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Syntrophobacter pfennigii sp. nov., new syntrophically propionate-oxidizing anaerobe growing in pure culture with propionate and sulfate

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Abstract A new strain of syntrophically propionate-oxidizing fermenting bacteria, strain KoProp1, was isolated from anoxic sludge of a municipal sewage plant. It oxidized propionate or lactate in cooperation with the hydrogen- and formate-utilizing *Methanospirillum hungatei* and grew as well in pure culture without a syntrophic partner with propionate or lactate plus sulfate as energy source. In all cases, the substrates were oxidized stoichiometrically to acetate and CO₂, with concomitant formation of methane or sulfide. Cells formed gas vesicles in the late growth phase and contained cytochromes *b* and *c*, a menaquinone-7, and desulforubidin, but no desulfoviridin. Enzyme measurements in cell-free extracts indicated that propionate was oxidized through the methylmalonyl CoA pathway. Protein pattern analysis by SDS-PAGE of cell-free extracts showed that strain KoProp1 differs significantly from *Syntrophobacter wolinii* and from the propionate-oxidizing sulfate reducer *Desulfobulbus propionicus*. 16S rRNA sequence analysis revealed a significant resemblance to *S. wolinii* allowing the assignment of strain KoProp1 to the genus *Syntrophobacter* as a new species, *S. pfennigii*.

Key words Syntrophic oxidation · Propionate · Sulfate reduction · *Syntrophobacter pfennigii* · Methylmalonyl CoA pathway

Introduction

In the absence of external electron acceptors such as oxygen, nitrate, iron (III), or sulfate, oxidation of propionate requires the activity of fermenting bacteria in syntrophic association with hydrogen-scavenging methanogenic bac-

teria (Zehnder 1978). Fermentation of propionate to acetate, CO₂, and hydrogen is a highly endergonic process (calculations of free energies after Thauer et al. 1977):

The hydrogen partial pressure has to be kept low by the partner organism to make the reaction energetically feasible, e.g., in syntrophic methanogenic propionate degradation:

However, the amount of free energy liberated during syntrophic propionate oxidation is still low and just in the range of the minimum energy quantum needed for ATP formation by each partner bacterium (Schink 1990, 1992). So far, only one defined syntrophic propionate-degrading culture, *Syntrophobacter wolinii*, has been described; this culture contains a methanogenic bacterium and a sulfate-reducing partner bacterium (Boone and Bryant 1980). It has been shown recently that the propionate-fermenting partner bacterium in this mixed culture could be grown in pure culture, either with pyruvate alone or with propionate plus sulfate as substrates (Wallrabenstein et al. 1994). Another propionate-degrading syntrophic anaerobe, strain MPOB, has been recently obtained in an enrichment culture with propionate plus fumarate as substrates, which were fermented to acetate, CO₂, and succinate (Stams et al. 1993); a thermophilic methanogenic propionate-oxidizing syntrophic enrichment culture has also been described (Stams et al. 1992).

The present study reports on the isolation and characterization of a new strain of syntrophically propionate-oxidizing fermenting bacteria that grows also in pure culture by propionate-dependent sulfate reduction and represents a new species within the genus *Syntrophobacter*.

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Materials and methods

Sources of organisms

Strain KoProp1 was isolated from anoxic sludge of the municipal sewage plant in Konstanz, Germany. *Syntrophobacter wolinii* (DSM 2805), a ternary coculture with *Desulfovibrio vulgaris* G11, *Methanospirillum hungatei*, and *Desulfobulbus propionicus* strain Lindhorst (DSM 2032), was obtained from the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). *Methanospirillum hungatei* strain SK was kindly provided by Prof. F. Widdel (Bremen, Germany).

Cultivation and isolation

All procedures for cultivation and isolation were essentially as described in earlier papers (Pfennig 1978; Widdel and Pfennig 1981). The mineral medium for cultivation, enrichment, and isolation contained 30 mM sodium bicarbonate as buffer, 1 mM sodium sulfide as reducing agent, the trace element solution SL 10 (Widdel et al. 1983), a selenite-tungstate-solution (Tschech and Pfennig 1984), and a 7-vitamin solution (Widdel and Pfennig 1981). The medium contained 0.5 g NaCl and 0.4 g $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$ per liter. The pH was 7.2–7.4. For isolation of a pure culture, the agar shake culture method (Pfennig 1978) and a dilution series in liquid media were used. Incubation temperature for strain KoProp1 was 37°C, whereas *S. wolinii* and *D. propionicus* were grown at 28°C.

