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Sodium-dependent succinate decarboxylation by a new anaerobic bacterium belonging to the genus *Peptostreptococcus*

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Abstract

An anaerobic bacterium was isolated from a polluted sediment, with succinate and yeast extract as carbon and energy sources. The new strain was Gram-positive, the cells were coccal shaped, the mol% G+C content of the genomic DNA was 29, and the peptidoglycan was of the L-ornithine–D-glutamic acid type. Comparative sequence analysis of the 16S rRNA gene showed the new strain to belong to the genus *Peptostreptococcus*. Succinate, fumarate, pyruvate, 3-hydroxybutyrate and lysine supported growth. Succinate was degraded to propionate and presumably CO_2, with a stoichiometric cell yield. Key enzymes of the methylmalonyl-CoA decarboxylase pathway were present. The methylmalonyl-CoA decarboxylase activity was avidin-sensitive and sodium dependent, and about 5 mM Na^+ was required for maximal activity. Whole cells, however, required at least 50 mM sodium for maximal succinate decarboxylation activity and to support the maximum growth rate. Sodium-dependent energy conservation coupled to succinate decarboxylation is shown for the first time to occur in a bacterium belonging to the group of Gram-positive bacteria containing the peptostreptococci and their relatives.

Introduction

Succinate degradation under anoxic conditions by *Propionigenium modestum* has been studied in some detail. The growth (Schink & Pfennig 1982) and metabolism (Hilpert et al. 1984) of *P. modestum* are sodium dependent. The energy metabolism is based on a sodium-translocating methylmalonyl-CoA decarboxylase which generates a sodium-motive force, which is then used to generate ATP via a membrane-bound adenosinetriphosphatase (ATPase) (Hilpert et al. 1984, Laubinger & Dimroth 1988). Although the ATPase of *P. modestum* is able to pump protons (Laubinger & Dimroth 1989), its physiological role is sodium translocation (Hilpert et al. 1984; Laubinger & Dimroth 1988). The methylmalonyl-CoA decarboxylase and ATPase require approximately 1 mM and 2 mM Na^+ for maximal activity, respectively (Hilpert et al. 1984). The minimum requirement for growth is 100–150 mM NaCl (Schink 1992). *Propionigenium* spp. (Schink & Pfennig 1982, Janssen & Liesack 1995) are related to *Fusobacterium nucleatum*, which catalyzes a sodium-dependent decarboxylation of glutaconyl-CoA, an intermediate of glutamate catabolism, to generate a transmembrane sodium gradient able to be utilized by the bacterium for ATP generation (Beatrix et al. 1990). *Propionigenium* spp. and *Fusobacterium* spp. belong to cluster XIX of Collins et al. (1994).

The decarboxylation of succinate can also generate metabolic energy in a range of other bacteria belonging to the group of Gram-positive bacteria with a Gram-negative cell wall structure. These include *Sporomusa*

spp. (Ollivier et al. 1985, Breznak et al. 1988, Dehning et al. 1989), *Selenomonas* spp. (Guangsheng et al. 1992, Schleifinger & Wolin 1973), *Phascolarctobacterium faecium* (Osawa et al. 1992, Del Dot et al. 1993), *Succiniclasticum ruminis* (van Gylswyk 1995), and *Veillonella parvula* (Janssen 1992, Willems & Collins 1995). Phylogenetically, these bacteria belong to cluster IX of Collins et al. (1994). Some of these organisms are only able to utilize succinate as a supplementary energy source, and cannot grow with succinate as the sole energy source.

In this communication we report on the isolation and characteristics of a succinate-decarboxylating bacterium which does not belong to these two phylogenetic groups, but represents a new taxon within another radiation of the Gram-positive bacteria, typified by the peptostreptococci [cluster XIII of Collins et al. (1994)]. Succinate decarboxylation and the ability to grow with this reaction has not been previously reported within this group.

