APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2001, p. 300–306 Vol. 67, No. 1
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.1.300–306.2001

Non-Growth-Associated Demethylation of Dimethylsulfoniopropionate by (Homo)acetogenic Bacteria

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Received 10 July 2000/Accepted 18 October 2000

The demethylation of the algal osmolyte dimethylsulfoniopropionate (DMSP) to methylthiopropionate (MTPA) by (homo)acetogenic bacteria was studied. Five *Eubacterium limosum* strains (including the type strain), *Sporomusa ovata* DSM 2662^T, *Sporomusa sphaeroides* DSM 2875^T, and *Acetobacterium woodii* DSM 1030^T were shown to demethylate DMSP stoichiometrically to MTPA. The (homo)acetogenic fermentation based on this demethylation did not result in any significant increase in biomass. The analogous demethylation of glycine betaine to dimethylglycine does support growth of acetogens. In batch cultures of *E. limosum* PM31 DMSP and glycine betaine were demethylated simultaneously. In mixed substrates experiments with fructose-DMSP or methanol-DMSP, DMSP was used rapidly but only after exhaustion of the fructose or the methanol. In steady-state fructose-limited chemostat cultures (at a dilution rate of 0.03 h^{-1}) with DMSP as a second reservoir substrate, DMSP was biotransformed to MTPA but this did not result in higher biomass values than in cultures without DMSP; cells from such cultures demethylated DMSP at rates of approximately 50 nmol min^{-1} mg of $protein^{-1}$, both after growth in the presence of DMSP and after growth in its absence. In cell extracts of glycine betaine-grown strain PM31, DMSP demethylation activities of 21 to 24 nmol min^{-1} mg of $protein^{-1}$ were detected with tetrahydrofolate as a methyl acceptor; the activities seen with glycine betaine were approximately 10-fold lower. A speculative explanation for the demethylation of DMSP without an obvious benefit for the organism is that the DMSP-demethylating activity is catalyzed by the glycine betaine-demethylating enzyme and that a transport-related factor, in particular a higher energy demand for DMSP transport across the cytoplasmic membrane than for glycine betaine transport, may reduce the overall ATP yield of the fermentation to virtually zero.

Certain marine sulfate-reducing bacteria belonging to the *Desulfobacterium-Desulfobacter* cluster of the δ-proteobacteria and possessing the oxidative CO dehydrogenase pathway for acetyl-coenzyme A oxidation use the algal osmolyte dimethylsulfoniopropionate [DMSP; $(CH_3)_2$-S^+-CH_2-CH_2-COO^-] for growth and convert it to methylthiopropionate (MTPA; CH_3-S-CH_2-CH_2-COO^- 40). These bacteria use a specific DMSP-tetrahydrofolate methyltransferase for the demethylation reaction (22, 23). Glycine betaine [$(CH_3)_3$-N^+-CH_2-COO^-], an N-containing structural analog of DMSP, was demethylated to dimethylglycine [$(CH_3)_2$-N-CH_2-COO^-] by these bacteria, but in cell extracts no activity was detected with tetrahydrofolate as a methyl acceptor and glycine betaine as a substrate. Both glycine betaine and DMSP are important osmolytes (2, 7, 13). DMSP is produced by many marine algae and some plants, where it is synthesized from methionine (12, 36). The occurrence of high DMSP concentrations in certain types of biological material and the possibility to convert DMSP to other sulfur-containing compounds, including MTPA, by using bacterial cultures, make DMSP of potential interest for the natural flavor industry (16, 17).

(Homo)acetogenic bacteria synthesize acetyl-coenzyme A from C_1 compounds with involvement of the reductive CO dehydrogenase pathway, which is the reverse of the route used by the sulfate-reducing bacteria for acetyl-coenzyme A oxidation. The acetyl-coenzyme A can be converted to acetate or to acetate plus longer acids such as butyrate (19). (For a discussion of the term acetogenic bacteria or acetogens, see reference 10). Since the discovery in 1981 that the acetogenic bacterium *Eubacterium limosum* can demethylate glycine betaine to dimethylglycine, with acetate and butyrate as fermentation products (34), many other acetogenic bacteria have been shown to grow by demethylation of betaine (19). A possible involvement of organisms such as *E. limosum* in DMSP demethylation in anoxic sediments was already suggested by Kiene and Taylor (27), but without direct experimental evidence. Recently, in our laboratory a slow demethylation of DMSP to MTPA was demonstrated in experiments with an *E. limosum*-like strain isolated from intertidal mud; growth of this strain was very poor (40). We show here that under certain conditions the biotransformation of DMSP to MTPA can be carried out at appreciable rates by this strain and by a number of other acetogenic bacteria; this process, however, does not support growth, in contrast to the analogous demethylation of glycine betaine.