Characterization

Gram staining was carried out using the method of Bartholomew (1962). For determination of the G+C content, DNA was purified on hydroxyapatite according to the method of Cashion et al. (1977). DNA digestion and subsequent analysis by HPLC were carried out according to Mesbah et al. (1989) and Tamaoka and Komagata (1984).

Cytochromes were determined in cell-free extracts prepared by French-press treatment and in the membrane and soluble fraction separated by ultracentrifugation ($120,000 \times g$, 1 h). Fractions were subjected to difference spectroscopy (dithionite-reduced *minus* air-oxidized) in a Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland). Isoprenoid quinones, extracted from dry cells according to Collins (1985) using petroleum ether:methanol (2:1, v/v) as solvent, were separated and isolated by analytical and preparative thin-layer chromatography. Quinones were identified by HPLC analysis according to the method of Kroppenstedt (1985). Desulfovibrin was determined according to Widdel and Pfennig (1981).

For determination of desulforubidin, cells grown with propionate plus sulfate (6 g wet cell mass) were suspended in 6 ml 10 mM Tris-HCl, pH 7.6. Cells were broken in a French pressure cell, and cell debris was removed by centrifugation at $5,000 \times g$ for 15 min. After ultracentrifugation ($120,000 \times g$, 1 h), the supernatant (soluble fraction) was applied to an anion-exchange column DE 52 (Whatman, Kent, UK) equilibrated with the same buffer. Proteins were eluted with a linear gradient (10–500 mM Tris-HCl in 4 h, HiLoadTM-System, Pharmacia, Freiburg, Germany). Desulforubidin was identified by its typical absorption spectrum between 300 and 700 nm (Lee et al. 1973).

Enzyme measurements

Enzymes were assayed in cell-free extracts of cells grown either in mixed or in pure culture. Cells were broken by French press treatment. After removal of cell debris at $5,000 \times g$, extracts were kept on ice until used. All enzyme measurements were carried out under nitrogen gas, excluding air oxygen as far as possible.

Acetate kinase (EC 2.7.2.1), phosphotransacetylase (EC 2.3.1.8), fumarase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37)

were assayed according to the methods of Bergmeyer (1974); propionate kinase was assayed analogous to acetate kinase with propionate as starter substrate. Succinate thiokinase (EC 6.2.1.5) was assayed according to Oberlies et al. (1980); succinate dehydrogenase (EC 1.3.99.1), methylmalonyl CoA: pyruvate transcarboxylase (EC 2.1.3.1), and malic enzyme (EC 1.1.1.40) were analyzed according to Stams et al. (1984). Fumarate reductase (EC 1.3.1.6) was analyzed according to Boonstra et al. (1975); pyruvate: ferredoxin oxidoreductase were analyzed according to Odom and Peck (1981). Carbon monoxide dehydrogenase (EC 1.2.99.2) was measured using the method of Diekert and Thauer (1978), and hydrogenase (EC 1.18.99.1) and formate dehydrogenase (EC 1.2.1.43?) were measured in the same manner with benzyl viologen as electron acceptor.

Chemical determinations

Propionate and acetate were assayed by gas chromatography, as described previously (Platen and Schink 1987), and methane was measured by gas chromatography according to Matthies and Schink (1992). Sulfide was determined according to Cline (1969); protein was quantified according to Bradford (1976).

Polyacrylamide gel electrophoresis

Samples were prepared and the separation gels (15 × 15 cm) were made according to standard procedures (Sambrook et al. 1989). Electrophoresis was carried out in a Mini-Protein II Dual Slab Cell (Bio-Rad, Richmond, Calif., USA). Protein (10–15 µg protein per lane) was separated in a 10% separation gel at a constant voltage of 200 V (15 V/cm). Gels were stained for 4 h with Coomassie Brilliant Blue [0.25% w/v Coomassie Brilliant Blue R250 in methanol:H₂O:glacial acetic acid (45:45:10, by vol)].

