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Analysis of genotypic variation in genes associated with virulence in *Aggregatibacter actinomycetemcomitans* clinical isolates

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Background and Objective: Although certain serotypes of *Aggregatibacter actinomycetemcomitans* are associated more with aggressive periodontitis than are other serotypes, the correlation between distinct lineages and virulence traits in this species is poorly understood. This study aimed to evaluate the polymorphism of genes encoding putative virulence factors of clinical isolates, and to correlate these findings with *A. actinomycetemcomitans* serotypes, genotypes and periodontal status of the hosts.

Material and Methods: Twenty-six clinical isolates from diverse geographic populations with different periodontal conditions were evaluated. Genotyping was performed using pulse-field gel electrophoresis. Polymorphisms in the genes encoding leukotoxin, Aae, ApaH and determinants for serotype-specific O polysaccharide were investigated.

Results: The isolates were classified into serotypes a–f, and exhibited three *apaH* genotypes, five *aae* alleles and 25 macrorestriction profiles. Two serotype b isolates (7.7%), obtained from Brazilian patients with aggressive periodontitis, were associated with the highly leukotoxic genotype; these isolates showed identical fingerprint patterns and *aae* and *apaH* genotypes. Serotype c, obtained from various periodontal conditions, was the most prevalent among Brazilian isolates, and isolates were distributed in two *aae* alleles, but formed a genetically distinct group based on *apaH* analysis. Cluster analysis showed a close relationship between fingerprinting genotypes and serotypes/*apaH* genotypes, but not with *aae* genotypes.

Conclusion: Apart from the deletion in the *ltx* promoter region, no disease-associated markers were identified. Non-JP2-like strains recovered from individuals with periodontal disease exhibited considerable genetic variation regarding *aae*/*apaH* genotypes, serotypes and *Xho*I DNA fingerprints.

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Aggregatibacter (formerly *Actinobacillus) actinomycetemcomitans* is a gram-negative, nonmotile, capnophilic cocobacillus that colonizes the human oral cavity. *A. actinomycetemcomitans* is strongly implicated in the etiology of localized aggressive periodontitis, but it can also be found in patients with chronic periodontitis and in healthy subjects (1). The association with disease is dependent on *A. actinomycetemcomitans* virulence and host susceptibility (2).

A particular lineage among serotype b strains produces high levels of leukotoxin (ltx), and is associated with localized aggressive periodontitis in people of African descent (3–5). However, the presence of low-leukotoxin-producing *A. actinomycetemcomitans* in subjects with aggressive periodontitis (6) suggests that other virulence determinants are involved in pathogenesis.

Although genetic diversity within *A. actinomycetemcomitans* has been shown, and a lineage associated with enhanced virulence (JP2 clone) was found to be related to aggressive disease (7,8), very little is known on the correlation between genetic diversity and virulence genes involved in tissue attachment, invasion, evasion of the host defense and bone destruction (9–12).

The interaction with epithelial cells of the oral crevicular sulcus is essential for a periodontopathogenic organism. The adhesion of *A. actinomycetemcomitans* to epithelial cells is mediated by the autotransported bacterial outer membrane proteins Aae (13) and ApiA (also known as Omp100) (14). To date, at least four different alleles of *aae* are known, and they differ in the number of copies of a 135-bp repeat sequence present in the region of *aae* encoding the effector domain (13).

A. actinomycetemcomitans can be internalized in epithelial cells, and the invasion efficiency varies among isolates (15). As a result of internalization, the bacteria may evade the host immune response, penetrate the mucosal epithelial cell layer and reach the underlying connective tissues. Thus, the cytoplasm of buccal epithelial cells may function as an extracrevicular reservoir of periodontopathogenic bacteria, and the intracellular levels of bacteria are not affected by the mechanical periodontal therapy associated with the use of antimicrobial agents (16). The invasion process is not only affected by adhesins that mediate cell–cell interaction (17) but also by other factors. Studies have suggested that *apaH* may be required for *A. actinomycetemcomitans* invasion. Homologs of the chromosomally encoded *apaH* in *A. actinomycetemcomitans* are associated with the invasion process in other pathogenic bacteria (18–21). An *A. actinomycetemcomitans apaH*-deficient mutant exhibited loss of invasiveness to KB cells, whereas expression of recombinant ApaH resulted in an invasive phenotype to KB cells *in vitro* (22).

