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Characterisation of *Eubacterium*-like strains isolated from oral infections

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The genus *Eubacterium* currently includes a heterogeneous group of gram-positive, non-spore-forming anaerobic bacilli, many of which are slow growing, fastidious and generally unreactive in biochemical tests. As a consequence, cultivation and identification of isolates are difficult and the taxonomy of the group remains indifferent. In this study, 105 isolates from odontogenic infections, infections associated with dental implants or saliva from healthy subjects and provisionally assigned to the genus *Eubacterium* were subjected to phenotypic and genotypic analysis. Ninety-one of the isolates were identified as belonging to one of 14 previously described species: *Atopobium parvulum* (5 isolates), *A. rimae* (29), *Bulleidia extructa* (2), *Cryptobacterium curtum* (1), *Dialister pneumosintes* (1), *Eubacterium saburreum* (2), *E. sulci* (8), *E. yurii* subsp. *yurii* (1), *Filifactor alocis* (3), *Lactobacillus uli* (1), *Mogibacterium timidum* (13), *M. vescum* (6), *Pseudoramibacter alactolyticus* (6) and *Slackia exigua* (13). The remaining 14 isolates did not correspond to existing species. This study confirms the diversity of organisms provisionally assigned to the genus *Eubacterium* by conventional identification methods. This group of organisms is frequently isolated from oral infections but their role in the aetiology of these conditions has yet to be determined.

Introduction

Gram-positive, non-spore-forming, anaerobic bacilli are frequently isolated from oral infections such as periodontitis [1, 2] and dento-alveolar infections [3]. At present, many of these isolates are assigned to the genus *Eubacterium*, which comprises a heterogeneous collection of species with DNA G + C contents ranging from 30 to 55 mol % and has largely been defined by default [4]. Because many of these strains are slow growing and generally unreactive in conventional biochemical tests, it has proved difficult to identify them positively. Recent taxonomic studies of this group have led to the proposal of a number of new genera to accommodate species that were originally placed in the genus *Eubacterium* but are distinct at the genus level from the type species *E. limosum*. These include *Collinsella aerofaciens* [5], *Mogibacterium timidum*

[6], *Pseudoramibacter alactolyticus* [7], *Slackia exigua* and *Eggerthella lenta* [8]. Novel species have also been proposed either within the genus *Eubacterium* or in related or novel genera, both in the low G + C gram-positive and Actinobacteria divisions, such as *E. minutum* [9], *Cryptobacterium curtum* [10], *M. vescum* [6] and *Bulleidia extructa* [11].

The aim of this study was to identify definitively 105 isolates from oral infections, provisionally assigned to the genus *Eubacterium*, by means of a comprehensive range of phenotypic and genotypic analyses.

Materials and methods

A total of 105 isolates of anaerobic, gram-positive non-spore-forming rods from the oral cavity, provisionally identified as belonging to the genus *Eubacterium* [12], were included in the study. Isolates were from odontogenic infections (84 isolates), infections associated with dental implants (13) and saliva from healthy subjects (8) and were isolated at the National Public Health Institute in Helsinki, Finland.

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Isolates were grown at 37°C on Fastidious Anaerobe Agar (FAA, LabM) supplemented with horse blood 5% under anaerobic conditions (N₂ 80%, H₂ 10%, CO₂ 10%). Colonial morphologies were determined after incubation for 7 days by means of a plate microscope. Cell morphologies were recorded after Gram's staining of 3-day-old cultures.

Fermentation tests were performed with pre-reduced, anaerobically sterilised (PRAS) sugars according to the methods of Holdeman *et al.* [12] except that the PRAS media were prepared in an anaerobic workstation with pre-reduced distilled water. Other biochemical tests, including indole production, hydrolysis of aesculin and arginine and growth stimulation by Tween 80 0.5% and arginine 0.5%, were performed as described by Holdeman *et al.* [12] and Summanen *et al.* [13].

Bacterial isolates were grown in PYG broth and short-chain volatile and non-volatile fatty acids were extracted by standard methods [12] and analysed by gas chromatography with a capillary column coated with CP-Wax 58 solid phase.

