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Characterization of *Propionispira arboris* gen. nov. sp. nov., a Nitrogen-fixing Anaerobe Common to Wetwoods of Living Trees

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A new species in the family *Bacteroidaceae* is described, isolated from alkaline wetwoods of poplar trees. It was a non-sporing, curved rod that formed long spiral filaments, especially when grown with N_2 as the sole nitrogen source. This obligate anaerobe had peritrichous flagellation and an outer-wall membranous layer. The DNA G + C content was 36·7 ± 1·0 mol %. Cell extracts displayed absorption peaks for type *b* cytochromes in oxidized versus reduced difference spectra. Nitrogenase was detected by acetylene reduction in cells grown on glucose in the absence of combined nitrogen. This species fermented lactate and a variety of saccharides. Propionate, acetate and CO_2 were major products, with succinate and ethanol formed in trace quantities. The optimal pH and temperature for growth were 6·0–6·5 and 30–33 °C, respectively. The name *Propionispira arboris* gen. nov. sp. nov. is proposed for the type strain 12B4, which has been deposited as ATCC 33732.

INTRODUCTION

Most descriptions of Gram-negative, helical to spiral shaped, chemoorganotrophic rods concern aerobic *Spirillum* species (Buchanan & Gibbons, 1974). In addition, an aerobic nitrogen-fixing genus, *Azospirillum*, has been recognized (Tarrand *et al.*, 1978), and studies of obligately anaerobic organisms have resulted in the descriptions of *Anaerobiospirillum* (Davis *et al.*, 1976) and *Methanospirillum* (Ferry *et al.*, 1974) both with polar flagellation, and *Pectinatus* with lateral comb-like flagellation (Lee *et al.*, 1978). Both *Anaerobiospirillum succiniciproducens* (Davis *et al.*, 1976) and *Pectinatus cerevisiiphilus* (Lee *et al.*, 1978) ferment carbohydrates; however, the former produces succinate and the latter propionate as the major fermentation end product.

Recently, the wetwood disease syndrome of living hardwood trees has been attributed to high population densities [i.e. $\sim 10^7$ cells (g wood)$^{-1}$] of anaerobic bacteria (Schink *et al.*, 1981 *a*, *b*). Important metabolic activities expressed by wetwood anaerobes included methanogenesis (Zeikus & Ward, 1974), nitrogen fixation (Schink *et al.*, 1981 *a*) and pectinolysis (Schink *et al.*, 1981 *b*). Moreover, a wide diversity of bacteria can be isolated from wetwoods; the prevalent chemoorganotrophs include *Bacteroides*, *Clostridium*, *Erwinia*, *Edwardsiella*, *Klebsiella* and *Lactobacillus* species (Schink *et al.*, 1981 *a*).

The present study describes a new species of obligately anaerobic, Gram-negative, helical-shaped rods that are commonly enriched from alkaline wetwoods in a carbohydrate medium devoid of combined nitrogen.

METHODS

Chemicals. All chemicals used were reagent grade and were obtained from either Mallinckrodt (Paris, Ky., U.S.A.) or Sigma. Pectin was a gift of Sunkist Growers (Corona, Calif., U.S.A.). Agar, tryptone and yeast extract were obtained from Difco. All gases were obtained from Matheson (Joliet, Ill., U.S.A.) and were purified free of oxygen by passage over heated (370 °C) copper filings.

Inocula and bacterial strains. Wetwood inocula were obtained from various cottonwood trees (*Populus deltoides*

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Bartr.) located in Wisconsin, U.S.A. A wetwood sample obtained from the largest cottonwood tree in Columbus, Wisconsin, was used for enrichment and isolation of strain 12B4. Procedures for anaerobic sampling and processing of wetwood were as described previously (Zeikus & Ward, 1974; Schink *et al.*, 1981*a*). *Pectinatus cerevisiiphilus* strain CCCB-1022 was obtained from Dr Sharon Moore, Adolph Coors, Golden, Colo., U.S.A.