This study aimed to evaluate the polymorphism of genes encoding putative virulence factors (*ltx, aae* and *apaH*) of clinical isolates, and to correlate these findings with *A. actinomycetemcomitans* serotypes, genotypes and periodontal status of the hosts.

Material and methods

Bacterial strains and growth condition

Two reference strains (HK 1651 and JP2) and 26 clinical isolates of *A. actinomycetemcomitans* were evaluated. Seventeen isolates were obtained from Brazilian patients attending the Clinics of the School of Dentistry of São Paulo University and of Camilo Castelo Branco University (São Paulo). Six of these isolates (G10-2, G42-3, G59-3, G103-1, G104-2 and G105-2) were obtained from patients with chronic periodontitis and six (Aa1, G111-1, G121-2, S3-2, S4-1 and S4-6) were obtained from patients with aggressive periodontitis. Five samples (C12-2, C12-2, C21-1, C27-1 and C51-2) were isolated from healthy students, from samples collected during an epidemiological study of 11- to 12-year-old children in São Paulo, Brazil (23). The strains from Sweden (181, 194 and 250) were obtained from samples from subjects with aggressive periodontitis and were isolated in the Department of Microbiology, School of Dentistry, University of Gothenburg, Sweden. Isolates from Kenya (420, 443, 707 and 721) were obtained from subjects without periodontal bone loss or light periodontal disease (24) and isolates from Japan (1519 and 1583) originated from inactive sites of patients with deep pockets (25). Serotype reference strains SA 1151, SA 1350, SA 3632 and SA 781 were generously given by Dr Sirkka Asikainen and were used as controls in serotype determination by PCR. The reference strains SUNY 465 (one copy of the *aae* repeat) and HK 1651 (three copies of the *aae* repeat) were used as controls for *aae* genotyping. This study was approved by the Human Research Ethics Committee of the University of São Paulo (São Paulo, Brazil). All samples were kept in 20% glycerol, in a −80°C freezer. The frozen stocks were inoculated on TSYE agar [tryptic soy agar containing 0.6% (w/v) yeast extract] and incubated for 48 h at 37°C in a 10% CO_2 chamber.

Fingerprinting

Chromosomal DNA was prepared in agarose blocks (26) and cleaved with *Xho*I (27). Bacterial suspensions, at an optical density at 610 nm of ~ 3.7–4.0 (2.5×10^9 colony-forming units/mL), were treated with lysis solution [50 mM Tris–HCl (pH 8.0), 50 mM EDTA, 2.5 mg/mL of lysozyme and 1.5 mg/mL of proteinase K), added to an equal volume of 1.2% molten Incert agarose (FMC BioProducts, Rockland, ME, USA) containing 1% sodium dodecyl sulfate and poured into a CHEF Disposable Plug Mold (Bio-Rad Laboratories, Hercules, CA, USA). The proteolysis was performed with 0.5 M EDTA, 1% sarcosyl and 400 μg/mL of proteinase K, overnight at 50°C. After washing, DNA was restricted with *Xho*I (New England Biolabs, Hitchin, UK) according to Eriksen *et al.* (27).

Electrophoresis was performed in 1.2% agarose gels (SeaKem Gold agarose; Cambrex Bio Science, Workingham, UK), by using the contour homogeneous electric fields device (CHEF-DR III; Bio-Rad); the pulse time was increased from 1 s to 30 s

over 24 h at 6 V/cm (200 V). Ethidium bromide-stained gels were photographed under ultraviolet light.

The macrorestriction fingerprints generated by pulse-field gel electrophoresis (PFGE) were analyzed using BIONUMERICS Software (Applied Maths, Austin, TX, USA). A tolerance in the band position of 1% was applied for comparison of PFGE patterns. The similarities of isolates were determined using the Dice coefficient and clustered by the unweighted method with arithmetic averages (UPGMA). Dendrograms were constructed to reflect the similarities among strains. Clusters were defined above the 70% similarity level (28).