Chemicals

All chemicals used were of analytical grade and were obtained from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), and Serva (Heidelberg, Germany).

Results

Isolation and characterization of strain KoProp1

A propionate-degrading methanogenic enrichment culture inoculated with anoxic sewage sludge, originally supplied with 50 mM and later with 20 mM propionate as sole organic substrate and no sulfate, was repeatedly transferred into fresh medium for more than 4 years before purification was attempted. Over the years, a stable mixed culture had developed that consisted mainly of fat egg-shaped rods and *Methanospirillum hungatei*-like fluorescing cells. In agar-shake dilution series in the presence of 1 mM acetate and a lawn of *M. hungatei* strain SK, large yellowish lens-shaped colonies developed after 10 weeks of incubation, together with many small, white colonies of varying shape. The large colonies consisted of the fat rods prevailing in the enrichment culture, together with *M. hungatei*. However, the number of colonies that developed in the agar-shake dilution tubes was much lower than the number of cells in the enrichment culture, and they grew up only to the fifth dilution tube at most. A subsequent second dilution of a single

Fig. 1 Phase-contrast photomicrographs of strain KoProp1 in co-culture with *Methanospirillum hungatei*. **a** Fresh culture; **b** aged culture showing gas vacuoles; **c** pure culture grown with propionate and sulfate. Bar equals 10 μ m for all three panels

colony did not lead to growth of colonies, not even after 15 weeks of incubation, regardless of whether dithionite, fresh or predigested yeast extract, or additional vitamins were added. The colonies obtained after single dilutions still contained small numbers of thin non-motile rods, as proven after cultivation in the presence of 0.5% yeast extract and 0.5% peptone. Since purification was not possible in agar shakes, we tried purification by series dilution in liquid medium. After many unsuccessful efforts, we finally isolated a defined binary culture consisting of the egg-shaped rods and *M. hungatei*.

Physiological studies revealed that our culture also reduced sulfate (see below). After repeated transfers in min-

eral media containing propionate and sulfate, and 5 mM bromoethane sulfonate for inhibition of methanogens, new dilutions in liquid media were made to obtain strain KoProp1 finally in pure culture.

Cells of strain KoProp1 were motile, slightly egg-shaped rods, $2.2\text{--}3.0 \times 1.0\text{--}1.2$ μ m in size (Fig. 1a). Motility was observed only in the early exponential growth phase. Cells stained gram-negative; the G+C content of the DNA was 57.3 ± 0.2 mol%. Spore formation was never observed, but cells formed gas vacuoles in the late exponential growth phase (Fig. 1b), which could be destroyed by pressure shock treatment in a hypodermic syringe. Cells grown in pure culture with propionate plus sulfate were slightly larger than syntrophically grown cells (Fig. 1c). Growth was possible between 20 and 37°C, with an optimum at 37°C. The strain was strictly anaerobic; no growth occurred after exposure to air oxygen. Addition of dithionite (50–100 μ M) helped to re-

Fig. 2a, b Growth of strain KoProp1 with 10 mM propionate as substrate. **a** Methanogenic coculture with *M. hungatei*; **b** pure culture with propionate plus 10 mM sulfate. (Filled circles cell density, triangles propionate, squares acetate, filled diamonds methane, open diamonds sulfide)

Table 1 Growth yields and stoichiometry of substrate conversion by strain KoProp1. Cell dry matter formed was calculated from turbidity at 578 nm using the conversion factors $0.1 \text{ OD}_{578} = 20.1 \text{ mg/l}$ for the syntrophic culture and $0.1 \text{ OD}_{578} = 17.6 \text{ mg/l}$ for the culture grown with propionate plus sulfate, which were both determined by direct measurements in 1 l cultures. Substrate assimilated into cell material was calculated using the following equations:

Substrate	Substrate degraded (μmol)	Cell dry matter formed (mg)	Substrate assimilated (μmol)	Products formed (μmol)			Carbon recovery (%)	Electron recovery (%)	Growth yield (g mol ⁻¹)
				Acetate	Methane	Sulfide			
<i>a</i> Mixed culture									
Propionate	2000	3.3	50.7	1880	1330	n.d. ^c	95	84	1.6
Lactate	800 ^a	3.1	42.2	750	350	n.d.	98	101	3.9
Propanol	1000 ^a	2.4	29.2	990	1010	n.d.	97	87	2.4
<i>b</i> Pure culture									
Propionate + Sulfate ^b	1000	3.0	47.0	920	n.d.	720	98	109	3.0

^a Substrate added

^b 1000 μmol sulfate was added

sume growth after transfers. The pH range of growth was 6.2–8.0; the pH optimum was 7.0–7.3. In the methanogenic coculture at 37°C with 20 mM propionate as substrate, the growth rate was 0.066 day^{-1} ($t_d = 10.5$ days, Fig. 2a). Propionate was degraded also in the absence of a syntrophic partner when sulfate was supplied, which was stoichiometrically reduced to sulfide. Under these conditions, the growth rate was 0.07 day^{-1} ($t_d = 10$ days; Fig. 2b). In both cases, propionate was oxidized incompletely to acetate and CO_2 (Table 1). Sulfite (2 mM) or thiosulfate (10 mM) could serve as alternative electron acceptors; fumarate or nitrate was not reduced. Besides propionate, also lactate could be oxidized by the pure culture and the methanogenic coculture; propanol was only oxidized by the coculture (Table 1).

Cytochromes, quinones, and sulfite reductases

Syntrophically grown cells of strain KoProp1 contained cytochromes of the *b*- and *c*-type. Cytochrome *c* (maxima at 419, 522, and 553 nm) was detected in the cytoplasmic fraction, whereas cytochrome *b* (maxima at 429, 529, and 561) was membrane-associated.

From cells grown in methanogenic coculture, a menaquinone-7 was isolated as sole isoprenoid quinone. In cells of a pure culture of *M. hungatei*, no lipophilic substance of such properties was detected. No desulfovibrin was found in cells grown with propionate plus sulfate. Instead, desulforubidin was detected by its absorption spectrum with maxima at 394 and 541 nm after enrichment.

Enzymes of propionate degradation

Enzymes involved in propionate degradation by strain KoProp1 were assayed in cell-free extracts (Table 2). High ac-

n.d., not determined. *Substrates not degraded* (either in pure culture or in coculture): glucose, fructose, xylose, arabinose, ethanol, methanol, pyruvate, succinate, fumarate, oxaloacetate, malate, acrylate, butyrate, valerate, caproate. H_2/CO_2 + acetate, formate + acetate were not used by the pure culture

Table 2 Enzyme activities measured in cell-free extracts of syntrophically grown strain KoProp1 and of *Methanospirillum hungatei* as control. Values are given as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$

Enzyme	Coculture with KoProp1	<i>M. hungatei</i>
Propionate kinase	40	< 5
Methylmalonyl CoA:pyruvate	80	< 5
Transcarboxylase		
Succinate thiokinase	270	< 5
Succinate dehydrogenase ^a	350	< 5
Fumarate reductase ^b	3,420	< 5
Fumarase	1,860	< 1
Malate dehydrogenase (NAD^+)	260	25
Malic enzyme	< 5	< 5
Pyruvate:ferredoxin	210	< 5
Oxidoreductase ^c		
Phosphotransacetylase	50	< 5
Acetate kinase	30	< 5
Hydrogenase ^c	3,100	950
Formate dehydrogenase ^c	13,600	11,100

^a With $\text{K}_3\text{Fe}(\text{CN})_6$ as electron acceptor

^b With dithionite-reduced benzyl viologen as electron donor

^c With benzyl viologen as electron acceptor

tivities of fumarate reductase and fumarase were found; lower activities of methylmalonyl CoA:pyruvate transcarboxylase, succinate dehydrogenase, malate dehydrogenase, and pyruvate:ferredoxin oxidoreductase were detected, indicating that propionate was oxidized through the methylmalonyl CoA pathway. High activities of hydrogenase and formate dehydrogenase are probably required for electron transfer between the syntrophic partner bacteria.