Culture techniques and media. Anaerobic procedures used for cell cultivation and media preparation were as described previously (Zeikus *et al.*, 1980; Schink *et al.*, 1981*a*). The low phosphate buffered basal medium (LPBB medium) described by Zeikus *et al.* (1979) was used, with a N_2/CO_2 (95 : 5) gas phase. When needed, carbohydrates (0·5%, w/v), tryptone (1%, w/v) or yeast extract (0·3%, w/v) were autoclaved separately and added by syringe to the final concentrations indicated. The medium of Ng *et al.* (1977) was used to examine cellulose fermentation, and medium 77 (Postgate, 1963) was used to test sulphate reduction. Ammonium chloride was omitted from LPBB medium when cells were examined for nitrogen fixation. Routine cell maintenance and experimental analyses were performed in anaerobic culture tubes (23 ml volume, 18 × 142 mm; Bellco Glass, Vineland, N.J., U.S.A.) containing 10 ml medium, and sealed with black rubber bungs. Prior to inoculation, culture medium was reduced by the addition of sodium sulphide (0·05%, w/v final concentration). For isolation of colonies in either anaerobic roll tubes or in Petri dishes maintained in an anaerobic chamber (Coy Products, Ann Arbor, Mich., U.S.A.) LPBB medium was supplemented with 0·5% (w/v) glucose, 0·1% (w/v) yeast extract and 2·0% (w/v) purified agar. Large quantities of cells were grown in glass carboys that contained 15 l LPBB medium, 0·5% glucose and 0·1% yeast extract. Cultures were routinely incubated without shaking at 30 °C unless indicated in the text.

Cellular characterization. A Carl Zeiss photomicroscope was used for phase-contrast and bright-field observations including determination of cell size. Agar-coated glass slides were used to obtain long spiral filaments in focus for photomicroscopy. Flagella were stained by the techniques of Mayfield & Inniss (1977) and examined by phase-contrast microscopy.

The preparation of cells for thin sectioning and electron microscopy was as described by Zeikus & Bowen (1975). For analysis of carbohydrate storage material in cells, the techniques of Hanker (1964) were used prior to thin sectioning. The procedures described by Ben-Bassat & Zeikus (1981) were used for negative staining. All preparations were examined with a Hitachi HU 11E electron microscope.

DNA was isolated and purified from lysozyme-treated cells by the method of Marmur (1961). DNA base compositions were calculated according to the method of DeLey (1970) in 0·015 M-NaCl and 0·0015 M-trisodium citrate as determined in a Gilford model 250 spectrophotometer equipped with a model 2527 thermoprogrammer. *Escherichia coli* DNA VIII (lot no. 57C-6830, Sigma) served as standard. DNA composition reported represents the mean of four separate determinations. The percentage G + C of the *E. coli* standard was 52·8 ± 1·0%.

Cytochromes were identified in air versus dithionite-reduced difference spectra of cell extracts analysed with a Beckman model 25 scanning spectrophotometer. Cell extracts were prepared by sonicating a suspension of 1 g wet weight cells in 5 ml 50 mM-potassium phosphate buffer and retaining the supernatant after centrifugation at 10 000 *g* for 15 min in a Sorvall centrifuge. Protein was determined by the Lowry method.

Growth and metabolic characterization. Growth was determined by measuring the increase in turbidity at 660 nm. Absorbance was estimated directly by insertion of the anaerobic culture tubes into a Spectronic 20 spectrophotometer (Bausch & Lomb).

Growth on various substrates was examined in liquid cultures after one week of cultivation. Physiological reactions were determined by use of assay kits (API Systems 20E and 20A; Analytab Products, Plainview, N.Y., U.S.A.).

Fermentation products were measured directly in liquid or gas samples removed from the culture tubes by syringe. Alcohols and acids were measured by gas chromatography using a flame ionization detector as described by Zeikus *et al.* (1979). All gases were quantified by the gas chromatography–thermal conductivity detection methods of Nelson & Zeikus (1974). A Perkin Elmer Series 3 high-performance liquid chromatograph with a Bio-Rad organic acid analysis column (no. 125-0140) was used to detect succinate. Lactate was measured spectrophotometrically by the D- or L-lactate dehydrogenase assay procedure of Bergmeyer (1965). Glucose consumption was determined by reducing sugar analysis with dinitrophenylsalicylic acid (Miller *et al.*, 1960).

Nitrogen fixation was determined by the acetylene reduction test (Stewart *et al.*, 1967). Late-exponential phase grown cells were gassed with N_2/CO_2 (95 : 5, v/v) and 0·4 ml acetylene was added to each experimental culture tube. The formation of ethylene with time was detected by the gas chromatography–flame ionization detection methods of Nelson & Zeikus (1974).