PCR assays

A. actinomycetemcomitans chromosomal DNA was extracted using standard methods (29). The primers used in this study are listed in Table 1.

Leukotoxin promoter subtypes— ltx genotyping was performed with the primers LtxA and LtxB (30). The PCR was performed under the following conditions: a 4-min initial denaturation at 94°C; 25 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C) and extension (1 min at 72°C); and a final extension (10 min at 72°C). An amplicon of 492 bp was indicative of the 530-bp deletion, characteristic of high leukotoxin production isolates (JP2-like), whereas an amplicon of 1022 bp indicated a genotype associated with a low level of leukotoxin production.

Serotypes— Serotyping was performed with primers: P15 and P16 for serotype a; P11 and P14 for serotype b; P12 and P14 for serotype c; P17 and P18 for serotype d; P19 and P20 for serotype e; and P13 and P14 for serotype f (10). The PCR was performed under the following conditions: a 5-min initial denaturation at 94°C; 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 48°C) and extension (1 min at 72°C); and a final extension (5 min at 72°C). DNA from reference strains *A. actinomycetemcomitans* ATCC 29523 (serotype a), FDC Y4 (serotype b), SA 1151 (serotype c), SA 781 (serotype d), SA 1350 (serotype e) and SA 1350 (serotype f) were used as controls.

aae *genotypes—* An internal fragment of *aae* was amplified by PCR with the primers INT3 and INT5 (13). The PCR was performed under the following conditions: a 5-min initial denaturation at 94°C; 40 cycles of denaturation (30 s at 94°C), annealing (30 s at 52.5°C) and extension (30 s at 72°C); and a final extension (2 min at 72°C). The strains were classified based on the number of copies of a repeat sequence (135 bp).

apaH *genotypes— apaH* genotyping was performed with the primers apaH1 and apaH2, as proposed by Saarela *et al.* (9). The PCR was performed under the following conditions: a 3-min initial denaturation at 94°C; 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 52°C) and extension (1 min at 72°C); and a final extension (2 min at 72°C). Amplification products were characterized by restriction fragment length polymorphism (RFLP) using *Sph*I and *Nhe*I, according to the manufacturer's instructions (Invitrogen Life Technologies, São Paulo, SP, Brazil).

In order to confirm the polymorphism of *apaH*, the PCR products of selected isolates – G111-1 (*apaH* genotype I), G10-2 (*apaH* genotype II) and C21-1 (*apaH* genotype III) – were sequenced. The PCR fragments were cloned into a pCR II TOPO TA 4.0 Cloning kit (Invitrogen) and transformants were selected after overnight growth at 37°C in Luria–Bertani agar containing 100 μg/mL of ampicillin. The inserts were sequenced using MegaBACE 1000 (GE Healthcare, Piscataway, NJ, USA), following the manufacturer's recommendations, using a DYEnamic ET Dye Terminator kit (with Thermo SequenaseTM II DNA Polymerase). Data were analyzed with the aid of MEGALIGN (Dnastar version 3.03–2002).

The DNA sequences determined in this study have been deposited in the GenBank database under the codes GU056781, GU056783 and GU056782).

Table 1. Primers used in this study

Primer name	Primer sequences (5′ → 3′)	References
ltxA	TCC ATA TTA AAT CTC CTT GT	Zambon *et al.* (30)
ltxB	AAC CTG ACT ACA GTA TT	
P11	TCT CCA CCA TTT TTG AGT GG	Kaplan *et al.* (31)
P12	GAA ACC ACT TCT ATT TCT CC	
P13	CCT TTA TCA ATC CAG ACA GC	
P14	ARA AYT TYT CWT CGG GAA TG	
P15	TGG GTC ATG GGA GGT ACT CC	
P16	GCT AGG AAC AAA GCA GCA TC	
P17	TGG AAC GGG TAT GGG AAC GG	
P18	GGA TGC TCA TCT AGC CAT GC	
P19	ATT CCA GCC TTT TGG TTC TC	
P20	TGG TCT GCG TTG TAG GTT GG	
INT5	AAG TTG CCC GAG TAA ATC G	Rose *et al.* (13)
INT3	CCG GGA CTT CTC ACG TTT AAC	
apaH1	ATT TAA TCG GCG ACC TGC AC	Saarela *et al.* (9)
apaH2	TGT CTT CCC AAC GTA GCA TG	

