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Genetic diversity and toxic activity of *Aggregatibacter actinomycetemcomitans* isolates

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Introduction: Very little is known of the diversity and expression of virulence factors of serotypes of *Aggregatibacter actinomycetemcomitans*. Toxic activity on Chinese hamster ovary (CHO) cells and *cdt and ltx* genotyping were evaluated in *A. actinomycetemcomitans* serotypes.
Methods: Forty-one *A. actinomycetemcomitans* isolates were analysed for CHO cell growth inhibition. Genotyping was performed by polymerase chain reactions specific to the *ltx* promoter region, serotype-specific and *cdt* region and by sequencing of *cdtB*.
Results: *cdtABC* was detected in 40 strains. Analysis of the *cdtA* upstream region revealed 10 *cdt* genotypes. Toxicity to CHO cells was detected for 92.7% of the isolates; however, no correlation between the toxic activity and the *cdt* genotype was detected. Serotype **c** was more prevalent among Brazilian samples (68.0%). Four serotype **b** isolates from subjects with aggressive periodontitis were associated with high leukotoxin production and exhibited moderate to strong toxic activity in CHO cells, but were classified in different *cdt* genotypes. High levels of toxicity in CHO cells were not associated with a particular serotype; 57.1% of serotype **a** isolates presented low toxicity to CHO cells whereas the highly toxic strains belonged to serotypes **b** and **c**. Sequencing of *cdtB* revealed a single nucleotide polymorphism of amino acid 281 but this was not related to the toxic activity in CHO cells.
Conclusion: Differences in prevalence of the low and highly cytotoxic strains among serotypes reinforce the hypothesis that serotype **b** and **c** isolates of *A. actinomycetemcomitans* are more virulent than serotype **a** strains.

Key words: *Aggregatibacter actinomycetemcomitans*; cytolethal distending toxin; leukotoxin; serotype

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Aggregatibacter actinomycetemcomitans is an important pathogen in aggressive periodontitis and has a variety of virulence factors such as production of leukotoxin, cytolethal distending toxin (CDT), bacteriocins, adhesins, and lipopolysaccharide (26).

A. actinomycetemcomitans isolates are classified into six serotypes, **a** to **f** (1, 27, 41). Serotypes **a**, **b**, **c**, and **f** occur more frequently in the oral cavity than serotypes **d** and **e** (35, 48, 53) and serotype **b** is frequently associated with aggressive periodontitis (1, 41, 54). However, differences in serotype distribution have been shown among African, Asian, Europeans, and North and South American populations (1, 25, 48, 50, 53).

Leukotoxin is a member of the RTX (repeats in toxin) family produced by *A. actinomycetemcomitans* that targets neutrophils, monocytes, and subset lymphocytes (30, 37). The *ltx* operon is formed by four genes: *ltxA* is the structural gene, and the three remaining genes (*ltxB*, *ltxC*, and *ltxD*) are required for activating and transporting the toxin (30). All isolates of *A. actinomycetemcomitans* exhibit the *ltx* operon, but differences in the *ltx* promoter region account for differences in leukotoxin expression among the isolates. The *ltx* operon of isolates with high titers of leukotoxin production (JP2-like) presents a deletion of 530 base pairs (bp) resulting in 10–20 times higher leukotoxin expression than the low leukotoxic strains (7, 55). These highly leukotoxic strains descend from a common ancestor, exhibit *O*-polysaccharide antigen **b** (21), and are usually related to aggressive periodontitis (2, 8, 25).

CDT is a member of the heat-labile protein cytotoxins produced by a variety of

gram-negative pathogens associated with mucosal surfaces (10, 45). It is a secreted tripartite AB_2 toxin, in which CdtB is the active toxic unit 'A' containing a domain with *DNaseI*-like activity (31) and CdtA and CdtC comprise the binding 'B' subunit required for the delivery of CdtB into the target cell (9, 32). Culture filtrates of CDT-producing strains cause cytoplasm distension, arresting at G0/G1 or G2/M cell cycle phases, and induce programmed cell death in several cultured cell lines (39, 45).

A. actinomycetemcomitans is the only oral bacterium known to produce CDT (52) but the intensity of the toxic activity varies among strains of this species (17). The *cdtABC* operon in *A. actinomycetemcomitans* FDC Y4 is formed also by two additional open reading frames located upstream from *cdtA* (34, 42, 43, 46) which are believed not to be necessary for the toxic activity (43). Most isolates present all three CDT-encoding genes (17) but the region upstream from *cdtA* is highly polymorphic (28), suggesting that the region was originated by horizontal transfer (34). Despite this polymorphism, no association between CDT activity and genotyping of this region was established, except that a single nucleotide polymorphism (SNP) at position 281 of *A. actinomycetemcomitans cdtB* was involved with increased specific activity of the CDT holotoxin (38).

Although the association of CDT with pathogenesis is not fully understood, evidence suggests that this toxin may represent a bacterial adaptation affecting the interaction between the bacterium and the host immune system in chronic diseases (18–20, 40, 51). Given that CDT was associated with the persistence of infection in animal models (40), and with increased expression of RANKL (receptor activation of nuclear factor-κB ligand) and consequently osteoclastogenesis (5), the association of CDT activity and genetic diversity within *A. actinomycetemcomitans* should be better evaluated. For this reason, this study aimed to analyse the inhibitory activity of bacterial lysates of *A. actinomycetemcomitans* on cell growth and to compare these data with the polymorphism of the *cdt* region, *ltx* genotypes, and serotypes.

