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Phosphate-starvation induced changes in *Thiobacillus ferrooxidans*

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Abstract: We have analysed the response of the acidophilic chemolithotroph *Thiobacillus ferrooxidans* to phosphate starvation. Cultivation of the bacteria in the absence of added phosphate induced a remarkable filamentation of the cells. Polyacrylamide gel electrophoresis revealed several proteins whose levels increased upon phosphate limitation, as well as some polypeptides that were exclusively synthesized under this growth limitation. One of the proteins whose level increased by the lack of phosphate was apparently an acid phosphatase with a pH optimum of about 3.8, and a molecular mass of 26 kDa, which was located in the periplasm. The N-terminal sequence of a 26 kDa protein derepressed by starvation, which may correspond to the *T. ferrooxidans* phosphatase, showed 30% and 35% identity with the known sequence of *Lysobacter enzymogenes* and *Escherichia coli* alkaline phosphatases, respectively.

Key words: Phosphate starvation; *Thiobacillus ferrooxidans*; Acid phosphatase

Introduction

Thiobacillus ferrooxidans is a Gram-negative, acidophilic chemolithotrophic bacterium capable of oxidizing ferrous iron or reduced sulfur compounds [1]. The lack of some essential nutrients such as phosphorus will affect the rate of oxidation reaction of ferrous salts or elemental sulfur [2], and therefore could be a rate-limiting factor in industrial processes [3].

The response to phosphorus deprivation is well characterized in the heterotroph *Escherichia coli* [4,5]. To cope with conditions of low phosphate concentrations, *E. coli* has developed an emergency system known as the *pho* regulon [4–6]. This regulon consists of a large number of genes coding for products that allow the micro-organism to scavenge traces of usable phosphate sources from the environment. These gene products are the proteins within the cell envelope that transport phosphate into the cytoplasm or the Pi starvation-derepressed proteins, such as alkaline phosphatase, the pore protein PhoE, and a high-affinity phosphate transport system (PST).

We have recently described changes in the expression of an outer membrane protein when *T. ferrooxidans* is starved for phosphate [7]. In the present report we have extended our studies on the molecular response of *T. ferrooxidans* to

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phosphate limitation and describe an acid phosphatase activity which may be part of a Pi-scavenging system in this industrially important chemolithotrophic acidophile.

Materials and Methods

Microorganisms and growth conditions

The chemolithotrophic microorganism employed in this study was *T. ferrooxidans* strain R2. Bacteria were grown at pH 1.5 and at 30°C as described previously [8]. Growth under phosphate-limiting conditions was in the same modified 9K medium, except that the phosphate salt was omitted.

Polyacrylamide gel electrophoresis (PAGE)

For one-dimensional slab sodium dodecyl sulfate (SDS)-PAGE, samples were mixed with Laemmli sample buffer [9] and boiled for 10 min. Electrophoresis was performed on 7 to 15% linear gradient SDS polyacrylamide gels as described previously [8,10]. Two-dimensional (2-D) electrophoresis with non-equilibrium pH gradient (NEPHGE) was performed as described by O'Farrell [11] as used before [8].

Microsequencing of proteins

Proteins of interest were recovered from Coomassie brilliant blue-stained and heat-dried 2-D gels by excising the protein spots. After rehydration and concentration of the spots by SDS-PAGE, the proteins were electroblotted onto a polyvinylidene difluoride membrane [12] and were subjected to microsequencing by the Protein Chemistry Laboratory at Hoffmann-La Roche Inc., Nutley, NJ.

Electron microscopy

For negative staining, 50 μl of bacterial suspensions containing about 1×10^{10} bacteria per ml in 0.005 M sulfuric acid were placed on Formvar-coated copper grids (200 mesh). After blotting the excess fluid, the cells were stained with 2% sodium phosphotungstate (pH 6.8) for 1 min. Observations were done on a Carl Zeiss D-7082 Oberkochen EM 109 electron microscope.

Phosphatase activity in solution and in non-denaturing gels

To determine the phosphatase activity in cell-free extracts, cells (100 mg wet weight) were resuspended in 1 ml of a buffer containing 10% Triton X-100, 10 mM Tris · HCl, pH 7.4, 1 M $MgCl_2$ and 1 mM phenylmethylsulphonyl fluoride (PMSF), and after incubating at 4°C for 45 min with vigorous stirring, the unbroken cells and debris were separated by centrifugation for 10 min at $12\,000 \times g$. The resulting supernatant was used as the source of enzymatic activity.