Materials and methods

Isolation and characterization

The estuarine and freshwater media used in this study have been described (Janssen & Harfoot 1990). Unless noted otherwise, L-isomers of organic and amino acids, and D-isomers of sugars were used. For all cultures other than enrichments, sulfide reductant (Janssen & Harfoot 1990) was used. Enrichment media contained cysteine/sulfide reductant (Janssen & Harfoot 1990). The agar deep method for isolating pure cultures was described by Pfennig (1978). Estuarine cooked meat medium consisted of commercial cooked meat medium (Difco, Detroit, Michigan, USA) supplemented with 15 g NaCl per litre. All incubations were at 34 °C unless otherwise noted.

The amino acid stock solution of Hudson et al. (1989) with the addition of 0.8 g L-tryptophan per litre and with the pH adjusted to 7.0 with NaOH was filter-sterilized and added at 10 ml per litre of medium where noted. The 10 vitamin supplement was that of Janssen & Harfoot (1990)

The measurement of succinate and propionate by HPLC, analysis of cytochromes, cell dry mass determinations, and the tests for catalase, oxidase, urease, esculin hydrolysis, and indole production, and the methods for electron microscopy were described by Janssen & Harfoot (1990). Nitrate reduction was determined as described by Smibert & Krieg (1981). Sulfide production was tested using a copper precipitation assay (Cord-Ruwisch 1985).

Determination of the G+C content of genomic DNA

DNA was isolated from cells using 100/G genomic tips (Qiagen, Hilden, Germany), as described by the manufacturer. The purified DNA, in 5 ml QF buffer (Qiagen), was precipitated by the addition of 5 ml 2-propanol, followed by mixing and then centrifugation at 20000 × *g* for 20 min at 4 °C. The buffer/2-propanol mixture was decanted and allowed to drain, and the pellet resuspended in 100 µl 1.5 mM citrate buffer (pH 7.0 with NaOH) containing 15 mM NaCl. The digestion of the purified DNA, and determination of the mol% G+C ratio were carried out using the following modifications of the methods of Mesbah & Whitman (1989) and Mesbah et al. (1989). A 50 µl sample of a DNA preparation or standard was heated for 2 min at 100°C, then cooled immediately in an ice bath. Once cool, 100 µl 30 mM acetate buffer (pH 5.3 with NaOH) and 10 µl 20 mM $ZnSO_4$ were added, then 10 µl of 340 U nuclease P1 per ml(Sigma, Deisenhofen, Germany; prepared in 30 mM acetate buffer, pH 5.3 with NaOH, containing 0.5 mM $ZnSO_4$). The solution was well mixed, and incubated at 30°C for 60 min. After the incubation, 20 µl of 500 U alkaline phosphatase per ml(Type VII-NT, Sigma; prepared in 100 mM glycine buffer, pH 10.4 with NaOH) was added, and the mixture well mixed. After a further incubation at 37 °C for 6 h, 10 µl 400 mM triethylamine (pH 5.1 with H_3PO_4) was added.

The nucleosides were separated by reversed-phase HPLC on a Superspher RP18 (4 µm) 200 × 4.6 mm column (Grom, Herrenberg, Germany) with 20 mM triethylamine (pH 5.1 with H_3PO_4) containing 12% (v/v) methanol, at a flow rate of 1 ml per min. The elution of the nucleosides was followed at 270 nm using a UVIS 204 variable wavelength detector (Linear Instruments, Freemont, California, USA), and the data collected and peaks integrated using Axxiom 747 software (Axxiom, Moorpark, California, USA). Standards of individual bases (Sigma) allowed the detection of contaminating ribonucleosides, and identification of the thymidine and deoxyguanosine peaks. Standard DNAs (Sigma) from *Clostridium perfringens*, *Escherichia coli*, and *Micrococcus luteus* were dissolved at 1 mg per ml in 1.5 mM citrate buffer (pH 7.0 with NaOH and containing 15 mM NaCl), and digested and analyzed as described above, and used to prepare a standard curve for deter-

age, with a random-order input of sequences, and the global rearrangement option.

