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Betaine: New Oxidant in the Stickland Reaction and Methanogenesis from Betaine and L-Alanine by a *Clostridium sporogenes-Methanosarcina barkeri* Coculture

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Growing and nongrowing cells of *Clostridium sporogenes* fermented betaine with L-alanine, L-valine, L-leucine, and L-isoleucine as electron donors in a coupled oxidation-reduction reaction (Stickland reaction). For the substrate combinations betaine and L-alanine and betaine and L-valine balance studies were performed; the results were in agreement with the following fermentation equation: 1 R- $CH(NH_2)$-COOH + 2 betaine + 2 $H_2O \rightarrow$ 1 R-COOH + 1 CO_2 + 1 NH_3 + 2 trimethylamine + 2 acetate. Growth and production of trimethylamine were strictly dependent on the presence of selenite in the medium. With cell suspensions it was shown that *C. sporogenes* was unable to catabolize betaine as a single substrate. Betaine, however, was reduced to trimethylamine and acetate under an atmosphere of molecular hydrogen. For the reduction of betaine by cell extracts of *C. sporogenes*, dimercaptans such as 1,4-dithiothreitol could serve as electron donors. No betaine reductase activity was detected in cells grown in a complex medium without betaine. The pH optimum of betaine reductase was at pH 7.3. When *C. sporogenes* was cocultured with *Methanosarcina barkeri* strain Fusaro on betaine together with L-alanine, an almost complete conversion of the two substrates to CH_4, NH_3, and presumably CO_2 was observed.

Trimethylamine has been shown to be a growth substrate for strains of the genus *Methanosarcina* which convert this compound into CH_4, NH_3, and CO_2 (15, 33). Production of trimethylamine is accomplished in anaerobic habitats by bacterial reduction of trimethylamine-*N*-oxide (30) and by cleavage of choline (3, 14). The contribution of choline to methane production via trimethylamine was demonstrated in the rumen (22, 25), in bacterial enrichments (15), and in a coculture of *Methanosarcina barkeri* and *Desulfovibrio* sp. strain G1. (K. Fiebig, Ph.D. thesis, University of Göttingen, 1981). Recently, the importance of trimethylamine as precursor of methane in salt marsh sediments was reported (24).

Betaine has also been shown to give rise to a rapid production of methane in bacterial enrichments (15). On the basis of its chemical structure it can be assumed that trimethylamine is formed from betaine as an intermediate during methanogenesis. However, the first pathway that became known for the anaerobic breakdown of betaine did not support this view. *Eubacterium limosum* was found to degrade this compound to *N,N*-dimethylglycine, acetate, and butyrate (21).

In this report we show that *Clostridium sporogenes* is able to form trimethylamine and acetate from betaine in an oxidation-reduction reaction (Stickland-type reaction [1, 23, 29]), in which betaine acts as an electron acceptor for the oxidation of certain amino acids. Furthermore, methane production from betaine in combination with L-alanine via trimethylamine is demonstrated in a coculture of *C. sporogenes* and *M. barkeri* strain Fusaro.

MATERIALS AND METHODS

Organisms. Cultures of *C. sporogenes* (DSM795) and *Methanosarcina barkeri* strain Fusaro (DSM804) were obtained from the German Collection of Microorganisms, Göttingen.

Growth media and cultivation. For medium preparation and cultivation anaerobic cultural techniques were employed (5). All media were prepared under an atmosphere of 100% N_2. Traces of oxygen were removed from N_2 by passing it through a Pyrex column packed with a copper catalyst and heated to approximately 350°C. The growth temperature was 37°C. Hungate tubes (16 by 125 mm; Bellco Glass Inc., Vineland, N.J.) containing 5 ml of medium were used to maintain the two organisms in pure culture and coculture. Substrates such as betaine and trimethylamine were heat sterilized. All amino acids were filter sterilized under N_2 and were injected into the autoclaved medium with a hypodermic syringe. All media

were reduced with sterile 0.03% (wt/vol) sodium sulfide before inoculation.

C. sporogenes was routinely grown in a basal medium containing the following in 1.0 liter of distilled water: K_2HPO_4, 2.28 g; $FeSO_4 \cdot 7H_2O$, 2 mg; $CaCl_2 \cdot 2H_2O$, 4 mg; $MgCl_2 \cdot 6H_2O$, 10 mg; NaCl, 80 mg; biotin, 0.02 mg; nicotinic acid, 1 mg; *p*-aminobenzoic acid, 0.05 mg; $Na_2SeO_3 \cdot 5H_2O$, 0.26 mg; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2 g; acid-hydrolyzed casein hydrolysate (E. Merck AG, Darmstadt, West Germany), 2 g; resazurin, 1 mg. L-Alanine and betaine were added to the basal medium in a final concentration of 25 and 50 mmol per liter, respectively.

The organism was anaerobically mass cultured in 20-liter carboys. After 20 h of growth cells were harvested and collected by continuous-flow centrifugation under a steady stream of N_2 and stored under an N_2 atmosphere at −70°C. For experiments with cell suspensions, freshly harvested cells from stationary-phase cultures were washed once with anaerobic 0.2 M potassium phosphate buffer (pH 7.4) and resuspended in the same buffer.

