

Population Structure and Genetic Diversity of *Actinobacillus actinomycetemcomitans* Strains Isolated from Localized Juvenile Periodontitis Patients

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The phylogeny of 20 *Actinobacillus actinomycetemcomitans* strains isolated from patients with localized juvenile periodontitis (LJP) was investigated by using partial sequence analysis of 16S rRNA genes, arbitrarily primed PCR (AP-PCR), and four additional PCR assays that amplified polymorphic regions in the leukotoxin (*lkt*), cytolethal distending toxin (*cdt*), major fimbrial subunit (*flp-1*), and serotype-specific O polysaccharide gene clusters. Our analysis also included four strains isolated from healthy subjects and nine reference strains. We found that *A. actinomycetemcomitans* strains comprised three major phylogenetic lineages. One lineage consisted of serotype b strains, a second lineage consisted of serotype c strains, and a third lineage consisted of serotype a, d, e, and f strains. 16S rRNA sequences within each lineage were highly conserved (<1% base substitutions), whereas sequences between lineages were exceptionally divergent (1.9 to 5.0% substitutions). Two strains exhibited 16S rRNA sequences that were even more distantly related to those of the three major lineages (2.7 to 6.7% substitutions), indicating that additional minor lineages or variants exist. The distribution of 16S rRNA sequences and *lkt*, *cdt*, *flp-1*, and AP-PCR genotypes was consistent with a clonal population structure, with little evidence of assortative recombination between strains of different serotypes. Strains from all three major lineages were recovered from LJP patients, suggesting that phylogenetically diverse strains of *A. actinomycetemcomitans* carry pathogenic potential.

Actinobacillus actinomycetemcomitans is a gram-negative, capnophilic coccobacillus that colonizes the human oral cavity (24). *A. actinomycetemcomitans* is implicated in the etiology of localized juvenile periodontitis (LJP), a severe and rapid form of periodontal disease that affects more than 70,000 predominantly African-Americans in the United States annually (42). Infrequently, *A. actinomycetemcomitans* can enter the submucosa and cause extraoral infections including endocarditis, bacteremias, and abscesses (21). *A. actinomycetemcomitans* produces adhesive pili which cause tight adherence to surfaces such as glass, plastic, and saliva-coated hydroxyapatite (8, 20), a property that has been shown to play an important role in the ability of *A. actinomycetemcomitans* to colonize the mouths of rats (10). *A. actinomycetemcomitans* also produces several potential virulence factors, including leukotoxin, a 116-kDa secreted lipoprotein that specifically kills human polymorphonuclear leukocytes and macrophages (26), and cytolethal distending toxin, an immunosuppressive factor which is homologous to a family of toxins expressed by several other gram-negative bacteria (37). Little is known about the role of these and other potential virulence factors in the pathogenesis of LJP (11).

Numerous studies on the genetic diversity of *A. actinomycetemcomitans* strains isolated in Europe, Japan, and the United States have been reported (1, 3, 6, 12, 14–16, 28, 29, 34, 40, 43). These studies showed that there is considerable genetic variation among natural isolates of *A. actinomycetemcomitans* and suggested that variation in the virulence potential of strains

may exist. For example, studies on the distribution of *A. actinomycetemcomitans* serotype a to e strains showed that serotype a and b strains are frequently isolated from LJP patients, serotype c strains are more common in nonoral infections and in healthy people, and serotype d and e strains are rare in all populations (1, 15, 28, 43). Studies on the leukotoxin gene showed that strains carrying a 530-bp deletion in the leukotoxin gene promoter that results in 10- to 20-fold-higher levels of leukotoxin production are almost exclusively isolated from LJP patients, suggesting that these strains may have an increased virulence potential (3, 12, 14–16). Other studies have shown an association between disease and certain restriction fragment length polymorphism genotypes (6) or arbitrarily primed PCR (AP-PCR) genotypes (1, 28).

The serologic specificity of *A. actinomycetemcomitans* serotype a to e strains is defined by five structurally and antigenically distinct O polysaccharide (O-PS) components of their respective lipopolysaccharide molecules (27, 31, 32). We recently identified a sixth *A. actinomycetemcomitans* serotype, designated serotype f, which synthesizes an O-PS molecule that is structurally distinct from those of serotype a to e strains but which shows immunological cross-reactivity with the O-PS molecule of serotype b strains (22). We also analyzed the DNA sequence of the gene cluster responsible for the synthesis of serotype f O-PS and showed that it is homologous to the serotype b, c, and e O-PS gene clusters (22). The aim of the present study was to clarify the genetic relationship between serotype f strains and the five previously identified *A. actinomycetemcomitans* serotypes. This report describes our analysis of 33 *A. actinomycetemcomitans* clinical isolates and reference strains, including strains representing all six of the currently

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TABLE 1. *A. actinomycetemcomitans* strains analyzed in this work

Name	Serotype	Strain ^a						Subject ^b				
		<i>lkt</i>	<i>flp-1</i>	<i>cdt</i>	AP-PCR	16S rRNA type	GenBank accession no.	Source or reference	Age (yr)	Sex	Race	LJP
DF2200	a	652	o	2	1	I	AF355071	This work	27	F	Afr	+
NJ3800	a	652	1	2	2	II	AF355072	This work	7	F	Cau	+
NJ4600	a	652	o	2	1	I	AF355078	This work	15	M	Afr	+
NJ5000	a	652	2	2	1	I	AF355079	This work	22	F	Afr	+
NJ6000	a	652	o	2	1	I	AF355080	This work	13	F	Afr	-
ATCC 29523	a	652	2	2	1	I	M75038	ATCC	?	M	?	?
SUNYab75	a	652	o	o	1	I	AF355067	SUNY	17	F	Cau	+
NJ1000	b	JP2	1	1	3	II	AF355081	This work	18	F	Afr	+
NJ3500	b	JP2	1	2	3	II	AF355082	This work	22	F	Afr	+
NJ4500	b	JP2	1	2	3	II	AF355083	This work	19	F	Afr	+
NJ5400	b	JP2	1	2	3	II	AF355084	This work	11	F	Afr	+
NJ8800	b	JP2	1	2	3	II	AF355085	This work	6	F	Afr	+
NJ5800	b	JP2	1	2	3	II	AF355086	This work	13	M	Afr	-
JP2	b	JP2	1	2	4	II	AF355087	Penn	25	M	Afr	+
NK1651	b	JP2	1	2	3	II	AGSP	SUNY	18	M	Afr	+
ATCC 29524	b	652	1	o	3	II	M75037	ATCC	50	F	Afr	?
Y4	b	652	1	1	3	II	M75035	ATCC	?	?	?	+
DF2000	c	652	o	4	3	III	AF355076	This work	8	F	Afr	+
DF2300	c	652	2	o	3	III	AF355077	This work	40	M	Afr	+
NJ2300	c	652	2	4	5	III	AF355088	This work	30	M	Cau	+
NJ2700	c	652	2	4	3	III	AF355068	This work	18	M	Afr	+
NJ6700	c	652	2	3	3	IV	AF355089	This work	18	F	Afr	+
NJ7200	c	652	2	4	3	III	AF355090	This work	19	F	Afr	+
NJ7500	c	652	2	3	5	III	AF355091	This work	30	M	Afr	+
NJ9300	c	652	2	o	3	III	AF355092	This work	14	F	Afr	+
NJ8400	c	652	2	o	3	III	AF355093	This work	22	M	Cau	-
Aa307	c	652	2	nt	nt	III	AF355094	SUNY	60	M	Cau	-
IDH781	d	652	2	nt	nt	I	AF355069	SUNY	10	M	Cau	-
NJ9500	e	652	o	nt	6	V	AF355073	This work	22	M	Cau	-
IDH1705	e	652	2	nt	nt	I	AF355066	SUNY	32	M	Cau	-
CU1000	f	652	o	3	1	I	AF355070	22	13	F	Afr	+
NJ9100	f	652	o	3	1	I	AF355074	This work	21	M	His	+
NJ9200	f	652	2	3	1	I	AF355075	This work	23	M	Asi	+

