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Bacterial diversity in aphthous ulcers

L. Marchini[1,2,3], M. S. Campos[1], A. M. Silva[1], L. C. Paulino[4], F. G. Nobrega[1,3]
[1]Laboratory of Molecular Genetics and Genomics, University of Vale do Paraíba, São José dos Campos, São Paulo, Brazil, [2]Department of Dentistry, University of Taubaté, Taubaté, São Paulo, Brazil, [3]Microbiology Graduate Program, Biomedical Sciences Institute, University of São Paulo, São Paulo, Brazil, [4]Department of Medicine, New York University School of Medicine, New York, NY, USA

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Introduction: Recurrent aphthous ulcers are common lesions of the oral mucosa of which the etiology is unknown. This study aimed to estimate the bacterial diversity in the lesions and in control mucosa in pooled samples using a culture-independent molecular approach.
Methods: Samples were collected from ten healthy individuals and ten individuals with a clinical history of recurrent aphthous ulcers. After DNA extraction, the 16S ribosomal RNA bacterial gene was amplified by polymerase chain reaction with universal primers; amplicons were cloned, sequenced and matched to the GenBank database.
Results: A total of 535 clones were analyzed, defining 95 bacterial species. We identified 62 putative novel phylotypes. In recurrent aphthous ulcer lesions 57 phylotypes were detected, of which 11 were known species. Control samples had 38 phylotypes, five of which were already known. Only three species or phylotypes were abundant and common to both groups (*Gemella haemolysans*, *Streptococcus mitis* strain 209 and *Streptococcus pneumoniae* R6). One genus was found only in recurrent aphthous ulcer samples (*Prevotella*) corresponding to 16% of all lesion-derived clones.
Conclusion: The microbiota found in recurrent aphthous ulcers and in the control groups diverged markedly and the rich variety of genera found can provide a new starting point for individual qualitative and quantitative analyses of bacteria associated with this oral condition.

Key words: 16S RNA gene sequence; aphthous ulcers; microbial diversity

F.G. Nobrega, Laboratório de Genética Molecular e Genomas, IP UNIVAP, Avenida Shishima Hifumi, 2911, Urbanova, SJ Campos, São Paulo, 12244-000, Brazil
Tel./fax: +55 12 39471119;
e-mail: fgdnobre@univap.br
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Recurrent aphthous ulcers are one of the most common oral mucosal lesions in the general population (6). The incidence of this pathology can affect 50% of the population, depending on the sample evaluated (22). Clinical manifestations include minor (ulcers ≤10 mm in diameter), major (ulcers >10 mm in diameter) and herpetiform (showing multiple small pinpoint ulcers) recurrent aphthous ulcers (27).

Minor recurrent aphthous ulcers are the most prevalent form (80% of all recurrent aphthous ulcers) and their clinical features include round or oval shallow ulcers, with a grayish white pseudomembrane in the center, enveloped by a thin erythematous halo (14). Minor recurrent aphthous ulcers occur on non-keratinized mucosal surfaces and generally appear as a single ulcer, although multiple ulcers have been found in some cases. These ulcers usually heal within 10–14 days without scarring (23). However, they generally cause considerable pain and discomfort, and can interfere with many oral functions such as speaking, eating and swallowing (5).

Regardless of its clinical significance, the primary cause these ulcers remains unknown. Consequently, the treatment is still palliative (14). Recent hypotheses postulate that recurrent aphthous ulcers are a consequence of an autoimmune reaction against oral epithelium. It has been suggested that this autoimmune reaction could be a cross-reaction immune response, activated by heat-shock proteins released by oral bacteria and targeting similar peptides in the oral epithelium (11, 29). However, the micro-organisms present in these lesions have so far only been investigated with culture-based techniques (4), which are known to underestimate bacterial diversity (16).

