Construction of mutants of *Actinobacillus actinomycetemcomitans* defective in serotype b-specific polysaccharide antigen by insertion of transposon Tn916

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Mutants of *Actinobacillus actinomycetemcomitans* strain Y4 defective in the capsular-like serotype b-specific polysaccharide antigen (SPA) were constructed by inserting the transposon Tn916. Southern blot analysis suggested that the transposon was inserted into a variety of different sites on the chromosome. Whole cells from two mutants (strains ST1 and ST2) lacked reactivity with a monoclonal antibody to SPA of *A. actinomycetemcomitans* Y4 (mAb S5) in enzyme-linked immunosorbent assay, but those from another nine mutants (e.g. strains ST3 and ST5) reacted very weakly with mAb S5. Immunodiffusion tests showed that mAb S5 or rabbit antiserum against whole cells of strain Y4 produced a fused precipitin band with purified SPA and autoclaved extract from strain Y4, but no precipitin band with autoclaved extracts from these four mutants. The hydrolysate of autoclaved extract from strain Y4 contained equal amounts of rhamnose and fucose, component sugars of SPA. The hydrolysates of autoclaved extracts from strains ST1 and ST2 contained a trace amount of rhamnose, but not fucose. Those of autoclaved extracts from strains ST3 and ST5 contained a trace amount of fucose, but not rhamnose. All of these SPA-defective mutants reacted with a mAb to lipopolysaccharide of strain Y4. The cell hydrophobicity of SPA-defective mutants was higher than that of the parent strain. These mutant clones will be useful for analysing the gene complex responsible for the synthesis of SPA of *A. actinomycetemcomitans* and the regulation of expression of the polysaccharide.

Introduction

Actinobacillus actinomycetemcomitans is associated with localized juvenile periodontitis and adult periodontitis (Tanner *et al.*, 1979; Slots & Genco, 1984; Slots & Schonfeld, 1991). The organisms are serologically classified into three serotypes, a, b and c (Zambon *et al.*, 1983). In subjects with periodontal disease, serotype b is the most frequently isolated (Zambon *et al.*, 1983; Asikainen *et al.*, 1991). Serotype-specific antigens (SPAs) of *A. actinomycetemcomitans* are polysaccharides consisting of 6-deoxyhexoses (Amano *et al.*, 1989; Shibuya *et al.*, 1991). SPA of *A. actinomycetemcomitans* serotype b is a

polymer consisting of a disaccharide repeating unit, $\rightarrow 3$ - α -D-fucopyranosyl-(1 $\rightarrow$ 2)- β -L-rhamnopyranosyl-(1 $\rightarrow$) (Amano *et al.*, 1989). Patients with localized juvenile periodontitis have a high level of antibodies to SPA of *A. actinomycetemcomitans* serotype b (Ebersole *et al.*, 1983; Califano *et al.*, 1989; Sims *et al.*, 1991). The ability of SPA from serotype b to induce the release of interleukin-1 by murine macrophages is higher than that of SPAs from serotypes a and c (Takahashi *et al.*, 1991). However, the periodontopathic mechanism of SPA of serotype b is poorly understood.

Tn916, which encodes tetracycline resistance (Tc^r), is a conjugative transposon on the chromosome of *Enterococcus faecalis* DS16 (Gawron-Burke & Clewell, 1982; Clewell, 1986). The transposon is inserted into several different sites on the chromosomes of *Ent. faecalis* (Gawron-Burke & Clewell, 1982), *Streptococcus sanguis* (Cawron-Burke & Clewell, 1984), *Streptococcus mutans* (Procino *et al.*, 1988; Caufield *et al.*, 1990), *Streptococcus*

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Abbreviations: SPA, serotype-specific antigen; THY broth, Todd-Hewitt broth supplemented with 1% (w/v) yeast extract; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% (v/v) Tween 20; mAb, monoclonal antibody.

pneumoniae (Watson & Musher, 1990), *Haemophilus influenzae* (Kauc & Goodgal, 1989) and *Haemophilus parainfluenzae* (Kauc & Goodgal, 1989). The insertion of Tn916 into the bacterial chromosome has been reported to be useful for mutagenesis (Procino *et al.*, 1988; Caufield *et al.*, 1990; Watson & Musher, 1990). In this study, SPA-defective mutants of serotype b *A. actinomycetemcomitans* were constructed by Tn916 insertion.

