

The family *Coriobacteriaceae*: reclassification of *Eubacterium exiguum* (Poco *et al.* 1996) and *Peptostreptococcus heliotrinreducens* (Lanigan 1976) as *Slackia exigua* gen. nov., comb. nov. and *Slackia heliotrinreducens* gen. nov., comb. nov., and *Eubacterium lentum* (Prevot 1938) as *Eggerthella lenta* gen. nov., comb. nov.

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16S rRNA gene sequences were determined for *Eubacterium exiguum* and *Peptostreptococcus heliotrinreducens*. These species were found to be closely related and, together with *Eubacterium lentum*, to constitute a branch of the *Coriobacteriaceae*. Two new genera are proposed on the basis of phenotypic characteristics and 16S rRNA gene sequence comparisons: *Slackia* to include the bile-sensitive species *Eubacterium exiguum* and *P. heliotrinreducens*, and *Eggerthella* to include the bile-resistant *Eubacterium lentum*. It is proposed that *Eubacterium exiguum* and *Peptostreptococcus heliotrinreducens* are transferred to the genus *Slackia* gen. nov. as *Slackia exigua* gen. nov., comb. nov. (type strain ATCC 700122^T) and *Slackia heliotrinreducens* gen. nov., comb. nov. (type strain NTCC 11029^T), respectively, and *Eubacterium lentum* is transferred to the genus *Eggerthella* gen. nov. as *Eggerthella lenta* gen. nov., comb. nov. with *Eggerthella lenta* as the type species.

Keywords: *Coriobacteriaceae*, *Eubacterium*, *Peptostreptococcus*, *Slackia*, *Eggerthella*

INTRODUCTION

The Gram-positive, non-sporulating, anaerobic bacilli comprising the genus *Eubacterium* are a heterogeneous collection of organisms. This is largely because the genus is defined by default and has become a depository for a large number of unrelated taxa (Moore & Holdeman Moore, 1986). On the basis of 16S rRNA sequence analysis, the majority of species currently classified as *Eubacterium*, including the type species *Eubacterium limosum*, belong to the low G + C Gram-positive phylum (Cheeseman *et al.*, 1996; Willems & Collins, 1996). However, some species and unnamed

taxa provisionally identified as *Eubacterium* have DNA of high G + C content (Nakazawa & Hoshino, 1994). One such species is *Eubacterium exiguum* (Poco *et al.*, 1996), formerly designated *Eubacterium* D6 (Moore *et al.*, 1982, 1983), *Eubacterium* S-group (Sato *et al.*, 1993) or *Eubacterium* Cluster 2 (Wade *et al.*, 1990, 1994). *Eubacterium exiguum* is frequently found in periodontitis and periapical infections (Wade, 1997) but is difficult to identify because it is generally unreactive in conventional biochemical tests. In this, it resembles *Eubacterium lentum* (Moore *et al.*, 1971), an organism found in human faeces, with which it is often confused (Wade *et al.*, 1990). *Eubacterium lentum* is only rarely reported as being isolated from the mouth when tests capable of distinguishing it from *Eubacterium exiguum* are performed (Wade *et al.*, 1990).

The GenBank accession numbers for the 16S rDNA of *Slackia exigua* and *Slackia heliotrinreducens* are AF101240 and AF101241, respectively.

Murdoch & Mitchelmore (1989) described the frequent isolation from oral abscesses of strains of *Peptostreptococcus heliotrinreducens*, which were identified on the basis of enzyme profiles. However, the type strain of *P. heliotrinreducens* was isolated from a sheep rumen and Goodacre *et al.* (1996) showed by Py-MS that the isolates identified as *P. heliotrinreducens* by Murdoch & Mitchelmore (1989) were, in fact, *Eubacterium exiguum*. In a phylogenetic study of the genus *Peptostreptococcus* based on 16S rDNA sequence comparisons, Li *et al.* (1994) found *P. heliotrinreducens* to be dissimilar to all other *Peptostreptococcus* species and, further, unlike any other prokaryotic sequence available at that time. Unfortunately, the sequence for this species was not deposited in the sequence databases and remains unavailable.

The aim of this study was to use 16S rDNA sequence analysis to clarify the taxonomic positions of *Eubacterium exiguum*, *P. heliotrinreducens* and *Eubacterium lentum*.

METHODS

Bacterial strains. *Eubacterium lentum* NCTC 11813^T and *P. heliotrinreducens* NCTC 11029^T were obtained from the NCTC; *Eubacterium exiguum* ATCC 700122^T and *P. heliotrinreducens* ATCC 29202^T were obtained from the ATCC. Bacteria were maintained on Fastidious Anaerobe Agar (LabM) supplemented with 5% horse blood and incubated under anaerobic conditions.

