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Zinc and copper play a role in coaggregation inhibiting action of *Porphyromonas gingivalis*

M. Tamura[1,2], K. Ochiai[1,2]
[1]Department of Microbiology, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan, [2]Division of Immunology and Pathobiology, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan

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Background/aim: We investigated the mechanisms of adherence of salivary and serum proteins, which mimic gingival crevicular fluid (GCF), to *Porphyromonas gingivalis*, and the effects of these adhered proteins on coaggregation and hemagglutination properties.
Methods: The amounts of salivary and serum proteins adhering to *P. gingivalis* were determined using ^{3}H-labeled and non-labeled proteins. The coaggregation between *P. gingivalis* and *Streptococcus oralis* or *Streptococcus gordonii* was observed. Hemagglutination was evaluated using sheep erythrocytes. Proteins that interacted with zinc or copper in saliva and serum and on *P. gingivalis* were examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
Results: The amount of salivary or serum proteins that adhered to the surface of *P. gingivalis* strains was increased by cations, especially zinc and copper ions. The pretreatment of bacterial cells with salivary or serum proteins before the assay inhibited coaggregation with gram-positive bacteria and hemagglutination. These phenomena were enhanced by the presence of zinc or copper ions during the pretreatment of *P. gingivalis* with proteins. We detected protein bands that were related to these cations in saliva and serum and on *P. gingivalis.*
Conclusions: Our findings suggest that zinc and copper ions markedly enhanced the adhesion and accumulation of salivary and serum proteins on cells of *P. gingivalis* and inhibited the coaggregation and hemagglutination of *P. gingivalis*. These cations might be useful for limiting the settlement of *P. gingivalis* in the gingival sulcus with the goal of preventing periodontal disease.

Key words: coaggregation; copper; oral fluid; *Porphyromonas gingivalis*; zinc

Kuniyasu Ochiai, Department of Microbiology, Nihon University School of Dentistry, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Tel.: +81 3 3219 8125;
fax: +81 3 3219 8317;
e-mail: Ochiai@dent.nihon-u.ac.jp

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Adhesion of oral bacteria to the surfaces of oral tissues has been observed in the first stage of the accumulation of dental plaque, which is implicated in periodontal disease as well as dental caries (23, 30). Many studies have speculated that periodontal disease is related to the occurrence of general disorders (15, 22, 25). The onset of periodontal disease may be related to the adhesion and establishment of periodontal pathogens in the gingival sulcus (6). Consequently, removing or reducing the number of periodontal pathogens in the gingival sulcus is important for preventing periodontal disease.

The tissues in the gingival sulcus are constantly bathed with saliva and gingival cervicular fluid (GCF), and periodontal pathogens that settle on the inside of the gingival sulcus are suspended in this fluid before settlement and are invariably covered with the same molecules, especially proteins. Some studies have reported that the presence of saliva or serum inhibits the adhesion and hemagglutination of periodontal pathogens (18, 21, 27).

It is important to consider how these oral fluids that coat periodontal pathogens affect the adhesion and aggregation of these bacteria that are observed during the initial step in the outbreak of periodontal diseases and are related to the formation/accumulation of the periodontal pathogenic plaque. By intentionally increasing the amount of adhered proteins on periodontal pathogens, it may be possible to

inhibit the outbreak and progression of periodontal disease.

Based on an examination of this hypothesis, we reported that cation-bridge binding played an important role in the adhesion of serum proteins that mimicked GCF to *Prevotella intermedia* and *Prevotella nigrescens*, both of which are periodontal pathogens (29). We also found that zinc and copper ions markedly enhanced the adhesion and accumulation of serum proteins on these two periodontal pathogens and greatly inhibited the pathogens' adhesion and hemagglutination.

In the present study, we examine the interactions of proteins originating in saliva and serum and possessing a high affinity for the surface of *Porphyromonas gingivalis* that are associated with the outbreak and progression of periodontal disease (23, 24, 26, 30, 31). We also investigated a method for increasing protein adhesion to bacterial cells by examining the effects of zinc and copper ions on the coaggregation and hemagglutination of *P. gingivalis*. In addition, we examined the presence of zinc- and copper-complexed proteins in saliva and serum and on *P. gingivalis*.

Materials and methods

Bacterial cells

Microorganisms used in this study were as follows – *P. gingivalis* : strains ATCC 33277, ATCC49417, and FDC381; gram-positive: *Streptococcus oralis* ATCC9811, *Streptococcus gordonii* ATCC10558, and *Actinomyces naeslundii* ATCC12104. *P. gingivalis* cells were inoculated and grown in Gifu anaerobic medium (GAM) broth (Nissui, Tokyo, Japan) under anaerobic conditions (Becton Dickinson Microbiology Systems, Sparks, MD) at 37°C overnight. *Streptococcus* strains inoculated in Todd–Hewitt broth (Becton Dickinson) and *Actinomyces* inoculated in tryptic soy broth (Becton Dickinson) were grown under aerobic conditions at 37°C overnight. The bacterial cells were harvested in the late logarithmic phase by centrifugation at 5000 ***g*** at 4°C for 15 min and washed twice in phosphate-buffered saline (PBS; 5.0 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) (29). Then, the bacterial cells were resuspended, and the cells were quantified using optical density (OD) at 550 nm and a standard curve determined by microscopy.

