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REGULATION AND ENERGIZATION OF NITRATE TRANSPORT IN A HALOPHILIC *Pseudomonas stutzeri*

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Nitrate transport and its regulation by oxygen was studied in denitrifying halophilic *Pseudomonas stutzeri*, strain Zobell, and a Tn-5 transposon nitrite reductase mutant of this organism. The rate of nitrate transport was found to be 130 nanomoles nitrate min^{-1} mg $protein^{-1}$ and 150 nanomoles nitrate min^{-1} mg $protein^{-1}$ in the wildtype and the nitrite reductase mutant respectively as compared to 26.4 nanomoles nitrate min^{-1} mg $protein^{-1}$ in a non-halophilic *Pseudomonas stutzeri*. Asparagine was found to be the best energy source for nitrate uptake. The ratio of nitrate import to nitrite export was established by measuring extracellular nitrate and nitrite concentrations using HPLC/UV analysis. There was a 1.3:1 (NO_3^-/NO_2^-) exchange. High concentrations of nitrate during growth was found to have a negative effect on nitrite metabolism. Oxygen exerted an inhibitory effect on nitrate uptake which was reversible and more pronounced in cells grown on low concentrations of nitrate compared to cells grown at high concentrations of nitrate.

Dissimilatory nitrate reduction exists in two forms: the first is represented by organisms such as *Escherichia coli*, which have the capacity to reduce nitrate to nitrite under anaerobic conditions thus supplementing energy derived from fermentation. The second form of dissimilatory nitrate reduction termed denitrification is found in many *Pseudomonas* species, where nitrate is ultimately reduced to dinitrogen (1,2,3).

The first step of the denitrification pathway is of interest because of its environmental impact which involves depletion of nitrogen from fertilizer thus decreasing agricultural productivity, removal of nitrate from freshwater and waste material, production of nitrogenous oxides and biogeochemical cycling of dinitrogen (1,3,4).

The capacity of an organism to reduce nitrate and other nitrogen oxides is known to be rapidly lost in the presence of oxygen (1,2,3,4,5,6,7,8,9). It has been found that oxygen regulates both forms of nitrate dissimilation at two levels: 1) Oxygen inhibits transcription of genes involved in the pathway (3,5) and 2) oxygen inhibits the transport of nitrate to the catalytic site of reduction (4). The latter form of oxygen regulation is immediate, reversible and can occur in a number of organisms capable of both forms of nitrate dissimilation (4,6,7,8,9).

To better understand the regulation of nitrate transport during nitrate dissimilation, it is important to define the mechanism by which nitrate is transported to the catalytic site and the mechanism by which oxygen regulates uptake in a variety of organisms. Studies so far have concentrated on typical mesophilic denitrifiers (7,8,9). In order to examine the possible diversity of nitrate transport systems, we chose to study this regulatory mechanism in the halophilic bacterium *Pseudomonas stutzeri* (formerly *Pseudomonas*

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perfectomarina). This investigation was aimed at characterizing nitrate transport with respect to oxygen regulation, energy requirements, nitrite export and the effect of initial nitrate concentration for growth on uptake using a denitrifying halophilic organism, *Pseudomonas stutzeri* and a Tn-5 transposon induced nitrite reductase mutant (MK202) of this organism (10).

MATERIALS AND METHODS

Organisms and Media. The organisms utilized in this study were *Pseudomonas stutzeri* ATCC 14405 (formerly *P. perfectomarina*) and a nitrite reductase mutant derived from *P. stutzeri*, MK202 which was obtained from W. Zumft, University of Karlsruhe (10). Both organisms were maintained on LB agar slants or grown in broth supplemented with 1% (w/v) sea salts and 10 mM or 20 mM KNO_3. In addition kanamycin (50 ug/ml) was incorporated in all media used for growth of the mutant strain.

Growth Conditions. In order to derepress and induce the nitrate respiration system the organisms were grown as follows: two large loopfuls of a 36-48 h culture were used to inoculate 150 ml of LB medium in 250 ml Erlenmeyer flasks. Cultures were incubated semi-anaerobically at 30°C for 4-6 h. A 20% transfer of starter culture was made into a 500 ml Erlenmeyer flask which was filled to the neck with the same medium. The culture was flushed with argon to establish anaerobic conditions, sealed with a stopper that allowed for gas release and incubated at 30°C for 12-14 h. The cultures were stirred with a magnetic stir bar to prevent clumping.

