Identification of a sheath-associated protein involved in phosphate transport in *Sphaerotilus natans*

Bryan Kaphammer* and Charles F. Kulpa Jr

Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556, USA

Summary. *Sphaerotilus natans* was shown to have a fourfold lower K'_m of phosphate transport when grown in medium containing 0.1 mM phosphate, compared to cells grown in 10.0 mM phosphate. Analysis of sheath proteins from cells grown at these two phosphate levels revealed a protein of 53 kDa present in the sheath of cells grown at a phosphate concentration of 0.1 mM. This sheath-associated, phosphate-regulated protein, designated SapP, was gel purified and used to raise a polyclonal antibody. Enzyme-linked immunosorbent assay was used to localize this protein to the surface of the sheathed cells. Phosphate uptake assays done in the presence of the antibody also showed a rise in the K'_m of phosphate transport in cells grown in 0.1 mM phosphate, indicating that this protein is involved in high-affinity phosphate transport.

Introduction

Sphaerotilus natans is a Gram-negative sheathed bacterium, characterized by the presence of a contiguous close-fitting sheath enclosing chains of rod-shape cells. The chemical composition of the sheath has been shown to be protein, carbohydrate, and lipid, and functions in the attachment of *S. natans* filaments to solid objects. Presumably this gives the organism a selective advantage in environments that contain low nutrient levels (Romano and Peloquin 1963).

The growth and physiology of *S. natans* have both economic and environmental importance in the area of waste-water treatment (Chiesa and Irvine 1985). The predominance of *S. natans* in an activated sludge environment results in poor settling characteristics of the sludge. Among the environmental conditions that can cause proliferation of this organism is low influent

phosphorus (Hattingh 1963). However, there is little information on the physiology of *S. natans* as it relates to low phosphate environments. Gaudy and Wolfe (1961) demonstrated that *S. natans* grows in a narrow range of phosphate concentrations, with 10 mM phosphate being optimal. The concentration of phosphate affects the amount of biomass, but not sheath formation.

Phosphate starvation has been shown to result in the synthesis of several proteins in *Escherichia coli* (Tomassen and Lugtenberg 1980). One of these proteins (PhoE) is an outer-membrane, anion-specific pore that facilitates the diffusion of organic and inorganic phosphate through the outer membrane (Korteland et al. 1982). This study was undertaken to examine if phosphate starvation results in synthesis of new sheath proteins, and if so, whether these proteins are involved in phosphate transport in *S. natans*.

Materials and methods

Bacterial strains, media, and growth conditions. The *S. natans* strain used was ATCC 13338. Cultures were routinely maintained on solid medium composed of 5 g/l casitone, 1 g/l glucose, 1 g/l yeast extract, and 15 g/l agar (CGY). Phosphate limited and non-limited cultures were grown in a defined medium containing 5.2 g/l HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1 g/l glucose, 0.1 g/l $MgSO_4$-$7H_2O$, 0.36 g/l NH_4Cl_2, and 0.01 g/l $CaCl_2$. The pH was adjusted to 7.2 and the medium was autoclaved. After cooling, 1 ml sterile $FeCl_3$-EDTA solution (2 g/l $FeCl_3$, 3 g/l EDTA) was added, followed by the addition of filter-sterilized vitamin B_{12} to a final concentration of 5 μg/l. This medium was designated HGM. Potassium phosphate buffer was added to 0.1 mM for phosphate-limited growth and 10.0 mM for non-limited growth. Erlenmeyer flasks (125 ml) containing 50 ml HGM at a phosphate concentration of 10 mM or 0.1 mM were inoculated with 1 ml of a turbid suspension of *S. natans* and incubated at 30° C on an orbital shaker at 150 rpm for 24 h. These cultures were used as inoculum for fresh HGM (100 ml in a 250-ml erlenmeyer flask) at phosphate concentrations of 10 mM or 0.1 mM and incubated at 30° C on an orbital shaker at 150 rpm to mid-log phase. These cultures were used in all experiments that required phosphate-limited and non-limited cells.

Bacterial growth rates. Cultures of *S. natans* were grown under phosphate-limiting and non-limiting conditions and 1-ml samples

* *Present address:* 1301 Catherine Medical Science II 5605, University of Michigan, Ann Arbor, MI 48109, USA

Offprint requests to: C. F. Kulpa Jr

were collected at various times. The samples were washed and resuspended in sterile distilled water. Each sample was assayed for total protein by the Lowry method (Lowry et al. 1951). Bovine serum albumin (BSA) was used as a standard.

