

Structural and Genetic Analyses of O Polysaccharide from *Actinobacillus actinomycetemcomitans* Serotype f

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The oral bacterium *Actinobacillus actinomycetemcomitans* is implicated as a causative agent of localized juvenile periodontitis (LJP). *A. actinomycetemcomitans* is classified into five serotypes (a to e) corresponding to five structurally and antigenically distinct O polysaccharide (O-PS) components of their respective lipopolysaccharide molecules. Serotype b has been reported to be the dominant serotype isolated from LJP patients. We determined the lipopolysaccharide O-PS structure from *A. actinomycetemcomitans* CU1000, a strain isolated from a 13-year-old African-American female with LJP which had previously been classified as serotype b. The O-PS of strain CU1000 consisted of a trisaccharide repeating unit composed of L-rhamnose and 2-acetamido-2-deoxy-D-galactose (molar ratio, 2:1) with the structure $\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1-3)-2-O-(}\beta\text{-D-GalpNAc)-}\alpha\text{-L-Rhap-(1}\rightarrow$. O-PS from strain CU1000 was structurally and antigenically distinct from the O-PS molecules of the five known *A. actinomycetemcomitans* serotypes. Strain CU1000 was mutagenized with transposon IS903 ϕ kan, and three mutants that were deficient in O-PS synthesis were isolated. All three transposon insertions mapped to a single 1-kb region on the chromosome. The DNA sequence of a 13.1-kb region surrounding these transposon insertions contained a cluster of 14 open reading frames that was homologous to gene clusters responsible for the synthesis of *A. actinomycetemcomitans* serotype b, c, and e O-PS antigens. The CU1000 gene cluster contained two genes that were not present in serotype-specific O-PS antigen clusters of the other five known *A. actinomycetemcomitans* serotypes. These data indicate that strain CU1000 should be assigned to a new *A. actinomycetemcomitans* serotype, designated serotype f. A PCR assay using serotype-specific PCR primers showed that 3 out of 20 LJP patients surveyed (15%) harbored *A. actinomycetemcomitans* strains carrying the serotype f gene cluster. The finding of an *A. actinomycetemcomitans* serotype showing serological cross-reactivity with anti-serotype b-specific antiserum suggests that a reevaluation of strains previously classified as serotype b may be warranted.

Actinobacillus actinomycetemcomitans is a gram-negative, capnophilic coccobacillus that colonizes the human oral cavity (25). *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 (63). *A. actinomycetemcomitans* is also a member of the clinically important HACEK group of bacteria (*Haemophilus parainfluenzae*, *Haemophilus aphrophilus*, and *Haemophilus paraprothophilus*, *A. actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) (4). These fastidious, gram-negative organisms are considered normal flora of the human oral cavity but can infrequently enter the submucosa and cause extraoral infections including endocarditis, bacteremias, and abscesses (16, 23, 29, 33, 55).

Serological investigations with polyclonal rabbit antisera have led to the recognition of five *A. actinomycetemcomitans* serotypes, designated a to e (1, 15, 39, 44, 47, 56, 64). Approximately 3 to 9% of *A. actinomycetemcomitans* isolates do not react with any of the five serotype-specific antisera (40). Dif-

ferent serotypes have been shown to be associated with periodontal health, periodontitis, and nonoral infections (1, 2, 7, 17, 65) and with different racial, ethnic, and geographic populations (18, 19, 20, 30, 31, 32), suggesting that serotype-specific strain differences may be responsible for host specificity and virulence. The immunodominant outer membrane antigen of *A. actinomycetemcomitans* is located in the high-molecular-mass O polysaccharide (O-PS) component of lipopolysaccharide (LPS) (6, 38, 49, 58). *A. actinomycetemcomitans* LPS may be an important virulence factor (9, 12, 24, 64) and vaccine candidate (51).

The chemical structures of the *A. actinomycetemcomitans* serotype a to e antigenic O-PS molecules are known (42, 43), and the DNA sequences of the genes involved in their synthesis have been described (35, 36, 50, 61, 62). In this report we analyze the O-PS antigen from *A. actinomycetemcomitans* CU1000, a strain isolated from a 13-year-old African-American female with classical symptoms of LJP (12). We show that strain CU1000 contains an O-PS component that is structurally, antigenically, and genetically distinct from those of the five known *A. actinomycetemcomitans* serotypes. We propose that strain CU1000 be assigned to a new *A. actinomycetemcomitans* serotype, designated serotype f.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *A. actinomycetemcomitans* strain CU1000 was isolated in 1992 in New York City from the first-molar site of

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a 13-year-old, medically healthy, African-American female with classical symptoms of LJP (12). Strain CU1000 was the source of LPS and O-PS for structural analysis. Strain CU1000N, an isogenic nalidixic acid-resistant derivative of CU1000 (22), was used for transposon mutagenesis. *A. actinomycetemcomitans* strains SUNYab75 (ATCC 43717 [American Type Culture Collection]), Y4 (ATCC 43718), NJ2700 (D. H. Fine laboratory collection), IDH781 (provided by H. Reynolds, State University of New York at Buffalo), and NJ9500 (D. H. Fine laboratory collection) are serotypes a, b, c, d, and e, respectively. A total of 20 *A. actinomycetemcomitans* clinical strains isolated from 20 different LJP patients (average age, 18.4 years; range, 6 to 40 years) were from the laboratory collection of D. H. Fine. All *A. actinomycetemcomitans* strains were preserved as -70°C frozen stocks in 10% dimethyl sulfoxide. Bacteria were grown on Trypticase soy agar (TSA; BD Biosystems) supplemented with 6 g of yeast extract and 8 g of glucose/liter. Broth cultures were in Trypticase soy broth (TSB) supplemented as described above. For large-scale preparation of LPS, strain CU1000 was grown in brain heart infusion broth (Difco) incubated with constant agitation (200 rpm). All *A. actinomycetemcomitans* strains were grown at 37°C in 10% CO_2 . When appropriate, media were supplemented with 3 μg of chloramphenicol, 20 μg of nalidixic acid, or 20 μg of kanamycin/ml or 2 mM isopropyl- β -D-thiogalactopyranoside (IPTG).

Escherichia coli strain SK338 (22) was used for transposon mutagenesis of *A. actinomycetemcomitans*. *E. coli* SK338 harbors plasmids pVJT128 (carrying a chloramphenicol resistance marker and the cryptic IS903 ϕ kan transposon) and pRK21761 (an *oriT*-defective derivative of RK2) and was grown at 37°C in Luria-Bertani medium (48) supplemented with chloramphenicol and kanamycin (50 $\mu\text{g}/\text{ml}$ each).

Large-scale preparation of LPS and O-PS for structural analysis. Bacterial cultures were chilled to 4°C , killed by the addition of phenol to a final concentration of 2%, and harvested by Sharples continuous centrifugation. The collected bacteria (41 g wet weight) were washed in 0.9% NaCl and extracted with 50% aqueous phenol for 12 min at 60 to 70°C . Following the addition of 2 volumes of water, the cooled mixture (4°C) was subjected to low-speed centrifugation to remove solid material and the clear extract was dialyzed against running tap water until it was free of phenol. The lyophilized dialysate was dissolved in 10 mM Tris (pH 8.0, 35 ml) and treated sequentially with 200 μg of DNase and 60 μg of RNase/ml for 3 h at 37°C , followed by proteinase K (Sigma) at a concentration of 200 $\mu\text{g}/\text{ml}$ for a further 10 h. The digest was dialyzed against tap water, and the dialysate was subjected to ultracentrifugation at $105,000 \times g$ at 4°C for 12 h to yield LPS as a gel pellet, which was dissolved in distilled water and lyophilized (402 mg). Solutions of the LPS (2% [wt/vol]) in 2% acetic acid were hydrolyzed at 100°C for 2 h, and, following removal of precipitated lipid A, the lyophilized water-soluble products were dissolved in 0.05 M pyridinium acetate (pH 4.6, 4 ml) and chromatographed on a Sephadex G-50 column (3 by 92 cm) equilibrated with the same buffer. Collected samples (10 ml) were analyzed for neutral glycoside by the phenol-sulfuric acid method (8) and for 2-amino-2-deoxyhexose (14).

