Phosphate-starvation-inducible proteins in *Bacillus subtilis*: a two-dimensional gel electrophoresis study

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A two-dimensional (2-D) gel electrophoresis study of *Bacillus subtilis* strain 168 identified 20 proteins that are strongly induced in response to phosphate starvation. The induction of nine of these phosphate-starvation-induced (Psi) proteins was dependent on a functional PhoR protein. PhoR is the histidine sensor-kinase component of a phosphate-concentration-sensing two-component regulatory system which, together with its partner response regulator PhoP, controls the expression of genes in the Pho regulon. Genes encoding PhoR-dependent Psi proteins are therefore likely to be members of the Pho regulon. Spo0A~P, the response regulator of the signal transduction pathway required for the induction of sporulation, has previously been shown to negatively affect the induction of the Pho regulon by repressing the *phoP-phoR* operon. The induction pattern of some PhoR-dependent Psi proteins was altered in a *spo0A* mutant such that their synthesis continued for longer than was found with the wild-type. The most abundant Psi protein, Psi1-3, was characterized by N-terminal sequencing of internal peptide fragments and shown to have a high similarity to an *Escherichia coli* protein which is involved in phosphate uptake during phosphate starvation.

Keywords: *Bacillus subtilis*, phosphate starvation, Pho regulon, stress proteins, alkaline phosphatase

INTRODUCTION

In soil, the natural environment of the Gram-positive, endospore-forming bacterium *Bacillus subtilis*, micro-organisms are subjected to a variety of stresses and nutrient deprivations. Among the most commonly encountered is phosphorus limitation, since inorganic phosphate (P_i) is one of the major growth-limiting substrates in natural environments (Hulett, 1993). The importance of phosphorus in cellular metabolism is reflected in the development, in *B. subtilis* and many other bacterial species, of highly efficient mechanisms for conserving it and for recovering P_i from organic sources such as nucleic and teichoic acids (Grant, 1979). In *B. subtilis*, these mechanisms are induced transiently in response to phosphate starvation, but are repressed once sporulation is induced.

The best-studied response to phosphate starvation is the induction of alkaline phosphatases (APases), which catalyse the hydrolysis of organic and inorganic phosphate monoesters to release P_i (Coleman, 1992). *B. subtilis* has a multi-gene family for the production of at least four APases (Chesnut *et al.*, 1991; Hulett *et al.*, 1990, 1991). The genes for two APases have been mapped and sequenced. PhoA, the main APase, is induced specifically in response to phosphate starvation whilst PhoB is phosphate-stress-induced during sporulation (Chesnut *et al.*, 1991; Birkey *et al.*, 1995). The genes for both enzymes are induced by a two-component signal transduction pathway, comprising a histidine sensor-kinase, PhoR, and its partner response regulator, PhoP. PhoR is a membrane-bound protein that appears to sense low P_i both at the outer surface of the cell membrane and within the cytoplasm (Shi *et al.*, 1995). It responds by auto-phosphorylation at a histidine residue in the C-terminal half of the protein. PhoR~P then provides a substrate for the activation of PhoP to PhoP~P, which in turn activates the expression of genes in the Pho regulon,

Abbreviations: APase, alkaline phosphatase; 2-D, two-dimensional.

including the *phoP-phoR* operon itself (Hulett *et al.*, 1994; Mueller & Harwood, unpublished data).

Although a great deal of information has accumulated in recent years on the control of the Pho regulon, and on *phoA*, *phoB*, *phoP* and *phoR* in particular, little is known about the control of other Psi proteins. We have therefore undertaken a systematic study of the proteins induced in response to phosphate starvation using two-dimensional (2-D) gel electrophoresis of radiolabelled proteins. By using a *phoR* mutant, we have identified nine Psi proteins that appear to belong to the Pho regulon.

