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Phosphate uptake by phosphorus-starved cells of the cyanobacterium *Phormidium laminosum*

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Phosphorus(P)-starved cells of the cyanobacterium *Phormidium laminosum* have been investigated in relation to their phosphate uptake characteristics. P-deficient cells showed much higher phosphate uptake rates from ultrapure water supplemented with this anion than P-sufficient ones. After 9 days of starvation in P-free medium, the total cellular P content of P-deficient cells was approximately five times lower than that of cells grown in the presence of phosphate. Phosphate uptake by P-deficient cells occurred in both light and dark under aerobic conditions. In anaerobiosis, light was required for uptake, suggesting that the necessary energy could be derived from the respiratory electron transport chain. Phosphate uptake in P-deficient cells was sensitive to vanadate, suggesting the involvement of a plasma membrane ATPase.

Key words: *Phormidium laminosum*, phosphate uptake, phosphorus-starved, photosynthesis, vanadate.

In spite of its abundance in nature, phosphate is frequently the limiting nutrient in many environments since it is mainly found in forms not readily available such as insoluble salts (Wagner *et al*. 1995; Eberl *et al*. 1996). Nevertheless, phosphorus (P) in municipal, agricultural and industrial effluents is an important contributory factor to eutrophication and its removal from those effluents is a matter of increasing concern.

Cyanobacteria are photosynthetic prokaryotes whose dominant mode of nutrition is photoautotrophy and thus growth, with its reliance on light as the primary energy source, proceeds via the fixation of CO_2 and the acquisition of other simple inorganic nutrients such as phosphate (Mann 1994). In fact, phosphate is widely accepted as the main nutrient controlling the development of natural populations of cyanobacteria in many freshwater environments (Mann 1994).

The uptake of phosphate by cyanobacteria has already been characterized in several cyanobacterial strains showing that the kinetic parameters for phosphate transport vary considerably among the cyanobacteria, depending also on the composition of the medium and the nutritional state of the cells (Marco & Orús 1988; Thiel 1988; Garbisu *et al*. 1993). In particular, cultivation of cyanobacteria under P-limited conditions leads to higher rates of phosphate uptake (Thiel 1988). Many cyanobacteria, to deal with P-limiting conditions, store polyphosphate reserves and induce the synthesis of extracellular phosphatases to obtain phosphate from organic substrates present in the surrounding medium (Wagner *et al*. 1995).

Phosphate flow into cyanobacteria is closely linked to photosynthetic ATP formation and both its uptake as well as its intracellular accumulation in the form of polyphosphate granules are, therefore, energy-dependent processes (Falkner *et al*. 1984).

P-sufficient cells of the thermophilic filamentous cyanobacterium *Phormidium laminosum* have already been studied in relation to their capacity to take up phosphate from water (Garbisu *et al*. 1993; Hall *et al*. 1993; Garbisu *et al*. 1994). Since our *P. laminosum* strain was originally isolated from hot spring waters, some of which are quite deficient in phosphate (Castenholz 1969), it was of interest to study its capacity to take up phosphate from water under phosphate starvation. The effects on phosphate uptake rate of aerobiosis as compared with anaerobiosis and the presence or absence of light were also determined. Finally, the effect of sodium vanadate, a universal inhibitor of the P-ATPases, on phosphate uptake by P-deficient *P. laminosum* cells was investigated. To our

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knowledge, this is the first report on the phosphate uptake characteristics of a thermophilic cyanobacterium growing in a P-limited environment.

Materials and Methods

Phormidium laminosum (Agardh) Gomont (strain OH-1-pCl$_1$) was originally obtained from Prof. R.W. Castenholz (University of Oregon, Eugene). Cultures were grown in medium D (Castenholz 1969) in 2-l Erlenmeyer flasks at 45 °C in an orbital shaker at 100 rev min^{-1} under continuous cool white fluorescent light at an irradiance of 70 μmol photon m^{-2} s^{-1}. The phosphate concentration in medium D was 780 μm. Sodium bicarbonate (0.5 g l^{-1}) was supplemented to the culture medium as an extra carbon source.

