

## Formation of *N,N*-Dimethylglycine, Acetic Acid, and Butyric Acid from Betaine by *Eubacterium limosum*

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Two bacterial strains that grow anaerobically on betaine were isolated from enrichment cultures and identified as strains of *Eubacterium limosum*. In a mineral medium supplemented with yeast extract and Casitone, the doubling time of *E. limosum* strain 11A on betaine was 6 h at 37°C. The molar growth yield amounted to 9 g of dry cell mass per mol. Betaine was fermented in accordance with the following equation: 7 betaine + 2 CO<sub>2</sub> → 7 *N,N*-dimethylglycine + 1.5 acetate + 1.5 butyrate. *E. limosum* also grew on methanol and choline. The former was converted to acetate and butyrate, and the latter was converted to *N,N*-dimethylethanolamine, acetate, and butyrate. The conditions for the quantitative determination of *N,N*-dimethylglycine by capillary tube isotachopheresis have been determined.

Betaine is a constituent of plant and animal tissues. Many salt-tolerant plant species, particularly species of the *Chenopodiaceae*, *Gramineae*, *Solanaceae* and *Compositae* families, accumulate glycinebetaine in considerable concentrations (32). In sugar beets (*Beta vulgaris*), betaine accounts for 1.6% of the dry weight (10). Betaine is thought to act as a nontoxic cytoplasmic osmoticum which maintains the intracellular osmotic balance between cytoplasm and sodium chloride in vacuoles (16, 32). Likewise, it is involved in regulating the osmotic balance of intracellular fluids in marine invertebrates (3). Both in plants and mammalian tissues, betaine plays a dominant role as an intermediate in the transport of methyl groups for transmethylation. It is progressively demethylated through *N,N*-dimethylglycine and sarcosine to glycine with the concomitant formation of active 1-carbon fragments (15).

The ability to decompose betaine is widespread among aerobic microorganisms. Representatives of the genera *Pseudomonas*, *Agrobacterium*, *Arthrobacter*, and *Micrococcus* (1, 12, 23, 33) are able to grow with betaine as the sole carbon and nitrogen source. The observation that all betaine-utilizing bacteria oxidize betaine, *N,N*-dimethylglycine, and sarcosine at the same rate provides indirect evidence that aerobic decomposition of betaine in bacteria takes place in the same way as it does in higher organisms (23).

The anaerobic metabolism of betaine has been largely neglected. Hayward and Stadtman (17) isolated pure cultures able to grow anaerobically

on *N*-methyl compounds such as betaine and choline. However, only the choline-utilizing bacterium was studied further. It converted 1 mol of choline to 1 mol of trimethylamine and 0.5 mol each of acetic acid and ethanol. Later, the organism was identified as a strain of *Desulfovibrio desulfuricans* (31). Recently, the formation of methane from *N*-methyl compounds by mixed cultures was studied (18), and it was found that choline as well as betaine is a good substrate for methanogenesis by mixed populations. Since methanogenic bacteria are unable to utilize betaine, it was of interest to isolate anaerobic organisms which can grow with this compound. Such organisms have been obtained. Their identification as *Eubacterium limosum* and the overall fermentation process are described in this report.

### MATERIALS AND METHODS

**Medium and cultivation.** For medium preparation and cultivation, anaerobic cultural techniques were employed (6, 20). The medium in which the organism was grown was similar to that described by Hippe et al. (18) and contained the following (per liter): K<sub>2</sub>HPO<sub>4</sub>, 0.348 g; KH<sub>2</sub>PO<sub>4</sub>, 0.277 g; NH<sub>4</sub>Cl, 0.5 g; MgSO<sub>4</sub>·7 H<sub>2</sub>O, 0.5 g; CaCl<sub>2</sub>·2 H<sub>2</sub>O, 0.25 g; NaCl, 2.25 g; FeSO<sub>4</sub>·7 H<sub>2</sub>O, 2 mg; resazurin, 1 mg; vitamin solution (34), 10 ml; trace elements solution (28) without disodium-ethylenediaminetetraacetic acid and FeSO<sub>4</sub>, 3 ml; yeast extract (Difco Laboratories), 0.5 g; Casitone (Difco), 2 g; NaHCO<sub>3</sub>, 0.85 g; betaine (1 M), 50 ml; and Na<sub>2</sub>S·9 H<sub>2</sub>O, 0.3 g. Betaine and the reducing agent were heat sterilized separately as concentrated aqueous solutions under N<sub>2</sub> and were injected into the autoclaved medium with a hypodermic syringe. The final concentration of betaine was 50 mM. For growth