MATERIALS AND METHODS

Microorganisms, media, and cultivation. Bacterial strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) unless otherwise indicated. *E. limosum* PM31 (isolated by J. H. F. G. Heijthuijsen in our laboratory 20), *E. limosum* DSM 20543^T, *E. limosum* DSM 20517, *E. limosum* DSM 2594 (strain 11A), "*Butyribacterium methylotrophicum*" Marburg (obtained from J. G. Zeikus), and *Sporomusa sphaeroides* DSM 2875 were grown in 120-ml vials containing 50 ml of medium with the following composition (per liter): 1.0 g of NaCl, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NH_4Cl, 0.3 g of KCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1.0 g of yeast extract (Difco, Detroit, Mich.), and 0.5 mg of resazurin. The medium was also composed of 0.1 μM Na_2SeO_3, 0.1

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μM Na_2WO_4, and 1 ml of a trace elements solution (28). After autoclaving, the basal medium was supplemented with 1 ml of a vitamin solution (42), 2 ml of a phosphate buffer (KH_2PO_4, 1.58 M; K_2HPO_4, 0.93 M), 50 ml of 1 M sodium bicarbonate, 4 ml of 0.5 M sodium sulfide, and substrate as indicated. The vials were gassed with an oxygen-free mixture of N_2-CO_2 (80:20 [vol/vol]). Incubation temperature was 37°C, except for *S. sphaeroides* (30°C). *Acetobacterium woodii* DSM 1030 and *Sporomusa ovata* DSM 2662 were cultured at 30°C in medium 135 and medium 311, respectively, as described previously (9).

DMSP and glycine betaine demethylation by cell suspensions and chemostat cultures. For cultivation of strain PM31 in fructose-limited chemostats, a culture vessel with a working volume of 730 ml was used. The following conditions were employed: a dilution rate of 0.03 h^{-1}, a reservoir medium (for composition see above) with 3 mM fructose, pH 7.2 (kept constant by automatic titration with 2 N NaOH), a temperature of 37°C, a gas phase above the reservoir and culture N_2-CO_2 (80:20 [vol/vol]). The medium reservoir was slowly stirred to avoid a possible loss of precipitated trace elements. At steady state (after at least five volume changes) the medium feed was stopped, and DMSP from an anoxic 1 M stock solution was added or cells were removed anoxically from the culture vessel, and 50 ml was transferred into 120-ml vials in an anaerobic glove box equipped with a palladium catalyst (R020; BASF, Ludwigshafen, Germany) under an atmosphere of N_2-H_2 (approximately 95:5 [vol/vol]). The vials were gassed with N_2-CO_2 (80/20% [vol/vol]) and used in experiments with protein synthesis inhibitors. Cell suspension experiments with batch-grown strain PM31 were done with anoxically harvested cells that had been grown on various concentrations of glycine betaine or glycine betaine-DMSP. These cells were washed once or twice with complete sulfide-reduced medium without yeast extract or with anoxic 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol.

Cell extract preparation and enzyme measurements. Extracts of cells (3 to 10 mg of protein/ml) grown on medium with 15 mM DMSP, 15 mM glycine betaine, or 15 mM glycine betaine plus 15 mM DMSP were prepared under anoxic conditions as described by Hensgens et al. (21) with the following minor modifications: the cells were washed and suspended in 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM dithiothreitol and passed three times through a French pressure cell. The enzyme assays were performed in an anaerobic glove box; the assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.2), 2 mM dithiothreitol, 2.5 mM titanium(III)–10 mM nitrilotriacetic acid, 3 mM tetrahydrofolate, 0.2 mM cyanocobalamin, 2 mM ATP, 8 mM $MgCl_2$, and cell extract in a total volume of 1 ml. After 10 min of incubation at 37°C, the reaction was started by the addition of 10 mM substrate. Reactions were stopped with 60 mM HCl and, after approximately 15 min, the reaction mixture was centrifuged (5 min, 2,000 × *g*) in the glove box. The supernatants were transferred to airtight vials, and the tetrahydrofolate and methyltetrahydrofolate concentrations were measured by high-performance liquid chromatography (HPLC).

