CHAPTER IV

DISCUSSION

Osmoregulation in *A. halophytica.*

Microorganisms must be able to adapt to changes in the osmolarity of their environment. To adapt to these changes, bacteria accumulate some compounds, named compatible solutes, that confer protection against the deleterious effect of the low water activity (Canovas et al., 1998). Most halotolerant cyanobacteria accumulate glycine betaine to protect cells against osmotic stress. The glycine betaine accumulation is an indication that the cells are adapting to the changes of external osmolarity.

The salt tolerance of *A. halophytica* was studied by following the growth rate of cells in salt stress condition. When the cells were transferred to the medium with higher NaCl concentration, the growth of the cells was decreased. Our data showed *A. halophytica* could adapt to a broad range of salt concentrations from 0.5 to 2.0 M NaCl (Figure 3.2). We found that externally provided choline and glycine betaine functioned as efficient osmoprotectants for *A. halophytica* (Figure 3.3). Choline and glycine betaine stimulated the growth of *A. halophytica* over the whole range of salinities. The enhancement of growth by the two compounds was nearly identical at optimal salinity, but choline was less efficient than glycine betaine both below and

above the optimal salinity. The fact that many bacteria prefer to transport external compatible solutes rather than to carry out the energetically more expensive de novo synthesis (Galinski, 1995) may account for the accumulation glycine betaine in *A. halophytica* at 2 M NaCl.

The cessation of growth due to salt stress probably occurred as a result of energy being diverted to the initial adjustment of cellular volume by regulating the influx and efflux of certain ions. The influx of Na^+ and K^+ in *A. halophytica* in 100% seawater medium has previously been shown to occur mostly in the first 24 hour (Reed et al., 1984). Furthermore uptake and extrusion of of Na^+ in *Synecocystis* PCC 6714 have also been shown to occur during the initial period of hypersaline treatment (Reed et al., 1985). It remains to be clarified how the movement of various solutes in and out of *A. halophytica* affects the osmoregulation of the cells. Results similar to those observed in *A. halophytica* have been recently reported for the effect of salt stress on growth of a salinity-adapted cyanobacterium, *Spirulana platensis* (Vonshak et al, 1996). Cells exhibited a lag period in growth when stressed with 0.5 M or 0.75 M NaCl. However, normal growth resumed after the lag period. It is also noteworthy that the lag period after a salt- shock has also been observed in green algae, *Dunaliella salina* (Borowitzka et al., 1990).

Determination of [*methyl*-^{14}C]choline uptake system.

Choline uptake by *A. halophytica* in response to osmotic stress occurred via an inducible transport system. To study the effect of salinity on the uptake of choline, cells were incubated with 0.5 or 2.0 M NaCl and 50 μM [*methyl*-^{14}C]choline. There is

substantial choline uptake of *A. halophytica* in osmotic stress condition, and this choline uptake activity is stimulated about three-fold when the osmolarity of the stress condition is raised by the addition of NaCl. Figure 3.16 shows that the rapid uptake of [*methyl*-^{14}C]choline occurred during the first 30 min of incubation but the uptake increased slowly after 60 min. The pattern of [*methyl*-^{14}C]choline uptake was similar for control and stress condition. After uptake, choline was used by *A. halophytica* as an energy substrate, a source of cell carbon and a precursor of the osmoprotectant glycine betaine. Salt stress was identified as one variable which favored the conversion of choline to glycine betaine and retention of glycine betaine in *A. halophytica*, presumably for its compatible solute functions. Choline uptake by osmotically upshocked cells occurred only after a considerable lag and could be prevented by chloramphenicol (Figure 3.7). This stimulation is a true osmotic effect, since it can be triggered by increase with osmoticum (sorbitol, mannitol and sucrose). (Figuer 3.8).