NMR spectroscopy. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian Inova 500 spectrometer equipped with standard Varian software. Carbohydrate samples were exchanged twice with D_2O and redissolved in 99.9% D_2O . Measurements were made at 30°C . Proton spectra were obtained using a spectral width of 2.5 kHz and a 90° pulse. Chemical shifts are reported relative to those for internal acetone (2.225 ppm for ^1H and 31.07 ppm for ^{13}C). The two-dimensional (2D) homonuclear and heteronuclear experiments correlated spectroscopy (COSY [3]), nuclear Overhauser effect spectroscopy (NOESY [27]), and heteronuclear single quantum coherence (HSQC [26]) were performed under standard conditions.

GC. Gas chromatography (GC) was performed with a Hewlett-Packard 5890A gas chromatograph fitted with a flame ionization detector by means of a capillary column (0.25 mm by 30 m; fused silica DB-17) and a temperature program (either 180 [delay of 2 min] to 240°C at $2^{\circ}\text{C}/\text{min}$ for alditol acetate derivatives or 200 to 240°C at $1^{\circ}\text{C}/\text{min}$ for methylated alditol acetate derivatives). GC-mass spectrometry (GC-MS) was performed under the same conditions using a Hewlett-Packard 5985B GC-MS system and an ionization potential of 70 eV. Retention times of derivatives were recorded relative to those for hexa-*O*-acetyl-D-glucitol (t_G) or 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (t_{GM}).

PC. Paper chromatography (PC) was performed on water-washed Whatman 3MM paper using butan-1-ol-pyridine-water (10:3:3 by volume) for neutral sugars and butan-1-ol-ethanol-water (4:1:5; top layer) for amino sugars. Detection was made with periodate-alkaline AgNO_3 spray reagent, and mobilities are quoted relative to those for D-galactose (R_{Gal}).

Gel electrophoresis. Deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) was performed on separating gels of 14% acrylamide and 9% sodium deoxycholate. Gels were silver stained after oxidation with periodate (54).

O-PS deamination. O-PS (60 mg) was N deacetylated by treatment with 4 M NaOH (5 ml) containing NaBH_4 (3 mg) at 100°C for 3 h. The diluted solution was neutralized in the cold with acetic acid, dialyzed against water, and lyophilized. The product (42 mg) in 30% acetic acid (4 ml) was treated with 5% aqueous sodium nitrite (4 ml) and kept at 20°C for 45 min. The reaction mixture was passed through a column of Rexyn 101(H^+) ion-exchange resin (6 ml), and the concentrated eluate was collected as the void volume fraction eluting from a Sephadex G-50 column (20 mg).

Small-scale preparation of LPS for enzyme-linked immunosorbent assays (ELISAs). LPS was prepared from test strains using the hot aqueous phenol method (57) with slight modifications. Briefly, cells from TSB cultures were washed two times with phosphate-buffered saline and pelleted in a 1.5-ml microcentrifuge tube ($5,000 \times g$ for 1 min). One hundred microliters of water (68°C) per 10 mg of cells (wet weight) was added, and the cell pellet was homogenized with a disposable polypropylene pellet pestle (Kontes). An equal volume of phenol (68°C) was added, and the tube was then vortexed for 5 s and incubated at 65°C for 15 min and at 10°C for 10 min. After centrifugation ($5,000 \times g$ for 5 min), the upper aqueous phase containing crude LPS was transferred to a new tube and diluted with 3 volumes of water.

ELISAs. All of the following steps were carried out at room temperature. The wells of a flat-bottom Nunc-Immuno microtiter plate were coated (in triplicate) with 50 μl of LPS solution overnight, washed three times with water, and blocked for 30 min with blocking buffer (phosphate-buffered saline containing 0.05% Tween 20, 1 mM EDTA, and 0.25% bovine serum albumin). The wells were washed two times with water, and 50 μl of primary anti-Y4 (serotype b) rabbit antiserum (provided by J. Zambon, State University of New York at Buffalo) or anti-CU1000 (serotype f) rabbit antiserum (12) (both diluted 1:1,000 in blocking buffer) was added. The plate was incubated for 2 h, and the wells were then washed two times with water, blocked for an additional 10 min, and rewashed. Fifty microliters of a solution of goat anti-rabbit immunoglobulin M (IgM) plus IgG alkaline phosphatase-conjugated antibody (Fisher; diluted 1:2,000 in blocking buffer) was added to each well, and the plate was incubated an additional 2 h. The wells were washed two times with water, blocked for an additional 10 min, and rewashed. Seventy-five microliters of a 1-mg/ml solution of *p*-nitrophenyl phosphate (in 10% diethanolamine-0.05 mM MgCl_2 , pH 9.8) was added to each well, and the plate was incubated for 45 min. The end product was measured in a Bio-Rad model 3550 microplate reader set at 405 nm.

IS903 ϕ kan mutagenesis. Donor strain (*E. coli* SK338; washed three times with phosphate-buffered saline) and recipient strain (*A. actinomycetemcomitans* CU1000N) cells were mixed in a ratio of 1:10, spotted on TSA plates, and incubated at 37°C in 10% CO_2 for 16 h. The mating mixture was scraped from the plate with a sterile loop, resuspended in 1 ml of TSB, and plated on TSA supplemented with chloramphenicol and nalidixic acid to select for transconjugants. Approximately 40 colonies of *A. actinomycetemcomitans* transconjugants carrying plasmid pVJT128 were resuspended in 1 ml of TSB, plated on TSA containing chloramphenicol and IPTG, and grown for 24 h to allow transposition. Colonies were pooled and plated on TSA supplemented with kanamycin to select for transposon mutants. Colony morphology of the transposon mutants was observed after 2 days of growth using an Olympus IMT-2 inverted microscope at $\times 40$. Three mutants (JK1002, JK1005, and JK1022) that displayed a colony morphology that was rougher than the wild-type CU1000 rough-colony phenotype (12) were streaked to purity and frozen.

Cloning and sequencing the IS903 ϕ kan transposon insertion sites. Genomic DNA was prepared from mutants JK1002, JK1005, and JK1022 using a DNeasy tissue kit (Qiagen) according to the instructions provided by the manufacturer. Inverse PCR (53) was performed in 0.5-ml thin-wall tubes using primers *kanStart* (5'-GTTTCCCGTTGAATATGGCTGGG-3') and *kanStop* (5'-GCAGTTTCA TTTGATGCTCGA-3'), which hybridize within the transposon and which are oriented upstream and downstream, respectively (52). The reaction mixture contained 50 pmol of each primer, 10 μl of $10\times$ PCR buffer, 1 mM deoxynucleoside triphosphates, 2.5 U of *Taq* DNA polymerase (PE Biosystems), and 100 ng of *Hind*III-digested and ligated genomic DNA as a target in a 100- μl reaction volume overlaid with 100 μl of light mineral oil. Thirty cycles of PCR were performed under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. In this experiment, DNAs from mutants JK1005 and JK1022 both yielded a 1.2-kb PCR product. The ends of the JK1005 and JK1022 PCR products were made flush using *E. coli* DNA polymerase (Klenow fragment) and ligated into the *EcoRV* site of plasmid LITMUS28 (New England BioLabs) as described previously (48). Plasmid DNAs were prepared using a QIAprep spin miniprep kit (Qiagen) and subjected to DNA sequence analysis on an ABI model 377 PRISM automated sequencer. The DNA sequences of these two fragments indicated that the transposons in mutants JK1005 and JK1022 inserted 100 bp apart into the same 1.2-kb *Hind*III

