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The *Azotobacter chroococcum* nitrate permease is a multicomponent system

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Nitrate uptake by *Azotobacter chroococcum* ATCC 4412 was sensitive to osmotic shock. Cytoplasmic membrane preparations from nitrate-grown cells exhibited three polypeptide components of 52, 49 and 44 kDa, respectively, that were missing from, or very much decreased in, N_2-fixing or ammonium-grown cells. The *A. chroococcum* TR1 strain, which is deficient in nitrate uptake but exhibits normal levels of nitrate reductase (EC 1.6.6.1) and nitrite reductase (EC 1.6.6.4), lacked the 44 kDa membrane-bound protein while exhibiting the other two polypeptide components. Transfer of ammonium-grown *A. chroococcum* cells to a medium containing nitrate as the sole nitrogen source and [^{35}S]methionine resulted in parallel development of nitrate uptake activity and the above mentioned 52, 49, and 44 kDa polypeptides radioactively labelled. *A. chroococcum* MCD1, a pleiotropic mutant unable to use nitrate as a nitrogen source, had none of the membrane proteins inducible by nitrate in the wild-type cells. The results strongly suggest that a multicomponent system transports nitrate in *A. chroococcum*.

Introduction

Using the short-half-life isotope ^{13}N, it has been shown that, in heterotrophic bacteria, nitrate transport is the first step of nitrate assimilation [1,2]. The energy requirement of the uptake process was estimated from nitrate utilization by the cells with unlabelled nitrate [3]. More recently, a cytoplasmic membrane protein of approximately 47 kDa has been proposed as a component of nitrate transport in cyanobacteria [4,5], the microorganisms from which the gene encoding this transport element has been cloned, sequenced, and mutagenized [6]. The nucleotide sequence of the eukaryote *Aspergillus nidulans crnA* gene, that encodes the permease for nitrate uptake by this filamentous fungus, has also been determined [7]. The predicted *crnA* protein of about 51 kDa exhibited hydrophobic domains characteristic of a transmembrane protein, as is the case for other transport proteins [7].

In all microorganisms tested, the regulation of the nitrate uptake depends on expression of nitrate transport repressed by NH_4^+. In heterotrophic bacteria, when NH_4^+ was present in the transport assay [^{13}N]-nitrate uptake was inhibited [1,2]. In cyanobacteria, the cytoplasmic membrane protein associated with the uptake process can be synthesized in the absence of nitrate [4,5]. It has been claimed that, in *A. nidulans*, expression of the gene *crnA* is subject to nitrate induction through the regulatory gene *nirA* [7], but other authors maintain that nitrate is not required to derepress the nitrate transporter *crnA* gene [8].

Bacterial active transport systems can be divided into two groups: those which are osmotic shock-resistant with one single membrane protein, and those which are shock-sensitive and have a membrane-bound protein complex plus a soluble periplasmic protein [9]. The shock-sensitive permeases are also called periplasmic permeases. Whether the bacterial nitrate transport falls into one of these two groups appears not to be known yet.

We report here that nitrate uptake by the strictly aerobic, N_2-fixing heterotrophic bacterium *Azotobacter chroococcum* was sensitive to osmotic shock, a treatment that did not affect ammonia utilization or the cells' viability and that had no effect on the activities of nitrate reductase and nitrite reductase. We found that the polypeptide composition of cytoplasmic membranes changed in response to the nitrogen source available to the cells. Incorporation of [^{35}S]methionine into proteins and use of the *A. chroococcum* TR1 mutant, that is defective in nitrate transport [10], and the *A. chroococcum* MCD1 strain, a pleiotropic mutant unable to use nitrate as a nitrogen source [11,12]

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Abbreviations: DCCD, *N-N'*-dicyclohexycarbodiimide; Mops, 3-morpholinepropane sulphonic acid.

suggest that nitrate transport into *A. chroococcum* cells is mediated by a multicomponent system tightly bound to the cytoplasmic membrane.