studies on various nitrogen-containing compounds, betaine was replaced by the respective substrate. The medium was prepared under a  $N_2$ - $CO_2$  (80:20; vol/vol) atmosphere; traces of oxygen were removed from gas streams by passage over a reduced-copper column heated to approximately 350°C.

To enrich and isolate organisms that degrade betaine, yeast extract and Casitone were omitted from the medium. Hungate tubes (18 by 125 mm; Bellco Glass, Inc.) containing 5 ml of medium and 1 g of mud sample were incubated at 37°C. Gas production, as measured with hypodermic syringes by the method of Hippe et al. (18) started after 4 days. When it ceased producing gas, betaine was added to give again a final concentration of 50 mM. Gas production started now after 1 day. After 1 week, 0.5 ml of the culture was transferred to 5 ml of medium. After five more weekly transfers under similar conditions, 0.5-ml portions of appropriate dilutions were streaked on agar in flat bottles by the method of Braun et al. (5). The agar medium consisted of the complete medium with 2% agar. After incubation at 37°C for 2 weeks, 24 colonies were picked and inoculated into 5 ml of medium in Hungate tubes. After 2 weeks, all tubes were turbid, but gas was not produced. Streaking of appropriately diluted portions on agar, isolation and transfer of single colonies into 5 ml of medium, and incubation were repeated twice. Two strains capable of growth on betaine were isolated by this procedure. Identification of these strains was done by the method of Holdeman et al. (19).

Cells used for nutritional studies were cultured anaerobically at 37°C in Hungate tubes that contained 5 ml of medium. Growth was indicated by an increase in the optical density at 600 nm, which was quantified directly in a Bausch & Lomb Spectronic 20 or 88 photometer.

The growth experiment was performed at 37°C in a 10-liter glass carboy that was sealed with a butyl rubber stopper. The medium was made anaerobic by passing an  $N_2$ - $CO_2$  stream (80:20) through it for 3 h; the medium was then inoculated with 5% cell suspension of *E. limosum* strain 11A. Portions of the culture were withdrawn anaerobically. The optical density at 600 nm was measured in a Zeiss PM4 spectrophotometer with cuvettes of 1-cm light path. Samples having an absorbance at 600 nm of more than 0.3 were appropriately diluted with medium. Cells were harvested by centrifugation at  $13,000 \times g$  for 15 min at 4°C. The clear supernatant fluid was stored at -20°C until substrates and fermentation end products were determined.

**Determination of dry weight and protein.** Portions of the culture (20 to 100 ml) were centrifuged at  $13,000 \times g$  for 15 min at 4°C, and the supernatant fluids were discarded. The cells were washed once with 20 ml of 0.1% (wt/vol) sodium chloride, suspended in 5 ml of distilled water, quantitatively transferred to small, preweighed vessels of aluminum, and dried at 90 to 95°C for 24 to 48 h. After cooling to room temperature in a desiccator, the vessels were weighed.

The protein content of whole cells was determined by the biuret method, performed in accordance with Schmidt et al. (30), with bovine serum albumin as a standard.

**Gas chromatographic procedures.** Acetic and butyric acids were quantified by gas chromatography with a Perkin Elmer model 900 chromatograph, equipped with a glass column (2 m by 2 mm) packed with Porapak QS (Riedel de Haën) and a flame ionization detector. The injector temperature was 200°C, the oven temperature was 170°C, and the detector temperature was 200°C. Nitrogen was used as carrier gas at a flow rate of 35 ml  $min^{-1}$ . Before injection, 20  $\mu$ l of 2 N HCl was added to 200  $\mu$ l of cell-free culture medium in stoppered 2-ml glass vials to release the free acids from their salts. After it was shaken vigorously, 3  $\mu$ l of the liquid sample was used for analysis. Evaluation of peaks was done with a Hewlett-Packard 3370 B integrator. The reference standard solution contained 0.5 to 10 mM acetic and butyric acids in uninoculated medium.

