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Adherence of *Aggregatibacter actinomycetemcomitans* via serotype-specific polysaccharide antigens in lipopolysaccharides

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Introduction: Gram-negative *Aggregatibacter actinomycetemcomitans* is recognized as an important periodontal pathogen. A striking property of this bacterium is its ability to form a tenacious biofilm adhering to abiotic surfaces. Both fimbrial and non-fimbrial adhesins are believed to be responsible for this ability. In our study, specific markerless mutants in the biosynthesis genes of cell surface polysaccharides were constructed with the Cre-*loxP* recombination system to identify non-fimbrial adhesin(s).
Methods: Non-fimbriated *A. actinomycetemcomitans* strain ATCC29523 (serotype a) was used to construct a deletion mutant of serotype-a specific polysaccharide antigen (SPA-a) in lipopolysaccharide (LPS). The LPS was purified through a polymyxin B column following phenol extraction, and verified by silver staining following sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by immunoblot analysis using rabbit antisera raised against SPA-a. Strains were grown in broth for 2 days and examined for the adherence of bacterial cells on the glass surface.
Results: Strain ATCC29523 formed a thin film of bacterial growth on the glass surface. The deletion of SPA-a affected its ability to form this thin film. When this mutant was rescued with the wild-type SPA-a gene cluster, its adherence-positive phenotype was restored.
Conclusion: SPA-a in the LPS molecule appears to promote the adherence of *A. actinomycetemcomitans* cells to abiotic surfaces.

Key words: adherence; *Aggregatibacter actinomycetemcomitans*; biofilm; lipopolysaccharide

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In natural environments, bacteria often adhere to inert or living surfaces to form a biofilm. The biofilm can be described as a structured community of cells encased in a self-produced matrix. The matrix is a complex mixture of macromolecules that include polysaccharides, proteins, and DNA (5, 6, 8, 19, 28, 40, 45, 47).

The gram-negative facultative bacillus *Aggregatibacter actinomycetemcomitans*, recognized as an important periodontal pathogen (3), has been observed to form biofilms. In broth culture, this bacterium autoaggregates and forms a tenacious biofilm on the glass or plastic surfaces of culture vessels (13, 41). The adherence and autoaggregation are so dramatic that broth cultures of *A. actinomycetemcomitans* often show no turbidity, while the tightly adherent microcolonies on the vessel surfaces are resistant to dislodging by vigorous shaking or vortex mixing.

Fimbriae are thought to be responsible for both the adherence and autoaggregation of *A. actinomycetemcomitans* (12). In addition, *A. actinomycetemcomitans* might express non-proteinaceous, non-fimbrial adhesins. Fine et al. (7) showed that the adherence of *A. actinomycetemcomitans* to abiotic surfaces was disrupted by treatment of the bacteria with periodate (a carbohydrate-oxidizing reagent) but not by treatment with trypsin. A more recent study by Inoue et al. (11) demonstrated that a non-fimbriated *A. actinomycetemcomitans* strain adheres and forms biofilm on glass

culture vessels or coverslips. This adherence was also affected by treatment with periodate, but not by treatment with protease. These reports suggest that polysaccharides on the surface of *A. actinomycetemcomitans* might function as adhesins. The identity of the non-fimbrial adhesin(s) of *A. actinomycetemcomitans* remains to be determined.

Several surface polysaccharides of *A. actinomycetemcomitans* have been identified. Kaplan et al. (16) identified that the gene locus *pgaABCD* is responsible for the synthesis of a linear polysaccharide of *N*-acetyl-D-glucosamine that functions in aggregation, but not adherence, of *A. actinomycetemcomitans*. On the other hand, *A. actinomycetemcomitans* strains are classified into six distinct serotypes (a–f), based on structurally and immunologically distinct serotype-specific polysaccharide antigens (SPAs). The structures and genetic determinants of SPAs have been elucidated in detail (1, 15, 21–26, 32, 36, 48–50). The structure of lipopolysaccharide (LPS) consists of a lipid A portion, a core oligosaccharide region, and an O-antigen polysaccharide chain of repeating oligosaccharide units. *A. actinomycetemcomitans* SPAs are recognized as LPS O-antigens (17, 24, 46). Specifically pertinent to serotype a, Suzuki et al. (36) identified a gene cluster for the synthesis of serotype-a specific polysaccharide antigen (SPA-a) and confirmed the functions of this gene cluster in the expression of SPA-a. Furthermore, they proved a biochemical function of some genes in the cluster to synthesize SPA-a, using the gene products overproduced in *Escherichia coli* (37).

