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Betaine Fermentation and Oxidation by Marine *Desulfuromonas* Strains

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Two bacterial strains were dominant in anaerobic enrichment cultures with betaine (*N*,*N*,*N*-trimethylglycine) as a substrate and intertidal mud as an inoculum. One was a coccoid bacterium which was a trimethylamine (TMA)-fermenting methanogen similar to *Methanococcoides methylutens*. The other strain, a rod-shaped, gram-negative, motile bacterium, fermented betaine. On the basis of its ability to oxidize acetate and ethanol to CO_2 with sulfur as an electron acceptor, its inability to reduce sulfate and sulfite, its morphology, the presence of *c*-type cytochromes, and other characteristics, the isolated strain PM1 was identified as *Desulfuromonas acetoxidans*. Although only malate and fumarate were known as substrates for fermentative growth of this species, the type strain (DSM 684) also fermented betaine. Strain PM1 grew with a doubling time of 9.5 h at 30°C on betaine and produced approximately 1 mol of TMA per mol of betaine, 0.75 mol of acetate, and presumably CO_2 as fermentation products but only in the presence of selenite (100 nM). In this fermentation, betaine is probably reductively cleaved to TMA and acetate, and part of the acetate is then oxidized to CO_2 to provide the reducing equivalents for the initial cleavage reaction. In the presence of sulfur, betaine was converted to TMA and presumably CO_2 with the formation of sulfide; then, only traces of acetate were produced.

Betaine (*N*,*N*,*N*-trimethylglycine) plays an important role as a compatible solute in certain plants and animals and many eubacteria (8, 12, 13, 21, 25), where its concentration can attain values as high as 1.2 M. When betaine is released into the environment either by death of the organisms or excretion, it becomes available as a substrate for microbial growth.

Several bacteria metabolize betaine aerobically (e.g., see reference 11). Most likely, betaine is first oxidized to glycine with *N*,*N*-dimethylglycine and sarcosine as intermediates. In *Arthrobacter* sp. strain P1, two of the methyl groups are metabolized via formaldehyde by way of the ribulose-monophosphate cycle and glycine is converted to serine with the third formaldehyde (14).

Three types of anaerobic betaine-supported growth have been reported. *Eubacterium limosum* ferments betaine as shown in equation 1:

$$7 \text{ betaine} + 2CO_2 \rightarrow 7\ N,N\text{-dimethylglycine} + 1.5 \text{ acetate} + 1.5 \text{ butyrate} \quad (1)$$

In this fermentation, the C_2 skeleton of betaine remains intact and the fatty acids produced are derived from CO_2 and one of the methyl groups of betaine (16). Betaine can also undergo a reductive cleavage to yield trimethylamine (TMA) and acetate. This reaction was discovered in *Clostridium sporogenes* (17). In this organism, betaine functions as an electron acceptor in a Stickland-type reaction with, for example, alanine as an electron donor:

$$\text{alanine} + 2 \text{ betaine} + 2H_2O \rightarrow 3 \text{ acetate} + CO_2 + NH_3 + 2 \text{ TMA} \quad (2)$$

Certain enrichments with betaine as a substrate led to the isolation of strains of *Sporomusa*, a new type of spore-forming bacterium. *Sporomusa ovata* ferments betaine to acetate, TMA, dimethylglycine, NH_3, and CO_2 (15). It can reductively cleave betaine and sarcosine, and it obtains the reducing equivalents for these reactions in the conversion of methyl groups to CO_2. The C_2 skeleton of the betaine molecule remains intact.

Here, we report on a novel type of anaerobic betaine dissimilation which was discovered in *Desulfuromonas* strains. In this fermentation, the *N*,*N*,*N*-trimethyl moiety of the molecule is liberated as TMA and the C_2 unit (acetate) is partly oxidized to CO_2 to provide the reducing equivalents for the reduction of betaine. Acetate is formed as a fermentation product.

MATERIALS AND METHODS

Organisms. Strain PM1 was isolated from a sample of black intertidal mud from sediments (upper 3 cm) of the Dutch Wadden Sea at Peasens Moddergat, The Netherlands. *Desulfuromonas acetoxidans* (DSM 864) was obtained from the German Collection of Microorganisms, Braunschweig, Federal Republic of Germany.