Treatment of saliva and serum

Whole unstimulated saliva was collected from donors after obtaining informed consent. To mimic GCF, the saliva samples along with human serum samples (Nippon Bio-Test Laboratories Inc., Tokyo, Japan) were heated at 56°C for 60 min to inactivate any enzymes and then clarified by centrifugation. The samples were filtered (0.22-μm pore size; Millipore, Billerica, MA) and dialyzed against PBS to remove inorganic substances that can influence protein and bacterial adhesion. The samples were then lyophilized and stored at −80°C until use. The protein concentration was estimated with a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Labeling of salivary and serum proteins

The labeling of both salivary and serum proteins with ^{3}H-labeled formaldehyde (NEN Life Science Products Inc., Boston, MA) was performed as described by Jentoft and Dearborn (10). Briefly, after labeling the proteins, the protein-containing solution was dialyzed for 3 days with a physiological saline solution to eliminate excess radiolabel. The radioactivity of the solution was measured directly in a scintillation counter (Packard BioScience, Meriden, CT), and the protein concentration was calculated from the radioactivity.

Adhesion of salivary and serum proteins to bacterial cells

The assay was performed using two different methods. The first involved a previously described experiment that used labeled proteins (28). Briefly, 1.0 mg of labeled salivary or serum proteins was added to 1×10^{9} *P. gingivalis* cells. After incubation at room temperature for 60 min, the bacterial cell pellet was washed and transferred to a scintillation vial and counted, which allowed the amount of adhered protein to be calculated. To investigate the effect of cation-bridge binding, hydrophobic interactions, and lectin–ligand binding on the adhesion of proteins, 1.0 mmol/l of several cations, hydrophobic inhibitors, and sugars were used. We also investigated the effect of heat-treating the bacterial cells at 80°C for 5 min on the amount of adhered protein.

In the second method, we relied on the difference in the applied protein and adhered protein; we examined the amount of adhered, non-labeled protein on bacterial cells obtained by ethylenediaminetetraacetic acid (EDTA) elution. Briefly, 1.0 ml PBS containing 2.0 mg of salivary or serum proteins was added to 2.5×10^{9} *P. gingivalis* cells in polypropylene microcentrifuge tubes (Sarstedt, Rommelsdorf, Germany). Following incubation at room temperature for 60 min, the cell pellet was harvested after centrifugation. After washing the bacterial cells, 1.0 ml PBS containing 1.0 mM EDTA was added to the cell pellet, and the mixture was incubated for 15 min. After centrifugation, the amount of adhered protein eluted from the bacterial cells into the supernatant was estimated using a protein assay kit (Bio-Rad). This supernatant was also used as a sample for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The attachment assays were performed in triplicate, and the experiments were performed four times.

Coaggregation assay

For coaggregation assay, we mixed 50-μl multiple dilutions of *P. gingivalis* suspension (maximum OD 1.0) in coaggregation buffer (21) with 50 μl of *S. oralis*, *S. gordonii* (OD 1.0), or *A. naeslundii* (OD 0.5) suspensions in 96-well microtiter plates. After incubation at room temperature for 60 min, a coaggregation score ranging from 0 (no change in turbidity and no visible coaggregate) to +4 (maximum coaggregation, with large coaggregates settling immediately, leaving a water-clear supernatant) was given for each pair visually (12). The coaggregation assay was performed in triplicate, and the experiment was performed four times.

Porphyromonas gingivalis that were pretreated with saliva or serum for 1 h and washed with PBS in the absence or presence of 1.0 mM $ZnCl_2$ or $CuCl_2$ were used to examine the effect of pretreatment with either protein on the coaggregation assay.

Hemagglutination assay

The hemagglutination assay was performed with sheep erythrocytes in a round-bottomed microtitration plate. We mixed 50-μl multiple dilutions of *P. gingivalis* suspension (maximum OD 1.0) with 50 μl of 2% washed sheep erythrocytes in PBS. After incubation at room temperature for 60 min, the bacterial dilutions that inhibited hemagglutination were determined visually. The hemagglutination assay was performed in triplicate, and the experiment was performed four times. Hemagglutination was also assessed for the bacterial cells treated with saliva or serum as described above.

Cation-binding proteins in saliva and serum

The cation-binding proteins in saliva and serum were obtained by EDTA elution after treating the bacterial cells with saliva or serum as described above. We used this elution as a sample of cation-binding proteins in saliva and serum for SDS–PAGE.

