First pupl. in: Applied and Environmental Microbiology 75 (2009), 14, pp. 4821-4828

Syntrophic Degradation of Cadaverine by a Defined Methanogenic Coculture[▽]

Julia Roeder and Bernhard Schink*

Fachbereich Biologie, University of Konstanz, D-78457 Constance, Germany

A novel, strictly anaerobic, cadaverine-oxidizing, defined coculture was isolated from an anoxic freshwater sediment sample. The coculture oxidized cadaverine (1,5-diaminopentane) with sulfate as the electron acceptor. The sulfate-reducing partner could be replaced by a hydrogenotrophic methanogenic partner. The defined coculture fermented cadaverine to acetate, butyrate, and glutarate plus sulfide or methane. The key enzymes involved in cadaverine degradation were identified in cell extracts. A pathway of cadaverine fermentation via 5-aminovaleraldehyde and crotonyl-coenzyme A with subsequent dismutation to acetate and butyrate is suggested. Comparative 16S rRNA gene analysis indicated that the fermenting part of the coculture belongs to the subphylum *Firmicutes* but that this part is distant from any described genus. The closest known relative was *Clostridium aminobutyricum*, with 95% similarity.

Cadaverine is a biogenic primary aliphatic amine. Together with other biogenic amines, like putrescine or spermidine, it is formed during oxygen-limited decomposition of protein-rich organic matter by decarboxylation of amino acids or by amination of aldehydes and ketones (8, 27, 30, 42, 53, 54). These putrid-smelling and, at higher concentrations (100 to 400 mg per kg), often toxic compounds play a major role in food microbiology, e.g., as flavoring constituents in the ripening of cheese or as contaminants of fish and meat products, wine, and beer (24, 29, 49).

Little is known about the degradation of primary amines. Mono- and diamine oxidases of higher organisms and bacteria (23, 41, 64) initiate aerobic degradation, leading to the respective formation of aldehyde, ammonia, and hydrogen peroxide as products (28). Alternatively, in a putrescine-degrading mutant of *Escherichia coli*, putrescine is degraded by a putrescine–2-oxoglutarate transaminase and a subsequent dehydrogenase to form 4-aminobutyrate, which is further metabolized via succinate (43).

Anaerobic degradation of primary amines could follow basically similar pathways. The released reducing equivalents can be disposed of in a manner similar to that described for primary alcohols (9, 15, 16). In the absence of external electron acceptors, such as sulfate or nitrate, incomplete oxidation of cadaverine to fatty acids or dicarboxylic acids could be coupled to syntrophic methane production, homoacetogenesis, or reductive synthesis of long-chain fatty acids (1, 25, 31).

In the present study, we describe a new isolate of strictly anaerobic bacteria which oxidizes cadaverine syntrophically with the methanogen *Methanospirillum hungatei* and forms acetate, butyrate, glutarate, and methane as products. The enzymes involved in the degradation of cadaverine were identified, and a catabolic pathway is proposed.

* Corresponding author. Mailing address: Fachbereich Biologie, University of Konstanz, D-78457 Constance, Germany. Phone: 49 7531 882140. Fax: 49 7531 884047. E-mail: Bernhard.Schink@uni-konstanz.de.

MATERIALS AND METHODS

Sources of organisms. The coculture LC 13D was isolated from the enrichment culture La Cad, which was originally inoculated with sediment taken from the Lahn river in Marburg, Germany. *Methanospirillum hungatei* M1h (DSM 13809) was isolated from digested sludge obtained from the sewage plant at Gottingen, Germany. *Acetobacterium woodii* (DSM 2396) and *Desulfovibrio vulgaris* strain Marburg (DSM 2119) were taken from the strain collection of our laboratory. *Methanobrevibacter arboriphilus* DH1 (DSM 1125) and *Clostridium aminobutyricum* (DSM 2634) were purchased from DSMZ, Braunschweig, Germany.

Media and growth conditions. All procedures for cultivation and isolation were conducted as previously described (44, 47, 62). The sulfate-reducing partner *Desulfovibrio vulgaris* was exchanged for *Methanospirillum hungatei* in a liquid dilution series, similar to what was described previously for an agar dilution series (62) in 27-ml serum tubes filled with liquid medium, gassed with an 80% N_2-20% CO_2 gas mixture with 5 mM cadaverine as a substrate and 2 mM acetate as an additional carbon source, and sealed with butyl rubber stoppers fitted with aluminum caps. The mineral salts medium for isolation and further cultivation was CO_2-bicarbonate buffered (30 mM) and reduced with 1 mM sulfide. It contained 1 ml selenite-tungstate solution, 1 ml of trace element solution SL 13 (40) per liter, and 0.5 ml per liter 7-vitamines solution (63). The pH was adjusted to 7.2 to 7.4. For growth of the syntrophic cadaverine-oxidizing coculture, the medium contained 5 mM cadaverine and about 100 μM Ti(III)-nitrilotriacetic acid as a further reducing agent. Unless specified otherwise, the cultures were incubated at 28°C in the dark without shaking.