$H_2$  was determined by gas chromatography as described recently (4).

**Identification of nitrogen-containing fermentation products.** Primary and secondary amines were determined by spectrophotometry (8, 22), and ammonia was determined with glutamate dehydrogenase (9).

Thin-layer chromatography was used for the separation of betaine, choline, and the tertiary amines trimethylamine, *N,N*-dimethylethanolamine, and *N,N*-dimethylglycine, and a modification of the method of Eneroth and Lindstedt (13) was followed. Cell-free medium (2  $\mu$ l) or standard solution (5.0 to 10 mM) was applied as a spot to the starting line on glass plates (10 by 20 cm) coated with silica gel (silica gel 60 plates; E. Merck AG). Ascending development was carried out in a solvent-saturated chamber. The mobile phase was allowed to ascend 17 cm on the plates. After they were dried at room temperature, the plates were put into a jar with iodine vapor. Brown spots appeared after approximately 3 h; their  $R_f$  values were determined and compared with those of authentic samples.

**Determination of betaine.** For the quantitative determination of betaine, we used the procedure of Focht and Schmidt (14), which is based upon the precipitation of betaine reineckate, and then we measured the red color of the reineckate ions colorimetrically at 525 nm. Interfering ions such as *N,N*-dimethylglycine and ions of the culture medium that would be coprecipitated with betaine by ammonium reineckate were removed by ion-exchange chromatography, in accordance with the method of Carruthers et al. (7).

**Isotachophoretic methods.** Capillary tube isotachopheresis was used for the quantitative determination of *N,N*-dimethylglycine. A Shimadzu Ip 1B isotachophoretic analyzer, equipped with a PGD-1 potential gradient detector and a counterflow attachment (Shimadzu Seisakusho, Kyoto, Japan), was employed. The separations were carried out in a capillary tube (20 cm long; 0.5 mm in diameter) which was maintained at a constant temperature of 10°C. The migration current was 100  $\mu$ A. The leading electrolyte consisted of 0.05 M 2-amino-2-methyl-1,3-propanediol and hydrochloric acid at pH 9.7. Polyvinyl alcohol (0.4%) was added to sharpen the zone boundaries. The terminating electrolyte was 0.01 M  $\beta$ -alanine and  $Ba(OH)_2$  at pH 10.9. The electrolytes were prepared under an atmosphere of nitrogen and were stored

under nitrogen. Total assay time was about 25 min. Portions of the cell-free culture medium were diluted either 10 or 20 times with distilled water, and 3- $\mu$ l samples were subjected to isotachopheresis. Quantitative determinations were done by measuring the zone length. Standard solutions contained between 0.25 to 3.0 mM *N,N*-dimethylglycine in uninoculated medium.

Trimethylamine was determined with the same apparatus. The conditions were as follows: migration current, 100  $\mu$ A; leading electrolyte, 0.01 M potassium acetate and acetic acid (pH 4.3) and 0.2% Triton X-100; terminating electrolyte, 0.01 M histidine and acetic acid (pH 4.3); temperature, 20°C.

**Cellular characterization.** Deoxyribonucleic acid (DNA) was isolated by the method of Marmur (25). The cells were grown on peptone-yeast extract medium, which was supplemented with 1% glucose and 10 mM lysine. The base composition of the DNA was determined from its thermal denaturation temperature (24). The DNA hybridization was measured from renaturation rates (11). A Gilford 250 spectrophotometer was used.

**Chemicals.** The chemicals used were reagent grade and were purchased from Merck AG. *N,N*-Dimethylglycine was from Fluka AG.

