Helsinki University of Technology Department of Chemical Technology
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PATHWAYS OF GLYCINE BETAINE SYNTHESIS IN TWO EXTREMELY HALOPHILIC BACTERIA, *ACTINOPOLYSPORA HALOPHILA* AND *ECTOTHIORHODOSPIRA HALOCHLORIS*

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ABSTRACT

Glycine betaine is a solute which is able to restore and maintain the osmotic balance of living cells. In this thesis, the glycine betaine synthesis in two extremely halophilic bacteria *Actinopolyspora halophila* and *Ectothiorhodospira halochloris* is investigated.

A. halophila synthesized remarkably high intracellular concentrations of glycine betaine. The highest glycine betaine concentration, determined at 24% (w/v) NaCl, was 33% of the cellular dry weight.

The data presented in this work indicate that the *de novo* synthesis of glycine betaine proceeds *via* the threefold methylation of glycine. *S*-adenosylmethionine acts as the methyl group donor in the reactions. The genes encoding this pathway were cloned and successfully expressed in *Escherichia coli*. In *E. halochloris,* glycine sarcosine *N*-methyltransferase (GSMT) and sarcosine dimethylglycine *N*-methyltransferase (SDMT) catalyze the reaction sequence. In *A. halophila* all three methylation reactions appear to be catalyzed by a fusion protein. The methyltransferases from the two bacteria show high sequence homology.

Furthermore, it was demonstrated that in addition to the glycine methylation pathway, *A. halophila* has the ability to oxidize choline to glycine betaine. Choline was first oxidized to betaine aldehyde in a reaction in which H_2O_2 –generation and oxygen consumption are coupled. Betaine aldehyde was oxidized further to glycine betaine in a reaction in which $NAD(P)^+$ was reduced.

The GSMT and SDMT of *E. halochloris* were expressed in *E. coli*, purified, and some of their enzymatic properties were characterized. Both enzymes had high substrate specificity and pH optima near physiological pH. No evidence of cofactors was found. The enzymes showed Michaelis-Menten kinetics for their substrates. The apparent K_m and V_{max} values were determined for all substrates, when the other substrate was present in saturating concentrations. Both enzymes were strongly inhibited by the reaction product *S*-adenosylhomocysteine. Glycine betaine inhibited the methylation reactions only at high concentrations.

Finally, it was demonstrated that the expression of the *E. halochloris* methyltransferase genes in *E. coli* results in glycine betaine accumulation and improves salt tolerance.

PREFACE

This work was carried out in the Cultor Technology Center (CTC) during the years 1996-1998 and continued in the Laboratory of Bioprocess Engineering, Department of Chemical Technology, Helsinki University of Technology during 1999-2001.

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LIST OF PUBLICATIONS

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II. Nyyssölä, A., Kerovuo, J., Kaukinen, P., von Weymarn, N. and Reinikainen, T., Extreme halophiles synthesize betaine from glycine by methylation, *J. Biol. Chem.* **275** (2000) 22196-22201.

III. Nyyssölä, A., Reinkainen, T. and Leisola, M., Characterization of glycine sarcosine *N*-methyltransferase and sarcosine dimethylglycine *N*-methyltransferase, *Appl. Environ. Microbiol.* **67** (2001) 2044-2050.

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ABBREVIATIONS

AdoHcy	*S*-Adenosyl-L-homocysteine
AdoMet	*S*-Adenosyl-L-methionine
GSMT	Glycine sarcosine methyltransferase
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
NAD^+	β-Nicotinamide adenine dinucleotide
$NADP^+$	β-Nicotinamide adenine dinucleotide phosphate
ORF	Open reading frame
PCR	Polymer chain reaction
SDMT	Sarcosine dimethylglycine methyltransferase
SDS-PAGE	Sodium sulfate-polyacrylamide gel electrophoresis

CONTENTS

1 INTRODUCTION

During the last decade there has been considerable interest in the transport, synthesis and stress-relieving effects of the so-called compatible solutes. These compounds play a vital role in the osmotic regulation of living organisms. This study is concerned with the best-known compatible solute, glycine betaine.

The first part of the introduction deals with the strategies that microorganisms utilize for survival in saline habitats. Commonly, microorganisms use compatible solutes for this purpose. Compatible solutes are low molecular weight organic compounds, which can either be taken up from the environment or synthesized by osmoregulating cells. The distribution of various compatible solutes among bacteria is described.

Glycine betaine has been shown to protect a wide variety of organisms and macromolecules against different stresses. Although the functioning of compatible solutes on the molecular level is still not fully understood, a review of the predominant theories on the mechanisms of stress-protection is presented.

Glycine betaine is produced from simple carbon sources by a number of halophilic bacteria. The two possible pathways of glycine betaine synthesis are the glycine methylation pathway and the choline oxidation pathway. The existing experimental data on the enzymology and regulation of glycine betaine synthesis in bacteria are presented.

As well as in bacteria, glycine betaine also acts as a compatible solute in plants. However, many plants, including some commercially important crops, are not capable of glycine betaine synthesis. The most interesting application of the genes of compatible solute synthesis is therefore their use in the metabolic engineering of stress-tolerant plants. Finally, some developments in this field are presented.

1.1 Adaptation of microorganisms to saline environments

Microbes are able to live in environments over a wide range of water activities, from fresh water to concentrated brines. The capability to adapt to low water activity of the environment is thus of great importance for the proliferation and survival of microbes. Many highly saline habitats occur naturally, such as salt or soda lakes, salt marshes, marine ponds and coastal lagoons, others are man-made such as salterns and saltworks (Trüper and Galinski, 1986).

Since biological membranes are readily permeable to water molecules, organisms encountering salt-stress are in danger of being dehydrated. Without balancing of the osmotic pressure and restoration of turgor pressure, the increasing salinity of the external medium leads to shrinkage of the cell. As a possible consequence, the concentrations of intracellular solutes could attain growth-inhibiting or even toxic levels (Csonka, 1989). Hence, the cytoplasmic water activity should be maintained near the water activity of the ambient environment without disturbing the physiological functions of the cell.

Two different strategies exist in nature for balancing the osmotic pressure and the intracellular water activity: The KCl-type (Lanyi, 1974) and the compatible solute type strategy (Brown, 1976).

Osmotic balance in microbes can be maintained by accumulation of high cytoplasmic concentrations of KCl. As a consequence, the intracellular enzymes and macromolecules must be adapted to high ionic strength. In fact, intracellular enzymes of the microbes using the KCl-strategy are not only fully active at high KCl concentrations, but also require monovalent cations (preferably K^+) for stability. It has been shown that bacteria using this strategy have intracellular proteins with higher proportion of acidic amino acids and lower proportion of non-polar residues than proteins usually (Lanyi, 1974).

The KCl-strategy is typical for halophilic, anaerobic, heterotrophic eubacteria (*Haloanaerobiales*) (Oren, 1986; Rengpipat *et al.*, 1988; Oren *et al.*, 1997) and extremely halophilic archaebacteria (*Halobacteriales*) (Lanyi, 1974), with the exception of extremely halophilic methanogens. The anaerobic heterotrophic halophiles also accumulate significant amounts of NaCl.

Extremely large amounts of KCl can be accumulated by halophilic archaebacteria. Intracellular concentrations of several molal K^+ have been reported for some *Halobacterium* strains (Lanyi, 1974).

A far more common strategy among microbes is the restoration of osmotic balance by the accumulation of low molecular weight organic compounds. These compounds were named compatible solutes by Brown (1976), because of their compatibility with cellular metabolism even at high concentrations. However, as has been pointed out by Galinski and Trüper (1994) this classical definition does not take into account the fact that these compounds are not merely inert cytoplasmic solutes. They are also efficient stabilizers of intracellular enzymes and other macromolecules. In addition to microbes animals and plants also use compatible solutes for maintaining the osmotic balance (Yancey *et al.*, 1982).

The osmolarity of the environment can change for example due to evaporation or rain. The compatible solute strategy allows the cells flexibility under such circumstances. The cells maintain osmotic balance by adjusting their intracellular osmolarity. Since compatible solutes are compatible with enzyme action over a wide range of concentrations, there is no need for modification of a vast group of proteins when the salt concentration of the environment changes. The intracellular compatible solute concentration can in principle be varied by means of transport, synthesis or catabolism. Consequently, bacteria using the compatible solute strategy can tolerate fluctuations in ambient salinity far better than bacteria using the KCl-strategy. The latter are strictly confined to those environments in which the salinity of the environment is both high and relatively stable (Yancey *et al*., 1982).

It was suggested by Oren (1999) that the disadvantage of organic osmolyte synthesis is that it consumes more energy than the accumulation of KCl. It should however be also noted that the compatible solute pool could be used for growth when the osmolarity of the medium decreases. Hence compatible solutes would act as carbon and energy reserves for the cell and the energy used for their synthesis would not be completely lost. Still, whether compatible solutes or KCl are used as osmolytes, halophilic life is energetically expensive, since in both cases the cell has to maintain steep osmolyte gradients across the cell membranes (Oren, 1999).

1.2 Distribution of compatible solutes in microorganisms

Microbes using the compatible solute strategy are usually able to accumulate several different compounds simultaneously in response to osmotic stress. Some of them can be synthesized and some are transported from the medium. The composition of the intracellular compatible solute pool can vary considerably depending on the growth conditions and availability of extracellular osmolytes (Galinski and Herzog, 1990; Sowers *et al.*, 1990; Severin *et al.*, 1992). Uptake of compatible solutes from the environment is preferred over *de novo* synthesis (Galinski and Trüper, 1994; Miller and Wood, 1996), because of the high energy cost of their synthesis (Oren, 1999).

Compatible solutes belong to various classes of organic compounds with different chemical structures. Nevertheless, some common characteristics can be summed up. Compatible solutes are usually low molecular weight compounds. Since they must be accumulated in high concentrations in the cytoplasm, they are typically highly soluble in water. Zero net charge of the compound is an advantage, because such solutes do not cause electrostatic perturbation of enzyme action. In addition, solutes that are uncharged at physiological pH can be accumulated without a counterion. Polyols, sugars and zwitter-ionic amino acid derivatives fulfill these requirements and are the main classes of compatible solutes (Trüper and Galinski, 1986; Galinski, 1993).

The most important compatible solutes of microorganisms are presented below (compiled following Trüper and Galinski, 1986 and Galinski, 1995). It should be noted, however, that new compatible solutes have been found steadily during the past decade (Galinski, 1993; Galinski, 1995).

Polyols:
Polyols such as sorbitol, arabinitol, erythritol and especially glycerol are typical compatible solutes of eukaryotes, such as xerotolerant yeasts (Brown, 1976; Adler *et al.*, 1985), fungi in general (Blomberg and Adler, 1992; Luxo *et al.*, 1993), and algae (Wegman, 1986; Ben-Amotz and Avron, 1983). As pointed out by Oren (1999), glycerol is an excellent compatible solute, because it is one of the least costly organic osmolytes to synthesize and its solubility in water has no limit. Microorganisms can accumulate glycerol in extremely high concentrations in saline environments. For example, the halophilic alga *Dunaliella salina* produces glycerol up to 50% of the cellular dry weight (Ben-Amotz and Avron, 1983). Bacteria do not usually appear to use polyols as compatible solutes (Galinski and Trüper, 1994). One exception is *Pseudomonas putida*, which produces mannitol in response to osmotic stress (Kets *et al.*, 1996).

Glucosyl glycerol and sugars:
Glucosyl glycerol is the compatible solute of less halotolerant cyanobacteria living in fresh water (Mackay *et al.*, 1984). It has also been reported for the purple bacterium *Rhodobacter sulfidophilus* (Severin *et al.*, 1992) and for the heterotroph *Pseudomonas mendocina* (Pocard *et al.*, 1994).

Sugars (mainly trehalose and sucrose) are widespread osmolytes both in halophilic and nonhalophilic microorganisms (Mackay *et al.*, 1984; Giæver *et al*., 1988; D´Amore *et al.*, 1991; Severin *et al.*, 1992; Xu *et al.*, 1998). However, their concentrations appear to remain rather low under all conditions (<0.5 M) (Galinski, 1993). Some novel disaccharides have also been identified. 2-Sulfotrehalose is a compatible solute in haloalkaliphilic archaebacteria (Desmarais *et al.*, 1997) and mannosucrose (β-fructofuranosyl-α-mannopyranoside) is synthesized by *Agrobacterium tumefaciens* in response to osmotic stress (Smith *et al.*, 1990).

Ectoines:
Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is the predominant compatible solute synthesized *de novo* in halotolerant and halophilic aerobic heterotrophic eubacteria (Wohlfarth *et al*., 1990; Severin *et al.*, 1992). It has also been detected as a component of the compatible solute pool in anaerobic phototrophic bacteria (Galinski *et al.*, 1985). The hydroxy derivative of ectoine is more common in Gram-positive heterotrophs.

Amino acids:
Most amino acids are poorly suitable as compatible solutes, because they exist in charged form at physiological pH values. In addition, most amino acids do not have the high water solubility that is required of compatible solutes (Trüper and Galinski, 1986). Nevertheless, there are some amino acids that act as compatible solutes. The best-known example is proline. The ability to uptake proline from the growth medium as a compatible solute is widespread among microbes (Csonka, 1989). Some *Bacillus* strains have been shown to increase their proline content in response to osmotic stress also in the absence of exogenous proline (Tempest *et al.*, 1970; Galinski, 1993). Furthermore, some halophilic algae produce proline as a compatible solute (Wegmann, 1986).

Despite its low solubility in uncharged form, α-glutamine has been detected as the compatible solute of moderately halophilic *Corynebacterium* strains (Frings *et al.*, 1993). The more soluble β-isomer of glutamine is a component of the compatible solute pools of halotolerant and halophilic methanogenic archaebacteria, in which it can reach 0.5 M cytoplasmic concentration (Lai *et al.*, 1991). *Escherichia coli* has been shown to accumulate K^+ ions as a response to increased osmolarity (Epstein, 1986). Electroneutrality is achieved partly by the synthesis of glutamate as the counter ion (Larsen *et al.*, 1987).

N-acetylated diamino acids:
Ornithine and lysine have a positive charge at physiological pH. *N*-Acetylation turns these compounds into uncharged zwitterions, which makes them more suitable as compatible solutes (Galinski, 1995). N^{ε}-Acetyl lysine and its homologue N^{δ}-acetyl ornithine are synthesized by *Sporosarcina halophila* (Galinski and Trüper, 1994) and some bacilli (del Moral *et al.*, 1994; Galinski and Trüper, 1994). The β-form, N^{ε}-acetyl-β-lysine, is a compatible solute of halophilic and halotolerant methanogens (Sowers *et al.*, 1990; Lai *et al.*, 1991).