DNA sequencing was suggested as a powerful tool for a better understanding of the participation of micro-organisms in the etiology of recurrent aphthous ulcers (26). Modern molecular methods that allow organism identification without cultivation would disclose the real diversity of micro-organisms, from pathogenic to commensal bacteria (24). By sequencing the 16S ribosomal RNA gene, the presence of many previously unidentified bacteria was revealed in the gingival sulcus, an exhaustively studied microbial niche (16). Broad-range 16S ribosomal DNA analysis

has been successfully used to study bacterial diversity in many oral conditions, such as periodontitis (9, 13, 20), endodontic infections (25, 28), noma lesions (21), halitosis (15), dental caries (7, 19) and healthy sites (1, 16).

Since this method has contributed to a better understanding of bacterial diversity in all the environmental studies so far undertaken (31), the present study was designed to compare micro-organisms from the oral mucosa of subjects without a history of recurrent aphthous ulcers with those present in ulcers from patients with minor recurrent aphthous ulcers, using pooled samples, to gain initial comparative knowledge of the corresponding microbial populations.

Material and methods

Subject selection

Ten patients with a history of recurrent aphthous ulcers and at least one ulcer over the buccal mucosa at the time of sampling were included in the group of affected subjects. Ten other individuals who had never reported to suffering from recurrent aphthous ulcers were used as healthy controls. No subject in the study was a chronic alcoholic or diabetic. None of them had received antibiotics in the preceding 3 months. The study was previously approved by the institutional review board (CEPSH 22/2003), and informed consents were obtained from all subjects. The control group was composed of seven females and three males, with an average age of 29.3 ± 12.5 years. The subjects with ulcers were five females and five males with an average age of 27 ± 6.3 years. Two patients presented with multiple ulcers but the majority had only one. None of the subjects with ulcers were using medicines to treat ulcers at the time of sampling or before sampling.

Sampling procedures

The samples were collected by swabbing (Catch-All, Epicentre, Madison, WI) over the ulcers, all of which were localized in the buccal mucosa. The lesions were sampled at the time they were fully developed and painful, usually within 3–5 days from the initial symptoms. Just one ulcer was sampled if the subject had multiple lesions, and whenever possible that sample was taken from the lesion closest to the buccal fold. In subjects without recurrent aphthous ulcers, healthy mucosa at the buccal fold was sampled. Swabs were directly immersed in 500 µl QuickExtract DNA extraction solution (Epicentre). The samples were vortexed, incubated at 65°C for 30 min, vortexed again and incubated at 98°C for 16 min with occasional mixing.