Biochemical tests. Fermentation tests were performed using pre-reduced, anaerobically sterilized sugars according to the methods of Holdeman *et al.* (1977), except that distilled water for broth preparation was pre-reduced in an anaerobic workstation. Sensitivity to bile was determined by the plate method described by Summanen *et al.* (1993). Other biochemical tests were performed as described by Holdeman *et al.* (1997).

Metabolic-end-product analysis. Bacterial strains were grown in peptone/yeast extract/glucose broth and short-chain volatile and non-volatile fatty acids extracted by standard methods (Holdeman *et al.*, 1977). Analysis was performed by gas chromatography with a capillary column packed with a 10% free fatty acid solid phase.

Enzyme profiles. Enzyme profiles were generated with the Rapid ID32A anaerobe identification kit (bioMérieux) according to the manufacturer's instructions. Bacteria were harvested from blood agar plates [Blood Agar Base No. 2 (LabM) + 5% horse blood] incubated anaerobically at 37 °C for 72 h.

Protein profiles. Protein profiles of whole-cell proteins were generated by SDS-PAGE using 10–15% gradient gels and the PhastSystem (Pharmacia) as described previously (Slayne *et al.*, 1990), except that the cells were pre-treated with lysozyme (50 µg ml⁻¹) for 3 h at 37 °C.

DNA isolation and PCR amplification. DNA was released from bacteria by combining 1 µl of a dense suspension of bacteria with 20 µl Gene Releaser (BioVentures) and following the manufacturer's microwave protocol. PCRs were performed in thin-walled tubes with a Perkin-Elmer 480 thermal cycler, GeneAmp7 PCR Reagent Kit and AmpliWax PCR Gem 100s. The 20 µl from the Gene Releaser was combined with 1 µM primers and other reagents in the Hot Start protocol suggested by Perkin-Elmer. Forward primer C75 (*Escherichia coli* numbering 7–27) 5' GAGAGTTTGATYCTGGCTCAG 3' and reverse primer C90 (1501–1483) 5' GTTACGACTTCACCCTCT 3' were used. The following conditions were used for amplification: denaturation at 72 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 45 s, with 5 s added for each elongation step. A total of 30 cycles were performed, followed by a final elongation step at 72 °C for 15 min. The purity of the product was determined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and viewed under long-wavelength UV light.

Purification of PCR products. The amplified DNA was purified by precipitation with PEG 8000 (Kusukawa *et al.*, 1990). After removal of the AmpliWax, 0.6 vols 20% PEG 8000 (Sigma) in 2.5 M NaCl were added and the mixture incubated at 37 °C for 10 min. The sample was centrifuged for 15 min at 15000 g and the pellet was washed with 80% ethanol and pelleted as before. The pellet was air-dried and dissolved in 30 µl distilled water, and used for cycle sequencing as described below.

Sequencing methods. The DNA sample from PCR was directly sequenced using a cycle-sequencing kit (*fmo17* DNA Sequencing System; Promega), following the manufacturer's protocol. The eight sequencing primers are shown in Table 1. Primers were end-labelled with ³³P (NEN DuPont) using the manufacturer's protocol. Approximately 100 ng purified

Table 1. Sequencing primers used in this study

Name	Sequence (5' → 3')	Position*	Orientation
C75	GAGAGTTTGATYCTGGCTCAG	8–23	Forward
B34	RCTGCTGCCTCCCGT	344–358	Reverse
B35	GTRTTACCGCGGCTGCTG	519–536	Reverse
B36	GGACTACCAGGGTATCTA	789–806	Reverse
C01	GGTTGCGCTCGTTGCGGG	1096–1113	Reverse
C31	GGAATCGCTAGTAATCG	1337–1353	Forward
X91	CCCGGGAACGTATTCACCG	1369–1387	Reverse
C90	GTTACGACTTCACCCTCT	1501–1483	Reverse

* Numbering based upon the sequence of *E. coli*.

