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Fermentative degradation of putrescine by new strictly anaerobic bacteria

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Abstract. Three strains of new strictly anaerobic, Gram-positive, non-sporeforming bacteria were isolated from various anoxic sediment samples with putrescine as sole carbon and energy source. Optimal growth in carbonate-buffered defined medium occurred at 37° C at pH 7.2 – 7.6. The DNA base ratio of strain NorPut1 was 29.6 ± 1 mol% guanine plus cytosine. In addition to a surface layer and the peptidoglycan layer, the cell wall contained a second innermost layer with a periodic arrangement of subunits. All strains fermented putrescine to acetate, butyrate, and molecular hydrogen; the latter originated from both oxidative putrescine deamination and 4-aminobutyraldehyde oxidation. In defined mixed cultures with methanogens or homoacetogenic bacteria, methane or additional acetate were formed due to interspecies hydrogen transfer. Also 4-aminobutyrate and 4-hydroxybutyrate were fermented to acetate and butyrate, but no hydrogen was released from these substrates. No sugars, organic acids, other primary amines or amino acids were used as substrates. Neither sulfate, thiosulfate, sulfur, nitrate nor fumarate was reduced. Most of the enzymes involved in putrescine degradation could be demonstrated in cell-free extracts. A pathway of putrescine fermentation via 4-aminobutyrate and crotonyl-CoA with subsequent dismutation to acetate and butyrate is suggested.

Key words: Anaerobic degradation – Primary amine oxidation – Cell wall structure – Putrescine – Interspecies hydrogen transfer – Diamines

Primary aliphatic amines are formed during oxygen-limited decomposition of organic matter rich in protein. Clostridia and some enterobacteria produce biogenic amines, e.g., putrescine or cadaverine, by decarboxylation of the respective amino acids (Brock 1979; Knoke and Bernhardt 1985). These putrid-smelling and often highly toxic compounds ("ptomaines") are also released in food, e.g., in ripening cheese (Lindner 1986).

In natural environments, primary amines do not accumulate, but few is known about their decomposition. Aerobic degradation starts with oxidative deamination by amine oxidases as described for the inactivation of biogenic amines in vertebrates (Karlson 1984), leading to the respective aldehyde, ammonia, and H_2O_2 as products. Another way of elimination of the NH_2-group is transamination as described for a putrescine-degrading mutant of *Escherichia coli:* Putrescine is degraded by a putrescine:2-oxoglutarate-transaminase and a dehydrogenase to form 4-aminobutyrate which is further metabolized through succinate via the citric acid cycle (Prieto-Santos et al. 1986).

Anaerobic degradation of primary amines by fermenting bacteria could follow a basically similar route, and dispose of the released reducing equivalents in a similar manner as described for primary aliphatic alcohols. In the absence of external electron acceptors such as sulfate or nitrate, incomplete oxidation to fatty or dicarboxylic acids could be coupled to syntrophic methane formation, homoacetogenesis, or reductive synthesis of long-chain fatty acids. It was of general interest to find out whether the fermentative degradation of primary amines takes comparable routes.

In the present study, new isolates of strictly anaerobic bacteria are described which degrade putrescine fermentatively and form acetate, butyrate, and H_2 as products. Enzymes involved in putrescine degradation were measured, and a catabolic pathway is proposed.

Materials and methods

Sources of organisms

Three strains were isolated in pure culture from enrichment cultures inoculated with mud samples:

Strain NorPut1 from anoxic brackish water sediment of the estuary of the Norsminde fjord near Aarhus, Denmark.

Strain FrPut1 from anoxic mud of a polluted ditch near Konstanz, FRG.

Strain MaPut1 from anoxic marine black mud of Rio Marin, a channel located in the city of Venice, Italy.

Methanospirillum hungatei strain M1h was isolated from digested anaerobic sludge of the sewage plant at Göttingen, FRG, and cultivated in freshwater medium with 5 mM acetate under H_2/CO_2 gas mixture (80%/20%).

Desulfovibrio vulgaris strain Marburg was kindly provided by Prof. R. K. Thauer, Marburg, FRG.

Acetobacterium woodii (DSM 1030) was obtained from the Deutsche Sammlung für Mikroorganismen, Braunschweig, FRG.

Media and growth conditions

All procedures for cultivation and isolation were as previously described (Widdel and Pfennig 1981; Schink and Pfennig 1982; Schink 1984). The mineral salts medium for

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isolation and further cultivation was carbonate-buffered (30 mM) and sulfide-reduced (1 mM), and contained 1 ml of selenite-tungstate solution and 1 ml of trace element solution SL 10 (Widdel et al. 1983) per liter, as well as 0.5 ml/l of 7-vitamins solution (Widdel and Pfennig 1981). The pH was adjusted to 7.2–7.4. Growth experiments were carried out in 27 ml-serum tubes filled with medium, gassed with N_2/CO_2 gas mixture (90%/10%), and sealed with butyl rubber stoppers. Growth was followed by measuring turbidity at 450 nm wavelength with a Spectronic 20 spectrophotometer (Milton Roy, NY, USA). All cultures were incubated at 30° C.

