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Characterization of Two Phosphate Transport Systems in *Acinetobacter johnsonii* 210A

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The transport of P_i was characterized in *Acinetobacter johnsonii* 210A, which is able to accumulate an excessive amount of phosphate as polyphosphate (polyP) under aerobic conditions. P_i is taken up against a concentration gradient by energy-dependent, carrier-mediated processes. *A. johnsonii* 210A, grown under P_i limitation, contains two uptake systems with K_t values of 0.7 ± 0.2 μM and 9 ± 1 μM. P_i uptake via the high-affinity component is drastically reduced by *N,N'*-dicyclohexylcarbodiimide, an inhibitor of H^+-ATPase, and by osmotic shock. Together with the presence of P_i-binding activity in concentrated periplasmic protein fractions, these results suggest that the high-affinity transport system belongs to the group of ATP-driven, binding-protein-dependent transport systems. Induction of this transport system upon transfer of cells grown in the presence of excess P_i to P_i-free medium results in a 6- to 10-fold stimulation of the P_i uptake rate. The constitutive low-affinity uptake system for P_i is inhibited by uncouplers and can mediate counterflow of P_i, indicating its reversible, secondary nature. The presence of an inducible high-affinity uptake system for P_i and the ability to decrease the free internal P_i pool by forming polyP enable *A. johnsonii* 210A to reduce the P_i concentration in the aerobic environment to micromolar levels. Under anaerobic conditions, polyP is degraded again and P_i is released via the low-affinity secondary transport system.

Enhanced biological phosphorus removal from domestic wastewaters in full-scale activated-sludge plants is currently perceived to hinge on the provision of alternate stages in which the activated sludge is subjected to anaerobic and aerobic conditions (40). A characteristic feature of such plants is that P_i, after being released from the biomass in an anaerobic stage, is reincorporated in the biomass during aeration, together with part or all of the influent P_i (16). Significant numbers of polyphosphate (polyP)-accumulating bacteria, especially from the gram-negative genus *Acinetobacter*, have been isolated from activated sludge in which biological phosphorus removal has been observed (14, 29). Similar to activated sludge, polyP-accumulating *Acinetobacter* spp. take up P_i under aerobic conditions and release it anaerobically (10).

One of these strains, *Acinetobacter johnsonii* 210A, is able to accumulate up to 300 mg of P_i per g (dry weight). The extent of P_i accumulation depends on growth rate, carbon and energy source, limiting nutrients, and temperature (46). When oxidative phosphorylation is impaired (e.g., in the absence of oxygen or an electron donor), polyP is degraded and P_i is released into the medium (43). Two enzymes are involved in the degradation of polyP: (i) polyphosphatase and (ii) polyP:AMP phosphotransferase (44). The latter enzyme has been characterized recently (8). In combination with adenylate kinase, this enzyme enables the organism to conserve the energy from the phosphate bonds in polyP and to use the accumulated polymer as a source of ATP when energy cannot be obtained otherwise (45).

The possible role of *Acinetobacter* spp. in the enhanced biological phosphorus removal from domestic wastewaters raised questions about the involvement of P_i transport systems in the uptake and release of P_i in *A. johnsonii* 210A. The nature and properties of P_i transport systems have been investigated in other bacterial strains, including *Escherichia coli* (36, 37, 49), *Pseudomonas aeruginosa*, (24, 30), *Lactococcus lactis* (31), and *Micrococcus lysodeikticus* (12). In *E. coli* and *P. aeruginosa*, two major P_i transport systems with low and high affinity for P_i are present (36).

In the work reported here, the presence of two P_i transport systems in *A. johnsonii* 210A is demonstrated. One system is an inducible, ATP-dependent, binding-protein-dependent permease enabling the organism to reduce the P_i concentration in the aerobic environment to micromolar concentrations. The other system is a constitutive, reversible secondary transport system which mediates the efflux of P_i under anaerobic conditions.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DNP, α-dinitrophenol; Δp, proton motive force; ΔpH, transmembrane proton gradient; Δψ, membrane potential; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PQQ, 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]-quinoline-2,7,9-tricarboxylic acid; TCA, trichloroacetic acid; and TPP^+, tetraphenylphosphonium ion.

Organism and culture conditions. *A. johnsonii* 210A was grown aerobically at 30°C in a buffered medium (pH 7.2) containing 20 mM Na-butyrate, 20 mM NH_4Cl, 5 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2 \cdot 2H_2O$, 10 mM KCl, 2 ml of

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trace element solution per liter, and 50 mM Tris-HCl. The composition of the trace element solution has been described by Van Groenestijn et al. (45). Sterile NaP_i, pH 7.2, was added to a final concentration of 5 mM (high-P_i medium) or 20 μM (low-P_i medium) for cultivation of high-P_i-grown and low-P_i-grown cells, respectively. *E. coli* K-12 was grown under P_i limitation in a minimal medium with glucose and 0.1% (wt/vol) yeast extract as the phosphorus source (17). For growth in medium with excess P_i, 5 mM NaP_i buffer was added. Cells at the logarithmic phase were harvested by centrifugation (7,000 × *g*, 10 min). The pellet was washed and resuspended as indicated below.

