CHAPTER I

INTRODUCTION

Salinity is one of the important limiting factors in crop productivity and much effort has been made to understand salt tolerance (Bohnert and Jenzen, 1996; Zhu et al., 1997). Although the ability to adapt to fluctuations in the external salinity is fundamental to survival of organisms, the mechanisms responsible for osmotic adaptation have been largely unknown. Upon the changing salinity of environment, all microorganisms, need to balance their cytoplasm with the osmotic pressure exerted by the external medium. To adjust the internal osmotic status that enables organisms to survive in hypersaline environment, several strategies have been reported:

(i) Regulation of ions

(ii) The accumulation of organic molecules

(iii) Quenching of active oxygen

(iv) Chaperone for protein folding (stress protein)

(v) Molecular evolution of the salt tolerance enzyme

(vi) The modification of membrane lipid composition.

The first two strategies will be focused in this study.

Regulation of ions

When organisms are subjected to a sudden increase in hyperosmotic conditions, they usually respond with an adaptation reaction. Upon the exposure to high salinity, extrusion of sodium ion is needed to balance and maintain sodium level. Hence all cells have sodium extrusion via the Na^+/H^+ antiporters which catalyze the

exchange of Na^+ for H^+ across membranes. The primary energy source for this system in most organisms is the proton electrochemical gradient ($\Delta\mu_H+$) across the cytoplasmic membrane. This proton electrochemical gradient is derived either from respiratory electron transport or at the expense of ATP formed during substrate-level phosphorylation by activity of the membrane ATPase (Oren, 1999). The Na^+/H^+ antiporters are membrane proteins that are essential for maintenance of the balance between Na^+ and K^+ in plant, fungal and bacterial cells, in particular when the organism lacks primary Na^+ pumps or when the Na^+ pumps are not operative (Inaba et al., 2001). The Na^+/H^+ antiporters play a variety of functions such as:

(i) Establishment of an electrochemical potential of Na^+ across the cytoplasmic membrane, this being the driving forces for Na^+-coupled processes such as Na^+/solute symport and Na^+/driven flagella rotation (Vimont and Borche, 2000).

(ii) Extrusion of Na^+ and Li^+, which are toxic if they accumulate to high concentrations in cells (Dover et al., 1996; Dover and Padan, 2001).

(iii) A role in pH homeostasis, i.e., regulate intracellular pH under alkaline pH condition. In the mutant lacking Na^+/H^+ antiporter activity the capacity to regulate its intracellular pH (pH_i) is lost (Karpel et al., 1991).

(iv) Cell volume regulation (Blumward et al., 2000; Orlowski and Grinstein, 1997; Padan and Schudiner, 1996).

(v) Morphogenesis, it has been reported in yeast cells, *Candida albicans* that the deletion of *chn1* which encoded Na^+/H^+ antiporter resulted in the ratardation of growth and highly elongation morphology in a

significant fraction of cells under conditions that normally support yeast growth (Soong et al., 2000).

In *E. coli*, physiological and molecular genetic studies of the Na^+/H^+ antiporters system have been studied and contribute extensively to the understanding of the physiology of antiporters. Three antiporters in *E. coli*, NhaA, NhaB and ChaA, are known and their functional characteristics have been well described (Padan and Schuldiner, 1996; Rosen, 1986). Six homologous Na^+/H^+ antiporters (exchangers) (NHE1-6) have been found in animals (Orlowski and Grinstein, 1997). In plants and yeast, the vacuole type (NHX1 and AtNHX1) (Nass et al., 1997; Nass and Roa, 1998; Gaxiola et al., 1999; Wells and Roa, 2001) and the plasma membrane type (SOD2 and SOS1) (Jia et al., 1992; Shi et al., 2000) Na^+/H^+ antiporters have been reported. The homology between *E. coli* antiporters and eucaryotic ones was very low suggesting the independent evolution among Na^+/H^+ antiporter genes.

It has been reported that NhaA catalyzes the specific exchange between Na^+ (Li^+) for H^+. Its function is indispensable for adaptation to high salinity, for resistance to Li^+ toxicity, and for growth (Dover et al., 2001; Rosen, 1986). The antiporter activity of NhaA is enhanced at alkaline pH and very low at neutral and acidic pH (Inoue et al., 1995). *Nha*A has been mapped at 0.1 min on *E.coli* chromosome (Karpel et al., 1991). The transcription of this gene is induced by sodium ion and regulated by protein NhaR and affected by H-Ns (Dover and Padan, 2001). The protein NhaR with a molecular mass 34.2 kDa was encoded by *nha*R gene (located 59 nucleotides downstream of *nha*A). It shows homology to a large family of positive regulator in prokaryotes, the LysR-OxyR family (Carmel et al., 1997). The protein encoded by

nhaA is predicted to have a putative secondary structure consisting of 12 transmembrane segments connected by hydrophilic loops (Vimont and Berche, 2000). The *nhaA* homologous gene has also been reported in *Salmonella entiritidis* (Pinner et al., 1992), *Vibrio parahaemolyticus* (Kurada et al., 1994), *Vibrio alginolyticus* (Vimont and Berche, 2000) and *Haemophilus influenza* (Fleischmann et al., 1995).