TABLE 1. *A. actinomycetemcomitans* serotype-specific PCR assay

Reaction	Primer	Sequence ^a (5'-3')	Serotype	PCR product size (bp)	Region amplified ^b	GenBank accession no.
1	P11	TCTCCACCATTTTGTAGTGG	b	333	12780-13112	AB002668
	P12	GAAACCACCTCTATTCTCC	c	268	12808-13075	AB010415
	P13	CCTTTATCAATCCAGACAGC	f	232	4813-5044	AF213680
	P14	ARAYTTYTCWTCGGGAATG				
2	P15	TGGGTCATGGGAGGTAATCC	a	293	8021-8313	AB046360
	P16	GCTAGGAACAAAGCAGCATC				
3	P17	TGGAACGGGTATGGGAACGG	d	411	11462-12034	AB041226
	P18	GGATGCTCATCTAGCCATGC				
4	P19	ATTCCAGCCTTTTGGTTCTC	e	311	17118-17427	AB030032
	P20	TGGTCTGCGTTGTAGGTTGG				

^a R = A or G; Y = C or T; W = A or T.

^b GenBank nucleotide sequence coordinates.

fragment and in the same orientation (see Fig. 6). PCR analysis of genomic DNA from mutant JK1002 using primers *kan*Stop (see above) and P1 (5'-TTAAACAATAATGAGTAACCC-3'), which hybridizes within the *Hind*III fragment and which is oriented upstream (see Fig. 6), showed that the transposon in JK1002 inserted 900 bp upstream from the transposon in JK1005 and in the same orientation as the transposons in JK1005 and JK1022.

Cloning and sequencing the CU1000 O-PS gene cluster. Primer pairs P1 (see above) and P2 (5'-GGATGATATTGTAGTTAATG-3'), P3 (5'-CATTAACCAATATCATCC-3') and P4 (5'-GTAAACCGAGTTTATCGGC-3'), and P5 (5'-CGATAAACTCGGTTTAGGATA-3') and P6 (5'-CAAGAYTTAYGGCTGGTAAG-3') were used to amplify by PCR three overlapping fragments (755 bp, 1.3 kb, and 3.4 kb, respectively) upstream from the transposon insertion site in JK1002 (Fig. 6). Primer pairs P7 (5'-GGATTAGAAGAAGATATTGG-3') and P8 (5'-CTTGCCATTGCTGGTCTGCG-3'), and P9 (5'-ACAACGCAGACCAGCAATGG-3') and P10 (5'-AAACTCCGCCAAATCGGTGC-3') were used to amplify two overlapping fragments (5.2 and 2.9 kb, respectively) downstream from the JK1022 transposon insertion site. PCR conditions were as described above, except that 5 ng of CU1000 genomic DNA was used as a target. All five of these PCR products were cloned into the *Eco*RV site of LITMUS28 and subjected to DNA sequence analysis as described above. Universal and internal primers were used to sequence both strands of all plasmids.

Serotype-specific PCR assay. Four PCRs were performed on each clinical isolate (Table 1). All reaction mixtures contained 50 pmol of each of the indicated primers and 3 ng of genomic DNA in a 100- μ l volume as described above. PCR conditions were 30 cycles at 94°C for 30 s and 55°C for 30 s. Reaction 1 was used to detect serotype b, c, and f sequences in a single multiplex reaction mixture containing primers P11, P12, P13, and P14. Primer P14 is a universal forward primer that hybridizes to ORF11 of serotypes b, c, and f (Fig. 6). Primers P11, P12, and P13 are serotype b-, c-, and f-specific reverse primers, respectively, that hybridize to serotype-specific sequences at the 3' end of ORF11. When combined with P14, primers P11, P12, and P13 produce PCR products of 333, 268, and 232 bp, respectively. Reaction 2 was used to detect serotype a with primers that hybridize to ORF8 of the serotype a gene cluster (50) and produce a 293-bp PCR product. Reaction 3 detected serotype d with primers that hybridize to ORF9 of the serotype d gene cluster (35) and produce a 411-bp PCR product. Reaction 4 detected serotype e with primers that hybridize to ORF3 of the serotype e gene cluster (see Fig. 6) (61) and produce a 311-bp PCR product. A 10- μ l aliquot of each reaction mixture was electrophoresed through a 5% acrylamide-0.17% bisacrylamide gel in 1 \times Tris-borate-EDTA buffer, and the PCR products were visualized by staining with ethidium bromide (48). Assays were validated with DNAs from strains SUNYab75, Y4, NJ2700, IDH781, NJ9500, and CU1000 (serotypes a, b, c, d, e, and f, respectively). DNA from each of these strains produced a single PCR product of the predicted size in the appropriate reaction, and no DNA produced a PCR product in more than one reaction. Results of the serotype-specific PCR assay were consistent with those of conventional seroclassification using serotype-specific rabbit antisera (data not shown).

DNA sequence accession number. The DNA sequence of the CU1000 O-PS gene cluster was deposited in GenBank under accession no. AF213680.

RESULTS

Chemical analysis of O-PS from *A. actinomycetemcomitans* strain CU1000. *A. actinomycetemcomitans* strain CU1000 cells were extracted by a modified hot aqueous phenol method (21) and, following sequential treatment with RNase, DNase, and protease K and ultracentrifugation, yielded a precipitated gel, which was dissolved in water and lyophilized to yield LPS (10% yield based on dry cell weight). The LPS on DOC-PAGE analysis gave a typical smooth-type LPS ladder pattern with a band spacing indicative of an O-PS composed of a repeating trisaccharide unit (41).

The LPS was hydrolyzed by hot dilute acetic acid to give an insoluble lipid A (21%) and a water-soluble product, which on Sephadex G-50 gel filtration chromatography yielded a high-molecular-weight O-PS fraction (40%; K_{av} , 0.02), a core oligosaccharide (14%; K_{av} , 0.14), and a low-molecular-weight fraction containing 3-deoxy-D-manno-oct-2-ulosonic acid and phosphate (12%; K_{av} , 0.95).

The O-PS had the following characteristics: $[\alpha]_D$, -36.8° (c 0.3, water); analytical composition results for C, 41.40%; H, 6.04%; N, 1.84%; ash, nil. The O-PS on hydrolysis and GC-MS of derived alditol acetate derivatives was found to be composed of rhamnose and 2-amino-2-deoxy-galactose in the molar ratio of 2:1. The two component glycoses were separated by preparative PC, and the collected glycoses were identified as L-rhamnose and 2-amino-2-deoxy-D-galactose. The L-rhamnose fraction (R_{Gal} , 2.90) had an $[\alpha]_D$ of +7° (c 0.2, water) on reduction (sodium borodeuteride [NaBD₄]), and acetylation afforded penta-O-acetyl-L-rhamnitol-1-d, which gave a single peak on GC (t_G , 0.60) corresponding in retention time and MS analysis results to that of a reference sample; on methanolysis (2.5% methanol-HCl, 1 h, 100°C) the fraction gave methyl α -L-rhamnopyranoside having an $[\alpha]_D$ of -60.2° (c 0.1, water). The aminoglycoside fraction was characterized as its *N*-acetyl derivative 2-acetamido-2-deoxy-D-galactose, having an $[\alpha]_D$ of +82° (c 0.3, water), and on reduction (NaBD₄) and acetylation afforded 1,3,4,5,6-penta-O-acetyl-2-acetamido-2-deoxy-D-galactitol-1-d with a GC retention time (t_G , 1.33) and MS spectrum identical with those of a reference sample. The 2-acetamido-2-deoxy-D-galactose was also characterized by GC-MS as its trimethylsilyl 2-acetamido-2-deoxy-3,4,6-tri-O-trimethyl-

