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Short communication

DNA–DNA relatedness and phylogenetic positions of *Slackia exigua, Slackia heliotrinireducens, Eggerthella lenta,* and other related bacteria

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Recently, two asaccharolytic *Eubacterium* species, *Eubacterium exiguum and Eubacterium lentum*, and *Peptostreptococcus heliotrinreducens* have been reclassified as *Slackia exigua, Eggerthella lenta and Slackia heliotrinireducens* in the novel genera on the basis of 16S rDNA sequence analysis. But DNA–DNA relatedness among these species and other related bacteria have not been reported yet. DNA–DNA relatedness is the standard arbiter and the recommended method for the designation and evaluation of new species, particularly closely related ones. In the present study, DNA–DNA hybridization studies were performed on *S. exigua, S. heliotrinireducens and E. lenta* together with the other bacterial species in the related genera. The phylogenetic relationships of these species were also investigated by comparison analysis of 16S rDNA sequence data. In the DNA–DNA hybridization studies, *S. exigua* showed a DNA homology level of 33% to *S. heliotrinireducens and* 11% to *E. lenta*. DNA–DNA homology between *S. heliotrinireducens and E. lenta* was 10%. But these three species showed very low homology (less than 5%) to the related asaccharolytic species such as *Eubacterium* and *Mogibacterium*. In conclusion, the DNA–DNA relatedness data together with the evolutionary data in the present paper further support the reclassification of *Eubacterium exiguum, Peptostreptococcus heliotrinreducens* and *Eubacterium lentum* as *Slackia exigua*, *Slackia heliotrinireducens* and *Eggerthella lenta*, respectively.

Key words: DNA–DNA relatedness; *Eggerthella lenta*; *Slackia exigua*; *Slackia heliotrinireducens*

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Two novel genera, *Slackia* and *Eggerthella*, have been proposed on the basis of 16S rDNA sequence analysis, and *Eubacterium exiguum* and *Eubacterium lentum* have been transferred to these genera as *Slackia exigua* and *Eggerthella lenta*, respectively (9). *Peptostreptococcus heliotrinreducens* has been reclassified as *Slackia heliotrinireducens* at the same time. These three bacterial species have been shown to fall into a cluster related to species of *Atopobium* within the same family, *Coriobacteriaceae*.

The genus *Eubacterium* comprises uniform or pleomorphic nonspore-forming gram-positive rods, which are obligate anaerobic bacteria. They do not produce propionic acid, lactic acid or succinic acid as a major end product. This broad definition of the genus *Eubacterium*, which is derived by differentiation from other anaerobic genera on the basis of negative fermentation characteristics (4), has for a long time provided a convenient classification for the collection of diverse organisms (1, 5). It was inevitable that there was considerable heterogeneity among the species of this genus. It has also been reported that at the present time the genus contains many bacterial species and groups which are phylogenetically unrelated (5). Some *Eubacterium* species, especially asaccharolytic species, have been transferred to

the novel genera such as *Pseudoramibacter* (11), *Collinsella* (3), *Holdemania* (12) and *Mogibacterium* (6) according to their phylogenetic characteristics.

S. exigua (7), which belongs to the high G + C gram-positive phylum (1,11), is asaccharolytic and significantly associated with periodontitis, endodontic infections and oral abscesses but is rarely found in oral health (8). Another asaccharolytic species, *Eggerthella lenta* (4), is found primarily in feces, is rarely isolated from the mouth and most resembles *S. exigua*. These two species are clearly distinct from *Eubacterium limosum,* which is the type species of the genus *Eubacterium* and has low G + C content (4). *S. heliotrinireducens* is bile-sensitive and was originally isolated from the sheep rumen; it also resembles *S. exigua* phenotypically (9).