RESULTS

Isolation and cultivation

The organism was selectively enriched from alkaline wetwoods of mature cottonwood trees in LPBB medium that contained pectin as the energy source. When the enrichment culture fluid

was examined by phase-contrast microscopy it contained some curved rods that moved very fast, and in a manner characteristic of *Spirillum* species. Transfer of primary enrichments to arabinose/LPBB medium without combined nitrogen greatly enhanced cell numbers relative to other species. The organism was then readily isolated by dilution in agar roll tubes and by picking and re-streaking individual colonies. Colonies developed rapidly (1–2 d) on LPBB/0·5% glucose/0·1% yeast extract agar plates in the anaerobic glove box chamber. Colonies were uniformly round and became light brown when stored. Stock cultures were maintained on LPBB/0·5% sodium lactate/0·1% yeast extract medium, and transferred weekly. Liquid cultures lost viability after 2 weeks' storage. Permanent stocks were kept for more than 6 months by freezing anaerobic cell suspensions in 20% (v/v) glycerol at −80 °C.

Cellular properties

Exponential phase cultures appeared as actively motile curved rods when observed microscopically (Fig. 1*a*). Long spiral cell filaments were readily observed in cultures growing in the absence of combined nitrogen (Fig. 1*b*) or in late-exponential phase cultures grown in the presence of ammonium. Spores were not observed and old cultures became granular before cell lysis occurred. The cells stained Gram-negative and lacked catalase. Growth was inhibited by cycloserine, penicillin, streptomycin, tetracycline or chloramphenicol (each at 100 μg ml^{-1}); 2% NaCl; and sodium azide (500 μg ml^{-1}).

Stained cells observed by bright-field microscopy showed peritrichous flagellation. This was confirmed by transmission electron microscopic analysis of negatively stained cells (Fig. 2*a*, *b*). Control preparations with *Pectinatus cerevisiiphilus* showed flagellar insertion only on the concave side of the cells. In thin sections (Fig. 3*a*, *c*) a typical Gram-negative cell architecture comprising an outer-wall membrane and a poorly discernible inner-wall layer was evident. Granular cytoplasmic inclusions were confirmed as carbohydrate reserve material by specific staining (Fig. 3*b*). The base composition of the DNA was 36·7 ± 1·0 mol % G + C. Difference spectra of crude cell extracts indicated the presence of a type *b* cytochrome with α, β and γ bands at 560–562, 530–535 and 429 nm respectively (Fig. 4).

Growth and metabolic properties

The organism displayed the cardinal growth temperatures of a mesophile with an optimum of 30–35 °C, and no growth was observed above 45 °C or below 4 °C. The optimum pH for growth in LPBB medium with 0·5% glucose was 6·0–6·5, and no growth was detected below 4·5 or above 8·0.

A wide variety of compounds were fermented, including arabinose, cellobiose, galactose, glucose, lactose, mannose, mannitol, raffinose, galacturonate, sorbitol, sucrose, glycerol, lactate and amygdalin. Inositol, maltose, melibiose, melezitose, rhamnose, trehalose, arabinogalactan, cellulose, mannose, pectin, polygalacturonic acid, starch, xylan, citrate, pyruvate, tartrate, aesculin, salicin, Casamino acids, gelatin and tryptone were not fermented.

The growth rate of the organism was dependent on both the specific energy source and the nutrient nitrogen source (Fig. 5). The specific growth rate (μ) was much higher on glucose in complex media (0·18 h^{-1}) than on ammonium chloride (0·13 h^{-1}) or N_2 (0·03 h^{-1}). Notably, the growth rate on LPBB medium with 0·5% sodium lactate and N_2 as nitrogen source was only 0·003 h^{-1}. The cell yield on any substrate tested was highest in complex medium and lowest when N_2 was the sole source of nitrogen. Cultures grown on glucose in LPBB medium with N_2 alone exhibited an acetylene reducing activity of 5–21 nmol ethylene formed h^{-1} ml^{-1} (A_{660} was 0·35). The addition of ammonium chloride to cultures inhibited nitrogenase activity. Neither growth with N_2 as the sole nitrogen source nor acetylene reduction activity was detected in control experiments with *Pectinatus cerevisiiphilus*.