Growth experiments. Growth experiments were carried out either with 120-ml serum bottles or with 27-ml serum tubes filled with medium, gassed with an 80% N_2-20% CO_2 gas mixture, and sealed with butyl rubber stoppers fitted with aluminum caps. For specific-growth tests, the culture was inoculated into freshwater mineral medium with 5 mM cadaverine with or without 2 mM acetate, 5 mM cadaverine with or without 10 mM 2-bromoethanesulfonate, 5 mM cadaverine with or without 0.05% yeast extract, or 0.05% yeast extract alone. Growth was followed by measuring turbidity at a 578-nm wavelength with a Camspec model M107 spectrophotometer (Camspec, Ltd., Cambridge, United Kingdom). Product formation was measured by high-performance liquid chromatography (HPLC) and gas chromatography. To determine whether the electron transfer was based on transport of hydrogen, formate, or both, *Methanospirillum hungatei* was replaced by *Methanobrevibacter arboriphilus* as the partner in the liquid dilution series as described above. Possible unspecific side effects of bromoethanesulfonate were checked by addition of 10 mM 2-bromoethanesulfonate to the sulfate-reducing coculture LC 13D in the presence of sulfate.

Cytological characterization. Cultures were routinely examined with a Zeiss Axiophot phase-contrast microscope (Zeiss, Germany). Photomicrographs were taken on agarose-coated slides (by using a modified version of the method in reference 59). Gram staining was carried out according to the method of Bartholomew (2), with *Acetobacterium woodii* and *Desulfovibrio vulgaris* as references. Cytochromes were assayed for with French press cell extracts of cadav-

erine-grown cells through redox difference spectroscopy with a Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland) at a 350- to 900-nm wavelength.

Chemical analyses. Acetate, butyrate, and glutarate were separated by HPLC on an Aminex HPX-87 H ion exchange column (Bio-Rad Laboratories GmbH, Munich, Germany) and detected by a model ERC-7512 refraction detector (ERC Gesellschaft für den Vertrieb wissenschaftlicher Geräte mbH, Regensburg, Germany). The column was operated at 40°C, and 5 mM H_2SO_4 was the eluent at a flow rate of 0.6 ml per min. Methane was determined with a Carlo Erba 6000 Vega Series 2 gas chromatograph (Carlo Erba Instruments, Milan, Italy) with a steel column (2 m by 4 mm) packed with a 60/80-mesh molecular sieve (5 Å; Serva, Heidelberg, Germany) and a flame ionization detector with a column temperature of 120°C, a detector temperature of 150°C, and carrier gas nitrogen at a flow rate of 60 ml per min.

Sulfide was analyzed by the methylene blue method (10) and nitrite by Merckoquant test strips (Merck, Darmstadt, Germany). Fumarate reduction was assayed by scanning UV spectra at 220 nm with a Uvikon 860 spectrophotometer. Protein was quantified with bicinchoninic acid (bicinchoninic acid protein assay kit; Pierce, Rockford, IL.).

Substrate utilization tests. Growth experiments using various substrates were carried out with 27-ml serum tubes filled with 10 ml medium, under a headspace of 80% N_2-20% CO_2. Substrates were added from filter-sterilized or autoclaved stock solutions. Product formation was measured by HPLC and gas chromatrography.

Temperature, pH, and NaCl ranges for growth. For determination of the temperature optimum, cultures were incubated at 4 to 55°C. For the assay of the pH dependence of growth, a buffer system consisting of citrate, Tris-HCl, and potassium phosphate (10 mM each) was used in the pH range of 4.0 to 8.5. To determine the effect of salt concentrations on growth, the culture was inoculated alternatively into brackish and saltwater medium (by using a modified version of the method in reference 63).

Preparation of cell extracts. All manipulations were performed inside an anoxic chamber (Coy Laboratory products Inc., MI) under a 95% N_2-5% H_2 atmosphere. All centrifugation steps were carried out anoxically. All buffers and reagents were prepared under anoxic conditions. Cultures (1 liter) were harvested in the late-exponential growth phase by centrifugation at 8,600 × *g* and 4°C for 20 min in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments, Wilmington, DE). The cell pellet was washed twice with an equal amount of anoxic 50 mM Tris-HCl buffer, pH 8.0. Cells were resuspended in anoxic buffer to give a final volume of 5 ml, and a protease inhibitor cocktail (50 μl per ml; Sigma, Darmstadt, Germany) was added to the suspension. Cells were disrupted three times in a cooled, anoxic French pressure cell (SLM Aminco Spectronic Instruments, Silver Spring, MD) at 137 MPa. After addition of 832 U per ml DNase I (Sigma), cell debris, DNA, and undisrupted cells were removed from the homogenate by centrifugation at 14,500 × *g* at 4°C for 20 min under anoxic conditions. The supernatant was used for enzyme assays.

Alternatively, mutanolysin (Sigma) was used to prepare cell extracts. Briefly, 2.5 U of mutanolysin per ml was injected into the serum vials with the cell suspension. The suspension was incubated at 37°C for 30 min. To remove intact cells, including the methanogenic partner, the suspension was centrifuged at 14,500 × *g* at 4°C for 20 min. The supernatant was used for enzyme assays.