## RESULTS

The two strains isolated from the enrichment cultures formed circular (1.5 to 2.0 mm in diameter), entire, raised, translucent yellowish to brownish colonies on betaine agar. They were strict anaerobic, nonmotile, nonsporeforming, gram-positive rods measuring 0.6 to 0.9  $\mu$ m in width and 2.5 to 4.0  $\mu$ m in length. Cell morphology varied from single or paired straight rods to swollen and club-shaped cells, or bifid forms. The organisms produced large amounts of butyrate and acetate during growth, indicating that they belonged to the genus *Eubacterium*. This was supported by results obtained in a characterization by Holdeman et al. (19) and in additional experiments. From the data summarized in Table 1, it was concluded that both isolates were strains of *E. limosum*. Strains 2A and ATCC 10825 showed a high degree of correspondence, whereas strain 11A differed in the following properties. The guanine-plus-cytosine content of its DNA was higher. The DNA-DNA homologies with *E. limosum* ATCC 10825 and with strain 2A were lower than that between the latter two strains. During growth in a peptone-yeast extract medium, isovalerate and isobutyrate were not formed, but propionate was among the products.

Growth on betaine was studied in detail with *E. limosum* strain 11A. The presence of 0.05% yeast extract and 0.2% Casitone was required for maximal growth rates. The doubling time was 6.0 h, as compared with 23 h in a mineral medium. No growth was observed in mineral me-

dium when  $\text{NH}_4\text{Cl}$  was omitted. Bicarbonate was essential for growth. The optimal conditions were 15 mM  $\text{NaHCO}_3$  under an atmosphere containing 20%  $\text{CO}_2$  and 80%  $\text{N}_2$ . A pH between 7.4 and 7.6 and a temperature of approximately 39°C were found to result in the fastest growth.

Of several methyl compounds tested, the organism grew only on betaine, choline, and methanol. Growth was not detectable on primary, secondary, and tertiary amines such as methylamine, glycine, ethanolamine, dimethylamine, sarcosine, *N*-methylethanolamine, trimethylamine, *N,N*-dimethylglycine, and *N,N*-dimethylethanolamine. Betaine and methanol were fermented with a doubling time of 6.0 h, whereas choline was utilized rather slowly (doubling time, 16.5 h).

The breakdown of betaine did not produce ammonia or glycine. *N,N*-Dimethylglycine was identified as the nitrogen-containing fermentation product. Identification was done by thin-layer chromatography of the culture medium in two different solvent systems, with authentic *N,N*-dimethylglycine as the reference (Table 2).

Correspondingly, choline was degraded to *N,N*-dimethylethanolamine. In both cases, neither trimethylamine nor primary or secondary amines were found among the products by colorimetric methods (8, 22). Thus, *E. limosum* strain 11A was only able to remove one methyl group from compounds such as betaine and choline. *E. limosum* strains 2A and ATCC 10825 produced the same products as strain 11A when grown with betaine, choline, and methanol, respectively.

Before balance studies of the betaine fermentation could be performed, a method for the quantitative determination of *N,N*-dimethylglycine had to be worked out. Capillary tube isotachopheresis was used. The procedure described above allowed the determination of *N,N*-dimethylglycine in a concentration range of 0.5 to 6.0 nmol per 3- $\mu$ l sample volume. The deviation in the slope of the calibration line was less than 2%.

Growth of *E. limosum* strain 11A on betaine was followed by measuring optical density, protein content, and dry weight of the cells in portions withdrawn from the culture. In addition, the concentrations of substrate and products were determined (Fig. 1). The molar growth yield, as obtained from the slope of the plot of the increase in dry weight versus betaine consumption, was 9 g of dry cell mass per mol of betaine. It is apparent that the decrease in betaine concentration during growth was associated with a corresponding increase of the *N,N*-dimethylglycine concentration. Acetate and butyrate were formed at different rates, but the

TABLE 1. Physiological characteristics of isolated strains 2A and 11A and of *E. limosum* ATCC 10825<sup>a</sup>

