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New motile anaerobic bacteria growing by succinate decarboxylation to propionate

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Abstract. Three strains of new anaerobic, gram-negative bacteria which grew with succinate as sole source of carbon and energy were isolated from anoxic marine and freshwater mud samples. Cells of the three strains were small, non-spore-forming, motile rods or spirilla. The guanine-plus-cytosine content of the DNA of strain US2 was 52.6 ± 1.0 mol%, of strain Ft2 63.5 ± 1.4 mol%, and of strain Ft1 62.6 ± 1.0 mol%. Succinate was fermented stoichiometrically to propionate and carbon dioxide. The growth yields were 1.2 – 2.6 g dry cell mass per mol succinate degraded. Strains US2 and Ft2 required 0.05% w/v yeast extract in addition to succinate for reproducible growth. Optimal growth occurred at 30° – 37° C and pH 6.8 – 8.0. Addition of acetate as cosubstrate did not stimulate growth with any strain. Strain Ft2 grew only under strictly anaerobic conditions, whereas strains US2 and Ft1 tolerated oxygen up to 20% in the headspace. Strains US2 and Ft2 grew only with succinate. Strain Ft1 also converted fumarate, aspartate, and sugars to propionate and acetate. This strain also oxidized propionate with nitrate to acetate. Very low amounts of a *c*-type cytochrome were detected in propionate plus nitrate- or glucose-grown cells of this strain ($0.4\ \mu g \times g\ protein^{-1}$). Moderate activities of avidin-sensitive methylmalonyl-CoA decarboxylase were found in cell-free extracts of all strains.

Key words: Succinate fermentation – Decarboxylation energy – Na^+ bioenergetics – Flagellar motors – Propionate oxidation – Methylmalonyl-CoA

Succinate is an important end product of anaerobic fermentations (Gottschalk and Andreesen 1979). Aerobically it is oxidized completely to carbon dioxide in the citric acid cycle. Many sulfate-reducing bacteria can oxidize succinate (Widdel 1988), and phototrophic purple bacteria assimilate succinate as cell carbon source (Biebl and Pfennig 1981). Some rumen bacteria such as *Selenomonas ruminantium* (Scheifinger and Wolin 1973) or *Veillonella alcalescens* (Yousten and Delwiche 1961) have been shown to decarboxylate succinate to propionate in a reaction not linked to growth. A strictly anaerobic bacterium, *Propionigenium modestum*, was isolated which can grow on the basis of this decarboxylation reaction (Schink and Pfennig 1982b). The reaction sequence of succinate degradation includes succinyl-CoA, methylmalonly-CoA, and propionyl-CoA (Hilpert et al. 1984). The small free energy change of the decarboxylation reaction ($\Delta G_0' = -20.5$ kJ/mol succinate; Thauer et al. 1977) is converted into a sodium ion gradient which drives synthesis via a sodium ion dependent ATPase (Hilpert et al. 1984). *Propionigenium modestum* is a saltwater isolate and requires at least 10 g sodium chloride per liter medium for growth. It was the aim of the present study to look for new bacterial strains which ferment succinate under different conditions. In the present paper three new strains conserving energy from succinate decarboxylation are described.

Materials and methods

Sources of organisms

The new strains were isolated from enrichment cultures inoculated with mud samples from the following sources: Strain US2 from marine sediment of the Sippewissett salt marshes, close to Woods Hole, MA, USA; strains Ft2 and Ft1 from digested anaerobic sludge of a municipal sewage plant at Marburg-Cappel, FRG.

Propionibacterium freudenreichii ssp. *shermanii* (DSM 20270) and *Escherichia coli* K12 (DSM 498) were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

Media and growth conditions

All procedures for cultivation and isolation were as previously described (Widdel and Pfennig 1981; Schink and Pfennig 1982a; Schink 1984). The mineral salts medium for isolation and further cultivation was carbonate-buffered (30 mM) and sulfide-reduced

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(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 per liter of 7-vitamins solution (Widdel and Pfennig 1981). The pH was adjusted to 7.1 – 7.3. Growth experiments were carried out either in 22 ml screw-cap tubes completely filled with medium, or in 120 ml serum bottles filled with 60 ml medium under an atmosphere of N_2/CO_2 (90%/10%). All growth experiments were carried out at least in duplicates at 30°C unless stated otherwise