Enzyme assays. All enzyme assays were carried out with cell extracts of cadaverine-grown cells under anoxic conditions, using a model 100-40 spectrophotometer (Hitachi, Tokyo, Japan) under an N_2 atmosphere at 28°C and special glass cuvettes or quartz cuvettes fitted with rubber stoppers.

Hydrogenase was assayed with benzyl viologen and H_2 (on the basis of reference 14). Phosphate acetyltransferase, acetate kinase, β-ketothiolase, glutamate dehydrogenase, and 3-hydroxybutyryl-coenzyme A (CoA) dehydrogenase were measured using standard methods (5, 38). Butyrate kinase was assayed by the acetate kinase assay, using butyrate as a substrate. Crotonase was determined following the crotonyl-CoA absorption decrease at 263 nm (36). Glutaryl-CoA dehydrogenase was assayed with ferricyanide as an electron acceptor (by using a modified version of the method in reference 50). Glutaconyl-CoA decarboxylase was assayed using a discontinuous approach, measuring the formation of CO_2 with different electron acceptors {NAD^+, phenazine methosulfate, $K_3[Fe(CN)_6]$, each at 40 mM}, using a gas chromatograph with a thermal conductivity detector and helium as a carrier gas. Serum bottles (25-ml volume) were closed with butyl rubber stoppers, fitted with aluminum caps, and filled with 100% N_2. The 1-ml assay mixture contained 500 mM 2-(*N*-morpholino) ethanesulfonic acid buffer, pH 6.0, 10 mM glutarate, 0.1 mM butyryl-CoA, 0.1 mM electron acceptor, and 0.85 mg protein. At intervals of 30 min, 300-μl samples of headspace gas were injected on the column. Cadaverine–2-oxoglutarate transaminase was assayed following the formation of a dye from *o*-aminobenzaldehyde and 5-aminovaleraldehyde, which is the product of the enzyme reaction (by using a modified version of the method in reference 26). All enzyme assays were carried out with 0.01 to 0.1 mg protein per assay unless noted otherwise.

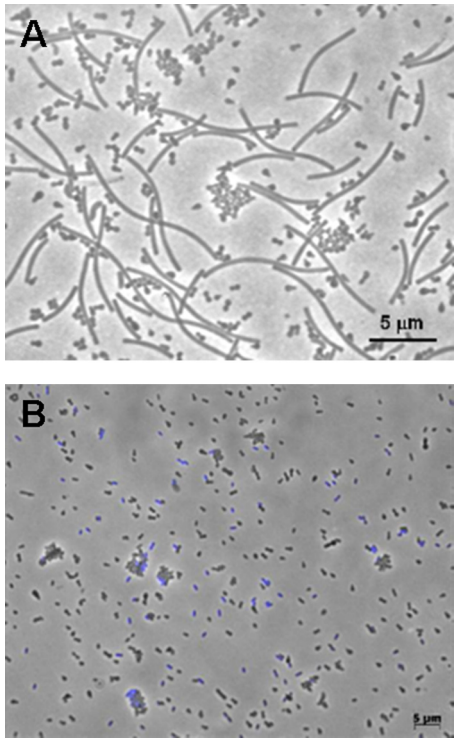


FIG. 1. Phase-contrast photomicrographs of defined methanogenic cocultures growing with cadaverine. (A) Coculture LC 13 M with *Methanospirillum hungatei* as a partner. Bar = 5 μm. (B) False-color overlaid phase-contrast photomicrograph of strain LC 13R with *Methanobrevibacter arboriphilus* as the partner organism, which is shown in blue. Bar = 5 μm.

DNA extraction, purification, and amplification of the 16S rRNA gene. Genomic DNA was extracted after addition of mutanolysin (5 U per ml) to a cell suspension and subsequent incubation at 37°C for 30 min. Cell debris was removed by centrifugation at 15,000 × *g* for 5 min. The DNA was purified from the supernatant by using a Qiagen purification kit and used as a template for PCR amplification of the 16S rRNA gene. The PCR primers used for amplification were 27F, 533F, 1492R, and 907R. The PCR conditions consisted of preheating for the initial denaturation step at 94°C for 3 min, primer annealing at 55°C for 30 s, an extension step at 68°C for 1.3 min, a run comprising 32 cycles, and a final extension step at 68°C for 10 min.

16S rRNA gene sequence determination and phylogenetic analysis. Purified PCR products were sequenced by GATC Biotech AG, Konstanz, Germany. Sequences were aligned using the DNA star program (version 5.06) to reconstruct the entire 16S rRNA gene. The complete contig sequence was inserted into the NCBI BLAST search engine and the ARB database SILVA (April 2008 version). A phylogenetic tree was constructed by selecting from the ARB database the relatives closest to the GenBank hits.

Chemicals. All chemicals were of reagent grade quality and obtained from Fluka, Neu-Ulm, Germany; Merck, Darmstadt, Germany; Sigma, Deisenhofen, Germany; and Boehringer, Mannheim, Germany. Gases were obtained from Messer-Griesheim, Darmstadt, Germany.

Nucleotide sequence accession number. The sequence obtained from the phylogenetic analysis was deposited in GenBank under accession number FJ981591.

