Oral Microbiology Immunology 2008: 23: 112–118
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Microbiological diversity of generalized aggressive periodontitis by *16S rRNA* clonal analysis

M. Faveri[1,2], M. P. A. Mayer[1], M. Feres[2], L. C. de Figueiredo[2], F. E. Dewhirst[3], B. J. Paster[3]

[1]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [2]Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, SP, Brazil, [3]Department of Molecular Genetics, Forsyth Institute, Boston, MA, USA

Faveri M, Mayer MPA, Feres M, de Figueiredo LC, Dewhirst FE, Paster BJ. Microbiological diversity of generalized aggressive periodontitis by 16S rRNA *clonal analysis.*
Oral Microbiol Immunol 2008: 23: 112–118. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Background/aim: The purpose of this study was to determine the bacterial diversity in the subgingival plaque of subjects with generalized aggressive periodontitis by using culture-independent molecular methods based on 16S ribosomal DNA cloning.
Methods: Samples from 10 subjects with generalized aggressive periodontitis were selected. DNA was extracted and the *16S rRNA* gene was amplified with the universal primer pairs 9F and 1525R. Amplified genes were cloned, sequenced, and identified by comparison with known *16S rRNA* sequences.
Results: One hundred and ten species were identified from 10 subjects and 1007 clones were sequenced. Of these, 70 species were most prevalent. Fifty-seven percent of the clone (40 taxa) sequences represented phylotypes for which no cultivated isolates have been reported. Several species of *Selenomonas* and *Streptococcus* were found at high prevalence and proportion in all subjects. Overall, 50% of the clone libraries were formed by these two genera. *Selenomonas sputigena*, the species most commonly detected, was found in nine of 10 subjects. Other species of *Selenomonas* were often present at high levels, including *S. noxia*, *Selenomonas* sp. EW084, *Selenomonas* sp. EW076, *Selenomonas* FT050, *Selenomonas* sp. P2PA_80, and *Selenomonas* sp. strain GAA14. The classical putative periodontal pathogens, such as, *Aggregatibacter actinomycetemcomitans*, was below the limit of detection and was not detected.
Conclusion: These data suggest that other species, notably species of *Selenomonas*, may be associated with disease in generalized aggressive periodontitis subjects.

Key words: aggressive periodontitis; subgingival microbiota; uncultivable bacteria; 16S ribosomal identification

Marcia P.A. Mayer, Department of Microbiology, Institute of Biomedical Sciences, Av. Lineu Prestes 1374, São Paulo, SP 05508-900, Brazil
Tel.: +55 11 3091 7348;
fax: +55 11 30917354;
e-mail: mpamayer@icb.usp.br
Accepted for publication May 4, 2007

Aggressive periodontitis, previously known as early onset periodontitis (4, 5), is a specific type of periodontitis with clearly identifiable clinical and laboratory findings that are sufficient to differentiate it from other forms of periodontitis (30). Normally it occurs in individuals younger than 30 years and is characterized by a relatively rapid loss of attachment and alveolar bone affecting many teeth in addition to permanent first molars and incisors (30, 43, 52). Furthermore, aggressive periodontitis occurs in otherwise healthy individuals and tends to run in families (5, 30). According to the classification of periodontal disease, aggressive periodontitis can be further characterized by extent and severity. Thus, localized and generalized forms, as well as slight, moderate, and severe forms, are recognized (4, 5, 30, 52). Microbiological criteria were not mentioned in the current classification as primary features separating aggressive periodontitis from other forms of periodontal disease (30).

The role of oral microbiota in the etiology of various inflammatory periodontal diseases has been well established, and specificity may exist between certain bacterial species or groups and the various forms of periodontal disease (2, 18, 23, 25, 34, 35, 47). There have been studies evaluating the microbiota associated with aggressive periodontitis using culture, polymerase

chain reaction, and other methodologies, and available data have implicated *Aggregatibacter actinomycetemcomitans* [formally *Actinobacillus actinomycetemcomitans*, (38)] as the main pathogen of this infection (6, 12, 13, 18, 46, 51, 56, 57), although not in all populations (8, 15, 32, 48, 54). However, after 21 years of age, the relative frequency of detection of *A. actinomycetemcomitans* in cases of aggressive periodontitis seems to decrease, with a concomitant increase in the prevalence and mean counts of other periodontal pathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia, Peptostreptococcus micros, Fusobacterium* species, *Selenomonas sputigena* and spirochaetes (2, 13, 21, 22, 25, 31, 37, 55).

By using molecular methods, over 700 bacterial species have been detected in the oral cavity but only about 50% of oral bacteria have been cultivated (1, 39). This raises the interesting possibility that uncultivated and as-yet-uncharacterized species that have remained undetected may also participate in the etiology of oral diseases, including generalized aggressive periodontitis (GAgP).

The purpose of the present study was to determine the microbial diversity of subgingival plaque samples from periodontally untreated subjects with GAgP using *16S rRNA* clonal analysis.