METHODS

Bacterial strains. *B. subtilis* 168 (*trpC2*) was obtained from the Bacillus Genetic Stock Center (BGSC), Ohio, USA. *B. subtilis phoR* was constructed by transformation of strain *B. subtilis* 168 with chromosomal DNA from *B. subtilis* MH5124 (Hulett *et al.*, 1994). The genotype is *trpC2 phoRΔBaII::Tet^r*. *B. subtilis spo0A* was constructed by transformation of strain *B. subtilis* 168 with chromosomal DNA from *B. subtilis* BGK2 (using the plasmid pSKS2). The genotype is *trpC2 spo0A12::Tet^r*. *B. subtilis* strain GCH871 was constructed by a single crossover integration of the plasmid *pDYphoR12* (Mueller & Harwood, unpublished data) in strain 168. The new strain contains a mutant allele of *phoR* (*phoR12*) behind the wild-type *phoPR*-promoter and the wild-type allele of *phoR* behind the P_{SPAC} -promoter. The lesion in *phoR12* is a single base change leading to an Arg to Ser transition at residue 532 (Seki *et al.*, 1988). *B. subtilis* ML6 (*trpC2 sigB HindIII-EcoRV::cat*) was obtained from R. Losick (Igo *et al.*, 1987).

Culture conditions and enzyme assay. The cells were grown in a minimal medium prepared as described previously (Stülke *et al.*, 1993). The concentration of KH_2PO_4 was 0.28 mM for phosphate limitation and 0.56 mM for control conditions.

APase activity was measured in cells that had been grown in the low-phosphate medium. Growth was assessed by measuring OD₅₀₀ of the culture. APase activity was assayed as described by Nicholson & Setlow (1990). The APase activity of colonies growing on the surface of agar plates was detected by overlaying the colonies with filter paper moistened with the chromogenic substrate *o*-nitrophenol phosphate.

Transformation. Chromosomal DNA (10 µg) was mixed with 500 µl competent cells of *B. subtilis* 168 and cultured as described by Sambrook *et al.* (1989). Aliquots (100 µl) of the competence mixture were plated out on LB plates with tetracycline (17 µg ml⁻¹) and cultured at 37 °C overnight. Putative transformants were checked for the Trp⁻ phenotype and for the expression of APase. The Spo⁻ phenotype of the *spo0A* mutants was tested on 2 × SG agar (Nicholson & Setlow, 1990).

Pulse labelling and 2-D gel electrophoresis. *phoR* and *spo0A* strains and the wild-type were grown in low-phosphate medium and at different growth points (exponential phase, transition phase, and 0.5 h, 1 h and 2 h into stationary phase) 2 ml culture was transferred into 10 ml flasks and labelled with 10 µCi (3.7×10^5 Bq) L-[³⁵S]methionine for 3 min. Sample preparation for 2-D PAGE was carried out as described by Hecker & Voelker (1990) except that an equal amount of radioactively labelled proteins (1×10^6 c.p.m.) was resuspended in E1-PMSF solution (described in the protocol for the Investigator 2-D Electrophoresis System; Millipore). Proteins were separated using 2-D PAGE as described by Hecker & Voelker (1990).

The rate of incorporation of L-[³⁵S]methionine was determined by TCA precipitation: 20 µl aliquots from the extract were applied to Whatman no. 2 paper, dried, washed with ice-cold 10% (w/v) TCA for 10 min, washed with ice-cold 5% TCA for 10 min, washed twice with 96% (v/v) ethanol for 10 min and finally dried. The amount of radioactivity incorporated was measured in a liquid scintillation counter (TRI-CARB; Packard).

Computer-supported analysis of synthesized proteins. 2-D gels were exposed to phosphor screens (Molecular Dynamics) for 24 h and scanned with the PhosphorImager system (Molecular Dynamics) with 200 µm resolution. Scan modifications, gel image procedure, spot editing and spot quantification were made with the PDI-software PDQuest 5.0, based on a UNIX-based computer system (J. Bernhardt, unpublished data).

Preparative 2-D PAGE and sequencing of peptides after internal cleavage. Proteins eluted from Coomassie-stained preparative 2-D gels (Antelmann *et al.*, 1995) were treated with cyanogen bromide to cleave the protein behind methionine residues. The resulting peptides were separated on one-dimensional preparative gels, eluted and prepared for N-terminal sequencing (Matsudaira, 1989). Peptide sequencing, based on the Edman method (Ausubel, 1993), was performed with the Applied Biosystems A473a protein sequencer.