Nucleotide sequence accession numbers

The 16S rDNA sequence of strain 9succ1 has been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X90471. The EMBL and Genbank data base accession numbers for sequences used as references in phylogenetic treeing (Fig. 7) are as follows: *Propionigenium modestum*: X54275; *Propionigenium maris*: X84049; *Fusobacterium nucleatum*: X55401; *Clostridium butyricum*: M59085; *Clostridium histolyticum*: M59094; *Clostridium perfringens*: M69264; *Selenomonas ruminantium*: M62702; *Phascolarctobacterium faecium*: X72865; *Succiniclasticum ruminis*: X81137; *Veillonella parvula*: X84005; *Sporomusa paucivorans*: M59117; *Sporomusa termitida*: M61920; *Helcococcus kunzii*: X69837; *Escherichia coli*: J01695. The following sequences were obtained from the DNA Data Bank of Japan: *Peptostreptococcus asaccharolyticus*: D14138; *Peptostreptococcus indolicus*: D14147; *Peptostreptococcus magnus*: D14149; *Peptostreptococcus micros*: D14143.

Peptidoglycan analysis

Preparation of cell walls and determination of the peptidoglycan structure was carried out by the methods of Schleifer & Kandler (1972), modified by using thin layer chromatography on cellulose sheets instead of paper chromatography. Briefly, 1 mg of freeze-dried cell wall material was hydrolyzed in 0.2 ml of 4 M HCl at 100 °C for 16 hours (total hydrolysate) and 45 min (partial hydrolysate). Diamino acids were identified from the total hydrolysate by one-dimensional chromatography using methanol:pyridine:water:10 M HCl (32:4:7:1, v/v/v/v). Amino acids and peptides in the partial and total hydrolysates were identified by their mobilities and staining characteristics with ninhydrin spray, after two dimensional chromatography (Schleifer & Kandler 1972). The resulting "fingerprints" were compared with those of known peptidoglycan structures.

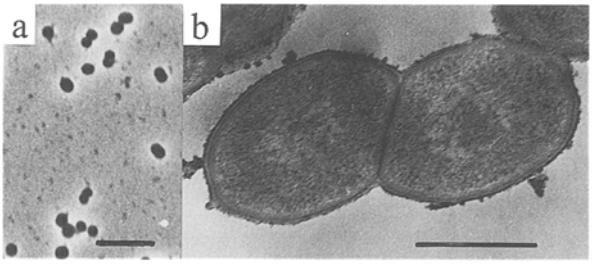

Figure 1. Morphology of strain 9succ1. (a) Phase contrast micrograph. Bar = 5 μm. (b) Electron micrograph of ultrathin section of strain 9succ1, showing the wall structure and the division point. Bar = 0.5 μm

Results

Enrichment and isolation of strain 9succ1

Enrichment cultures in estuarine medium with 20 mM succinate and 1.0 g yeast extract per litre were inoculated with sediment from a polluted pond. After 8 days incubation at 34 °C, all of the added succinate had been degraded to propionate. The enrichment cultures were subcultured twice into the same medium, then diluted into agar deep dilution series. Colonies developed within a week, and a pure culture was obtained by dilution of selected colonies into further agar deep series using estuarine medium with 20 mM succinate and 1.0 g yeast extract per litre. The pure culture obtained from one colony was designated strain 9succ1. The strain was further cultivated in freshwater medium, using sulfide reductant instead of cysteine/sulfide, and supplemented with 20 mM succinate and 1.0 g yeast extract per litre.

Morphological and biochemical characteristics

The cells of strain 9succ1 were cocci, 0.7 to 0.9 μm in diameter (Fig 1a), and occurred singly, in pairs or in short chains of up to 6 cells. The cells were non-motile, and negatively-stained whole cell electron micrographs revealed no flagella. No spores were ever observed, even when inoculated into estuarine cooked meat medium. No viable subcultures could be produced from a ten day-old culture after incubation at 80 °C for 5 minutes. Cells of strain 9succ1 stained Gram-positive, and thin sections revealed a single-layered Gram-positive cell wall structure (Fig. 1b).