Table 2. Sources and accession numbers of strains examined

Species	Strain or clone	GenBank accession no.
<i>Atopobium minutum</i>	ATCC 33267 ^T	X67148
<i>Atopobium parvulum</i>	ATCC 33793 ^T	X67150
<i>Atopobium rimae</i>	ATCC 49626 ^T	X67149
<i>Bifidobacterium breve</i>	ATCC 15700 ^T	M58731
<i>Bifidobacterium longum</i>	ATCC 14707 ^T	M58739
<i>Coriobacterium glomerans</i>	ATCC 49209 ^T	X79048
<i>Eubacterium lentum</i>	ATCC 25559 ^T	RDP*
<i>Eubacterium exiguum</i>	ATCC 700122 ^T	AF101240
Nitropropanol-degrader	NPOH1	U43492
<i>Peptostreptococcus heliotrinreducens</i>	ATCC 29202 ^T	AF101241

* Sequence available from the Ribosomal Database Project.

DNA from the PCR was used for sequencing. Reaction products were loaded onto 8% polyacrylamide/urea gels, electrophoresed and detected by exposure to X-ray film for 24 h.

16S rDNA data analysis. A program set for data entry, editing, sequence alignment, secondary structure comparison, similarity matrix generation and dendrogram construction for 16S rDNA data was written in Microsoft Quick BASIC for use on PC-compatible computers (Paster & Dewhirst, 1988). Sequences were entered and aligned as previously described (Paster & Dewhirst, 1988). Our sequence database contains approximately 1000 sequences determined in our laboratory and another 500 obtained from GenBank or the Ribosomal Database Project (Maidak *et al.*, 1997). Reference strains used in the 16S rDNA analysis are given in Table 2. Similarity matrices were constructed from the aligned sequences by using only those sequence positions from which 90% of the strains had data. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes & Cantor (1969). Phylogenetic trees were constructed using the neighbour-joining method of Saitou & Nei (1987).

Estimation of G + C content of DNA. Bacteria were grown for 7 d in peptone/yeast extract/glucose broth supplemented with 0.5% arginine. The cells were harvested by centrifugation and washed twice in 0.9% NaCl, 50 mM EDTA, pH 8. One gram (wet wt) of cells was then suspended in 8 ml 0.9% NaCl, 50 mM EDTA, pH 8. Lysozyme was added to 50 µg ml⁻¹ and the suspension incubated for 30 min at 37 °C. Proteinase K was then added to 50 µg ml⁻¹ and the suspension incubated for 1 h at 37 °C, followed by the addition of 1/10 vol 20% SDS and incubation for 2 h at 37 °C. An equal volume of phenol/chloroform was added and mixed with the suspension, which was then incubated on ice for 15 min. After centrifugation, the supernatant was removed, mixed with an equal volume of chloroform and incubated on ice for a further 15 min. The DNA was then precipitated with 2 vols ice-cold ethanol and removed by spooling. The spooled DNA was washed in 70% ethanol and then dissolved in distilled water. RNase was added to 50 µg ml⁻¹ and incubated for 30 min at 37 °C. Following a further chloroform extraction, the DNA was re-precipitated

in ethanol and then dried before being dissolved in distilled water.

DNA solutions were prepared to 1 mg ml⁻¹ and heated at 100 °C for 10 min and then rapidly cooled in an ice bath. The denatured DNA solution (50 µl) was then mixed with 50 µl nuclease P₁ solution (2 units ml⁻¹ in 40 mM sodium acetate buffer containing 0.2 mM ZnCl₂, pH 5.3). The mixture was then incubated at 50 °C for 1 h after which the hydrolysates and a standard solution containing equimolar amounts of dCMP, dAMP, dGMP and dTMP were subjected to HPLC through a LiChroCART 250-4 column (Merck). Peak heights were measured and the mol% G + C content was calculated according to the formula of Katayama-Fujimura *et al.* (1984).

RESULTS AND DISCUSSION

The phylogenetic relationships between the strains included in the study are shown in Fig. 1. *Eubacterium lentum*, *Eubacterium exiguum* and *P. heliotrinreducens* fell into a cluster related to species of *Atopobium* within the family *Coriobacteriaceae* in the high-G + C Gram-positive phylum. The position of the *Coriobacteriaceae* in this phylum is well established (Rainey *et al.*, 1994) and was confirmed in preliminary analyses that included representatives of the high- and low-G + C Gram-positive bacteria and other phyla. The cluster also includes *Coriobacterium glomerans*, an endosymbiont of the red soldier bug (Haas & König, 1988), and an unnamed nitropropanol-degrading organism (Anderson *et al.*, 1997). The phenotypic relatedness of *Eubacterium exiguum* and *P. heliotrinreducens*

