CHAPTER I

INTRODUCTION

The osmotic strength of the environment is an important physical parameter that influences the ability of organisms to proliferate in a given habitat. Although the ability to adapt to fluctuations in the external osmolarity is fundamental to survival of organisms, the mechanisms responsible for osmotic adaptation have been largely unknown. There are remarkable similarities between bacteria and plants in their cellular responses to osmotic stress, because organisms from both kingdoms accumulate the same set of cytoplasmic solutes upon exposure to conditions of hyperosmolarity. Thus, it is likely that there will be close parallels in the mechanisms that these organisms employ to regulate responses to osmotic stress (Csonka and Hanson, 1991).

Exposure of cells to high external osmolarity results in an efflux of water from the interior. The decrease in the internal water content brings about a reduction in the turgor pressure and a shrinkage of the cytoplasmic volume. As a consequence, the concentrations of all the intracellular metabolites increase, thus cause a reduction in the intracellular water activity. In the absence of active osmotic adjustment by the cell, the cytoplasmic volume would shrink until water activity of the interior equaled that of the exterior. This passive alteration of cell volume is not adequate for adaptation to changes in the osmolarity of the environment, because an elevation in the concentrations of various intracellular molecules may be inhibitory to cellular processes e.g., inhibitors of specific enzymes may reach deleterious concentrations or the increase in the concentrations of ions may become toxic (Yancey et al., 1982).

Instead of a passive volume regulation, organisms respond to osmotic stress by increasing the concentrations of a limited number of solutes. Thus, the water activity of cell interior can be reduced, and consequently cell volume and turgor can be restored near their prestress values without an increase in the concentration of all cytoplasmic components. Since the molecules that are accumulated during conditions of osmotic stress are not greatly inhibitory to cellular processes, they have been termed compatible solutes (Csonka, 1989).

Cyanobacteria, oxygenic photosynthetic prokaryotes from which plasmids of photosynthetic eukaryotes (algae and plants) are derived, inhabit a variety of environments including those in which extreme conditions such as high temperatures and/or high salinity are found (Nomura et al., 1995). The critical demands of cyanobacteria exposed to high salinity, i.e., accumulation of osmoprotectants and extrusion of sodium ions, are met through immediate activation and/or long term (protein synthesis-dependent) adaptation of various processes:

1. uptake and endogenous biosynthesis of osmotica, the nature and amount of which are strain and salt concentration-dependent;
2. enhancement of H^+-ATPase activity and active extrusion of sodium ions;
3. probable modifications of membrane lipid composition; and
4. increased energetic capacity, at the level of cyclic electron flow around photosystemI (through routes induced under these conditions) and cytochrome c oxidase (Joset et al., 1996).

One mechanism of osmotic stress adaptation commonly found in many organisms involves intracellular accumulation of organic compounds, called osmolytes, which contribute to a counterbalancing osmotic pressure (Csonka and Hanson, 1991). Osmolytes are often termed compatible solutes because they do not produce adverse effects upon protein structure and solubility, protein-protein interactions, enzyme-substrate interactions, or protein-nucleic interactions, as would

high concentrations of inorganic salt (Yancey et al., 1982). Hence, intracellular accumulation of osmolytes, which can occur either by *de novo* synthesis or by transport from the growth medium, confers tolerance to hyperosmotic stress (Csonka, 1989)

1.1 Compatible solutes

The prominent compatible solutes found in bacteria are K^+ ions, the amino acids glutamate, glutamine, proline, γ-aminobutyrate, and alanine, the quaternary amines glycine betaine and other fully *N*-methylated amino acid derivatives, and the sugars sucrose, trehalose, and glucosylglycerol (Imhoff, 1986; Reed et al., 1986). These compounds are accumulated at high intracellular concentrations and unable to cross the cell membranes rapidly without the aid of transport systems. The most part of compatible solutes do not carry a net electrical charge near pH 7.0. This property can be rationalized to be beneficial because uncharged molecules can be accumulated to high intracellular concentrations without greatly disturbing the structures of cellular macromolecules. However, K^+ ions and glutamate are noteworthy exception to this generalization, and these two solutes may not offer as effective protection against hyperosmotic stress as some of the uncharged metabolites (Sutherland et al., 1986).