Determination	Strain 2A	Strain 11A	<i>E. limosum</i> <sup>b</sup>
Arabinose	w	w	w
Betaine	+	+	+
Erythritol	a	a	a <sup>w</sup>
Esculin hydrolysis	+	+	+
Fructose	a	a	a
Galactose	a	w	- <sup>a</sup>
Gelatin digestion	-	-	- <sup>+</sup>
Glucose	a	a	a
Glycerol	w	w	- <sup>w</sup>
Glycogen	-	w	-
Lecithinase	-	-	-
Lipase	-	-	-
Indole	-	-	-
Inositol	-	w	-
Lactate	+	+	+
Lactose	w	w	- <sup>w</sup>
Maltose	w	w	- <sup>a</sup>
Mannose	w	a	- <sup>a</sup>
Methanol	+	+	+
Pyruvate	+	+	+
Raffinose	w	w	-
Rhamnose	w	w	-
Ribose	w	w	a <sup>w</sup>
Starch pH	-	-	- <sup>w</sup>
Sucrose	a	w	- <sup>w</sup>
Xylose	w	w	v
H <sub>2</sub> production	+	+	+
Products from peptone-yeast extract	a,ib,b,iv	(a),p,b	a,ib,b,iv,l,s
Products from peptone-yeast extract glucose	L,A,b,s	L,A,b	L,A,b,(s)
Motility	-	-	-
Guanine-plus-cytosine content (mol%)	46.4	48.1	46.0
DNA-DNA homology (%)			
Strain 2A	-	53	77
Strain 11A	53	-	63

<sup>a</sup> Abbreviations: A, acetic acid (concentration, >10 mM); a (in products), acetic acid (concentration, <10 mM); a (in assays for substrate utilization), acid (pH, <5.5); b, butyric acid; ib, isobutyric acid; iv, isovaleric acid; L, lactic acid (concentration, >10 mM); 1, lactic acid; PY, peptone-yeast extract; p, propionic acid; s, succinic acid; v, variable acid production; w, weak acid production (pH 5.5 to 6.0); -, negative reaction or no acid; +, growth or positive reaction.

<sup>b</sup> The results for *E. limosum* were taken from reference 25, except the data for growth on betaine and methanol, for products formed from peptone-yeast extract and peptone-yeast extract glucose, and for DNA-DNA homology. Any of the above used as superscripts indicate reaction of 10 to 40% of the strains.

TABLE 2. Identification by thin-layer chromatography of *N,N*-dimethylglycine and *N,N*-dimethylethanolamine as fermentation products

Compound	<i>R<sub>f</sub></i> value	
	Solvent 1 <sup>a</sup>	Solvent 2 <sup>b</sup>
Betaine	0.415	0.150
Trimethylamine	0.327	ND <sup>c</sup>
<i>N,N</i> -Dimethylglycine	0.547	0.175
Choline	0.232	ND
<i>N,N</i> -Dimethylethanolamine	0.362	ND
Culture medium 1 <sup>d</sup> (strain 11A)	0.546	0.175
Culture medium 1 (ATCC 10825)	0.547	0.176
Culture medium 2 <sup>d</sup> (strain 11A)	0.362	ND
Culture medium 2 (ATCC 10825)	0.362	ND

<sup>a</sup> Solvent 1, Acetone-methanol-hydrochloride; 90:10:10 (vol/vol).

<sup>b</sup> Solvent 2, Chloroform-methanol-NH<sub>3</sub>/H<sub>2</sub>O; 50:50:4:4 (vol/vol).

final concentrations were in the same range. The carbon and redox balances of the betaine fermentation are given in Table 3. It was correct only if the fixation of 28 mol of CO<sub>2</sub> per 100 mol of betaine degraded was assumed. The redox balance was calculated by a procedure in which the oxidation numbers for all carbon and nitrogen atoms of each compound were determined and added up to give the oxidation degree (27). Based on the data in Table 3, the redox balance was 1.01.

<sup>c</sup> ND, Not determined.

<sup>d</sup> The organisms were grown in mineral medium supplemented with 50 mM betaine (culture medium 1) or 50 mM choline (culture medium 2). 2 μl of cell-free medium was used for thin-layer chromatography.