Characterization of isolates

Gram staining was carried out according to Bartholomew (1962). *Acetobacterium woodii* and *Pelobacter acidigallici* were used as controls.

Flagella were visualized by staining after Blenden and Goldberg (1965).

Spore formation was checked for after growth in freshwater medium with 20% soil extract and 5 mg $MnSO_4$ per liter. Alternatively, 1.5% tryptone and 0.1% xylose were added as well (modified after Hollaus and Sleytr 1972).

Cytochromes were assayed in French press cell extracts of putrescine-grown cells. Crude extracts were subjected to difference spectroscopy in a Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland).

The DNA base composition was determined with the thermal denaturation method according to De Ley et al. (1970) after extraction according to Mandel et al. (1970). *Escherichia coli* strain B (NCTC 10537) was taken as reference.

For electron microscopic examinations, cell material was fixed, dehydrated, ultrathin-sectioned and post-stained as described (Walther-Mauruschat et al. 1977). Electron micrographs were taken with a Philips EM 301 electron microscope at calibrated magnifications.

Chemical analyses

Acetate, butyrate, and methane were assayed by gas chromatography as described (Platen and Schink 1987). Hydrogen was determined with a Carlo Erba Vega 6000 gas chromatograph with thermal conductivity detector and a steel column (2 m × 4 mm) packed with 60/80 mesh molecular sieve (5 Å, Serva, Heidelberg, FRG), detector temperature 130° C, column temperature 50° C, carrier gas nitrogen, 78 ml/min.

Formation of sulfide from sulfate or sulfur was analyzed by the methylene blue method (Cline 1969). Ammonia formation was determined by indophenol formation with phenol and hypochlorite (Chaney and Marbach 1962). Formation of nitrite from nitrate was assayed by azo dye formation with sulfanilic acid and α-naphthylamine (Procházková 1959). Fumarate reduction to succinate was assayed by scanning UV spectra in a Uvikon 860 spectrophotometer. Fumarate in 98% H_2SO_4 has an absorption maximum at 220 nm whereas succinate does not absorb at this wavelength. Protein in crude cell extracts was determined by dye formation with $CuSO_4$ in alkaline solution (modified after Zamenhoff 1957).

Enzyme assays

All enzyme assays were carried out with crude French press cell extracts of putrescine-grown cells under anoxic conditions using a spectrophotometer model 100-40 (Hitachi, Tokyo, Japan). Hydrogenase was assayed with methylviologen as electron acceptor and H_2 as substrate (modified after Diekert and Thauer 1978). Phosphate acetyl transferase and acetate kinase were measured by standard methods (Bergmeyer 1974). In the acetate kinase assay, the Mg^{2+} concentration was increased to 5 mM. Butyrate kinase was assayed by the acetate kinase assay using butyrate as substrate. Crotonase was determined following the crotonyl-CoA absorption decrease at 263 nm (Moskowitz and Merrick 1969). β-keto thiolase was measured according to Lynen and Ochoa (1953) with a cell extract dialyzed against potassium phosphate buffer (50 mM, pH 7.2, reduced with 2.5 mM dithioerythrite) to outdilute coenzyme A. 4-aminobutyraldehyde dehydrogenase was assayed photometrically with benzylviologen as electron acceptor and butyraldehyde as substrate (Odom and Peck 1981). 4-hydroxybutyrate dehydrogenase was measured photometrically by following the oxidation of NADH with succinicsemialdehyde. The assay mixture contained: potasium phosphate buffer, pH 7.2, 50 mM; NADH, 0.3 mM; succinicsemialdehyde, 18 mM; cell extract with 0.01–0.03 mg protein. The reaction was started by addition of succinicsemialdehyde. Putrescine: 2-oxoglutarate transaminase was assayed following the formation of a dye from o-aminobenzaldehyde and 4-aminobutyraldehyde which is the product of the enzyme reaction (modified after Kim 1964; Holmstedt et al. 1961). Butyryl-CoA dehydrogenase was quantified by following the oxidation of NADH with crotonyl-CoA (modified after Tischer et al. 1979). 3-hydroxybutyryl-CoA dehydrogenase was determined as NADH oxidation with acetoacetyl-CoA according to Lynen and Ochoa (1953). Alternatively, this enzyme was measured by APAD reduction with different substrates (modified after Bergmeyer 1974). Butyryl-CoA: acetate CoA transferase was measured in a coupled enzyme assay using butyryl-CoA and acetate as substrates (modified after Hilpert et al. 1984). All enzyme assays were carried out with 0.01–0.1 mg protein per assay. They were measured photometrically in a spectrophotometer model 100-40 (Hitachi, Tokyo, Japan).

