***Roseburia intestinalis* sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces**

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Five strains of butyrate-producing, anaerobic, Gram-positive bacteria were isolated from human faecal material. These strains were slightly curved rods that showed motility by means of multiple subterminal flagella. The DNA G + C content of the strains was 29–31 mol%. A detailed investigation of the phenotypic and phylogenetic characteristics of the strains revealed that they represent a novel species of anaerobic, low-G+C-content, butyrate-producing bacterium that shows net acetate utilization during growth on media containing carbohydrates and short-chain fatty acids. The 16S rRNA gene sequences of the five isolates were determined and they confirmed that these strains were closely related to each other. Phylogenetic analysis indicated that the most closely related species are *Eubacterium rectale*, *Eubacterium oxidoreducens* and *Roseburia cecicola*, members of cluster XIVa of the *Clostridium* subphylum of Gram-positive bacteria, although they share less than 95% sequence identity with the novel strains. It is proposed that a novel species, *Roseburia intestinalis* sp. nov., be created, with strain L1-82^T (= DSM 14610^T = NCIMB 13810^T) as the type strain.

Keywords: *Roseburia intestinalis*, human faeces, butyrate, low-G + C bacteria, cluster XIVa

INTRODUCTION

Butyrate produced by fermentation in the human colon is considered to have health-promoting properties (von Engelhardt *et al.*, 1998; Scheppach *et al.*, 1995). There is, however, only limited information concerning bacterial strains capable of butyrate production in the human gut. Barcenilla *et al.* (2000) isolated butyrate-producing bacteria from human faeces and showed that the vast majority fell within the low-G + C cluster XIVa as defined by Collins *et al.* (1994). However, based on 16S rDNA sequence analysis, more than 50% of the strains examined were less than 95% related to known species. Barcenilla *et al.* (2000) also categorized the butyrate-producing strains based on their restriction profiles with the

enzyme *AluI*. One particular ribogroup considered here, ribogroup 1, which accounted for 13 of the 74 strains isolated, formed a distinct cluster related to *Roseburia cecicola*, *Eubacterium oxidoreducens* and *Eubacterium rectale* on the basis of phylogenetic analysis of 16S rDNA sequences.

The genus *Eubacterium* was defined by Prévot to include isolates from human faeces that possessed properties beneficial to human health (reviewed by Prévot, 1966). Later, Moore & Holdeman Moore (1986) described *Eubacterium* as a convenient but diverse taxon, based mainly on bacteria that could not be assigned to other genera. *Eubacterium* species have now been found in a range of other habitats including animal faeces (Moore *et al.*, 1987; Mott & Brinkley, 1979), the rumen (Bryant, 1959), the oral cavity (Holdeman *et al.*, 1980) and infections (Sans & Crowder, 1973). Certain species may be recovered from the soil or sewage (Moore & Holdeman Moore, 1986). They are obligately anaerobic, Gram-positive, non-spore-forming rods. Many species use sugars, but

Abbreviation: SCFA, short-chain fatty acids.

The GenBank accession numbers for the 16S rDNA sequences of the *R. intestinalis* strains are AJ270478–AJ270480, AJ312385 and AJ312386.

the genus also includes non-saccharolytic species. Most species produce butyric acid and hydrogen gas. Many produce lactate, but neither propionate nor succinate is a major product (Bokkenheuser *et al.*, 1979; Krumholz & Bryant, 1986; reviewed by Andreesen, 1992). The species *R. cecicola*, reported from the mouse intestine (Stanton & Savage, 1983a), shares many of these characteristics and is most closely related to *E. rectale*. Based on 16S rDNA sequence analyses, most *Eubacterium* species and *R. cecicola* belong to the *Clostridium coccoides* cluster of low-G + C-content Gram-positive bacteria (Willems *et al.*, 1996). Interest in the role of these species in the human gut is increasing because of evidence that this cluster accounts for 40–50% of eubacterial sequences in studies of randomly cloned 16S rDNA from human faeces (Suau *et al.*, 1999; Franks *et al.*, 1998).

Although the contribution of *Eubacterium* and its relatives to colonic metabolism has still to be elucidated, it is considered that these species may account for much of the butyrate produced in the human large intestine (Barcenilla *et al.*, 2000). Butyrate is an important nutrient for colonocytes, as well as a signalling molecule with a central role in cell differentiation and apoptosis (von Engelhardt *et al.*, 1998; Scheppach *et al.*, 1995). *Eubacterium* species also contribute to other important processes within the human gut: for example, *Eubacterium ramulus* (Simmering *et al.*, 1999) forms phenolic acids from a range of flavonoids including quercetin, rutin and luteolin (Schneider & Blaut, 2000) and is likely to be one of the most important flavonoid-transforming bacteria in the gut (Schneider *et al.*, 2000).

