FINAL REPORT

NITRATE TRANSPORT OF THE HALOPHILIC CYANOBACTERIUM, APHANOTHECE HALOPHYTICA GROWN UNDER NORMAL AND SALT STRESS CONDITIONS

BY

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Abstract

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Project Title : Nitrate transport of the halophilic cyanobacterium, *Aphanothece halophytica* grown under normal and salt stress conditions

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The transport of nitrate by *Aphanothece halophytica* grown under normal and salt-stress conditions was investigated. Nitrate transport was monitored by measuring nitrate remaining in the assay medium using an anion-exchange high performance liquid chromatography. Kinetic studies revealed that the affinities of nitrate to normal and salt-stress cells were not significantly different, K_s = 416 and 450 μM respectively. However, the maximum velocity of nitrate uptake for normal cells was about 2 fold of that for salt-stress cells. Both cells required the presence of Na^+ for the uptake of nitrate. Ammonium inhibited nitrate uptake whereas the presence of methionine sulfoximine was unable to release the inhibition by ammonium. Nitrite was found to competitively inhibit nitrate uptake with a K_i value of 84 μM. Both chloride and phosphate anions had no effect on nitrate uptake. DL-glyceraldehyde, an inhibitor of CO_2 fixation, reduced nitrate uptake. Monensin inhibited nitrate uptake in concentration-dependent manner suggesting an involvement of a Na^+-electrochemical gradient. Amiloride, an inhibitor of Na^+/H^+ antiporter, reduced nitrate uptake. Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and N, N′-dicyclohexylcarbodiimide (DCCD) each severely inhibited nitrate uptake suggesting that a pH gradient generated by H^+/ATPase played a role in the transport of nitrate. Exogenously added glucose or lactate led to an increase of nitrate uptake rendering a further support for the involvement of ATP and electrochemical potential for the transport of nitrate into *A. halophytica*.

Key Words : Nitrate transport, Cyanobacteria, Salt stress

บทคัดย่อ

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ชื่อโครงการ : การขนส่งไนเตรตของไซยาโนแบคทีเรียมชนิดชอบความเค็ม, *อะฟาโนทีคี ฮาโลฟิทิคา* ที่เจริญภายใต้สภาวะปกติและสภาวะความเครียดของเกลือ

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ได้ทำการศึกษาการขนส่งไนเตรตเข้าสู่เซลล์ของไซยาโนแบคทีเรียมชนิดชอบความเค็ม, *อะฟาโนทีคี ฮาโลฟิทิคา* ซึ่งเจริญภายใต้สภาวะปกติและสภาวะความเครียดของเกลือ โดยทำการตรวจวัดปริมาณไนเตรตที่เหลือในสารละลายด้วยวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี พบว่า เซลล์ที่เจริญภายใต้ 2 สภาวะดังกล่าวจับตัวกับไนเตรต โดยมีค่า K_s ที่ไม่แตกต่างกันนัก กล่าวคือมีค่า 416 และ 450 ไมโครโมลาร์ตามลำดับ สำหรับเซลล์ที่เจริญภายใต้สภาวะปกติและสภาวะความเครียดของเกลือ อย่างไรก็ตามพบว่าความเร็วสูงสุดของการนำไนเตรตเข้าสู่เซลล์ที่เจริญภายใต้สภาวะปกติมีค่าประมาณ 2 เท่าของเซลล์ที่เจริญภายใต้สภาวะความเครียดของเกลือ การนำไนเตรตเข้าสู่เซลล์ต้องอาศัยการทำงานร่วมกันของ Na^+ ด้วย ทางด้านการศึกษาผลของอิออนต่าง ๆ พบว่าแอมโมเนียมยับยั้งการนำไนเตรตเข้าสู่เซลล์ โดยที่การยับยั้งนี้ยังคงมีอยู่ถึงแม้ว่าจะเติมสารเมไธโอนีนซัลฟอกซิมีนลงไปก็ตาม สำหรับไนไตรต์นั้นพบว่าสามารถยับยั้งการนำไนเตรตเข้าสู่เซลล์แบบแข่งขันโดยมีค่า K_i 84 ไมโครโมลาร์ ในขณะที่คลอไรด์และฟอสเฟตไม่มีผลต่อการนำไนเตรตเข้าสู่เซลล์ กลีเซอราลดีฮัยด์ซึ่งเป็นสารยับยั้งกระบวนการตรึงคาร์บอนไดออกไซด์สามารถลดอัตราการนำไนเตรตเข้าสู่เซลล์ ในด้านการศึกษาแหล่งพลังงานของการนำไนเตรตเข้าสู่เซลล์นั้น ทั้งโมเนนซินและอะมิโลไรด์ ซึ่งยับยั้งกระบวนการก่อให้เกิด Na^+ และ H^+ เกรเดียนท์ตามลำดับ สามารถลดอัตราการนำไนเตรตเข้าสู่เซลล์ ในทำนองเดียวกัน คาร์บอนิลไซยาโนด์คลอโรฟินิลไฮดราโซน (ซีซีซีพี) ซึ่งเป็นสารที่ทำลายแรงผลักดันเนื่องจากโปรตอน สามารถยับยั้งการนำไนเตรตเข้าสู่เซลล์ ในส่วนของไดไซโคลเฮกซิลคาร์โบไดอิไมด์ (ดีซีซีดี) ซึ่งเป็นสารยับยั้งการย่อยสลายเอทีพีก็สามารถยับยั้งการนำไนเตรตเข้าสู่เซลล์ นอกจากนั้นยังพบอีกว่าการเติมแหล่งพลังงานจากภายนอกในรูปของกลูโคสหรือแลคเตทสามารถเพิ่มอัตราการนำไนเตรตเข้าสู่เซลล์ได้ จากข้อมูลทั้งหมดข้างต้นสามารถบ่งชี้ว่าแหล่งพลังงานในรูปของเอทีพีหรือในรูปของความต่างศักย์ของเคมีไฟฟ้ามีบทบาทต่อการนำไนเตรตเข้าสู่เซลล์ *อะฟาโนทีคี ฮาโลฟิทิคา*

คำหลัก : การขนส่งไนเตรต, ไซยาโนแบคทีเรีย, ความเครียดของเกลือ

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Nitrogen Assimilation

After carbon, oxygen and hydrogen, nitrogen is the most abundant element in all living cells. Nitrogen is a constituent of a large number of important compounds found in all living cells. Particular notable examples are amino acids, proteins (enzymes) and nucleic acids (RNA and DNA), while in other examples polyamines and chlorophylls, may play a major role in some organisms. Most animals do not have the capacity to assimilate inorganic nitrogen, nor to synthesize half the amino acids found in protein, unless assisted by bacteria (e.g. in the rumen of sheep and cattle).

Nitrogen is available to all living cells in the three basic forms: free nitrogen gas, and as combined inorganic or organic compounds. The ability to reduce or fix nitrogen gas is found only among the prokaryotes and exclusively in the photosynthetic cyanobacteria. Nitrogen fixation is the conversion of gaseous nitrogen (N_2) to ammonia. Nitrogen fixation requires nitrogenase, an oxygen-sensitive irons-, sulphur-, molybdenum-containing enzyme complex that also brings about the reduction of other substrates containing triple covalent bonds (nitrous oxide, cyanides, isocyanides, cyclopropene, and acetylene).

Nitrate is a major source of nitrogen for photosynthetic organisms including cyanobacteria, algae and plants (Guerrero et al, 1990). Nitrate assimilation takes place by three sequential steps: (1) nitrate transport into the cell by a specific nitrate permease; (2) reduction to nitrite by assimilatory nitrate reductase; and (3) further reduction to ammonium by

assimilatory nitrite reductase. The resulting ammonium is then incorporated into central metabolism through the action of glutamine synthetase and glutamate synthase.

Upon entering the cells, nitrate is converted to nitrite by nitrate reductase. There are two types of nitrate reductase known. The first, and better-known enzyme, is found in eukaryotic algae. This nitrate reductase complex is similar to that found in fungi and higher plants, consisting of haem (cytochrome *b-557*), flavin adenine dinucleotide (FAD), and molybdenum (Mo) as a prosthetic group. The best characterized algae enzyme is from *Chlorella*; this enzyme has a molecular mass of about 350,000 and a complex structure (Solomonson and Barber, 1990). The enzyme catalyzes the reduction of nitrate to nitrite by reduced pyridine nucleotides according to the reaction:

$$NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NAD(P)^+ + H_2O$$

With the higher plant enzyme there is evidence that Mo is contained in a small complex (molecular mass less than 30,000) that can be fairly easily separated from the bulk of the enzyme. Haem is present as a cytochrome *b-557* which is reduced by NADH and reoxidized by NO_3^-; cyanide stops its reoxidization by NO_3^- but not its reduction by NADH. The pathway of electrons from NAD(P)H to nitrate through nitrate redutase from eukaryotes may be depicted as:

$$NAD(P)H \longrightarrow (FAD \longrightarrow \text{cyt } b\text{-}577 \longrightarrow Mo) \longrightarrow NO_3^-$$

The pyridine nucleotide specificity of the enzyme differs in different algae. Nitrate reductase is always active with NADH as the electron donor and, in many algae, only with this. Some algae, however, are not able to utilize NADPH.

The second type of nitrate reductase is found in prokaryotic cells including cyanobacteria. The prokaryotic nitrate reductase is simpler and smaller (molecular mass of about 75,000); it also contains

molybdenum but not flavin or cytochrome (Manzano et al, 1976). The important difference from the enzyme of eukaryotes is that it does not use pyridine nucleotide as electron donor but reduced ferredoxin. It therefore catalyzes the reaction:

$$NO_3^- + 2Fd + 2H^+ \longrightarrow NO_2^- + 2Fd + H_2O$$

Activation of cytosolic nitrate reductase has been studied in depth over the last 20 years. In higher plants, there are a number of examples of nitrate reductase activation by ferricyanide, light, and higher levels of CO_2, but it seems that redox potential ($NADH/NAD^+$) could be an important regulator of proportion of nitrate reductase found in active form (Solomonson and Barber, 1990). There is a light/dark modulation of nitrate reductase, via nitrate reductase phosphorylation / dephosphorylation reaction, dependent on Mg^+ and ATP and requiring regulator proteins. These proteins are a protein kinase (PK) which phosphorylates nitrate reductase but without inactivating it, an inhibitor protein (IP) which binds to phospho-nitrate reductase and inactivates it, and a protein phosphatase (PP) which reactivates nitrate reductase (Glaab and Kaiser, 1995).

The reduction of NO_2^- to NH_4^+ is catalyzed by ferredoxin nitrite reductase, characteristic of photosynthetic organisms (the second type of nitrite reductase, NAD(P)H-nitrite reductase is found in nonphotosynthetic organisms). This enzyme appears to be much the same in algae and in leaves of higher plants. It is a small molecule with a molecular mass of 60,000-70,000. This enzyme contains sirohaem which is an iron tetrahydroporphyrin and where NO_2^- probably attaches, it also contains an iron-sulfur center which participates in electron transport. The reaction catalyzed is:

$$NO_2^- + 6\,Fe + 8\,H^+ \longrightarrow NH_4^+ + 6\,Fe + 2H_2O$$

In the leaves of higher plants, nitrite reductase has been localized definitely in the chloroplast; information about its localization in algae cells is lacking, but some results suggest a close linking between nitrite reduction and the photochemical reaction in the chloroplast.

1.2 Regulation of nitrate assimilation

As the assimilation of nitrogen into protein requires both energy and organic skeletons, it is not surprising that there are major interactions between N-assimilation and photosynthetic metabolism (Bassham et al, 1981). The assimilation of both NH_4^+ and NO_3^- is dependent on photosynthesis, that is assimilation requires light and CO_2; removal of either of these prevents assimilation. *Chlamydomonas reinhardtii* does not assimilate nitrate and ammonium unless a suitable source of carbon is provided. Suitable sources are CO_2 in light or, in darkness, acetate or the carbon reserves accumulated in nitrogen-starved cells. Nitrate metabolism is strongly light dependent in green algae. Nitrate uptake can be stimulated by light, leading to indirect activation of nitrate reductase. The enzyme nitrate reductase can also be directly activated by light; light can furthermore induce nitrate reductase synthesis.

Nitrate uptake is generally believed to be the rate-limiting step in nitrate assimilation, and the overall rate of nitrate assimilation is tightly regulated at the nitrate uptake step by the metabolic carbon/nitrogen status of cells (Flores and Herrero, 1994). It has been known for a long time that ammonium suppresses nitrate uptake in many algae species and also in various higher plants. It has been demonstrated many times that in the presence of both ammonium and nitrate in the medium, the NH_4^+ is assimilated first, and only when it has gone is NO_3^- utilized. Preferential uptake of NH_4^+ has been shown often for both marine and

freshwater algae. However, there have been few reports showing preferential assimilation of nitrate; this phenomenon has been reported e.g. for *Pandorina* and *Haematococus*. There are several reasons for this preferential assimilation of ammonium. Active nitrate reductase is not formed in the presence of NH_4^+ nor is the NO_3^- uptake system. And even if active nitrate reductase and an NO_3^- uptake system are present, the addition of NH_4^+ can lead to a rapid cessation of NO_3^- utilization. There are at least three mechanisms by which nitrate reductase activity can disappear from cells. These include two sorts of reversible inactivation phenomena and an irreversible loss of enzyme due, presumably, to degradation. The effect of addition of NH_4^+ to cells assimilating NO_3^- is complex. The first effect appears to be an inhibition of NO_3^- uptake but this is followed by loss of nitrate reductase (and nitrite reductase) activities. The loss of nitrate reductase activity will be partly due to reversible inactivation, and partly due to irreversible loss of enzyme with the rate of proteolytic breakdown of nitrate reductase possibly being greater in the presence of NH_4^+. At the same time addition of NH_4^+ stops the synthesis of nitrate reductase. The regulation of the formation of nitrite reductase has received much less attention.

1.3 Nitrate uptake in cyanobacteria

Nitrate is a major source of nitrogen for cyanobacteria. Nitrate assimilation in cyanobacteria involves two steps: (i) the uptake process and (ii) enzymic reduction. The enzyme involved in the reduction of nitrate to nitrite in cyanobacteria is ferredoxin-dependent nitrate reductase (E.C. 1.7.7.2). It consists of a single polypeptide of 58-85 kDa, a molybdenum cofactor, four non-heme iron and four labile sulfide (two Fe_2S_2 clusters) per molecule (Mikami and Ida, 1984). The enzyme

nitrite reductase (E.C. 1.7.2.1), which mediates the reduction of nitrite to ammonium, is a polypeptide of 52-68 kDa, having Fe_4S_4 cluster and a siroheme as prosthetic group. Ammonium resulting from nitrate an nitrite is assimilated mostly through the GS/GOGAT pathway rendering glutamate, the principal nitrogen donor in the biosynthesis of other organic nitrogen compounds. The transport of nitrate has been the least understood step of nitrate assimilation, although its importance has been recognized for a long time (Beevers and Hageman, 1983). The first unequivocal demonstration of a gene responsible for nitrate transport and of its essential role in nitrate assimilation was done in the cyanobacterium *Synechococus* sp. PCC7942 (Omata et al, 1989), and the subsequent works have shown the structure of the nitrate transporter of the cyanobacterium (Omata et al, 1993). Clusters of genes involved in cyanobacterium *Synechococus* sp. PCC 7942 nitrate assimilation have been mapped. They are in an operon that is regulated by nitrogen availability and are cotranscribed. The operon consists of two nitrogen regulated genes nirB and ntcB, one structural gene nirA for nitrite reductase, four genes for nitrate transport (nrtA, nrtB, nrtC, and nrtD), and one structural gene NarB for nitrate reductase. Nitrate transport is essential for the growth of the cyanobacterium at physiological concentrations of nitrate and has been shown to be involved in the active transport of nitrite as well.

Comparison of the structures of nitrate transporters from eukaryotic and prokaryotic, photosynthetic and non- photosynthetic organisms indicate that the nrt nitrate/nitrite transporter represents a prokaryotic nitrate transporter distinct from the nitrate transporter of eukaryotes. The activity of the nitrate-assimilation system of cyanobacteria is regulated transcriptionally in response to changes in nitrogen availability ; biosynthesis of the NrtA protein, expression of

nitrate-transporting ability, and expression of NR and NiR activities are all subjected to nutritional repression by ammonium (Herrero et al, 1981). In *Synechococus* sp. PCC 7942, the concerted regulation of the activities related to nitrate assimilation is ascribed to co-transcription of gene nirA-nrtBCD-narB as a single operon. (Suzuki et al, 1992). Ammonium, either added to the medium or generated internally by reduction of nitrate, negatively regulates the transcription of the nirA operon. Since L-methionine sulfoximine (MSX), an inhibitor of ammonium fixation by glutamine synthetase, induces development of NR and NiR activities in *Synechococus* (Herrero et al, 1981), it has been assumed that a nitrogenous compound(s), resulting from the assimilation of ammonium, inhibits the expression of the nitrate assimilation system (Guerrero and Lara, 1987). Nitrate assimilation by cyanobacteria is subjected not only to transcriptional regulation but also to post-translational regulation (Guerrero and Lara, 1987). Addition of ammonium to the medium causes prompt cessation of nitrate uptake in nitrate-utilizing cells of *Synechococcus* sp. PCC 6301, a strain closely related to the strain PCC 7942 (Lara et al, 1987). As in the regulation of the nirA operon, fixation of ammonium to Glu is required for the negative effects of ammonium to prevail on the activity of nitrate transport. It is supposed that a metabolite(s) of Glu acts as a negative effector of nitrate transport.

By monitoring intracellular accumulation of nitrate in *Synechococus* sp. PCC 7942, Rodriguez et al showed a selective and strong dependence of nitrate transport on millimolar concentrations of Na^+ (Rodriguez et al, 1992). Monensin, an ionophore that collapses the eletrochemical gradient of Na^+, was shown to depress nitrate transport. Based on detailed kinetic studies on the sodium-dependent nitrate transport, Rodriguez et al proposed a sodium/nitrate symport driven by

the energy of eletrochemical gradient of Na^+ as the most likely mechanism of nitrate transport.

1.4 Relationship of cyanobacteria salt tolerance and nitrate

Salt overloading in soils is by far the major hindrance for plant growth, and a crucial problem for agriculture. Each organism displays a salinity tolerance range, which includes its optimal growth conditions, though larger ranges of salt concentrations (resistance range) may still be compatible with cell survival. Crop plants are particularly limited in their tolerance range, whereas other photosynthetic organisms, including microalgae and cyanobacteria, may display wider acclimation capacities. Exposure of cells to salt concentrations that are physiologically above those present intracellularly threatens them via two deleterious effects, namely, increase of both the osmotic pressure and the ion concentration. The water potential decreases, leading to loss of water by the cells, and simultaneously to influx of ions into the cytoplasm. Stress from NaCl, in particular, causes a dramatic increase in the concentration of inorganic ions.

Cyanobacteria, the only prokaryotes performing oxygenic photosynthesis and probable ancestors of chloroplasts, constitute valuable models for the study of the molecular mechanisms involved in tolerance to high salinity, or to its corollary, drought, a major agricultural problem. The critical demands of cyanobacteria exposed to high salinity, i.e., accumulation of osmoprotectors and extrusion of sodium ions, are met through immeiate activation and/or long term (protein synthesis-dependent) adaptation of various processes: (i) uptake and endogenous biosynthesis of osmotica, the nature and amount of which are strain and salt concentration dependent (ii) probable modifications of membrane lipid composition (iii) increased energetic

capacity, at the level of cyclic electron flow around photosystem I (through routes induced under these conditions) and cytochrome c oxidase, and (iv) enhancement of H^+-ATPase activity and active extrusion of sodium ions.

Extrusion of sodium ions phenomena have an important influence on the halotolerance of various organisms. Cyanobacteria do not accumulate Na^+ although a transient net Na^+ uptake may occur in response to hypersaline upshock (Reed et al, 1985). It was shown further that the ability to curtail Na^+ influx can also be induced by certain environmental factor like alkaline pH or presence of combined nitrogen in the form of nitrate or ammonium in the growth medium (Reddy et al, 1989). Presence of certain nitrogenous compounds in the growth medium significantly enhanced the salt tolerance of freshwater cyanobacterium *Anabaena* sp. strain L-31 as well as the brackish water cyanobacterium *Anabaena torulosa*. Amoung these, nitrate, ammonium, and glutamine were most effective followed by glutamate and aspartate. These nitrogenous compounds also inhibited Na^+ influx in both *Anabaena* sp. with the same order of effectiveness as that observed for protection against salt stress. The inhibition of Na^+ influx on addition of the nitrogenous substances was rapid; nitrate and ammonium inhibited Na^+ influx competitively. Uptake of nitrate and ammonium increased during salt stress but was not correlated with growth. Intracellular levels of nitrate and ammonium were found to be inadequate to constitute a major component of the internal osmoticum. These suggest that inhibition of Na^+ influx by nitrate and/or amonium is part of a mechanism for protection of cyanobacteria against salt stress.

1.5 Objectives of the project

So far the knowledge on nitrate uptake by cyanobacteria has been very limited. The effect of salinity on the uptake of nitrate is poorly understood. Moreover, the source of the driving force for the transport of nitrate is still controversial. These, coupled to our interest in the mechanism of salt tolerance in cyanobacteria, prompted us to investigate the uptake of nitrate by a unique halotolerant cyanobacterium *Aphanothece halophytica* which is capable of growth in a wide range of salinity. Specifically the objectives of this project are as follows :

1.5.1 Study the kinetics of nitrate uptake by *A. halophytica* grown under non-stress and salt-stress conditions

1.5.2 Study the regulatory aspects of nitrate uptake by non-stressed and salt-stressed *A. halophytica*

1.5.3 Study the energetic aspects of nitrate uptake by non-stressed and salt-stressed *A. halophytica*

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains

Aphanothece halophytica was initially isolated from Solar Lake in Israel. The organism was kindly provided by Dr. T. Takabe of Research Institute of Meijo University, Japan.

2.2 Culture of *A. halophytica*

Ten percent inoculum of *Aphanothece halophytica* was inoculated into a 250 ml flask containing 100 ml of Turk Island salt solution + modified BG_{11} medium (see Appendix I) with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on a rotary shaker with 160 rpm at 30°C, 60 $\mu Em^{-2}s^{-1}$ of continuous irradiance. After 10 days, 10 ml culture was inoculated into the same medium containing either 0.5 M NaCl or 2.0 M NaCl and shaken on a rotary shaker (160 rpm) at 30°C with 2000 lux of continuous illumination.

2.3 Determination of nitrate uptake

2.3.1 Determination of nitrate content by HPLC

The nitrate content was determined by anion-exchange HPLC method using analytical Hypersil-10 sax column (10 μm porous silica gel packing 250 mm x 4.6 mm i.d.) recommended by Romero et al. (1989). HPLC analyses were performed with HP series 1050, 4 pumps and UV detector, which was set at 210 nm. 30 mM potassium dihydrogen phosphate buffer pH 3 was

used as mobile phase and operated at the flow rate of 1.0 ml/min.

2.3.2 Time courses of nitrate uptake

Ten percent of *Aphanothece halophytica* were inoculated into a 250 ml flask containing 100 ml of Turk Island salt solution + modified BG_{11} medium with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on a rotary shaker with 160 rpm at 30°C, 60 $\mu Em^{-2}s^{-1}$ of continuous irradiance. After 10 days, 10 ml culture was inoculated into the same medium containing either 0.5 M NaCl (normal) or 2.0 M NaCl (salt stress) and shaken on the rotary shaker (160 rpm) at 30°C with 60 $\mu Em^{-2}s^{-1}$ of continuous irradiance. After 10 days, the culture was centrifuged at 2000 g for 10 min and the cell pellet was suspended in 0.5 M sorbitol, 25 mM Hepes-KOH buffer pH 8.3, 12 mM $NaHCO_3$ containing either 0.5 M NaCl or 2.0 M NaCl followed by the addition of 100 μM $NaNO_3$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by rapid filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1

2.4 Characterization of nitrate uptake

2.4.1 Kinetics of nitrate uptake

A. halophytica was grown in the medium, harvested and osmotic stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll /ml. To

determine the kinetic constants of the nitrate uptake system, initial rates of uptake were determined over a wide range of nitrate concentrations (100 to 1000 μM $NaNO_3$) using cells grown under normal and salt stress conditions (0.5 M and 2.0 M NaCl). The half saturation value (K_s) and maximum velocity (V_{max}) were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot, i.e., 1/[s] vs 1/[v].

2.4.2 Na^+ requirement of the uptake system

Cell were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. Initial rates of uptake were determined in the presence of various Na^+ concentrations using NaCl as a source.

2.5 Effect of various ions on nitrate uptake

2.5.1 Effect on NH_4^+ on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of nitrate and ammonium with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of ammonium concentrations on nitrate uptake,

initial rates of uptake were determined in the presence of various NH_4^+ concentrations using NH_4Cl as a source. To study the effect of L-methionine D, L-sulfoximine (MSX, an inhibitor of ammonium assimilation) to remove negative effect of NH_4^+, preincubation of the cells with 1 mM MSX for 30 min in the dark was done before assay. The assay was started by simultaneous addition of nitrate and ammonium with irradiance. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1.