It has been reported that the levels of 16S rDNA sequence similarity in *S. exigua*, *S. heliotrinireducens* and *E. lenta* range from 89.9 to 94.7% (9). At such levels of 16S rDNA sequence similarity, DNA–DNA hybridization is the most important and final criterion in confirming the genetic relationships among these bacterial species. In current bacteriology the phylogenetic definition of a species is strains with approximately 70% or greater DNA–DNA relatedness (10). The aim of the present study was, by using DNA–DNA hybridization analysis, to investigate the genetic interrelationship of *S. exigua, S. heliotrinireducens*, *E. lenta* and other closely related bacterial species that are asaccharolytic anaerobic gram-positive rods (AAGPR). The present study, we also validated the phylogenetic positions of these species in the evolutionary tree based on a comparison study of 16S rDNA sequences.

Bacterial strains used for this study were type strains of *S. exigua* ATCC 700122^T, *S. heliotrinireducens* ATCC 29202^T and *E. lenta* ATCC 25559^T. Also examined were other type strains, *Cryptobacterium curtum* ATCC 700683^T, *Eubacterium brachy* ATCC 33089^T, *Eubacterium minutum* ATCC 700079^T, *Eubacterium nodatum* ATCC 33099^T, *Eubacterium saphenum* ATCC 49989^T, *Mogibacterium diversum* ATCC 700923^T, *Mogibacterium neglectum* ATCC 700924^T, *Mogibacterium pumilum* ATCC 700696^T, *Mogibacterium timidum* ATCC 33093^T and *Mogibacterium vescum* ATCC 700697^T, all reported to be microorganisms of AAGPR and genetically closely related bacteria (5–7).

The strains were cultured in brain heart infusion broth under strictly anaerobic conditions for 7–10 days in an anaerobic glove box containing 80% N_2, 10% H_2, and 10% CO_2. The bacterial cells were harvested by centrifugation, washed with 10 mM sodium phosphate-buffered saline (pH 7.2), and stored at − 20°C until they were used. Chromosomal DNA was isolated from these bacterial cells and purified following a modification of the Marmur protocol (5). DNA–DNA hybridization was performed by the membrane filter method as described previously (5). Briefly, reference DNA was labeled by using a multiprime DNA labeling kit and [α-^{32}P] dCTP, and then purified with a Nick column (Amersham Pharmacia Biotech., UK). About 40 μg of unlabeled single-stranded DNA immobilized on each nitrocellulose membrane filter and 0.015 μg of labeled reference DNA were reassociated in a solution containing 0.08% (w/v) SDS, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) Ficoll 400, BSA (Fraction V; Sigma), and 1 mL of 6× SSC (1 × SSC is 0.15 M NaCl plus 15 mM sodium citrate). After incubation overnight at 60°C, the filters were washed and dried. The radioactivity was measured with a liquid scintillation counter. Triplicate tests were performed for each assay, and the results were normalized to 100% for the homologous DNA.

S. exigua ATCC 700122^T exhibited 33% DNA–DNA relatedness with *S. heliotrinireducens* ATCC 29202^T (Table 1), indicating that these two strains are members of a same genus but different species. Our DNA–DNA hybridization studies also showed that *E. lenta* ATCC 25559^T had 11% and 10% DNA homology with *S. exigua* ATCC 700122^T and *S. heliotrinireducens* ATCC 29202^T (Table 1), respectively. These very low homology values suggest that strain ATCC 25559^T belongs to a different genus from strain ATCC 700122^T and ATCC 29202^T. These hybridization studies also showed that *S. exigua* ATCC 700122^T, *S. heliotrinireducens* ATCC 29202^T and *E. lenta* ATCC 25559^T have very low levels (less than 5%) of DNA relatedness to the other AAGPR species examined in the present study (Table 1).