RESULTS

Enrichment and isolation. Enrichment cultures with freshwater medium (50 ml) containing 5 mM cadaverine as the sole organic substrate were inoculated with 5 ml of anoxic sediment

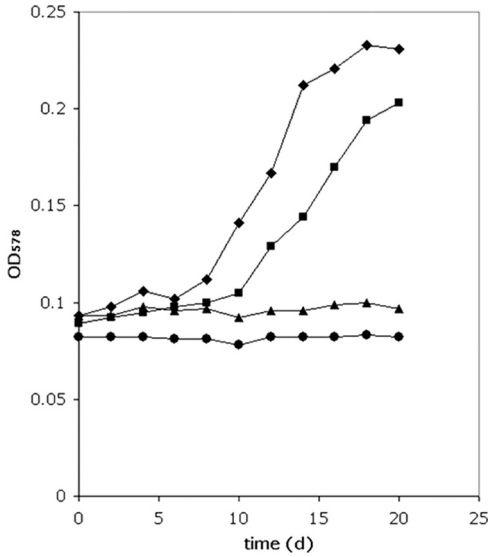


FIG. 2. Growth of the coculture LC 13 M under different conditions. Experiments were performed at 28°C with 12- ml serum bottles gassed with an 80% N_2-20% CO_2 gas mixture, sealed with butyl rubber stoppers, and fitted with aluminum caps. Symbols: ◆, 5 mM cadaverine; ■, 5 mM cadaverine plus 2 mM acetate for assimilation of the methanogenic partner; ▲, 5 mM cadaverine plus 2 mM acetate plus 10 mM bromoethane sulfonate; ●, sterile control. OD_{578}, optical density at 578 nm; d, days.

from the river Lahn in Marburg, Germany. Gas production started after 1 to 4 weeks. After five transfers, the enrichment culture contained several shorter and longer rod-shaped bacteria, few spirilla, and a dominant short rod-shaped bacterium. Isolation of cadaverine-degrading bacteria was attempted using an agar shake series with 5 mM cadaverine and 2 mM acetate in the presence or absence of either *Acetobacterium woodii*, *Methanospirillum hungatei*, or *Desulfovibrio vulgaris* cells, the last with 10 mM sulfate as an electron acceptor. Exclusively in the presence of *Desulfovibrio vulgaris*, lens-shaped, slightly yellowish colonies surrounded by small satellite colonies developed, which were again subjected to an agar dilution series with *D. vulgaris* as a partner. After two further agar dilution series, the yellowish colonies were transferred into liquid medium containing 5 mM cadaverine, 10 mM sulfate, and 5% inoculum of *D. vulgaris*, resulting in the defined coculture LC 13D.

Purity was checked by microscopic control and by growth tests with AC medium (1:10 diluted; Difco Laboratories, Detroit, MI) in which only the isolated cell types were observed. The sulfate-reducing partner was exchanged for *Methanospirillum hungatei* strain M1h via a liquid dilution series, analogous to the agar dilution series, with liquid medium containing 5 mM cadaverine, 2 mM acetate, and a 5% inoculum of *M. hungatei* M1h. The resulting methanogenic coculture, LC 13 M, is described in the present work.

Cytological properties of the coculture LC 13 M. The cadaverine-oxidizing cells in the coculture LC 13 M were coccoid-to-pear-shaped short rods, about 1×0.5 μm in size (Fig. 1A), and stained gram-positive. Cells occurred in single numbers, in pairs, or in aggregates. Motility was observed only in growing cultures. Cells moved in a tumbling manner and formed terminal endospores. The cadaverine-oxidizing partner in this coculture is referred to as strain LC 13R. Strain LC 13R could also be cocultivated with *Methanobrevibacter arboriphilus*, which oxidizes only hydrogen and cannot oxidize formate (Fig. 1B).

Physiology. The coculture LC 13 M grew well in freshwater and brackish water medium but not in saltwater medium. Phosphate was tolerated up to 50 mM. In the presence of 0.05% yeast extract, the cells grew faster and the cell yield increased by approximately 20%. No growth was found with yeast extract alone (Fig. 2). Growth was optimal ($\mu = 0.005\ h^{-1}$, doubling time = 140 h) at 28°C, and the temperature limits were 22 and 37°C. The pH optimum was at 7.0 to 7.5; weak growth was still possible at pHs 6.0 and 8.0. Bromoethanesulfonate had no effect on the growth of the sulfate-reducing coculture LC 13D.