^a Boldfacing denotes the 20 LJP strains isolated in our laboratory. The serotype, *lkt*, *flp-1*, *cdt*, and AP-PCR columns show the results of PCR assays described in the text. o, no PCR product; nt, not tested. 16S rRNA types correspond to those shown in Fig. 2. The GenBank accession number is for the 16S rRNA sequence. AGSP, Actinobacillus Genome Sequencing Project (www.genome.ou.edu/act.html). Source: ATCC, American Type Culture Collection; SUNY, J. Zambon and H. Reynolds, State University of New York at Buffalo; Penn. E. T. Lally, University of Pennsylvania, Philadelphia.

^b Race: Cau, Caucasian; Afr, African or African-American; His, Hispanic; Asi, Asian. LJP: +, LJP; -, periodontally healthy. ?, Not known.

known serotypes, by using four genotype-specific PCR assays and a comparison of partial 16S rRNA sequences.

MATERIALS AND METHODS

***A. actinomycetemcomitans* strains.** The strains analyzed in this study are listed in Table 1. Strains designated NJ, DF, and CU were isolated from cheek, tongue, saliva, or subgingival plaque samples obtained from subjects in New York City and Newark, N.J., between 1992 and 2000. All subjects were unrelated except for one pair of half siblings (NJ5500 and NJ5800). LJP was diagnosed according to the criteria described by Baer (2). Strains were identified as *A. actinomycetemcomitans* by the following criteria: (i) characteristic star-positive colony morphology on AAGMBV selective medium (9); (ii) CO₂ required for growth; (iii) catalase positive; (iv) tight adherence to surfaces when grown in broth (9); (v) PCR amplification products with primers that hybridize to *lktCA* of strain JP2 (25), *cdtABC* of strain Y4 (37), and *flp-1* or *flp-2* of strain CU1000 (20); and (vi) amplification of a diagnostic PCR product in an *A. actinomycetemcomitans* serotype-specific PCR assay (22). *Haemophilus aphrophilus* strains NJ8500 and NJ8700 were distinguished from *A. actinomycetemcomitans* by the following criteria: (i) yellowish colony on AAGMBV selective medium; (ii) catalase negative; (iii) absence of PCR products with *lktCA*, *cdtABC*, and *flp-1/flp-2* primers; (iv) absence of a PCR product in the *A. actinomycetemcomitans* serotype-specific PCR assay; (v) 16S rRNA sequence >97% identical to that of *H. aphrophilus*

ATCC 33389 (5) (see Fig. 2); and (vi) presence of an *fmr* homologue >97% identical to that of *H. aphrophilus* ATCC 13252 (13). Strains were preserved as -70°C frozen stocks in 10% dimethyl sulfoxide and grown in Trypticase soy broth supplemented with yeast extract and glucose as previously described (9).

PCR assays. Genomic DNA was prepared from test strains by using a DNeasy tissue kit (Qiagen, Valencia, Calif.) according to the instructions provided by the manufacturer. PCR primers were purchased from Biosource International (Camarillo, Calif.) or Integrated DNA Technologies (Coralville, Iowa). Unless otherwise indicated, PCR assays were performed in 0.5-ml thin-walled tubes containing 10 µl of 10× PCR buffer (500 mM KCl; 100 mM Tris-HCl, pH 8.3; 150 mM MgCl₂), 1 mM deoxynucleoside triphosphates, 2.5 U of *Taq* DNA polymerase (Applied Biosystems, Foster City, Calif.), and 5 ng of genomic DNA as a target in a 100-µl reaction volume overlaid with 100 µl of mineral oil. A 10-µl aliquot of each PCR was electrophoresed through a 1.0 or 1.8% agarose gel in 1× TAE buffer or a 5% acrylamide-0.17% bisacrylamide gel in 1× Tris-borate-EDTA buffer, and the PCR products were visualized by staining with ethidium bromide (36). The specific primers and reaction conditions for the PCR assays were as follows.

