Oral Microbiology Immunology 2006: 21: 385–391
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Characterizing the specific coaggregation between *Actinobacillus actinomycetemcomitans* serotype c strains and *Porphyromonas gingivalis* ATCC 33277

N. Suzuki[1,2], Y. Nakano[3], Y. Kiyoura[2]
[1]Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College, Sawara-ku, Fukuoka, Japan, [2]Department of Oral Bacteriology, Ohu University School of Dentistry, Tomitamachi, Koriyama, Japan, [3]Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Higashi-ku, Fukuoka, Japan

Suzuki N, Nakano Y, Kiyoura Y. Characterizing the specific coaggregation between Actinobacillus actinomycetemcomitans *serotype c strains and* Porphyromonas gingivalis *ATCC 33277.*
Oral Microbiol Immunol 2006: 21: 385–391. © Blackwell Munksgaard, 2006.

A visual coaggregation study showed specific interspecies coaggregation between an *Actinobacillus actinomycetemcomitans* serotype c strain and *Porphyromonas gingivalis* strains ATCC 33277 and 381. We mutagenized *A. actinomycetemcomitans* SUNYaB 67 (serotype c) with transposon IS*903*ϕ*kan* and isolated three transposon insertion mutants that had a reduced ability to aggregate with *P. gingivalis* ATCC 33277. The three transposon insertions in the mutant strains mapped to the genes at ORF12, ORF13 and ORF16 of the gene cluster responsible for producing serotype c-specific polysaccharide antigen (SPA). Western blot analysis with serotype c-specific antibody showed that these strains did not produce the high-molecular-mass smear of SPA. Furthermore, two SPA-deficient mutants and an SPA-producing mutant were constructed. The two SPA-deficient mutants were deficient for ORF12 and ORF14, which are necessary for the synthesis of serotype c-SPA, and the SPA-producing mutant was deficient for ORF17, which is not related to SPA synthesis. The ORF12- and ORF14-deficient mutants showed reduced ability to aggregate with *P. gingivalis* ATCC 33277, while the ORF17-deficient mutant aggregated with ATCC 33277 to the same extent as wild-type SUNYaB 67. Our findings suggest that serotype c-SPA of *A. actinomycetemcomitans* mediates coaggregation with *P. gingivalis* ATCC 33277.

Key words: *Actinobacillus actinomycetemcomitans*; coaggregation; polysaccharide; *Porphyromonas gingivalis*; serotype antigen

Y. Kiyoura, Department of Oral Bacteriology, Ohu University School of Dentistry, 31-1 Misumido, Tomitamachi, Koriyama 963-8611, Japan
Tel.: +81 24 932 8931;
fax: +81 24 933 7372;
e-mail: kiyusu@s5.dion.ne.jp
Accepted for publication May 1, 2006

Actinobacillus actinomycetemcomitans is a non-motile, Gram-negative, capnophilic fermentative coccobacillus that has been implicated in the etiology and pathogenesis of localized juvenile periodontitis (4, 40, 48), adult periodontitis (38), and severe non-oral human infections (28). Strains of *A. actinomycetemcomitans* are classified into six serotypes: a, b, c, d, e and f (8, 14, 32, 50). Their serological specificity is defined by the polysaccharide on the surface of the organism (15). Of these serotypes, serotype b is most frequently isolated from subjects with localized juvenile periodontitis (4, 50) who have elevated serum antibody levels to serotype b-specific polysaccharide antigen (SPA) of *A. actinomycetemcomitans* (37). Nevertheless, serotype b

is a minor strain of *A. actinomycetemcomitans* in Asia, including Japan (45), Korea (6) and China (20). By contrast, serotype c strains are frequently recovered from subgingival sites in periodontitis patients in Asian countries (6, 20, 45), while they are uncommon in the USA (50). Serotype c-SPA is also one of the immunodominant antigens of the organism in periodontitis patients (5) and induces the release of interleukin-1 by murine macrophages (42). The differences between the frequency distributions of *A. actinomycetemcomitans* serotypes among populations (4, 20, 45, 50) suggests that the virulence and pathogenic role of the bacterium in inducing periodontitis may differ among serotypes.

Porphyromonas gingivalis is a black-pigmented, gram-negative, anaerobic rod bacterium, which is one of the important pathogens in adult-onset periodontitis (39, 49) and oral malodor (26). Previously, we examined the relationship between the detection of *P. gingivalis* and the distribution of *A. actinomycetemcomitans* serotypes in Japanese adults using polymerase chain reaction (PCR) (47). Although some investigators have reported that *A. actinomycetemcomitans* and *P. gingivalis* are rarely detected in the same periodontal site (1, 30, 41), our data suggest that the frequency of *A. actinomycetemcomitans* serotype c is significantly higher in *P. gingivalis*-positive sites than in *P. gingivalis*-negative sites, in comparison with serotypes a, b, d and e (47). Furthermore, the deeper the periodontal pockets are, the greater the frequency of detecting both *P. gingivalis* and *A. actinomycetemcomitans*. These results suggest that *A. actinomycetemcomitans* serotype c strains have a possible association with *P. gingivalis* in the periodontal pockets. In this study, we describe the specific relationship producing the intergeneric coaggregation of *A. actinomycetemcomitans* serotype c and *P. gingivalis*, and identify a factor in *A. actinomycetemcomitans* serotype c strains that is responsible for their coaggregation with *P. gingivalis* ATCC 33277.