When the substrate concentration was varied the choline uptake system followed typical Michaelis-Menten kinetics. Using Lineweaver-Burk transformation of the data, the apparent K_m's of control and stress condition were 278.6 and 256.4 μM respectively, the V_{max}'s were 17.9 and 35.7 nmol/min/mg protein, respectively. Choline transport systems have been described in a variety of microoraganisms, including a marine pseudomonad (Snipes et al., 1974) *P. aeruginosa* (Salvano et al., 1989), *E.coli*, (Styrvold et al., 1986), *R. meliloti* (Pocard et al., 1983) and *S. cerevisiae* (Hosaka et al, 1980).

The uptake of choline by *A. halophytica* was susceptible to a variety of inhibitory agent (Table 3). Effect of inhibitors for ATPase activity, proton motive force and Na^+ electrochemical gradients on the initial rate of [*methyl*-^{14}C]choline uptake via osmotic stress condition were studied. Uncouplers of oxidative phosphorylation, such as dinitrophenol, which dissipate the electrochemical gradient of proton acrosss the cytoplasmic membrane, effectively reduced the initial rates of choline uptake. Equally severe inhibition was apparent by the respiratory inhibitor potassium cyanide, the sulfhydryl group reagents, *N*-methylmalemide and sodium *p*-chloromercuribenzoate that affect protein structure. Moreover, sodium arsenate and sodium fluoride, which inhibit reactions involving high-energy phosphate bonds could also reduce choline uptake. These results provide further evidence on the dependency of choline uptake on the availability of energy. We suggest that the main driving force in uptake is the electrochemical proton gradient generated by respiration.

From the results of the competition studies (Table 2), it appears that *N*-methyl groups on one end of the molecule and the alcohol or aldehyde group (or at least a neutral group) on the other end are important in the recognition of choline by the uptake system. It appears that the charge of the molecule is important in that the most effective competitors are positively charged (acetylcholine and glycine betaine aldehyde), whereas zwitterionic compounds closely related to choline (phosphorylcholine and glycine betaine) are less effective.

Indeed, a detailed genetic and biochemical analysis of choline-glycine betaine pathway in *E.coli* has proven that choline has no osmoprotective properties (Styrvold et al., 1986). *B.subtilis* thus shares ability to oxidize choline to glycine betaine for

osmoprotective purposes with a number of gram-negative and gram-positive bacteria (Abee et al,1990; Bernard et al., 1986; D'Souza-Ault et al., 1993; Kaenjak et al., 1993; Lanfald and Strom, 1986; Le Rudulier et al., 1984 and Rozwadowski et al., 1991). This oxidation is a two step process with glycine betaine aldehyde as an intermediate. In microroganisms, it can involve either a soluble choline oxidase that is proficient for both enzymatic reactions and a combination of a membrane-bound choline dehydrogenase (which also can oxidize glycine betaine aldehyde to glycine betaine) and soluble glycine betaine aldehyde dehydrogenase with a high degree of substrate specificity (Rozwadowski et al., 1991).

A sudden osmotic upshock stimulates immediately the activity of preexisting choline transporters, and subsequent de novo synthesis is required to achieve maximal choline uptake activity. Thus, the expression of structural gene is essential for the response to changes in medium osmolarity. Both a modulation of the activity of the choline transporter and a stimulation at the level of gene expression contribute also to the increase in choline transport observed in *E.coli* under high osmolarity growth conditions (Echoo, 1988 and May et al., 1986). A number of environmental factors (osmolarity, availability of oxygen and temperature) and the presence of choline in the growth medium all influence the transcription of the betT gene, which encodes the *E.coli* choline transport protein (Echoo, 1988). High osmolarity, low phosphate concentrations and the availability of choline are also known to stimulate the expression of the gene(s) for an effective choline transport system in *Staphylococcus aureus* that serves to scavenge choline from environment for synthesis of the osmoprotectant glycine betaine (Kaenjak et al., 1993).

Recently the uptake of choline with subsequent conversion to glycine betaine has been reported for *Synecococcus* PCC 7942 transformed with *E.coli* bet genes (Nomura et al., 1995). Choline uptake by *Synecococcus* PCC 7942 was also strongly inhibited by the uncoupler suggesting the presence of an energy-dependent transport system in *Synecococcus*. Previously a glycine betaine uptake system has been reported in *A. halophytica* (Moor et al., 1987).