Results

In the analysis of the *ltx* promoter region, reference strains JP2 and HK1651 showed 492-bp PCR products, which were characteristic of high leukotoxin production (JP2-like genotype). Out of 26 clinical isolates, two strains (G111-1 and G121-1) (7.7%), obtained from Brazilian subjects with aggressive periodontitis, showed similar 492-bp PCR products, and were classified as JP2-like genotype (data not shown). In contrast, twenty-four clinical isolates (92.3%) showed 1022-bp PCR products, and were classified as having a non-JP2-like genotype (data not shown). In the *Xho*I DNA fingerprint analysis, however, JP2 and HK1651 showed different DNA-ladder

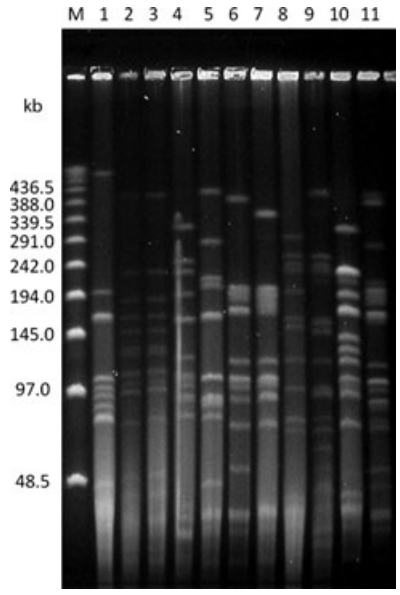


Fig. 1. *Xho*I DNA fingerprinting of *Aggregatibacter actinomycetemcomitans* strains resolved by pulse-field gel electrophoresis (PFGE). M represents the molecular size marker (bacteriophage ladder), the sizes of which are shown in kilobases on the left. The *A. actinomycetemcomitans* strains were: lane 1, G 104-2; lanes 2 and 3, both HK 1651; lane 4, JP2; lane 5, C51-2; lane 6, C21-1; lane 7, G103-1; lane 8, 181; lane 9, 1583; lane 10, 443; and lane 11, C12-2.

patterns (Fig. 1 lanes 2–4). *Xho*I DNA fingerprints of G111-1, G121-1 and JP2 were identical (data not shown), whereas twenty-four clinical isolates showed diverse macrorestriction profiles, which were also different from those of JP2 and HK 1651 (Fig. 1).

Serotype-specific genotyping was performed by PCR using six pairs of primers. Three isolates (11.5%) were classified as serotype a: two (C21-1 and C27-1) from Brazilian subjects with a healthy periodontal status and one (707) from a Kenyan subject. The five isolates (19.2%) classified as serotype b included Brazilian isolates from patients with aggressive periodontitis (G111-1 and G121-2) and chronic periodontitis (G104-2), and two isolates from Sweden (181 and 194). Seventeen isolates (65.3%) were classified as serotype c. Most of the Brazilian clinical isolates ($n = 12$; 70.5%) were classified as serotype c, including isolates from patients with aggressive periodontitis (Aa1, S3-2, S4-1 and S4-6), chronic periodontitis (G10-2, G42-3, G59-3, G103-1 and G105-2), and healthy subjects (C12-2, C12-2 and C51-2). One clinical isolate from Japan (1583) was classified as serotype f. Serotypes d and e were not detected.

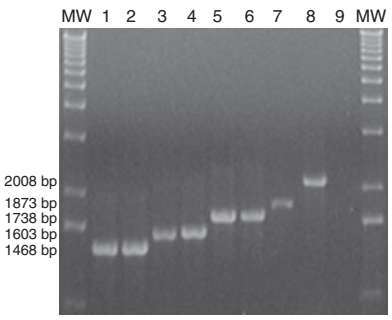


Fig. 2. PCR of the INT5-INT3 fragment from several *Aggregatibacter actinomycetemcomitans* strains demonstrating five *aae* alleles. Lanes 1 and 2: strains SUNY 465 and 443 showed a product of 1468 bp; lanes 3 and 4: strains Aa1 and G103-1 showed a product of 1603 bp; lanes 5 and 6: strains HK 1651 and G 111-1 showed a product of 1738 bp; lane 7: strain 707 showed a product of 1873 bp; lane 8: strain 1583 showed a product of 2008 bp; and lane 9: negative control. MW represents the molecular size marker (1 kb plus DNA ladder), the sizes of which are shown on the left.