Material and methods

Subject population

Ten subjects with GAgP were selected from the population referred to the periodontal clinic of Guarulhos University (Guarulhos, SP, Brazil). A complete clinical examination was performed, including medical and dental histories, an intra-oral examination, and a full-mouth periodontal probing. The protocol for all procedures was approved by The Institutional Committee of Ethics in Clinical Research of the Guarulhos University and University of São Paulo. All the subjects signed the committee-approved informed consent.

Inclusion and exclusion criteria

Inclusion criteria were as follows: subjects with GAgP were <30 years old, had >15 natural teeth and exhibited at least six sites with pocket depth and attachment level measurements >5 mm localized in the first molars and/or incisors, and three other sites with the same clinical characteristics in non-first molars/incisors teeth. These sites were located in different teeth.

Exclusion criteria were as follows: previous periodontal therapy, pregnancy, nursing, smoking, any systemic condition that could affect the progression of periodontal disease or that required antibiotic coverage for routine dental therapy, and antibiotic therapy in the previous 12 months.

Clinical examination

The clinical examination was performed by one trained and calibrated examiner according to Araujo et al. (3). Plaque accumulation (0/1), gingival bleeding (0/1), bleeding on probing (0/1), suppuration (0/1), pocket depth (mm) and clinical attachment level (mm) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual) in all teeth, excluding third molar, at baseline visit. Pocket depth and clinical attachment level measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL).

Microbiological examination

Sample collection

Subgingival plaque samples were collected from one site per subject with pocket depth ≥7 mm. An additional periodontitis site and one periodontally healthy site (pocket depth ≤3 mm; bleeding on probing negative) in one subject were also sampled. The selected sites were randomized. After the clinical parameters had been recorded, the supragingival plaque was removed and the samples were taken with individual sterile Gracey curettes and immediately placed in separate polypropylene tubes containing 50 μl TE [10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6].

Bacterial lysis

For bacterial lysis, subgingival plaque samples were directly suspended in 50 μl TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6) and 0.5% Tween-20. Proteinase K (200 μg/ml) (Roche Applied Science, Indianapolis, IN) was added to the mixture. The samples were then incubated at 55°C for 2 h and proteinase K was inactivated by heating at 95°C for 5 min.

Polymerase chain reaction amplification of *A. actinomycetemcomitans 16S rRNA* genes

A. actinomycetemcomitans 16S rRNA genes were amplified under standardized conditions using a species-specific primer set (forward primer, 5′-GTT TAG CCC TGG TGC CCG AAG-3′; reverse primer, 5′-TGA CGG GCG GTG TGT ACA AGG-3′) (33). Primers were synthesized commercially (Invitrogen, San Diego, CA). Polymerase chain reactions (PCR) were performed in thin-walled tubes with a GeneAmp PCR system 9700 (ABI, Foster City, CA). Five microliters of the lysed sample was added to a reaction mixture (final volume, 25 μl) containing 25 pmol of each primer, 40 nmol deoxynucleoside triphosphates, and 2.5 U of platinum *Taq* polymerase (Invitrogen). In a hot-start protocol, the samples were preheated at 95°C for 4 min, followed by amplification under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. A total of 30 cycles were performed; followed by a final elongation step at 72°C for 5 min. *A. actinomycetemcomitans* ATCC 43718 and ATCC 29523 strains were used as positive controls. The results of the PCR amplification were examined by electrophoresis in a 1% agarose gel. The presence of *A. actinomycetemcomitans* was determined by a distinct band of 547 base pairs (bp), which was sequenced.

PCR amplification of universal *16S rRNA*

The *16S rRNA* was amplified under standardized conditions using a modified universal primer set (forward primer 9F: 5′-GAG TTT GAT YMT GGC TCA G-3′; reverse prime 1525R: 5′-GAA GGA GGT GWT CCA DCC-3′) as described by Paster et al. (39). Primers were synthesized commercially (Invitrogen). PCR was performed in thin-walled tubes with a GeneAmp PCR system 9700 (ABI). One microliter of the lysed sample was added to a reaction mixture (final volume, 50 μl) containing 20 pmol each primer, 40 nmol deoxynucleoside triphosphates, and 1 U platinum *Taq* polymerase (Invitrogen). In a hot-start protocol, the samples were preheated at 95°C for 4 min, followed by amplification under the following conditions: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 1.5 min, with an additional 15 s for each cycle. A total of 30 cycles were performed; followed by a final elongation step at 72°C for 15 min. The products of the PCR amplification were examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

Cloning procedures

Cloning of PCR-amplified DNA was performed using the TOPO TA cloning kit (Invitrogen) according to the

manufacturer's instructions. The sizes of the inserts were determined in a PCR using an M13 (–20) forward primer and an M13 reverse primer (Invitrogen). Before sequencing of the fragments, the PCR-amplified *16S rRNA* gene fragments were purified and concentrated according to Paster et al. (39).