rDNA sequencing and sequence comparison. DNA of strain PM31 and "*B. methylotrophicum*" for 16S rRNA gene sequence analysis was extracted and amplified as described previously (40). The PCR product was purified using the Wizard PCR purification system (Promega, Madison, Wis.) and subsequently sequenced on an ABI310 automated sequencer (Perkin-Elmer, Norwalk, Conn.) using the dye-terminator cycle sequencing method of Perkin-Elmer in combination with custom primers based on the conserved regions of the 16S rRNA gene. Reaction conditions for the cycle sequencing reaction were according to the manufacturer's manual. The similarity of the sequences was determined by alignment of 1,460 nucleotides using the DCSE program of De Rijk and De Wachter (8) and subsequently calculating the number of common nucleotides. The 16S ribosomal DNA (rDNA) sequence of *E. limosum* DSM 20543^T was obtained from GenBank.

Analytical procedures. DMSP was determined as acrylate after conversion to dimethylsulfide and acrylate by overnight treatment with 1 M NaOH (44). Acrylate, MTPA, and mercaptopropionate were analyzed by HPLC (22). Mercaptopropionate was measured after reduction of the sample with up to 20 mM tributylphosphine. Betaine and dimethylglycine were measured as described elsewhere (18), with acetonitrile-water (80:20 [vol/vol]) as a mobile phase instead of acetonitrile with a 10 mM sodium phosphate buffer (pH 7.5). Tetrahydrofolate and methyltetrahydrofolate were measured by HPLC with UV detection at 280 nm as described by Stupperich and Konle (38). Homocysteine and methionine were assayed by HPLC after derivative formation with *ortho*-phthalaldehyde (11). Fructose was measured by HPLC using a Polyspher OA HY column (Merck, Darmstadt, Germany) and refractometric detection; the flow rate of the mobile phase (0.01 N H_2SO_4) was 0.6 ml/min. The detection limit for fructose was 20 μM. Protein in cell extracts was determined according to the method of Bradford (3) using the Bio-Rad reagent with bovine serum albumin as a standard. The protein content of whole cells was measured after treatment with 1 M NaOH at 100°C for 10 min according to the method of Lowry et al. (30). The optical densities (ODs) of cultures were measured in a 1-cm cuvette in a Starrcol colorimeter (Hoorn, The Netherlands) at 660 nm. An OD at 660 nm (OD_{660}) value of 1.0 corresponds to 0.26 mg of protein per ml. Cell carbon was determined as described previously (18). Dimethyl sulfide and methanethiol were assayed by gas chromatography as described previously (41, 39).

Chemicals. DMSP was synthesized from acrylic acid and DMS (5) or obtained from CASS (Groningen, The Netherlands). MTPA was obtained by alkaline hydrolysis of its methylester (Aldrich, Steinheim, Germany). 5,6,7,8-Tetrahydrofolic acid was obtained from Sigma (St. Louis, Mo.) or Schircks Laboratories (Jona, Switzerland); 5-*methyl*-5,6,7,8-tetrahydrofolic acid was from Merck (Darmstadt, Germany). Cyanocobalamin was purchased from Sigma. Titanium(III)-nitrilotriacetic acid stock solutions were prepared according to the method of Moench and Zeikus (33).

Nucleotide sequence accession numbers. The 16S rDNA sequences of "*B. methylotrophicum*" and *E. limosum* PM31 were deposited with GenBank under accession numbers AF064241 and AF064242, respectively.