Chemicals

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

Results

Enrichment and isolation

Enrichment cultures with saltwater, brackish water and freshwater medium (50 ml) containing 10 mM putrescine as substrate were inoculated with 5 ml of anoxic mud from various sources. Gas production started after 1–4 weaks. In subcultures on the same medium, turbidity developed within 3–8 days. After four transfers, isolation of putrescine-degrading bacteria was attempted in agar shake series in the absence or presence of a lawn of either *Acetobacterium*

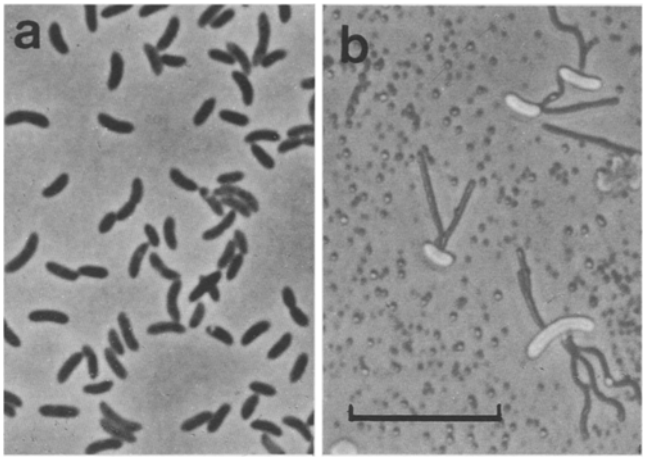

Fig. 1a, b. Phase contrast photomicrographs of strain NorPut1. **a** Vegetative cells; **b** vegetative cells with stained flagella. Bar equals 10 μm

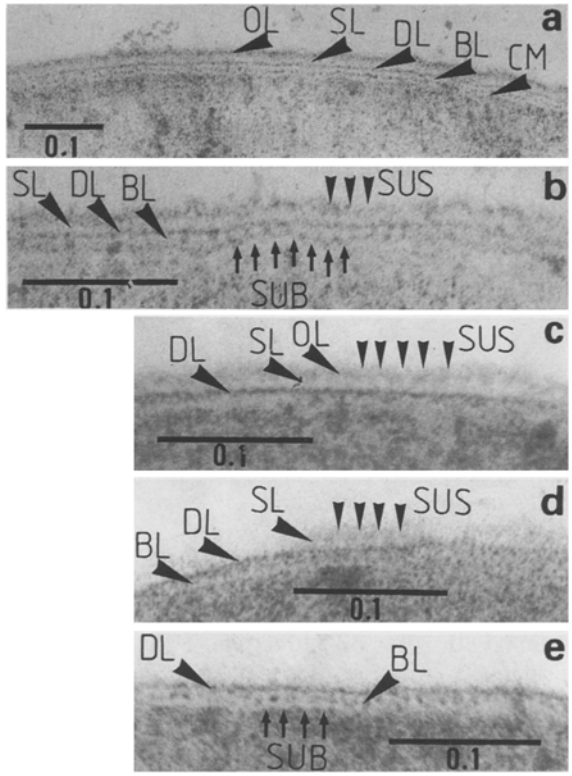

Fig. 2a–e. Ultrathin sections exhibiting cell envelope structures. *BL* bright (electron-translucent) wall layer; *CM* cytoplasmic membrane; *DL* inner dark (electron-dense) wall layer; *OL* outermost (electron-dense) wall layer; *SL* periodic surface layer; *SUB* periodically arranged subunits in *BL*; *SUS* periodically arranged subunits in *SL*. **a** The cell envelope contains several wall layers. **b** Subunits of similar size can be seen both in the surface layer and in an inner wall layer (*BL*). These two wall layers are separated by a thin dark wall layer (*DL*). **c** Surface layer with subunits (covered by the outermost electron-dense layer) and inner dark layer are visible. **d** and **e** Cells seen in ultrathin sections often showed partial (**d**) or complete (**e**) removal of the periodic surface layer (*SL*). Bars refer to sizes in μm

woodii or *Desulfovibrio vulgaris* cells, the latter with sulfate as electron acceptor. In three cases lens-shaped, slightly yellowish colonies surrounded by small satellite colonies of the hydrogen-oxidizing partner bacteria developed which were again subjected to agar shake series dilution without the hydrogen oxidizing bacteria. Pure cultures were isolated from the last positive dilution tube (strains NorPut1, MaPut1, FrPut1). Purity was checked by microscopical control and by growth tests in AC medium (Difco, USA) in which only the isolated cell types were observed.

