Adaptive response of the archaeon Sulfolobus acidocaldarius BC65 to phosphate starvation

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The adaptive response of the archaeon Sulfolobus acidocaldarius BC65 to phosphate starvation was studied. When cells were subjected to phosphate limitation, their growth was affected. In addition, the levels of synthesis and/or the degree of phosphorylation of several proteins changed, as detected by two-dimensional nonequilibrium pH gradient electrophoresis of cells labelled in vivo with [35S]methionine and [35S]cysteine, or H2 32PO4. After another growth-restricting treatment, a heat shock, a general inhibition of protein synthesis was observed. Under phosphate starvation conditions, a 36 kDa protein became phosphorylated without its synthesis being significantly modified, suggesting a probable regulatory role during adaptation of the cell to the change in the external environment. In Southern blot analysis with specific probes from very conserved regions of the phoR and phoB genes from Escherichia coli, a positive hybridization with S. acidocaldarius BC65 chromosomal DNA fragments was found. This suggested the presence in S. acidocaldarius BC65 of genes related to the E. coli genes involved in the phosphate starvation response system. This appears to be the first evidence of the possible existence of a two-component sensory system in a micro-organism from the archaean kingdom Crenarchaeota.

Keywords: Sulfolobus acidocaldarius, Archaea, phosphate starvation, two-component sensory systems, adaptive response

INTRODUCTION

Bacteria sense and respond to their environment by means of signal transduction across their membranes. A major mechanism of signal transduction widespread in these cells is the so-called two-component sensory systems (Bourret et al., 1991; Stock et al., 1989; Alex & Simon, 1994; Volz, 1995). The first component consists of a sensor, a histidine kinase that autophosphorylates a histidine residue in response to an environmental stimulus. The phosphate group is then transferred to an aspartate residue on the second component, the response regulator, which effects a cellular response. There are many signalling pathways in bacteria that are regulated by two-component systems, the best-studied being osmoregulation, nitrogen assimilation, chemotaxis and phosphate starvation (Volz, 1995; Torriani-Gorini et al., 1994).

The domain Archaea is a distinct group of prokaryotes, differing phylogenetically as much from bacteria (domain Bacteria) as they do from eukaryotes (domain Eukarya) (Wheelis et al., 1992; Woese, 1987; Woese et al., 1990). The archaean signal transduction systems are scarcely known, but evidence exists that the phototactic sensory systems in the archaean Halobacterium halobium (kingdom Euryarchaeota) could be similar to those of bacteria (Alam & Hazelbauer, 1991; Alam et al., 1989; Spudich, 1993; Rudolph & Oesterhelt, 1995). Very recently, two-component sensory systems have also been discovered in the domain Eucarya (Chang et al., 1993; Ota & Varshavsky, 1993; Maeda et al., 1994).

We have been studying some adaptive responses in chemolithotrophic, acidophilic micro-organisms, specifically the heat shock, phosphate starvation and chemotaxis responses (Seeger & Jerez, 1992, 1993; Acuña et al., 1992; Jerez, 1988; Amaro et al., 1991). Therefore, we have become interested in finding out whether the Archaea might possess sensory responses that could be regulated by systems homologous to the two-component bacterial ones. We chose to study Sulfolobus acidocaldarius, a species belonging to the archaean kingdom Crenarchaeota, which could represent the archaic ancestral ecotype (Brock,
1991). We analysed the response of the archaeon *S. acidocaldarius* BC65 to phosphate starvation since the PhoB family of regulators is one of the most highly conserved of the bacterial two-component sensory systems (Volz, 1995). The results reported here suggest that this archaeon could respond to phosphate starvation in a way homologous to the bacterial systems.

**METHODS**

**Bacterial strains and growth conditions.** The micro-organisms employed in this study were *S. acidocaldarius* BC65, kindly provided by Paul Norris, and *S. acidocaldarius* DSM 639, the type strain. They were grown at pH 2.5 and 70 °C with rotatory shaking (120 r.p.m.) in a medium that contained (1 l): 0.4 mg (NH₄)₂SO₄, 0.5 g MgSO₄·7H₂O, 0.2 g K₂HPO₄ and 50 g elemental sulfur prills as energy source (Arradondo et al., 1994). The initial inoculum was a stationary-phase cell suspension containing 10⁶ micro-organisms ml⁻¹ and was usually 10⁸ (v/v, final volume). Under these conditions, the generation time for both micro-organisms was around 20 h. Growth under phosphate-limiting conditions was in the same medium, except that the phosphate salt was omitted. Thus, only the trace amount of phosphate contained in the pure salts and sulfur (all from Merck) employed was available, which was usually a maximum of 5 × 10⁻² g l⁻¹.

