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Formation and breakdown of glycine betaine and trimethylamine in hypersaline environments

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Abstract

Glycine betaine is accumulated as a compatible solute in many photosynthetic and non-photosynthetic bacteria – the last being unable to synthesize the compound – and thus large pools of betaine can be expected to be present in hypersaline environments. A variety of aerobic and anaerobic microorganisms degrade betaine to among other products trimethylamine and methylamine, in a number of different pathways. Curiously, very few of these betaine breakdown processes have yet been identified in hypersaline environments. Trimethylamine can also be formed by bacterial reduction of trimethylamine N-oxide (also by extremely halophilic archaeobacteria). Degradation of trimethylamine in hypersaline environments by halophilic methanogenic bacteria is relatively well documented, and leads to the formation of methane, carbon dioxide and ammonia.

> Die ganze Salzmasse roch, besonders an frischen Bruchstellen, stark nach Trimethylamin. An ein präfermiertes Vorhandensein dieser Base im Salz ist nicht zu denken. Die Annahme liegt näher, dass sich dieselbe aus Cholin bildet. (The whole salt mass smelled strongly of trimethylamine, in particular at new fracture planes. It cannot be imagined that the trimethylamine was originally present in the salt. It seems more probable that it was formed from choline) (Schweinfurth & Lewin 1898).

The above sentences were written almost a hundred years ago in a paper on the properties of the alkaline salt lakes of Wadi Natrun (Egypt). They clearly demonstrate that the importance of biological processes in hypersaline environments in which methylated amines are involved has been recognized already for a long time.

During the last decades the reasons for the importance of methylated amines in the microbiology of saline environments have become clear: it was discovered that one of the major organic solutes accumulated by halophilic and halotolerant bacteria is glycine betaine (abbreviated as betaine in the following). It can thus easily be imagined that excess betaine is degraded with the release of trimethylamine, which in its turn is a carbon and energy source for methanogenic bacteria.

In view of the above it is noteworthy that, though quite a lot a knowledge has accumulated on the mode of formation of betaine, its degradation, and the fate of the methylated amines, the existing information relating to those hypersaline environments in which betaine can be expected to be a readily available substrate is extremely scarce.

In this review I attempt to summarize our understanding of the microbiological processes in which

betaine and methylated amines are involved and their relative importance in nature, with special emphasis on hypersaline environments.

Occurrence and formation of betaine in saline environments

Betaine is one of the most widespread osmotic solutes in microorganisms, and it can be stated that a large number of really halophilic eubacteria depend primarily on glycine betaine as compatible solute, enabling them to withstand the high osmotic pressure of their surrounding medium (Trüper & Galinski 1989). However, of the many halophilic eubacteria containing betaine, only two groups seem to be able of de novo synthesis of the compound: the cyanobacteria and a few anaerobic photosynthetic bacteria (the genus *Ectothiorhodospira).*

Among the cyanobacteria a wide variety of osmotic solutes has been identified, but those strains that grow at the highest salt concentrations (typically above 10%) contain betaine. Betaine has been found in different genera, such as *Calothrix, Dactylococcopsis, Gloeocapsa,* the *Lyngbia – Phormidium – Plectonema* group, *Synechococcus* (Reed et al. 1986), *Synechocystis* (Mohammad et al. 1983), *Aphanothece* (Moore et al. 1987), and *Spirulina* (Gabbay-Azaria et al. 1988). Intracellular betaine concentrations may be as high as 2 M (Mohammad et al. 1983). Two *Calothrix* strains were found to form another quaternary amine compound: glutamate betaine (Mackay et al. 1984). Also the halophilic anoxygenic phototrophic bacteria may accumulate betaine (Oren et al. 1989; Trüper & Galinski 1989), in *Ectothiorhodospira halochloris* concentrations of 1.6 M and higher were reported in cells grown on CO_2 and acetate as sole carbon sources (Galinski & Trüper 1982). Enzymatic studies on the mode of betaine formation in bacteria are scarce; in *Arthrobacter globiformis* betaine is produced via choline and betaine aldehyde (Ikuta et al. 1977), a pathway similar to that known from higher plants (Delwiche & Bregoff 1958). However, the assumption that the same pathway is used by all bacteria is not necessarily justified: *Ectothiorhodospira* probably produces betaine by transmethylation of glycine with S-adenosylmethionine (I. Tschichholz and H.G. Trüper, personal communication).