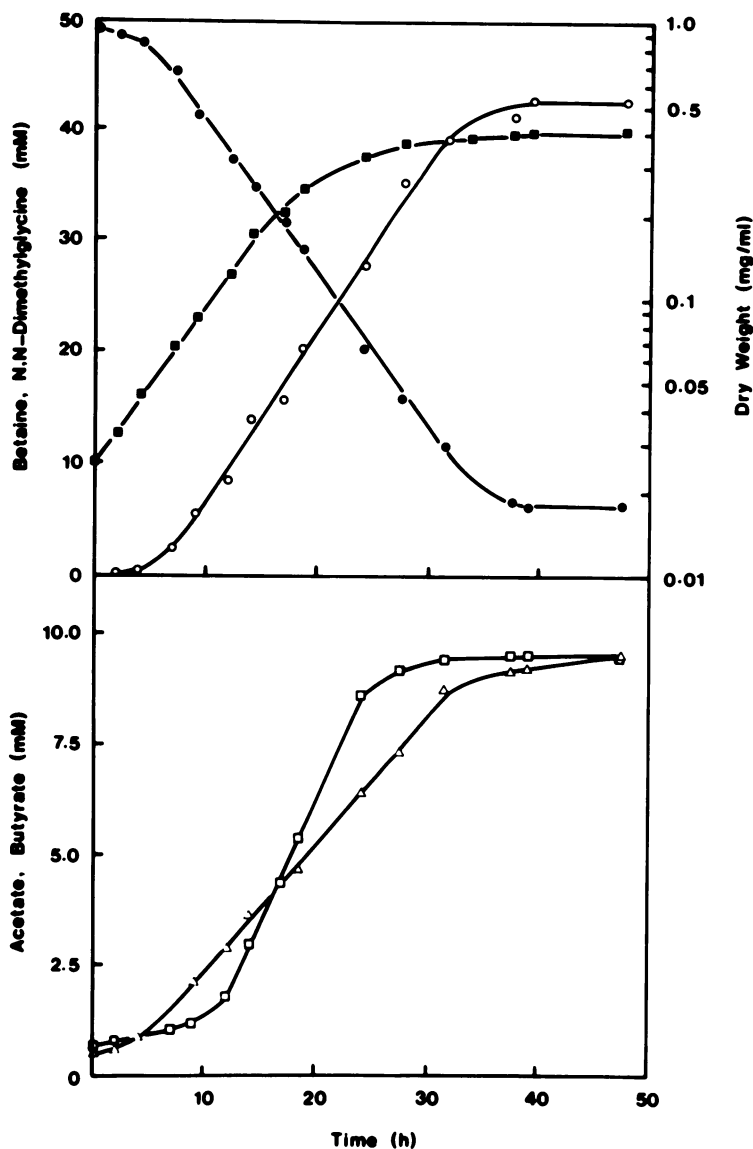


FIG. 1. Formation of *N,N*-dimethylglycine, acetate, and butyrate during growth of *E. limosum* strain 11A on 50 mM betaine. Cells were grown at 37°C in a 10-liter carboy under a  $N_2$ - $CO_2$  (80:20; vol/vol) atmosphere. Determinations were done as described in the text. The value for the optical density at the end of growth was 1.55, and the protein content at the end of growth was 0.18 mg/ml. The pH decreased from 6.7 to 5.0 during growth. Symbols: ■, dry weight; ●, betaine; ○, *N,N*-dimethylglycine; □, acetate; △, butyrate.

## DISCUSSION

Surprisingly, the breakdown of betaine and choline by *E. limosum* is not analogous to the breakdown of choline by *Desulfovibrio desulfuricans*, which cleaves choline to trimethylamine and acetaldehyde (17). Betaine and choline were demethylated by *E. limosum* to yield *N,N*-dimethylglycine and *N,N*-dimethylethanolamine, respectively. The demethylating enzyme sys-

tems seem to be very specific, because 1 mol of *N,N*-dimethylglycine was formed per mol of betaine consumed. Further demethylated compounds such as sarcosine or glycine were not detected in the culture medium and also did not serve as growth substrates for the *E. limosum* strains.

The methyl group removed from betaine was converted to a mixture of acetate and butyrate. The redox and carbon balances of substrate and

TABLE 3. Balance of betaine fermentation<sup>a</sup>

Substrate and product	Mol/100 mol of substrate	Mol of C/100 mol of substrate)	Oxidation degree	Oxidation degree (mol/100 mol)
<b>Substrate</b>				
Betaine .....	100	500	-7	-700
CO <sub>2</sub> .....	28.3 <sup>b</sup>	28.3	+4	+113.2
<b>Product</b>				
<i>N,N</i> -Dimethylglycine .....	101.4	405.7	-5	-586.8
Acetate .....	20.9	41.7	0	0
Butyrate .....	21.9	84.9	-4	-84.9

<sup>a</sup> Carbon balance, 100.8 %; redox balance, 1.01.