Methods

Materials

ADP, DCCD, Mops, and L-glutamic dehydrogenase (EC 1.4.1.3) (Type II, from bovine liver) were purchased from Sigma. NADPH was from Boehringer. [^{35}S]methionine was from New England Nuclear. Electrophoresis reagents and molecular weight markers were from Bio-Rad. All other chemicals were of analytical grade from Merck.

Organisms and cultures conditions

A. chroococcum ATCC 4412 (from the Valencia University Collection, Valencia, Spain), *A. chroococcum* TR1 [10] and *A. chroococcum* MCDl, (a gift from R. Robson, Brighton, UK) were grown heterotrophically on nitrogen-free Burk's medium supplemented with sucrose (0.5%) as the sole energy and carbon source. When necessary this medium was supplemented with ammonium chloride, potassium nitrate or ammonium nitrate at the concentration indicated in each case. Growth conditions were as previously described [13]. Mid-logarithmic-growth cells (A_{560} of approx. 0.5) were used for each experiment.

Osmotic shock procedure

Cell suspensions were harvested by centrifugation at $12000 \times g$ for 10 min at 4°C, washed with 10 mM Tris-HCl buffer (pH 8.0), and resuspended (1 g wet weight per 80 ml) in 20% (w/v) sucrose containing 30 mM Tris-HCl buffer (pH 8.0). Following 10 min of gentle agitation (87 rpm in a gyratory shaker) at 30°C, the cells were pelleted by centrifugation at $12000 \times g$ at 4°C and the supernatant fluid was removed. The well-drained pellet was rapidly mixed with a volume of 15% (w/v) sucrose containing 30 mM Tris-HCl buffer (pH 7.5) equal to that of the original volume of the suspension and incubated for 5 min at 30°C. The mixture was centrifuged as above, the supernatant discarded and the sediment resuspended with 50 mM Mops-KOH (pH 7.5) containing 7% (w/v) sucrose. The resulting mixture (approx. 70 μg cell protein/ml) was used as osmotically shocked cells. Viability of these cells was determined by plating on solid Burk's medium.

Isolation of cytoplasmic-membrane fractions

Cytoplasmic membranes were prepared from cells grown for 2–4 h, as specified in each case, in media containing, as indicated, the appropriate nitrogen source in the absence or in the presence of [^{35}S]-methionine. They were isolated as described by Kaback [14] with some modifications. A 5 ml aliquot of cell suspension was centrifuged at $12000 \times g$ for 10 min (all centrifugations were at 4°C) and the pellet washed twice with ice-cold 10 mM Tris-HCl buffer (pH 8.0). Cells were resuspended (1 g wet weight per 80 ml) at room temperature in 20% (w/v) sucrose containing 30 mM Tris-HCl (pH 8.0), 10 mM potassium EDTA (pH 7.0), and 1 mg/ml lysozyme. To avoid any further protein synthesis, translation was prevented by addition of chloramphenicol (10 μg/ml). The mixture was incubated at 30°C for 30 min. Spheroplasts were collected by centrifugation at $12000 \times g$ for 40 min, resuspended in 10 μl of 20% (w/v) sucrose containing 100 mM potassium phosphate buffer (pH 6.6) and 20 mM $MgSO_4$, and then poured directly into 300 vol. (3 ml) of 50 mM potassium phosphate buffer (pH 6.6) that had been equilibrated at 37°C (lysis buffer). After 15 min of vigorous swirling at 37°C, potassium EDTA (pH 7.0) at a final concentration of 10 mM was added, and stirring continued for 15 min. 15 mM (final concentration) $MgSO_4$ was then added with stirring prolonged for 15 min. The lysate was centrifuged at $20000 \times g$ for 40 min and the pellet resuspended in an ice-cold solution of 100 mM potassium phosphate buffer (pH 6.6) containing 10 mM EDTA and centrifuged at $40000 \times g$ for 50 min. The pellet was resuspended in 100 mM potassium phosphate buffer (pH 6.6) containing 10 mM EDTA and centrifuged at $800 \times g$ for 30 min to remove cell debris. The supernatant fluid was carefully decanted and centrifuged at $40000 \times g$ for 50 min. The membranes so obtained were resuspended in 100–200 μl of sample buffer [15] for electrophoresis.