Medium and cultivation. The bacteria were cultivated in a basal medium which contained the following components (per 950 ml of demineralized water): NaCl, 25.0 g; $MgCl_2 \cdot 6H_2O$, 3.0 g; KCl, 0.3 g; NH_4Cl, 0.5 g; $CaCl_2$, 0.08 g; resazurin, 0.5 mg; trace element solution SL9 (23), 1 ml; and a solution containing sodium selenite (0.1 mM) and sodium tungstate (0.1 mM), 1 ml. After being autoclaved, the basal medium was supplemented with 1 ml of a vitamin solution (6), 1 ml of a yeast extract solution (10% [wt/vol]), 5 ml of a phosphate buffer (KH_2PO_4, 0.63 M; K_2HPO_4, 0.37 M), 50 ml of sodium bicarbonate solution (1 M), and 2 ml of sodium sulfide solution (0.5 M). Substrates were added to the culture bottles from sterile neutralized stock solutions (1 M) to give the desired concentrations. The pH of the complete medium was adjusted to 7.0 with 2 N HCl. All incubations were at 30°C.

Enrichment and isolation procedures. A 4-ml mud sample was added to 40 ml of sterile medium with betaine (20 mM) but without yeast extract and incubated in bottles (100 ml) sealed with butyl rubber stoppers and crimp caps, with a gas

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atmosphere consisting of N_2 and CO_2 (80:20, vol%). Turbidity developed usually within 1 to 2 weeks. After several transfers into fresh medium, samples were taken for the isolation procedures. Two techniques were used: the roll tube method described by Hungate (7) and a direct isolation on agar plates. The agar medium used for both methods consisted of the complete betaine medium with 2% agar (Noble; Difco Laboratories, Detroit, Mich.). The agar plates were prepared in an anaerobic cabinet, and after being streaked the plates were placed in an anaerobic jar. The jar was gassed with a mixture of N_2 and CO_2 (80:20, vol%) to remove the cabinet atmosphere, which contained relatively high amounts of H_2 (10 vol%), and approximately 0.1 vol% H_2S gas was added as a potential sulfur source and to keep the agar plates reduced. For nutritional studies, crimp-cap sealed bottles (100 ml) were used which contained 40 ml of medium; when no abundant gas production was expected, completely filled screw-cap bottles (40 ml) were used. Sulfur was added to the medium in excess from an autoclaved suspension of sulfur flower.

Growth measurements. To determine the time course of substrate consumption and product formation with betaine or with betaine and sulfur, *D. acetoxidans* PM4 was grown in 600-ml bottles containing 500 ml of the medium with 0.02% yeast extract and either 40 mM betaine (experiment carried out in triplicate) or 3 mM betaine and sulfur (experiment carried out in duplicate) with a gas atmosphere of N_2-CO_2 (80:20, vol%). Samples (2 ml) were withdrawn with a syringe, and the amounts of betaine, *N,N*-dimethylglycine, sarcosine, methylamines, acetate, and sulfide and the optical density were determined.

Determination of cell carbon. Portions of the culture (30 ml) were centrifuged at 10,000 × *g* for 10 min at 4°C. The cells were washed twice with 30 ml of sodium phosphate buffer (100 mM, pH 4.0) that contained 25 g of NaCl and 3.0 g of $MgCl_2 \cdot 6H_2O$ per liter. Cell carbon was determined with a model 915A Total Carbon Analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Gas chromatographic procedures. Volatile fatty acids were analyzed with a Packard gas chromatograph model 437 (Packard B.V., Delft, The Netherlands) equipped with a flame ionization detector and a Shimadzu integrator (Chromatopac C-R1B). A 1.4-m glass column (inner diameter, 2 mm) was used with Chromosorb WAW 100/120 mesh coated with 10% SP-1000 and 3% H_3PO_4 (Chrompack Nederland B.V., Middelburg, The Netherlands). The flow rate of the carrier gas, N_2, was 25 ml/min. The temperatures of injection port, column, and detector were 175, 140, and 175°C, respectively. The volatile fatty acids were determined by thoroughly mixing 0.2 ml of culture supernatant with 0.3 g of NaCl, 0.1 ml of internal standard solution, 0.02 ml of concentrated formic acid, and 0.3 ml of diethyl ether and by injecting 2.0 μl of the ether phase on the column.