Johnson (2) has proposed that groups of bacteria with an intragroup DNA homology of 80–90% and an intergroup homology of 60–70% could be considered different subspecies within a species. Also, Johnson has stated that strains showing a homology of 20–60% could be the closely related species in the same genus. Using these criteria with the DNA–DNA hybridization carried out in the present study, *S. exigua* ATCC 700122^T and *S. heliotrinireducens* ATCC 29202^T can be assigned to two different species in the same genus, and *E. lenta* ATCC 25559^T to a different genus. These data are consistent with the results obtained from comparison study of the 16S rDNA sequence similarities.

For the cycle sequence method of 16S rDNA sequence analysis, a Thermo Sequenase Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used with the 11 universal primer sets labeled with Cy-5, following the protocol described previously (6). These sequences of the 16S rDNAs were analyzed with a DNA sequencer (ALFexpress, Amersham Pharmacia Biotech.). The segmented nucleotide sequences of 16S rDNA were connected by using SEQMAN in LASERGENE computer program (DNA Star, Madison, WI). MEGALIGN of LASERGENE program, and CLUSTAL W and NJPLOT programs were used to establish the sequence similarities and to construct

Table 1. Levels of DNA–DNA relatedness

Source of DNA	Homology (%) with labeled DNA*		
	700122	29202	25559
Slackia exigua ATCC 700122^T	100		
Slackia heliotrinireducens ATCC 29202^T	33	100	
Eggerthella lenta ATCC 25559^T	11	10	100
Cryptobacterium curtum ATCC 700683^T	4	5	4
Eubacterium saphenum ATCC 49989^T	1	3	3
Eubacterium nodatum ATCC 33099^T	1	1	1
Eubacterium brachy ATCC 33089^T	1	3	2
Eubacterium minutum ATCC 700079^T	2	1	1
Mogibacterium timidum ATCC 33093^T	2	2	3
Mogibacterium pumilum ATCC 700696^T	2	ND	1
Mogibacterium vescum ATCC 700697^T	1	ND	2
Mogibacterium neglectum ATCC 700924^T	2	ND	2
Mogibacterium diversum ATCC 700923^T	1	ND	1

*All values were normalized to 100% for the homologous reactions.
ND: Not detected.