2.5.2 Effect of anions on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of nitrate and nitrite (or nitrate and chloride, or nitrate and phosphate) with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of nitrite concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various nitrite concentrations.

2.5.3 Effect of DL-glyceraldehyde (DLG) on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation of the cells with 30 mM DLG for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of DLG concentrations on nitrite uptake, initial rates of uptake were determined in the presence of various DLG concentrations.

2.6 Effect of various inhibitors on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 an 12 mM $NaHCO_3$. Preincubation of the cells with 20 μM monensin (or 100 μM amilorie or 50 μM DCCD or 10 μM CCCP) for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of various inhibitors concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various inhibitors concentration.

CHAPTER 3

RESULTS

3.1 Characterization of nitrate uptake system

3.1.1 Kinetics of nitrate uptake

We first determine the concentration needed to saturate the uptake of nitrate by *A. halophytica* under normal and salt stress conditions (0.5 M and 2.0 M NaCl). Figure 3.1 shows that nitrate uptake rates under normal condition were higher than those under salt stress condition. When the cells were assayed with 100 μM $NaNO_3$ the linear increase of nitrate uptake occurred during the first 2 min and the uptake was rather constant after that. Initial uptake rates were determined over a wide range of nitrate concentration (100 to 1000 μ M). The nitrate uptake system was saturable and displayed typical Michaelis-Menten type kinetics. The Lineweaver-Burk transformation of the data under these conditions was performed (Fig. 3.2) and the line of best fit was obtained by using a least squares linear regression. The apparent K_s values for normal and salt stress conditions were 416 and 450 μM respectively, the maximum velocity values (V_{max}) were 9.1 and 5.3 μmol/min/mgChl respectively.

3.1.2 Effect of Na^+ on nitrate uptake

It has been reported that sodium is required for Nrt-dependent nitrate uptake in *Synechococcus* sp. strain PCC 7942 (Rodriguez et al, 1994). We therefore determined whether nitrate uptake by *A. halophytica* is influenced by external sodium. The rate of nitrate uptake

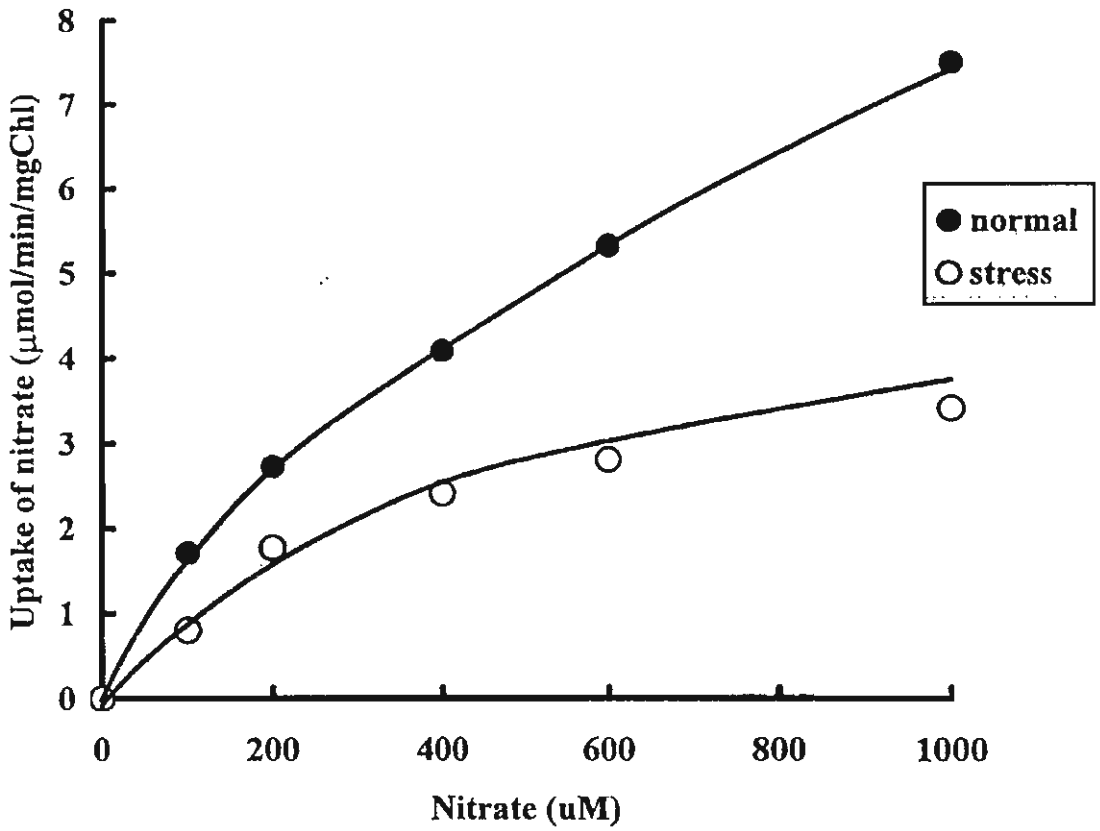

Figure 3.1 Kinetics of nitrate uptake by A.halophytica under normal and salt stress conditions

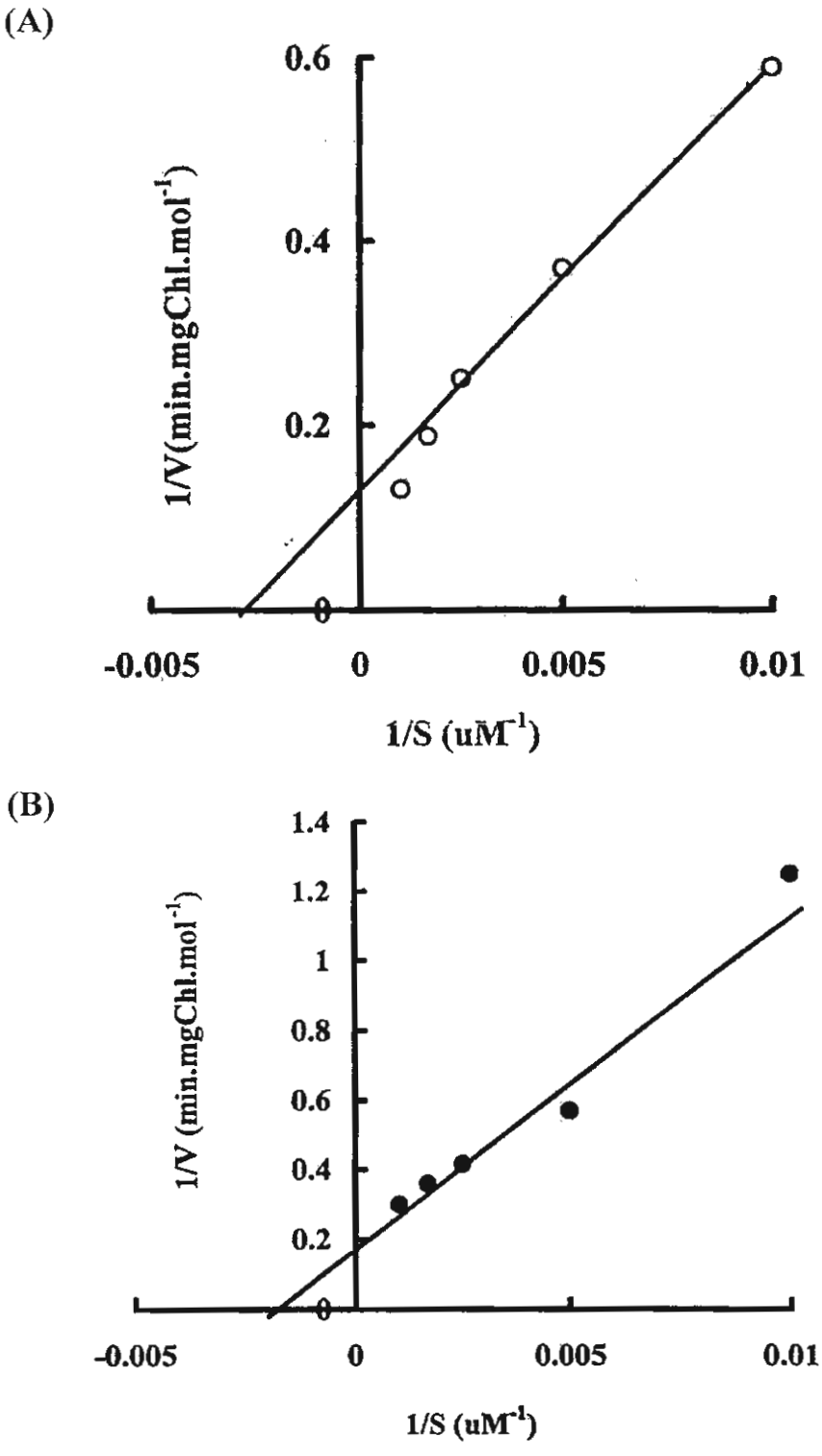

Figure 3.2 Lineweaver-Burk transformation of the data from cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M (B)

was determined in reaction mixtures containing 0 to 10 mM NaCl. Figure 3.3 show that Na^+ could activate nitrate uptake in both normal and salt stress conditions. At a fixed concentration of nitrate used, the uptake of nitrate appeared to reach saturation at 1 mM Na^+ for both conditions. In addition, nitrate uptake was not inhibited by Na^+ as high as 10 mM.

3.2 Effect of various ions on nitrate uptake

3.2.1 Effect of NH_4^+ on nitrate uptake

Figure 3.4 shows the effects of 100 μM ammonium chloride on nitrate uptake in the presence and absence of mM L-methionine sulfoximine (MSX). At 100 μM ammonium chloride, the inhibition of nitrate uptake was hardly observed in the first 2 minutes for both normal and salt stress conditions. However, slightly lower uptake occurred after 2 minutes. MSX did not appear to release the inhibition by ammonium for both normal and salt stress conditions. When higher than 100 μM ammonium chloride was tested, i.e. at 200 μM ammonium, nitrate uptake rate was reduced for both normal and salt stress conditions (Fig. 3.5). Again, MSX had no effect on the release of inhibition by ammonium.

3.2.2 Effect of NO_2^- on nitrate uptake

Nitrite, an alternative nitrogen source, behaves as an effective competitive inhibitor of nitrate uptake. Figure 3.6 shows the effect of 100 μM $NaNO_2$ on nitrate uptake. In both cases (normal and salt stress conditions) the addition of nitrite to the uptake media inhibited the net uptake of nitrate. Figure 3.7 illustrates the response of nitrate uptake rate to four concentrations of nitrite (50, 100, 200, and 400 μM). In

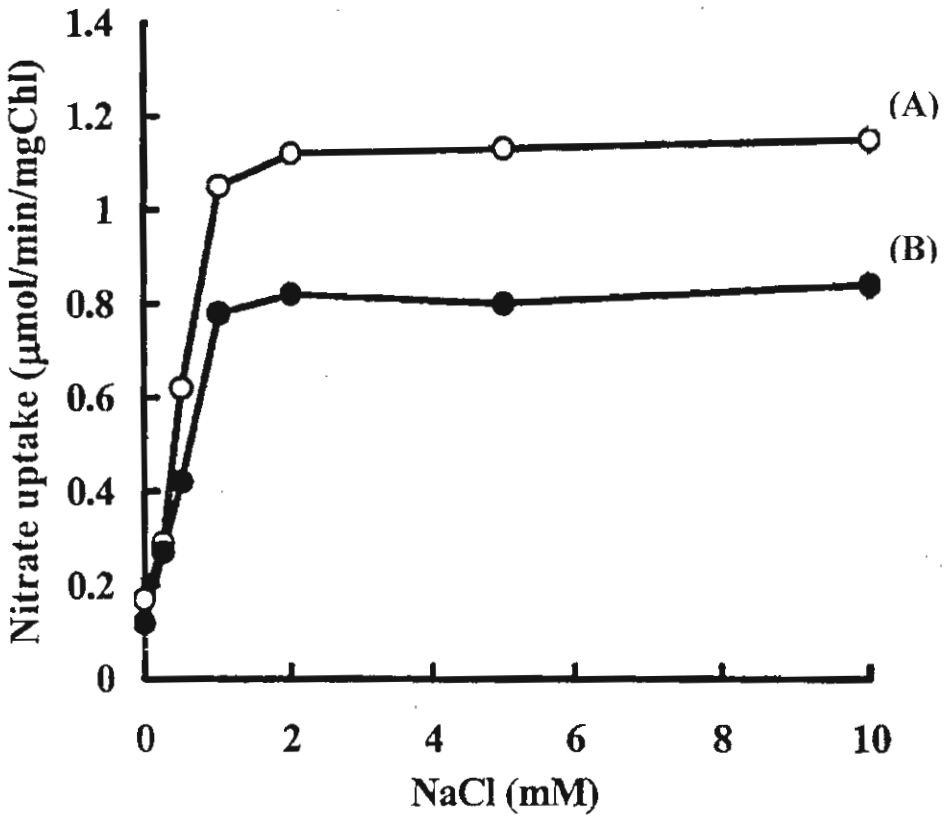

Figure 3.3 Effect of Na^+ concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

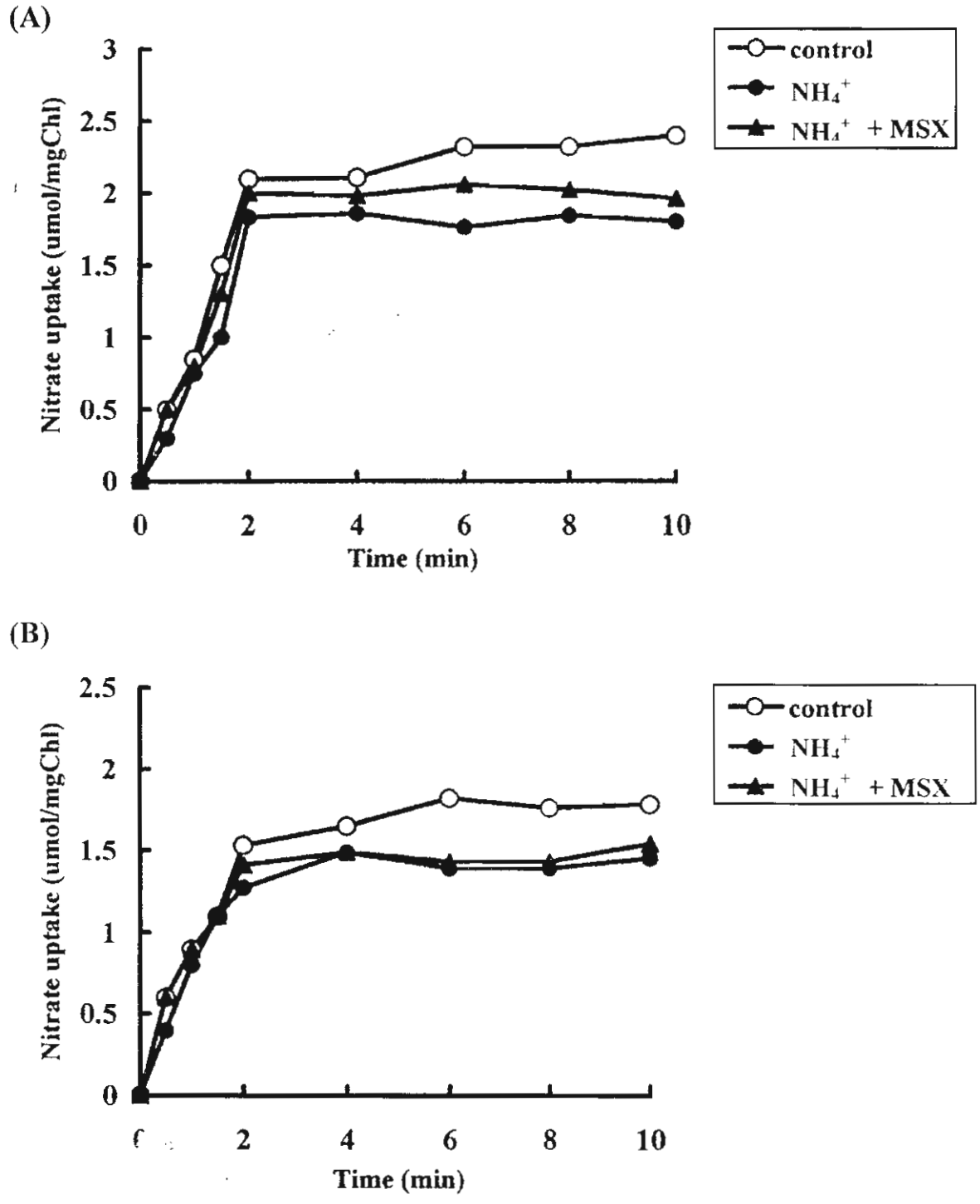

Figure 3.4 Effect of ammonium and MSX on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

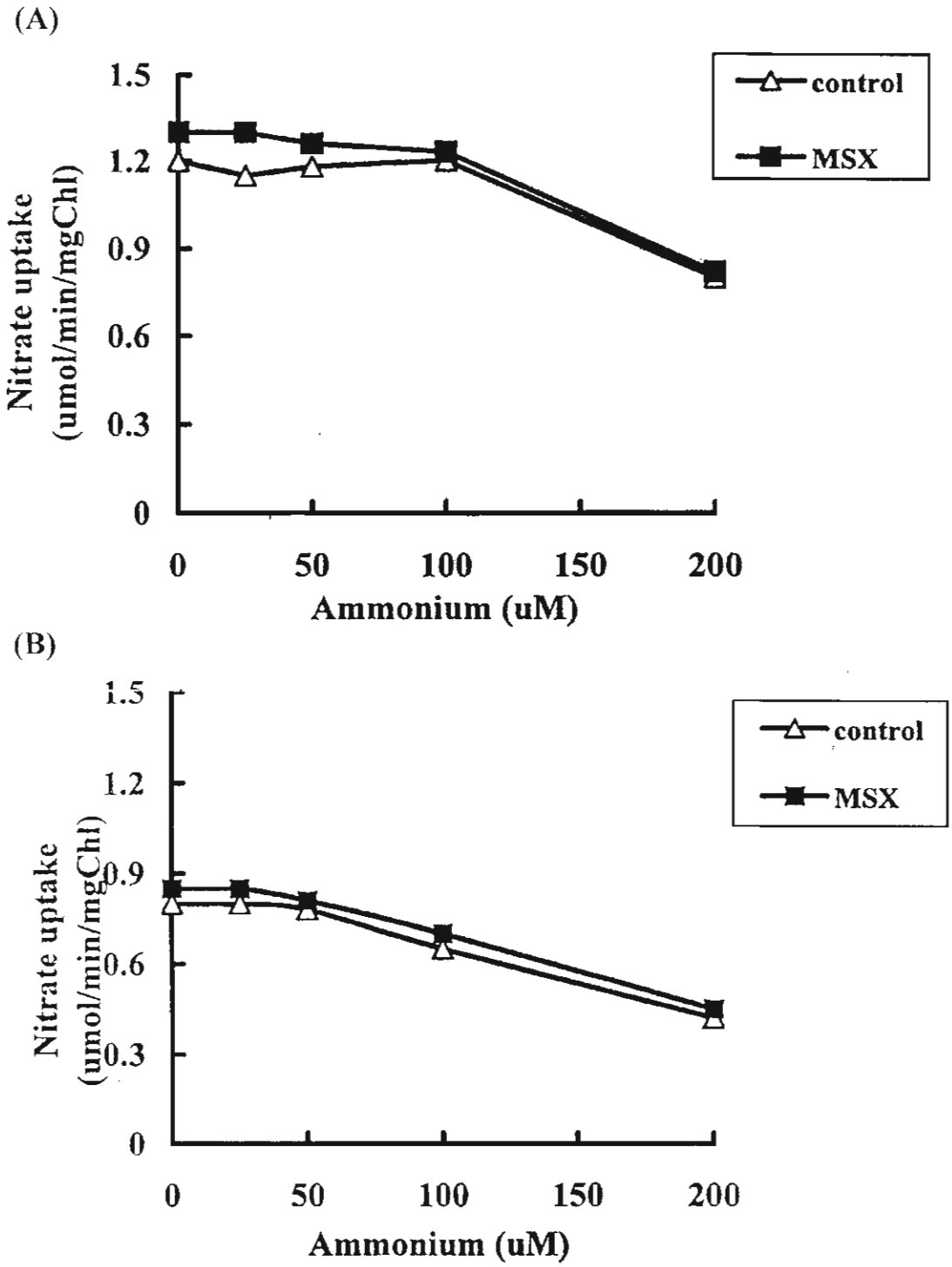

Figure 3.5 Effect of ammonium concentration with or without MSX on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

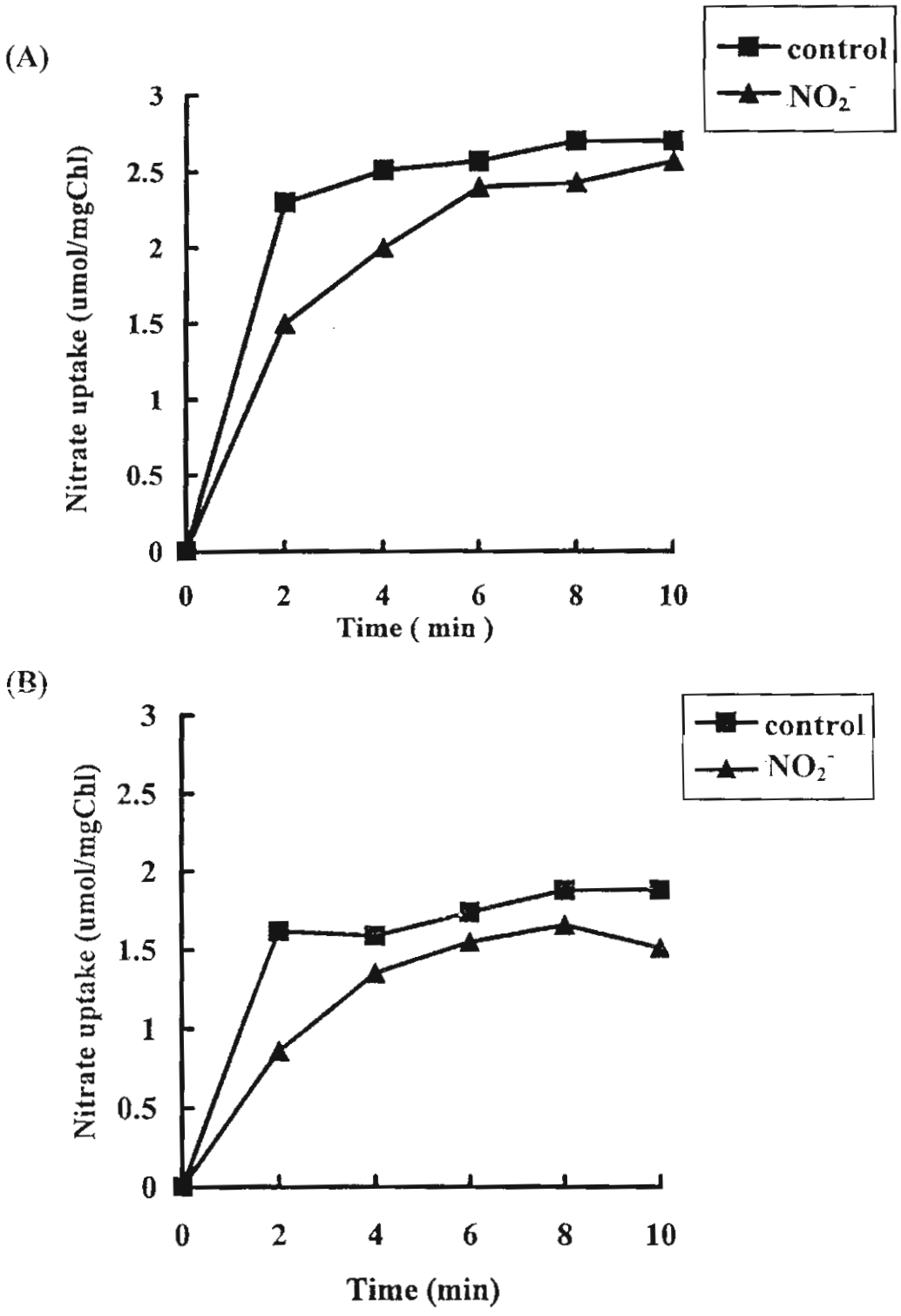

Figure 3.6 Effect of nitrite on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