Phosphate-grown cells were harvested during the exponential phase of growth, washed twice with P-free medium D and, finally, resuspended in this medium. The resulting cell suspension was maintained under the same conditions used to grow the cells for a further 10 days. Subsequently, an aliquot from this culture was used to inoculate fresh P-free medium D which was then incubated as described above. Time zero in the experiments corresponded to the time when this last subculture was inoculated.

Chlorophyll was estimated according to MacKinney (1941). Protein was estimated by the method of Peterson (1983). Phosphate was determined spectrophotometrically according to Murphy & Riley (1962). Total P content of the cells was determined by inductively coupled plasma spectrometry (ICP) using a Perkin-Elmer (Branchburg, NJ, USA) Plasma 40 Emission Spectrophotometer after drying the cells at 50 °C in an oven and, subsequently, completely digesting the organic matter with hot (80 °C) concentrated HNO_3. ICP data were expressed as a percentage (w/w) of P using dry cells as the 100% value.

Photosynthesis of whole *P. laminosum* cells was measured as net O_2-exchange in a Clark-type electrode (Rank Brothers, Cambridge, UK) at 25 °C under saturating orange-red light as described previously (Garbisu *et al*. 1990). O_2-uptake was used to measure photosystem I (PSI) activity with the following added in sequence: 3 mM methyl viologen, 12 mM sodium azide, 15 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 2.5 mM reduced glutathione and 0.3 mM 2,6 dichlorophenolindophenol. Photosystem II (PSII) activity was measured by quantifying the O_2-evolution by whole cells in a total volume of 3 ml of cells plus the reaction mixture containing 4 mM potassium ferricyanide and 3 mM 2,6 dimethyl-benzoquinone.

For phosphate uptake assays, cells were harvested by centrifugation or filtration through glass-fibre (GF/C Whatman) filters; they were then washed with Milli-Q (Millipore, Bedford, MA, USA) ultrapure water to remove all traces of culture medium. The cells were resuspended in the same water, placed in 250-ml Erlenmeyer flasks and supplemented with 70 μM Na_2HPO_4 (this concentration was used because it is very close to the maximum limit found at four different water treatment plants) (Abel 1989). Finally, the flasks were incubated under the same conditions used to grow the cells. Samples of 1 ml were withdrawn at timed intervals and centrifuged immediately at 15,000 × *g* for 10 min at 4 °C. The clear supernatants were analysed for phosphate and the uptake rate was estimated by measuring phosphate disappearance from the external medium.

The effect of anaerobiosis and light on phosphate uptake from water was studied by flushing the cultures with N_2 and covering the flasks with aluminium foil, respectively. Sodium vanadate was supplemented to the cultures to investigate the involvement of a plasma membrane ATPase on phosphate uptake.

In all the experiments, ultrapure water was used as a standardized system which allowed us to study the phosphate uptake capacity of the cells without interference from other ions. It should also be pointed out that since the cells were resuspended in water, cell growth appeared to be negligible during the experiments (data not shown). For comparison purposes, phosphate uptake was referred to biomass in all cases.

All experiments were repeated a minimum of five times with consistent results and values from a representative experiment are given. This was done to compensate for the variability of results due to the different states of P-starvation reached by the cultures under the same growth conditions because, although these conditions were standardized as much as possible, different cultures appeared to reach different degrees of P-starvation under the same experimental conditions. The degree of P-starvation reached by the cells appeared to be the main, if not the only, cause of the differences found among experiments.

For observation in scanning electron microscopy (SEM), cells were fixed and dehydrated as described by Shi (1987). Subsequently, the samples were dried in a critical point drying apparatus (Sandri-780, Tousimis Research Corporation, Rockville, MD, USA) using liquid CO_2. Samples were examined in a Hitachi S-510 SEM (Hitachi Scientific Instruments, Japan) at an accelerating voltage of 20 kV.

Results

Effect of P-starvation on Photosynthetic Activity and Phosphate Uptake

The photosynthetic activity of whole cells (net O_2-exchange, PSI and PSII) during the first 2 weeks of incubation in medium D containing or lacking phosphate is shown in Figure 1. Although P-sufficient and P-deficient cells showed similar PSII activities, the values for both total and PSI activity were somewhat different.