Transport assays. Cells, washed and resuspended in 20 mM (K)PIPES (pH 7.0) containing 10 mM $MgSO_4$ and 50 μg of chloramphenicol per ml, were stored on ice and used within 2 h. Transport assays were performed at 30°C. Cells were diluted in 100 μl of air-saturated buffer to about 0.5 mg of protein per ml. The suspension was kept aerobic by flushing with water-saturated air. Cells were preincubated for 3 min with 2 μM PQQ, after which 20 mM glucose was added. Two minutes later uptake was started by the addition of ^{32}P-labeled KP_i (0.33 to 1.47 TBq/mol) or ^{14}C-labeled L-lysine at concentrations as specified in the figure legends. At given time intervals, 2 ml of ice-cold 0.1 M LiCl was added and the samples were filtered immediately through cellulose-nitrate filters. Filters were washed once with 2 ml of 0.1 M LiCl. The radioactivity on the filters was measured with a liquid scintillation counter. To remove contaminating P_i, the glassware used for $^{32}P_i$ transport assays was kept in chromic acid and rinsed 15 times with distilled water before use. ^{32}P-labeled KP_i was filtered through a 0.45-μm-pore-size cellulose-nitrate filter prior to use, in order to remove $^{32}P_i$ adsorbed to particles (27).

EDTA treatment of intact cells. To permeabilize the outer membrane, cells were washed three times with 20 mM (K)PIPES (pH 7.0) containing 50 μg of chloramphenicol per ml and suspended in this buffer at an A_{660} of about 30. After 3 min of preincubation of the cell suspension at 30°C, 1 mM sodium EDTA (pH 7.0) was added. $MgSO_4$ was added 10 min later to a final concentration of 10 mM. Cells were washed once with 20 mM (K)PIPES (pH 7.0) containing 10 mM $MgSO_4$ and 50 μg of chlorampenicol per ml, stored on ice, and used within 2 h.

Determination of Δp. The Δψ (interior negative) was determined from the distribution of TPP^+, using a TPP^+-selective electrode (39). The standard assay was done at 30°C with EDTA-treated cells in 1 ml of oxygen-saturated buffer [50 mM (K)PIPES, 10 mM $MgSO_4$, 50 μg of chloramphenicol per ml, pH 7.0] in the presence of 4 μM TPP^+. Cells were supplied with metabolic energy by glucose oxidation as described in "Transport assays." Measurements were corrected for nonspecific probe binding to the cells (25). The pH gradient across the membrane was calculated from the increase in Δψ upon the addition of nigericin, assuming a complete interconversion of the ΔpH into the Δψ (11).

Osmotic shock, preparation of periplasmic protein fractions, and P_i-binding experiments. Cells were exposed to an osmotic shock procedure, essentially as described by Neu and Heppel (28). Cells were washed three times with 20 mM (K)PIPES (pH 7.5) containing 50 μg chloramphenicol per ml and suspended to an A_{660} of about 5.0 in 20 mM (K)PIPES (pH 7.5) containing 0.75 M sucrose and 1 mM EDTA. This cell suspension was incubated for 10 min at 20°C to induce plasmolysis. Cells were collected by centrifugation (7,000 × *g*, 20 min). Periplasmic proteins were released upon resuspension of the cell pellet in 50 volumes of 0.1 mM $MgSO_4$ at 20°C. The osmotic shock-treated cells were collected by centrifugation and used for transport assays. The supernatant (shock fluid) was concentrated 20-fold via ultrafiltration under N_2 pressure as described by Abee et al. (1) and dialyzed extensively at 4°C against 20 mM (K)PIPES (pH 7.5). Binding of $^{32}P_i$ to concentrated shock fluid was measured according to the method of Richarme and Kepes (35), by the addition of 10 μM $^{32}P_i$ (2.81 TBq/mol) to 0.5 ml of shock fluid (0.3 mg of protein per ml). After incubation for 10 min at 20°C, proteins were precipitated by the addition of 4 ml of an ice-cold saturated ammonium sulfate solution. The mixture was immediately passed through a nitrocellulose filter, which was washed twice with 2 ml of ice-cold saturated ammonium sulfate. The radioactivity retained on the filter was measured by scintillation counting. In control experiments, binding of $^{32}P_i$ to the filter and to bovine serum albumin was determined.