Furthermore, saliva and serum after treatment with *P. gingivalis* ATCC33277 were also used as samples for the influence of the proteolytic activities of *P. gingivalis* against saliva and serum.

Cation-binding proteins on bacterial cells

The proteins on bacterial cell surfaces were collected by incubation in 0.1% CHAPS (Wako Ltd., Tokyo, Japan) at 4°C for 30 min. The supernatant was obtained by centrifugation at 8000 ***g*** at 4°C for 3 min and then filtered (0.22-μm pore size; Millipore) and dialyzed. The assay was carried out with a HiTrap™ Chelating HP column (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's directions. The column had a volume of 1.0 ml and was washed with 5.0 ml distilled water; a 0.1 M metal salt solution of $FeCl_2$, $ZnCl_2$, $CuCl_2$, or $NiCl_2$ was loaded in the column to bind these cations. The column was then washed with distilled water and equilibrated with binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.2), and 1.0 mg of the sample from the bacterial cell surfaces dissolved in binding buffer was loaded onto the column. After washing with 10 ml of binding buffer to eliminate unbound proteins, the cation-binding proteins were eluted with 1.0 ml of elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, 50 mM EDTA, pH 7.2). We used the elutant as a sample of the cation-binding proteins on bacterial cells for SDS–PAGE.

SDS–PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to Laemmli (14). We used 20 μl of the solution extracted from the cells with EDTA or eluted from the HiTrap™ Chelating HP column as the samples. The samples were mixed with SDS reducing buffer and boiled for 10 min, and electrophoresis was performed using a Tris–glycine 12.0% (weight/volume) resolving gel and molecular weight standard (Bio-Rad). All samples were also run on SDS–PAGE. The gels were stained with silver nitrate for analysis.

Statistical analyses

Statistical evaluations were performed using Mann–Whitney *U*-tests.

Results

Amount of adhered salivary or serum proteins on bacterial cells

The amount of labeled salivary or serum proteins adhered to *P. gingivalis* is shown in Fig. 1. The amount of non-labeled proteins adhered to *P. gingivalis* that eluted with EDTA is also given in Fig. 2. Although the amounts of adhered proteins indicated by the two methods were different, we observed that the amount of protein was increased by the presence of cations, especially those of zinc and copper, for all the strains tested. Heat treatment inhibited the adhesion of proteins to the bacterial cells. In contrast, the presence of other substances, hydrophobic inhibitors and sugars, had little effect on the adhesion of proteins to bacterial cells (data not shown).

Coaggregation assay

The effect of diluting the *P. gingivalis* suspension on coaggregation inhibition is shown in Table 1. Coaggregation was inhibited when all the *P. gingivalis* strains tested were treated with protein. Moreover, the presence of zinc or copper ions when bacterial cells were treated with either protein strongly blocked coaggregation and zinc and copper ions had a concentration-dependent effect on coaggregation. Conversely, zinc and copper ions promoted the coaggregation of non-treated *P. gingivalis* to gram-positive bacteria (data not shown).

