Contribution of *Streptococcus mutans* Strains with Collagen-Binding Proteins in the Presence of Serum to the Pathogenesis of Infective Endocarditis

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**ABSTRACT** *Streptococcus mutans*, a major pathogen of dental caries, is considered one of the causative agents of infective endocarditis (IE). Recently, bacterial DNA encoding 120-kDa cell surface collagen-binding proteins (CBPs) has frequently been detected from *S. mutans*-positive IE patients. In addition, some of the CBP-positive *S. mutans* strains lacked a 190-kDa protein antigen (PA), whose absence strengthened the adhesion to and invasion of endothelial cells. The interaction between pathogenic bacteria and serum or plasma is considered an important virulence factor in developing systemic diseases; thus, we decided to analyze the pathogenesis of IE induced by *S. mutans* strains with different patterns of CBP and PA expression by focusing on the interaction with serum or plasma. CBP-positive (CBP+/PA−) strains showed prominent aggregation in the presence of human serum or plasma, which was significantly greater than that with CBP+/PA-positive (PA+) and CBP-negative (CBP−/PA+) strains. Aggregation of CBP+/PA− strains was also observed in the presence of a high concentration of type IV collagen, a major extracellular matrix protein in serum. In addition, aggregation of CBP+/PA− strains was drastically reduced when serum complement was inactivated. Furthermore, an *ex vivo* adherence model and an *in vivo* rat model of IE showed that extirpated heart valves infected with CBP+/PA− strains displayed prominent bacterial mass formation, which was not observed following infection with CBP+/PA+ and CBP−/PA+ strains. These results suggest that CBP+/PA− *S. mutans* strains utilize serum to contribute to their pathogenicity in IE.

**KEYWORDS** *Streptococcus mutans*, blood, infective endocarditis

Gram-positive cocci, such as *Staphylococcus aureus* and viridans group streptococci, are major pathogens in infective endocarditis (IE) (1, 2). One of the important steps for the pathogenesis of IE is invasion of pathogenic bacteria into the bloodstream (1). Viridans group streptococci invade the bloodstream following professional dental treatment as well as routine daily oral care (3, 4) and are associated with a risk for IE in subjects with underlying heart disorders, though these bacteria are predominantly phagocytosed and eliminated in healthy humans (5).

*Streptococcus mutans*, classified as a viridans group streptococcus, is a major pathogen of dental caries and is occasionally isolated from the blood of IE patients (6). In 1993, approximately 10 to 20% of *S. mutans* strains were demonstrated to possess collagen-binding activity, which was regarded to be advantageous for binding to collagenous tissue, such as dentin (7). Thereafter, 120-kDa collagen-binding proteins (CBPs), which were classified as Cnm and Cbm, were identified on the cell surfaces of some *S. mutans* strains in 2004 and 2012, respectively (8, 9).
The distribution of S. mutans strains expressing Cnm in the human oral cavity is approximately 10 to 20% (10), whereas that of strains expressing Cbm is only 2 to 3% (9). However, bacterial DNA encoding CBPs was detected in more than 60% of S. mutans-positive heart valve specimens (11). CBP-positive (CBP+) S. mutans strains displayed binding to type I, III, and IV collagens (11), which are major components of cardiovascular tissues. In addition, these strains showed properties of high levels of adhesion to and invasion of human vein endothelial cells (11). These properties were drastically reduced in collagen-binding gene-knockout strains (11). CBP (especially Cbm)-positive S. mutans strains also possess properties of binding to and aggregation with fibrinogen, which acts as a bridging molecule to allow the bacterium to bind to platelets, resulting in platelet aggregation (12).

A 190-kDa protein antigen (PA), also known as SpaP, P1, and antigen I/II, is associated with sucrose-independent initial adhesion of S. mutans to tooth surfaces (13). Approximately 3% of S. mutans clinical strains, all of which were CBP positive, did not express PA, as demonstrated by Western blot analyses, and displayed extremely weak expression of PA in reverse transcription-PCR (RT-PCR) analyses (14, 15). Though the putative PA amino acid sequences of most PA-negative (PA−) S. mutans strains were highly conserved, 20-bp deletions in the promoter regions or stop codons following frameshifts were noted in some of these strains (12, 15). PA-negative S. mutans strains displayed decreased susceptibility to phagocytosis by human polymorphonuclear leukocytes and caused the development of bacteremia for longer durations (15, 16). In addition, CBP+/PA− strains showed enhanced binding activity to collagen compared with CBP+/PA-positive (PA+) and CBP-negative (CBP−)/PA− strains (14), and the collagen-binding activity of a PA-knockout mutant strain of a CBP+/PA− strain was significantly greater than that of the parent strain. These results imply that PA may act in an antagonistic manner with CBPs, though the mechanisms of such interactions remain to be elucidated.

Recently, the aggregation properties of pathogenic bacteria, which sometimes require serum or plasma, have been reported to be important virulence factors for systemic diseases (17–19). Following the suspension of bacteria in human or animal plasma, bacterial aggregation was closely linked to biofilm formation, which facilitates antibiotic tolerance and resistance to phagocytosis for the bacteria (18, 20). As for S. mutans, interactions with fibrinogen, platelets, and complement, in addition to other virulence properties, such as endothelial cell adhesion/invasion and collagen-binding activity, have been reported (7, 10, 12, 21, 22). In the present study, we analyzed the aggregation properties of S. mutans strains with different patterns of CBP and PA expression following suspension in the fluid phases of blood, such as serum and plasma. In addition, the contribution of the aggregation properties induced by S. mutans strains to the pathogenesis of IE was investigated using ex vivo and in vivo models.