N-derivatized glutamine amides:
In these compounds the free amino residue of glutamine is either acetylated or carbamoylated. *N*-α-carbamoyl-L-glutamine amide has been found in the halophilic phototrophic sulfur bacterium *Ectothiorhodospira marismortui*, in which it is synthesized up to 0.5 M (Oren *et al.*, 1991). *N*-α-acetylglutaminyl glutamine amide is the only neutral dipeptide known to have osmotic function. It has been detected in the solute pools of *Rhizobium meliloti* (Smith and Smith, 1989), *Pseudomonas* strains (Smith and Smith, 1989; D´Souza-Ault *et al.*, 1993; Pocard *et al.*, 1994) and purple bacteria (Severin *et al.*, 1992).

Betaines:
Betaines are fully *N*-methylated derivatives of amino acids. Glycine betaine is probably the most common betaine used as a compatible solute, but some other betaines also play an important role in osmotic regulation. Glutamate betaine is synthesized by some cyanobacteria living in hypersaline environments (Mackay *et al.*, 1984) and proline betaine is the product of osmoregulating marine algae (Mason and Blunden, 1989). The ability to act as compatible solutes appears to be a common property of betaines. For example, a wide variety of betaines have been shown to alleviate the osmotic stress of *E. coli* when provided exogenously (Larsen *et al.*, 1987; Randall *et al.*, 1996; Peddie *et al.*, 1999).

The osmoprotective properties of glycine betaine have been studied extensively. Since its isolation as the osmotolerance-conferring component in yeast extract by Dulaney *et al.* in 1968, there have been numerous reports on its transport, stress-relieving effects and synthesis. Many different bacteria are able to accumulate it from the growth medium.

The synthesis of glycine betaine from simple carbon sources is rare among halophilic heterotrophic bacteria, but common among halophilic phototrophs (Mackay *et al*., 1984; Severin *et al.*, 1992) and halophilic, methanogenic archaebacteria (Lai *et al.*, 1991). However, many bacteria are able to synthesize it from exogenously provided choline by oxidation. The osmoprotective effects and biosynthesis of glycine betaine will be reviewed in detail later in this section.

Glycine betaine is generally considered to be the most compatible of the compatible solutes (Warr *et al.*, 1984; Csonka, 1989; Trüper and Galinski, 1990; Galinski, 1993). It is perhaps the most widely distributed organic osmolyte in nature. It is found not only in bacteria (Galinski and Trüper, 1994), but also in plants (Gorham, 1995) and animals (Yancey *et al.*, 1982; Anthoni *et al.*, 1991).

1.3 Properties and uses of glycine betaine

Glycine betaine (hereafter abbreviated as betaine) is named after the best-known betaine-producer, sugar beet (*Beta vulgaris*). Betaine was isolated from sugar beet already in 1866. Sugar beet contains 1 - 1.5% betaine per cellular dry weight, depending on the growth phase and strain (von Steinmetzer, 1972). Betaine is currently produced by chromatographic separation from sugar beet molasses (Heikkilä *et al.*, 1982).

Chemically, betaine is an *N,N,N*-methylated derivative of glycine (Fig. 1). It has the properties of an ideal compatible solute. It is highly soluble in water: 1.6 $g \cdot g^{-1}$ H_2O, it has zero net charge at physiological pH (Trüper and Galinski, 1986) and its molecular weight is relatively low: 117.15 $g \cdot mol^{-1}$.

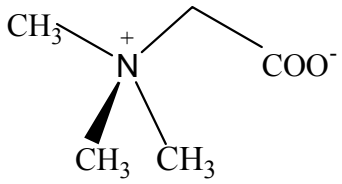

Figure 1. Glycine betaine

Betaine and its salts have commercial uses in pharmaceuticals, dental products, cosmetics, and especially as a feed ingredient. In animal cells, betaine acts as a methyl group donor. It is able to methylate homocysteine back to methionine in a reaction in which dimethylglycine is formed. The synthesized methionine can then be used as a methyl group donor in the synthesis of *S*-adenosyl-L-methionine (AdoMet), the universal methyl group donor in all organisms. One of the remaining methyl groups of dimethylglycine is used in the further methylation of methyltetrahydrofolate, which can then also be used for the synthesis of methionine from homocysteine. Because of these reactions, betaine provides an efficient way to supply methyl groups in the diet and is therefore used in feed (reviewed by Virtanen and Rumsey, 1996).

High substrate concentrations used in fermentation media can cause osmotic stress to the production strains. Betaine has been shown to alleviate the osmotic stress of some industrially important bacteria (Kawahara *et al.*, 1990; Farwick *et al.*, 1995). The ability of betaine to function as a compatible solute can thus be utilized in the fermentation industry. Betaine is commercially sold for this purpose (Nutristim®, Danisco Corp.)

1.4 Betaine as a stress protectant

1.4.1 Stress-protection of cells by exogenously provided betaine

A wide variety of microbes are able to accumulate betaine from the environment in response to osmotic stress. In many natural habitats betaine is available for microbes which are unable to synthesize it (King, 1988; Gauthier and Le Rudulier, 1990; Ghoul *et al.*, 1990). Betaine can be leaked from damaged betaine-producers or it can be released from these organisms due to a decrease in salinity (Oren, 1990). Efflux systems of betaine have been described for betaine-producers such as

Ectothiorhodospira halochloris[1] (Tschichholz and Trüper, 1990) and cyanobacteria (Moore *et al.*, 1987). Strains from the whole halotolerance scale of bacteria are able to use exogenous betaine as a compatible solute. However, it should be noted that there are also many bacteria of which growth is not stimulated by betaine under osmotic stress (Csonka, 1989).

Reported examples of bacterial strains which benefit from betaine accumulation from the environment under high osmolarity include *Agrobacterium tumefaciens* (Smith *et al.*, 1990), *Azospirillum brasilense* (Riou and Le Rudulier, 1990), *Bacillus subtilis* (Imhoff, 1984), *Brevibacterium* strains (Kawahara *et al.*, 1990; Jebbar *et al.*, 1995), *Corynebacterium glutamicum* (Farwick *et al.*, 1995), many enteric bacteria (Le Rudulier and Bouillard, 1983), *Erwinia chrysanthemi* (Gouesbet *et al.*, 1995), many lactic acid bacteria (Hutkins *et al.*, 1987; Molenaar *et al.*, 1993; Glaasker *et al*., 1996), *Listeria monocytogenes* (Ko *et al.*, 1994), *Pseudomonas* strains (D´Souza-Ault *et al.*, 1993; Pocard *et al.*, 1994), *Rhizobiaceae* strains (Csonka, 1989; Miller and Wood, 1996; Boncompagni *et al.*, 1999), *Rhodobacter sphaeroides* (Abee *et al.*, 1990), *Staphylococcus auereus* (Graham and Wilkinson, 1992), *Thiobacillus ferrooxidans* (Kieft and Spence, 1988), many *Vibrio* strains (Csonka *et al.*, 1989) and *Yersinia enterocolitica* (Park *et al.*, 1995). In addition, the ability to uptake betaine from the medium is especially common for halophilic bacteria of many different genera (Wohlfarth *et al.*, 1990; Severin *et al.*, 1992).

Betaine does not function solely as an inert osmolyte; it has been shown to protect bacteria, not just against salt-stress but also against other stress factors such as drying (when combined with salt stress) (Kets and Bont, 1994), heat (Caldas *et al.*, 1999) and cold (Ko *et al.*, 1994). In addition, betaine has a small protective effect on *E. coli* at low pH and high osmolarity (Chambers and Kunin, 1985) and at high pH (Smirnova and Oktyabrsky, 1995).

Typically, betaine concentrations from 0.5 mM to 2 mM in the medium are sufficient for maximal osmoprotection. In some cases extremely small betaine concentrations in the environment have been found to confer the cells osmotolerance. For example, even 10 μM betaine ameliorates the osmotic stress of *P. aeruginosa* at 0.7 M NaCl (D´Souza-Ault *et al.*, 1993) and as little as 1 nM has been show to stimulate the growth of *E. coli* at 0.8 M NaCl (Cosquer *et al.*, 1999). Betaine can be accumulated in high intracellular concentrations in bacteria living at high osmolarity. For example, *E. coli* contains 250 mM betaine when cultivated with 0.25 mM betaine and 0.65 M NaCl (Perroud and Le Rudulier, 1985). *Klebsiella pneumoniae* accumulates 615 mM betaine when grown in the presence of 1 mM betaine and 0.8 M NaCl (Le Rudulier and Bouillard, 1983). Moderately halophilic, heterotrophic bacteria growing in high salt concentrations can accumulate betaine even in molar concentrations (Imhoff and Rodriguez-Valera, 1984). It can thus be concluded that betaine is transported against substantial concentration gradients in osmoregulating bacterial cells.

Among microbes, the betaine uptake systems of *E. coli* and *S. typhimurium* are the most intensively investigated. The uptake of betaine is a highly complex process. According to the hypothesis presented by Higgins and co-workers it is

[1] The name *Ectothiorhodospira halochloris* is used in this study. *Ectothiorhodospira halochloris* has been renamed by Imhoff and Süling (1996) as *Halorhodospira halochloris*.

linked to K^+ transport (Sutherland *et al.*, 1986; Higgins *et al.*, 1987). In *E. coli* (Gowrishankar, 1985; Gowrishankar, 1986) and *S. typhimurium* (Cairney *et al.*, 1985a; Cairney *et al.*, 1985b) the accumulation of betaine occurs by means of two transport systems. ProP is a low affinity system and ProU a high affinity, binding protein-dependent (Barron *et al.*, 1987) system. In addition to betaine, these transport mechanisms also have affinity for proline and ectoine (Jebbar *et al.*, 1992).

In *E. coli*, K^+ is the principal osmolyte at moderate salt-stress, but as the osmolarity of the medium increases, mainly betaine is transported. K^+ is taken up by the constitutive low-affinity system Trk and the high-affinity system Kdp (Rhoads *et al.*, 1976). When transport by Trk is insufficient for the maintenance of turgor pressure of the cells under osmotic stress, lowering of turgor induces the *Kdp* operon (Laimins *et al.*, 1981). The accumulation of K^+ by Kdp leads to the induction of *proU* and its expression is increased 100- to 400 fold (Cairney *et al.*, 1985b; Sutherland *et al.*, 1986). The betaine transport system ProU is active only when the osmolarity of the medium is high (Cairney *et al.*, 1985b). The expression of the constitutive low-affinity-system ProP is also increased under high osmolarity, but to a much lesser degree than the expression of ProU (Cairney *et al.*, 1985a). However, ProP also plays an important role in osmotic regulation. The changes in osmolarity are responded to very rapidly by ProP-mediated transport of betaine (Milner *et al.*, 1988).

The osmotolerance-enhancing potential of exogenously provided betaine is not confined to bacterial cells. Some eukaryotic microorganisms can also use betaine as an osmoprotectant. The fungus *Penicillium fellutanum* (Park and Gander, 1998) and bakers yeast, *Saccharomyces cerevisiae* (Thomas *et al.*, 1994) have been shown to benefit, at least to some extent, from betaine when osmotically stressed.

Betaine acts also as an osmoprotectant in hybridoma cell (Øyaas *et al.*, 1994a) and chinese hamster ovary cell cultures (Ryu *et al.*, 2000). For some of these cell-lines the inclusion of betaine in hyperosmolar medium has been shown to result in greater antibody production than in cultures with physiological osmolarity (Øyaas *et al.*, 1994b; Ryu *et al.*, 2000).

The osmolarity in mammalian tissues is normally kept within narrow limits. An exception, however, is the kidney medulla. High osmolarity and urea concentrations occur in kidney medulla with the normal urinary concentrating mechanism. Betaine originating from the diet accumulates in renal medullary cells, both as a compatible solute and as a protectant against urea (Garcia-Perez and Burg, 1991).

Exogenous betaine also increases the productivity of some crop plants which are not able to synthesize betaine. The foliar applications of betaine have been reported to increase the above-ground biomass and grain yields of maize and sorghum under low water activity (Agboma *et al.*, 1997).

1.4.2 Stress-protection of cell macromolecules by betaine

Since betaine can be accumulated in high intracellular concentrations, it should not perturb the action of intracellular macromolecules. In fact, it has been shown *in*

vitro that the action of many intracellular enzymes is unaffected by high concentrations of betaine (Pollard and Wyn Jones, 1979).

Furthermore, betaine stabilizes macromolecules when they are subjected to different stresses. Betaine has been shown to protect enzymes against heat (Paleg *et al.*, 1981; Nash *et al.*, 1982; Arakawa and Timasheff, 1983; Laurie and Stewart, 1990; Lippert and Galinski, 1992; Santoro *et al.*, 1992; Caldas *et al.*, 1999), freezing (Carpenter and Crowe, 1988; Lippert and Galinski, 1992), salt inhibition and inactivation (Pollard and Wyn Jones, 1979; Pavlicek and Yopp, 1982a; Pavlicek and Yopp, 1982b; Warr *et al.*, 1984; Manetas *et al.*, 1986; Gabbay-Azaria *et al.*, 1988; Murata *et al.*, 1992), and cold inactivation (Krall *et al.*, 1989). Betaine has also been reported to protect membrane lipids against heat destabilization (Jolivet *et al.*, 1982; Rudolph *et al.*, 1986) and freezing stress (Coughlan and Heber, 1982; Zhao *et al.*, 1992).

Many of the macromolecules investigated in the above-mentioned studies are of plant origin. Stress-protection by betaine has been considered as evidence indicating that the introduction of betaine synthesis into plants could be used for improving their tolerance against drought and salinity. Therefore the protective effects of betaine on plants and plant enzymes have been of particular interest. The genetic engineering of stress tolerance into plants will be considered later in this review.