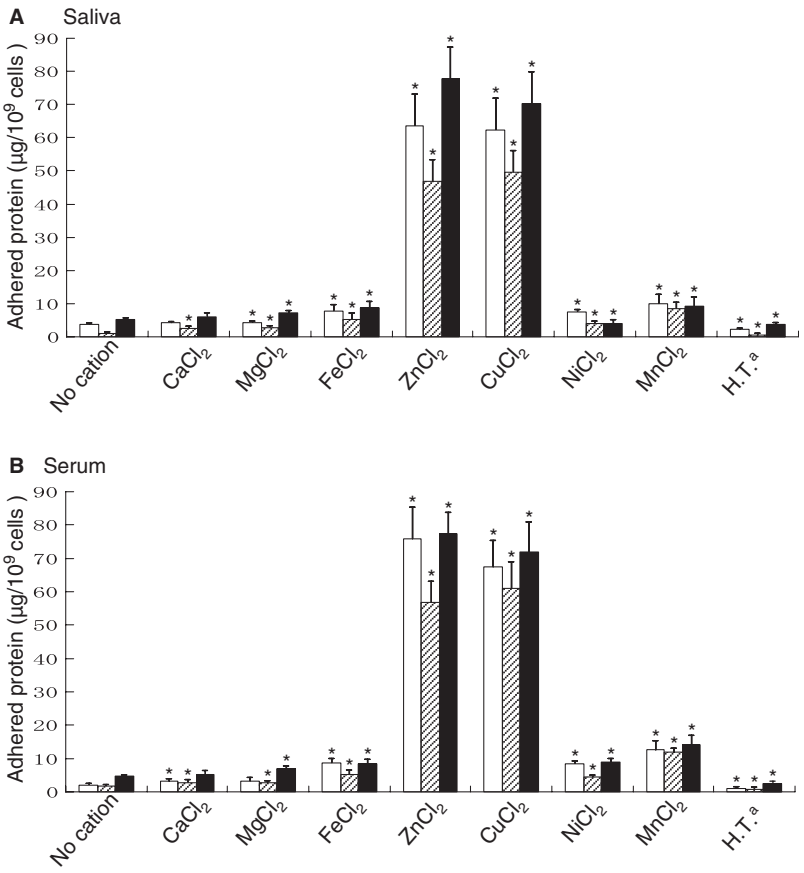

Fig. 1. The effects of cations and heating on the amount of ^{3}H-labeled salivary or serum proteins adhered to *Porphyromonas gingivalis*. (A) The amount of salivary proteins adhered to ATCC33277 (□), ATCC49417 (▨), and FDC381 (■). (B) The amount of serum proteins adhered to the tested strains. Final concentration of cation is 1.0 mM. Data are expressed as the amount of adhered protein (mean ± SD) from four different experiments with triplicate samples. $^*P < 0.05$, significantly different from no cation. [a]Heat-treated bacterial cells.

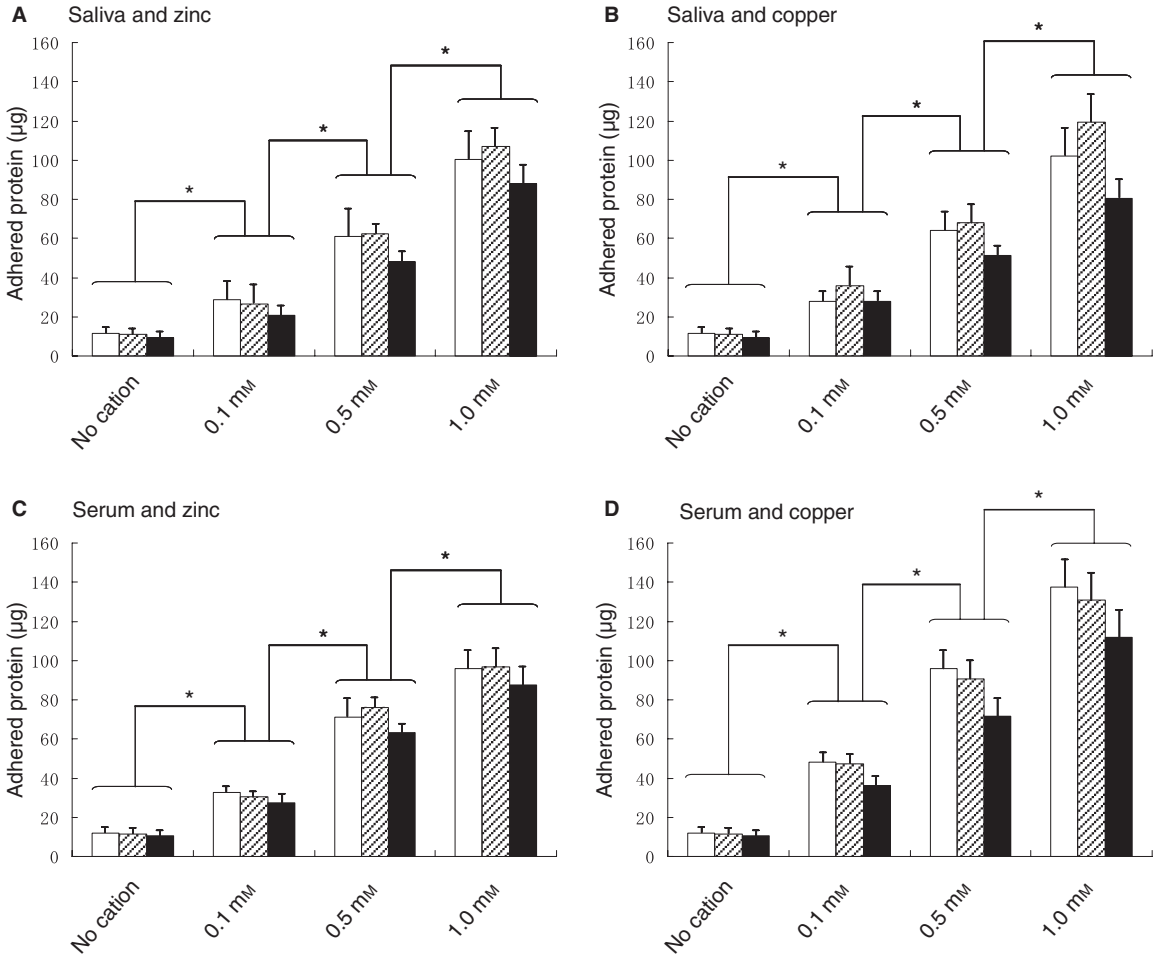

Fig. 2. The effects of zinc and copper on the amount of non-labeled salivary and serum proteins adhered to *Porphyromonas gingivalis*. (A) The effect of zinc on the amount of salivary proteins adhered to ATCC33277 (□), ATCC49417 (▨), and FDC381 (■). (B) The effect of zinc on the amount of serum proteins adhered to the cells of the tested strains. (C) The effect of copper on the amount of serum proteins adhered to the cells of the tested strains. (D) The effect of copper on the amount of serum proteins adhered to the cells of the tested strains. Data are expressed as the amount of adhered protein (mean ± SD) from four different experiments with triplicate samples. *$P < 0.05$, significantly different from each other.