**RESULTS**

**Aggregation properties in the presence of human serum or plasma.** Bacterial aggregation rates in the presence of serum and plasma for strain TW295 (CBP+/PA−) were elevated in a time-dependent manner and plateaued at approximately 6 h (Fig. 1A and B), whereas strains MT8148 (CBP−/PA+) and NN2094 (CBP+/PA+) exhibited significantly lower aggregation activities at each time point. However, the self-aggregation rates were similar among these strains (Fig. 1C). Based on these initial findings, we analyzed the serum and plasma aggregation activities of the 45 clinical strains (15 strains each displaying the CBP−/PA+, CBP+/PA−, and CBP+/PA− phenotypes) using a standard incubation time of 6 h. The mean aggregation rates in the presence of serum and plasma were significantly higher in the CBP+/PA− group than in the CBP+/PA+ and CBP+/PA+ groups (P < 0.001) (Fig. 1D). However, the mean self-aggregation rates were not significantly different among the groups. The aggregation rates in the presence of serum and plasma were similar in all groups, and a regression analysis between the aggregation rates in the presence of serum and plasma showed a
significant positive correlation ($r = 0.761, P < 0.001$) (Fig. 1E). These findings indicated that the results obtained for the aggregation rates in the presence of plasma and serum were quite similar. Interestingly, aggregation was confirmed in TW295 mixed with whole blood obtained from a volunteer (Fig. 2). No aggregation was observed in strain TW295CND mixed with whole blood, whereas strain TW295comp showed bacterial mass formation. Based on these results, we considered that the aggregation properties of S. mutans strains were primarily influenced by serum, and aggregation in the presence of serum was therefore examined in greater detail.

**Aggregation properties in the presence of serum obtained from various subjects.** As shown in macroscopic, stereoscopic microscopic, and scanning electron microscopic images (Fig. 3A), no aggregation was observed for strain MT8148 (CBP−/PA+) in the presence of serum obtained from a human volunteer. Strains NN2094 (CBP+/PA+) and TW295 (CBP+/PA−) showed slight and prominent aggregation, respectively. When the aggregation properties of the CBP+/PA− strains and the CBP-knockout strains were compared, the CBP+/PA− strains showed significant aggregation properties, which were absent in the CBP-knockout mutant strains (Fig. 3B). The aggregation properties were analyzed with five different serum samples isolated from five healthy volunteers, which revealed that MT8148 exhibited negligible aggregation activity and NN2094 showed weaker aggregation activity than TW295 (Fig. 3C). In contrast, TW295 displayed prominent aggregation activity with all serum samples. Similar results were also observed using bovine serum (Fig. 3D; see also Fig. S2 in the supplemental material). Next, to investigate the effect of collagen in serum, the aggregation properties of these strains were evaluated in the presence of type IV collagen, which is prevalent in the basement membrane and identified in the serum of healthy humans at a concentration of <0.14 μg/ml. The aggregation of TW295 in the
presence of type IV collagen at each concentration was significantly higher than that of MT8148 and NN2094 (P < 0.05) (Fig. 3E). TW295CND did not show prominent aggregation in the presence of type IV collagen, whereas TW295comp restored the aggregation activity (Fig. 3F). Though high rates of TW295 aggregation were observed in the presence of high concentrations of type IV collagen (1.4 and 14 μg/ml), the rate of aggregation of TW295 in the presence of low concentrations of type IV collagen (<0.14 μg/ml, the concentration found in healthy human serum) was significantly lower than that in the presence of serum. These results indicated that type IV collagen is one of the factors required for bacterial aggregation but that other important factors for the aggregation induced by CBP+/PA− strains may exist. Thus, we analyzed aggregation activities in the presence of complement-inactivated serum obtained from a volunteer to examine the effects of complement, which revealed that the aggregation activity of TW295 in the presence of inactivated serum was much lower than that in the presence of (activated) serum (P < 0.001) (Fig. 3G). Such a reduction in aggregation activity by inactivation of complement was also observed in TW295comp (Fig. 3H). These results indicated that complement is an important factor in bacterial aggregation induced by CBP+/PA− strains.