Betaine is also present in a wide variety of chemoorganotrophic halophilic bacteria, and even in non-halophiles when subjected to salt stress, all organisms unable of de novo synthesis of the compound. Concentrations of between 0.5 and 1.2 M have been measured inside a number of moderately halophilic eubacteria grown at 20% salt: *Vibrio costicola, Alcaligenes* sp., *Alteromonas, Pseudomonas, Acinetobacter, Chromobacterium marismortui, Micrococcus halobius,* and *Micrococcus* sp. (Imhoff & Rodriguez-Valera 1984). Betaine is also accumulated by members of the *Halomonadaceae* when grown in the presence of yeast extract (Wohlfarth et al. 1990). In the moderately halophilic bacterium Ba1 (isolated from the Dead Sea, and probably also belonging to the genus *Halomonas*) the addition of betaine protects the respiratory system against damage at high salt concentrations (Rafaeli-Eshkol & Avi-Dor 1968). In non-halophiles such as *Escherichia coli, Klebsiella,* other members of the *Enterobacteriaceae,* and other bacterial groups, the addition of betaine to the medium not only increases growth rates at any salt concentration, but also extends the growth range towards higher salt concentrations (Bernard et al. 1986; Le Rudulier & Bouillard 1983). The addition of betaine to the plating medium restores the ability of osmotically stressed *E. coli* cells to form colonies (Roth et al. 1988). A number of halophilic or halotolerant methanogenic bacteria (*Methanogenium cariaci, Methanogenium anulus, Methanohalophilus zhilinae, Methanohalophilus mahii* and *Methanococcus voltae*) were also reported to accumulate betaine from their medium (Robertson et al. 1990). Curiously, aerobic halophilic archaeobacteria (the *Halobacterium* group) were reported to contain between 1 and 20 mM betaine (taken up from the medium), which is membrane-associated, and fulfills a yet unknown role, probably not connected with osmoregulation (Nicolaus et al. 1989).

Little is known to what extent betaine and related compounds are available to organisms unable to produce it in their natural habitats. In a cyanobac-

terial mat at a salinity of 9–13% (Western pond - Virgin Islands), dominated by *Spirulina,* betaine was found to make up 20% of the total nitrogen in the top 1 cm of the sediment (King 1988). Betaine may leak from unhealthy cells of cyanobacteria or photosynthetic bacteria that produce it. Moreover, as the betaine concentration in the cell is a function of the salinity of the medium, a sudden drop in salinity may be accompanied by the excretion of betaine into the surrounding medium. Such an excretion of betaine as a reaction to a salinity decrease was shown in *Ectothiorhodospira;* this excretion is an overshoot reaction and is corrected by immediate partial re-uptake (Tschichholz & Trüper 1990). A similar phenomenon was reported in the halophilic cyanobacteria *Aphanothece halophytica, Dactylococcopsis* and *Synechocystis* (Moore et al. 1987).

Degradation of betaine

Much more is known on the breakdown of betaine under anaerobic than under aerobic conditions. Intuitively one may expect that aerobic breakdown will release trimethylamine, the remaining carbon skeleton becoming available for assimilatory purposes. The ability to decompose betaine is widespread in microorganisms, but little information seems to exist on the pathway of degradation. *Rhizobium meliloti* uses betaine for growth in a minimal medium (at low salinity only), and when methyl [^{14}C]-betaine was added, up to 83% of the radioactivity was found in the ethanol-insoluble fraction, suggesting that also the methyl groups are incorporated into cell material (Le Rudulier & Bernard 1986). The products of betaine degradation in *Rhizobium* were identified as dimethylglycine, sarcosine, glycine and serine (Bernard et al. 1986). At high salinities betaine catabolism is blocked in this organism, betaine being conserved as an osmoprotectant (Le Rudulier & Bernard 1986).

Anaerobically betaine can be degraded in a number of ways: by fermentation, by reduction - using external electron donors such as certain amino acids (the Stickland reaction) or other compounds, or by oxidation with sulfate or elemental sulfur as electron acceptors.