Materials and methods

Bacterial strains and growth conditions

P. gingivalis ATCC 33277, W50, W83 and 381 were cultured at 37°C anaerobically (85% N_2, 10% H_2, 5% CO_2) in GAM broth (Nissui Medical, Tokyo, Japan) and on 5% sheep blood agar plates (trypticase soy agar; Becton Dickinson, Franklin Lakes, NJ) supplemented with 5.0 μg/ml hemin and 1.0 μg/ml menadione. *A. actinomycetemcomitans* ATCC 29523 (serotype a), Y4, JP2 (serotype b), NCTC 9709, NCTC 9710, SUNYaB 67 (serotype c), IDH 781, 3381 (serotype d) and IDH 1705 (serotype e) were cultured at 37°C in a CO_2-enriched atmosphere in THY broth (Todd–Hewitt broth supplemented with 1.0% yeast extract; Becton Dickinson) or on THY agar plates. When required, the medium was supplemented with 5 μg/ml chloramphenicol, 75 μg/ml rifampicin, 6.25 μg/ml kanamycin and 25 μg/ml spectinomycin.

Escherichia coli DH5α was used in the DNA manipulations. *E. coli* DH1 containing pVJT128 (44) and pRK21761 (36), designated KD59, which was kindly provided by Prof. K. M. Derbyshire (Division of Infectious Disease, Wadsworth Center, New York State Department of Health, NY), was used as the donor for mutagenesis of *A. actinomycetemcomitans* SUNYaB 67 with transposon IS*903*φ*kan*. The *E. coli* strains were grown aerobically at 37°C in 2× YT medium (yeast/tryptone; Becton Dickinson) or on a 2× YT agar plate. Where appropriate, the medium was supplemented with 20 μg/ml chloramphenicol, 25 μg/ml kanamycin and 50 μg/ml ampicillin.

DNA manipulation

DNA fragment preparation, agarose gel electrophoresis, DNA labeling, ligation and bacterial transformation were performed using the methods of Sambrook et al. (33).

Southern blot hybridization

Southern blot hybridization was performed non-radioactively with a digoxigenin (DIG)-labeled *kan* gene in IS*903*φ*kan* as the probe. This DIG-labeled PCR probe was constructed using PCR DIG Labeling Mix (Roche Diagnostics GmbH, Penzberg, Germany) and oligonucleotide primers OKD110 and OKD115 (44), in accordance with the supplier's instructions. Other procedures involved in Southern blot hybridization were performed as previously described (33).

DNA sequencing and database search

DNA was sequenced using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). Sequence homology between the nucleotide fragments cloned into pMCL210 (24) and those in the GenBank database was determined using BLAST on the server at the National Center for Biotechnology Information, Bethesda, MD (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences involved in the synthesis of serotype c-specific polysaccharide antigen from *A. actinomycetemcomitans* NCTC 9710 have been submitted to the DDBJ/EMBL/GenBank under accession no. AB010415 (25).

Western blot analysis

Cells harvested from the wild-type and mutant strains of *A. actinomycetemcomitans* SUNYaB 67 were mixed with 0.1 mol Tris–HCl (pH 6.8) containing 2% [weight/volume (w/v)] sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol, and boiled at 100°C for 15 min. Serotype-specific polysaccharide was extracted using 500 μg/ml proteinase K (Sigma, St Louis, MO). The samples were analysed by 8.0% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting. After blotting, the membrane was incubated with rabbit antiserum raised against whole cells of *A. actinomycetemcomitans* NCTC 9710 (serotype c) (35). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Zymed Laboratories, San Francisco, CA) were used for detection. Serum against whole cells of serotype c strain NCTC 9710 was extensively pre-absorbed with cells of serotypes a (ATCC 29523), b (Y4), d (IDH 781) and e (IDH 1705) to remove non-specific reactivity.

Coaggregation assays

Visual assay

A visual coaggregation assay (7) was used to determine the potential coaggregation of *A. actinomycetemcomitans* and *P. gingivalis* strains. Bacterial cells were harvested by centrifugation and washed twice with coaggregation buffer containing 1 mmol sodium phosphate buffer (pH 7.2), 0.1 mmol $CaCl_2$, 0.1 mmol $MgCl_2$ and 150 mmol NaCl. Then, the cell suspensions in coaggregation buffer were adjusted to an optical density (OD_{590}) of approximately 0.6 using a double-wave spectrophotometer (Shimazu, Tokyo, Japan). Equal volumes (1.0 ml) of each cell suspension were added to a glass test tube and mixed for at least 10 s on a vortex mixer; they were then stood at room temperature for 20 h under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2). Tubes containing each cell suspension (1.0 ml) with an equal volume of coaggregation buffer were incubated as controls. Coaggregation scores from

0 (no change in turbidity and no visible coaggregates) to + 4 (maximum coaggregation with a clear-water supernatant) were monitored visually using the scale described by Kolenbrander et al. (16). Average coaggregation scores were determined based on at least five reactions.

Spectrophotometric assay

To measure the coaggregation between the wild-type or mutant strains of *A. actinomycetemcomitans* SUNYaB 67 (serotype c) and *P. gingivalis* ATCC 33277, we used a spectrophotometric assay. The coaggregation assay was performed as described above. After the coaggregation assay, the glass test tube was rotated on its axis gently 50 times and then the supernatant fluid was analysed spectrophotometrically at 590 nm. The differences in the OD_{590} between the 0-h and 20-h tubes were calculated. Tubes containing just the individual cell suspension were used as controls. The percentage of coaggregation was calculated as [(OD_{590} difference of mixed sample – OD_{590} difference of control sample)/OD_{590} difference of control sample] × 100. The coaggregation score was obtained as the average of the percentages of seven or eight independent reactions.