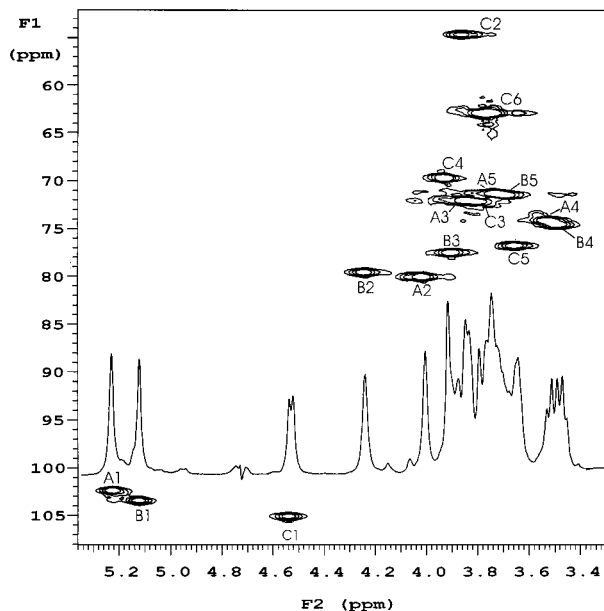


FIG. 1. Partial HSQC and one-dimensional proton NMR spectra of the LPS O-PS from *A. actinomycetemcomitans* strain CU1000 showing H/C cross-peak assignments for glucose residues A [α -L-Rhap-(1-)], B [α -L-Rhap-(1-)], and C [β -D-GalpNAc-(1-)].

silyl- α -D-galactopyranoside and - β -D-galactopyranoside derivatives (41).

The ^1H NMR spectrum of the O-PS (Fig. 1) showed inter alia signals for three anomeric protons at 5.220 ($J_{1,2}$, ~ 1 Hz), 5.123 ($J_{1,2}$, 2.2 Hz), and 4.550 ppm ($J_{1,2}$, 7.3 Hz), a signal at 2.073 ppm (3H) from an acetamido substituent, and signals at 1.332 (3H) and 1.640 ppm (3H) arising from the 6-deoxy functions of the identified L-Rha constituents. The ^{13}C NMR spectrum of the O-PS (Fig. 2) showed inter alia anomeric carbon signals at 103.99 ($J_{\text{C1-H1}}$, 161 Hz), 102.34 ($J_{\text{C1-H1}}$, 175 Hz), and 101.28 ppm ($J_{\text{C1-H1}}$, 174 Hz), carbon signals (underlined) at 23.27 (NHCOCH₃), 175.25 (NHCOCH₃), and 53.54 ppm (C-2)

arising from the 2-acetamido-2-deoxy function of the identified D-GalNAc component residue and two methyl signals at 17.66 and 17.33 ppm arising from the two L-Rha residues. The composition and NMR data were consistent with the O-PS being a polymer of a regular repeating trisaccharide unit.

GC-MS analysis of the reduced (NaBD₄) and acetylated hydrolysis products of methylated O-PS identified 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol-1-d (t_{GM} , 0.87), 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-L-rhamnitol-1-d (t_{GM} , 1.13), and 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-1-d (t_{GM} , 2.69) (1:1:1), showing the repeating trisaccharide unit to contain a 2-substituted L-Rhap residue, a 2,3-disubstituted L-Rhap residue, and a terminal D-GalpNAc residue glycosidically linked to the branched L-Rhap residue.

The complete structural characterization of the O-PS, involving linkage position and anomeric configuration assignments, was obtained by the application of (2D) ^1H and ^{13}C NMR spectroscopy. The anomeric proton signals of the glycosyl residue in the NMR spectrum of the O-PS were arbitrarily assigned A to C in order of their decreasing chemical shifts, and their subspectra were identified from H-1 connectivities to corresponding ring proton resonances and the magnitude of vicinal coupling constants (Table 1). The proton subspectra of residues A and B were consistent with a *manno* configuration having small $J_{1,2}$ (~ 1 Hz) and $J_{2,3}$ (~ 4 Hz) coupling constants and a large $J_{3,4}$ (9.3 Hz) coupling constant. Further consideration of the corresponding $J_{\text{C1-H1}}$ values for residues A (174 Hz) and B (175 Hz) led to them both being identified as α -L-Rhap residues. Glucose C was identified as a β -D-GalpNAc residue from an HSQC spectrum (Fig. 1), in which H-2C correlated with the carbon resonance at 53.54 ppm (C-2C), and from its characteristic anomeric coupling constants $J_{\text{C1-H1}}$ (160 Hz) and $J_{1,2}$ (7.3 Hz).

The sequence and glycosidic linkage positions within the O-PS were established from observed transglycosidic nuclear Overhauser effects (NOEs), which were seen between H-1A (α -L-Rhap) and H-3B (α -L-Rhap) and its own H-2A, which,

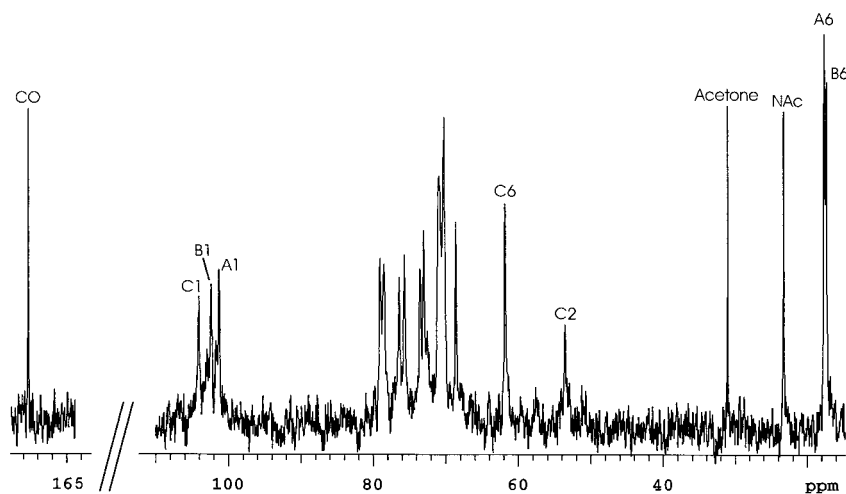


FIG. 2. ^{13}C NMR spectrum of the LPS O-PS from *A. actinomycetemcomitans* strain CU1000 showing carbon atom resonances for glucose residues A [α -L-Rhap-(1-)], B [α -L-Rhap-(1-)], and C [β -D-GalpNAc-(1-)]. NAC, *N*-acetyl.

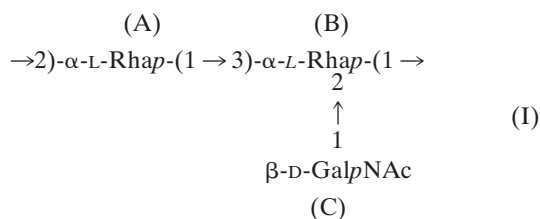
TABLE 2. ^1H and ^{13}C NMR chemical shifts for the LPS O-PS of *A. actinomycetemcomitans* CU1000^a

Glycose unit	Chemical shift (ppm) (coupling constant [Hz]) for:											
	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	C-1 (J_{C1-H1})	C-2	C-3	C-4	C-5	C-6
-2)- α -L-Rhap-(1- (A)	5.222 (~1)	4.021 (~4)	3.842 (9.4)	3.527 (9.3)	3.738	1.342 (3.9)	101.28 (174)	78.93	70.88	73.24	70.16	17.66
2,3)- α -L-Rhap-(1- (B)	5.123 (2.2)	4.249 (4.5)	3.904 (9.4)	3.513 (9.3)	3.692	1.256 (4.3)	102.34 (175)	78.44	76.36	73.45	70.37	17.33
β -D-GalpNAc-(1- (C)	4.550 (7.3)	3.863 (9.5)	3.805 (~4)	3.933 (~1)	3.666	3.760	103.99 (161)	53.44	71.03	68.57	75.64	61.80

^a Chemical shifts were recorded at 30°C.

together with NOEs observed between H-1B and H-2A and its own H-2B, indicated that the O-PS had a linear backbone structure composed of a repeating disaccharide having the structure $\rightarrow 2)\text{-}\alpha\text{-L-Rhap-(1}\rightarrow 3)\text{-}\alpha\text{-L-Rhap-(1}\rightarrow$.