<sup>b</sup> Value calculated to fit the carbon balance.

products made it necessary to assume that CO<sub>2</sub> was fixed in this process. The following fermentation equation was in agreement with the experimental data: 7 betaine + 2 CO<sub>2</sub> → 7 *N,N*-dimethylglycine + 1.5 acetate + 1.5 butyrate.

That CO<sub>2</sub> can be used by *E. limosum* as an additional hydrogen acceptor was already observed by Barker and co-workers (2, 29) when they studied the fermentation of glucose and of lactate to butyrate. Since *E. limosum* removed only one methyl group each from betaine and choline, it was obviously necessary to test methanol as the growth substrate. All three *E. limosum* strains grew with methanol and produced a mixture of acetate and butyrate from it. The formation of these products from methanol has also been reported for a newly isolated anaerobic rod (35).

Plants, animals, and aerobic microorganisms generally oxidize choline to betaine via betaine aldehyde. Betaine is then progressively demethylated to glycine (15, 21, 26). The results presented here demonstrate that *N,N*-dimethylglycine is also an intermediate in the anaerobic decomposition of betaine. It, of course, has to be catabolized further, and in conjunction with this, enrichment cultures have been observed to be able to produce methane from *N,N*-dimethylglycine (18).

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#### LITERATURE CITED

- Aurich, H., W. Rotzsch, and E. Strack. 1963. Assimilation von (-)-Carnitin durch *Pseudomonas ovalis*. *Acta Biol. Med. Ger.* 11:274-280.
- Barker, H. A., M. D. Kamen, and V. Haas. 1945. Carbon dioxide utilization in the synthesis of acetic and butyric acids by *Butyribacterium rettgeri*. *Proc. Natl. Acad. Sci. U.S.A.* 31:335-360.
- Bowling, R. D., and G. N. Somero. 1979. Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. *J. Exp. Zool.* 208:137-152.
- Braun, K., and G. Gottschalk. 1981. Effect of molecular hydrogen and carbon dioxide on chemo-organotrophic growth of *Acetobacterium woodii* and *Clostridium acetitum*. *Arch. Mikrobiol.* 198:294-298.
- Braun, M., S. Schoberth, and G. Gottschalk. 1979. Enumeration of bacteria forming acetate from H<sub>2</sub> and CO<sub>2</sub> in anaerobic habitats. *Arch. Mikrobiol.* 120:201-204.
- Bryant, M. P. 1972. Commentary on the Hungate technique for the culture of anaerobic bacteria. *Am. J. Clin. Nutr.* 25:1324-1328.
- Carruthers, A., J. E. T. Oldfield, and H. J. Teague. 1960. The removal of interfering ions in the determination of betaine in sugar-beet juices and plant material. *Analyst* 85:272-275.
- Clark, S. J., and D. J. Morgan. 1956. The spectrophotometric determination of secondary aliphatic amines. *Mikrochim. Acta* 46:578-582.
- Da Fonseca-Wollheim, F., H. U. Bergmeyer, and J. Gutman. 1974. Ammoniak, p. 1850-1853. In H. U. Bergmeyer (ed.), *Methoden der enzymatischen Analyse*, 3rd ed. Verlag Chemie GmbH, Weinheim, Germany.
- Davies, W. L., and H. C. Dowden. 1936. The betaine content and nitrogen distribution of beet molasses and other beet by-products. *J. Soc. Chem. Ind. London* 55:175-179.
- De Ley, J., H. Cattoir, and A. Reynaerts. 1970. The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* 12:133-142.
- Den Dooren De Jong, L. E. 1927. Über protaminophage Bakterien. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2* 71:193-232.
- Eneroth, P., and G. Lindstedt. 1965. Thin-layer chromatography of betaines and other compounds related to carnitine. *Anal. Biochem.* 10:479-485.
- Focht, R. L., and F. H. Schmidt. 1956. Colorimetric determination of betaine in glutamate process end liquor. *J. Agric. Food Chem.* 4:546-548.
- Greenberg, D. M. 1961. Biosynthesis of amino acids and related compounds, p. 173-235. In D. M. Greenberg (ed.), *Metabolic pathways*, vol. 2. Academic Press, Inc., New York.
- Hall, J. L., M. R. Harvey, and T. J. Flowers. 1978. Evidence for the cytoplasmic localization of betaine in leaf cells of *Suaeda maritima*. *Planta* 140:59-62.
- Hayward, H. R., and T. C. Stadtman. 1959. Anaerobic degradation of choline. I. Fermentation of choline by an anaerobic cytochrome-producing bacterium, *Vibrio cholonicus* n. sp. *J. Bacteriol.* 78:557-561.
- Hippe, H., D. Caspari, K. Fiebig, and G. Gottschalk. 1979. Utilization of trimethylamine and other N-methyl compounds for growth and methane formation by *Methanosarcina barkeri*. *Proc. Natl. Acad. Sci. U.S.A.* 76:494-498.
- Holdeman, L. V., E. P. Cato, and W. E. C. Moore.