RESULTS

Conversion of DMSP by pure cultures of acetogenic bacteria. Recently, we reported that the acetogenic strain PM31, which had been isolated from intertidal mud and had been tentatively identified as *E. limosum*, was able to demethylate DMSP to MTPA, but growth was very poor and the conversion was slow (40). Here we describe in more detail the demethylation of DMSP by this bacterium. When 5% of a culture (grown on 15 mM DMSP–15 mM betaine) was inoculated into medium with 15 mM DMSP and 0.1% yeast extract, 5.6 mM MTPA was produced from 6.0 mM DMSP after 163 h of incubation (Fig. 1). Other products in this culture were 1.7 mM acetate and 0.7 mM butyrate. Other possible sulfur-containing endproducts such as dimethylsulfide, methanethiol, and mercaptopropionate were not detected. The increase in OD in these cultures (OD_{660} = 0.12) was not higher than in cultures without DMSP and therefore was most likely due to utilization of components from the yeast extract. This increase took place during the first 40 h; in this period only 1.4 mM MTPA had been formed, again indicating that growth could not have been supported by DMSP demethylation. Incubations with 15 mM glycine betaine resulted in a maximum OD of 0.28 (Fig. 1A), a value considerably above the control with only yeast extract; products of the growth on glycine betaine were 16.1 mM dimethylglycine, 6.6 mM acetate, and 0.8 mM butyrate. The growth yield on glycine betaine in this experiment was 3.0 g (dry weight) of cells/mol of glycine betaine. In earlier work (40) DMSP had been tested for its use as a growth substrate by acetogens; since it now appeared that DMSP utilization was not associated with growth, we reinvestigated a possible biotranformation of DMSP to MTPA by other acetogens. Besides strain PM31, several other acetogenic bacteria were indeed found to be able to demethylate DMSP; *E. limosum* DSM 20543^T, *E. limosum* DSM 20517, *E. limosum* DSM 2594, *S. ovata* DSM 2662, *S. sphaeroides* DSM 2875, *A. woodii* DSM 1030, and "*B. methylotrophicum*" produced MTPA from DMSP at rates that are comparable with the DMSP demethylation rates in cultures of strain PM31. Also, cultures of these strains showed no significant increase in optical density when DMSP was added to the medium. This is in agreement with the observation of Van der Maarel et al. (40) that these pure cultures were unable to grow on DMSP. Importantly, with all of these bacteria the demethylation of glycine betaine to dimethylglycine did support growth.

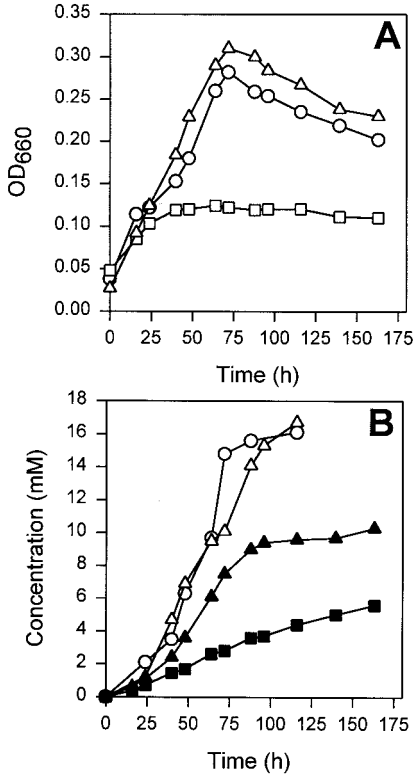

FIG. 1. (A) Growth of strain PM31 on 15 mM glycine betaine (○), 15 mM DMSP (□), or 15 mM DMSP plus 15 mM glycine betaine (△). (B) Product concentrations. Dimethylglycine levels during growth on 15 mM glycine betaine (○) or on 15 mM DMSP plus 15 mM glycine betaine (△) and MTPA levels during growth on 15 mM DMSP (■) or on 15 mM DMSP plus 15 mM glycine betaine (▲) are shown.

Characteristics and phylogenetic position of strain PM31. Strain PM31 was chosen as the model organism for the detailed studies described below because it originates from an environment where DMSP is known to occur. Strain PM31 is strictly anaerobic, salt tolerant, nonmotile, nonsporeforming, gram positive, and rod shaped (0.6 to 0.9 μm by 2.0 to 3.0 μm); it was isolated from an enrichment culture inoculated with anoxic intertidal sediment of the Wadden Sea (The Netherlands) with 10 mM vanillate as a substrate (20). It showed good growth in freshwater medium at 37°C on several substrates, including methanol, H_2-CO_2 (80:20 [vol/vol]), glucose, fructose, various methoxylated aromatic compounds, and glycine betaine. Acetate and butyrate were the main fermentation products.

The 16S rRNA genes of strain PM31 and "*B. methylotrophicum*" were found to differ in only one nucleotide position (99.9% similarity). Based on their 16S rRNA gene sequences, strain PM31 and "*B. methylotrophicum*" are very closely related to the type strain of *E. limosum* (similarities of 99.5 and 99.4%, respectively). These data and its phenotypic properties support assignment of strain PM31 to *E. limosum*. It is also evident that "*B. methylotrophicum*" is in fact an *E. limosum* strain, which is in agreement with the phenotypic similarity. Its major difference from *E. limosum*, namely, its ability to produce spores, has been questioned by Cato et al. (4).