Amplification of *aae* revealed five different alleles with between one and five copies of the 135-bp repeat sequence, resulting in products ranging from 1468 to 2008 bp in size (Fig. 2). Alleles with two copies of the repeat sequence were present in 50% of non-JP2-like strains, whereas three copies of the repeat sequence were present in all JP2-like strains.

The amplification using *apaH* primers resulted in a 771-bp product for each *A. actinomycetemcomitans* isolate studied. According to RFLP using *Sph*I and *Nhe*I, the 771-bp *apaH* PCR products were classified into three genotypes. In genotype I, the 771-bp *apaH* PCR fragment was cut into 347- and 424-bp fragments by *Sph*I digestion and into 104- and 667-bp fragments by *Nhe*I digestion, as seen in FDC Y4. In genotype II, the 771-bp *apaH* PCR fragment was cut into 129-, 218- and 424-bp fragments by *Sph*I digestion, but the 771-bp fragment remained intact following digestion with *Nhe*I (i.e. no restriction occurred). In genotype III, the 771-bp fragment remained intact following digestion with either *Sph*I or *Nhe*I (i.e. no restriction occurred) (Fig. 3). The sequence analyses of *apaH* genotypes I, II and III (GU056781, GU056783 and GU056782) revealed that the recogni-

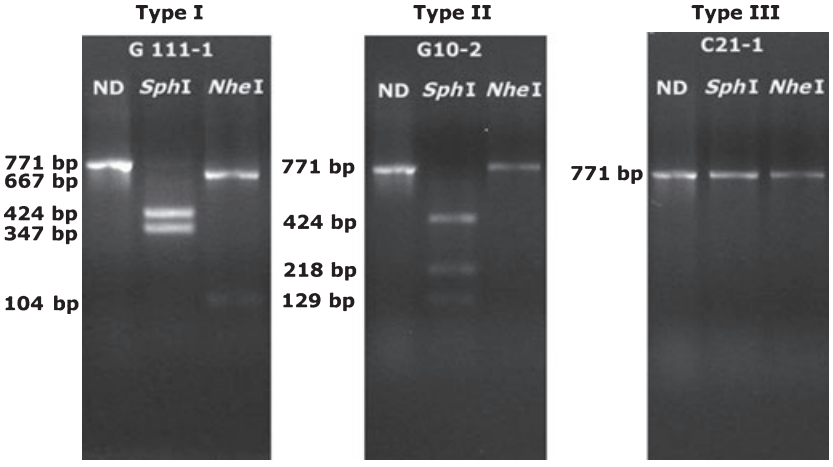
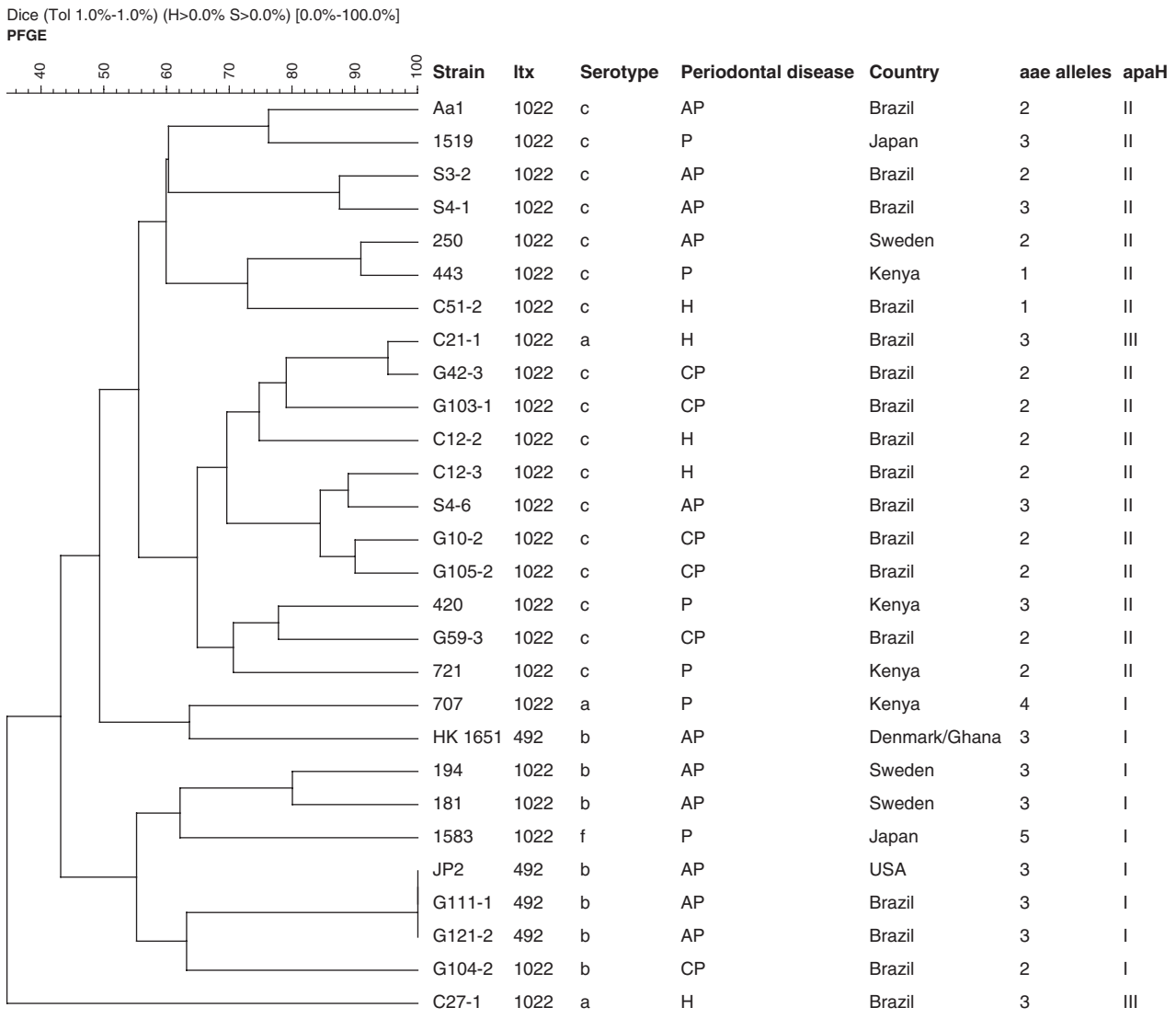