Polyacrylamide gel electrophoresis

For further differentiation between strain KoProp1, *Syntrophobacter wolinii*, and *Desulfobulbus propionicus* as a representative of propionate-oxidizing sulfate reducers, the protein patterns of cell-free extracts of these three strains were compared and were found to be somewhat different (Fig. 3). *S. wolinii* grown with pyruvate (Fig. 3, lane a) or propionate plus sulfate (Fig. 3, lane b) gave identical protein patterns, although some bands differed in their intensity. The protein pattern of strain KoProp1 (Fig. 3, lane c) differed from that of *D. propionicus* (Fig. 3, lane d) and of *S. wolinii* by the absence of many bands in the high-molecular-weight range. The differences between *D. propionicus* and *S. wolinii* were less striking, but *D. propionicus* contained at least five specific proteins documented by strong bands that were not detected in *S. wolinii*.

Discussion

Physiology and biochemistry

In the present communication, a new isolate of syntrophically propionate-oxidizing fermenting bacteria is described that can also be grown in pure culture with propionate plus sulfate. With these properties, the new isolate resembles *Syntrophobacter wolinii* which has recently

been described to be phylogenetically related to sulfate-reducing bacteria (Harmsen et al. 1993), to be able to reduce sulfate and to grow in pure culture with propionate plus sulfate (Wallrabenstein et al. 1994). These syntrophically fermenting bacteria differ substantially from *Desulfobulbus propionicus* (Widdel and Pfennig 1982) and *D. elongatus* (Samain et al. 1984), which also oxidize propionate incompletely with sulfate as electron acceptor, but are unable to couple propionate oxidation with syntrophic hydrogen release in the absence of sulfate. Strain KoProp1 differs from *S. wolinii* in its morphology and its inability to grow by fermentation of pyruvate. Moreover, strain KoProp1 can grow with lactate in pure and mixed culture. It also forms gas vesicles in the late growth phase, an ability which has so far not been described for any of these syntrophically fatty-acid-fermenting bacteria. The ecological function of such gas vesicles remains unclear; it is difficult to envisage a function for gas vesicles in sediments where such bacteria are typically found.

Similar to *S. wolinii*, strain MPOB (Stams et al. 1993) and other syntrophically fatty-acid-oxidizing anaerobes such as *Syntrophus buswellii* and *S. gentianae* (Wallrabenstein et al. 1995), strain KoProp1 did not grow in pure culture in agar shakes and had to be purified in dilution series with liquid media, a technique that required substantially more effort. We do not know what inhibits growth of single cells in agar. Since the strain was able to grow in agar in larger assemblages with other bacteria, we assume that toxic agar constituents rather than the exposure to enhanced temperatures during the dilution process were the reason for this failure.

Strain KoProp1 shares with *S. wolinii* its capacity for incomplete propionate oxidation. Propionate is oxidized stoichiometrically to acetate and CO₂; the acetate residue remains untouched. With this, these two bacteria differ again from strain MPOB, which can combine propionate, oxidation with fumarate reduction and can also catalyze in the presence of sulfate a complete oxidation of propionate including the acetate residue through the carbon monoxide dehydrogenase pathway (Plugge et al. 1993). Carbon monoxide dehydrogenase was not detected in strain KoProp1.

Our enzyme studies demonstrate that strain KoProp1 uses the methylmalonyl CoA pathway for propionate oxidation. The same pathway is used by sulfate-reducing propionate oxidizers, such as *D. propionicus* (Stams et al. 1984) and the syntrophic fermenters *S. wolinii* (Houwen et al. 1990) and another enrichment culture (Houwen et al. 1987), and it appears also to be the predominant pathway used by microbial communities in anoxic sediments (Schink 1985). Indications of a different pathway not involving a symmetrical intermediate have been published earlier for a sewage sludge sample (Tholozan et al. 1988), but were never substantiated further. Oxidation of propionate through the methylmalonyl CoA pathway involves oxidation steps in succinate dehydrogenase, malate dehydrogenase, and pyruvate:ferredoxin oxidoreductase, of which the first step represents a special problem with respect to its high standard redox potential; release of hy-