The main products of carbohydrate fermentation were propionate, acetate and CO_2. Ethanol and succinate were only formed in trace quantities when glucose was fermented (Table 1). However, ethanol was a more significant end product of xylose metabolism, and succinate was a

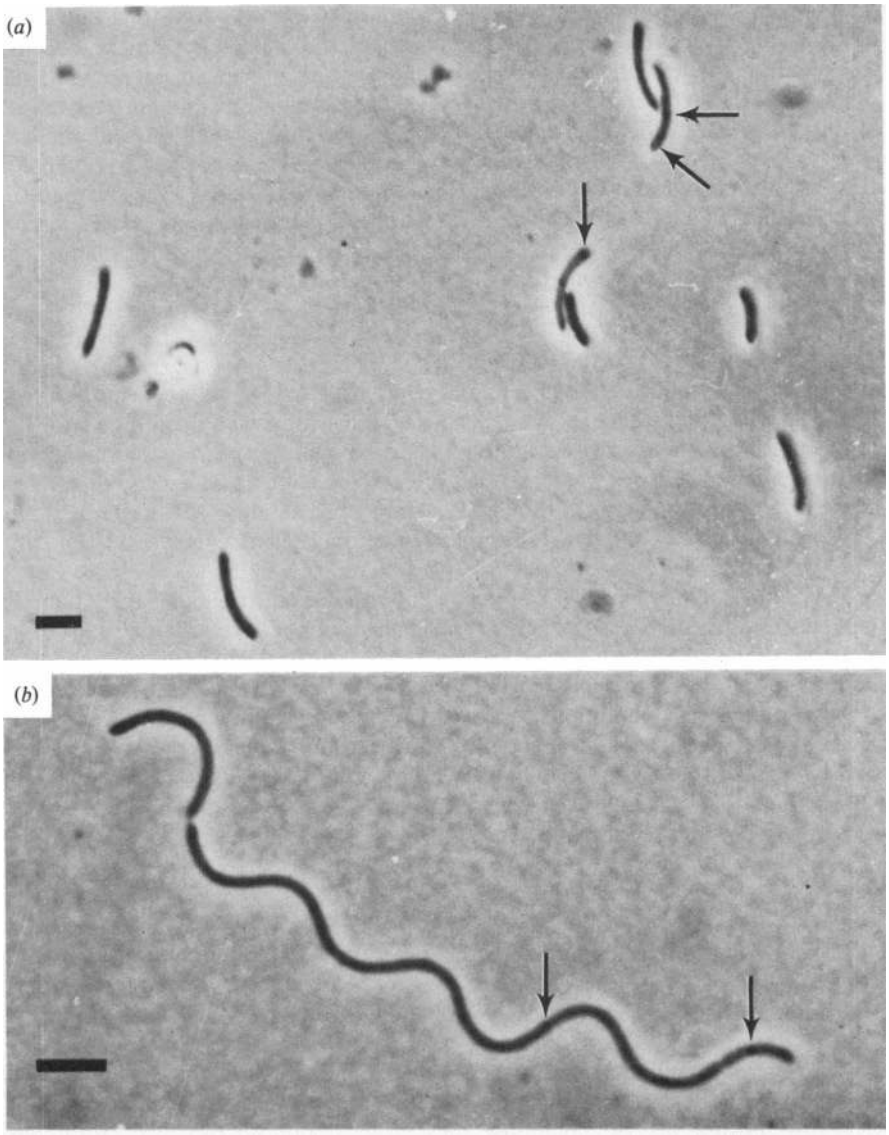

Fig. 1. Phase-contrast photomicrographs of strain 12B4. (*a*) Exponential phase cells grown on LPBB medium with glucose (0·5%) and yeast extract (0·1%). Arrows point to granular reserve material. (*b*) A spiral shaped cell present in an exponential phase culture grown in LPBB medium with 0·5% (w/v) sodium lactate and N_2 as sole nitrogen source. The bar markers represent 5 μm.

major end product when fumarate was added to glucose/LPBB medium. Neither H_2, lactate nor other soluble or gaseous compounds were detectable fermentation products.

The dependence of end product formation on growth is shown in Fig. 6. At the end of the time course the fermentation balance measured was

$$227\ \text{nmol glucose} \rightarrow 300\ \text{nmol propionate} + 161\ \text{nmol acetate} + 142\ \text{nmol } CO_2$$

This accounted for a carbon recovery of 100% and an oxidation–reduction index of 0·95.