Materials and methods

This study was approved by the ethical committee in research in humans of the University of São Paulo, São Paulo, Brazil.

Bacterial strains and growth conditions

Reference strains (HK 1651, ATCC 29523, FDC Y4, and JP2) and 41 clinical isolates of *A. actinomycetemcomitans* were evaluated. Twenty-five isolates were obtained from Brazilian subjects, five from patients with chronic periodontitis, 12 from patients with aggressive periodontitis and eight from healthy adolescents. Strains from Sweden ($n = 7$) were obtained from subjects with aggressive periodontitis. Strains from Kenya ($n = 4$) were isolated from subjects without periodontal bone loss or with light periodontal disease (12). Strains from Japan ($n = 5$) were isolated from inactive sites of patients with deep pockets (13). Serotype reference strains SA 1151(serotype **c**), SA 1350 (serotype **e**), SA 3632 (serotype **f**), and SA 781 (serotype **d**), kindly donated by Dr. Sirkka Asikainen, were used as controls in polymerase chain reactions (PCR). All samples were kept in 20% glycerol, in a −80°C freezer. The frozen stocks were inoculated on tryptic soy agar with 0.6% weight/volume yeast extract, and incubated for 48 h at 37°C in a 10% CO_2 chamber.

Escherichia coli DH5α *pBluescript*, used as a negative control, was inoculated in Luria–Bertani agar (100 μg/ml ampicillin) and incubated for 24 h at 37°C.

Genotyping

DNA extraction was performed by standard methods (3) and samples were stored at −20°C for PCR analysis. All reactions consisted of 50 ng chromosomal DNA, 50 pM of each primer, 1 U *Taq* DNA polymerase (Invitrogen Life Technologies, São Paulo, SP, Brazil), 5 mM dNTP, 1 × 10 PCR buffer, and 1.5 mM $MgCl_2$ in a total volume of 25 μl. The products were submitted to electrophoresis in 1 or 1.5% weight/volume agarose gel (Invitrogen) in Tris–acetate–ethylenediaminetetraacetic acid (Invitrogen) buffer, at 60 V for 2 h and the amplicons were visualized with an ultraviolet transilluminator. Negative and positive controls were used in every set of reactions.

ltx genotyping was performed with the primers LtxA (5′-TCCATATTAAATCTCC TTGT-3′) and LtxB (5′–AACCTGATAA CAGTATT-3′) (55). The temperature profile in the thermocycler was an initial denaturation at 97°C for 4 min and 25 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

cdt genotyping was performed with primer pairs CDT1 (5′-GTCAACGAA GCTCCCAAGAACGCT-3′ and 5′-TGTA CCTCTCCTTAGATCCATCCT-3′), CDT2 (5′-ACGTTCACCACCCAGTAACAGGAT-3′ and 5′-TTCGCCATAACGTCAACGTAG TAA-3′), and CDT3 (5′-ATCCCGGGAAAC GGGTAACGG-3′ and 5′-ACAACAAGCA ACGTTAGGTCTGTG-3′) (28). The temperature profile was an initial denaturation at 97°C for 5 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. When using CDT2 and CDT3 primer pairs, the annealing temperature was increased to 60°C.

cdtABC detection was performed with primers CDTA forward (5′-GGTTTA GTGGCTTGT-3′) and CDTC reverse (5′-CCTGATTTCTCCCCA-3′) (17). The amplification conditions were an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min.

Serotype-specific genotyping was performed with primers P15 (5′-TGGG TCATGGGAGGTACTCC-3′) and P16 (5′-GCTAGGAACAAAGCAGCATC-3′) for serotype **a**; P11 (5′-TCTCCACCAT TTTTGAGTGG-3′) and P14 (5′-AR AAYTTYTCWTCGGGAATG-3′) for serotype **b**; P12 (5′-GAAACCACTTC TATTTCTCC-3′) and P14 (5′-ARAAY TTYTCWTCGGGAATG-3′) for serotype **c**; P17 (5′-TGGAACGGGTATGG GAACGG-3′) and P18 (5′-GGATGCT CATCTAGCCATGC-3′) for serotype **d**; P19 (5′-ATTCCAGCCTTTTGGTTCTC-3′) and P20 (5′-TGGTCTGCGTTGT AGGTTGG-3′) for serotype **e**; and P13 (5′-CCTTTATCAATCCAGACAGC-3′) and P14 (5′-ARAAYTTYTCWTCGGG AATG-3′) for serotype **f** (27). The amplification conditions were an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. DNA from the 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 3632 (serotype **f**) were used as controls.

Detection of SNP in *cdtB*

Seven *A. actinomycetemcomitans* strains exhibiting different levels of toxicity to epithelial cells were selected for sequencing part of *cdtB* to detect the SNP (38). Primers Mut281cdtBfw (5′-CGCCAACA GAACCGACTCA-3′) and Mut281cdtBrv (5′-CGCAAGCTAATACGTGGAGGAG-3′) were used to amplify a 317-bp fragment of

cdtB. The amplification conditions were an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 40°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. 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 ampicillin. The inserts were sequenced using MegaBACE 1000 (GE Healthcare, Piscataway, NJ) following the manufacturer's recommendations using DYEnamic ET Dye Terminator kit (with Thermo Sequenase™ II DNA Polymerase). Data were analysed with the aid of MegAlign (DNASTAR version 3.03–2002).