TMA, dimethylamine, and methylamine were analyzed with a Packard gas chromatograph model 438 (flame ionization detector) and a Shimadzu integrator (Chromatopac C-R3A). A 2.8-m glass column (inner diameter, 2 mm) was filled with Pennwalt 231 methylamine packing (Alltech Associates, Deerfield, Ill.). The flow rate of the carrier gas, N_2, was 25 ml/min. The temperatures of injection port and detector were 110 and 175°C, respectively. The temperature of the column was controlled with a time program (40°C for 5 min followed by 100°C for 1 min). The amines were determined by mixing 0.1 ml of culture supernatant with 0.01 ml of 4 N NaOH and injecting 2.0 μl on the column.

Methane was analyzed with a Pye Unicam 104 gas chromatograph equipped with a thermal conductivity detector and a 2.3-m glass column (inner diameter, 2 mm) with Porapak Q (Chrompack). The flow rate of the carrier gas, N_2, was 25 ml/min. Injection port, column, and detector were kept at room temperature, and the detector current was 100 mA. Samples were taken directly from the gas phase of the cultures with a pressure lock syringe.

Methanol was analyzed with a Packard gas chromatograph model 427 (flame ionization detector). A 1.4-m glass column (inner diameter, 2 mm) was used with Porapak Q (Chrompack). The flow rate of the carrier gas, N_2, was 25 ml/min. The temperatures of injection port, column, and detector were 200, 150, and 200°C, respectively. Methanol was determined by injecting 2 μl from a culture supernatant directly on the column.

Determination of betaine, *N,N*-dimethylglycine, and sarcosine by high-performance liquid chromatography. Betaine, *N,N*-dimethylglycine, and sarcosine were analyzed on a Waters high-performance liquid chromatography system (Waters Associates, Milford, Mass.) equipped with a 10-cm Hypersil APS-2-amino column (Chrompack). The flow rate of the mobile phase (acetonitrile, 10 mM sodium phosphate buffer [pH 7.5]; 75:25, vol%) was 0.6 ml/min. The *N*-methyl compounds were determined after removal of the cells and the sulfide by injecting 3 μl on the column. The sulfide was removed from 1-ml samples by adding 50 μl of 1 N HCl and gassing with N_2 for 5 min. Subsequently, the samples were neutralized by the addition of 50 μl of 1 N NaOH. The peaks were detected at 205 nm with an absorbance detector (model 480) and processed with a Waters data module (model 730).

Morphological characterization and Gram stain. The morphological properties of the strains were examined by phase-contrast microscopy and by electron microscopy. For the Gram stain, cells from the exponential phase were used with *Butyribacterium methylotrophicum* and *Sporomusa ovata* as controls. The Gram reaction was compared with the lysis of cells in 3% KOH (5) and with the aminopeptidase test (2) using Bactident Aminopeptidase strips (E. Merck AG, Darmstadt, Federal Republic of Germany).

For negative staining intact cells were fixed for 60 min in a mixture of 1.5% (vol/vol) formaldehyde and 4% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 0°C, washed once in distilled water, and negatively stained with 1% aqueous uranyl acetate. For thin sectioning, the cells were prefixed as described above, washed for 15 min in the cacodylate buffer, and postfixed for 90 min in a solution of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in the same buffer at room temperature. After poststaining in 1% (wt/vol) uranyl acetate for 4 h, the cells were dehydrated in a graded ethanol series and embedded in Epon 812, sectioned with a diamond knife on a microtome (LKB Instruments, Inc., Rockville, Md.), and examined in a Philips EM 300 electron microscope.

Cytochromes and optical density measurements. The presence of *c*-type cytochromes was determined from dithionite-reduced versus air-oxidized spectra (1). The optical densities of cultures were measured in a 1-cm cuvette in a Vitatron colorimeter at 663 nm.

Chemicals. All chemicals were reagent grade and purchased from E. Merck AG, except *N,N*-dimethylglycine and sarcosine (Sigma Chemical Co., St. Louis, Mo.).