Fig. 3. PCR-RFLP of the *apaH* gene. The 771-bp amplification products are shown (ND, not digested). Amplicons of strain G111-1 showed two restriction sites for *Sph*I, leading to products of 347 and 424 bp, and two restriction sites for *Nhe*I, leading to products of 104 and 667 bp. Amplicons of strain G10-2 showed products of 129, 218 and 424 bp after *Sph*I digestion, but were not digested with *Nhe*I. Amplicons of C21-1 were not digested by any of the studied endonucleases.



Strain	ltx	Serotype	Periodontal disease	Country	aae alleles	apaH
Aa1	1022	c	AP	Brazil	2	II
1519	1022	c	P	Japan	3	II
S3-2	1022	c	AP	Brazil	2	II
S4-1	1022	c	AP	Brazil	3	II
250	1022	c	AP	Sweden	2	II
443	1022	c	P	Kenya	1	II
C51-2	1022	c	H	Brazil	1	II
C21-1	1022	a	H	Brazil	3	III
G42-3	1022	c	CP	Brazil	2	II
G103-1	1022	c	CP	Brazil	2	II
C12-2	1022	c	H	Brazil	2	II
C12-3	1022	c	H	Brazil	2	II
S4-6	1022	c	AP	Brazil	3	II
G10-2	1022	c	CP	Brazil	2	II
G105-2	1022	c	CP	Brazil	2	II
420	1022	c	P	Kenya	3	II
G59-3	1022	c	CP	Brazil	2	II
721	1022	c	P	Kenya	2	II
707	1022	a	P	Kenya	4	I
HK 1651	492	b	AP	Denmark/Ghana	3	I
194	1022	b	AP	Sweden	3	I
181	1022	b	AP	Sweden	3	I
1583	1022	f	P	Japan	5	I
JP2	492	b	AP	USA	3	I
G111-1	492	b	AP	Brazil	3	I
G121-2	492	b	AP	Brazil	3	I
G104-2	1022	b	CP	Brazil	2	I
C27-1	1022	a	H	Brazil	3	III