Table 1. Substrates tested for growth and fermentation by strain 9succ1. Numbers given after each substrate indicate initial concentration (20 mM unless noted otherwise). Tested in the presence of 1.0 g yeast extract per litre.

Substrates supporting growth:	Organic end products[a]
Succinate	propionate
Fumarate	malate[b], acetate, propionate
Pyruvate	formate, acetate, butyrate
DL-3-Hydroxybutyrate	acetate, butyrate
Lysine	acetate, butyrate
Yeast extract (1.0 $g.l^{-1}$)	acetate, propionate, butyrate[c]

Substrates not utilized:

Oxalate, malonate, glutarate, adipate, pimelate, malate, maleate, tartrate, glycolate, α-ketoglutarate, citrate, DL-lactate, acrylate, formate, DL-2-hydroxybutyrate, ethanol, ethane-1,2-diol, butane-2,3-diol, acetoin, glucose (5), galactose (5), fructose (5), ribose (5), xylose (5), maltose (5), lactose (5), sucrose (5), cellobiose (5), mannitol (5), glycerol (5), sorbitol (5), alanine, arginine, aspartate, threonine, glutamate, cysteine, glycine, tryptophan

[a] Corrected for the end products on yeast extract alone
[b] Not always formed
[c] Other minor products were detected but not identified

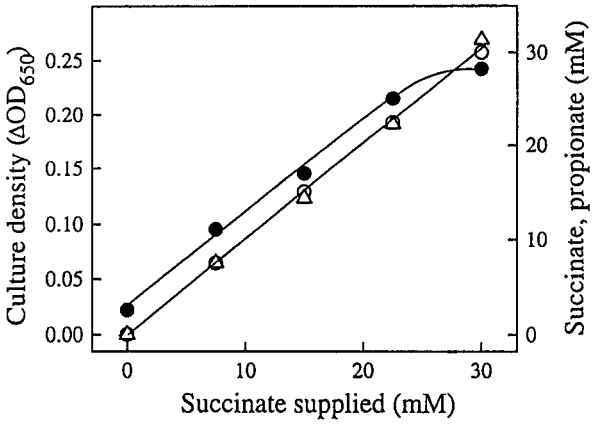

Figure 2. Growth of strain 9succ1 on a range of succinate concentrations in the presence of 1.0 g yeast extract per litre. Symbols: (○) succinate degraded, (△) propionate formed, (●) culture density at 660 nm

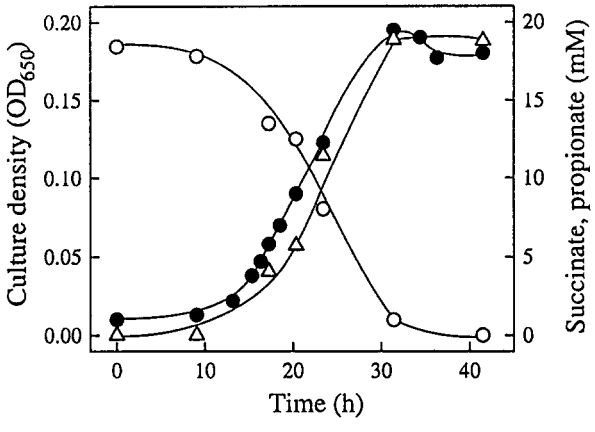

Figure 3. Growth curve of strain 9succ1 on succinate in the presence of 1.0 g yeast extract per litre. Symbols: (○) succinate, (△) propionate, (●) culture density at 660 nm

No cytochromes were detected in cell-free extracts of succinate-grown cultures. The mol% G+C content of the genomic DNA was 29, as determined by HPLC analysis of hydrolyzed DNA. The peptidoglycan of strain 9succ1 was of the L-ornithine–D-glutamate type.