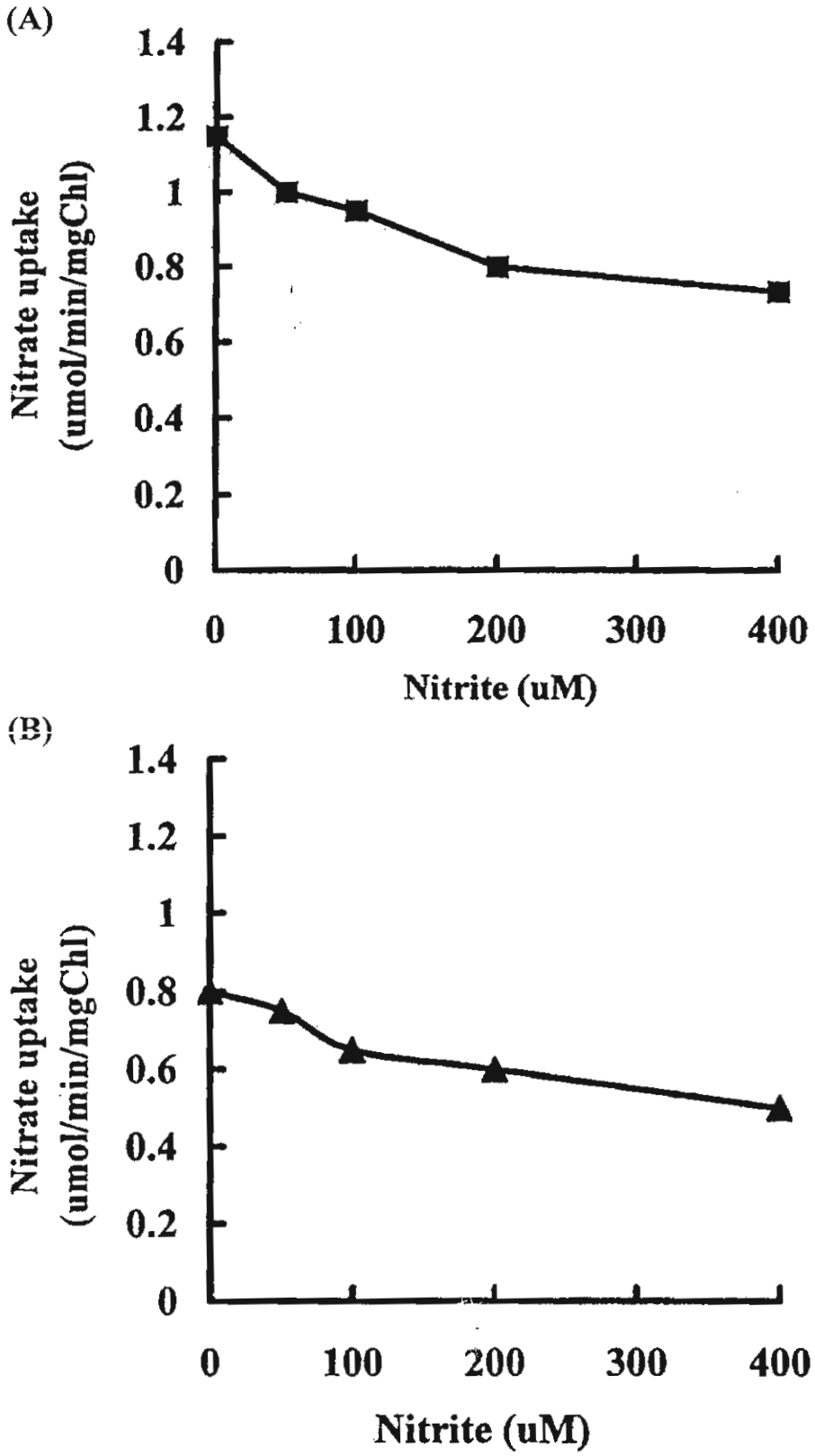

Figure 3.7 Effect of nitrite concentration on nitrate uptake with cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

comparison to the maximum rate (no nitrite), increasing the concentration of NO_2^- in the uptake solution gradually inhibited the uptake of NO_3^-. The inhibition by nitrite appeared to be competitive because the regression lines of the double reciprocal plots at different concentrations of nitrite approached a common intercept (Fig. 3.9, inset). The value of inhibition constant, K_i, obtained by plotting the concentration of nitrite versus slope, was 84 μM (Figs. 3.8 and 3.9). This indicates that nitrite binds to the nitrate transporter with very high affinity.

3.2.3 Effect of other anions on nitrate uptake

Previous studies on several genotypes of the genus *Hordeum* compared net uptake of nitrate from the solution containing equimolar concentration of chloride. The kinetics of net nitrate uptake suggested that the presence of chloride might inhibit nitrate uptake (Smith 1973). We tested the hypothesis by adding nitrate with various anions to the assay medium. Figure 3.10 shows the effects of chloride and phosphate on nitrate uptake. Both 100 μM chloride and 100 μM phosphate did not inhibit nitrate uptake compared with the control treatment in both normal and salt stress conditions. The results suggest that chloride and phosphate have no effect on nitrate uptake in *A. halophytica*.

3.3 Effect of various inhibitors on nitrate uptake

3.3.1 Effect of DL-glyceraldehyde (DLG)

As previously reported in *Synechococcus* sp. PCC 7942 (Rodriguez et al, 1998), nitrate uptake was severely inhibited by preincubation of the cells with DLG (a selective inhibitor of CO_2 fixation). We have tested this hypothesis in *A. halophytica* cells by

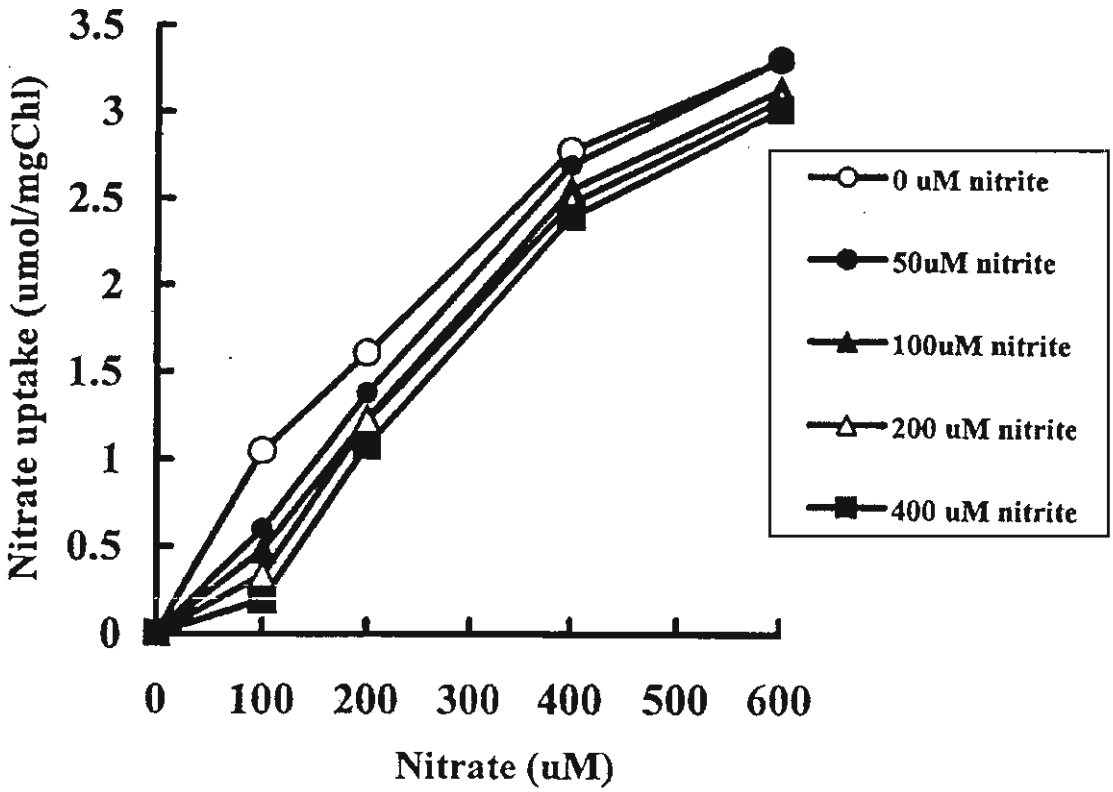

Figure 3.8 Kinetics of nitrate uptake as influenced by various concentrations of nitrite

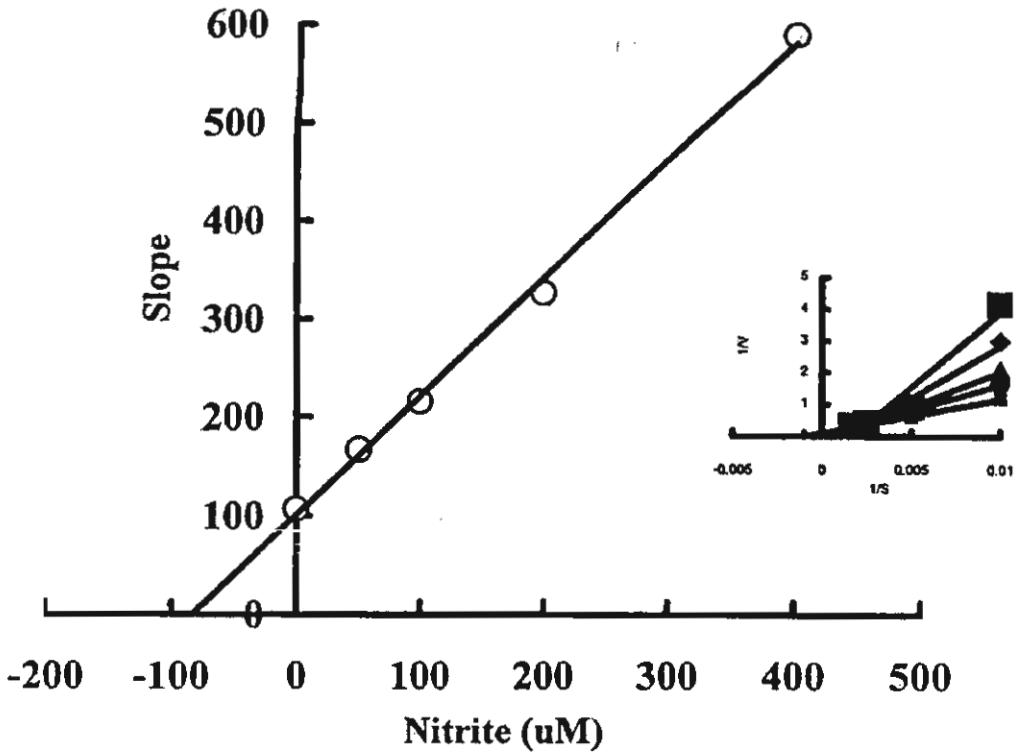

Figure 3.9 Relationship between various nitrite concentrations and slopes obtained from double reciprocol plots

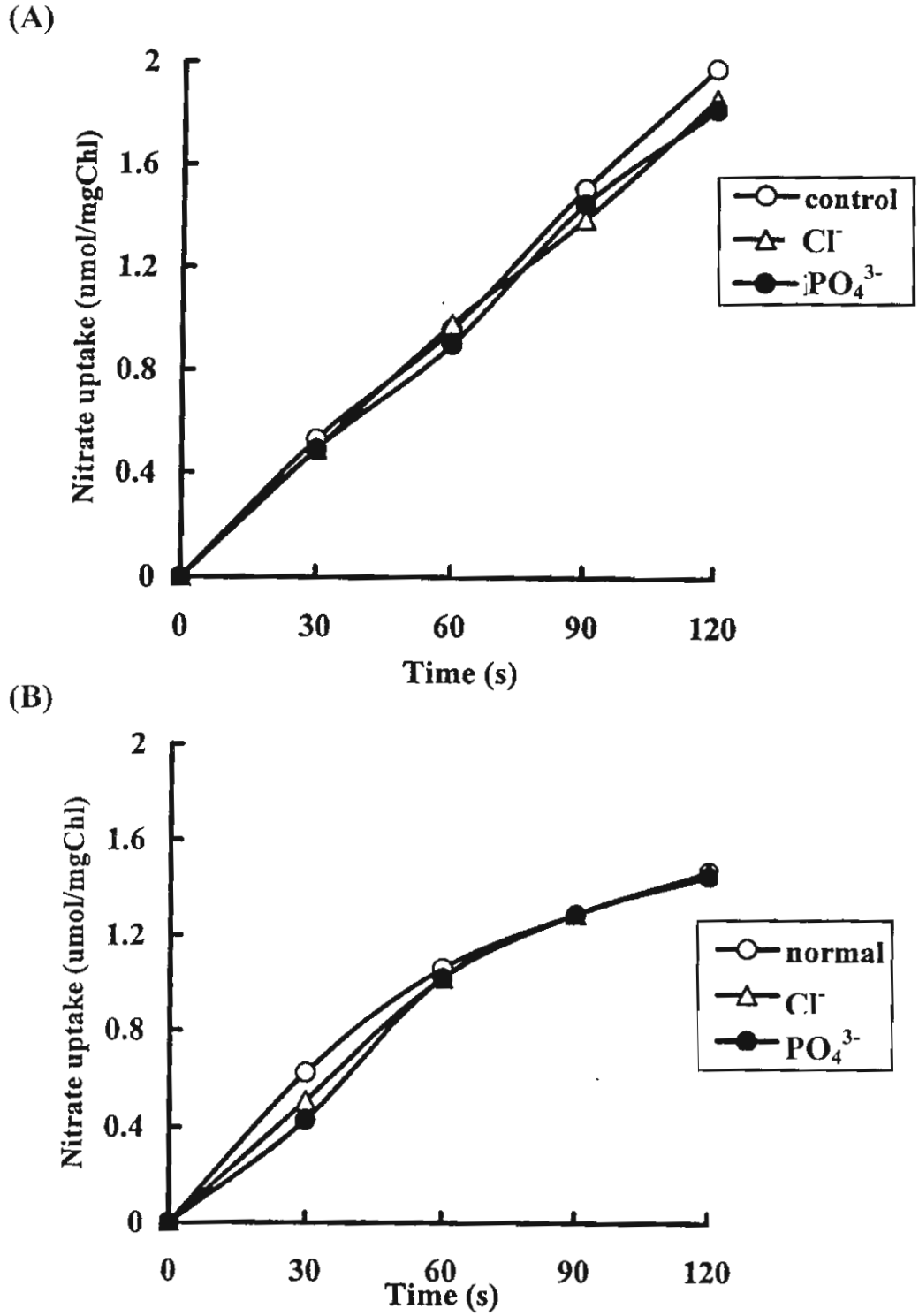

Figure 3.10 Effect of various anions on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

preincubation of the cells with 30 mM DLG for 30 min in the dark prior to the nitrate uptake assay. In both normal and salt stress cells the data show that when DLG, a selective inhibitor of CO_2 fixation, was present in the assay medium, cells of *A. halophytica* consumed nitrate from the medium, but the rate of nitrate uptake was slower than that in the absence of DLG (Fig. 3.11). The nitrate uptake rates declined with increasing concentration of DLG (Fig. 3.12). These results strongly indicated the dependence of nitrate uptake upon active carbon assimilation. The results presented here confirm that nitrate uptake in *A. halophytica* is partly dependent on active CO_2 fixation.

3.3.2 Effect of metabolic inhibitors on nitrate uptake

The effects of various inhibitors that interfere with ion gradients, i.e. disrupting Na^+ electrochemical gradients (monensin), inhibiting Na^+/H^+ antiportor and/or Na^+ channel (amiloride), blocking proton channel (DCCD) and protonophore (CCCP) on the initial rate of nitrate uptake were examined. Inhibitors were preincubated with the cells for 30 min prior to the nitrate uptake assay. The results are shown in Figs. 3.13, 3.15, 3.17, 3.19. All inhibitors inhibited nitrate uptake in both normal and salt stress conditions. In comparison to the maximum rate obtained without inhibitors, the nitrate uptake rates declined with increasing concentration of inhibitors (Figs. 3.14. 3.16, 3.18, 3.20).

The concentrations of monensin, amiloride, DCCD and producing 50% inhibition of nitrate uptake in normal condition were 23, 245, 32, and 22 μM, respectively. The concentrations of monensin, amiloride, DCCD and CCCP producing 50% inhibition of nitrate uptake in salt stress condition were 25, 160, 75, and 18 μM, respectively. CCCP and DCCD appeared to be a more potent inhibitor of nitrate uptake in *A. halophytica*. Inhibition of nitrate uptake was almost

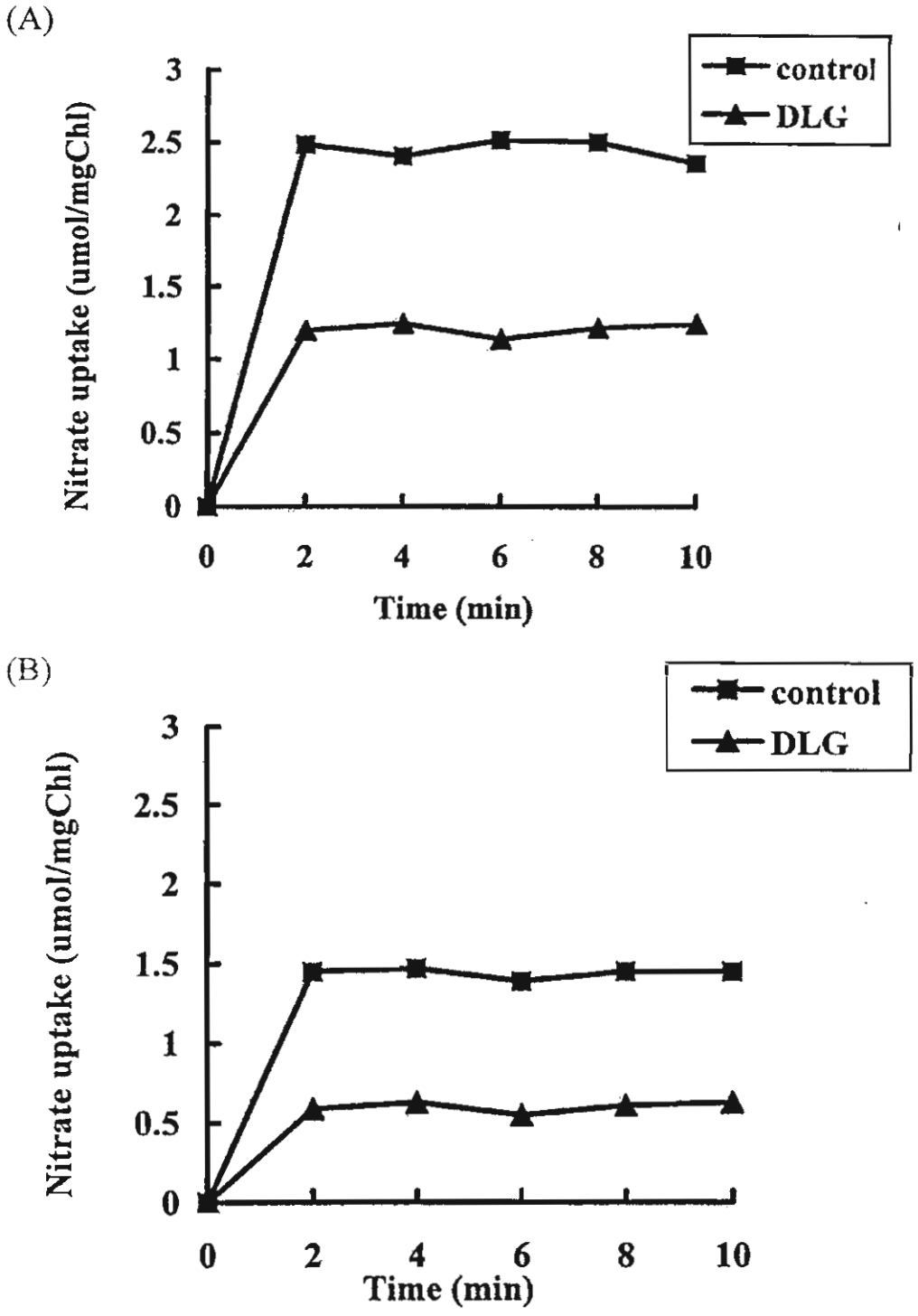

Figure 3.11 Effect of DLG on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

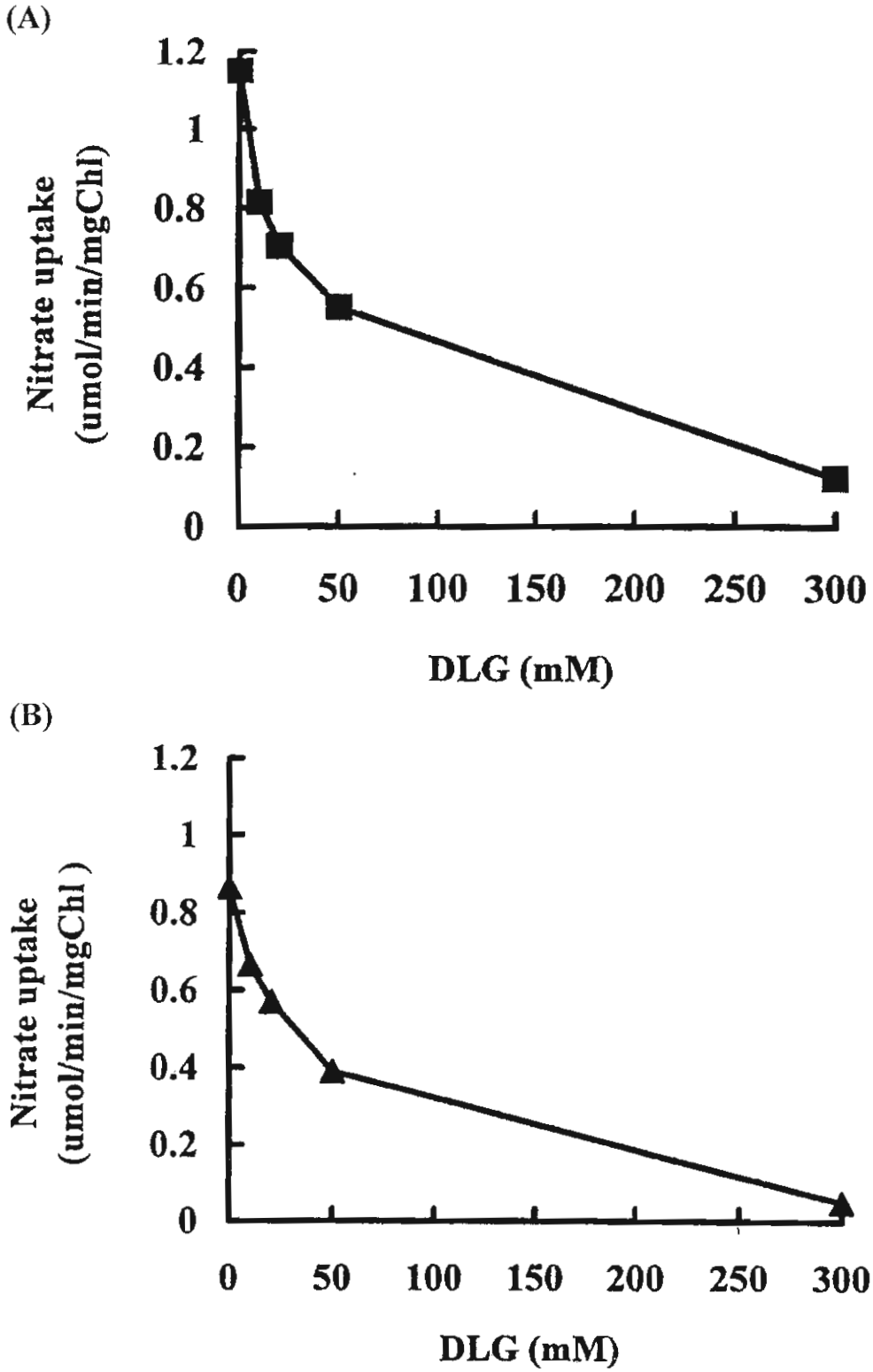

Figure 3.12 Effect of DLG concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

