CHAPTER I

INTRODUCTION

The osmotic strength of the environment is one of the physical parameters that determine the ability of organisms to proliferate in a given habitat. Although the ability to adapt to fluctuations in the external osmolarity is fundamental to the survival of organisms, the mechanisms responsible for osmotic adaptation have been elucidated only relatively recently. There are remarkable similarities between bacteria and plants because organisms from both kingdoms accumulate the same set of cytoplasm 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 response to osmotic stress (Csonka and Hanson, 1991).

The determination of saline spectrum is very important for the characterization of the type of bacteria to be regarded as halotolerant or extreme, moderate and slight halophiles. According to the salt concentration required for optimum growth, the bacteria were classified as non halophilic (grow below 0.2 M NaCl), slightly halophilic (grow at 0.2 to about 1.0-1.2 M NaCl), moderately halophilic (grow at about 1.0-1.2 to 2.0-2.5 M NaCl) and extremely halophilic bacteria (grow at 2.0-2.5 M NaCl or more). Halophile is demonstrated by the requirement of a high salt concentration for optimum growth; however, halotolerance, qualitatively and quantitatively, describes the ability to grow at a salt concentration higher than optimum. However, the degree of tolerance of bacteria depends on the composition of the growth medium. The halophilic eubacteria are usually found in numerous saline

habitats with different salinities, e.g. hypersaline soils, inland marshes, bottom sediments or other different habitats. They are present in non-saline habitats, in the same manner that non-halophilic bacteria are present in hypersaline environments (Zahan, 1997).

The bacteria, which usually grow in non-saline conditions, may exhibit a great modification in cell morphology when subject to high salt stress. The swelling, elongation and shrinkage (reduction in cell volume) are characteristic features of sensitive bacteria under salt stress. However, it has been recently found that the cell and cytoplasmic volume of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum* (a salt-tolerant bacterium) spontaneously decrease upon hyperosmotic shock (Skjerdal et al., 1995). The modification in cell morphology under salt stress is a familiar response of some gram-negative bacteria such as *Azotobacter vinelandii* (Knowles and Smith, 1971), *Escherichia coli* (Baldwin et al., 1988), *Pseudomonas fluorescens* (Parente and Silva, 1984) and *Rhizobium* (Zahran, 1991). The root-nodule bacteria of the genus *Rhizobium*, isolated from salt-affected soils, have shown some salt-induced alterations. The cell shape, the synthesis pattern of protein and lipopolysaccharide as well as the genomic structure of these bacteria were modified. These alterations may affect the symbiosis between rhizobia and their legume hosts. The morphology of some gram-negative bacteria of saline soil has been reported to be modified, cells were elongated several times when subjected to 10-20% NaCl. The gram-positive bacteria (e.g. *Bacillus* and *Staphylococcus*) modified their cell structure under salts stress conditions. Cells of *Bacillus* were elongated and thickened, and the formation of more chains (streptobacilli) was stimulated at

10% NaCl, and the cell volume of *Staphylococcus* was reduced at 10-20% NaCl (Zahran, 1997).

Salt tolerant bacteria usually exhibit structural modifications to cope with salt stress. One important aspect of structural adaptations is the change in composition of the cell envelope and membranes. The stretched state of the wall and the internal osmotic pressure of bacteria are usually affected by the biophysical properties of the stress bearing peptidoglycan (Koch, 1984). Changes in composition of bacterial membranes, which might be caused by environmental factors, are thought to act as an adaptive response to maintain membrane stability and function (Imhoff and Theimann, 1991). In fact, structural adaptations of membrane involve alterations in the composition and synthesis of proteins, lipids and fatty acid (Thiemann and Imhoff, 1991). Some bacteria occasionally show slight cell modifications, but more profound changes in cellular properties of bacteria only occur at concentrations above 2 M NaCl as an adaptation to salt stress. Electron micrographs of thin sections, however, have not revealed any ultrastructural differences in membranes of the anaerobic bacteria (*Halobacteroides halobius* and *Halobacteroides acetoethylicus*) that could account for the ability of these bacteria to inhabit ecological niches high in salt (Lowe et al., 1993).

Osmotic adaptation in microorganisms-two strategies

The ability to adapt to fluctuations in external osmotic pressure (osmoadaptation) and development of specific mechanism to archive this (osmoregulation) are fundamental to the survival of cells. Most cells maintain an osmotic pressure in the cytoplasm that is higher than that of the surrounding environment, resulting in an outward directed pressure, turgor, whose maintenance is essential for cell division and growth. Changes in environmental osmolarity can trigger the flux of water across the cytoplasm membrane. Thus, to avoid lysis under low osmolarity or dehydration under mechanisms that permit timely and efficient adaptation to changes in environmental osmolarity (Deana et al., 1999).