As shown in Figure 2, P-deficient cells took up phosphate from ultrapure water supplemented with 70 μM of this anion at much higher rates than P-sufficient cells. In fact, the phosphate uptake rate values for P-sufficient cells were negligible in comparison with those of P-deficient cells. The capacity of cells for phosphate uptake increased markedly with the time of P-starvation during the first 7 days of incubation and then decreased. Although the maximum phosphate uptake rate was observed at approximately 7 days of starvation, this value varied slightly depending on the physiological status of the cells at the beginning of the starvation period.

After 9 days in P-free medium D, the total cellular P content of P-deficient cells was already approximately five times lower than that of cells grown in the presence of phosphate [0.19 and 0.93% (weight of P/weight of dry cells) for P-deficient and P-sufficient cells, respectively].

When phosphate uptake assays from water were carried out using both P-sufficient and P-deficient cells,

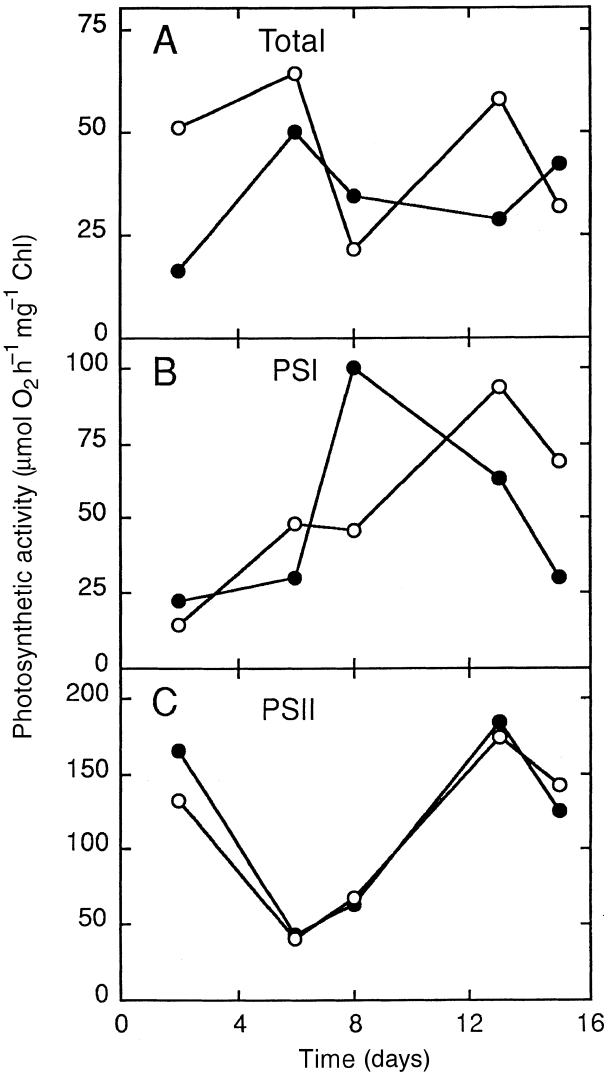

Figure 1. Effect of P starvation on photosynthetic activity of *P. laminosum*. Cells were incubated for 15 days in medium D containing 780 μM phosphate (●) or in P-free medium D (○). (A) Total (i.e., without using artificial electron mediators) photosynthetic activity. (B) PSI photosynthetic activity. (C) PSII photosynthetic activity.

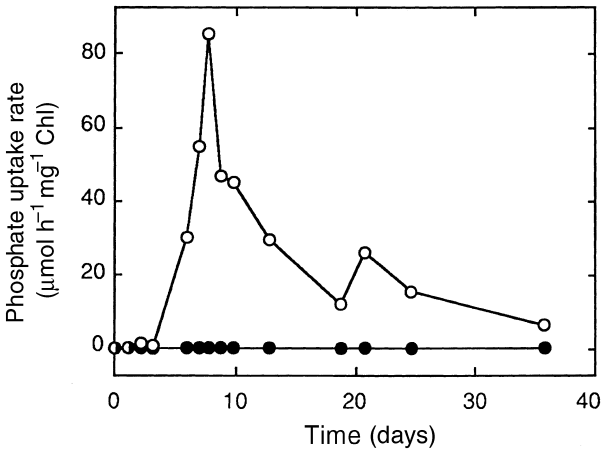

Figure 2. Effect of P-starvation on the rate of phosphate uptake from water by *P. laminosum*. Cells were incubated for up to 37 days in medium D containing 780 μM phosphate (●) or in the absence of phosphate (○). At timed intervals cells were taken from the cultures and their phosphate uptake capacity from ultrapure water evaluated. These uptake assays were carried out using an initial phosphate ion concentration of 70 μM.