Sheath isolation and protein analysis. Sheath material was isolated by modifying the method of Romano and Peloquin (1963). Modifications included incubating the cultures at 30° C in the lysozyme-EDTA buffer instead of at 37° C, and then pelleting the sheath by ultracentrifugation at 40 000 g for 30 min. The sheath pellet was washed three times with 30 mM TRIS-HCl, pH 7.0.

Proteins were extracted from the sheath by resuspending purified sheath from a 1.5-l culture in 10 ml extraction buffer, which consisted of 6.3 mM TRIS-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol. The sheath proteins were solubilized by repeated passage through an 18-gauge hypodermic needle followed by passage through a 23-gauge needle. This sheath suspension was subjected to ultracentrifugation at 40 000 g for 30 min. The supernatant, which contained the extracted sheath proteins, was saved and 10 ml extraction buffer was added to the pellet. The pellet was resuspended by passage through an 18-gauge hypodermic needle followed by passage through a 23-gauge needle, and then subjected to ultracentrifugation at 40 000 g for 30 min. The supernatant was saved and 10 ml of extraction buffer was used to resuspend the pellet by passage through the same needles followed by ultracentrifugation at 40 000 g for 30 min. The supernatant from this extraction was combined with the other supernatants and the proteins were precipitated by adding 150 ml ice-cold acetone. This acetone mixture was aliquoted to eight 30-ml Corex tubes, which were then incubated for 30 min at −70° C. The precipitated proteins were pelleted by centrifugation at 12 000 g for 15 min. Supernatants were discarded and 2 ml buffer containing 2% SDS in 6.2 mM TRIS-HCl, pH 6.8, was added to each pellet and the tubes were boiled for 10 min to solubilize the proteins.

Sheath proteins from cells grown in HGM with 0.1 mM and 10 mM phosphate were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970), with 10% polyacrylamide gel.

Gel purification of SapP. Preparation SDS-PAGE was performed on sheath proteins of *S. natans* grown in HGM with 0.1 mM phosphate. The proteins in the gel were visualized by the KCl method of Hagar and Burgess (1980). The protein band corresponding to SapP was cut out of the gel and electroeluted. The eluted protein was precipitated by the acetone precipitation method described by Hagar and Burgess (1980).

Immunization procedure. Two-hundred micrograms of gel-purified SapP in 250 μl normal saline was emulsified with 250 μl Freund's Complete Adjuvant. The emulsion was injected into a young adult female New Zealand White rabbit both intramuscularly (400 μl) and intraperitoneally (100 μl). On days 14, 21, 28, and 35 the rabbit was injected intramuscularly with 100 μg gel-purified SapP in 100 μl normal saline, emulsified with 100 μl Incomplete Freund's Adjuvant. On day 42, 100 μg gel-purified SapP was injected intramuscularly and intraperitoneally. On day 49, the serum was collected.

Immunoblotting procedure. Immunoblotting of SDS-PAGE gels was done by the method of Towbin et al. (1979). The nitrocellulose, which contained the sheath proteins, was incubated for 1 h at 37° C in 20 mM TRIS-HCl, pH 7.5, 500 mM NaCl (TBS) to which gelatin had been added to 2%. The TBS-2% gelatin was discarded and anti-SapP antiserum was added at a concentration of 1% in TBS-2% gelatin. The blot was allowed to incubate for 90 min at room temperature followed by five washes of 6 min each in TBS containing 0.05% Tween 20. After the last wash was discarded goat-anti-rabbit alkaline phosphatase at a concentration of 0.5% in TBS-2% gelatin was added and the blot was allowed to incubate at room temperature for 90 min followed by five washes of 6 min each in TBS containing 0.05% Tween 20.

Goat-anti-rabbit alkaline phosphatase bound to the blot was detected by the nitro blue tetrazolium, 5-bromo-4-chloro-indolyl-phosphate method of Blake et al. (1984).