Purity of the cytoplasmic membrane preparations was checked by measuring glutamine synthetase (EC 6.3.1.2) activity and nitrate reductase activity, both soluble enzymes, as well as their respiratory capacity. None of those enzymes contaminated the membrane preparations. NADH-dependent O_2 uptake was 155 nmol/min per mg protein and no respiration in the absence of an externally added substrate was observed.

Measurement of nitrate, nitrite and ammonium uptake

Mid-exponential phase cells, or osmotically shocked cells (see above), were used for each experiment. Cultures were harvested by centrifugation at $12000 \times g$ for 10 min at 4°C, washed once with 50 mM Mops-KOH (pH 7.5) and resuspended to 70 μg cell protein/ml in the same buffer. Assays were done with continuous shaking at 30°C in conical flasks open to the air. The experiments were started by the addition of either 0.5 mM KNO_3, 0.2 mM $NaNO_2$, or 0.2 mM NH_4Cl, and at intervals thereafter uptake was assayed by following the disappearance of the corresponding ion from the medium after rapid removal of bacteria by filtration in a Millipore system with Whatman glass microfibre paper. When the passive influx of nitrite uptake [16] was to be determined, the assay mixture contained 100 μM

DCCD and it was incubated for 10 min at 30°C before starting the uptake assay. All the results are representative of three separate experiments on different batches of bacteria.

Enzyme assays

Nitrate reductase [12], nitrite reductase [12], and glutamine synthetase [13] activities, were determined in permeabilized cells.

Analytical methods

Samples of cytoplasmic membranes containing approx. 60 μg protein, or 40000 cpm in the experiments with [^{35}S]methionine, were subjected to SDS-PAGE on 12% gels. Electrophoresis was performed in gel slabs according to Laemmli [15]. Protein markers were electrophoresed in parallel. The proteins were visualized with Coomassie brilliant blue R-250. Fluorograms of in vivo-labelled polypeptides were obtained as described by Bonner and Laskey [17]. Nitrate, nitrite, ammonium and whole-cell protein were estimated by methods as referred to in Ref. [12].

Results and Discussion

Nitrate (nitrite) transport in A. chroococcum is an osmotic shock-sensitive system

Nitrate uptake activity in *A. chroococcum* cells was severely inhibited when the bacteria were subjected to an osmotic shock. As shown in Table I, cells suspended in 15% (w/v) sucrose, collected, and then resuspended with 7% (w/v) sucrose lost 50% of their nitrate consumption activity, the inhibition being total when the initial sucrose concentration was 20% (w/v) . By contrast, the osmotic shock treatment caused only slight inhibition (17%) of ammonium consumption which was not affected at all when the starting sucrose concentration was 15%. This lack of effect on ammonium consumption is in agreement with published data demonstrating that osmotically shocked cells of *Escherichia coli* and *Klebsiella pneumoniae* are active in [^{14}C]methylammonium (an NH_4^+ analog) transport [18]. Furthermore, cell viability was not affected by shock treatment under the specified conditions. However, cell lysis occurred when bacteria coming from a 15–20% (w/v) sucrose medium were resuspended with sugar concentrations smaller than 7% (w/v). Nitrate reductase activity of the osmotically shocked cells (7.8 nmol/min per mg protein), as measured in situ, was 85% of that of the control cells. From these results, and from the fact that nitrite reductase, the second enzyme of the nitrate-reducing system, was not affected by the shock treatment either (see below), we conclude that nitrate transport in *A. chroococcum cells* is an osmotic shock-sensitive system.

TABLE I

Osmotic shock sensitivity of nitrate uptake and ammonium uptake by A. chroococcum ATCC 4412 cells

Cells grown under air in the presence of 10 mM KNO_3 were osmotically shocked with sucrose as described in the text. Activities of nitrate uptake and ammonium uptake are expressed as nmol ion taken up/min per mg protein. Other experimental conditions as in Methods.