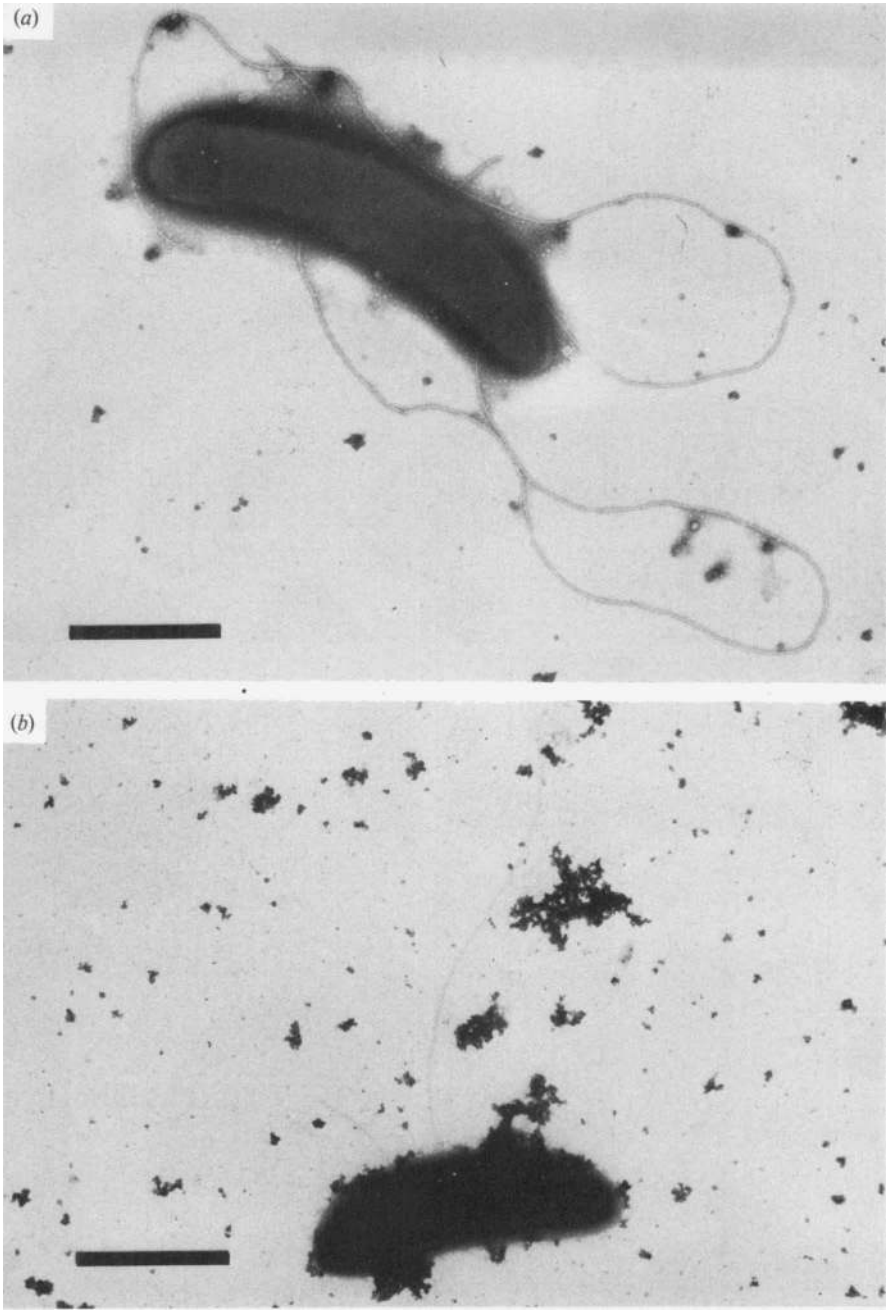

Fig. 2. (*a*, *b*) Electron micrographs of phosphotungstic acid stained cells showing random flagella placement of strain 12B4. The bar markers represent 1 μm.