Growth cell inhibition activity

Cell lysates

Cells of *A. actinomycetemcomitans* isolates and *E. coli* DH5α *pBluescript* (negative control) were suspended in phosphate-buffered saline (pH 7.5, 0.8% NaCl), washed once and lysed by sonication for 40 s at maximum potency, four times. The lysates were centrifuged at 14,000 ***g*** for 5 min at 4°C and sterilized by filtration through 0.22 μm pore size filters. The protein concentration of the cell lysates was determined using the Bradford protocol (6). Aliquots were kept frozen at −80°C until use.

Cell culture

Chinese hamster ovary cells (CHO) and oral carcinoma cells (KB) were inoculated into Dulbecco's modified Eagle's minimal essential medium (DMEM-AS; Embriocare, Campinas, SP, Brazil) containing penicillin (1.664 U/ml), streptomycin (745 U/ml), and sodium bicarbonate (2.20 g/l) supplemented with 10% volume/volume fetal calf serum (DMEM-AS) and incubated for 48 h at 37°C in 5% CO_2.

Toxic activity assay

CHO cells suspensions (4×10^2 cells) were added to 60 × 15-mm plates and incubated for 24 h. Aliquots containing 1, 2, and 5 μg/ml of each bacterial lysate were added to the cells and kept on ice for 15 min. Cells were washed once with DMEM and 4 ml DMEM-AS were added, followed by incubation for 7 days in 5% CO_2 at 37°C.

The colonies were fixed with 10% (volume/volume) formaldehyde for 1 min, stained with 10% (weight/volume) crystal violet and counted; the number of surviving cells was calculated. All experiments were performed in triplicate and the toxic activity was determined as mean percentage of CHO cell growth inhibition in relation to the control without the addition of bacterial lysate. The toxic activity was considered positive when inhibitory values were higher than those obtained for lysate of *E. coli* DH5α *pBluescript* (negative control).

Determination of CDT activity by blockade of cell cycle at G2

To compare the data concerning inhibition of colony formation by *A. actinomycetemcomitans* CDT (15) and CDT activity, the percentages of cells in G2 phase of mitosis were evaluated by flow cytometric analysis after interaction with lysates of strains JP2, FDC Y4, and HK 1651. KB cells were plated in 96-well plates (1×10^4 cells/well) in DMEM-AS and incubated for 2 h. *A. actinomycetemcomitans* lysates were added at protein concentrations of 100, 200, and 400 μg/ml. Cells were kept on ice for 15 min, centrifuged twice to remove the supernatant and added with 100 μl of medium. After incubation for 48 h in 10% CO_2 at 37°C, the nuclei were stained with propidium iodide (Sigma-Aldrich, St Louis, MO) (10,000 events) and cell cycle arrest was determined using a FacsCalibur flow cytometer (Becton Dickinson, Flanklin Lakes, NJ). As a negative control, lysate from *E. coli pBluescript* was also tested.

Statistical analysis

Statistical analysis was performed using the STATGRAPHICS PLUS 5.0 program (StatPoint Technologies, Warrenton, VA). Analysis of variance was employed to assess differences in the toxic activity according to serotype, CDT genotype, or periodontal condition of the host. Significance was considered for $P < 0.05$.

Results

Genotyping

Among 41 clinical isolates, DNA from only four (9.7%), obtained from Brazilian subjects with aggressive periodontitis, resulted in a PCR product of approximately 492 bp, which was indicative of the 530-bp deletion, and were classified as having a JP2-like genotype (Table 1). A representative gel of these results is shown in Fig. 1.

Analysis of the *cdt* region allowed the determination of the presence of *cdtABC*, the variability in the *cdtA* upstream region (*cdt* genotyping) and the SNP in amino acid 281 in *cdtB*. *cdtABC* were detected in all tested strains, except for one isolate from Japan. However, *cdtB* was amplified for this strain and sequencing showed that it was conserved in this genome (Table 1).

cdt genotyping using primer pairs located upstream from *cdtA*, as shown in Fig. 2, allowed classification of reference strains FDC Y4, HK 1651, and ATCC 29523 as expected (28). Ten different *cdt* genotypes were detected, as shown in Tables 1 and 2 and Fig. 3. Consequently, 11 *cdt* genotypes were reported, because type 4 described by Kaplan et al. (28) was not found in the studied isolates. PCR with DNA of three (7.3%) of the 41 isolates studied did not yield amplicons. Most isolates were classified as genotype *cdt* 3 (n = 13, 31.7%).

Serotype-specific genotyping, as shown in Fig. 4, was performed by PCR using six primer pairs. Five isolates (12.9%) were classified as serotype **a**, three from Brazilian subjects with healthy periodontal status, one from Sweden, and one from Kenya. The nine isolates (21.9%) classified as serotype **b** included Brazilian isolates from aggressive periodontitis (n = 4) and chronic periodontitis (n = 1), three isolates from Sweden, and one from Japan. Twenty-six isolates (63.4%) were classified as serotype **c**. Most of the Brazilian clinical isolates (n = 17, 68.0%) were classified as serotype **c**, including isolates from aggressive periodontitis (n = 8), chronic periodontitis (n = 4), and healthy subjects (n = 5). Serotypes **d**, **e**, and **f** were not detected and one clinical isolate from Japan could not be classified. Data are shown in Table 1.