(i) **Serotype assay.** Four PCRs were used to amplify unique sequences in the gene clusters responsible for the synthesis of serotype a-, b-, c-, d-, e-, and f-specific O-PS molecules as previously described (22). The first PCR utilized four primers that hybridized to the serotype b-, c-, and f-specific O-PS gene clusters, resulting in the amplification of 333-, 268-, and 232-bp PCR products for

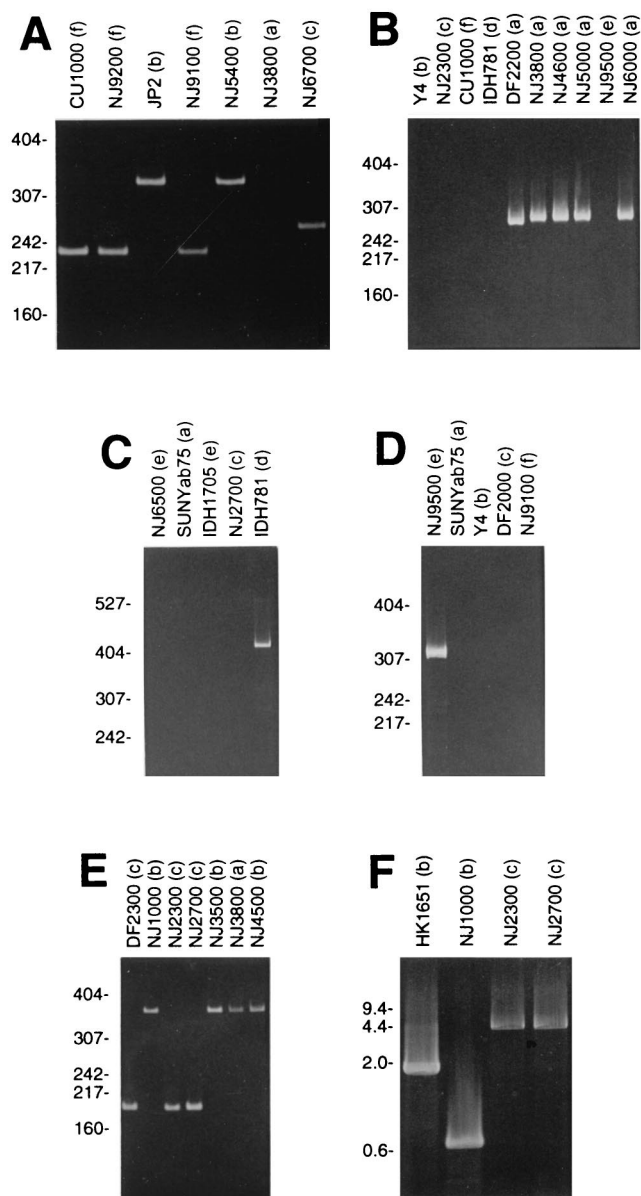


FIG. 1. Examples of PCR assays utilized in this study. Lane labels indicate strain name with serotype in parentheses. (A) Serotype b-, c-, and f-specific quadruplex reaction; (B) serotype a-specific reaction; (C) serotype d-specific reaction; (D) serotype e-specific reaction; (E) *flp-I*-specific reaction; (F) *cdt*-specific reaction. Panel F shows the PCR products amplified with primer pair P1. The sizes of molecular weight standards run in an adjacent lane are shown on the left. Molecular weight standard sizes are given in base pairs for panels A to E and in kilobase pairs for panel F. Panels A to E show 5% acrylamide gels; panel F shows a 1.0% agarose gel.

serotype b, c, and f strains, respectively (Fig. 1A). The remaining three PCRs amplified sequences in the serotype a-, d-, and e-specific O-PS gene clusters individually, resulting in the synthesis of 293-, 411-, and 311-bp PCR products for serotype a, d, and e strains, respectively (Fig. 1B to D). Assays were validated with DNAs from strains SUNYab75, Y4, NJ2700, IDH781, NJ9500, and CU1000 (serotypes a to f, respectively). Seroclassification of serotype a, b, c, and f strains was confirmed by enzyme-linked immunosorbent assay with anti-serotype a-, b-, c-, and f-specific rabbit antisera as previously described (22).

(ii) *lkt* assay. The characteristic 530-bp deletion present in the leukotoxin promoter of highly leukotoxic strains was detected by using a PCR assay as

previously described (16). PCR products were analyzed on 5% acrylamide gels as described above. Strains that amplified a 504-bp PCR product were classified as strain JP2-like (highly leukotoxic); those that amplified a 1,034-bp PCR product were classified as strain 652-like (minimally leukotoxic).

(iii) *flp-I* assay. PCR primers 5-AGCCACTTTCATCTGCGCTGG-3 and 5-ATTGCATCTAGTCTCTTTTCG-3 were used to amplify a region located ca. 0.5 kb upstream from the *flp-I* start codon (corresponding to bp 1448 to 1626 in GenBank accession no. AB005741). Thirty PCR cycles of 94°C, 55°C, and 72°C (1 min each) were performed. Strains that amplified a 363-bp PCR product were classified as type 1 (strain NK1651-like); those that amplified a 179-bp PCR product were classified as type 2 (strain 304-like) (Fig. 1E).

(iv) *cdt* assay. PCR primer pairs P1 (5-GTCAACGAAGCTCCCAAGAAGCCT-3 and 5-TGTACCTCTCCTTAGATCCATCCT-3), P2 (5-ACGTTCCACCA CCCAGTAACAGGAT-3 and 5-TTCGCCATAACGTCAACGTAGTAA-3), and P3 (5-ATCCGGGAAACGGGTAACGG-3 and 5-ACAACAAGCAACG TTAGGTCTGTG-3) were used to amplify three regions upstream from the *cdtABC* cytolethal distending toxin gene cluster. PCR primer pair P1 amplified the region corresponding to bp 1 to 667 of the *cdtABC* sequence of strain Y4 reported by Sugai et al. (37). PCR primer pair P1 amplified a 1,893-bp PCR product in strain NK1651, due to a 1,226-bp sequence located 607-bp upstream from the *cdtA* start codon in strain NK1651 (Actinobacillus Genome Sequencing Project, www.genome.ou.edu/act.html) that is absent in strain Y4 (37). PCR primer pairs P2 and P3 hybridized within the 1,226-bp fragment present in strain NK1651. PCRs were incubated at 94°C for 5 min, followed by 30 cycles of PCR at 94°C, 55°C, and 72°C (1 min each), with a final elongation step of 72°C for 5 min. When primer pairs P2 and P3 were used, the annealing temperature was changed from 55 to 60°C. Four *cdt* genotypes (designated 1 to 4 in Table 1) were defined as follows: strains that amplified a 667-bp PCR product with primer pair P1 were classified as type 1; those that amplified a 1,893-bp PCR product with primer pair P1, a 1,272-bp PCR product with primer pair P2, and a 932-bp PCR product with primer pair P3 were classified as type 2; those that amplified a 932-bp PCR product with primer pair P3 were classified as type 3; those that amplified a 4.4-kb PCR product with primer pair P1, a 1,272-bp PCR product with primer pair P2, and a 932-bp PCR product with primer pair P3 were classified as type 4 (Fig. 1F).