Sequential and simultaneous utilization of substrates by batch cultures of *E. limosum* PM31. Demethylation of DMSP and glycine betaine in cultures of strain PM31 occurred simultaneously (Fig. 1B). In cultures inoculated with 5% of a glycine betaine (15 mM)-DMSP (15 mM) pregrown culture, the change in dimethylglycine concentration was similar to those of cultures supplemented with only glycine betaine; the production of MTPA was slower. In such cultures glycine betaine and DMSP were demethylated stoichiometrically to dimethylglycine and MTPA, respectively. Under these conditions the demethylation of DMSP was faster than in cultures supplemented with only DMSP (Fig. 1B). In batch fermentor cultures (pH kept constant at 7.2) with medium containing 15 mM fructose and 30 mM DMSP, strain PM31 rapidly demethylated DMSP after a growth-supporting utilization of the fructose (Fig. 2); during the more or less linear decrease in the DMSP concentration between 40 and 60 h the demethylation rate was 50 nmol min^{-1} mg of $protein^{-1}$. Similarly, in experiments with methanol and DMSP as substrates, DMSP was only demethylated after virtual exhaustion of the methanol. Also in these experiments, no or negligible growth was observed when DMSP was demethylated. In cultures of strain PM31 with fructose and glycine betaine, first fructose was used and subsequently the glycine betaine was demethylated. All other tested acetogenic bacteria (see Materials and Methods) also showed faster DMSP demethylation in media containing a true growth substrate and DMSP.

DMSP and glycine betaine demethylation by cells of *E. limosum* strain PM31. Cells obtained from glycine betaine-DMSP-yeast extract- or DMSP-yeast extract-grown batch cultures, washed in medium without yeast extract, did not demethylate DMSP or, after a lag phase of many hours, did so at rates lower than 5 nmol min^{-1} mg of $protein^{-1}$ despite the use of anaerobic techniques throughout the manipulations. To obtain active cells, experiments were carried out with fructose-limited chemostat cultures, where at steady state at a dilution rate of 0.03 h^{-1} the medium flow was stopped and DMSP was added. At steady state the fructose concentration was below the detection level (<20 μM). DMSP demethylation occurred at a rate of approximately 50 nmol min^{-1} mg of $protein^{-1}$ and started immediately after the addition of DMSP. Over the period during which the DMSP was demethylated there was a small OD_{660} decrease, again showing that DMSP did not support growth. The apparent K_m value for DMSP was approximately 2 mM as calculated from the substrate depletion curve.

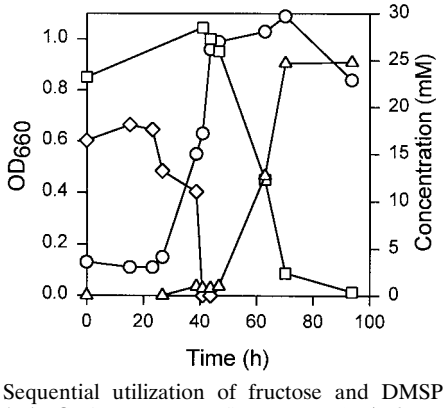

FIG. 2. Sequential utilization of fructose and DMSP by strain PM31. Symbols: ○, OD_{660}; □, DMSP; △, MTPA; ◇, fructose.

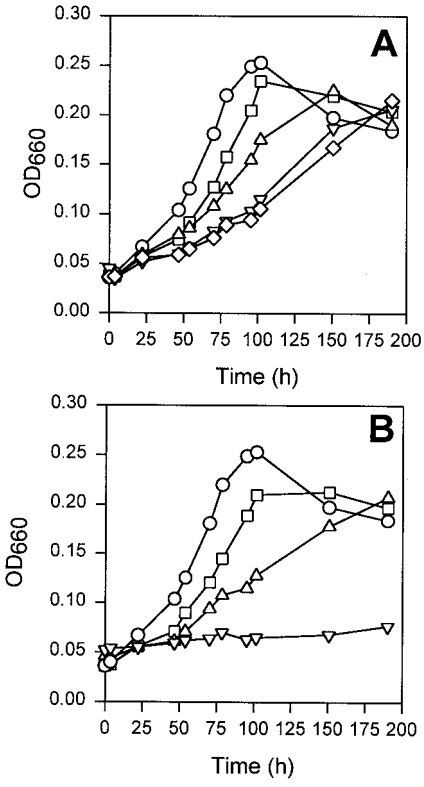

FIG. 3. Effect of MTPA (A) and dimethylglycine (B) on the growth of strain PM31 on glycine betaine (15 mM). The symbols in panel A indicate the control (○) or addition at $t = 0$ of 5 (□), 10 (△), 15 (▽), or 20 (◇) mM MTPA. The symbols in panel B indicate the control (○) or addition at $t = 0$ of 5 (□), 10 (△), or 20 (▽) mM dimethylglycine.