Cells used for nutritional studies were cultured in Hungate tubes. Growth was followed by measuring the optical density at 600 nm with a Bausch & Lomb Spectronic 88 spectrophotometer. The growth experiments were performed at 37°C in 1-liter bottles that were sealed with butyl rubber stoppers. The basal medium supplemented with substrates as indicated below was inoculated with a 5% cell suspension of *C. sporogenes*. At 3- to 4-h intervals the gas atmosphere of the culture was analyzed (see below), and samples of the culture were withdrawn. The optical density at 600 nm was measured in a Zeiss PM4 spectrophotometer with cuvettes of 1-cm light path. Cells were harvested by centrifugation at 13,000 × *g* for 15 min at 4°C. The clear supernatant fluid was stored at −20°C until substrates and fermentation products were determined.

M. barkeri and the coculture of *M. barkeri* and *C. sporogenes* were grown in the basal medium described for *M. barkeri* by Hippe et al. (15), except that 100 mg of nicotinic acid per liter and 0.26 mg of $Na_2SeO_3 \cdot 5H_2O$ per liter were added. Yeast extract and Casitone were replaced by Trypticase (0.2%, wt/vol) and acid-hydrolyzed casein hydrolysate (0.2%, wt/vol; Merck). $NaHCO_3$ was not included in the medium. For pure cultures of *M. barkeri* the medium was supplemented with 100 mmol of trimethylamine per liter. The coculture was grown on basal medium with 25 mmol of L-alanine per liter together with 50 mmol of betaine per liter as substrates. The coculture was started by inoculating a Hungate tube containing 5 ml of the medium mentioned above with 0.25 ml each of a liquid culture of *C. sporogenes* and *M. barkeri*. It was maintained by transferring 0.5 ml of the culture into 5 ml of the same medium every 6 days. The coculture experiment was performed without shaking in 1-liter bottles closed with black butyl rubber stoppers. Samples were withdrawn as described above for the experiments with pure cultures of *C. sporogenes*.

Analytical methods. Ammonia was determined enzymatically with glutamate dehydrogenase (10). For the quantitative determination of betaine the samples were subjected to ion-exchange chromatography by the method of Carruthers et al. (6). The betaine content in the filtrate was estimated by the colorimetric method of Focht and Schmidt (12).

Capillary tube isotachophoresis was used for quantitation of the amino acids glycine, L-alanine, and L-valine (17). A capillary tube isotachophoretic analyzer IP-2A (Shimadzu Seisakusho Ltd., Kyoto, Japan) equipped with a potential gradient detector was employed. The separation of the three amino acids was carried out at 20°C in a two-stage migration tube system consisting of a 4-cm tube with 1.0-mm inner diameter (first migration tube) and a 10-cm tube with 0.5-mm inner diameter (second migration tube). The migration current was 250 μA in the first stage and 125 μA until the end of the analysis. The leading electrolyte consisted of 0.01 M hydrochloric acid and 0.02 M 2-amino-2-methyl-1,3-propanediol; 0.5% polyvinylalcohol was added to sharpen the zone boundaries. The terminating electrolyte was 0.01 M β-alanine and $Ba(OH)_2$ at pH 10.5. The electrolytes were prepared under an N_2 atmosphere and were stored under nitrogen. Total assay time was about 15 min. Samples (8 μl) appropriately diluted with water were subjected to isotachophoresis. Quantitative analysis was done by measuring the zone length (millimeters) with the aid of the differential curves and by comparison of sample zone length to those of standards. Standard solutions contained up to 3.5 mmol of each of the amino acids per liter in uninoculated medium.

In cell-free culture medium methylamine, dimethylamine, and trimethylamine were quantified by gas chromatography. Liquid samples (2 μl) alkalized by the procedure of Hippe et al. (15) were injected into a Perkin Elmer 900 gas chromatograph equipped with a flame ionization detector. Separation of the three amines took place in a 2-m by 2-mm glass column on Carbopack B (60-80 mesh) modified with 4% Carbowax 20 M and 0.8% KOH (Supelco Inc., Bellefonte, Pa.). The inlet port and column were heated to 110°C, and the detector was maintained at 200°C. N_2 (10 ml/min) was used as the carrier gas. The evaluation of peaks was done with a Hewlett-Packard 3370 B integrator. The reference standard solution contained 0.25 to 50 mmol of methylamines per liter in uninoculated medium. Ethanol, acetate, propionate, isobutyrate, isovalerate, 2-methylbutyrate, and valerate were also separated and quantified by gas chromatography with the same apparatus. All conditions were as described by Müller et al. (21).

Gas analysis. The total gas production of cultures was followed by measuring the gas formed with hypodermic syringes as described by Chung (7). In pure cultures of *C. sporogenes*, H_2 and CO_2 were analyzed in the atmosphere over the culture with a Perkin Elmer Fractometer F 20 H equipped with a hot wire detector and a 2-m steel column (1/8-in. [ca. 7.8-mm] inner diameter) packed with molecular sieve 5Å (Merck). The injector and the detector were heated to 220°C, and the oven temperature was maintained at 175°C. N_2 was used as the carrier gas at a flow rate of 60 ml min^{-1}. After the gas phase above the liquid was allowed to reach equilibrium with atmospheric pressure a sample of 100 μl was taken from the culture vessel with a gas-tight pressure-lock syringe (Precision Sampling Corp., Baton Rouge, La.) and was injected on the column. The analysis of the chromatographic data was done with an Autolab System IV B integrator (Spectra Physics, Santa Clara, Calif.).