**Ex vivo adherence model with bovine heart valve specimens.** In the presence of serum, pathological observations following Gram staining revealed that no bacteria were observed following infection with strain MT8148, whereas bacterial adhesion was confirmed following infection with NN2094 (Fig. 4A). TW295 induced considerable bacterial mass formation, which was drastically reduced when TW295CND was used for infection in the presence of serum. Furthermore, TW295comp also induced bacterial mass formation. The numbers of adherent cells following infection with TW295 were the highest among these strains, with the differences from the numbers for the other strains being statistically significant (Fig. 4B). When the numbers of adherent cells following infection with the 45 clinical strains (15 strains each displaying the CBP+/PA−, CBP+/PA+, and CBP−/PA− phenotypes) were analyzed, those of the CBP+/PA+ and CBP+/PA− groups were significantly higher than those of the CBP−/PA+ group (P < 0.05 and P < 0.001, respectively) (Fig. 4C). In addition, the mean cell numbers for the
FIG 3 Aggregation of *S. mutans* strains in the presence of serum obtained from various subjects. (A) Representative images of *S. mutans* strains in the presence of serum obtained from a healthy volunteer. (Left) Macroscopic images; (middle) stereoscopic microscopic images (bars = 20 μm); (right) scanning electron microscopic images (bars = 5 μm). (B) Aggregation rates of CBP+/PA+ strains and their CBP-inactivated mutant strains in the presence of serum obtained from a healthy donor; (C) aggregation of *S. mutans* strains in the presence of serum obtained from five different volunteers; (D) aggregation of *S. mutans* strains in the presence of serum samples obtained from six different bovines; (E) aggregation of *S. mutans* strains in the presence of type IV collagen at various concentrations; (F) aggregation of TW295 and its mutant strains in the presence of type IV collagen at various concentrations; (G) aggregation of *S. mutans* strains in the presence of serum or complement-inactivated serum; (H) aggregation of TW295 and its mutant strains in the presence of serum or complement-inactivated serum. There were significant differences between the groups, which were determined using ANOVA with the Bonferroni correction. *, P < 0.05; **, P < 0.01; ***, P < 0.001. The bars in panels B to H represent standard deviations.
FIG 4 Evaluation of the ex vivo adherence model with bovine heart valve specimens. (A) Representative histopathological images following Gram staining of tissue sections of bovine heart valves infected with \textit{S. mutans} strains in the presence of serum. Bars = 50 \mu m. (B) Rates of \textit{S. mutans} adhesion to bovine heart valves in the presence of serum. There were significant differences in the rates, which were determined using ANOVA with the Bonferroni correction. **, \( P < 0.01 \) versus the TW295 group; ***, \( P < 0.001 \) versus the TW295 group; ###, \( P < 0.001 \) versus the NN2094 group. (C) Adhesion rates of \textit{S. mutans} clinical strains classified by expression of CBP and PA. Each closed circle represents the mean value for each bacterial strain. Horizontal bars indicate the mean values for the groups. There were significant differences in the rates, which were determined using ANOVA with the Bonferroni correction. *, \( P < 0.05 \); ***, \( P < 0.001 \). (D) Representative histopathological images following Gram staining of tissue sections of bovine heart valves infected with TW295 in the presence of type IV collagen or serum. Bars = 50 \mu m. (E, F) Rates of TW295 adhesion to bovine heart valves in the presence of type IV collagen (E) and inactivated serum (F). There were significant differences in the rates, which were determined using ANOVA with the Bonferroni correction. ***, \( P < 0.001 \) versus the bacterium-only group; ##, \( P < 0.01 \) versus the serum group; ###, \( P < 0.001 \) versus the serum group. The bars in panels B, E, and F represent standard errors.
CBP⁺/PA⁻ group were significantly higher than the mean cell numbers for the CBP⁺/PA⁺ group ($P < 0.05$). When type IV collagen was added instead of serum in the ex vivo model, prominent bacterial mass formation was observed for TW295 in the presence of high concentrations of type IV collagen (Fig. 4D and E), though the numbers of adherent cells were significantly lower than those in the presence of serum ($P < 0.01$). In addition, bacterial cell numbers of TW295 were drastically reduced when serum complement was inactivated (Fig. 4D and F). However, complement did not solely induce bacterial adherence since the number of adherent cells in the presence of inactivated serum was significantly higher than that in the absence of inactivated serum.

**Virulence in a rat model of IE.** Pathological observations from Gram staining showed that administration of MT8148 and NN2094 did not induce the formation of vegetative bacterial masses in transversely sectioned heart specimens from all rats, which were extirpated after euthanasia on day 7 after infection (Fig. 5A). In contrast, the formation of considerable vegetation was observed following infection with TW295. No bacteria were observed in heart valves infected with TW295CND, whereas rats infected with TW295comp showed bacterial mass formation. As for bacterial clearance in the blood of rats, TW295 and TW295comp were recovered from rats up to 7 days after infection, while TW295CND was recovered until 6 h after infection (Table 1). Next, the rates of vegetation formation induced by TW295 infection were analyzed by extirpating the heart valve specimens at 1, 3, and 7 days after infection. Prominent bacterial masses were not observed in the heart valves extirpated on days 1 and 3, though hypertrophy of the endocardium was observed on day 3 (Fig. 5B). Conversely, considerable bacterial masses were formed by day 7, and these showed histopathological scores significantly higher than those determined on days 1 and 3 ($P < 0.05$) (Fig. 5C). Hematoxylin-eosin-stained sections of heart valve lesions showed that all histopathological scores (e.g., scores for infiltration of inflammatory cells, hypertrophy of the endocardium, hypertrophy of the annulus, and acceleration of fibrosis) except the score for fibrin-like deposition increased in a time-dependent manner (Fig. 53).

**DISCUSSION**

Some pathogenic bacteria possess the ability to adhere to host cells, developing into masses called biofilms (23). In IE, biofilms localized on cardiac valves (termed vegetations) enable bacteria to be protected from and resist host defenses or antibiotic treatment (24, 25). The aggregation induced by pathogenic bacteria in the heart valve is closely related to the deterioration of the condition of patients with IE (26, 27). In the present study, we analyzed the aggregation properties of *S. mutans*, which invades the bloodstream and interacts with serum, by focusing on the cell surface proteins CBP and PA.

*S. mutans* strains are classified into four serotypes, serotypes c, e, f, and k, on the basis of the chemical composition of their cell surface rhamnose-glucose polymers (28). Serotype c is the major type in oral isolates from healthy subjects, with a distribution frequency of approximately 70 to 75%, followed by serotype e (distribution frequency, approximately 20%). Though the distribution frequencies for serotypes f and k are lower than 5%, a high frequency of non-c serotypes has been identified in *S. mutans*-positive heart valve specimens extirpated from patients with cardiovascular diseases (29, 30). Thus, we investigated the specific characteristics of strains with minor serotypes using more than 500 *S. mutans* clinical strains, which revealed that the cmn gene, encoding Cmm, is prominent in serotype f strains, whereas the cbm gene, encoding Cbm, in oral strains is predominant in serotype k strains (9).