16S rRNA sequencing

Quarter dye chemistry (BigDye Terminator Cycle Sequencing Kit with Amplitaq DNA polymerase FS) was used with 80 μM primers and 1.5 μl PCR product in a final volume of 20 μl. Cycle sequencing was performed with a Gene amp PCR system 9700 (ABI) with 25 cycles of denaturation at 96°C for 10 s, annealing at 55° for 5 s, and extension at 60°C for 4 min. The sequencing reactions were run on an ABI 3100 DNA sequencer (ABI).

16S rRNA sequencing and data analysis of unrecognized inserts

A total of 1007 clones with a *16S rRNA* insert of the correct size of approximately 1500 bases were analyzed. The number of *16S rRNA* clones per subject that were sequenced ranged from 71 to 96, with an average of 84 ± 6.2. A sequence of approximately 500 bases was obtained to determine identity or approximate phylogenetic position. For identification of the closest relatives, the sequences of unrecognized inserts were compared with the *16S rRNA* sequences of over 10,000 microorganisms in our database and over 100,000 sequences in the Ribosomal Database Project (11) and GenBank. The similarity matrices were corrected for multiple base changes at a single position by the method of Jukes and Cantor (20). Similarity matrices were constructed from the aligned sequences using those sequence positions for which data were available for 90% of the tested clones. Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (44). TREECON, a software package for Microsoft Windows, was used for the construction and drawing of evolutionary trees (53). Chimeric sequences were identified using the Chimera check program in RDP, by treeing analysis, or by base signature analysis. Identification of chimeras was obtained in four sequences, and these sequences were not examined for phylogenetic analysis.

RESULTS

Clinical data

Demographic characteristics and clinical parameters are presented in Table 1. The mean age of the studied population was 24.1 years, ranging from 20 to 27 years. The full-mouth mean probing pocket depth and clinical attachment levels were 4.67 ± 0.63 and 4.45 ± 0.97 mm, respectively. The percentage of diseased sites was high, with a mean of 59.3% sites showing a probing pocket depth >4 mm per subject. The mean probing pocket depth and clinical attachment level of diseased sites were 8.5 and 8.8 mm, respectively.

Microbiological data

A. actinomycetemcomitans was detected by PCR in seven of the 10 subjects. The amplicons were sequenced and revealed specificity for *16S rRNA* of *A. actinomycetemcomitans*. Samples 1, 4, and 9 were negative for the presence of *A. actinomycetemcomitans* (Fig. 1).

The phylogenetic identity of 1007 *16S rRNA* gene clones was determined by sequencing from 459 to 562 bp in each clone. A level of 98.5% sequence identity was used as the cut-off for identification of a specific taxon. Overall, 110 bacterial taxa/species were detected; 70 of the most prominent taxa are shown in Fig. 1. Taxa that were found only once in a single subject were excluded from the phylogenetic analysis. Forty of these 70 more prevalent taxa (57%) have not yet been cultivated and were designated as 'clones'. The taxa detected fell into four bacterial phyla, namely, the *Firmicutes* (including *Streptococcus*, *Eubacterium*, *Peptostreptococcus*, *Selenomonas* and related genera), the *Spirochaetes* (including *Treponema*) the *Actinobacteria* (including *Actimomyces* and related genera) and the *Bacteroidetes* (including genera *Porphyromonas* and *Capnocytophaga*). *A. actinomycetemcomitans* was not detected in any of the samples by clonal analysis.

Differences in bacterial profiles for each subject are also presented in Fig. 1. Species of *Selenomonas* and *Streptococcus* were found in high prevalence and proportion in all subjects. Overall, 50% of the clone libraries consisted of these two genera. *S. sputigena*, the species most commonly detected, was found in nine of the 10 subjects. In three of these subjects, *S. sputigena* was detected at high levels, comprising 18–30% of the total. Other species of *Selenomonas* were often present in high levels, including *S. noxia*, *Selenomonas* sp. EW084, *Selenomonas* sp. EW076, *Selenomonas* FT050, *Selenomonas* sp. P2PA_80, and *Selenomonas* sp. strain GAA14. *Streptococcus anginosus*, *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus* sp. FX003 and *Streptococcus cristatus* were found in high levels and proportions in four different subjects. Other predominant bacterial species detected were *Dialister invisus*, *Anaeroglobus geminatus* (formally *Megasphaera* sp. BB166), *Veillonella* sp. AA050, *Gemella morbillorum*, *Gemella* sp. strain 933-88, and *Capnocytophaga granulosa.*

To analyze the variability among sites in a single subject, two sites with pocket depth ≥7 mm and one site with pocket depth ≤3 mm were sampled. A total of 233 clones were analyzed in these three samples. Figure 2 shows the distribution and prevalence of bacterial phyla in the two disease sites and in one healthy site in the same patient. Twenty-one different species were found in the healthy site and 43 species were found in both diseased sites. *Veillonella parvula* was found in higher proportion in the healthy site and *Selenomonas* sp. EW076, *Selenomonas* sp. GAA14 and *Eubacterium saphenum* were found in higher proportions in the diseased sites. Only *Selenomonas* sp. P2PA_80 and *Streptococcus mitis* were found in the three samples in the same subject.