IS*903*φkan mutagenesis of A. actinomycetemcomitans

To isolate mutants of *A. actinomycetemcomitans* SUNYaB 67 (serotype c) that were not able to aggregate with *P. gingivalis* ATCC 33277 cells, transposon IS*903φkan* (44) was used. *E. coli* KD59 was used as a donor in mating with *A. actinomycetemcomitans* SUNYaB 67Rif, which was isolated as a spontaneous rifampicin-resistant (75 μg/ml) mutant of strain SUNYaB 67. Conjugal transfer was performed using the method of Thomson et al. (44). After the performance of two coaggregation assays between kanamycin-resistant *A. actinomycetemcomitans* cells and five times the concentration of *P. gingivalis* ATCC 33277, the *A. actinomycetemcomitans* cells that remained in the fluid were plated onto THY medium with kanamycin (6.25 μg/ml) at 37°C in 5% CO_2. A total of 49 kanamycin-resistant transformants were screened using the visual coaggregation assay with *P. gingivalis* ATCC 33277. Southern blot hybridization analysis of the coaggregation-deficient mutants was performed with the DIG-labeled *kan* gene in IS*903φkan* as the probe, and the *Hin*dIII fragments extracted from an agarose gel were cloned into plasmid vector pMCL210 (24).

Genomic integration

Insertional mutagenesis of the ORFs in the cluster responsible for producing serotype c-SPA was performed by homologous recombination of the target genes and selected by resistance to spectinomycin. The chromosomal DNA of *A. actinomycetemcomitans* NCTC 9710 containing genes disrupted by insertion of the spectinomycin gene (25) was introduced into *A. actinomycetemcomitans* SUNYaB 67 cells by electroporation. The electroporation was performed in a Gene Pulser apparatus (Bio-Rad, Hercules, CA) set to 12.5 kV/cm, and the cells were plated onto THY–spectinomycin (25 μg/ml) medium.

Results

Characterization of coaggregation between *A. actinomycetemcomitans* serotype c and *P. gingivalis*

The coaggregation scores between the nine *A. actinomycetemcomitans* strains, including five serotypes (a, b, c, d and e), and the four *P. gingivalis* strains, are listed in Table 1. As a control, a cell suspension containing each *A. actinomycetemcomitans* strain and coaggregation buffer showed no visible aggregation (score 0 to + 1). Of the four control tubes containing *P. gingivalis* strains, those containing strains W50 and W83 showed no change in turbidity and visible aggregation and no coaggregation with any *A. actinomycetemcomitans* serotype strains. In contrast, the tubes containing *P. gingivalis* ATCC 33277 and 381 alone showed high aggregation that scored + 4 and + 3, respectively, and they coaggregated strongly with *A. actinomycetemcomitans* strains NCTC 9709, NCTC 9710 and SUNYaB 67 (serotype c), but did not bind to any other serotype strains of *A. actinomycetemcomitans*. In the cell suspensions containing each strain of *A. actinomycetemcomitans* serotype c and *P. gingivalis* strains ATCC 33277 or 381, the supernatant became clear (Fig. 1). Rough tiny clusters that were difficult to remove when rocking the tube adhered to the wall of the glass test tube. At the bottom of the tube, the clusters accumulated and formed an adhesive biofilm. Consequently, we counted planktonic cells in the supernatant from the cell suspension of

Table 1. Coaggregation scores between *A. actinomycetemcomitans* and *P. gingivalis*[a]

	P. gingivalis				
	ATCC 33277	W50	W83	381	CB[b]
A. actinomycetemcomitans serotype					
ATCC 29523(a)	0–1	0–1	0	1	0
Y4(b)	0–1	0–1	0	1–2	0
JP2(b)	0–1	ND[c]	ND	ND	0
NCTC 9709(c)	3–4	ND	ND	ND	0
NCTC 9710(c)	3–4	0–1	0	3–4	0
SUNYaB 67(c)	3–4	ND	ND	3–4	0
IDH781 (d)	0–1	1	0	1–2	0–1
3381 (d)	0–1	ND	ND	1	0
IDH1705 (e)	0–1	0–1	0	2	0
CB	4	1	0	3	

[a]Coaggregation scored as described in Materials and Methods, read at 20 h following vortex mixing.
[b]CB: coaggregation buffer.
[c]ND: not done.

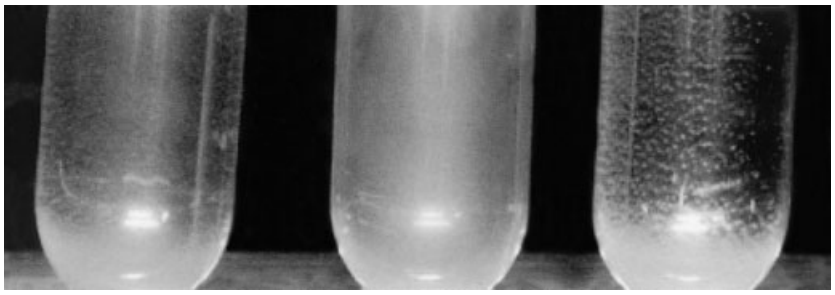

Fig. 1. Coaggregation reaction between *Actinobacillus actinomycetemcomitans* SUNYaB 67 (serotype c) and *Porphyromonas gingivalis* ATCC 33277. Equal volumes (1.0 μl) of cell suspensions of each strain or coaggregation buffer (CB) were added to a glass test tube and allowed to stand in anaerobic conditions at room temperature for 20 h. The tubes contain *P. gingivalis* ATCC 33277 and CB (left), *A. actinomycetemcomitans* SUNYaB 67 and CB (center), and ATCC 33277 and SUNYaB 67 (right).