It is proposed that the isolates reported here should be placed in a novel species, named *Roseburia intestinalis* sp. nov. in view of its habitat and its relationship to *R. cecicola*, with strain L1-82^T as the type strain.

METHODS

Anaerobic methods and media. Five butyrate-producing strains (L1-82^T, L1-952, L1-8151, L1-81 and L1-93) were isolated from the highest countable dilution of healthy infant faecal samples in roll tubes of anaerobic M2GSC medium (Miyazaki *et al.*, 1997) as described by Barcenilla *et al.* (2000). Anaerobic culture methods were those of Bryant (1972) using Hungate culture tubes, sealed with butyl rubber septa (Bellco Glass Inc.). Media were prepared and maintained anaerobically using O₂-free CO₂. The isolates were routinely maintained by growing for 16–18 h at 37 °C in 7.5 ml aliquots of M2GSC medium.

Morphology. The cellular morphology of the novel isolates was determined by Gram-staining exponential- and stationary-phase cultures as described by Holdeman *et al.* (1977). Motility was determined by examination of 16 h old cultures by phase-contrast microscopy. The presence of flagella was observed by scanning electron microscopy (SEM) following the procedure described by Stewart *et al.* (1990).

Substrate utilization and hydrolysis. Substrate utilization was determined by adding a final concentration of 0.5% of

stock (10%, w/v) filter-sterilized sugar solutions to YCFA medium dispensed in 7.5 ml amounts in Hungate tubes. The medium consisted of (in 100 ml) 1 g casitone, 0.25 g yeast extract, 0.4 g NaHCO₃, 0.1 g cysteine, 0.045 g K₂HPO₄, 0.045 g KH₂PO₄, 0.09 g NaCl, 0.009 g MgSO₄·7H₂O, 0.009 g CaCl₂, 0.1 mg resazurin, 1 mg haemin, 1 µg biotin, 1 µg cobalamin, 3 µg *p*-aminobenzoic acid, 5 µg folic acid and 15 µg pyridoxamine. Final concentrations of short-chain fatty acids (SCFA) in the medium were 33 mM acetate, 9 mM propionate and 1 mM each of isobutyrate, isovalerate and valerate. All components were added aseptically while the tubes were flushed with CO₂. Heat-labile vitamins were added after the medium was autoclaved to give a final concentration of 0.05 µg thiamin ml⁻¹ and 0.05 µg riboflavin ml⁻¹. YCFA supplemented with a carbon source provided a convenient alternative to rumen-fluid medium for the cultivation of the strains in this study. Growth was measured spectrophotometrically as OD₆₅₀. Twelve different arylamidase activities were tested using the Rapid ID-32A system (bioMérieux). The compounds tested were arginine arylamide (A), proline A, leucine glycine A, phenylalanine A, leucine A, pyroglutamic acid A, tyrosine A, alanine A, glycine A, histidine A, serine A and glutamyl glutamic acid A. Aesculin hydrolysis was determined as described by Duncan *et al.* (1998).

Fermentation product analysis. Acid production was determined by capillary GC (Richardson *et al.*, 1989) and the gaseous products were analysed by packed-column GC (Rumney *et al.*, 1995).

DNA preparation and DNA base composition. DNA was extracted from 24 h old cultures grown on M2GSC medium following the method of Ausubel *et al.* (1994). Chromosomal DNA was purified by standard methods (Sambrook *et al.*, 1989), with the G + C content determined using the thermal denaturation procedure as described by Johnson (1981).

16S rDNA sequencing and phylogenetic analysis. For amplification of 16S rDNA, a universal primer set was used that corresponded to positions 8–27 (forward primer) and 1492–1510 (reverse primer) of the *Escherichia coli* numbering system (Weisburg *et al.*, 1991). The PCR operating conditions were as described by Wood *et al.* (1998). Direct sequencing of the amplified DNA fragments was performed using an automated ABI 377 sequencer as described previously (Hold *et al.*, 2001). Similarity of the 16S rDNA sequences from the five strains to sequences of other organisms was compared with all sequence data in GenBank and EMBL using the BLAST algorithm (Gish & States, 1993) and the Ribosomal Database Project (Maidak *et al.*, 1994). Nucleotide sequences were aligned with reference 16S rDNA gene sequences and phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) via the PHYLIP package (Felsenstein, 1989) using DNADIST for distance analysis (Kimura, 1980).