(v) AP-PCR assay. AP-PCR (41) was performed by using Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia, Piscataway, N.J.) according to the instructions supplied by the manufacturer. AP-PCR products were analyzed on 5% polyacrylamide gels as described above. Three of the six AP-PCR primers supplied with the kit (RAPD analysis primers 2 [5-GTTTCGCTCC-3], 3 [5-GT AGACCCGT-3], and 4 [5-AAGAGCCCGT-3]) amplified AP-PCR products that were clearly and reproducibly polymorphic in the strains tested. These included 780- and 470-bp AP-PCR products amplified by RAPD analysis primer 2, a 625-bp AP-PCR product amplified by RAPD analysis primer 3, and a 530-bp AP-PCR product amplified by RAPD analysis primer 4. Six AP-PCR genotypes (designated 1 through 6 in Table 1) were defined as follows: AP-PCR genotype 1 amplified the 625- and 780-bp AP-PCR products; AP-PCR genotype 2 amplified all four AP-PCR products; AP-PCR genotype 3 amplified the 470- and 530-bp AP-PCR products; AP-PCR genotype 4 amplified the 530-bp AP-PCR product; AP-PCR genotype 5 amplified the 470-, 530-, and 780-bp AP-PCR products; and AP-PCR genotype 6 amplified the 470-bp AP-PCR product.

16S rRNA sequencing. PCR primers 5-GCTTAACACATGCAAGTCGG-3 and 5-TGTGGCAGGAGTTAGCCG-3 were used to amplify a 478-bp region of the *A. actinomycetemcomitans* 16S rRNA gene corresponding to coordinates 46 to 523 of the *Escherichia coli* 16S rRNA sequence (5). The ends of the PCR products were made flush by using *E. coli* DNA polymerase (Klenow fragment) and ligated into the *EcoRV* site of plasmid LITMUS28 (New England Biolabs, Beverly, Mass.) as described previously (36). Plasmid DNAs were prepared by using a QIAprep Spin Miniprep kit (Qiagen) and subjected to DNA sequence analysis on an ABI model 377 PRISM automated sequencer. Sequences were aligned by using CLUSTALW 1.8 (19), and the alignment was manually adjusted to minimize the number of multistate positions.

Phylogenetic analysis. Phylogenetic trees were generated by using the software package PAUP 4.0b4a (Phylogenetic Analysis Using Parsimony), developed by D. L. Swofford (Sinauer Associates, Sunderland, Mass.), by using the heuristic search algorithm with default options. Attributes included those listed in Table 1, as well as the genotype of the *flp-I* structural gene as determined by DNA sequence analysis (J. B. Kaplan, unpublished data). The results of *lkt*, *flp-I*, and *cdt* PCR assays which failed to produce a PCR product were considered unknown. Strains carrying the JP2-like leukotoxin promoter were topologically constrained to a single clade, since these strains have previously been shown to be monophyletic (12, 14, 16). The phylogram was rooted between the serotype {b,c} and {a,d,e,f} clusters, since these two groups have previously been shown

11111111111111111111222222222222 222222233333333333444444 444 444444 444

Species	Strain	7777 7888888899 992233344555688888899900001111222234 56677890112333345778034445 566 777777 789 0247 801278912 67090257845672347890391789016856721 74589546370367836471524565 824 012347 890
<i>Actinobacillus actinomycetemcomitans</i>	I DF2200 (a)	TGAA-GAAGCTTCT-TGAGACTGGGACCGAGGGGACTCCTTAGGAGAGGT-GAGGAGTGACGCCGATAAGCTAGATT-GGT-CATGCA-CAT
	NJ4600 (a)
	NJ5000 (a)
	NJ6000 (a)
	SUNYab75 (a)
	ATCC29523 (a)
	IDH781 (d)
	IDH1705 (e)
	CU1000 (f)
	NJ9100 (f)G
	NJ9200 (f)A
	II Y4 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ3800 (a)A.....G.....T.....T.....A.....G.....AT.....A..
	NK1651 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ1000 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ3500 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ4500 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ5400 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ5800 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	JP2 (b)A.....G.....T.....T.....A.....G.....AT.....A..
	NJ8800 (b)A.....G.....T.....T.....A.....G.....AT.....A..
ATCC29524 (b)A.....G.....T.....T.....A.....G.....AT.....A..	
ATCC29522 (b)G.....T.....T.....A.....G.....AT.....A..	
III NJ8400 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
NJ2700 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
DF2000 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
DF2300 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
NJ7200 (c)	C.....T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
NJ7500 (c)T.AGTACTA.C.....A.....A.....T.T.....C.A.AG.....A..	
NJ9300 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
Aa307 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....A..	
NJ2300 (c)T.AGTACTA.....AG.....A.....T.T.....C.A.AG.....T..	
NCTC9710 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AGA.....A..	
IV NJ6700 (c)T.AGTACTA.....A.....A.....T.T.....C.A.AG.....T.....CCGA-.....AACGATC	
V NJ9500 (e)	...AGT.AGTACTA.....A.....A.....T.A.T.....G.GAT.....TCAC.	
<i>Haemophilus aphrophilus</i>	NJ8700 NJ8300 ATCC33389T.AGTACTA.....G.....A.....T.T.....C.A.AG.....G.T.....T.A.....C.T.G.....ATCT.AGT-TACTA.....G.....A.....T.T.....C.A.AG.....AG.....T.....T.A.....C.T.....GCAA.....ATCT.AGTACTA.....-G.....A.....T.T.....C.A.AG.....AG.....T.....T.A.....C.T.....GCAA.....ATC
<i>Haemophilus paraphrophilus</i>	ATCC29241T.AGTACTA.....-G.....A.....T.T.....-A.AG.AG.....AT.....T.A.....-.....C.T.G.....CAA.....ATC
<i>Haemophilus influenzae</i>	ATCC33391A.....G.....TTA.....A.T.....TGAGA.....AG.....G.....TTGT.A.TATA.....G.....AT.....A.....CAT.....A..
<i>Pasteurella multocida</i>	NCTC10322A.....G.....T.....A.....G.....TGTATTCT.AGGGT.....ACA.....G.....T.....T.A.TATA.....G.....A.....GAT-A.....AGTCAT
<i>Haemophilus segnis</i>	ATCC33393A.....G.....T.....A.....G.....A.....GC.A.....AG.....G.....T.....T.A.....G.....-A.....CGA.....G.....-CGATC