Table 1. Periodontal variables of generalized aggressive periodontitis subjects

Variables	Mean values ± SD
Age (range)	24.1 ± 3.10 (20–27)
Gender (female/male)	8/2
Plaque accumulation (%; full mouth)	44.3 ± 12.1
Gingival bleeding (%; full mouth)	8.8 ± 6.3
Bleeding on probing (%; full mouth)	71.3 ± 25.4
Suppuration (%; full mouth)	5.41 ± 2.21
Mean probing pocket depth (mm; full mouth)	4.67 ± 0.63
Mean clinical attachment level (mm; full mouth)	4.45 ± 0.97
Mean number of sites with probing depth ≤3 mm	40.7 ± 8.05
Mean number of sites with probing depth 4–6 mm	39.5 ± 5.12
Mean number of sites with probing depth ≥7 mm	19.8 ± 3.23
Mean probing pocket (mm; sample sites)	8.5 ± 1.5
Mean clinical attachment level (mm; sample sites)	8.8 ± 1.4

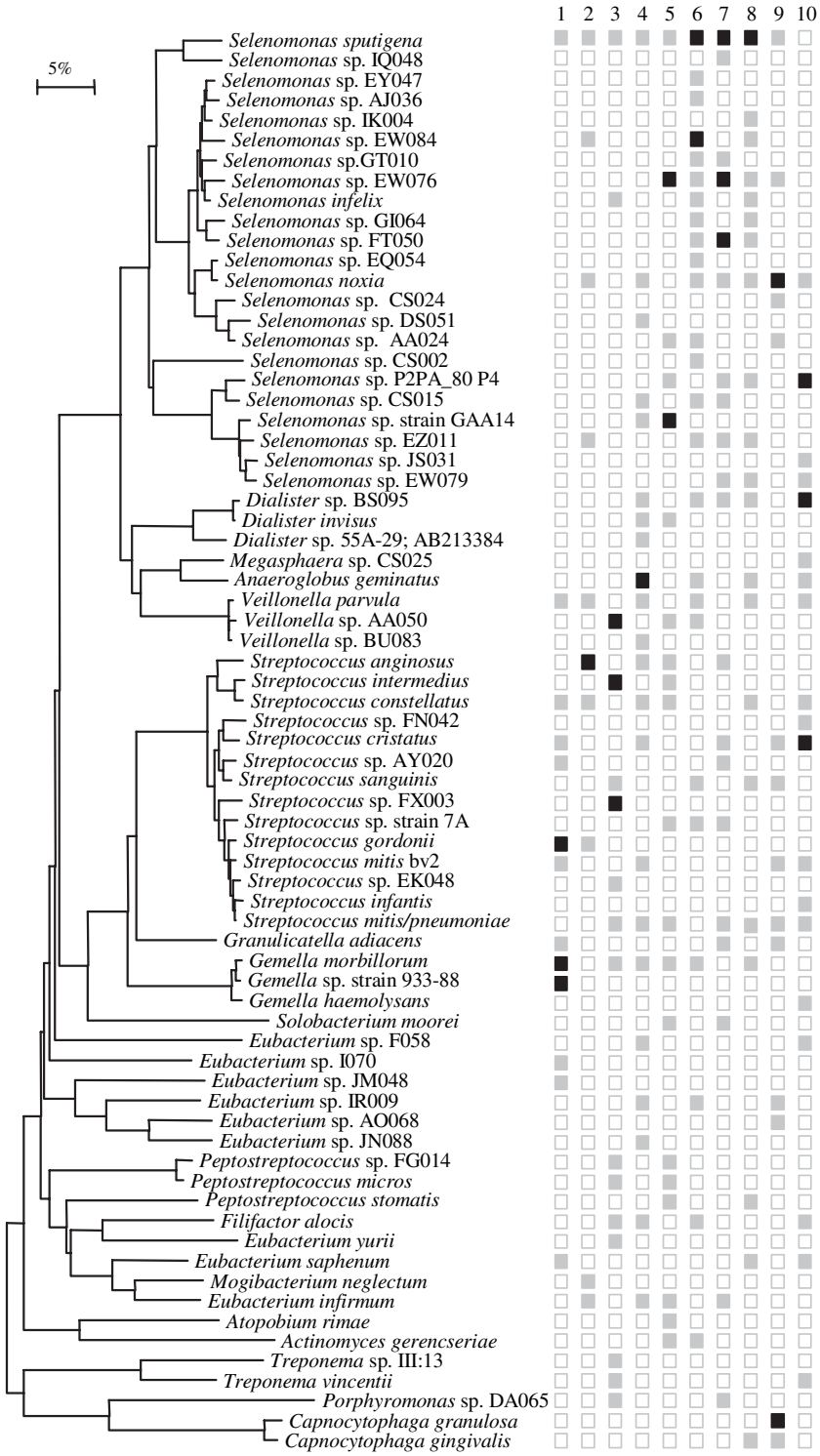

Fig. 1. Phylogenetic tree of bacterial phylotypes detected in generalized aggressive periodontitis subjects. Each column of boxes represents the bacterial profile/subject. Grey-shaded boxes indicate presence of species detected at <10% of the total number of clones analyzed. Black-shaded boxes indicate presence of species detected at >10% of the total number of clones analyzed. Clear boxes indicate that species were not detected (below the limit of detection). The marker bar represents a 5% difference in nucleotide sequence.