All three strains were morphologically very similar in size, shape, and Gram reaction, as well as in their physiological properties. Therefore, only one strain, NorPut1, was chosen for further characterization.

Characterization of the brackish water isolate NorPut1

Cytological properties. The cells of strain NorPut1 were slightly curved rods, $0.7-0.8 \times 1.9-2.7$ μm in size, with rounded ends (Fig. 1a). Motility could only be observed in growing cultures. The cells moved in a tumbling manner by 3–5 flagella which were inserted on the concave side of the cell (Fig. 1b). Strain NorPut1 stained Gram-negative, but the KOH test showed no slime formation, a reaction typical of Gram-positive bacteria.

Electron microscopic examination of ultrathin sections of the cells indicated that no outer membrane typical of the cell wall of Gram-negative bacteria existed (Fig. 2a). The cell wall was seen to consist of a surface layer (SL) (Sleytr 1976; Sleytr and Glauert 1982) covered by unstructured amorphous electron-dense material (OL) (Sleytr et al. 1988) (Fig. 2a, c). The surface layer (SL) appeared to be attached to a thin electron-dense layer (DL) (Fig. 2c) probably representing the peptidoglycan layer of the wall (Wahlberg et al. 1987). An unusual feature of the cell wall was the occurrence of a second, innermost wall layer (BL) (Fig. 2a, b, d, e) exhibiting a periodic arrangement of subunits (SUB) (Fig. 2b, e) similar in size to those (SUS) (Fig. 2b, c, d) of the surface layer (SL).

Spore formation could never be observed, neither in defined medium nor in special sporulation media. The guanine-plus-cytosine content of the DNA of the strain NorPut1 was 29.6 ± 1.0 mol%. No cytochromes could be detected in redox difference spectra of crude cell extracts.

Physiology. Strain NorPut1 grew well in freshwater mineral medium and in saltwater medium containing up to 2% NaCl and 0.3% $MgCl_2 \times 6\ H_2O$ (w/v). Phosphate concentrations up to 50 mM were tolerated. For growth in the defined medium, no supplines other than vitamines were required. In the presence of 0.05% yeast extract, the cells grew faster and the cell yield increased by 20%. No growth occurred with yeast extract alone. The highest cell densities were obtained with 30 mM putrescine. Optimal growth (μ = 0.044 h^{-1}, t_d = 16 h) occurred at 37°C, the temperature limits were 12°C and 50°C. The pH-optimum was at pH 7.2–7.6; weak growth was still possible at pH 5.1 and 8.0.

The only substrates degraded were putrescine, 4-aminobutyrate, and 4-hydroxybutyrate. Neither sugars, amino acids, organic acids, alcohols, nor other amines were used (Table 1). Neither nitrate, sulfate, sulfur, thiosulfate, nor fumarate could be used as electron acceptor during putrescine fermentation. Externally added electron donors, e.g. H_2 or formate, were not oxidized during the fermentation of 4-aminobutyrate or putrescine. Products of putrescine fermentation were acetate, butyrate and molecular hydrogen. During fermentation of 4-aminobutyrate and 4-hydroxybutyrate, only acetate and butyrate were formed.

Table 1. Stoichiometry of fermentation and growth yields of strains NorPut1 and MaPut1

Substrate	Substrate degraded[c] (μmol)	Optical density reached ΔE_{450}	Cell dry mass formed[a] (mg)	Substrate assimilated[b] (μmol)	Products formed (μmol)				Electron recovery (%)	Molar growth yield (g/mol)
					Acetate	Butyrate	H_2	Ammonia		
Strain *NorPut1*										
Putrescine	94.0	0.19	0.29	2.1	56	61	146	188	97.1	3.2
4-Amino-butyrate	103.0	0.14	0.21	1.9	110	41	0	103	93.9	2.1
4-Hydroxy-butyrate	100.0[d]	0.15	0.23	2.1	112	42	2	–	99.0	2.3
Strain MaPut1										
Putrescine	93.5	0.31	0.68	5.2	22	77	119	187	98.0	7.3
4-Amino-butyrate	112.0	0.23	0.50	4.6	122	43	0	112	96.0	4.5
4-Hydroxy-butyrate	100.0[c]	0.22	0.48	4.4	110	39	0	–	97.6	4.8