Choline oxidation by choline dehydrogenase

A. halophytica exhibited the activities of choline dehydrogenase (which catalyzes the oxidation of choline to betaine aldehyde) and betaine aldehyde dehydrogenase (which catalyzes the oxidation of betaine aldehyde to glycine betaine). The presence of choline dehydrogenase and betaine aldehyde dehydrogenase activities from cell grown under normal condition and salt stress supported the notion that glycine betaine is synthesized by a two step oxidation of choline via betaine aldehyde. Choline dehydrogenase was mainly present in membrane fraction whereas betaine aldehyde dehydrogenase was found mainly in cytoplasmic fraction. Formation of these enzymes was also regulated by the NaCl concentration of the medium. These results suggested that NaCl concentration of the medium could induce the biosynthesis of both enzymes.

In *E.coli* grown in the presence of choline, the stimulation of choline dehydrogenase activity was 30-fold as compared to 10-fold for betaine aldehyde dehydrogenase activity when cells were subjected to salt stress (Lanfald and Strom, 1986). However, in the absence of choline both dehydrogenases were stimulated by

salt stress to about the same extent; i.e. a 2.5-fold increase compared to the background non-stress levels. It is obvious that not only the external osmotic pressure, but also the internal choline content, contributes to the activation of both dehydrogenase in *E.coli*. Further study in *A. halophytica* with regard to the molecular mechanism for the activation of the two dehydrogenases is important.

Choline dehydrogenase from *A. halophytica* was also inhibited by substrate analog. Aldehyde compound such as glycine betaine aldehyde was a strong enzyme inhibitor. This suggested that the aldehyde functional group of the substrate might play an important role in binding to the active site of choline dehydrogenase.The effect of salt on *A. halophytica* choline dehydrogenase activity was tested. It was found that the monovalent cations such as Na^+ and K^+ at low concentration (0.05 to 0.1 M) could increase choline dehydrogenase activity, with maximal activity at 0.1 M. At the higher concentration (0.25 to 1.0 M) choline dehydrogenase was inhibited by both Na^+ and K^+.

Evidence for periplasmic binding protein

In order to evaluate the role of the periplasm for cyanobacterial salt adaptation, we investigated the protein composition of cell comparment in *A. halophytica*. By cold osmotic shock treatment, the total protein was released from either control cells or cells adapted to 2.0 M NaCl. SDS-PAGE revealed the released periplasmic fraction (Figure 3.14). These results indicated that the cold osmotic shock treatment is suitable for selectively releasing periplasmic proteins from *A. halophytica*. Only when cells were grown at NaCl concentrations higher than

2.0 M did the shock fluid become bluish, indicating cell lysis. Therefore, cells grown at 2.0 M NaCl were used throughout the experiments as salt-adapted. The composition of periplasmic proteins obtained from cells adapted to 0.5 M and 2.0 M NaCl showed clear differences in five proteins with apparent molecular masses of 19.9, 26.2, 34.6, 47.8 and 83.9 kDa (designated PP 1, PP 2, PP 3, PP 4 and PP 5). The protein with apparent molecular masses of 19.9, 26.2 and 47.8 (designated PP 1, PP 2 and PP 4) represented the most abundant proteins in all periplasmic preparations (Figure 18, lanes 1-4), proteins with apparent molecular masses of 34.6 and 63.9 kDa (designated PP 3 and PP 5) represented the periplasmic fraction proteins that differed significantly between cells adapted to 0.5 M and 2.0 M NaCl (Figure 3.14, lanes3 and 4).

Since it is well known that in different gram-negative bacteria, choline uptake activity may occur through periplasmic protein dependent transport system, fraction of periplasmic obtained from cells grown at low or high osmolarity were subjected to non-denaturing PAGE in the presence of [*methyl*-^{14}C]choline as described in materials and methods. In 0.5 M NaCl grown cells a weak band was observed and in 2.0 M NaCl grown cells strongly enhanced band was observed (Figure 3.16).