Methods

Bacterial strains. *A. actinomycetemcomitans* strains ATCC 29523 (serotype a), Y4 (serotype b) and NCTC 9710 (serotype c) were obtained from Y. Murayama (Okayama University, Okayama, Japan), Y. Yamamoto (Sunstar Corp., Osaka, Japan) and the National Collection of Type Cultures (London, UK), respectively. These strains were grown in Todd-Hewitt broth (Difco) supplemented with 1% (w/v) yeast extract (THY broth) at 37 °C in a 5% CO₂ atmosphere. *Escherichia coli* strain K235 was kindly provided by J. R. McGhee, University of Alabama at Birmingham, AL, USA, and grown aerobically in Trypticase soy broth (BBL) at 37 °C. *E. coli* DH1 (F-*recA1 endA1 gyrA96 thi-1 hsdR17 supE44*) containing plasmid pAM120 (pGL101::Tn916) was kindly supplied by D. B. Clewell, University of Michigan, Ann Arbor, MI, USA, and grown aerobically in LB broth (Lennox, 1955) containing ampicillin (25 µg ml⁻¹) and tetracycline (6 µg ml⁻¹). Transformant strains of *A. actinomycetemcomitans* were grown in THY broth containing tetracycline (2 µg ml⁻¹).

Preparation of antigens. Lyophilized cell suspensions (10 mg ml⁻¹) of *A. actinomycetemcomitans* strains in phosphate-buffered saline (0.15 M-NaCl, 0.01 M-sodium phosphate, pH 7.2; PBS) were autoclaved at 120 °C for 15 min, and centrifuged at 1600 g at 4 °C. After centrifugation, the supernatants were collected and used as autoclaved extract. Phenol/water extracts from *A. actinomycetemcomitans* were prepared from cell suspensions (10 mg ml⁻¹) by the modification by Inzana (1983) of the method of Westphal & Jann (1965). SPA was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 by autoclaving (Amano *et al.*, 1989). The extract was purified by chromatography on columns of DEAE-Sephadex A-25 (Pharmacia) and Sephacryl S-300 (Pharmacia) and lyophilized (Amano *et al.*, 1989).

Preparation of antibodies. Antiserum against whole cells of *A. actinomycetemcomitans* Y4 was prepared in a rabbit by intravenous injections of formalin-killed whole cells. A monoclonal antibody (mAb) directed to SPA of *A. actinomycetemcomitans* Y4 (mAb S5) and one directed to lipopolysaccharide (LPS) of strain Y4 (mAb L2) were prepared and purified as described previously (Koga *et al.*, 1990b).

Construction and isolation of SPA-defective mutants. Plasmid pAM120 was isolated from *E. coli* DH1 containing the plasmid as described by Sambrook *et al.* (1989). The plasmid preparation was purified twice by CsCl/ethidium bromide gradient centrifugation (Sambrook *et al.*, 1989). *A. actinomycetemcomitans* Y4 was grown overnight in THY broth. The overnight culture (0.5 ml) was diluted into 5 ml fresh THY broth, and incubated at 37 °C in a 5% (v/v) CO₂ atmosphere for about 4 h until an OD₅₅₀ of 0.1–0.2 was attained. Samples (0.2 ml) were transferred to sterile glass culture tubes, and 1 µg of pAM120 DNA was added. After mixing gently, the mixtures were diluted with 1 ml fresh THY broth and allowed to stand at 37 °C in a 5% CO₂ atmosphere for 4 h. The cultures were then diluted with PBS, plated on THY agar containing tetracycline (2 µg ml⁻¹), and incubated at 37 °C for 3 d. Sterile nitrocellulose membrane filters were placed on colonies on the

agar at 37 °C for 2 h, washed with PBS, and blocked with 3% (w/v) bovine serum albumin (BSA) for 1 h. The filters were reacted with mAb S5 (at a 1:400 dilution in PBS) at 25 °C for 1 h, and washed with PBS. The antibody bound to the immobilized antigens was detected by a solid-phase immunoassay with horseradish-peroxidase-conjugated goat anti-mouse immunoglobulin G (at a 1:1000 dilution in PBS; Cappel) (Koga *et al.*, 1986). Clones that failed to react with mAb S5 were isolated as SPA-defective mutants.