Isolation and characterization

Pure cultures were obtained by repeated application of the agar shake culture method (Pfennig 1978). Purity was checked microscopically and also by growth tests in complex medium (AC-medium, Difco-Laboratories, Detroit, MI, USA). Growth was followed by measuring turbidity at 400 nm wavelength with a Spectronic 20 Spectrophotometer (Milton Roy, Rochester, NY, USA) or in 1-ml cuvettes in a spectrophotometer model 100-40 (Hitachi, Tokyo, Japan). Gram staining was carried out according to Bartholomew (1962) with *Propionibacterium freudenreichii* and *Escherichia coli* as controls. The KOH test according to Gregersen (1978) was applied in addition. Spore formation was checked after growth in medium with 20% soil extract, 1% Trypton, 0.1% Xylose, and 0.5% $MnSO_4$ according to Hollaus and Sleytr (1972). Cytochromes were assayed in cell-free extracts as well as in membrane preparations obtained by ultracentrifugation (1 h at 150000 *g*). Samples were subjected to difference spectroscopy (dithionite-reduced minus air-oxidized) in an Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland). The DNA base composition was determined by thermal denaturation according to De Ley et al. (1970) after extraction described by Marmur (1961). *Escherichia coli* strain K12 was taken as reference.

Chemical analyses

Acetate and propionate were assayed by gas chromatography with a Carlo Erba 6000 gas chromatograph (Milano, Italy) as described (Platen and Schink 1987). Succinate was determined as dimethylester by capillary gas chromatography (Dehning and Schink 1989). H_2 was determined with a thermal conductivity detector and a steel column (2 m × 4 mm) packed with 60/80 mesh molecular sieve (5 A, 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 determined half-quantitatively (Cord-Ruwisch 1985). Formation of nitrite from nitrate was assayed by azo dye formation with sulfanilic acid and α-napthylamine (Procházková 1959).

Enzyme assays

Methylmalonyl-CoA decarboxylase activity in crude cell extracts was assayed photometrically at 25°C (modified after Hilpert and Dimroth 1983). The cuvette contained in 1 ml: 100 mM potassium phosphate buffer, pH 7.0, 20 mM NaCl, 5 U phosphotransacetylase, and 0.1 mM methylmalonyl-CoA. Control experiments were carried out with crude cell extract preincubated for 15 min at room temperature with 0.1 mg avidin or with 0.1 mg avidin pretreated with 0.1 mg biotin.

Protein in crude cell extracts was determined with $CuSO_4$ in alkaline solution (Zamenhoff 1957).

Chemicals

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

Results

Enrichment and isolation

Enrichment cultures with freshwater and saltwater medium (50 ml) containing 20 mM di-sodium succinate with or without 5 mM sodium acetate were inoculated with 5 ml of anoxic mud samples. Turbidity developed within 1 – 2 weeks. In subcultures with the same substrate, turbidity developed within 2 – 5 days and the cell population got more and more uniform. After 5 – 6 transfers, the fastest growing cultures were diluted in two subsequent agar shake series each. Three morphologically different pure cultures growing with succinate as substrate were isolated and further characterized. Strain US2 was isolated in saltwater medium whereas strains Ft2 and Ft1 were freshwater isolates. Two strains (US2 and Ft2) required 0.05% yeast extract for growth.

Morphology and cytological properties

Cells of strain US2 were small, slightly curved rods, 0.2 – 0.3 × 2 – 3 μm in size (Fig. 1 a). Cells of strain Ft2 were small spirilla, 0.2 μm × 2 – 10 μm (Fig. 1 b). Cells of strain Ft1 were short, slightly curved rods, 0.5 × 2 – 3 μm in size, often occurring in pairs (Fig. 1 c). All strains were very motile in fresh cultures. All strains stained gram-negative and the KOH test showed slime formation typical of gram-negative bacteria. Spore formation was never observed, neither in defined medium nor in a special sporulation medium. The guanine-plus-cytosine content of the DNA of strain US2 was 52.6 ± 1 mol%, of strain 63.5 ± 1.4 mol%, and of strain Ft1 62.6 ± 1 mol%.