TABLE 1. Effect of various reductants on growth of *C. sporogenes* with betaine and formation of trimethylamine[a]

Substrate added	Growth[b]	Trimethylamine (mmol per liter)
None	0.28	0
Betaine	0.38	5.2
Betaine + H_2[c]	0.42	10.8
Betaine + L-alanine[d]	0.82	25.2
Betaine + L-valine	0.79	24.5
Betaine + L-leucine	0.77	24.0
Betaine + L-isoleucine	0.80	24.8

[a] Where indicated, basal medium was supplemented with 25 mmol of betaine per liter and with 25 mmol of one of the amino acids per liter. Culture tubes containing 5 ml of medium were incubated for 48 h at 37°C.

[b] Optical density at 600 nm.

[c] H_2 as gas phase.

[d] The optical density was <0.38 on medium without betaine, but supplemented with one of the amino acids indicated.

In the coculture experiments H_2 was analyzed with the same procedure and apparatus, but the oven temperature was 75°C to separate H_2 from CH_4. Methane was determined with the Perkin Elmer 900 gas chromatograph by the method of Hippe et al. (15).

The amount of H_2, CO_2, and CH_4 (micromoles) formed per milliliter of culture during an experiment was computed by the method of Mah et al. (19). The theoretical solubilities of the gases and, in the case of CO_2, the effect of the bicarbonate equilibrium at the respective pH of the medium were taken into consideration. All values were corrected for the decreasing volume of the culture due to samples taken for analysis as described by McInerney and Bryant (20).

Preparation of cell extracts and enzyme assays. When needed, cells were thawed, suspended in 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-KOH buffer (pH 7.3) containing 1 mM MgK_2-EDTA and 1 mM 1,4-dithiothreitol and washed twice in the same buffer. After the addition of a small amount of DNase, the cells were disrupted by passing them once through an Aminco French pressure cell (13,500 N/cm^2). The broken cells were directly collected in a stoppered, anaerobic bottle which was fitted with a syringe-tube connector to the French pressure cell. Cell debris was removed by centrifugation for 30 min at 30,000 × *g* at 4°C. The supernatant fraction was used as such, or 2-ml samples were dialyzed against 2.0 liters of 20 mM TES-KOH buffer (pH 7.3) containing 1 mM MgK_2-EDTA for 4 h at 4°C. The cell extracts were stored at −70°C. Protein was estimated by the method of Beisenherz et al. (2). Manipulations such as filling of cell-free extracts into the centrifuge tubes or the French pressure cell or dialysis were performed in a flexible, anaerobic glove box (type A; Coy Laboratory Products, Inc., Ann Arbor, Mich.) containing an atmosphere of 98.5% N_2 and 2.5% H_2. The amount of oxygen was kept below 2 ppm (2 μg/g) measured with a couloximeter (Chemical Sensor Development, Inc., Torrance, Calif.).

Betaine reductase activity was determined by following trimethylamine formation from betaine. For routine assays each reaction mixture (1 ml) contained 60 mM TES-KOH buffer (pH 7.3), 20 mM K_2HPO_4, 4 mM AMP, 4 mM ADP, 6 mM $MgCl_2$, and 40 mM 1,4-dithiothreitol. The incubation tubes (2-ml glass vials) were flushed with nitrogen and stoppered with serum caps. After the addition of 2.0 to 11.0 mg of protein per ml of assay mixture and a preincubation of 6 min at 30°C, the reaction was started with 40 mM betaine and incubated at 30°C. Samples (100 μl) were withdrawn at 2-min intervals, and the reaction was stopped with 10 μl of 4 N HCl. Denaturated protein was sedimented by centrifugation. Samples (5 μl) of the supernatant were used for the quantitative determination of trimethylamine by capillary tube isotachophoresis. The same apparatus mentioned above was used.

The leading electrolyte consisted of 0.01 M potassium acetate in water-methanol (50:50, vol/vol). The pH was adjusted to 4.0 with acetic acid. Triton X-100 (0.2%) purified over a mixed-bed ion exchanger (Merck) was added. The terminating electrolyte was 0.01 M histidine in water-methanol (50:50 vol/vol). The pH was adjusted to 4.3 with acetic acid. The separations were carried out in a 20-cm tube (1.0-mm inner diameter) and a 10-cm tube (0.5-mm inner diameter) at 20°C. The migration current was 150 μA for 28 min and 100 μA until the end of the analysis. The total assay time was about 50 to 55 min. The standard solutions contained up to 3.0 mM trimethylamine in crude extract.

For determination of glycine reductase activity, crude extracts were prepared in 20 mM *N*-tris(hydroxymethyl)methylglycine–KOH buffer under anaerobic conditions (32). The activity was determined by following [1-^{14}C]acetate production from [1-^{14}C]glycine with 1,4-dithiothreitol as the electron donor (28). The assay contained 40 μmol of [1-^{14}C]glycine per ml of assay mixture (5,550 dpm of glycine per μmol). The reaction mixtures were incubated in nitrogen-flushed, stoppered test tubes (2-ml glass vials) for 5 min at 30°C.