An observed NOE between H-1C (β -D-GalpNAc) and H-2B, along with interresidue NOEs to its own H-2C, H-3C, and H-5C confirmed that residue C was a nonreducing β -D-GalpNAc end group glycosidically linked to residue B and that the repeating trisaccharide of the O-PS had the structure (I)



Confirmatory proof of the above structure was obtained from nitrous acid degradation of the N-deacetylated O-PS to yield a high-molecular-weight homopolymer having an $[\alpha]_{\text{D}}$ of -75° (c 1.0, water) and composed of only L-rhamnose. GC-MS analysis of the methylated L-rhamnan gave two products identified as 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-L-rhamnitol-1-d (t_{GM} , 0.87) and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol-1-d (t_{GM} , 0.94) in the molar ratio 1:1, confirming the 2- and 3-monosubstitutions of the α -L-Rhap residues and also the substitution of the β -D-GalpNAc end group at the 2 position of the backbone 3-substituted α -L-Rhap residue (B) in the native O-PS. 2D NMR experiments (Table 2) on the N-deacetylated O-PS confirmed the repeating disaccharide nature of the L-rhamnan backbone chain and thus the proposed structure of the antigenic O chain of *A. actinomycetemcomitans* strain CU1000 LPS.

Reactivity of CU000 LPS with anti-Y4 and anti-CU1000 rabbit sera. *A. actinomycetemcomitans* CU1000 was initially identified serologically as a serotype b strain (12). To demonstrate that LPS from strain CU1000 is antigenically distinct from that of a serotype b strain, LPS from strains CU1000 and Y4 (serotype b) were tested in an ELISA using anti-CU1000 and anti-Y4 rabbit antisera (Fig. 3). Binding of rabbit antisera to CU1000 and Y4 LPS was serotype specific, although a small amount of cross-reactivity was observed. This cross-reactivity could be due to epitopes residing in structures common to both CU1000 and serotype b O-PS antigens (see below).

Construction of O-PS mutants in strain CU1000N. Gram-negative bacteria that are unable to synthesize the O-antigenic side chains of LPS generally exhibit a rough colony morphology (5, 45). *A. actinomycetemcomitans* strain CU1000N was mutagenized with transposon IS903 ϕ kan, which carries a cryptic kanamycin resistance gene that is expressed only after suc-

cessful transposition into an actively transcribed gene (52), and three kanamycin-resistant mutants (out of ~3,000) that displayed a dry, rough colony morphology on TSA plates were selected. All three mutants (designated JK1002, JK1005, and JK1022) exhibited wild-type growth rates and adherence properties in TSB (11) (data not shown). LPS preparations from all three mutants displayed reduced reactivity with anti-CU1000 rabbit antisera as measured by ELISA (Fig. 4). The residual antigenic cross-reactivity displayed by LPS prepared from the mutants may be due to reactivity of antibodies with partially assembled O-PS components or the presence of minor amounts of immunoreactive molecules that copurified with the LPS (49). LPS preparations from strain CU1000 and the three mutants were analyzed by DOC-PAGE (Fig. 5). Strain CU1000 produced a smooth-type LPS with extended O-antigen side chains, whereas LPS from the three mutants consisted of a core oligosaccharide with O-antigen side chains short or absent. These data were confirmed by analyzing hydrolyzed LPS preparations from CU1000 and the three mutants using Sephadex G-50 chromatography (data not shown). Strain CU1000 yielded an O-antigen fraction which was absent in the corresponding products of the mutants. Proton NMR spectra of core oligosaccharides from all three mutants were identical to those of strain CU1000 (data not shown). These data indicate that mutant strains JK1002, JK1005, and JK1022 are deficient in LPS O-antigen side chain biosynthesis.

DNA sequence of the CU1000 O-PS gene cluster. DNA sequence analysis indicated that the transposons in mutants JK1002, JK1005, and JK1022 inserted into a region that was homologous to the *A. actinomycetemcomitans* serotype b, c,

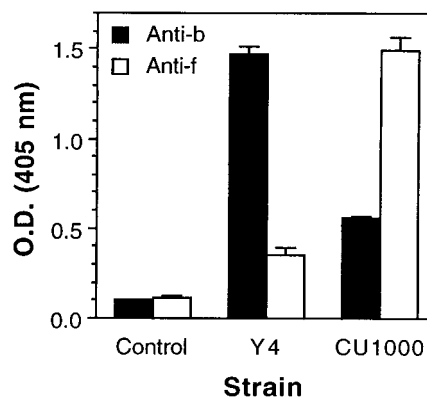


FIG. 3. Reactivities of anti-serotype b and anti-serotype f rabbit antisera with LPS prepared from *A. actinomycetemcomitans* strains Y4 (serotype b) and CU1000 (serotype f) as measured by ELISA. Control wells contained no LPS. The mean values (\pm standard errors) for triplicate wells are shown. O.D., optical density.

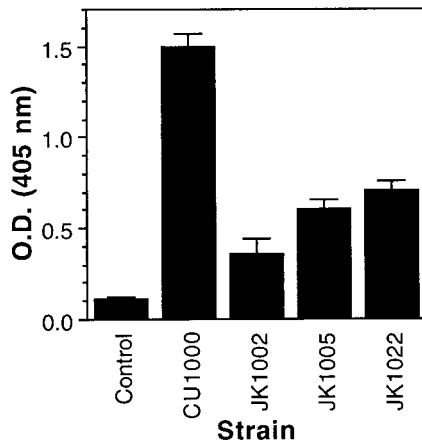


FIG. 4. Reactivities of anti-serotype f rabbit antisera with LPS prepared from *A. actinomycetemcomitans* strain CU1000 and three LPS mutants (JK1002, JK1005, and JK1022) as measured by ELISA. Control wells contained no LPS. The mean values (\pm standard errors) for triplicate wells are shown. O.D., optical density.

and e O-PS gene clusters (36, 61, 62). Primers that hybridize to conserved sequences in the serotype b, c, and e O-PS gene clusters and flanking regions were used to amplify by PCR the region surrounding the transposon insertion sites from strain CU1000 as described in Materials and Methods. DNA sequence analysis of a 13.1-kb region surrounding the transposon insertion sites revealed a cluster of 14 closely spaced or overlapping open reading frames (ORFs) that were all transcribed in the same orientation and in the same direction as the transposon insertions in mutants JK1002, JK1005, and JK1022. The DNA sequences of the CU1000 gene cluster and predicted amino acid sequences were clearly homologous to those of the O-PS gene clusters from strains Y4, NCTC9710, and IDH1705 (Fig. 6), indicating that this region contained the serotype f O-PS gene cluster.

Comparison of the serotype b, c, e, and f O-PS gene clusters.