Very low amounts of a cytochrome were detected only with strain Ft1 in crude cell extracts as well as in the cytoplasmic fraction of propionate plus nitrate- or glucose-grown cells. From the absorption bands at 552 and 420 nm, it was identified as a *c*-type cytochrome. Assuming a molecular weight of 12000 and an extinction coefficient for the α-peak of 19.0 mM^{-1} cm^{-1} (Chance and Williams 1955), a cytochrome *c* content of 0.4 μg per g protein was calculated.

Growth conditions and physiology

All strains fermented succinate stoichiometrically to propionate (Table 1) and carbon dioxide (not determined). The growth rates with 20 mM succinate were 0.045 h^{-1} ($t_d = 15$ h) with strain US2, 0.025 h^{-1} ($t_d = 28$ h) with strain Ft2, and 0.05 h^{-1} ($t_d = 14$ h) with strain Ft1. Optimal growth occurred at 30 – 37°C and pH 6.8 – 8.0. The temperature limits were 24 and 45°C, the pH range was 6.3 – 8.4. Phosphate concentrations up to 50 mM did not influence growth. Optimal growth occurred with succi-

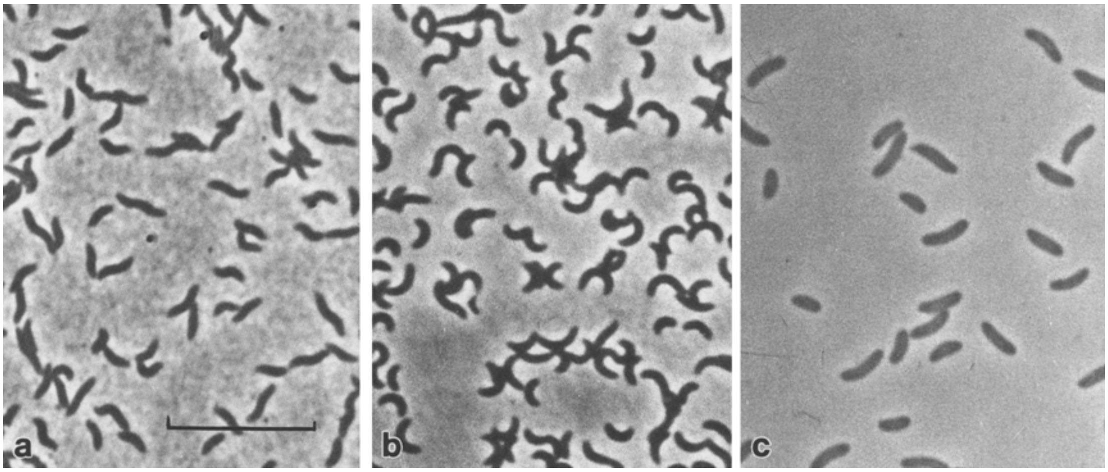

Fig. 1a–c. Phase contrast photomicrographs of strain US2, Ft2 and Ft1. *Bar* equals 10 µm for all panels

Table 1. Fermentation stoichiometries and growth yields of the strains US2, Ft2 and Ft1 with succinate as substrate

Strain	Substrate degraded[a] [µmol]	Cell dry mass formed[b] [mg]	Substrate assimilated[c] [µmol]	Propionate formed [µmol]	Electron recovery [%]	Molar growth yield [g/mol]
US2	1000	1.23	14.5	1010	102	1.2
Ft2	795	2.04	24.1	745	97	2.6
Ft1	925	2.25	26.6	890	99	2.4

Experiments were carried out in 120-ml serum bottles filled half with medium under N_2/CO_2 (90%/10%) gas mixture and sealed. Strains US2 and Ft2 required 0.05% yeast extract in addition to succinate for growth

[a] Substrate degraded was calculated from the difference of substrate concentration at the beginning and the end of the experiment

[b] Cell dry mass was calculated via cell turbidity using the conversion factors 0.1 OD_{400} = 49 mg dry mass per liter (US2), 0.1 OD_{400} = 37 mg dry mass per liter (Ft2), 0.1 OD_{400} = 30 mg dry mass per liter (Ft1), which were obtained by direct gravimetric determination in 500 ml cultures grown with succinate