1.5 The mechanism of stress-protection

The leading theory explaining the protective effects of compatible solutes is the preferential exclusion model (Arakawa and Timasheff, 1985). According to this model, compatible solutes are excluded from the hydration layers of proteins. Folded, native proteins have a smaller surface area than their unfolded, denaturated forms. The volume of the compatible solute-free layer on the surface of the protein would therefore increase if the protein were denaturated. However, the formation of the layer means increased order and therefore decreased entropy. Since the system tends to minimize the volume of this thermodynamically unfavorable layer, native conformation and subunit aggregation are stabilized. In other words, the following equilibrium (1) is set to the left:

$$\text{Native} \Leftrightarrow \text{Denaturated} \qquad (1)$$

The model presented by Wiggins (1990) of the properties of water in biological systems was later used by Galinski (1993) to describe the formation of the compatible solute-free layer. This explanation is based on different structures of the water populations on the surfaces of proteins and in the bulk of the solution. The structure of liquid water varies as a result of different degrees of hydrogen bonding between the water molecules. Water near the surfaces of proteins is denser (and less structured) than in the bulk. Since compatible solutes have a strongly bonded hydration shell, they fit better in the less dense (and more structured) bulk water. As a consequence of this preference, a compatible solute-free layer is formed on the surface of the protein (Galinski, 1993). Furthermore, because of their hydrophobic

moieties, compatible solutes do not destroy the structure of low-density water, as do inorganic ionic solutes such as K^+ (Wiggins, 1990). The formation of compatible solute-free layers on lysozyme and bovine serum albumin has been demonstrated for betaine and other compatible solutes (Arakawa and Timasheff, 1983; Arakawa and Timasheff, 1985).

Betaine stimulates the growth of *E. coli* at high osmolarity (Le Rudulier and Bouillard, 1983). To moderate osmotic stress, *E. coli* responds mainly by accumulating K^+ from the environment. However, high concentrations of K^+ are potentially deleterious to the function of intracellular enzymes. In particular, cations such as K^+ are known to reduce DNA-protein affinities (Record *et al.*, 1978).

As proposed by Sutherland *et al*. (1986), betaine stimulates growth for two reasons. The uptake of betaine decreases the cytoplasmic concentration of K^+ (Sutherland *et al.*, 1986; Cayley *et al.*, 1992) and reduces therefore its inhibitory effects. In addition, growth is stimulated because the accumulated betaine protects intracellular enzymes against the detrimental effects of K^+.

In the view of Cayley *et al.* (1992), the perturbation of DNA-protein interactions by increased concentrations of K^+ is, however, not a plausible explanation for the growth inhibition of *E. coli*. The cell shrinkage resulting from osmotic stress also leads to increased concentrations of DNA and proteins, a phenomenon that has been termed marcromolecular crowding. Macromolecular crowding should compensate for the deleterious effects of K^+ on DNA-protein interactions. Consequently, Cayley *et al.* (1992) offered an alternative explanation for the osmoprotective function of betaine in *E. coli*.

As shown by Cayley *et al.* (1991; 1992), there exists a remarkable correlation between the volume of free cytoplasmic water and the growth rate of salt-stressed *E. coli* both in the presence and absence of compatible solutes. On the basis of these results Cayley *et al.* (1992) suggested that the volume of free water in *E. coli* is the main parameter determining the growth-rate under osmotic stress. As mentioned above, the reduction of the cell volume under osmotic stress leads to macromolecular crowding. Macromolecular crowding also has adverse effects on cellular processes. It retards the diffusional motion of the substrates and therefore reduces the rates of enzymatic reactions (Muramatsu and Minton, 1988; Minton, 2001).

The accumulation of compatible solutes increases cell volume significantly. For example the amount of cytoplasmic water is 50% higher in cells growing in a medium with 0.5 M NaCl and 1 mM betaine than without betaine (Cayley *et al.*, 1992). The hypothesis presented by Cayley and co-workers is also consistent with the preferential exclusion model. Betaine replaces other, less compatible solutes such as trehalose, glutamate and K^+. It is likely that these compounds are excluded from macromolecule surfaces to a lesser degree than betaine, *i.e.* they are more evenly distributed in the cytoplasm. The accumulation of betaine increases the volume of the compatible solute-free layers on proteins and therefore also the total volume of cytoplasmic water (Cayley *et al.*, 1992). The reason for the growth stimulation of osmotically stressed *E. coli* cells by betaine is that betaine restores the cell volume closer to that of the unstressed cells and thereby prevents the impairment of cellular functions due to macromolecular crowding.

However, Garner and Burg (1994) pointed out that the explanation of Sutherland *et al.* (1986), based on the reduction of cytoplasmic K^+ concentration and protection provided by betaine against its deleterious effects, cannot be ruled out completely. As already stated, betaine not only increases the volume of cytoplasmic water, but also decreases the intracellular concentration of K^+. Compatible solutes are (by definition) compounds that are compatible with enzyme action even at high concentrations. Therefore replacement of K^+ and other perturbing solutes with betaine can stimulate growth. It is not possible to assess how much of the growth stimulation comes from these effects, and how much from the alterations in macromolecular crowding that result from the restoration of cell volume (Garner and Burg, 1994).

The preferential exclusion model is widely accepted, because it is supported by considerable experimental evidence. However, the model does not explain why betaine is the most effective stimulator of growth in high salt concentrations (Csonka, 1989). Osmolyte-free layers are formed on the surfaces of proteins in the solutions of many different amino acids (Arakawa and Timasheff, 1983; Arakawa and Timasheff, 1985), yet there are great differences in the protective properties of these solutes. There is also variation in the degree of protection which the same compatible solute confers to different enzymes. This suggests that enzyme - compatible solute specific interactions also exist (Galinski, 1993).

The protectiveness of betaine appears to depend on the degree of methylation of its nitrogen atom. It has been shown both on the cellular (Le Rudulier *et al.*, 1984; Øyaas *et al.*, 1994a) and the enzyme (Pollard and Wyn Jones, 1979) level that in the series betaine-dimethylglycine-sarcosine-glycine the more methylated derivatives of glycine are the more effective protectants. It should however be emphasized that it is at present not possible to predict the compatibility of a solute from its chemical structure. The mechanisms of compatible solute function are still not fully understood (Oren, 1999).

1.6 Betaine biosynthesis in bacteria

1.6.1 Conversion of choline to betaine

The ability to oxidize choline to betaine is very common in nature. It has been reported to take place in animals (Tsuge *et al.*, 1980; Yancey *et al.*, 1982), plants (Rhodes and Hanson, 1993) and microbes. The reaction proceeds in two steps with betaine aldehyde as the intermediate. Choline oxidation is not necessarily related to osmotic adaptation. Choline, in the form of phosphatidyl choline, is a common component of cell lipids and therefore ubiquitous in nature. Consequently, many microorganisms are able to use it as a carbon and nitrogen source for growth.

In aerobic bacteria betaine is an intermediate in the catabolic pathway of choline. The degradation of choline begins with its oxidation to betaine, which is then demethylated further to glycine (2) (Shieh, 1964; Kortstee, 1970; White and Demain, 1971):

Choline → Betaine aldehyde → Betaine → Dimethylglycine → Sarcosine (Monomethylglycine) → Glycine → Serine → Pyruvate (2)

The roles of choline in osmotic regulation and as a substrate for growth are not necessarily mutually exclusive. Some strains, such as *Rhizobium meliloti*, are able to use betaine as a compatible solute and also to degrade it *via* the above pathway (2) (Smith *et al.*, 1988). When the salt concentration of the medium is low, the catabolic activities of the pathway are high. However, when the osmolarity of the environment increases, the reaction sequence stops at betaine. Betaine is subsequently accumulated as a compatible solute in the cytoplasm. Choline also stimulates the growth of *Pseudomonas aeruginosa* under osmotic stress. However, unlike *R. meliloti*, *P. aerugionosa* is also capable of utilizing choline as a carbon and nitrogen source at high osmolarity (Lisa *et al.*, 1994). *E. coli* and *Bacillus subtilis* also have choline oxidizing activity, but contrary to *R. meliloti* and *P. aerugionosa*, they cannot use choline as a substrate for growth (Perroud and Le Rudulier, 1985; Boch *et al.*, 1994). Betaine functions solely as a compatible solute in these bacteria.

Depending on the strain, bacteria have different enzymatic systems for the oxidation of choline. The first step is catalyzed by the soluble flavoprotein choline oxidase in *Arthrobacter globiformis* (Ikuta *et al.*, 1977) and *Alcaligenes* sp. (Ohta *et al.*, 1983) and by a membrane-bound choline dehydrogenase in *P. aeruginosa* (Nagasawa *et al.*, 1975), *E. coli* (Landfald and Strøm, 1986), and in the moderate halophile *Vibrio costicola* (Choquet *et al.*, 1991). The membrane-bound choline dehydrogenase from *E. coli* has also been shown to mediate the oxidation of betaine aldehyde to betaine, but with lower efficiency (Landfald and Strøm, 1986). The oxidation of betaine aldehyde is catalyzed by a NAD^+-dependent betaine aldehyde dehydrogenase in several strains (Nagasawa *et al*., 1976; Falkenberg and Strøm, 1990; Choquet *et al.*, 1991; Mori *et al*., 1992; Boch *et al.*, 1997).

According to the unpublished results reported by Galinski (1995), choline oxidation is a common property of halophilic heterotrophic bacteria, which normally produce ectoine as their compatible solute. This is expected, since the transformation of choline to betaine is energetically more favorable than the *de novo* synthesis of compatible solutes.

1.6.2 De novo synthesis of betaine

On the basis of experiments by Imhoff and Rodriguez-Valera (1984), it was previously believed that betaine is the main compatible solute of halophilic heterotrophic eubacteria. It has, however, become evident that betaine found in these bacteria was transported from the medium and not synthesized *de novo* (Severin *et al.*, 1992). Complex media components, such as yeast extract and

peptone, contain considerable amounts of betaine (1 - 3% of the dry weight) that can be taken up by heterotrophic bacteria (Galinski and Trüper, 1994). In fact, betaine synthesis from simple carbon sources has proven to be quite rare among heterotrophic bacteria (Galinski and Trüper, 1994). In the extensive study by Severin *et al.* (1992), the extremely halophilic actinomycete *Actinopolyspora halophila* and a related isolate were the only heterotrophic strains in which betaine synthesis could be detected. Most heterotrophic halophiles are ectoine producers.

However, betaine synthesis from simple carbon sources is common among halophilic phototrophic eubacteria. It has been shown both for halophilic phototrophic anaerobes (Severin *et al*., 1992) and for halophilic cyanobacteria (Mohammed *et al.*, 1983; Mackay *et al.*, 1984; Reed *et al.*, 1984; Gabbay-Azaria *et al.*, 1988). Betaine synthesis is characteristic for cyanobacteria living in high salt concentrations, whereas less halophilic strains synthesize sugars and glycosyl glycerol in response to osmotic stress (Mackay *et al.,* 1984). In addition to these eubacteria halophilic methanogenic archaebacteria (*Methanohalophilus*-strains) also synthesize betaine as their osmolyte (Lai *et al.*, 1991).

Depending on the salinity of the growth medium, betaine-producers can accumulate extremely high cytoplasmic concentrations of betaine. For example, in the presence of 4.4 M NaCl the intracellular betaine concentration of the extremely halophilic archaebacterium *Methanohalophilus* Z7302 can be as high as 4.1 M (Lai and Gunsalus, 1992). The extremely halophilic sulfur bacterium *Ectothiorhodospira halochloris* synthesizes 2.5 mol betaine · (kg cytoplasmic water)$^{-1}$ at 240 g NaCl · (kg H_2O)$^{-1}$ (Galinski and Herzog, 1990) and the halophilic cyanobacterium *Synchocystis* DUN52 produces 3.0 M betaine when the bacterium is cultivated at 200 g sea salt · l^{-1} (Mohammed *et al.*, 1983).

The pathways of *de novo* synthesis of betaine in bacteria have not been fully characterized. The alternative routes are the direct methylation of glycine to betaine and the methylation of ethanolamine to choline followed by its further oxidation to betaine. The biosynthesis of betaine thus requires the threefold methylation of glycine or ethanolamine. The methyl groups would originate from the universal methyl group donor AdoMet. According to Atkinson (1977) methylation reactions are among the most energy-consuming processes in nature. The regeneration of one active methyl group of AdoMet costs the cells 12 ATP molecules. As in the production of compatible solutes in general, betaine synthesis is also energetically costly for the cell, especially if the betaine can not be used as an energy reserve.

There are no reports on the pathway of betaine synthesis in *A. halophila*, the only heterotrophic bacterium known to produce betaine *de novo*. However, there exists experimental evidence for the pathways of betaine synthesis in some other betaine-producers. The studies based on ^{13}C-NMR are consistent with betaine being synthesized *via* the glycine methylation pathway in the methanogenic archaebacterium *Methanohalophilus portucalensis* FDF1 (Roberts *et al.*, 1992). In addition, enzyme activities of the threefold methylation of glycine have been found in the cell extract prepared from this strain (Lai *et al.*, 1999).

It is also believed that in the extremely halophilic phototrophic anaerobe *Ectothiorhodospira halochloris*, betaine synthesis proceeds *via* the methylation of glycine. The results from ^{13}C-labeling studies by Galinski (1986) are in accordance with this assumption. In addition, the three methylation reactions of betaine

synthesis have been identified in this strain, although partly by indirect methods (Tschichholz-Mikus, 1994). However, as with the *Methanohalophilus* strains, none of the methyltransferases catalyzing the reactions have been purified to homogeneity. It has been suggested both for *M. portucalensis* FDF1 and *E. halochloris* that the glycine for betaine synthesis is generated from isocitrate *via* the glyoxylate cycle (Trüper and Galinski, 1990; Roberts *et al.*, 1992). The glycine methylation pathway is presented in Figure 2A.