Fig. 4. Dendrogram showing *Xho*I pattern similarity of 28 *Aggregatibacter actinomycetemcomitans* strains, their serotypes, genotypes (*ltx*, *aae* and *apa*H), origin and periodontal condition of the host: H, periodontally healthy, no alveolar bone loss; P, periodontitis; AP, aggressive periodontitis; and CP, chronic periodontitis. *ltx* promoter genotypes: fragments of approximately 492 bp are indicative of the JP2 clone, whereas amplification with DNA of other strains resulted in a fragment of approximately 1022 bp.

tion sites for *Sph*I and *Nhe*I had disappeared as a result of synonymous changes in genotype II and III. Alignment of the expected translated proteins revealed that all genotypes exhibited conserved domains for the diadenosine tetraphosphatase and the RGD sequence (data not shown).

In general, cluster analysis showed a close relationship between PFGE genotypes and serotypes, except for serotype a isolates, which were distributed in three different clusters (Fig. 4). Serotype c formed a genetically distinct group comprising *apaH* genotype II isolates.

Discussion

The association of *A. actinomycetemcomitans* with periodontal disease is dependent on its virulence traits and host/environmental-related factors (2). Despite the evidence that the highly toxic JP2-like *A. actinomycetemcomitans* serotype b is more virulent, non-JP2-like serotype b and other serotypes are commonly isolated from patients with aggressive periodontitis in subjects of non-African descent (6). In the present study, most strains of *A. actinomycetemcomitans* isolated from patients with periodontal disease, including aggressive periodontitis, were minimally leukotoxic. This study examined various typing systems for *A. actinomycetemcomitans* and their association with periodontal disease status, and no disease-associated markers were identified, except for the *ltx* genotype of JP2-like strains. These findings support the hypothesis that most types of *A. actinomycetemcomitans* are capable of inducing periodontal disease. Variable regions in the genome, such as those encoding genomic islands, would probably account for differences in the pathogenic potential of a strain. Recent data

reported that the genome of strain D11S-1, a serotype c isolate, presents five genomic islands (H to L) not found in the genome of strain HK1651, a JP2-like serotype b isolate (32). Similarly, the annotation of the genome of the serotype a strain, DS7-1, revealed four genomic islands not found in the other sequenced strains (33).

In the present study, non-JP2-like strains were included in serotypes a, b, c and f. Serotype c was the most prevalent serotype among Brazilian *A. actinomycetemcomitans* isolates, and they were isolated from various periodontal conditions, including aggressive periodontitis, which is in accordance with previous studies (12,34,35). In addition, a strong response to this serotype may be found in the sera of patients with aggressive periodontitis (36). Serotype distribution patterns of subgingival *A. actinomycetemcomitans* may vary among subjects of different race or ethnicity, and serotype c is also the most prevalent *A. actinomycetemcomitans* serotype in subgingival dental plaque in the USA (37).

As expected, a high genetic diversity was found among non-JP2-like strains comprising different serotypes. All strains were discriminated by PFGE, showing different fingerprint patterns. Apart from serotype a strains, cluster analysis showed an association between fingerprint genotypes and serotypes. DNA fingerprint analysis tended to cluster serotype b and serotype c isolates in different groups, suggesting that these lineages may present genetic features that could result in differences in virulence. An early study by multilocus enzyme electrophoresis also demonstrated that serotypes comprise distinct subpopulations, although serotype a isolates were classified in genetically very different divisions (7).