We previously analyzed the relationship between serotype and the patterns of CBP and PA expression using *S. mutans* clinical strains (9, 14, 15). In those studies, most serotype c strains showed the CBP⁺/PA⁻ phenotype (>90% of approximately 400 serotype c strains), which was the highest frequency at which that phenotype was observed among all serotypes. In addition, the distribution rate for the CBP⁺/PA⁺ phenotype in serotype e strains was nearly 10% of approximately 100 serotype e strains,
which was higher than that in the other serotypes. Conversely, of more than 10 serotype k strains tested, most exhibited the CBP+/PA− phenotype. Based on this background, we selected MT8148 (CBP+/PA−, serotype c), NN2094 (CBP+/PA+, serotype e), and TW295 (CBP+/PA+, serotype k) as representative clinical strains.

Most CBP-positive strains classified as serotype f or k showed unique patterns of expression of other proteins which are closely related to bacterial virulence. For example, most CBP-positive strains were negative for gbpA, which encodes glucan-binding protein A, resulting in lower glucan-binding activity for CBP-positive strains.
than for CBP-negative strains (31). In addition, approximately 25% of CBP-positive strains lacked PA, and all of these strains were serotype f or k (14, 15). These CBP+/PA− strains showed lower sucrose-dependent adhesion and cellular hydrophobicity than the other S. mutans strains. Conversely, PA-negative strains showed decreased susceptibility to phagocytosis by human polymorphonuclear leukocytes, and the strains were recovered from blood for a longer duration in a rat bacteremia model (16). Though GbpA-negative/PA-negative strains appear to be less cariogenic because of decreased sucrose-dependent adhesion and cellular hydrophobicity (14, 31), CBP-positive strains could exhibit stronger cariogenicity via attachment to dentin and dental pulp cells because type I collagen is the major organic component of these cells (8, 32, 33). Thus, mechanisms for the development of dental caries may differ between CBP-negative and CBP-positive strains.

S. aureus and viridans group streptococci are the most common organisms responsible for IE (1, 2). S. aureus forms vegetations by adhering to the endothelial cell surface and subsequently aggregating in the presence of plasma (34). Recently, viridans group streptococci have been reported to display properties of adhesion to and invasion of human vascular endothelial cells (35, 36). S. mutans, classified as a viridans group streptococcus, also showed properties of high levels of adhesion to endothelial cells, which was mainly observed in CBP-positive S. mutans strains, with the distribution of such strains being approximately 10 to 20% of all S. mutans strains (9, 21). In addition, the adhesion of CBP-positive S. mutans strains to human vascular endothelial cells was enhanced by the inactivation of PA in parental CBP-positive strains (14). We previously observed prominent masses of aggregated bacteria following infection of human vascular endothelial cells with CBP+/PA− strains (11). Thus, we analyzed the aggregation properties of S. mutans strains with different CBP and PA expression patterns by focusing on their interactions with serum.

Though the detailed mechanisms for the property of high levels of collagen binding of CBP+/PA− strains remain unclear, two hypotheses appear to be relevant. First, both PA and CBP have been reported to possess a collagen-binding ability (8, 9, 37). The binding properties of the clumping factor A protein of S. aureus were antagonized by a high-molecular-weight staphylococcal surface protein (20). Thus, we speculate that 190-kDa PA may likewise interfere with 120-kDa CBP in a similar manner. Another possibility is that a larger amount of CBP than PA is anchored on the cell surface in strains with low levels of PA expression, since both of these proteins are anchored by the LPXTG motif. Further studies should be performed on the basis of these hypotheses.

The components of serum are similar to those of plasma; however, only plasma contains clotting factors, such as fibrinogen, which is one of the major extracellular matrix proteins in blood (38). In this study, CBP+/PA− S. mutans strains showed prominent aggregation in the presence of both human serum and plasma. We previously reported that CBP+/PA− strains (mainly Cbm-positive/PA-negative strains) showed adhesion and aggregation activities in the presence of fibrinogen (12). However, aggregation in the presence of fibrinogen plateaued at 24 h after incubation, which was much slower and weaker than the aggregation observed in the presence of serum. These results indicated that serum is a major factor in aggregation by CBP+/PA− S. mutans. Therefore, we performed detailed analyses regarding the aggregation properties of S. mutans strains, primarily in the presence of serum.

### TABLE 1 Bacterial counts in rat blood

<table>
<thead>
<tr>
<th>Infection strain</th>
<th>Mean no. of CFU/ml of blood ± SE (no. of rats from which bacteria were recovered) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>TW295</td>
<td>1,453 ± 902 (7)</td>
</tr>
<tr>
<td>TW295CND</td>
<td>816 ± 187 (7)</td>
</tr>
<tr>
<td>TW295comp</td>
<td>864 ± 154 (7)</td>
</tr>
</tbody>
</table>

*aSignificant difference in relation to the parent strain at the same time point (P < 0.05, as determined using ANOVA with the Bonferroni correction).*
Blood contains several kinds of extracellular matrix proteins, such as fibronectin, fibrinogen, type IV collagen, vitronectin, and elastin, all of which except fibrinogen are contained in both plasma and serum (39–41). It has been reported that some of the cell surface proteins of S. aureus bind and aggregate following interaction with extracellular matrix proteins in the blood (42, 43). We previously analyzed the properties of CBP-positive S. mutans strain binding to type I, III, and IV collagens, since these collagen types are major components of cardiovascular tissues (11). Approximately three-fourths of the collagen in human heart valve is type I, while one-fourth is type III (44). Type IV collagen is present in the serum of healthy humans at a concentration of 0.14 g/ml (45) and is also prevalent in the basement membrane of blood vessels (46). However, elevated levels of type IV collagen in the serum of patients with systemic diseases, such as liver disease and diabetes, have been reported (47, 48). Thus, we analyzed the aggregation properties of CBP+/PA− S. mutans in the presence of various concentrations of type IV collagen.