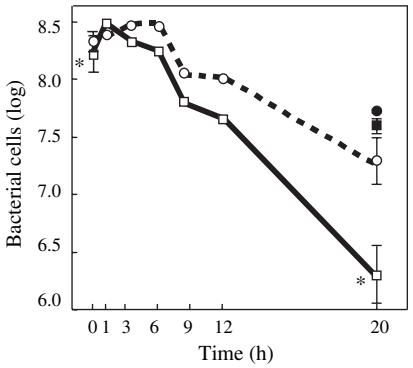

Fig. 2. Counts of cell-forming units of *Actinobacillus actinomycetemcomitans* SUNYaB 67 cells in the supernatant from a mixture with an equal volume of *P. gingivalis* ATCC 33277 cell suspension (open squares) or coaggregation buffer (open circles). (Closed square) and (closed circle) indicate total *A. actinomycetemcomitans* cells (planktonic and biofilm) in the reaction mixture at 20 h obtained by vortexing. $*P < 0.05$ when compared with planktonic cells of *A. actinomycetemcomitans* between 0 and 20 h.

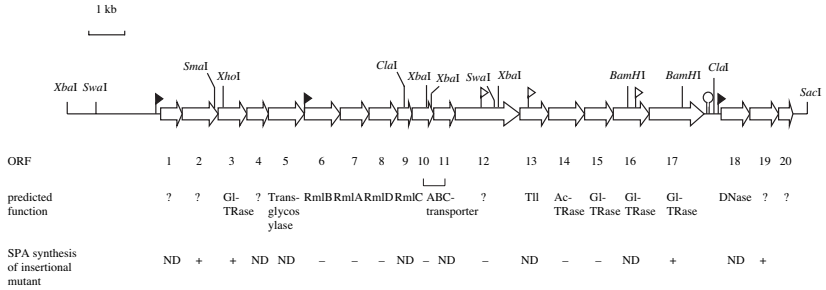

Fig. 3. Restriction map and genetic organization of the gene cluster responsible for the production of SPA of *Actinobacillus actinomycetemcomitans* serotype c. Solid arrows indicate open reading frames (ORFs). Putative promoters and a terminator are represented as solid arrowheads and a hairpin, respectively. The open arrowheads indicate the location and direction of transcription of the IS*903*φ *kan* insertions in the three different mutant strains. Ac-TRase, aceryltransferase; Gl-TRase, glycosyltransferase; Tll, dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase; DNase, deoxynuclease; ND, not done because ORF could not be inactivated.

A. actinomycetemcomitans SUNYaB 67 and *P. gingivalis* ATCC 33277 (Fig. 2). There were consistently fewer *A. actinomycetemcomitans* cells in the supernatant mixed with *P. gingivalis* cells than with *A. actinomycetemcomitans* alone and there was a significant difference between the 0-h and 20-h planktonic cells ($P < 0.05$), while there was no significant reduction in planktonic cells in the suspension of *A. actinomycetemcomitans* alone. All the *A. actinomycetemcomitans* cells contained in the cell suspension at 20 h were obtained by vortexing and there was no difference between the mixture with *P. gingivalis* and *A. actinomycetemcomitans* alone (Fig. 2). Therefore, the survival of *A. actinomycetemcomitans* was not affected by the presence of *P. gingivalis* ATCC 33277. The number of planktonic *P. gingivalis* ATCC 33277 did not change, despite the presence of *A. actinomycetemcomitans* (data not shown). These findings suggest that *A. actinomycetemcomitans* serotype c-cells have the ability to bind specifically to *P. gingivalis* ATCC 33277 and coaggregate, while *P. gingivalis* ATCC 33277 aggregates with itself, but is not affected by the presence of *A. actinomycetemcomitans* cells.

Isolation and characterization of *A. actinomycetemcomitans* coaggregation-deficient mutants

To isolate *A. actinomycetemcomitans* coaggregation-deficient mutants, visual coaggregation assays were performed using each of 49 kanamycin-resistant transformants from *A. actinomycetemcomitans* SUNYaB 67 and *P. gingivalis* ATCC 33277. Three coaggregation-deficient derivatives of *A. actinomycetemcomitans* SUNYaB 67 were isolated and these mutant strains were designated SZ41, SZ47 and SZ49. The tubes containing each mutant strain and *P. gingivalis* ATCC 33277 were similar to that containing the wild-type serotype c and ATCC 33277, as shown in Fig. 1. However, when these test tubes were rocked, the clusters on the glass surface and the bottom of the tube readily fell off (data not shown); indicating the reduction in adhesion. Southern hybridization analysis using the DIG-labeled *kan* gene in IS*903*φ*kan* as a probe hybridized with 3.0-, 4.6- and 3.0-kb *Hin*dIII fragments from the chromosomal DNA of SZ41, SZ47 and SZ49, respectively. DNA sequencing of these *Hin*dIII fragments cloned into pMCL210 revealed that the insertion from each mutant strain was located in a gene responsible for the biosynthesis of serotype c-SPA in *A. actinomycetemcomitans* (Fig. 3). The transposon in SZ49 was part of open reading frame 13 (ORF13) encoding dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (23), which forms dTDP-6-deoxy-L-talose. The transposons in SZ41 and SZ47 were parts of ORF12 and ORF16, respectively, which share 26% identity with RgpF of *Streptococcus mutans* (25) and 56% identity with RfbN of *Salmonella typhimurium* (11), respectively. Western blot analysis with rabbit antiserum against whole cells of *A. actinomycetemcomitans* NCTC 9710 showed that these mutants did not produce the high-molecular-mass smear of SPA (Fig. 4). Next, the coaggregation values of each coaggregation-deficient mutant strain and *P. gingivalis* ATCC 33277 were measured for the 0- and 20-h reaction