Discussion

The main objective of this study was to characterize the microbial diversity in a homogeneous group of young Brazilian adults who had been diagnosed with GAgP. Regarding the clinical characteristics, the subjects with GAgP showed advanced disease, and 59.3% of the sites had pocket depths >4 mm. Similar figures have been reported by other authors for GAgP (23, 37, 55).

Previous studies that used the *16S rRNA* cloning and sequencing strategy have shown that there are more than 700 species

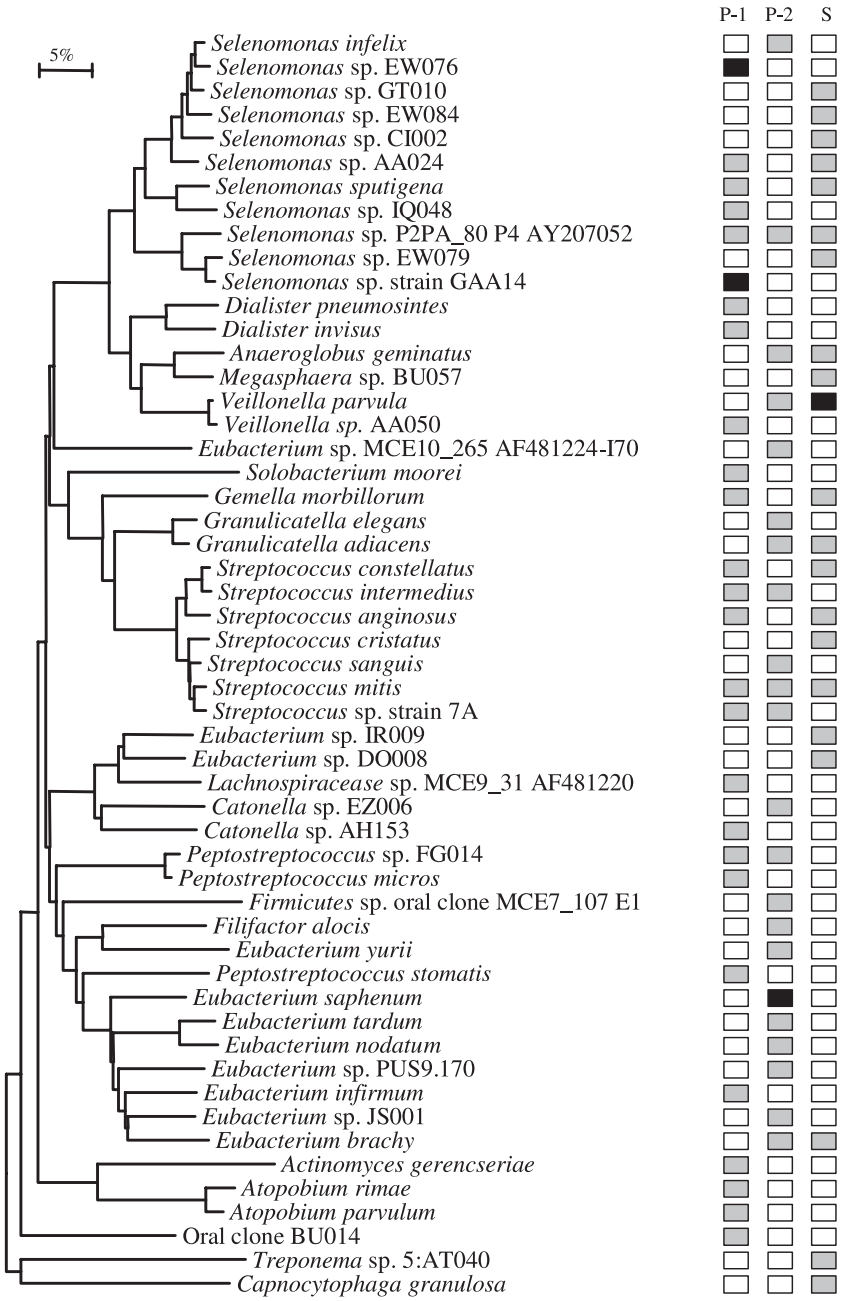

Fig. 2. Phylogenetic tree of bacterial phylotypes detected in two disease sites (D-1 and D-2) and one healthy site (S) in the same subject with generalized aggressive periodontitis. Each column of boxes represents the bacterial profile/subject. Grey-shaded boxes indicate presence of species detected at <10% of the total number of clones analyzed. Black-shaded boxes indicate presence of species detected at >10% of the total number of clones analyzed. Clear boxes indicate that species were not detected (below the limit of detection). The marker bar represents a 5% difference in nucleotide sequence.