Compatible solutes can be accumulated by bacteria by synthesis or transport from the culture medium. There are differences in the effects of various compatible solutes on the osmotic stress tolerant cells. Some can elicit dramatic stimulation of the growth rates of the cells in media of high osmolarity when they are added to the culture, whereas others have no detectable effects on the growth of the cells. The reason for this difference between the two types of compatible solutes is not understood, but it may be that solutes which can alleviate osmotic inhibition can be

accumulated to higher internal levels by transport or are less toxic at high internal concentrations than solutes which do not have similar effects. Alternatively, it may be that the substances which can alleviate osmotic inhibition have some special interactions with cellular macromolecules resulting in increased stability of these macromolecules in cell grown in media of high osmolarity. Regardless of the molecular mechanism responsible for their ability to overcome osmotic inhibition, solutes which have this property are sometimes called osmoprotectants (Csonka, 1989).

Osmoregulation in cyanobacteria grown under salt stress involves the accumulation of organic and inorganic solutes. Fresh water cyanobacteria accumulate simple saccharides predominantly sucrose and trehalose; marine strains accumulated glucosylglycerol, while hypersaline strains accumulate glycine betaine (Table 1.1).

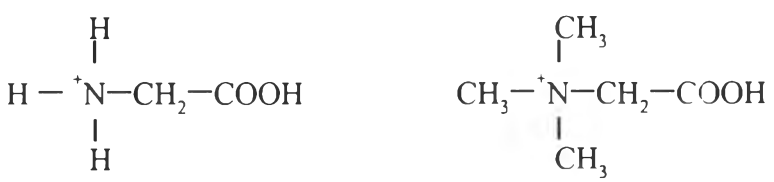
The unicellular cyanobacterium *Aphanothece halophytica* is a highly halotolerant organism that can grow at high external NaCl concentration up to 3.0 M (Takabe et al., 1988). The osmotic adaptation of this organism was first studied by Miller et al. (1976) who showed that *A. halophytica* accumulated up to 1.0 M K^+ in response to increasing external salinity. Reed et al. (1986) demonstrated that glycine betaine is the major solute accumulated up to nearly 2.0 M followed by K^+ in *A. halophytica* grown in high salinity. Furthermore, the variation for glycine betaine accumulation in response to changes in extracellular salinity is much greater than that observed with K^+. In addition, Sibley and Yopp (1985) reported that *A. halophytica* synthesize glycine betaine as a major osmolyte at high levels of external salinity.

Table 1.1 Major organic osmoregulatory solutes of cyanobacteria (Mackay et al.,1984).

Taxomic assignment	Sucrose	Trehalose	Glucosyl-glycerol	Glycine betaine	Maximum salt tolerance (g NaCl/l)	Strain origin
Chamaesiphon PCC6605	-	+	-	-	< 4.8	Stream water
Anabaena PCC7122	+	-	-	-	14.3	Pond water
Synechococystis PCC6701	+	-	-	-	28.5	Fresh water
Synechococystis PCC6906	-	-	+	-	70.4	Hypersaline lake
Dermocarpa	-	-	+	-	104.8	Supralittoral
Myxosarcina PCC7312	-	-	+	-	108.8	Intertidal
Calothrix PCC7426	-	-	+	-	132.1	Intertidal
Synechococcus PCC7418	-	-	-	+	150.0	Hypersaline lake
Spirulina	-	-	-	+	≥150.7	Salt lake

1.2 Glycine betaine

An important osmoprotectant compound accumulated by organisms under conditions of hyperosmolarity is glycine betaine (*N*,*N*,*N*-trimethylglycine), a derivative of amino acid glycine (Fig. 1.1). Higher plants and some other prokaryotes are able to carry out *de novo* synthesis of glycine betaine. Most other bacteria are not able to do so, and therefore they are dependent on the transport of this compound for its accumulation (Koo et al., 1991; Peter et al., 1996; Riou and Le Rudulier, 1990). Sakaguchi (1960) first reported that exogenous glycine betaine can stimulate the respiration rate of a halophilic bacterium, Ba-1, in media of elevated NaCl concentration. Subsequently, Le Rudulier and Bouillard (1983) observed that this compound is a potent osmoprotectant for members of family *Enterobacteriaceae*. Perroud and Le Rudulier (1985) found that the intracellular concentrations of glycine betaine maintained by *E. coli* were proportional to the osmolarity of the medium. The transport of glycine betaine was observed to be stimulated by osmotic stress in *Staphylococcus aureus* (Pourkomailian and Booth, 1994), *Listeria monocytogenase* (Ko et al., 1994), *Rhodobacter sphaeroides* (Abee et al., 1990) and *Lactobacillus acidophilus* (Hutchins et al., 1987).