Experiments were carried out in 27 ml serum tubes filled with 10 ml culture, headspaces gassed with N_2/CO_2 gas mixture (90%/10%)
[a] Cell dry matter was calculated via cell turbidity using the conversion factors 0.1 OD_{450} = 16 mg dry matter per liter (NorPut1) and 0.1 OD_{450} = 23 mg dry matter per liter (MaPut1), which were obtained by direct gravimetric determinations in 500 ml cultures grown with putrescine
[b] Substrate assimilated was calculated using the formula ($C_4H_8O_2N$) for cell material
[c] Substrate degraded was calculated by determination of ammonia formed
[d] Substrate added
The following substrates were not degraded by strain NorPut1 in the presence of 5 mM acetate (if not indicated otherwise, the substrates were added to 10 mM, sugars to 5 mM): 1,3-diaminopropane, cadaverine, β-alanine, ethylamine, ethylenediamine, ethanolamine, methylamine (5 mM), betaine, choline, aniline (2 mM), casamino acids (0.1%), glycine, glucose, fructose, arabinose, xylose, glucosamine, lactose, sucrose, formate, lactate, malate, fumarate, succinate, oxalate (4 mM), malonate, glycerol, 1,4-butanediol, 1,3-propanediol, methanol (5 mM), H_2/CO_2 (80%/20%). Degradation of ethylamine, ethyleneamine, methylamine, aniline, and amino acids was tested also in the presence of H_2 as external electron donor

Growth yields and stoichiometry

The stoichiometry of substrate utilization was measured with all three strains isolated. The results obtained with strain NorPut1 and strain MaPut1 are presented in Table 1. The values obtained with strain FrPut1 were similar to those with strain NorPut1. The amounts of fermentation products agreed with complete conversion of the substrates according to the following equation:

Putrescine:

Strain NorPut1

$$10\ H_3NCH_2(CH_2)_2CH_2NH_3^{2+} + 26\ H_2O \rightarrow 6\ CH_3COO^- + 7\ CH_3(CH_2)_2COO^- + 20\ NH_4^+ + 16\ H_2 + 13\ H^+ \quad (1)$$

Strain MaPut1

$$20\ H_3NCH_2\ (CH_2)^2CH_2\ NH_3^{2+} + 46\ H_2O \rightarrow 6\ CH_3COO^- + 17\ CH_3(CH_2)_2COO^- + 40\ NH_4^+ + 26\ H_2 + 23\ H^+ \quad (2)$$

4-Aminobutyrate:
Both strains

$$2\ H_3NCH_2(CH_2)_2COO + 2\ H_2O \rightarrow 2\ CH_3COO^- + 1\ CH_3(CH_2)_2COO^- + 2\ NH_4^+ + 1\ H(3)$$

4-Hydroxybutyrate:
Both strains

$$2\ HOCH_2(CH_2)_2COO^- + 2\ H_2O \rightarrow 2\ CH_3COO^- + 1\ CH_3(CH_2)_2COO^- + 1\ H^+ \quad (4)$$

Effect of hydrogen on putrescine fermentation

Experiments in defined mixed cultures were carried out with strain NorPut1 and either *Acetobacterium woodii* or *Methanospirillum hungatei* as hydrogen-scavening partner organism. The results obtained are summarized in Table 2. In mixed cultures with hydrogen-oxidizing bacteria the putrescine fermentation pattern of strain NorPut1 changed. No hydrogen was formed, and acetate to butyrate ratio was 2:1 as compared to 6:7 in pure culture. Putrescine degradation in mixed cultures agreed with the following equations:

$$2\ H_3NCH_2(CH_2)_2CH_2NH_3^{2+} + 2\ HCO_3^- + 2\ H_2O \rightarrow 3\ CH_3COO^- + 1\ CH_3(CH_2)_2COO^- + 4\ NH_4^+ + 2\ H^+ \quad (5)$$

$$2\ H_3NCH_2(CH_3)_2CH_2NH_3^{2+} + 1\ HCO_3^- + 3\ H_2O \rightarrow 2\ CH_3COO^- + 1\ CH_3(CH_2)_2COO^- + 1\ CH_4 + 4\ NH_4^+ + 2\ H^+ \quad (6)$$

Excess hydrogen added to a culture of strain NorPut1 growing with putrescine delayed and inhibited growth considerably (Fig. 3).

Enzymes involved in putrescine degradation

Enzymes were assayed in crude cell extracts prepared from putrescine-grown cells of strain NorPut1. Hydrogenase, 4-aminobutyraldehyde dehydrogenase, 4-hydroxybutyrate dehydrogenase, acetate kinase, phosphate acetyl transferase,

Table 2. Growth yields and stoichiometry of putrescine fermentation by strain NorPut1 in coculture with *Acetobacterium woodii* or *Methanospirillum hungatei*

Co-organism	Putrescine degraded (μmol)	Acetate (μmol)	Butyrate	Methane	Ammonia	Electron recovery (%)	Growth yield (g/mol putrescine utilized)
A. woodii	94.8	138	46	–	189.6	100	5.1
M. hungatei	468.0	499	205	396	936.0	110	2.6