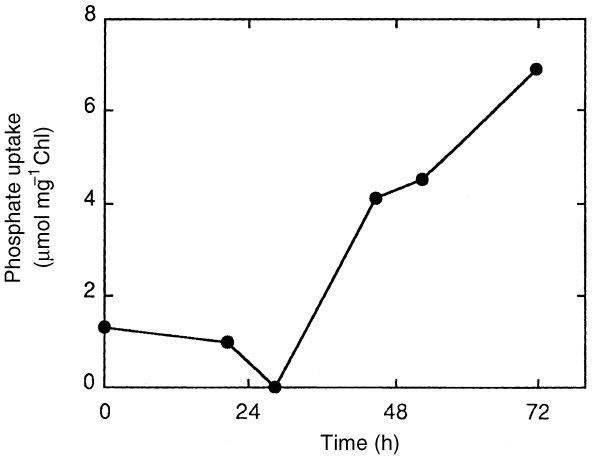

Figure 3. Time-course of phosphate uptake by P-sufficient cells of *P. laminosum*.

the former appeared to excrete phosphate into the external medium during the first 30 h of incubation (Figure 3). Such an effect was not observed when P-deficient cells were used to study phosphate uptake from water (Figure 4 and Figure 5).

Effect of Light and Anaerobiosis on Phosphate Uptake from Water

P-deficient cells took up phosphate in the presence or absence of light at similar rates (Figure 4). The effect of anaerobiosis on phosphate uptake from water was studied by flushing the flasks with N_2 20 min after the addition of this anion. In the dark, P-deficient cells required the presence of an air atmosphere to take up phosphate at a rate similar to that found in illuminated cultures (Figure 5).

Vanadate Sensitivity of Phosphate Uptake

Vanadate, a universal inhibitor of the P-ATPases (Pedersen & Carafoli 1987) was found to inhibit the uptake process (Figure 6). When P-deficient cultures were supplemented with 2 mM sodium vanadate 20 min before the addition of phosphate, a decrease in phosphate uptake rate of approximately 45% was observed. Similarly, 3 mM sodium vanadate caused a ~80% decrease in the phosphate uptake rate of P-deficient cells.

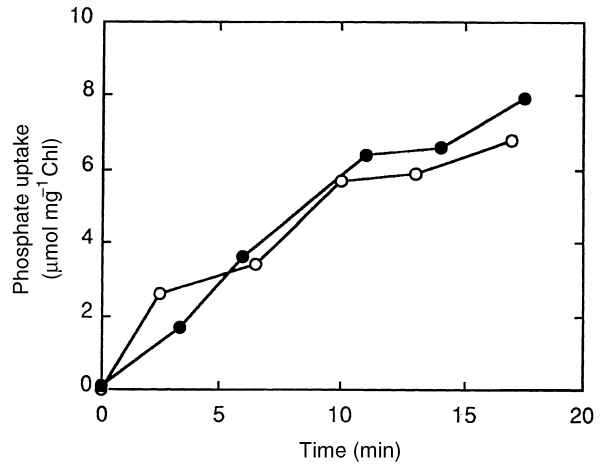

Figure 4. Effect of light on phosphate uptake time-course by P-deficient (9 days incubation in P-free medium) cells of *P. laminosum*. A light intensity of 70 μmol photon m^{-2} s^{-1} was used. Dark conditions were obtained by covering the flasks with aluminium foil. ○ – Light conditions; ● – dark conditions.