Treatment	Nitrate uptake	Ammonium uptake
None	31.6	9.9
Sucrose 15%	16.4	10.8
Sucrose 20%	0	7.3

Nitrite consumption by *A. chroococcum* cells was also measured. As we have recently described [16], nitrite uptake is made up of two components, a passive diffusion and an active transport of nitrite that uses the nitrate transport system. At appropriate pH values, and in the presence of an inhibitor of oxidative phosphorylation or of F_0F_1-ATPases, these two components can be conveniently separated. Table II shows that the active component of nitrite uptake, 9.4 nmol/min per mg protein, decreased approx to 25% of this value when the *A. chroococcum* wild-type cells were subjected to a sucrose concentration change from 18% to 7%. On the other hand, the passive influx of nitrite into these cells was not affected by the osmotic shock. As also indicated in Table II, nitrite uptake by the *A. chroococcum* TR1 mutant was the same in osmotically shocked cells as in control cells. Nitrite reductase activity, as measured in situ, of *A. chroococcum* cells (6.5 nmol/min per mg protein) was not inhibited by the osmotic shock treatment (9 nmol/min per mg protein). Even though the nitrite uptake activity was not determined, the respiratory NADH-nitrite oxidoreductase activity was also shown to remain in the osmotically sensitive spheroplast obtained from *E. coli* [19]. Hence, the bacterial active nitrite transport is also an osmotic shock-sensitive system.

Proteins involved in nitrate transport in A. chroococcum

Since, in *A. chroococcum* cells, the assimilatory nitrate uptake system is inducible by nitrate and repressible by ammonium [20], we studied the protein composition of membranes from bacteria grown on different nitrogen sources. Electrophoretic techniques before and after incorporation of radioisotopes into proteins were employed. For these experiments, the wild-type cells and the mutant strains *A. chroococcum* TR1 (see above) and MCD1, which, as mentioned above, are unable to use nitrate as nitrogen source, were used.

Fig. 1 shows the polypeptide composition of cytoplasmic membrane preparations from wild-type *A. chroococcum* cells grown in media containing different

TABLE II

Osmotic shock sensitivity of nitrite uptake by cells of A. chroococcum ATCC 4412 and mutant TR1

Cells grown under air in the presence of 10 mM KNO_3 were osmotically shocked with sucrose as described in the text. Activities of nitrite uptake in the absence (active component) and in the presence of 100 μM DCCD (passive influx) are expressed as nmol NO_2 taken up/min per mg protein. Other experimental conditions as in Methods.

Strain	Treatment	Nitrite uptake	
		active component	passive influx
ATTC 4412	none	9.4	8.5
ATCC 4412	sucrose 18%	2.5	10.0
TR1	none	– [a]	13.0
TR1	sucrose 18%	– [a]	10.0

[a] The active component is missing from TR1 cells [16].

nitrogen sources (lanes 1–3), and the polypeptide pattern of cytoplasmic membranes from the mutant strain TR1 (lane 4) grown at the expense of molecular nitrogen in the presence of nitrate. The polypeptide profile of nitrate-grown wild-type cells (lane 3) exhibited three major protein bands of 52, 49 and 44 kDa that were absent or present at very low levels in N_2-fixing cells (lane 1) and ammonium-grown cells (lane 2). The cytoplasmic membrane preparations from TR1 cells lacked the 44 kDa polypeptide; the 52 and 49 kDa protein components were present at significant levels. Since diazotrophically [12] and ammonium-grown *A. chroococcum* cells [20] cannot take up nitrate, and TR1 cells suffer from the same incapacity [16], these results suggested that the 52, 49, and 44 kDa membrane-bound polypeptides are involved in the nitrate assimilation system of *A. chroococcum*, most likely at the level of nitrate transport. To test this possibility, the levels of the 52, 49 and 44 kDa cytoplasmic membrane polypeptides, and nitrate uptake activity were analyzed after transferring ammonium-grown bacteria to media containing [^{35}S]methionine and different nitrogen sources. Development of nitrate reductase activity was also followed.