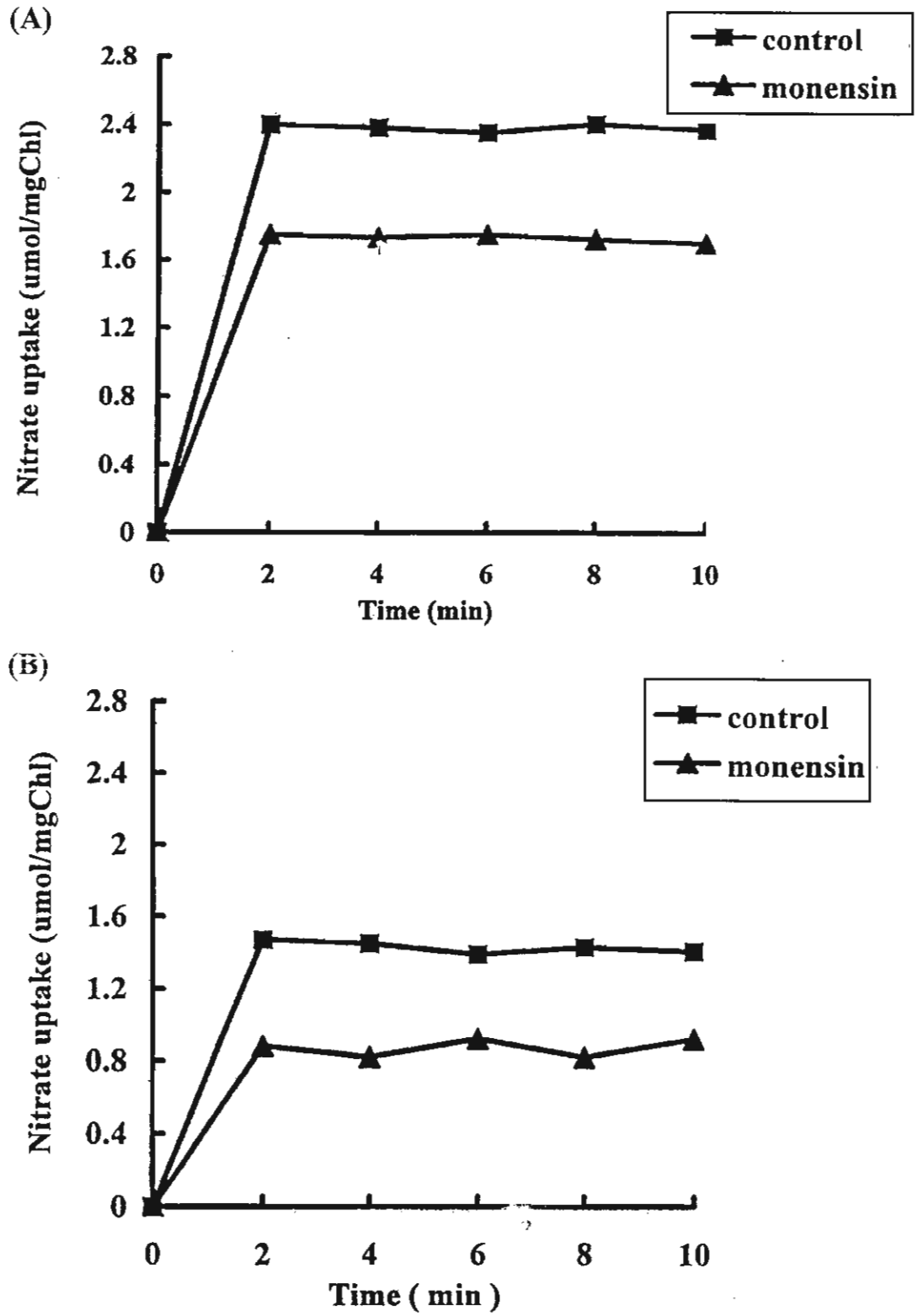

Figure 3.13 Effect of monensin on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

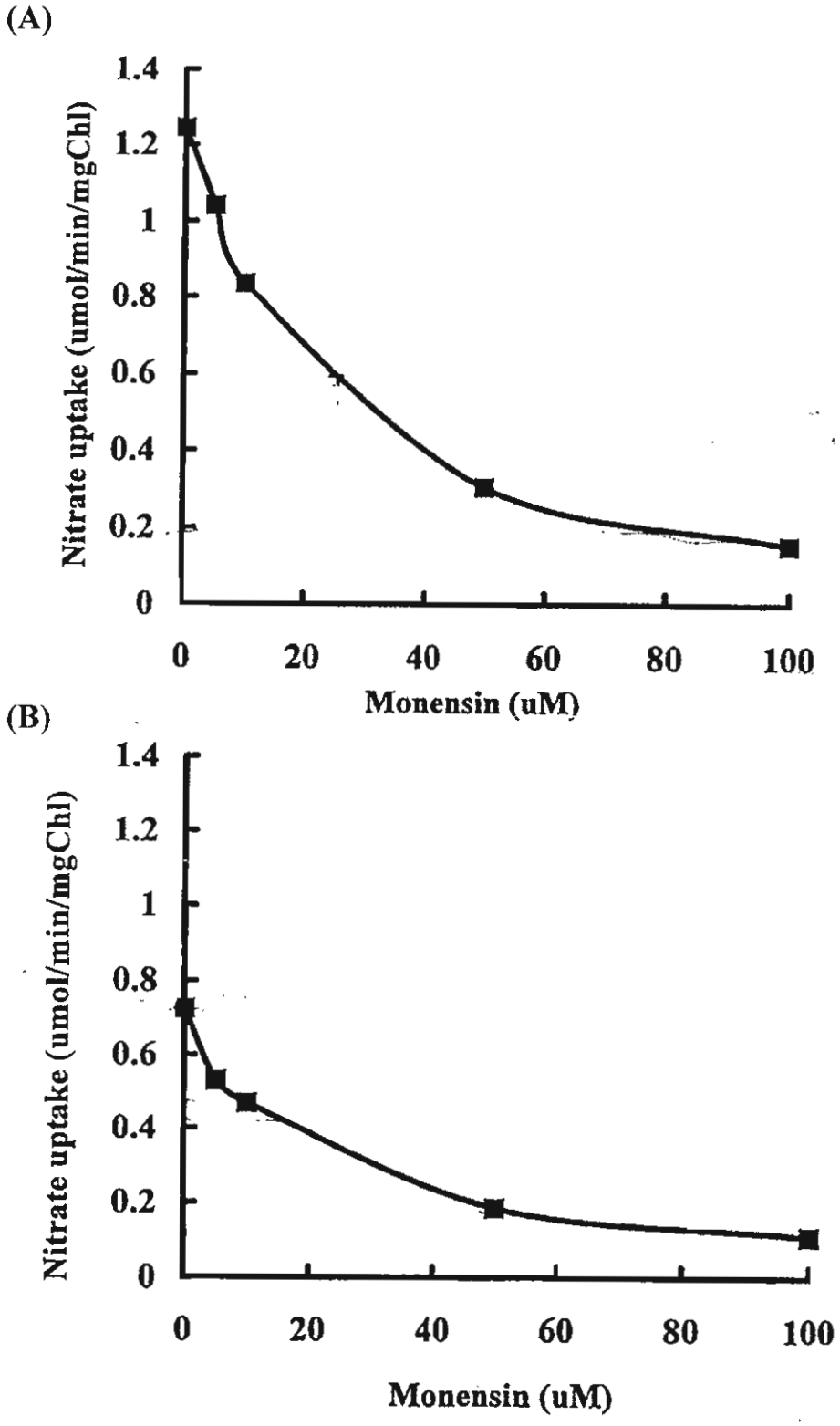

Figure 3.14 Effect of monensin concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

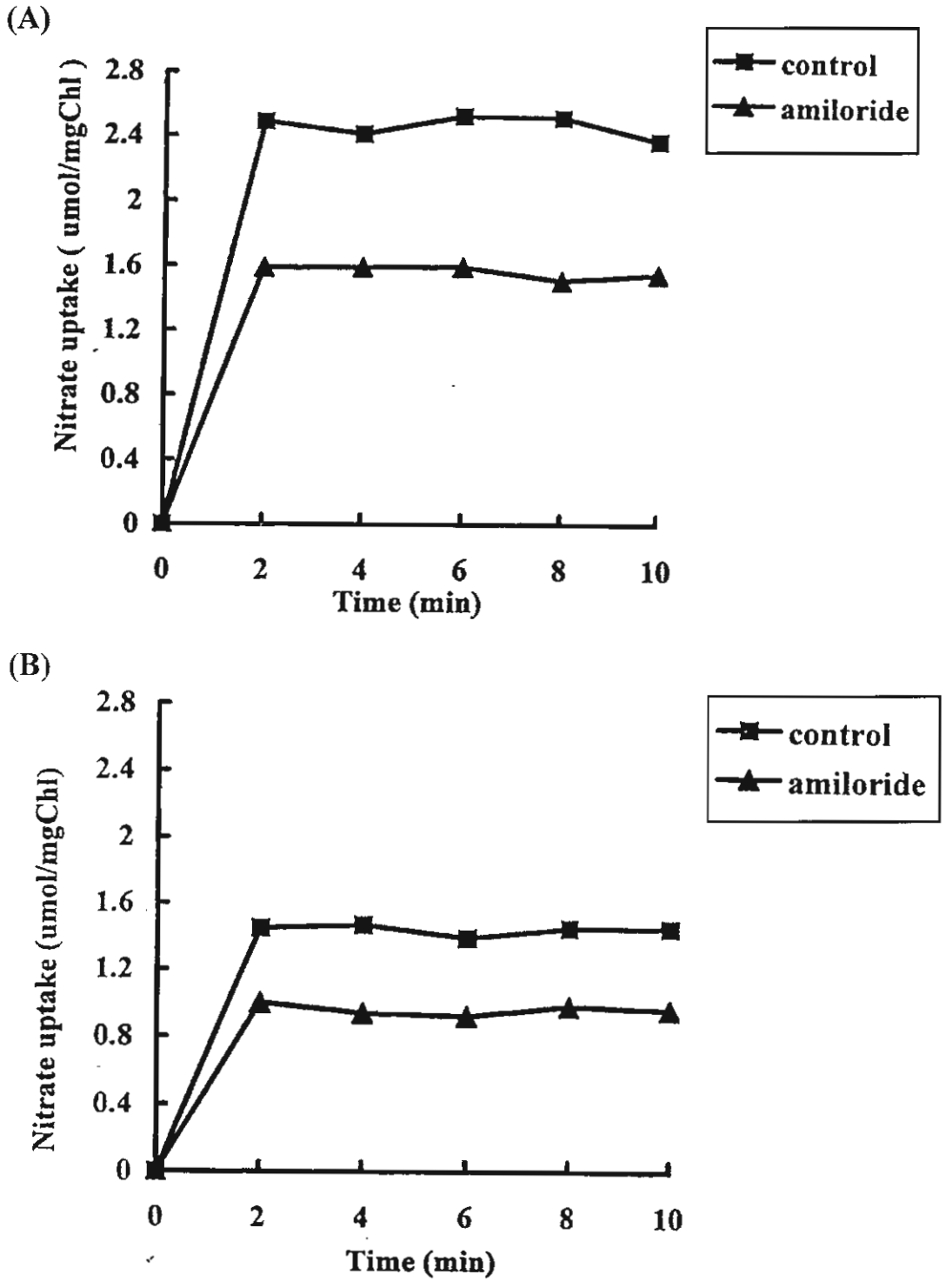

Figure 3.15 Effect of amiloride on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

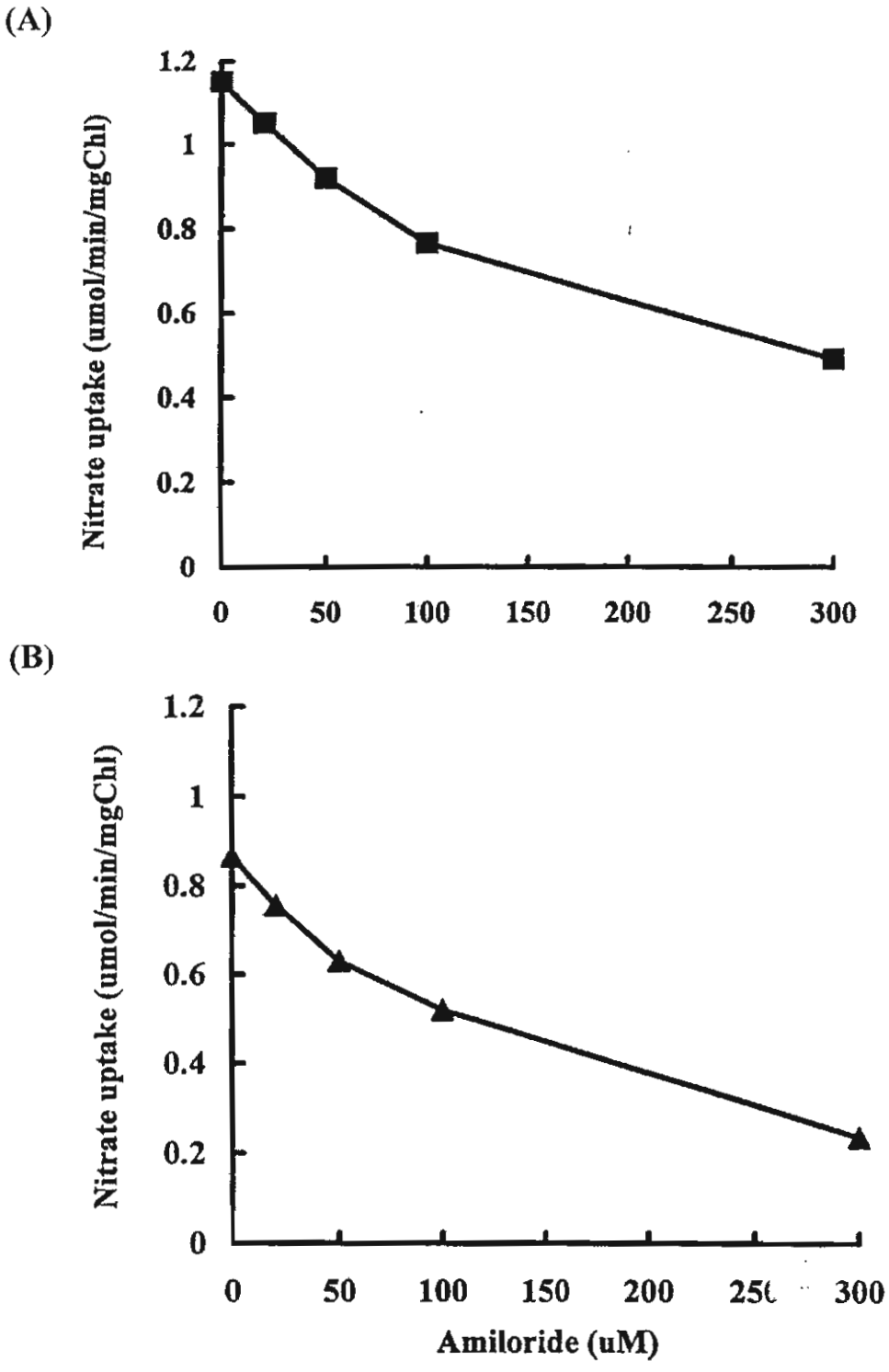

Figure 3.16 Effect of amiloride concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

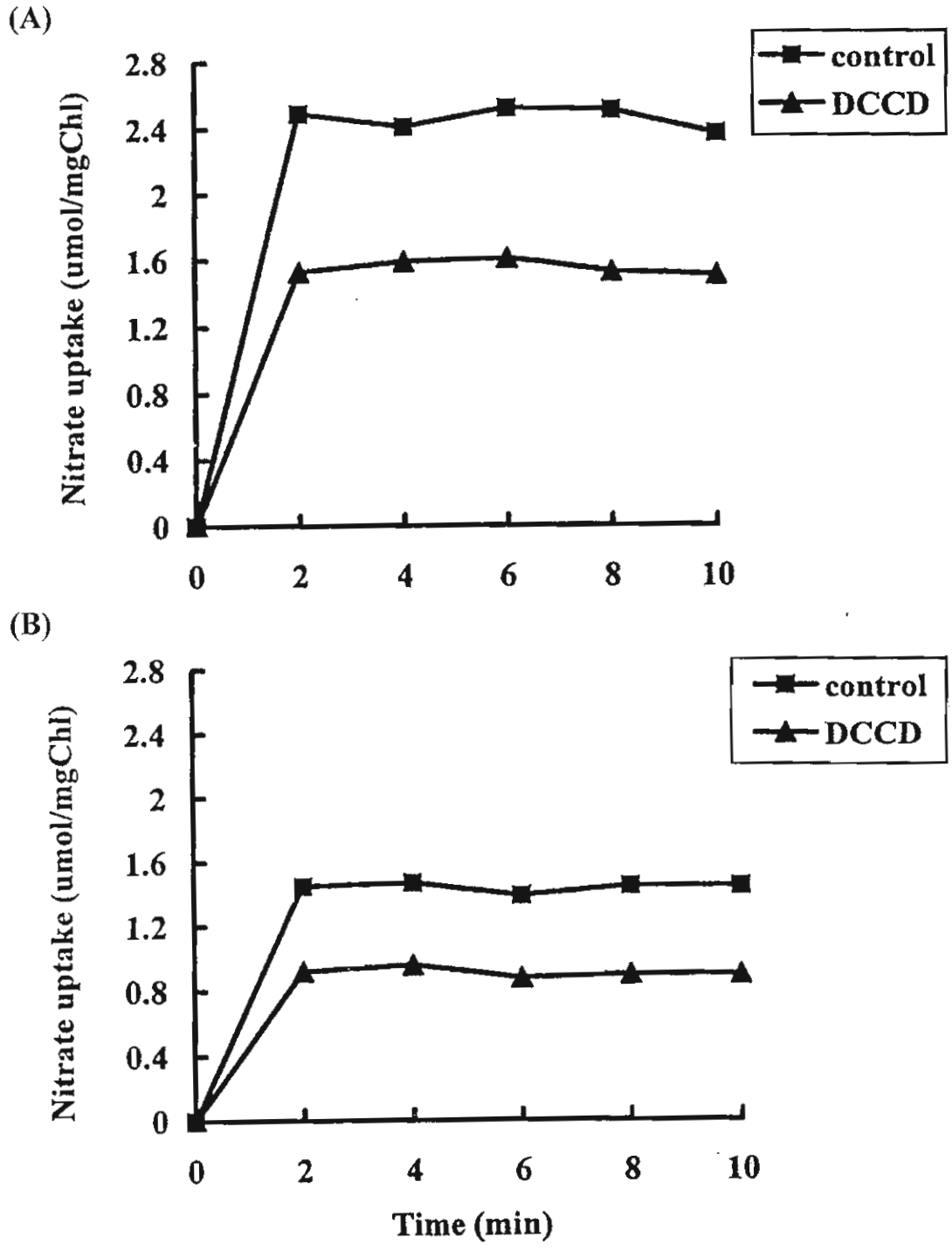

Fig3.17 Effect of DCCD on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

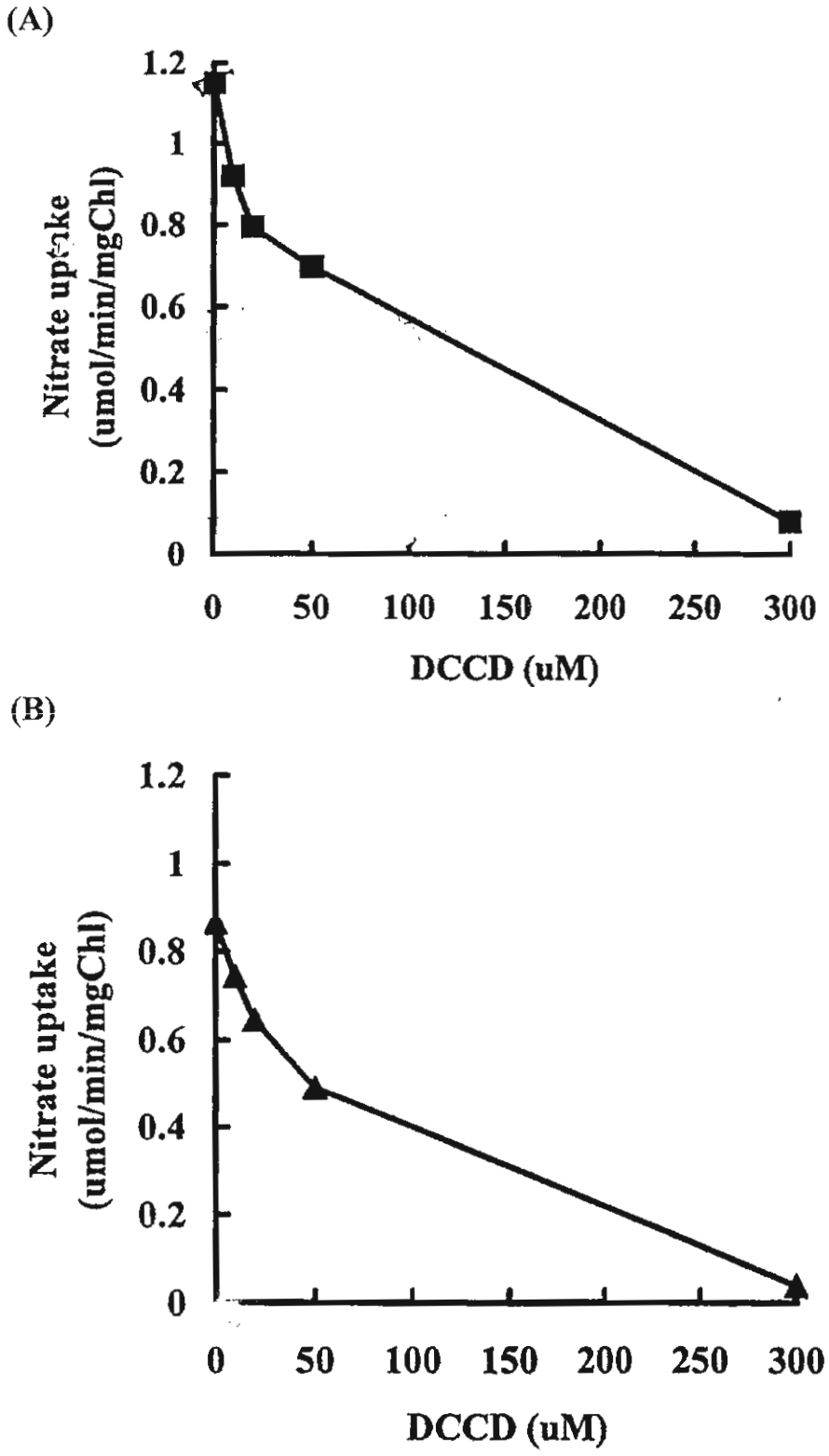

Figure 3.18 Effect of DCCD concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

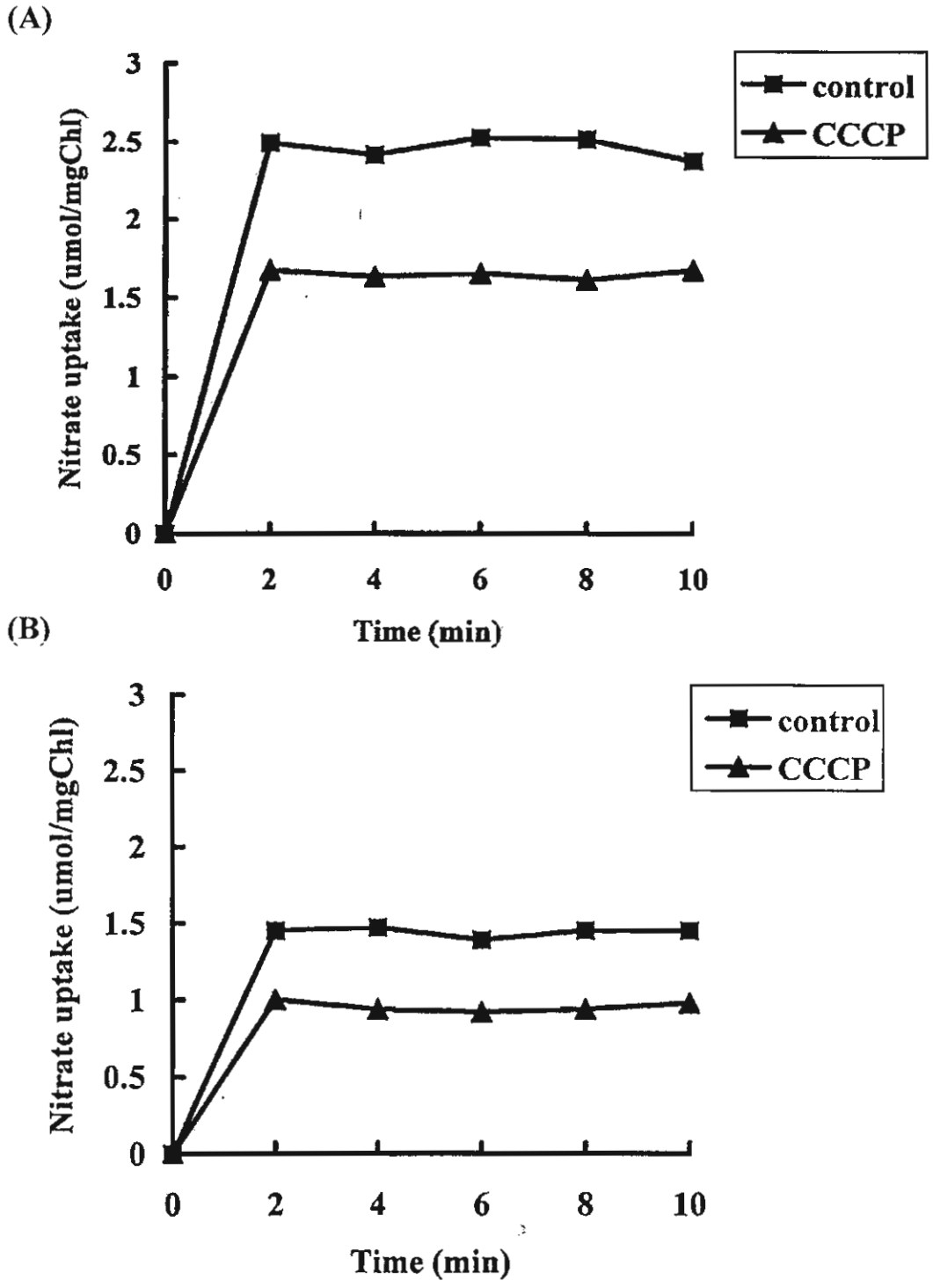

Figure 3.19 Effect of CCCP on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