in the oral cavity, with 400 species in the subgingival crevice alone (1, 28, 29, 39, 40). To our knowledge, the present study represents the first report using these molecular techniques to describe the bacterial diversity in subjects with GAgP. One hundred and ten bacterial taxa were detected, and the 70 more prevalent species/taxa are shown (Fig. 1). Of the 70 clones, 57% of sequences represented phylotypes for which no cultivated isolates have been reported. This represents a higher ratio of as-yet-uncultivated to unrecognized bacterial species than the approximately 40% previously shown in other oral infections and in healthy subjects (1, 26, 29, 39, 41, 45). A similar study analyzing subjects with aggressive periodontitis from an older population reported a more diverse microbial population, and detected known periodontal pathogenic organisms, such as *P. gingivalis* and *Treponema socranskii* subsp *buccale*. Differences in the primer pairs used between both studies, as well as differences in the studied populations, may have accounted for the differences in the results (19).

It was noteworthy that species of *Selenomonas* dominated the diseased sites of subjects with GAgP. *S. sputigena* was the most frequently detected bacterial species, present in nine of the 10 subjects, often at high levels of about 20% of the total

bacterial population. This gram-negative, multiflagellated, motile, anaerobic rod (27) has been previously associated with necrotizing ulcerative periodontitis (16), rapidly progressive periodontitis (24), and active periodontitis lesions (17, 49). Other predominant *Selenomonas* species were *Selenomonas* sp. oral clone EW084, *Selenomonas* sp. oral clone EW076, *Selenomonas* sp. oral clone FT050, *Selenomonas* sp. strain GAA14, *Selenomonas* sp. oral clone P2PA_80, and *Selenomonas noxia*. All of these have been previously associated with oral infections (14, 29, 39, 41). The heavy colonization of *Selenomonas* species observed in GAgP subjects was somehow unexpected; however, some reports indicate that an increased number of motile bacteria have been observed in active periodontal disease sites (36, 49). Therefore, the high prevalence and proportion of species of *Selenomonas* suggest a role for these species in the etiology of aggressive periodontitis.

Three other species found in high levels were *Anaeroglobus geminatus*, *Dialister invisus*, and *C. granulosa*. Both *A. geminatus* and *D. invisus* have been previously found in higher numbers in the subgingival plaque of subjects with periodontitis compared with healthy subjects (28, 29, 39). Recently, *C. granulosa* has been associated with chronic periodontitis (9, 10).

In addition to subject-to-subject variability, significant differences were also observed within a single subject (Fig. 2). Such variation in subgingival microbial profiles from site to site in the same subject, and from subject to subject, had been described recently by Teles et al. (50) using checkerboard DNA–DNA hybridization and by other authors using culture and molecular techniques (24, 35). Teles et al. (50) suggested that subjects differ in their subgingival microbiota and may have different periodontal pathogens that could be associated to their disease.

It was interesting that *A. actinomycetemcomitans*, considered a putative pathogen in GAgP, was not detected in the sequence analyses of *16S rRNA* libraries. However, using PCR with species-specific primers, seven of 10 subjects were positive for *A. actinomycetemcomitans.* This species has been previously reported in subgingival plaque as a relatively small proportion of the microbiota (23, 35, 57). For example, culture studies showed that *A. actinomycetemcomitans* was present in proportions ranging between 2% and 5% of the total cultivable subgingival flora (35, 46, 57). With its high prevalence and strong virulence, despite the low proportion, *A. actinomycetemcomitans* is still considered as associated to the etiology of the disease, because even at low levels, this species could be harmful to the periodontium (7, 42).

This descriptive study illustrates the breadth of bacterial diversity in subjects diagnosed with GAgP. Even though superior to cultivation methods for exploring microbial diversity, DNA extraction, PCR, and cloning have been reported as potential causes of bias (14). Although only a limited number of subjects and sites were analyzed, it was shown that the classical putative periodontal pathogens were below the limit of detection. These data suggest that other species, notably species of *Selenomonas*, may be associated with disease in GAgP subjects. However, it is premature to draw definitive conclusions about the specific bacterial species or complexes of species that are associated with GAgP. We are currently developing DNA probes for approximately 300 known species and novel phylotypes for use in DNA microarray formats (40). Consequently, microbial profiles of a statistically significant number of samples from aggressive periodontitis can be readily determined to identify and compare those species or complexes of species that are associated with this form of periodontal disease.