The only substrates degraded were putrescine, lysine, 5-aminovalerate, γ-aminobutyrate, β-alanine, crotonate, glutarate, and butyrate. The following substrates were not degraded (unless indicated otherwise, the substrates were added to give a 5 mM final concentration): ethylamine, ethanolamine, glycine,

TABLE 1. Stoichiometry of substrate fermentation by the methanogenic coculture LC 13 M[a]

Substrate	OD_{578}	Concn of substrate provided (mM)	Cell dry mass (mg/liter)	Concn of substrate assimilated[c] (mM)	Product concn (mM)				% Electron recovery	Molar growth yield[d] (g/mol)
					Acetate	Butyrate	Glutarate	Methane		
Cadaverine[b]	0.151	5	37.8	0.25	1.5	3.5	0.25	5	95	7.9
Cadaverine plus 2 mM acetate[b]	0.121	5	30.3	0.2		3	0.1	5	76	6.3
Crotonate	0.137	5	34.3	0.33	8.5	0.98		0.15	105	7.3
Lysine	0.153	5	38.3	0.25	6.32	0.86		0.4	63	8.1
Putrescine	0.107	5	26.8	0.26	4.29	0.91		0.55	68	5.7
γ-Amino-butyrate	0.087	5	21.8	0.21	7.8	1.49		0.13	107	4.6
β-Alanine	0.048	5	12.0	0.16	2.94			0.18	53	2.5
5-Amino-valerate	0.067	5	16.8	0.13	0.52	2.74	0.14	0.43	68	3.5
Butyrate	0.029	5	7.3	0.071	0.1			0.77	4	1.5

[a] Except where noted otherwise, experiments were carried out using 27-ml serum tubes filled with 10 ml culture, with headspace gassed with a 20% N_2-80% CO_2 gas mixture. Cell dry mass was calculated using the conversion factor wherein 0.1 optical density (OD) unit is equal to 25 mg dry matter per liter.
[b] Carried out using 120-ml serum bottles filled with 50 ml culture, with headspace gassed with an N_2-CO_2 gas mixture.
[c] The amount of substrate assimilated was calculated using the formula ($C_4H_7O_3$) for cell material.
[d] Growth yields were calculated on the basis of the formed products.

TABLE 2. Activities of enzymes in French-pressed cell extracts involved in cadaverine degradation by the coculture LC 13 M[a]

Enzyme	EC no.[b]	Sp act (U/mg protein)[c]
Cadaverine–2-oxo-glutarate aminotransferase	?	0.10
Glutamate dehydrogenase	1.4.1.3	0.09
5-Aminovaleraldehyde dehydrogenase	?	3.63*/10.95**
H_2-Fd oxidoreductase	1.12.7.2	0.77
Glutaryl-CoA dehydrogenase	1.3.99.7	0.15
Glutaconyl-CoA decarboxylase	4.1.1.70	ND
Butyryl-CoA dehydrogenase	1.3.99.2	0.04
3-Hydroxybutyryl-CoA dehydrogenase	1.1.1.157	0.03
Crotonase	4.2.1.17	3.0
β-Ketothiolase	2.3.1.19	0.20
Phosphate acetyltransferase	2.3.1.8	0.37
Acetate kinase	2.7.2.1	0.09

[a] All assays were carried out at 25°C except where otherwise noted. The physiological activity of the growing coculture LC 13 M was 21.1 nmol cadaverine per min per mg cell protein.
[b] ?, unknown.
[c] * indicates that benzyl viologen was used as an electron acceptor; ** indicates that methyl viologen was used as an electron acceptor. ND, not detectable.

aniline, phenylalanine, Casamino Acids (0.1%), aspartate, formate plus 2 mM acetate, valerate, citrate, fumarate, glutarate plus 2 mM acetate, malate, malonate, oxalate plus 2 mM acetate, succinate plus 2 mM acetate, butanol, 1,4-butandiol, choline, ethylene glycol, glycerine, methanol, 1,3-propan-diol, arabinose, fructose, galactose, glucosamine, glucose, saccharose, and xylose. Neither nitrate, fumarate, nor sulfate was used as an electron acceptor for cadaverine degradation. In the presence of 10 mM bromoethanesulfonate, no growth was observed. The products of cadaverine degradation were acetate, butyrate, and methane, with traces of glutarate. In growth assays with 5 mM cadaverine plus 2 mM acetate, no acetate was formed. During fermentation of γ-aminobutyrate, crotonate, lysine, putrescine, and 5-aminovalerate, only acetate, butyrate, and methane were formed. Butyrate and β-alanine were fermented to acetate and methane only.

Methanogenic fermentation of cadaverine by culture LC 13 M was stoichiometrically almost complete (Table 1) and agreed with the following equation. $\Delta G_0'$ values were calculated on the basis of published tables (12, 55), as follows:

$$5\ C_5H_{16}N_2^{2+} + 0.75\ CO_2 + 11.25\ H_2O \rightarrow 2.375\ C_2H_3O_2^- + 0.25\ C_5H_6O_4^{2-} + 3.5\ C_4H_7O_2^- + 10\ NH_4^+ + 5.75\ CH_4 + 6.375\ H^+$$

$$\Delta G_0' = -103.2\ \text{kJ per mol cadaverine} \quad (1)$$

Also, fermentations of crotonate and γ-aminobutyrate were complete, according to the following empirical transformation reactions:

$$5\ C_4H_5O_2^- + 6.5\ H_2O + 0.75\ CO_2 \rightarrow 8\ C_2H_3O_2^- + C_4H_7O_2^- + 0.75\ CH_4 + 4\ H^+$$

$$\Delta G_0' = -57.5\ \text{kJ per mol crotonate} \quad (2)$$

$$5\ C_4H_9O_2N + 3\ H_2O + 2\ CO_2 \rightarrow 7\ C_2H_3O_2^- + 1.5\ C_4H_7O_2^- + 5\ NH_4^+ + 2\ CH_4 + 8.5\ H^+$$

$$\Delta G_0' = -130.9\ \text{kJ per mol γ-aminobutyrate} \quad (3)$$

Fermentations of lysine, 5-aminovalerate, β-alanine, and putrescine were incomplete, with acetate, butyrate, and methane as the most important products.