1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Anaerobe Laboratory, Blacksburg.
20. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. In J. R. Norris and D. W. Ribbons (ed.), *Methods in microbiology*, vol. 3B. Academic Press, Inc., New York.
21. **Ikuta, S., K. Matuura, S. Imamura, H. Misaki, and Y. Horitui.** 1977. Oxidative pathway of choline to betaine in the soluble fraction prepared from *Arthrobacter globiformis*. *J. Biochem.* **82**:157-164.
22. **Kakác, B., and Z. J. Vajdák.** 1974. Handbuch der photometrischen Analyse organischer Verbindungen, bd. 1 and 2. Verlag Chemie GmbH, Weinheim, Germany.
23. **Kortstee, G. J. J.** 1970. The aerobic decomposition of choline by microorganisms. *Arch. Mikrobiol.* **71**:235-244.
24. **Mandel, M., and J. Marmur.** 1968. Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA, p. 195-206. In S. P. Colowick and N. O. Kaplan (ed.), *Methods in enzymology*, vol. 12B. Academic Press, Inc., New York.
25. **Marmur, J. A.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208-218.
26. **Nagasawa, T., and Y. Kawabata.** 1975. Choline dehydrogenase of *Pseudomonas aeruginosa* A-16. *Agric. Biol. Chem.* **39**:1513-1514.
27. **Pauling, L.** 1962. *Die Natur der chemischen Bindung.* Verlag Chemie GmbH, Weinheim, Germany.
28. **Pfennig, N., and K. D. Lippert.** 1966. Über das Vitamin B<sub>12</sub>-Bedürfnis phototropher Schwefelbakterien. *Arch. Mikrobiol.* **55**:245-256.
29. **Pine, L., and H. A. Barker.** 1954. Tracer experiments on the mechanism of acetate formation from dioxide by *Butyribacterium rettgeri*. *J. Bacteriol.* **68**:216-226.
30. **Schmidt, K., S. Liaaen-Jensen, and H. G. Schlegel.** 1963. Die Carotinoide der Thiorhodaceae. *Arch. Mikrobiol.* **46**:117-126.
31. **Senez, J. C., and M. C. Pascal.** 1961. Dégradation de la choline par les bacteries sulfato-reductrices. Identification de *Desulfovibrio desulfuricans* et de *Vibrio cholerae*. *Z. Allg. Mikrobiol.* **1**:142-149.
32. **Storey, R., N. Ahmad, and R. G. Wyn Jones.** 1977. Taxonomic and ecological aspects of the distribution of glycinebetaine and related compounds in plants. *Oecologie (Berlin)* **27**:319-332.
33. **Strack, E., R. Noack, H. Aurich, G. Focke, and I. Lorenz.** 1962. Untersuchungen über den Abbau von Carnitin durch *Pseudomonas pyocyanea* A 7244. *Acta Biol. Med. Ger.* **9**:115-125.
34. **Wolin, E. A., R. S. Wolfe, and M. J. Wolin.** 1964. Viologen dye inhibition of methane formation by *Methanobacillus omelianskii*. *J. Bacteriol.* **87**:993-998.
35. **Zeikus, J. G., L. N. Lynd, T. E. Thompson, J. A. Kryzcki, P. J. Weimer, and P. W. Hegge.** 1980. Isolation and characterization of a new, methylotrophic, acidogenic anaerobe, the Marburg strain. *Curr. Microbiol.* **3**:381-386.