Uptake Studies. The cells were harvested by centrifugation at 10,410 x g for 15 min at 25°C; the pellet was washed twice with room-temperature 20 mM Tris-HCl buffer (pH 7.4), containing 1% NaCl (w/v) and chloramphenicol (100 ug ml^{-1}). Cell suspensions made to a density of 0.50 g (wet wt.) per 100 ml of 20 mM Tris-HCl buffer (pH 7.4) with 1% NaCl (w/v), were placed in a glass reaction vessel, closed to the atmosphere with ports for gas flushing, substrate addition and sample withdrawal. To study the best suitable energy source and the effect of NO_3^- concentration on NO_3^- uptake, washed cells were divided into 1 ml aliquots at a concentration of 0.3 g wet weight and resuspended in 10 ml of the same buffer as described above; appropriate concentrations of energy sources were subsequently added.

The cell suspensions were continuously flushed with argon to maintain anaerobic conditions. Vigorous stirring helped prevent clumping and thus maintained a homogenous suspension. Uptake of nitrate was initiated by the addition of the appropriate concentration of nitrate. The effect of oxygen on nitrate uptake was done by flushing the cell suspension with air at 120-150 ml min^{-1}. K_m determinations from the data presented in Fig.2, were made using Statpro (Penton Software, NY).

Determination of Nitrate and Nitrite. Extracellular nitrate and nitrite concentrations were determined at specific time intervals. During uptake, 0.2 ml samples were filtered through 0.45 u ACRO LC 13 discs (Gelman). The filtrates were then analyzed for nitrate and nitrite using HPLC/U.V. analysis (11). A Dionex Series 2000 Ion Chromatograph with an HPIC/AS4 strong anion exchange column connected to a pressure pulsed amperometric detector was employed. The eluant used was 35 mM NaCl. The column was operated at a pressure of 72.8 bar. Separation of sample components was monitored with a Waters Model 481 UV detector at a wavelength of 215 nm. The output terminals were connected to an SP4620 integrator. Samples were injected through a 50 ul loop using a 2 cc tuberculin syringe. Nitrate and nitrite were quantitated by peak height.

Protein Determinations. The amount of protein in the samples was determined by the Coomassie Blue method as described by Spector (12). Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Nitrate Transport. A variety of energy sources were tested to determine which best supports the uptake of nitrate in cell suspensions. The choice of energy sources was based on a previous study of carbon sources for denitrification by the halophilic *Pseudomonas stutzeri* (13). Table 1 indicates that nitrate uptake in cell suspensions of *Pseudomonas stutzeri* depends on an energy source. The order of effectiveness of the compounds tested was asparagine > formate > glycerol > glucose > malate. These results are consistent with the previous physiological studies on *P. stutzeri* (13) and other pseudomonads (14), which

Table 1: Effect of energy sources on rates of nitrate uptake in *Pseudomonas stutzeri*

Energy Source	Rate*
None	27
Asparagine	134
Formate	108
Glycerol	104
Glucose	61
Malate	45

*Rates are reported in nanomoles nitrate min^{-1} mg $protein^{-1}$.
Nitrate (final concentration: 600 uM) was used to initiate uptake. Samples were removed at 30 sec intervals over a period of 10 min., filtered and analyzed for extracellular nitrate and nitrite by HPLC/UV analysis.

have demonstrated that basic amino acids and amides rather that carbohydrates or organic acids are preferred for denitrification. The requirement for an energy source for nitrate uptake is most likely a result of the direct link between nitrate reduction and nitrate transport rather than an energy requirement specifically to support transport.

The nitrate transport system was found to obey saturation kinetics with respect to nitrate concentration (Fig.1). Maximal velocity was reached at 600 uM nitrate. The estimated K_m was = 306 uM.