When DMSP and glycine betaine, both at 10 mM, were added to a fructose-limited chemostat culture, both compounds were demethylated at the same time at similar rates (data not shown).

With 3.0 mM fructose and 9.0 mM DMSP in the medium reservoir at a dilution rate of 0.03 h^{-1}, at steady state there was 0.9 mM DMSP left and 8.0 mM MTPA produced; the OD values of fructose-limited cultures with DMSP in the reservoir were almost the same as without DMSP (0.47 versus 0.50). When 10 mM DMSP was added to such a culture immediately after stopping the medium flow, approximately the same DMSP demethylation rate was found as with the culture grown in the absence of DMSP (53 nmol min^{-1} mg of $protein^{-1}$). These results show that the DMSP demethylation system does not require induction by DMSP. This was confirmed by using tetracycline as an inhibitor of de novo protein synthesis; other inhibitors of protein synthesis did not block growth at all (rifampin at 4 μg/ml) or inhibited DMSP demethylation completely in cells that were able to demethylate DMSP (chloramphenicol at 25 to 100 μg/ml). Tetracycline (20 μg/ml) blocked the growth of strain PM31 on fructose and strongly reduced the rate of fructose degradation. When cells were growing in batch culture in a medium with initial concentrations of 3 mM fructose and 20 mM DMSP and already producing MTPA (after exhaustion of the fructose), the addition of 20 μg of tetracycline per ml did not affect the demethylation of DMSP. When, after growth on fructose (in the absence of DMSP), DMSP and tetracycline were added simultaneously, strain PM31 was still able to demethylate DMSP. With cells obtained from fructose-limited chemostat cultures similar results were obtained.

Yeast extract is known to contain 1 to 3% (wt/wt) glycine betaine (13); 1 g of yeast extract per liter in the medium may therefore lead to an initial glycine betaine concentration of 0.27 mM. Such a concentration might be sufficient for the induction of the glycine betaine demethylation system if it is inducible and, because of the structural similarity of DMSP and glycine betaine, of a specific DMSP demethylating enzyme if such an enzyme exists. We therefore tried to culture strain PM31 in chemostat cultures without yeast extract in the reservoir medium. After 9.4 volume changes there was considerably more wall growth and a lower OD (0.3) than under culture conditions with yeast extract present, but the residual fructose concentration remained below the detection limit. These cells were able to demethylate DMSP in the presence of 10 μg of tetracycline per ml, albeit very slowly (approximately 1 nmol min^{-1} mg of $protein^{-1}$; the control without tetracycline demethylated DMSP at 7.5 nmol min^{-1} mg of $protein^{-1}$).

Effects of MTPA and dimethylglycine on the growth of *E. limosum* strain PM31. Dimethylglycine and MTPA both have an inhibitory effect on the growth on betaine of strain PM31 (Fig. 3). This effect was small at initial dimethylglycine or MTPA concentrations of 5 mM but became pronounced at initial concentrations of 10 mM or higher. The effect of MTPA was not stronger than that of dimethylglycine. The effect on growth with 3 mM fructose appeared to be smaller than on growth with betaine, but during growth on betaine an increasing concentration of dimethylglycine is produced. Up to 10 mM dimethylglycine or MTPA hardly affected the growth on fructose, but 20 mM dimethylglycine or MTPA strongly reduced the growth rate, and the final OD of the culture was approximately 25% lower than in the control. These data exclude a far stronger inhibitory effect of MTPA than that of dimethylglycine on the growth as an explanation for the inability of strain PM31 to utilize DMSP as a growth-supporting substrate.