Enzyme profiles were generated with the Rapid ID 32A identification system for anaerobes (bioMérieux) according to the manufacturer's instructions except where indicated in the text. Bacteria were harvested from blood agar plates (Blood Agar Base No. 2, LabM, with horse blood 5%) incubated anaerobically at 37°C for 72 h.

Protein profiles of whole-cell proteins were generated by SDS-PAGE with 10–15% gradient gels and the PhastSystem (Pharmacia) as described previously [14] except that the cells were pre-treated with lysozyme 50 µg/ml for 2 h at 37°C and vortex mixed for 2 min before boiling.

DNA was isolated from the bacteria by a method optimised for gram-positive bacteria [15]. The 16S rRNA genes were amplified by PCR with primers 27F and 1525R [16] in a Uno II Thermocycler (Biometra, Maidstone, Kent) with PCR Buffer (Bioline, London) containing 1.5 mM MgCl₂, 200 µM dNTPs, 1 mM of each oligonucleotide primer, 1 U *Taq* DNA polymerase (Bioline) and template DNA in a total volume of 100 µl. Thirty amplification cycles were performed with a denaturing temperature of 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 2 min. PCR products were sequenced directly with the CEQTM 2000 Dye Terminator Cycle Sequencing Kit (Beckman Coulter) and 60 ng of template DNA, according to the manufacturer's instructions. Sequencing was performed with an automated sequencer (CEQTM 2000 DNA Analysis System, Beckman Coulter) with primers 27F, 342R, 357F, 519R, 907R, 926F, 1100R, 1114F, 1392R and 1525R [16].

Sequences were connected by using DNASIS (Hitachi)

and then submitted to the Ribosomal Database Project II (RDP) [17] *via* the world-wide web for provisional identification in the Sequence_Match programme. From the phylogenetic position indicated by Sequence_Match, related sequences were selected and aligned with the type strains of the species by means of CLUSTAL W [18].

Results and discussion

The results of the identification process are shown in Table 1 along with differential phenotypic characteristics of the species. The isolates were provisionally grouped on the basis of colony and gram-strain morphologies, fermentation of glucose, end-products of fermentation in PYG broth and enzyme profiles obtained with the Rapid ID 32A identification system. Partial 16S rRNA gene sequence analysis was performed on 72 of the 105 isolates. All isolates of *Atopobium parvulum*, *A. rimae* and *Lactobacillus uli* and all strains of *M. timidum* and *M. vesicum* were sequenced as they were difficult to differentiate by phenotypic characteristics. Twenty isolates representative of the remaining 11 groups were sequenced to confirm identifications.

Ninety-one isolates were identified as belonging to one of 14 previously described species. A further 13 isolates constituted a homogeneous group which appeared to represent a novel species. The homogeneity of the group was confirmed by protein profile analysis performed on 9 of the 13 isolates. Phylogenetic analysis of the sequence data obtained for 8 of the 13 isolates assigned this group to the clostridial subphylum within the low G + C gram-positive division. The closest match indicated by the Sequence_Match programme of the RDP was *B. extructa* [11] with a S_{ab} value of 0.74. Another single isolate (AHS 5884) also appeared to represent an as yet undescribed species. It was most closely related to *M. timidum* with a S_{ab} value of 0.66. Further work is necessary on these 14 unidentified isolates to clarify their phylogenetic position and to fully define their phenotypic characteristics.

Thirty-four isolates were identified as belonging to the genus *Atopobium*. This genus within the Actinobacteria was created to include the former species *Streptococcus parvulus*, *L. rimae* and *L. minutus* as *A. parvulum*, *A. rimae* and *A. minutum* [19]. *A. parvulum* and *A. rimae* have been isolated from human gingival crevices and periodontal pockets [20]. Since their description, reports of their isolation have been rare, but here they were the most frequent genus found. The species are phenotypically very similar, with growth in PYG broth enhanced by the addition of Tween 80, and acetate, major amounts of lactate and occasionally trace amounts of succinate produced as end-products of glucose fermentation. Olsen *et al.* [20] compared the

Table 1. Identification and differential characteristics of 105 oral isolates provisionally identified as *Eubacterium* spp.