Enzyme-linked immunosorbant assay (ELISA). Whole cell ELISA was performed by placing 50 μl of an *S. natans* cell suspension, which had been grown in HGM with 0.1 mM or 10 mM phosphate, washed twice with sterile distilled water, and resuspended in buffer consisting of 0.1 M ammonium acetate, 0.1 M ammonium carbonate, pH 8.2, to a concentration of 30 μg/ml total cellular protein, into each well of a microtiter plate. Plates were incubated at 37° C until dry. ELISA buffer (50 μl), consisting of 10 mM Na$_2$HPO$_4$, pH 7.2, 1% BSA, 0.5% Tween 20, and 0.02% sodium azide, was added and the plates were incubated for 30 min at room temperature. The ELISA buffer was removed and 50 μl anti-SapP antiserum or pre-immune serum diluted in ELISA buffer to 1:1, 1:10, 1:100, 1:200, 1:300, 1:600, or 1:1000 was added. Plates were incubated for 1 h at room temperature, washed five times with ELISA buffer followed by the addition of 50 μl of a 1:1000 dilution of goat-anti-rabbit alkaline phosphatase in ELISA buffer to each well. Plates were incubated for 1 h at room temperature, washed five times with ELISA buffer, and then 50 μl of a 1 mg/ml solution of *p*-nitrophenyl-phoshate in substrate buffer (9.7% diethanolamine, 0.1 g/l MgCl$_2$·6H$_2$O, 0.2 g/l sodium azide, pH 9.8) was added to each well. After incubation for 30 min the reaction was stopped by the addition of 25 μl of 1 M NaOH. The optical density of each well was read at a wavelength of 405 nm.

Phosphate uptake kinetics. Cultures (100 ml) of *S. natans* were grown under phosphate-limited and non-limited conditions to mid-log phase, harvested, washed twice with uptake buffer (5.2 g/l HEPES, pH 7.2, 1 g/l glucose, 0.1 g/l MgSO$_4$·7H$_2$O, 0.36 g/l NH$_4$Cl$_2$, 0.01 g/l CaCl$_2$) and finally resuspended in 40 ml of uptake buffer. Uptake was initiated by adding 200 μl radiolabeled ^{32}P (1.0 Ci/mmole) to 1.8 ml cells. Samples (100 μl) were removed every 10 s and placed on 0.45-μm Gelman Merricel (Fisher Scientific, Co., Itasca, IL) filters, which had been soaked for 24 h in 0.01 M phosphate buffer, pH 7.0. Each filter was immediately washed with 6 ml uptake buffer and air dried. Radioactivity was detected by placing filters in 10 ml scintillation fluid (53 ml 2,5-diphenyloxazole(PPO)-phenyloxazoylphenyloxazolylphenyl (PO-POP) concentrate/l toluene, obtained from National Diagnostics (Highland Park, N. J., USA) and counted in a Beckman (Palo Alto, Calif.) LS 7000 scintillation counter. Background was determined by performing the assays without cells.

Phosphate uptake assays were also performed in the presence of anti-SapP antibody or pre-immune serum at a dilution of 1:10 in uptake buffer. Cells were incubated with the antibody solutions for 30 min and then assayed for phosphate uptake as before.

Results

Phosphate-limited and non-limited growth

A defined medium was designed (HGM), which contained all the necessary nutrients with the exception of phosphate. *S. natans* was grown in HGM with either 0.1 mM or 10 mM phosphate as described in Materials and methods. Growth curves were constructed and generation times were calculated to be 4.4 h for cells grown in 0.1 mM phosphate and 3.8 h for cells grown in 10 mM phosphate. The final cell mass, after 72 h incubation was 75 μg/ml for the 0.1 mM phosphate culture and 126 μg/ml for the 10 mM phosphate culture. These data suggest that *S. natans* grown in HGM with 0.1 mM phosphate had its growth limited due to the amount of phosphate in the medium.

Table 1. Phosphate uptake kinetics of *Sphaerotilus natans*

Phosphate concentration (mM)[a]	Antibody[b]	K_m' (μM)	V_{max} (nmol/s/mg dry weight)
10.0	None	16.90 ± 0.2	0.14 ± 0.01
0.1	None	4.46 ± 0.7	0.19 ± 0.02
10.0	AS	16.60 ± 3.9	0.10 ± 0.02
0.1	AS	22.60 ± 3.4	0.28 ± 0.06
10.0	PI	14.60 ± 2.8	0.08 ± 0.04
0.1	PI	3.56 ± 1.1	0.12 ± 0.03

[a] Phosphate concentration (mM) in the growth media
[b] Cells were incubated with anti-sheath-associated, phosphate-regulated protein (AS) or pre-immune (PI) antisera before phosphate uptake assays as described in Materials and methods

Phosphate uptake kinetics

Phosphate uptake experiments were performed to examine differences in phosphate transport kinetics in *S. natans* grown under phosphate-limiting and non-limiting conditions. Experiments were run at six phosphate concentrations ranging from 0.7 μM to 4.0 μM. Data were corrected for background and rates of phosphate transport were calculated. There was no significant difference in the V_{max} between the two populations (Table 1). However, there was a fourfold K_m' of transport effect (Table 1) indicating that there is a difference in phosphate permeability between *S. natans* grown at 10 mM or 0.1 mM phosphate (Nikaido 1979).