Chemicals. All chemicals used were of the highest purity commercially available and were purchased from Merck. 2-Amino-2-methyl-1,3-propanediol was obtained from Fluka, Buchs, Switzerland. 1,4-Dithiothreitol, *N*-tris(hydroxymethyl)methylglycine, TES, and 2-*N*-morpholineethanesulfonic acid buffers were from Sigma Chemical Co., St. Louis, Mo. Enzymes, ADP, AMP, NADH, and NADPH were from Boehringer, Mannheim, Germany. Bovine serum albumin was from Serva, Heidelberg, Germany. [1-^{14}C]glycine (58 mCi/mmol) was obtained from Amersham-Buchler, Braunschweig, Germany.

RESULTS

Growth of *C. sporogenes* on betaine together with amino acids. When several clostridial species were tested for the formation of trimethylamine from betaine, it was found that *C. sporogenes* produced some trimethylamine from that compound. The amount was increased under an atmosphere of molecular hydrogen and reached maximal values when an amino acid such as L-alanine, L-valine, L-leucine, or L-isoleucine was also present in the medium at substrate levels (Table 1). Under these conditions growth was

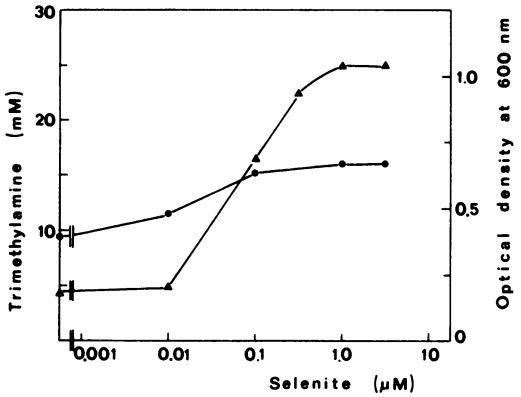

FIG. 1. Effect of selenite on growth of *C. sporogenes* and trimethylamine formation from betaine. Basal medium was supplemented with 25 mM L-alanine and 25 mM betaine. Symbols: ●, optical density at 600 nm; ▲, trimethylamine.

stimulated, and the final optical density was twice as high as in the absence of betaine. L-Serine, L-phenylalanine, L-ornithine, L-arginine, and L-histidine did not support the formation of trimethylamine from betaine.

Growth on betaine together with one of the aliphatic amino acids was strictly dependent on the trace element selenium. At 1 μM selenite maximal formation of trimethylamine was observed, and the turbidity of the culture reached maximal values (Fig. 1). To show the effect of selenite, glassware, stoppers, syringes, and needles were used which had not had any contact with this chemical before. The requirement for selenium appeared to be specific. The addition of molybdate, tungstate, nickel, and stannate failed to stimulate growth.

The time course of substrates consumed and fermentation products formed during growth of *C. sporogenes* on 15 mmol of L-valine per liter and 45 mmol of betaine per liter is shown in Fig. 2. From 1 mol of L-valine 1 mol each of isobutyrate, CO_2, and presumably NH_3 was produced. The decrease in betaine concentration was associated with a corresponding increase of equimolar concentrations of trimethylamine and acetate. The oxidation of 1 mol of L-valine provided reducing power for the reduction of 2 mol of betaine. Residual betaine in the medium was not utilized. Only traces of H_2 (1.8 mmol/liter) were formed; they were neglected in balance calculations, because comparable amounts of H_2 were produced in media without substrates as well. The high carbon yield and even redox value apparent from the data summarized in Table 2 indicated that all major products had been recovered. These results demonstrated that beta-

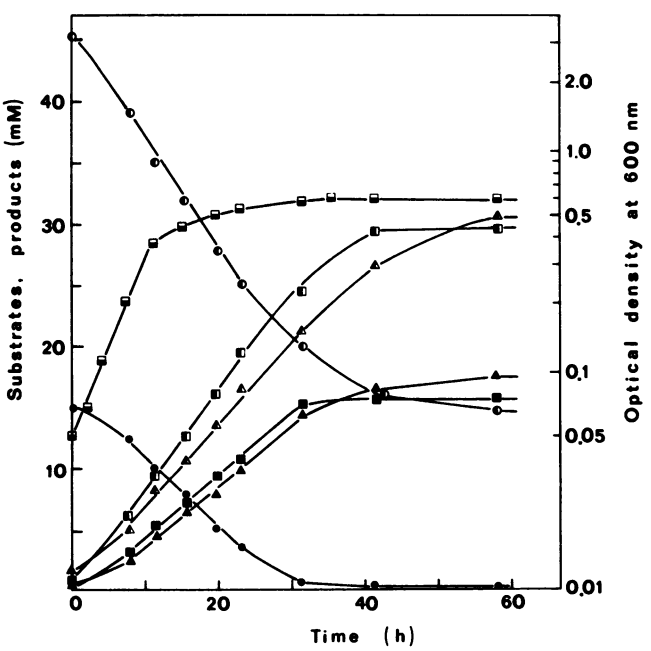

FIG. 2. Growth of *C. sporogenes* on a medium containing 15 mM L-valine and 45 mM betaine and time course of product formation. The organism was grown anaerobically at 37°C in a 1-liter flask and was inoculated with a culture grown on the same substrate combination. Symbols: ▣, optical density at 600 nm; ◐, betaine; ●, L-valine; ■, CO_2, ▲, isobutyrate; ◧, trimethylamine; ◭, acetate.