Microbial life can be found over the whole range of salt concentrations from freshwater and marine biotropes to hypersaline environments with NaCl concentrations up to saturation. Halophilic and halotolerant microorganisms are found in three domains of life: *Archaea*, *Bacteria* and *Eucarya*. Colonization of hypersaline environments such as salt lakes and salted food products by these microorganisms is often highly successful, and salt-loving and/or salt tolerant microorganisms may reach high population densities in such ecosystem.

Since biological membranes are permeable to water, cells cannot maintain the water activity of their cytoplasm higher than that of the surrounding brine, because this would lead to a rapid loss of water to the environment. Therefore, any microorganism living at high salt concentrations may be expected to keep its

cytoplasm at least isoosmotic with the extracellular environment. Buildup of a turgor pressure requires a hyperosmotic cytoplasm (Oren, 1999).

Microorganisms do not possess active transport mechanisms for water; hence, turgor is adjusted by controlling the pool of osmotically active solutes in the cytoplasm. Two basic schemes of adaptation to high osmolality have been identified.

1. The accumulation of very high intracellular concentrations of ions, a strategy followed frequently by extreme halophilic archaea and by halotolerant bacteria whose entire physiology has been adapted to a high saline environment (Ventosa et al.,1998).

2. The intracellular amassing of osmotically active compounds that are highly congruous with cellular functions, the so-called compatible solutes. These organic solutes are accumulated by many microorganisms through synthesis or through uptake from the environment to counteract the out flow of water under hypertonic growth conditions. Osmoprotectants are operationally defined as exogenously provided organic solutes that enhance bacterial growth in media of high osmolality. These substances may themselves be compatible solutes, or they may act as precursor molecules that can be enzymatically converted into these compounds. The intracellular amassing of compatible solutes is not restricted to the prokaryotic world but is also widely used as an adaptive strategy in fungal, plant, animal and even human cells to offset the deleterious effects of high osmolality and high ionic strength. Furthermore, the nature of the osmolytes that are accumulated during water stress is evolutionarily well conserved across the kingdoms, reflecting fundamental

constraints on the kind of solutes that are compatible solutes with macromolecular and cellular functions (Le Rudulier et al., 1984).

Compatible solutes: characteristics and function

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 the reduction in the turgor pressure and shrinkage of the cytoplasmic volume. As a consequence of the decrease in the cytoplasmic volume, the concentrations of all the intracellular metabolites increase and thus cause a reduction in the intracellular water activity. In the absence of of active osmotic adjustment by the cell, the cytoplasmic volume would shrink until the water activity of the interior equaled that of the exterior. 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) passive alteration of the cell volume is not adequate for adaptation to changes in the osmolarity of the environment (Laszlo, 1989).

Instead of a passive volume regulation, organisms generally respond to osmotic stress by increasing the concentrations of a limited number of solutes. Thus the water activity of the cell interior can be reduced and consequently cell volume and torgor can be restored near their prestress values without an across the board increase in the concentrations of all cytoplasmic components (Brown et al., 1972).

A variety of bacteria and archaea have been examined for compatible solute production by means of high performance liquid chromatography and nuclear magnetic resonance methods. The spectrum of compatible solutes used by microorganisms comprise only a limited number of compounds: sugar (e.g. trehalose and sucrose), polyols (e.g. glycerol and glucosylglycerol), free amino acids (e.g. proline and glutamate), derivatives thereof (e.g. proline betain and ectoine), quaternary amines and their sulfonium analogues (e.g. glycine betaine, carnitine and dimethylsulfoniopropinate), sulfate ester (e.g. choline-*O*-sulfate), and *N*-acetylated diamino acids and small peptides (e.g. *N*-acetylornithine and *N*-acetylglutaminylglutamine amide). In general, compatible solutes (Figure 1) are highly soluble molecules and do not carry net charge at physiological pH. In contrast to inorganic salts, they can reach high concentrations without disturbing vital cellular functions such as DNA replication, DNA-protein interaction and the cellular metabolic machinery (Strom and Kaasen, 1993). Many microorganisms accumulate compatible solutes as metabolically inert stress compounds, and in those instances where these substances can be metabolized, sensitively balanced regulatory mechanisms ensure their high level accumulation under hypertonic conditions. A spectrum of compatible solutes is usually used by given microorganism for osmoregulatory purposes, and the composition of the solutes pool can vary in response to growth phase and growth medium (Galinski and Truper, 1994). The accumulation of compatible solutes not only allows the cells to withstand a given osmolality but also expands the ability of microorganisms to colonies ecological niches that are otherwise strongly inhibitory for their proliferation. Depending on the type, compatible solutes can also protect microorganisms against stresses other than dehydration. An example is the increased cold tolerance conferred by the