**Labelling of archaeal proteins in vivo.** One hundred millilitres of a control culture (8 × 10⁸ cells ml⁻¹) or a phosphate-starved culture that had reached 3 × 10⁷ cells ml⁻¹ (see Fig. 1) were incubated for 2 h with 14.8 × 10⁶ Bq [³²P]methionine and [³⁵S]cysteine Pro-mix (specific activity 3.7 × 10⁹ Bq mmol⁻¹; Amersham). For heat shock treatment, the same volume of control cells grown in the presence of phosphate was subjected to a heat shock at 80 °C for 2 h in the presence of the same radioactive amino acids. The total radioactivity incorporated into hot trichloroacetic-acid-precipitable material was determined by scintillation counting. For radioactive labelling of archaeal cells with H₃₂PO₄, we used 100 ml of a culture which had been grown in the medium with the amount of phosphate reduced to 1/100 normal concentration in the case of control cells, and with no phosphate in the case of phosphate-starved cells. Reducing the phosphate concentration to 0.002 g l⁻¹ in the control treatment was done to avoid dilution of the radioactive label. Under these conditions, we obtained the same growth observed with the normal medium. Once the control cells had reached 8 × 10⁷ and the phosphate-limited cells 3 × 10⁶ cells ml⁻¹, respectively, 29.6 × 10⁶ Bq H₃₂PO₄ (specific activity 7.4 × 10⁸ mmol⁻¹), Comisión Chilena Energetica Nuclear was added and incubation at 70 °C was continued for 2 h.

**Two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE).** Total cell proteins were separated by two-dimensional NEPHGE, as described by O’Farrell et al. (1977), employing ampholines (pH 3–10) from Bio-Rad Laboratories. The cell samples (3.5 mg wet wt cold cells, or the same amount of cells containing approximately 2 × 10⁶ c.p.m. [³²P]methionine/[³⁵S]cysteine) were resuspended in 100 μl sonication buffer (10 mM Tris/HCl, pH 7.4; 5 mM MgCl₂; 50 μg pancreatic RNasea ml⁻¹), sonicated and treated with DNase (50 μg ml⁻¹) (O’Farrell et al., 1977). The mixture was then lyophilized and dissolved in lysis buffer as described previously (Chamorro et al., 1988). NEPHGE gels were electrophoresed for 5–5 h at 400 V. The second dimension was a SDS-polyacrylamide gel (11.5 %, w/v, acrylamide). Molecular mass standards for the second dimension were from Bio-Rad laboratories.

**Construction of oligonucleotide probes and hybridization assays.** Chromosomal DNA was extracted by a phenol/chloroform protocol modified according to Barns et al., 1994. Digestions of *S. acidocaldarius* BC65 and DSM 635 genomic DNA with restriction enzymes (from Promega) were done as described by Sambrook et al. (1989). For the Southern hybridization assays, we employed single-stranded probes corresponding to nucleotides 13–63 of the phoB gene and 1643–1692 of the *phoR* gene from *Escherichia coli* [5'-ATTCTGTCGTAGAA-GATGAAGCTCCAATTGCAGAATGTCGCTTG-3' (Makino et al., 1986a) and 5'-ACTTTTTTGCCAACG-TGAGCCATGAGTTACGTACGCCATTGACCGTGTT-A-3' (Makino et al., 1986b), respectively]. The hybridization was performed at 50 °C in the presence of 5 × NaCl/citrate buffer (0.75 M NaCl, 0.075 sodium citrate, pH 7.0), 0.1 % sodium laurylsarcosinate and 0.02 % SDS. The probe was synthesized in the Center of Oligonucleotides of the University of Chile and was labelled with [γ-³²P]ATP by the exchange reaction (Sambrook et al., 1989).