Table 1. Bacteria identified in recurrent aphthous ulcer group samples

No.	Blast results	GenBank accession no.	%Id	Base pairs
1	*Actinobacillus pleuropneumoniae* MCCM 00189	AF224283	98	773
1	Bacterium oral clone ASCD05[1]	DQ272508[4]		1560
14	*Gemella haemolysans*[3]	L14326	99	807
2	*Gemella morbillorum*	L14327	98	487
3	*Granulicatella elegans*	AF016390	98	799
2	*Granulicatella* sp. oral clone ASCA05[1]	DQ341469[4]		1621
1	*Granulicatella* sp. oral clone ASCB09[1]	AY953251[4]		1560
1	*Granulicatella* sp. oral clone ASCC02[1]	AY923126[4]		1510
1	*Haemophillus* sp. oral clone ASCD02[2]	DQ366687	97	1095
4	*Haemophilus* ASCB07[2]	DQ366688	96	1203
2	*Haemophilus quentini*	AF224307	99	601
2	*Haemophilus segnis*	AF224299	98	821
1	*Haemophilus* sp. oral clone ASCA07[1]	AY923117[4]		1555
2	*Haemophilus* sp. oral clone ASCA10[1]	AY923120[4]		1508
5	*Haemophilus* sp. oral clone ASCB01[1]	DQ272505[4]		1555
3	*Haemophilus* sp. oral clone BJ095[3]	AY005033	99	729
2	*Neisseria* sp. R-22841	AJ786809	99	804
1	*Peptococcus* sp. oral clone MCE10_265 E1	AF481224	99	482
2	*Porphyromonas* sp. oral clone ASCC08[2]	DQ366689	97	1224
1	*Porphyromonas* sp. oral cloneASCG09[2]	DQ366690	95	1098
6	*Prevotella* sp. oral clone ASCB10[2]	DQ366691	96	1269
1	*Prevotella* sp. oral clone ASCD07[1]	AY953252[4]		1530
35	*Prevotella* sp. oral clone ASCG10[1]	AY923148[4]		1537
1	*Prevotella* sp. oral clone ASCG12[1]	DQ272511[4]		1496
1	*Streptococcus anginosus* strain 1012	AF104679	99	452
1	*Streptococcus* sp. oral clone ASCA01[2]	DQ366692	97	1202
1	*Streptococcus* sp. oral clone ASCD03[2]	DQ366693[4]		731
3	*Streptococcus mitis* clone 4C3	AM157420	99	431
35	*Streptococcus mitis* strain 209[3]	AJ295853	99	831
2	*Streptococcus mitis* strain Sm91	AY518677	98	1192
37	*Streptococcus pneumoniae* R6[3]	AE008546	99	803
1	*Streptococcus salivarius*	AF459433	99	1549
1	*Streptococcus* sp. oral clone ASCB02[1]	AY923121[4]		1558
2	*Streptococcus* sp. oral clone ASCA03[1]	DQ272504[4]		1556
1	*Streptococcus* sp. oral clone ASCA04[1]	AY923116[4]		1557
10	*Streptococcus* sp. oral clone ASCA09[1]	AY923119[4]		1559
2	*Streptococcus* sp. oral clone ASCB04[1]	AY923123[4]		1550
1	*Streptococcus* sp. oral clone ASCB06[1]	AY923124[4]		1560
34	*Streptococcus* sp. oral clone ASCB12[1]	AY923125[4]		1558
6	*Streptococcus* sp. oral clone ASCC01[1]	DQ272506[4]		1569
1	*Streptococcus* sp. oral clone ASCC04[1]	AY923127[4]		1556
2	*Streptococcus* sp. oral clone ASCC05[1]	AY923128[4]		1555
1	*Streptococcus* sp. oral clone ASCC12[1]	DQ272507[4]		1564
1	*Streptococcus* sp. oral clone ASCD01[1]	AY923129[4]		1554
1	*Streptococcus* sp. oral clone ASCD09[1]	AY923130[4]		1553
1	*Streptococcus* sp. oral clone ASCD10[1]	DQ272509[4]		1552
3	*Streptococcus* sp. oral clone FX003	AY134901	99	822
1	Swine manure bacterium RT-18A	AY167955	100	815
1	Uncultured bacterium clone ASCG08[2]	DQ366694	95	682
3	Uncultured bacterium clone MP104-1109-b17	DQ088801	98	1130
2	Uncultured bacterium clone rRNA269	AY959042	99	808
1	Uncultured bacterium clone rRNA374	AY959147	98	1246
1	Uncultured bacterium clone ASCA02[2]	DQ366695	97	806
1	Uncultured bacterium oral clone ASCD11[1]	AY923131[4]		1467
6	*Veillonella ratti*	AF186071	99	784
1	*Veillonella* sp. oral clone ASCA08[1]	AY923118[4]		1577
7	*Veillonella* sp. oral clone ASCB03[1]	AY923122[4]		1573

No., number of clones obtained; % Id, per cent identity; Base pairs, number of base pairs sequenced.
[1]Novel phylotypes, [2]partial sequences, [3]phylotypes observed in both groups.
[4]Clones obtained in the present study.

Amplification of 16S ribosomal DNA

Samples from the subjects with recurrent aphthous ulcers were pooled by taking 40 µl from each individual sample. The same was done with the control samples.