RESULTS

Coccoid immotile cells and a lower number of smaller motile rods were the two dominant types of bacteria that were enriched in a betaine (20 mM) medium inoculated with

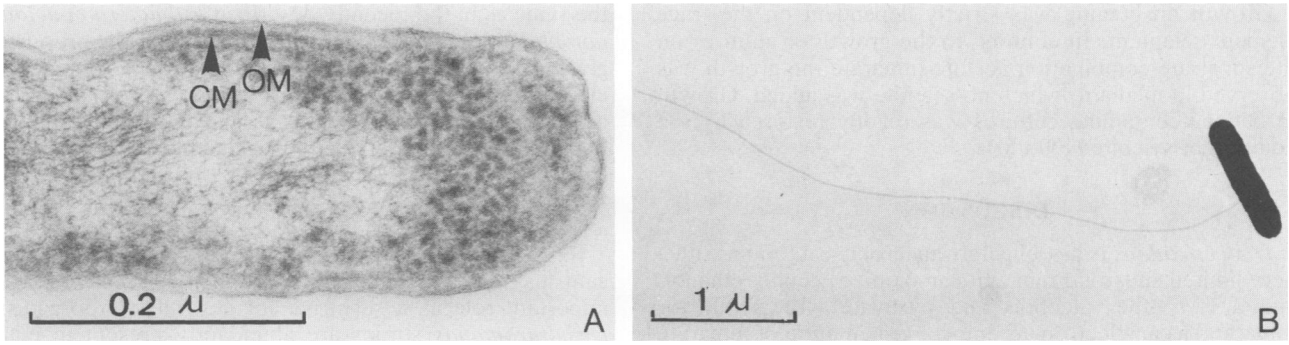

FIG. 1. Electron micrographs of *D. acetoxidans* PM1. (A) Ultrathin section; OM, outer membrane; CM, cytoplasmic membrane. (B) Negatively stained cell.

intertidal mud. The coccoid cells were conspicuously similar to *Methanococcoides methylutens* (22). The presence of such a methanogen would explain the large amounts of methane that were detected in the headspaces of the cultures. Pure cultures of the coccoid organism and the rod-shaped bacteria were obtained both via betaine roll tubes under an N_2-CO_2 (80:20, vol%) atmosphere and via anaerobic betaine plates. The coccoid organism did not grow on betaine but did grow on TMA (10 mM), dimethylamine (10 mM), methylamine (20 mM), and methanol (50 mM) and fermented these substrates with the formation of methane. Methanol fermentation was virtually in agreement with equation 3:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 \quad (3)$$

The isolate did not grow on H_2-CO_2 (80:20, vol%), formate (25 mM), acetate (20 mM), dimethylglycine (20 mM), or sarcosine (20 mM). All of these characteristics support the view that the coccoid organism that probably grew on one of the products formed by the motile rods is a strain of *M. methylutens* or possibly of the rather similar *Methanolobus tindarius*, though the latter organism was described as being motile (10).

The rod-shaped bacterium (strain PM1) grew on betaine (20 mM, no yeast extract added) in pure culture. Cells were 2 to 3 μm long and 0.6 μm wide and stained gram negative, which was in agreement with the gram-negative cell wall structure (Fig. 1A). Cells possessed no more than one flagellum in a subpolar-to-lateral position (Fig. 1B). Young colonies of strain PM1 on betaine plates were yellow and later became orangish brown. The latter color was also observed in the pellets of liquid cultures that were centrifuged. Fumarate (10 mM) was the only other substrate that supported fermentative growth of strain PM1. In the presence of elemental sulfur, however, the strain appeared to oxidize acetate (10 mM), pyruvate (10 mM), ethanol (10 mM), propanol (10 mM), and butanol (10 mM) and produced high concentrations of sulfide (up to 12 mM). H_2-CO_2 (80:20, vol%), formate (10 mM), propionate (10 mM), butyrate (10 mM), valerate (10 mM), lactate (10 mM), malate (10 mM), succinate (10 mM), alanine (10 mM), aspartate (10 mM), glutamate (10 mM), Casamino Acids (0.1%), yeast extract (0.1%), methanol (20 mM), glycerol (10 mM), glucose (10 mM), fructose (10 mM), choline (10 mM), *N*,*N*-dimethylglycine (10 mM), methylamines (10 mM), and sarcosine (10 mM) were not suitable substrates in the presence of sulfur (no growth after 4 weeks of incubation). Sulfate (20 mM), sulfite (5 mM), thiosulfate (10 mM), and nitrate (10 mM) were not used as electron acceptors, and strain PM1 did not grow in acetate-sulfur medium under air. The morphological and physiological characteristics of strain PM1, in particular the growth on acetate with sulfur as electron acceptor, allow the identification of strain PM1 as a *D. acetoxidans* strain (20). The type strain of this species (strain DSM 684) also grew on betaine (20 mM). Both the type strain and PM1 contain high concentrations of *c*-type cytochromes (with absorption maxima at 523 and 553 nm). The time course of growth, substrate utilization, and product formation of *D. acetoxidans* on betaine is shown in Fig. 2. Per mol of betaine, 0.99 (± 0.03) mol of TMA and 0.71 (± 0.03) mol of acetate were formed. Dimethylglycine, sarcosine, methylamine, and dimethylamine were not detected as fermentation products. Growth was exponential only in the early phase (10 to 30 h). In this period, the specific growth rate was 0.073 h^{-1}, corresponding to a doubling time of 9.5 h. The amount of cell carbon produced was 0.125 mol (± 0.011) per mol of betaine. In cultures grown on betaine in the presence of sulfur, the amount of TMA formed per mol of betaine was 0.94 (± 0.13) mol while the amounts of acetate and sulfide formed were 0.23 (± 0.01) and 1.7 (± 0.3) mol, respectively.