The present study tested the hypothesis that SPA might function as a non-fimbrial adhesin in a non-fimbriated *A. actinomycetemcomitans* serotype a strain ATCC29523. Specific markerless gene deletion mutants in the biosynthesis of SPA-a and the *N*-acetyl-D-glucosamine polysaccharide were constructed with the Cre-*loxP* recombination system.

Materials and methods

Bacterial strains and culture conditions

The *A. actinomycetemcomitans* strains used in this study are listed in Table 1. These strains were grown on a modified sTSB agar (3% trypticase soy broth, 0.3% yeast extract, 5% heat-inactivated horse serum, and 1.5% agar) (42), or in mTSB broth (3% trypticase soy broth and 0.6% yeast extract) (44) at 37°C in 5% CO_2. When needed, the medium was supplemented with 50 μg/ml spectinomycin (Spe) or 6 μg/ml tetracycline (Tc). The *E. coli* plasmids were propagated in DH5α strain as described previously (31).

For broth culture of *A. actinomycetemcomitans* strains, bacterial colonies from agar plates were collected and transferred to a tube with 2.5 ml mTSB broth. Bacterial cells were cultured overnight and then subcultured (1 : 100) into fresh mTSB broth. After 2 days incubation, the adherence of *A. actinomycetemcomitans* cells to the glass surface was evaluated as described below.

Table 1. Plasmids and *Actinobacillus actinomycetemcomitans* strains used in this study

Plasmid/strain	Characteristics
Plasmid	
pBluescript KS	Purchased plasmid (Stratagene, La Jolla, CA), Amp^r
pB-Lox2	pBluescript KS with a pair of *loxP* sequences
pK-Spe	Spe^r cassette (18, 42)
pLox2-Spe	pB-Lox2 with Spe^r cassette from pK-Spe, inserted into the *loxP*-intervening region
pPK1	*A. actinomycetemcomitans*/*E. coli* shuttle plasmid (33), Spe^r
pAT1	pPK1 with *tet(O)* gene (20)
pAT-Cre	pAT1 with Cre recombinase gene (35)
Strains	
ATCC29523	Serotype a, non-fimbrial, natural transformable
Y4	Serotype b, non-fimbrial
SA15L	ATCC29523 Δorf2–12 in a gene cluster for the biosynthesis of SPA-a (36), replaced by Spe^r cassette flanked by a pair of *loxP* sequences
SA16ML	SA15L ΔSpe^r cassette by pAT-Cre, markerless mutant with one *loxP* sequence
SA21CL	SA16ML Δone *loxP* sequence, replaced by *orf2–12* in a SPA-a gene cluster (36) and Spe^r cassette flanked by a pair of *loxP* sequences
SA28CML	SA21CL ΔSpe^r cassette by pAT-Cre, markerless mutant with one *loxP* sequence
HM17L	ATCC29523 Δ*pgaBC* (16), replaced by Spe^r cassette flanked by a pair of *loxP* sequences
HM23ML	HM17L ΔSpe^r cassette by pAT-Cre, markerless mutant with one *loxP* sequence

Plasmids

Plasmids used in this study are listed in Table 1. The plasmid pLox2-Spe was constructed, in which the Spe-resistant (Spe^r) cassette was flanked by *loxP* sites. Two DNAs were synthesized: 5′-TCGA CACCACGTGGATCCATAACTTCGTA TAAGATCTGCTATACGAAGTTATGAT ATCATAACTTCGTATAATGTATGCTA T-ACGAAGTTATGTCGACACGTGGT G-3′; 5′-GATCCACCACGTGTCGACA TAACTTCGTATAGCATACATTATACG AAGTTATGATATCATAACTTCGTATA GCAGATCTTATACG-AAGTTATGGAT CCACGTGGTG-3′ (*loxP* sites are underlined). These two DNA fragments were annealed, and cloned into the pBluescript KS (Stratagene, La Jolla, CA) at the *Bam*HI and *Sal*I sites. Three of the resultant plasmids were sequenced to confirm the construct, and one of them was designated as pB-Lox2. An *Eco*RI-cut Spe^r cassette from pK-Spe (18, 42) was inserted in the *Eco*RV site between the *loxP* sites in pB-Lox2 to produce pLox2-Spe.

The *E. coli–A. actinomycetemcomitans* shuttle plasmid pAT-Cre was constructed based on the pPK1 (33). Briefly, a 2.4-kilobase (kb) DNA containing the *tet(O)* gene (20) was amplified with primers Tc-USS and Tc-D2, listed in Table 2. The DNA was treated with T4 polynucleotide kinase and DNA polymerase Klenow fragment (New England BioLabs, Beverley, MA), and ligated to the 2.6-kb *Bam*HI/*Kpn*I fragment from pPK1 to produce pAT1. A 1.1-kb DNA containing the Cre recombinase gene (35) was amplified with primers Cre-X1 and Cre-X2 (Table 2). The DNA was inserted at the *Xba*I site in pAT1 to produce pAT-Cre.