Fermentative pathways ofter yield *N,N*-dimethylglycine as one of the degradation products. Thus, the obligately anaerobic *Eubacterium limosum* ferments betaine to *N, N*-dimethylglycine, acetate and butyrate, the last two products being made from the methyl groups split off from the nitrogen atom of betaine (Müller et al. 1981). The marine isolate *Sporomusa* degrades betaine to trimethylamine, acetate, CO_2 and *N,N*-dimethylglycine:

1.5 betaine + $3H_2O$ → 1.5 *N,N*-dimethylglycine + 1.5 CO_2 + 9 [H]
0.5 betaine + $3H_2O$ → 0.5 acetate + 0.5 NH_3 + 1.5 CO_2 + 7 [H]
8 betaine + 16 [H] → 8 acetate + 8 trimethylamine

Part of the *N-N*-dimethylglycine is later consumed, the reducing power required being derived from oxidation of methyl groups (Möller et al. 1984);

2 dimethylglycine → 1 betaine + 1 sarcosine
8 dimethylglycine + $16H_2O$ → 8 sarcosine + $8CO_2$ + 48 [H]
1 betaine + 2 [H] → 1 acetate + 1 trimethylamine
9 sarcosine + 18 [H] → 9 acetate + 9 methylamine
$7CO_2$ + 28 [H] → 3.5 acetate

In the sulfur-reducing bacterium *Desulfuromonas* (able to oxidize betaine with sulfur as electron acceptor, see below) a reductive cleavage of betaine to trimethylamine and acetate occurs in the absence of elemental sulfur, part of the acetate being oxidized to CO_2 to provide the reducing equivalents required for the initial cleavage (Heijthuijsen & Hansen 1989a).

Other anaerobes perform a reductive cleavage of betaine, using other compounds as the electron donors. Thus, *Clostridium sporogenes* performs a Stickland reaction with betaine as electron acceptor (with the formation of acetate and trimethylamine), alanine, valine, leucine and isoleucine serving as potential electron donors (Naumann et al. 1983); alternatively externally supplied hydrogen may donate the necessary electrons. Likewise, *Eubacterium acidaminophilum* cleaves betaine to acetate and trimethylamine, with either other amino acids as electron donors, or with hydrogen, for-

mate, or malate (Hormann & Andreesen 1989; Zindel et al. 1988).

An alternative strategy for anaerobic betaine breakdown is oxidation with sulfate or elemental sulfur as electron acceptor. From an anaerobic enrichment on betaine and sulfate, using marine sediment as inoculum, *Desulfobacterium* strains were isolated, forming *N,N*-dimethylglycine and sulfide in a 1: 1 ratio, one methyl group of the betaine being oxidized to CO_2. The same isolates also grow on a lactate/sulfate medium (Heijthuijsen & Hansen 1989b). *Desulfobacterium autotrophicum* degraded betaine according to:

$$\text{betaine} + 0.75\,SO_4^{2-} \rightarrow \textit{N,N}\text{-dimethylglycine}^- + CO_2 + 0.75\,HS^- + 0.25\,H^+ + H_2O$$

(demethylation of betaine, followed by oxidation of the methyl group to CO_2, the reducing equivalents being used to reduce sulfate) (Heijthuijsen & Hansen 1989b).

Several *Desulfuromonas* strains were found to be able to use betaine as energy source, which is oxidized to CO_2 and trimethylamine, elemental sulfur serving as the electron acceptor (Heijthuijsen & Hansen 1989a).

The relative importance of above processes in nature is not well known. Experiments performed with marine sediments (Lowes Cove - Maine) suggested that most betaine was degraded in a 1: 1 stoichiometry to trimethylamine and acetate; betaine metabolism via the Stickland reaction or by demethylation such as in *Eubacterium limosum* did not account for the major part of betaine degradation in these sediments, a conclusion based on the low levels of butyrate formation, and the lack of a measurable dimethylamine pool (King 1984). However, the last argument may not be a valid one, as – as discussed above – the metabolism of *N,N*-dimethylglycine may yield trimethylamine and monomethylamine, rather than dimethylamine (Möller et al. 1984). It remains to be established if betaine breakdown to *N,N*-dimethylglycine by *Desulfobacterium* is quantitatively important in sediments.