Prominent aggregation of bacterial suspensions of CBP+/PA− strains was observed in the presence of relatively high concentrations of type IV collagen (>1.4 μg/ml) compared with that normally observed in serum. Though the aggregation rates obtained with high concentrations of type IV collagen were similar to those obtained with serum, the ex vivo model showed that bacterial mass formation in the presence of type IV collagen was lower than that in the presence of serum. These results indicated that type IV collagen is an important factor but not the sole factor for the induction of biofilm formation on heart valves by CBP+/PA− strains.

The S. mutans strains in our laboratory stock isolated from the blood of patients with bacteremia or IE were divided into CBP-positive and CBP-negative strains (49). The survival in blood of CBP-negative strains isolated from blood was analyzed in our recent study by focusing on the orphan regulator protein named CovR (22). CovR plays an important role in the adhesion of S. mutans to complement C3b, which leads to activation of phagocytosis by opsonization. In that study, CBP-negative blood isolates showed reduced expression of CovR and lower levels of adhesion to C3b than oral isolates, which increased survival in a rat model of IE. Collagen-binding protein Cna in S. aureus, which is homologous to CBP of S. mutans, with approximately 55% homology in the collagen-binding domain (8, 9), was reported to bind to complement C1q, resulting in the blockage of the classical pathway and inhibition of complement activation (50). The binding of Cna-positive S. aureus to complement was shown to be related to decreased susceptibility to phagocytosis. It was also demonstrated that a recombinant CBP of S. mutans adhered to C1q, which led to inhibition of the classical pathway (50). However, the authors analyzed recombinant CBP, which was not purified from the full-length CBP but from a partial sequence, including the collagen-binding domain (this domain is a critical part but makes up less than half of the full-length sequence). Thus, we analyzed the relationship between complement and CBP of S. mutans using CBP-positive clinical strains. In addition, we showed that CBP-positive strains were isolated from blood in the rat model of IE over a longer period. According to the results from research by Kang et al. (50) and our study, CBP-positive strains adhere to complement, leading to aggregation and blockage of the classical pathway, followed by decreased susceptibility to phagocytosis.

Our ex vivo analysis revealed that the binding abilities of CBP+/PA− strains in the presence of inactivated serum were significantly lower than those in the presence of (activated) serum, which indicated that the dysfunction of complement induced by CBP+/PA− strains is an important factor for bacterial mass formation on heart valves. The major relevant outcome of the heat treatment of serum is the inactivation of complement; however, other serum components, such as vitamins, amino acids, and hormones, may be damaged by heating. Thus, it is difficult to minimize the involvement of serum components other than complement following heat treatment. Further studies may be important to determine which serum components and complement are closely related to the aggregation property induced by CBP+/PA− S. mutans strains.

We developed an ex vivo adherence model, which may be a useful tool to evaluate
the virulence of many bacterial strains for screening purposes. We used heart valves extracted from bovine hearts in the ex vivo assays because tissue valves (sometimes called “bioprotetic” valves), which are widely used in heart valve replacement, are crafted from valves obtained from bovine or porcine hearts (51). In addition, we used easily accessible bovine serum available as a commercial product in the ex vivo assays, since the aggregation activities of S. mutans strains in the presence of human serum were quite similar to those in the presence of bovine serum. We utilized the ex vivo model to evaluate the virulence of S. mutans in the presence of serum. The interaction between bacteria and host factors other than serum on the heart valves can be analyzed by simply exchanging serum for other components. The reproducibility of the experiments was confirmed by using bovine heart valves and bovine serum, which were kept at −20°C until use, which was within a few months.

The ex vivo model is more clinically relevant than in vitro assays, such as the classical biofilm assays, collagen-binding assays, and the settling of aggregates on treated slides (i.e., poly-L-lysine-coated slides), since collagen or poly-L-lysine is completely different from the affected tissues. In addition, the ex vivo model is inexpensive, can provide results relatively quickly, and does not require specialized equipment, unlike cell culture techniques. Animal experiments, including experiments using the rat model of IE, are widely used as one of the most reliable ways to assess human disease and biology (52). However, animal experiments have disadvantages, such as potential ethical concerns and the need for the use of specialized techniques and animals in predefined growth stages, which makes it difficult to analyze a large number of samples. These limitations can be overcome by using the ex vivo model. We previously evaluated the virulence of S. mutans strains by using larvae of the wax moth (Galleria mellonella), which was constructed as a model of systemic bacterial infection on the basis of evidence of a correlation of systemic bacterial infection in the wax moth with that in mammals (53). Though the method of evaluation with this model is relatively simple, the survival rate of the wax moth is not an accurate reflection of the infection of heart valves.