Enzymes involved in cadaverine degradation. Enzymes were assayed under anoxic conditions in cell extracts prepared from cadaverine-grown cells of culture LC 13 M (Table 2). Cadaverine–2-oxoglutarate aminotransferase, glutamate dehydrogenase, glutaryl-CoA dehydrogenase, β-ketothiolase, butyryl-CoA dehydrogenase, phosphate acetyltransferase, acetate kinase, and hydrogenase showed activities sufficient for involvement in the dissimilatory metabolism in comparison to the calculated physiological activity of growing cells (21 nmol per min per mg cell protein) (Table 2). Glutaconyl-CoA decarboxylase could not be detected so far. NADH oxidation by an NADH-Fd oxidoreductase activity was measured with benzylviologen or methylviologen as an electron acceptor.

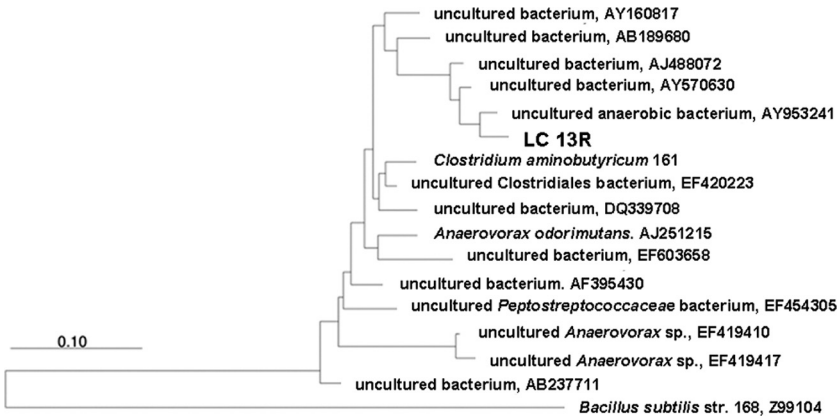
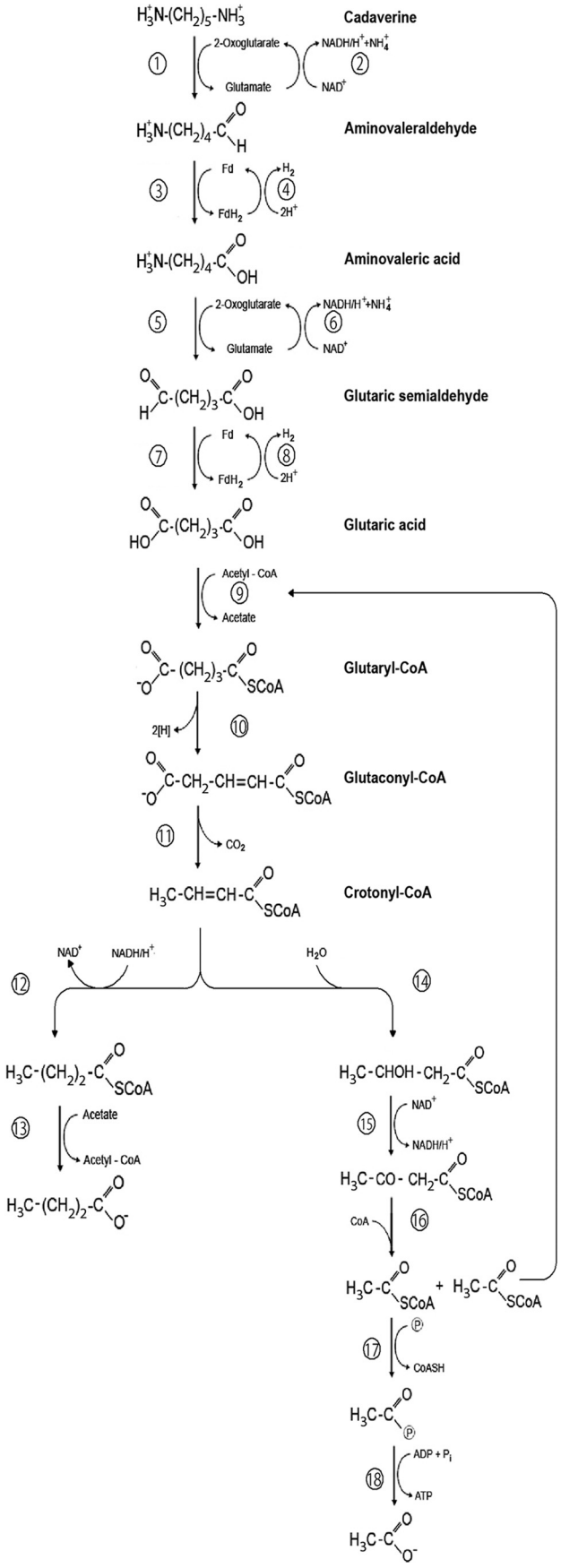


FIG. 3. Phylogenetic position of the fermenting strain LC 13R in the culture LC 13 M within the *Firmicutes* subphylum. The tree was based on the 16S rRNA gene sequence of strain LC 13R and was constructed using the parsimony method. Bar = 0.10 base substitutions per nucleotide position.