**RESULTS AND DISCUSSION**

**Effect of phosphate starvation on growth of *S. acidocaldarius* BC65**

The effect of lack of phosphate on the growth of *S. acidocaldarius* BC65 is shown in Fig. 1. Cells stopped growing much earlier, and the final number of cells was one-sixth that of cultures grown in the presence of phosphate. Moreover, when the cells grown under phosphate-limited conditions were subcultured in a medium without phosphate, there was no further growth (data not shown). Whether *S. acidocaldarius* BC65 possesses phosphate reserves in the form of polyphosphates, as has been observed in other micro-organisms (Dawes, 1985), is not clear at present.
Table 1. Relative levels of synthesis and phosphorylation of proteins from *S. acidocaldarius* BC65 grown under normal or phosphate-limited conditions

<table>
<thead>
<tr>
<th>Spot</th>
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<th>Phosphorylation</th>
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The approximate molecular masses were determined by measuring the relative migration distances in the second dimension on the gel shown in Fig. 2.

Changes in total protein synthesis pattern after phosphate starvation and heat shock

Total proteins from *S. acidocaldarius* BC65 grown with sulfur as energy source and with 35S-labelled amino acids were separated by two-dimensional NEPHGE and subjected to autoradiography. The results are shown in Fig. 2. We observed some proteins whose synthesis was consistently induced under phosphate starvation (arrowheads). These could represent members of a phosphate starvation response system. Conversely, the synthesis of proteins of interest were numbered arbitrarily. Numbers on the left of the gels indicate the positions and molecular masses in kDa of markers. Arrowheads and squares indicate proteins whose synthesis was consistently induced or decreased under phosphate limitation, respectively. Circles indicate phosphoproteins.

**Fig. 2.** Effect of phosphate starvation and heat shock on the total protein synthesis pattern in *S. acidocaldarius* BC65. The micro-organism was grown under normal (a) or phosphate-limited (b) conditions, or was grown under normal conditions and then subjected to a heat shock (c), and was then incubated with 35S-labelled amino acids for 2 h. Total cell proteins were separated by two-dimensional NEPHGE with a pH gradient of 3-10 (right to left) and visualized by autoradiography. Proteins
several polypeptides (enclosed by squares) was inhibited when the cells were grown in the absence of phosphate. The proteins marked by circles are phosphoproteins (see below), the synthesis of which did not change under phosphate limitation. A summary and relative quantification of these results is shown in Table 1.

When *S. acidocaldarius* cells were subjected to another growth-restricting treatment, a heat shock for 2 h at 80 °C, a completely different pattern of protein synthesis was obtained, as shown in Fig. 2(c). As we (Jerez, 1988) and others (Trent et al., 1990) have described previously for *S. acidocaldarius* and *Sulfobolus sibatae*, respectively, a heat shock caused a decrease in the synthesis of most cellular proteins, with the preferential synthesis of a few proteins. The most actively synthesized proteins at 80 °C were two in the 50–55 kDa range, as described for *S. sibatae* by Trent et al. (1990). Therefore, some or most of the protein synthesis changes observed under phosphate limitation may be specific for this condition and not for any growth-limited state.

### Analysis of phosphoproteins and their changes during phosphate starvation

The protein phosphorylation patterns of *S. acidocaldarius* BC65 under normal growth and phosphate starvation are shown in Fig. 3. The degree of phosphorylation of some phosphoproteins (spots X, Y and W) changed, whereas that of others (for example spots a, b and c), was not significantly modified. The coordinates of these last spots in Fig. 2 are not known at present; therefore their levels of synthesis were not determined by image process analysis (Table 1). In general, the molecular mass range of the phosphoproteins observed was similar to those previously described for *S. acidocaldarius* under normal growth conditions (Skorko, 1984). The phosphoprotein Y became greatly phosphorylated under phosphate starvation conditions. This protein could not be visualized by Coomassie-Blue-staining, and was detected only by radioactive labelling, suggesting that it is normally present in very low amounts in archaeal cells. As shown in Fig. 2, its migration was slightly shifted towards the acid side of the gel under phosphate starvation conditions, as is that of other phosphoproteins (compare spot Y in Fig. 2(a) and 2(b)).