FIG. 2. Polymorphisms in 16S rRNA sequences. Numbers on top correspond to coordinates of *E. coli* 16S rRNA sequence (5). Letters in parentheses indicate serotype. Bases occupying the indicated positions in the *A. actinomycetemcomitans* DF2200 sequence are shown. Gaps (indicated by hyphens) were inserted after *E. coli* positions 77, 93, 250, 455, 469, and 477. A dot indicates that the base is identical to that in DF2200. Roman numerals indicate five 16S rRNA types discussed in the text. The GenBank accession numbers for strains not listed in Table 1 are as follows: *A. actinomycetemcomitans* ATCC 29522, M75036; NCTC9710, M75039; *H. aphrophilus* NJ8700, AF355095; NJ8300, AF355096; ATCC 33389, M75041; *H. paraphrophilus*, M75042; *H. influenzae*, M35019; *P. multocida*, M35018; and *H. segnis*, M75043.

to be genotypically distinct based on genomic DNA fingerprinting, restriction fragment length polymorphisms, and multilocus enzyme electrophoresis (15, 16, 34). Rooting the tree with a hypothetical distant outgroup placed the root on branch 4 of the phylogram (Fig. 2), which did not alter the conclusions drawn from the analysis. Because of insufficient data or a high level of divergence or assortative recombination, strains NJ3800 and NJ9500 could not be confidently positioned in the phylogram and were therefore excluded from the analysis.

Nucleotide sequence accession numbers. The GenBank accession numbers of the 16S rRNA sequences reported in this study are listed in Table 1 and in the legend of Fig. 2.

RESULTS

***A. actinomycetemcomitans* strains.** Table 1 lists the 33 *A. actinomycetemcomitans* strains analyzed in this study. A total of 20 of these strains were isolated in our laboratory from specimens obtained from localized juvenile periodontitis patients (referred to as the 20 LJP strains and indicated by boldface in Table 1). Our analysis also included four *A. actinomycetemcomitans* strains isolated from the oral cavities of healthy subjects (NJ5800, NJ6000, NJ8400, and NJ9500) and nine reference strains (ATCC29523, SUNYab75, ATCC29524, Y4, JP2, NK1651, Aa307, IDH781, and IDH1705).

Serotypes. All 33 *A. actinomycetemcomitans* strains amplified one of the six O-PS gene clusters in the serotype-specific PCR assay (Table 1). The serotypes of the nine reference

strains were consistent with the reported seroclassifications of these strains (7, 34, 35, 43). Among the 20 LJP strains, four were serotype a (20%), five were serotype b (25%), eight were serotype c (40%), and three were serotype f (15%). The four strains isolated from healthy subjects included one each of serotypes a, b, c, and e.

Leukotoxin promoter. All 33 *A. actinomycetemcomitans* strains amplified a PCR product in the leukotoxin-specific PCR assay. Of the 33 strains, 8 carried the 530-bp deletion in the leukotoxin promoter (highly leukotoxic), including 5 of the 20 LJP strains, 1 strain isolated from a healthy subject (NJ5800), and reference strains JP2 and NK1651. All highly leukotoxic strains were serotype b, but two serotype b reference strains (ATCC 29524 and Y4) contained the minimally leukotoxic promoter. It has been shown that highly leukotoxic strains of *A. actinomycetemcomitans* are frequently isolated from LJP patients (12, 14), Africans and African-Americans (3, 12), and younger subjects (12). In our study, highly leukotoxic strains comprised 25% of the isolates from LJP patients (5 of 20) and 25% of the isolates from healthy subjects (1 of 4). All six of these highly leukotoxic strains were isolated from African-Americans. Subjects harboring highly leukotoxic strains were younger than those harboring minimally leukotoxic strains (14.8 versus 20.3 years old), but the difference was

not statistically significant ($P = 0.18$, unpaired two-tailed t test).

***flp-1* upstream region.** Comparison of the DNA sequence upstream from *flp-1* in strain NK1651 (serotype b) (Actinobacillus Genome Sequencing Project [www.genome.ou.edu/act.html]) with the sequence upstream from the *flp-1* gene of strain 304 (serotype a) (18) revealed a 184-bp sequence located 0.5 kb upstream from the *flp-1* start codon in strain NK1651 that was absent in strain 304. The effect of this 184-bp sequence on the expression of *flp-1* or other genes is not known. We used a PCR assay to detect the presence of this 184-bp sequence in all 33 test strains (Table 1). Eight strains failed to produce a PCR product in this assay. The structure of the *flp-1* upstream regions in these eight strains is not known. Of the remaining 25 strains, 11 contained the 184-bp sequence (designated type 1 in Table 1), including all 10 of the serotype b strains and 1 serotype a strain (NJ3800). The remaining serotype a strains and all serotype c, d, e, and f strains lacked the 184-bp sequence (type 2 in Table 1). Among the 20 LJP strains, subjects harboring strains containing the 184-bp sequence (type 1) were significantly younger than those harboring strains with the strain 304-like *flp-1* upstream region (type 2) (13.7 ± 6.2 versus 23.8 ± 8.1 years old; $P = 0.02$).

***cdt* upstream region.** Twenty-four strains could be classified as *cdt* genotypes 1 to 4 based on the *cdt*-specific PCR assay (Table 1). The distribution of the four *cdt* genotypes correlated with serotypes: *cdt* genotype 1 was found only in serotype b strains; *cdt* genotype 2 was found only in serotype a and b strains; *cdt* genotype 3 was found only in serotype c and f strains; and *cdt* genotype 4 was found only in serotype c strains. Over one half of the strains belonged to the *cdt* genotype 2. The structure of the *cdtABC* upstream region in the five strains that failed to amplify a PCR product in this assay is not known.

AP-PCR. Of the 33 test strains, 30 were analyzed by using AP-PCR genotyping. The distribution of the six detected AP-PCR genotypes correlated with serotypes: AP-PCR genotypes 1 and 2 were found only in serotype a and f strains, and AP-PCR genotypes 3, 4 and 5 were found only in serotype b and c strains. AP-PCR genotype 6 was found in only one serotype e strain. A total of 25 of the 30 strains tested (83%) belonged to AP-PCR genotypes 1 and 3.