Table 1. Coaggregation assay

	ATCC33277			ATCC49417			FDC381		
P. gingivalis	*S. oralis*	*S. gordonii*	*A. naeslundii*	*S. oralis*	*S. gordonii*	*A. naeslundii*	*S. oralis*	*S. gordonii*	*A. naeslundii*
No. treated	+3	+3	+4	+3	+3	+4	+4	+4	+4
Saliva-treated	+2	+2	+3	+2	+2	+3	+3	+3	+4
$ZnCl_2$ (mM)									
0.5	+1	+2	+2	+2	+1	+2	+2	+2	+2
1.0	0	+1	+2	+2	+1	+1	+2	+2	+3
$CuCl_2$ (mM)									
0.5	+1	+1	+2	+2	+1	+2	+2	+2	+2
1.0	0	0	+1	0	0	+2	+2	+2	+2
Serum-treated	+2	+2	+3	+2	+2	+3	+3	+3	+3
$ZnCl_2$ (mM)									
0.5	+1	+1	+2	+1	+2	+2	+2	+2	+2
1.0	0	0	+2	0	0	+2	+2	+2	+2
$CuCl_2$ (mM)									
0.5	+1	+1	+2	+1	+1	+2	+2	+2	+2
1.0	0	0	+1	0	0	+2	+1	+1	+2

Data are expressed as the coaggregation score from the mean of four different experiments with triplicate samples.
Bacteria: *Actinomyces naeslundii*, *Porphyromonas ginigavalis*, *Streptococcus gordonii*, *Streptococcus oralis*.

Hemagglutination assay

The effect of diluting the *P. gingivalis* suspension on hemagglutination inhibition is shown in Table 2. Pretreatment with proteins inhibited hemagglutination, and the presence of zinc or copper ions when bacterial cells were treated with either protein strongly blocked hemagglutination. Zinc and copper had a concentration-dependent effect on hemagglutination, which suggests that they affect hemagglu-

Table 2. Hemagglutination assay

	Porphyromonas gingivalis		
	ATCC33277	ATCC49417	FDC381
No. treated	20	10	20
Saliva-treated	10	5	10
$ZnCl_2$ (mM)			
0.5	4	2	4
1.0	1	1	1
$CuCl_2$ (mM)			
0.5	4	2	4
1.0	1	1	1
Serum-treated	10	5	10
$ZnCl_2$ (mM)			
0.5	5	2	5
1.0	2	1	2
$CuCl_2$ (mM)			
0.5	4	2	5
1.0	2	1	2

Data are expressed as dilutions that inhibited the hemagglutination property from the mean of four different experiments with triplicate samples.

tination in a manner similar to their influence on coaggregation.

Cation-binding proteins in saliva and serum

The proteins that adhered to bacterial cells with zinc or copper ions in saliva or serum are shown in Fig. 3; their identity is based on silver staining of SDS–PAGE gels. We observed several protein bands in saliva or serum that had adhered to and were eluted from cells of *P. gingivalis* with EDTA. Different protein band molecular masses and patterns appeared to be present in the adhered proteins in saliva and serum. The number of adhered protein bands in serum was higher than that in saliva when zinc or copper was added. In the case of ATCC33277, we detected 50-kDa and 44-kDa proteins that adhered with zinc, but not copper, in saliva. In serum, we detected four zinc-related bands and four copper-related bands. In the case of ATCC49417, we observed 50-kDa and 45-kDa proteins that adhered with zinc and four copper-related bands in saliva, and we recognized a 45-kDa zinc-related band and four copper-related bands in serum. Among the saliva from FDC381, we found 55-kDa and 34-kDa proteins with affinity for copper. We detected three zinc-related bands and six copper-related bands in serum.

Saliva and serum treated with *P. gingivalis* ATCC33277 were identified by silver staining of SDS–PAGE gels, as shown in Fig. 4. There was little influence on saliva, but we observed moderate degradation of proteins in serum by protease. Interestingly, most of the effect was lost in the presence of zinc and copper ions . Therefore, zinc and copper may obstruct the protease activity of *P. gingivalis*.