SNP analysis of H281 *cdtB*

The sequencing data analysis at position H281 of *cdtB* gene of seven *A. actinomycetemcomitans* clinical isolates representative of each serotype and toxic activity was compared with the data of two reference strains, FDC Y4 [GenBank accession no. AF006830, Mayer et al. (34)] and HK 1651 (http://www.oralgen.lanl.gov). The analysis of SNP at amino acid 281 of CDTB showed that the mutation of H281 to R281, previously associated with increased CDT activity, was found in five of the seven tested clinical isolates (Table 1).

Toxic activity assay

The toxic activity assessed by inhibition of cell growth was compared with the blockade of the cell cycle in G2 promoted by CDT in cell lysates of strains FDC Y4, JP2, and HK 1651. The percentage of cells

Table 1. Distribution of clinical isolates and reference strains according to serotypes, *ltx* promoter genotype, *cdt* genotype, toxic activity in CHO cells, geographical location, and periodontal condition of the host

				Host	
	ltx promoter[1]	CDT genotype	Toxic activity (%)	Geographical location	Periodontal condition
Serotype a					
ATCC 29523	1022	2	0–25		(Blood)
C2-1[2]	1022	3	25–35	Brazil	H
C21-1	1022	3	0–25	Brazil	H
C27-1	1022	3	0–25	Brazil	H
707	1022	3	>65	Kenya	P
162	1022	10	35–65	Sweden	AP
Serotype b					
FDC Y4[3]	1022	1	25–35	USA	AP
HK 1651[3]	492	2	35–65	Denmark/Ghana	AP
JP_2	492	2	35–65	USA	AP
1515	1022	2	25–35	Japan	P
S06-7	492	11	>65	Brazil	AP
G111-1[3]	492	6	>65	Brazil	AP
A26	492	2	35–65	Brazil	AP
G121-2[3]	492	7	35–65	Brazil	AP
161	1022	3	>65	Sweden	AP
181	1022	1	>65	Sweden	AP
194	1022	8	35–65	Sweden	AP
G104-2	1022	6	35–65	Brazil	CP
Serotype c					
1522[2]	1022	8	0–25	Japan	P
1523[2]	1022	8	25–35	Japan	P
1519	1022	8	35–65	Japan	P
443	1022	0	>65	Kenya	P
721	1022	3	35–65	Kenya	P
722	1022	9	35–65	Kenya	P
S3-2	1022	3	>65	Brazil	AP
G92-1	1022	9	>65	Brazil	AP
G101-2	1022	7	>65	Brazil	AP
G121-3[2]	1022	2	>65	Brazil	AP
Aa1	1022	5	35–65	Brazil	AP
S3-6	1022	6	35–65	Brazil	AP
S4-1	1022	3	35–65	Brazil	AP
S4-6	1022	10	35–65	Brazil	AP
254	1022	3	25–35	Sweden	AP
183	1022	11	35–65	Sweden	AP
250	1022	3	35–65	Sweden	AP
G59-3	1022	6	>65	Brazil	CP
G42-3	1022	3	35–65	Brazil	CP
G103-1	1022	7	35–65	Brazil	CP
G105-2	1022	1	35–65	Brazil	CP
C12-3	1022	3	>65	Brazil	H
C51-2	1022	6	>65	Brazil	H
C54-2	1022	8	>65	Brazil	H
C19-4	1022	0	35–65	Brazil	H
C34-2	1022	3	35–65	Brazil	H
Not serotypeable					
1525[2,4]	1022	0	35–65	Japan	P

[1]The fragments of approximately 492 base pairs are indicative of JP2 clone whereas amplification with DNA of other strains resulted in a fragment of approximately 1022 base pairs.
[2]Mut 281*cdtB* CGT (R).
[3]Mut 281*cdtB* CAT (H).
[4]cdtABC not detected, but presence of *cdtB* was confirmed.
H, healthy periodontium, no alveolar bone loss; P, periodontitis; AP, aggressive periodontitis; CP, chronic periodontitis.

at G2 when 200 μg/ml of lysate of strain FDC Y4 were used was 26.9%, whereas these values for strain HK 1651 and JP2 were 82.7% and 76.0% (Fig. 5). The comparison of these data with those obtained by estimation of growth inhibition (Table 1), revealed that in both assays strain FDC Y4 was less toxic to the epithelial cells than strains JP2 or HK 1651, suggesting that the inhibitory effect revealed by epithelial cell growth could be used to assess CDT activity.

Most of the studied strains were able to inhibit CHO cell growth when 5 μg/protein of bacterial lysate/plate were used, as shown in Table 1. Only three clinical isolates (7.3%) and reference strain ATCC 29523 were equal or less toxic to CHO cells than the *E. coli* used as a negative control. Twenty clinical isolates (48.8%) and reference strains HK 1651 and JP2 presented moderate toxic activity (between 35 and 65%). Fourteen (34.2%) clinical isolates exhibited strong toxic activity (>65% of growth inhibition). Two of four JP2-like isolates were moderately toxic and two were highly toxic to CHO cells. However, three of eight isolates from healthy subjects were also considered highly toxic (Table 3).

