***Eubacterium tucumanense* sp. nov.: an Anaerobic Gram-positive Non-sporeformer Isolated from an Anaerobic Digester**

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An anaerobic bacterium, strain ED₃, able to grow on a wide variety of substrates has been isolated from the contents of an anaerobic digester. Cells were non-motile, rod shaped and presented a Gram-positive envelope. Ethanol, methanol, hydrogen plus carbon dioxide, pentoses and a variety of carbohydrates were utilized for growth. Strain ED₃ grew best at 37 °C and at pH 6.8–7.5. The mol % G + C of the DNA was 44.0 (*T_m*). Strain ED₃ was named *Eubacterium tucumanense* sp. nov. and is the type strain (ATCC 49281) for this species.

INTRODUCTION

The stillage ('vinaza') obtained after the distillation of alcohol produced by yeast fermentation of molasses is a highly concentrated organic wastewater (chemical oxygen demand about 50–100 g oxygen l⁻¹) that contains excretion products of yeast, yeast cell debris, other compounds present in the sugar-cane juice and pentose sugars. We are interested in organisms that would ferment the pentoses in the anaerobic digester ecosystem, as a first step for the formation of methane.

Some members of the genus *Eubacterium* ferment pentoses. This genus comprises a large group of strictly anaerobic Gram-positive bacilli which are catalase-negative, non-sporeforming, pleomorphic, and motile or non-motile. They may be saccharoclastic or nonsaccharoclastic but all are chemo-organotrophic. They do not produce large amounts of propionate or lactate, but mixtures of volatile organic acids, from carbohydrates and peptone. Several species are more difficult to grow in liquid than solid media. There are significant variations in the fermentation products when they utilize carbohydrates in defined medium without yeast extract and peptone (Wilkins *et al.*, 1974). Most of the known species produce butyric acid as the principal product of fermentation, together with small quantities of acetic, lactic and succinic acids. Nevertheless, nine species of *Eubacterium* do not produce butyrate in defined medium when they ferment different carbohydrates (Wegienek & Reddy, 1982).

The genus *Eubacterium* can be readily distinguished from morphologically similar genera of anaerobic Gram-positive bacteria, for example *Acetobacterium* and *Clostridium*. Members of the genus *Acetobacterium* differ from those of the genus *Eubacterium* in their ability to grow by the anaerobic oxidation of hydrogen and the reduction of CO₂ to acetic acid, as well as in their ability to carry out only homoacetic fermentations on a limited number of substrates. The mol % G + C of the DNA is 39 (buoyant density) to 43 (*T_m*). Species of *Clostridium* in which spores are difficult to detect can be differentiated from *Eubacterium* by demonstration of heat- or alcohol-resistance.

Thirty-four species of the genus *Eubacterium* have been described (Moore & Holdeman-Moore, 1986). The mol % G + C of the DNA of the type species, *E. limosum*, is 47 (*T_m*). The mol % G + C of the DNA of other species examined is 30–55 (*T_m*), indicating that the genus is very heterogeneous. Here we describe a new species, *E. tucumanense*, isolated from an anaerobic digester.

METHODS

Isolation of strain ED₃. Strain ED₃ was isolated by enrichment culture on mineral medium (Balch *et al.*, 1979) plus xylose, from the contents of an upflow anaerobic sludge bed reactor used for the treatment of stillage from distilleries that produce alcohol from the fermentation of sugar-cane molasses. Serum bottles (50 ml) containing 10 ml Balch's medium plus 0.05% xylose, with a nitrogen gas phase, were inoculated with 0.1 ml of the contents of the anaerobic digester and incubated for 15 d at 37 °C. Dilutions in agar roll tubes with the same medium were incubated for 24 d at 37 °C. The colonies which developed were punctiform, and less than 1 mm in diameter. Some were picked and subjected to a new agar dilution series. Isolated colonies were transferred to Balch's medium, without agar, containing 0.5% xylose, and nitrogen as above, inside an anaerobic chamber (COY Manufacturing Co.) filled with N₂/CO₂/H₂ (80:10:10, by vol.). The culture was transferred monthly.

Media. All culture media were prepared as described by Miller & Wolin (1974). Balch's medium (Balch *et al.*, 1979) contained, per litre: resazurin (1%, w/v), 4 ml; mineral solution no. 1, 50 ml; mineral solution no. 2, 50 ml; trace minerals solution (Balch *et al.*, 1979), 10 ml; vitamin solution (Wolin *et al.*, 1963), 10 ml; FeSO₄ · 7H₂O (0.2% in 0.07% HCl), 1 ml. Mineral solution no. 1 contained (g l⁻¹): K₂HPO₄, 6. Mineral solution no. 2 contained (g l⁻¹): KH₂PO₄, 6; (NH₄)₂SO₄, 6; NaCl, 12; MgSO₄ · 7H₂O, 2.45; CaCl₂ · 2H₂O, 1.50. Roll tubes were prepared by adding 2% (w/v) agar to the same medium. For biochemical tests, PY and PYG media were prepared as described in the VPI Anaerobic Manual (Holdeman & Moore, 1975).