Cation-binding proteins on bacterial cells

The protein samples bound to the HiTrap™ Chelating HP column from the surfaces of the bacterial cells and identified based on silver staining of SDS–PAGE gels are shown in Fig. 5. We detected several protein bands in the bacterial cell surface proteins that bound and were eluted from the HiTrap™ Chelating HP column treated with cations by EDTA. In the surface proteins from ATCC33277, we did not detect any protein band that bound iron, but we found 11 that bound zinc, 15 with affinity for copper, and 14 with affinity for nickel. We detected a 50-kDa protein that clearly had zinc-binding bands and 56-, 48-, and 26.5-kDa proteins that had copper-binding bands. In the surface proteins from ATCC49417, we did not detect any protein band that bound iron, but we found eight that bound zinc, 12 with affinity for copper, and nine with affinity for nickel. We recognized a 21.2-kDa protein that was a zinc-binding band and 44-kDa and 25.5-kDa proteins that were copper-binding bands. Among the surface proteins from FDC381, no iron-binding protein band was detected, but 13 were found with affinity for zinc, 30 for copper, and 20 for nickel. We detected a 25.5-kDa protein that had zinc-binding bands, and 33-, 27.5-, 25-, and 24.5-kDa proteins that formed copper-binding bands.

Discussion

One virulence factor of periodontal pathogens, their settlement to oral surfaces, soft tissues, and hard tissues in the gingival sulcus, is believed to be the first step in the initiation of the pathogenesis of periodontal disease (6). Therefore, the prevention of the adhesion/accumulation of periodontal pathogens to gram-positive bacteria on teeth or on oral epithelial cells in the gingival sulcus is very important for the control of outbreaks of periodontal disease. The oral cavity is always damp, so periodontal pathogens, including *P. gingivalis*, are suspended in oral fluid and covered with the proteins from oral fluid. The adherence of body fluid proteins to bacteria has been described (3), and some studies have reported that the presence of saliva or serum inhibits the adhesion and hemagglutination of periodontal pathogens (18, 21, 27).

Based on these findings, we investigated the mechanisms of the adherence of salivary and human serum proteins to the surface of *P. gingivalis*, the effects of cations and adhered salivary and serum proteins on the coaggregation with gram-positive early colonizers, and the effects on hemagglutination to confirm our hypothesis.

The amount of labeled proteins adhered to the cells of all tested *P. gingivalis* was increased by the presence of cations, especially those of zinc and copper. Cation-bridge binding appears to play an important role in the adhesion of salivary and serum proteins to *P. gingivalis*. On the other hand, the results from the heat treatment experiments suggest that the protein components of bacterial cell surface play an important role in these protein adhesions. Accordingly, zinc and copper ions seemed to promote the increased adhesion of salivary and serum proteins and the resultant accumulation of these proteins (4, 7, 16, 20). Hydrophobic binding and lectin–ligand binding (1) did not appear to be important to the adhesion mechanisms, while heat-sensitive components, such as proteins on the surface of bacteria, did play an important role in the adhesion. The results for *P. gingivalis* were similar to the findings for *P. intermedia* and *P. nigrescens* and the amount of labeled serum proteins that adhered to *P. gingivalis* was approximately the same as the amounts that adhered to *P. intermedia* and *P. nigrescens* (29).

Experiments that used proteins labeled with radioactive materials have been reported; however, the research groups used cyanoborohydride and formaldehyde to label the ϵ-amino groups of lysyl residues and the α-NH_2 terminus. Doubt exists as to the ability of these procedures to label all proteins and allow accurate determination of the quantity of adhered proteins. Therefore, we investigated an EDTA-elution method that was simple to