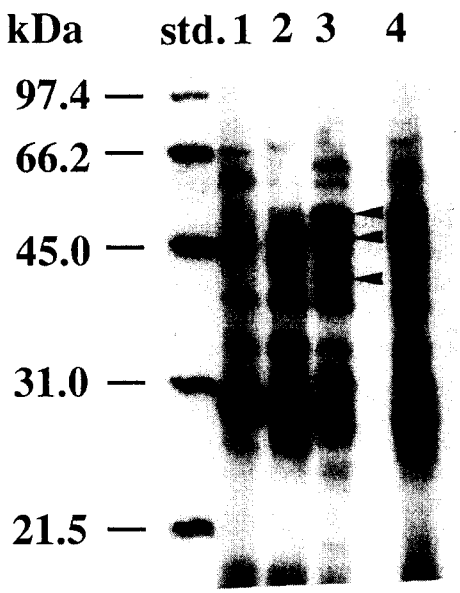

Fig. 1. Cytoplasmic membrane proteins in *A. chroococcum* strains. Cytoplasmic membranes were prepared from cells that had been subcultured twice for 12 h in media containing the specified nitrogen sources. Lanes 1–3 contained cytoplasmic membrane proteins from wild-type ATCC 4412 cells grown either under N_2-fixing conditions (lane 1) or in the presence of 10 mM NH_4Cl (lane 2) or 8 mM KNO_3 (lane 3), and, lane 4, from mutant TR1 cells grown at the expense of air nitrogen in the presence of 8 mM KNO_3. Arrows point to the polypeptides of 52, 49, and 44 kDa. The sizes of the six molecular mass markers are shown on the left margin. Other experimental conditions are as indicated in Methods.

Fig. 2 shows that in wild-type cells transferred to a medium containing nitrate (lane 2), the 52 and 49 kDa polypeptides were heavily labelled. The 44 kDa protein was labelled very weakly, however, and it was only detected as a thin band, most likely due to a low methionine content. Although not shown, a protein profile identical with that in lane 2 of Fig. 2 was obtained when ammonium-grown *A. chroococcum* cells were transferred to a medium containing nitrite instead of nitrate. In cells transferred to medium lacking a combined nitrogen source (lane 1), i.e. under N_2-fixing conditions, the 52, 49, and 44 kDa polypeptides appeared very weakly labelled compared to what would be expected for nitrate-induced proteins. Cells that had been again transferred to ammonium, lane 3, exhibited lower levels of these polypeptides than when transferred to nitrate medium. This is in favour of nitrate acting as an inducer. *A. chroococcum* wild-type cells transferred to ammonium nitrate-containing medium (lane 4) incorporated somewhat more radioactivity into the 52, 49 and 44 kDa polypeptides than cells transferred to medium with ammonium alone. This can be interpreted as being caused by some nitrate, due to the high concentration used (10 mM), entering the cells during the experiment, in spite of the well-known ammonium inhibition of nitrate uptake [1,21].

The fact that MCD1 did not exhibit the 52, 49 and 44 kDa polypeptides at all (lane 5) lends support to the proposal that these are components of the nitrate transport system in *A. chroococcum*. As shown in Fig. 2 (lane 6), the 44 kDa protein was missing from cytoplasmic membranes of the TR1 mutant incubated in the presence of nitrate. As also depicted, the 52 and 49 kDa proteins present in the nitrate-grown wild-type cells appeared in the TR1 strain at high levels. The proposal [10] that TR1 was a transport-deficient mutant and not a regulatory mutant is thus supported by these results.

Table III indicates the activities of nitrate uptake and nitrate reductase in those cell suspensions from which the membrane preparations illustrated in Fig. 2 were obtained. Wild-type cells transferred to nitrate medium had developed nitrate uptake activity and nitrate reductase activity appreciably, both parameters reaching similar magnitude. Transfer to ammonium nitrate-containing medium approximately halved the values of these parameters, but only negligible nitrate uptake activity and nitrate reductase activity appeared in the wild-type cells transferred to ammonium medium or combined nitrogen-free medium. While it did not take up nitrate at all, the TR1 mutant nitrate reductase activity nearly equated to the wild-type cells (Table III) in agreement with previously published data [10]. In contrast, the MCD1 strain was without the activities of nitrate reductase and nitrate uptake. Therefore, in *A. chroococcum*, the synthesis of the three membrane-bound proteins and development of nitrate uptake activity appear to be inducible by nitrate (nitrite). Although the nitrate reducing system of this bacterium shares this pattern of regulation ([20], and this work), both nitrate reductase and nitrite reductase are soluble proteins [21].