The clinical isolates considered highly toxic (cell growth inhibition >65%) were equally distributed among serotypes **b** and **c** (4/12 serotype **b** and 9/26 serotype **c**) but were less frequently found among serotype **a** strains (1/5 serotype **a** strains). The frequency of strains showing cell growth inhibition (0–25%) similar to the negative control (*E. coli* DH5α *pBluescript*) or low levels (25–35%) was higher in serotype **a** strains (3/5 strains) than among the other serotypes (2/12 strains of serotype **b**, and 3/26 strains of serotypes **c**).

Variance analysis indicated an absence of differences in toxic activity in CHO cells of *A. actinomycetemcomitans* isolates with the studied periodontal condition of the hosts, or with the *cdt* genotypes. There was a tendency to an increased toxic activity (mean value ± SD in assay using 5 μg protein/ml) of strains belonging to serotype **c** (58.24 ± 21.13%) and **b** (51.57 ± 17.91%) when compared with serotype **a** strains (38.38 ± 24.09%). However, analysis of variance revealed that the differences between groups were not significant ($P = 0.097$).

The toxic activity varied among strains independently from the *ltx* genotype, as previously reported for serotype **b** reference strains FDC Y4 and HK 1651. In addition, there was no correlation between toxic activity and SNP at amino acid 281.

Discussion

Serotype **b** strains *of A. actinomycetemcomitans* are more frequently found in subjects with localized aggressive periodontitis (1). A genetic variation in the promoter of the operon *ltx* can be detected among serotype **b** isolates from people of African descent (2, 8, 24), and this genotype, associated with the production of high titers of leukotoxin, is closely related to aggressive periodontitis in these populations. However, other aspects correlating virulence and *A. actinomycetemcomitans* serotypes are poorly understood.

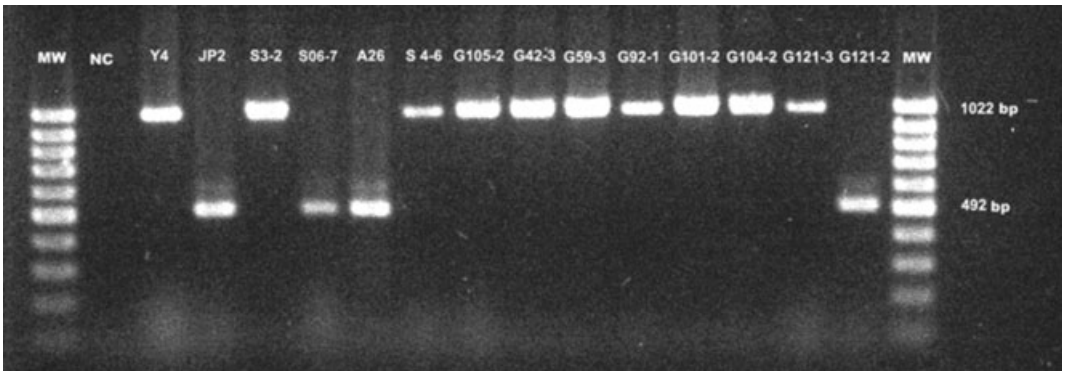

Fig. 1. Gel electrophoresis representative of polymerase chain reaction products of the *ltx* operon promoter region using DNA from *Aggregatibacter actinomycetemcomitans*. NC, negative control. Positive controls JP2 and FDC Y4 (Y4) strains. Remaining wells represent clinical isolates from Brazilian patients. In MW: 100 bp Mass Ladder (Invitrogen).

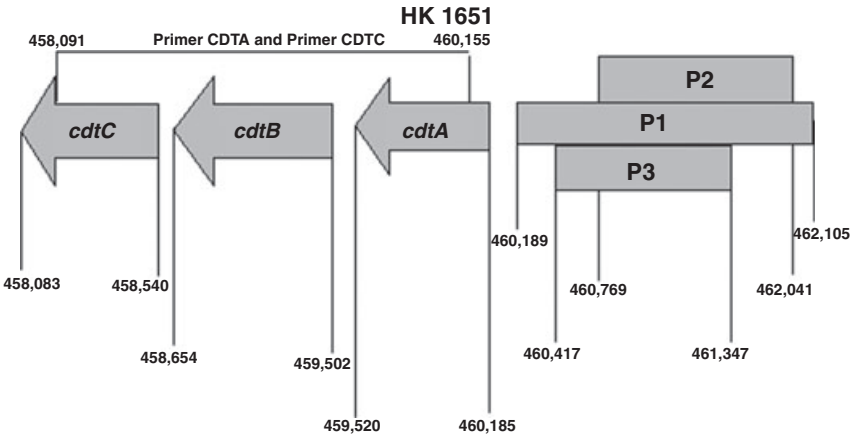

Fig. 2. Location of the polymerase chain reaction (PCR) primer pairs CDT1, CDT2, and CDT3 used to amplify the upstream region from the *cdtABC* gene cluster in the genome of strain HK 1651 (http://www.oralgen.lanl.gov). PCR primer pair CDT1 amplified a 1893-base pair (bp) PCR product for strain HK 1651 (667 of strain FDC Y4); PCR primer pair CDT2 amplified a 1272-bp fragment and CDT3 a 932-bp fragment for strain HK 1651, but resulted in no amplicon for strain FDC Y4.