The serotype b, c, e, and f O-PS gene clusters each contained a set of four genes (*rmlBADC*) at the beginning of the cluster (ORF6, ORF7, ORF8, and ORF9 in that order) that encode the four enzymes responsible for the four-step biosynthesis of dTDP-L-rhamnose from glucose-1-phosphate. dTDP-L-rhamnose is the activated nucleotide sugar form of L-rhamnose, which is widely distributed in O antigens of gram-negative bacteria (28, 60). Homologous *rmlBADC* genes have been detected in a wide range of bacteria, although the gene order may vary from species to species (28). The *rmlBADC* genes were highly conserved (>90% amino acid identity) among *A. actinomycetemcomitans* serotypes b, c, e, and f, except for *rmlD* from serotype c, whose product showed only 60% amino acid identity to the *rmlD* gene products of the other three serotypes (35, 36). Although significantly divergent, both Y4 and NCTC9710 RmlD gene products are capable of converting dTDP-6-deoxy-L-lyxo-4-hexulose to dTDP-L-rhamnose, the last step in the dTDP-L-rhamnose pathway (34).

Two genes (*wzm* and *wzt*) that encode a hydrophobic integral-membrane protein component and a hydrophilic ATPase component, respectively, of an ATP-binding cassette (ABC) membrane transporter (10) immediately followed *rmlBADC* in

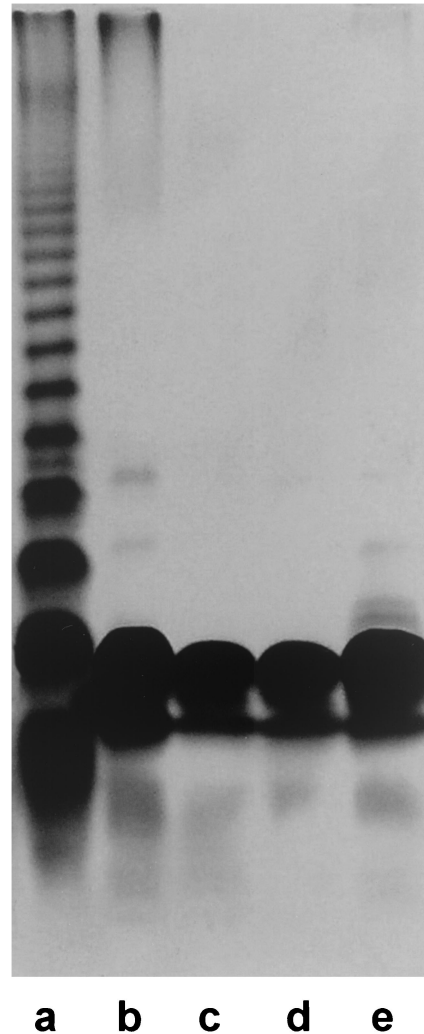


FIG. 5. DOC-PAGE profiles of LPS from *Salmonella enterica* serovar Milwaukee (lane a) and *A. actinomycetemcomitans* strains CU1000 (lane b), JK1002 (lane c), JK1005 (lane d), and JK1022 (lane e). Each lane contained 10 μ g of LPS in saline plus 0.002% bromophenol tracking dye. LPS was visualized by silver staining.

the serotype b, c, e, and f O-PS gene clusters (ORF10 and ORF11 in that order; Fig. 6). These two genes showed 40 to 60% amino acid identity among the four serotypes. ABC transporters have been shown to be involved in the translocation of exopolysaccharides in a variety of gram-negative bacteria (46), and *wzm* and *wzt* have been shown to be necessary for the expression of *A. actinomycetemcomitans* serotype b, c, and e O-PS antigens in *E. coli* (36, 61, 62). One of the transposon mutations in *A. actinomycetemcomitans* strain CU1000 that resulted in reduced levels of LPS synthesis (mutant JK1002) was located in *wzt*.

The *wzm* and *wzt* genes were followed by a group of two to four genes that are unique to each serotype (Fig. 6). The O-PS gene cluster from strain CU1000 contained two unique genes, ORFf1 and ORFf2. ORFf1 encodes a putative glycosyltransferase that is homologous to the N terminus of the serotype c ORFf1 gene product (27% amino acid identity in a region of >300 residues). ORFf1 has been shown to be necessary for the

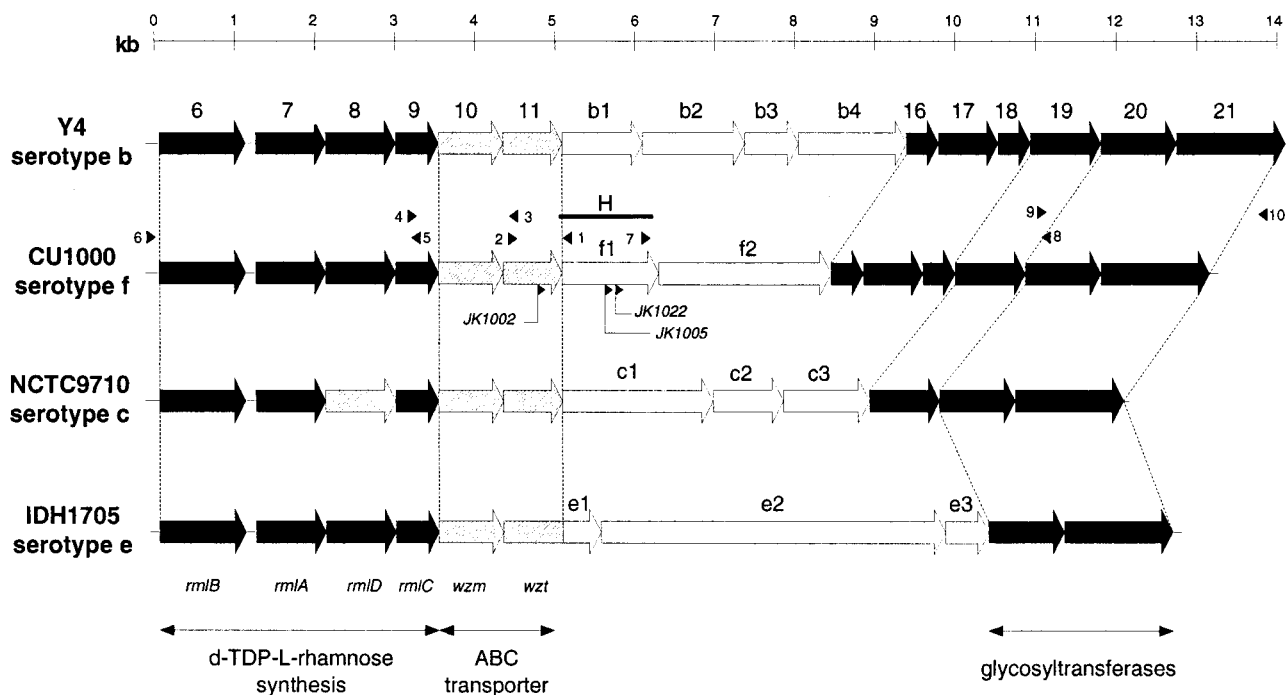


FIG. 6. Comparison of the serotype-specific O-PS gene clusters from *A. actinomycetemcomitans* strains Y4, CU1000, NCTC9710, and IDH1705. Top line, scale in kilobases (kb). Arrows, ORFs and direction of transcription. Black arrows, ORFs with greater than 90% identity among strains; hatched arrows, ORFs with 40 to 60% identity; open arrows, ORFs with no corresponding gene in the other strains. Dashed lines demarcate homologous regions. ORFs numbered 6 to 11 and 16 to 21 correspond to ORFs 6 to 11 and 16 to 21 in reference 62, respectively. Bar H, position of the *Hind*III fragment amplified by inverse PCR. Numbered arrowheads above the CU1000 sequence, position and orientation of primers P1 to P10; arrowheads below CU1000 ORF11 and ORFf1, insertion sites and direction of transcription of transposon IS903 ϕ kan insertion mutations in JK1002, JK1005, and JK1022. The names and functions of genes shared by all four clusters are indicated at the bottom.