Fig. 3 Polyacrylamide gel electrophoresis of cell-free extracts of anaerobic propionate oxidizers. **a** *Syntrophobacter wolinii* grown with pyruvate; **b** *S. wolinii* grown with propionate plus sulfate; **c** strain KoProp1 grown with propionate plus sulfate; **d** *Desulfobulbus propionicus* grown with propionate plus sulfate; **e** Protein standards with (top to bottom) 94, 67, 30, and 20.1 kDa molecular mass

drogen from this oxidation step requires a hydrogen partial pressure as low as 10^{-10} Pa (Schink and Friedrich 1994), which cannot be maintained by methanogenic bacteria (Schink 1992). It has been suggested, therefore, that syntrophic propionate oxidation should involve an energy-dependent reversed electron transport step fueled by ATP hydrolysis at the cytoplasmic membrane. Indications of such a reversed electron transfer step were obtained in our lab with strain KoProp1; hydrogen release from propionate by intact resting cells is inhibited by addition of the protonophore carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) or the ATPase inhibitor *N,N'*-dicyclohexyl carbodiimide (DCCD), but suitable control experiments could not be performed (Dörner 1992). Recent studies have provided hints that electrons from succinate dehydrogenation are transferred to the partner bacterium in the form of formate (Dong et al. 1994). The high formate dehydrogenase activity we found in our mixed culture may indicate that formate plays an important role in electron transfer also in this culture.

Phylogeny and taxonomy

Comparison of 16S rRNA sequences of various syntrophically propionate-oxidizing bacteria in defined or non-defined cultures has shown that all these bacteria are related to each other and are also closely linked to sulfate-reducing bacteria (Harmsen et al. 1993, 1995). *S. wolinii* and the strains KoProp1 and MPOB are closely related, all within 4% estimated sequence divergence. Closest relatives were found among the sulfate-reducing bacteria, with *Desulfoarculus baarsii* (6.7%) and *Desulfobulbus* sp. (10% sequence divergence). On the basis of these sequence data and the physiological and morphological differences mentioned above, it appears justified to describe strain KoProp1 as a new species within the genus *Syntrophobacter*, as *S. pfennigii* sp. nov. A formal description of this strain follows: *Syntrophobacter pfennigii* . pfen.ni'gi.i. M. L. gen. n. after Prof. Dr. Norbert Pfennig, a German microbiologist who has contributed substantially to the understanding of syntrophic relationships and of the physiology of sulfate-reducing bacteria.

Rod- to egg-shaped cells, $2.2\text{--}3.0 \times 1.0\text{--}1.2$ mm in size, with rounded ends, single, in pairs or in chains. Gram-negative. Endospores not formed. Gas vesicles visible in the late growth phase.

Strictly anaerobic chemoorganotroph. Growth by fermentation or by reduction of sulfate. Pure cultures oxidize propionate or lactate with sulfate as electron acceptor. No growth with glucose, fructose, xylose, arabinose, ethanol, methanol, pyruvate, succinate, fumarate, oxaloacetate, malate, acrylate, butyrate, valerate, caproate. H_2/CO_2 +acetate and formate+acetate are not used by the pure culture. Propionate and lactate are fermented to acetate and CO_2 in the presence of hydrogen- and/or formate-utilizing methanogenic bacteria (e.g. *Methanospirillum hungatei*). The coculture also oxidizes propanol. Sulfite or thiosulfate serve as alternative electron acceptors, nitrate is not

reduced. Growth requires mineral media with a strong reductant (dithionite).

Selective enrichment in reduced freshwater mineral medium with propionate as substrate. pH range: 6.2–8.0; optimum at pH 7.0–7.3. Temperature range: 30–37°C; optimum at 37°C. DNA base ratio: 57.3 ± 0.2 mol% G+C. Cytochromes *b* and *c*, menaquinone-7, and desulforubidin present. Habitat: Anaerobic sewage sludge.

Type strain: strain KoProp1, DSM 10092, deposited with the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

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