DISCUSSION

The isolation of this bacterium extends the diversity of known obligately anaerobic bacteria. These spiral-shaped cells could be regarded as the anaerobic counterpart to *Spirillum lipoferum*, the aerobic nitrogen-fixing curved rods first reported by Beijerinck (1925). Although this aerobic

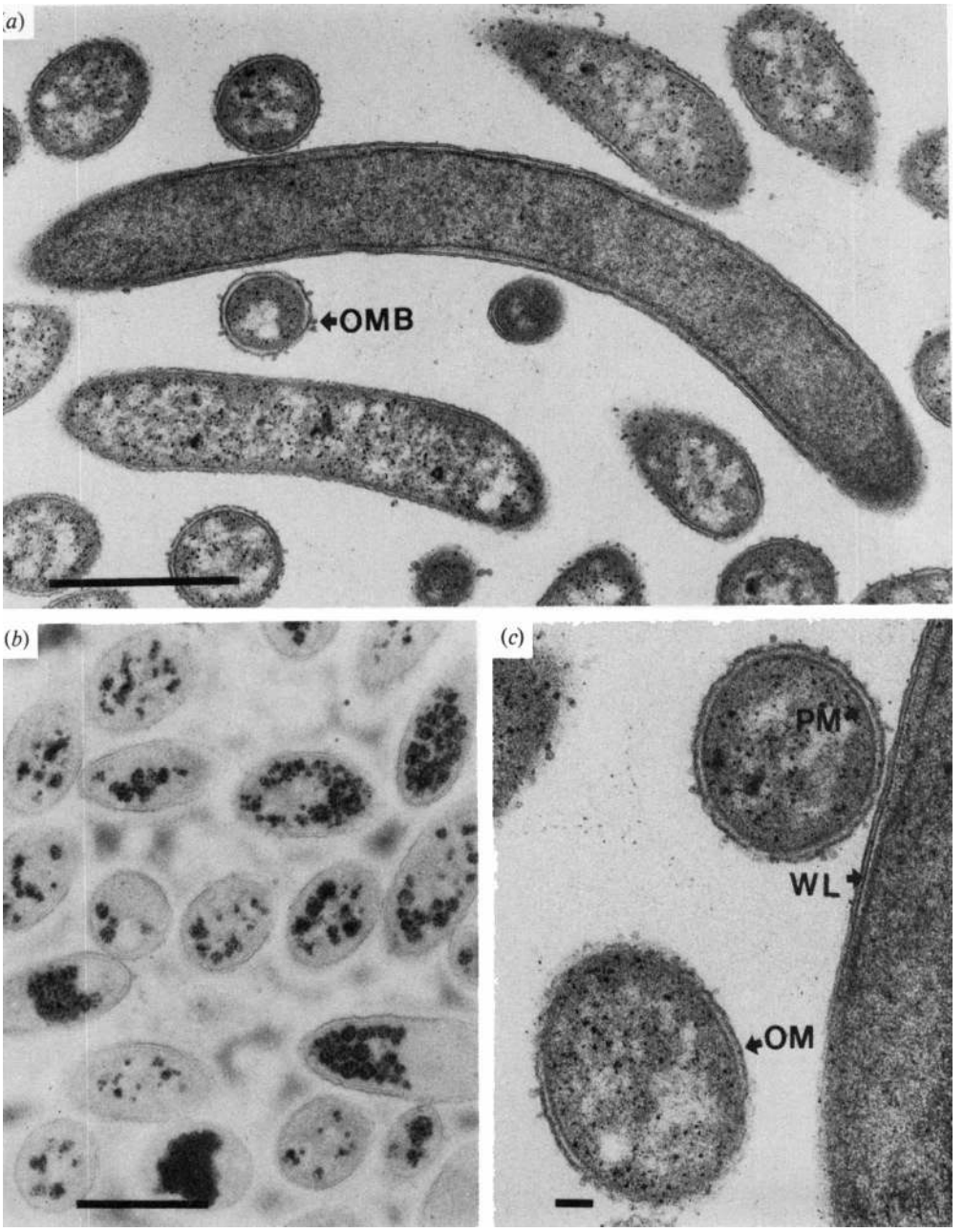

Fig. 3. Electron micrographs of strain 12B4 thin sections. (*a*) Grazing sections of cells showing general ultrastructural features and outer membranous blebs (OMB). (*b*) Cells showing carbohydrate storage material after specific staining and fixation procedures. (*c*) High magnification illustrating typical Gram-negative cell wall ultrastructure including outer membranous wall layer (OM), inner wall layer (WL) and the cytoplasmic membrane (PM). The bar markers represent 1 μm in (*a*) and (*b*) and 0·1 μm in (*c*).

species is now recognized as *Azospirillum lipoferum* (Tarrand *et al.*, 1978), it also contained granular storage material. However, the bacterium described here is an obligate anaerobe and forms a carbohydrate reserve material, not poly-β-hydroxybutyric acid.