[c] Assimilation of substrates into cell material was calculated using the formula $\langle C_4H_7O_3\rangle$ for cell material, and the following assimilation equation for succinate: 17 $\text{succinate}^{2-} + 32\,H_2O \rightarrow 14\,\langle C_4H_7O_3\rangle + 12\,HCO_3^- + 22\,OH^-$; thus, 11.8 µmol succinate was degraded to form 1 mg cell material

nate at concentrations of 20 to 40 mM. The saltwater isolate US2 grew in medium containing 0.1–2.0% NaCl (w/v) and 0.015–0.3% $MgCl_2 \times 6\,H_2O$ (w/v), respectively, but growth ceased after 3–4 transfers in freshwater medium (containing 0.1% NaCl and 0.015% $MgCl_2$). The freshwater isolates Ft2 and Ft1 tolerated NaCl up to 1% (w/v) and $MgCl_2$ up to 0.15% (w/v).

Strain Ft2 grew only under strictly anaerobic conditions, whereas strains US2 and Ft1 tolerated traces of oxygen as visualized in oxygen gradient tube cultures. Growth of strain Ft1 was possible even under air in a phosphate-buffered medium with succinate as substrate.

Addition of acetate as cosubstrate did not stimulate growth with any strain.

Strains US2 and Ft2 were specialized for succinate fermentation only and did not ferment any other organic substrates including other dicarboxylic acids, sugars, lactate, pyruvate, glycerol, ethanol, ethylene glycol, acetoin, and 2,3-butandiol. H_2 or formate was not oxidized, nitrate, sulfate, thiosulfate, sulfite, or sulfur was not reduced.

Unlike these strains, strain Ft1 exhibited a broader metabolic versatility. It fermented fumarate, aspartate, and sugars to propionate and acetate (Table 2). Weak growth occurred with malate, pyruvate, or glycerol as substrates.

The amount of fermentation products agreed with complete conversion of the substrates according to the following equations:

$$\text{succinate}^{2-} + H_2O \rightarrow \text{propionate}^- + HCO_3^- \quad \Delta G'_0 = 20.5\ \text{kJ/mol succinate}$$

$$3\ \text{fumarate}^{2-} + 6\,H_2O \rightarrow 2\ \text{propionate}^- + \text{acetate}^- + 4\,HCO_3^- + H^+ \quad \Delta G'_0 = -81.1\ \text{kJ/mol fumarate}$$

$$3\ \text{aspartate}^- + 6\,H_2O \rightarrow 2\ \text{propionate}^- + \text{acetate}^- + 4\,HCO_3^- + 3\,HN_4^+ + H^+ \quad \Delta G_0{}' = -51.3\ \text{kJ/mol aspartate}$$

$$3\ \text{glucose/fructose} \rightarrow 4\ \text{propionate}^- + 2\ \text{acetate}^- + 2\,HCO_3^- + 8\,H^+ \quad \Delta G'_0 = -308.1\ \text{kJ/mol hexose}$$

$$4\ \text{xylose/arabinose} \rightarrow 4\ \text{propionate}^- + 3\ \text{acetate}^- + 2\,HCO_3^- + 9\,H^+ \quad \Delta G'_0 = -264.0\ \text{kJ/mol pentose.}$$

Addition of hemin at concentrations up to 100 µg/l did not enhance growth yields with fumarate or aspartate. In growth experiments with 20 mM succinate plus 2–8 mM sodium nitrate, growth of strain Ft1 was enhanced by nitrate (Fig. 2). Nitrate was reduced stoichiometrically to nitrite. Figure 3 shows that also propionate could be oxidized to acetate while nitrate was reduced to nitrite.

Table 2. Fermentation stoichiometries and growth yields of strain Ft1

Substrate	Substrate degraded [µmol]	Cell dry mass formed [mg]	Substrate assimilated[a] [µmol]	Products formed [µmol]		Electron recovery [%]	Molar growth yield [g/mol]
				propionate	acetate		
Fumarate	220	1.85	25.3	129	64	99	8.4
Aspartate	220	1.91	26.2	140	62	105	8.7
Glucose	220	4.28	29.1	240	119	95	19.5
Fructose	220	4.60	31.3	253	116	99	20.9
Xylose	220	3.69	30.3	194	145	103	16.8
Arabinose	220	3.69	30.3	184	130	96	16.8