Experiments with *Acetobacterium woodii* were carried out as described in Table 1 whereas the experiments with *Methanospirillum hungatei* were run in half-filled 100 ml serum bottles, the headspace gassed with N_2/CO_2 gas mixture (90%/10%)

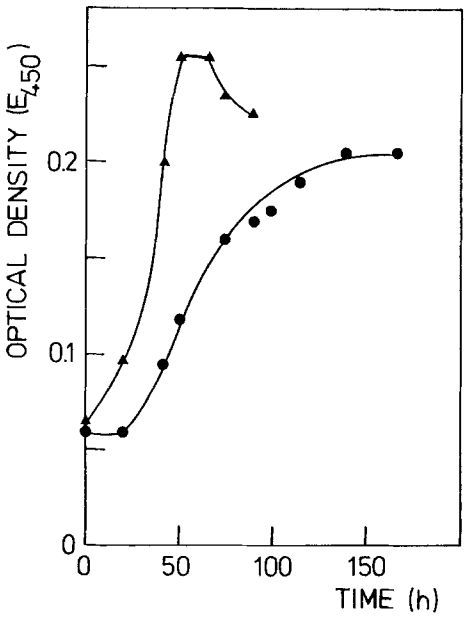

Fig. 3. Effect of excess hydrogen on putrescine fermentation by strain NorPut1. Experimental conditions are described in Table 1. (▲) growth under N_2/CO_2; (●) growth under H_2/CO_2; OD_{450} optical density at 450 nm

crotonase, β-ketothiolase and butyryl-CoA:acetate CoA transferase showed activities sufficient for involvement in dissimilatory metabolism (Table 3). The activity of 4-aminobutyraldehyde dehydrogenase could not be enhanced by adding coenzyme A. Putrescine:2-oxoglutarate aminotransferase, butyryl-CoA dehydrogenase and 3-hydroxybutyryl-CoA dehydrogenase were detected at low activities (Table 3) as well as NAD-dependent 3-hydroxybutyryl-CoA dehydrogenase measured with APAD and vinylacetate as substrates in the presence of acetyl-CoA (0.032 μmol · min^{-1} · mg $protein^{-1}$). For the latter enzyme, no activity was detected with crotonate, crotonyl-CoA and 3-hydroxybutyryl-CoA. Putrescine:2-oxoglutarate aminotransferase, butyryl-CoA dehydrogenase and NAD-dependent 3-hydroxybutyryl-CoA dehydrogenase (with APAD and vinylacetate) did not exhibit significant catabolic activities and showed no linear relationship between activity and the amount of protein added. Carbon monoxide dehydrogenase and butyrate kinase could not be detected.

Discussion

Physiology

In the present study, three strains of putrescine-fermenting bacteria were enriched and isolated from marine, brackish, and limnic anoxic sediment samples. They all were bacteria typical of an estuarine environment where salt concentrations change periodically, and they all tolerate concentrations up to 2% NaCl and 0.3% $MgCl_2$ (w/v). Nonetheless, their optimal growth temperature was at 37°C indicating that they could as well be typical inhabitants of the intestinal system of warm-blooded higher animals where primary amines are common substrates. The range of substrates used as sole carbon and energy source was very small: only putrescine, 4-aminobutyrate and 4-hydroxybutyrate were used, and were fermented to acetate, butyrate and, in the case of putrescine, also molecular hydrogen. Thus, putrescine is not syntrophically oxidized to the corresponding dicarboxylic acid succinate as originally expected, but dismutated to acetate, butyrate, and hydrogen, by a pure culture.

The pathway of substrate degradation was analyzed with strain NorPut1 by enzyme measurements in cell-free extracts. The results are summarized in a hypothetical degradation pathway (Fig. 4). Putrescine:2-oxoglutarate aminotransferase and 4-aminobutyraldehyde dehydrogenase were detected which oxidize putrescine to 4-aminobutyrate. 4-Aminobutyraldehyde oxidation was coupled to benzylviologen reduction and was independent of coenzyme A in our test system. ATP synthesis coupled to this oxidation step has to be postulated on the basis of cell yield measurements (see below), however, the mechanism involved remains unclear at present.