Markerless mutations

Target genes in *A. actinomycetemcomitans* ATCC29523 strain were deleted with a Cre-*loxP* recombination system (see Figs 1A and 2 for details). Table 2 lists the sequences of the primers used for deletion or complementation of target genes by polymerase chain reaction (PCR). Two sets of primers were used to amplify two DNA fragments flanking the target gene: SApre5/SApre2 and SApst7/SApst4 for the deletion of the gene cluster producing SPA-a, and HMpre1/HMpre6 and HMpst7/HMpst8 for the deletion of *pgaBC*. The recognition sites of the restriction enzyme *Dra*III were incorporated into the primers adjoining the target gene. The amplicons were mixed and digested with *Dra*III, and ligated to the

Table 2. Sequences of primers used for deletion or complementation of target genes

Primer	Sequence (5′ → 3′)[1,2]	Annealing site
Tc-USS	AAAGTGCGGTTTAAATTACGAAGGAGGAG	Upstream of *tet(O)* gene (20)
Tc-D2	TACACCACGTGCAAGCTGTTAAGCTAACTT	Downstream of *tet(O)* gene (20)
Cre-X1	CCTCTAGACCCT-GGAGCCGATATGTCC	Upstream of Cre gene (35)
Cre-X2	CGATCTAGAAATCCTT-TCTCATATGTC	Downstream of Cre gene (35)
SApre2	ATCACGTGGTGCTTTCTCACAAGCTTC	ORF2 (36)
SApre5	CAGGGAAAGTTCTGTCGCTC	ORF1 (36)
SApre6	CACACGTGGTGAAAAATAGGAAAAGC	Downstream of ORF12 (36)
SApst4	GGCGTAGCGACATTGTCTTA	ORF14 (36)
SApst7	GTTCACGTGGTGTTGAAGGGGAAAGG	ORF12 (36)
HMpre1	CGCGGTTATGTGACATCCGA	*pgaA* gene (16)
HMpre6	CACACGTGGTGAACACAAATGAAACG	*pgaB* gene (16)
HMpst7	CTCACGTGGTGATTATGGATTTAC	*pgaD* gene (16)
HMpst8	CGTAATGAGCACCTTGTTCTTC	Downstream of *pgaD* (16)

[1]In the Tc-USS primer, the nine-base core sequence of the uptake signal sequence (44) is underlined.
[2]Other underlined sequences are the restriction sites of either *Dra*III or *Xba*I for specific ligation to the antibiotic-resistant cassette or plasmid.

Spe^r cassette (which was flanked by two *loxP* sites) derived from plasmid pLox2-Spe by *Dra*III digestion. The recombinant fragment was directly used for natural transformation of the ATCC29523 strain. The basic method of natural transformation was as described previously (42, 44). Transformation was verified by PCR analysis using primers flanking the conjoining site and primers in the target gene. In the ATCC29523 strain, the SPA-a gene cluster and *pgaBC* were replaced by the Spe^r cassette flanked by a pair of *loxP* sequences, and SA15L and HM17L were generated, respectively.

Plasmid pAT-Cre was next introduced into the primary mutant by natural transformation as described previously (42, 44). Tc-resistant colonies were identified and streaked on a sTSB plate without antibiotics for 2 days. A small amount of a bacterial colony was plated to obtain single colonies, which were subsequently examined for Spe-resistance and Tc-resistance. The selected colonies were found to be sensitive to both antibiotics, which was indicative of the loss of the Spe^r marker and the pAT-Cre plasmid. PCR examination with flanking primers suggested that all these colonies had lost the Spe^r gene via recombination at the *loxP* sites. The markerless mutants of the SPA-a gene cluster and *pgaBC* were designated as strains SA16ML and HM23ML, respectively.

The resultant SPA-a deletion mutant (strain SA16ML) had the region between the open reading frame (ORF) 2 (including part of ORF2) and ORF12 (including part of ORF12) replaced with a 34-base pair (bp) *loxP* in *A. actinomycetemcomitans* strain ATCC29523 (Fig. 1A). ORF2–ORF12 products are believed to function as a nucleotide–sugar synthetase (ORF2), ABC-transporters (ORF3 and -4), glycosyltransferases (ORF5, -6, -10, -11, and -12), a nucleotide–sugar reductase (ORF7), an acetyltransferase (ORF8) and a nucleotide–sugar dehydratase (ORF9) (36). Deletion of these ORFs was expected to result in the elimination of SPA-a from *A. actinomycetemcomitans* strain ATCC29523. In mutant strain HM23ML the genes *pgaBC*, which are part of the *pgaABCD* operon involved in the synthesis of a linear polymer of *N*-acetyl-D-glucosamine in *A. actinomycetemcomitans* (16), were deleted (Fig. 2).