Counterflow of $^{32}P_i$ and ^{14}C-lysine. Intact cells, grown in high-P_i medium, were deenergized by incubation for 12 h at 30°C in 20 mM (K)PIPES (pH 7.0) containing 10 mM $MgSO_4$, 50 μg of chloramphenicol per ml, and 2.5 mM DNP. Cells were washed twice with 20 mM (K)PIPES (pH 7.0) containing 30 mM sodium azide and 50 μg of chloramphenicol per ml and suspended in this buffer to an A_{660} of about 30. After the addition of 1 mM sodium EDTA, the cell suspension was incubated at 30°C for 10 min. Subsequently, the cells were washed and resuspended to a concentration of 0.5 mg of protein per ml in loading buffer (pH 7.5) containing 100 mM (K)PIPES, 10 mM $MgSO_4$, 30 mM sodium azide, 50 μg of chloramphenicol per ml, and 20 μM CCCP. Cells were loaded for 3 h at 20°C with 10 mM KP_i or 3 mM L-lysine. Control cells were incubated in a buffer without KP_i or L-lysine. Cells were concentrated to 20 mg of protein per ml and diluted 200-fold in 400 μl of loading buffer containing a final concentration of 10 μM $K^{32}P_i$ (3.91 TBq/mol) or 16 μM L-[U-^{14}C]lysine (1.26 TBq/mol). The uptake of the radiolabeled substrates was monitored in time at 30°C as described in "Transport assays."

Analytical procedures. Intracellular ATP concentrations were determined by the luciferin-luciferase method of Jetten et al. (22). The assay of alkaline phosphatase activity in concentrated shock fluid with *p*-nitrophenylphosphate as the substrate was carried out according to the method of Yashphe et al. (50). The respiration rate was measured with a Clark-type oxygen electrode. Protein was determined by the method of Lowry et al. (26) with bovine serum albumin as a standard. Soluble phosphates were extracted from cells by incubation in cold 5% (wt/vol) TCA for 20 min according to the method of Hellingwerf et al. (19). P_i in the cells was assayed according to the method of Avron (5) as a phosphomolybdate complex extracted into an organic phase of isobutanol-benzene.

Materials. Cellulose-nitrate filters (0.45-μm pore size) were supplied by Millipore, Etten-Leur, The Netherlands, and Schleicher und Schuell, Dassel, Germany. $^{32}P_i$ (carrier free) and L-[U-^{14}C]lysine (11.5 TBq/mol) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, United Kingdom. The ATP bioluminescence constant light signal kit was from Boeringer Mannheim. Other chemicals were reagent grade and obtained from commercial sources.

RESULTS

Active P_i transport. (i) Energy-dependent uptake of P_i. Washed cells of *A. johnsonii* 210A, grown in high-P_i medium with butyrate as the carbon and energy source, took up P_i in

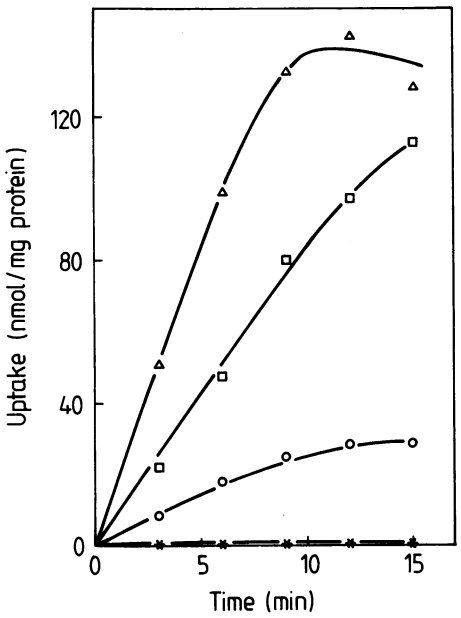

FIG. 1. Uptake of 100 μM P_i by washed cells of *A. johnsonii* 210A, high-P_i-grown in the absence (○) and presence of 20 mM butyrate (□) or 20 mM glucose–2 μM PQQ (△) or by cells treated with 1% chloroform (*).

the absence of an exogenous energy supply (Fig. 1). Initial experiments revealed the presence of a membrane-bound glucose dehydrogenase in *A. johnsonii* 210A, like in *Acinetobacter lwoffi* (47), which requires PQQ for activity (48). Although *A. johnsonii* 210A cannot grow on glucose with or without PQQ or on gluconate, the addition to washed cells of 20 mM glucose plus 2 μM PQQ resulted in an increase of the respiration rate from 41 to 145 nmol of O_2 per min per mg of protein and a stimulation of the Δp from −90 to −152 mV (inside negative and alkaline). In the presence of 20 mM butyrate, the oxidation rate was 83 nmol of O_2 per min per mg of protein and the Δp was −115 mV (inside negative and alkaline). The rate of P_i uptake was stimulated in proportion with the respiration rate and the Δp (Fig. 1).