To measure the phosphatase activity released by osmotic shock, *T. ferrooxidans* were osmotically disrupted by adapting the method of Baumann et al. [13]. The sediment was discarded, and the final supernatant was used to measure phosphatase activity. All solutions employed contained 1 mM PMSF.

Alkaline and acid phosphatases were assayed using the substrate *p*-nitrophenylphosphate as described by Torriani [14]. The alkaline phosphatase activity was determined in the presence of 1 M Tris · HCl, pH 8.8 and the acid phosphatase was assayed in 0.1 M sodium acetate, pH 4.0. All activities were expressed as μmol of product formed per min per mg protein.

The phosphatase activity was detected in non-denaturing gels by using the system with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoxilphosphate (BCIP) [15].

Results and Discussion

The effect of low phosphate on growth and morphology of T. ferrooxidans cells

When *T. ferrooxidans* is transferred to a phosphate-free medium, only a four-fold decrease in the total number of cells has been observed (Seeger and Jerez, unpublished results). A more significant inhibition of bacterial growth and total iron-oxidation rate due to phosphate deficiency was seen only after a second subculture in phosphate-free medium. These results suggested to us the possibility that *T. ferrooxidans* may also have the capacity to store phosphorus, perhaps in the form of the polyphosphate granules described for other microorganisms [16].

Moreover, after several subcultures of *T. ferrooxidans* in the absence of phosphate, there was a striking filamentation of the cells as shown in Fig. 1. The length of the Pi-starved cells reached 8–9 μm, and therefore they experienced a 10-fold enlargement compared with the microorganisms grown in the presence of phosphate (see C and D in Fig. 1).

The simplest possible explanation of this phenomenon, is that in the absence of phosphate, the synthesis of DNA is inhibited, and as a consequence, cell division is affected, as occurs with inhibitors of DNA gyrase such as nalidixic acid. As pointed out by Roszack and Colwell, under these temporary stressful conditions, cell mass would increase, resulting in lengthwise cell growth without division [17]. On the other hand, with micro-organisms such as *E. coli*, phosphate starvation results in the reduction of the cell size [18].

Effect of phosphate starvation on the proteins synthesized by T. ferrooxidans

When cells of *T. ferrooxidans* were grown in the absence of phosphate, several proteins of high and low apparent molecular mass showed an increased synthesis (Fig. 2, arrows). Proteins with similar electrophoretic migrations, especially in the 60 to 90 kDa region, are also induced in *T. ferrooxidans* under heat shock conditions, in the presence of ethanol or upon a pH shift [8,10]. In addition, growing cells under limiting phosphate conditions produced smaller amounts of a few proteins (Fig. 2, arrowheads).

Detection of an acid phosphatase activity in T. ferrooxidans

Upon phosphate starvation, several bacteria greatly stimulate the synthesis of phosphatase activities that degrade phosphorylated com-