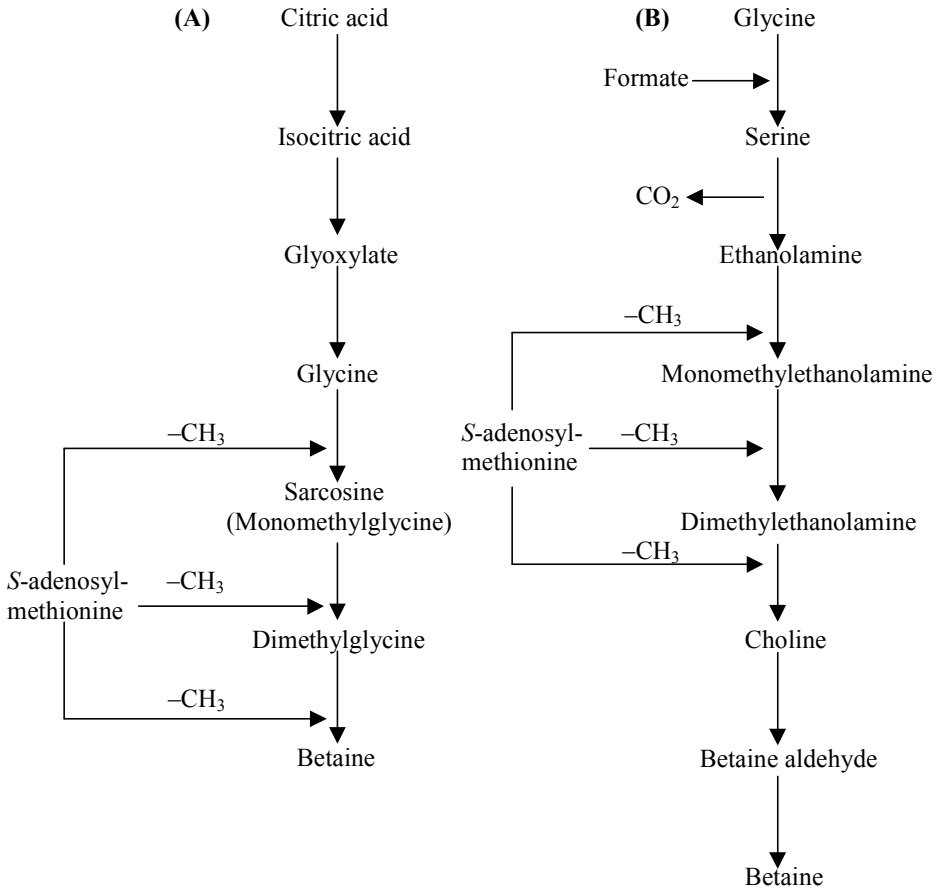

Figure 2. Alternative pathways of betaine synthesis in bacteria. **(A)** Glycine *N*-methylation pathway. **(B)** *N*-methylation of ethanolamine followed by oxidation of choline to betaine.

The pathway of betaine synthesis in cyanobacteria still remains to be conclusively established. On the basis of preliminary evidence from inhibitor studies by Yopp *et al.* (1984), it has been suggested that, in contrast to other bacteria, betaine is synthesized *via* the ethanolamine-choline-pathway in cyanobacteria. Furthermore, choline and betaine aldehyde dehydrogenase activities involved in the oxidation of choline to betaine were recently found in the halophilic cyanobacterium *Aphanothece halophytica* (Incharoensakdi and Wutipraditkul, 1999). The putative route of betaine synthesis in cyanobacteria is presented in Figure 2B.

The existing information on the regulation mechanisms of betaine production is very scanty. However, Sibley and Yopp (1987) proposed an interesting hypothesis concerning the regulation of betaine synthesis in *A. halophytica*. As stated earlier, three methylation reactions occur in the biosynthesis of betaine. Sibley´s and Yopp´s model is based on the (presumed) strong inhibitory effect of the methylation reaction product *S*-adenosylhomocysteine (AdoHcy) on the methyltransferase activities.

AdoHcy is degraded in a reversible reaction by AdoHcy hydrolase. When the salt concentration of the environment increases, *A. halophytica* accumulates K^+ to a high concentration. AdoHcy hydrolase is inhibited by the increased concentration of K^+ in the direction of AdoHcy synthesis, but is unaffected in the direction of AdoHcy hydrolysis. Hence, AdoHcy is degraded, and its inhibiting effect on betaine synthesis is relieved. Betaine stimulates the synthesis of AdoHcy in the presence of high concentration of K^+, but not in its absence. Therefore the higher betaine concentration leads to stimulation of AdoHcy synthesis and consequently to decreased rate of betaine synthesis. According to this hypothesis betaine would thus regulate its own synthesis in a feedback manner. It remains to be seen whether betaine synthesis is actually regulated in this way in *A. halophytica* and other betaine-producers. The hypothesis cannot be established without information on how K^+ and betaine affect the performance of the methyltransferases.

Galinski and Herzog (1990) investigated the effects of nitrogen limitation on the compatible solute pool of *E. halochloris*. In addition to the nitrogen-containing osmolytes betaine and ectoine, *E. halochloris* synthesizes the disaccharide trehalose as its compatible solute. When cultivated under nitrogen limitation, *E. halochloris* metabolizes ectoine. Betaine is partly replaced by trehalose, but this is a result of decreased synthesis, not of degradation. The authors suggested that the inability of *E. halochloris* to metabolize betaine indicates that the role of betaine is not only of an inert osmolyte, but that it also has indispensable protective functions.

The ability to uptake betaine from the medium appears to be common among betaine-producers. Betaine uptake has been reported for *Methanohalophilus* strains (Lai *et al.*, 1991), *E. halochloris* (Peters *et al.*, 1992), and *A. halophytica* (Moore *et al.*, 1987). Again, betaine uptake is beneficial because it is energetically less expensive than *de novo* synthesis. Furthermore, according to Oren (1999) the uptake mechanisms may also be used for salvaging compatible solutes that are leaked to the medium through the membranes of betaine producers.

1.7 Betaine synthesis in plants

1.7.1 De novo synthesis of betaine in natural producers

Many plants produce betaine in response to osmotic stress. Betaine synthesis is especially common in angiosperms such barley, sugar beet and spinach. However, many taxa have hitherto not been systematically investigated for their ability to synthesize betaine (Gorham, 1995).

In plants, betaine is synthesized from choline, which is a product of the successive methylation reactions of ethanolamine derivatives. Choline has two

fates. In addition to being oxidized to betaine in betaine-producers, it is used for the synthesis of phosphatidyl choline, which is a component of cell lipids. The ethanolamine is formed from serine. Depending on the plant species, the methylation of ethanolamine to choline can occur either at the free base, phospho-base or at the phosphatidyl-base level (Rhodes and Hanson, 1993). It has been shown that in betaine-producers, such as barley, spinach and sugarbeet, mainly phospho-derivatives are methylated (Hitz *et al.*, 1981; Coughlan and Wyn Jones, 1982; Hanson and Rhodes, 1983; Weretilnyk and Summers, 1992). However, there exists evidence that in sugar beet (Hanson and Rhodes, 1983) and spinach (Coughlan and Wyn Jones, 1982) the choline for betaine synthesis is hydrolyzed directly from phosphocholine, whereas in barley phosphocholine is first incorporated into phosphatidylcholine before it enters the free choline pool (Hitz *et al.*, 1981). The pathway of betaine synthesis in plants is presented in Figure 3.

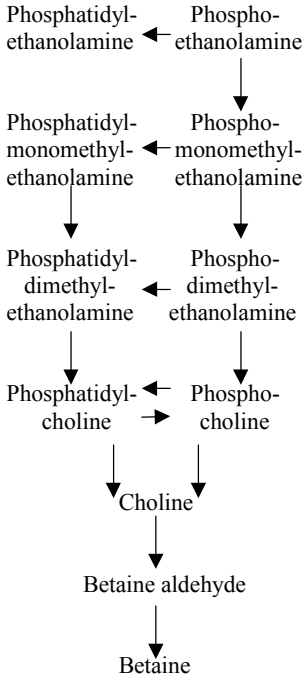

Figure 3. Betaine synthesis in plants *via* the methylation of ethanolamine derivatives followed by oxidation of choline to betaine (drawn following Nuccio *et al.*, 1998).

As in many bacteria, in plants choline is also oxidized to betaine in two steps with betaine aldehyde as the intermediate. However, the enzyme catalyzing the first oxidation step in plants differs from the bacterial choline oxidases and dehydrogenases. It has been shown that in spinach, sugar beet and amaranth the oxidation of choline is catalyzed by a ferredoxin-dependent choline monooxygenase (Lerma *et al.*, 1988, Brouquisse *et al.*, 1989, Rathinasabapathi *et al.*, 1997; Russell *et al.*, 1998). Betaine aldehyde is oxidized further to betaine by an NAD^+-dependent betaine aldehyde dehydrogenase (Weretilnyk and Hanson, 1989; Wood *et al.*, 1996). In chenopods, the enzymes for choline oxidation are localized in the chloroplasts (Hanson *et al.*, 1985).

1.7.2 Engineering of betaine synthesis into non-producers

Osmotic stress is one of the most important environmental factors limiting plant productivity. It is mainly caused by drought and salinity. Although irrigation increases crop yields, its use is not without problems. Water is a limited resource, which is also needed for other purposes. Furthermore, the accumulation of salts in the soil as a result of irrigation has been a problem for agriculture for thousands of years (Boyer, 1982). Consequently, there has been considerable interest in the genetic engineering of stress tolerant plants. Single genes coding for the synthesis of compatible solutes, such as proline, polyols, trehalose and betaine have therefore been introduced into many plants (Nuccio *et al.*, 1999).

Salt-, drought- (Rhodes and Hanson, 1993; Gorham, 1996), and cold-stress (Naidu *et al.*, 1991; Kishitani *et al.*, 1994) have been shown to enhance betaine accumulation in plants capable of synthesizing it. In addition, there exists substantial experimental evidence indicating that betaine protects plant macromolecules against various stress factors (as presented earlier in this study).

These findings have led to the assumption that the engineering of betaine synthesis into crop plants unable to synthesize it could be used to improve their stress-tolerance (McCue and Hanson, 1990; Le Rudulier *et al.*, 1984). Hence spinach choline mono-oxygenase and *E. coli* choline dehydrogenase have been introduced into tobacco (Lilius *et al*., 1996; Nuccio *et al.*, 1998) and bacterial choline oxidases have been introduced into tobacco (Huang *et al.*, 2000), *Brassica napu*s (Huang *et al.*, 2000), *Arabidopsis* (Hayashi *et al.*, 1997; Alia *et al.*, 1998; Huang *et al.*, 2000) and rice (Sakamoto *et al.*, 1998).

However, the levels of betaine in the transgenic plants have been significantly lower than in those naturally accumulating it, although in some cases improved stress tolerance has been reported. The supply of choline for betaine synthesis is limited, because it is converted almost exclusively to phosphatidyl choline. The choline synthesis itself is constrained at the first methylation step of phosphoethanolamine (Nuccio *et al.*, 1998). As suggested by Nuccio *et al.* (1998; 1999), it is reasonable to assume that the main source of choline in non-producers is from the turnover of phosphatidylcholine. However, the free choline thus formed is rapidly and virtually irreversibly converted into phosphocholine, which acts as a reserve for the synthesis of phosphatidylcholine.

Organisms use rigid metabolic networks for maintaining optimal distribution of metabolites. These networks resist redistribution of metabolic fluxes (Stephanopoulos and Vallino, 1991). It is believed that the reactions of choline metabolism in non-betaine producing plants form a rigid metabolic network, which makes it difficult to direct choline to betaine synthesis (Nuccio *et al.*, 1999).

2 AIMS OF THIS STUDY

The topic of the present work is the *de novo* synthesis of betaine in extremely halophilic bacteria. Two extreme halophiles, *Actinopolyspora halophila* and *Ectothiorhodospira halochloris*, were chosen as the objects of this study. There are no reports on the pathway and characteristics of betaine synthesis in *A. halophila*. For *E. halochloris* some evidence exists suggesting that in this bacterium betaine is synthesized *via* the threefold methylation of glycine. However, no enzymes catalyzing the reaction have been isolated.

The general purpose of the work was to provide a new potential alternative for the metabolic engineering of industrial microbes and stress-tolerant crops.

The specific aims of the study were:

- to establish the methyltransferase pathway of betaine synthesis in both bacteria
- to determine whether *A. halophila* is able to convert choline to betaine in addition to the *de novo* pathway
- to purify the methyltransferases catalyzing the reactions of betaine synthesis and to characterize their properties
- to clone and sequence the methyltransferase genes
- to express the betaine pathway in *E. coli* and to investigate the effect of the pathway on the osmotic stress tolerance of the cells.

3 MATERIALS AND METHODS

Only a brief summary of the most essential materials and methods used in this study is presented here. In addition, it should be noted that some of the methods were modified during the course of the work. For more detailed descriptions the reader is referred to the supplemented original papers I-IV.

3.1 Bacterial strains and growth conditions

In order to produce cells for the isolation of the methyltransferases, *Actinopolyspora halophila* (DSM 43834) was cultivated in the complex medium described by Sehgal and Gibbons (1960).

Betaine transport and synthesis in *A. halophila* was investigated using a semisynthetic medium containing per liter of distilled water: 150 – 240 g NaCl, 10 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 3.4 g trisodium citrate$\cdot 2H_2O$, 2 g NH_4Cl, 0.84 g $K_2HPO_4 \cdot 3H_2O$, 20 g glucose, 50 mg $FeSO_4 \cdot 7H_2O$, 9.1 g Tris, 1 ml vitamin solution VA (Imhoff and Trüper, 1977), 1 g casamino acids, and 100 mg yeast extract. The pH of the medium was adjusted to 7.5 with HCl. *A. halophila* cultures were grown aerobically at 37 ^{o}C.

Ectothiorhodospira halochloris (ATCC 35916) was grown anaerobically under light as described by Tschichholz and Trüper (1990). The stress-tolerance experiments with *E. coli* XL1-blue MRF´ (Qiagen) were carried out in the mineral medium MM63 (Miller, 1972) supplemented with 1.5 ml $\cdot$ l^{-1} vitamin solution VA (Imhoff and Trüper, 1977) at 37 ^{o}C.

3.2 Dry weight, betaine, trehalose, and glucose determinations

Cells of *A. halophila* were separated from the culture by filtration onto weighed filter papers. The filter papers were washed with a solution containing the same concentration of NaCl as was the total concentration of salts in the medium. The papers were dried, weighed and extracted with water at 70 ^{o}C for 90 min. The suspensions were centrifuged and the amount of NaCl present in the dry weight was determined by titrating potentiometrically an aliquot of the supernatant with 0.1 M $AgNO_3$. Betaine was analyzed from the supernatant by high-performance liquid chromatography (HPLC) using an Aminex HPX-87C column (Bio-Rad) at 80 ^{o}C with 5 mM $Ca(NO_3)_2$ (0.6 ml min^{-1}) as the eluent. Trehalose and glucose were analyzed by HPLC under the same conditions as described above, except that deionized water was used as the eluent and salts were removed in a deashing Micro-Guard pre-column (Bio-Rad).

The recombinant *E. coli* cells were separated by centrifugation and the dry weight determined essentially as described above. For the HPLC analyses of intracellular solutes of *E. coli*, the cells were extracted with perchloric acid using a modification of the method described by Sutherland and Wilkinson (1971).

3.3 Enzyme activity assays

3.3.1 H_2O_2-generating activity assays

H_2O_2-generating activity was assayed using the method described by Lartillot (1987) with the exception that the reaction mixture was supplemented with 20% (w/v) glycerol. In this assay, the H_2O_2 formed is measured spectroscopically using the peroxidase - 4-hydroxybenzoate - 4-aminoantipyrine system as the H_2O_2-acceptor. The reaction mixtures were incubated at 37 ^{o}C and the absorbance was monitored at 506 nm. The enzyme activities were calculated as nmol of H_2O_2 generated per minute.