Glycine **Glycine betaine**

Figure 1.1 Structure of glycine and glycine betaine.

1.3 The role of glycine betaine

Glycine betaine has been demonstrated to be a major osmoticum in widely different organisms, both prokaryotes (Csonka, 1989) and eukaryotes (Rhodes and Hanson, 1993). In higher plants, some plants in families *Chenopodaceae*, *Amaranthaceae*, and *Gramineae*, for example, accumulate glycine betaine in response to salt stress or drought (Brouquisse et al., 1989; Ishitani et al., 1995). Hanson et al. (1985) reported that glycine betaine synthesis occurs in chloroplasts of spinach leaves. Subsequently, Robinson and Jones (1986) reported the accumulation of glycine betaine up to 0.3 M in spinach chloroplast to provide osmotic adjustment during salt stress.

The osmoprotective role of the glycine betaine is evident in a number of diverse microbial systems, including enteric bacteria (Koo et al., 1991; Le Rudulier and Bouillard, 1983), soil bacteria (Peter et al., 1996; Smith et al., 1988), halophilic bacteria (Galinski and Truper, 1982), methanogenic archaebacteria (Robertson et al., 1990), and cyanobacteria (Gabbay-Azaria et al.,1988; Moore et al.,1987). It has been suggested that glycine betaine functions as an osmolyte in the mammalian renal system as well (Bagnasco et al., 1986; Garcia-Perez and Burg, 1991).

High concentration of salts have been reported to inhibit the activity of many enzymes in both prokaryote and eukaryote (Matoh et al., 1987). Glycine betaine is also known to protect enzymes from the inhibitory effects of high salt centration. Higher plants are able to compartmentalize the accumulated salts, Na^+ and Cl^- in particular, in the vacuole and thereby prevent the inhibition of enzyme activities. In contrast, the soluble enzymes in prokaryotic organisms as well as cytoplasmic enzymes are directly exposed to any osmoregulatory substances (Matoh et al.,1987). Incharoensakdi et al. (1986) reported that glycine betaine masks inhibitory effect of Cl^- on the enzyme activity of ribulose 1,5-bisphosphate carboxylase/oxygenase and prevents the enzyme dissociation into constituent subunits in *A. halophytica*. Glycine

betaine 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). Furthermore, glycine betaine protects photosystem II complex from the salt-induced dissociation of extrinsic proteins and inactivation of the oxygen-evolving machinery of spinach (Murata et al., 1992). In cyanobacterium *Synechocystis* PC6803, glycine betaine is effective both in the stabilization and stimulation of the oxygen-evolving machinery and the synthesis of ATP (Mamedov et al., 1991). Therefore, glycine betaine is involved in not only osmoregulation but also stabilization of enzymes in the cells grown in high salinities.

Besides the physiological role as an osmoprotectant, glycine betaine also functions in general metabolism where methyl groups derived from it are incorporated into alkaloids in plants (Byerrum et al., 1956), into methionine in mammals (Skiba et al.,1982) and microorganisms (Smith et al., 1988; White and Demain, 1971), and into cobalamine (vitamin B12) in microorganisms (White and Demain, 1971). Furthermore, glycine betaine and its precursor, choline can be used as a carbon and nitrogen source by some microorganisms (Pocard et al.,1997). Methyl groups liberated by glycine betaine catabolism in these microbial systems may be assimilated or oxidized to carbon dioxide via one-carbon metabolism pathways (Livering et al., 1987).