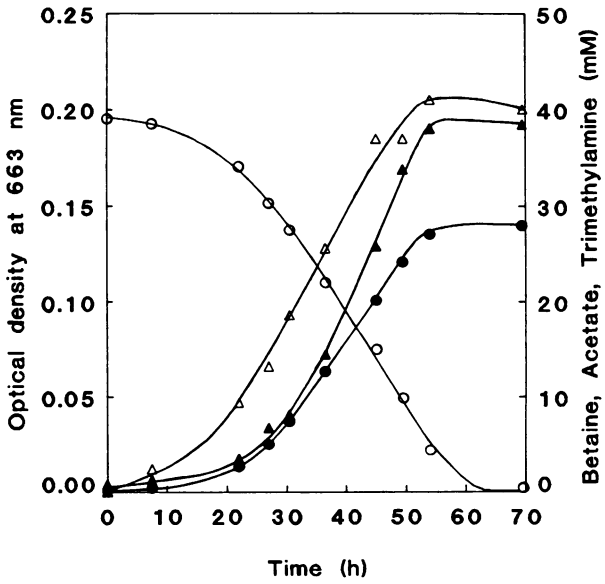

FIG. 2. Formation of acetate and trimethylamine during growth of *D. acetoxidans* PM1 on 40 mM betaine (0.01% yeast extract). Symbols: △, optical density (at 663 nm); ▲, trimethylamine; ○, betaine; ●, acetate.

Growth on betaine was strictly dependent on the trace element selenium. In contrast to the growth of cultures on the substrate combination acetate-fumarate, no growth was observed in media to which no selenite was added. Growth in betaine-containing cultures was readily restored by the addition of selenite (100 nM).

DISCUSSION

D. acetoxidans is an obligately anaerobic bacterium with a very limited substrate range; it can oxidize acetate, ethanol, and a few other alcohols and pyruvate with sulfur (or fumarate) as an electron acceptor. Fermentation of fumarate or malate has been reported as an alternative energy-yielding process (19). In this study, we have shown that at least some *Desulfuromonas* strains can both ferment and oxidize betaine. The fermentation was virtually in agreement with equation 4:

$$\text{betaine} + 0.5H_2O \rightarrow \text{TMA} + 0.75\ \text{acetate} + 0.5CO_2 \quad (4)$$

$$\text{betaine} + 2H \rightarrow \text{TMA} + \text{acetate} \quad (5)$$

$$0.25\ \text{acetate} + 0.5H_2O \rightarrow 0.5CO_2 + 2H \quad (6)$$

This result is interesting for two reasons. It adds a new compound to the list of energy substrates for *Desulfuromonas* strains, and it proves the existence of another pathway by which betaine can be degraded in anaerobic sediments. This type of betaine fermentation can be described as a reductive cleavage of the compound (equation 5) and an oxidation of part of the acetate formed (equation 6) for the production of the reducing equivalents for the cleavage reaction. In the presence of sulfur as an electron acceptor, the rest of the acetate initially formed in the betaine cleavage can be oxidized to CO_2 with the formation of sulfide as shown in equations 7 and 8:

$$0.75\ \text{acetate} + 1.5H_2O \rightarrow 1.5CO_2 + 6H \quad (7)$$

$$6H + 3S^0 \rightarrow 3H_2S \quad (8)$$

Desulfuromonas strains have a complete tricarboxylic acid cycle (4) which allows them to oxidize acetate anaerobically. The electrons generated in the acetate oxidation are used to reduce the betaine and, in the presence of sulfur, also to reduce this electron acceptor. Some of these electrons are thermodynamically unfavorable because of the difference in redox potential between the S^0-H_2S couple (−270 mV) and the fumarate-succinate couple (30 mV). Yet growth occurs, and it is assumed (4, 18) that the succinate dehydrogenase reaction is coupled to sulfur reduction via reversed electron transport. The precise redox potential of the betaine-(TMA + acetate) couple is not known to us, but it is likely to be close to the −10-mV level of the glycine-(acetate + NH_3) couple. Therefore, we would expect a higher molar growth yield during growth on betaine than during growth on acetate with sulfur. The amount of biomass produced was 3.0 g/mol of betaine if we assume that the carbon content of the cell material is about 50%. To compare this yield with that on acetate-sulfur, we have to multiply the growth yield on betaine by 4, because only 0.25 mol of acetate is oxidized per mol of betaine reduced. Then the yield on betaine (12.0 g/mol of acetate oxidized) is approximately three times higher than on acetate with sulfur (4.2 g/mol of acetate; 20).

Desulfuromonas strain PM1, *Sporomusa* strains (15), and *C. sporogenes* (17) all require selenium during growth on betaine, and all metabolize betaine via a reductive cleavage to acetate and trimethylamine. The role of selenium may be the same as in the recently described *Eubacterium acidaminophilum* (26), which can oxidize formate in the presence of glycine, sarcosine, or betaine as electron acceptors. The electrons from the oxidation of formate are probably channelled to three different reductases via a selenium-containing membrane-bound protein (W. Freudenberg, K. Hormann, M. Rieth, and J. R. Andreesen, in *Proceedings of the 4th International Symposium on Selenium in Biology and Medicine*, in press).

Since betaine occurs in significant concentrations in organisms that inhabit marine sediments, it may play an important role as a substrate for several microorganisms. *Desulfuromonas* strains are abundantly present in marine sediments (24), which is not surprising in view of the high rates of sulfate reduction and the rapid biotic and abiotic conversion of hydrogen sulfide to sulfur in the presence, and under certain conditions also in the absence, of oxygen. *Desulfuromonas* strains are excellent acetate oxidizers with sulfur as an electron acceptor (19, 20), and the strategy displayed by *Desulfuromonas* strains in betaine utilization is in accordance with this characteristic. The formation of TMA by *Desulfuromonas* strains explains the growth in the enrichment cultures of methanogens that resemble *M. methylutens*. High counts of *M. methylutens* were found in sediments of a salt marsh dominated by *Spartina alterniflora*, which contains betaine as an osmoregulator (3). In general, betaine and choline are considered to be the main sources of TMA in anaerobic sediments (9). Our enrichment media could not give rise to the development of sulfate-reducing bacteria, because sulfate was omitted from the media. Still, sulfate-reducing bacteria might be important directly or indirectly in situ; King (9) showed that both methanogens and sulfate-reducing bacteria play an indirect role in the decomposition of betaine and choline in sediments from the intertidal region of Lowes Cove, Maine. Evidence was also presented to suggest that in the upper 10-cm layer betaine is split into TMA and acetate in a 1:1 ratio. These results indicate that the role of *Desulfuromonas* strains in the degradation of betaine in such sediments would be limited because with *Desulfuromonas* a maximal acetate/TMA ratio of 0.75 can be expected. However, King's experiments were carried out with anaerobic sediment slurries under conditions where recycling of elemental sulfur is unlikely or even impossible. In the presence of elemental sulfur, which is easily formed by biotic or abiotic processes in the upper sediment layers, the role of *Desulfuromonas* strains may be important. Until now, the role of *D. acetoxidans* in the degradation of betaine remains speculative, but it is clear that the metabolism of betaine in anoxic systems is a complex process in which many species may be involved. This complexity is also illustrated by the recent isolation in our laboratory of a betaine-degrading, sulfate-reducing bacterium (manuscript in preparation) and by the work of Fendrich (C. Fendrich, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1988). She found that in deeper layers of the sediments of the German Wadden Sea, betaine was mainly converted to TMA and acetate, whereas in the top layer of the sediment bacteria dominated that formed dimethylglycine as an additional product.

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