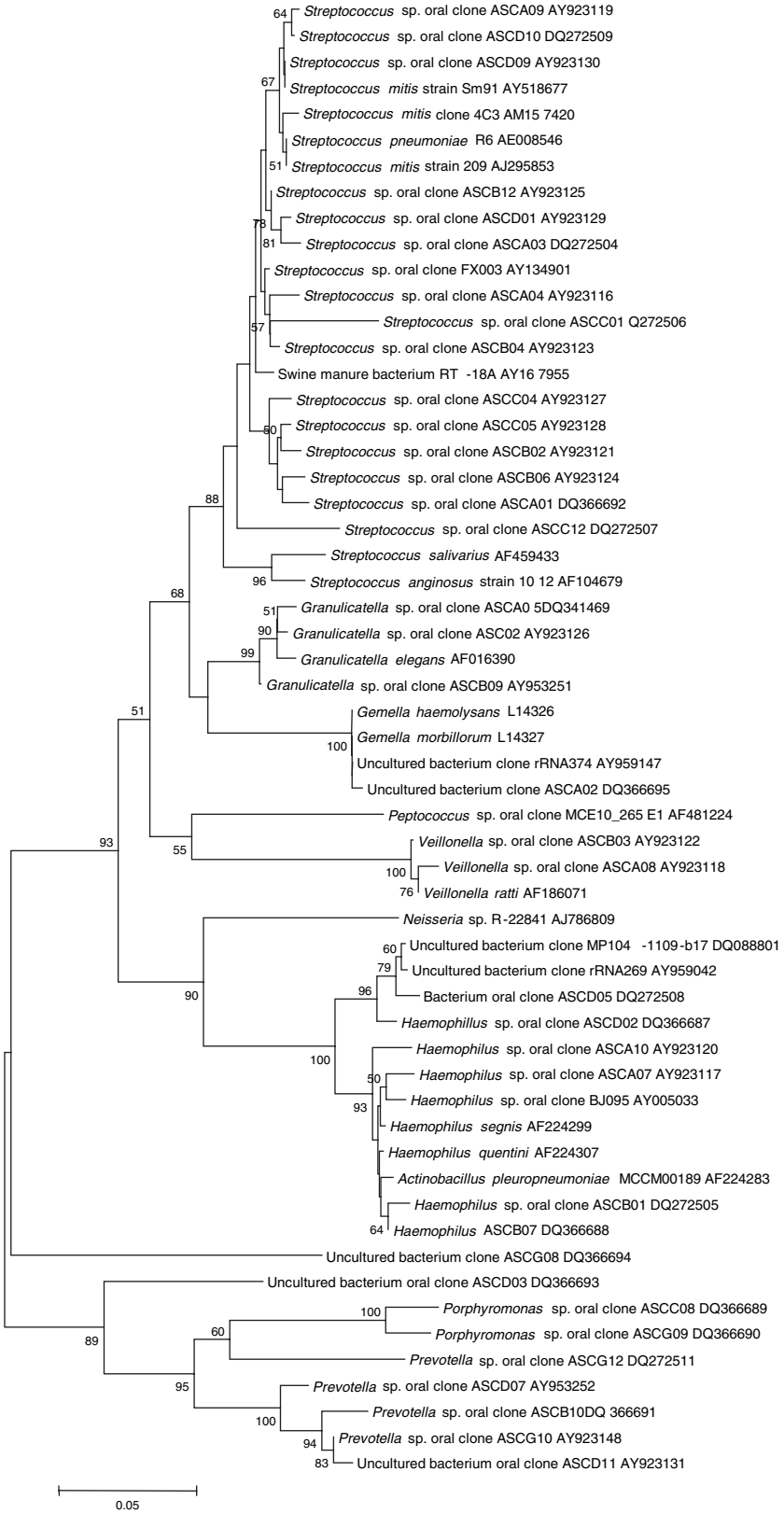

Fig. 1. Neighbor-joining tree based on partial 16S rDNA sequences from the recurrent aphthous ulcer group. The matrix of distances was calculated using the Jukes-Cantor algorithm. Bootstrap values are based on 500 replicates (values ≥ 50% are shown). The code that starts with the letters ASC marks the new phylotypes identified in the project. Final codes correspond to GenBank accession numbers.