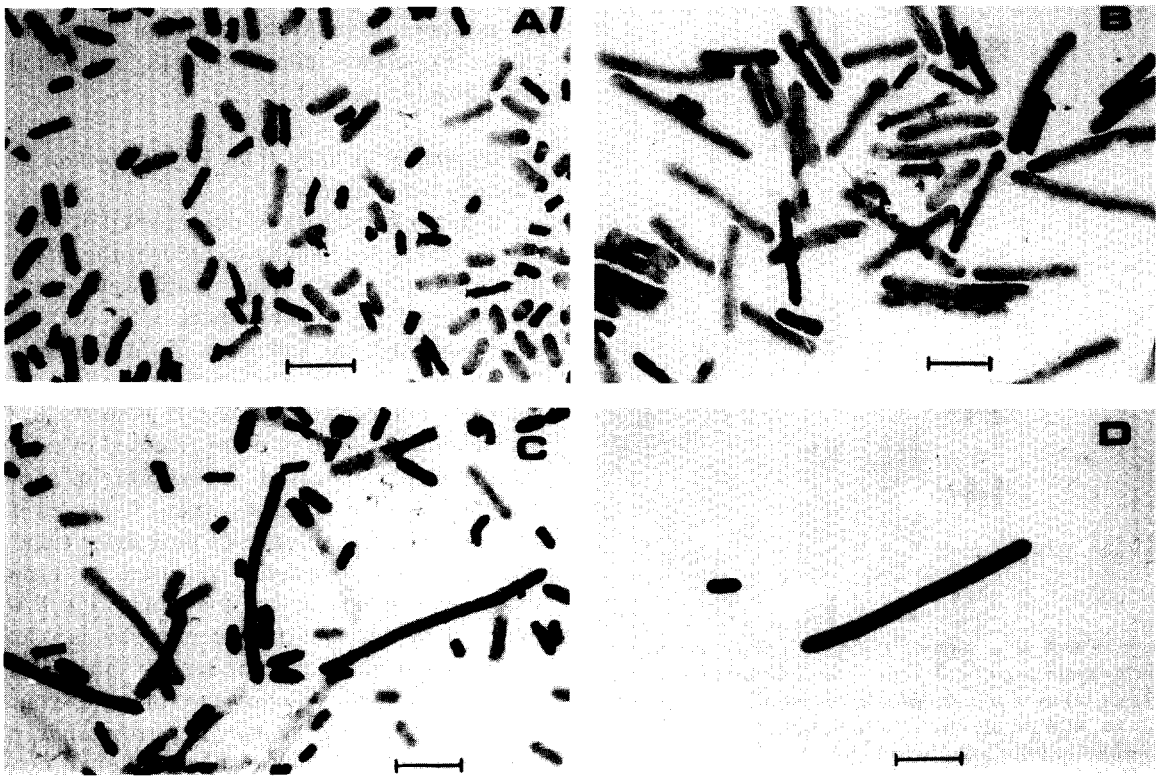

Fig. 1. Effect of the lack of phosphate on *T. ferrooxidans* cell morphology. *T. ferrooxidans* grown under normal conditions (**A**), in the absence of phosphate for four subcultures (**B**) or a mixture of both kinds of cells (**C**, **D**) were subjected to negative staining and electron microscopic analysis. Bars, 2 μm.

pounds that cannot be metabolized unless they are first converted to inorganic phosphate [14,19,20]. We therefore analyzed the presence of phosphatase activity in *T. ferrooxidans* cell-free extracts at different incubation pHs as shown in Fig. 3. It is clear that *T. ferrooxidans* possessed an acid rather than alkaline phosphatase activity which was increased by growing the cells in the absence of phosphate. The pH optimum for this activity was around 3.8 (Fig. 3, insert). The phosphatase activity was strongly inhibited by 100 mM phosphate (70%), slightly inhibited by 10 mM sodium fluoride (35%) and it was not affected by the presence of 5 mM dithiothreitol. Therefore, it shares properties common to both acid and alkaline phosphatases [14,19–21].

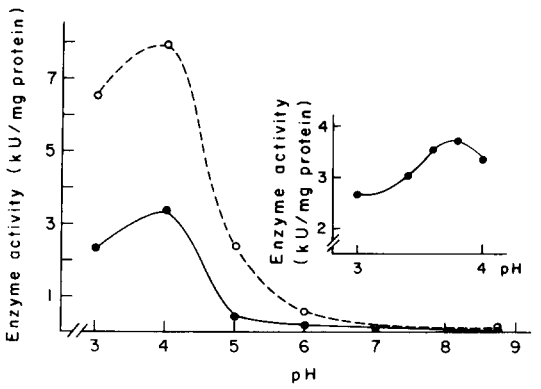

Fig. 3. Phosphatase activity as a function of pH. Phosphatase activity was measured in cell-free extracts from *T. ferrooxidans* grown in the presence (●) or in the absence (○) of phosphate. In the insert, the activity was measured in cell-free extracts from bacteria grown in the presence of phosphate. Conditions of the assay and unit definition were as described in Materials and Methods.

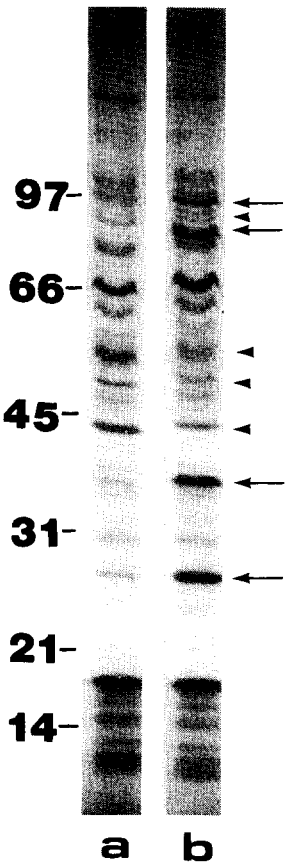

Fig. 2. Effect of phosphate starvation on the pattern of proteins synthesized by *T. ferrooxidans*. Cells grown in the presence (a) or in the absence (b) of phosphate were examined by SDS-PAGE and the polypeptides stained with Coomassie brilliant blue. Arrows indicate the main polypeptides with increased levels of synthesis and arrowheads, some of the polypeptides whose levels decreased upon phosphate starvation. Numbers to the left indicate molecular mass markers in kDa.