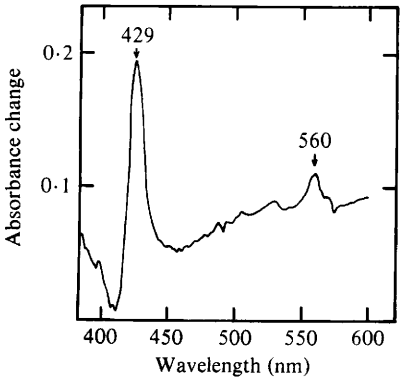

Fig. 4. Sodium dithionite-reduced versus air-oxidized difference spectrum of crude extract of strain 12B4. The protein content of the preparation was 2·2 mg ml^{-1}.

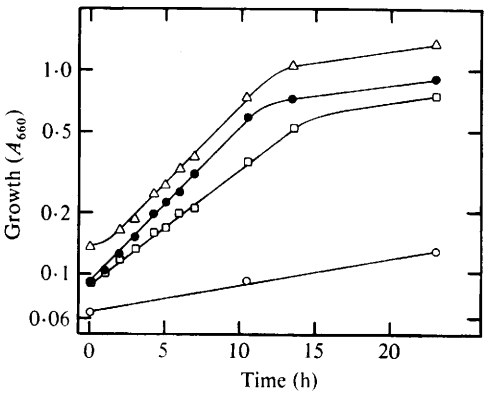

Fig. 5

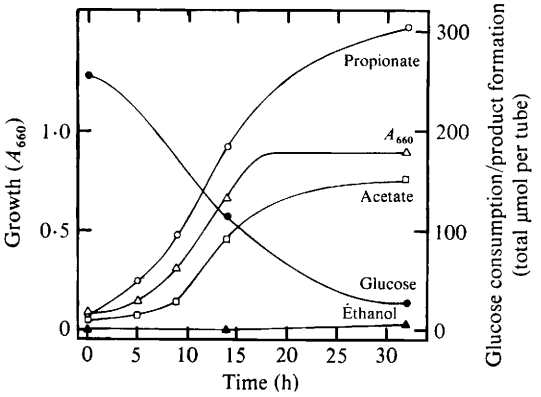

Fig. 6

Fig. 5. Relation between medium nitrogen sources and growth of strain 12B4 on glucose. △, LPBB + 1% (w/v) tryptone + 0·3% (w/v) yeast extract; ●, LPBB + 0·3% (w/v) yeast extract; □, LPBB; ○, LPBB with N_2 as sole nitrogen source.

Fig. 6. Glucose fermentation time course for strain 12B4. Experiments were performed in anaerobic culture tubes that contained 10 ml LPBB and 0·5% (w/v) glucose.

Table 1. *Relation between energy source and end products formed by strain 12B4*

Experiments were done in anaerobic culture tubes that contained 10 ml LPBB medium. Substrate concentrations (mM) added were: glucose, 25; arabinose, 33; xylose, 33; lactate, 90 and fumarate, 40.

	Fermentation products (total μmol per tube)			
Substrate	Ethanol	Acetate	Propionate	Succinate
Arabinose	6	203	370	ND
Xylose	47	156	280	ND
Lactate	8	255	460	ND
Glucose	<1	161	300	<1
Glucose/fumarate	<1	240	400	356

ND, Not determined.

This is the first report of a propionic acid-forming anaerobe able to fix nitrogen. At present, two pathways are recognized for the formation of propionic acid (Zeikus, 1980). *Propionibacterium* and *Selenomonas* species utilize fumarate as an intermediary metabolite, whereas *Bacteroides*, *Megasphaera* and *Clostridium* species utilize acrylyl-CoA. The fermentation balance for the new isolate was very close to classical propionibacteria results; namely, 3 glucose → 4 propionate + 2 acetate + 2CO_2. The *Propionibacterium–Selenomonas* pathway is suggested for

strain 12B4 for the following reasons. First, hydrogen was not detected as an end product. Secondly, fumarate addition greatly stimulated propionate formation during glucose fermentation. Lastly, a type *b* cytochrome was identified which is the same as that reported in *Propionibacterium* (de Vries *et al.*, 1973; Schwartz & Sporkenbach, 1975) and *Selenomonas* (de Vries *et al.*, 1974).