TABLE 2. Balance of betaine fermentation by *C. sporogenes* in the presence of increasing amounts of L-valine[a]

Expt	Concn (mol/100 mol of betaine)								Carbon balance (%)	O/R balance[b]
	Substrates		Products							
	L-Valine	Betaine	Isobutyrate	CO_2	NH_3[c]	Trimethylamine	Acetate	Ethanol		
A	52	100	53.5	51.6	52.0	98.7	97.9	0	99.8	0.99
B	82.4	100	81.8	86.8	82.4	99.6	71.8	27.3	99.8	0.97
C	93.3	100	88.9	100.7	93.3	98.5	71.9	27.8	98.1	0.92
D	142	100	137.7	138.5	142.0	98.1	52.3	50.0	98.0	0.90

[a] *C. sporogenes* was grown in basal medium supplemented with the following: (A) 15 mM L-valine plus 45 mM betaine, (B) 27 mM L-valine plus 56 mM betaine, (C) 50.4 mM L-valine plus 54 mM betaine, and (D) 100 mM L-valine plus 26 mM betaine. The substrate and product concentrations were determined at intervals of 3 to 4 h over a period of 70 h. The balances were calculated from the values at the end of product formation. All values were corrected for any product formed in the absence of the substrates. Betaine was completely consumed in experiments C and D, and L-valine was completely consumed in experiments A, B, and C. The following were not consumed: 15 mM betaine in experiment A, 23.2 mM betaine in experiment B, and 6 mM L-valine in experiment D.

[b] The oxidation/reduction (O/R) balance was calculated on the basis of the oxidation numbers of all atoms of each compound (21, 26).

[c] Calculated from the amount of L-valine consumed.

ine and L-valine were fermented together in a Stickland-type reaction.

Ethanol was usually also among the products (Table 2). Only when betaine was present in the medium in a threefold excess over L-valine, was it not detectable (Table 2). Upon the addition of equal amounts of valine and betaine to the growth medium 0.3 mol of ethanol and 0.72 mol of acetate were formed per mol of betaine reduced (Table 2). With L-valine and betaine in a ratio of 4:1 the pattern of fermentation products shifted to the formation of equal amounts of ethanol and acetate (Table 2). Surprisingly, ethanol was also produced when the medium was supplemented with 1 equivalent of L-valine and 2 equivalents of betaine (Table 2). Under these conditions L-valine was completely degraded to isobutyrate, NH_3, and CO_2, whereas only 60% of the betaine was fermented, indicating that the electrons derived from L-valine oxidation were preferentially used for the reductive cleavage of betaine, but apparently they were also employed to some extent for the conversion of part of the acetate formed from betaine to ethanol. Analogous results were obtained when L-valine was replaced by L-alanine. The products of L-alanine oxidation were acetate, NH_3, and CO_2.

Stickland reaction with betaine in cell suspen-

TABLE 3. Reduction of betaine and glycine by cell suspensions of *C. sporogenes*[a]

Cells	Substrates	Products (mmol per liter)			
		NH_3	Acetate	Trimethylamine	Isobutyrate
Betaine grown	Betaine	0.5	5.75	5.8	ND
	Betaine + H_2[b]	0.6	52.5	51.5	ND
	Glycine + H_2[b]	51.5	47.0	1.4	ND
	L-Alanine + betaine	22.6	78.8	48.4	0
	L-Valine + betaine	27.7	24.9	48.5	24.5
	L-Alanine + glycine	74.4	78.2	0.63	ND
Glycine grown	Glycine + H_2[b]	50.1	49.5	0.45	ND
	Betaine + H_2[b]	0.3	0.6	0.55	ND
	L-Alanine + glycine	76.5	77.0	0.46	ND
	L-Alanine + betaine	0.58	0.82	0.64	ND

[a] The reaction mixtures contained 40 mM potassium phosphate buffer (pH 7.4), 0.03% $Na_2S \cdot 9H_2O$, 80 mg (wet weight) of washed cells per ml of assay sample, 50 mM oxidant (betaine or glycine), and 25 mM reductant (L-alanine or L-valine) where indicated. The assay was incubated under N_2 gas phase at 37°C. Samples were withdrawn at intervals of 30 min for 3.5 h. Before harvesting cells were grown on 25 mM L-alanine together with 50 mM betaine in experiment A or with 50 mM glycine in experiment B. ND, Not determined. CO_2 production was not determined.

[b] H_2 as gas phase.