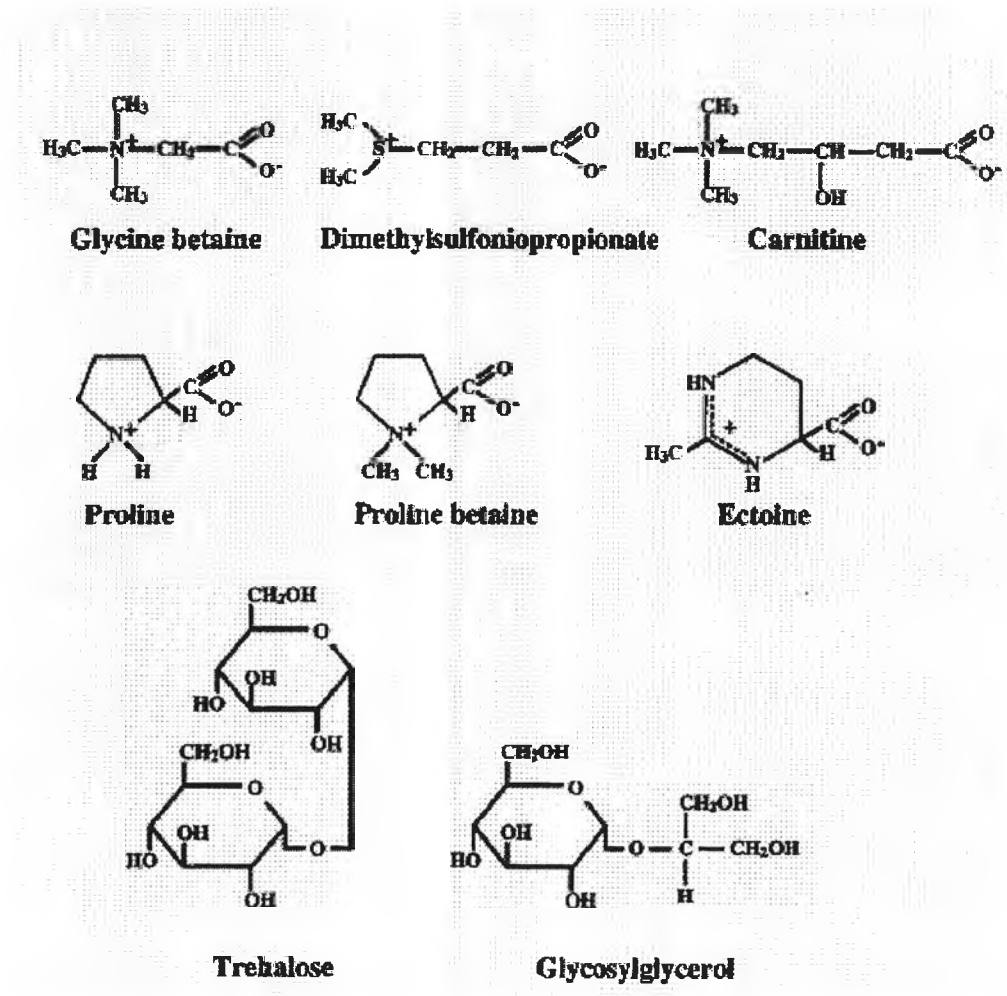


Figure 1.1 Structure of select osmoprotectants

accumulation of glycine betaine in the food-borne pathogen *Listeria monocytogenes* (Ko et al., 1994).

Compatible solutes serve a dual function in osmoregulating cell. Because microorganisms frequently accumulate them up to molar concentrations, compatible solutes lower the cytosolic osmotic potential and hence make major contributions to the restoration and maintenance of turgor. The free cytoplasmic volume (unbound water, in contrast to water bound by macromolecules) is a key determinant for cell growth (Cayley et al., 1992). Compatible solutes such as glycine betaine and proline increase the cytoplasmic volume and free water content of the cell at high osmolarity, and their accumulation thus permits continued cell proliferation under unfavorable condition. Compatible solutes also serve as stabilizers of proteins of high ionic strength. This protective property is not fully understood, but according to the preferential exclusion model, osmoprotectants are kept away from the immediate vicinity of proteins, resulting in a preferential hydration of protein surface. This solvent distribution leads to a situation in which the disruption of water structure in the hydration shell of proteins by local or global unfolding of the polypeptide chain is energetically unfavorable, and hence the native conformations of proteins are stabilized.