Restoration of function of deleted genes in the mutants

Downstream genes of the SPA-a cluster are ORF13 and ORF14, which putatively encode the xylose operon regulatory protein and Na/H antiporter, respectively (36). Although the sequence data analysis suggested that their transcriptional directions were opposite to the SPA-a cluster (36), it has not been verified biochemically or genetically. Therefore, a downstream polar effect of the mutation in strain SA16ML was examined by the restoration of deleted gene fragment. The deleted region in mutant strain SA16ML was too large to be easily complemented with a cloning vector in *A. actinomycetemcomitans*. We elected to rescue strain SA16ML using a copy of the SPA-a gene cluster, and thereby generated the restored strain SA28CML (Fig. 1B). Briefly, the DNA fragment of the deleted region in strain SA16ML SPA-a with its upstream sequence was obtained by PCR (primers SApre5/SApre6) using genomic DNA of *A. actinomycetemcomitans* ATCC29523 strain as the template. The downstream region of the deleted region was also amplified by PCR (primers SApst7/SApst4). The amplicons were mixed and digested with *Dra*III, and then ligated to a Spe^r cassette released from plasmid pLox2-Spe as described above. After ligation, the mixture (which contained a recombinant fragment of Spe^r flanked by the deleted regions in the SPA-a gene cluster) was used to transform the mutant strain SA16ML and to select for Spe^r transformants. One of the Spe^r transformants was designated as strain SA21CL and was selected for further use. The Spe^r marker in strain SA21CL was removed by Cre recombinase using pAT-Cre as described above to generate strain SA28CML, which retained a copy of the *loxP* site after recombination. The *loxP* site in SA28CML served as a marker to distinguish it from the original gene cluster in the wild-type strain ATCC29523. The starting point of the downstream sequence next to the *loxP* site in strain SA28CML was identical to that in the mutant strain SA16ML. This could obviate concern about downstream polar effects from the insertion of *loxP*.

Preparation of LPS

LPS samples were prepared from strains Y4, ATCC29523, SA16ML, and SA28CML. Bacterial colonies cultured on two sTSB agar plates for 1 day were harvested and resuspended in 1 ml saline buffer. The concentration of bacterial cells in the sample was adjusted to OD_{600} = 25. After the collection of bacterial cells by centrifugation, LPS was extracted using a LPS extraction kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's procedure and then applied to the polymixin B immobilized column (Detoxi-Gel™ AffinityPak™ Pre-packed Columns; Pierce, Rockford, IL) for further purification. The LPS bound to the column was eluted with 1% sodium deoxycholate, purified by ethanol precipitation, and dispersed in 10 mM Tris–HCl buffer (pH 8.0).

Characterization of purified LPS

Purified LPS samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4–20% gels (Bio-Rad Laboratories, Hercules, CA) and subsequent silver staining, as described by Tsai and Frasch (39). The amount of LPS loaded for each strain was adjusted based on the number of cells (determined by OD_{600}). Rabbit antisera raised against *A. actinomycetemcomitans* serotype a, a kind gift from S. Asikainen (30), was used for the Western blot, and the