Experiments were carried out in 22-ml screw-cap tubes filled completely with medium. Calculations of substrate degradation and cell dry mass formation as in Table 1

[a] The following assimilation equations were used for calculation:

$17\ \text{fumarate}^{2-} + 42\ H_2O \rightarrow 12\langle C_4H_7O_3\rangle + 20\ HCO_3^- + 14\ OH^-$

$17\ \text{aspartate}^- + 42\ H_2O \rightarrow 12\langle C_4H_7O_3\rangle + 20\ HCO_3^- + 14\ H_2O + 17\ NH_3 + 3H^+$

$17\ \text{glucose/fructose} + 6\ OH^- \rightarrow 24\langle C_4H_7O_3\rangle + 6\ HCO_3^- + 18\ H_2O$

$17\ \text{xylose/arabinose} + 5\ OH^- \rightarrow 20\langle C_4H_7O_3\rangle + 5\ HCO_3^- + 15\ H_2O$

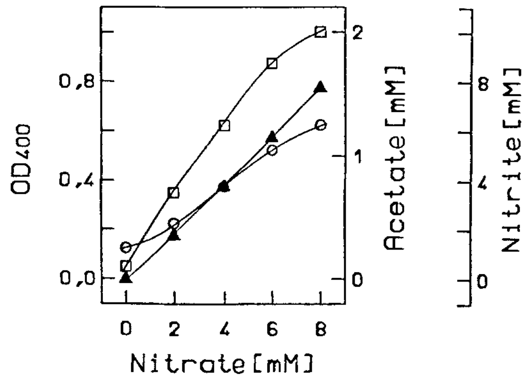

Fig. 2. Growth of strain Ft1 with 20 mM succinate in the absence and presence of nitrate. Experiments were performed at 30°C in 22-ml screw-cap tubes. *Symbols*: ○ cell density, □ acetate, ▲ nitrite. OD_{400}: optical density at 400 nm

No other inorganic electron acceptor such as sulfate, thiosulfate, sulfite, or sulfur was reduced; neither H_2 nor formate was oxidized by strain Ft1.

Enzyme activities

The key enzyme of succinate fermentation, methylmalonyl-CoA-decarboxylase, was assayed in French-press cell extracts prepared from succinate-grown cells of all three strains. With strains US2 and Ft1, an activity of 0.33 U/mg protein was detected. If extracts were preincubated with avidin, only 25% of this activity was found. Avidin preincubated with biotin had no inhibitory effect. With strain Ft2, the methylmalonyl-CoA decarboxylase activity was 0.25 U/mg protein; this activity was only slightly inhibited by avidin.

Discussion

Physiology

Three new strains of succinate-fermenting bacteria were enriched and isolated from anoxic mud samples in the present study, showing that the ability of *Propionigenium modestum* to grow by decarboxylation of succinate to propionate was not an entirely unique phenomenon. Moreover, *Sporomusa termitida* and *Sporomusa malonica* were recently shown to grow by succinate decarboxylation as well (Breznak et al. 1988; Dehning et al. 1989), and also a thermophilic bacterium was isolated which can grow by succinate fermentation (Stams and Zehnder, pers. communication). Two of our new isolates as well as the *Sporomusa* species and the thermophilic isolate are freshwater organisms. Our medium contained only 0.1% NaCl (w/v) and 0.015% $MgCl_2 \times 6H_2O$ (w/v) showing that enhanced NaCl concentrations are not a necessary prerequisit of energy-conserving succinate fermentation. Molar growth yields of 1.2 to 2.6 g dry cell mass per mol succinate were calculated which are in the same range as the one determined for *Propionigenium modestum* (2.4 g dry cell mass per mol succinate). These values correlate with the small free energy change of succinate decarboxylation ($\Delta G_0' = 20.6$ kJ/mol succinate). The yield differences observed may be due to different efficiencies of the energy-conserving systems involved in these new isolates.