In the present study, genotyping and CHO cell growth inhibition were determined in 41 isolates of *A. actinomycetemcomitans* of different periodontal conditions and from geographically diverse regions.

Most studied isolates from Brazil were classified as serotype **c** (68%), including those from subjects with aggressive periodontitis, as previously reported by Tinoco et al. (50) and Teixeira et al. (48). Serotype **b** strains were strongly correlated with patients with aggressive periodontitis and included the genotype associated with the increased production of leukotoxin. On the other hand, isolates from healthy subjects belonged to serotypes **a** or **c** (Table 1), differing from other data obtained in Brazil, which associated serotype **b** strains with health (48).

Several studies have associated the highly toxic JP2-like *A. actinomycetemcomitans* serotype **b** with subjects with aggressive periodontitis (7, 14, 25, 55). Phylogenetic analysis suggested that the highly conserved JP2 clone of *A. actinomycetemcomitans* originated in the northern Mediterranean part of Africa and spread to West Africa and to other continents (22). However, distinct lineages of the JP2-like clone could still be associated with Arabic and African populations and most likely developed tropism for these hosts. Nevertheless, in the present study, one of the subjects harboring the JP2-like clone was Caucasian, possibly through the intense miscegenation occurring in Brazil (21).

Population genetic studies by multilocus enzyme electrophoresis and multilocus sequence typing revealed that *A. actinomycetemcomitans* shows a basically clonal population structure and except for the JP2 clone, no difference in disease association among the distinct evolutionary lineages was demonstrated (23, 29). However, differing from what is expected for an opportunistic pathogen, the high prevalence of serotype **c** and JP2-like and non-JP2-like serotype **b** strains in patients with aggressive periodontitis may suggest the presence of other bacterial virulence factors in these isolates and/or a variation

Table 2. Number of isolates according to *cdt* genotype and size of amplicons (approximated base pair values obtained from gels)

	CDT1	CDT2	CDT3	
Genotype *cdt*	667 bp, 1893 bp, and 4.4 kb	1272 bp	932 bp	Number of samples $n = 41$ (%)
0[1]	–	–	–	3 (7.3)
1[1]	667	–	–	2 (4.9)
2[1]	1893	1272	932	3 (7.3)
3[1]	–	–	932	13 (31.7)
4[1]	4.4	1272	932	0
5	1893	–	–	1 (2.4)
6	667	–	932	5 (12.2)
7	–	1272	–	3 (7.3)
8	4.4	–	932	5 (12.2)
9	4.4	–	–	2 (4.9)
10	1893	–	932	2 (4.9)
11	–	1272	932	2 (4.9)
				Total 41

[1]*cdt* genotype described by Kaplan et al. (28).
–, absence of polymerase chain reaction products.
bp, base pairs; kb, kilobases.

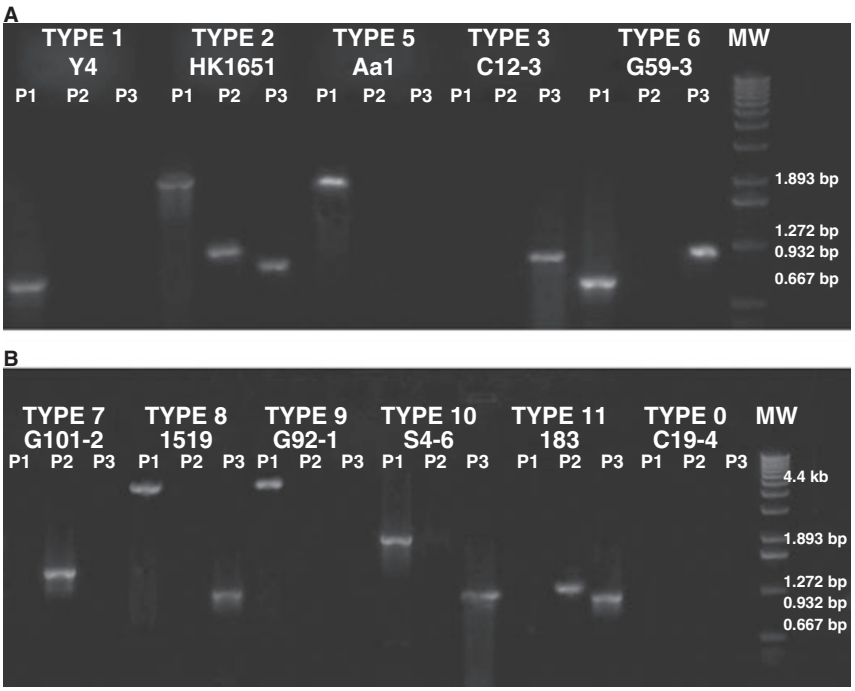

Fig. 3. Gel electrophoresis of polymerase chain reaction products using DNA of *Aggregatibacter actinomycetemcomitans* as template and primer pairs CDT1, CDT2, and CDT3, homologous to the *cdtA* upstream region, representing the *cdt* genotypes 1, 2, 5, 3, and 6 (in A) and 7 to 10 and 0 (in B). In MW: 1 kilobase Mass Ladder (Invitrogen).