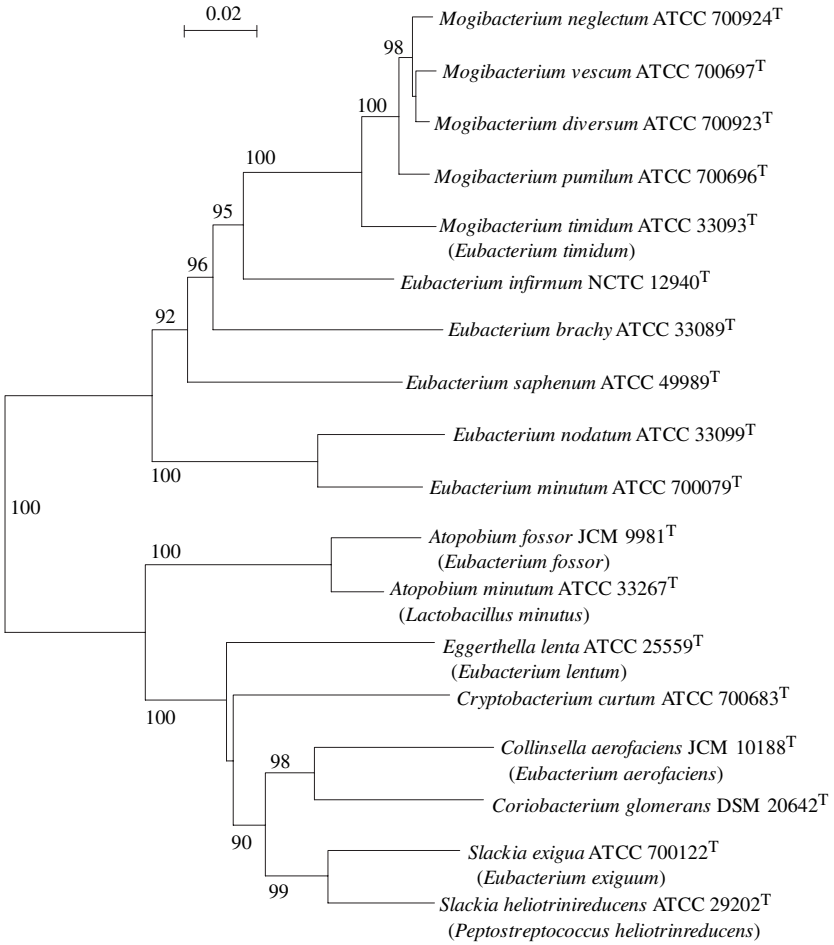

Figure 1. Evolutionary tree based on 16S rRNA gene sequence comparisons, showing the phylogenetic positions of *Slackia exigua*, *Slackia heliotrinireducens* and *Eggerthella lenta*, respectively. The dendrogram was created by using the neighbor-joining method.

an evolutionary tree for the neighbor-joining method. Confidence values were also assessed by CLUSTAL W with bootstrap analysis.

The sequences of *S. exigua* ATCC 700122^T, *S. heliotrinireducens* ATCC 29202^T and *E. lenta* ATCC 25559^T were compared with those of AAGPR species, including *C. curtum, E. brachy, E. minutum, E. nodatum, E. infirmum, Eubacterium saphenum, M. diversum, M. neglectum, M. pumilum, Mogibacterium timidum, M. vescum*, and other related gram-positive bacteria selected from the GenBank database by the sequence similarity search using the BLAST algorithms. The phylogenetic interrelationships of these bacterial species included in the study are shown in Fig. 1. According to the evolutionary tree, *S. exigua* and *S. heliotrinireducens* could be clearly distinguished from any species in the same cluster. Their closest phylogenetic neighbors were the genus *Collinsella* or/and the genus *Coriobacterium*. *E. lenta* form a distinct branch exhibiting a specific phylogenetic position in the evolutionary tree, and the 16S rDNA sequence is most similar to that of previously established *C. curtum*.

Wade et al. (9) reported a 94.7% level of 16S rDNA sequence similarity between *S. exigua* and *S. heliotrinireducens*. Based on this sequence similarity data, the new genus *Slackia* was created for these bacterial strains. *E. lenta* is also reported to have 89.9% and 90.8% 16S rDNA sequence similarities with *S. exigua* and *S. heliotrinireducens* (9), respectively. Our present data agree with these previous reports. It is also noteworthy that all *Eubacterium* species examined in this study showed no close phylogenetic affinity with *E. limosum* which is the type species of genus *Eubacterium* (data not shown). These results may indicate that the genus *Eubacterium* is incoherent and that these oral asaccharolytic *Eubacterium* species need reassignment to different genera.

Many bacterial species of AAGPR, including *S. exigua, S. heliotrinireducens* and *E. lenta,* are difficult to culture and identify because they are inert in most conventional biochemical tests (5, 7) and some AAGPR species have been reported to be only distinguishable from other AAGPR species by the sequences of the 16S rDNA and DNA relatedness values (6). Sequence analysis of the *16S rRNA* gene can be used effectively for identification and is extremely useful for the determination of branching orders in evolution, which is difficult to achieve by using DNA–DNA hybridization. But it has been also reported that the reliability of DNA–DNA hybridization analysis may be superior to comparison of the 16S rDNA sequence when used for classification of recently diverged strains, since DNA–DNA hybridization measures the homology in total genomic sequences (13).

In the present paper, on the basis of DNA–DNA relatedness data together with evolutionary data obtained from the comparisons of the 16S rDNA sequences, we provide further support for the reclassification of *E. exiguum, Peptostreptococcus heliotrinreducens* and *E. lentum* as *S. exigua*, *S. heliotrinireducens* and *E. lenta*, respectively. The DNA–DNA relatedness data are also very useful for identification of the AAGPR species from human clinical specimens.

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