Phylogenetic analysis. Nearly the complete (1,369-bp) 16S rRNA gene sequence of the cadaverine-oxidizing bacterium strain LC 13R was determined. The sequence was aligned in the ARB database SILVA (26.09.2007), and a tree was constructed with the closest relatives. On the basis of the phylogenetic analysis (Fig. 3), strain LC 13R was found to be a member of the subphylum *Firmicutes*, and the closest relative was *Clostridium aminobutyricum*, with 95% sequence identity. *C. aminobutyricum* did not degrade cadaverine in syntrophic coculture with *M. hungatei*.

DISCUSSION

Isolation of a cadaverine-degrading methanogenic coculture. In the present study, we describe a novel, strictly anaerobic, cadaverine-oxidizing bacterium as the fermenting part of the defined coculture LC 13 M, which was isolated from a freshwater sediment sample. If 2-bromoethanesulfonate was added, cadaverine was not degraded, indicating that cadaverine oxidation depends on syntrophic cooperation with an electron-scavenging partner. Interestingly, it was not possible to isolate the fermenting partner directly with the methanogen *Methanospirillum hungatei* in an agar dilution series, but only with the sulfate-reducing bacterium *Desulfovibrio vulgaris*, although colonies of cadaverine degraders were formed in dilution series with methanogenic or sulfate-reducing partners at the same numbers and to the same dilution steps. However, the cadaverine-oxidizing bacterium grew well with *Methanospirillum hungatei* in a liquid dilution series in the absence of sulfate. The same strategy had been used in the past with the first isolations of bacteria that syntrophically oxidize butyrate (35, 37), propionate (7), or benzoate (39). Obviously, the most difficult step in obtaining defined methanogenic cocultures in these cases is the resumption of growth after transfer into the liquid medium, rather than growth inside the agar. One may speculate that, just in this critical situation, the syntrophically fermenting bacterium depends on a sulfate-reducing partner that maintains a substantially lower hydrogen partial pressure than methanogens can (11).

Physiology. The range of substrates used by the coculture was small: only cadaverine, putrescine, lysine, 5-aminovalerate, γ-aminobutyrate, β-alanine, and crotonate were used, and they fermented to acetate, butyrate, and methane (to acetate plus methane with β-alanine only) and traces of glutarate (only with cadaverine). Growth of the coculture with crotonate was possible in the presence of bromoethane sulfonate, indicating that crotonate dismutation to acetate and butyrate did not require

FIG. 4. Hypothetical pathway of cadaverine degradation by the methanogenic coculture LC 13 M. Numbers in circles refer to the following enzymes: 1, cadaverine–2-oxoglutarate aminotransferase; 2, glutamate dehydrogenase; 3, 5-aminovaleraldehyde dehydrogenase; 4, H_2-Fd oxidoreductase; 5, 5-aminovalerate–2-oxoglutarate aminotransferase; 6, same as 2; 7, glutaric semialdehyde dehydrogenase; 8, same as 4; 9, acetyl-CoA–glutarate CoA transferase; 10, glutaryl-CoA dehydrogenase; 11, glutaconyl-CoA decarboxylase; 12, butyryl-CoA dehydrogenase; 13, butyryl-CoA–acetate CoA transferase; 14, crotonase; 15, 3-hydroxybutyryl-CoA dehydrogenase; 16, β-ketothiolase; 17, phosphate acetyltransferase; 18, acetate kinase.

interspecies electron transfer to a partner organism, as has been shown in the past with numerous syntrophically fatty acid-oxidizing syntrophic bacterial strains (4). Since strain LC13R grew well also with *Methanobrevibacter arboriphilus* instead of *Methanospirillum hungatei*, we conclude that the electrons are transferred primarily via hydrogen rather than via formate. This concept appears to be true also for syntrophic oxidation of butyrate (51, 60), glycolate (18, 46), and benzoate (48), whereas syntrophic degradation of propionate also includes formate as an electron carrier, perhaps simultaneous with hydrogen (13, 17).

The pathway of substrate degradation was analyzed by enzyme measurements with cell extracts of the coculture. The results are summarized in a hypothetical degradation pathway (Fig. 4). Activities of cadaverine–2-oxoglutarate aminotransferase and glutamate dehydrogenase indicate that cadaverine degradation starts with a transamination reaction. Although the subsequent reactions could not be detected, a subsequent dehydrogenation step, another transamination, and dehydrogenation have to follow to form glutarate, which was also detected as a side product (Table 1). Glutarate has to be activated to glutaryl-CoA, and subsequently, glutaryl-CoA dehydrogenase forms glutaconyl-CoA. Unfortunately, we were not able to measure glutaconyl CoA decarboxylase activity in either continuous or discontinuous assays. Nonetheless, this step has to take place, forming crotonyl-CoA as an intermediate. Further dismutation of crotonyl-CoA to acetate and butyrate follows well-known reaction sequences, for which almost all of the respective enzyme activities were demonstrated to occur in cell extracts.