A phosphatase activity from T. ferrooxidans is released by osmotic shock

Bacterial phosphatases derepressed by the lack of phosphate are usually located in the periplasmic space [6]. Since many periplasmic proteins are released by osmotic shock ([13] and references therein), we subjected *T. ferrooxidans* to this treatment, and analyzed the proteins released by non-denaturing-PAGE as shown in Fig. 4A. Several bands of proteins released by osmotic rupture can be observed. A few proteins showed a higher level of synthesis when *T. ferrooxidans* was grown in the absence of phosphate (lane b). When the same proteins released by osmotic shock were separated by non-denaturing PAGE followed by a very sensitive phosphatase detection-reaction in the gel, the results shown in Fig. 4B were obtained. The presence of a phosphatase activity was evident in the *T. ferrooxidans* proteins released by osmotic shock although no significant increase in the activity was observed in *T. ferrooxidans* cells grown in the absence of phosphate (lane d). Under these conditions, we de-

tected both *E. coli* alkaline and wheat germ acid phosphatases (not shown).

To determine the relative molecular mass of the phosphatase activity detected on these gels, the band containing the activity was excised from a non-denaturing gel similar to the one shown in Fig. 4B. The protein was then re-run on an SDS-PAGE followed by Coomassie brilliant blue staining as shown in Fig. 4C, lane f. The approximate molecular mass of this protein was 26 kDa and it comigrated with an intense band with the same molecular mass present in the osmotic extract from *T. ferrooxidans* (lane e) and whose level of synthesis was raised upon phosphate starvation. The estimated molecular mass of 26 kDa for the *T. ferrooxidans* phosphatase appears to be unusually small compared with other phosphatases. Most alkaline phosphatases reported in the literature have subunits with molecular mass of at least 40 kDa [23]. The *E. coli* acid phosphatase has a molecular mass of 44.64 kDa [22]. However, a phosphatase with a pH optimum of 7.5 and a molecular mass of 26–30 kDa has been described for *Lysobacter enzymogenes* [24].

Several acid phosphatases have been described in different bacteria [20–22]. Nevertheless, it is not easy to explain the physiological role of an enzyme such as the *E. coli* acid phosphatase with a pH optimum of 2.5 [21,22]. On the contrary, *T. ferrooxidans* is an acidophilic bacterium, living normally between pH 1.5 and 4.0 [1], and it has been suggested that in this micro-organism, the periplasmic pH is similar to the external pH [25]. Therefore, the described acid phosphatase would be acting under 'physiological' conditions.

Possible identification of a phosphatase by microsequencing of protein spots from 2-D gels

Since *T. ferrooxidans* showed a phosphatase activity in non-denaturing gels, having a molecular mass of about 26 kDa in SDS-PAGE (Fig. 4), we analyzed the protein spots in 2-D NEPHGE