The ex vivo model has some limitations, such as the viability of tissues and a lack of blood flow and tissue perfusion, which are observed in patients with IE. Future studies based on the ex vivo model could include improvements, such as the use of flow chambers to better simulate the circulation. In addition, the heart valve lacks host defense systems. The ex vivo model cannot simulate injury to the endothelium, which appears to be the main disadvantage of the model. However, some highly virulent bacteria, such as S. aureus, colonize the heart valves without causing damage to the endothelium through interaction with host factors (1). Similar limitations were also observed in an ex vivo model using porcine heart valves to evaluate biofilm formation following infection by Enterococcus faecalis (26). Though it is difficult to overcome these limitations at this time, we propose the use of this ex vivo adherence model to screen large numbers of bacterial strains, and an improved model based on this system may be developed in future studies.

In the evaluation of porcine heart valve colonization by E. faecalis, 100 µg/ml of gentamicin was used to avoid contamination (26). In addition, gentamicin (300 µg/ml) and metronidazole (200 µg/ml) were used to kill Gram-negative bacteria, such as Porphyromonas gingivalis, in a cell culture study (54). We selected penicillin at a concentration of 50 µg/ml and gentamicin at a concentration of 300 µg/ml on the basis of the concentrations needed to kill adherent bacteria in a cell culture experiment, as previously described (11). In that study, these antibiotics were mainly used to kill S. mutans. Though we confirmed that the bovine heart specimens were sterile, we should select antibiotics and their concentrations more carefully in future studies.

Experimental rat models of IE have been reported, and most of these involved catheters surgically placed across heart valves via the carotid artery followed by bacterial administration via the blood circulation (55–57). These reports investigated the virulence of IE induced by CBP-negative S. mutans strains because CBP-negative strains were widely used in laboratory studies due to their frequent distribution in the oral cavity. In addition, many studies were carried out before the identification of
CBP-positive strains, which was first reported in 2004 (8). In the latter study, CBP-negative strains were identified in injured heart valves in rat models of IE, a finding which was not concordant with the results of the present study. The differences between the results of our study and those of the previous study may be due to variations in the experimental procedures, such as the use of distinct bacterial infection dosages, the timing of bacterial inoculation, euthanasia protocols, and variations in the choice of catheters and guide wires. Another explanation for the discordance in the results may be variation of the expression of cell surface proteins, in addition to CBP, in each S. mutans strain examined.

Though the presence of CBP-negative strains in injured heart valves was not confirmed in the present study, a previous study showed that CBP-negative strains were found in vegetations in a rat model of IE (56). When we administered 10-fold higher concentrations of the CBP-negative strain (1 $\times$ 10$^9$ CFU/body) than the strain used in the present study (1 $\times$ 10$^8$ CFU/body) (22), bacteria were found in the injured heart valves. This result indicated that a high concentration of a CBP-negative strain invading the blood can induce vegetation formation. In addition, a wax moth larva virulence assay revealed that the survival rate observed in the CBP-negative strain-infected group was significantly lower than that observed in the uninfected group (12). In fact, CBP-negative strains have been isolated from the blood of IE patients (49). Thus, CBP-negative S. mutans strains are also related to virulence in IE (56, 58, 59), though the intensity of the pathogenicity and the mechanisms of IE development appear to be different from those for CBP-positive S. mutans strains.

In our previous study, we also compared the virulence in a rat model of IE using CBP-positive S. mutans strain SA31 and its genetically manipulated strains (CBP-knockout and -complemented mutant strains), which demonstrated that the S. mutans strains expressing CBP were involved in bacterial mass formation (12). In the present study, we showed that the CBP$^+$/PA$^-$ S. mutans strains greater virulence than the CBP$^+$/PA$^+$ and CBP$^-$/PA$^-$ strains, which were clinically isolated strains. In addition, virulence factors for a rat model of IE infected with a CBP$^+$/PA$^-$ S. mutans strain were evaluated at 1, 3, and 7 days after bacterial infection and revealed that the severity of infection on the basis of histopathological findings became more prominent in a time-dependent manner. Considering these results, bacterial mass formation by CBP$^+$/PA$^-$ S. mutans strains in the rat model of IE was not simply induced by bacterial adhesion to endothelial cells immediately after bacterial administration. Two virulence properties of CBP$^+$/PA$^-$ S. mutans strains, such as platelet aggregation via fibrinogen, which was found in our previous study (12), and serum aggregation properties, which were observed in the present study, may be important for bacterial accumulation in endothelial cells.

In conclusion, we found that expression of the CBP$^+$/PA$^-$ phenotype in S. mutans strains induced aggregation in the presence of serum, likely involving type IV collagen and complement. Such aggregation may be an important factor for bacterial mass formation on heart valves, which was confirmed by ex vivo and in vivo models. These results could help to explain why CBP$^+$/PA$^-$ strains showed properties of high levels of adhesion to endothelial cells and why the gene encoding CBP is frequently detected in S. mutans-positive heart valve specimens extirpated from IE patients.

MATERIALS AND METHODS

Ethics statement. This study was conducted with full adherence to the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the Osaka University Graduate School of Dentistry (approval no. H26-026). Prior to specimen collection, the subjects were informed of the study contents, and written informed consent was obtained from all participants. All rats were treated humanely in accordance with National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Osaka University Graduate School of Dentistry (approval no. 24-019-0). Animals were maintained and handled in accordance with guidelines for animal research.

S. mutans strains and growth conditions. S. mutans MT8148 (CBP$^-$/PA$^-$) and NN2094 (CBP$^+$/PA$^-$), isolated from the oral cavity of a Japanese child (28, 60), were used. TW295 (CBP$^+$/PA$^-$), isolated from the blood of a patient with bacteremia after tooth extraction, and its CBP-knockout mutant strain,
TW295CND, were also analyzed (28, 31). TW295 has properties of high levels of binding to collagen and fibrinogen (10, 12), whereas NN2094 displays only collagen-binding properties, in which its level of binding to fibrinogen is significantly lower than that of TW295 (12, 14). The CBP-negative strains, MT8148 and TW295CND, did not express properties of binding to these extracellular matrix proteins (10, 12, 31).