Southern hybridization. Cellular DNA from *A. actinomycetemcomitans* was prepared as described by Okahashi *et al.* (1989) and digested with HindIII, EcoRI, or HindIII and SacI. The DNA fragments were separated by 0.7% (w/v) agarose gel electrophoresis, and transferred to nylon membranes (Okahashi *et al.*, 1989). pAM120 DNA biotinylated with the PhotoGene Nucleic Acid Detection System (Bethesda Research Laboratories) was used as a probe. Hybridization on nylon membranes was performed with 50% (v/v) formamide at 42 °C (Okahashi *et al.*, 1989).

Immunological procedures. Enzyme-linked immunosorbent assay (ELISA) was performed as described by Koga *et al.* (1990b). In brief, whole cells (0.1 mg ml⁻¹) in 50 mM-carbonate/bicarbonate buffer (pH 9.6) were added to the wells of flat-bottomed polystyrene microtitre plates (100 µl each). After centrifuging the plates, 0.5% (v/v) glutaraldehyde solution (100 µl) was added to each well, and incubated for 15 min at room temperature. The plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST). After a final wash, 1% (w/v) BSA in PBST (PBST-BSA) was added (100 µl per well), and the plates were incubated at 25 °C for 1 h. Excess BSA was removed by three washes with PBST, and then serial twofold dilutions of mAb S5 or mAb L2 were added (100 µl per well). After incubation for 1 h at 25 °C, unbound antibodies were washed out with PBST and alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin G (Zymed) at a 1:1000 dilution in PBST-BSA was added (100 µl per well). The plates were incubated at 25 °C for 1 h, washed with PBST, and *p*-nitrophenyl phosphate (1 mg ml⁻¹) dissolved in diethanolamine buffer (pH 9.6) containing 0.02% (w/v) sodium azide and 0.5 mM-MgCl₂ was then added (100 µl per well). After incubation at 37 °C for 1 h, the A₄₀₅ was measured.

For Western blot analysis, the autoclaved extract or phenol/water extract from *A. actinomycetemcomitans* was mixed with an equal volume of 0.2 M-Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.4 mM-EDTA, and heated at 100 °C for 5 min. The mixture (20 µl) was electrophoresed at 25 mA per gel at room temperature for 1.5 h using a 12.5% (w/v) resolving and a 3% (w/v) stacking gel (90 × 80 × 1 mm) containing 0.1% (w/v) SDS, and transferred to a nitrocellulose sheet by the method of Towbin *et al.* (1979). After blocking with PBST-BSA, the sheet was treated with mAb S5 or mAb L2 at a 1:400 dilution in PBST. The antibody, bound to the immobilized replica antigens on the sheet, was detected with horseradish-peroxidase-conjugated goat anti-mouse immunoglobulin G (E·Y Laboratories) at a 1:400 dilution in PBST. Low-range prestained SDS-polyacrylamide gel electrophoresis standards (Bio-Rad) were used as standards in Western blotting. Immunodiffusion was performed in 1% (w/v) Noble agar (Difco) in PBS.

Hydrophobicity. Cells of *A. actinomycetemcomitans* were suspended in PUM buffer (Rosenberg *et al.*, 1980) to an OD₅₅₀ of 0.6. Samples (3 ml) were placed in test tubes, and hexadecane (300 µl) was added. The tubes were then vortex-mixed for 1 min and allowed to stand until the phases separated. The OD₅₅₀ of the lower, aqueous phase was measured. Adsorption was calculated as the percentage loss in OD₅₅₀ relative to that of the initial cell suspension (Koga *et al.*, 1990a).

Sugar composition. The autoclaved extract (5 mg) was hydrolysed with 400 µl of 2 M-HCl at 100 °C for 5 h. The hydrolysate was dried by evaporation, and suspended in 100 µl distilled water containing xylose

(30 µg ml⁻¹) as an internal standard. Sugar composition in the hydrolysate was analysed with a high-performance liquid chromatograph (model LC-4A; Shimadzu) equipped with a postcolumn labelling system for reducing sugars and an anion-exchange column (Shimpack ISA-07; Shimadzu) (Amano *et al.*, 1989).