In the most cases, microorganisms regulate their osmotic solute content separately from their biosynthetic and energy generating processes. Thus, *Rhizobium* (a bacterium with a relatively low salt tolerance) can synthesize glycine betaine from choline or take up glycine betaine from the medium. While glycine betaine can serve as both a nutrient and an osmoprotector, its degradation is repressed at high salt

concentrations, when it serves as an osmoticum only (Fougere and Le Rudulier, 1990). *Halorhodospira* cells use glycine betaine only as osmotic solute. The compound cannot serve as a nitrogen source, even under the most stringent nitrogen depletion. The physiological basis of this one way only biosynthetic pathway is not understood, but it emphasizes the importance of betaine not only as an inert solute but also as a cytoplasmic protectant. However, nitrogen starvation induces the breakdown of ectoine, mobilizing two nitrogen atoms per molecule; trehalose then replaces ectoine as the osmotic solute (Galinski and Herzog, 1990). The advantage of being able to choose between different osmotic solutes is obvious. Many halophilic and halotolerant microorganisms maintain "cocktails" of osmotic solutes, and the regulation of the synthesis of each of the solutes is optimized according to the needs of the cells (Ventosa et al., 1998 and Galinski, 1995).

Transport of compatible solutes

In addition to accumulating compatible solutes by endogenous synthesis, a large variety of *Bacteria* and *Archaea* have developed the ability to acquire preformed osmoprotectants from exogenous sources. These compounds are released into ecosystems by primary microbial producers from osmotically downshocked cells; by decaying microbial, plant and animal cells; by root exudes and by mammals in their excretion fluids (Galinski and Truper 1994 and Ventosa et al., 1998). Transporters for osmoprotectancts (Table 1) have evolved to meet the special demands imposed by their physiological tasks. In natural ecosystems, the supply of osmoprotectants and their biosynthetic precursors is varying and generally very low, with concentrations

usually in the nanomolar to micromolar range (Kiene et al., 1998). Therefore, osmoprotectant transporters commonly exhibit very high affinity for their major substrates, and their capacity is geared to permit accumulation of compatible solutes to molar concentration. In addition, they function effectively at high osmolarity and at high ionic strength, conditions that frequently impair the performance of transporters for nutrients (Roth et al., 1985). To take advantage of the spectrum of osmoprotectants available in their habitat, microorganisms often possess several transport systems, some of which exhibit broad substrate specificity. Tranporters for osmoprtectants have been most fully investigated at the molecular level in the gram-negative enteric bacteria *E.coli* and in the gram-positive soil bacteria *B.subtilis*.

The role of glycine betaine

A very important quaternary ammonium compound commonly used in cellular osmoregulation is glycine betaine (*N,N,N*-trimethylglycine), a derivative of amino acid glycine (Figure 2), is evident in a number of diverse microbial systems, including enteric bacteria (Andersen et al., 1988), soil bacteria (Smith et al., 1988), halophiliic bacteria (Galinski and truper, 1982), cyanobacteria (Mackey et al., 1984) and metanogenic archae bacteria (Robertson et al., 1990). In higher plant, some plants in families *Chenopodaceae Amaranthaceae*, and *Gramineae*, for example, accumulate glycine betaine in response to salt stress or drought (Brouquisse et al., 1989 and Ishitani et al., 1995). Hanson et al (1985) reported that glycine betaine synthesis occurs in chloroplasts of spinach leaves. Subsequently, Robinson and Jones (1986)

Table 1. Uptake systems for compatible solutes

Organism	System	Type of mechanism	Substrate spectrum	Major substrate(s)[a]	Regulation at the level of:	
					Expression	Activity
E.coli	ProP	H^+ symport	Broad	GB, PB, Pro, Car, Ect, others	+	+
	ProU	ABC transporter[b]	Broad	GB, PB, Pro, others	+	+
	BetT	Secondary transport[c]	Narrow	Cho	+	+
B.subtilis	OpuA	ABC transporter	Meduim	GB, PB, others	+	ND[d]
	OpuB	ABC transporter	Narrow	Cho	+	ND
	OpuC	ABC transporter	Broad	GB, PB, Pro, Car, others	+	ND
	OpuD	Na^+ symport	Narrow	GB, others	+	+
	OpuE	Na^+ symport	Narrow	Pro	+	-

[a] Car, carnitine; Cho, choline; Ect, ectoine; GB, glycine betaine; PB, proline betaine; Pro, proline.

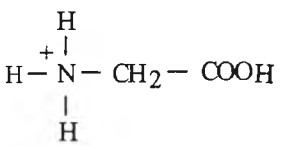
[b] ABC, ATP-binding cassette.

[c] The cotransported ion (Na^+ or H^+) is not known.

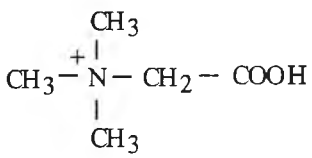
[d] ND,not determined

reported the accumulation of glycine betaine up to 0.3 M in spinach chloroplast provide osmotic adjustment during salt stress.