Isolation of DNA and determination of base composition. DNA was extracted and purified with phenol, according to the method of Saito & Miura (1963). The mol % guanine plus cytosine was determined according to Mandel & Marmur (1968), measuring the absorbance at 260 nm. The melting point (*T_m*) was obtained graphically. DNA from *Escherichia coli* (Sigma) was used as standard. *T_m* was related to G + C using the equation: mol % G + C = (*T_m* - 53.9)2.44.

Analytical techniques. Growth of strain ED₃ in liquid cultures was measured using a spectrophotometer (Metrolab Serie UV-Vis), recording the optical density at 560 nm.

Gases were analysed using a gas chromatograph (Gow-Mac Serie 550) equipped with a thermal conductivity detector. A column of silica gel (180 × 0.3 cm) was used. Operational conditions were: injection volume, 0.05 ml; injection temperature, 80 °C; detector temperature, 100 °C; column temperature, 90 °C; carrier gas, nitrogen at 10 ml min⁻¹.

Volatile fatty acids were determined with a gas chromatograph (Gow-Mac Serie 752) equipped with a flame ionization detector and a glass column (180 × 0.3 cm) of 10% SP-1200/H₃PO₄ on Chromosorb. Operational conditions were: injection volume, 200 µl (supernatant of a 72 h culture); injection temperature, 110 °C; column temperature, 100 °C; carrier gas, nitrogen at 20 ml min⁻¹. Hydrogen flow rate, 60 ml min⁻¹ and air flow rate, 400 ml min⁻¹.

Electron microscopy. Cells were fixed with glutaraldehyde (2.5%, w/v) in 0.1 M-phosphate buffer pH 7.8 and then subjected to 1% (w/v) OsO₄, dehydrated in acetone and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate (2.0%, w/v) for 20 min and lead citrate (2.0%, w/v) for 5 min, and then observed with an electron microscope (Zeiss 109).

RESULTS AND DISCUSSION

Strain ED₃ conformed to the major characteristics of the genus *Eubacterium*: non-sporing Gram-positive rods, obligately anaerobic, chemo-organotrophic, producing mixtures of organic acids, including butyric, acetic and formic acids, from carbohydrates or peptone.

The structure of the cell envelope of strain ED₃ is shown in Fig. 1. The cell wall has a typical Gram-positive structure, with some resemblance to those reported for *Acetobacterium woodii* (Mayer & Schoberth, 1977) and '*Butyribacterium methylophilicum*' (Zeikus *et al.*, 1980). Cells were usually present in pairs. No spores were seen either by phase-contrast microscopy or after staining with malachite green. No growth occurred after heating the culture at 70–80 °C for 10 min.

Colonies on the roll tubes were punctiform, circular, entire, convex and translucent, reaching about 0.5 mm in diameter after 21 d incubation at 37 °C. Growth was enhanced by adding 15% (v/v) rumen fluid to the medium. Cultures were catalase negative. Biochemical tests were performed on PY medium as described by Holdeman & Moore (1975) (Table 1).

Strain ED₃ used CO₂ plus H₂ for growth, acetate being the main product; the doubling time in these conditions was 43 h. Doubling times with sugars as carbon source were: xylose, 26 h; arabinose, 24 h; glucose, 10 h; and fructose, 12 h. The organism slowly fermented ethanol (doubling time 72 h). The fermentation balance with xylose as carbon source is shown in

Fig. 1. Electron micrograph of thin sections of organism ED₃. Magnification ×41200.

Table 1. *Biochemical tests of organism ED₃ and Eubacterium limosum*

Symbols: —, negative reaction for 90–100% of strains; +, positive reaction for 90–100% of strains; ±, pH 6.0–7.0; A, acid (pH below 5.5); w, weak reaction (pH 5.5–5.9, sugars); d, 40–60% of strains positive; numbers (hydrogen) represent abundant (4) to negative on a — to 4 scale.

Characteristics	Organism ED ₃	<i>E. limosum</i>
Cells motile	—	—
Acid produced from:		
Arabinose	w	—A
Cellobiose	A	—
Fructose	A	A
Glucose	A	A
Lactose	w	—
Maltose	w	—w
Mannitol	w	—A
Mannose	w	—w
Melezitose	w	—
Melibiose	—	—
Raffinose	w	—
Rhamnose	w	—
Sucrose	w	—
Trehalose	w	—
Xylose	w	d
Aesculin hydrolysed	±	+
Starch hydrolysed	+	±
Gelatin digested	+	d
H ₂ produced	4	4
Fatty acids produced*:		
PYG (cultures)	A, B	A, B, L, s, ib, iv
PY (cultures)	A, p, ib, (b, iv)	A, IV, IB, b, (p)

* a, acetate; b, butyrate; p, propionate; ib, isobutyrate; iv, isovalerate; l, lactate; s, succinate. Capital letters represent an amount of product equal to or greater than 1 meq per 100 ml culture; small letters represent an amount of product less than 1 meq per 100 ml of culture; products in parentheses are not produced uniformly.