expression of serotype c O-PS in *E. coli* (36). ORFf1 was also homologous (26 to 27% amino acid identity) to *Streptococcus mutans* *rgpFc* (59), *Vibrio anguillarum* *virA* (37), and gene *y4gN* from the *Rhizobium* sp. plasmid pNGR234a (13), all of which are involved in the expression of exopolysaccharide antigens. Two of the transposon mutations in *A. actinomycetemcomitans* strain CU1000 that resulted in reduced levels of LPS synthesis (those of JK1005 and JK1022) were located in ORFf1. ORFf2 has no known function and no homologues in the GenBank database, although homologues of ORFf2 were found in the unfinished genome sequences of *Enterococcus faecalis* and *Clostridium acetobutylicum* (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html). The function of these genes is not known. Among the genes unique to the serotype b, c, and e O-PS gene clusters are the following: ORFb3 (*fcd*), encoding dTDP-4-keto-6-deoxy-D-glucose reductase, which catalyzes the conversion of dTDP-4-keto-6-deoxy-D-glucose to dTDP-D-fucose, the activated nucleotide sugar form of the D-fucose present in serotype b O-PS (60); ORFc2 (*tll*), encoding dTDP-6-deoxy-L-lyxo-4-hexulose reductase, which catalyzes the conversion of dTDP-4-keto-L-rhamnose to dTDP-6-deoxy-L-talose, the activated nucleotide sugar form of the 6-deoxy-L-talose present in serotype c O-PS (34); ORFb1 and ORFe2, encoding putative glycosyltransferases (61, 62); ORFc3 and ORFe3, encoding putative acetyltransferases (36, 61); ORFb2 and ORFe1 (which is fused to ORF11), which are homologous to genes of unknown function present in O-antigen operons of

other bacteria; ORFb4, which has no known function and no homologues in the GenBank database.

A set of three genes (ORF16, ORF17, and ORF18) was present in the serotype b and f O-PS gene clusters but not in the serotype c and e gene clusters (Fig. 6). The products of these three genes displayed >90% amino acid identity between serotypes b and f. ORF17 encodes a putative glycosyltransferase (62), ORF16 is homologous to genes of unknown function in other bacterial O-antigen operons, and ORF18 has no known function and no homologues in GenBank. Two or three highly conserved (>90% amino acid identity) putative glycosyltransferases (encoded by ORF19, ORF20, and ORF21) were located at the distal end of all four O-PS gene clusters (36, 61, 62).

The serotype b, c, and e O-PS gene clusters contain a region of low G+C content (ORF10 to ORF19 in serotypes b and c, and ORF10 to ORFe3 in serotype e) (36, 61, 62). These regions display <30% G+C, compared to 37 to 42% G+C for *rmlBADC*, 42 to 43% G+C for ORF20 and ORF21, and 46% for the entire *A. actinomycetemcomitans* genome (35). The corresponding region of the CU1000 O-PS gene cluster (ORF10 to ORF19) also displayed a low G+C content (29%), compared to 41% G+C for *rmlBADC* and 42% G+C for ORF20 and ORF21.

The DNA sequences of the regions flanking the CU1000 O-PS gene cluster were >90% identical to the corresponding regions flanking the serotype b, c, and e gene clusters (data not

strains previously classified as serotype b may be warranted. Our findings indicate that serotype f may account for some strains that have been previously classified as serotype b and that serotype f may constitute a significant portion of strains isolated from LJP patients. Because of possible cross-reactivity between serotypes b and f, a serotype-specific PCR assay such as the one described in this report may be more accurate than conventional serotyping with polyclonal rabbit antisera for seroreclassification of *A. actinomycetemcomitans* serotype b isolates.

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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.
- Asikainen, S., C.-H. Lai, S. Alaluusua, and J. Slots. 1991. Distribution of *Actinobacillus actinomycetemcomitans* serotypes in periodontal health and disease. *Oral Microbiol. Immunol.* **6**:115–118.
- Bax, A., R. Freeman, and G. Morris. 1981. Correlation of proton chemical shifts by two-dimensional Fourier transform NMR. *J. Magn. Reson.* **42**:164–168.
- Berbari, E. F., F. R. Cockerill, and J. M. Stickelberg. 1997. Infective endocarditis due to unusual or fastidious microorganisms. *Mayo Clin. Proc.* **72**:532–542.
- Brink, B. A., J. Miller, R. W. Carlson, and K. D. Noel. 1990. Expression of *Rhizobium leguminosarum* CFN42 genes for lipopolysaccharide in strains derived from different *R. leguminosarum* soil isolates. *J. Bacteriol.* **172**:548–555.
- Califano, J. V., H. A. Schenkein, and J. G. Tew. 1992. Immunodominant antigens of *Actinobacillus actinomycetemcomitans* serotype b in early-onset periodontitis patients. *Oral Microbiol. Immunol.* **7**:65–70.
- Dogan, B., M. H. Saarela, H. Jousimies-Somer, S. Alaluusua, and S. Asikainen. 1999. *Actinobacillus actinomycetemcomitans* serotype e—biotypes, genetic diversity and distribution in relation to periodontal status. *Oral Microbiol. Immunol.* **14**:98–103.
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric methods for the determination of sugars and related substances. *Anal. Chem.* **28**:350–356.
- Ebersole, J. L., and D. Cappelli. 1994. Gingival crevicular fluid antibody to *Actinobacillus actinomycetemcomitans* in periodontal disease. *Oral Microbiol. Immunol.* **9**:335–344.
- Fath, M. J., and R. Kolter. 1993. ABC transporters: bacterial exporters. *Microbiol. Rev.* **57**:995–1017.
- Fine, D. H., D. Furgang, J. 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.
- Fine, D. H., D. Furgang, H. C. Schreiner, P. Goncharoff, J. Charlesworth, G. Ghazwan, P. Fitzgerald-Bocars, and D. H. Figurski. 1999. Phenotypic variation in *Actinobacillus actinomycetemcomitans* during laboratory growth: implications for virulence. *Microbiology* **145**:1335–1347.
- Freiberg, C., R. Fellay, A. Bairoch, W. T. Broughton, and L. R. Perret. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* **387**:394–401.
- Gatt, R., and E. R. Berman. 1965. A rapid method for the estimation of aminosugars on a micro scale. *Anal. Biochem.* **15**:167–171.
- Gmür, R., H. McNabb, T. J. M. van Steenberg, P. Baehni, A. Mombelli, A. J. van Winkelhoff, and B. Guggenheim. 1993. Seroreclassification of hitherto nontypeable *Actinobacillus actinomycetemcomitans* strains: evidence for a new serotype e. *Oral Microbiol. Immunol.* **8**:116–120.
- Grance, C. J., R. E. Levitz, H. Katz-Pollak, and L. R. Brettman. 1988. *Actinobacillus actinomycetemcomitans* prosthetic valve endocarditis. *Rev. Infect. Dis.* **10**:922–929.
- Haraszthy, V. I., 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.
- 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.
- 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.
- Hölttä, P., S. Alaluusua, M. Saarela, and S. Asikainen. 1994. Isolation frequency and serotype distribution of mutans streptococci and *Actinobacillus actinomycetemcomitans*, and clinical periodontal status in Finnish and Vietnamese children. *Scand. J. Dent. Res.* **102**:113–119.
- Johnson, K. G., and M. B. Perry. 1975. Improved techniques for the preparation of bacterial lipopolysaccharides. *Can. J. Microbiol.* **22**:29–34.
- Kachlany, S. C., P. J. Planet, M. K. Bhattacharjee, E. Kollia, R. DeSalle, D. H. Fine, and D. H. Figurski. 2000. Nonspecific adherence by *Actinobacillus actinomycetemcomitans* requires genes widespread in *Bacteria* and *Archaea*. *J. Bacteriol.* **182**:6169–6176.
- 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.
- Kiley, P., and S. C. Holt. 1980. Characterization of the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4 and N27. *Infect. Immun.* **30**:862–873.
- King, E. O., and H. W. Tatum. 1962. *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. *J. Infect. Dis.* **111**:85–94.
- Kogan, G., and D. Uhrin. 2000. Current NMR methods in the structural elucidation of polysaccharides, p. 73–131. In Atta-w-Rahman (ed.), *New advances in chemistry*. Harwood Academic Publishers, Amsterdam, The Netherlands.
- Kumar, A., R. R. Ernst, and K. Wurthrich. 1980. A 2-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complex proton-proton cross-relaxation in networks of biological molecules. *Biochem. Biophys. Res. Commun.* **95**:1–6.
- Li, Q., and P. R. Reeves. 2000. Genetic variation of dTDP-L-rhamnose pathway genes in *Salmonella enterica*. *Microbiology* **146**:2291–2307.
- Martine, B. F., B. M. Derby, G. N. Budzilovich, and J. Ransohoff. 1967. Brain abscesses due to *Actinobacillus actinomycetemcomitans*. *Neurology* **17**:833–837.
- McNabb, H., A. Mombelli, R. Gmür, S. Mathey-Dinc, and N. P. Lang. 1992. Periodontal pathogens in the shallow pockets of immigrants from developing countries. *Oral Microbiol. Immunol.* **7**:267–272.
- Mombelli, A., R. Gmür, J. Frey, J. Meyer, K. Y. Zee, J. O. W. Tam, E. C. M. Lo, J. DiRienzo, N. P. Lang, and E. F. Corbet. 1998. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in young Chinese adults. *Oral Microbiol. Immunol.* **13**:231–237.
- Mombelli, A., R. Gmür, N. P. Lang, E. Corbet, and J. Frey. 1999. *Actinobacillus actinomycetemcomitans* in Chinese adults. *J. Clin. Periodontol.* **26**:505–510.
- Muhle, L., J. Rau, and J. Ruskin. 1979. Vertebral osteomyelitis due to *Actinobacillus actinomycetemcomitans*. *JAMA* **142**:1824–1825.
- Nakano, Y., N. Suzuki, Y. Yoshida, T. Nezu, Y. Yamashita, and T. Koga. 2000. Thymidine diphosphate-6-deoxy-L-lyxo-4-hexulose reductase synthesizing dTDP-6-deoxy-L-talose from *Actinobacillus actinomycetemcomitans*. *J. Biol. Chem.* **275**:6806–6812.
- Nakano, Y., Y. Yoshida, N. Suzuki, Y. Yamashita, and T. Koga. 2000. A gene cluster for the synthesis of serotype d-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochim. Biophys. Acta* **1493**:259–263.
- Nakano, Y., Y. Yoshida, Y. Yamashita, and T. Koga. 1998. A gene cluster for 6-deoxy-L-talan synthesis in *Actinobacillus actinomycetemcomitans*. *Biochim. Biophys. Acta* **1442**:409–414.
- Norqvist, A., and H. Wolf-Watz. 1993. Characterization of a novel chromosomal virulence locus involved in expression of a major surface flagellar sheath antigen of the fish pathogen *Vibrio anguillarum*. *Infect. Immun.* **61**:2434–2444.
- 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.
- 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.
- 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.
- Perry, M. B. 1964. The separation, determination and characterization of 2-amino-2-deoxy-D-glucose and 2-amino-deoxy-D-galactose in biological materials by gas-liquid chromatography. *Can. J. Biochem.* **42**:451–460.
- 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.