Identification (number of strains)	Glucose fermentation	Metabolic end- products*	Tween 80 stimulation	Arginine stimulation	Indole production	Aesculin hydrolysis	Arginine hydrolysis	Rapid ID 32A profile									
<i>Atopobium parvulum</i> (5)	+	a,L,(s)	+	-	-	v	-	0	1/5 ^v	0	2	0	1/5	3	7	0	5
<i>Atopobium rimae</i> (29)	+	a,L,(s)	+	-	-	v	v	2/0	0/4	0/1	0/1/3	0	1/5/0	7/6	7/1/3	0	5
<i>Bulleidia extructa</i> (2)	+	a,L,(s)	+	-	-	-	+	2	0	0	0	0	1	2	0	0	0
<i>Cryptobacterium curtum</i> (1)	-	none	-	+	-	-	+	2	0	0	0	0	1	2	4	0	0
<i>Dialister pneumosintes</i> (1)	-	(a)	-	-	-	-	-	0	0	0	0	0	1	0	0	0	0
<i>Eubacterium saburreum</i> (2)	+	A,b,l	-	-	+	+	-	5	5	1	0/5	2	0	4	0	0/2	0
<i>Eubacterium sulci</i> (8)	-	(a),B	-	-	-	-	-	0	0	0	0	0	4	0	0	0	0
<i>Eubacterium yurii</i> subsp. <i>yurii</i> (1)	w	a,B	-	- ^w	+	-	-	0	0	0	0	2	1	0	0	0	0
<i>Filifactor alocis</i> (3)	-	a,B	-	-	-	-	+	0/2	0	0	0	0	1	2/0	0	0	0
<i>Lactobacillus uli</i> (1)	+	a,L,(s)	+	-	-	+	+	2	0	0	2	0	1	2	7	0	5
<i>Mogibacterium timidum</i> (13)	-	(a),PAA	-	-	-	-	-	0	0	0	0	0	2	0	0	0	0
<i>Mogibacterium vescum</i> (6)	-	(a),PAA	-	-	-	-	-	0	0	0	0	0	2	0	0	0	0
<i>Pseudoramibacter alactolyticus</i> (6)	+/w	a,B,C	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0
<i>Slackia exigua</i> (13)	-	none	-	+	-	-	+	2	0	0	0	0	2/3/7	3	7	0	5
Unidentified group (13)	+	a,L,(s)	+	-	-	v	+	6	5	0/1	0	0	1/3	2	0	0	0/1
Unidentified isolate AHS 5884	-	paa	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0

v, variable reactions; w, weak reaction.

*a, acetic acid; p, propionic acid; b, butyric acid; c, caproic acid; l, lactic acid; s, succinic acid; paa, phenyl acetic acid. Capital letters indicate major products; parentheses indicate trace amount or variable production.

sugar fermentation reactions of *A. parvulum* and *A. rimae*. The only significant difference was that all of 82 isolates of *A. parvulum* fermented lactose whereas only two of 67 isolates of *A. rimae* tested were positive. The present study also found differences in the enzyme profiles obtained with the Rapid ID 32A identification system (Table 1), although the enzyme profiles could be quite variable. One isolate of *L. uli* was found in this study and was identified by 16S rRNA gene sequence analysis. *L. uli* is phenotypically similar to *A. parvulum* and *A. rimae*. Olsen *et al.* [20] reported that all 48 strains of *L. uli* tested were negative for trehalose fermentation in PRAS media without added serum, whereas all 82 strains of *A. parvulum* and 66 of 67 strains of *A. rimae* were positive. Furthermore, these workers isolated *L. uli* from human gingival crevices, especially from patients with periodontitis.