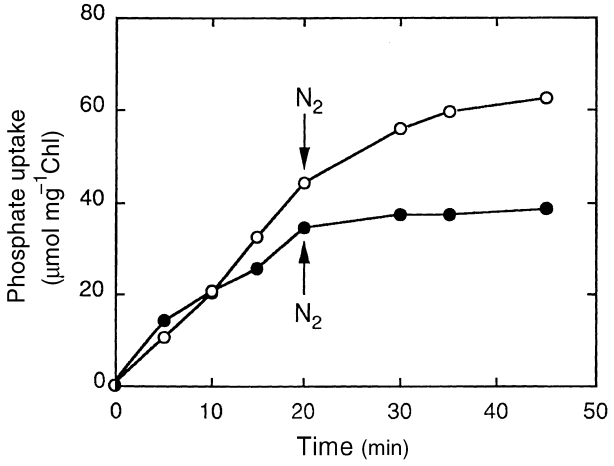

Figure 5. Effect of light and gas phase on phosphate uptake time-course by P-deficient (9 days incubation in P-free medium) cells of *P. laminosum*. The atmosphere at time zero was air. At 20 min of phosphate uptake (arrows), the flasks were bubbled with N_2 to remove the air from the cultures and the phosphate uptake was monitored for a further 25 min. ○ – Light conditions; ● – dark conditions.

In contrast, vanadate appeared not to have a negative effect on the photosynthetic O_2-evolution e.g., photosynthetic activity of whole cells expressed in μmol O_2 $h^{-1}\mu g^{-1}$ Chl; 0.83, 2.48 and 2.13 for the control (no vanadate), 2 mM vanadate and 3 mM vanadate cultures, respectively.

Scanning Electron Microscopy

SEM of P-sufficient and P-deficient *P. laminosum* cells revealed that cell surface morphology was not affected by the lack of phosphate in the surrounding culture medium (Figure 7).

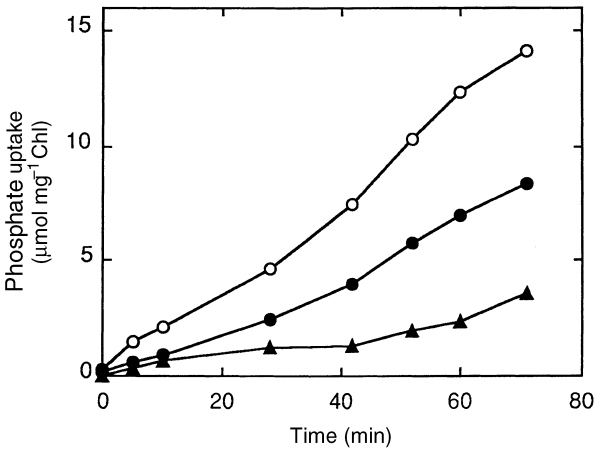

Figure 6. Phosphate uptake time-course by P-deficient (9 days incubation in P-free medium) cells of *P. laminosum* in the presence and absence of vanadate. Vanadate (2 or 3 mM) was added to the cultures 25 min before beginning the phosphate uptake assays. ○ – No vanadate (control); ● – 2 mM vanadate; ▲ – 3 mM vanadate.

Discussion

During their growth in P-free medium, and unlike N-starved cells, the typical green coloration of *P. laminosum* cultures did not change to yellowish, a phenomenon termed chlorosis which is due to the rapid catabolism of all pigments, mainly phycobiliproteins and to a minor degree chlorophyll (Fresnedo *et al.* 1991; Garbisu *et al.* 1992). Fresnedo *et al.* (1991) showed that, although N-starved *P. laminosum* cells had a similar size and shape to nitrate-grown cells when observed under the SEM, the exocellular layer of mucilage rapidly increased with starvation. In the case of P-deficient cells, not only did macroscopic observation of cultures show no difference between P-sufficient and P-deficient cultures but SEM analysis revealed similar size, shape and cell surface morphologies (Figure 7).

P-sufficient and P-deficient cells showed similar PSII activities during the first 2 weeks of incubation in P-containing and P-free medium (Figure 1). By contrast, the values for total photosynthetic activity and that of PSI were slightly different between cells growing in P-containing and P-free medium. In previous studies, P deficiency has been correlated with lowered photosynthetic rates (Smith 1983; Prasad & Kashyap 1991). Nevertheless, in our study, a great diversity and in some cases apparently contradictory results were found when quantifying the photosynthetic electron transport rates. This could arise from the fact that the values of the photosynthetic electron transport rate depend on the state of cell growth.