In addition, a total of 45 S. mutans clinical strains selected from our laboratory stock (15 strains each displaying the CBP /PA− , CBP+/PA− , and CBP+/PA+ phenotypes) as well as CBP-knockout mutant strains were tested (Table 2). All strains were confirmed to be S. mutans on the basis of the observation of a rough colony morphology on mitis-salivarius agar (Difco Laboratories, Detroit, MI, USA) plates containing bacitracin (0.2 U/ml; Sigma Chemical Co., St. Louis, MO, USA) and 15% (wt/vol) sucrose (MSB agar), as well as 16S rRNA sequence analysis with the primers 8UA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′), as described previously (64). For routine growth, all strains were cultured overnight in brain heart infusion broth (Difco Laboratories). For the culturing of knockout mutant strains with inactivated genes encoding CBPs (31), brain heart infusion broth (Difco Laboratories) was supplemented with erythromycin (10 μg/ml) as required.

Western blotting using antisera against CBP and PA was performed to confirm the expression of these proteins in all S. mutans strains, as described previously (9, 15). Sequences encoding CBP in all CBP-positive strains were analyzed, and all CBP-positive strains were confirmed to have no mutations (9, 10). In addition, transcription of the gene encoding CBP was confirmed by reverse transcription-PCR (RT-PCR) analysis, as described previously (9, 10), though the promoter region has not been identified. The sequences encoding PA in the S. mutans strains used in the present study were also determined as described previously (15). In addition, transcription of the gene encoding PA was confirmed by RT-PCR analysis, as described previously (15). Mutations and 20-bp deletions in the promoter region were identified in PA-negative strains, all of which were positive for CBP expression (12, 15).

Construction of a Cnm-complemented strain. A complemented strain of TW295 was generated, as described previously (65). First, the entire length of cnm was amplified using primers cnm-3F (5′-GAC AAA CTT TGG AAA AAT CAG GGC GGG-3′) and cnm-1730R (5′-ATG GGC TGC CAG TCC ACT TCC TTC ATC-3′), which contain the restriction sites for HindIII and BamHI, respectively (underlined). The amplified DNA fragment was then digested with BamHI and HindIII and cloned into plasmid pDL278 (66). Transformation into Cnm-knockout strain TW295CND was performed according to the protocol of Lindler and Macrina (67). Overnight cultures of TW295CND were inoculated into Todd-Hewitt medium supplemented with 10% heat-inactivated horse serum (Invitrogen, Carlsbad, CA, USA) and incubated for 2 h. Approximately 200 μg of the plasmid was added to growing liquid cultures, and the samples were incubated for 2 h at 37°C. The cells were then collected by centrifugation and plated on mitis-salivarius agar containing spectinomycin (1 mg/ml) and erythromycin (10 μg/ml) to select for the complemented mutant TW295comp strain, followed by anaerobic incubation at 37°C for 48 h. Confirmation of the presence of pDL278 in the cnm gene in the TW295comp strain was performed by PCR analysis, and confirmation of the collagen binding of that strain was performed by collagen-binding assays.

Preparation of serum and plasma. Human serum and plasma were prepared from the blood collected from volunteers according to the methods described previously (68). To prepare serum, whole blood from six healthy male volunteers was collected in sterile test tubes and stored at room temperature to allow clotting for 30 min. Clots were removed by centrifugation at 3,000 × g for 10 min, followed by collection of the supernatant (serum). For human plasma, whole blood from a healthy male volunteer was collected in a sterile test tube containing heparin, followed by centrifugation at 3,000 × g for 10 min, and the supernatant (plasma) was used immediately. The serum and plasma samples were used immediately for the analyses described below. Six bovine serum samples obtained from different animals were purchased from Gibco (Grand Island, NY, USA) and Nichirei Bioscience Inc. (Tokyo, Japan). If necessary, these human and bovine serum samples were heated at 56°C for 30 min to prepare complement-inactivated serum, which was kept at −20°C prior to use.

Aggregation assays in the presence of serum or plasma. Aggregation assays were carried out in the presence of serum and plasma by the previously described method for aggregation assays in the presence of fibrinogen, with some modifications (12), and the self-aggregation assays were performed as described previously, with some alterations (69). Cultures of S. mutans strains MT8148, NN2094, and TW295 at the stationary growth phase were collected by centrifugation at 3,000 rpm for 10 min. The cultures were washed and resuspended in phosphate-buffered saline (PBS) to reach an optical density at 600 nm (OD600) of approximately 0.6. For the self-aggregation assay, the bacterial suspension was incubated at 37°C for 1.5, 3, 4.5, 6, 7.5, and 9 h. The self-aggregation rates were calculated as follows: [(OD600 at the initial time point of the bacterial suspension − OD600 at the examined time point of the bacterial suspension)/OD600 at the initial time point of the bacterial suspension] × 100%. For the aggregation assays in the presence of serum or plasma, the bacterial suspensions, at an OD600 of 0.6, were added to serum or plasma at a final concentration of 10%, and the mixtures were incubated at 37°C for 1.5, 3, 4.5, 6, 7.5, and 9 h. The aggregation rates in the presence of serum or plasma were calculated as follows: [(OD600 at the examined point of the bacterial suspension without serum or plasma − OD600 at the examined time point of the bacterial suspension with serum or plasma)/OD600 at the initial time point of the bacterial suspension with serum or plasma] × 100%. These aggregation rates for the three tested strains plateaued at approximately 6 h; thus, the aggregation activities of the 45 S. mutans clinical strains were examined under a fixed incubation time of 6 h.