TABLE 4. Influence of the growth substrate on the glycine and betaine reductase activities in crude extracts of *C. sporogenes*[a]

Growth substrates	Sp act (μmol · min^{-1} per mg of protein)	
	Glycine reductase	Betaine reductase
L-Alanine + glycine	0.168	<0.001
L-Alanine + betaine	0.015	0.048

[a] *C. sporogenes* was grown in the complex medium with 25 mM L-alanine together with 50 mM betaine or 50 mM glycine. Crude extracts were prepared under anaerobic conditions in 20 mM TES-KOH buffer (pH 7.3) for determination of betaine reductase activity as measured by the rate of trimethylamine production, and in 20 mM *N*-tris(hydroxymethyl)methylglycine–KOH buffer (pH 8.2), for determination of glycine reductase activity as determined from the rate of [1-^{14}C]acetate formed from [1-^{14}C]glycine.

sions. Cells of *C. sporogenes* which had been grown in a complex medium either with L-alanine and betaine or with L-alanine and glycine as substrates were suspended in buffer and tested as to their ability to use betaine and glycine as electron acceptors. Washed cell suspensions of betaine-grown cells exhibited only a slight ability to ferment betaine as a single substrate. Reducing equivalents derived from H_2 or the amino acids L-alanine and L-valine were required for cleavage of 1 mol of betaine to 1 mol each of trimethylamine and acetate (Table 3). The slight reduction of betaine observed in the absence of hydrogen donors was probably caused by endogenous, oxidizable substrates in the cells. When L-alanine or L-valine was present as a single substrate, likewise less than 10% of the amount was oxidized as compared with the amount oxidized together with betaine. Glycine, too, acted as an electron acceptor and was reduced to acetate and NH_3 in the presence of H_2 or L-alanine.

However, washed cell suspensions of glycine-grown cells could only use glycine, but not betaine, as a hydrogen acceptor, which indicates that specific components of the transport system for betaine or of the betaine-reducing enzyme system (or of both) were only synthesized with betaine present in the medium (Table 3).

Reduction of betaine in cell extracts. The results of Table 3 show that there existed an analogy between betaine reduction and glycine reduction as carried out by cell suspensions. However, glycine-grown cells could not reduce betaine. To find out whether this was due to the substrate specificity of the reductase systems involved, cell extracts were investigated. The betaine reductase activity was measured under an atmosphere of N_2 by determining isotachophoretically the amount of trimethylamine formed. The reaction was started with 40 mM betaine after a preincubation of the assay mixture containing all components and cell extracts for 6 min. Samples were withdrawn at 2-min intervals over a period of 12 min.

Crude extracts prepared from glycine-grown cells reduced glycine by using 1,4-dithiothreitol as a hydrogen donor, but were unable to reduce betaine (Table 4). In contrast, crude extracts of cells grown with betaine as the substrate exhibited activity and had a largely diminished activity with glycine (Table 4). These data suggested that the betaine-reducing system was inducible and different from the glycine reductase. In analogy to the glycine reductase system (28), extracts of betaine-grown cells used 1,4-dithiothreitol as an artificial electron donor for the reductive cleavage of betaine when added at substrate levels.

The influence of pH on the enzyme activity was investigated with 0.06 M potassium phosphate buffer, 0.06 M Tris-hydrochloride buffer, 0.06 M *N*-tris(hydroxymethyl)methylglycine–KOH buffer, 0.06 M 2-*N*-morpholineethanesulfonic acid–KOH buffer, and 0.06 M TES-KOH buffer at pH values in the range of the buffer capacity of the respective buffer systems. At the same pH values, the highest enzyme activities were observed with TES-KOH buffer. The pH optimum was at pH 7.3 with this buffer. In analogy to the assay for the determination of the glycine reductase activity (28, 32), 20 mM K_2HPO_4, 4 mM ADP, 4 mM ADP, 4 mM AMP, and 6 mM $MgCl_2$ were routinely added to the assay mixture. Under optimal conditions the time course of betaine reduction was linear over incubation periods up to 8 min (Fig. 3). A specific activity of 0.046 μmol of trimethylamine formed per min per mg of protein was obtained. A linear relationship between the amount of betaine reduced and extract concentrations was found over a range of 2.0 to 11.0 mg of protein per ml of incubation mixture.

Compared with the activity of 1,4-dithiothreitol as a hydrogen donor, we observed 47.5% activity with 2-mercaptoethanol, 28.8% activity with 2,3-dimercaptopropanol-1, and 62% activity with NADH. Betaine was not reduced with NADPH, 6,8-dimercaptooctanoate, or glutathione (reduced).

Extracts prepared under anaerobic conditions in 20 mM Tris-hydrochloride buffer (pH 7.1) or in 20 mM *N*-tris(hydroxymethyl)methylglycine–KOH (pH 8.2) and stored at 4°C lost all activity within 4 h. Preparations in 20 mM TES-KOH (pH 7.3) retained 62% activity after 4 h and 20% activity after 8 h. Stability was somewhat improved by adding 5 mM choline; 10% (wt/vol) sucrose, 10% (wt/vol) glycerol, or 2 mM phenylmethylsulfonyl fluoride had no effect. Enzyme

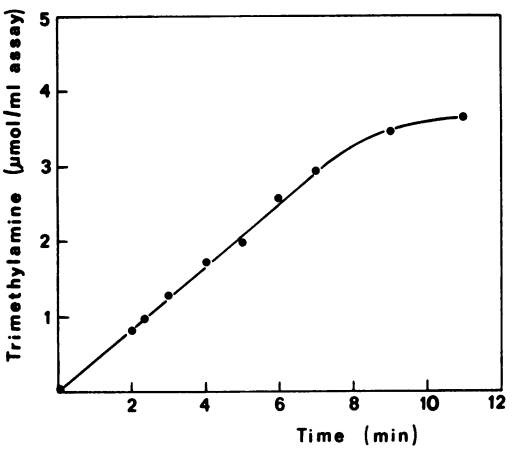

FIG. 3. Time course of the formation of trimethylamine from betaine as catalyzed by crude extracts. The reactions were carried out with 9.03 mg of protein of nondialyzed extracts per ml of incubation mixture.