16S rRNA sequencing. Figure 2 shows the nucleotide polymorphisms between coordinates 46 and 523 in the 16S rRNA sequences of 35 *A. actinomycetemcomitans* strains and seven strains of closely related bacteria. This section of the 16S rRNA molecule is the most informative for determining the relatedness of bacteria because it contains several variable regions (30, 39). The 35 *A. actinomycetemcomitans* strains include the 33 strains listed in Table 1, as well as reference strains ATCC29522 (serotype b) and NCTC9710 (serotype c). Nucleotide sequences were derived from published sources (5) or were determined in our laboratory. The nucleotide sequence of the 16S rRNA gene from strain NK1651 was obtained from the Actinobacillus Genome Sequencing Project (www.genome.ou.edu/act.html). There are 92 sites that are polymorphic in at least one of the 42 strains shown in Fig. 2, and 52 sites that are polymorphic in at least one of the 35 *A. actinomycetemcomitans* strains. Three major *A. actinomycetemcomitans* 16S rRNA types were observed (labeled I to III in Fig. 2). Nucleotide sequences were highly conserved within

each 16S rRNA type (0.0 to 0.8% nucleotide substitutions) but highly divergent between types (1.9 to 5.0% substitutions). In addition, strains NJ6700 (serotype c) and NJ9500 (serotype e) contained 16S rRNA sequences (designated types IV and V in Fig. 2, respectively) that were more divergent (2.7 to 6.7% substitutions) from those of the three major types. The distribution of the three major 16S rRNA types was serotype specific: type I included all serotype a, d, e, and f strains; type II included all serotype b strains and one serotype a strain (NJ3800); and type III included all serotype c strains. The sequence of the type III 16S rRNA contained a large region (coordinates 77 to 226 in Fig. 2) that was homologous to the corresponding region of the 16S rRNAs of *H. aphrophilus* and *H. paraphrophilus*. This region corresponds to the stem regions designated helices 6 to 18 of the 16S rRNA molecule (helix numbering according to Van de Peer et al. [39]). The 16S rRNA sequence from strain NJ6700 contained this same region, but it also contained a sequence homologous to helix 18 of the *H. segnis* 16S rRNA (coordinates 455 to 490). The 16S rRNA sequence from strain NJ9500 contained a sequence homologous to helix 6 of the *H. aphrophilus* and *H. paraphrophilus* 16S rRNAs (coordinates 77 to 92) and a sequence homologous to helix 18 of the *Pasteurella multocida* 16S rRNA (coordinates 455 to 478). These interspecific homologous regions may have resulted from retention of homologous sequences in some strains with concomitant DNA sequence evolution in other strains or from horizontal gene transfer between *A. actinomycetemcomitans* and other closely related oral bacteria (38).

Phylogenetic analysis of *A. actinomycetemcomitans* strains. Phylogenetic analysis of *A. actinomycetemcomitans* strains based on the six attributes listed in Table 1 (serotype, 16S rRNA type, and *lkt*, *flp-1*, *cdt*, and AP-PCR genotypes) and the genotype of the *flp-1* structural gene (Kaplan, unpublished) was performed, and three clusters were observed (Fig. 3). One cluster consisted of serotype b strains, a second cluster consisted of serotype c strains, and a third cluster consisted of serotype a, d, e, and f strains. Serotype b and c strains formed distinct, monophyletic groups within the serotype {b,c} cluster (branches 1 and 2, respectively, in Fig. 3). Our analysis also revealed that serotype f strains were closely related to serotypes a, d, and e, confirming that serotype f strains constitute a unique *A. actinomycetemcomitans* serotype distinct from serotype b (22). The tree shown in Fig. 3 also suggests that the phylogenetic relatedness of strains within the serotype {a,d,e,f} cluster is {a,d} {e,f} (branches 3 and 4, respectively), although more data are needed to confidently resolve the branching pattern in this part of the tree. Highly leukotoxic strains of *A. actinomycetemcomitans* that carry the 530-bp deletion in the leukotoxin promoter (JP2-like strains in Table 1) have previously been shown to comprise a monophyletic group based on multilocus enzyme electrophoresis and genomic DNA fingerprinting (16), DNA sequence analysis (14), and AP-PCR genotyping (12). Our data suggest that the 530-bp deletion in the leukotoxin promoter occurred in a branch within the serotype b cluster (branch 5 in Fig. 3). Our findings are in good agreement with previous phylogenetic analyses of *A. actinomycetemcomitans* (15, 34).

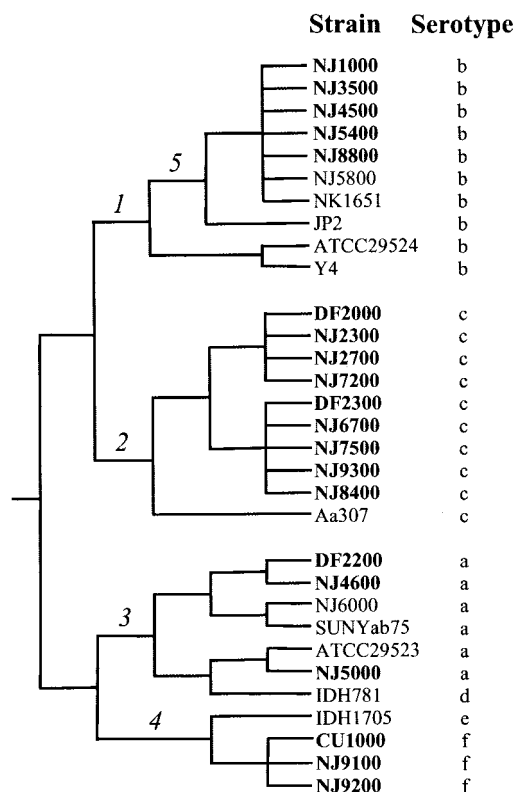


FIG. 3. Phylogeny of *A. actinomycetemcomitans*. Boldface type indicates strains isolated from LJP patients in this study. Numbers 1 to 5 denote branches discussed in the text. Horizontal branch lengths are not to scale and do not metrically represent evolutionary change.

DISCUSSION

The distribution of PCR genotypes among the strains analyzed in our study was consistent with previous population genetic analyses of *A. actinomycetemcomitans* which showed considerable genetic diversity both between and within serotype a to e strains, with little evidence of assortative recombination between strains of different serotypes (1, 7, 15, 28, 34). Our data revealed five types of *A. actinomycetemcomitans* 16S rRNA sequences which displayed 0.0 to 6.7% sequence divergence, higher than the amount of interspecific 16S rRNA divergence observed many other bacteria. 16S rRNA sequence divergence of 1 to 2% corresponds to a species-level difference for most bacteria (30). We recently identified a strain isolated from the gingival pocket of a rapidly progressive periodontitis patient in Japan (strain OEN12-5, serotype e) that contains a 16S rRNA sequence identical to that of strain NJ9500 (also serotype e) (J. B. Kaplan and S. Kokeguchi, unpublished data), suggesting that 16S rRNA type V constitutes a distinct and geographically diverse *A. actinomycetemcomitans* clade. Because of the small number of strains analyzed to date, it is possible that other *A. actinomycetemcomitans* 16S rRNA types exist.