DMSP demethylating activities in cell extracts of *E. limosum* PM31. DMSP-tetrahydrofolate methyltransferase was identified as the key enzyme in DMSP demethylation in sulfate-reducing bacteria (22). Using a modified assay system we also detected DMSP-tetrahydrofolate methyltransferase activity in the acetogenic strain PM31, but the activities were lower than in the sulfate reducers (Table 1). DMSP-tetrahydrofolate

TABLE 1. DMSP and glycine betaine demethylation with tetrahydrofolate as a methyl acceptor in cell extracts of strain PM31[a]

Substrate(s)[b]	Mean DMSP-demethylating activity ± SD (nmol min^{-1} mg of $protein^{-1}$)	Mean betaine-demethylating activity ± SD (nmol min^{-1} mg of $protein^{-1}$)
DMSP	10.4 ± 1.5	1.0 ± 0.4
Glycine betaine	24.2 ± 3.0	2.2 ± 0.3
Glycine betaine plus DMSP	20.9 ± 0.8	2.6 ± 0.2

[a] Results are the means of three experiments ± the standard deviation, except for DMSP-demethylating activities with DMSP (the mean of five experiments) and glycine betaine as a growth substrate (the mean of eight experiments).
[b] All media contained 0.1% yeast extract.

methyltransferase activities of approximately 21 to 24 nmol min^{-1} mg of $protein^{-1}$ were detected with cell extracts of glycine betaine- or glycine betaine-DMSP-grown strain PM31. Activities with glycine betaine as substrate were significantly lower, even in cells grown on glycine betaine (Table 1). No activity was detected without titanium-nitrilotriacetic acid. ATP and Mg^{2+} were not obligatory for activity but did stimulate the DMSP demethylating activities in cell extracts of strain PM31. When cyanocobalamin was omitted from the assay mixture, DMSP demethylating activities were approximately 10 to 30% lower. Hydroxycobalamin did not have a stimulatory effect on the DMSP demethylation activities. In extracts of cells that had been grown on fructose or on fructose and DMSP (harvested when they were demethylating DMSP) no or negligible DMSP-tetrahydrofolate methyltransferase (and no activity with betaine) could be detected.

A DMSP or glycine betaine demethylating activity with homocysteine as a methyl acceptor (glycine betaine-homocysteine methyltransferase) was not found. In titanium-nitrilotriacetic acid-reduced assays with cell extracts of glycine betaine-grown strain PM31 and with glycine betaine or DMSP as a substrate, we did not detect a decrease in homocysteine concentration or an increase in methionine concentration, not even after incubations for 2 h.

DISCUSSION

This study shows that demethylation of DMSP to MTPA under anoxic conditions can not only be performed by certain sulfate-reducing bacteria of the *Desulfobacter-Desulfobacterium* cluster of the δ proteobacteria (40) but also by several acetogenic bacteria. These acetogenic bacteria are also able to demethylate the N-containing analog of DMSP, glycine betaine but, unlike DMSP, glycine betaine was a growth substrate, whereas DMSP was only biotransformed without supporting growth. In view of this finding and the poor K_m value for DMSP (millimolar range) of *E. limosum* PM31 compared to the value for the sulfate-reducing bacteria (micromolar range), the observed demethylation of DMSP by such acetogens is probably of no or very limited ecological significance. The demethylation of DMSP to MTPA at a rate of 50 nmol min^{-1} mg of $protein^{-1}$, as shown with strain PM31, is of clear biotechnological relevance, however (16). Biologically produced MTPA can be used in the production of natural flavors.

Since the demethylation of DMSP is of no obvious benefit for the organism, the process is most probably catalyzed by an enzyme which is normally involved in another demethylation of a structurally related true substrate such as glycine betaine. In analogy with the biochemistry of the metabolism of other methylated substrates in acetogens (methanol, methoxylated aromatics; see references 25, 26, and 38), one would expect the presence of a DMSP- and a glycine betaine-demethylating enzyme which would feed the methyl group into the methyl branch of the Wood-Ljungdahl (reductive CO dehydrogenase) pathway for acetyl-coenzyme A synthesis. Recently, the *O*-demethylase from *Acetobacterium dehalogenans* was purified and shown to consist of four components that were all required for the efficient catalysis of the methyl transfer from phenyl methyl ethers to tetrahydrofolate (25, 26). Purification of the DMSP- and betaine-demethylating system(s) will be necessary to reveal whether these systems are indeed identical and of a similar complexity to the *O*-demethylating system. The low activities of a glycine-betaine methyltransferase we detected are only a first indication for the nature of the methyltransferase reaction. The media used contained yeast extract because the strain grew poorly in its absence; therefore, glycine betaine was always present in low concentrations in the media. Under these conditions DMSP demethylation by cells grown under fructose limitation does not require de novo protein synthesis. Furthermore, betaine and DMSP were demethylated simultaneously. The low activities of the methyltransferase reaction with glycine betaine did not allow a detailed kinetic analysis of the effect of DMSP on the activity. There are some examples in the literature that show that DMSP can indeed be a substrate (and sometimes with higher activities!) for a betaine-utilizing enzyme, but this is not a general rule. Mammalian betaine-homocysteine methyltransferase is known to be active toward DMSP (14). In *Sinorhizobium meliloti* dimethylsulfonioacetate, the acetate analog of DMSP, is demethylated via the glycine betaine demethylating system, which in this organism is thought to be a glycine betaine-homocysteine methyltransferase (37), but DMSP is not demethylated by *S. meliloti* and is used only as an osmoprotectant (35). Similarly, in extracts of *Pseudomonas denitrificans* betaine and dimethylsulfonioacetate can function as methyl donors for homocysteine methylation, whereas DMSP cannot (43). In the sulfate-reducing bacterium strain WN the demethylation of DMSP is catalyzed by a DMSP-tetrahydrofolate methyltransferase which is not active toward glycine betaine (22, 23).