3.3.2 $NAD(P)^+$-reducing activity assays

The NAD^+ and $NADP^+$-reducing activities with choline and betaine aldehyde as the substrates were assayed by measuring the change in absorbance at 340 nm at 37 ^{o}C. The reaction mixtures typically contained 50 mM potassium phosphate buffer pH 7.5, 5 mM NAD^+ or $NADP^+$, and 20 mM choline or 20 mM betaine aldehyde. The enzyme activities were calculated as nmol of $NAD(P)^+$ reduced per minute.

3.3.3 Methyltransferase activity assays

A modification of a previously reported assay (Cook and Wagner, 1984) was used. After the methylation of glycine, sarcosine or dimethylglycine, the unreacted methyl group donor *S*-adenosyl-L-[*methyl*-^{14}C] methionine was adsorbed to charcoal and the remaining methylation products were determined by scintillation counting.

The reaction mixture (100 µl) contained typically 250 mM glycine or 25 mM sarcosine in glycine sarcosine methyltransferase (GSMT) assays and 80 mM sarcosine or 50 mM dimethylglycine in sarcosine dimethylglycine methyltransferase (SDMT) assays; 150 mM Tris-HCl pH 7.4; 2 mM *S*-adenosyl-L-methionine (AdoMet) (with 910 µCi · l^{-1} *S*-adenosyl-L-[*methyl*-^{14}C] methionine, Amersham Pharmacia Biotech); and enzyme sample (1/4 of the total volume). The reaction mixture was incubated at 37 ^{o}C and the reaction was stopped by adding 75 µl of charcoal suspension (133 g · l^{-1} in 5% w/v trichloroacetic acid) and incubated for 10 min at 0 ^{o}C. After centrifugation for 10 min, 75 µl of the supernatant was removed for assay in a liquid scintillation counter. The enzyme activity was calculated as µmoles of methyl groups transferred per min.

The substrate specificity was studied at 37 ^{o}C using the radiometrical methyltransferase assay described by Ogawa and Fujioka (1982). In this assay the unreacted AdoMet is precipitated with phosphotungstic acid and adenosine. The composition of the reaction mixture was the same as above.

3.4 Purification of the native methyltransferases

3.4.1 Purification of A. halophila sarcosine dimethylglycine methyltransferase (SDMT)

Harvested *A. halophila* cells were suspended in disruption buffer (1 g cells, wet weight, in 1.5 ml buffer). The cells were disrupted by sonication and the cell-free extract was clarified by centrifugation. Adenosine-Sepharose affinity chromatography was used for the purification of SDMT. The affinity column was prepared from 5´AMP-sepharose 4B (Pharmacia Biotech). The swollen gel (1 g dry weight) was equilibrated with buffer and treated for 2 h at 37 °C with 100 units of Calf Intestinal Alkaline Phosphatase (Finnzymes, Espoo, Finland). The cell-free extract was applied to the affinity column, which was then washed with 20 mM Tris-HCl, pH 7.5. The protein bound to the column was eluted with 1 mM AdoMet.

3.4.2 Purification of E. halochloris glycine sarcosine methyltransferase (GSMT)

Harvested *E. halochloris* cells were suspended in disruption buffer (1 g cells, wet weight, in 1.5 ml buffer). The cells were disrupted by sonication and the cell-free extract was clarified by centrifugation. Ammonium sulfate was added to the cell-free extract to achieve 20% saturation and the suspension was incubated for 30 min at 0 °C. The solution was centrifuged and the supernatant applied to a Butyl Sepharose 4 FF (Pharmacia Biotech) column pre-equilibrated with 20% saturated ammonium sulfate in 20 mM Tris-HCl, pH 7.5. The column was washed with the equilibration buffer and eluted with a linear gradient of 20 to 0% saturated ammonium sulfate. The active fractions were pooled and ammonium sulfate was removed by gel filtration (Sephadex G-25, Pharmacia Biotech). The sample was applied to a DEAE-Memsep 1000 HP (Millipore) column pre-equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed and eluted with a linear NaCl gradient (0 - 1 M). The active fractions were pooled and concentrated by ultrafiltration. The concentrated sample (100 µl) was applied to a Superose 12 HR 30 (Pharmacia Biotech) column. The elution buffer used was 20 mM Tris-HCl, pH 7.5 containing 150 mM NaCl. The fractions containing GSMT activity were again collected and concentrated by ultrafiltration.

3.5 Cloning of the genes

The genomic DNAs from *A. halophila* and *E. halochloris* were isolated (Ausubel *et al.*, 1991) and digested partially with *SacI*. The partially digested fragments were ligated to *SacI* digested dephosphorylated λ ZapII arms (Stratagene, La Jolla, CA) and packaged to λ particles using Gigapack III Gold packing extract (Stratagene) according to the protocol provided by the manufacturer.

The *N*-terminal and tryptic peptides were purified and sequenced at the Institute of Biotechnology (Helsinki, Finland) by using a Perkin Elmer/Applied Biosystems

Procise 494A protein sequencing system as described previously (Kerovuo *et al.*, 1998). Degenerate primers were designed on the basis of *N*-terminal and tryptic peptide sequences of *E. halochloris* GSMT and *A. halophila* SDMT. Chromosomal DNA was used as the template in the polymerase chain reactions (PCR). The PCR-fragments were labeled with *redi*prime DNA labeling system (Amersham) according to the instructions given by the manufacturer and used as probes to screen the genomic libraries of the strains. The positive λ clones were cored and excised with ExAssist helper phage (Stratagene) to obtain phagemids. The obtained phagemids were used to transform *E. coli* SOLR´ cells (Stratagene). The plasmid DNAs were purified and sequenced.

3.6 Expression of the methyltransferases

The gene fragments coding for the methyltransferases were amplified by PCR with insertion of *Nco*I at the 5´ end and *Bgl*II at the 3´ end. The template of the PCR reactions was chromosomal DNA of *A. halophila* or the plasmid used for the sequencing of the "betaine operon" of *E. halochloris* (see above). The amplified PCR fragments were purified and cloned into *Nco*I/*Bgl*II cut pQE-60 expression vectors (Qiagen). The resulting plasmids were transformed into *E. coli* XL-1 Blue MRF´ as described previously (Hanahan, 1983). The expression vectors used in this study are summarized in Table 1.

Table 1. Methyltransferase expression vectors[a].

Name	Proteins expressed	His_6-tag
PAGSMTh	GSMT of *A. halophila*	Yes
PASDMTh	SDMT of *A. halophila*	Yes
PAFUh	GSMT-SDMT fusion of *A. halophila*	Yes
PEGSMTh	GSMT of *E. halochloris*	Yes
PESDMTh	SDMT of *E. halochloris*	Yes
PEGSMT[b]	GSMT of *E. halochloris*	No
PESDMT[b]	SDMT of *E. halochloris*	No
PEhFU[c]	GSMT and SDMT of *E. halochloris*	In SDMT

[a]All vectors were derivatives of PQE-60 (Qiagen).
[b]These vectors were used in the production of the methyltransferases for characterization.
[c]pEhFU was co-expressed with pREP4 (Qiagen).

The expression of the methyltransferases was carried out in Luria-Bertani broth using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as the inducer. The cells were separated by centrifugation, washed and disrupted by sonication. The cell debris was removed by centrifugation and the supernatant (cell-free extract) was used for further experiments.

3.7 Purification of recombinantly produced GSMT and SDMT of *E. halochloris*

3.7.1 Purification of GSMT

Ammonium sulfate was added to the cell-free extract to achieve 25% saturation and the solution was incubated for 45 min at 0 °C. The suspension was centrifuged and the supernatant was applied to a Butyl Sepharose 4 FF (Amersham Pharmacia Biotech) column pre-equilibrated with 25% saturated ammonium sulfate in 20 mM Tris-HCl, pH 7.5. The column was washed with the equilibration buffer and eluted with a linear gradient of 25 to 0% saturated ammonium sulfate. Salts were removed from the enzyme sample by ultrafiltration and dilution. The sample was applied to a DEAE Sepharose FF (Amersham Pharmacia Biotech) column pre-equilibrated with 20 mM Tris-HCl pH 7.5. The column was washed with the equilibration buffer and eluted with a linear NaCl gradient from 0 M to 1 M NaCl. The active fractions were pooled and concentrated by ultrafiltration.

3.7.2 Purification of SDMT

Ammonium sulfate fractionation at 40% saturation was carried out as described above. The supernatant from this purification step was diluted to 25% saturated ammonium sulfate and applied to a Phenyl Sepharose (high sub) column (Amersham Pharmacia Biotech) pre-equilibrated with 25% saturated ammonium sulfate in 20 mM Tris-HCl, pH 7.5. The column was washed with the equilibration buffer and eluted with a linear gradient of 25 to 0% saturated ammonium sulfate. The active fractions were pooled, purified further with DEAE and concentrated as above.

3.8 Some additional methods

Oxygen consumption was measured polarographically at 37 ºC using an YSI 5300 Biological Oxygen Monitor (YSI Incorporated, Yellow Springs, Ohio, USA). Thin-layer chromatography analysis of choline and betaine was performed as described in Speed and Richardson (1968). Initial-velocity data were analyzed by nonlinear least-squares regression using the program DYNAFIT (Kuzmic, 1996). The molecular weights of GSMT and SDMT were estimated by analytical gel filtration with a Superose 12-HR-30 (Amersham Pharmacia Biotech) column according to the instructions given by the manufacturer. Metals were analyzed by inductively coupled plasma emission spectrometry using a Thermo Jarell Ash AtomScan 16 (Thermo Jarell Ash Corp., Franklin, MA) instrument at Danisco-Cultor Innovation Center (Kirkkonummi, Finland).

4 SUMMARY OF THE RESULTS

4.1 Intracellular compatible solute concentrations of *A. halophila* (I)

Betaine, trehalose and glucose were determined by HPLC from extracts that were prepared from dried *A. halophila* cells grown in different salt concentrations. As shown in Figure 4, betaine was synthesized in extremely high intracellular concentrations, increasing from 1.7 mmol · (g dry weight)$^{-1}$ (or 20%) to 2.8 mmol · (g dry weight)$^{-1}$ (or 33%) over a salinity range from 15 to 24% (w/v). Trehalose accounted for a much smaller proportion of the compatible solute pool; its concentration was between 0.15 mmol · (g dry weight)$^{-1}$ (or 5.1%) and 0.28 mmol · (g dry weight)$^{-1}$ (or 9.7%). In contrast to betaine, the trehalose concentration was highest at 15% (w/v) NaCl and lowest at 21 and 24% (w/v) NaCl. Glucose was also detected in the cell extracts, but its concentrations were very low, less than 0.04 mmol · (g dry weight)$^{-1}$, at all NaCl-concentrations.

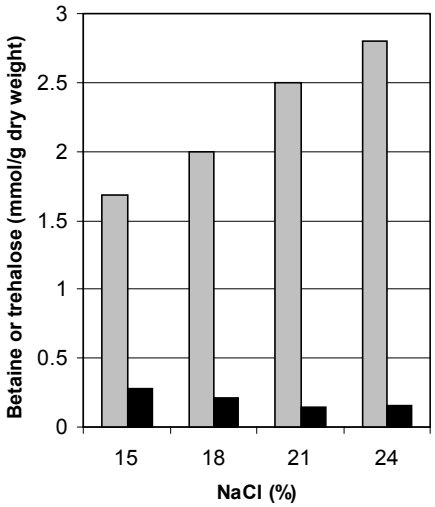

Figure 4. Intracellular betaine (gray) and trehalose (black) concentrations of *A. halophila* cultivated in various salt concentrations. Means of three independent determinations. The standard deviations were within 7%.

4.2 Uptake and recycling of betaine through the medium by *A. halophila* (I)

A. halophila was grown in the presence of 18% (w/v) NaCl and 0.5 mM [*methyl*-^{14}C]-betaine (74 nCi·µmol^{-1}). The amount of radioactivity taken up by the cells was determined by scintillation counting. The maximum amount of radioactivity incorporated by the cells represented approximately 90% of the total radioactivity in the culture. No loss of total radioactivity of the culture was observed during the experiment. The results indicate that *A. halophila* effectively takes up betaine.

The recycling of betaine was investigated under the same conditions. *A. halophila* was grown until virtually all [*methyl*-^{14}C]betaine had disappeared from the medium. After this the culture was divided in two, and to one half 15 mM unlabeled betaine was added. As shown in Figure 5, the addition of an excess of unlabeled betaine led to rapid release of the radiolabeled betaine taken up by the cells. Two hours after the addition, approximately 24% of the accumulated [*methyl*-^{14}C]betaine was detected in the medium. No loss of radiolabeled betaine was observed without the addition of unlabeled betaine (data not shown). The results indicate that betaine is recycled through the medium.

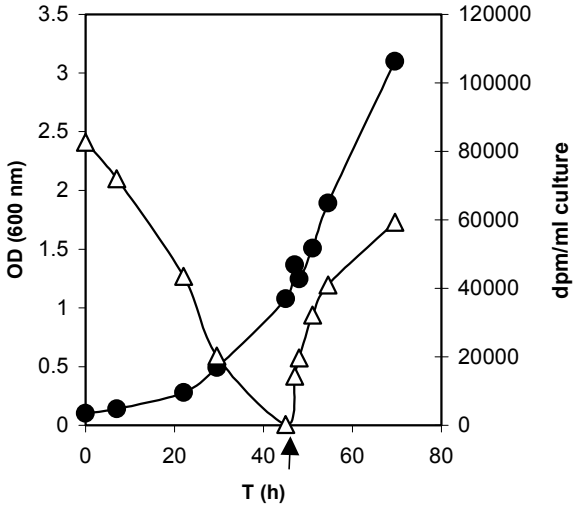

Figure 5. Circulation of betaine through the medium. [*methyl*-^{14}C]Betaine in the medium (Δ) and bacterial growth (●). Growth medium containing 18% (w/v) NaCl was supplemented with 0.5 mM [*methyl*-^{14}C]betaine. At 46 h, 15 mM unlabeled betaine was added to the medium (see arrow).

4.3 Methylation of glycine to betaine by *E. halochloris* and *A. halophila* cell extracts (II)

The hypothesis in this work was that betaine is synthesized *via* the threefold methylation of glycine in *A. halophila* and *E. halochloris*. Glycine would first be methylated to sarcosine and then further to dimethylglycine and betaine.