Three of the five *A. actinomycetemcomitans* 16S rRNA types identified in our study (types III to V) contained sequences that were homologous to sequences in the 16S rRNAs of *H. aphrophilus* and *H. paraphrophilus*, a finding consistent with the close evolutionary relationship of *A. actinomycetemcomitans* to

these species (4, 5, 17, 33). Our PCR genotyping data, however, indicated that *A. actinomycetemcomitans* 16S rRNA types III to V were more closely related to other *A. actinomycetemcomitans* strains than to *H. aphrophilus* or *H. paraphrophilus*. 16S rRNA type III strains include type strain NCTC9710, which has also been shown to be more closely related to other *A. actinomycetemcomitans* strains than to *H. aphrophilus* or *H. paraphrophilus*, based on DNA-DNA hybridization (4, 33) and partial *infB* sequence comparisons (17). These data support the classification of 16S rRNA types III to IV as *A. actinomycetemcomitans*.

Previous studies showed that highly leukotoxic (JP2-like) strains are isolated almost exclusively from LJP patients (12, 14), suggesting that these strains may have an increased virulence potential. Highly leukotoxic strains also comprise approximately one-half of the strains isolated from Africans and African-Americans but only 0 to 2% of strains isolated from Europeans and Asians (3, 12, 14, 15). These findings led to the hypothesis that LJP may have two different etiologies and epidemiologies: in Caucasians LJP may be associated with diverse *A. actinomycetemcomitans* clones acting as opportunistic pathogens, whereas in some Africans and African-Americans LJP may be associated with JP2-like strains acting as exogenous pathogens (23). It is also possible that JP2-like strains are acting as opportunistic pathogens that are simply more prevalent in Africans and African-Americans, who have an increased susceptibility to LJP for other reasons, perhaps related to host determinants. In the present study, all six JP2-like strains were isolated from African-Americans, but JP2-like strains constituted less than one-third (5 of 16) of the strains isolated from African-American LJP patients. The fact that phylogenetically diverse strains were recovered from LJP patients in our study supports the hypothesis that *A. actinomycetemcomitans* plays the role of an opportunistic pathogen (14, 15, 34). Since all data presented to date have been cross-sectional in nature, it is impossible to determine the temporal relationship of JP2-like strains to disease. This question can only be resolved in a prospective longitudinal study that measures the frequency of conversion from health to disease in a sufficient number of healthy subjects who carry JP2-like and non-JP2-like strains. Resolution of this question could have implications for treatment strategies (23).

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REFERENCES

- Asikainen, S., C. Chen, and J. Slots. 1995. *Actinobacillus actinomycetemcomitans* genotypes in relation to serotypes and periodontal status. *Oral Microbiol. Immunol.* **10**:65-68.
- Baer, P. N. 1971. The case for periodontitis as a clinical entity. *J. Periodontol.* **42**:516-519.
- Contreras, A., T. Rusitanonta, C. Chen, W. G. Wagner, B. S. Michalowicz, and J. Slots. 2000. Frequency of 530-bp deletion in *Actinobacillus actinomycetemcomitans* leukotoxin promoter region. *Oral Microbiol. Immunol.* **15**:338-340.
- Coykendall, A. L., J. Setterfiels, and J. Slots. 1983. Deoxyribonucleic acid relatedness among *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and other *Actinobacillus* species. *Int. J. Syst. Bacteriol.* **33**:422-424.
- Dewhirst, F. E., B. J. Paster, I. Olsen, and G. J. Fraser. 1992. Phylogeny of