Oxidative deamination of 4-aminobutyrate would lead to succinicsemialdehyde which is reduced to 4-hydroxybutyrate by a very active dehydrogenase. The further degradation involves dehydration and activation to the CoA derivative, and isomerization to crotonyl-CoA. Unfortunately, we were unable to detect the respective enzyme activities and it remains unclear whether isomerization of the vinylacetyl-CoA to the crotonyl residue occurs with the free acids or their CoA derivatives. A vinylacetyl-CoA: crotonyl-CoA isomerase was reported to be active in 4-aminobutyrate degradation by *Clostridium aminobutyricum* (Hardman and Stadtman 1963), and in ethanol-acetate fermentation by *Clostridium kluyveri* (Bartsch and Barker 1961). In extracts of strain NorPut1, an NAD-dependent 3-hydroxybutyryl-CoA dehydrogenase activity could be proven with

Table 3. Enyzmes involved in putrescine degradation by strain NorPut1

Enzyme	EC number	Specific activity (μmol · min^{-1} · mg protein^{-1})
Hydrogenase[a]	1.18.99.1	5.85
4-Aminobutyraldehyde dehydrogenase[b]	1.2.1.19	1.44
4-Hydroxybutyrate dehydrogenase[c]	1.1.1.61	3.3
Acetate kinase	2.7.2.1	0.44
Phosphate acetyl transferase	2.3.1.8	4.2
Crotonase	4.2.1.17	2.1
β-Ketothiolase	2.3.1.19	2.9
Butyryl-CoA:acetate CoA transferase	2.8.3.8	1.05
Putrescine:2-oxoglutarate aminotransferase	?	0.061
Butyryl-CoA dehydrogenase	1.3.99.2	0.028
3-Hydroxybutyryl-CoA dehydrogenase[c]	1.1.1.157	0.06

[a] Methylviologen-dependent
[b] Benzylviologen-dependent
[c] NAD^+-dependent

vinylacetate, APAD, and acetyl-CoA as substrates indicating that vinylacetate is activated to its CoA derivative before isomerization by an acetyl-CoA:vinylacetate CoA transferase. Such an enzyme was also assumed to be active in succinate metabolism of *Clostridium kluyveri* (Stadtman 1953; Kenealy and Waselefsky 1985).

The further dismutation of the crotonyl residue to acetate and butyrate follows well-known reaction sequences which were all proven by demonstration of the respective enzyme activities in cell extracts. Unlike in *Clostridium aminobutyricum,* the CoA-group of butyryl-CoA is transferred to acetate with our strain by butyryl-CoA:acetate CoA transferase to form acetyl-CoA. Hydrogen was an important product of putrescine fermentation by all three isolates, but not of 4-aminobutyrate or 4-hydrxoybutyrate fermentation. Obviously, it is formed from the reducing equivalents released in oxidative deamination via glutamate as amino group carrier, and in aminobutyraldehyde oxidation to aminobutyric acid. The former ones arise at $E_0' = -110$ mV, the latter at $E_0' < -500$ mV. Whereas the low-potential electrons are quantitatively released as molecular hydrogen via a methylviologen-reducing hydrogenase, the former ones need an acceptor of a potential considerably higher than that of protons, and give rise to more butyrate instead of acetate formation [Table 1 and Eqs. (1–4)]. The ratio of butyrate over acetate fermentation depended considerably on the size of the culture headspace or on the presence of hydrogen-consuming partner organisms. How-