In order to demonstrate the existence of the glycine methylation pathway, methyltransferase activities were determined by using *S*-adenosylmethionine (AdoMet) as the methyl group donor and glycine, sarcosine or dimethylglycine as the methyl group acceptors. As shown in Table 2, both *A. halophila* and *E. halochloris* cell extracts showed methyltransferase activity on glycine, sarcosine, and dimethylglycine.

Table 2. Methyltransferase activities on glycine, sarcosine, and dimethylglycine in *A. halophila* and *E. halochloris* cell-free extracts. AdoMet was used as the methyl group donor. *A. halophila* cells were disrupted in the growth medium. The errors were estimated to be within 25%.

Organism	Activity ($\mu mol \cdot min^{-1} \cdot ml^{-1}$)		
	Glycine	Sarcosine	Dimethylglycine
E. halochloris	0.058	0.23	2.6
A. halophila	0.023	0.34	0.93

Furthermore, the reaction mixtures were analyzed by HPLC. Fractions were collected from the eluent and their radioactivity was measured by scintillation counting. The expected methylation reaction products were identified from the reaction mixtures with glycine, sarcosine and dimethylglycine as the substrates (data not shown).

4.4 Purification of the native methyltransferases (II)

In order to characterize the enzymology of the methylation pathway, the methyltransferases were purified from *A. halophila* and *E. halochloris* cell-free extracts. *A. halophila* cell extract was fractionated with Adenosine-Sepharose affinity chromatography and a pure enzyme, showing activity on sarcosine (0.36 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and dimethylglycine (1.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was obtained. There was no activity on glycine. The enzyme was named as sarcosine dimethylglycine methyltransferase (SDMT) according to its substrate specificity. It should be noted that the specific activities presented here are only approximate estimates. Unfortunately, it became later evident that these values were not determined in the strictly linear range. Because of this, there were approximately 20 - 25% errors in the results.

As estimated by sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the molecular mass of SDMT was 32 kDa. Gel filtration under non-denaturing conditions gave similar results, which indicates that the protein is a monomer. Since the activity on glycine was very unstable, the isolation of methyltransferases having glycine methylation activity was not successful.

However, the enzymatic activity on glycine was found to be stable in the *E. halochloris* cell extract. After chromatographic purification, a 38 kDa protein was isolated. The enzyme had activity on glycine (0.52 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and sarcosine (0.19 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). There was no activity on dimethylglycine. The enzyme was accordingly named glycine sarcosine methyltransferase (GSMT).

4.5 Synthesis of betaine by GSMT and SDMT *in vitro* (II)

The enzyme activity data suggested that two enzymes with partially overlapping substrate specificity can catalyze the threefold methylation of glycine. This hypothesis was confirmed by the results from an *in vitro* synthesis experiment using purified enzymes. *E. halochloris* GSMT and *A. halophila* SDMT were incubated in

a reaction mixture containing glycine and [^{14}C-methyl]-AdoMet and the reaction mixtures were analyzed by HPLC. Fractions were collected from the eluent and their radioactivity was measured by scintillation counting. The HPLC of the reaction mixture showed radioactive peaks corresponding to the retention times of sarcosine, dimethylglycine and betaine (Fig. 6).

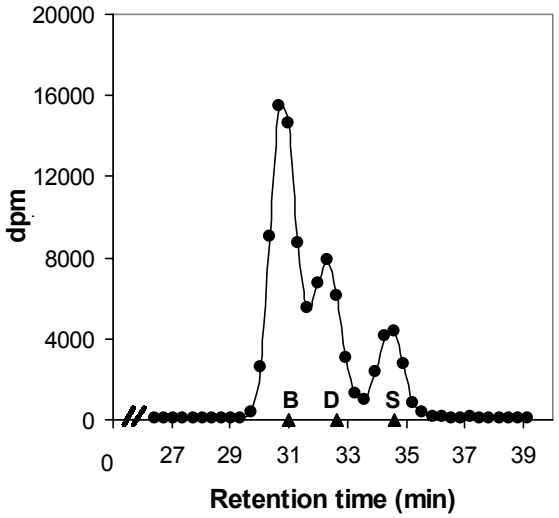

Figure 6. Synthesis of methylation products *in vitro* using purified *E. halochloris* GSMT and *A. halophila* SDMT. The reaction mixture contained 0.53 mg·ml^{-1} GSMT, 0.7 mg·ml^{-1} SDMT, 8 mM [^{14}C-methyl]AdoMet, and 1.25 mM glycine. The reaction products were analyzed by HPLC. (●) Radioactivity of the eluent. The retention times of the standards (sarcosine (S), dimethylglycine (D) and betaine (B)) are shown by triangles on the x axis.

4.6 Cloning of the methyltransferase genes (II)

Positive clones with 3.5 (*A. halophila*) and 5.0 kilobase pair (*E. halochloris*) inserts were isolated and the inserts were sequenced. The *E. halochloris* clone contained 2 open reading frames (ORFs) coding for the methyltransferases, which were 807 base pairs and 840 base pairs in length. The *A. halophila* clone had only one ORF (1698 base pairs), which had significant sequence homology with the *E. halochloris* ORFs. Further sequence analysis and comparison to the peptide sequences of the purified proteins revealed that the first gene of the *E. halochloris* clone coded for GSMT (Fig. 7). A homologous sequence was found in the *N*-terminal part of the *A. halophila* gene. The *N*-terminal amino acid sequence of the purified *A. halophila* SDMT can be identified in the middle of the same ORF, suggesting that the *C*-terminal part of the gene encodes the SDMT. Thus in *A. halophila* the GSMT and SDMT are synthesized from a single gene, and SDMT is probably a proteolytic processing product. In *E. halochloris* separate genes encode GSMT and SDMT.

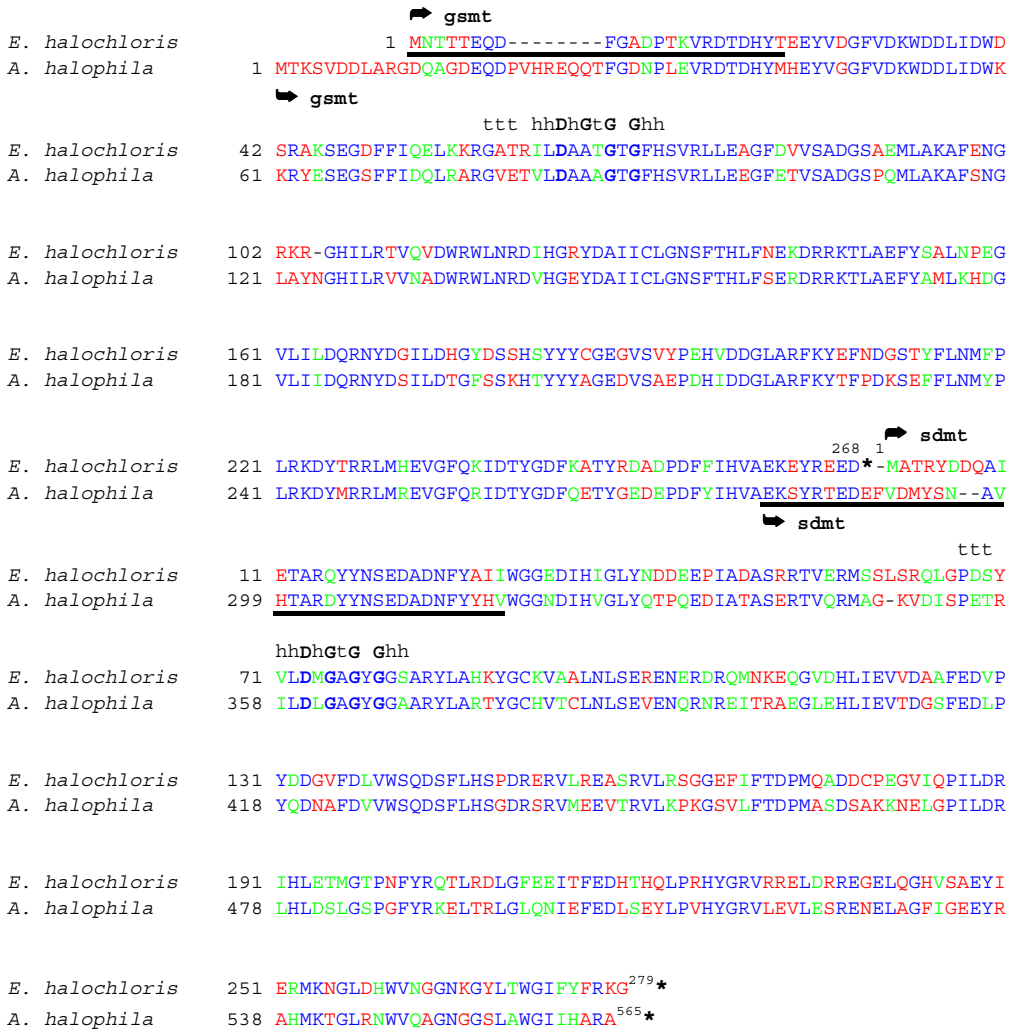

Figure 7. Homology comparison of the amino acid sequences of the GSMT and SDMT enzymes of *E. halochloris* and *A. halophila*. Identical amino acids are shown in blue, similar amino acids are shown in green, and non-matching amino acids are shown in red. The *N*-termini of the native proteins isolated from the cell extracts are underlined. The putative SAM binding site consensus sequence (tttxhhDhGtGxGhh) can be found in the amino acid sequences of all proteins. (t=polar or turn forming, h=hydrophobic, x=any amino acid).

As presented in Figure 7 the consensus sequence for a SAM binding site, as suggested by Bork *et al.* (1992), can be found from all methyltransferases.

4.7 Expression of the methyltransferases in *E. coli* (II)

The functionality of the enzymes was further confirmed by overexpressing the isolated genes in *E. coli* under *E. coli* phage T5 promoter (Table 3). The *E. halochloris* GSMT and SDMT were both expressed in active form. The substrate specificity of GSMT was identical to that of the native protein. In addition, the *E. halochloris* SDMT was shown to have similar substrate specificity to that of the *A. halophila* SDMT. The functionality of the *A. halophila* enzymes could be demonstrated only partially. When the *A. halophila* SDMT-GSMT gene was expressed in *E. coli,* only a very low level of SDMT activity was detected. A protein corresponding to the size of GSMT-SDMT fusion was, however, found in the cell pellet (data not shown). Truncated *A. halophila* GSMT and SDMT were designed on the basis of sequence homology with *E. halochloris* genes. Whereas expression of the truncated GSMT was not successful in *E. coli*, truncated SDMT was successfully expressed in a soluble form. The soluble protein had activity on sarcosine and dimethylglycine and a trace of activity on glycine.

Table 3. Methyltransferase activities in cell extracts of *E. coli* transformed with *E. halochloris* and *A. halophila* GSMT and SDMT. The genes were overexpressed using PQE-60 expression vector. The errors were estimated to be within 25%.

Protein	Activity ($\mu mol \cdot min^{-1} \cdot ml^{-1}$)		
	Glycine	Sarcosine	Dimethylglycine
E. halochloris			
GSMT	0.58	0.19	0
SDMT	0.006	1.3	5.6
A. halophila			
SDMT-$GSMT_{1-565}$ [a]	0	0.028	0.072
$GSMT_{1-281}$ [a] (truncated)	0	0	0
$SDMT_{282-565}$ [a] (truncated)	0.017	0.77	1.5

[a]The amino acid number of truncated *A. halophila* proteins - see Fig. 7.

4.8 Choline oxidation in *A. halophila* (I)

4.8.1 Uptake of choline

Uptake studies with *A. halophila* were carried out in a growth medium containing 18% (w/v) NaCl. The medium was supplemented with 0.5 mM [*methyl*-^{14}C]choline chloride (75 $nCi \cdot \mu mol^{-1}$). The amount of radioactivity taken up by the cells was determined by scintillation counting. Choline was effectively taken up by *A. halophila*. The maximum amount of radioactivity incorporated was 70% of the total amount of radioactivity in the culture. No loss of total radioactivity of the culture was observed during the experiment.

In order to examine whether the choline taken up by the cells is converted to betaine, cells collected at 72 h were analyzed by thin-layer chromatography as described in Materials and Methods. Betaine aldehyde, phosphatidylcholine and choline do not migrate under the conditions used. From the autoradiograph of the developed thin-layer chromatography plate, only a substance corresponding to the

retention time of betaine could be detected (data not shown.). This indicates that choline is oxidized to betaine by *A. halophila*.

4.8.2 The choline oxidation system of A. halophila

H_2O_2-generating and $NAD(P)^+$-reducing activities of *A. halophila* cells grown in a medium supplemented with 10 mM choline and in a medium without choline are presented in Table 4. All activities could be clearly detected only in cells grown in the presence of choline. This indicates that the enzymes catalyzing the oxidation of choline and betaine aldehyde are produced inducibly. No $NAD(P)^+$-reducing activities were detected with 3 mM or 50 mM choline as the substrate in any of the cell-free extracts.

Table 4. Enzyme activities (H_2O_2 generation and $NAD(P)^+$ reduction) related to choline oxidation in cells grown in the presence or absence of 10 mM choline. Activities were assayed from cell-free extracts. NAD^+ and $NADP^+$ were used at 5 mM.

Type of activity	Substrate	Activity [nmol·min^{-1}·(mg protein)$^{-1}$]	
		Choline (10 mM)	No choline
H_2O_2 gen.	20 mM Choline	33	0
H_2O_2 gen.	20 mM Betaine aldehyde	11	0
NAD^+ red.	3 mM Betaine aldehyde	120	6.7
$NADP^+$ red.	3 mM Betaine aldehyde	51	0

Both oxidoreductase activities displayed Michaelis-Menten kinetics for their substrates. The apparent K_m –value determined for the H_2O_2-generating activity was 5.0 ± 0.5 mM with choline as the variable substrate.

The apparent K_m –values calculated from the initial velocity data were 0.36 ± 0.04 mM for betaine aldehyde and 0.56 ± 0.05 mM for NAD^+ with 5 mM NAD^+ and 3 mM betaine aldehyde as the co-substrates, respectively. Since the activities on other substrates were very low, their K_m-values could not be reliably determined.

The relationship of H_2O_2 generation to oxygen consumption was also examined. Oxygen consumption was monitored polarographically and H_2O_2 generation colorimetrically as described in Materials and Methods. The reactions were carried out at 37 ^oC with 50 mM choline as the substrate. The experimentally determined ratio of moles of H_2O_2 generated to moles of O_2 consumed was 0.90:1.0. The results suggest that oxygen consumption is coupled with H_2O_2 generation in the enzymatic reaction.