activity was lost very quickly in the presence of traces of O_2 in the buffer used or in the assay mixture. At −70°C extracts retained their activity for at least 2 weeks. Dialysis of the extracts against 20 mM TES-KOH buffer (pH 7.3) at 4°C for 4 h lowered the activity to about one-half of that detected in crude extracts after 4 h. In dialyzed extracts the interference of assays by residual substrate was largely reduced, and betaine was cleaved to equimolar amounts of trimethylamine and acetate. The reductase activity was 0.011 μmol min^{-1} per mg of protein as measured by the rate of acetate production, and 0.013 μmol min^{-1} per mg of protein as determined from the rate of trimethylamine formation.

Methanogenesis of betaine and L-alanine by a coculture. *C. sporogenes* catabolized 51.2 mmol of betaine in combination with 48.7 mmol of L-alanine as the hydrogen donor to 49 mmol of NH_3, 48.7 mmol of CO_2, 15 mmol of ethanol, 49.5 mmol of trimethylamine, and 85 mmol of acetate. Therefore, most of the organic carbon was present in the form of the two methanogenic substrates trimethylamine and acetate, and a coculture of *C. sporogenes* and *M. barkeri* should bring about the conversion of most of the carbon of betaine and L-alanine into methane and CO_2. *M. barkeri* alone was unable to grow on these compounds. However, methanogenesis occurred when *M. barkeri* was cocultured with *C. sporogenes* on the betaine–L-alanine combination (Fig. 4). Two phases of substrate conversions and methanogenesis could be distinguished. In the first phase of 5.5 days, all of the betaine and L-alanine was degraded by *C. sporogenes*. All of the trimethylamine formed in between was methanogenized via dimethylamine and methylamine as intermediates by *M. barkeri* as described for the pure culture of this organism (15). Trimethylamine formation and consumption proceeded simultaneously, for its maximal concentration reached only 21 mM as compared with 50 mM observed with a pure culture of *C. sporogenes*. At the same time 15.6% of the expected acetate was used by *M. barkeri* and apparently contributed to growth and methane formation. The rate of methane formation in the first phase was 26.5 mmol per liter per day. At the end of the first phase 14.8 mmol of ethanol per liter and 63.6 mmol of acetate per liter were left in the culture medium. Although in the second phase acetate was methanogenized at a rate of 1.6 mmol of CH_4 per liter per day and a substrate consumption rate of 1.2 mmol per liter per day as compared with 4.9 mmol per liter per day in the first phase, ethanol remained in the medium at the same concentration.

A pure culture of *C. sporogenes* produced 1.8 mmol of H_2 per liter. In the coculture only 0.25 mmol of H_2 per liter, corresponding to a partial pressure of 500 kPa, was detected after 12.5 h. The possibility is not excluded that *M. barkeri* utilized a small amount of H_2 under these conditions. After 12.5 h, there was no further bacterial production or degradation of H_2.

DISCUSSION

C. sporogenes is known to be capable of satisfying its energy requirements by means of coupled oxidation-reduction reactions between appropriate pairs of amino acids (Stickland reaction [1, 23, 29]). The reduction of the major electron acceptors glycine and proline, for instance, can be coupled to the oxidative deamination and decarboxylation of alanine, valine, leucine, and isoleucine, yielding the respective volatile fatty acids acetic acid, isobutyric acid, isovaleric acid, and 2-methylbutyric acid (4, 11, 13). The amino acids mentioned above have now been shown to serve also as hydrogen donors for the reduction of betaine. Molecular hydrogen can also function as reductant, but growth was only stimulated when one of the aliphatic amino acids was present in the medium in addition to betaine. The experiment carried out with L-alanine and L-valine as hydrogen donors allowed the formulation of the following fermentation equations: 1 R-CH(NH_2)-COOH + 2 H_2O → 1 RCOOH + 1 CO_2 + 1 NH_3 + 4 (H) and 2 betaine + 4 (H) → 2 trimethylamine + 2 acetate. This result is interesting for two reasons. It adds a new compound to the list of oxidants participating in the Stickland reaction, and it proves the existence of another reaction yielding trimethylamine under anaerobic conditions. The reductive cleavage of betaine generates the two meth-