NhaB is the second antiporter system in *E.coli* and has been mapped at 25.5 min. NhaB by itself confers a limited sodium tolerance to the cells but becomes essential when the lack of NhaA activity limits growth. This gene encodes a membrane protein with predicted 504 amino acids and molecular mass 55,500 Da. Hydropathic analysis of the sequence indicates the presence of 12 putative transmembrane helices (Pinner et al., 1992). The properties of NhaB protein is different from NhaA in both ion specificity and pH sensitivity (Karpel et al.,1991).

ChaA, the third antiporter system, functions as a calcium/proton antiporter and also have the capacity to extrude sodium ion under physiological conditions (Ohyama et al., 1994) suggesting its role in calcium ion circulation at alkaline pH. *Cha*A is proposed to be the structural gene for a Ca^{2+}/H^{+} antiporter whose overexpression leads to resistance to growth inhibition by both calcium and sodium (Ivey et al., 1994).

In cyanobacterium, the existence of a concentration gradient of Na^{+} directed inward, which is dependent on the photosynthetic or respiratory metabolism, has been demonstrated (Padan and Vitterbo, 1986). These results indicate the existence of an active transport system extruding Na^{+} from cyanobacterium cells. From the complete nucleotide sequence of cyanobacterium *Synechocystis* sp. PCC 6803, it was suggested that *Synechocystis* sp. PCC 6803 contains at least five Na^{+}/H^{+} antiporters (Kaneko et. al., 1996). The first study of functional characterization of cyanobacterium antiporter,

SynNhaP, encoded by *synnha*P has been reported (Hamada et al., 2001). SynNhaP shows homology to eucaryotic and a new unique Na^+/H^+ antiporter of procaryotic (NhaP from *Pseudomonas aeruginosa*) ones (Utsugi et al., 1998), but not to the NhaA, NhaB and ChaA. Furthermore, SynNhaP contains a conserved Asp138 in trans-membrane (TM) spanning region and relatively long C-terminal hydrophilic tail important for the carrier activity. The long C-terminal tails are believed to play a role in the regulation of transport activity in animals (Orlowski and Grinstein, 1997; Wakabayashi et al., 1992). These facts suggest that the cyanobacterial antiporters would provide a model system for the study of structural and functional properties of eucaryotic Na^+/H^+ antiporters.

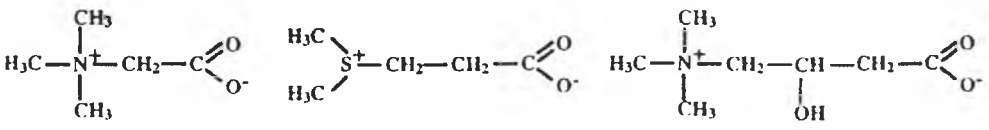
To date, only a few functional residues, especially the residues involved in the cation transport, have been identified in Na^+/H^+ antiporter proteins. The importance of Asp138 in SynNhaP (Hamada et al., 2001) and Asp133, Asp163, and Asp164 in NhaA (Inoue et al., 1995 ; Nakamura et al., 1995) have been reported. The Na^+/H^+ antiporter has been thought to exchange specifically between H^+ and Na^+ or Li^+, but some antiporters exhibited low exchange activity between H^+ and Li^+ (Utsugi et al., 1998). The *E. coli* ChaA has been reported to have an exchange activity between Ca^{2+} and H^+ as well as Na^+ and H^+ at alkaline pH (Ivey et al., 1994), but not examined in detail. It is not clear by which factors ion specificity are determined. To identify the conserved sequences for the cation transport in Na^+/H^+ antiporters, cloning of antiporter with novel ion specificity is a prerequisite.

The accumulation of organic molecule (compatible solute)

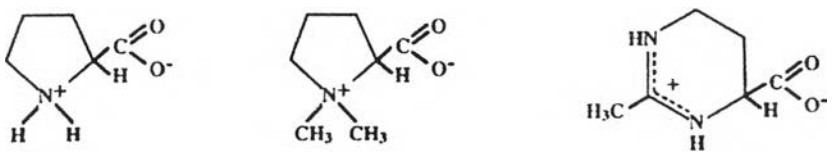
Under osmotic stress, organisms commonly accumulate potassium ion in large amounts and this is rapidly taken up from the environment via turgor-responsive mechanism. Potassium ion is the prominent compatible solutes found in bacteria, transiently accumulated and is superseded by the cocommitted other compatible solute such as proline or glycinebetaine (Kempf and Bremer, 1998).