Fig. 4. Western blot analysis of the coaggregation-deficient mutants and the wild-type serotype c strain of SUNYaB 67. Cell lysates treated with proteinase K were run on SDS–polyacrylamide (8%) gels, and transferred to a nitrocellulose membrane. The membrane was treated with rabbit antiserum raised against NCTC 9710 cells. The samples in lanes 1–3 were prepared from the coaggregation-deficient mutants (SZ41, SZ47 and SZ49, respectively) and that in lane 4 was prepared from wild-type strain of SUNYaB 67.

samples. The coaggregation values of every cell suspension of each mutant and *P. gingivalis* ATCC 33277 were markedly lower than that of the wild-type strain *A. actinomycetemcomitans* SUNYaB 67 and *P. gingivalis* ATCC 33277 (Fig. 5A). Because *P. gingivalis* ATCC 33277 aggregates with itself, negative coaggregation values (indicating that the OD_{590} differ-

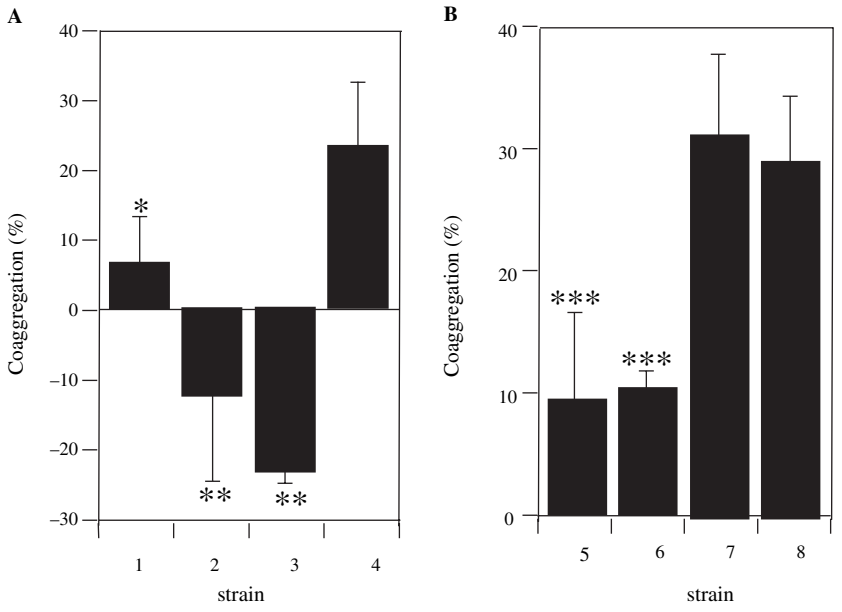

Fig. 5. Coaggregation value of *Porphyromonas gingivalis* ATCC 33277 and the spontaneous *Actinobacillus actinomycetemcomitans* coaggregation-deficient mutants (A) and ORF-deficient mutants of the genes that synthesize serotype c-SPA (B) derived from SUNYaB 67 (serotype c). Lanes 1–3 contain the spontaneous coaggregation-deficient mutants of SUNYaB 67, lanes 4 and 8 contain the wild-type SUNYaB 67, and lanes 5–7 contain mutants deficient in the ORFs for the synthesis of serotype c-SPA. Lane 1, strain SZ41; lane 2, strain SZ47; lane 3, strain SZ49; lane 4, wild-type SUNYaB 67; lane 5, ORF12-deficient; lane 6, ORF14-deficient; lane 7, ORF17-deficient; lane 8, wild-type SUNYaB 67. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ when compared to the value for wild-type SUNYaB 67.

ence of the mixed sample was lower than the OD_{590} difference of the control sample) were obtained for SZ47 and SZ49.

Coaggregation values of SPA-deficient strains with *P. gingivalis* ATCC 33277

To demonstrate that serotype c-SPA of *A. actinomycetemcomitans* is directly involved in the coaggregation with *P. gingivalis* ATCC 33277, two SPA-deficient mutant strains and one SPA-producing mutant strain derived from *A. actinomycetemcomitans* SUNYaB 67 were prepared by electroporation using the chromosomal DNA from the ORF12-, ORF14-, and ORF17-deficient strains in *A. actinomycetemcomitans* NCTC 9710 (25). Neither ORF13 nor ORF16 could be inactivated, although we tried to construct mutants. Immunodiffusion reactions using autoclaved extracts from whole cells of wild-type *A. actinomycetemcomitans* SUNYaB 67 and its mutants with anti-*A. actinomycetemcomitans* NCTC 9710 serum revealed that insertional activation of ORF12 and ORF14, but not of ORF17, resulted in the loss of the ability of *A. actinomycetemcomitans* SUNYaB 67 cells to produce SPA (data not shown). These findings suggest that ORF12 and ORF14 are essential for SPA biosynthesis, and that the ORF17 gene is located adjacent to the genes that are indispensable for serotype c-SPA synthesis but is unrelated to the synthesis of serotype c-SPA. Figure 5B shows the coaggregation values between these insertional mutants of *A. actinomycetemcomitans* SUNYaB 67 and *P. gingivalis* ATCC 33277. The coaggregation values of the cell suspensions containing either the ORF12- or ORF14-deficient mutant and *P. gingivalis* ATCC 33277 were reduced by approximately one-third in comparison with that of the wild-type *A. actinomycetemcomitans* SUNYaB 67 and *P. gingivalis* ATCC 33277. By contrast, the ORF17-deficient strain aggregated with *P. gingivalis* ATCC 33277 as well as the wild-type strain SUNYaB 67 did.

