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Original

Attitudes Towards HIV-Infected Patients, Knowledge Related to HIV/Universal Precautions, and Infection Control Practices of Japanese Dentists

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Abstract: The aim of this study was to explore factors influencing attitudes towards HIV-infected patients, knowledge related to human immunodeficiency virus (HIV)/universal precautions, and infection control practices (ICPs) of Japanese dentists, and to analyze the relationship between the attitude toward HIV-infected patients and knowledge related to HIV/universal precautions, and infection control practices in a population of Japanese dentists. Data on attitudes towards HIV-infected patients, knowledge related to HIV/universal precautions, and ICPs were obtained from 3,316 dentists in Aichi Prefecture, Japan, through questionnaires, which were then statistically analyzed. Overall, an age of 49 years or younger, specialist in oral surgery, and seeing 36 or more patients per day were positive significant predictors for attitudes towards HIV-infected patients, knowledge related to HIV/universal precautions, and ICPs. Those aged 49 years or younger were more likely to report desirable responses for all attitude items than older dentists. Those seeing 36 or more patients per day were more likely to report desirable ICPs in all but one item than those seeing 35 or less. In addition to being younger, specialty in oral surgery, seeing 36 or more patients per day, and having a higher knowledge index were significant positive associating factors for a higher infection control practice index. Dentists who are younger, have a specialty in oral surgery, or see more patients per day showed positive attitudes for dental care provision toward HIV-infected patients, greater knowledge related to HIV/universal precautions, and better compliance for ICPs. Knowledge related to HIV/universal precautions was positively related to ICPs.

Key words: Dentist, Infection control practice, Attitude, Knowledge, Infectious disease

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Introduction

The number of newly reported human immunodeficiency virus (HIV)-infected patients and newly reported AIDS patients has been increasing every year (HIV infection: 397 in 1997 to 1,021 in 2009; AIDS: 250 in 1997 to 431 in 2009), and, at present, there are more than 10,000 HIV-infected patients in Japan[1]. Furthermore, the number of hepatitis C virus (HCV) carriers is estimated to comprise about 2% of the entire population in Japan, the highest incidence among developed countries. Given these circumstances, dental care for patients with infectious diseases is of marked concern in Japan. Japanese dentists now have a higher risk of exposure to pathogens via blood or saliva while providing dental care than ever before. The provision of dental health care to persons infected with HIV has been deemed an ethical responsibility[2-5]. The necessity for dentists to be willing to provide dental care for patients with infectious disease and to perform infection control practices (ICPs) has been increasing.

In many countries, there have been reports documenting the status of dentists' attitudes, knowledge of HIV/AIDS patients, and ICPs[6-19]. Factors influencing attitudes and ICP compliance have been analyzed[20-27]. However, little information is available about the influence of demographic factors on dentists' attitude toward HIV-infected patients, knowledge related to HIV/

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universal precautions, and ICPs, or on the association among attitudes, knowledge about infectious diseases, and ICPs in Japan's dentists. To our knowledge, only one study has investigated the association between age and ICPs[28]. In recent years, a general clinical training system for dental school graduates has been introduced in Japan, and the Japan Dental Association has promoted training for practitioners performed by each local Dental Association. The necessity for introducing ICPs in these training programs is now proposed.

The present study has explored the factors influencing attitudes towards HIV-infected patients, knowledge related to HIV/universal precautions, and ICPs in a population of Japanese dentists. Furthermore, we analyzed the relationship between attitude, knowledge, and ICPs.

Study Population and Method

1. Study population and procedure for data collection

This study was conducted involving dentists from Aichi Prefecture between August and October 2008. Since the cumulative HIV infection rate in Aichi Prefecture was 6.05/100,000 of the population, which is similar to that of Japan (6.47/100,000 of the population), we considered Aichi Prefecture as representative of Japan in terms of HIV infection. A self-administered questionnaire was sent to all 3,316 directors of dental offices listed as members of the Aichi Prefecture Dental Association. The self-administered questionnaire was accompanied by a letter of endorsement signed by the President of the Aichi Prefecture Dental Association and a letter of introduction signed by the research team emphasizing the importance of this study and ensuring the anonymity of responders. One thousand nine hundred and twenty-five (58.1%) were returned.

2. Sociodemographic factors

Sociodemographic factors included gender, age, specialty in oral surgery, and the number of patients treated per day. The distribution of subjects by sociodemographic factors is shown in Table 1. Under Japan's dental health service system, dentists are able to establish private dental offices offering services in the specialties of dentistry, oral surgery, pedodontics, or orthodontics. In terms of specialty in oral surgery, we classified dentists into two groups, 'specialists' and 'general dentists'. 'Specialists' were dentists who have established private dental offices which include oral surgery as a specialty and offer relatively difficult oral surgery treatment as well as restorative and prosthodontic treatments. 'General' dentists were those who have established private dental offices that do not include oral surgery as a specialty and mainly perform restorative and prosthodontic treatment, as well as basic oral surgery such as tooth extraction. The number of patients treated per day was counted for each private dental office.