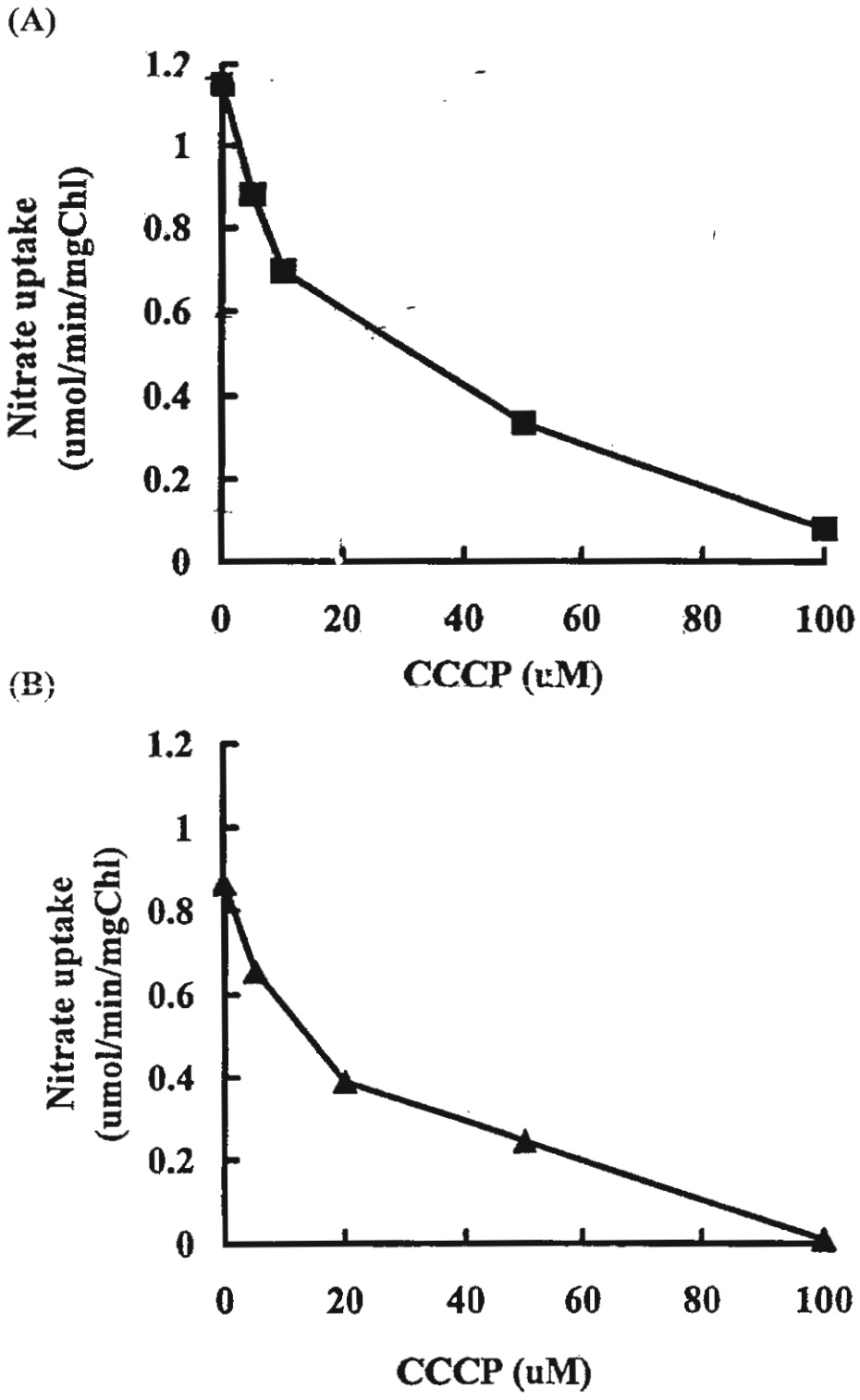

Figure 3.20 Effect of CCCP concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

complete in the presence of 300 μM CCCP and 300 μM DCCD (Figs, 3.18, 3.20). These results seem to suggest that a pH gradient generated by H^+/ATPase drives nitrate uptake in *A. halophytica*.

The monensin effect indicates that nitrate uptake relies on the maintenance of a sodium electrochemical gradient across the plasmalemma, which may represent an immediate source of energy for active nitrate uptake. Amiloride treatment of *A. halophytica* resulted in a decreased rate of nitrate uptake. These data suggest that nitrate uptake in *A. halophytica* cells is partly attributed to the Na^+/H^+ antiporter activity.

3.3.3 Combined effects of metabolic inhibitors on nitrate uptake

Treatments that decrease ATP synthesis (i.e. by DCCD) and treatments dissipating the proton and Na^+ gradients (i.e. by CCCP and monensin) led to the reduced nitrate uptake. We therefore investigated further how each individual inhibitor of driving force when present alone or in combination would affect nitrate uptake. Figure 3.21 shows that either monensin or CCCP or DCCD could reduce nitrate uptake by about 50% when compared to that in the absence of inhibitor. When two inhibitors were present together in any combination, only about 10% of nitrate uptake activity was detected. Interestingly, the presence of three inhibitors together almost completely abolished nitrate uptake.

3.4 Effect of energy source on nitrate uptake

In order to test whether exogenously added energy source can influence nitrate uptake, glucose an lactate were employed for this purpose. Glucose or lactate could increase nitrate uptake to a similar extent (Fig. 3.22). When glucose and lactate were present together, there was hardly any further increase of nitrate uptake. *Aphanothece*

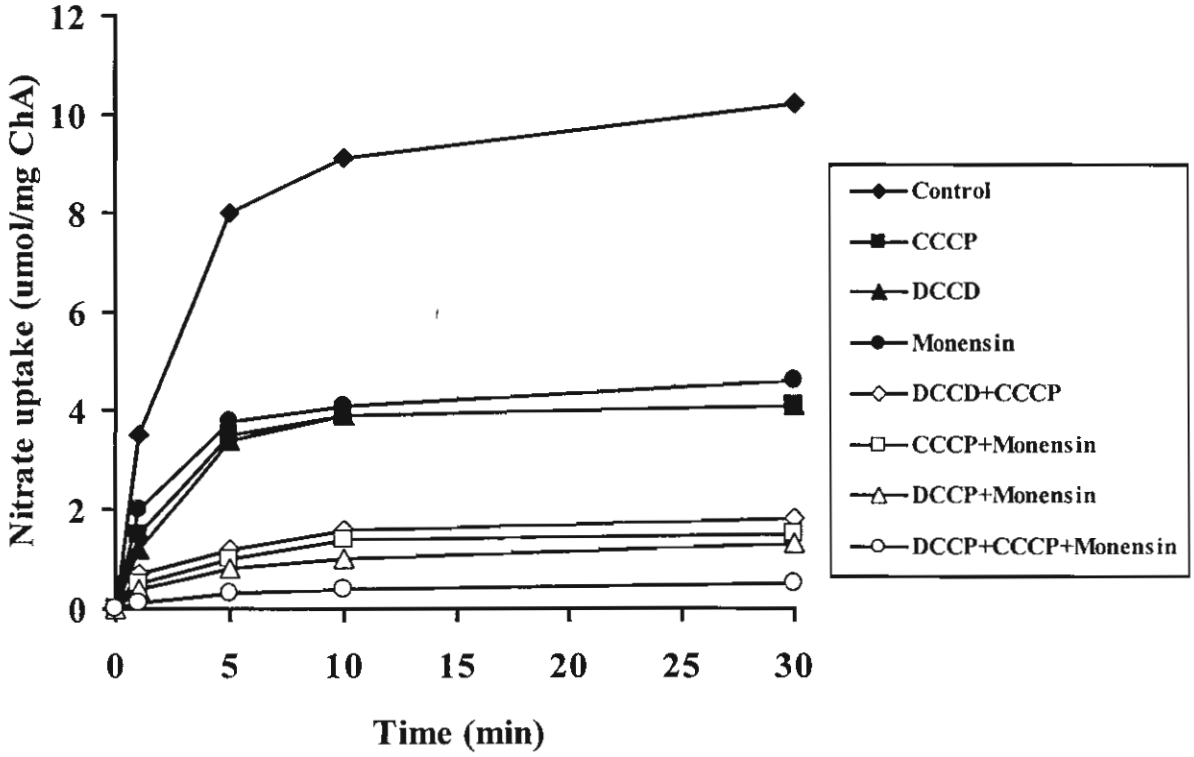

Figure 3.21 Effect of various inhibitors on nitrate uptake by normal cells

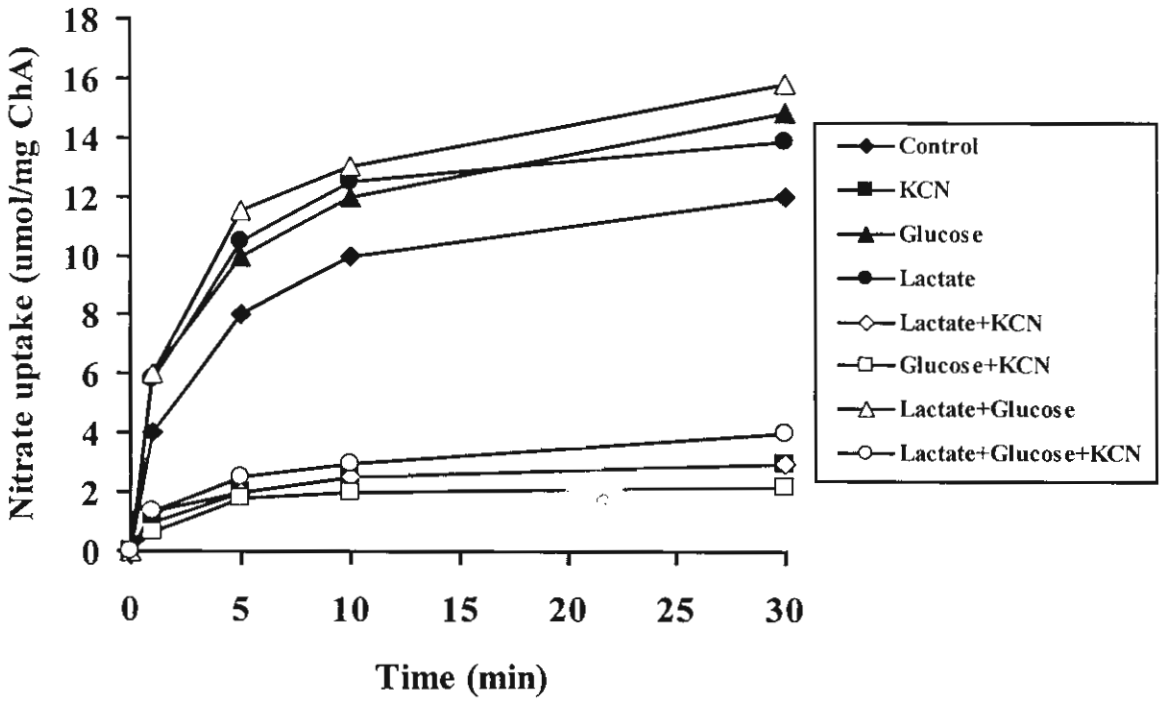

Figure 3.22 Effect of KCN, lactate and glucose on nitrate uptake by normal cells

halophytica cells without energy source supplementation exhibited considerable nitrate uptake. This was probably due to the transport driven by endogenous energy source. This contention was supported by the abolition of nitrate uptake in the presence of KCN, an inhibitor of an energy-yielding respiration. The strong inhibitory effect of KCN on nitrate uptake prevailed regardless of the presence of glucose alone or in combination with lactate. The residual nitrate uptake observed even in the presence of KCN might arise as a result of passive diffusion of nitrate into the cells.

CHAPTER 4

DISCUSSION

Characterization of nitrate uptake system

The effect of salinity on nitrate uptake has been studied in the halotolerant cyanobacterium *Aphanothece halophytica* with reduced nitrate reductase activity and hence the transported nitrate nitrate inside the cells would be minimally reduced. This has been accomplished by tungstate treatment of *A. halophytica* to generate cells with reduced levels of nitrate reductase activity. Molybdenum is a prosthetic group of cyanobacterial nitrate reductase with an essential role in catalysis (Guerrero and Lara, 1987). Under conditions of molybdenum deprivation, tungsten can be incorporated into newly synthesized apoprotein in place of molybdenum, leading to the formation of an inactive nitrate reductase (Lara et al, 1987). Figure 3.1 shows that nitrate uptake in normal condition is higher than that in salt stress condition. Furthermore, the low level of nitrate uptake observed at higher concentrations of external nitrate is probably a case of passive diffusion.

When nitrate concentration was varied the nitrate uptake system followed typical Michaelis-Menten kinetics. Using Lineweaver-Burk transformation of the data, *A. halophytica* showed K_s values in normal and salt stress conditions of 416 and 450 μM respectively. Similarity in K_s values suggest that salt stress causes no alteration on the binding site of the nitrate transport protein. The K_s values for nitrate uptake by *A. halophytica* was much hither than that reported for a filamentous cyanobacterium *Anabaena* sp. PCC 7120 (Ks = 31 μM) (Rai and Tiwari, 1999). In this respect it should be noted that in the present study 0.5 M sorbitol was present

in the assay medium of nitrate uptake to maintain the turgor pressure of *A. halophytica*. The possibility that sorbitol might hinder the binding of nitrate to the transporter cannot be ruled out. However, ***A. halophytica*** also showed a uniphasic nitrate uptake system similar to ***Anabaena*** sp. 7120.

The data presented in this study clearly showed that *A. halophytica* had a reduced uptake of nitrate when grown under salt stress (Fig. 3.1). This is in contrast with the previous report in a nitrogen-fixing *Anabaena torulosa* showing an increase nitrate uptake when grown in 170 mM NaCl (Reddy et al, 1989). The increase of nitrate uptake in *A. torulosa* was related to the increased salt tolerance of the cells via the inhibition of Na^+ influx which is proposed to be a major mechanism for protection of *A. torulosa* against salt stress. In the present study, log-phase *A. halophytica* grown under salt stress was used. At this phase of growth salt stress adaptation via the accumulation of glycine betaine has been complete. This was substantiated by the previous observation that the accumulation of glycine betaine already reached mixiumum after 5 days of growth in the medium containing 1.5 M NaCl (Ishitani et al, 1993). Therefore, a reduced nitrate uptake by *A. halophytica* grown under salt stress may not be related to salt stress adaptation. Another contributing factor for the salt stress protection is via the regulation of intracellular Na^+. Recently it has been reported that the efflux of Na^+ catalyzed by a Na^+ / H^+ antiporter appeared to be a major mechanism for salt stress protection of *A. halophytica* (Waditee et al, 2001 and Waditee et al, 2002).

Na^+ requirement for nitrate uptake

The uptake of nitrate by *A. halophytica* was dependent on the presence of Na^+ as illustrated in Fig. 3.3 This finding agrees with a previous study in *Anacystis nidulans* R2 (Rodriguez et al, 1994). The study showing that monensin, a dissipator of the electrochemical potential for Na^+, could

inhibit nitrate uptake also supported the requirement of Na^+ for nitrate uptake by *A. halophytica* (Fig. 3.14). Not only is nitrate uptake dependent on Na^+ but the uptakes of other solutes also require Na^+ as coupling ions in a number of organisms, most notably in alkalophilic and halophilic bacteria (Rosen, 1986). Stimulation of the uptake of glycine betaine (Moore et al, 1987) and choline (Incharoensakdi and Karnchanatat, 2003) by Na^+ has also been shown in *A. halophytica*.

Inhibitory effect of NH_4^+ on nitrate uptake

Previous studies on nitrate uptake by some cyanobacteria and microalgae have shown the inhibitory effect of ammonium and that the inhibition was found to be mediated by ammonium assimilation products (Omata, 1995). Our results in Fig. 3.5 also give further support for nitrate uptake inhibition by ammonium. However, it is not certain whether the inhibition of nitrate uptake by ammonium has any connection with the process of ammonium assimilation. We found little or no effect of methionine sulfoximine, an ammonium assimilation inhibitor, on the release of nitrate uptake from ammonium inhibition. There exists the possibility that the effect of ammonium on nitrate uptake observed in *A. halophytica* was through stimulation of nitrate efflux rather than inhibition of nitrate influx. A recent study in barley roots demonstrated the increased nitrate efflux upon exposure to ammonium (Kronzucker et al, 1999). Nevertheless, it is reasonable to expect that ammonium might have direct effect on the transport systems as well as effects at the level of transcription via products of ammonium assimilation. It was demonstrated many times that in the presence of both ammonium and nitrate in the medium, the ammonium is assimilated first, and only when it has gone is nitrate utilized. However there have been few reports showing preferential assimilation of nitrate; this phenomenon bas been reported e.g. for *Pandorina* and *Haematococcus*.

Effect on NO_2^- and anions on nitrate uptake

Nitrite is a particularly interesting candidate for regulation because it appears to be taken up by the same transporters as nitrate (Aslam et al, 1992). The kinetics of nitrate uptake inhibition by nitrite showing low K_i value (Figs. 3.8, 3.9) suggest that the inhibition is of competitive type, i.e. both nitrate and nitrite are taken up by the same uptake system. This is in agreement with previous studies in other cyanobacteria, for example *Synechococcus* sp. PCC 7942 with a nitrate-nitrite bispecific transporter (Maeda and Omata, 1997), *Anacystis nidulans* R2 (Rodriguez et al, 1992). However, a very recent study in a filamentous, heterocystous canobacterium, Nostoc ANTH indicated the existence of separated nitrate and nitrite uptake systems (Bhattacharya et al, 2002). This was based on the results of a chlorate-resistant mutant of Nostoc ANTH which lacked nitrate uptake activity but retained nitrite uptake capacity. Nitrite-specific active transport system has also been recently reported in *Synchococcus* sp. PCC 7942 (Maeda et al, 1998) Whether *A. halophytica* contains nitrite-specific transport system remains a subject of further study.

Both chloride and phosphate ha no effect on the uptake of nitrate by *A. halophytica* (Fig. 3.10). This suggests that the reduced nitrate uptake under salt stress condition is not due to chloride ion. Rather, it may be due to the deleterious effect of high sodium ion. Another possibility of reduced nitrate uptake caused by salt stress is due to its osmotic effect rather than an ionic effect.

Reduction of nitrate uptake by an inhibitor of CO_2 fixation

As the assimilation of nitrogen into protein requires both energy and organic skeleton, it is not surprising that there are major interactions between N-assimilation and photosynthetic metabolism. The assimilation of both

ammonium an nitrate is dependent on phostosynthesis, that is assimilation requires light and CO_2; removal of either of these prevents assimilation. To investigate the relationship between nitrate assimilation an carbon dependence in *A. halophytica*, we tested whether the interruption of CO_2 fixation affected nitrate uptake. It turned out that DL-glyceraldehyde which is an inhibitor of CO_2 fixation caused a reduction in the nitrate uptake (Fig. 3.12). This indicated the strict dependency of nitrate uptake in *A. halophytica* on active CO_2 fixation. This is logical since CO_2 fixation will provide carbon skeletons as acceptors of ammonium arising from nitrate. The same phenomenon of positive relationship between nitrate uptake an CO_2 fixation was also observed in *Synechococcus* sp. PCC 7942 (Rodriguez et al, 1998). Both nitrate uptake an CO_2 fixation require energy for the process. Although the source of energy for nitrate uptake in cyanobacteria remains unsolved, the Na^+-dependent active transport or the Na^+ / NO_3^- symport system has been suggested which relies on Na^+ electrochemical potential as a driving force (Lara et al, 1993). This would obviate the need for the nitrate uptake process to compete for another energy source, ATP, which as a result would be utilized mainly for CO_2 fixation. Consequently, in terms of energy requirement, the process of nitrate uptake and CO_2 fixation will proceed without conflict. This indirectly reinforces the contention that nitrate uptake in cyanobacteria is Na^+-dependent, at least in *A. nidulans* R2 and *A. halophytica*.

Effect of various inhibitors on nitrate uptake

In an attempt to resolve the nature of nitrate uptake in *A. halophytica* we examined its response to several metabolic inhibitors. The uncouples carbonylcyanide-*m*-chlorophenylhydrazone an the ATPase inhibitor N, N-dicyclohexylcarbodiimide each severely inhibited nitrate uptake in *A. halophytica* in both normal and salt stress conditions (Figs. 3.18, 3.20)

These results suggest that a pH gradient generated by H^+/ATPase drives nitrate uptake in *A. halophytica*.

The plasma membranes of various prokaryotes have Na^+/H^+ antiproter activity. We could not ignore the possibility that the uptake of nitrate into the cells was due to a Na^+/H^+ antiport driven by a pH gradient. Amiloride has been used as an inhibitor of the Na^+/H^+ antiport and/or Na^+ channel blacker (at different concentration), in a wide variety of eukaryotic systems (Krulwich, 1983). Amiloride treatment of *A. halophytica* cells resulted in a decreased rate of nitrate uptake (Fig. 3.16). These data suggest that nitrate uptake in *A. halophytica* cells is caused by the Na^+/H^+ antiporter. However, there have been reports showing that nitrate transport in *Anacystis* is stimulated by amiloride (at pH 8) consistent with inhibition of a Na^+-importing/H^+-extruding antiport (Lara et al, 1993 and Kaplan et al, 1989). Increased Na^+-dependent nitrate transport activity in amiloride-treated cells indicates that the electrochemical gradient of Na^+ required for nitrate transport is not generated by the Na^+/H^+ antiport, but by other means, the sodium circuit may thus be the primary chemiosmotic event in cyanobacteria plasma membranes, at least in cells or species grown at or adapted to alkaline pH (Miller et al, 1984, Brown et al, 1990, Ritchie, 1992).

Nitrate uptake in *A. halophytica* is sensitive to monensin, an ionophore that collapses the electrochemical gradient for sodium, the inhibition being higher than 50% for monensin concentration as low as 30 μM (Fig. 3.14). These observations indicate that nitrate uptake in *A. halophytica* relies on the maintenance of an electrochemical gradient of Na^+ across the plasma membrane, which might represent the immediate source for active nitrate transport. Monensin is a carboxylic polyether ionophore that, in artificial system, causes the electroneutral exchange of Na^+ for H^+ and thereby collapses the Na^+ gradient between the cells and the medium (Pressman, 1976). *Synechococcus* cells also actively extrude Na^+ through an Na^+/H^+

antiporter (Blumwald et al, 1984) or a primary Na^+ pump (Brown et al, 1990). The active extrusion of Na^+ creates an electrochemical potential for Na^+ (Ritchie, 1992). The energy conserved in an electrochemical potential for Na^+ may, therefore, serve as an energy source for the secondary active transport of anions. The Na^+ electrochemical potential comprises an electrical component and a chemical component. The inhibitory effects of monensin and amiloride, which dissipate a chemical component, on nitrate uptake in *A. halophytica* suggest that at least this component of the electrochemical potential of Na^+ plays a role in this transport process. Indeed, an Na^+/H^+ antiporter has been reported to play a role in the extrusion of Na^+ which is one of the adaptive mechanisms for salt tolerance in *A. halophytica* (Waditee et al, 2001).

Energy source of nitrate uptake

It appeared evident from the results showing the inhibitory effect by DCCD, CCCP and monensin that both ATP and electrochemical potential contribute to the driving force for nitrate uptake (Fig, 3.21). Another line of supporting evidence for this notion came from the experiments using glucose (which can generate ATP by respiration) and lactate (which can feed electrons to the electron transport chain, hence producing proton potential across the membrane), both of which could increase nitrate uptake (Fig. 3.22).

In summary, overall results in this project indicate that *A. halophytica* showed the difference in V_{max} of nitrate uptake between non-stress and salt-stress cells. Other parameters tested did not elicit different responses between the two cell types. Under salt stress *A. halophytica*, in a short term response, would utilize Na^+ / H^+ antiporter to extrude Na^+ out of the cytoplasm in exchange for H^+ (Waditee et al, 2001 and Waditee et al, 2002). However, in a long term response to salt stress the uptake of nitrate should

proceed normally. The synthesis and accumulation of an osmolyte glycine betaine can then finally be responsible for salt stress protection in *A. halophytica*. With regard to an energetic aspect, nitrate transport by *A. halophytica* relies on the driving force derived from ATP and from electrochemical potential involving Na^+ and H^+ gradients.