The uptake of P_i was severely impaired by preincubation of the cells with the respiratory chain inhibitor CN^-, the uncoupler DNP or CCCP, or the H^+-ATPase inhibitor DCCD (Table 1). A total collapse of the Δp in EDTA-treated cells after the addition of a combination of the ionophores nigericin and valinomycin in the presence of glucose-PQQ resulted in a complete abolition of the uptake of P_i (Table 1). The EDTA treatment itself had no effect on the uptake of P_i and was given to facilitate the incorporation of the ionophores into the cytoplasmic membrane of this bacterium. When the cell membranes were permeabilized by a treatment with chloroform, no P_i was taken up (Fig. 1).

These results indicate that the uptake of P_i in *A. johnsonii* 210A is an energy-dependent process. Because of the strong stimulation of the P_i uptake rate in the presence of glucose-PQQ, this substrate was used for energization of cells in further experiments.

(ii) Fate of intracellular P_i. Soluble phosphates were extracted from high-P_i-grown cells of *A. johnsonii* 210A as described in Materials and Methods. The total amount of P_i entering energized cells increased approximately linearly in time for at least 10 min. Most of this P_i was incorporated rapidly into cold TCA-soluble organic phosphates and into TCA-insoluble phosphates. A minor fraction remained in the cells as P_i. Assuming an internal volume of these cells of 3 μl/mg of protein (7), an internal P_i concentration of 3 mM can be calculated, implying a 40-fold accumulation of P_i under these conditions.

TABLE 1. Effect of inhibitors on P_i uptake in *A. johnsonii* 210A[a]

Inhibitor(s)[b]	mM	Uptake (%)
None (control)		100
CN^-	0.5	8
	1.0	5
DNP	0.5	54
	1.0	24
CCCP	0.02	32
DCCD	0.005	30
Valinomycin + nigericin[c]		2

[a] Cells grown in high-P_i medium were washed, treated with 1 mM EDTA, and diluted to a protein concentration of 0.3 mg/ml, as described in Materials and Methods. After addition of 20 mM glucose and 2 μM PQQ, the initial rate of P_i uptake over the first 60 s was determined at a P_i concentration of 100 μM. Cells incubated without inhibitors (control) took up P_i at a rate of 18 nmol/min/g of cell protein (100%).

[b] Inhibitors were added 5 min prior to the uptake experiments, except for DCCD, which was added 45 min prior to the uptake experiment.

[c] Valinomycin and nigericin were added to final concentrations of 1.5 and 0.15 μM, respectively.

Effect of P_i deprivation on P_i uptake and accumulation. The rate of uptake of P_i in cells grown in high-P_i medium was 15 to 25 nmol of P_i per min per mg of protein. After transfer of these cells into a P_i-free medium, the rate of P_i uptake gradually increased to as much as 100 to 150 nmol of P_i per min per mg of protein during incubation for 4 h. This stimulation was inhibited in the presence of chloramphenicol. Since chloramphenicol had no effect on the P_i uptake rate itself, it is concluded that the stimulation reflects de novo protein synthesis. Washed cells grown in low-P_i medium took up P_i at a rate of 100 to 150 nmol of P_i per min per mg of protein. This rate was hardly stimulated by the addition of an exogenous source of energy. The high endogenous respiration rate of these cells (358 nmol of O_2 per min per mg of protein) indicates the presence of an internal energy reserve. Recently it has been reported that *A. johnsonii* 210A cells form poly-β-hydroxybutyrate when grown at a low P_i concentration (9). Most likely, the oxidation of this polymer supplies the energy for the uptake of P_i.

An intensive accumulation of polyP, known as the overplus phenomenon (18) or as polyP supersynthesis (23), has been observed in several microorganisms after the addition of P_i to phosphorus-starved cells. The fate of internalized P_i under these conditions has not yet been investigated. Low-P_i-grown cells of *A. johnsonii*, which took up P_i at a rate of 120 nmol/min/mg of protein, maintained an internal concentration of free P_i of 1.5 mM, comparable to internal concentrations found in high-P_i-grown cells. However, within 2 min of uptake a considerable amount of P_i (more than 85%, versus 15% in high-P_i-grown cells) was found in the TCA-insoluble fraction (data not shown). In *M. lysodeikticus* this fraction was shown to be composed mainly of polyphosphate (12, 13). Although most of the P_i taken up by the low-P_i-grown cells was subsequently metabolized, a maximal concentration gradient of 150 was obtained at an external P_i concentration of 10 μM, showing that P_i was taken up against a concentration gradient.