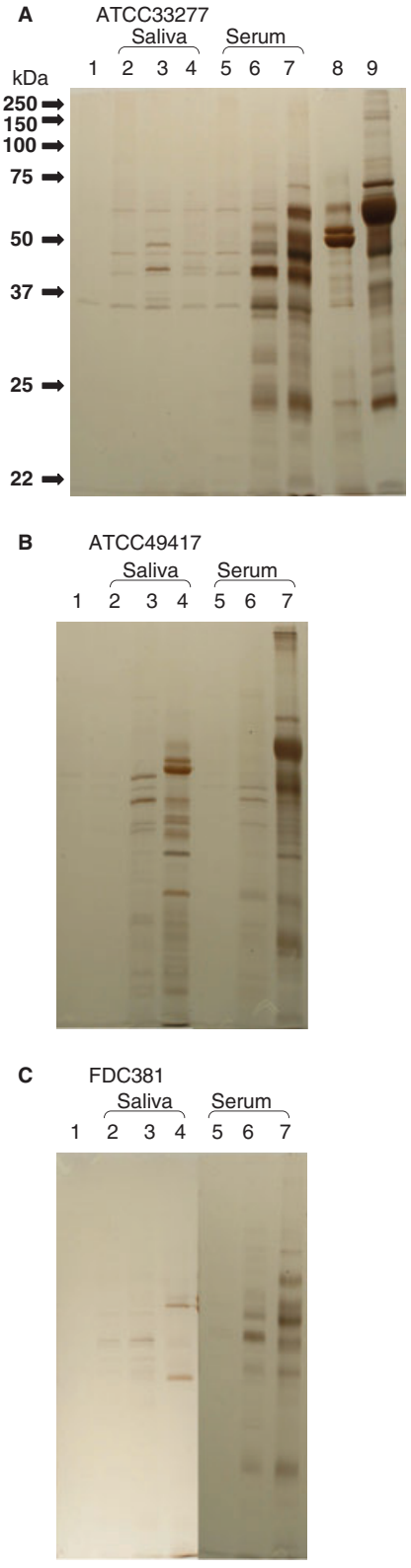

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ethylenediaminetetraacetic acid (EDTA) extracted cation-binding proteins in saliva and serum that adhered to *Porphyromonas gingivalis* ATCC33277 (A), ATCC49417 (B), and FDC381 (C). Lane 1, EDTA extract from *P. gingivalis*; lane 2, salivary proteins adhered to bacterial cells; lane 3, adhered salivary proteins with $ZnCl_2$; lane 4, adhered salivary proteins with $CuCl_2$; lane 5, serum proteins adhered to bacterial cells; lane 6, adhered serum proteins with $ZnCl_2$; lane 7, adhered serum proteins with $CuCl_2$; lane 8, saliva; lane 9, serum.

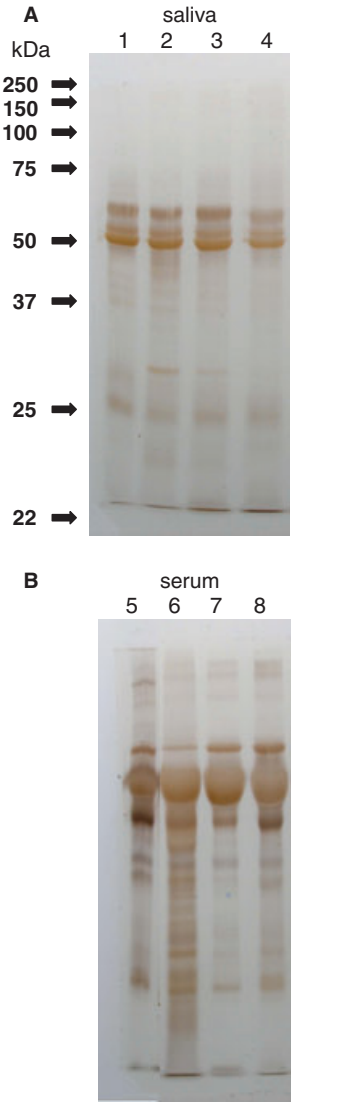

Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of saliva and serum treated with *Porphyromonas gingivalis* ATCC33277. Lanes : 1, salivary proteins; 2, salivary proteins treated with *P. gingivalis*; 3, salivary proteins treated with *P. gingivalis* and $ZnCl_2$; 4, salivary proteins treated with *P. gingivalis* and $CuCl_2$; 5, serum proteins; 6, serum proteins treated with *P. gingivalis*; 7, serum proteins treated with *P. gingivalis* and $ZnCl_2$; 8, serum proteins treated with *P. gingivalis* and $CuCl_2$.

perform for confirmation. As the same phenomenon was observed with both the labeled and non-labeled protein methods, we reaffirmed that zinc and copper ions increased the amount of protein that adhered to *P. gingivalis*.

We should consider whether saliva and serum are affected by the proteolytic activity of *P. gingivalis*, such as gingipain. We observed the moderate degradation of proteins in serum, but not in saliva, using SDS–PAGE. This difference may depend on the constituent proteins in each. As this protease activity was slightly weak under aerobic conditions, it may be necessary to perform the experiment under anaerobic conditions to reproduce the environment of the gingival sulcus. Surprisingly, the protease activity disappeared in the presence of zinc and copper ions. The inhibitory effect of zinc on the protease activity of *P. gingivalis* has been reported (2) so it was thought that a similar phenomenon was caused. The inhibition of protease activity by zinc and copper might be considered one of the reasons for the increases in proteins adhering to *P. gingivalis*. On the other hand, when *N*-tosylo-L-lysine chloromethyl ketone, which obstructs the proteolytic activities including gingipain of *P. gingivalis*, was added to an adhesion assay, the amount of protein adhering to *P. gingivalis* was increased in the case of calcium, magnesium, iron and nickel ions (data not shown). The results for zinc and copper ions were different, especially an increase of proteins was hardly seen in the case of copper. However, the amount of adhered proteins in the presence of zinc and copper ions was higher than for other cations.