RESULTS AND DISCUSSION

Cell growth, methionine incorporation and synthesis of APase

The wild-type (Fig. 1), and the *phoR* and *spo0A* mutants of *B. subtilis* 168 (data not shown) were characterized with respect to their growth kinetics, rate of incorporation of methionine and APase activities. Phosphate-limited cells stopped exponential growth at OD₅₀₀ 0.9–1.1, but cells grown with an excess of phosphate at OD 2.0–2.1 (shown

Fig. 1. Growth of *B. subtilis* strain 168 under high- (○) and low- (Δ) phosphate conditions. The arrows indicate the times at which samples were taken for pulse labelling: 1, exponential phase (control); 2, transition phase; 3–5, 0.5 h, 1 h and 2 h, respectively, after the onset of phosphate starvation. ■, APase activity and ▽, percentage incorporation of L-[³⁵S]methionine measured in cultures grown under phosphate starvation.

Fig. 2. For legend see page 3166.

for the wild-type in Fig. 1). In all three strains, the rates of incorporation of methionine declined towards the end of the exponential growth phase, but the two mutants

showed a more drastic decline. APase was induced in response to phosphate starvation in the wild-type (Fig. 1) and threefold hyperinduced in the *spo0A* mutant, as

Fig. 2. 2-D gel electrophoresis patterns of proteins isolated from cells of *B. subtilis* 168 (a, b) and *B. subtilis* 168 *phoR* (c, d). Cells were sampled during exponential phase (control) (a, c) and 0.5 h after the onset of phosphate starvation (b, d). *PhoR*-dependent (class I) Psi proteins are boxed and *PhoR*-independent (class II) Psi proteins are underlined.

described by Jensen *et al.* (1993). As expected, APase was not induced in the *phoR* mutant.

Pattern of protein synthesis in phosphate-starved cells

The proteins synthesized by *B. subtilis* strain 168 following phosphate starvation were revealed by 2-D gel electrophoresis and autoradiography. Computer analysis showed that the rate of synthesis of more than 60 proteins appeared to be increased in response to phosphate starvation. The increased rate of synthesis of 20 of these proteins was significant enough to identify these proteins as phosphate-starvation-induced or Psi proteins (Fig. 2).

In comparison with the wild-type, the synthesis of 9 of the 20 Psi proteins appeared to be impaired in the *phoR*-deletion mutant (Fig. 2b, d). Comparison of the wild-type and *phoR* mutant revealed two classes of Psi proteins: class I proteins, which are no longer synthesized or are barely synthesized in the *phoR* strain (Psi1-3, Psi8, Psi28, Psi34, Psi38, Psi39, Psi40, Psi41 and Psi42), and class II proteins, which are induced in both strains (Psi7, Psi9, Psi10, Psi12, Psi13, Psi14, Psi25, Psi26, Psi27, Psi32 and Psi36).

Class I Psi proteins are encoded by genes that appear to be PhoP/R-dependent and therefore to belong to the Pho regulon (Fig. 3). Class II proteins seem to be PhoR-independent and therefore do not belong to the Pho regulon (Fig. 4). Some of the class II proteins (e.g. Psi9, Psi10, Psi27 and Psi36) also appear to be induced after glucose starvation but at a lower rate (data not shown).

Studies on the kinetics of induction showed that most Psi proteins are induced immediately in response to phosphate exhaustion (e.g. Psi1-3, Psi26, Psi28 and Psi39); others are induced after a short delay.

We have compared the protein patterns of non-growing phosphate-starved cells and cells grown in a chemostat under phosphate limitation. About half of the Psi proteins (Psi1-3, Psi8, Psi9, Psi10, Psi28, Psi34, Psi38, Psi39 and Psi41) were also synthesized at an elevated rate in steady-state cells growing under phosphate limitation in the chemostat (data not shown).

Influence of *spo0A*, *phoR*⁺ and SigB

The pattern of PhoR-dependent Psi proteins induced in a *spo0A* strain was different to that of the wild-type. Some of the class I Psi proteins continued to be synthesized in

Fig. 3. Rates of synthesis of PhoR-dependent (class I) Psi proteins from wild-type (■) and *phoR* (□) strains of *B. subtilis* 168. Cells were sampled during exponential phase (control, co), transition phase (t.p.) and 0.5 h, 1 h and 2 h after the onset of phosphate starvation. Results are means of 4 determinations and bars represent $\pm$ SEM.

Fig. 4. Rates of synthesis of PhoR-independent (class II) Psi proteins from wild-type (■) and *phoR* (□) strains of *B. subtilis* 168. For labelling of x-axis, see Fig. 3 legend. Results are means of 4 determinations and bars represent $\pm$ SEM.