TABLE III

Activity levels of nitrate uptake and nitrate reductase in A. chroococcum wild-type and mutant strains TR1 and MCD1 following transfer of ammonium-grown cells to media containing different nitrogen sources

Just before the isolation of the cytoplasmic membrane preparations described in Fig. 2, the corresponding cell suspensions were analyzed for activities of nitrate uptake and nitrate reductase. Other experimental conditions as in Fig. 2 and Methods.

A. chroococcum strain	Nitrogen source	Nitrate uptake (nmol/min per mg)	Nitrate reductase (mU/mg)
ATCC 4412	N_2 (air)	3	2
	NH_4Cl	1	2
	NH_4NO_3	6	4
	KNO_3	12	9
TR1	N_2 (air) plus KNO_3	0	7
MCD1	N_2 (air) plus KNO_3	0	0

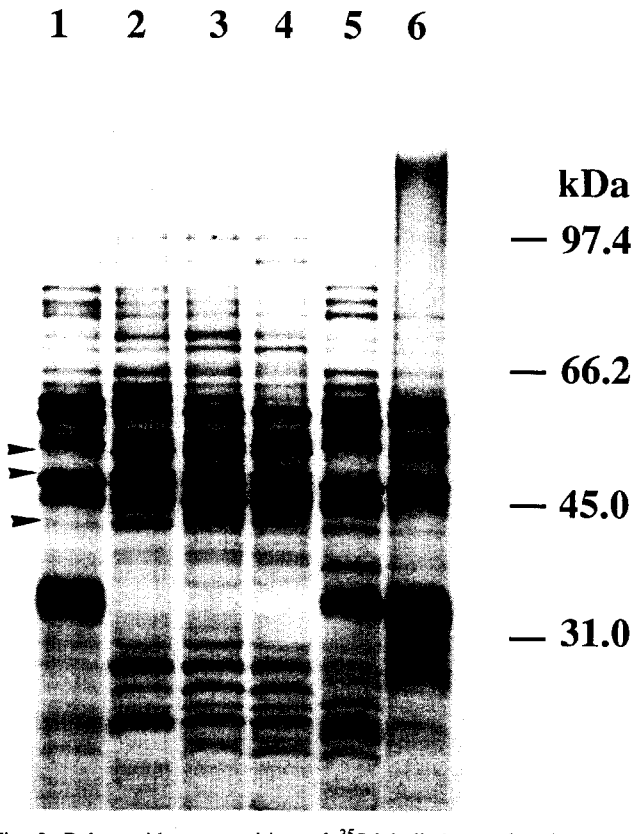

Fig. 2. Polypeptide composition of ^{35}S-labelled cytoplasmic membranes upon transfer of ammonium-grown cells of *A. chroococcum* wild-type and mutant strains to media containing different nitrogen sources. Cytoplasmic membranes were prepared from ammonium-grown cells that had been collected at an A_{560} of approx. 0.8 and then subcultured for 2 h (A_{560} 0.3) in a medium containing 5 μCi/ml [^{35}S]-methionine and the indicated nitrogen source. Lanes 1–3 contained cytoplasmic membranes proteins from wild-type cells transferred to combined nitrogen-free medium (lane 1), 10 mM KNO_3 (lane 2), 10 mM NH_4Cl (lane 3), 10 mM NH_4NO_3 (lane 4). Lane 5 contained cytoplasmic membrane proteins from strain MCD1 transferred to nitrate (8 mM KNO_3) medium, and lane 6 from strain TR1 also transferred to this last medium. Other experimental conditions are given in Methods.

These results strongly support that *A. chroococcum* nitrate transport has a membrane-bound multicomponent system, most likely a polypeptide complex consisting of three proteins. Furthermore, the osmotic shock sensibility of nitrate uptake suggests that some periplasmic protein component might be involved in nitrate transport as well. Work is now in progress to determine what role, if any, some periplasmic space element may play on the conveyance of nitrate to the membrane-bound polypeptide components.

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