- 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. *J. Bacteriol.* **174**:2002–2013.
6. DiRienzo, J. M., J. Slots, M. Sixou, M.-A. Sol, R. Harmon, and T. L. McKay. 1994. Specific genetic variants of *Actinobacillus actinomycetemcomitans* correlate with disease and health in a regional population of families with localized juvenile periodontitis. *Infect. Immun.* **62**:3058–3065.
 7. Dogan, B., M. Saarela, and S. Asikainen. 1999. Genotyping of *Actinobacillus actinomycetemcomitans* serotype d isolates based on polymerase chain reaction. *Oral Microbiol. Immunol.* **14**:387–390.
 8. Fine, D. H., D. Furgang, J. B. Kaplan, J. Charlesworth, and D. H. Figurski. 1999. Tenacious adhesion of *Actinobacillus actinomycetemcomitans* strain CU1000 to salivary-coated hydroxyapatite. *Arch. Oral Biol.* **44**:1063–1076.
 9. Fine, D. H., D. Furgang, H. C. Schreiner, P. Goncharoff, J. Charlesworth, G. Ghazwan, P. Fitzgerald-Bocarsly, and D. H. Figurski. 1999. Phenotypic variation in *Actinobacillus actinomycetemcomitans* during laboratory growth: implications for virulence. *Microbiology* **145**:1335–1347.
 10. Fine, D. H., P. Goncharoff, H. Schreiner, K. M. Chang, D. Furgang, and D. Figurski. 2001. Colonization and persistence of rough and smooth colony variants of *Actinobacillus actinomycetemcomitans* in the mouths of rats. *Arch. Oral Biol.* **46**:1065–1078.
 11. Fives-Taylor, P. M., D. M. Meyer, K. P. Mintz, and C. Brissette. 1999. Virulence factors of *Actinobacillus actinomycetemcomitans*. *Periodontol.* **2000** **20**:136–167.
 12. Haraszthy, V. L., G. Hariharan, E. M. B. Tinoco, J. R. Cortelli, E. T. Lally, E. Davis, and J. J. Zambon. 2000. Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. *J. Periodontol.* **71**:912–922.
 13. Hattori, T., K. Takahishi, T. Nakanishi, H. Ohta, K. Fukui, S. Taniguchi, and M. Takigawa. 1996. Novel FNR homologues identified in four representative oral facultative anaerobes: *Capnocytophaga ochracea*, *Capnocytophaga sputigena*, *Haemophilus aphrophilus*, and *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **137**:213–220.
 14. Haubek, D., J. M. DiRienzo, E. M. B. Tinoco, J. Westergaard, N. J. Lopez, C.-P. Chung, K. Poulsen, and M. Kilian. 1997. Racial tropism in a highly toxic clone of *Actinobacillus actinomycetemcomitans* associated with juvenile periodontitis. *J. Clin. Microbiol.* **35**:3037–3042.
 15. Haubek, D., K. Poulsen, S. Asikainen, and M. Kilian. 1995. Evidence for absence in northern Europe of especially virulent clonal types of *Actinobacillus actinomycetemcomitans*. *J. Clin. Microbiol.* **33**:395–401.
 16. Haubek, D., K. Poulsen, J. Westergaard, G. Dahlén, and M. Kilian. 1996. Highly toxic clone of *Actinobacillus actinomycetemcomitans* in geographically widespread cases of juvenile periodontitis in adolescents of African origin. *J. Clin. Microbiol.* **34**:1576–1578.
 17. Hedegaard, J., H. Okkels, B. Bruun, M. Kilian, K. K. Mortensen, and N. Nørskov-Lauritsen. 2001. Phylogeny of the genus *Haemophilus* as determined by comparison of partial *infB* sequences. *Microbiology* **147**:2599–2609.
 18. Inoue, T., I. Tanimoto, H. Ohta, K. Kato, Y. Murayama, and K. Fukui. 1998. Molecular characterization of low-molecular-weight component protein, Flp, in *Actinobacillus actinomycetemcomitans* fimbriae. *Microbiol. Immunol.* **42**:253–258.
 19. Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson. 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23**:403–405.
 20. Kachlany, S. C., P. J. Planet, R. DeSalle, D. H. Fine, D. H. Figurski, and J. B. Kaplan. 2001. *flp-I*, first representative of a new pilin gene subfamily, is required for nonspecific adherence of *Actinobacillus actinomycetemcomitans*. *Mol. Microbiol.* **40**:542–554.
 21. Kaplan, A. H., D. J. Weber, E. Z. Oddone, and J. R. Perfect. 1989. Infection due to *Actinobacillus actinomycetemcomitans*: 15 cases and review. *Rev. Infect. Dis.* **11**:46–63.
 22. Kaplan, J. B., M. B. Perry, L. L. MacLean, D. Furgang, M. E. Wilson, and D. H. Fine. 2001. Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. *Infect. Immun.* **69**:5375–5384.
 23. Kilian, M. 1998. Clonal basis of bacterial virulence, p. 131–142. *In* B. Guggenheim and S. Shapiro (ed.), *Oral biology at the turn of the century*. S. Karger AG, Basel, Switzerland.
 24. King, E. O., and H. W. Tatum. 1962. *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *J. Infect. Dis.* **111**:85–94.
 25. Kraig, E., T. Dailey, and D. Kolodrubetz. 1990. Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. *Infect. Immun.* **58**:920–929.
 26. Kolodrubetz, D., T. Dailey, J. Ebersole, and E. Kraig. 1989. Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **57**:1465–1469.
 27. Page, R. C., T. J. Sims, L. D. Engel, B. J. Moncla, B. Bainbridge, J. Stray, and R. P. Darveau. 1991. The immunodominant outer membrane antigen of *Actinobacillus actinomycetemcomitans* is located in the serotype-specific high-molecular-mass carbohydrate moiety of lipopolysaccharide. *Infect. Immun.* **59**:3451–3462.
 28. Paju, S., P. Carlson, H. Jousimies-Somer, and S. Asikainen. 2000. Heterogeneity of *Actinobacillus actinomycetemcomitans* strains in various human infections and relationships between serotypes, genotype, and antimicrobial susceptibility. *J. Clin. Microbiol.* **38**:79–84.
 29. Paju, S., M. Saarela, S. Alaluusua, P. Fives-Taylor, and S. Asikainen. 1998. Characterization of serologically nontypeable *Actinobacillus actinomycetemcomitans* isolates. *J. Clin. Microbiol.* **36**:2019–2022.
 30. Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst. 2001. Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183**:3770–3783.
 31. Perry, M. B., L. L. MacLean, J.-R. Brisson, and M. E. Wilson. 1996. Structures of the antigenic O-polysaccharides of lipopolysaccharides produced by *Actinobacillus actinomycetemcomitans* serotypes a, c, d and e. *Eur. J. Biochem.* **242**:682–688.
 32. Perry, M. B., L. L. MacLean, R. Gmür, and M. E. Wilson. 1996. Characterization of the O-polysaccharide structure of lipopolysaccharide from *Actinobacillus actinomycetemcomitans* serotype b. *Infect. Immun.* **64**:1215–1219.
 33. Potts, T. V., T. Mitra, T. O'Keefe, J. J. Zambon, and R. J. Genco. 1986. Relationships among isolates of oral haemophilii as determined by DNA-DNA hybridization. *Arch. Microbiol.* **145**:136–141.
 34. Poulsen, K., E. Theilade, E. T. Lally, D. R. Demuth, and M. Kilian. 1994. Population structure of *Actinobacillus actinomycetemcomitans*: a framework for studies of disease-associated properties. *Microbiology* **140**:2049–2060.
 35. Saarela, M., S. Asikainen, S. Alaluusua, L. Pyhälä, C.-H. Lai, and H. Jousimies-Somer. 1992. Frequency and stability of mono- or poly-infection by *Actinobacillus actinomycetemcomitans* serotypes a, b, c, d or e. *Oral Microbiol. Immunol.* **7**:277–279.
 36. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 37. Sugai, M., T. Kawamoto, S. Y. Peres, Y. Ueno, H. Komatsuzawa, T. Fujiwara, H. Kurihara, H. Suginaka, and E. Oswald. 1998. The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. *Infect. Immun.* **66**:5008–5019.
 38. Ueda, K., T. Seki, T. Kudo, T. Yoshida, and M. Kataoka. 1999. Two distinct mechanisms cause heterogeneity in 16S rRNA. *J. Bacteriol.* **181**:78–82.
 39. Van de Peer, Y., S. Chapelle, and R. De Wachter. 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Res.* **24**:3381–3391.
 40. van Steenberg, T. J. M., C. J. Bosch-Tijho, A. J. van Winkelhoff, R. Gmür, and J. de Graaff. 1994. Comparison of six typing methods for *Actinobacillus actinomycetemcomitans*. *J. Clin. Microbiol.* **32**:2769–2774.
 41. Williams, J. G. K., A. R. Kubelik, J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **22**:6531–6536.
 42. Zambon, J. J. 1985. *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J. Clin. Periodontol.* **12**:1–20.
 43. Zambon, J. J., J. Slots, and R. J. Genco. 1983. Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. *Infect. Immun.* **41**:19–27.