Strain 9succ1 was both catalase and oxidase negative. Esculin and urea hydrolysis, indole formation from L-tryptophan, and H_2S production from L-cysteine were all negative.

Cultural characteristics

When grown in estuarine medium with 20 mM succinate and 1.0 g yeast extract per litre, liquid cultures were of white uniform turbidity, but cells tended to lyse after more than 3 weeks. Growth occurred when the medium was not reduced, but was not possible under air. There was no growth when yeast extract was omitted; a 10-vitamin supplement plus an amino acid mixture could not replace the requirement for yeast extract. The optimum temperature for growth

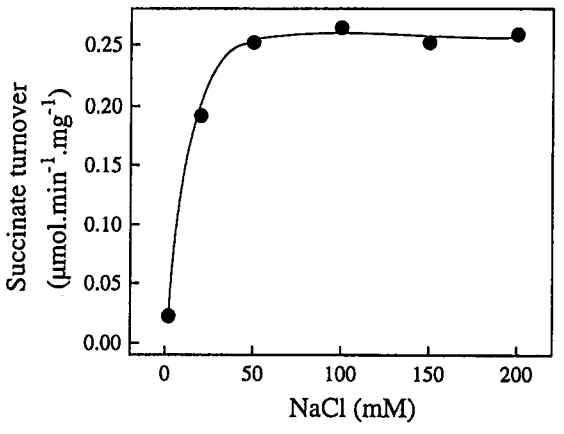

Figure 4. Rate of propionate formation from succinate by strain 9succ1 at various NaCl concentrations. The ionic strength was held constant at 350 mM NaCl-plus-KCl

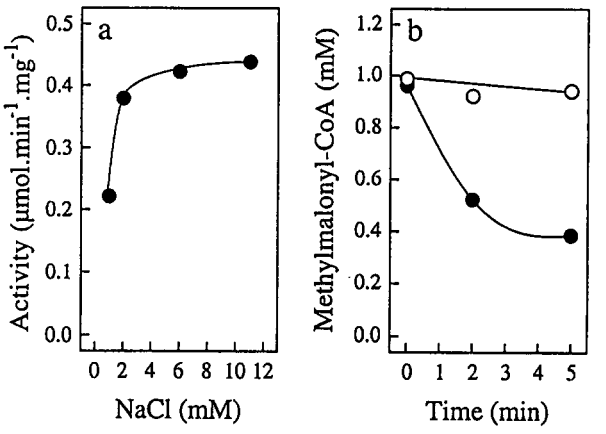

Figure 5. (a) Relationship between methylmalonyl-CoA decarboxlyase activity in a crude cell-free extract of strain 9succ1 and the NaCl concentration in the assay. (b) Time course of methylmalonyl-CoA decarboxylation by vesicle preparations of strain 9succ1 at low ($\leq$ 30 μM) levels of Na^+ (○), and at 10 mM added NaCl (●)

was 37 °C. Growth was still possible at 40 °C, but not at 43 °C.

Nutritional characteristics

Strain 9succ1 grew on a limited range of substrates in the presence of 1.0 g yeast extract per litre (Table 1). No carbohydrates were fermented and only a limited range of organic and amino acids were utilized: succinate, fumarate, pyruvate, 3-hydroxybutyrate and lysine. Growth on yeast extract alone was poor. There was no growth on H_2 plus CO_2 (80:20; v:v). When grown on formate plus fumarate there was no utilization of the formate, and the fumarate was fermented as it was when supplied alone. The organic end products of catabolism were formate, acetate, propionate, and butyrate, depending on the substrate. Fumarate was sometimes partly converted to malate.

Nitrate, thiosulfate (each tested with succinate), sulfate or sulfur (each tested with succinate and with lactate) were not reduced.