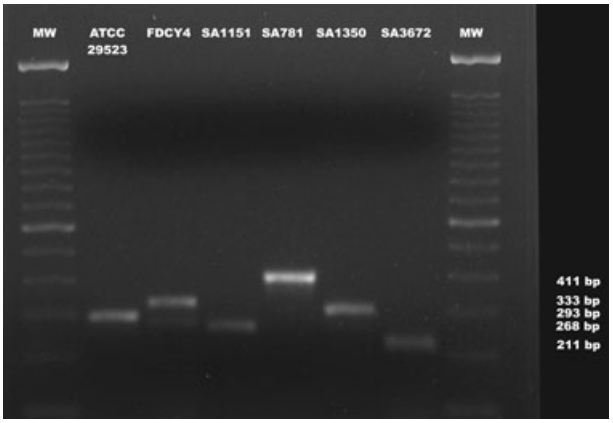

Fig. 4. Gel electrophoresis of polymerase chain reaction products obtained with primer pairs for serotype genotyping and template DNA of strains ATCC 29523 (serotype **a**), FDC Y4 (Y4) (serotype **b**), SA 1151 (serotype **c**), SA 781 (serotype **d**), SA 1350 (serotype **e**), and SA 3632 (serotype **f**). In MW: 100 base pairs Mass Ladder (Invitrogen).

in host response (21, 28, 50). Variable regions in the genome, such as those encoding genomic islands, would probably account for differences in the pathogenic potential of a strain. In *A. actinomycetemcomitans* FDC Y4, the *cdt* genes are adjacent to a region named GIY4-1, which is absent in HK 1651 and exhibits several features of being originated by horizontal transfer (16, 34). Base composition analyses of the genome of strain HK 1651 revealed that besides the *cdt* cluster, seven other regions could also be considered genomic islands, including the tight adherence gene cluster (*tad*), the *O*-antigen biosynthesis transport gene cluster, the leukotoxin gene cluster, the lipooligosaccharide biosynthesis enzyme cluster, and three uncharacterized regions (http://www.oralgen.lanl.gov). We therefore aimed to evaluate variations among strains in the *cdt* locus and to compare them with the CDT activity. In addition, the *O*-antigen biosynthesis cluster and the leukotoxin cluster were analysed by using serotype-specific primers and a pair that amplified the *ltx* promoter region, respectively.

Although the association of CDT with pathogenesis is not fully understood, this toxin may represent a bacterial adaptation affecting the interaction between the bacterium and the host immune system in chronic diseases (40). CDT targets several types of cells including epithelial cell, fibroblasts (4), and cells of the immune system such as antigen-presenting cells (51), lymphocytes (42), and macrophages (18), and was correlated with persistent infection (40, 43).

All the studied strains presented the *cdtABC* genes, except for a Japanese isolate in which *cdtB* was detected and shown to be conserved by sequencing (data not shown). The absence of *cdtABC* in *A. actinomycetemcomitans* isolates was reported for isolates in Japan (52) and China (33, 47), but our data confirmed that *cdtABC* are frequently found in the genome of *A. actinomycetemcomitans* (17, 33, 52) indicating that other aspects besides the absence of *cdtABC* genes would be influencing the toxic activity of the isolates. The *cdtA* upstream region is extremely variable (28, 34, 43) and in this study 10 distinct *cdt* genotypes were determined. Diversity in this region was higher than reported by Kaplan et al. (28). Strains of the JP2-like clone were classified in distinct *cdt* genotypes, showing further diversity within this lineage, as also shown by others (28).

CDT activity was evaluated by the number of surviving CHO cells after contact with the bacterial lysate. Hence, because this study estimated cell viability after contact with cell lysate, the number of surviving cells was dependent not only on the interruption of cell cycle but also on apoptosis (44, 45). As previously shown, strain JP2 showed a higher activity than FDC Y4 (17, 34). The similar activity of strains HK 1651 and JP2, higher than FDC Y4, was also confirmed by determination of the percentage of cells in G2 phase by flow cytometry. Hence, the data obtained by cell cycle analysis correlated with the growth inhibitory effect determined in these strains, the latter could be considered CDT activity, as assessed by others (15, 38).

Three isolates (7.3%) and ATCC 29523 did not exhibit toxic activity higher than 25% when the highest protein concentration/well was used (5 μg/ml). Among these, two samples were isolated from healthy subjects. On the other hand, six of