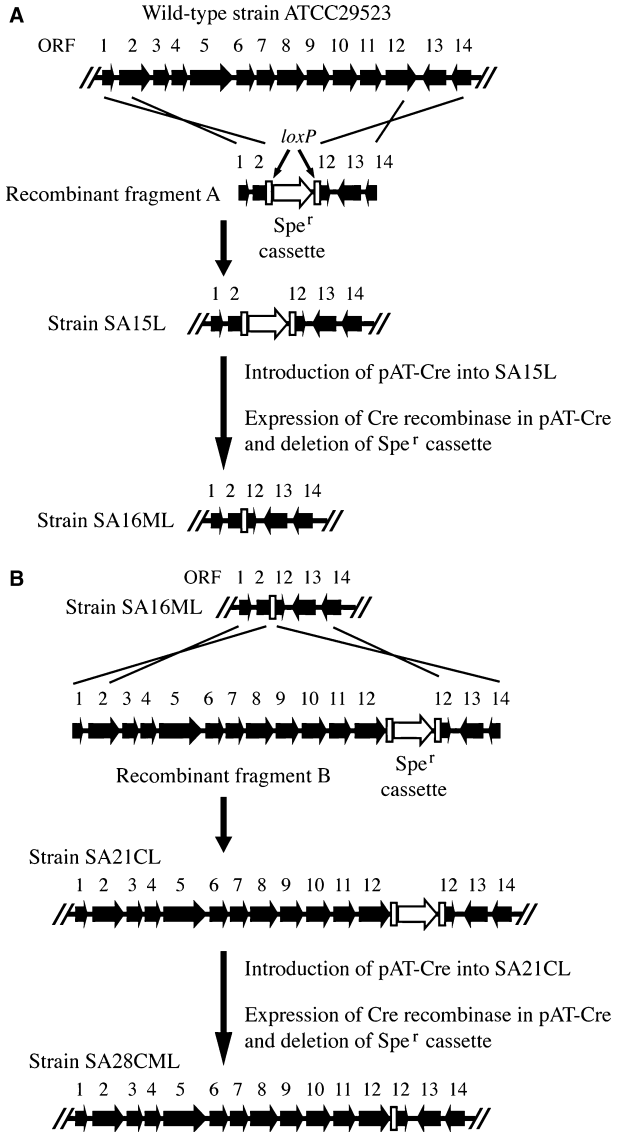

Fig. 1. Markerless mutation procedure for generating a deletion-mutant of the SPA-a gene cluster (A) and its complementation procedure (B). (A) According to the elucidated sequence of the SPA-a gene cluster (36), two DNA fragments flanking the ORF2–12 genes were amplified and ligated to the Spe^r cassette derived from pLox2-Spe (white rectangles flanking Spe^r cassette are the *loxP* sequence). Constructed fragment (Recombinant fragment A) was used as donor DNA for natural transformation of ATCC29523 strain. The ORF2–12 genes of the transformant were replaced by the Spe^r cassette to generate a mutant SA15L. Plasmid pAT-Cre was introduced and recombination at one pair of *loxP* sites eliminated the Spe^r cassette. The mutant obtained was designated SA16ML. (B) For the complementation of deleted genes in SA16ML, the DNA fragment of the SPA-a gene cluster (ORF1–12) was amplified by PCR using genomic DNA of *Actinobacillus actinomycetemcomitans* ATCC29523 strain. The downstream region of the deleted sequence (ORF12–14) was also amplified by PCR. The amplicons were ligated to the Spe^r cassette derived from pLox2-Spe. The recombinant fragment (Recombinant fragment B) was directly used for the complementation of markerless mutant SA16ML through natural transformation, and the generated strain was designated as SA21CL. Plasmid pAT-Cre was introduced and recombination at one pair of *loxP* sites eliminated the Spe^r cassette. The obtained mutant was designated as SA28CML.

SPA-a was detected with horseradish peroxidase-conjugated mouse antirabbit IgG.

Bacterial adherence assay

Strains ATCC29523, HM23ML, SA16ML, and SA28CML were assayed for bacterial adherence. The bacteria were grown in broth for 2 days in the conditions described above. Non-adherent cells were removed by vigorous vortex agitation. The glass tube was further rinsed with mTSB broth to remove the residual non-adherent cells. Adherent cells in the glass tube were stained with ethidium bromide and visualized under ultraviolet light (12). To verify the bacterial cells adhering to the glass surface, Gram-stain was applied to a glass coverslip located in the broth culture. Following incubation of bacterial biofilms for 2 days, the coverslips were washed with saline buffer to remove non-adherent bacterial cells. Gram-stained coverslips were observed with an optical light microscope (BX50-DP70; Olympus, Tokyo, Japan) according to standard protocol.

Results

SDS-PAGE and Western blot assays of purified LPS

Purified LPS samples prepared with polymixin B columns were examined by silver-staining the gels after SDS–PAGE (Fig. 3A). The quantities of LPS samples were adjusted based on equivalence cell numbers used for LPS extractions. LPS from the wild-type parental strain ATCC29523 (lane 2) revealed glycoforms with a ladder-like pattern similar to that previously reported in other *A. actinomycetemcomitans* strains (9). Mutant strain SA16ML (deletion of SPA-a) showed a similar SDS–PAGE pattern to that of the wild-type strain (compare lanes 2 and 3).