Kinetic parameters of P_i uptake. The uptake of P_i in high-

TABLE 2. Kinetic parameters of P_i uptake in *A. johnsonii* 210A[a]

Cells	Low-affinity uptake		High-affinity uptake	
	K_t (μM P_i)	V_{max} (nmol of P_i/min/mg of protein)	K_t (μM P_i)	V_{max} (nmol of P_i/min/mg of protein)
High P_i grown	41 ± 7	15 ± 3	4 ± 1	12 ± 4
Osmotic shock treated	31 ± 6	10 ± 3	—[b]	—
DCCD treated	34 ± 7	5 ± 2	—	—
Low P_i grown	9 ± 1	15 ± 5	0.7 ± 0.2	80–120[c]

[a] The kinetics of P_i uptake was analyzed in energized high-P_i-grown control cells, shocked cells, cells treated with 15 nmol of DCCD per mg of protein, and low-P_i-grown cells, using Lineweaver-Burk and Eadie-Hofstee plots. Initial velocities in high- and low-P_i-grown cells were determined over the first 20 and 8 s, respectively, at a P_i concentration between 0.025 and 500 μM. Values are means from four separate experiments.

[b] —, high-affinity P_i uptake system could not be detected under these conditions.

[c] V_{max} was dependent on growth conditions.

and low-P_i-grown cells, energized by glucose-PQQ, was linear for at least 60 and 8 s, respectively, in the range of 0.025 to 500 μM P_i. The kinetic parameters of this uptake, K_t and V_{max}, were determined via linear regression analysis of Lineweaver-Burk and Eadie-Hofstee plots. The results show the presence of two transport systems in high- and low-P_i-grown cells (Table 2).

Presence of a binding-protein-dependent P_i transport system. (i) Effect of DCCD. In order to examine the presence of an ATP-driven P_i transport system, the effect of the H^+-ATPase inhibitor DCCD on the internal ATP concentration, the Δp, and P_i uptake was studied in EDTA-treated, high-P_i-grown cells. In a control experiment, the internal ATP concentration increased from 0.5 to 2.8 mM within 2 min after glucose-PQQ addition to washed cells. In a parallel experiment in which the cells were preincubated with 15 nmol of DCCD per mg of protein, a decrease in the ATP concentration was observed, from 2.8 to 0.4 mM. The ΔpH remained constant at −10 mV (inside alkaline) in DCCD-treated cells, whereas the $\Delta\psi$ was stimulated from −139 to −180 mV (inside negative). Since the oxidation of glucose-PQQ results primarily in the generation of a Δp, which is partly used for ATP synthesis by the H^+-ATPase, the higher Δp and the lower internal ATP concentration in DCCD-treated cells are indications for the inhibition of H^+-ATPase. Uptake of L-lysine, which is mediated by a secondary transport system in this organism (48), was not affected by up to 30 nmol of DCCD per mg of protein. P_i uptake was strongly inhibited by 15 nmol of DCCD per mg of protein (Table 1). Only one component, resembling the low-affinity transport system kinetically, could be demonstrated in DCCD-treated cells (Table 2). This result indicates that phosphate bond energy is required for the energization of the high-affinity P_i uptake system.

(ii) Effect of osmotic shock and binding of P_i to concentrated shock fluid. ATP plays a role in the energization of periplasmic binding-protein-dependent transport systems in gram-negative bacteria (2). These transport systems are called osmotic shock sensitive because of the loss of the periplasmic binding proteins into the medium by osmotic shock (15). P_i uptake in cells of *A. johnsonii* 210A grown in high-P_i (Fig. 2A) and in low-P_i medium (Fig. 2B) was inhibited by an osmotic shock by approximately 45 and 80%, respectively. Shocked cells maintained an intact cytoplasmic membrane and a constant internal pH, as was shown in high-P_i-grown cells in which the respiration rate, the $\Delta\psi$, and the ΔpH remained constant at 108 nmol of O_2 per min per mg of protein, −100 mV (inside negative), and −12 mV (inside alkaline), respectively, before and after osmotic shock. This conclusion is consistent with the observed insensitivity of the uptake of L-lysine to the shock procedure in high-P_i-grown cells (Fig. 2C). Kinetic analysis of P_i uptake in shocked cells strongly suggests a specific inhibition of the high-affinity P_i uptake system by osmotic shock (Table 2).