Results

Construction and characterization of SPA-defective mutants

To define the frequency of normally occurring spontaneous SPA-defective variants, we screened about 1500 colonies of *A. actinomycetemcomitans* Y4. However, all colonies strongly reacted with a mouse mAb to SPA of strain Y4 (mAb S5) in colony immunoblotting. To construct SPA-defective mutants, plasmid pAM120 containing transposon Tn916 was used to transform *A. actinomycetemcomitans* Y4. The frequency of transformation was about 2×10^{-7} transformants (Tc^r) per viable cell. In colony immunoblotting, 11 clones among 1000 Tc^r transformants gave negative or a very weak reaction with mAb S5, and were isolated as SPA-defective mutants. Reactivity of mAb S5 and mAb L2 against whole cells of these clones was determined by ELISA. Two clones (strains ST1 and ST2) lacked reactivity with mAb S5, and 9 clones (strains ST3 to ST11) reacted very weakly with it (Table 1). mAb S5 did not react with whole cells of *A. actinomycetemcomitans* strains ATCC 29523 (serotype a) and NCTC 9710 (serotype c), or *E. coli* K235. mAb L2 reacted with whole cells of all strains of *A. actinomycetemcomitans* used in this study, but not with those of *E. coli* K235 (Table 1). This mAb reacted more

Table 1. Reactivity of mAb S5 and mAb L2 against whole cells of *A. actinomycetemcomitans* and *E. coli*

Strain (serotype)	ELISA titre (A_{405})*	
	mAb S5	mAb L2
<i>A. actinomycetemcomitans</i>		
Y4 (b)	1.19	1.10
ST1	0.03	2.76
ST2	0.03	2.62
ST3	0.19	2.63
ST4	0.15	2.82
ST5	0.20	2.47
ST6	0.12	2.67
ST7	0.24	2.71
ST8	0.21	2.70
ST9	0.26	2.56
ST10	0.26	2.53
ST11	0.24	2.68
ATCC 29523 (a)	0.06	0.67
NCTC 9710 (c)	0.02	0.32
<i>E. coli</i>		
K235	0.03	0.06

* Whole cells (10 µg) were allowed to react with mAb S5 (1:32000 dilution) or mAb L2 (1:128000 dilution). Each value represents the mean of triplicate assays.

strongly with whole cells of SPA-defective mutants than with those of the parent strain Y4, perhaps due to the exposure of LPS on their cell surfaces by the loss of SPA.

Immunodiffusion tests showed that mAb S5 or rabbit antiserum against whole cells of strain Y4 produced a fused precipitin band with purified SPA and autoclaved extract from *A. actinomycetemcomitans* Y4, but no precipitin band with autoclaved extracts from strains

Fig. 1. Immunodiffusion of autoclaved extracts of *A. actinomycetemcomitans* strains against rabbit antiserum to whole cells of strain Y4 (A) and mAb S5 (B). Wells: 1, SPA purified from autoclaved extract of strain Y4; 2, autoclaved extract of strain Y4; 3, autoclaved extract of strain ST1; 4, autoclaved extract of strain ST2; 5, autoclaved extract of strain ST3; 6, autoclaved extract of strain ST5.

Fig. 2. Western blot analysis of autoclaved extracts of *A. actinomycetemcomitans* strains. Immobilized antigens transferred to a nitrocellulose sheet by an electrophoretic blotting procedure were allowed to react with mAb S5. The antibody was detected by solid-phase immunoassay with horseradish-peroxidase-conjugated goat anti-mouse IgG. Lanes: 1, strain Y4; 2, strain ST1; 3, strain ST2; 4, strain ST3; 5, strain ST5.

ST1, ST2, ST3 and ST5 (Fig. 1). Autoclaved extracts and phenol/water extracts from *A. actinomycetemcomitans* strains were analysed by Western blotting using mAb S5 and mAb L2, respectively. In the autoclaved extract from strain Y4, multiple high-molecular-mass bands reacted with mAb S5, but the autoclaved extracts from strains ST1, ST2, ST3 and ST5 did not react with this mAb (Fig. 2). In the phenol/water extracts from all strains of *A. actinomycetemcomitans* used in this study, diffuse low-molecular-mass bands reacted with mAb L2 (Fig. 3). These results indicate that these SPA-defective mutants as well as the parent strain Y4 have the ability to produce LPS.

Sugar composition of autoclaved extracts from *A. actinomycetemcomitans* strains was determined by high-performance liquid chromatography. Rhamnose, fucose, galactose, glucose, glucosamine and three other unidentified sugars were detected in the autoclaved extract from strain Y4 (Table 2). The hydrolysates of autoclaved extracts from strains ST1 and ST2 contained a trace

Fig. 3. Western blot analysis of phenol/water extracts of *A. actinomycetemcomitans* strains. Immobilized antigens on a nitrocellulose sheet were allowed to react with mAb L2. Lanes are as in Fig. 2.