Suggestions for further work

The present study has shown that in *A. halophytica* salt stress could reduce the uptake of nitrate by about one-half compared to that without salt stress. The reduced uptake could be due to the consequence of the adaptation of cells in response to salt stress by extruding Na^+ out of the cells via the function of Na^+/H^+ antiporter. In view of the fact that the uptake of nitrate requires Na^+ as a coupling ion and the tendency by the cells to extrude Na^+ under salt stress, the reduced nitrate uptake is not unexpected. The next step is to explore how the nitrate after entry into the cells can be assimilated further. Specifically, we should investigate how salt stress affects the function of nitrate reductase, an enzyme that catalyzes the formation of nitrite from nitrate. The information obtained will pave way for a better understanding of nitrogen metabolism in *A. halophytica* under salt stress.

REFERENCES

Aslam M, Travis RL, Huffaker RC (1992) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. Plant Physiol 99 : 1124-1133

Bassham JA, Larsen PO, Lawyer AL, Cornwell KL (1981) Relationships between nitrogen metabolism and photosynthesis. In : Bewley JD (ed) Nitrogen and carbon metabolism. London : Junk, pp 135-163

Beevers L, Hageman RH (1983) Uptake and reduction of nitrate : bacteria and higher plants. In : Lauchli A, Bieleski RL (eds) Encyclopedia of plant physiology, new series, vol 15 A. Berlin : Springer Verlag, pp 351-375

Bhattacharya J, Singh AK, Rai AN (2002) Isolation and characterization of a chlorate-resistant mutant (C10-R) of the symbiotic cyanobacterium *Nostoc* ANTH : heterocyst formation and N_2-fixation in the presence of nitrate, and evidence for separate nitrate and nitrite transport systems. Curr Microbiol 45 : 99-104

Flores E, Herrero A (1994) Assimilatory nitrogen metabolism and its regulation. In : Bryant DA (ed) The molecular biology of cyanobacteria. Dordrecht : Kluwer, pp 487-517

Blumwald E, Wolosin, J M & Packer, L. (1984). Na+/H+ exchange in the cyanobacterium *Synechococcus* 6311. Biochem Biophys Res Commun 122 : 452-459

Brown I I, Fadeyey, S I, Severina, I I & Skulachev, V P (1990). Light-dependent ΔμNa-generation and utilization in the marine cyanobacterium *Oscillatoria brevis*. FEBS Lett 270 : 203-206

Campbell WH (1999) Nitrate reductase structure, function and regulation : bridging the gap between biochemistry and physiology. Annu Rev Plant Physiol Plant Mol Biol 50 : 277-303

Glab J, Kaiser WM (1995) Inactivation of nitrate reductase involves NR protein phosphorylation and subsequent binding of an inhibitor protein. Planta 195 : 514-518

Guerrero MG, Lara C (1987) Assimilation of inorganic nitrogen. In : Fay P, van Baalen C (eds) The Cyanobacteria. Amsterdam : Elsevier, pp 163-186

Guerrero MG, Romero JM, Rodriguez R, Lara C (1990) Nitrate transport in cyanobacteria. In : Ullrich WR, Rigano C, Fuggi A, Aparicio PJ (eds) Inorganic nitrogen in plants and microorganisms. Berlin : Springer Verlag, pp 79-85

Herrero A, Flores E, Guerrero MG (1981) Regulation of nitrate reductase levels in the cyanobacteria *Anacystis nidulans*, *Anabaena* sp. strain 7119 and *Nostoc* sp. strain 6719. J Bacteriol 145 : 175-180

Huber JL, Huber SC, Campbell WH, Redinbaugh MG (1992) Reversible light/dark-modulation of spinach leaf nitrate reductase involves protein phosphorylation. Arch Biochem Biophys 296 : 58-65

Incharoensakdi A, Karnchanatat A (2003) Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*. Biochim Biophys Acta 1621 : 102-109

Ishitani M, Takabe T, Kojima K, Takabe T (1993) Regulation of glycine betaine accumulation in the halotolerant cyanobacterium *Aphanothece halophytica*. Aust J Plant Physiol 20 : 693-703

Kaplan A, Scherer S, Lerner M (1989) Nature of the light-induced H^+ efflux and Na^+ uptake in cyanobacteria. Plant Physiol 89 : 1220-1225

Krulwich TA (1983) Na^+/H^+ antiporters. Biochim Biophys Acta 726 : 245-264

Lara C, Rodriguez R, Guerrero MG (1993) Sodium-dependent nitrate transport and energetics of cyanobacteria. J Phycol 29 : 389-395

Lara C, Romero JM, Guerrero MG (1987) Regulated nitrate transport in the cyanobacterium *Anacystis nidulans*. J Bacteriol 169 : 4376-4378

Maeda S, Okamura M, Kobayashi M, Omata T (1998) Nitrite specific active transport system of the cyanobacterium *Synechococcus* sp. strain PCC 7942. J Bacteriol 180 : 6761-6763

Maeda S, Omata T (1997) Substrate binding lipoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in the transport of nitrate and nitrite. J Biol Chem 272 : 3036-3041

Manzano C, Candau P, Gomez-Moreno C, Relimpio AM, Losada M (1976) Ferredoxin-dependent photosynthetic reduction of nitrate and nitrite by particles of *Anacystis nidulans*. Mol Cell Biochem 10 : 161-169

Mikami B, Ida S (1984) Purification and properties of ferredoxin-nitrate reductase from the cyanobacterium *Plectonema boryanum*. Biochim Biophys Acta 791 : 294-304

Miller A G, Turpin D H, Canvin D T (1984) Na^+ requirement for growth, photosynthesis, and pH regulation in the alkalotolerant cyanobacterium *Synechococcus leopoliensis* J Bacteriol 159 : 100-106

Moore DJ, Reed RH, Stewart WDP (1987) A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine-synthesizing cyanobacteria. Arch Microbiol 147 : 399-405

Omata T, Andriesse X, Hirano A (1993) Identification and characterization of a gene cluster involved in nitrate transport of the cyanobacterium *Synechococcus* sp. PCC 7942. Mol Gen Genet 236 : 193-202

Omata T. Ohmori M, Arai N, Ogawa T (1989) Genetically engineered mutant of the cyanobacterium *Synechococcus* sp. PCC 7942 defective in nitrate transport. Proc Natl Acad Sci (USA) 86 : 6612-6616

Pressman BC (1976) Biological applications of ionophores. Annu Rev Biochem 45 : 501-530

Rai AK, Tiwari SP (1999) Mutants of the cyanobacterium *Anabaena* sp. PCC 7120 altered in nitrate transport and reduction. Curr Microbiol 39 : 237-243

Reddy BR, Apte SK, Thomas J (1989) Enhancement of cyanobacterial salt tolerance by combined nitrogen. Plant Physiol 89 : 204-210

Reed R H, Warr S R C, Richardson D L, Moore D J, Stewart W D P (1985) Multiphasic osmotic adjustment in a euryhaline cyanobacterium. FEMS Microbiol Lett 28 : 225-229

Ritchie RJ (1992) Sodium transport and the origin of the membrane potential in the cyanobacterium *Synechococcus* R-2 (*Anacystis nidulans*) PCC 7942. J Plant Physiol 139 : 320-330

Rodriguez R, Guerrero MG, Lara C (1994) Mechanism of sodium / nitrate symport in *Anacystis nidulans* R2. Biochim Biophys Acta 1187 : 250-254

Rodriguez R, Lara C, Guerrero MG (1993) Nitrate transport in the cyanobacterium *Anacystis nidulans* R2. Kinetic and energetic aspects. Biochem J 282 : 639-643

Rodriguez R, Kobayashi M, Omata T, Lara C (1998) Independence of carbon and nitrogen control in the post translational regulation of nitrate transport in the cyanobacterium *Synechococcus* sp. strain PCC 7942. FEBS Lett 432 : 207-212

Appendix 1

Growth Medium for *A. halophytica*

1). Turks Island Salt Solution

A solution of 5 l (made with distilled water) of Turks Island Salt Solution consists of the following components :

A). KCl	3.33 g
B). $MgCl_2 . 6H_2O$	2.75 g
C). $CaCl_2 . 2H_2O$	7.33 g
D). $MgSO_4 . 7H_2O$	34.70 g
E). NaCl	140.80 g

2). BG 11 plus NO_3 solution

The components of BG 11 plus NO_3 solution are as follows :

A). $NaNO_3$	150.0 g/l
B). KH_2PO_4	4.0 g/l
C). $MgSO_4 . 7H_2O$	75.0 g/l
D). $CaCl_2 . 7H_2O$	36.0 g/l
E). Na_2CO_3	20.0 g/l
F). Citric acid	6.0 g/l
G). EDTA . Na_2	1.0 g/l
H). $FeSO_4 . 7H_2O$	6.0 g/l

I). Trace element A_5 solution consisting of the following components in 1 l solution

H_3BO_3	2.68 g
$MlnCl_2 . 4H_2O$	1.81 g
ZnSO4 . $7H_2O$	0.22 g
$Na_2 MoO_4 . 2H_2O$	0.39 g
CuSO4 . $5H_2O$	0.079 g

Rodriguez R, Lara C, Guerrero MG (1992) Nitrate transport in the cyanobacterium *Anacystis nidulans* R2 : kinetic and energetic aspects. Biochem J 282 : 639-643

Romero JM, Lara C, Guerrero MG (1989) Determination of intracellular nitrate. Biochem J 259 : 545-548

Rosen BP (1986) Recent advances in bacterial ion transport. Annu Rev Microbiol 40 : 263-286

Smith FA (1973) The internal control of nitrate uptake into excised barley roots with differing salt contents. New Phytol 72 : 769-782

Suzuki I, Omata T, Sugiyama T (1992) Gene expression and regulation of nitrate assimilating enzymes in *Synechococcus* PCC 7942. In : Murata N (ed) Research in photosynthesis, vol. 4. Dordrecht : Kluwer, pp 75-78

Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Takabe T (2001) Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+ / H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. J Biol Chem 276 : 36931-36938

Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Takabe T, Takabe T (2002) Overexpression of Na^+ / H^+ antiporter confers salt tolerance on a freshwater cyanobacterium, making it capable of growth in sea water. Proc Natl Acad Sci (USA) 99 : 4109-4114

$Co(NO_3)_2 . 6H_2O$ 0.049 g

The growth medium is prepared by adding 50 ml of item 2.A and 5 ml each of items 2.B to 2.I to 5 l of Turks Island Salt Solution.

Output

1. International Publications

1.1 Incharoensakdi, A., Wangsupa J. Nitrate uptake by the halotolerant cyanobacterium *Aphanothece halophytica* grown under non-stress and salt-stress conditions. Current Microbiology (2003) (in press)

1.2 Incharoensakdi, A., Karnchanatat A. Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*. Biochimica et Biophysica Acta (2003) 1621 : 102-109

1.3 Incharoensakdi, A., Laloknam, S. Nitrate uptake in *Aphanothece halophytica* is driven by both electrochemical potential and ATP. (To be submitted to Current Microbiology)

2. Scientific Meetings (as an invited speaker)

2.1 Glycine betaine accumulation and Na^+/H^+ antiporter are responsible for salt talerance in a halotolerant cyanobacterium *Aphanothece halophytica*. Presented for Protein Research Network Symposium 2002 on Protein Structure and Molecular Enzymology, at Faculty of Science, Mahidol University, 29-30 August, 2002.

2.2 Mechanism of salinity tolerance in cyanobacteria : regulation via osmoprotectant and ion homeostasis. Presented for the first National Conference on Algae and Plankton, at Kasetsart University, 20-21 March, 2003.

3. Award Received

3.1 Recipient of the research award from the National Research Council of Thailand for the year 2002 (Section : chemical and pharmaceutical science)

Addendum

Two in-press papers are herewith enclosed :

1. Current Microbiology (2003)
2. Biochimica et Biophysica Acta (2003) 1621 : 102-109

CURRENT MICROBIOLOGY Vol. • (2003), pp. 000–000
DOI: 10.1007/s00284-002-4000-6

Current Microbiology
An International Journal

Nitrate Uptake by the Halotolerant Cyanobacterium *Aphanothece halophytica* Grown under Non-Stress and Salt-Stress Conditions

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Abstract. We have compared the characteristics of nitrate uptake by *Aphanothece halophytica* grown under non-stress and salt-stress conditions. Both cell types showed essentially similar patterns of nitrate uptake toward ammonium, nitrite, and DL-glyceraldehyde. Although the affinities of nitrate to non-stress cells and salt-stress cells were not significantly different, i.e., K_s = 416 and 450 μM respectively, the V_{max} value for non-stress cells was about twofold of that for salt-stress cells (9.1 vs 5.3 μmol min^{-1} mg^{-1} Chl). Nitrate uptake by *A. halophytica* was found to be dependent on Na^+. Ammonium inhibited nitrate uptake, and the presence of methionine sulfoximine could not release the inhibition by ammonium. Nitrite appeared to competitively inhibit nitrate uptake with a K_i value of 84 μM. Both chloride and phosphate anions did not affect nitrate uptake. DL-Glyceraldehyde, an inhibitor of CO_2 fixation, caused a reduction in the uptake of nitrate.

Nitrate is an essential nitrogen source for growth in many heterotrophic and photosynthetic organisms including cyanobacteria. The rate-limiting step of nitrate assimilation is its active transport into the cells prior to its reduction to ammonium by the sequential action of nitrate reductase and nitrite reductase [2]. The resulting ammonium is further incorporated into the amide nitrogen of glutamine, thus initiating amino acid biosynthesis.

Our laboratory has been using the halotolerant cyanobacterium *Aphanothece halophytica* for the study of the mechanism of salt tolerance. We found that carbon assimilation in terms of photosynthetic CO_2 fixation increased in *A. halophytica* under salt stress [20]. On the other hand, salt stress also caused an increase in glycine betaine, a quaternary nitrogenous compound, in *A. halophytica* subjected to salt stress [4]. So far there have been very few studies in connection with the relationship of salt tolerance to nitrate assimilation in cyanobacteria. Previously it was reported that *Anabaena torulosa* cultures exposed to salt stress showed an increased rate of nitrate uptake [15].

In the present work, we compared the characteristics of nitrate uptake in *A. halophytica* grown under non-stress and salt-stress conditions. We found that *A. halophytica* grown under salt-stress conditions exhibited a reduction in nitrate uptake rate compared with that under non-stress condition.

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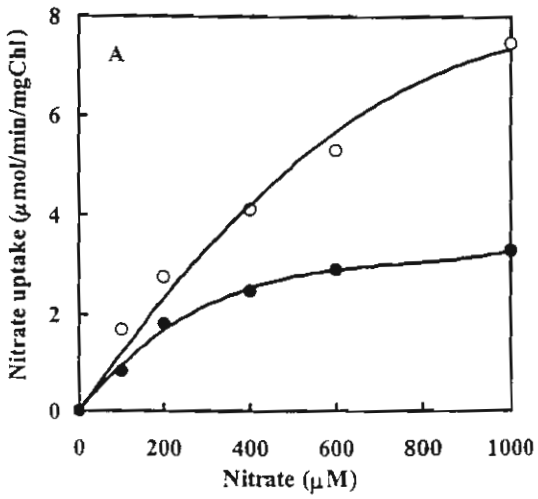
Materials and Methods

Organism and culture. *Aphanothece halophytica* was grown photoautotrophically in BG 11 medium supplemented with 18 mM $NaNO_3$ as described previously [3]. A slight modification was made in which molybdenum in the medium was replaced with tungsten to induce the cells to synthesize nitrate reductase in an inactive form [7]. Cells were grown in a 250-ml flask containing 100 ml medium on a rotary shaker at 30°C without CO_2 supplementation. Continuous illumination was provided by cool white fluorescent lamps at an irradiance of 60 μEm^{-2} s^{-1}. The concentration of NaCl was adjusted to 0.5 M for non-stress cells and 2.0 M for salt-stress cells.

Assay of nitrate uptake. Log-phase cells were washed with 25 mM Hepes-KOH buffer, pH 8.3, containing 12 mM $NaHCO_3$ and 0.5 M sorbitol, and were suspended in the same buffer at a chlorophyll (Chl) concentration of 25 μg/mL determined as described by Mackinney [9]. The reaction was started by the addition of 100 μM $NaNO_3$ or KNO_3 to the suspension kept at 30°C in the light with an irradiance of 60 μEm^{-2} s^{-1}. After 2 min of incubation, the duration in which nitrate uptake increased linearly, the cell suspension was rapidly filtered through a 0.45-μm membrane filter. The nitrate content remaining in the filtrate was determined by anion-exchange high performance liquid chromatography (Hypersil-10 Sax column, 250 mm × 4.6 mm). The values shown in the figures represent the averages of two independent experiments whose variations were less than 15% of the average.



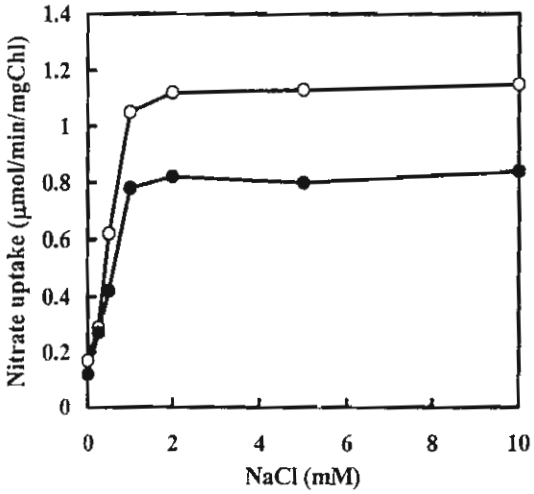


Fig. 2. Initial rates of nitrate uptake as a function of sodium concentrations by non-stress (○) and salt-stress (●) cells. The assay of nitrate uptake was done as described in Materials and Methods, but with no addition of 12 mM $NaHCO_3$, and KNO_3 was used instead of $NaNO_3$ in the assay medium.

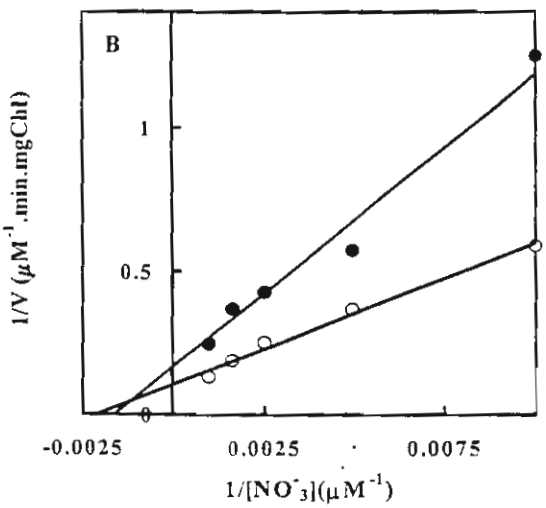


Fig. 1. Kinetics of nitrate uptake by *A. halophytica* grown under non-stress (○) and salt-stress (●) conditions. (**A**) Initial rates of nitrate uptake as a function of external nitrate concentration. (**B**) Lineweaver-Burk plots of the initial rates of nitrate uptake. The lines drawn are those derived from regression analysis of the data.

Results

Kinetics of nitrate uptake. The rates of nitrate uptake by *A. halophytica* as a function of external nitrate concentration are shown in Fig. 1A. Nitrate uptake rates increased with increasing concentrations of nitrate for both non-stress and salt-stress cells. Cells under salt stress showed a lower nitrate uptake rate than those under non-stress at all external nitrate concentrations. The kinetics of uptake for both cells were of Michaelis-Menten types. The apparent K_s values determined from the Lineweaver-Burk plots (Fig. 1B) for non-stress and salt-stress cells were 416 and 450 μM respectively, whereas the maximal velocities (V_{max}) were 9.1 and 5.3 μmol min^{-1} mg^{-1} Chl, respectively.

Next, we tested whether nitrate uptake was dependent on the presence of Na^+. Figure 2 shows that Na^+ could activate nitrate uptake in both non-stress and salt-stress cells. At a fixed concentration of nitrate used (100 μM), the uptake of nitrate appeared to reach saturation at 1 mM Na^+ for both cell types. Furthermore, nitrate uptake was not inhibited by Na^+ as high as 10 mM.

Inhibition of nitrate uptake by ammonium. The presence of higher than 100 μM ammonium in the assay medium containing 100 μM nitrate led to a reduction of nitrate uptake rate for non-stress cells, whereas for salt-stress cells inhibition of nitrate uptake was observed at higher than 50 μM ammonium (Fig. 3). Ammonium was slightly more effective for the inhibition of nitrate uptake in the salt-stress cells than in the non-stress-cells, i.e., about one-half of nitrate uptake was inhibited in salt-stress cells, whereas about one-third was inhibited in non-stress cells at 200 μM ammonium. The inhibition of nitrate uptake was nearly complete at 300 μM ammonium in both cell types. We also tested the effect of methionine sulfoximine on the prevention of ammonium inhibition of nitrate uptake. It was found that preincubation of the cells with 1 mM methionine sulfoximine did not release

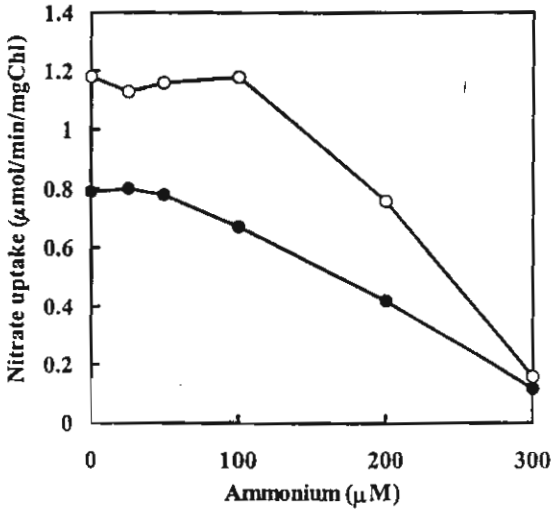


Fig. 3. Initial rates of nitrate uptake as a function of ammonium concentrations by non-stress (○) and salt-stress (●) cells.

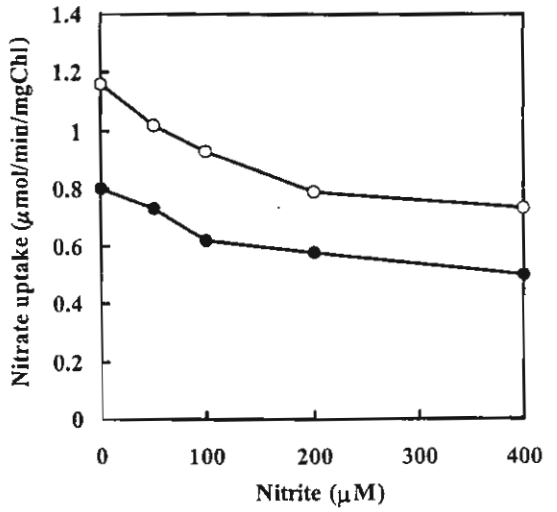


Fig. 4. Initial rates of nitrate uptake as a function of nitrite concentrations by non-stress (○) and salt-stress (●) cells.

the inhibition of nitrate uptake by 300 μM ammonium for both non-stress and salt-stress cells (data not shown).

Effect of anions on nitrate uptake. Nitrite could inhibit nitrate uptake in both non-stress and salt-stress cells (Fig. 4). About one-third of nitrate uptake was inhibited by 400 μM nitrite in both cells. Increasing concentration of nitrite resulted in a gradual decline in the nitrate uptake. By measuring the nitrate uptake rate at various nitrate and nitrite concentrations, the plot between inhibitor concentrations and the slopes obtained from double reciprocal plots yielded the inhibition constant (K_i) of 84 μM. This indicates that nitrite binds to the nitrate transporter with higher affinity than does nitrate. We also

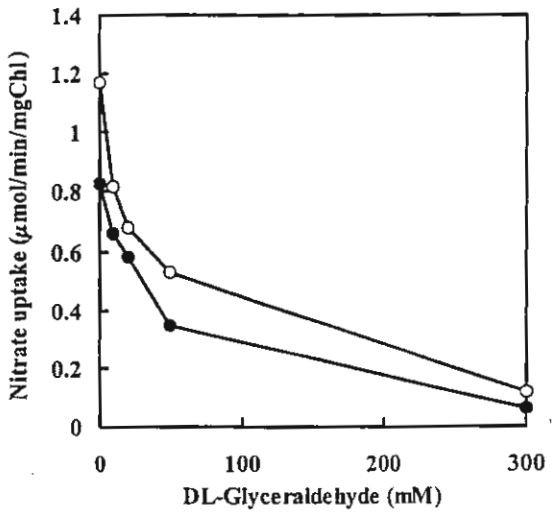


Fig. 5. Initial rates of nitrate uptake as a function of DL-glyceraldehyde concentrations by non-stress (○) and salt-stress (●) cells. The reaction mixture without nitrate was preincubated with DL-glyceraldehyde in the dark for 30 min before the addition of 100 μM $NaNO_3$ to initiate nitrate uptake.

tested the effect of chloride and phosphate anions on nitrate uptake, and the results showed that these two anions at equimolar concentration with nitrate were without effect for both non-stress and salt-stress cells (data not shown).