Breakdown of betaine under hypersaline conditions is poorly documented. An obligately anaerobic, halophilic, betaine-degrading gram-negative eubacterium was recently isolated from the Arabat split (East Crimea, USSR) from a hypersaline cyanobacterial mat dominated by *Microcoleus chtonoplastes*. The organism, named *Acetohalobium arabaticum* (Zhilina & Zavarzin 1990a; Zhilina & Zavarzin 1990b) grows at salinities from 10 to 25% (optimally at 15–18% NaCl and 38–40° C). The isolate has a versatile metabolism: it can grow as a chemolithotrophic homoacetogen on H_2+ CO_2, as a methylotroph on trimethylamine (see below), and as a chemoorganotroph, using betaine, lactate, pyruvate or histidine. During growth on betaine acetate is formed as the major product, with minor amounts of methylamines only as methylamines are further degraded to acetate and ammonia.

Another halophilic anaerobe able to form trimethylamine from betaine is *Sporohalobacter lortetii* (Oren, unpublished results). This organism is a spore-forming gram-negative obligately anaerobic bacterium which produces gas vacuoles attached to the endospores; 16S rRNA sequence data suggest a possible relationship with the genus *Sporomusa* (Oren et al. 1987). No degradation of betaine could be demonstrated in other members of the *Haloanaerobiacae* (Oren, unpublished results).

Other sources of methylated amines

Betaine is not the only possible source of trimethylamine in nature. Another, possible major source is the breakdown of choline, a process performed by several bacterial groups, among others sulfate reducers.

Another precursor for trimethylamine may be trimethylamine N-oxide. This compound may have been formed by oxidation of trimethylamine, e.g. by methylotrophic bacteria (Barrett & Kwan 1985), but is also present in high concentrations in a variety of marine fish species, where it serves as an osmotic solute: it was found in concentrations of up to 2–4% of the dry weight in teleost fishes, and 3–7% in elasmobranchs (Yancey et al. 1982).

A wide variety of aerobic and facultatively an-

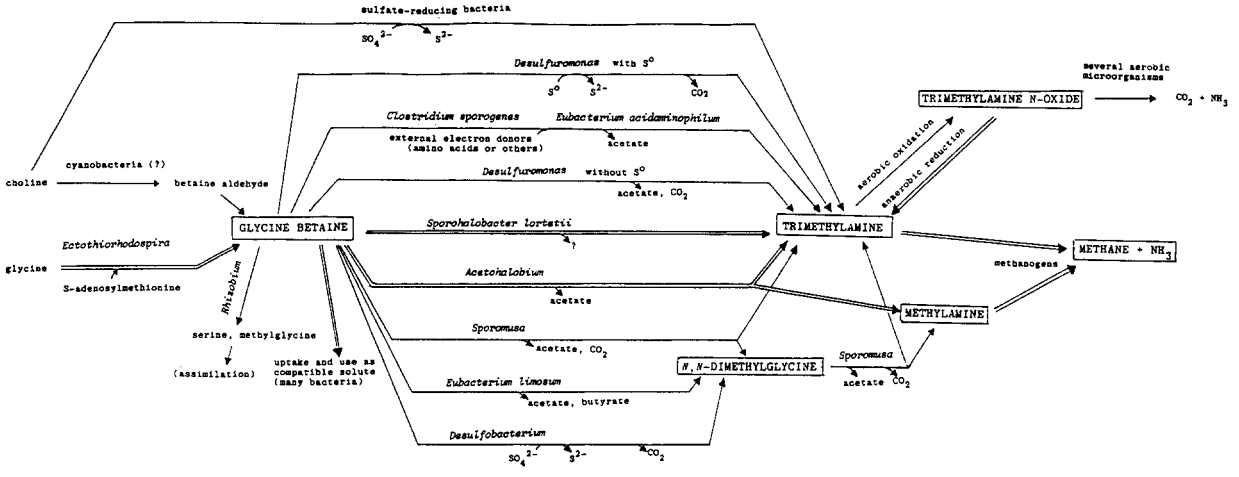

Fig. 1. Microbial transformations of betaine and trimethylamine (for details see text). Those processes that have been identified in hypersaline (> 10% salt) environments and/or in laboratory cultures of bacteria growing at such high salt concentrations are marked with double arrows.

aerobic bacteria are able to use trimethylamine N-oxide as terminal electron acceptor in their respiration (Madigan et al. 1980; Strøm et al. 1979). Lately the reduction of trimethylamine N-oxide was also demonstrated in the extremely halophilic archaeobacteria of the genera *Halobacterium, Haloferax* and *Haloarcula,* bacteria that grow at salinities of 15% and higher, up to NaCl saturation (Oren & Trüper 1990). From an ecological point of view this process may be of some importance, as halobacteria are often found growing on dried or salted fish.