Table 2. Sugar composition of autoclaved extracts from whole cells of *A. actinomycetemcomitans* strains

Strain	Content (μg per mg of whole cells)*				
	Rha	Fuc	Glc	Gal	GlcN
Y4	0.13	0.13	0.65	0.54	0.36
ST1	0.02	<0.01	0.54	0.43	0.30
ST2	0.02	<0.01	0.55	0.36	0.19
ST3	<0.01	0.04	0.58	0.31	0.24
ST5	<0.01	0.04	0.60	0.30	0.40

* Each value represents the mean for four assays. Three other unidentified sugars were detected by high-performance liquid chromatography, but their contents in strains ST1, ST2, ST3 and ST5 were almost the same as those in strain Y4.

amount of rhamnose, but not fucose. Those of autoclaved extracts from strains ST3 and ST5 contained a trace amount of fucose, but not rhamnose. The autoclaved extracts from all the mutants contained galactose, glucose, glucosamine and three unidentified sugars.

Cell-surface hydrophobicity of *A. actinomycetemcomitans* strains was determined by their adsorption to hexadecane. The hydrophobicity of SPA-defective

Fig. 4. Southern hybridization analysis of *A. actinomycetemcomitans* strains. Chromosomal DNAs from *A. actinomycetemcomitans* strains were digested with *Eco*RI (a) or *Hind*III (b). Hybridization was done with the biotinylated pAM120 DNA probe at 42 °C in 50% (v/v) formamide. Lanes: 1, strain Y4; 2, strain ST1; 3, strain ST2; 4, strain ST3; 5, strain ST5.

Table 3. Cell hydrophobicity of *A. actinomycetemcomitans* strains

Strain	Hydrophobicity (%)*
Y4	8.6 ± 1.9
ST1	20.5 ± 1.5
ST2	16.4 ± 1.8
ST3	23.1 ± 2.3
ST5	24.9 ± 1.7

* The bacterial suspension (3 ml) was mixed with 0.3 ml hexadecane. Adsorption was calculated as the percentage loss in OD₅₅₀ relative to that of the initial cell suspension. Each value represents the mean ± standard deviation for five assays.

strains ST1, ST2, ST3 and ST5 was higher than that of the parent strain Y4 (Table 3).

Analysis of Tn916 insertions

To confirm the integration of Tn916 into chromosomal DNA, we carried out Southern blot analysis of SPA-defective mutants by using biotinylated pAM120 DNA as a probe. The transposon contains one *Hind*III site and one *Sac*I site, but no *Eco*RI site (Senghas *et al.*, 1988). The biotinylated plasmid DNA hybridized with a 21–23 kb *Eco*RI fragment of chromosomal DNA from strains ST1, ST2, ST3 and ST5, but not with the chromosomal DNA from strain Y4 (Fig. 4a). The probe hybridized with two *Hind*III fragments of chromosomal

DNAs from these mutants (Fig. 4b). The sizes of hybridized *Hind*III fragments from strains ST1, ST2, ST3, and ST5 were 14 and 17 kb, two 17 kb, 14 and 22 kb, and 10 and 16 kb, respectively. To confirm the overlapping of hybridized *Hind*III fragments from strain ST2, chromosomal DNA from this strain was digested with both *Hind*III and *Sac*I. The probe hybridized with three *Hind*III–*Sac*I fragments of 1, 9 and 17 kb (data not shown). These findings suggest a single insertion of Tn916 into different locations within the genome of *A. actinomycetemcomitans*.

Discussion

A. actinomycetemcomitans strain Y4 (serotype b) produces capsular-like SPA that is composed of 44% L-rhamnose and 49% D-fucose in repeating disaccharide units, and a trace amount of fatty acid (Amano *et al.*, 1989). In the present study, 11 SPA-defective mutants of *A. actinomycetemcomitans* Y4 were constructed by insertion of transposon Tn916. Southern blot analysis suggested that Tn916 was inserted into a variety of different sites on the chromosome. ELISA showed that whole cells of two mutant clones completely lacked reactivity with a mAb to SPA (mAb S5). Those of the other clones reacted very weakly with mAb S5, suggesting that in these clones Tn916 might have inserted into sites that regulate the expression of SPA or that these clones might produce an mAb S5-reactive antigen

different from SPA. Autoclaved extracts from clones that reacted very weakly with mAb S5 in ELISA failed to react with this mAb in immunodiffusion tests and Western blot analysis. This discrepancy might be due to the difference in sensitivity among the assays.