Discussion

Periodontitis is thought to be caused by a microflora complex that includes the putative periodontopathic bacteria *P. gingivalis*, *A. actinomycetemcomitans*, *Treponema denticola*, *Prevotella intermedia*, *Tannerella forsythia* (formerly *Bacteroides forsythus* or *Tannerella forsythensis*) (18) and *Fusobacterium nucleatum* (9, 43). These bacteria are isolated together from affected periodontal sites (41) and it has been suggested that periodontitis is initiated by cooperation between the different species. Bacterial coaggregation has been recognized as the most important process in the development of multi-species biofilms (29). Many synergistic relationships between *P. gingivalis* and other periodontopathic bacteria depend on cell-to-cell interactions (10, 34) and on soluble components of the bacteria (46). For *A. actinomycetemcomitans*, only a cell-to-cell interaction with *F. nucleatum* has been reported (31); this report is the first to describe cell–cell coaggregation between *A. actinomycetemcomitans* and *P. gingivalis* and the coaggregation factor of the *A. actinomycetemcomitans* serotype c strain that interacts with other periodontopathic bacteria.

Various compounds on the surface of *A. actinomycetemcomitans* have important roles in oral biofilm formation. Non-specific, tenacious surface attachment is dependent on the presence of long, bundled adhesive pili (fimbriae) that form on the cell surface (12). Mutations in *flp-1*, which encodes the major fimbrial protein subunit, result in cells that fail to produce fimbriae or attach to surfaces. Kaplan et al. (13) reported that the genes responsible for the synthesis of serotype f-specific O polysaccharide (O-PS) of lipopolysaccharide were required for biofilm cell detachment that involves the release of cells from inside a biofilm colony, but were not required for surface attachment or biofilm colony formation. It has been reported that *A. actinomycetemcomitans* strains with mutations of the outer membrane protein 100 (Omp100), which localizes on the cell surface, show decreased adhesion and invasion efficiency to KB cells – it is 60% that of the wild-type (3). In this study, we found that three coaggregation-deficient mutants of *A. actinomycetemcomitans* had reduced coaggregation efficiency with *P. gingivalis*. These findings suggest that the serotype-SPA plays a role in biofilm formation in *A. actinomycetemcomitans*, in addition to various other factors including O-PS, Omp100, SPA and other unidentified factors. Most of these studies have rarely examined the effects of saliva and serum components. When saliva, serum and other bacteria are added to these reactions, the results should be more complex.

The serotypes a-SPA and c-SPA of *A. actinomycetemcomitans* have unique structural features and consist solely of

rare 6-deoxyhexoses: 6-deoxy-D-talose and 6-deoxy-L-talose, respectively (28, 35). D-Fucose, which is found in serotype b-SPA, is also rare. Rosen et al. (31) reported that the β-*N*-acetyl-D-galactosamine in *A. actinomycetemcomitans* serotype b O-PS is recognized by *F. nucleatum* lectin. Therefore, the serotype-specific polysaccharides in *A. actinomycetemcomitans* have a role in relation to the differences in virulence, pathogenesis and biofilm formation among the different serotypes of this organism.

Interestingly, of the four *P. gingivalis* strains studied here, *P. gingivalis* ATCC 33277 and 381 had the ability to bind to the *A. actinomycetemcomitans* serotype c strains, while *P. gingivalis* W50 and W83 did not coaggregate with any *A. actinomycetemcomitans* strain. Nakagawa et al. (21, 22) classified *P. gingivalis* into six types (I to V and Ib) based on the differences in the *fimA* genes encoding the subunit of the long (major) fimbriae, FimA. Both *P. gingivalis* ATCC 33277 and 381 are type I *fimA* strains and W50 and W83 are type IV *fimA* strains. The FimA of *P. gingivalis* ATCC 33277 and 381 may affect coaggregation with *A. actinomycetemcomitans* serotype c strains. *P. gingivalis* fimbriae bind to various oral surfaces, such as salivary components (19) and epithelial cells (2), and have also been reported to participate in coaggregation with other oral bacteria (10, 17, 27). The long fimbriae of *P. gingivalis*, predominantly composed of FimA, interact with glyceraldehyde-3-phosphate dehydrogenase on *Streptococcus oralis* (17). MfaI, which is the major protein subunit of the short fimbriae, bound to the SspB streptococcal surface polypeptide of *Streptococcus gordonii* (27). Recently, FimA-deficient and MfaI-deficient mutants derived from *P. gingivalis* ATCC 33277 were kindly provided by Prof. Fuminobu Yoshimura, Department of Microbiology, Aichi-Gakuin University, and we are currently investigating the adhesion factor of *P. gingivalis* that is responsible for coaggregation with *A. actinomycetemcomitans* serotype c-SPA.