1.4 The synthesis of glycine betaine

The ability to synthesize glycine betaine is found in several bacteria (Canovas et al., 1998; Mori et al., 1992; Osteras et al., 1998), higher plants (Hanson and Wyse, 1982; McCue and Hanson, 1992; Wood, 1996) and mammals (Chern and Pietruszko, 1995; Gracia-Perez and Burg, 1991). Biosynthesis of glycine betaine results from oxidation of choline via a two-step reaction with betaine aldehyde as the intermediate

(Fig. 1.2). This series of reaction may be catalyzed by three different enzymatic systems. In microorganisms and mammals a membrane-bound choline dehydrogenase (EC 1.1.99.1) is employed in conjunction with a soluble betaine aldehyde dehydrogenase (EC 1.2.1.8) (Andresen et al., 1988; Gracia-Perez and Burg, 1991; Lamark and Strom, 1986). Plants utilize a soluble choline monooxygenase in combination with betaine aldehyde dehydrogenase (Burnet et al., 1995; Hanson et al., 1994; Rathinasabapathi et al., 1997) The third choline oxidation system, as yet found only in microorganisms, involves a soluble choline oxidase (EC 1.1.3.17) that is capable of catalyzing both reactions (Deshnium et al., 1995; Rozwadowski et al., 1991).

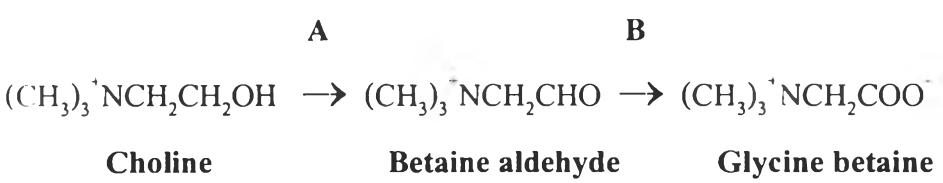


Figure 1.2 Choline-glycine betaine pathway. Enzymes involved are

A : choline dehydrogenase or choline monooxygenase,

B : betaine aldehyde dehydrogenase

1.5 Glycine betaine synthesis of *E. coli*

The choline-glycine betaine pathway of *E. coli* comprises two dehydrogenases. The first enzyme is an oxygen-dependent choline dehydrogenase, which can catalyze the oxidations of choline to betaine aldehyde and betaine aldehyde to glycine betaine at approximately the same rate. This enzyme is independent of soluble cofactors, membrane-bound, and probably electron-transfer-linked. In addition. *E. coli* has a soluble NAD^+-dependent betaine aldehyde dehydrogenase

(Landfald and Strom, 1986). This enzyme has been purified and shown to be highly specific for betaine aldehyde (Boyd et al., 1991; Falkenberg and Strom, 1990). *E. coli* has a high-affinity transport system for choline with a K_m value of 8 μM (Styrvold et al., 1986). The *bet* genes encoding all of these proteins have been cloned from *E. coli* by genetic complementation of the salt sensitive phenotype of *bet* deletion mutants (Andresen et al., 1988). The nucleotide sequence and biochemical data revealed that the *bet* region of *E. coli* comprises four genes. *betA* and *betB* code for choline dehydrogenase and betaine aldehydrogenase, respectively. *betT* codes for a proton-motive force-driven, high-affinity transport system for choline, and *betI* for a repressor involved in the regulation of *bet* genes for choline. The *betTIBA* genes cluster in *E. coli* chromosome within a region of 5.9 kilobases, with *betI* located upstream of and transcribed divergently to the tandemly linked *betIBA* genes (Lamark et al.,1991).

The *bet* genes of *E. coli* are expressed only under aerobic conditions. They are induced by osmotic stress, but for full expression, choline (e.g. 1 mM) is required in the growth medium (Landfald and Strom, 1986). Studies using *lac* operon fusions with the *bet* genes revealed that they are regulated at the transcriptional level by glycine betaine and temperature, and also by osmolarity, oxygen, and choline (Eshoo, 1988).