Strains US2 and Ft2 were specialized on succinate fermentation only and did not grow with any other substrate tested. Unlike these two specialists, strain Ft1 showed a broader substrate spectrum and fermented also fumarate, aspartate, glucose, fructose, arabinose, and xylose to acetate and propionate. Weak growth occurred also with malate, pyruvate, and glycerol as substrates. Growth yields with fumarate and aspartate were higher than with succinate because additional energy can be conserved by the acetate-kinase reaction. However, these growth yields (8.4–8.7 g/mol) are small if compared to those observed with *Propionibacterium* sp. with lactate (10.2–12.9 g/mol; Stouthamer 1980), and this difference is even more expressed for growth with glucose (19.5–20.9 vs. 50–70 g/mol). These comparably low growth yields suggest that strain Ft1 does not conserve energy by electron transport in fumarate reduction, similar to other recently isolated gram-negative propionate-forming bacteria (Schink and Pfennig 1982b; Schink et

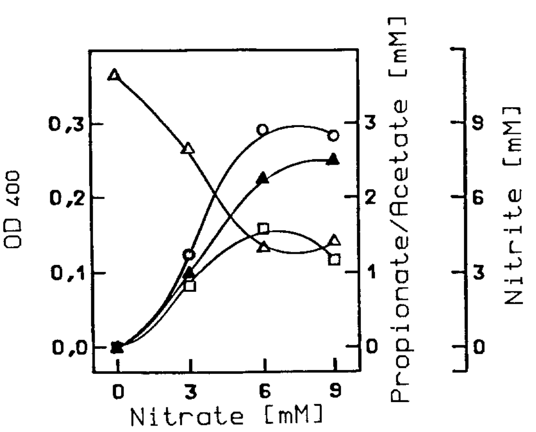

Fig. 3. Growth of strain Ft1 with 3 mM propionate in the absence and presence of nitrate. Experimental conditions as described in Fig. 2. *Symbols*: ○ Cell density, △ propionate, □ acetate, ▲ nitrite. OD_{400}: optical density at 400 nm

al. 1987). This view is further supported by the obvious lack of significant amounts of a *b*-type cytochrome.

Growth of strain Ft1 with succinate was enhanced by addition of nitrate which was reduced stoichiometrically to nitrite. Also propionate, the end product of fermentative metabolism, could be oxidized to acetate and carbon dioxide while nitrate was reduced to nitrite. So far no nitrate-reducing, succinate-decarboxylating bacterium was described. *Selenomonas ruminantium* can reduce nitrate to nitrite (de Vries et al. 1973), and *Veillonella alcalescens* reduces nitrate to ammonia (Stouthamer 1980), but neither one can grow with succinate only.

In *Propionigenium modestum*, ATP synthesis during succinate fermentation is driven directly by a sodium ion gradient (Laubinger and Dimroth 1989) which is generated upon decarboxylation of methylmalonyl-CoA (Hilpert et al. 1984). With our new isolates, enzyme activities of the methylmalonlyl-CoA-decarboxylase could be detected which were inhibited by avidin indicating a biotin enzyme as described for *Veillonella alcalescens* (Hilpert and Dimroth 1983) and *Propionigenium modestum*. It remains to be elucidated whether also in our new isolates all energy-related membrane functions are sodium dependent. Preliminary experiments with ionophores and antiporter inhibitors (not shown) indicated that ATP synthesis and flagellar rotation are driven by a proton gradient.

Taxonomy

The three new succinate-fermenting strains differ in many respects from the first succinate-fermenting organism, *Propionigenium modestum*. They differ in morphology, motility, DNA base ratio, substrate spectrum and most likely in the energy coupling system. Morphologically, they resemble *Campylobacter* species which are as well gram-negative, motile, curved rods. Both strain Ft1 and *Campylobacter* species contain cytochromes and are able to reduce nitrate, but *Campylobacter* forms succinate and does not degrade it. Also the DNA base ratio of *Campylobacter* with 29–38 mol% is considerably lower than that of strain Ft1 and therefore an assignment to this genus is not possible. *Sporomusa* species ferment succinate, however, they form spores, which none of the new isolates does. There are similarities between the new isolates and the rumen bacterium *Selenomonas ruminantium* with respect to gram-staining reaction, DNA base ratio, and degradation of succinate, but *Selenomonas ruminantium* cannot grow with succinate. The taxonomic assignment of these new isolates will be treated in a later communication. Strains US2 and Ft1 were deposited with the Deutsche Sammlung von Mikroorganismen, Braunschweig, under the numbers 5848 and 5849, respectively.

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