The growth yield of strain 9succ1 was proportional to the amount of succinate degraded (Fig. 2). Direct gravimetric determination of the cell dry mass after growth on succinate plus yeast extract, and after growth on yeast extract alone allowed calculation of a molar growth yield of 6.0 g dry cell mass per mol succinate, at 300 mM Na^+. After correction for the end-products formed on yeast extract, 21.3 mmol succinate per litre was degraded to 21.0 mmol propionate per litre (means of 10 independent cultures). The increase in optical density occurred concomitant with succinate degradation and propionate formation (Fig. 3). Under these conditions (pH 7.2, 300 mM Na^+, 34 °C, 1 g yeast extract per litre), the doubling time was about 4 h (μ = 0.17 h^{-1}).

Sodium requirements

Strain 9succ1 grew over a sodium chloride range of 10 mM (the lowest tested) to 900 mM. The final growth yield was maximal at 300 to 550 mM. There was no growth at 1000 mM. The growth rate of strain 9succ1 on succinate was independent of sodium ion concentrations between 50 mM and 200 mM ($\mu \cong 0.15\ h^{-1}$), while the growth rate decreased at concentration lower than 50 mM (not shown). Addition of 20 μM monensin completely inhibited growth of strain 9succ1 at 50 mM Na^+ with succinate and yeast extract or with 3-hydroxybutyrate plus yeast extract as the growth substrates. Controls to which only ethanol, the solvent for the monensin stock solution, was added showed no growth inhibition. The rate of propionate production from succinate by cell suspensions of strain 9succ1 was lower at low NaCl concentrations (Fig. 4), even though the buffer was supplemented with KCl to ensure an equal osmotic strength in all experiments. The rate of succinate metabolism by strain 9succ1 was maximal at NaCl concentrations greater than 50 mM, and was about 0.25 μmol·min^{-1}·mg $protein^{-1}$.

Methylmalonyl-CoA decarboxylase (EC 4.1.1.41) at 0.437 μmol·min^{-1}.mg $protein^{-1}$ (in the pres-

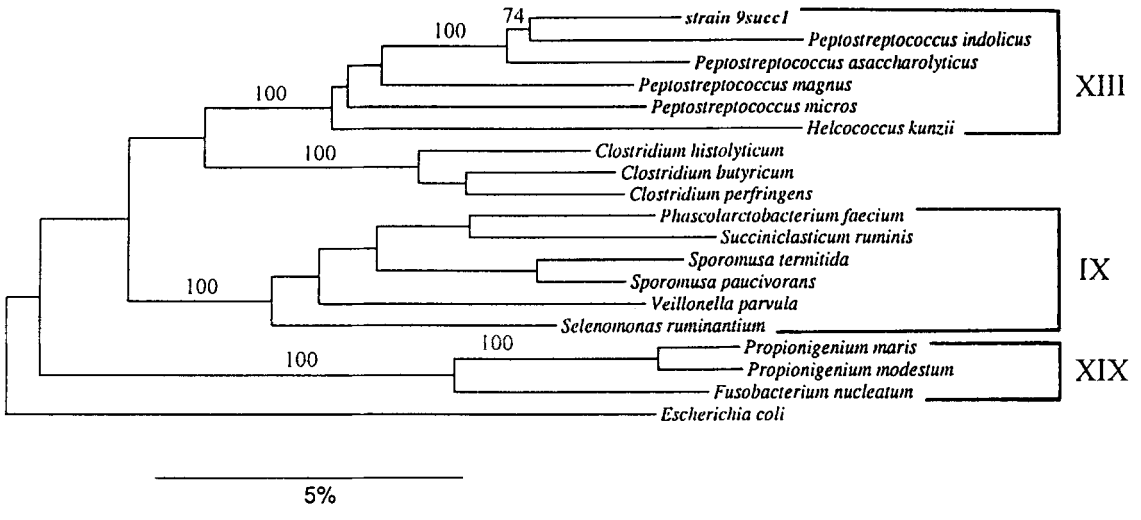

Figure 6. Phylogenetic tree constructed for strain 9succ1 and 17 reference organisms. The 16S rDNA sequence from *Escherichia coli* was used to root the tree. The numbers indicate the significance (percentage of outcomes) of the branching points as derived from a bootstrap analysis of 1000 replicas using the neighbour-joining method implemented in "MEGA" (Kumar et al. 1993). Bar = 5% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting two species. The Roman numerals refer to the clusters of Collins et al. (1994)