Neisseria meningitidis serogroup B produces a capsular polysaccharide that is composed of α -2,8-linked N-acetylneuraminic acid (Jennings, 1990). Frosch *et al.* (1989) have recently cloned the gene complex encoding all determinants of the biosynthetic pathway of the capsule of the organism. Using transposon and deletion mutagenesis, they presented evidence for the existence of distinct genetic functions that are essential for its synthesis and transport from the cytoplasm to the periplasm and further to the cell surface (Frosch *et al.*, 1989). No autoclaved extracts from SPA-defective mutants of *A. actinomycetemcomitans* reacted with monoclonal and polyclonal antibodies to SPA in immunodiffusion tests and Western blot analysis. Similar results were obtained with sonic extracts from these mutants (data not shown). These findings indicate that the lack of SPA in these mutants is not due to the inactivation of the gene for the transport of the polysaccharide.

Polysaccharide capsules of various organisms, such as *Bacteroides fragilis* (Kasper *et al.*, 1984), *H. influenzae* (Moxon & Vaughn, 1981), *Vibrio vulnificus* (Wright *et al.*, 1990) and *S. pneumoniae* (Watson & Musher, 1990), have been reported to be important for their pathogenicity. Takahashi *et al.* (1991) reported that SPA of *A. actinomycetemcomitans* serotype b strongly induces the release of interleukin-1 by murine macrophages. This finding suggests that the SPA of *A. actinomycetemcomitans* may play a critical role in the pathogenesis of the organism.

The role of cell-surface hydrophobicity in mediating bacterial adherence to tooth surfaces and host cell surfaces has recently received increased attention (Rosenberg & Doyle, 1990). Most capsular polysaccharides are hydrophilic (Moxon & Kroll, 1990). The presence of capsular polysaccharide has been reported to reduce the cell hydrophobicity of *Pasteurella multocida*, *Streptococcus pyogenes*, *Acinetobacter calcoaceticus*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Rosenberg & Doyle, 1990). SPA-defective mutants of *A. actinomycetemcomitans* were more hydrophobic than the parent strain. Hydrophilic SPA of *A. actinomycetemcomitans* may interfere with the hydrophobic interaction between the organism and the host cell. However, the possibility also exists that the SPA of *A. actinomycetemcomitans* might participate in the lectin-like interaction between the organism and the host cell.

SPA of *A. actinomycetemcomitans* serotype b was recently reported to be a portion of the O-polysaccharide

of LPS (Page *et al.*, 1991; Wilson & Schifferle, 1991). In this study, whole cells of all SPA-defective mutants reacted strongly with a mAb to LPS (mAb L2). Western blot analysis showed that mAb L2 recognized a low-molecular-mass component in phenol/water extracts from the parent strain Y4 and its SPA-defective mutants. These findings seem to support the concept that the SPA of *A. actinomycetemcomitans* is the polysaccharide portion of LPS and that the mutants are unable to synthesize complete LPS molecules but they are still able to synthesize the LPS core. However, the major component sugars of the purified Y4 LPS are glucose, L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, galactose and glucosamine, but fucose and rhamnose are not detectable (Koga *et al.*, 1991; Masoud *et al.*, 1991). On the other hand, SPA of strain Y4 is composed of fucose and rhamnose, and L-glycero-D-mannoheptose, D-glycero-D-mannoheptose, glucosamine and 3-hydroxymyristic acid, which are chemical markers of LPS, are not detectable (Amano *et al.*, 1989). In this study, the rhamnose and fucose contents of autoclaved extracts from the mutants, as compared with the autoclaved extract from strain Y4, were very low. However, the hydrolysates of autoclaved extracts from all SPA-defective mutants contained large amounts of glucose, galactose and glucosamine. In immunodiffusion assays, the two precipitin lines that formed when rabbit antiserum against whole cells of strain Y4 was reacted with purified Y4 SPA and with purified Y4 LPS crossed completely (unpublished data). Further work is needed to determine whether SPA of *A. actinomycetemcomitans* serotype b is related to LPS.

The Tn916-induced mutant clones described here should prove very useful for clarifying the role of SPA of *A. actinomycetemcomitans* in the virulence of the organism and locating the genetic determinants for the biosynthesis of SPA. In addition, fragments containing the transposon in the chromosomes of SPA-defective mutants will be useful for cloning the gene complex responsible for the synthesis of SPA and the regulation of expression of the polysaccharide.

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