The physiological properties of the bacterium reported here suggest that it is well-adapted to the wetwood ecosystem. Alkaline wetwoods are notably anaerobic, low in fixed nitrogen, abundant in saccharides and contain numerous bacterial fermentation products including propionate (Zeikus & Ward, 1974; Schink *et al.*, 1981*a*). This bacterium can ferment a variety of wood sugars including galacturonate released by pectinolytic activity (Schink *et al.*, 1981*a*), and under the nitrogen-fixing conditions (Schink *et al.*, 1981*a*) associated with the wetwood disease syndrome in living trees. This organism may therefore function in the supply of fixed nitrogen to other wetwood species and account in part for the formation of propionate in this novel microbial ecosystem, which also contains *Methanobacterium arbophilicum* (Zeikus & Henning, 1975).

The cellular and metabolic properties of the bacterium suggest that it may be affiliated to the family *Bacteroidaceae* (Buchanan & Gibbons, 1974). However, since it forms only propionate, acetate, CO_2 and traces of succinate and ethanol, generic assignment to *Bacteroides*, *Fusobacterium*, *Leptotrichia*, *Butyrivibrio*, *Succinivibrio*, *Succinimonas*, *Lachnospira*, *Selenomonas* or *Anaerobiospirillum* is not possible. It differs dramatically from propionate-forming *Selenomonas* species by its low DNA G + C content (36·7 versus 53–61%) and peritrichous flagellation, and bears closest resemblance to *Pectinatus cerevisiiphilus*. However, the latter produces lactate and succinate from glucose, has a DNA G + C content of 39·8% and has a 'comb-like' insertion of flagella only on the concava side of the cell (Lee *et al.*, 1978). Moreover, *Pectinatus cerevisiiphilus* does not fix nitrogen or grow in a mineral medium on amygdalin, lactose, sorbitol or sucrose. Nonetheless, further macromolecular taxonomic studies are needed on the generic designations of these two species.

The catabolic activity of this bacterium may be of industrial interest. For example, the organism, unlike *Propionibacterium* species, can grow without vitamins, yeast extract and, if necessary, ammonium. Perhaps this could be of importance to the production of vitamin B_{12} or propionic acid.

Propionispira arboris gen. nov. and sp. nov.

Pro.pi.o.ni.spi.ra. M.L. n. *acidum propionicum* propionic acid, L. fem.n. *spira* coil L. fem.n. *arbor* tree, *arboris* of a tree.

Morphology. Curved to helical rods 1 × 7 μm. Exponential-phase cells very motile by peritrichous flagellation. Forms long spiral cells when grown under N_2-fixing conditions. Gram-negative and endospores not formed. Colonies on agar surfaces are smooth and 1–3 nm in diameter.

Cellular characteristics. DNA base composition of 36·7 ± 1 mol% G + C. Cytochrome *b* and nitrogenase are present and catalase is absent. Double-layered cell wall architecture with outer wall membrane present.

Growth characteristics. Optimum temperature for growth is 30–35 °C, maximum < 46 °C and minimum > 4 °C. pH optimum for growth is 6·0–6·5; no growth above 8·0 or below 4·5. Obligate anaerobe. Complete inhibition by either 2% (w/v) NaCl, 250 μg sodium azide ml^{-1}, or 100 μg cycloserine ml^{-1}, penicillin, streptomycin, tetracycline or chloramphenicol.

Metabolic characteristics. Chemoorganotroph. Utilizes a wide variety of compounds as energy sources including lactate, glucose, lactose, sorbitol, xylose, and amygdalin. Fermentation end products are propionic acid, acetic acid and carbon dioxide. Ethanol and succinate are formed under special conditions. Fumarate is reduced to succinate or propionate, but sulphate and nitrate are not reduced.

Habitat. Alkaline wetwoods associated with living poplar trees. Type strain 12B4. This strain was isolated from wetwood of a mature cottonwood tree located in Columbus, Wisconsin, U.S.A. and has been deposited in the American Type Culture Collection (ATCC 33732), Rock-

ville, Md., U.S.A. and the Deutsche Sammlung von Mikroorganismen (DSM 2179), Göttingen, Germany.

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