Identification of SapP

Analysis of sheath proteins by SDS-PAGE was complicated by the fact that these proteins are associated with the carbohydrates of the sheath. An extraction procedure was developed allowing easy analysis of the sheath proteins. The results of SDS-PAGE of these sheath proteins are shown in Fig. 1, lanes B and C. This analysis shows that there is a 53 kD phosphate-regulated, sheath-associated protein in the sheath of cells grown in 0.1 mM phosphate, but not in the sheath of cells grown in 10 mM phosphate. This 53 kDa protein was designated SapP.

Localization of SapP

The protein band corresponding to SapP was visualized, cut out of the preparative polyacrylamide gel, eluted and a polyclonal antiserum was raised to this denatured protein. The anti-SapP serum was analyzed for its specificity to SapP by using it as the primary antibody in immunoblots of sheath proteins from *S. natans* grown under phosphate-limited and non-limited conditions (Fig. 1, lanes D and E). These results demonstrate that the anti-SapP antiserum is specific for SapP, and the SapP is only found in sheath prepara-
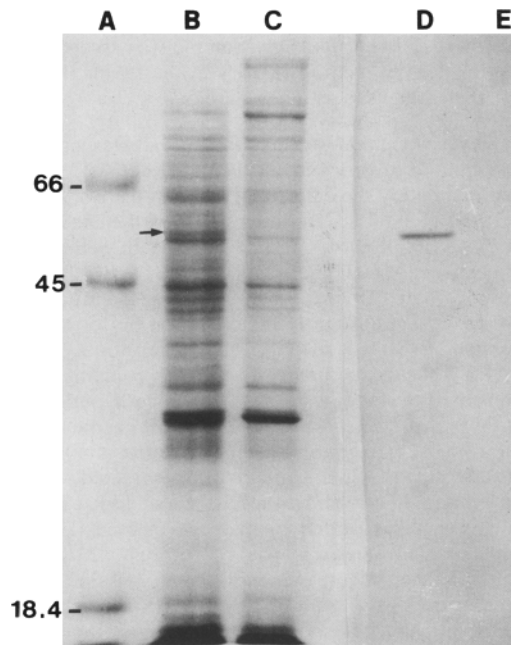
Fig.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot of *Sphaerotilus natans* sheath proteins using anti-sheath-associated, phosphate regulated protein (SapP) antiserum. *Lanes A, B,* and *C* are SDS-PAGE stained with Coomasie blue: *A,* molecular weight standards in kilodaltons; *B,* sheath proteins from cells grown in HGM (see Materials and methods) with 0.1 mM phosphate; *C,* sheath proteins from cells grown in HGM with 10 mM phosphate; *Lane D,* immunoblot of lane B; *Lane E:* immunoblot of lane C

tions of cells grown under phosphate-limited conditions.

Since SapP is a sheath-associated protein and the sheath is the outermost layer, the question was asked whether SapP could be detected on the surface of whole cells. An ELISA assay was done using *S. natans* grown under phosphate-limited and non-limited conditions. The results in Fig. 2 show that SapP can be detected on the surface of cells grown under phosphate-limited conditions. There was some reaction with cells grown under non-limited conditions which could indicate a basal level of SapP expression.

Phosphate transport in the presence of anti-SapP

SapP has been demonstrated to be a phosphate-regulated, sheath-associated protein. However, the question remains whether SapP is directly involved in phosphate transport. If so, it may be possible to hinder SapP by blocking its active site with antibody. Phosphate uptake experiments were performed in the presence of anti-SapP antiserum, and parallel experiments were also done using the same concentration of pre-immune serum as a control.