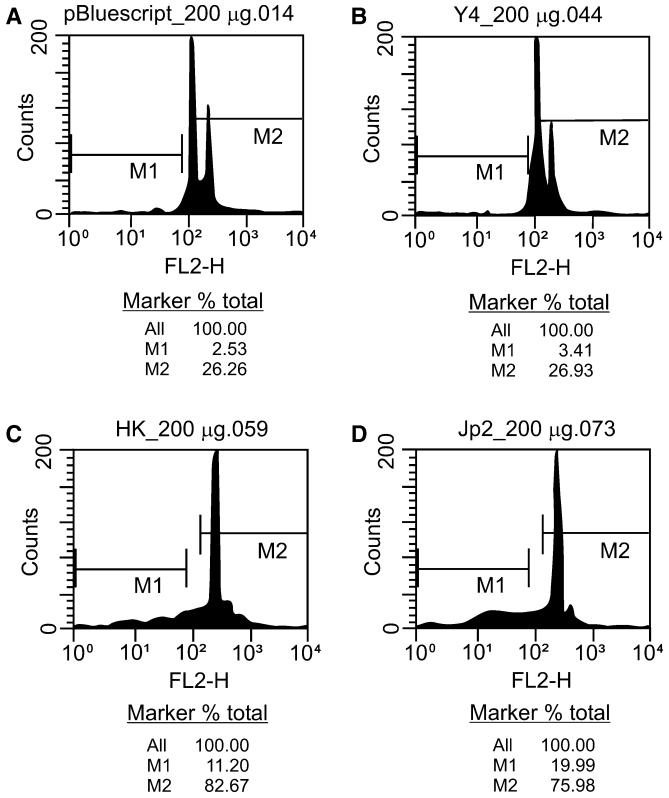

Fig. 5. Effect of *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin (CDT) on the cell cycle of KB cells. KB cells (1×10^4) were treated with *A. actinomycetemcomitans* FDC Y4 (B), HK 1651 (C), and JP2 (D) lysates in a concentration of 200 μg/ml for 48 h post intoxication. DNA profiles were obtained by fluorescence-activated cell sorting analysis of propidium iodide-stained nuclei. G2 phase ($n = 4$) was represented in M2. *Escherichia coli pBluescript* lysate (A) was used as a negative control.

12 isolates from patients with aggressive periodontitis inhibited growth of more than 65% of CHO cells.

Although high levels of toxicity to CHO cells were not associated with a particular serotype, 57.1% isolates of serotype **a** presented low toxicity to CHO cells (<35% inhibition) whereas the highly toxic strains (inhibition >65%) were more often found among serotype **b** and **c** isolates.

The higher toxic activity could be the result of higher expression of the toxin by different strains or of an increased activity promoted by amino acid changes in these proteins. Recently, Nishikubo et al. (38) demonstrated that a single nucleotide polymorphism in the amino acid at position 281 of *A. actinomycetemcomitans cdtB* gene would be involved with increased titer of the CDT holotoxin. When H281, as observed in strain FDC Y4, is substituted by an arginine, the CDT activity is elevated. However, analysis of the *cdtB* sequences of seven isolates with different inhibitory activities on CHO cells revealed no correlation between toxicity and this SNP. Four isolates, belonging to serotypes **a** or **c**, presented an arginine at position 281 (R281), but only one of them was highly inhibitory to CHO cells. On the other hand, the two serotype **b** clinical isolates exhibited H281 (similar to the serotype **b** reference strains FDC Y4 and HK 1651) and were moderately or highly toxic to CHO cells. Consequently, despite the low number of studied isolates, it seems that other factors besides the single nucleotide polymorphism (amino acid 281) in *cdtB* are involved in the differences in inhibitory activity to CHO cells promoted by *A. actinomycetemcomitans*. Other virulence factors, such as CagE and GroEL-like protein (36, 49), as well as differences in the expression of *cdt*, may account for these differences in the cytotoxic effect. The latter hypothesis is emphasized by the observation that strains HK 1651 and FDC Y4, both presenting H281 in *cdtB* differ in their ability to block the cell cycle at G2 phase, which is characteristic of CDT activity, as shown by flow cytometry.

Early studies by multilocus enzyme electrophoresis (23) and phylogenetic analysis based on sequences of housekeeping genes (22) demonstrated that serotypes **b** and **c** are more closely related than serotype **a** isolates. Recently, the comparison of the genome of strains of different serotypes revealed that the genome of a serotype **c** isolate was largely collinear with HK 1651 (serotype **b**), whereas the serotype **a** isolate showed a greater rearrangement (11). It would be reasonable to expect that certain virulence factors are encoded and expressed in a more similar way among strains of serotypes **b** and **c** than in serotype **a** isolates. The association of serotypes **b** and **c** with aggressive disease and of serotype **a** with healthy subjects, as shown in several studies including this one, and the differences in prevalence of the highly cytotoxic strains among serotypes reported here, reinforce the hypothesis that serotype **b** and **c** isolates of *A. actinomycetemcomitans* are more virulent than serotype **a** strains.

Table 3. Percentage of Chinese hamster ovary (CHO) cell inhibition promoted by 5 μg/ml cell lysates of *Aggregatibacter actinomycetemcomitans* according to the geographical location of strain isolation and periodontal condition of the host

	CHO cell inhibition					
Origin	Periodontal condition	0–25%[1]	25–35%	35–65%	>65%	Total
Sweden	Aggressive periodontitis	0	1	4	2	7
Japan	Aggressive periodontitis	1	2	2	0	5
Kenya	Periodontitis	0	0	2	2	4
Brazil	Healthy	2	1	2	3	8
	Chronic periodontitis	0	0	4	1	5
	Aggressive periodontitis	0	0	6	6	12
Total		3	4	20	14	41
Reference strains		(ATCC 29523)	(FDC Y4)	(HK 1651) (JP2)	0	4

[1]Similar to *Escherichia coli* DH5α *pBluescript* control.

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SA 3632 (serotype **f**). This study was supported by CAPES and FAPESP grant 03/08598–0.

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