ence of 10 mM NaCl), succinate: propionyl-CoA CoA-transferase (EC 2.8.3.–) at 3.89 μmol.min^{-1}.mg protein^{-1} and ATPase (EC 3.6.1.3) at 0.126 μmol.min^{-1}.mg protein^{-1} (in the presence of 2.5 mM NaCl) were measured in crude cell-free extracts. The methylmalonyl-CoA decarboxylase activity in crude cell-free extracts was stimulated by the addition of NaCl (Fig. 5a), and membrane vesicle preparations containing only low levels of Na^+ ($\leq$ 30 μM) catalyzed virtually no methylmalonyl-CoA decarboxylation without added NaCl (Fig. 5b). Avidin treatment of crude cell-free extracts completely abolished methylmalonyl-CoA decarboxylase activity.

Phylogenetic analysis

The nucleotide sequence of almost the complete 16S rRNA gene (1405 bases) of strain 9succ1 was determined. Phylogenetic analysis of strain 9succ1 based on comparative 16S rDNA sequence analysis revealed its membership within the clostridial cluster XIII of Collins et al. (1994). This grouping is characterized by members of the genus *Peptostreptococcus* and by *Helcococcus kunzii* (Fig. 6). Within this cluster, the species most closely related to strain 9succ1 are *P. asaccharolyticus* and *P. indolicus*, as well as *P. lacrimalis* (not shown in Fig. 6).

Discussion

A new strain of succinate-decarboxylating anaerobe, designated strain 9succ1, was enriched and isolated from a freshwater site using an estuarine medium, although sodium at lower concentrations also supported good growth (see below). Strain 9succ1 had a requirement for yeast extract that could not be replaced by vitamin and amino acid mixtures. The cell yield was proportional to the amount of succinate decarboxylated to propionate, and the utilization of succinate was closely coupled with the increase in cell density. This indicates that succinate decarboxylation served as the energy source for growth. The high, almost stoichiometric, recovery of propionate suggests that components of the yeast extract served as precusors for cell matter. Strain 9succ1 fermented succinate in agreement with the following equation [ΔG_0' value calculated after Thauer et al. (1977)]:

$$\text{succinate}^{2-} + H_2O \rightarrow \text{propionate}^- + HCO_3^-$$

$$\Delta G_0' = -20.6\text{kJ.mol(succinate)}^{-1}$$

From the specific growth rate and specific growth yield, a succinate turnover rate in growing cultures of 0.24 μmol·min^{-1}·mg protein^{-1} can be calculated. Both the methylmalonyl-CoA decarboxylase and suc-

cinate: propionyl-CoA CoA transferase activities measured were high enough to account for this substrate turnover rate. In addition, the succinate turnover rate by cell suspensions at non-limiting sodium concentrations was similar to that in growing cultures, at about 0.25 μmol·min^{-1}·mg protein^{-1}. The methylmalonyl-CoA decarboxylase activty in crude cell-free extracts was stimulated by sodium ions. Activity was almost absent in membrane vesicle preparations with ≤ 30 μM Na^+, but could be stimulated by adding NaCl. Avidin completely inhibited the methylmalonyl-CoA decarboxylase activity. These observations imply the enzyme to be a typical biotin-containing, sodium-translocating methylmalonyl-CoA decarboxylase (Dimroth 1987).