Acknowledgments

This study was supported in part by Research Grants 2006/52890-6 and 05/59443-2 from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, Brazil).

References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005: **43**: 5721–5732.
2. Albandar JM, Brown LJ, Loe H. Putative periodontal pathogens in subgingival plaque of young adults with and without early-onset periodontitis. J Periodontol 1997: **68**: 973–978.
3. Araujo MW, Hovey KM, Benedek JR et al. Reproducibility of probing depth measurement using a constant-force electronic probe: analysis of inter- and intraexaminer variability. J Periodontol 2003: **74**: 1736–1740.
4. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999: **4**: 1–6.
5. Armitage GC. Periodontal diagnoses and classification of periodontal diseases. Periodontol 2000 2004: **34**: 9–21.
6. Bonta Y, Zambon JJ, Genco RJ, Neiders ME. Rapid identification of periodontal pathogens in subgingival plaque: comparison of indirect immunofluorescence microscopy with bacterial culture for detection of *Actinobacillus actinomycetemcomitans*. J Dent Res 1985: **64**: 793–798.
7. Bragd L, Dahlen G, Wikstrom M, Slots J. The capability of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* to indicate progressive periodontitis; a retrospective study. J Clin Periodontol 1987: **14**: 95–99.
8. Celenligil H, Ebersole JL. Analysis of serum antibody responses to periodontopathogens in early-onset periodontitis patients from different geographical locations. J Clin Periodontol 1998: **25**: 994–1002.
9. Ciantar M, Gilthorpe MS, Hurel SJ, Newman HN, Wilson M, Spratt DA. *Capnocytophaga* spp. in periodontitis patients manifesting diabetes mellitus. J Periodontol 2005: **76**: 194–203.
10. Ciantar M, Spratt DA, Newman HN, Wilson M. *Capnocytophaga granulosa* and *Capnocytophaga haemolytica*: novel species in subgingival plaque. J Clin Periodontol 2001: **28**: 701–705.
11. Cole JR, Chai B, Farris RJ et al. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 2005: **33**: 294–296.
12. Cortelli JR, Cortelli SC, Jordan S, Haraszthy VI, Zambon JJ. Prevalence of periodontal pathogens in Brazilians with aggressive or chronic periodontitis. J Clin Periodont 2005: **32**: 860–866.
13. Darby IB, Hodge PJ, Riggio MP, Kinane DF. Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. J Clin Periodontol 2000: **27**: 417–424.
14. de Lillo A, Ashley FP, Palmer RM et al. Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. Oral Microbiol Immunol 2006: **21**: 61–68.
15. Gajardo M, Silva N, Gomez L et al. Prevalence of periodontopathic bacteria in aggressive periodontitis patients in a Chilean population. J Periodontol 2005: **76**: 289–294.
16. Gmur R, Wyss C, Xue Y, Thurnheer T, Guggenheim B. Gingival crevice microbiota from Chinese patients with gingivitis or necrotizing ulcerative gingivitis. Eur J Oral Sci 2004: **112**: 33–41.
17. Haffajee AD, Socransky SS, Ebersole JL, Smith DJ. Clinical, microbiological and immunological features associated with the treatment of active periodontosis lesions. J Clin Periodontol 1984: **11**: 600–618.
18. Haraszthy VI, Hariharan G, Tinoco EM et al. Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. J Periodontol 2000: **71**: 912–922.
19. Hutter G, Schlagenhauf U, Valenza G et al. Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. Microbiology 2003: **149**: 67–75.

20. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, ed. Mammalian Protein Metabolism. New York, NY: Academic Press Inc., 1969: 21–132; chapter 3.
21. Kamma JJ, Contreras A, Slots J. Herpes viruses and periodontopathic bacteria in early-onset periodontitis. J Clin Periodontol 2001: **28**: 879–885.
22. Kamma JJ, Nakou M, Baehni PC. Clinical and microbiological characteristics of smokers with early onset periodontitis. J Periodontal Res 1999: **34**: 25–33.
23. Kamma JJ, Nakou M, Gmur R, Baehni PC. Microbiological profile of early onset/aggressive periodontitis patients. Oral Microbiology Immunology 2004: **19**: 314–321.
24. Kamma JJ, Nakou M, Manti FA. Predominant microflora of severe, moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. J Periodontal Res 1995: **30**: 66–72.
25. Kamma JJ, Nakou M, Manti FA. Microbiota of rapidly progressive periodontitis lesions in association with clinical parameters. J Periodontol 1994: **65**: 1073–1078.
26. Kazor CE, Mitchell PM, Lee AM et al. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J Clin Microbiol 2003: **41**: 558–563.
27. Kolenbrander PE, Andersen RN, Moore LV. Coaggregation of *Fusobacterium nucleatum*, *Selenomonas flueggei*, *Selenomonas infelix*, *Selenomonas noxia*, and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. Infect Immun 1989: **57**: 3194–3203.
28. Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. J Dent Res 2003: **82**: 338–344.
29. Kumar PS, Griffen AL, Moeschberger ML, Leys EJ. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J Clin Microbiol 2005: **43**: 3944–3955.
30. Lang N, Barthold PM, Culliman M et al. Consensus report: aggressive periodontitis. Ann Periodontol 1999: **4**: 53.
31. Loesche WJ, Syed SA, Schmidt E, Morrison EC. Bacterial profiles of subgingival plaques in periodontitis. J Periodontol 1985: **56**: 447–456.
32. Lopez NJ. Occurrence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in progressive adult periodontitis. J Periodontol 2000: **71**: 948–954.
33. Macheleidt A, Muller HP, Eger T, Putzker M, Zoller L. Clonal diversity of *Actinobacillus actinomycetemcomitans* isolates from young adults with minimal periodontal disease. J Periodontal Res 1999: **34**: 179–187.
34. Melvin WL, Assad DA, Miller GA, Gher ME, Simonson L, York AK. Comparison of DNA probe and ELISA microbial analysis methods and their association with adult periodontitis. J Periodontol 1994: **65**: 576–582.
35. Moore WE, Holdeman LV, Cato EP et al. Comparative bacteriology of juvenile periodontitis. Infect Immun 1985: **48**: 507–519.
36. Moore WE, Holdeman LV, Smibert RM, Hash DE, Burmeister JA, Ranney RR. Bacteriology of severe periodontitis in young adult humans. Infect Immun 1982: **38**: 1137–1148.
37. Mullally BH, Dace B, Shelburne CE, Wolff LF, Coulter WA. Prevalence of periodontal pathogens in localized and generalized forms of early-onset periodontitis. J Periodontal Res 2000: **35**: 232–241.
38. Norskov-Lauritsen N, Kilian M. Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. Int J Syst Evol Microbiol 2006: **56**: 2135–2146.
39. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
40. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000 2006: **42**: 80–87.
41. Paster BJ, Russell MK, Alpagot T et al. Bacterial diversity in necrotizing ulcerative periodontitis in HIV-positive subjects. Ann Periodontol 2002: **7**: 8–16.
42. Rams TE, Listgarten MA, Slots J. Utility of 5 major putative periodontal pathogens and selected clinical parameters to predict periodontal breakdown in patients on maintenance care. J Clin Periodontol 1997: **23**: 346–354.
43. Ranney RR. Classification of periodontal diseases. Periodontol 2000 1993: **2**: 13–25.
44. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987: **4**: 406–425.
45. Sakamoto M, Rocas IN, Siqueira JF Jr, Benno Y. Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. Oral Microbiol Immunol 2006: **21**: 112–122.
46. Slots J, Feik D, Rams TE. *Actinobacillus actinomycetemcomitans* and *Bacteroides intermedius* in human periodontitis: age relationship and mutual association. J Clin Periodontol 1990: **17**: 659–662.
47. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
48. Takeuchi Y, Umeda M, Ishizuka M, Huang Y, Ishikawa I. Prevalence of periodontopathic bacteria in aggressive periodontitis patients in a Japanese population. J Periodontol 2003: **74**: 1460–1469.
49. Tanner A, Maiden MF, Macuch PJ, Murray LL, Kent RL Jr. Microbiota of health, gingivitis, and initial periodontitis. J Clin Periodontol 1998: **25**: 85–98.
50. Teles RP, Haffajee AD, Socransky SS. Microbiological goals of periodontal therapy. Periodontol 2000 2006: **42**: 180–218.
51. Tinoco EM, Lyngstadaas SP, Preus HR, Gjermo P. Attachment loss and serum antibody levels against autologous and reference strains of *Actinobacillus actinomycetemcomitans* in untreated localized juvenile periodontitis patients. J Clin Periodontol 1997: **24**: 937–944.
52. Tonetti MS, Mombelli A. Early-onset periodontitis. Ann Periodontol 1999: **4**: 39–53.
53. Van der Peer Y, De Wacheter R. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 1994: **10**: 569–570.
54. Wang Z, Yang S, Shang J. Serotype distribution of *Actinobacillus actinomycetemcomitans*. Zhonghua Kou Qiang Yi Xue Za Zhi 1997: **32**: 7–9.
55. Ximenez-Fyvie LA, Almaguer-Flores A, Jacobo-Soto V, Lara-Cordoba M, Sanchez-Vargas LO, Alcantara-Maruri E. Subgingival microbiota of periodontally untreated Mexican subjects with generalized aggressive periodontitis. J Clin Periodontol 2006: **33**: 869–877.
56. Yang HW, Asikainen S, Dogan B, Suda R, Lai CH. Relationship of *Actinobacillus actinomycetemcomitans* serotype b to aggressive periodontitis: frequency in pure cultured isolates. J Periodontol 2004: **4**: 592–599.
57. Zambon JJ, Christersson LA, Slots J. *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. J Periodontol 1983: **54**: 707–711.