S. natans grown under phosphate-limited conditions and assayed in the presence of anti-SapP showed a fivefold higher K_m' than the same cells assayed in the presence of pre-immune serum (Table 1). Cells grown
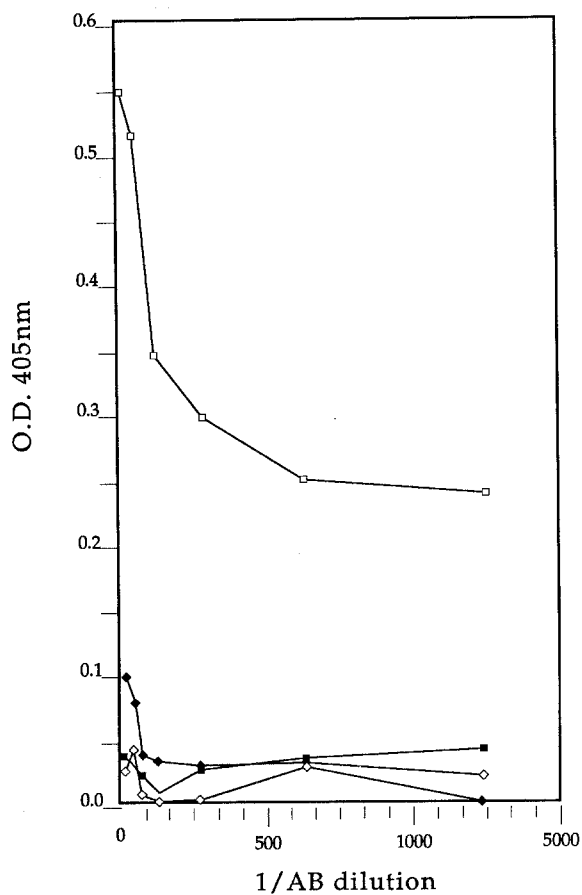
Fig. 2. Enzyme-linked immunosorbent assay (ELISA) of *S. natans* whole cells: □, cells grown in HGM with 0.1 mM phosphate and assayed with anti-SapP; ◆, cells grown in HGM with 10 mM phosphate and assayed with anti-SapP; ■, cells grown in HGM with 0.1 mM phosphate and assayed with pre-immune serum; ◇, cells grown in HGM with 10 mM phosphate and assayed with pre-immune serum

under non-limiting conditions showed no difference in the K'_m when assayed in the presence of either anti-SapP or pre-immune serum (Table 1). The V_{max} of both populations was only slightly affected by the presence of the anti-SapP antibody.

The fivefold K'_m effect seen in the phosphate-limited cells indicates that anti-SapP is specifically interfering with phosphate transport. Since the anti-SapP antiserum has been shown to be specific for the SapP protein it can be concluded that SapP is involved in phosphate transport.

Discussion

Phosphate transport was studied as a model of the role of the sheath in the physiology of *S. natans*. The available data indicate that *S. natans* could become predominant in a waste-water environment that was limited in phosphate (Hattingh 1963). These data suggested that *S. natans* has a higher affinity for phosphate relative to the other organisms in the environment.

The results of phosphate transport assays suggest that there is an inducible phosphate transport system in *S. natans*. The fourfold K'_m effect between cells grown under phosphate-limited and non-limited conditions suggests that there is a change in the permeability of the outer envelope of the cell (the outer envelope being the outer membrane and sheath). This type of K'_m effect has been demonstrated in several *E. coli* outer membrane proteins mutants (Korteland et al. 1982; Nikaido 1979).

This is the first report of a sheath protein that is involved in phosphate transport and raises questions concerning the mechanism of action of SapP. The sheath proteins could be outer membrane proteins that are associated with the carbohydrates of the sheath. The association of Gram-negative outer-membrane proteins with the carbohydrate structure of the cell wall has been documented (Tokunaga et al. 1979). The ELISA results demonstrate that SapP is exposed on the surface of the sheath, and the phosphate transport experiments done in the presence of anti-SapP antibody demonstrate that SapP has a role in high-affinity phosphate transport in *S. natans*. The nature of this role can only be speculated. SapP could aid in phosphate uptake by directing phosphate to specific membrane proteins involved in transport. This could be accomplished in several ways. If SapP were a phosphate-binding protein it could sequester phosphate and make it available to the membrane transport systems. SapP could also interact with outer-membrane pore proteins, thereby effectively increasing the surface area of the membrane and thus the velocity of diffusion.

The fact that anti-SapP antibody interferes with phosphate transport suggests that it is a phosphate-binding protein. Attempts to block transport of cefsulodin (a *beta*-lactam antibiotic) through the *E. coli* anion specific pore protein, PhoE, using both monoclonal and polyclonal antisera were not successful (van der Ley et al. 1985), suggesting that important recognition sites are not located on the outer surface of this pore protein. If SapP were a pore protein, it could be speculated that its important recognition sites would also be unavailable to antibody and thus anti-SapP would not have affected phosphate transport.

The sheath structure itself would be a good location for binding proteins, allowing *S. natans* to scavenge the available nutrients from the environment, making them unavailable for the other members of the community. This could be an explanation for *S. natans* becoming predominant in low phosphate environments.

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