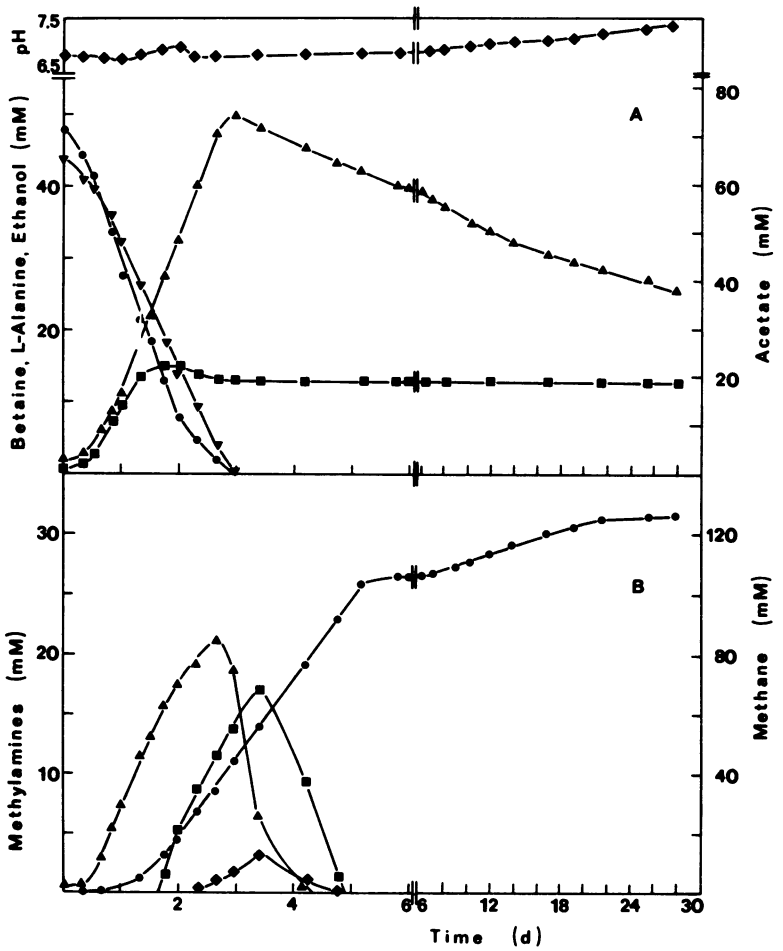

FIG. 4. Time course of the degradation of betaine in combination with L-alanine by *C. sporogenes* cocultured with *M. barkeri*. Each of two 1-liter culture bottles containing 540 ml of medium with 44 mM betaine and 47 mM L-alanine was inoculated with 40 ml of a mixed culture grown on the same substrates for 6 days. Values are mean values obtained from these two cultures. (A) Symbols: ▼, betaine; ●, L-alanine; ▲, acetate; ■, ethanol; ◆, pH. (B) Symbols: ●, methane; ▲, trimethylamine; ◆, dimethylamine; ■, methylamine.

anogenic substrates trimethylamine and acetate, and it is now conceivable that methane is rapidly produced by bacterial enrichments upon the addition of that compound (15). A coculture of *C. sporogenes* and *M. barkeri* on betaine plus L-alanine in a ratio of 2:1 led to an almost complete conversion of these substrates to NH_3, CO_2, and CH_4. The ethanol produced by *C. sporogenes* was left in the medium. The coculture was apparently unable to utilize the electrons generated during the oxidation of L-alanine for the reduction of CO_2 to CH_4 and did not influence the conversion of acetate to ethanol by *C. sporogenes*. It should be noted that growing cells of *C. sporogenes* degraded L-alanine (L-valine) together with betaine in accordance with the fermentation equations mentioned above only when betaine was present in the medium in a threefold excess over L-alanine (L-valine). If the excess of betaine was smaller, ethanol was formed as an additional product.

The fermentation of betaine and L-alanine by the coculture gave rise to the same products found in pure culture of *C. sporogenes*. This indicates that the mixed culture did not develop any syntrophic relation. The methanogen just grew in response to trimethylamine and acetate production. Similar results were obtained with a coculture of *Desulfovibrio* strain G1 and *M. barkeri* on choline (K. Fiebig, Ph.D. thesis).

In both coculture experiments methanogenesis occurred in two distinct phases. All of the trimethylamine and part of the acetate were simultaneously degraded by *M. barkeri* in the

first phase. The rate of acetate degradation in the presence of trimethylamine in the first methanogenic phase was much faster than the rate of acetate degradation alone in the second methanogenic phase. A simultaneous consumption of acetate and methanol during growth of different strains of the genus *Methanosarcina* was observed by several authors (16, 18, 27, 34, 35). Under these conditions acetate was preferentially assimilated and oxidized, accompanied by an increased reduction of methanol to methane (18, 35). After methanol depletion, the residual acetate was primarily used for methanogenesis (18, 35).

The involvement of selenium in the glycine reductase system of *Clostridium sticklandii* (8, 32) and *C. sporogenes* (9) is well established. In this report we have shown that cells of *C. sporogenes* were only able to use betaine as oxidant when selenite was present in the growth medium. This indicates a role of selenium in the betaine reductase system, too. Both reductase systems are distinct from one another. Cells grown on a complex medium containing glycine were found to contain glycine reductase activity. When glycine was replaced in this medium by betaine, betaine reductase activity was readily detectable. Some glycine reductase activity was also present in these cells. This probably was so because the complex constituents of the medium contained glycine. Suspensions of cells grown in a complex medium with glycine did, therefore, catabolize glycine, but not betaine, in the presence of hydrogen donors, whereas cell suspensions of betaine-grown cells reduced betaine and also glycine.

Both reductases can use dithiols as artificial hydrogen donors (28). The possibility is also not excluded that betaine reduction is associated with ATP synthesis from ADP and P_i as has been shown for the glycine reductase of *C. sticklandii* (31). The high instability of the betaine-reducing system in crude extracts has so far hindered attempts to purify the enzyme and to study it in more detail.

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