Compatible solutes are generally defined as organic compounds of low molecular weight that have no inhibitory effects on metabolism when present at high concentration. They are highly soluble molecules and do not carry a net charge at physiological pH. In contrast to inorganic salts, they can reach high intracellular concentrations without disturbing vital functions such as DNA-protein interactions, and the cellular metabolic machinery (Strom and Kaasen 1993; Yancey, 1982; Record et al. 1998). A spectrum of compatible solutes is usually used by a given microorganism for osmoregulatory purposes, and the composition of the solute pool can vary in response to growth phase and growth medium (Galinski and Truper, 1994).

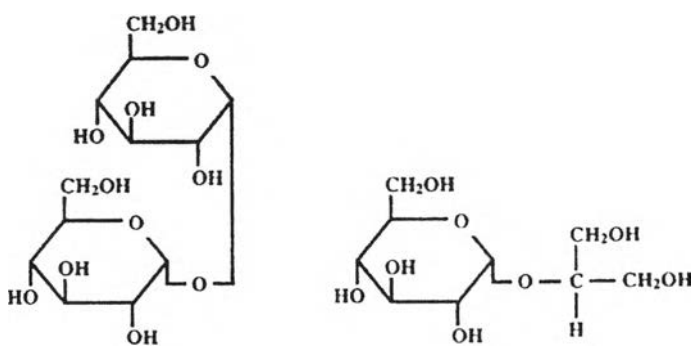
There are several types of compatible solutes (Fig.1.1): sugars (e. g. trehalose), polyols (e. g. glycerol, arabitol, mannitol, glycosylglycerol), free amino acids (e.g. proline and glutamate), derivatives thereof (e. g. prolinebetaine, ectoine), quaternary amines and their sulfonium analogs (e. g. glycinebetaine, carnitine and dimethylsulfoniopropionate), low-molecular weight nonionic carbohydrates (sucrose, trehalose), free amino acids and their derivatives (proline, glutamate, glycine, γ-amonobutyrate, taurine) , unique organic zwitter ion (tetrahydropyrimidine such as ectoine) , methylamines (glycinebetaine and trimethylamine-N-oxide).



Glycine betaine Dimethylsulfoniopropionate Carnitine



Proline Proline betaine Ectoine



Trehalose Glycosylglycerol

Figure 1.1 Structure of some compatible solutes

Accumulation of compatible solutes is a particularly ubiquitous response. It serves a dual function in osmoregulating cells and also serves as stabilizer of proteins and cells components against the denaturing effects of high ionic strength (Kempf and Bremer, 1998). The current understanding is that these compatible solutes maintain and equilibrium between macromolecule surface areas and the water phase by resisting drastic changes in intracellular water density (Martin et al., 1999). These compatible solutes are accumulated by many organisms through synthesis or uptake from the environment to counteract the outflow of water under hypertonic growth condition (Galinski and Truper, 1994). The uptake systems for compatible solutes have been well described in Bacteria. Transporters for osmoprotectant have evolved to meet the special demands imposed by their physiological task. In *E.coli*, permeation of osmoprotectant across outer membrane is accomplished by passive diffusion through nonspecific porins. However, the across of cytoplasmic membrane is only possible via osmoprotectant transporter (Kempf and Bremer, 1998). It has been reported that *Bacillus subtilis*, gram-positive bacteria, also has high-affinity and substrate-specific transport system (Kempf and Bremer, 1998; Whatmore et al., 1990; Kappes et al., 1996). In addition to transpart systems, compatible solutes can accumulate through synthesis. Biosynthesis of potent compatible solutes: trehalose, proline and glycinebetaine has been reported (Bremer and Kramer, 2000; Boch, 1996; Kappes et al., 1996).

Glycinebetaine: characteristics and functions

One of potent osmolytes accumulated by organisms under conditions of hyperosmolarity is glycinebetaine (*N, N, N*-trimethylglycine), a trimethylated

derivative of the amino acid glycine. It has been well described for osmoprotective effects in plant and bacteria (Csonka and Hanson, 1991; Rhodes and Hanson, 1993).

High concentration of glycinebetaine is not inhibitory to many enzymes and partial protection in freezing, heating or drying condition has been reported (Murata et al., 1992). Incharoensakdi et al (1986) reported that glycinebetaine masks inhibitory effect of chloride ion on the enzyme activity of ribulose 1, 5-biphosphate carboxylase/oxygenase and prevents the enzyme dissociation into constituent subunits in *A. halophytica*. Glycinebetaine is also shown to specifically protect glucose-6-phosphate dehydrogenase activity to retain full activity in the presence of high salt in *Spirulina subsalsa* (Gabbay-Azaria et. al., 1988). From the study in intact thylakoid membrane of spinach, glycinebetaine penetrates through lumen to stabilize and protect Mn^{2+}-cluster (catalytic site of photosynthesis in spinach) (Mohanty et al., 1993; Papageogior et al., 1991).