1.6 Glycine betaine biosynthesis in plants

In plants, the biosynthesis of glycine betaine is oxygen dependent and has been localized to chloroplast (Hanson et al., 1985). Glycine betaine is synthesized by the two-step oxidation of choline via the intermediate betaine aldehyde (Arakawa et al., 1987; Lerma et al., 1988). The first step is catalyzed by choline monooxygenase, a ferredoxin-dependent stromal enzyme (Burnet et al., 1995) that converts choline to

betaine aldehyde (Brouquisse et al., 1989; Rathinasabapathi et al., 1997). Choline monooxygenase is in fact unique to plants. In other organisms, the choline-oxidizing enzymes are oxidases (Ohta-Fukuyama et al., 1980) or dehydrogenases (Landfald and Strom, 1986). Furthermore, choline monooxygenase is both soluble and insensitive to choline (Brouquisse et al, 1989). The second step, as well as mammal and bacteria, is mediated by betaine aldehyde dehydrogenase (EC 1.2.1.8), a NAD^+-dependent dehydrogenase specific to betaine aldehyde (Weigel et al., 1986; Weretilnyk and Hanson, 1989). Both choline monooxygenase and betaine aldehyde dehydrogenase are soluble chloroplast enzymes (Brouquisse et al., 1989). The accumulation of glycine betaine is induced by drough (Ladyman et al., 1980) and salinity (Arakawa et al., 1992; Hanson and Wyse, 1982) Glycine betaine accumulates primarily in leaves of stressed plants (Hanson et al., 1985). The stress-induced accumulation is associated with increases in the activities of choline monooxygenase (Brouquisse et al., 1989) and betaine aldehyde dehydrogenase (Weretilnyk and Hanson, 1990). The observed increase in betaine aldehyde dehydrogenase activity is accompanied by increases in the levels of betaine aldehyde dehydrogenase protein and mRNA (McCue and Hanson, 1992; Weretilnyk and Hanson, 1990).

1.7 Expression of betaine aldehyde dehydrogenase gene in different hosts

The enzymes involved in glycine betaine synthesis have recently gained considerable biotechnological attention in connection with attempts to genetically engineer plants with increased salt tolerance and resistance to drought (Holmstrom et al.,1994; Rathinasabapati et al., 1994). The cDNA clones corresponding to betaine aldehyde dehydrogenase have been cloned from spinach (Weretilnyk and Hanson, 1990), sugar beet (McCue and Hanson, 1992), barley (Ishitani et al., 1995) and sorghum (Wood et al., 1996). Plant betaine aldehyde dehydrogenase cDNAs

(Rathinasabapathi et al., 1994) and a bacterial betaine aldehyde dehydrogenase gene (Holmstrom et al., 1994) have been expressed in transgenic tobacco plants. *Synechococcus* sp. PCC7942 cells transformed with *E. coli betTIBA* gene accumulated glycine betaine to about 80 mM and acquired an increased tolerance to salt stress (Nomura et al., 1995). In addition, salt-stress decreased the ribulose 1,5-bisphosphate carboxylase activity in *Synechococcus* sp. PCC7942 control cells, but not much in *betTIBA*-containing cells (Nomura et al., 1998).

The unicellular cyanobacterium *A. halophytica* is a highly halotolerant organisms. It can grow at high external NaCl concentration up to 3.0 M (Takabe et al., 1988). One of the mechanisms enabling the halotolerance is the accumulation of glycine betaine. Glycine betaine is a major osmoprotectant, accumulating up to 2.0 M in response to changes of extracellular salinity (Reed et al., 1984). Biosynthesis of glycine betaine results from the conversion of choline to betaine aldehyde by choline dehydrogenase, and betaine aldehydrogenase converts betaine aldehyde to glycine betaine (Kumarb, 1997; Wutipraditkul, 1998). In *E. coli* and *R. meliloti*, genes coding for these two enzymes are called *betA* and *betB*, respectively (Styrvold et al., 1986; Pocard, et al., 1997). The choline-glycine betaine synthesis pathway is an important facet of the process of cellular adaptation of *A. halophytica* to high-osmolarity stress. When *A. halophytica* was cultured under salt stress, the activity of betaine aldehyde dehydrogenase in the cells increased (Kumarb, 1997). The level of glycine betaine inside the cells was also increased. Therefore, choline-glycine betaine pathway was an important mechanism of salt tolerance in *A. halophytica*. To study the system in detail, an attempt to clone *betB* gene was considered. The objective of this thesis is to clone the *betB* gene that codes for betaine aldehyde dehydrogenase of *A. halophytica*.