4.9 Purification and characterization of recombinantly produced methyltransferases of *E. halochloris* (III)

Since it proved to be extremely difficult to purify sufficient amounts of native GSMT and SDMT for characterization, they were produced in *E. coli*. Both recombinant enzymes were purified to homogeneity by ammonium sulfate

fractionation, hydrophobic interaction chromatography and ion exchange chromatography (data not shown). The molecular masses estimated from the gel were 42 kDa and 36 kDa for GSMT and SDMT respectively, whereas for the native GSMT the value was 38 kDa (see 4.4). The values calculated from the amino-acid sequences are 31 kDa for GSMT and 32 kDa for SDMT. Estimates for the molecular masses of the enzymes obtained by analytical gel filtration were 40 kDa for GSMT and 25 kDa for SDMT. These results suggest that both recombinant enzymes are monomers.

4.10 Characterization of recombinant GSMT and SDMT (III)

4.10.1 pH-optima

The effect of pH on the activities of the purified enzymes was determined using the following buffers: 125 mM potassium phosphate (pH 4.9 to 6.2), 125 mM triethanolamine (pH 5.8 to 8.3), and 125 mM Tris-HCl (pH 8.0 to 9.0). The maximal activities obtained in the range of the triethanolamine buffer used were around pH 7.4 on glycine and around 7.9 on sarcosine with GSMT and around 8.0 on sarcosine and around 7.6 on dimethylglycine with SDMT. The pH optimum of SDMT appears to depend on the buffer used. With Tris-HCl as the buffer, the optimum for the sarcosine activity of SDMT was near pH 9.0, which differs from the optimum obtained with the triethanolamine buffer.

4.10.2 Substrate specificity

GSMT has strict specificity for glycine and sarcosine and SDMT for sarcosine and dimethylglycine as the methyl group acceptors. Neither ethanolamine, monomethylethanolamine nor any of the L-amino acids (alanine, asparagine, aspartate, cysteine, glutamate, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine or valine) tested at 25 mM were *N*-methylated by GSMT or SDMT.

4.10.3 Cofactor analysis

No evidence of cofactors was found. Incubation of GSMT or SDMT with 2.7 mM Ca^{2+}, Mg^{2+} or 13.3 mM EDTA had no significant effect on any of the activities. Furthermore, no Mn, Co nor Zn and only insignificant traces of Ca and Mg were detected when purified GSMT and SDMT were analyzed by inductively coupled plasma emission spectroscopy.

4.10.4 Inhibition of enzymatic activities

The reaction product, *S*-adenosylhomocysteine (AdoHcy) is known to be a strong competitive inhibitor of many methyltransferases (Heady and Kerr, 1973; Schneider and Vance, 1979; Upmeier *et al.*, 1988). The concentrations of AdoHcy causing 50% inhibition are presented in Table 5. The results indicate that GSMT and SDMT are also very susceptible to inhibition by AdoHcy.

Table 5. Inhibition of GSMT and SDMT by AdoHcy and dimethylglycine[a].

Enzyme	Methyl group acceptor	Concn inhibiting 50% (mM)	
		AdoHcy	Dimethylglycine
GSMT	250 mM Glycine	0.5	38
	25 mM Sarcosine	0.4	49
SDMT	80 mM Sarcosine	0.5	N.d.[b]
	50 mM Dimethylglycine	2.3	N.d.[b]

[a]All reaction mixtures contained 2 mM AdoMet as the methyl group donor.
[b]N.d. Not determined.

The inhibitory effects of dimethylglycine on GSMT and glycine on SDMT were also studied. These compounds were not methylated by the enzymes under the conditions used. As presented in Table 5, dimethylglycine, the product of the methylation of sarcosine, was a relatively poor inhibitor of GSMT. Glycine also inhibited the activities of SDMT only at high concentrations. At 50 mM it had no effect on the sarcosine activity but inhibited 27% of the activity on dimethylglycine. At 200 mM it inhibited 13% of the sarcosine activity and 56% of the dimethylglycine activity. The results indicate that the affinity of glycine for SDMT is low.

Phototrophically growing *E. halochloris* accumulates betaine up to 2.5 mol betaine · (kg cytoplasmic water)$^{-1}$ (Galinski and Herzog, 1990). The effects of betaine on the methyltransferase activities were determined at 0.5 M and 2 M. At 0.5 M betaine had a small activating effect on the methyltransferases with sarcosine (relative activity 120% for both GSMT and SDMT) and dimethylglycine (relative activity 130%) as the substrates, but no effect on the activity of GSMT on glycine. At 2 M betaine inhibited 60 - 70% of the methyltransferase activities.

4.10.5 Kinetic properties

GSMT catalyzes the reaction sequence glycine-sarcosine-dimethylglycine and SDMT the sequence sarcosine-dimethylglycine-betaine. In all the two-substrate reactions AdoMet acts as the methyl group donor. The apparent kinetic parameters for both substrates of every reaction step were determined with the other substrate present in excess. Both GSMT and SDMT displayed Michaelis-Menten kinetics for their substrates. The apparent K_m- and V_{max} -values determined are shown in Table 6

Table 6. Kinetic parameters of recombinant GSMT and SDMT of *E. halochloris*.

Enzyme	Substrate with varied concn	Substrate with fixed concn	Apparent K_m (mM)	V_{max}[a] (μmol· min^{-1}·mg^{-1})
GSMT	Glycine	AdoMet (4 mM)	18 ± 2	1.1
	AdoMet	Glycine (250 mM)	0.42 ± 0.04	1.0
	Sarcosine	AdoMet (4 mM)	2.3 ± 0.2	0.15
	AdoMet	Sarcosine (250 mM)	0.28 ± 0.04	0.12
SDMT	Sarcosine	AdoMet (2.5 mM)	(6.1 ± 1.0)[b]	(1.3)[b]
	AdoMet	Sarcosine (125 mM)	0.21 ± 0.02	1.1
	Dimethylglycine	AdoMet (2.25 mM)	4.9 ± 0.9	7.4
	AdoMet	Dimethylglycine (125 mM)	0.16 ± 0.01	6.1

[a]The errors were less than 10%.
[b]Approximate estimates. See results.

As presented in Table 6, in every methylation step the V_{max} –values determined for both substrates are close to each other. The initial velocity data fitted reasonably well with the hyperbolic curves both with high and low substrate concentrations, a fact that is also indicated by the standard errors presented in Table 6. However, the possibility of some minor substrate inhibition resulting from the saturating concentrations of the co-substrate used cannot be ruled out completely.

The specific activities presented earlier in this study for native GSMT and SDMT were obtained with 25 mM glycine, sarcosine or dimethylglycine and 1 mM AdoMet as the substrates (see 4.4). According to the results presented in Table 6, some of the substrates were not present in saturating concentrations in these assays. This appears to be especially true for the activities on glycine. Re-examining the linearity of the product formation in the enzymatic reactions revealed that our previous results were not obtained in the strictly linear range. In the case of the activity on glycine there is also the possibility that the strong inhibition by AdoHcy distorted the results, because the AdoMet concentration was close to its K_m-value.

Nevertheless, the specific activities of recombinant GSMT and SDMT were determined under the same conditions as earlier, in order to compare the activities with our previous results. The specific activities of purified recombinant GSMT were 0.16 μmol · min^{-1} · mg^{-1} on glycine and 0.075 μmol · min^{-1} · mg^{-1} on sarcosine and of purified recombinant SDMT they were 0.68 μmol · min^{-1} · mg^{-1} on sarcosine and 3.4 μmol · min^{-1} · mg^{-1} on dimethylglycine.

The specific activities of the recombinant GSMT were 3.3 and 2.5 times lower than for native GSMT with glycine and sarcosine as the substrates, respectively. The deviations from linearity of the product formation rates do not explain the differences between the specific activities. The clones were verified by sequencing, and therefore it is also highly unlikely that the lower activity could be a result of a mutation during the PCR amplification. However, taking into account the error margins, the ratios of the activities on different substrates correspond reasonably well with our previous results for native GSMT.

Since both enzymes catalyze two successive steps, it was necessary to determine whether the second methylation step would distort the results of the initial velocity determinations of the first one. Using the apparent K_m – and V_{max} –values for the sarcosine step of GSMT, it was estimated that the methylation of the newly formed sarcosine had only a negligible effect on the initial velocity determinations with glycine as the substrate. This was also corroborated by HPLC analyses of reactions with 4 mM AdoMet and 250 mM glycine; 4 mM AdoMet and 9 mM glycine; and 1 mM AdoMet and 25 mM glycine as substrates. In these reactions the amounts of dimethylglycine formed were only 3 - 7% of the total products (sarcosine + dimethylglycine). The reaction times were such that the final product concentrations were considerably higher than in any of the initial velocity determinations.

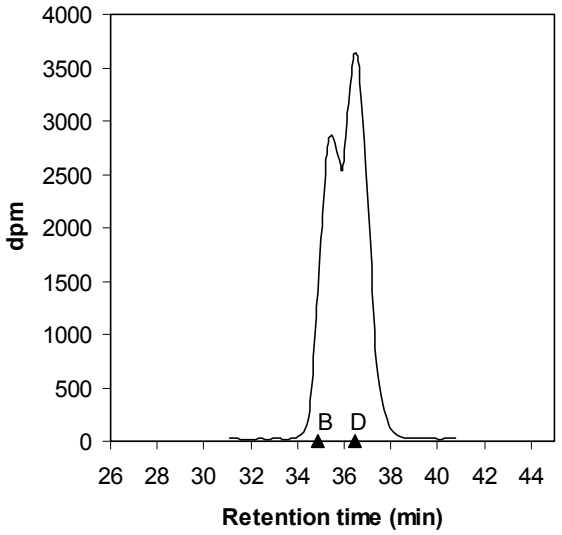

Figure 8. HPLC analysis of the radioactive methylation products with SDMT as the enzyme. Radioactive products were determined from fractions collected from the eluent. Reaction with 4 mM sarcosine and 4 mM *S*-adenosyl-L-[*methyl*-^{14}C] methionine as substrates. The retention times are show by triangles on the x-axis (D–dimethylglycine, B–betaine).

The situation was different in the case of the SDMT activity on sarcosine. As shown in the HPLC (Fig. 8) of the reaction mixture, the newly formed dimethylglycine is readily methylated to betaine with 4 mM sarcosine and 4 mM AdoMet as the substrates. Consequently, reliable values for the apparent K_m and V_{max} for the sarcosine activity with fixed AdoMet concentration could not be obtained with the methods used in this study, although the shortest possible reaction times were used. The apparent kinetic parameters of SDMT with sarcosine as the variable substrate presented in Table 6 are therefore only approximate estimates.

However, with 4 mM AdoMet and an excess of sarcosine (80 mM) as substrates the amount of betaine formed was negligible. Also with 0.11 mM and 1 mM AdoMet at this sarcosine concentration only barely detectable amounts of betaine were formed (data not shown). These results indicate that when saturating concentrations of sarcosine are used, the reaction catalyzed by SDMT stops at the dimethylglycine stage. It would seem plausible that under these conditions

sarcosine competitively inhibits the methylation of the newly formed dimethylglycine further to betaine. Because of this phenomenon it was possible to determine the apparent kinetic parameters for the AdoMet activity with saturating concentration of sarcosine as the co-substrate (Table 6).

4.11 Stress-relieving effects of the betaine pathway on *E. coli* (IV)

GSMT and SDMT of *E. halochloris* were expressed in *E. coli*, in order to investigate whether the osmotolerance of this microorganism could be improved by the introduction of the betaine pathway. The effects of betaine synthesis were also compared to the effects of exogenous addition of betaine.

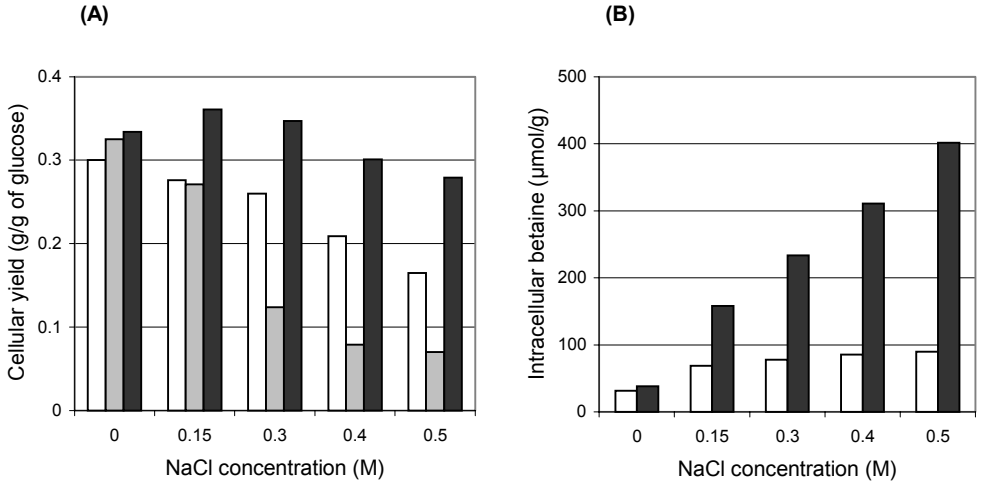

Figure 9. Influence of NaCl concentration on **(A)** cellular yield and **(B)** intracellular betaine concentration. *E. coli* was grown in mineral medium with 0.05 mM IPTG. Columns *white* pEhFU (genes for betaine synthesis); *gray* PQE-60 (cloning vector), *black* PQE-60 and 1 mM betaine.

As shown in Figure 9A, both endogenously synthesized and exogenously provided betaine stimulated the growth of *E. coli* in mineral medium with high NaCl-concentrations. However, the growth stimulation was clearly less effective for cells synthesizing betaine than for cells grown in the presence of betaine.

Figure 9B shows that the intracellular concentration of betaine correlates well with the concentration of NaCl in the medium, both with cell synthesizing and with cells taking up betaine. The level of betaine was much lower in cells synthesizing it than in cells accumulating it from the medium. At 0.5 M NaCl only 90 µmol betaine · (g dry weight)$^{-1}$ was accumulated in cells synthesizing betaine. The corresponding value in cells provided with exogenous betaine was 400 µmol betaine · (g dry weight)$^{-1}$.