Table 2. Bacteria identified in control group samples

No.	Blast results	GenBank accession no.	%Id	Base pairs
3	*Bergeyella* sp. oral clone ASCH01[1]	AY953258[4]		1535
1	*Capnocytophaga* sp. oral clone ASCH04[1]	AY953260[4]		1527
1	*Capnocytophaga* sp. oral clone ASCH05[1]	AY923149[4]		1529
1	*Fusobacterium* sp. oral clone ASCF06[1]	AY923141[4]		1512
1	*Fusobacterium* sp. oral clone ASCF11[1]	AY953256[4]		1558
35	*Gemella haemolysans*[3]	L14326	99	751
4	*Gemella* sp. oral clone ASCE02[1]	AY923133[4]		1569
2	*Gemella* sp. oral clone ASCF04[1]	AY923139[4]		1566
1	*Gemella* sp. oral clone ASCF12[1]	AY923143[4]		1570
1	*Granulicatella* sp. oral clone ASCG05[1]	AY923146[4]		1555
2	*Haemophilus* sp. oral clone ASCG06[1]	AY923147[4]		1560
2	*Haemophilus* sp. oral clone BJ095[3]	AY005033	99	689
1	*Kingella* sp. oral clone ASCH02[2]	DQ366697	96	924
1	*Lactobacillus gasseri* strain ATCC 33323	AF519171	99	588
2	*Porphyromonas* sp. oral clone ASCH03[1]	AY953259[4]		1533
2	*Streptococcus intermedius* strain ATCC27335	AF104671	99	735
32	*Streptococcus mitis* strain 209[3]	AJ295853	99	762
1	*Streptococcus pneumoniae* clone 4V4	AM157442	99	685
56	*Streptococcus pneumoniae* R6[3]	AE008546	99	686
1	*Streptococcus* sp. oral clone ASCE01[1]	AY923132[4]		1549
17	*Streptococcus* sp. oral clone ASCE03[1]	AY923134[4]		1554
2	*Streptococcus* sp. oral clone ASCE04[1]	AY953253[4]		1556
1	*Streptococcus* sp. oral clone ASCE05[1]	DQ272510[4]		1553
5	*Streptococcus* sp. oral clone ASCE06[1]	AY923135[4]		1559
30	*Streptococcus* sp. oral clone ASCE09[1]	AY923136[4]		1548
25	*Streptococcus* sp. oral clone ASCE10[1]	AY923137[4]		1467
1	*Streptococcus* sp. oral clone ASCE12[1]	AY923138[4]		1554
1	*Streptococcus* sp. oral clone ASCF05[1]	AY923140[4]		1548
1	*Streptococcus* sp. oral clone ASCF07[1]	AY953255[4]		1548
1	*Streptococcus* sp. oral clone ASCF09[1]	AY923142[4]		1554
3	*Streptococcus* sp. oral clone ASCG04[1]	AY923145[4]		1549
10	Uncultured bacterium clone K155	AY976601	98	758
1	Uncultured bacterium clone rRNA081	AY958854	98	677
4	Uncultured *Streptococcus* sp.	AY256519	99	694
4	Uncultured *Streptococcus* sp. clone 2.22	DQ016724	99	612
11	Unidentified oral bacterium AP60-55	AB028407	99	548
1	*Veillonella* sp. oral clone ASCG01[1]	AY923144[4]		1571
1	*Veillonella* sp. oral clone ASCG02[1]	AY953257[4]		1551

No., number of clones obtained; % Id, per cent identity; Base pairs, number of base pairs sequenced.
[1]New phylotypes, [2]partial sequences, [3]phylotypes observed in both groups.
[4]Clones obtained in the present study.