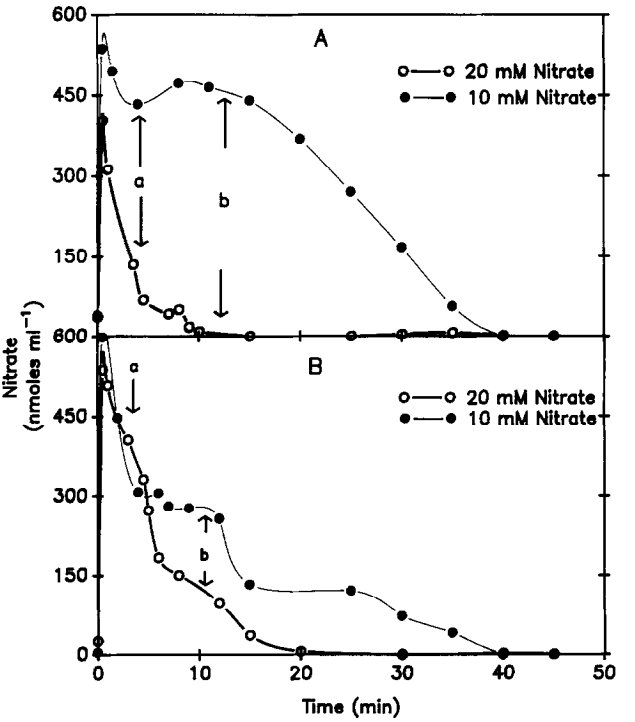

Fig.1. Effect of nitrate concentrations on nitrate uptake in *Pseudomonas stutzeri*. Nitrate concentrations ranging from 200-800 uM were added to 1 ml aliquots of the same batch of washed cells. NO_3^- and NO_2^- determinations were made as described in Fig.1. Rates are expressed as nanomoles NO_3^- min^{-1} mg $protein^{-1}$ and represent initial rates taken during the first 3 min of the reaction. K_m = 306 uM.

Table 2: Stoichiometric relationship between nitrate transport and nitrite export in mutant strain MK202

Nitrate nmoles/ml	Nitrite nmoles/ml	NO_3^-/NO_2^-
48	36	1.32
50	36	1.38
34	28	1.21
56	45	1.24
77	56	1.37
44	33	1.33
	Mean	1.30

Nitrate and nitrite concentrations represent initial extracellular concentrations. Cells were spiked with 600 uM nitrate and asparagine (10 mM) as electron donor. HPLC/U.V. analysis of filtered samples was used to follow disappearance of nitrite and accumulation of nitrite.

Evidence for a Nitrate/Nitrite exchange. The Tn-5 transposon nitrite reductase mutant (10), provided a means by which a mechanism for nitrate transport and its reduction to nitrite could be studied. It is clear from uptake studies that a stoichiometric exchange between nitrate import and nitrite export does take place (Table 2), in that NO_3^-/NO_2^- ratios approached 1.3:1. These results suggest that transport of nitrate is via a NO_3^-/NO_2^- antiport system, whereby extracellular nitrate is exchanged for intracellular nitrite formed as a result of nitrate reduction, thus maintaining electroneutrality. In addition, the rate of nitrate transport in MK202 was consistently greater than the wildtype organism (Table 3); this suggests that the rate of nitrate uptake is also regulated by intracellular concentrations of nitrite. A nitrate/nitrite exchange model for nitrate transport is also supported by physiological studies of nitrate and nitrite reduction in *Pseudomonas aeruginosa*, where it has been demonstrated that nitrite accumulates extracellularly in culture during denitrification until all the nitrate in the medium is metabolized. Subsequently, the nitrite is reduced (15).

Table 3: Nitrate uptake by wildtype and mutant cells grown on different concentrations of nitrate

Strain	NO_3^- Concentration	Rate*
Wildtype	10 mM	130
MK202	10 mM	150
Wildtype	20 mM	94
MK202	20 mM	108

*Rates are reported in nanomoles of nitrate min^{-1} mg $protein^{-1}$.
Disappearance of extracellular nitrate and its reduction to nitrite during uptake studies was analyzed by HPLC/U.V. analysis. The wild type and mutant strain (MK202) were grown either on 10 mM or 20 mM nitrate.

Effect of nitrate concentration during growth on nitrate transport and nitrite reduction. The rate of nitrate uptake was the same in wildtype and MK202 cell suspensions at each of the concentrations of nitrate in which they were grown (Table 3). When the wildtype was grown with 10 mM nitrate, the disappearance of nitrate in washed cell suspensions was stoichiometrically associated with nitrite export and nitrite accumulated extracellularly, until all of the nitrate was reduced, at which time nitrite was metabolized (Fig.2A). The same initial trend was observed in uptake studies of cell suspensions grown on 20 mM nitrate. However the extracellular nitrite accumulated with no further reduction (Fig.2B). The results observed with *Pseudomonas stutzeri* grown on 10 mM nitrate are consistent with physiological studies of denitrifying cultures of *Pseudomonas aeruginosa* in which nitrite accumulated and was subsequently reduced only after all the nitrate had disappeared from the culture medium (15).

The inability of *Pseudomonas stutzeri* grown on 20 mM nitrate to further reduce nitrite, is likely due to the sensitivity of this organism to the known toxic effects of nitrite on aerobic and anaerobic respiration (15,16). These results could therefore explain the findings of Zumft *et al.*, that denitrification in whole cells of *Pseudomonas stutzeri* was inhibited on the addition of 25 mM of nitrate (17).