RESULTS

Cell morphology, fermentation products and response to SCFA

The novel strains isolated from human faeces were Gram-variable-staining, slightly curved rods measuring approximately 0.5 × 1.5–5.0 µm (Table 1). Cells stained predominantly Gram-positive in young cultures (8–10 h). All five isolates were motile when examined by phase-contrast microscopy, with SEM of

Table 1. Phenotypic properties of strains of *Roseburia intestinalis* sp. nov.

Cells of all strains are motile rods and stain Gram-variable. All strains are negative for arylamidase, fermentation of mannitol, melezitose, rhamnose, ribose, trehalose and chicory inulin and indole, urease and catalase. All strains are positive for fermentation of glucose, raffinose, sucrose, xylose, starch and oat-spelt xylan, hydrolysis of aesculin and gas production from carbon dioxide and hydrogen. NT, Not tested; w, weak.

Property	L1-82 ^T	L1-952	L1-8151	L1-81	L1-93
Cell size (µm)	0.5 × 1.5–3.0	0.5 × 2.5–5.0	0.5 × 3.0–5.0	0.5 × 3.0–4.0	0.5 × 3.0–4.0
Flagella	Multiple	Multiple	Multiple	NT	NT
DNA G + C content (mol %)	31	29	29	NT	NT
Fermentation of:					
Arabinose	+	+	+	+	w
Cellobiose	w	+	+	+	+
Fructose	+	+	+	w	+
Maltose	w	+	+	+	+
Melibiose	w	+	w	w	w

Fig. 1. Scanning electron micrograph of *Roseburia intestinalis* sp. nov. L1-82^T showing a flagella bundle. Bar, 1 µm.**Table 2.** Fermentation products formed by strains of *Roseburia intestinalis* sp. nov.

Values are amounts of compounds produced/consumed (–), given in µmol ml^{–1} culture.

Compound	L1-82 ^T	L1-952	L1-8151	L1-81	L1-93
Formate	4.7	5.5	3.4	4.4	1.1
Acetate	–9.1	–10.1	–12.3	–14.2	–10.6
Butyrate	18.5	21.3	16.0	21.0	10.0
Lactate	10.2	7.1	5.5	3.6	5.7

strain L1-82^T demonstrating the presence of multiple flagella located subterminally (Fig. 1).

Analysis of fermentation products of cells grown in medium M2G showed that butyrate and L-lactate were

the main products, with more than 10 µmol butyrate ml^{–1} produced *in vitro*. Formate was also detected, but only as a minor product (Table 2). Hydrogen and carbon dioxide were also formed. Acetate was not essential for growth but the addition of between 20 and 100 mM acetate stimulated growth (maximum OD₆₅₀) by approximately 20 % and was utilized during growth on glucose (Duncan *et al.*, 2002). Branched-chain SCFA (isobutyrate and isovalerate) did not stimulate growth (data not shown). All strains were net acetate utilizers, removing between 9 and 14 µmol acetate ml^{–1} from the growth medium (Table 2).

Substrates fermented and hydrolysed

Aesculin was hydrolysed and weak fermentation of melibiose was detected. The substrates arabinose, cellobiose, fructose, maltose, raffinose, sucrose, xylose and starch were all fermented. Rhamnose, melezitose, mannitol, ribose, inulin and trehalose were not fermented, and arylamidase activity was not detected using the Rapid ID-32A system.

Molecular characterization and phylogenetic relationships

Incomplete 16S rDNA sequences of three ribotype 1 strains (L1-81, L1-952 and L1-93) were reported previously and shown to cluster together during phylogenetic analysis (Barcenilla *et al.*, 2000). Here, the 16S rDNA sequences of these three strains were completed and sequences were also determined for two further strains, L1-82^T and L1-8151. The five strains show more than 98.5 % sequence similarity to each other, but share < 95 % sequence identity with the most closely related species, *E. rectale*, *E. oxidoreducens* and *R. cecicola* (Fig. 2). The DNA G + C content determined for three of the strains was between 29 and 31 mol % (Table 1).

Fig. 2. Phylogenetic tree, constructed by the neighbour-joining method, based on 16S rDNA sequences of the novel isolates L1-82^T, L1-952, L1-8151, L1-81 and L1-93 and related bacteria. Sequence accession numbers are given in parentheses.

DISCUSSION

The results of the phenotypic and phylogenetic analyses clearly indicate that the novel strains (L1-82^T, L1-952, L1-8151, L1-81 and L1-93), isolated from human faeces, form a homogeneous group and represent a novel species within the genus *Roseburia*. These bacteria are Gram-positive, slightly curved rods, non-spore-forming, motile, anaerobic, butyrate-producing and show net utilization of acetate present in the growth medium. They are capable of utilizing a range of carbohydrates and produce several fermentation products, with butyrate and L-lactate identified as the major products.