Sakauchi (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 *Lactobacillus acidophilus* (Hutchins et al., 1987), *Listeria monocytogenase* (Ko et al., 1994), *Rhodobacter sphaeriodes* (Abee et al., 1990) and *Staphylococcus aureus* (Pourkomailian and Booth, 1994).



Glycine



Glycine betaine

Figure 1.2 Structure of glycine and glycine betaine

Glycine betaine also acumulates in the halophilic, photosynthetic bacterium *Ectothiorhodospira halochloris* and in several strains of cyanobacteria (Shkedy-Vinkler and Avi-Dor, 1975 and Reed et al., 1984). Mamedov et al (1991) reported that in cyanobacterium *Synecocystis* PCC 6830, glycine betaine was effective both in the stimulation and in the protection of the oxygen evolving machinery and the synthesis of ATP. In *Aphanothece halophytica*, a halotolerant cyanobacterium, glycine betaine was a major osmoticum accumulating inside the cells in response to changes in external salinity. In this organism, glycine betaine acts as an osmoprotectant (Reed et al., 1984).

High concentrations of salts have been reported to inhibit the activity of many enzymes in both prokaryote and eukaryote. Glycine betaine is also known to protect enzymes from the inhibitory effects of high salt concentration. Higher plants are able to compartmentalize the accumulated salts, Na^+ and Cl^- inparticular, in the vacuole and thereby prevent the inhibition of enzyme activities. In contrast, the soluble enzymes in prokaryotic organism as well as cytoplasmic enzymes are directly exposed to any osmoregulatory substances (Matoh et al., 1987). Incharoensakdi et al (1986) repoted that glycine betaine masks inhibitory effect of Cl^- on the enzyme activity of ribulose 1,5-bisphosphate carboxylase/oxyganase and prevents the enzyme dissociation into constituent subunits in *Aphanothece 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 machinary of spinach (Murata et al, 1992). Therefore, glycine betaine is involved in

not only osmoregulation but also stabilization of enzymes in the cells grown in high salinities. The function of glycine betaine as an osmolyte has also been reported in the mammalian renal system as well (Bagnasco et al., 1986). Besides this physiological role as an glycine betaine also functions in the general metabolism where methyl groups derived from it are incorperated into alkaloids in plants (Byerrum et al, 1956), into methionine in mammals (Skiba et al., 1982) and microorganisms, and into cabolamine (vitamin B_{12}) in microorganisms (White and Demain, 1971). Furthermore, glycine betaine 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 via metabolic pathway (Livering et al., 1987).

Accumulation of glycine betaine in cyanobacterium

Cyanobacteria, the oxygenic photosynthetic prokareotes from which plastids of photosynthetic eukareotes (algae and plants) derive, inhabit a variety of environments including those in which extreme conditions such as high temperatures and/or high salinity are found. Cyanobacteria that are able to grow in high salt concentaration environment maintain their cell turgor by accumulation of potassium ions and by synthesis and accumulation of low molecular weight organic osmoprotectant (Nomura et al., 1995). A close correlation between the major solute accumulated and the range of salt tolerance has been shown by analyzing osmoprotective compounds synthesized in about 130 strains (Mackay et al., 1994; Reed et al., 1984 and Reed and Stewart 1985). Three salt tolerance groups have been established. Strains with low tolerance (max 0.7 M) synthesize sucrose or trehalose,

strain with moderate salt tolerance (max 1.8 M) synthesize glucosylglycerol and strains with high salt tolerance synthesize betaine and glutamate betaine (Reed and Stewart, 1988). Osmoregulation involves the accumulation of both organic and inorganic solutes. However, a key factor in the adjustment of many cyanobacteria to hypersaline environment is the increase in the intracellular level of a species-specific low molecular weight organic solute. The role of low molcular weight carbohydrates in the maintenance of osmotic balance has been shown for several cyanobacteria as well as in many higher plants. There have been few studies on uptake of osmoprotective compounds by cyanobacteria, as is the case for other transport mechanisms of these photo autotropic organisms. An active transport system for glycine betaine has been found in cyanobacteria able to synthesize this compound (Moore et al., 1987).

Glycine betaine was fisrt shown to be the major osmoticum in a halotolerant cyanobacterium *Synechocytis* DUN52 (Mohammad et al., 1983). The unicellular cyanobacterium, *Aphanothece halophytica* is a highly halotelerant organism that can grow at high external NaCl concentration up to 3 M (Garlick et al., 1977 and Reed et al., 1984). It was demonstrated that glycine betaine is accumulated as the major osmoticum inside *A. halophytica* cells in response to changes in external salinity (Reed et al., 1984). High concentration of salts has been reported to inhibit the activity of many enzymes of both eucaryotic and procaryotic origin (Von Hipple and Schlelic, 1969). It has also previously been reported that salt inhibits enzyme activity of RuBisCo from *A. halophytica*, glycine betaine protects the enzyme against salt inhibition (Incharoensakdi et al., 1986) and is not harmful to the metabolic activities of the cell even at high concentration.