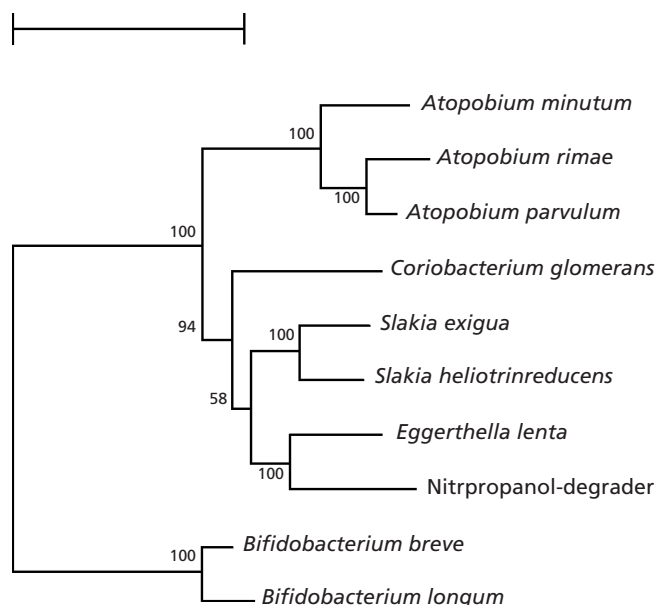


Fig. 1. 16S rDNA based phylogenetic tree for the family *Coriobacteriaceae*. Scale bar, 10% difference in nucleotide sequences as determined by measuring the lengths of the horizontal lines connecting two sequences. The numbers are the bootstrap values for the branches based on data obtained for 1000 trees.

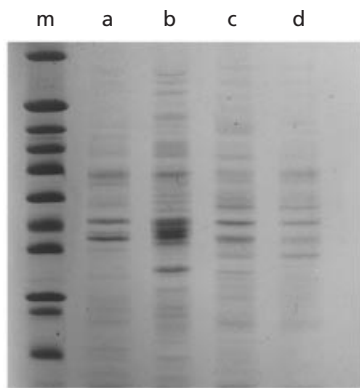


Fig. 2. Protein profiles of *Eggerthella lenta*, *Slackia exigua* and *Slackia heliotrinireducens*. Lane m, molecular mass markers; lane a, *Eggerthella lenta* NCTC 11813^T; lane b, *Slackia exigua* ATCC 700122^T; lane c, *Slackia heliotrinireducens* ATCC 29202^T; lane d, *Slackia heliotrinireducens* NCTC 11029^T.

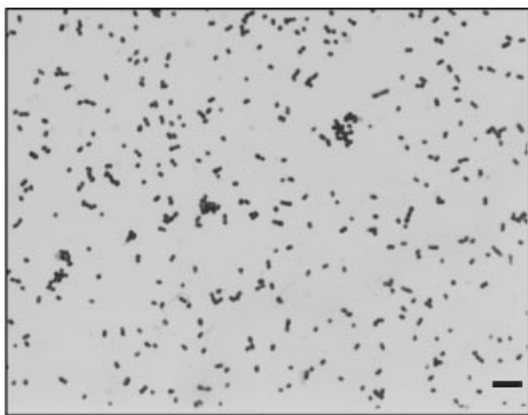


Fig. 3. Cellular morphology of *Slackia heliotrinireducens* (Gram-stained smear, bar, 5 μ m).

described previously (Goodacre *et al.*, 1996) was confirmed by the 16S rRNA sequence analysis, which showed them to be nearest neighbours in the tree; sequence similarity between the species was 94.7%. Both species showed less than 91% similarity to the other strains studied.

The G+C content of the DNA of *P. heliotrinireducens* has been reported as 36.1 mol% (Lanigan, 1976) and 34–36 mol% (Ezaki & Yabuuchi, 1986). In this study, we estimated the G+C content to be 61 mol%, which is consistent with its phylogenetic position. This degree of variation raises questions about the purity of the strain. We therefore obtained cultures from both the NCTC and ATCC and found them to be identical by enzyme and protein profiles (Fig. 2). The description of the phenotypic characters for *P. heliotrinireducens* (Lanigan, 1976) is consistent with those of the strains tested here. In addition, the cellular morphology seen in Gram-stained smears (Fig. 3) is identical to that seen in the micrograph presented by Lanigan (1976). The

cells are characteristically cocci and coccobacilli found in chains, singly and in clumps. We therefore believe that the strain examined in this study is the one deposited by Lanigan (1976), but cannot explain the differences observed in G+C values.