Based on these results, we examined the effect of zinc and copper ions on the coaggregation with gram-positive early colonizers and hemagglutination of *P. gingivalis* treated with saliva or serum. As for *P. gingivalis*, which is scarcely able to adhere to oral tissues directly, the ability to adhere to and coaggregate with gram-positive early colonizers, such as *Streptococcus* and *Actinomyces*, is very important for its settlement in the gingival sulcus (11–13, 19, 32). Furthermore, the hemagglutination ability of *P. gingivalis* is related to adhesion activity and nutrient acquisition. When *P. gingivalis* cells were treated with saliva or serum before the assay, coaggregation and hemagglutination of all the *P. gingivalis* strains tested was inhibited. When zinc or copper ions were added during the pretreatment of *P. gingivalis* cells with saliva or serum, both activities were markedly inhibited. The inhibition owing to the presence of both cations seemed to result from an increase in the adhesion and accumulation of proteins (4, 7, 16, 17, 20) on the surface of

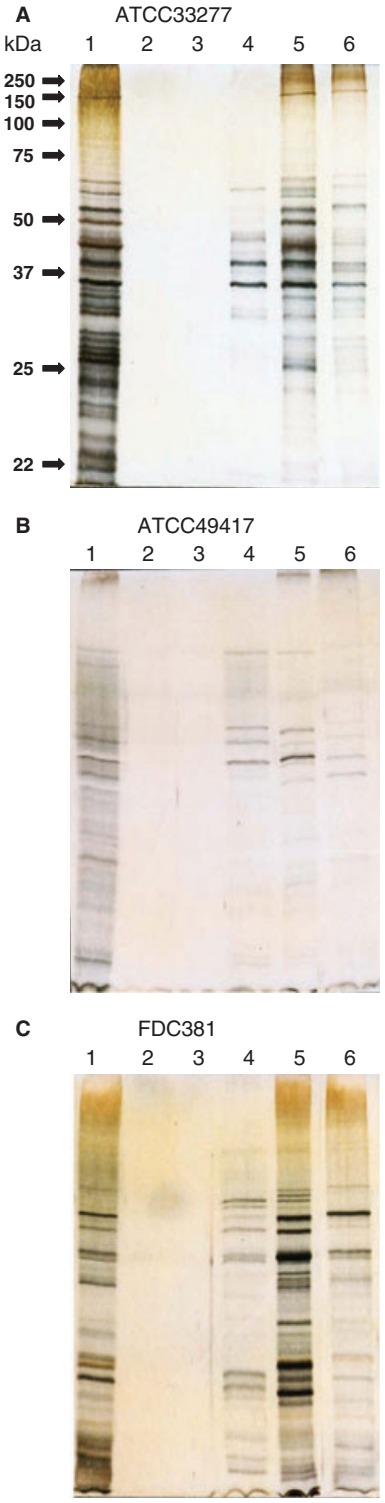

Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cation-binding proteins from the surface of *Porphyromonas gingivalis* ATCC33277 (A), ATCC49417 (B), and FDC381 (C). Lanes : 1, CHAPS extract from *P. gingivalis*; 2, extracted proteins that bound to no cation-binding chelating column; 3, extracted proteins to iron-binding column; 4, extracted proteins to zinc-binding column; 5, extracted proteins to copper-binding column; 6, extracted proteins to nickel-binding column.

P. gingivalis cells, which masked the aggregation and hemagglutination sites. We conclude that proteins with a high affinity for zinc or copper ions play important roles in the inhibition of coaggregation and hemagglutination of *P. gingivalis* in the general environment where bacterial cells are in contact with oral fluid. We suggest that the inhibition of coaggregation might be related to the obstruction of plaque accumulation and the prevention of transition to periodontal pathogenic plaque. Furthermore, the inhibition of hemagglutination may disturb the supply of hemin, a growth factor for *P. gingivalis* (5).

Therefore, we sought to identify zinc- or copper-related proteins in saliva and serum and on the cell surface of *P. gingivalis* by SDS–PAGE. Protein bands that showed the participation of zinc and copper in their adherence to bacterial cells were detected in saliva and serum using an EDTA-elution method. We observed different patterns for each sample. In addition, the different patterns of protein bands on the gels loaded with samples from bacterial cell surfaces implied the presence of different cation-binding proteins on the cells of each tested strain. It might be necessary to examine the relationship between these zinc- and copper-binding protein bands and fimbriae (8, 9). As the number of electrophoresis bands and patterns differed in each experiment, we suggest that specific zinc- and copper-related proteins might exist in saliva, serum, and on the cell surface of *P. gingivalis*.

The pattern of cation-binding proteins of *P. gingivalis* is also different from that of other periodontal pathogens (29). The effect of zinc and copper ions on the adhesion of salivary and serum proteins to bacterial cells is probably dependent on the interaction between the proteins related to zinc or copper ions in the saliva or serum and zinc- or copper-binding proteins on the bacterial cells. In addition, it seems likely that cation-binding proteins have specific binding capacities.

In conclusion, zinc and copper markedly enhanced the adhesion and accumulation of proteins on *P. gingivalis* and inhibited the coaggregation and hemagglutination of *P. gingivalis*. Full investigation of the cation-bridge binding system, including specific binding factors, will be important for clarifying the processes of bacterial adhesion and aggregation. Zinc and copper ions might be useful for limiting the settlement/colonization of *P. gingivalis* in the gingival sulcus with the goal of preventing periodontal disease.

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