Function of choline and metabolism

Choline [$(CH_3)_3N^+CH_2CH_2OH$] is a methylated nitrogen compound that is need for synthesis of the phospholipids in the cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, lipid cholesterol transport and metabolism (Zeisel and Blusztajn, 1994). Choline can be acethylated, phosphorylated, oxidized or hydrolyzed. There is no doubt that cells absolutely require choline and die by apoptosis when deprived of these nutrient (Albright, 1996). The debate as to whether the human diet must contain choline arises because there is a pathway (most active in the liver) for the de novo biosynthesis of the choline moiety via the sequential methylation of phosphatidylethanolamine using S-adenosylmethionine as the methyl donor. This ability to form choline moiety de novo means that some of the demand for choline can, in part, be met by using methyl groups derived from one carbon metabolism (via methyl-folate and methionine). Because of this metabolic interrelationship of choline, methionine, folate and vitamin B_6 and B_{12}, investigators need to show that, when the other nutrients are available in amounts sufficient to sustain normal growth and function, de novo synthesis rates are not adequate to meet the demand for choline before the designation of choline as essential is made (Zeisel, 2000). Only small amounts of choline are synthesized in the brain and the rest, absorbed from the diet, must be transported into the brain (Murakami, 2000). Choline is widely distributed in foods; the requirement is generally satisfied by both dietary and endogenous source, although choline deficiency has been reported. The most common signs of choline deficiency are fatty liver and hemorrhages kidney necrosis. Evidence for free radical activity in liver with

choline deficiency is reported and this may be related to the carcinogenesis process (Panfili, 2000).

In many plant species, notably member of Gramineae (barley) and Chenopodiaceae (sugar beet and spinach), Choline also serves as a precursor for the synthesis of the quaternary ammonium compound, glycine betaine [$(CH_3)_3{}^+NCH_2COO^-$]. More recently, choline has been shown to be the precursor for the synthesis of choline-*O*-sulfate in salinisized *Limonium* (Plumbaginaceae) (Hanson et al, 1991). Glycine betaine and choline-*O*-sulfate are believed to be the compatible cytoplasmic osmolytes whose accumulation plays a role in the adaptation of many plants to conditions of drought or salinity (Wyn Jones et al., 1977). Choline synthesis can be viewed not only as having an essential and ubiquitous role in plant phospholipid metabolism but as playing a fundamental role in the adaptation of many plants toward osmotic stress (Summer et al., 1993).

The fate of choline in aquatic systems is of interest because it contains nitrogen (C/N = 5) and is precursor of glycine betaine, one of the most potent osmoprotectants known (Csonka and Hanson, 1991; Le Rudulier et al., 1984 and Yancy et al., 1982). A variety of different bacteria including *Escherichia coli*, *Stapphylococcus aureus*, *Bacillus subtilus*, *Rhizobium meliloti*, *Rhodobacter sphaeroides* and *Vibrio costicola* oxidatively convert choline to glycine betaine (Abee et al., 1990; Boch et al., 1994; Choquet et al., 1991; Lanfald and Strom, 1986 and Pocard et al., 1989). Choline is oxidized to glycine betaine in a two step process with betaine aldehyde as an intermediate. In *Alcaligenes spp.*, a soluble choline oxidase can carry out both steps of choline oxidation (Ohta-Fukuyama et al., 1980), while in

E. coli and other bacteria, a membrane bound choline dehydrogenase is primarily responsible for oxidation to the aldehyde, which is further oxidized by a soluble betaine aldehyde dehydrogenase (Boch et al., 1994; Choquet et al., 1991 and Lanfald and Strom, 1986). The overall reaction requires NAD^+ and produces H_2O_2 in addition to glycine betaine.