Given that the activities of PSI and PSII were measured using artificial electron mediators, the amount of

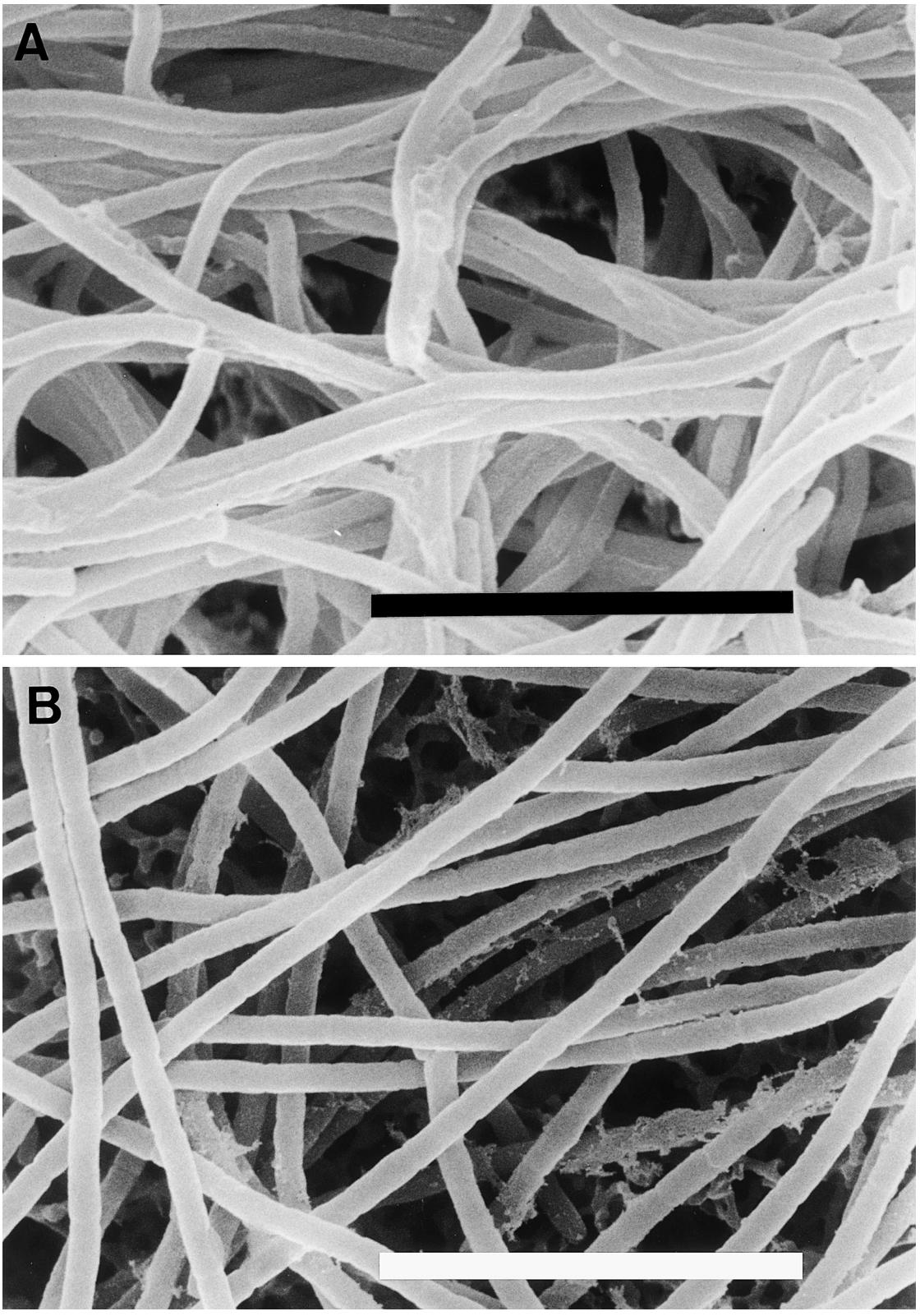

Figure 7. Scanning electron micrographs of late exponential (after 9 days incubation). (A) P-sufficient *P. laminosum* cells. (B) P-deficient *P. laminosum* cells. (Bar 10 μM in A and B.)

exopolysaccharide surrounding the cell surface, which in turn depends on the physiological and growth state of the cells, probably acted as an additional diffusion barrier to the entrance of the electron mediators into the cells and to gas-exchange (Garbisu *et al*. 1991).