The DNA content of each pool was concentrated by ethanol precipitation in the presence of glycogen (4 μg) as carrier, washed with 80% ethanol and suspended in 20 μl QuickExtract DNA extraction solution, followed by polymerase chain reaction amplifications for each pool, using generic primers for bacteria (D88 and E94) and specific primers for Spirochaetes (C90) and Bacteroidetes (F01), as described previously (20). Clones supposed to represent novel phylotypes were sequenced entirely by using primers B34 and F20 also (20). The reaction mixture, in a final volume of 50 μl, contained 20 pmol of each primer, 40 nmol deoxynucleoside triphosphates and 1 U platinum Pfx DNA polymerase (Life Technologies, Grand Island, NY). Five microliters of DNA template solution was added to the mixtures. Negative controls were performed with 5 μl sterile water. The thermal cycling was carried out in a polymerase chain reaction Express thermocycler (Hybaid, Middlesex, UK) and consisted of 30 cycles: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 90 s, with an additional 5 s for each cycle with a final elongation step at 72°C for 10 min. The polymerase chain reaction products were examined by agarose gel (1%) electrophoresis, stained with ethidium bromide and examined under ultraviolet light.

Cloning procedures

The amplicons were cloned using a Zero Blunt Cloning kit (Invitrogen, San Diego, CA), following the manufacturer's instructions. Transformation used One Shot chemically competent *Escherichia coli* cells. The transformed cells were selected on Luria–Bertani agar plates supplemented with 100 μg/ml ampicillin and were incubated overnight at 37°C. The colonies were collected with sterile toothpicks, and placed on 96-well microplates containing Luria-Bertani medium containing 100 μg/ml ampicillin and 8% glycerol and were grown overnight before storage at −80°C.

Plasmid DNA purification

Clones were recovered from frozen storage by inoculation with a 96-pin device onto LA agar plates and subsequent overnight incubation at 37°C. Fresh colonies were transferred to deep 96-well plates containing 750 μl of LA broth and incubated at 37°C for 24 h in a New Brunswick C24 incubator shaker (at 220 r.p.m.). Plasmid DNA purification was carried out by a boiling (microwave) method (18). The purified plasmid DNA was dissolved in 25 μl 10 mM Tris-HCl pH 7.5, 1 mM EDTA solution. The quality of the plasmid DNA purification was ascertained by electrophoresis in 1% agarose gels, after staining with ethidium bromide, and examination under ultraviolet light.

DNA sequencing

Purified plasmid DNA was sequenced using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit version 2.0 (Applied Biosystems, Foster City, CA). The sequencing reactions were performed with 4 μl plasmid DNA, 2 μl Big Dye terminator version 2.0, 2 μl 200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$ solution and 3.2 pmol of the M13 sequencing primer. Additional reactions for complete 16S rDNA sequencing of target clones used B34 and F20 primers, as described by Paster et al. (20). Reactions were performed in a PTC 100 thermocycler (MJ Research, Watertown, MA). The cycle sequencing amplification consisted of an initial denaturation at 96°C for 30 s, and 35 cycles at 96°C for 10 s, annealing at 50°C for 5 s and elongation at 60°C for 4 min. The amplification products were precipitated and dissolved in 10 μl formamide. DNA sequencing was carried out in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Sequences presenting 400 or more nucleotides of good quality, which was ascertained by inspecting the chromatogram, were used to determine the identity or approximate phylogenetic position. Full sequences were sought for clones that were