SPA-a is usually found as a high-molecular-weight smear that is not easily visualized by silver staining of SDS–PAGE gels. Therefore, we performed Western blot assays using polyclonal rabbit antisera raised against *A. actinomycetemcomitans* serotype a, and identified a smear band of SPA-a that was not detected in LPS from strain Y4 (a serotype b strain used as a negative control) (Fig. 3B, lanes 1 and 2). As expected, the SPA-a band was absent in LPS from mutant strain SA16ML (Fig. 3B, lane 3). The ladder-like pattern of bands in the low-molecular-weight region was commonly detected in strains ATCC29523, SA16ML, and Y4. The LPS profile of restored strains SA28CML (Fig. 3B, lanes 4) was indistinguishable from those in wild-type strain ATCC29523. The SPA-a appeared as a high-molecular-weight smear, whereas the fast migrating bands might represent molecules of lipid A with varying lengths of the core oligosaccharide. The above results demonstrated the deletion and the restoration of SPA-a in strain SA16ML and SA28CML, respectively.

Characteristics of broth-cultured *A. actinomycetemcomitans* strain ATCC29523 and derivative strains

Strain ATCC29523 grown in the broth for 2 days showed slightly turbid supernatant

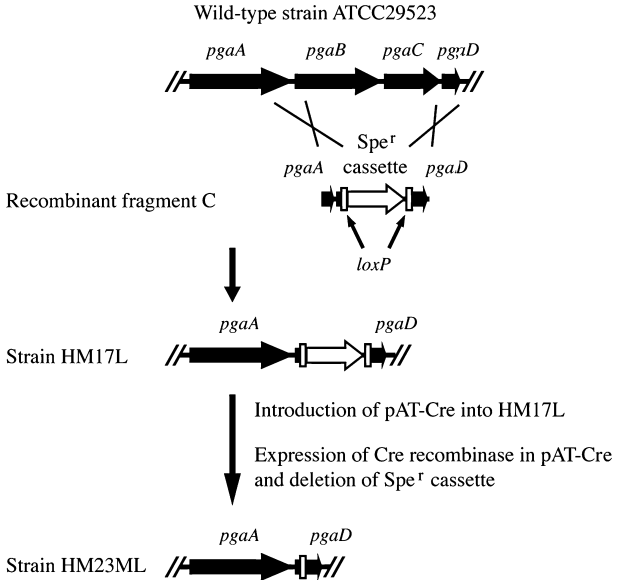

Fig. 2. Markerless mutation procedure for generating a deletion-mutant of *pgaBC* genes. Two DNA fragments flanking the *pgaBC* genes were amplified and ligated to the Spe^r cassette derived from pLox2-Spe (white rectangles flanking Spe^r cassette are the *loxP* sequence). The constructed fragment (Recombinant fragment C) was used as donor DNA for natural transformation of ATCC29523 strain. The *pgaBC* genes of the transformant were replaced by the Spe^r cassette to generate a mutant HM17L. Plasmid pAT-Cre was introduced and recombination at one pair of *loxP* sites eliminated the Spe^r cassette. The obtained mutant was designated HM23ML.

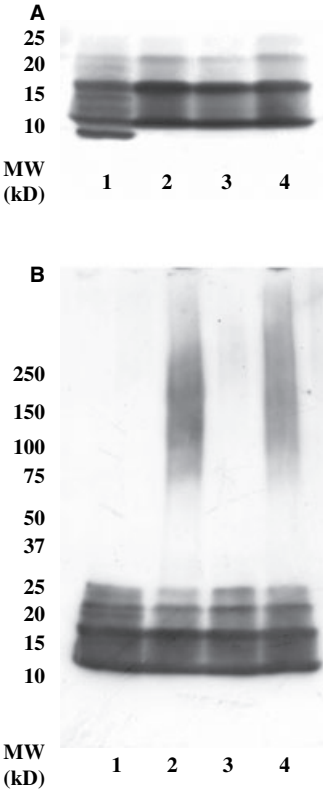

Fig. 3. (A) Silver-stained SDS–PAGE gel demonstrating the mutation or complementation of LPS genes and (B) Western blot membrane demonstrating the expression of SPA-a. Lane 1, Y4 (serotype b strain used as negative control); lane 2, ATCC29523 (serotype a and wild-type strain); lane 3, SA16ML (ΔSPA-a gene cluster in ATCC29523); lane 4, SA28CML (complementation of SPA-a gene cluster in SA16ML).

and loose aggregates settling down at the bottom of the glass tube (Fig. 4A, tube 1). The culture was removed after vigorous vortex agitation to loosen non-adherent bacteria and the tubes were rinsed. Adherent cells in the glass tubes were stained with ethidium bromide. A thin film of bacteria was seen on the surface of the glass tube (Fig. 4B, tube 1). Gram-stain showed that the thin film consisted of bacterial cells adhering to the glass surface (Fig. 5A).