Glycinebetaine: biosynthesis and degradation

Glycinebetaine is synthesized and accumulated by a wide range of organisms including bacteria, higher plants and animals (Csonka and Hanson, 1991; Rhodes and Hanson, 1993; Yancey et al., 1982). Biosynthesis of glycinebetaine results from the oxidation of choline via a two-step oxidation with betaine aldehyde as the intermediate. The series of reaction may be catalyzed by three different enzymatic systems. In microorganisms and mammals, a memebrane-bound choline dehydrogenase is employed in conjunction with a soluble betaine aldehyde dehydrogenase (Andreson et al., 1988; Lamark and Strom, 1986; Boch et al., 1996). Plants utilize a soluble choline monooxygenase in combination with betaine aldehyde

dehydrogenase (Hanson et al., 1994; Rathinasabapathi et al., 1997). Recently a three-step series of methylation reaction from glycine to glycinebetaine has been reported for two extreme halophiles (Nyyssola et al., 2000).

Although glycinebetaine was found to be nonmetabolized in many bacteria, its utililization as a growth substrate has also been reported in *Rhizobium meliloti* (Smith et al, 1988). The degradation of glycinebetaine has rarely been studied in plants and bacteria. The fate of glycinebetaine and its degradation pathway was first proposed in nonstressed *Rhizobium meliloti* (Smith et al, 1988) (Fig.1.2). Interestingly, a recent report has shown that in salt stress *Sinorhizobium meliloti*, the glycinebetaine level increased at an early exponential phase of growth and decreased at a late exponential phase (Talibart et al., 1997). In some marine bacteria, glycinebetaine is degraded upon the dilution stress (Ventosa et al., 1998).

Degradation of glycinebetaine was proposed to be due to the action of BHMT (EC 2.1.1.5) which is an enzyme catalysing a methyl transfer from glycinebetaine to homocysteine (Hcy) forming dimethylglycine and methionine, respectively. The functions of BHMT have been extensively studied in mammals such as horse, pig, and human and is shown to be a hexamer of 45 kDa (Durell et al., 1957; Finkelstein et al., 1967 ; Lee et al., 1992 ; Skiba et al., 1982 ; Sunden et al., 1997). In mammalian, BHMT plays an important role for the regulation of homocysteine metabolism and show high level in kidney and liver. The regulation by BHMT plays an important role for regulation of homocysteine level. The malfunction or deficiency of BHMT leads to hypercysteinemia or homocystinuria (the symptom of these malfuctions results in high level of homocysteine in urine).

Glycinebetaine

↓(1)

Dimethylglycine

↓(2)

Monomethylglycine

↓(3)

Glycine

↓(4)

Serine

↓(5)

Pyruvate

1 = glycinebetaine transmethylase (EC 2.1.1.5)

2=dimethylglycine dehydrogenase (EC 1.5.99.2)

3= monomethylglycine dehydrogenase (EC 1.5.99.1)

4= serine transhydroxylase (EC 2.1.2.1)

5= serine dehydratase (EC 4.2.1.13)

Figure 1.2 Proposed pathway of glycinebetaine degradation in *Rhizobium meliloti* (Smith et. al., 1988)

The organism used in this study is the unicellular cyanobacterium *A. halophytica.* This organism is a highly halotolerant organism that can grow in a wide range of salinity conditions from 0.25-3.0 M NaCl (Ishitani et al., 1993; Takabe et al., 1988). In general, there are a number of protective mechanisms for cells thriving in changing salinity environment. Previously, *A. halophytica* was shown to accumulate a compatible solute, glycinebetaine, upon the increase in the external salinity (Takabe et al., 1988; Lee et al., 1997). Recently, it has been shown that another mechanism employed by *A. halophytica* against high salinity was attributed to the function of DnaK protein as a chaperone for the stabilization of protein (Lee et al., 1997). It is therefore worthwhile investigating whether *A. halophytica* contains an Na^+/H^+ antiporter which may represent another mechanism for adaptation to changing salinity.

On the other hand, due to the fact that *A. halophytica* can exist in both increasing and decreasing salinity environment, it is therefore important to study the regulation of glycinebetaine under hypoosmotic conditions, whose information will further enhance our understanding of how *A. halophytica* copes with changing salinity conditions.

The objective of this thesis is 2-fold, namely the isolation of the Na^+/H^+ antiporter gene from *A. halophytica* and characterizing its function in the salt-sensitive mutant, *E.coli* TO114. Secondly, the characterization of betaine-homocysteine methyltransferase in this organism will be studied.