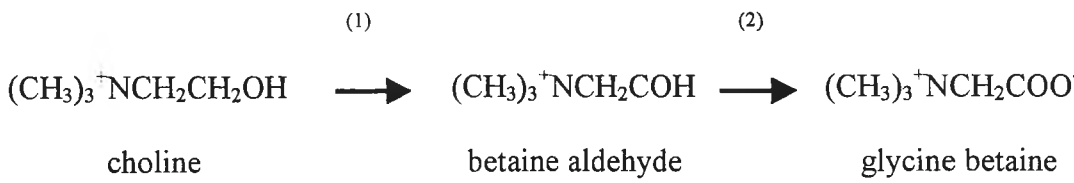
A supply of exogenous choline, and its subsequent conversion to glycine betaine, has been shown to confer osmotelerance to bacteria when cells are grown at other inhibitory osmolarities (Lanfald and Strom, 1986 and Le Rulier et al.,1984). On the other hand, choline has no osmoprotectant effects in mutants that are defective in their ability to convert choline to glycine betaine (Styrvold et al., 1986), indicating that conversion to glycine betaine is required for choline to be an osmoprotectant. Furthermore, choline uptake and oxidation activities are under osmotic control in a number of bacteria, with enhanced transport at high osmolarities (Boch et al, 1994; Choquet et al., 1991and Lanfald and Strom, 1986).

The genetic basis for choline uptake and dehydrogenation has been elucidated for *Escherichia coli* (Lamark et al., 1991) and recently for *Bacillus subtilis* (Boch et al., 1996). In *E. coli*, a gene cluster comprises the genes encoding a choline transporter (BetT), two dehydrogenase, an NADH-dependent glycine betaine aldehyde dehydrogenase (BetB), and an FADH-dependent choline dehydrogenase (BetA), which respondsible for the conversion of choline to glycine betaine aldehyde. In addition, a regulatory protein, BetI, is encoded by the *E.coli bet* gene cluster; BetI binds to the DNA region between the *betIBA* operon and *betT* gene and is respondsible for the choline dependent regulation of bet transcription (Lamark et al.,

1996 and Rocenes et al., 1996). In *B. subtilis*, an operon encodes two dehydrogenases, a glycine betiane aldehyde dehydrogenase (GbsA) that shows similarity to glycine betaine aldehyde dehydrogenase found in various other organisms, and a choline oxidase (GbsB) that belongs to a family of alcohol dehydrogenases and thus represents a novel type of choline dehydrogenating enzyme involved in glycine betaine biosynthesis. In contrast to *E. coli*, no genes for a choline transporter or a regulatory protein have been identified in the *gsb* locus (Rosenstein et al., 1999)

The choline-glycine betaine pathway

Despite the widespread occurrence of glycine betaine, the osmotic regulation is well understood only in several microbial systems and certain plant. Biosynthesis of glycine betaine results from oxidation of choline via a two-step reaction with betaine aldehyde as the intermediate (Figure 3). This series of reactions may be catalyzed by three-enzymatic system. In microorganisms and mamals a membrane bound choline dehydrogenase (EC 1.1.99.1) is employed in conjugation with a soluble betaine aldehyde dehydrogenase (EC 1.2.1.8) (Haubrich and Gerber, 1981; Lanfald and Strom, 1986; Nagasawa et al., 1976; Rotchid and Barron, 1954; Lin and Wu 1986; Wilken, 1970 and Wilken et al., 1970). Plants utilize a soluble choline monooxygenase in combination with betaine aldehyde dehydrogenase (Brouquisse et al., 1989 and Pan, 1988).



1: choline dehydrogenase or choline monooxygenase

2: betaine aldehyde dehydrogenase

Figure 1.3 Biosynthetic pathway of glycine betaine from choline via betaine aldehyde.

The glycine betaine synthesis has been studied in several bacteria, higher plants and marine animals (Lanfald and Strom, 1986, Reed et al., 1984, Weigel et al., 1986 and Dragolovich and Pierce, 1994). In most organisms, glycine betaine was formed by the oxidation of choline to betaine aldehyde, which is then oxidized to glycine betaine (Figure 3). Different enzymes such as FAD catalyzes the first step (choline to betaine aldehyde) linked choline dehydrogenase (EC1.1.99.9) in inner rat liver mitochondria membrane (Zhang et al., 1992). Choline oxidase (EC 1.1.3.17) is a new type of flavoprotein enzyme and was found in *Arthrobacter globiformis* (Ikuta et al., 1977). In all organisms studied, the final step in the synthesis of betaine from choline (betaine to glycine betaine) is catalyzed by a NAD^+-dependent betaine aldehyde dehydrogenase (EC1.2.1.8).

Choline dehydrogenase was discovered by in 1937. It has been most extensively studied in mammalian liver where it is found exclusively in mitochondria (Kimura and Singer 1962; Williams and Screenivasan 1953). The enzyme was located

on the matrix side of the inner mitochondrial membrane (Streumer-Svobodova and Drahata, 1997). Several groups have its isolation and purification (Chi-Shui and Ru-Dan, 1986; Haubrich and Gerber 1981; Redina and Singer 1959; Tsuga et al., 1980; Russell and Scopes, 1994) and it is generally believed to be a single-chain polypeptide in *Pseudomonas* strain with a molecular mass of approximately 60,000-70,000 Da (Russell and Scopes, 1994). It is thought to be linked to ubiquinone (coenzyme Q) in the electron transport chain to which it transfers the electrons from the oxidation reaction (Barrett and Dawson, 1975).

