO* and *ywmG* (now *csbD*) are members of the σ^B -GS regulon. No phosphate-starvation-repressed genes were detected.

All but two of the genes were induced during the transition from exponential phase to stationary phase (T_{-1} – T_0). The exceptions were *yhaX* and *yhbH*, both of which are PhoR-dependent genes. *yhbH* has a predicted σ^E promoter (Fawcett *et al.*, 2000), while analysis of the upstream region of *yhaX* reveals the presence of putative σ^E consensus sequence (5'-¹⁰⁵⁵⁹⁴⁵TTCTAAA-14 bp-CATATCCT¹⁰⁵⁵⁹⁷³-3'; coordinates according to Kunst *et al.*, 1997). When we transformed their respective mutants (BFA1631 and BFA1743) with a *sigE*-null mutation, both lost their ability to be induced in response to phosphate starvation (data not shown). These genes are therefore unique in being phosphate-starvation-induced via PhoP–PhoR from a sporulation-specific σ^E promoter or a promoter that requires the

product of a σ^E -dependent gene. One other member of the Pho regulon, namely *phoB*, is known to be transcribed during sporulation, but in this case from a phosphate-starvation-independent, sporulation-specific promoter (Chesnut *et al.*, 1991; Birkey *et al.*, 1994); *phoB* is also transcribed by a separate, phosphate-starvation-inducible σ^A promoter.

An interesting observation was the regulatory interactions between the Pho and σ^B -GS regulons. In a *sigB*-null mutant, the response of three of the four newly described members of the Pho regulon, namely *yhaX*, *yhbH* and *ytpP*, to phosphate starvation was enhanced about two- to fourfold (Fig. 2b). Similar results were obtained for three well-established members of the Pho regulon, i.e. *phoA*, *phoB* and *phoPR* (Fig. 4). The influence of σ^B on *phoPR* could account for its influence on the other members of the Pho regulon. The absence of σ^B had no effect on the expression of the remaining Pho-regulon gene *ykoL* (Fig. 2b), showing that a *sigB* mutation does not influence the expression of all members of the Pho regulon.

The situation with members of the σ^B -GS regulon was comparable to that of the Pho regulon. In a *phoR*-null mutant, the response of all five identified members of the σ^B -GS regulon, namely *csbD*, *ykzA*, *ysnF*, *yvgO* and *yheK*, to phosphate starvation was enhanced about five- to sevenfold (Fig. 2a). Similarly, the expression of well-established members of the σ^B -GS regulon, *ctc* and *sigB* itself, was enhanced by between two- and fourfold (Fig. 6). The influence of PhoP-PhoR on *sigB* could account for its influence on the other members of the σ^B -GS regulon.

We do not, as yet, understand the molecular mechanisms underlying the interactions between the Pho and σ^B -GS regulons. These regulons provide, respectively, specific and general responses to phosphate-starvation stress and contribute to the survival under phosphate limitation. The absence of one of these regulons is likely to enhance the strength of the signal sensed by the other regulon, resulting in its elevated expression. In the case of a defective σ^B -GS, the absence of σ^B may also reduce the sigma-factor competition for core RNA polymerase, leading to a general increase in the transcription of genes controlled by other sigma factors (such as σ^A and σ^E). However, analysis of the expression of a constitutive gene, *ysxC*, showed no evidence for sigma-factor competition (Fig. 3).

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