Breakdown of methylated amines

Though, as shown before, trimethylamine, and possibly other methylamines as well, may be important methanogenic precursors, especially in anaerobic hypersaline environments, there is a general paucity of information about the abundance of these compounds in hypersaline environments (Oremland and King 1989). In Mono Lake (California) (salinity 8.8%) the estimated in situ trimethylamine pool in the upper sediment layers was 1 μM (Oremland & King 1989). In Westend pond - Virgin Islands (9–13% salt) the laminated sediment, on which a *Spirulina* mat is present, and oxygen penetrates down to 2–4 mm, the trimethylamine concentration on top of the sediment was found to be around 6 μM, and decreased to 1 μM at a depth of 1 cm (King 1988).

Under aerobic conditions trimethylamine and other methylated amines can be completely oxidized (for a review see Large & Green 1984). The final fate of trimethylamine under anaerobic conditions in nature is almost always a breakdown into methane, CO_2 and ammonia (King 1984), and a variety of methanogenic bacteria are able to perform the process (Hippe et al. 1979; Oremland 1988). In freshwater or low salinity sediments methane formation from trimethylamine is probably of minor quantitative importance, most of the methane formed being derived from acetate or hydrogen/CO_2 ('competitive' substrates, also used as energy sources by sulfate-reducing bacteria). In hypersaline anaerobic sediments most or all methane is derived from 'non-competitive' substrates such as methylamines (Oremland 1988; Oren 1988). The highest salt concentration in which methanogenesis from hydrogen + CO_2 has been reported is 8.8% (Mono Lake, California) (Oremland & King 1989): most of methane there is derived from CO_2, but up to 33% may come from trimethylamine (probably an overestimation, as the concentration of radioactive tracer added (15 μM) greatly exceeded the in

situ pool concentration (1 μM). All methanogenic isolates known today to grow at salinities exceeding 10% use methylated amines and methanol as energy source, being unable to use acetate or hydrogen/CO_2 (Giani et al. 1984; Mathrani & Boone 1985; Oren 1988, Oren 1989; Paterek & Smith 1985; Zhilina 1986).

As mentioned above, the betaine-degrading halophilic anaerobe *Acetohalobium arabaticum* can also grown on trimethylamine as sole carbon and energy source with the production of acetate, with minor amounts of dimethylamine and monomethylamine (Zhilina & Zavarzin 1990b). The organism seems ineffective in competing against methanogens for trimethylamine, thus betaine breakdown by this organism will proceed to the formation of acetate and trimethylamine, the trimethylamine being utilized by methanogens. Thus, a coculture of *Acetohalobium arabaticum* and *Methanohalophilus* sp. (which is unable to use betaine) converts betaine quantitatively to acetate and methane (Zhilina & Zavarzin 1990b).

It has been suggested that in marine sediments a minor part of the trimethylamine may be broken down by sulfate-reducing bacteria (King 1984); no information is yet available on the nature of the organisms performing this process.

Concluding remarks

The different microbial processes discussed above, and summarized in Fig. 1, form the basis for the transformations of betaine and trimethylamine in nature. All above processes have been identified primarily in laboratory cultures, and relatively little information exists on their quantitative role in nature.

An important conclusion that can be drawn from the information presented is the paucity of information on the fate of the compounds discussed in hypersaline environments (defined here as salt concentrations exceeding 10%). While a compound such as betaine is characteristically present in high concentrations in halophilic and halotolerant microorganisms, most of the organisms known to degrade betaine in any of the pathways discussed are unable to grow at these high salt concentrations (though many of the organisms discussed are of marine origin). The recent discovery of anaerobic halophilic betaine degrading bacteria such as *Acetohalobium arabaticum* may enable us to understand the nature of the process in nature; however, no data are yet available on the quantitative importance of these organisms. Halobacteria do not grow on betaine - or at least do not produce trimethylamine from it (Oren, unpublished results). In Fig. 1 those processes that have been identified in hypersaline environments and/or in cultures of organisms growing above 10% salt are indicated, and it becomes clear that especially in those environments in which betaine is probably readily available, the relevant information on its breakdown and further fate is still lacking.

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Note added in proof

Recently a new halophilic *Clostridium* was described (*Clostridium halophilum*, Fendrich et al., Arch. Microbiol. 154: 127–132, 1990), growing optimally at 6% NaCl and degrading betaine in the Stickland reaction.