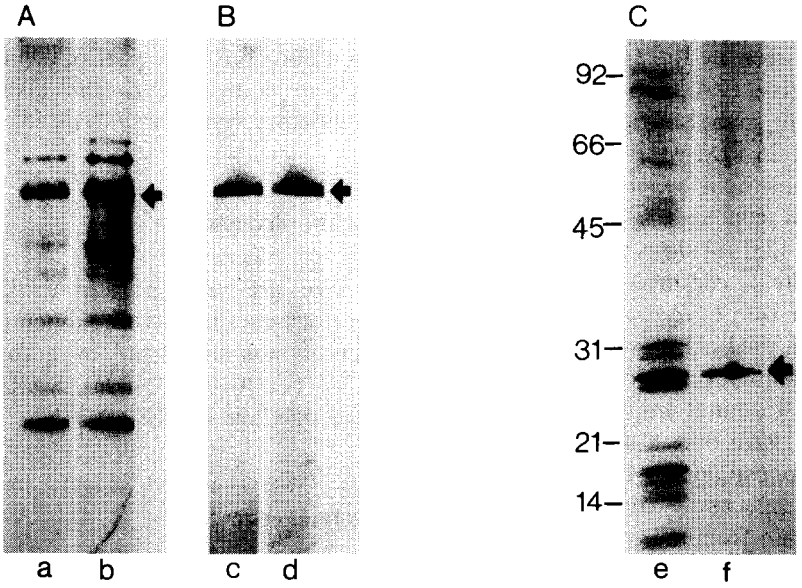

Fig. 4. Detection of a phosphatase activity in the proteins released from *T. ferrooxidans* by osmotic shock. Total proteins from *T. ferrooxidans* released by osmotic shock were subjected to non-denaturing PAGE and were stained with Coomassie brilliant blue (A) or developed with NBT-BCIP to detect phosphatase activity (B). Lanes a and c, proteins from *T. ferrooxidans* grown with phosphate; lanes b and d, proteins from the same bacteria grown in the absence of phosphate. The arrow indicates the protein with phosphatase activity. In (**C**), the band showing the phosphatase activity in the non-denaturing gel in (**B**), was excised from the gel and rerun in SDS-PAGE followed by staining with Coomassie brilliant blue. Lane e, proteins released from *T. ferrooxidans* grown in the absence of phosphate by osmotic rupture; lane f, band with phosphatase activity from the gel in (**B**). Numbers to the left indicate the molecular mass standards in kDa.

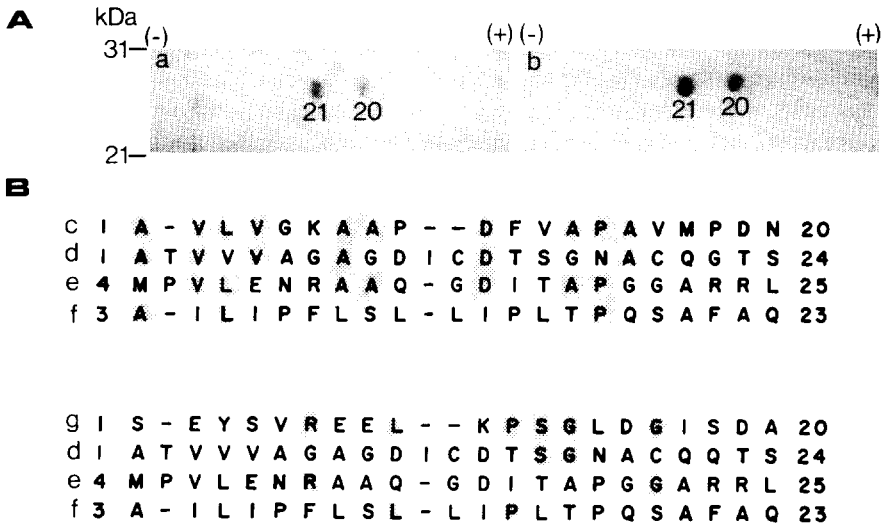

Fig. 5. Comparison of the N-terminal amino acid sequences of *T. ferrooxidans* spots 20 and 21 from 2-D gels. (**A**) Total proteins from *T. ferrooxidans* grown in the presence (a) or in the absence (b) of phosphate were separated by 2-D NEPHGE and stained with Coomassie brilliant blue. Spots were arbitrarily named. Only the region of the gel between 21 and 31 kDa is shown. (**B**) Spot 20 (c) and 21 (g) were microsequenced and compared with *L. enzymogenes* alkaline phosphatase (d), *E. coli* alkaline (e) and acid (f) phosphatases. The numbers indicate the location of the respective amino acids in the four protein sequences compared. The amino acid sequences of the *E. coli* alkaline phosphatase were taken from Bradshaw et al. [23], the acid phosphatase from Dassa et al. [22] and the *L. enzymogenes* alkaline phosphatase from Au et al. [24].

gels that were induced by the lack of phosphate (Seeger, M. and Jerez, C.A., unpublished results). The only ones with increased levels and with a molecular mass of 26 kDa were spots 20 and 21 (Fig. 5). The amino acid sequence of the first twenty amino acids from spot 20 was 35% identical to the *E. coli* [23] alkaline phosphatase and 30% identical to the *L. enzymogenes* [24] enzyme. In addition, when the *T. ferrooxidans* sequence was compared with the acid phosphatase from *E. coli*, only 15% identity could be seen. On the other hand, the N-terminal amino acid sequence of protein spot 21 gave only 8 and 11% identity with the alkaline phosphatases from *E. coli* and *L. enzymogenes*, respectively, and only 8% with the acid phosphatase from *E. coli*. This suggests that spot 20 may correspond to an acid phosphatase from *T. ferrooxidans*. Nevertheless, further studies will be required to confirm the identity of spot 20 as a *T. ferrooxidans* acid phosphatase.

It is possible that the acid periplasmic phosphatase and some of the other proteins are derepressed in *T. ferrooxidans* after phosphate starvation and constitute part of a potential scavenging system equivalent to the alkaline phosphatase regulatory system in *E. coli* [6].

Acknowledgements

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