Differentiation of *Roseburia intestinalis* sp. nov. from related species

In view of their phylogenetic and phenotypic similarities and their lack of relatedness to previously described species (Table 3), we propose that the five

strains reported here form a novel species, *Roseburia intestinalis* sp. nov. The most closely related known species are *R. cecicola* (Stanton & Savage, 1983a, b; Hespell, 1992), *E. rectale*, which has been subdivided into five groups based on their ability to ferment different sugars (Moore & Holdeman Moore, 1986; Andreessen, 1992), and *E. oxidoreducens* (Krumholz & Bryant, 1986). However, based on phenotypic properties, *R. intestinalis* is most similar to *E. rectale* subgroup II, while its 16S rDNA sequence indicates closest relatedness to *R. cecicola*.

The ability to ferment melezitose has been used to distinguish *E. rectale* from some other *Eubacterium* species (Moore & Holdeman Moore, 1986). Melezitose was not fermented by any of the *R. intestinalis* strains described here, but non-melezitose-fermenting strains of *E. rectale* have been reported (Moore & Holdeman Moore, 1986). The former type strain of *E. rectale*, ATCC 33656, was also reported to ferment inulin, a property shared with strain A1-86 of Barcenilla *et al.* (2000), shown previously to have 98% sequence similarity to *E. rectale* (data not shown). In contrast, inulin was not fermented by *R. intestinalis*. Direct comparison of *R. intestinalis* with representatives of the five *E. rectale* groups and *R. cecicola* could not be undertaken because the *E. rectale* isolates are not extant and *R. cecicola* is unavailable. All strains of *E. rectale* are reported to produce butyrate and lactate, with some acetate, whilst traces of propionate or succinate may be formed. *R. cecicola* is reported to produce only butyrate as a major fermentation product and *E. oxidoreducens* is reported to produce butyrate and acetate. In contrast, *R. intestinalis* utilizes acetate and produces butyrate, lactate and formate.

Examination of genomic DNA G+C content shows that *E. rectale* and *R. intestinalis* have similar genomic

Table 3. Phenotypic properties of *R. cecicola*, *R. intestinalis*, *E. rectale* and *E. oxidoreducens*

Data for reference taxa were taken from Moore & Holdeman Moore (1986) (*E. rectale*), Stanton & Savage (1983a) (*R. cecicola*) and Krumholz & Bryant (1986) (*E. oxidoreducens*). Cells of all taxa are rods. NR, Not reported.

Property	<i>R. intestinalis</i>	<i>R. cecicola</i>	<i>E. rectale</i>	<i>E. oxidoreducens</i>
Cell size (µm)	0.4–0.5 × 1.0–2.4	0.5 × 2.0–5.0	0.5–0.6 × 1.7–4.7	0.45 × 1.5–2.2
Gram stain	Variable	Negative	Variable	Positive
Flagella	Subterminal	Subterminal	Peritrichous	None
Motility	+	+	+	—
DNA G+C content (mol%)	29–31	42.3	30	35.7
Saccharolytic	+	+	+	—
Fermentation of:				
Inulin	—	NR	+	—
Melezitose	—	NR	+ / — *	—
Utilization of acetate	+	+	—	—
Major fermentation products†	BFL	B	BFLA	BA

* Dependent on subgroup.

† A, Acetate; B, butyrate; F, formate; L, lactate.

DNA G+C contents of between 29 and 31 mol%, compared with 35.7 mol% for *E. oxidoreducens* (Krumholz & Bryant, 1986) and 42 mol% for *R. cecicola* (Stanton & Savage, 1983a) (Table 3).

Description of *Roseburia intestinalis* sp. nov.

Roseburia intestinalis (in.tes.ti.nal'is. L. gen. n. *intestinalis* of the intestine, the presumed habitat of the isolates).

Gram-positive to Gram-variable rods, 0.4–0.5 × 1.0–5.0 µm, with DNA G+C content ranging from 29 to 31 mol%. Motile by means of multiple flagella, subterminally borne. Ferments glucose, arabinose, cellobiose, maltose, fructose, raffinose, sucrose, xylose, xylan and starch. Mannitol, melezitose, rhamnose, ribose, trehalose and inulin are not fermented. Aesculin is hydrolysed and arylamidase activity is not detected. Major fermentation products from glucose and acetate include butyrate, lactate, hydrogen and carbon dioxide. The type strain is strain L1-82^T (= NCIMB 13810^T = DSM 14610^T).

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