Eubacterium lentum and *Eubacterium exiguum* are both markedly distinct from *Eubacterium limosum*, the type species of the genus, and the other members of *Eubacterium sensu stricto*, which are found in the low-G+C Gram-positives (Willems & Collins, 1996). *Eubacterium lentum* is found primarily in human faeces and is bile-resistant, while *Eubacterium exiguum* is bile-sensitive and found in the mouth, mainly associated with oral infections (Poco *et al.*, 1996). *P. heliotrinireducens* is also bile-sensitive and was originally isolated from the sheep rumen, which may be its only habitat. The phenotypic characteristics of *Eubacterium exiguum*, *Eubacterium lentum* and *P. heliotrinireducens* are shown in Table 3. *Eubacterium exiguum* and *P. heliotrinireducens* differ by only a single test in the Rapid ID 32A test panel but can be distinguished easily by their protein profiles (Fig. 2), although it appears unlikely that they would be isolated from the same specimens and therefore confused.

On the basis of 16S rRNA sequence similarity and bile sensitivity, it is proposed that *Eubacterium exiguum* and *P. heliotrinireducens* be placed in a new genus, *Slackia*, as *Slackia heliotrinireducens* (we have taken this opportunity to correct the epithet) and that a second new genus, *Eggerthella*, be created to include *Eubacterium lentum*, which shares only 89.8 and 90.8% 16S rRNA sequence similarity with *Eubacterium exiguum* and *P. heliotrinireducens*, respectively. Whether the rumen nitropropanol-degrading organism should be included in the same genus as *Eubacterium lentum* will have to await a more uniform phenotypic characterization of both organisms.

Description of *Slackia* gen. nov.

Slackia (Slack'ia. M.L. fem. n. named to honour Geoffrey Slack, distinguished British microbiologist and dental researcher).

Cells are cocci, coccobacilli or short bacilli. Gram-positive, non-motile obligate anaerobes, which do not produce endospores. Bile-sensitive, hydrolyse arginine, do not produce catalase, urease or indole, and do not hydrolyse aesculin. Growth is stimulated by 0.5% arginine. Sugars are not fermented. The DNA base composition is 60–64 mol% G+C. The type species is *Slackia exigua*.

Description of *Slackia exigua* (*Eubacterium exiguum* Poco *et al.* 1996) comb. nov.

Slackia exigua (ex.i.gu'a. L. adj. *exigua* scanty, small, referring to the scanty or poor growth of this organism).

Characteristics of the species are as previously described by Poco *et al.* (1996). The type strain is ATCC

Table 3. Characteristics of *Eggerthella lenta*, *Slackia exigua* and *Slackia heliotrinireducens*

Test	<i>E. lenta</i>	<i>S. exigua</i>	<i>S. heliotrinireducens</i>
Rapid ID 32A profile	2000 0000 00	2000 0337 05	2000 0237 05
Nitrate reduction	+	—	+
Bile growth	4	—	—
Fermentation products from glucose*	(Acetate), (lactate), (succinate)	None detected	Acetate
G + C content (mol %)	62	60–64	61†
Morphology:			
Shape	Rods	Rods; single, clumps	Cocci and coccobacilli in chains and clumps
Size (µm)	0.2–0.4 × 0.2–2.0	0.5 × 1.0	0.8 × 0.8–1.2

* Fermentation products in parentheses indicates strain variation.

† Type strain, this study.

700122^T. The 16S rRNA gene sequence for the type strain has been deposited in GenBank under accession number AF101240.

Description of *Slackia heliotrinireducens* (*Peptostreptococcus heliotrinireducens* Lanigan 1976) comb. nov.

Slackia heliotrinireducens (he.li.o.trin.i.re.duc.ens. M.L. n. *heliotrinum* derived from heliotrine, a pyrrolizidine alkaloid; L. adj. *reducans* reducing M.L. adj. *heliotrinireducens* referring to the organisms ability to bring about oxidative cleavage of the heliotrine molecule).

Characteristics of the species are as previously described for *Peptostreptococcus heliotrinireducens* (Lanigan, 1976). The type strain is NCTC 11029^T. The 16S rDNA sequence for the type strain has been deposited in GenBank under accession number AF101241.

Description of *Eggerthella* gen. nov.

Eggerthella [Egg.er.thel'la. M.L. fem. n. named to honour Arnold Eggerth, who first described the organism later named *Eubacterium lentum* (Eggerth 1935)].

Characteristics of the genus are as previously described for *Eubacterium lentum* (Moore *et al.*, 1971). The type species is *Eggerthella lenta*.

Description of *Eggerthella lenta* (*Eubacterium lentum* Prevot 1938) comb. nov.

Eggerthella lenta (len'tum. L. neut. adj. *lentum* slow).

Characteristics of the species are as previously described by Moore *et al.* (1971). The type strain is NCTC 11813^T.

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