To investigate whether a P_i-binding protein was released by osmotic shock, P_i-binding experiments were performed with concentrated periplasmic protein fractions of high-P_i-grown and low-P_i-grown cells. Alkaline phosphatase, which

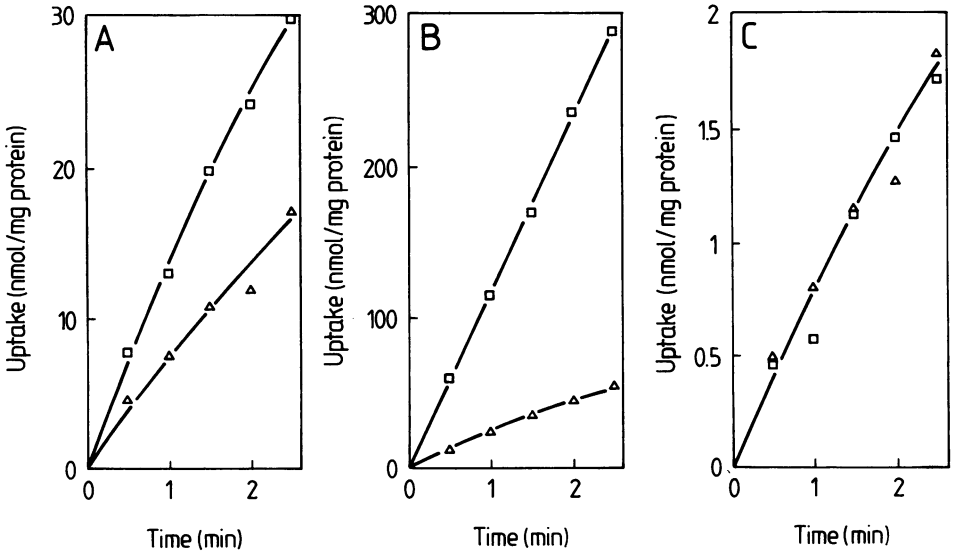

FIG. 2. Effect of an osmotic shock on the uptake of P_i and L-lysine in *A. johnsonii* 210A. The uptake of P_i and L-lysine was determined in control cells (□) and shocked cells (△) at concentrations of 100 μM P_i and 1.6 μM L-lysine. (A) P_i uptake in high-P_i-grown cells; (B) P_i uptake in low-P_i-grown cells; (C) uptake of L-lysine in high-P_i-grown cells.

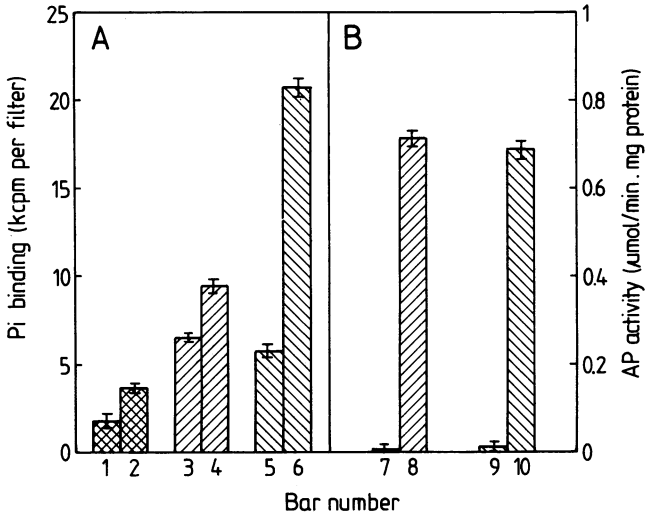

FIG. 3. P_i-binding and alkaline phosphatase (AP) activity in concentrated shock fluid of *E. coli* and *A. johnsonii* 210A. (A) Binding of 10 μM P_i to the filter (bar 1), to bovine serum albumin (bar 2), and to concentrated shock fluid of high- and low-P_i-grown cells of *A. johnsonii* 210A (bars 3 and 4) and high- and low-P_i-grown cells of *E. coli* (bars 5 and 6); (B) alkaline phosphatase activity in concentrated shock fluid of high- and low-P_i-grown cells of *A. johnsonii* 210A (bars 7 and 8) and of high- and low-P_i-grown cells of *E. coli* (bars 9 and 10). Each value is the mean of two separate determinations.

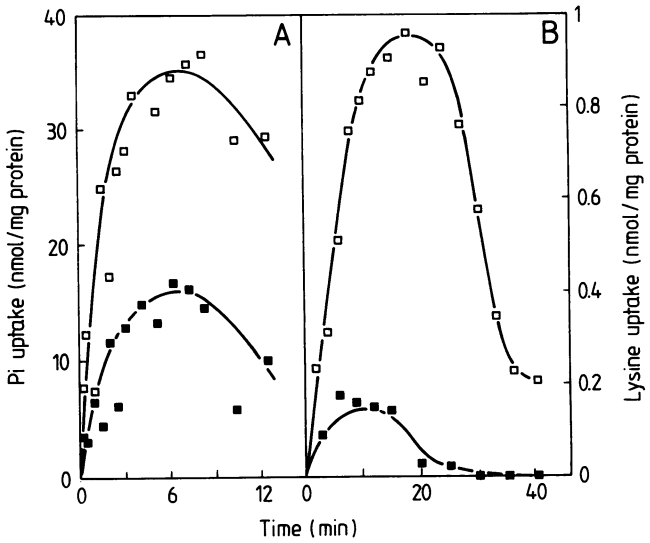

FIG. 4. Uptake of 10 μM P_i (A) and 16 μM L-lysine (B) via counterflow in deenergized cells of *A. johnsonii* 210A which were unloaded (■) or loaded with 10 mM P_i or 3 mM L-lysine (□).