Table 1 The distribution of subjects by sociodemographic factors

Variable	Number (percentage)
Gender	
Male	1,809 (95.1)
Female	94 (4.9)
Age	
-39	176 (9.4)
40-49	516 (27.5)
50-59	776 (41.3)
60-	410 (21.8)
Specialty of oral surgery	
Specialist	206 (11.0)
General	1,665 (89.0)
Number of patients a day	
≦15	292 (16.9)
16-25	548 (31.6)
26-35	467 (27.0)
36-45	251 (14.5)
46<	174 (10.0)

Missing data were excluded.

3. Variables measured

Dentists were asked to complete the questionnaire. Items of the questionnaire covered attitude (4 items), knowledge (3 items), and ICPs (9 items). Each item was dichotomously categorized as follows: attitude items (agree, disagree), knowledge items (yes, no), and ICP items (yes, no).

Summated rating scales were constructed for indices of attitude, knowledge, and ICPs. Positive behaviors were assigned a score of 1 and negative behaviors were assigned a score of 0. Correct knowledge was assigned a score of 1, and lack of knowledge was scored 0. If infection control practices were employed, the score

Table 2 The score of responses to questions concerning attitudes, knowledge, and infection control practice

Variable	Category	Number (percentage)
Attitude		
Willing to treat HIV patients in my practice	Agree	414 (22.1)
Willing to treat HIV patients in another practice	Agree	577 (31.8)
Refusal of HIV patients is ethically wrong	Agree	1,173 (63.3)
If I treat patients with HIV or AIDS, my other patients may be reluctant to continue in my care	Agree	1,258 (67.8)
Knowledge		
I know the meaning of Universal Precautions	Yes	258 (15.9)
Do you think HIV is transmitted via saliva contaminated with blood	Yes	1,569 (84.4)
I know the meaning of HAART	Yes	75 (4.0)
Infection control practice		
When treating patients, do you:		
Always wear protective eyewear	Yes	636 (33.6)
Always wear mask for treatment	Yes	1,689 (97.3)
Always wear gloves for treatment	Yes	1,248 (72.0)
Exchange handpiece for each patient	Yes	375 (21.7)
Provide education for preventing infection of clinical staff	Yes	1,448 (83.8)
Prepare office infection control manual	Yes	959 (55.9)
Usually participate in clinical lectures for infection control	Yes	1,353 (79.5)
HBV immunization (dentist)	Yes	1,130 (65.5)
HBV immunization (clinical staff)	Yes	343 (20.0)

Not all respondents completed every item.

was 1, and if they were not used, the score was 0. Thus, the sum score could range from 4 to 0 for attitude, 3 to 0 for knowledge, and from 9 to 0 for ICP. Each index was categorized into 'high' and 'low' as follows:

Attitude index 'high': 3 or more
'low' : 2 or less
Knowledge index 'high': 2 or more
'low' : 1 or less
ICP index 'high' : 5 or more
'low' : 4 or less

4. Ethics

Ethical approval for the study was provided by the Aichi Prefecture Dental Association. A consent form was used to obtain informed consent and clarify that no direct benefit could be expected from participating in the study. All data collected were confidential and anonymous.

5. Data analysis

To analyze the associations of sociodemographic factors with attitudes, knowledge, and ICPs, logistic regression models were used. The dependent variables consisted of each item of attitude, knowledge, and ICPs. Sociodemographic factors were used as independent variables for the analysis. Furthermore, we analyzed models by introducing the Attitude index (high/low) and Knowledge index (high/low), in addition to sociodemographic factors with dependent variables, the ICP index (high/low). Statistical analyses were carried out using SPSS. ver. 12. Differences at the 0.05 level were considered significant.