Acknowledgments

This work was supported by a research grant from the Fukushima Society for the Promotion of Medicine (N.S.). We thank Prof. K.M. Derbyshire, Division of Infectious Disease, Wadsworth Center, New York State Department of Health, and Prof. F. Yoshimura, Department of Microbiology, Aichi-Gakuin University, for helpful advice regarding this research.

References

1. Ali RW, Lie T, Skaug N. Early effects of periodontal therapy on the detection frequency of four putative periodontal pathogens in adults. J Periodontol 1992: **63**: 540–547.
2. Asai Y, Ohyama Y, Gen K, Ogawa T. Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. Infect Immun 2001: **69**: 7387–7395.
3. Asakawa R, Komatsuzawa H, Kawai T et al. Outer membrane protein 100, a versatile virulence factor of *Actinobacillus actinomycetemcomitans*. Mol Microbiol 2003: **50**: 1125–1139.
4. Asikainen S, Lai CH, Alaluusua S, Slots J. Distribution of *Actinobacillus actinomycetemcomitans* serotypes in periodontal health and disease. Oral Microbiol Immunol 1991: **6**: 115–118.
5. Califano JV, Schenkein HA, Tew JG. Immunodominant antigens of *Actinobacillus actinomycetemcomitans* serotypes a and c in high-responder patients. Oral Microbiol Immunol 1991: **6**: 228–235.
6. Chung HJ, Chung CP, Son SH, Nisengard RJ. *Actinobacillus actinomycetemcomitans* serotypes and leukotoxicity in Korean localized juvenile periodontitis. J Periodontol 1989: **60**: 506–511.
7. Cisar JO, Kolenbrander PE, McIntire FC. Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. Infect Immun 1979: **24**: 742–752.
8. Gmür R, McNabb H, van.Steenbergen TJ et al. Seroclassification of hitherto nontypeable *Actinobacillus actinomycetemcomitans* strains: evidence for a new serotype e. Oral Microbiol Immunol 1993: **8**: 116–120.
9. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994: **5**: 78–111.
10. Hashimoto M, Ogawa S, Asai Y, Takai Y, Ogawa T. Binding of *Porphyromonas gingivalis* fimbriae to *Treponema denticola* dentilisin. FEMS Microbiol Lett 2003: **226**: 267–271.
11. Jiang XM, Neal B, Santiago F, Lee SJ, Romana LK, Reeves PR. Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). Mol Microbiol 1991: **5**: 695–713.
12. Kachlany SC, Planet PJ, Desalle R, Fine DH, Figurski DH, Kaplan JB. *flp-1*, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. Mol Microbiol 2001: **40**: 542–554.
13. Kaplan JB, Meyenhofer MF, Fine DH. Biofilm growth and detachment of *Actinobacillus actinomycetemcomitans*. J Bacteriol 2003: **185**: 1399–1404.
14. Kaplan JB, Perry MB, MacLean LL, Furgang D, Wilson ME, Fine DH. Structural and genetic analyses of O polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. Infect Immun 2001: **69**: 5375–5384.
15. Koga T, Nishihara T, Amano K et al. Chemical and biological properties of cell-surface components of *Actinobacillus actinomycetemcomitans*. In: Hamada S, Holt SC, McGhee JR, ed. Pathogens and host immune responses. Tokyo: Quintessence Publishing Co., 1991: 117–127.
16. Kolenbrander PE, Andersen RN. Characterization of *Streptococcus gordonii* (*S. sanguis*) PK488 adhesin-mediated coaggregation with *Actinomyces naeslundii* PK606. Infect Immun 1990: **58**: 3064–3072.
17. Maeda K, Nagata H, Yamamoto Y et al. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. Infect Immun 2004: **72**: 1341–1348.
18. Maiden MF, Cohee P, Tanner AC. Proposal to conserve the adjectival form of the specific epithet in the reclassification of *Bacteroides forsythus* Tanner et al. 1986 to the genus *Tannerella* Sakamoto et al. 2002 as *Tannerella forsythia* corrig., gen. nov., comb. nov. Request for an Opinion. Int J Syst Evol Microbiol 2003: **53**: 2111–2112.
19. Malek R, Fisher JG, Caleca A et al. Inactivation of the *Porphyromonas gingivalis fimA* gene blocks periodontal damage in gnotobiotic rats. J Bacteriol 1994: **176**: 1052–1059.
20. Mombelli A, Gmür R, Lang NP, Corbert E, Frey J. *Actinobacillus actinomycetemcomitans* in Chinese adults. Serotype distribution and analysis of the leukotoxin gene promoter locus. J Clin Periodontol 1999: **26**: 505–510.
21. Nakagawa I, Amano A, Kimura RK, Nakamura T, Kawabata S, Hamada S. Distribution and molecular characterization of *Porphyromonas gingivalis* carrying a new type of *fimA* gene. J Clin Microbiol 2000: **38**: 1909–1914.
22. Nakagawa I, Amano A, Ohara-Nemoto Y et al. Identification of a new variant of *fimA* gene of *Porphyromonas gingivalis* and its distribution in adults and disabled populations with periodontitis. J Periodontal Res 2002: **37**: 425–432.
23. Nakano Y, Suzuki N, Yoshida Y, Nezu T, Yamashita Y, Koga T. Thymidine diphosphate-6-deoxy-L-*lyxo*-4-hexulose reductase synthesizing dTDP-6-deoxy-L-talose from *Actinobacillus actinomycetemcomitans*. J Biol Chem 2000: **275**: 6806–6812.
24. Nakano Y, Yoshida Y, Yamashita Y, Koga T. Construction of a series of pACYC-derived plasmid vectors. Gene 1995: **162**: 157–158.
25. Nakano Y, Yoshida Y, Yamashita Y, Koga T. A gene cluster for 6-deoxy-L-talan synthesis in *Actinobacillus actinomycetemcomitans*. Biochim Biophys Acta 1998: **1442**: 409–414.
26. Nakano Y, Yoshimura M, Koga T. Correlation between oral malodor and periodontal bacteria. Microbes Infect 2002: **4**: 679–683.
27. Park Y, Simionato MR, Sekiya K et al. Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. Infect Immun 2005: **73**: 3983–3989.
28. Perry MB, MacLean LM, Brisson JR, Wilson ME. Structures of the antigenic O-polysaccharides of lipopolysaccharides produced by *Actinobacillus actinomycetem-*