Analysis of aggregation in the presence of type IV collagen at a final concentration of 0.14 μg/ml, which is equal to the maximum concentration in the serum of healthy humans, instead of in the presence
of serum or plasma was performed. In addition, aggregation in the presence of 0.014, 0.14, 1.4, and 14 μg/ml of type IV collagen was examined.

The interactions between serum or whole blood and *S. mutans* strains MT8148, NN2094, and TW295 were observed with a light microscope following Giemsa staining as described previously, with some
modifications (28), in addition to by scanning electron microscopy (SEM), according to the method described previously (12). Briefly, bacterial samples reacted with serum for 6 h were fixed with 2% formaldehyde and 1% glutaraldehyde dehydrated with ethanol, then dried with t-butyl alcohol by the freeze-drying method. The dried samples were mounted on the stage and coated with osmium for conductive processing and then observed with an SEM (Hitachi S-4800; Hitachi High Technologies Corporation, Tokyo, Japan).

**Evaluation by use of an ex vivo adherence model with bovine heart specimens.** Hearts extirpated from healthy bovines were purchased from a butcher, and heart valves, such as the mitral valve, aortic valve, tricuspid valve, and pulmonary valve, were examined (see Fig. S1 in the supplemental material). Heart valve specimens were cut into sections of 5 mm by 5 mm and incubated in EBM-2 (Lonza, Walkersville, MD, USA) containing penicillin (50 μg/ml) and gentamicin (300 μg/ml) at 37°C for 3 h. Heart valve sections were washed three times with PBS to eliminate antibiotics before bacterial infection. Ten of these specimens were used to confirm sterilization following plating on blood agar. The 45 S. mutans strains were collected by centrifugation and adjusted to 1 × 10^8 CFU/ml with PBS. The S. mutans suspensions (1.0 ml) were then added to bovine serum at a final concentration of 10%, and the mixtures were incubated with four heart valve fragments per strain in 24-well tissue culture plates (Costar; Corning, NY, USA) at 37°C for 3 h. After incubation, pieces of the heart valves were washed three times by carefully moving the valve to other wells containing PBS to discard nonadherent bacterial cells, followed by sonication and vortexing to remove adherent S. mutans strains. The bacterial numbers were then serially diluted and added to MSB agar plates. After incubation at 37°C for 48 h, bacterial numbers were determined.

**Evaluation of virulence for establishment of IE in a rat model.** The virulence of the S. mutans strains was evaluated in the rat model of IE using a previously described method (12). Briefly, 51 male Sprague-Dawley rats (weight, 250 to 300 g each) were anesthetized with a mixture of xylazine and midazolam (0.1 ml/100 g of body weight). A sterile polyethylene catheter with a guide wire was surgically placed across the aortic valve of each animal via the right carotid artery, and the tip was positioned and placed at the aortic valve in the left ventricle. Bacterial suspensions of strain TW295 (1 × 10^8 CFU/body) in PBS were intravenously administered through the jugular vein of 18 rats. The hearts were then extirpated following euthanasia on days 1, 3, and 7 after bacterial infection (n = 6 for each day). In addition, 1 × 10^8 CFU of each strain MT8148 and NN2094 was administered via the jugular veins of the rats (n = 6 for each strain) with aortic valve impairment, and the hearts were extirpated following euthanasia on day 7 after bacterial infection. The extirpated hearts corresponding with the aortic valves were sectioned transversely, and Gram staining was performed. Differences in virulence were evaluated by comparing the levels of bacterial colonization in histopathological sections of the heart valves. In addition, hematoxylin-eosin staining of tissue sections was performed. Evaluation of pathological features was performed using these sections. Pathological features, including infiltration of inflammatory cells, hypertrophy of the endocardium and annulus, acceleration of fibrosis, as well as the presence of fibrin-like deposits and a bacterial mass, were determined using a previously described method (12).

Histopathological observations were scored as follows, as described previously (12): 0 for none, 1 for mild, 2 for moderate, and 3 for severe. All scoring evaluations were performed in a double-blind fashion by a pathologist (Applied Medical Research, Osaka, Japan). The remaining 21 rats were divided into three groups (TW295-, TW295CND-, and TW295comp-infected rats), and the same evaluations described above were conducted. Bacterial clearance was examined by measuring the numbers of bacteria in blood samples from the jugular vein, which were taken at 1 h, 3 h, 6 h, 24 h, and 7 days after the initial infection, as described previously (22). The blood samples were plated on MSB agar and incubated at 37°C for 48 h.

**Statistical analyses.** Statistical analyses were performed using the computational software package GraphPad Prism (version 6; GraphPad Software Inc., La Jolla, CA, USA).

Intergroup differences in each analysis were analyzed using analysis of variance (ANOVA) with the Bonferroni correction. Regression analysis was performed to compare the correlation of the ratio of aggregation in serum to that in plasma. A P value of less than 0.05 was considered statistically significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00401-17.

**SUPPLEMENTAL FILE 1,** PDF file, 1.5 MB.

**SUPPLEMENTAL FILE 2,** PDF file, 0.1 MB.

**SUPPLEMENTAL FILE 3,** PDF file, 2.2 MB.

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