5 DISCUSSION

5.1 The pathways of betaine synthesis in *A. halophila* and *E. halochloris*

Optimal growth of *A. halophila* and *E. halochloris* occurs at 15 - 20% (w/v) NaCl and at 14 - 27% NaCl, respectively (Gochnauer *et al.*, 1975; Imhoff and Trüper, 1977). Therefore these bacteria are usually classified as extreme halophiles. Both strains synthesize high intracellular concentrations of betaine. According to the results presented in this study, *A. halophila* contains up to 33% betaine of the cellular dry weight. *E. halochloris* has been reported to accumulate between 1.8 mol betaine · (kg cytoplasmic water)$^{-1}$ and 2.5 mol betaine · (kg cytoplasmic water)$^{-1}$ at a salinity range from 160 to 240 g NaCl · (kg water)$^{-1}$. The value 1.8 mol betaine · (kg cytoplasmic water)$^{-1}$ corresponds to 14% betaine of the cellular dry weight (Galinski and Herzog, 1990).

Betaine is clearly the major compatible solute of both strains. In addition to betaine *A. halophila* produces up to 9.7% trehalose of the cellular dry weight. Galinski and Herzog (1990) reported that besides 2.5 mol betaine · (kg cytoplasmic water)$^{-1}$ *E. halochloris* grown at 240 g · NaCl (kg water)$^{-1}$ contains 0.4 mol ectoine · (kg cytoplasmic water)$^{-1}$ and 0.1 mol trehalose · (kg cytoplasmic water)$^{-1}$.

On the basis of unpublished hybridization tests it has been suggested that *E. halochloris* does not have the genes for choline oxidation (Galinski and Trüper, 1994). This is in agreement with the data presented in this work suggesting that *E. halochloris* synthesizes betaine from glycine by methylation. The data also support the hypothesis that a similar pathway exists in *A. halophila*. In addition, evidence for the pathway in halophilic methanogens producing betaine *de novo* has been reported (Lai *et al.*, 1991; Lai *et al.*, 1999). Consequently, glycine methylation appears to be a common route for *de novo* synthesis of betaine in many different bacterial strains.

It was also shown in this study that unlike *E. halochloris*, *A. halophila* is capable of betaine production by choline oxidation. The data are compatible with choline being oxidized to betaine aldehyde by a choline oxidase and further to betaine by an NAD(P)$^{+}$-dependent betaine aldehyde dehydrogenase. Hence, *A. halophila* appears to be the only bacterium known to date that in addition to betaine synthesis by glycine methylation is also able to oxidize choline to betaine.

Since *E. halochloris* is an anaerobe, it is not surprising that it does not have oxygen-dependent activities of choline oxidation. However, *A. halophila* is a rare example of an aerobic heterotroph synthesizing betaine *de novo*. Choline is a ubiquitous compound in nature and the ability to use it for betaine synthesis is very common among aerobic bacteria. The transformation of choline to betaine is energetically less costly to *A. halophila* than betaine synthesis from simple carbon sources.

5.2 Betaine transport

As expected *A. halophila* was able to accumulate betaine from the medium in addition to choline uptake. Betaine transport has also been reported for *E. halochloris* (Peters *et al.*, 1992). A more interesting finding was that *A. halophila* both takes up and excretes betaine simultaneously. This type of mechanism, in which betaine is at the same time transported against a high concentration gradient and released back to the environment, has previously been demonstrated for osmoregulating *E. coli* cells (Lamark *et al.*, 1992). Hence, the betaine recycling mechanism appears to exist at both extremes of the halotolerance scale of bacteria, that is, in salt-sensitive strains such as *E. coli* as well as in extreme halophiles such as *A. halophila*. If compatible solutes are leaked through the membranes of bacteria producing them, as has been suggested by Oren (1999), the uptake mechanisms for salvaging them from the medium are truly important for the survival of these bacteria in saline habitats.

5.3 Enzymology of the glycine methylation pathway

The methyltransferases of *A. halophila* and *E. halochloris* are very homologous. However, despite the similarities the enzyme systems are different. Two separate methyltransferases are expressed in *E. halochloris.* Glycine sarcosine methyltransferase (GSMT) catalyzes the methylation steps from glycine to sarcosine (*N*-monomethylglycine) and from sarcosine to dimethylglycine. Sarcosine dimethylglycine methyltransferase (SDMT) catalyzes the steps from sarcosine to dimethylglycine and from dimethylglycine to betaine. AdoMet acts as the methyl group donor in the reactions. In *A. halophila* the whole reaction sequence appears to be catalyzed by a fusion protein, which possesses all three methyltransferase activities. Unfortunately, the functionality of the fusion protein could not be demonstrated. The activity on glycine was very unstable and a protein having only the *C*-terminal part of the fusion could be isolated from *A. halophila* cell extract. In addition, when expressed in *E. coli* the fusion protein formed inclusion bodies. It has been shown that the accumulation of compatible solutes into host cells can assist the correct folding of recombinant proteins that would otherwise form inclusion bodies (Barth *et al.*, 2000). Both *A. halophila* and *E. halochloris* contain high concentrations of compatible solutes. It is possible that the high intracellular compatible solute (betaine) concentration of *A. halophila* prevents the fusion protein from forming inclusion bodies. The phenomenon could also help to explain why the specific activity of the recombinantly produced *E. halochloris* GSMT was significantly lower than the activity of the native protein.

For characterization, the GSMT and SDMT of *E. halochloris* were expressed in *E. coli* and purified. Glycine methyltransferases have been isolated previously from different mammalian origins (Ogawa *et al.*, 1993). The *N*-terminal part of *A. halophila* fusion protein and the GSMT of *E. halochloris* were compared to glycine methyltransferases using the program BLAST (Altschul *et al.*, 1997). The comparison revealed a high degree of homology between these enzymes. The *A. halophila* GSMT has closest homology with human glycine methyltransferase

(36%, 51% similarity) and the *E. halochloris* GSMT with rat glycine methyltransferase (37%, 52% similarity). In addition, as the results with the recombinant *E. halochloris* enzymes show, many enzymatic properties are alike in mammalian glycine methyltransferases, GSMT, and also SDMT. These enzymes are very susceptible to inhibition by the reaction product AdoHcy and none of them appear to require cofactors (Heady and Kerr, 1973). Furthermore, all three methyltransferases are highly substrate-specific. No evidence for methylation of amino acids other than glycine by glycine methyltransferases or GSMT has been found.

However, the glycine methyltransferases do not catalyze the methylation of sarcosine (Heady and Kerr, 1973), whereas GSMT and SDMT methylate it further to dimethylglycine. In fact, this is the first report of an enzyme having sarcosine methyltransferase activity. An additional difference is that glycine methyltransferases have been reported to display sigmoidal kinetics with AdoMet as the variable substrate (Ogawa *et al.*, 1993). The sigmoidal rate behavior could not be detected for GSMT or SDMT in any of the initial velocity patterns.

The apparent K_m value of 18 mM determined for glycine was significantly greater than the ones determined for the other substrates, and also greater than those reported for the mammalian glycine methyltransferases (ranging from 0.13 mM for rat to 11 mM for pig) (Ogawa and Fujioka, 1982; Ogawa *et al.*, 1993). However, the apparent V_{max} of the glycine methylation step is six times greater than the value for the rat glycine methyltransferase (Ogawa and Fujioka, 1982). Furthermore the apparent K_m for AdoMet of the glycine step (0.42 mM) is higher than of the other steps and higher than the $S_{0.5}$ values (the substrate concentration at which the reaction rate is half of its maximal value) of the mammalian glycine methyltransferases (around 0.3 mM for human, rabbit and pig; 0.05 mM for rat) (Ogawa *et al.*, 1993).

A possible application of GSMT and SDMT is in improving the stress tolerance of plants. Betaine is a known protectant of cells and enzymes against various stresses (see Introduction). Comparing the kinetic parameters presented in this work to the levels of glycine and AdoMet in plant cells would therefore be of interest. It should however be noted that the working of the glycine methylation system is very complex and that care should be taken when parameters determined *in vitro* are applied to intracellular milieu. Betaine and salts can probably affect the activities of the methyltransferases significantly. In addition, the strong inhibiting effect of AdoHcy on the methyltransferase system cannot be evaluated before the determination of the K_i values.

The glycine concentrations in plants vary widely depending on plant species, cell organelle and metabolic activity. The apparent K_m of GSMT for glycine clearly exceeds the intracellular glycine concentrations (0.41 - 1.29 mM) reported for salinized tobacco cells (Binzel *et al.*, 1987) and the concentration of 2 mM in the cytosol and chloroplasts of illuminated spinach leaves (Bourguignon *et al.*, 1999). However, the apparent K_m for glycine of GSMT is lower than the concentration of 25 mM in the cytosol and only slightly over two times higher than 8 mM of glycine in the stroma of illuminated barley leaves. Consequently, the performance of GSMT would differ considerably in different plants and cell organelles.

Studies in which decreasing the AdoMet pool has been attempted, have shown that plants have a surprising capability of maintaining the intracellular AdoMet concentrations sufficient for growth despite adverse conditions (Good *et al.*, 1994; Ravanel *et al.*, 1998). This suggests that the regulatory mechanisms leading to AdoMet synthesis are highly flexible, which is in accordance with the fact that AdoMet is required for a wide variety of methylation reactions vital to the cell (Ravanel *et al.*, 1998). Such flexibility would be a benefit when part of the AdoMet flux is directed to betaine synthesis.

The concentration of AdoMet has been reported to be 14-30 μM in *Lemna paucicostata*, 40 μM in germinating pea seeds and 10-20 μM in turnip (Giovanelli *et al.*, 1980). The results presented in Table 6 suggest that in plants both GSMT and SDMT would be operating well below their K_m –values for AdoMet. Yet again, the situation is complicated by the compartmentalization of the plant cell. The available data suggest that AdoMet is synthesized in the plant cytosol and presumably transported from there to other cell compartments (Ravanel *et al.*, 1998). Therefore its concentration might vary considerably within the cell. If the AdoMet pool would limit the GSMT and SDMT catalyzed betaine synthesis in transgenic plants, its synthesis could most probably be increased by expressing one of the many plant *S*-adenosylmethionine (AdoMet) synthetases that have been cloned and sequenced (Peleman *et al.*, 1989; Schröder *et al.*, 1997).

5.4 Applications of the glycine methylation pathway

Betaine is being sold in thousands of tons annually. Since it is a bulk product, it would seem unlikely that it could be produced competitively by such slowly growing organisms as the extremely halophilic bacteria. Furthermore, these bacteria require vast concentrations of salt, which would be problematic in industrial scale. In this study, the betaine contents of *E. coli* cells synthesizing it recombinantly were extremely low. Therefore, even if the production of betaine by recombinant microbes would in theory be economical, it would most likely require extensive metabolic engineering in practice.

However, the glycine methylation pathway has many other possible applications. Betaine is widely used as an additive in the feed industry. Thus transgenic plants producing high concentrations of betaine would have a better nutritional value, and could therefore be used directly in feed without betaine supplementation.

The stress-relieving potential of betaine is not limited to plants. Many industrial microbes suffer from environmental stress under process conditions. For example, high substrate concentrations used in fermentation media can cause osmotic stress. As shown in this study with *E. coli*, the glycine methylation pathway has potential in increasing the osmotolerance of heterologous organisms. Hence, it could also be used in improving the stress-tolerance of commercially important microbes in industry.

However, many microorganisms are able to use exogenously added betaine as a compatible solute. Betaine is also commercially sold as an osmoprotective additive for use in fermentation media. Therefore the most attractive targets for the

introduction of the betaine pathway would be industrial microbes that are not able to accumulate betaine from the environment. One such microorganism is *Saccharomyces cerevisiae*. In high-gravity fermentations, in which very high concentrations of sugar are used, betaine has a small protective effect on *S. cerevisiae* (Thomas *et al.*, 1994). *S. cerevisiae* has been shown to produce small amounts of betaine (Dulaney *et al.*, 1968), but it does not seem to be able to accumulate it to any significant extent when osmotically stressed (Antti Nyyssölä, unpublished results).

The most interesting possibility is, however, the application of GSMT and SDMT in the genetic engineering of stress-tolerant plants. Drought, salinity, and low temperatures are among the most important environmental factors limiting plant productivity (Boyer, 1982). The stress-relieving effects of betaine on plants, and its stabilizing effects on plant macromolecules have been widely reported (see Introduction). Many important crops such as rice, potato, and tomato do not synthesize betaine and therefore introducing betaine synthesis into these plants has been a well-established target for metabolic engineering (McCue and Hanson, 1990). Hitherto, the strategy has been utilization of the choline oxidation pathway. Enzymes catalyzing the oxidation of choline to betaine have been introduced into many plants (Sakamoto *et al.*, 1988; Lilius *et al.*, 1996; Hayashi *et al.*, 1997; Alia *et al.*, 1998; Nuccio *et al.*, 1998; Huang *et al.*, 2000). However, the concentrations of betaine in the transgenic plants have been far from the levels found in natural producers, although amelioration of stress tolerance has been found in some cases.

It is believed that the reason for the low betaine contents of the trasgenic plants is the limited supply of choline. In tobacco the choline synthesis is constrained at the first methylation step of phosphoethanolamine and the choline itself is directed almost exclusively to phosphatidylcholine (Nuccio *et al.*, 1998). It has been suggested that the reactions of choline metabolism in non-betaine producing plants form a rigid metabolic network, which makes it difficult to direct choline to betaine synthesis (Nuccio *et al.*, 1999).

In higher plants the ethanolamine for choline synthesis is produced from glycine *via* serine (Stewart and Larher, 1988). Bypassing the glycine-choline pathway by the introduction of GSMT and SDMT would make it possible to directly engineer all the three methylation reactions of betaine synthesis and conceivably avoid the difficulties associated with the choline oxidation pathway. However, as shown in this study the heterologous expression of the methyltransferases can present difficulties. The specific activity of GSMT produced recombinantly was considerably lower than that of the native enzyme from *E. halochloris.*

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The data presented in this thesis suggest that the *de novo* synthesis of betaine proceeds *via* the threefold methylation of glycine in the extremely halophilic bacteria *Actinopolyspora halophila* and *Ectothiorhodospira halochloris*. The enzymology of betaine synthesis is described for both strains. In addition, the genes for the pathway were cloned and sequenced. The methyltransferases catalyzing the pathway in *E. halochloris* were produced recombinantly in *E. coli* and some of their enzymatic properties were characterized. In addition to the glycine methylation pathway, *A. halophila* was shown to produce betaine from exogenously provided choline.

Introduction of the glycine methylation pathway into *E. coli* resulted in increased osmotolerance. Hence, besides furthering the knowledge on halophilism in general, the results presented in this thesis could have use in the genetic engineering of stress-tolerant crops and industrial microbes. It would be interesting to compare the efficiency of the glycine methylation pathway with the choline oxidation pathway in improving the stress tolerance of plants. However, in order to accomplish this a great deal of further research would be necessary. It is currently not known whether the methyltransferases can be expressed in active form in plants.

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