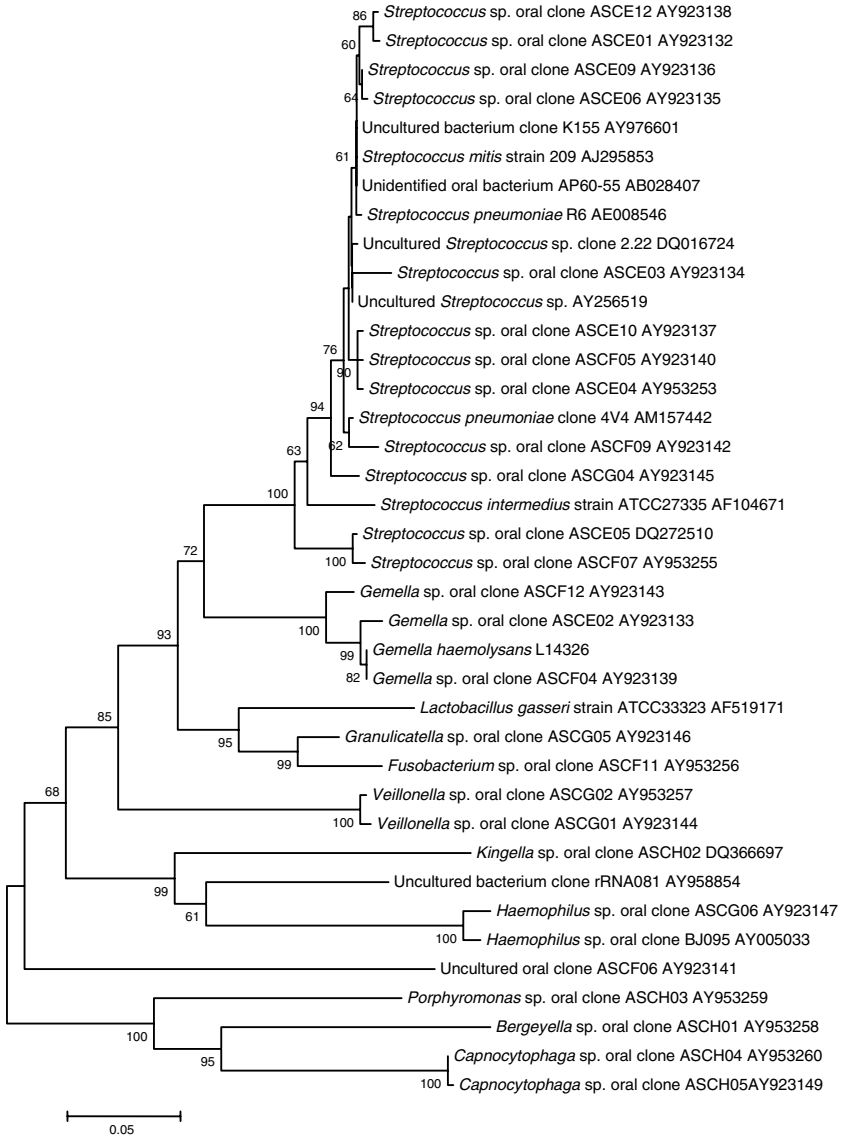

Fig. 2. Neighbor-joining tree based on partial 16S rDNA sequences from the control group. The matrix of distances was calculated using the Jukes-Cantor algorithm. Bootstrap values are based on 500 replicates (values ≥50% are shown). The code that starts with the letters ASC marks the new phylotypes identified in the project. Final codes correspond to GenBank accession numbers.

less than 98% similar to the closest known organisms. The sequences were converted to FASTA format and compared with data available at GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTn algorithm (3). Six chimeric sequences were identified using BELLEROPHON (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) (12) and discarded. The sequences were aligned using CLUSTALW (30) and phylogenetic analysis was performed using MEGA 2 (17). The matrix of distances was calculated using the Jukes-Cantor algorithm, and the neighbor-joining method was utilized to generate phylogenetic trees. Bootstrap resampling was based on 500 replicates.

Nucleotide sequence accession numbers

The DNA sequences of clones representing novel phylotypes were deposited at GenBank under accession numbers AY923116–AY923149, AY953251–AY953260, DQ272504–DQ272511, DQ366687–DQ366698 and DQ341469.

Results

Positive amplification was achieved with universal bacterial primers and Bacteroidetes primers, and 535 good quality sequences were obtained. Two hundred and sixty-six sequences came from patients with recurrent aphthous ulcers (Table 1), resulting in 57 phylotypes. Among them we identified 11 known species (*Actinobacillus pleuropneumoniae*, *Gemella haemolysans*, *Gemella morbillorum*, *Granulicatella elegans*, *Haemophilus quentini*, *Haemophilus segnis*, *Streptococcus anginosus*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus salivarius* and *Veillonella ratti*), represented by 109 clones. The remaining 157 clones fell into 46 different phylotypes, of which 36 (141 clones) were putative novel phylotypes. The phylogenetic tree in Fig. 1 was generated using the sequences from these 57 phylotypes.