comitans serotypes a, c, d and e. Eur J Biochem 1996: **242**: 682–688.
29. Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 2003: **11**: 94–100.
30. Riggio MP, Macfarlane TW, Mackenzie D, Lennon A, Smith AJ, Kinane D. Comparison of polymerase chain reaction and culture methods for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque samples. J Periodontal Res 1996: **31**: 496–501.
31. Rosen G, Nisimov I, Helcer M, Sela MN. *Actinobacillus actinomycetemcomitans* serotype b lipopolysaccharide mediates coaggregation with *Fusobacterium nucleatum*. Infect Immun 2003: **71**: 3652–3656.
32. Saarela M, Asikainen S, Alaluusua S, Pyhuälä L, Lai CH, Jousimies-Somer H. Frequency and stability of mono- or polyinfection by *Actinobacillus actinomycetemcomitans* serotypes a, b, c, d or e. Oral Microbiol Immunol 1992: **7**: 277–279.
33. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd edn, Vol. 3. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
34. Shaniztki B, Hurwitz D, Smorodinsky N, Ganeshkumar N, Weiss EI. Identification of a *Fusobacterium nucleatum* PK1594 galactose-binding adhesin which mediates coaggregation with periopathogenic bacteria and hemagglutination. Infect Immun 1997: **165**: 5231–5237.
35. Shibuya N, Amano K, Azuma J et al. Novel serotype-specific polysaccharide antigens from *Actinobacillus actinomycetemcomitans*. J Biol Chem 1991: **266**: 16318–16323.
36. Sia EA, Kuehner DM, Figurski DH. Mechanism of retrotransfer in conjugation: prior transfer of the conjugative plasmid is required. J Bacteriol 1996: **178**: 1457–1464.
37. Sims TJ, Moncla BJ, Darveau RP, Page RC. Antigens of *Actinobacillus actinomycetemcomitans* recognized by patients with juvenile periodontitis and periodontally normal subjects. Infect Immun 1991: **59**: 913–924.
38. Slots J, Bragd L, Wikström M, Dahlén G. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in destructive periodontal disease in adults. J Clin Periodontol 1986: **13**: 570–577.
39. Slots J, Genco RJ. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J Dent Res 1984: **63**: 412–421.
40. Slots J, Reynolds HS, Genco RJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological investigation. Infect Immun 1980: **29**: 1013–1020.
41. Socransky SS, Haffajee AD, Dzink JL, Hillman JD. Associations between microbial species in subgingival plaque samples. Oral Microbiol Immunol 1988: **3**: 1–7.
42. Takahashi T, Nishihara T, Ishihara Y et al. Murine macrophage interleukin-1 release by capsularlike serotype-specific polysaccharide antigens of *Actinobacillus actinomycetemcomitans*. Infect Immun 1991: **59**: 18–23.
43. Takamatsu N, Yano K, He T, Umeda M, Ishikawa I. Effect of initial periodontal therapy on the frequency of detecting *Bacteroides forsythus*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*. J Periodontol 1999: **70**: 574–580.
44. Thomson VJ, Bhattacharjee MK, Fine DH, Derbyshire KM, Figurski DH. Direct selection of IS*903* transposon insertions by use of a broad-host-range vector: isolation of catalase-deficient mutants of *Actinobacillus actinomycetemcomitans*. J Bacteriol 1999: **181**: 7298–7307.
45. Yamamoto M, Nishihara T, Koseki T et al. Prevalence of *Actinobacillus actinomycetemcomitans* serotypes in Japanese patients with periodontitis. J Periodontal Res 1997: **32**: 676–681.
46. Yoneda M, Yoshikane T, Motooka M et al. Stimulation of growth of *Porphyromonas gingivalis* by cell extracts from *Tannerella forsythia*. J Periodontal Res 2005: **40**: 105–109.
47. Yoshida Y, Suzuki N, Nakano Y, Shibuya K, Ogawa Y, Koga T. Distribution of *Actinobacillus actinomycetemcomitans* serotypes and *Porphyromonas gingivalis* in Japanese adults. Oral Microbiol Immunol 2003: **18**: 135–139.
48. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. J Clin Periodontol 1985: **12**: 1–20.
49. Zambon JJ, Reynolds HS, Slots J. Black-pigmented *Bacteroides* spp. in the human oral cavity. Infect Immun 1981: **32**: 198–203.
50. Zambon JJ, Slots J, Genco RJ. Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. Infect Immun 1983: **41**: 19–27.