From Figure 2 it can be concluded that phosphate removal is much faster in the case of P-deficient cells than P-sufficient cells. Subjecting the cells to a P-starvation period (to allow them to exhaust all their cellular P reserves) when studying P metabolism is a method frequently used to increase the rate of P uptake when the P source is replenished. Similar results, showing higher phosphate uptake rates under P-limited growth conditions, have been reported by other authors (Grillo & Gibson 1979; Thiel 1988; Wagner & Falkner 1992; Fernández-Valiente & Avendaño 1993). After approximately 7 days of P-starvation, the phosphate uptake rate progressively decreased, probably due to some degeneration of cell structures derived from a shortage of metabolic P.

The observation that P-sufficient cells excrete phosphate into the external medium (Figure 3) was not surprising since ''cytoplasmic P is readily exchangeable with external P, thereby complicating the interpretation of 'net' phosphate uptake. This is particularly true when the cells are grown in high-phosphate medium'' (Cembella *et al*. 1984). P-deficient cells did not excrete phosphate, probably because their cellular P reserves were diminished, as indicated by their total cellular P content i.e., after 9 days of starvation in P-free medium, the cellular P content of P-deficient cells was approximately five times lower than that of cells grown in the presence of phosphate as determined by ICP). However, this anomalous feature of phosphate metabolism i.e., efflux of phosphate occurring simultaneously with uptake) has never been clearly resolved (Whitton 1992).

Light was not a requirement for phosphate uptake by P-deficient cells (Figure 5). Other authors (Healey 1973; Istvánovics *et al*. 1993) also found that phosphate uptake was not strongly light-dependent in cyanobacterial cells. On the other hand, phosphate uptake by P-sufficient *P. laminosum* cells has been reported to be completely inhibited in darkness (Garbisu *et al*. 1993). Although previous publications have shown that the uptake and intracellular accumulation of phosphate is an energy-dependent process (Falkner *et al*. 1984), the source of this energy can vary and, therefore, the uptake is not necessarily directly dependent on light (Björk-Ramberg 1985).

However, in the light, phosphate flow into the cell is closely linked to photosynthetic ATP formation (Falkner *et al*. 1984) and the sensitivity of the phosphate uptake to vanadate (Figure 6) seems to suggest the involvement of a plasma membrane ATPase. Vanadate did not have a negative influence on photosynthetic O_2-evolution, indicating that the impaired phosphate incorporation was not due to a blockage of photosynthetic ATP formation. Similar results were found by Wagner & Falkner (1992) with *Anacystis nidulans* subjected to P-deficiency. On the contrary, Nalewajko *et al*. (1995) reported an at least two-fold reduction in the rate of photosynthesis in several cyanobacterial species (such as *Anabaena* sp, *Oscillatoria* sp. and *Nostoc* sp.) at 1 mM vanadate. However, it is still unclear how the photosynthetic energy is utilized for the incorporation process, especially under P-limited growth conditions (Wagner & Falkner 1992).

The effect of anaerobiosis on phosphate uptake in the dark suggests that the energy required for uptake comes from the respiratory electron transport chain since an aerobic atmosphere appears to be a requirement for dark uptake. Similar results were obtained when studying N uptake by N-starved *P. laminosum* cells (Garbisu *et al*. 1992). In cyanobacteria, the physiological function of respiration is mainly restricted to the maintenance of an adequate charge during dark periods (Scherer 1990). Although the rate of cyanobacterial respiratory electron transport is thought to be only 20% that of photosynthetic electron transport (Scherer 1990), in the case of P-deficient *P. laminosum* cells, we observed similar phosphate uptake rates in light and dark conditions under an aerobic atmosphere.

When cells were illuminated, they maintained the same uptake rates under an aerobic or anaerobic atmosphere, showing that the photosynthetically generated energy supply was sufficient to maintain the maximum uptake rate. In the light, photophosphorylated ATP and ATP generated from oxidative phosphorylation can support P transport simultaneously. In the dark, the latter source dominates but light-generated energy could also sustain uptake in the dark (Jansson 1988).

Further studies on the complex physiological roles of P, and in particular of inorganic polyphosphate (which accumulates intracellularly) to perform a multiplicity of functions in its many locations under P-limited conditions (Rao & Kornberg 1996), are required before a detailed understanding of the effects of phosphate starvation on the P metabolism of *P. laminosum* is fully realized.

Acknowledgements

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