Comparative analysis of the 16S rRNA gene of strain 9succ1 revealed its phylogenetic relationship with the genus *Peptostreptococcus*, within cluster XIII of Collins et al. (1994). Classification within the genus *Peptostreptococcus* is supported by its phenotypic similarity to other members of the genus *Peptostreptococcus* (Holdeman Moore et al. 1986, Ezaki et al. 1992), excluding *Peptostreptococcus productus*, which is unrelated to other *Peptostreptococcus* spp. (Collins et al. 1994), and has quite a different morphology and mol% G+C ratio (Holdeman Moore et al. 1986). The similarites between strain 9succ1 and true *Peptostreptococcus* spp. include: (i) the coccal morphology of strain 9succ1, shared with all true members of the genus *Peptostreptococcus* (Holdeman Moore et al. 1986); (ii) the single-layered cell wall with a thick peptidoglycan layer; (iii) a peptidoglycan of the L-ornithine–D-glutamic acid type; (iv) a mol% G+C ratio of 29, comparable to that of other true members of the genus, which have mol% G+C ratios of 27 to 35; (v) the production of acetate and butyrate as fermentation end products, typical of *Peptostreptococcus* species; and (vi) the inability to use carbohydrates as growth substrates.

Previously described bacteria able to grow or generate ATP coupled to the free energy change of succinate decarboxylation belong either to the *Fusobacterium-Propionigenium* line of decent [cluster XIX of Collins et al. (1994)], or to the group of Gram-positive bacteria with a Gram-negative cell wall structure [cluster IX of Collins et al. (1994)]. Members of the genus *Peptostreptococcus* are, however, not known to grow by decarboxylation of succinate (Holdeman Moore et al. 1986, Ezaki et al. 1992). In strain 9succ1, the decarboxylation of succinate is apparently coupled to energy conservation by a biotin-containing, sodium-pumping methylmalonyl-CoA decarboxylase, as has been shown to occur in *Propionigenium modestum* (Hilpert et al. 1984) and *Veillonella parvula* (Hilpert & Dimroth 1983). This, thus, represents a new mechanism of energy conservation within the genus *Peptostreptococcus*, although ATP synthesis via a sodium gradient generated by decarboxylation of glutaconyl-CoA during glutamate catabolism by *Peptostreptococcus asaccharolyticus* has been documented (Wohlfarth & Buckel 1985). Some transport processes are sodium dependent (Chen & Russell 1989, Beck & Russell 1994). Further investigations may well reveal a wide range of sodium-dependent processes within this genus.

The rate of succinate decarboxylation and the growth rate were dependent on the concentration of sodium; at sodium concentrations lower than 50 mM, the rate of succinate decarboxylation and the growth rate were both low. This could be the result of: (i) a sodium dependence of succinate import or propionate export, or both; (ii) a requirement for sodium by the methylmalonyl-CoA decarboxylase; or (iii) a requirement for sodium by an ATPase. The methylmalonyl-CoA decarboxylase of strain 9succ1 showed maximum activity at about 5 to 10 mM NaCl. Similarly, the methylmalonyl-CoA decarboxylases of *Propionigenium modestum* and *Veillonella parvula* are also sodium-pumping decarboxylases, requiring about 1 mM and 5 mM Na^+ for maximum activity, respectively (Hilpert et al. 1984, Hilpert & Dimroth 1983). In addition, the ATPase of *Propionigenium modestum* is stimulated by sodium, and maximal activity requires at least 2 mM Na^+ (Hilpert et al. 1984, Laubinger & Dimroth 1988). The transport of succinate by sodium symport has been reported to occur in *Selenomonas ruminantium* (Michel & Macy 1990), but this has been questioned in another study (Strobel & Russell 1991). Amino acid transport has been shown to be driven by transmembrane sodium gradients in *Peptostreptococcus* spp. (Chen & Russell 1989, Beck & Russell 1994). An alternative mechanism of succinate transport in strain 9succ1 could be a substrate-product antiport system (Anantharam et al. 1989, Konings et al. 1994).

Strain 9succ1 has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, under the accession number DSM 9536.

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