The 269 cloned sequences from control subjects (Table 2) identified 38 phylotypes. Five known species (*G. haemolysans*, *Lactobacillus gasseri*, *Streptococcus intermedius*, *S. mitis* and *S. pneumoniae*) were represented by 127 clones. The remaining 142 clones were grouped into 33 phylotypes, of which 26 (85 clones) were potentially novel phylotypes. The phylogenetic tree of the control group is shown in Fig. 2.

There was no amplification using primers specific for Spirochaetes in three independent experiments.

Discussion

DNA-based methods to search for microrganisms have been successfully and widely used to reveal an unexpected microbial diversity in environmental and human samples (13, 20, 24). Among these methods, 16S rDNA sequences are the most widely available data for investigations in bacterial diversity (8).

In the present paper, the numerous phylotypes identified ($n = 95$) add considerably to the very scant information previously available about the microbes associated with recurrent aphthous ulcers (4). Barile et al. (4) presented a mostly morphological description of the microbes present in the lesions and mentioned *Streptococcus* as the most likely genus beside the elusive L form of this microbe. Subsequently, using standard culture techniques, Donatsky et al. (10) found *Streptococcus*, coagulase-negative *Staphylococcus* and *Neisseria* in the lesions We did not find staphylococci in this study.

Only four species or phylotypes (*G. haemolysans*, *Haemophilus* sp. oral clone BJ095, *S. mitis* strain 209 and *S. pneumoniae* R6) were shared by the control and ulcer groups. These phylotypes represented 33.4% ($n = 89$) of recurrent

aphthous ulcer group clones and 46.4% ($n = 125$) of the control clones.

The control group presented a diversity that was comparable to that described previously using 16S rDNA sequencing for the buccal epithelium of healthy subjects (1), except for *Bergeyella*, *Capnocytphaga*, *Fusobacterium*, *Kingella* and *Lactobacillus*, which we detected as low-abundance genera represented by a few clones (nine clones or 3.3%). These genera were absent from the recurrent aphthous ulcer samples.

Prevotella is a genus that consistently appears only in recurrent aphthous ulcer samples and corresponds to 16% of all lesion-derived clones. Further studies with individual samples should be performed to confirm and eventually establish *Prevotella* as a genus with diagnostic value. *Veillonella* sp. is also more abundant in ulcer samples (5.3%) but two clones (0.7%) were identified in healthy mucosa. The ulcer group showed a greater bacterial diversity (57 phylotypes) compared to the control group (38 phylotypes). *Helicobacter pylori*, a bacterium suggested as possibly involved in the etiology of recurrent aphthous ulcers (2), was not detected in this study. It is possible that some organisms found in the ulcers may serve as triggers for the immune cross-reaction that has been hypothesized by some authors (11, 29) as a possible cause of recurrent aphthous ulcers.

Although the microbiota of the ulcer and control groups diverged, the present molecule-based study does not support *per se* a bacterial etiology. Nevertheless, it improves our knowledge of the bacterial community found in the ulcers and consequently tests the hypothesis that relies on bacterial causation (29). The present approach has limitations, i.e. the use of pooled samples. Nevertheless, we reasoned that the sampling of two pools would highlight the common species and the peculiarities between normal mucosa and aphthous ulcers over and above the reported individual variability (1). Considering the issue of site-to-site diversity, our samples originated from the same anatomical region: the non-keratinized mucosa of the buccal fold. The abundance of each species is indirectly and semi-quantitatively estimated from the number of corresponding cloned sequences in the original sample, but its accuracy depends, among other factors, on library size (31). The application of other methods, such as quantitative polymerase chain reaction on individual samples, will further refine and extend our knowledge of the bacterial community that is present in recurrent aphthous ulcers and its possible influence in the etiology of this condition.

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