The *pgaBC*-deletion mutant HM23ML exhibited turbid growth in broth culture (Fig. 4A, tube 2) without forming visible bacterial aggregates. The turbidity of the broth culture of strain HM23ML was greater than that of wild-type strain ATCC29523. However, strain HM23ML retained a thin film on the surface (Fig. 4B, tube 2; Fig. 5B) similar to wild-type strain ATCC29523, which was confirmed as a bacterial growth adhering on the surface.

On the other hand, a mutation of the SPA-a gene cluster showed a different result. The SA16ML mutant exhibited similar growth appearance in broth (Fig. 4A, tubes 3) to the wild-type strain ATCC29523 (Fig. 4A, tube 1). However, this mutant was not able to form a thin film on the glass surface (Fig. 4B, tubes 3; Fig. 5C). The generation of a strain SA28CML restored the phenotype seen in the wild-type strain ATCC29523 (Fig. 4B, tubes 4; Fig. 5D). These data suggest that SPA-a is required for the adherence ability of bacterial cells to the glass surface.

Discussion

Biofilm formation of bacterial cells is initiated by adherence to target surfaces. In general, the adherence is mediated by a number of distinct adhesins that are elements of the bacterial cell surface, such as fimbriae and glycocalyx polymers (5, 27, 28, 40, 47). In this study, we showed that the SPA of LPS was involved in the adherence of *A. actinomycetemcomitans* strain ATCC29523 to a glass surface in broth cultures (i.e. glass tubes). This phenomenon was also observed on the polypropylene and polystyrene surfaces (data not shown). Our results, however, disagree with those reported by Kaplan et al. (14), who demonstrated that the SPA of *A. actinomycetemcomitans* clinical isolate CU1000 (serotype f) was not required for adherence to a Petri dish surface. The discrepancy in results might be the result of different assay protocols or the use of different bacterial strains in the experiments. In particular, we noted that a SPA-a deletion mutant derived from a different non-fimbriated *A. actinomycetemcomitans* strain D7S-smooth (42) retained the ability for adherence in our assays (data not shown). It is possible that different strains of *A. actinomycetemcomitans* use different types of adhesins to form biofilms. The results of strain ATCC29523 adherence suggested that surface polysaccharide components might serve as adhesins in *A. actinomycetemcomitans*. Our finding that the *pgaABCD* locus did not contribute to adherence agrees with the results of Kaplan et al. (16).

Fresh clinical isolates of *A. actinomycetemcomitans* are invariably fimbriated and form small, rough-surface, translucent colonies with an internal star-shaped structure (7). After repeated *in vitro* passages, the rough-colony morphotype may yield non-fimbriated smooth-colony variants that grow as large, round, opaque colonies on agar (7). The expression of fimbriae is determined by the *flp* operon of 14 genes, *flp-1-flp-2-tadV-rcpCAB-tadZABCDEFG* (13). Smooth variants often contained mutations at the *flp* promoter region (43). Although strain ATCC29523 contained

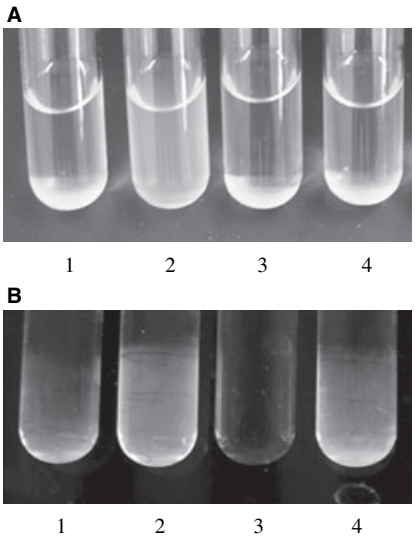

Fig. 4. (A) Broth culture in glass tubes and (B) visualization of adherence ability of *Actinobacillus actinomycetemcomitans* strains. After vigorous vortex agitation and rinsing of tubes in (A), adherent cells in the glass tubes were stained with ethidium bromide and visualized under ultraviolet light (B). Tube 1, ATCC29523 (wild-type strain); tube 2, HM23ML (Δ*pgaBC* in ATCC29523); tube 3, SA16ML (ΔSPA-a gene cluster in ATCC29523); tube 4, SA28CML (complementation of SPA-a gene cluster in SA16ML).