In general choline dehydrogenase activity is highest in the liver and kidney of the rat and other mammals although in humans its activity in the kidney is somewhat higher than in the liver (Streumer-Svobodova and Drahota, 1977). Some activity is also present in the mammalian brain but is negligible in other organs. Within the kidney, choline dehydrogenase activity has been localized to the proximal tubule (both convoluted and pars recta segments) and the inner medulla (Wirthensohn and Guder, 1982). Choline dehydrogenase was strongly induced and betaine aldehyde dehydrogenase less strongly induced by choline. The formation of these enzymes was also regulated by the NaCl concentration of growth medium, increasing with increasing NaCl concentrations. Intracellular glycine betaine concentration also increased with increasing choline and NaCl concentrations in the medium. This increase was almost completely blocked by chloramphenicol, which does not block the increase in salt tolerant active transport on transfer from a low to a high salt concentration (Choquet et al.,1991; Lanfald and Strom, 1986). The oxidation of choline to betaine aldehyde in rat liver mitochondrial preparation required no addition cofactors, suggesting that mitochondria contained an endogenous electron acceptor.

Oxygen might have been the ultimate electron acceptor because cyanide (an inhibitor of cytochrome oxidase) totally inhibited the formation of [*methyl*-^{14}C]betaine aldehyde from [*methyl*-^{14}C]choline. This inhibition could, however, be overcome by the addition of phenazine methosulfate (PMS). This would be expected if the electrons from choline dehydrogenase were shunted to PMS.

The periplasmic substrate-binding protein

The binding proteins are the most thoroughly analyzed of these transport components for obvious reason. Bacterial active transport systems can broadly into two classes, those which require the function of specific substrate-binding proteins and those which consist solely of membrane-bound components. These two classes of transport system were originally distinguished by the sensitivity of binding protein-dependent system to cold osmotic shock and by the nature of energy coupling to transport (Berger and Heppel, 1974).

Periplasmic, binding protein-dependent transport systems each require the function of several cytoplasmic membrane proteins. In gram-negative bacteria, a number of substrates (such as maltose and maltodextrins, histidine, oligopeptdes, phosphates, etc.) are concentrated into the cell by high affinity transport systems which include several proteins, and a common organization (Shuman, 1987). A central component of these systems is a binding protein (e.g. MalE for maltose and maltodextrins in *Escherichia coli*, HisP for histidine in *Salmonella typhimurium*, OppA for oligopeptides in *Salmonella typhimurium*) which is located in the periplasm between the outer and inner membranes. These water soluble "periplasmic binding

proteins" present high affinity for their specific substrates. Their concentration in the periplasm can reach very high values so that they increase the availability of substrates in the vicinity of the inner membrane. Upon specific interactions of the liganded periplasmic binding proteins with a complex of inner membrane proteins, the substrate is transferred to the complex and further transported into the cytoplasm (Gilson et al, 1988).

One of the major stress proteins accumulated in salt-adapted cells of the cyanobacterium *Synechosistis* sp. PCC 6803 has been identified as flavodoxin (Fluda and Hagemann, 1995). These previous investigations on salt stress proteins have been performed on total cell extracts. However, The cyanobacterial cell is composed of different compartments: periplasm, cytoplasm and lumen of thylakoids. The periplasm is particularly responsive to changes in salinity. The periplasmic of gram-negative bacteria contains proteins of several functions, such as for electron transport, substrate-binding, transport, degradation, cell wall biosynthesis and detoxification (Ferguson, 1992). Some of these proteins may be anchored to the cytoplasmic membrane or to the outer membrane; most are believed to be water-soluble and can be osmotic shock (Neu and Heppel, 1965). Among the periplasmic proteins that are members of the osmotic stimulation in *Escherichia coli*, the glycine betaine-binding protein of the ProU transport system (OsmY) and a periplasmic trehalose (TreA) have been identifiied (Gutierrez et al., 1989; Lucht and Bremer, 1994 and Yim and Villarejo, 1992).

In this study, the unicellular cyanobacterium, *Aphanothece halophytica* (this alga is classified into Chroococcales order, chroococcacean cyanobacteria subgroup, Geitler, 1932 and Stanier et al, 1971) was chosen as a source for the study of (a) choline function as an osmoprotectatn by oxidation to glycine betaine (b) uptake of choline by *A. halophytica* in normal and salt stressed condition (c) choline oxidation to betaine aldehyde and glycine betaine by monitoring the activities of enzymes and (d) isolation of choline binding protein from *A. halophytica*.