B. extructa is a recently described species of anaerobic gram-positive bacilli that was isolated from human periodontal pockets and a dento-alveolar abscess [3]. Analysis of 16S rRNA gene sequences of the isolates showed that they constituted a novel branch of the low G + C gram-positive division of the phylogenetic tree most closely related to *Holdemania filiformis* and *Erysipelothrix rhusiopathiae*. The two isolates identified in this study had the same phenotypic characteristics and protein profiles as the four strains described previously [11], with growth in broth enhanced by the addition of fermentable sugars and Tween 80, and with acetate, lactate and trace amounts of succinate as end-products of fermentation. The Rapid ID 32A enzyme profile along with protein profiles distinguished *B. extructa* from other species with similar phenotypic characteristics.

C. curtum is another recently described gram-positive anaerobic rod isolated from a periodontal pocket of an adult patient with periodontal disease and from a necrotic dental pulp [10]. The organism is asaccharolytic and unreactive in most of the conventional biochemical tests. No short-chain fatty acids are detected as end-products of glucose metabolism. In this study, the single isolate of *C. curtum* was from a patient with an odontogenic infection. Moore [21] summarised findings from a number of studies investigating the predominant microflora in periodontitis and identified several novel *Eubacterium* taxa. One of these groups, *Eubacterium* D40, is phenotypically identical to *C. curtum*. We have examined six strains of D40 and found them to have similar characteristics to the two strains studied by Nakazawa *et al.* [10] and to share >98% 16S rRNA gene sequence similarity with the type strain of *C. curtum* (unpublished data). Furthermore, we found that growth in PYG broth could be enhanced by arginine 0.5% and there was no growth in bile 20%. In the Rapid ID 32A identification system, the isolate identified in this study and the six D40 strains were all positive for arginine dihydrolase and arginine arylamidase, six of the seven

strains were positive for leucine arylamidase, five were positive for glycine arylamidase and one was positive for leucyl glycine arylamidase.

The species *Fusobacterium sulci* and *F. alocis* have recently been re-named as *E. sulci* and *Filifactor alocis* on the basis of 16S rRNA gene sequence analysis which showed that neither species belonged to the genus *Fusobacterium* [22]. Both these species were found to be more prevalent in the subgingival flora than in the supragingival flora and in diseased than in healthy sites [23]. The three isolates of *F. alocis* in this study and five isolates of *E. sulci* were recovered from odontogenic infections. One *E. sulci* isolate was from a patient with peri-implantitis and two were from saliva of healthy subjects. Both species could be distinguished from each other and other species in this study by their enzyme profiles and end-products of fermentation in PYG broth (Table 1).

The genus *Mogibacterium* was recently proposed to include *E. timidum* and two novel species, *M. vescum* and *M. pumilum*, all of which have been isolated from human periodontal pockets [6]. Thirteen isolates from this study were identified as *M. timidum*, 11 of which were isolated from odontogenic infections and two from peri-implantitis. 16S rRNA gene sequence analysis of a further six strains revealed that they had sequence similarity values over 454 unambiguously aligned bases ranging from 95.2% to 95.6% when compared to the type strain of *M. timidum*, but 99.3–99.6% sequence similarity with the *M. vescum* type strain, and hence were identified as *M. vescum*. Biochemically, there is little to distinguish between *M. timidum* and *M. vescum* as both are asaccharolytic, unreactive in conventional tests and produce phenyl acetate as an end-product of fermentation in PYG broth. In this study, all 19 isolates of *Mogibacterium* gave a positive reaction for the enzyme proline arylamidase only in the Rapid ID 32A identification system when it was incubated in an aerobic atmosphere, as recommended by the manufacturer. However, if incubated under anaerobic conditions, in addition to the proline arylamidase activity the 13 isolates of *M. timidum* also showed pyroglutamate arylamidase activity, whereas the 6 isolates of *M. vescum* did not. Protein profiles were also performed on 5 of the 13 isolates of *M. timidum* and the 6 isolates of *M. vescum* in this study. As previously reported [6], both species could be differentiated on whole-cell protein profiles. Differences in colony morphology were also observed by plate microscopy after incubation for 7 days on FAA; *M. timidum* colonies were circular, entire, convex and translucent with colony diameters of 0.3–0.5 mm, compared with *M. vescum* colonies which were circular, entire, umbonate with an opaque, off-white centre and colony diameters of 0.6–0.8 mm.