Table 2. *Xylose fermentation in mineral medium of ED₃ organisms*

	Xylose (μmol)	Acetate (μmol)	Propionate (μmol)	Cellular carbon (μmol)	Cell dry wt (g l^{-1})
Initial	417	—	—	—	—
Final	316	48	105	83	0.002

Carbon recovery: $\{[48(2) + 105(3) + 83] \div [101(5)]\} \times 100 = 97\%$

Electron recovery: $\{[48(8) + 105(14) + 83(4.22)*] \div [101(20)]\} \times 100 = 109\%$

* 4.22, number of electron equivalents μmol^{-1} (Harris & Adams, 1979).

Table 2. Lactate was not detected. Strain ED₃ was also capable of growing on stillage, producing acetate, butyrate and CO₂ as main fermentation products. These features, notably Gram-positive non-sporeforming rods which form acetate and butyrate as main fermentation products when grown in PYG medium, are consistent with strain ED₃ being assigned to the genus *Eubacterium* (Holdeman & Moore, 1975).

Strain ED₃ is different from all other *Eubacterium* species able to produce butyric acid: *E. alactolyticum*, *E. bifforme*, *E. budayi*, *E. cellulosolvens*, *E. cylindroides*, *E. hallii*, *E. limosum*, *E. monoliforme*, *E. multiforme*, *E. nitrogenes*, *E. plautii*, *E. tortuosum* and *E. ventriosum* do not produce acid from xylose, arabinose or melezitose. *E. hadrum*, *E. plexicaudatum*, *E. ramulus*, *E. rectale*, *E. ruminanthium* and *E. saburreum* do not digest gelatin. *E. brachy*, *E. dolichum* and *E. nodatum* are non-saccharolytic species and they do not hydrolyse gelatin.

The G + C values for strains of *Eubacterium* are between 26 and 48 mol % (Wegienek & Reddy, 1982). The melting point (T_m) for DNA from strain ED₃ was 72 °C (mean of three determinations), from which a DNA base composition of 44 mol % was derived. The butyric-acid-producing *Eubacterium* species have the following DNA G + C values (mol %): *E. bifforme*, 32 (T_m); *E. cylindroides*, 31 (T_m); *E. hallii*, 36–38 (T_m); *E. hadrum*, 32–33 (T_m); *E. plexicaudatum*, 44 (T_m); *E. ramulus*, 39 (T_m); *E. rectale*, 30 (T_m); and *E. nodatum*, 36–38 (T_m). The mol % G + C of the DNA for other butyric-acid-producing *Eubacterium* species has not been determined or reported because of poor growth and difficulty of preparing DNA.

Eubacterium limosum, which seems to be closely related to *Clostridium barkeri* and *Acetobacterium woodii* (Balch *et al.*, 1977; Tanner *et al.*, 1981) can use CO₂ plus H₂ for growth as can strain ED₃; however, the difference in DNA base composition (47 versus 44 mol %) indicates that they are distinct taxa. Moreover, *E. limosum* strains have not been reported to grow with ethanol as carbon source. Similarly, ethanol is not used by *Butyrivibrio methylotrophicum*, which is closely related to *Eubacterium*. The DNA base composition and growth on ethanol as well as pentoses led us to propose a new species for strain ED₃, which is described below.

Eubacterium tucumanense sp. nov. (tu.cu.man.en'se. M.L. adj. *tucumanense*, native to Tucumán; named after the province of Tucumán, where it was isolated). Cells are straight rods, 0.3–0.5 $\mu\text{m} \times 1.5$ –2 μm , usually present in pairs, non-motile. Cells stain Gram-positive and the cell wall is composed of a single layer. Colonies are punctiform and translucent, with a defined edge; they are about 0.5 mm in diameter after 21 d incubation at 37 °C. Cells can grow in defined mineral medium but yeast extract enhances growth. The following compounds support growth: methanol, ethanol, xylose, arabinose, fructose, CO₂ plus H₂. Cells produce acetate, propionate and hydrogen when grown on xylose. In PY medium, cells also produce small amounts of isobutyrate and isovalerate.

Obligate anaerobe. Optimal growth at 37 °C, grows also at 45 °C but does not grow at 55 °C.

The mol % G + C of the DNA of the type strain, ED₃, is 44. Strain ED₃ has been deposited at the American Type Culture Collection as ATCC 49281.

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