Fig. 5. Rates of synthesis of Psi1-3 and Psi28 in wild-type (■) and *spo0A* (□) strains of *B. subtilis* 168. For labelling of x-axis, see Fig. 3 legend. Results are means of 4 determinations and bars represent $\pm$ SEM.

the *spo0A* mutant after the induction ratio had already declined in the wild-type (see Psi1-3 and Psi28, Fig. 5).

B. subtilis strain GCH871 contains genes which encode a PhoR* protein that is functionally active at high phosphate concentrations. In this strain some class I Psi proteins were produced during exponential growth even under phosphate-deplete conditions (Psi1-3, Psi28, Psi38

and Psi39; data not shown). This was demonstrated particularly clearly for Psi1-3, the most abundant protein on 2-D gels (Fig. 6).

All Psi proteins could be detected on autoradiograms of proteins of the *sigB*-deletion strain *B. subtilis* ML6 (data not shown). They were induced in a SigB-independent manner and therefore do not belong to the SigB-

Fig. 6. Rates of synthesis of Psi1-3 in *B. subtilis* strains 168 (■) and GCH871 (*phoR12*) (□). Cells were grown under high-phosphate conditions (HiP), or under low-phosphate conditions and sampled during the transition phase (t.p.) or 1 h after the onset of phosphate starvation. Results are means of 4 determinations and bars represent $\pm$ SEM.

dependent stress regulon. SigB is the primary regulator controlling the expression of general stress proteins (Gsp) in *B. subtilis* strain IS58 (Völker *et al.*, 1994).

SigB-dependent general stress proteins slightly induced in response to phosphate starvation (e.g. RsbW, Hst23r, Hst23l, GsiB, Gsp14, Gsp26 and Gsp20u; Fig. 2b, d) were not considered in this study.

N-terminal sequencing of the main Psi protein, Psi1-3

The spot corresponding to Psi1-3 appeared to be composed of at least three spots on the gel presumably because of different isoelectric points. N-terminal sequencing of this protein was not possible because of the presence of blocking functional groups. However, two internal fragments were generated after cleavage with cyanogen bromide and their N-terminal amino acid sequences were determined. Peptide 1, containing two potential phosphorylation sites motifs (CK2-phosphosite, underlined; PKC-phosphosite, double underlined), had the sequence: N-terminus-AAAVN PDAGV KDISK DELKK IFTGK IKNXE LGGKD QKITL VNRPD-C-terminus. Peptide 2 had the sequence: N-terminus-**EE/GG**NPD ADIQV QAGGS GTGLS QVTEG-C-terminus.

The N-derived sequences showed 100% identity to regions of YzmB (SWISS-PROT P46338), a protein derived from DNA sequence data (EMBL 58414) of *B. subtilis*. YzmB shows similarity to *Escherichia coli* PstS, the phosphate-binding lipoprotein component of an ABC phosphate transporter (Rao & Torriani, 1990). Acyl diglyceride substituents required for anchoring at the outer surface of the cell membrane probably account for the failure to obtain an amino acid sequence from the original N-terminus. Sequences downstream from *yzmB* encode homologues for other components of the phosphate transporter, *yzmC* (*pstC*), *yzmD* (*pstA*) and *yzmE/F* (*pstB*).

In summary, we have obtained a global view of the proteins induced in response to phosphate starvation,

including proteins encoded by genes in the Pho regulon. In *B. subtilis* only a limited number of genes of the Pho regulon have been characterized so far (Hulett *et al.*, 1994), including *phoA*, *phoB* and the *phoR-phoP* operon. It is interesting that only 9 out of 20 Psi proteins depend on the PhoP/R two-component system and that at least 11 Psi proteins are still induced in a *phoR* mutant. This raises the possibility that these proteins are under the control of an alternative phosphate-starvation-sensing system, and in addition to *phoPR* two other potential systems have been identified from DNA sequencing studies (Sun *et al.*, 1996).

An essential but unsolved problem is the elucidation of the functions of the Psi proteins identified in this study. The main Psi protein visualized on the 2-D gels may belong to a high-affinity phosphate uptake system.

One of the main responses of *B. subtilis* to phosphate starvation is the replacement of teichoic acids with teichuronic acid. This has recently been shown to be under the control of PhoP/R (Mueller & Harwood, unpublished data) and consequently the genes required for teichuronic acid synthesis (*tua*) would be expected to be amongst those induced by phosphate starvation. The aim of future studies should be the detection and analysis of the Psi proteins that are involved in this switch.

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