Oxidative deamination of a primary amine to the respective aldehyde releases electrons at the E_0' (E_0 at pH 7.0) level of −130 mV (calculated with the pyruvate-alanine pair on the basis of reference 55). After transamination to glutamate, these electrons are transferred to NAD^+ ($E_0' = -320$ mV); the equilibrium of this reaction is far on the side of glutamate. To release these electrons via ferredoxin in the form of molecular hydrogen would require a substantial energy input, e.g., by Rnf-protein complexes (6, 22) or other means of a reversed electron transport, e.g., an NADH-quinone oxidoreductase, as was described to be involved in anaerobic metabolism of *Escherichia coli* (56, 57). Oxidation of aldehydes to the respective acids releases electrons at a very low redox potential ($E_0' = -635$ mV), which easily allows reduction of protons to hydrogen, even including ATP formation via the respective CoA derivatives.

Fermentation of cadaverine to glutarate, according to the equation $H_2N\text{-}(CH_2)_5\text{-}NH_2 + 4\ H_2O \rightarrow {}^-OOC\text{-}(CH_2)_3\text{-}COO^- + 2\ NH_4^+ + 4\ H_2$, is an endergonic reaction ($\Delta G_0' = +46$ kJ per mol, as estimated from the redox potentials mentioned above) which requires cooperation with, e.g., a hydrogen-utilizing methanogen to allow energy conservation and growth (45). In accordance with this calculation, inhibition of the methanogenic partner by bromoethane sulfonate prevented cadaverine degradation completely. Further degradation of glutarate via glutaconyl-CoA and crotonyl-CoA could end with butyrate as the only product. Fermenting bacteria growing with glutarate as their only substrate produce a mixture of butyrate and isobutyrate (32, 33). In the case of our culture LC 13 M, the presence of a hydrogen-scavenging partner allows for oxidation of the crotonyl residue at least partly to acetate, and it can also grow (very weakly) with glutarate or butyrate, fermenting these substrates to acetate (and CO_2). However, energy conservation by this mode of metabolism is low, and a delicate matter, as minor amounts of acetate already appear to impair energy conservation and growth.

Product inhibition by short-chain fatty acids is not uncommon (3, 20, 58, 61) and is probably due to the toxicity of the undissociated acids. Fukuzaki et al. (19) demonstrated that the degree of inhibition by fatty acids depended strongly on the concentration of their undissociated form. The undissociated acid can diffuse across the bacterial membrane, resulting in an intracellular-pH drop and in decreased ATP yields.

On the basis of the maximum optical density reached by the coculture with different substrates, the biomass formation, and the amount of substrate assimilation, we calculated the molar growth yield and the resulting ATP gain. With culture LC 13 M grown on 5 mM cadaverine, the molar growth yield is 8.2 g per mol cadaverine, corresponding to about 0.8 ATP per mol cadaverine if we assume an ATP-related yield of 10 g per mol ATP (52). If we further assume that synthesis of ATP requires about 60 to 70 kJ per mol under physiological conditions (55), the obtained growth yield is low compared to the calculated free energy change (−103.2 kJ per mol) (equation 1). In our hypothetical pathway (Fig. 4) of syntrophic cadaverine fermentation to a mixture of acetate and butyrate (equation 1), about 6 mol ATP per 5 mol cadaverine is formed by substrate level phosphorylation. Further ATP gains are possible in decarboxylation of glutaconyl-CoA to crotonyl-CoA. On the other hand, energy investments are required for proton reduction with the electrons obtained in the oxidation of the amines to the aldehyde residues and in the oxidation of glutaryl-CoA to glutaconyl CoA. A definitive understanding of the energy metabolism of this bacterium will be possible only when all these reactions have been characterized further with respect to possible energetical implications.

Taxonomy. The new cadaverine-degrading isolate is a strictly anaerobic, gram-positive, spore-forming bacterium. Physiologically, it resembles some clostridia, e.g., *Clostridium aminobutyricum*, as it ferments cadaverine mainly to butyrate and acetate. *C. aminobutyricum* is also the closest described relative, with 95% sequence identity at the 16S rRNA gene level. Like *C. aminobutyricum*, our isolate forms spores, but it does not ferment sugars at all. Our methanogenic coculture LC 13 M forms more acetate and less butyrate than *C. aminobutyricum* when growing on γ-aminobutyrate (21), due to its ability to use part of the released electrons for methane formation via interspecies hydrogen transfer. *Clostridium aminobutyricum* could not ferment cadaverine in either pure culture or coculture with *M. hingatei*. *Anaerovorax odorimutans* (31, 34) ferments putrescine but not cadaverine and does not resemble our isolate physiologically, although it appears to be distantly related, according to the 16S rRNA gene sequence similarity.

ACKNOWLEDGMENTS

We thank Carola Matthies for isolation of the sulfate-reducing coculture LC 13D, Oliver Geissinger for amino acid analysis, Antje Wiese and Matthias Becker for technical help, and Benjamin Griffin for stimulating discussions.

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