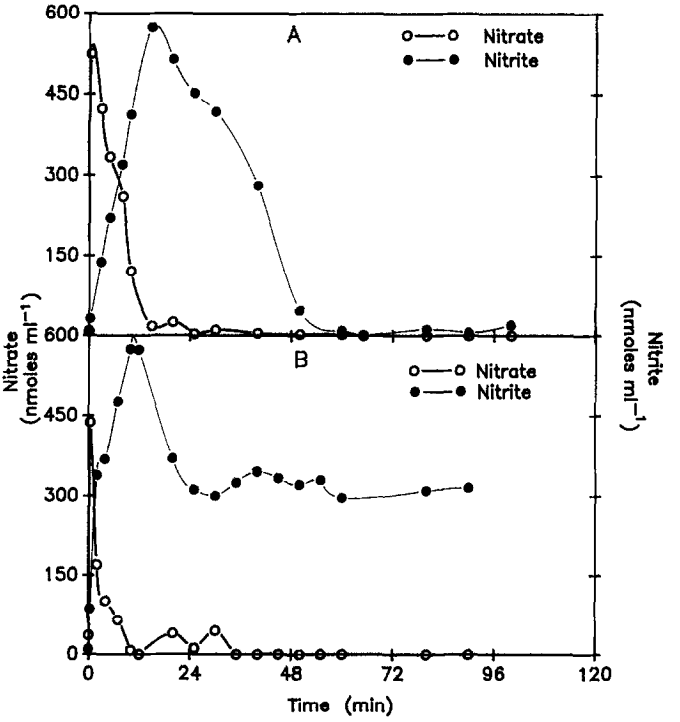

Fig.2. Nitrate uptake and nitrite export by cell suspensions of *Pseudomonas stutzeri*. (A) wildtype grown on 10 mM NO_3^-(B) wildtype grown on 20 mM NO_3^-. Harvested cells were suspended in 20 mM Tris-HCl buffer (pH 7.4), supplemented with 1 % NaCl, and flushed with argon for several minutes to make cells anaerobic. KNO_3 (600 uM) was added to initiate uptake. Samples were removed at regular time intervals over a period of a 10 min, filtered and analyzed for NO_3^- and NO_2^- by HPLC/U.V. analysis.

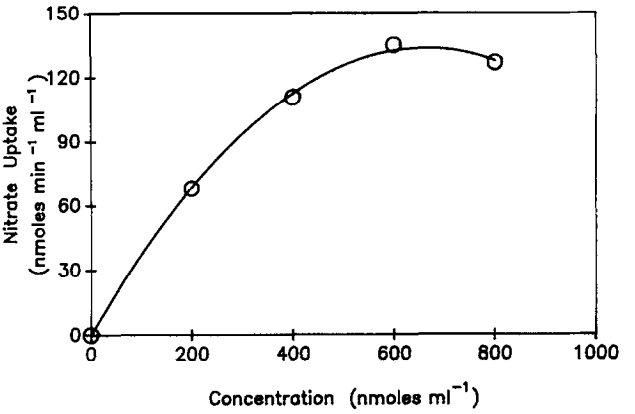

Fig.3. Effect of oxygen on nitrate uptake in *Pseudomonas stutzeri* (A) and mutant strain *MK202* (B). Cells were grown on 10 mM and 20 mM KNO_3. Cells were prepared and nitrate utilization was determined as described in Fig.2. At 3 min (a), air was bubbled through the suspension. After 9 min (b), aeration ceased and argon flushing resumed.

Oxygen regulation of nitrate uptake. Cells of the wildtype and the mutant *MK202* grown on 10 mM or 20 mM nitrate, exhibited different responses when exposed to oxygen during uptake studies (Fig.3). Upon aeration, uptake by cell suspensions grown anaerobically on 10 mM nitrate, were inhibited, however inhibition was not as immediate as oxygen inhibition seen in non-halophilic organisms. Nitrate uptake resumed to rates comparable with pre-oxygen treatment after anaerobic conditions were restored. However nitrate uptake of cell suspensions grown anaerobically with 20 mM nitrate, was not as drastically affected by oxygen (Fig. 3). These results along with those depicted in Fig.2, indicate that high initial nitrate concentrations in cultures change the physiological response of this halophile to both oxygen and nitrite dissimilation. This effect was found to be more pronounced in the nitrate reductase mutant compared to the wildtype.

In conclusion, asparagine was the best energy source for nitrate transport in *Pseudomonas stutzeri*. The requirement for an energy source is probably as a result of a direct link between nitrate transport and nitrate reduction rather than energy strictly to support transport. The transport system obeyed saturation kinetics with respect to nitrate with a K_m of 306 uM. There was a strict exchange of extracellular nitrate for intracellular nitrite at a stoichiometry of 1.3:1, suggesting an antiport system. This halophilic system was unique in its response to nitrate concentrations during growth in that at concentrations of 20 mM nitrate, nitrite reduction was prevented. Oxygen regulation was also affected by growth on 20 mM nitrate concentrations.

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