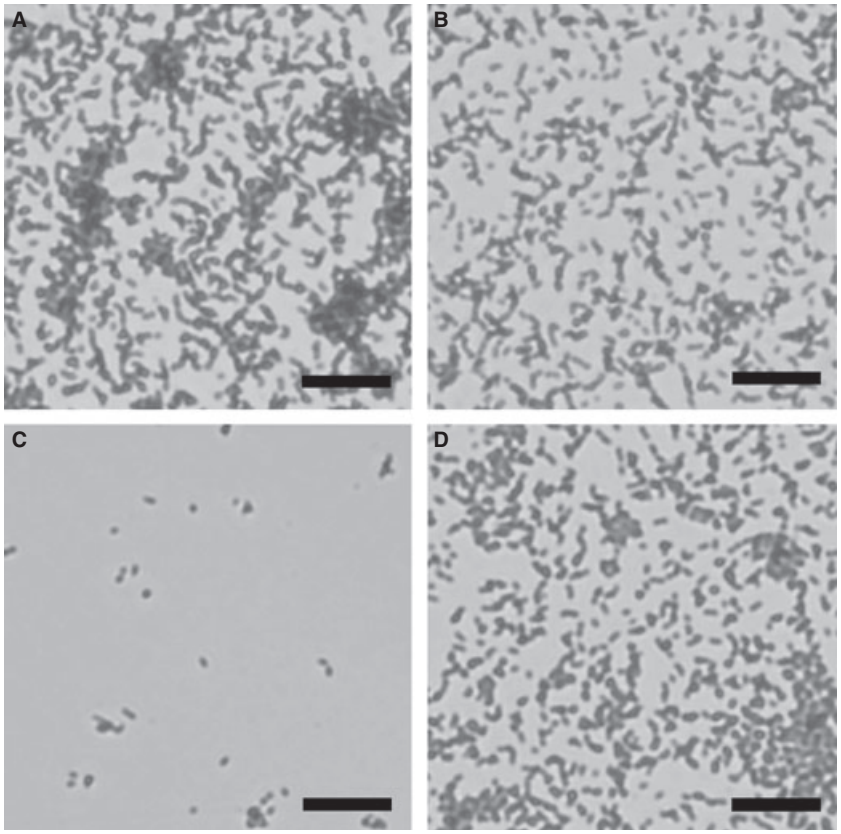

Fig. 5. Bacterial cells were grown on a glass coverslip located in the broth culture tubes. Following the removal of non-adherent cells from the coverslip by washing with saline buffer, Gram-stain was applied. Adherent cells on the surface were observed with an optical light microscope. (A) ATCC29523 (wild-type strain); (B) HM23ML (Δ*pgaBC* in ATCC29523); (C) SA16ML (ΔSPA-a gene cluster in ATCC29523); (D) SA28CML (complementation of SPA-a gene cluster in SA16ML). Bars represent 10 μm.

intact *flp-1* and *flp-2* gene loci with a wild-type promoter, and scant fimbriae were observed under transmission electron microscopy in our previous study (43), the colony of this strain appeared to be the smooth type on the agar plate. Thus, in this study the contribution of fimbriae to the adherence of strain ATCC29523 was assumed to be minimal.

In the conventional method, the function of a gene is assessed by deleting the target gene and replacing it with a marker for selection. However, cell physiology could be altered by known or unrecognized activities of the antibiotic-resistance marker. The need of additional markers for gene complementation of the mutant might further compound the problem. These issues can be obviated by the use of a markerless system for the construction of mutants. The Cre-*loxP* recombination system is a simple and powerful tool for DNA rearrangement in prokaryotic and eukaryotic cells (2, 4, 29, 38, 51). The Cre recombinase of bacteriophage P1 recognizes a 34-bp *loxP* site, which consists of an asymmetric 8-bp spacer flanked by 13-bp inverted repeats (34). When two *loxP* sites are in the same orientation in a DNA sequence, Cre-mediated recombination reaction results in the excision of the *loxP*-intervening region.

This study demonstrated the use of the Cre-*loxP* system for genetic manipulation of *A. actinomycetemcomitans*. The Cre-*loxP* system has already been used for other members of the Pasteurellaceae family (10). We also used this system to rescue the deletion mutant strain SA16ML. An important advantage is that the same downstream fragment (obtained by PCR) was used in generating the rescued mutant as well as the deletion mutant. Therefore, the phenotype in deletion mutant strain SA16ML cannot be attributed to a downstream polar effect if the phenotype is restored in the rescued strain SA28CML. To our knowledge, our study was the first to use the Cre-*loxP* recombination system for gene deletion and rescue of *A. actinomycetemcomitans.*

In conclusion, the deletion of SPA-a affected the ability of *A. actinomycetemcomitans* strain ATCC29523 to form a thin film on the glass surface of culture tubes. The O-antigen in the LPS molecule appears to be one of the adhesins of *A. actinomycetemcomitans* cells required for adherence to abiotic surfaces.

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