43. 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.
44. 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.
45. Rick, P. D. 1987. Lipopolysaccharide biosynthesis, p. 648–662. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
46. Rochetta, H. L., and J. S. Lam. 1997. Identification and functional characterization of an ABC transport system involved in polysaccharide export of A-band lipopolysaccharide in *Pseudomonas aeruginosa*. *J. Bacteriol.* **179**:4713–4724.
47. 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.
48. 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.
49. Sims, T. J., B. J. Moncla, R. P. Darveau, and R. C. Page. 1991. Antigens of *Actinobacillus actinomycetemcomitans* recognized by patients with juvenile periodontitis and periodontally normal subjects. *Infect. Immun.* **59**:913–924.
50. Suzuki, N., Y. Nakano, Y. Yoshida, H. Nakao, Y. Yamashita, and T. Koga. 2000. Genetic analysis of the gene cluster for the synthesis of serotype a-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochim. Biophys. Acta* **1517**:135–138.
51. Takamatsu-Matsushita, N., N. Yamaguchi, M. Kawasaki, Y. Yamashita, T. Takehara, and T. Koga. 1996. Immunogenicity of *Actinobacillus actinomycetemcomitans* b-specific polysaccharide-protein conjugate. *Oral Microbiol. Immunol.* **11**:220–225.
52. Thomson, V. J., M. K. Bhattacharjee, D. H. Fine, K. M. Derbyshire, and D. H. Figurski. 1999. Direct selection of IS903 transposon insertions by use of a broad-host-range vector: isolation of catalase-deficient mutants of *Actinobacillus actinomycetemcomitans*. *J. Bacteriol.* **181**:7298–7307.
53. Triglia, T., M. Peterson, and D. Kemp. 1988. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucleic Acids Res.* **16**:8186.
54. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
55. Vandepitte, J., H. De Geest, and P. Jousten. 1977. Subacute bacterial endocarditis due to *Actinobacillus actinomycetemcomitans*. *J. Clin. Pathol.* **30**:842–846.
56. van Steenberghe, T. J. M., C. J. Bosch-Tijhoh, 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.
57. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83–91. In R. L. Whistler (ed.), *Methods in carbohydrate chemistry*, vol. 5. Academic Press, New York, N.Y.
58. Wilson, M. E., and R. E. Schifferle. 1991. Evidence that the serotype b antigenic determinant of *Actinobacillus actinomycetemcomitans* Y4 resides in the polysaccharide moiety of lipopolysaccharide. *Infect. Immun.* **59**:1544–1551.
59. Yamashita, Y., Y. Tsukioka, K. Tomihisa, Y. Nakano, and T. Koga. 1998. Genes involved in cell wall localization and side chain formation of rhamnose-glucose polysaccharide in *Streptococcus mutans*. *J. Bacteriol.* **180**:5803–5807.
60. Yoshida, Y., Y. Nakano, T. Nezu, Y. Yamashita, and T. Koga. 1999. A novel NDP-6-deoxyhexosyl-4-ulose reductase in the pathway for the synthesis of thymidine diphosphate-D-fucose. *J. Biol. Chem.* **274**:16933–16939.
61. Yoshida, Y., Y. Nakano, N. Suzuki, H. Nakao, Y. Yamashita, and T. Koga. 1999. Genetic analysis of the gene cluster responsible for synthesis of serotype e-specific polysaccharide antigen in *Actinobacillus actinomycetemcomitans*. *Biochim. Biophys. Acta* **1489**:457–461.
62. Yoshida, Y., Y. Nakano, Y. Yamashita, and T. Koga. 1998. Identification of a genetic locus essential for serotype b-specific antigen synthesis in *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* **66**:107–114.
63. Zambon, J. J. 1985. *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J. Clin. Periodontol.* **12**:1–20.
64. 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.
65. Zambon, J. J., T. Umemoto, E. DeNardin, F. Nakazawa, L. A. Christersson, and R. J. Genco. 1988. *Actinobacillus actinomycetemcomitans* in the pathogenesis of human periodontal disease. *Adv. Dent. Res.* **2**:269–274.

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