Results

1. Attitudes, knowledge, and ICPs

Table 2 shows the distribution of all subjects by each item of attitude, knowledge, and ICPs. With regard to attitude, only 22.1 and 31.8% of respondents reported a willingness to treat HIV-infected patients in their practice and in another clinics, respectively, although 63.3% reported they should not refuse to treat HIV-infected patients. With regard to knowledge, only 15.9 and 4.0% knew the meaning of universal precautions and highly active antiretroviral therapy (HAART), respectively,

Table 3 Multiple regression of gender, age, specialty, and number of patients per day with attitude and knowledge toward HIV-infected patients and infection control practice

	Gender	Age	Specialty	Number of patients a day
Attitude				
Willing to treat HIV patients in my practice		2.7 (2.1–3.4) <0.001	1.7 (1.3–2.5) 0.001	
Willing to treat HIV patients in another practice		2.2 (1.7–2.7) <0.001	1.9 (1.4–2.7) <0.001	1.4 (1.1–1.7) 0.009
Refusal of HIV patients is ethically wrong		1.4 (1.1–1.7) 0.005		1.3 (1.1–1.6) 0.014
If I treat patients with HIV or AIDS, my other patients may be reluctant to continue in my care	0.6 (0.3–0.8) 0.034	0.6 (0.5–0.8) <0.001		
Knowledge				
I know the meaning of Universal Precautions		1.8 (1.3–2.4) <0.001	2.2 (1.5–3.2) <0.001	1.5 (1.1–2.0) 0.011
I think HIV is transmitted via saliva contaminated with blood				
I know the meaning of HAART		2.2 (1.3–3.7) 0.003	3.8 (2.2–6.7) <0.001	
Infection control practice				
Always wear protective eyewear for treatment		1.3 (1.0–1.6) 0.034	1.5 (1.1–2.0) 0.021	1.4 (1.2–1.8) 0.001
Always wear mask for treatment	4.0 (1.6–10.1) 0.003	3.0 (1.3–7.0) 0.010		
Always wear gloves for treatment	0.5 (0.2–0.9) 0.011	3.3 (2.5–4.3) <0.001		2.1 (1.7–2.7) <0.001
Exchange handpiece for each patient		1.6 (1.2–2.0) <0.001	1.5 (1.0–2.1) 0.026	1.7 (1.3–2.2) <0.001
Education for preventing infection of clinical staff	0.3 (0.1–0.7) 0.010		2.0 (1.1–3.4) 0.017	2.4 (1.8–3.2) <0.001
Preparing office infection control manual				1.8 (1.4–2.2) <0.001
Participation in clinical lecture for infection control		0.7 (0.5–0.9) 0.001		1.5 (1.4–2.1) 0.001
HBV immunization (dentist)		2.7 (2.1–3.4) <0.001	1.5 (1.4–2.1) 0.019	1.6 (1.3–1.9) <0.001
HBV immunization (clinical staff)			1.5 (1.0–2.1) 0.035	1.6 (1.3–2.1) 0.010

Not all respondents completed every item.
Upper: Odds ratios (95% confident intervals), bottom: p-value

compared to 84.4% who knew that HIV is transmitted via saliva contaminated with blood. Regarding ICPs, 97.3% reported wearing masks during dental treatment and 83.8% responded that they provide education about infection prevention for clinical staff. The least commonly reported ICP was HBV immunization for dental staff (20.0%) compared to HBV immunization for dentists (65.5 %).

2. Association of sociodemographic factors with attitude, knowledge, and ICPs using logistic regression analysis

Table 3 shows the associations of sociodemographic factors with attitude, knowledge, and ICPs using logistic regression analysis. Each item of attitude, knowledge, and ICPs was introduced into the regression models.

1) Attitude

Being aged 49 or younger and specialty in oral surgery were significant predictors of being willing to treat HIV-infected patients in their practice (age: OR=2.7, specialty: OR=1.7) and other clinics (age: OR=2.2, specialty: OR=1.9). The number of patients seen per day was also a significant predictor of being willing to treat HIV-infected patients in other clinics (OR=1.4). Age and the number of patients seen per day were significantly associated with the belief that refusing dental treatment for HIV patients is ethically wrong (age: OR=1.4, number of patients: OR=1.3). Men and the younger age group were less likely to fear that other patients may be reluctant to continue in their care when they treat HIV-infected patients (OR=0.6).

2) Knowledge

Age and specialty in oral surgery were significant predictors of being familiar with universal precautions (age: OR=1.8, specialty: OR=2.2) and HAART (age: OR=2.2, specialty: OR=3.8). The number of patients seen per day was significantly associated with being familiar with universal precautions (OR=1.5).

3) Infection control practice

All explanatory variables except gender were associated with wearing protective eyewear, changing handpieces after each treatment, and receiving HBV immunization (dentists). Being female, being younger, and seeing a greater number of patients were associated with being more likely to wear gloves (gender: OR=0.5, age: OR=3.3, number of patients: OR=2.1). Education for clinical staff on preventing infection was associated with all explanatory variables except age. Clinical staff vaccinated against HBV showed an association with specialty in oral surgery (OR=1.5) and number of patients a day (OR=1.6). Being male and younger were associated with an increased likelihood of wearing a mask (gender: OR=4.0, age: OR=3.0). Being older and seeing a greater number of patients were associated with being more likely to receive educational lectures (age: OR=0.7, number of patients: OR=1.5). Preparation of an office infection control manual was associated only with the number of patients treated a day (OR=1.8).