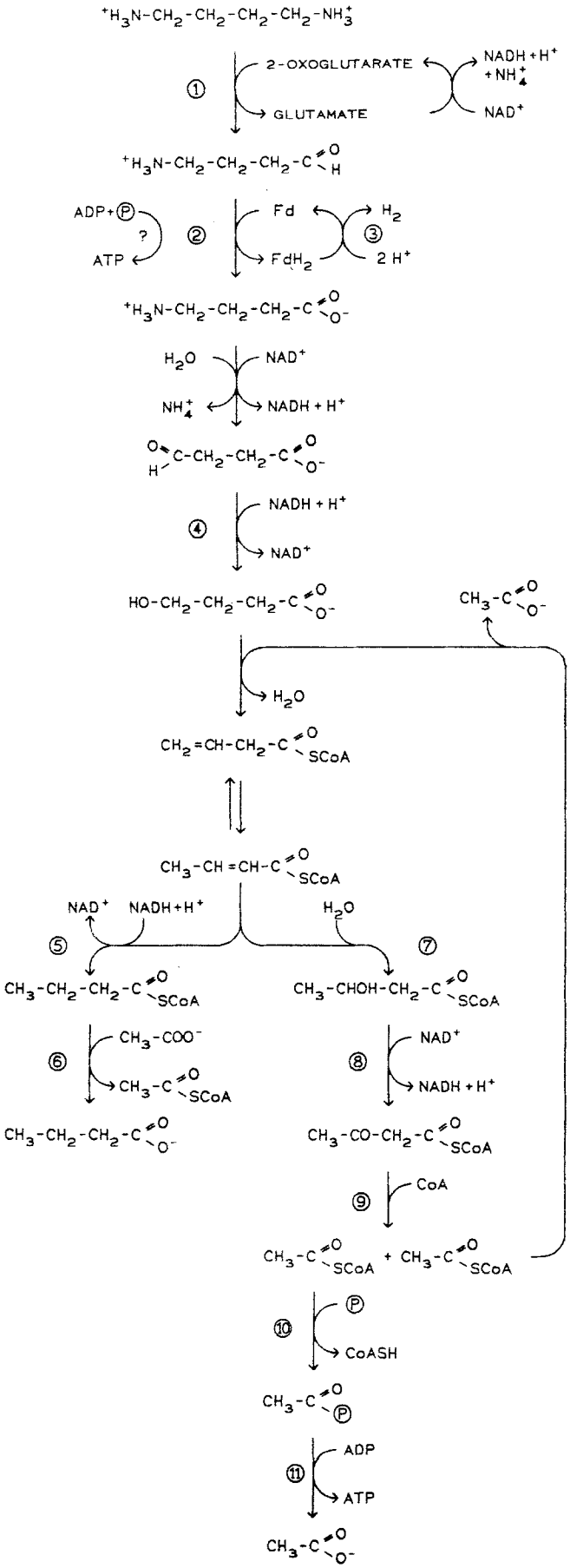

Fig. 4. Hypothetical pathway of putrescine degradation by strain NorPut1. Numbers in circles refer to the following enzymes which were detected in cell-free extracts: (*1*) putrescine:2-oxoglutarate aminotransferase; (*2*) 4-aminobutyraldehyde dehydrogenase; (*3*) hydrogenase; (*4*) 4-hydroxybutyrate dehydrogenase; (*5*) crotonyl-CoA dehydrogenase; (*6*) butyryl-CoA:acetate CoA transferase; (*7*) crotonase; (*8*) 3-hydroxybutyryl-CoA dehydrogenase; (*9*) β-ketothiolase; (*10*) phosphate acetyl transferase; (*11*) acetate kinase

ever, butyrate formation could not be prevented completely indicating that the terminal crotonyl-CoA dismutation was not significantly affected by the hydrogen partial pressure.

The overall energy balance of putrescine fermentation according to Eq. (1) releases about −107 kJ per mol putrescine, which would allow synthesis of more than 1 mol ATP per mol substrate (calculations after Thauer et al. 1977, and D'Ans and Lax 1983). The fermentation scheme in Fig. 4 in connection with Eqs. (1−4) would allow per substrate level phosphorylation an overall synthesis of 0.5 ATP per mol aminobutyrate or hydroxybutyrate, or of 1.15−1.3 ATP per mol of putrescine fermented, provided that the oxidation of aminobutyraldehyde is linked to ATP synthesis (see above). From experimentally determined cell yields Y_{ATP} values of 2.5−4.6 and 6.3−9.6 g per mol can be calculated with strains NorPut1 and MaPut1, respectively, which are low as compared to other bacteria (Stouthamer 1979). At present, we have no explanation for the significant difference in Y_{ATP} values between these two strains.

Taxonomy

The new putrescine-degrading isolates are strictly anaerobic, Gram-positive, non-sporeforming bacteria. Physiologically, they resemble *Clostridium aminobutyricum* which also ferments 4-aminobutyrate to acetate and butyrate (Hardman and Stadtman 1960). However, *Clostridium aminobutyricum* sporulates and ferments many sugars (Hardman and Stadtman 1960) but not putrescine. *Acetobacterium woodii, Clostridium barkeri* and *Eubacterium limosum* form a systematically homogenous group of Gram-positive, non-sporeforming strict anaerobes which are related to each other by high homologies of their 16S ribosomal RNAs (Tanner et al. 1981). They produce acetate or acetate and butyrate as fermentation products, and therefore resemble our new isolates. However, their DNA guanine-plus-cytosine contents (39−48.3 mol%) are considerably higher than that of our isolates (29.6 mol%), and therefore assignment to any of these genera is not possible either. A special feature of our strain NorPut1 is its unusual cell wall architecture. Most significantly, a second layer of periodically arranged subunits appears between the peptidoglycan layer and the cytoplasmic membrane. Occasionally, similar constructions of bacterial cell walls have been observed before with some *Clostridium* species (Sleytr 1976; Sleytr and Glauert 1976; Sleytr and Krebs 1971; Sleytr and Messner 1983, 1988), and with *Bacillus* sp. strain KL 1 (Wahlberg et al. 1987). For the clostridia it was assumed that the inner periodic protein layer is formed by reattachment of subunits (Sleytr 1976). With *Bacillus* sp. strain KL 1, the authors speculated whether the combination of the two periodic layers and the flagella might give rise for a moving apparatus enabling the cell to spread very fast on the surface of nutrient agar. With our strains, we did not observe spreading of this kind. Further work on the cell wall structure is needed before our isolates can be assigned correctly to a taxonomic entity.

Strain NorPut1 was deposited with the Deutsche Sammlung von Mikroorganismen, Braunschweig, under the number DSM 5092.

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