Effect of CO_2 fixation inhibitor on nitrate uptake. Preincubation of the cells with DL-glyceraldehyde, a selective inhibitor of CO_2 fixation, before nitrate uptake assay caused a reduction in the rate of nitrate uptake for both non-stress and salt-stress cells (Fig. 5). Nitrate uptake was almost completely inhibited when cells were preincubated with 300 μM DL-glyceraldehyde. The results indicated that the interruption of CO_2 fixation, which led to the deprivation of carbon, would inactivate nitrate transporter. Hence, it is likely that nitrate uptake [illegible] *halophytica* is highly dependent on active carbon assimilation.

Discussion

The data presented in this study clearly showed that *A. halophytica* had a reduced uptake of nitrate when grown under salt stress. This is in contrast with the previous report in a nitrogen-fixing *Anabaena torulosa*, showing an increased nitrate uptake when grown in 170 mM NaCl [15]. The increase of nitrate uptake in *A. torulosa* was related to the increased salt tolerance of the cells via the inhibition of Na^+ influx, which is proposed to be a major mechanism for protection of *A. torulosa* against salt stress. In the present study, log-phase *A. halophytica*

grown under salt stress was used. At this phase of growth, salt stress adaptation via the accumulation of glycine betaine has been complete. This was substantiated by the previous observation that the accumulation of glycine betaine already reached maximum after 5 days of growth in the medium containing 1.5 M NaCl [5]. Therefore, a reduced nitrate uptake by *A. halophytica* grown under salt stress may not be related to salt stress adaptation. Another contributing factor for the salt stress protection is via the regulation of intracellular Na^+. Recently, it has been reported that the efflux of Na^+ catalyzed by a Na^+/H^+ antiporter appeared to be a major mechanism for salt stress protection of *A. halophytica* [21, 22].

Half saturation (K_s) of nitrate uptake was very similar for both *A. halophytica* grown under non-stress and salt-stress conditions, i.e., 416 μM for the former and 450 μM for the latter. This suggests that salt stress causes no alteration on the binding site of the nitrate transport protein. The K_s value for nitrate uptake by *A. halophytica* was much higher than that reported for a filamentous cyanobacterium *Anabaena* sp. PCC 7120 (K_s = 31 μM) [14]. In this respect, it should be noted that in the present study 0.5 M sorbitol was present in the assay medium of nitrate uptake to maintain the turgor pressure of *A. halophytica*. The possibility that sorbitol might hinder the binding of nitrate to the transporter cannot be ruled out. However, *A. halophytica* also showed a uniphasic nitrate uptake system similar to that of *Anabaena* sp. 7120.

Previous studies on nitrate uptake by some cyanobacteria and microalgae have shown the inhibitory effect of ammonium and that the inhibition was found to be mediated by ammonium assimilation products [13]. Our results in Fig. 3 also give further support for nitrate uptake inhibition by ammonium. However, it is not certain whether the inhibition of nitrate uptake by ammonium has any connection with the process of ammonium assimilation. We found no effect of methionine sulfoximine, an ammonium assimilation inhibitor, on the release of nitrate uptake from ammonium inhibition (data not shown). There exists the possibility that the effect of ammonium on nitrate uptake observed in *A. halophytica* was through stimulation of nitrate efflux rather than inhibition of nitrate influx. A recent study in barley roots demonstrated increased nitrate efflux upon exposure to ammonium [6].

The uptake of nitrate by *A. halophytica* was dependent on the presence of Na^+, as illustrated in Fig. 2. This finding agrees with a previous study in *Anacystis nidulans* R2 [17]. A preliminary study using monensin, a dissipator of the electrochemical potential for Na^+, also supported the requirement of Na^+ for nitrate uptake by *A. halophytica* (unpublished results). Not only is nitrate uptake dependent on Na^+, but the uptakes of other solutes also require Na^+ as coupling ions in a number of organisms, most notably in alkalophilic and halophilic bacteria [19]. Stimulation of glycine betaine uptake by Na^+ has also been shown in *A. halophytica* [12].

The kinetics of nitrate uptake inhibition by nitrite showing low K_i value suggests that the inhibition is of a competitive type, i.e., both nitrate and nitrite are taken up by the same uptake system. This is in agreement with previous studies in other cyanobacteria; for example, *Synechococcus* sp. PCC 7942 with a nitrate–nitrite bispecific transporter [10], *Anacystis nidulans* R2 [16]. However, a very recent study in a filamentous, heterocystous cyanobacterium, *Nostoc* ANTH, indicated the existence of separate nitrate and nitrite uptake systems [1]. This was based on the results of a chlorate-resistant mutant of *Nostoc* ANTH, which lacked nitrate uptake activity but retained nitrite uptake capacity. A nitrite-specific active transport system has also been reported recently in *Synechococcus* sp. PCC 7942 [11]. Whether *A. halophytica* contains a nitrite-specific transport system remains a subject of further study.

To investigate the relationship between nitrate assimilation and carbon dependence in *A. halophytica*, we tested whether the interruption of CO_2 fixation affected nitrate uptake. It turned out that DL-glyceraldehyde, which is an inhibitor of CO_2 fixation, caused a reduction in the nitrate uptake (Fig. 5). This indicated the strict dependency of nitrate uptake in *A. halophytica* on active CO_2 fixation. This is logical, since CO_2 fixation will provide carbon skeletons as acceptors of ammonium arising from nitrate. The same phenomenon of a positive relationship between nitrate uptake and CO_2 fixation was also observed in *Synechococcus* sp. PCC 7942 [18]. Both nitrate uptake and CO_2 fixation require energy for the process. Although the source of energy for nitrate uptake in cyanobacteria remains unsolved, the Na^+-dependent active transport or the Na^+/NO_3^- symport system has been suggested which relies on Na^+ electrochemical potential as a driving force [8]. This would obviate the need for the nitrate uptake process to compete for another energy source, ATP, which as a result would be utilized mainly for CO_2 fixation. Consequently, in terms of energy requirement, the process of nitrate uptake and CO_2 fixation will proceed without conflict. This indirectly reinforces the contention that nitrate uptake in cyanobacteria is Na^+ dependent, at least in *A. nidulans* R2 and *A. halophytica*.

Overall results in the present study indicate that *A. halophytica* showed a difference in V_{max} of nitrate uptake between non-stress and salt-stress cells. Other parameters tested did not elicit different responses between the two cell types. Under salt stress, *A. halophytica*, in a

short-term response, would utilize Na^+/H^+ antiporter to extrude Na^+ out of the cytoplasm in exchange for H^+ [21, 22]. Since the uptake of nitrate appeared to require Na^+ as a coupling ion, the reduced nitrate uptake under salt stress seen in Fig. 1 was not unexpected. However, in a long-term response to salt stress, the uptake of nitrate should proceed normally. The synthesis and accumulation of an osmolyte glycine betaine can then finally be responsible for salt-stress protection in *A. halophytica.*

ACKNOWLEDGMENTS

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Literature Cited

1. Bhattacharya J, Singh AK, Rai AN (2002) Isolation and characterization of a chlorate-resistant mutant (Clo-R) of the symbiotic cyanobacterium *Nostoc* ANTH: heterocyst formation and N_2-fixation in the presence of nitrate, and evidence for separate nitrate and nitrite transport systems. Curr Microbiol 45:99–104
2. Guerrero MG, Romero JM, Rodriguez R, Lara C (1990) Nitrate transport in cyanobacteria. In: Ullrich WR, Rigano C, Fuggi A, Aparicio PJ (eds) Inorganic nitrogen in plants and microorganisms. Berlin: Springer Verlag, pp 79–85
3. Incharoensakdi A, Waditee R (2000) Degradation of glycinebetaine by betaine-homocysteine methyltransferase in *Aphanothece halophytica:* effect of salt downshock and starvation. Curr Microbiol 41:227–231
4. Incharoensakdi A, Wutipraditkul N (1999) Accumulation of glycinebetaine and its synthesis from radioactive precursors under salt-stress in the cyanobacterium *Aphanothece halophytica.* J Appl Phycol 11:515–523
5. Ishitani M, Takabe T, Kojima K, Takabe T (1993) Regulation of glycine betaine accumulation in the halotolerant cyanobacterium *Aphanothece halophytica.* Aust J Plant Physiol 20:693–703
6. Kronzucker HJ, Glass ADM, Siddiqi MY (1999) Inhibition of nitrate uptake by ammonium in barley: analysis of component fluxes. Plant Physiol 120:283–291
7. Lara C, Romero JM, Guerrero MG (1987) Regulated nitrate transport in the cyanobacterium *Anacystis nidulans.* J Bacteriol 169: 4376–4378
8. Lara C, Rodriguez R, Guerrero MG (1993) Sodium-dependent nitrate transport and energetics of cyanobacteria. J Phycol 29:389–395
9. Mackinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140:314–322
10. Maeda S, Omata T (1997) Substrate binding lipoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in the transport of nitrate and nitrite. J Biol Chem 272:3036–3041
11. Maeda S, Okamura M, Kobayashi M, Omata T (1998) Nitrite specific active transport system of the cyanobacterium *Synechococcus* sp. strain PCC 7942. J Bacteriol 180:6761–6763
12. Moore DJ, Reed RH, Stewart WDP (1987) A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine-synthesizing cyanobacteria. Arch Microbiol 147:399–405
13. Omata T (1995) Structure, function and regulation of the nitrate transport system of the cyanobacterium *Synechococcus* sp. PCC 7942. Plant Cell Physiol 36:207–213
14. Rai AK, Tiwari SP (1999) Mutants of the cyanobacterium *Anabaena* sp. PCC 7120 altered in nitrate transport and reduction. Curr Microbiol 39:237–243
15. Reddy BR, Apte SK, Thomas J (1989) Enhancement of cyanobacterial salt tolerance by combined nitrogen. Plant Physiol 89:204–210
16. Rodriguez R, Lara C, Guerrero MG (1992) Nitrate transport in the cyanobacterium *Anacystis nidulans* R2: kinetic and energetic aspects. Biochem J 282:639–643
17. Rodriguez R, Guerrero MG, Lara C (1994) Mechanism of sodium/nitrate symport in *Anacystis nidulans* R2. Biochim Biophys Acta 1187:250–254
18. Rodriguez R, Kobayashi M, Omata T, Lara C (1998) Independence of carbon and nitrogen control in the post translational regulation of nitrate transport in the cyanobacterium *Synechococcus* sp. strain PCC 7942. FEBS Lett 432:207–212
19. Rosen BP (1986) Recent advances in bacterial ion transport. Annu Rev Microbiol 40:263–286
20. Takabe T, Incharoensakdi A, Arakawa K, Yokota S (1988) CO_2 fixation and RuBisCO content increase in a highly halotolerant cyanobacterium *Aphanothece halophytica*, grown in high salinity. Plant Physiol 88:1120–1124
21. Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Takabe T (2001) Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+/H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail. J Biol Chem 276:36931–36938
22. Waditee R, Hibino T, Tanaka Y, Nakamura T, Incharoensakdi A, Takabe T, Takabe T (2002) Overexpression of Na^+/H^+ antiporter confers salt tolerance on a freshwater cyanobacterium, making it capable of growth in sea water. Proc Natl Acad Sci USA 99:4109–4114

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Salt stress enhances choline uptake in the halotolerant cyanobacterium *Aphanothece halophytica*

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Abstract

The uptake of [^{14}C]choline by a suspension of exponential-phase *Aphanothece halophytica* under various conditions has been studied. Salt stress was found to enhance the uptake of choline. The kinetics of choline transport followed the Michaelis–Menten relationship with apparent K_m values of 272 and 286 μM, maximum rates of transport (V_{max}) of 18 and 37 nmol/min/mg protein for unstressed and salt-stressed cells, respectively. Choline uptake under salt stress was significantly reduced in chloramphenicol-treated cells, suggesting that the activation by salt stress occurred via an inducible transport system. This was corroborated by the existence of the periplasmic choline binding protein, whose content was higher in cells grown under salt-stress condition. Exogenously provided choline significantly increased the growth rate of cells grown under salt stress, although less efficiently than glycine betaine. The presence of 1 mM choline in the growth medium conferred tolerance to high salinity on *A. halophytica* with the maintenance of high growth up to 1.5 M NaCl. The uptake of choline was Na^+-dependent, sensitive to various metabolic inhibitors as well as thiol-reactive agents. The results of competition studies suggested that *N*-methyl on one end of molecule and on the other end either an aldehyde, an alcohol or a neutral group were important features for substrate recognition.

Keywords: Choline uptake; Salt stress; Cyanobacteria

1. Introduction

High salinity in soils and in aqueous environment is an important physical factor that affects growth and survival of living organisms. In bacteria, salt stress can trigger the flux of water across the cytoplasmic membrane. Thus, to avoid dehydration under high salinity or lysis under low salinity growth conditions, bacteria must posses active mechanisms that allow them to adapt to changes in the concentration of salt in the environment [1,2]. Among these mechanisms, one of the most clearly established systems is the accumulation of organic compatible solutes or osmoprotectants like amino acids, sugars and betaines under conditions of high concentration of salt [2]. Such accumulations increase the cytoplasmic osmolarity without disturbing cellular metabolism [3]. The most universally adopted compatible solute is glycine betaine, which can be accumulated during salt stress or osmotic stress by a large variety of bacteria including cyanobacteria [4–6].

For the cyanobacteria, the type of compatible solutes synthesized or accumulated in the cells has been used to differentiate the degree of tolerance to external salinity. For example, strains with low salt tolerance (max 0.7 M) synthesize sucrose or trehalose, strains with moderate salt tolerance (max 1.8 M) synthesize glucosylglycerol and strains with high salt tolerance synthesize glycine betaine [7].

The accumulation of glycine betaine occurs either via transport from the environment or via biosynthesis from a two-step oxidation of choline with betaine aldehyde as an intermediate [4,6,8]. Choline itself can serve as an osmoprotectant in an organism provided that the organism possesses both choline uptake and choline oxidation activity. Mutants of *Escherichia coli* defective in their ability to convert choline to glycine betaine cannot grow at elevated osmotic strength [8]. The uptake of choline followed by two-step oxidation to glycine betaine is therefore an important element to confer tolerance to osmotic stress in microorganisms. The uptake of choline has been studied in a large

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number of bacteria [9–11] but so far no such study has been reported for cyanobacteria. Most of studies for the uptake of compatible solutes in cyanobacteria were done by Hagemann's group using a moderately halotolerant *Synechocystis* sp. PCC 6803, which accumulates glucosylglycerol in response to salt stress [12,13].

Aphanothece halophytica is a high salt tolerant cyanobacterium capable of growth in media containing NaCl concentration as high as 3.0 M [14]. The natural habitat of *A. halophytica* used in the present study is in Solar Lake, Israel. Previously we have shown that exogenously provided choline could confer salt tolerance on *A. halophytica* via the accumulation of glycine betaine [6]. Glycine betaine transport has been shown to be increased by *A. halophytica* grown in media of elevated osmotic strength [15]. This paper describes the influence of salt stress on choline uptake in *A. halophytica*. Evidence for the existence of a periplasmic choline binding protein is also presented for the first time in cyanobacteria.

2. Materials and methods

2.1. Chemicals

[*methyl*-^{14}C]Choline (58 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Dinitrophenol and potassium cyanide were obtained from E. Merck AG, Darmstadt, Germany. Acetylcholine, betaine aldehyde, chloramphenicol, *N*-ethylmaleimide, glycine betaine, gramicidin D, phosphorylcholine and sodium *p*-chloromercuribenzoate were purchased from Sigma Chemical Co., St. Louis, USA. Sodium arsenate, sodium fluoride, sorbitol and glycine were obtained from BDH, England. All reagents used were of analytical grade.

2.2. Growth conditions for A. halophytica

A. halophytica cells were grown photoautotrophically in BG_{11} medium supplemented with 18 mM $NaNO_3$ and Turk Island salt solution as described previously at an irradiation of 60 $\mu Em^{-2}\ s^{-1}$ [16]. Cotton-plugged 250-ml conical flasks containing 100 ml of medium were used and shaken on a rotary shaker at 30 °C. The concentration of NaCl in the growth medium was adjusted by adding an appropriate amount of NaCl to the medium, i.e. 0.5 M for unstress and 2.0 M for salt-stress conditions. Cell growth was followed by monitoring OD_{750}.

2.3. Transport assays

Cells at late log phase were harvested by centrifugation (8000 × *g*, 10 min), washed twice with 50 mM Hepes–NaOH buffer pH 7.5, and suspended to a concentration of ca. 0.1 mg cell protein/ml in the same buffer containing either 0.5 M NaCl (unstress) or 2.0 M NaCl (salt-stress). The uptake experiment was initiated by adding [*methyl*-^{14}C]choline with a specific activity of 0.1 μCi/μmol at a final concentration of 50 μM or otherwise stated. The cell suspension was incubated at 37 °C with shaking at 200 rpm. Aliquots were withdrawn at 1-min intervals and rapidly filtered through HAWP cellulose nitrate filters (0.45-μm pore size; Millipore). The filters were washed twice with 3 ml of buffer (same osmolarity as the assay buffer) and the radioactivity trapped in the cells was determined by counting with a scintillation counter. The rate of choline uptake was linear for at least 3 min depending on cell density and choline concentration. Initial choline uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of choline taken up per minute per milligram protein as determined by the method of Bradford [17] using bovine serum albumin as a standard. In inhibitory assays, the cells were preincubated with the inhibitor for 30 min at 37 °C before the addition of [*methyl*-^{14}C]choline. In competition experiments, cells were added to a mixture of labeled substrate and unlabeled analogs. Unless otherwise indicated, all data are averages of duplicate assays of two independent experiments whose values differ less than 10%.

2.4. Assay of periplasmic binding protein

Cells at late log phase grown either under normal growth conditions (0.5 M NaCl) or salt-stress conditions (2 M NaCl) were collected by centrifugation (8000 × *g*, 10 min) and washed twice with 10 mM Tris–HCl, pH 7.6 containing either 0.5 M or 2.0 M NaCl. Periplasmic proteins were released by osmotic shock according to Neu and Heppel [18]. Cells were resuspended in 20 ml of plasmolysis buffer containing 10 mM Tris–HCl pH 7.6, 0.5 or 2.0 M NaCl, 1.0 M sorbitol and 1 mM EDTA and shaken gently for 30 min at room temperature. Cells collected after centrifugation were resuspended in 10 ml of cold deionized water, frozen at −80 °C for 30 min and thawed at 37 °C for 30 min. After centrifugation the supernatant was centrifuged once more to remove remaining cells and used as periplasmic proteins fraction. Choline binding activity was detected by non-denaturing polyacrylamide gel electrophoresis (12%) using 50-μg periplasmic protein mixed with 10 μM [*methyl*-^{14}C]choline with an incubation at 20 °C for 30 min. The gels were quickly dried on Whatman 3 MM paper and autoradiographed with X-OMAT S Kodak films during 14 days.

3. Results

3.1. Protection against growth inhibition at high salt concentration by choline and glycine betaine

Growth of *A. halophytica* was retarded in the medium containing 2.0 M NaCl when compared to that containing 0.5 M NaCl (Fig. 1). The addition of either 1 mM choline

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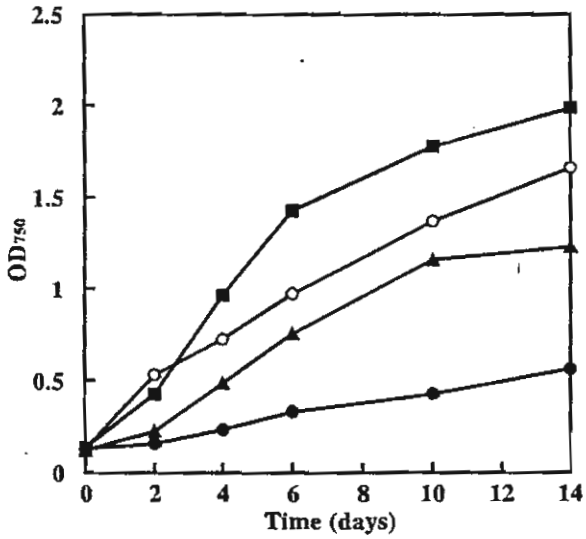

Fig. 1. Growth promoting effect of choline and glycine betaine on salt-stressed *A. halophytica*. Cells were grown under unstress condition, 0.5 M NaCl (○), under salt-stress condition 2.0 M NaCl (●), in 2.0 M NaCl plus 1 mM choline (▲), in 2.0 M NaCl plus 1 mM glycine betaine (■).

or glycine betaine alleviated the inhibitory effect. Glycine betaine showed a stronger protection than did choline. Growth under 2.0 M NaCl medium containing glycine betaine was even faster than that under normal growth conditions. Optimal stimulation of growth under salt stress was obtained at 1 mM choline and no stimulating effect was observed at higher than 10 mM choline (data not shown).

The growth rate of *A. halophytica* under different concentrations of NaCl with and without 1 mM choline is shown in Fig. 2. The growth rate was slightly stimulated by choline when NaCl was raised to 0.5 M. Growth of *A. halophytica* was inhibited at higher than 0.5 M NaCl in the absence of choline. The protective effect of choline against high salt concentration was observed when NaCl was increased up to 1.5 M. At higher than 1.5 M, the growth rate of *A. halophytica* decreased even in the presence of choline. Nevertheless, it is clear that up to 3 M NaCl the growth rates were higher in the presence than in the absence of choline.

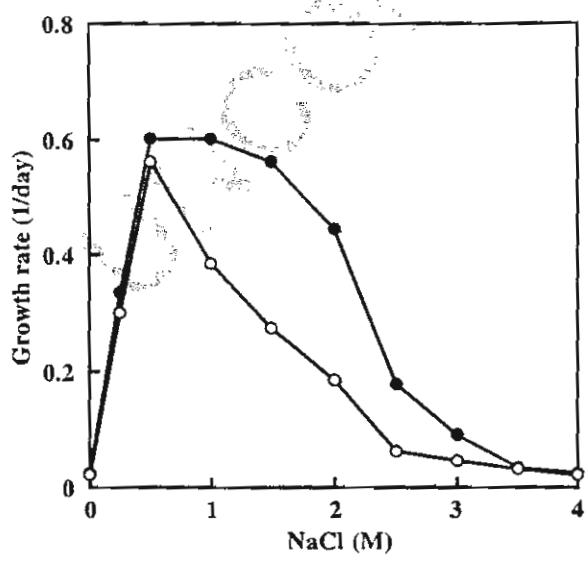

Fig. 2. Effect of choline on growth at high salinity. Cells were grown in media containing various concentrations of NaCl in the presence (●) or absence (○) of 1 mM choline. Growth rates were monitored by measuring OD_{750} and were expressed as (doubling time)$^{-1}$.

3.2. Kinetics of choline uptake

Incubation of *A. halophytica* cells under unstress and salt-stress conditions with a choline concentration from 25 to 700 μM resulted in saturable initial uptake rates for both conditions (Fig. 3A). Double reciprocal plots yielded a straight line (Fig. 3B), indicating that the uptake follows typical

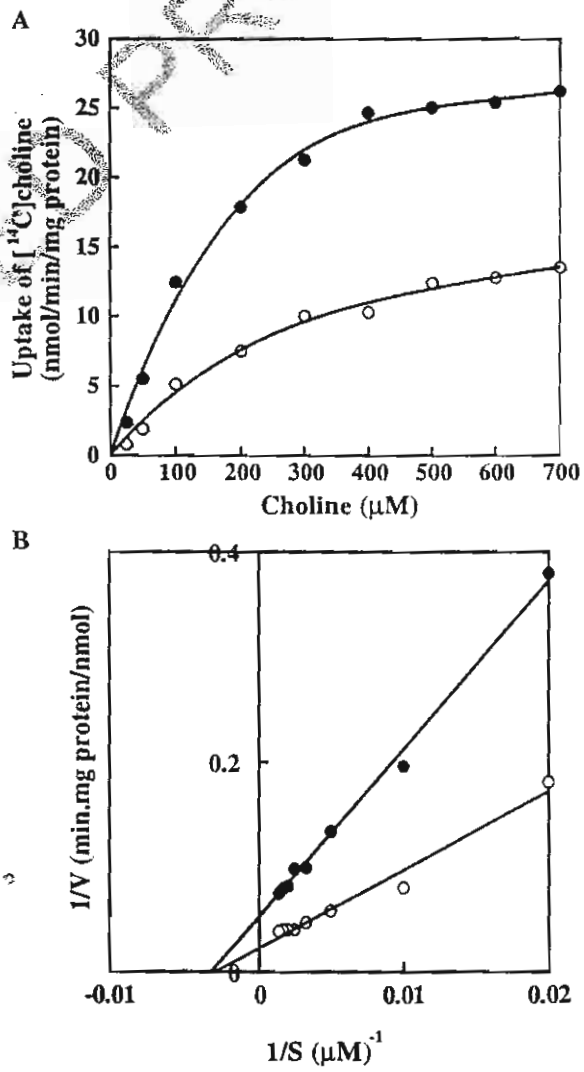

Fig. 3. Kinetics of choline uptake assayed under unstress condition, 0.5 M NaCl (○) or salt-stress condition, 2.0 M NaCl (●). (A) Substrate-saturable initial rates of choline uptake. (B) Lineweaver–Burk plot of the initial rates of choline uptake. The points shown are the means of three independent experiments, and the lines drawn are those derived from regression analysis of the data.