E. alactolyticus was reclassified as *Pseudoramibacter*

alactolyticus following 16S rRNA gene sequence analysis which showed only 91% sequence similarity between the type strains of *E. alactolyticum* and *E. limosum*, the type species for the genus *Eubacterium* [7]. *P. alactolyticus* has been isolated from root canals and from dental calculus and gingival crevices of patients with periodontal disease [4]. The six isolates in this study were from odontogenic infections. *P. alactolyticus* can be distinguished from *Eubacterium* and other *Eubacterium*-like species by the lack of enzymic activity in the Rapid ID 32A system and the formation of caproic acid as an end-product of fermentation in PYG broth (Table 1).

The genus *Slackia* was recently proposed to include the former *E. exiguum* and *Peptostreptococcus heliotrinreducens* as *S. exigua* and *S. heliotrinireducens*, with 94.7% 16S rRNA gene sequence similarity between the type strains of the two species [24]. Phylogenetic analysis placed both species within the family Coriobacteriaceae in the Actinobacteria (high G + C) division with the G + C content of their DNA ranging from 60 to 64 mol %. *S. exigua* has been isolated from human necrotic pulp samples, periapical infections and acute dento-alveolar abscesses [24]. The 13 isolates of *S. exigua* in this study were all from odontogenic infections. Protein profiles performed on 5 of the 13 strains confirmed the homogeneity of the group.

Two isolates of *E. saburreum* and one of *E. yurii* subsp. *yurii* were identified in this study. Both species are indole positive but could be differentiated in this study by aesculin hydrolysis and enzyme profiles (Table 1). The type strain of *E. saburreum* does not produce urease [4], whereas the two strains in this study were urease positive in the Rapid ID 32A system. Protein profiles performed on the two isolates also showed some differences when compared with the type strain of *E. saburreum*. Margaret and Krywolap [25] isolated *E. yurii* from periodontal pockets and found that it made up c. 1% of the cultivable flora of their subgingival plaque samples. Despite this, the isolation of *E. yurii* is rarely reported in the literature. Interestingly, they also reported that *E. yurii* produced propionate as an end-product of fermentation. The isolate studied here did not produce propionate despite good growth in PYG broth.

It was perhaps surprising that one isolate in this study was identified as *Dialister pneumosintes*, a gram-negative bacillus. However, *Eubacterium* cells may decolorise readily in Gram's staining and appear gram-variable or gram-negative [4]. *D. pneumosintes* also resembles some *Eubacterium* spp. in its morphology and lack of activity in conventional biochemical tests [26]. This has been noted previously in respect of strain SC3D which was originally identified as *Eubacterium* by conventional tests but was found by 16S rRNA gene sequence analysis to belong to *D. pneumosintes* (Nucleotide accession no. Z36297).

This study has emphasised the heterogeneity of the genus *Eubacterium*, with 10 genera being identified from a collection of 105 isolates provisionally assigned to the genus *Eubacterium*. Members of this genus have hitherto been identified as *Eubacterium* by default because they have failed to produce the major metabolic end-products that would have placed them within one of the other genera of gram-positive, non-spore-forming, anaerobic bacilli. However, as a result of the use of recently introduced techniques such as 16S rRNA gene sequencing, many new genera have been proposed to accommodate both novel species and former *Eubacterium* spp. that have been shown to be phylogenetically distinct from the type species *E. limosum*. The clinical importance of *Eubacterium* in oral infections is yet to be established. In the studies that supplied the isolates for this investigation, isolates provisionally identified as *Eubacterium* were isolated from 39% of the pus or tissue samples collected from odontogenic infections, consisting mostly of periapical abscesses with some cases of pericoronitis and infected sockets post-extraction, and in 65% of cases with peri-implantitis. In all cases, these organisms were found as a part of mixed oral microflora. The frequent isolation of species of this group from such infections indicates that they could play an important role. The recent and continuing improvements in the taxonomy of this group will allow consistent identification in different laboratories and greatly aid the elucidation of their precise role in disease.

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