The utilization of both DMSP and glycine betaine is completely inhibited as long as fructose is present in low millimolar concentrations but not when fructose is the limiting substrate in chemostat cultures. We do not know what mechanism underlies this phenomenon in our strain. In experiments with a different strain of *E. limosum*, Genthner and Bryant (15) observed a similar repression of the utilization of methanol, hydrogen, and isoleucine by 2 mM glucose, leading to their utilization after the glucose and a clear lag phase.

Why these bacteria grow on glycine betaine and show marginal or no growth on DMSP can only be speculated about. At the moment there are no reasons to believe that the intracellular conversion of DMSP and carbon dioxide to MTPA, acetate, and butyrate yields less ATP than the analogous fermentation of glycine betaine and carbon dioxide. Our value of the molar growth yield on glycine betaine was considerably lower (approximately 3.0 g [dry weight] of cells/mol) than the value (9 g/mol) reported by Müller et al. (34) for another strain of *E. limosum*, but the order of magnitude in both cases shows that less than one ATP per betaine is produced which can be used for biosynthesis (cf. reference 1). Three factors might contribute to differences in the amount of ATP available for biosynthesis as a result of betaine and DMSP fermentation: differences in the energetic costs of betaine and DMSP uptake, differences related to product (dimethylglycine and MTPA) export, and maintenance energy effects. The transport of DMSP across the membrane might be energetically more expensive than the transport of glycine betaine. How DMSP and glycine betaine are transported in *E. limosum* PM31 is not known, but both in *Escherichia coli* and *Bacillus subtilis* different mechanisms for glycine betaine transport exist (24, 31). In *E. coli*

glycine betaine can be transported across the membrane by the constitutive, low-affinity, proton-motive-force-driven system ProP and the inducible, high-affinity, ATP-consuming system ProU (31, 32). Differences in the energetic costs of glycine betaine and DMSP transport in *E. limosum* might strongly affect the growth yield. If DMSP would be transported mainly by an ATP-consuming system and glycine betaine mainly in symport with one proton, a considerable difference in molar growth yield might be expected. Interestingly, recent work has shown that in *B. subtilis* DMSP is not taken up by the proton-motive-force-driven secondary betaine transporter OpuD; DMSP is taken up only by the ABC transporters OpuA and OpuC [G. Nau-Wagner, M. Jebbar, C. Blanco, and E. Bremer, Abstr. 2nd Int. Symp. Biol. Environ. Chem. DMS(P) Related Compounds, p. 14, 1999]; however, in *E. coli* DMSP is taken up by both ProP and ProU (6). Differences in the mechanism of export of dimethylglycine and MTPA from the cells might also play a role. Because of the stronger structural similarity of butyrate and MTPA, the energy-consuming export system described for butyrate in *E. limosum* might also be involved in MTPA export and not in dimethylglycine export (29). Maintenance energy is known to affect the molar growth yields at low specific growth rates; the rates of DMSP and glycine betaine utilization did not differ so much that maintenance energy effects alone can easily explain the lack of growth on DMSP.

ACKNOWLEDGMENTS

This work was supported by Quest International, b.v., Naarden, The Netherlands.

We thank Lubbert Dijkhuizen for valuable suggestions and comments, Marc van der Maarel for sequencing the 16S rRNA genes, and Irma van der Veen and Manny Nienhuis for technical assistance.

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