Adhesion is the first step of the invasion process, and thus adhesins may influence the ability of bacteria to internalize in eukaryotic cells (17). Amplification of *aae* revealed five different alleles with between one and five copies of a 135-bp repeat sequence: these included the four different alleles previously reported (13) and a new one detected only in a serotype f isolate from Japan (which contained five copies of the repeat sequence). Alleles with two copies of the repeat sequence were present in 50% of non-JP2-like strains; whereas three copies were present in all JP2-like strains. The domain involved in binding Aae to buccal epithelial cells is situated in this variable repeated region (38), suggesting that an increased number of repeats may result in increased binding to target cells. The lack of an association between *aae* polymorphism and virulence may be indicative of a compensation promoted by other adhesins mediating cell interaction, such as Omp100 (14). Therefore, the functional significance of this genetic polymorphism, as well as the regulation of *aae* expression, should be further investigated.

The expression of other bacterial products, such as the production of diadenosine tetraphosphatase, encoded by *apaH*, may enhance bacterial survival within the cytoplasm of eukaryotic cells (21). The amplification using *apaH* complementary primers was obtained with all *A. actinomycetemcomitans* isolates tested. *A. actinomycetemcomitans apaH* is a homolog of genes associated with invasion of *Bartonella bacilliformis* (18), *Escherichia coli* K1 (19), *Salmonella enterica* (20) and *Rickettsia prowazekii* (21). PCR-RFLP analyses of *apaH* have previously revealed two genotypes among *A. actinomycetemcomitans* isolates (9). In the present study, a third genotype was found. The sequence analyses of *apaH* genotypes revealed that despite the absence of restriction enzyme recognition sites, the mutations resulted in the same amino-acid sequences in the translated proteins (synonymous), which may explain the lack of association between *apaH* polymorphism and virulence of *A. actinomycetemcomitans*. Moreover, alignment of the expected translated proteins revealed that all genotypes exhibited conserved domains for the diadenosine tetraphosphatase and the RGD sequence, characteristic of adhesins binding to integrin in eukaryotic surfaces. Thus, despite their sequence differences, the cell-cleansing function of Nudix hydrolases, which removes potentially deleterious endogenous metabolites (19), would be maintained in the three *apaH* genotypes.

Non-JP2-like strains exhibited considerable genetic variation regarding *aae*/*apaH* genotypes, serotypes and *Xho*I DNA fingerprints. The diversity of strains recovered from individuals with periodontal disease supports the conclusion that *A. actinomycetemcomitans* may play the role of an opportunistic pathogen in Caucasian individuals (6,10). On the other hand, JP2-like strains have been strongly associated with disease in hosts of African descent, being considered a particularly virulent subpopulation of *A. actinomycetemcomitans* (3–5,39).

The JP2 clone belongs to serotype b and is genetically very homogeneous, as demonstrated by analysis of housekeeping genes (Multi Loci Sequencing Typing) (8,27). In the present study, the JP2 clone was also homogenous in genes encoding factors related to interactions with host cells, such as *apaH* and *aae*. Besides enhanced production of leukotoxin in culture, the JP2 clone differs from other strains of *A. actinomycetemcomitans* by showing strong hemolytic activity (39). Moreover, members of the JP2 clone share a mutational event affecting the gene encoding the hemoglobin-binding protein (*hbpA*) (40). Although genetically highly conserved, members of the JP2 clone may show different patterns after PFGE analysis as a result of intragenic recombinations, failing to reveal the close phylogenetic relationships among JP2-like strains (27), as shown in the dendrogram for the HK 1651 strain. On the other hand, the *Xho*I fingerprinting patterns of G111-1 and G121-1 were identical to that of JP2, probably because of the potentially reversible nature of these events. Nevertheless, techniques that are sensitive to large inversions in the genome reveal that members of the clone have been diversifying since its initial emergence (39). In addition, previous studies have revealed that HK 1651 presented a nucleotide substitution in the *atpG* gene fragment that was not seen in other JP2-like isolates (8,27).

The polymorphisms of genes encoding putative virulence factors (*ltx, aae* and *apaH*) of clinical *A.*

actinomycetemcomitans isolates were evaluated in the present study. Apart from the deletion in the *ltx* promoter region, no disease-associated markers were identified. However, it is important to notice that the functional significance of these genetic polymorphisms, and their role in the pathogenesis of periodontal disease, should be further investigated.

Acknowledgements

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