As mentioned above, it is well known that many bacterial adaptive responses are regulated by phosphorylation of certain proteins comprising the so-called two-component sensory systems (Bourret et al., 1991; Stock et al., 1989; Alex & Simon, 1994; Volz, 1995). Two-component system response regulators are known to have a size range between about 28 and 50 kDa and are normally present in very low amounts in the cells. One could tentatively speculate that the 38 kDa protein Y that became phosphorylated under phosphate starvation could represent a member of a putative two-component sensory system in *S. acidocaldarius*. At present we do not know what type of phosphorylation of the *S. acidocaldarius* proteins is occurring. Although the characteristic aspartyl phosphate present in the two-component system has a short chemical half-life, in *E. coli* it has been possible to determine the in vivo phosphorylation of OmpR after SDS-PAGE of cell-free extracts from cells labelled in the presence of H₃²PO₄ (Forst et al., 1990).

### Probing the chromosomal DNA of *S. acidocaldarius* BC65 with oligonucleotide probes containing regions from *phoB* and *phoR*

If a two-component sensory system was mediating the phosphate starvation response in *S. acidocaldarius* BC65, we would expect that this archaeon contained *phoR*- and *phoB*-like genes in its genome. The probe used for *phoR* contained the site of autophosphorylation of the sensor (Bourret et al., 1991; Stock et al., 1989). The probe for *phoB* was constructed from one of the most conserved
Fig. 4. Southern blot of genomic DNA of *S. acidocaldarius* BC65 digested with different restriction enzymes. The probes used were *phoR* (a) and *phoB* (b) from *E. coli* and were labelled with [*γ-32P*]ATP. (a) Lanes: 1, 2 pg *E. coli* genomic DNA digested with *EcoRI*; 2-5, 10 pg genomic *S. acidocaldarius* BC65 DNA doubly-digested with *EcoRI* and either *HaeIII*, *Ddel*, *PstI* or *BanHI*, respectively. (b) Lanes: 1, 2 pg *E. coli* genomic DNA digested with *EcoRI*; 2-5, 10 pg genomic *S. acidocaldarius* BC65 DNA digested with *BamHI*, *PstI*, *EcoRI* or *Aval*, respectively. Numbers on the right of the gel in (b) indicate the positions and sizes in kbp of markers.

regions in the regulatory component superfamily (Volz, 1995) (see Methods for details). As shown in Fig. 4(a), the specific probe for the *phoR* gene hybridized with several fragments in the *S. acidocaldarius* BC65 genome, especially with a *HaeIII-EcoRI* fragment of approximately 2 kbp (lane 2) and a *PstI-EcoRI* fragment of approximately 0.5 kbp (lane 4). Other restriction fragments generated with different restriction enzymes gave less clear hybridizations (lanes 3 and 5). The *phoB* probe hybridized strongly with a 40 kbp *EcoRI* fragment of the *S. acidocaldarius* BC65 genome (Fig. 4(b), lane 4). Other hybridization reactions were also obtained with the fragments generated by different restriction enzymes (lanes 2, 3 and 5). When *S. acidocaldarius* DSM 639 genomic DNA was employed, the same results were obtained (data not shown).

Our results clearly show that under phosphate starvation conditions the global gene expression of *S. acidocaldarius* BC65 was modified. Synthesis of several proteins was induced whereas the levels of synthesis of numerous others was decreased. We also observed a change in the global protein phosphorylation pattern under these conditions. Some of the induced polypeptides could play important roles in the phosphate response of *S. acidocaldarius*, comprising a phosphate starvation response system. The Southern blot results strongly support the existence of *phoR* and *phoB*-like genes in this crenarchaeal micro-organism. This is supported by the very recent finding of a histidine kinase in a two-component system from an euryarchaeal micro-organism (Rudolph & Oesterhelt, 1995), which indicates that both archaeal kingdoms possess this class of sensorial systems. In evolutionary terms, the presence of two-component systems in the two principal evolutionary lineages (Bacteria and Archaea–Eucarya) (Chang et al., 1993; Ota & Varshavsky, 1993; Maeda et al., 1994) would indicate that these systems emerged in the common ancestor (assuming no gene transfer between domains). Since the domain *Archaea* is a slowly evolving group, one would expect it to have retained this type of sensory system.

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