is known to be present in the periplasm of gram-negative organisms (30, 42), served as a control for the presence of periplasmic proteins in the concentrated shock fluids. As an additional control, concentrated shock fluid was prepared from *E. coli*, in which the presence of a binding-protein-dependent P_i transport system has been reported (36). The results are very similar for the two organisms (Fig. 3). P_i-binding activity was detectable in concentrated shock fluids of high-P_i-grown cells of *A. johnsonii* 210A. However, along with the activity of alkaline phosphatase, the P_i-binding activity was appreciably higher in the concentrated shock fluid of low-P_i-grown cells. This result is consistent with the observation that P_i uptake in low-P_i-grown cells was more sensitive to osmotic shock than P_i uptake in high-P_i-grown cells (Fig. 2) and points to the presence of an inducible binding-protein-dependent transport system for P_i in *A. johnsonii* 210A.

Presence of a secondary P_i transport system. ATP-dependent uptake systems for solutes are in general unidirectional and should not allow counterflow of substrates. On the other hand, secondary transport systems are reversible and should allow an easy exchange of P_i inside and outside the cell in a deenergized state (1, 31, 32). In order to obtain more evidence for reversible secondary P_i and L-lysine transport, counterflow of P_i and L-lysine was studied in high-P_i-grown cells of *A. johnsonii* 210A which were depleted of endogenous energy reserves by an aerobic incubation in the presence of 2.5 mM DNP for 12 h at 30°C. Within 60 min of incubation with DNP, the internal ATP concentration was reduced from 1.5 mM to below 0.01 mM. Endogenous respiration and P_i uptake decreased to very low rates after 12 h of incubation. Upon addition of glucose-PQQ to these starved and washed cells, the respiration rate and P_i uptake rate were restored to levels comparable to those in energized cells, indicating that (i) DNP was removed effectively to allow an energization of the starved cells and (ii) the starved cells had retained the transport system in an active form.

The amounts of P_i and L-lysine taken up via counterflow in deenergized cells of *A. johnsonii* 210A loaded with 10 mM P_i or 3 mM L-lysine and diluted into media containing a final concentration of 10 μM $^{32}P_i$ and 16 μM L-[U-^{14}C]lysine, respectively, were significantly higher than in unloaded cells (Fig. 4). Counterflow activity of P_i and L-lysine clearly indicates the presence of reversible secondary transport systems for these substrates.

DISCUSSION

The uptake of P_i by *A. johnsonii* 210A is an active process. P_i uptake occurs against a concentration gradient, depends on the presence of an oxidizable energy source, and is inhibited by respiratory chain inhibitors and uncouplers of the oxidative phosphorylation.

Kinetic analysis reveals the presence of two P_i uptake systems with low and high affinity for P_i. The K_t values observed in low-P_i-grown cells (0.7 ± 0.2 μM and 9 ± 1 μM) are very similar to those reported for *E. coli* (0.7 and 9.2 μM) (27) and *P. aeruginosa* (1.1 and 10 μM) (24) but are sixfold lower than those observed in high-P_i-grown cells of *A. johnsonii* 210A (4 ± 1 μM and 41 ± 7 μM). High-P_i-grown cells contain polyP granules, which are absent in low-P_i-grown cells. Whether polyP granules and/or the slow degradation of polyphosphates in washed-cell suspensions of high-P_i-grown cells (43) affects the kinetics of P_i transport is unclear. However, since the high- and the low-affinity systems in both low- and high-P_i-grown cells are similarly affected by several treatments of the cells, it is concluded that the uptake of P_i in cells grown in low-P_i as well as in high-P_i medium is mediated by the same two transport systems. Comparison of the maximal uptake rates of the transport systems in low- and high-P_i-grown cells suggests the presence of a constitutive low-affinity system and an inducible high-affinity system. The maximal level of induction after transferring high-P_i-grown cells to P_i-free medium is obtained within 4 h, or approximately two cell divisions.

Strong evidence is obtained for the presence of a periplasmic binding-protein-dependent high-affinity P_i uptake system. The uptake of P_i in cells is inhibited by an osmotic shock, as is observed for all binding-protein-dependent systems but not for secondary transport systems (15). Kinetic experiments show the inactivation of the high-affinity transport system by osmotic shock. Further evidence comes from the demonstration of P_i-binding activity in concentrated periplasmic fractions of shocked cells. The observed induction of this activity under P_i limitation is consistent with its involvement in the high-affinity P_i uptake system.