Michaelis–Menten kinetics. The line of best fit was performed by using a least-squares linear regression. The apparent affinity constant (K_m) values of 272 ± 24 and 286 ± 28 μM and the maximal velocity (V_{max}) values of 18 ± 1.5 and 37 ± 2.2 nmol/min/mg protein were obtained for unstressed and salt-stressed cells, respectively.

3.3. Na^+ dependence of choline uptake

In order to test whether salt stress affects choline uptake, the cells initially grown in 0.5 M NaCl containing medium were extensively washed with 50 mM Hepes–NaOH buffer pH 7.5 to get rid of residual NaCl. As shown in Fig. 4 choline uptake rates increased with increasing NaCl concentration in the assay medium. In the absence of NaCl, only marginal choline uptake was detected. In order to test whether an osmotic stress contributes to the increase of choline uptake, we measured choline uptake by incubating the cells with varying amounts of NaCl together with an appropriate amount of sorbitol so that the final osmolarity was equivalent to 4 osM. It was found that the rates of choline uptake were higher in cells with osmotic stress than those without osmotic stress, i.e. compare upper curve with lower curve of Fig. 4. Nevertheless, it should be noted that in the absence of Na^+, choline uptake for *A. halophytica* with osmotic stress was not different from that without osmotic stress. This indicates that the activity of choline uptake is not caused by an osmotic stress. Stimulatory effect of osmotic stress could be observed under the condition that adequate Na^+ was available, i.e. for *A. halophytica* higher than 50 mM Na^+ was needed (Fig. 4).

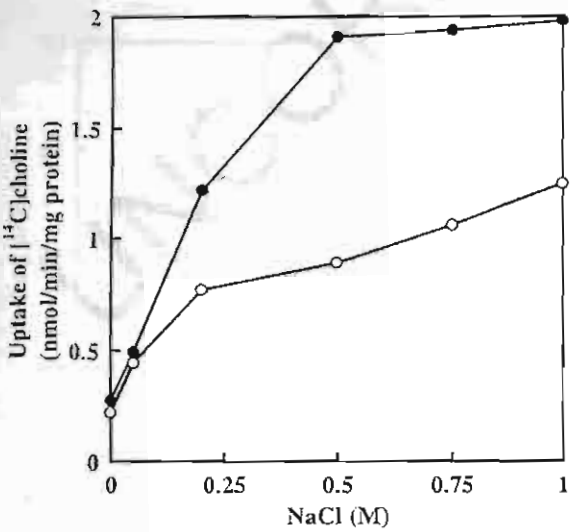

Fig. 4. Effect of different NaCl concentrations on choline uptake in the presence and absence of osmotic stress. Initial choline uptake rates were determined in the presence of various NaCl concentrations without osmotic stress (○) and with osmotic stress (●) in which the osmotic strength of the reaction mixture was kept constant at 4 osM by adding appropriate concentrations of sorbitol.

Table 1
Inhibition of choline uptake by unlabeled analogs[a]

Compound	Inhibition (%)	K_i (μM)[b]
Acetylcholine	78	118
Phosphorylcholine	21	329
Betaine aldehyde	88	101
Glycine betaine	17	365
Glycine	18	353

[a] *A. halophytica* was grown in normal 0.5 M NaCl. Cells were incubated for 3 min in a mixture containing 50 μM [methyl-^{14}C]choline and 5 mM unlabeled inhibitor. Data are given as the percent inhibition of the uninhibited uptake rate which was 1.3 nmol/min/mg protein.

[b] Inhibition constant (K_i) was determined from measurements at four concentrations of substrate and four concentrations of inhibitor. Each value is the mean of three independent experiments (SE was within 9%).

3.4. Specificity of choline uptake

The specificity of choline uptake in *A. halophytica* was studied by addition of 100-fold excess of various compounds into the assay medium and following their initial choline uptake rates (Table 1). The choline analog, acetylcholine, acted as an effective competitor for choline whereas phosphorylcholine did not. Betaine aldehyde, differing from choline by having an aldehyde-group instead of an alcohol group, showed strong inhibition of choline uptake. However, glycine betaine and glycine with a carboxyl group were poor competitors for choline uptake.

We further assessed the transporter specificity through competitive inhibition by measuring the choline uptake rates at various substrate and inhibitor concentrations. The plot between inhibitor concentrations and the slopes obtained from double reciprocal plots yielded the approximate value of inhibition constant (K_i) as shown in Table 1. It was evident that acetylcholine and betaine aldehyde with strong inhibition had high affinity to the transporter. In contrast, phosphorylcholine, glycine betaine and glycine had low affinity to the transporter which subsequently led to weak inhibition.

3.5. Effect of various inhibitors on choline uptake

N-Ethylmaleimide and sodium *p*-chloromercuribenzoate which modify the protein structures were effective inhibitors of choline uptake (Table 2). The inhibitors for ATP formation, sodium arsenate and sodium fluoride also reduced choline uptake, although sodium fluoride was less efficient. The interference of the electron transport by potassium cyanide and of the generation of proton motive force by dinitrophenol resulted in effective inhibition of choline uptake. Gramicidin D that collapses Na^+ gradients also showed considerable inhibition of choline uptake.

3.6. Induction of choline transport by salt stress

Because salt stress led to an increase in initial choline transport rate (Fig. 4), we therefore investigated whether

Table 2
Effect of metabolic inhibitors on the initial rate of choline uptake[a]

Inhibitor	Concentration	Percent inhibition
N-Ethylmaleimide	0.50 mM	97
Sodium *p*-chloromercuribenzoate	0.50 mM	94
Sodium arsenate	1.00 mM	95
Sodium fluoride	1.00 mM	63
Potassium cyanide	1.00 mM	83
Dinitrophenol	1.00 mM	79
Gramicidin D	1.00 μg/ml	74

[a] *A. halophytica* was grown for 30 min in normal 0.5 M NaCl. Cells were preincubated for 30 min in the presence of the indicated inhibitor before the addition of 50 μM [methyl-^{14}C]choline. Data are given as the percent inhibition of the uninhibited uptake rate which was 1.3 nmol/min/mg protein.

protein synthesis was involved in the stimulation of choline transport by salt stress. Choline transport was faster in stressed cells than in unstressed cells (Fig. 5). However, preincubation of the stressed cells with chloramphenicol (100 μg/ml) resulted in a significant decrease of choline transport. This suggests that the stimulation of transport by salt stress observed in the absence of chloramphenicol appeared to be dependent on de novo protein synthesis of either a transport protein or protein(s) regulating the activity of preexisting transport protein(s). Choline transport of unstressed cells in the presence of chloramphenicol was not significantly different from that in the absence of chloramphenicol, suggesting that the constitutive choline uptake was not affected by chloramphenicol. The observation that the increased choline transport was not completely abolished in the chloramphenicol-treated cells under salt stress could be ascribed to the possibility that chloramphenicol used at the concentration of 100 μg/ml could not completely inhibit protein synthesis. Indeed we found that virtually no choline transport occurred when cells were treated with 500 μg/ml chloramphenicol (data not shown).

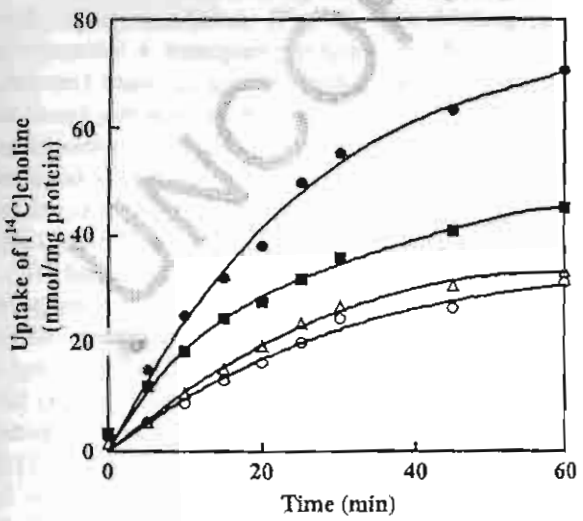

Fig. 5. Effect of chloramphenicol on choline uptake. Choline uptake was assayed under unstress condition, 0.5 M NaCl (○), under salt-stress condition, 2.0 M NaCl (●), in 0.5 M NaCl plus 100 μg/ml chloramphenicol (△), in 2.0 M NaCl plus 100 μg/ml chloramphenicol (■). In all cases, preincubation for 30 min prior to the addition of labeled choline to initiate the uptake was done.

3.7. Evidence for periplasmic choline binding protein

To analyze the existence of a choline binding protein, periplasmic fractions from unstressed and salt-stressed cells were subjected to nondenaturing polyacrylamide gel electrophoresis in the presence of [*methy*-^{14}C]choline. One single radioactive band was detected for cells under unstress and salt-stress conditions with more intense band observed for the latter (Fig. 6; lanes 2 and 3). The sample containing only [*methy*-^{14}C]choline but without periplasmic protein showed no band (Fig. 6; lane 1).

To verify that the periplasmic fractions obtained by cold osmotic shock used in this study contained periplasmic proteins and not cytoplasmic proteins, we checked for the marker enzyme activities [19]. With identical amounts of protein used, high activity of alkaline phosphatase was detected in the periplasmic fractions whereas none was detected in the total protein fractions. On the other hand, the specific activity of isocitric dehydrogenase which is a cytoplasmic marker enzyme accounted for less than 7% of that by the total protein fractions. These results demonstrated that the periplasmic fractions were not contaminated with cytoplasmic proteins. Furthermore, we also checked

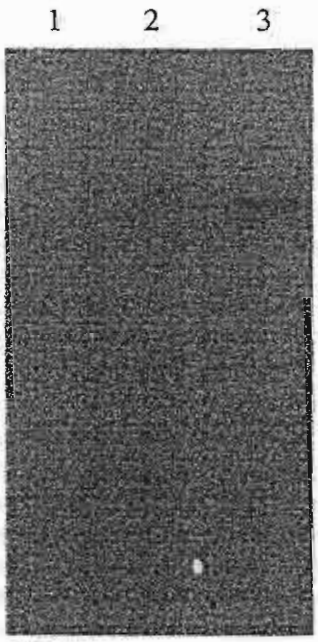

Fig. 6. Existence of choline binding protein from periplasmic fluid as revealed by autoradiography of nondenaturing gel electrophoresis. Lane 1 is sample without periplasmic proteins. Lanes 2 and 3 are samples of periplasmic proteins from cells grown under unstress (0.5 M NaCl) and salt-stress (2.0 M NaCl) conditions, respectively.

whether the cells depleted of periplasmic fractions were able to take up choline. Only low level of ^{14}C-choline could be detected in the periplasm-depleted cells after 30-min incubation, representing less than 5% of that by intact cells. This indicates that a choline binding protein was required for choline uptake and it was localized in periplasmic fractions.

4. Discussion

The results presented in this study clearly demonstrate the existence of a transport system for choline in *A. halophytica*. Exogenously provided choline could be transported into *A. halophytica* and could serve as a growth promoting substance when the cells were grown at high external salinity (Figs. 1 and 2). The protective role by choline against inhibition of growth at high salinity was not as efficient as that by glycine betaine, suggesting that choline per se is not an osmoprotectant. This was supported by our earlier observation that the transported choline was efficiently converted to betaine aldehyde and finally to glycine betaine in *A. halophytica* under high salinity [6]. Glycine betaine is also shown to be more efficient than choline in the stimulation of growth of the moderate halophilic bacterium *Halomonas elongata* over the range of salinity from 0.5 to 2.0 M NaCl [20].

The transport system for choline in *A. halophytica* is an active transport. This conclusion is based on the following three lines of evidence. First, the kinetic data indicate that choline transport is substrate-saturable and shows a Michaelis–Menten relationship (Fig. 3A), observations which support the involvement of a transport protein. Second, the existence of a periplasmic choline binding protein was demonstrated with increasing content in cells grown under high salinity conditions (Fig. 6). This binding protein is presumably a transport protein for choline since the increased transport activity under salt stress is due to an increased synthesis of transport protein (Fig. 5) rather than an activation of the existing transport protein. Third, choline transport is highly inhibited by various energy generation inhibitors including inhibitors, which modify protein structure (Table 2).

We have shown here that Na^+ was required for optimum transport activity of choline as only marginal level of choline uptake could be detected in the absence of Na^+, regardless of whether the cells were under osmotic stress or not (Fig. 4). Specific requirements of Na^+ for transport activities have been well documented in marine bacteria [21]. Very recently we have also shown that nitrate uptake in *A. halophytica* is dependent on Na^+ [22]. An increase of NaCl concentration resulted in higher choline uptake rate in *A. halophytica*. It seems likely that the rate of transport was affected by the concentration of NaCl providing Na^+ as a coupling ion for transport and Na^+ and Cl^- as sources of osmotic stress. In view of the fact that osmotic stress alone without Na^+ caused only modest choline uptake (Fig. 4), it is likely that the primary role of Na^+ is to act as a coupling ion. The result showing the inhibition of choline transport by gramicidin D (Table 2), a reagent which collapses Na^+-gradients, suggests an involvement of Na^+-gradients in the transport of choline by *A. halophytica*. In addition, dinitrophenol, an uncoupler disrupting the proton motive force, was a potent inhibitor of choline transport. Taken together, it may be that an Na^+-gradient is created in exchange for the proton gradient. Indeed, *A. halophytica* has recently been shown to contain an Na^+/H^+ antiporter with a major role for salt stress protection [23].

Previously, Na^+-activated glycine betaine transport in *A. halophytica* has been reported [15]. As *A. halophytica* is a halophilic cyanobacterium, its normal growth medium contains about 0.5 M NaCl. However, it can adapt to increasing external salinity by synthesis and accumulation of glycine betaine [6,24]. It is apparent that *A. halophytica* can rely on either the synthesis or the uptake of glycine betaine to osmoregulate against high external salinity. However, the latter process is preferable due to the fact that de novo synthesis is energetically more expensive than the transport process [25].

Choline transporter seems to be distinct from glycine betaine transporter in *A. halophytica* since glycine betaine was a poor competitor for choline transport (Table 1). It appears that *N*-methyl groups on one end of the molecule and an alcohol or aldehyde group on the other end are important for the affinity to the choline transporter. The charge of the molecule is also important in the recognition by the choline transporter because the most effective competitors are positively charged (acetylcholine, betaine aldehyde), whereas zwitterionic compounds closely related to choline (phosphorylcholine, glycine betaine) are less effective.

The data in Fig. 3B revealed that the choline transport system of *A. halophytica* had a low affinity for choline (K_m, 272 μM) which is in contrast to a rather high affinity for glycine betaine transporter (K_m, 2 μM) [15]. The levels of choline have been estimated to be in nanomolar range in coastal seawater [26]. The data on the levels of glycine betaine in seawater were not available. In general, the amount of glycine betaine available in the environments depends on many factors including the level of organic material, the rate of microbial degradation of dead cells and the atmospheric conditions. Presuming that the level of glycine betaine in seawater is very low, it is therefore likely that the functional glycine betaine transporter rather than choline transporter may play a role to allow *A. halophytica* to thrive in coastal seawater. However, choline transport in *A. halophytica* could clearly protect cells against high salinity stress provided that sufficient concentration of choline is available as shown in Fig. 2. There remains a possibility that the physiological role of this transporter is to transport choline for the supply of carbon and nitrogen sources under certain conditions. *Corynebacterium glutamicum*, a gram-positive soil bacterium, has previously been

shown to contain a specific proline carrier, Put P, which is not involved in osmoregulation but is responsible for proline utilization [27]. This Put P carrier also showed low affinity for proline, a similar property with respect to low affinity for choline by choline transporter in *A. halophytica*.

The present study seems to suggest that *A. halophytica* possesses only one transport system for choline with low affinity. However, kinetic data alone cannot conclusively exclude the possible multiple choline transport systems. Previously two or more choline transport systems have been found in *Pseudomonas aeruginosa* [28], *Rhizobium meliloti* [29] and *Bacillus subtilis* [30]. The fact that choline binding protein in the periplasmic fraction could be osmotically induced (Fig. 6) suggests that the transporter involved in choline uptake is an ATP-binding cassette (ABC) transporter. Since this kind of transporter usually has high affinity for its substrate, it is difficult to reconcile with the low affinity for choline suggested by kinetic data of the present study. However, recently Jebbar et al. [31] reported that ectoine was taken up by *B. subtilis* via the ABC-transport system Opu C with low affinity (K_m = 1.5 mM). Furthermore, another ABC-transport system Pro U in *E. coli* was also able to transport ectoine with low affinity (K_m = 200 μM) and this transport of ectoine appeared to involve a periplasmic binding protein [32].

At present it is unclear how *A. halophytica* with one system of low affinity choline transport can cope with high salinity environments. Worth mentioning in this regard is the fact that *A. halophytica* is able to synthesize glycine betaine de novo [24]. Hyperosmotic stress led to an increased accumulation of glycine betaine in *A. halophytica* without exogenous supply of choline. This suggests that choline-glycine betaine pathway might not significantly contribute to osmoregulation by *A. halophytica*. Although we previously showed that exogenously provided choline led to an increase of glycine betaine under hyperosmotic stress [6], we speculate that *A. halophytica* might as well synthesize glycine betaine via a three-step series of methylation reactions from glycine. This mechanism of glycine betaine synthesis has been reported in a number of bacteria and very recently in extreme halophiles [33,34]. The physiological role of choline transport of *A. halophytica* in natural habitats is likely to import choline as carbon and nitrogen sources. However, when high concentration of external choline is available, it can be taken up by low-affinity choline transporter and finally converted to glycine betaine for osmoregulatory function.

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References

[1] L.N. Conska, A.D. Hanson, Prokaryotic osmoregulation: genetics and physiology, Annu. Rev. Microbiol. 45 (1991) 569–606.
[2] D. Le Rudulier, A.R. Strom, A.M. Dandekar, L.T. Smith, R.C. Valentine, Molecular biology of osmoregulation, Science 224 (1984) 1064–1068.
[3] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Living with water stress: evolution of osmolyte systems, Science 217 (1982) 1214–1222.
[4] L.N. Conska, Physiological and genetic responses of bacteria to osmotic stress, Microbiol. Rev. 53 (1989) 121–147.
[5] R.H. Reed, J.A. Chudek, R. Foster, W.D.P. Stewart, Osmotic adjustment in cyanobacteria from hypersaline environments, Arch. Microbiol. 138 (1984) 333–337.
[6] A. Incharoensakdi, N. Wutipraditkul, Accumulation of glycine betaine and its synthesis from radioactive precursors under salt-stress in the cyanobacterium *Aphanothece halophytica*, J. Appl. Phycol. 11 (1999) 515–523.
[7] R.H. Reed, W.D.P. Stewart, The response of cyanobactria to salt stress, in: L.J. Rogers, J.R. Gallon (Eds.), Biochemistry of the Algae and Cyanobacteria, vol 12, Clarendon Press, Oxford, 1988, pp. 217–231.
[8] O.B. Styrvold, P. Falkenberg, B. Landfald, M.W. Eshoo, T. Bjornsen, A.R. Strom, Selection, mapping, and characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline-glycine betaine pathway, J. Bacteriol. 165 (1986) 856–863.
[9] A. Kaenjak, J.E. Graham, B.J. Wilkinson, Choline transport activity in *Staphylococcus aureus* induced by osmotic stress and low phosphate concentrations, J. Bacteriol. 175 (1993) 2400–2406.
[10] P. Boch, B. Kempf, E. Bremer, Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline, J. Bacteriol. 176 (1994) 5364–5371.
[11] E. Boncompagni, M. Osteras, M.C. Poggi, D. Le Rudulier, Occurrence of choline and glycine betaine uptake and metabolism in the family *Rhizobiaceae* and their roles in osmoprotection, Appl. Environ. Microbiol. 65 (1999) 2072–2077.
[12] S. Mikkat, M. Hagemann, A. Schoor, Active transport of glucosylglycerol is involved in salt adaptation of the cyanobacterium *Synechocystis* sp. strain PCC 6803, Microbiology 142 (1996) 1725–1732.
[13] S. Mikkat, U. Effmert, M. Hagemann, Uptake and use of osmoprotective compounds trehalose, glucosylglycerol, and sucrose by the cyanobacterium *Synechocystis* sp. Strain PCC 6803, Arch. Microbiol. 167 (1997) 112–118.
[14] T. Takabe, A. Incharoensakdi, K. Arakawa, S. Yokota, CO_2 fixation rate and RuBisCO content increase in the halotolerant cyanobacterium, *Aphanothece halophytica*, grown in high salinities, Plant Physiol. 88 (1988) 1120–1124.
[15] D.J. Moor, R.H. Reed, W.D.P. Stewart, A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine-synthesising cyanobacteria, Arch. Microbiol. 147 (1987) 399–405.
[16] A. Incharoensakdi, T. Takabe, T. Akazawa, Effect of betaine on enzyme activity and subunit interaction of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Aphanothece halophytica*, Plant Physiol. 81 (1986) 1044–1049.
[17] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[18] H.C. Neu, L.A. Heppel, The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts, J. Biol. Chem. 240 (1965) 3685–3692.
[19] S. Fulda, S. Mikkat, W. Schroder, M. Hagemann, Isolation of salt-induced periplasmic proteins from *Synechocystis* sp. Strain PCC 6803, Arch. Microbiol. 171 (1999) 214–217.
[20] D. Canovas, C. Vagas, L.N. Csonka, A. Ventosa, J.J. Nieto, Osmoprotectants in *Halomonas elongata*: high-affinity betaine transport system and choline-betaine pathway, J. Bacteriol. 178 (1996) 7221–7226.

[21] R.A. Macleod, Marine microbiology far from the sea, Annu. Rev. Microbiol. 39 (1985) 1–20.
[22] A. Incharoensakdi, J. Wangsupa, Nitrate uptake by the halotolerant cyanobacterium *Aphanothece halophytica* grown under non-stress and salt-stress conditions, Curr. Microbiol. (in press).
[23] R. Waditee, T. Hibino, Y. Tanaka, T. Nakamura, A. Incharoensakdi, T. Takabe, Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+/H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail, J. Biol. Chem. 276 (2001) 36931–36938.
[24] M. Ishitani, T. Takabe, K. Kojima, T. Takabe, Regulation of glycine betaine accumulation in the halotolerant cyanobacterium *Aphanothece halophytica*, Aust. J. Plant Physiol. 20 (1993) 693–703.
[25] E.A. Galinski, Osmoadaptation in bacteria, Adv. Microb. Physiol. 37 (1995) 273–328.
[26] M.A. Roulier, B. Palenik, F.M.M. Morel, A method for the measurement of choline and hydrogen peroxide in sea water, Mar. Chem. 30 (1990) 409–421.
[27] H. Peter, A. Bader, A. Burkovski, C. Lambert, R. Kramer, Isolation of the *putP* gene of *Corynebacterium glutamicum* and characterization of a low-affinity uptake system for compatible solutes, Arch. Microbiol. 168 (1997) 143–151.
[28] M.A. Salvano, T.A. Lisa, C.E. Domenech, Choline transport in *Pseudomonas aeruginosa*, Mol. Cell. Biochem. 85 (1985) 81–89.
[29] J.A. Pocard, T. Bernard, L.T. Smith, D. Le Rudulier, Characterization of three choline transport activities in *Rhizobium meliloti*: modulation by choline and osmotic stress, J. Bacteriol. 171 (1989) 531–537.
[30] R.M. Kappes, B. Kempf, S. Kneip, J. Boch, J. Garde, J. Meier-Wagner, E. Bremer, Two evolutionarily closely related ABC transporters mediated the uptake of choline for the osmoprotectant glycine betaine in *Bacillus subtilis*, Mol. Microbiol. 32 (1992) 203–206.
[31] M. Jebbar, C. von Blohn, E. Bremer, Ectoine functions as an osmoprotectant in *Bacillus subtilis* and is accumulated via the ABC-transport system Opu C, FEMS Microbiol. Lett. 154 (1997) 325–330.
[32] M. Jebbar, R. Talibart, K. Gloux, T. Bernard, C. Blanco, Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics, J. Bacteriol. 174 (1992) 5027–5035.
[33] E.A. Galinski, H.G. Truper, Microbial behaviour in salt-stressed ecosystems, FEMS Microbiol. Rev. 15 (1994) 95–108.
[34] A. Nyyssola, J. Kerovuo, P. Kaukinen, N. von Weymarn, T. Reinikainen, Extreme halophiles synthesize betaine from glycine by methylation, J. Biol. Chem. 275 (2000) 22196–22201.