Binding-protein-dependent transport systems are energized by a high-energy phosphate bond (2–4, 20, 21). P_i transport in *A. johnsonii* 210A can be energized by the oxidation of glucose via a membrane-bound, PQQ-dependent glucose dehydrogenase. Since *A. johnsonii* 210A is unable to grow on glucose ± PQQ or on gluconate as the sole carbon source, glucose oxidation can result only in ATP synthesis coupled to the Δp by a membrane-bound H^+-ATPase. Inhibition of the H^+-ATPase by DCCD results in a stimulation of the Δp, a sevenfold decrease of the internal ATP concentration, and a drastic reduction in the uptake of P_i via the high-affinity transport system. These effects of DCCD point to the involvement of ATP, or a related compound, in the energization of P_i uptake via the binding-protein-dependent transport system.

The low-affinity uptake of P_i is hardly affected by osmotic shock or by DCCD, but dissipation of the Δp by valinomycin-nigericin decreases the activity strongly, suggesting that it is mediated by a Δp-dependent secondary transport system. In contrast to ATP-dependent, binding-protein-dependent uptake systems which are usually unidirectional, secondary transport systems mediate reversible transport (1, 31, 32). Counterflow of P_i in deenergized cells loaded with 10 mM P_i confirmed the presence of a reversible (secondary) transport system for P_i.

The P_i uptake systems in *A. johnsonii* 210A resemble the two major transport systems of *E. coli*: the high-affinity P_i-specific transport system and the constitutive low-affinity P_i transport system (37, 49). In both organisms, the two systems are present in cells grown in high-P_i medium. In low-P_i medium, the rate of P_i uptake via the high-affinity system is increased by a factor of 6 to 10, just as the activity of alkaline phosphatase. This phenomenon is well documented for *E. coli*, where the *pho* regulon is an interlocking assembly of genes, transport systems, and enzymes dedicated to the singular purpose of ensuring that the cell obtains an adequate supply of P_i for growth under adverse conditions (33, 36, 41). The enhanced activity of the binding-protein-dependent transport system and of alkaline phosphatase in cells of *A. johnsonii* 210A under P_i limitation suggests the presence of a similar regulatory mechanism to scavenge the last traces of P_i and phosphorus-containing nutrients from the surrounding medium.

In *A. johnsonii* 210A, regulation of expression of the high-affinity P_i uptake apparently takes place via a mechanism of (de)repression of protein synthesis as described in *E. coli*. The ability of *A. johnsonii* 210A to accumulate polyP during logarithmic growth may result in P_i uptake characteristics which differ from those of other microorganisms. In *E. coli* (27) and *Bacillus cereus* (38), the uptake of P_i in time is biphasic: an initial high rate is followed by a lower one. This biphasic kinetics was explained by the presence of two transport systems, of which the primary transport system is subjected to inhibition when the primary pool of P_i within the cells is filled up. Since phosphate bond-driven transport systems are essentially unidirectional and can catalyze the uptake of solutes to much higher accumulation levels than secondary transport systems, *trans*-inhibition acts as a regulatory device to prevent solute accumulation to unacceptably high internal levels. This type of regulation of transport activity, usually not found for secondary transport systems, has been described for the major potassium transport system of *Enterococcus faecalis* (6), the potassium transport systems TrkA and Kup of *E. coli* (34), and the P_i uptake system of *L. lactis* (31). In contrast to the biphasic uptake of P_i in *E. coli* and *B. cereus*, monophasic uptake of P_i is maintained in *A. johnsonii* 210A. P_i uptake is linear in time until a maximal level is reached. However, the bulk of P_i that is taken up is rapidly metabolized and most likely incorporated into polyP in low- and high-P_i-grown cells. As a result of this incorporation into a polymer, a low intracellular P_i concentration can be maintained. It is very likely that the free-P_i concentration is too low to result in *trans*-inhibition of the ATP-dependent high-affinity uptake system, allowing the organism to efficiently take up large amounts of P_i.

The results in this paper allow the following conclusions to be drawn: (i) *A. johnsonii* 210A is able to reduce the P_i concentration in its environment to micromolar levels (or lower) because of the presence of an inducible high-affinity P_i uptake system in combination with its ability to synthesize and accumulate polyP, and (ii) P_i efflux is mediated by a low-affinity secondary transport system.

The secondary transport system could be involved in the anaerobic energy metabolism of *A. johnsonii* 210A. Besides a conservation of metabolic energy liberated from the cleavage of polyphosphate via a direct enzymatic synthesis of ATP, metabolic energy could additionally be conserved by the generation of an electrochemical ion gradient across the cytoplasmic membrane, when P_i is excreted together with ions.

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