Table 4 Multiple regression of gender, age, specialty, number of patients per day, attitude index, and knowledge index with infection control practice index

Variables	OR	95%CI	p-value
Gender	1.1	0.6-2.0	0.806
Age	1.5	1.2-2.0	0.002
Specialty	1.5	1.0-2.1	0.041
Number of patients	1.6	1.2-2.1	<0.001
Attitude	1.3	0.9-1.7	0.135
Knowledge	2.2	1.6-3.1	<0.001

Not all respondents completed every item.

3. Association of infection control practice with attitude and knowledge

We explored factors associated with a high ICP index using logistic regression analysis with explanatory factors consisting of the Attitude index, Knowledge index, and four sociodemographic variables (gender, age, specialty, and number of patients) (Table 4). Having a high Knowledge index, being younger, specialty in oral surgery, and seeing more patients were significantly positively associated with a high ICP index score.

Discussion

Relationships between sociodemographic factors and attitude, knowledge, and ICPs were analyzed using multiple logistic regression analyses. Female dentists were more likely to think that patients would be reluctant to continue in their care if they treated HIV-infected patients. They were also more likely to wear gloves and provide their clinical staff with education about preventing infection than men, and were less likely than men to wear masks. In McCarthy's report[23], women were more likely to wear gloves, which is consistent with our findings; however, in contrast with our results, they found that women were more likely to wear masks and less likely to fear that other patients may be reluctant to continue in their care if they treated HIV-patients.

Younger dentists had a significantly or relatively higher proportion of desirable responses than older dentists in most items on attitudes and ICPs. Increased numbers of HIV-infected patients in Japan and the advent of new infectious diseases have led to a stronger emphasis on the prevention of infection in undergraduate education. Younger dentists who have been

better educated about the prevention of infection during their studies are considered to have more knowledge about ICPs and better attitudes towards the prevention of infectious diseases than older dentists. Similar results have been reported regarding the routine use of gloves and HBV immunization of dentists[22,27]. Several studies have reported that younger dentists were more willing to provide dental care for patients with HIV infection[22,27,29]. Our results are consistent with these studies.

Overall, specialists in oral surgery exhibited a higher proportion of desirable attitudes, correct knowledge, and compliance with desirable ICPs than general dentists, confirming the findings of another report[27]. Most specialists in oral surgery in Japan have undergone post-graduate training at a university or hospital covering the prevention of infection during surgical treatment and treatment of patients with infectious diseases. On the other hand, dentists who did not major in oral surgery had fewer opportunities to learn about infectious diseases and ICPs. Lueveswanij *et al.* reported that educational intervention could change knowledge, attitudes, and ICPs of oral health personnel with regard to AIDS[30]. These findings suggest that specific education and training about infection control could change attitudes and infection control practices of dental health workers.

Dentists treating a smaller number of patients a day showed less compliance in wearing gloves during treatment, providing education and HBV immunization for clinical staff, and preparing a manual than their counterparts. Some studies reported that dentists who see more patients per day showed favorable compliance with ICPs[7,17]. Our results were consistent with these reports. Dentists treating a smaller number of patients per day may see using gloves during treatment as a financial burden, since a small patient base is linked with a small income. Other studies have reported that some dentists see ICPs as a financial burden[22,23]. However, providing education for staff and preparing infection control manuals are not expensive undertakings. This group showed less willingness to treat HIV-infected patients, suggesting that they feel they cannot afford to invest their time in infectious disease prevention. On the other hand, dentists with many patients may treat a large number with blood-borne pathogens, so they are more likely to be prepared by adopting infection control measures in their practice. These results emphasize the importance of educating dentists who see a small number of patients about employing measures to prevent the spread of infectious diseases.

It has been argued that knowledge and attitudes may influence adults' health behavior more or less over a long period of time[31-34]. Since we hypothesized that favorable attitudes and knowledge would lead to favorable ICPs, we analyzed the association of attitudes and knowledge with ICPs. The knowledge index had a significant association with the ICP index. Among the knowledge items, knowledge for 'universal precautions' and 'HAART' showed a significant relationship with the ICP index (universal precautions: $p<0.001$, HAART: $p<0.001$), which contributed to the association of the Knowledge index with the ICP index. This is of particular importance, given that an understanding of the concept of universal precautions may provide the motivation for dentists to undertake ICPs in their practice. To our knowledge, this is the first report describing the association of knowledge with ICPs in dentists, and further investigation is needed to analyze this association in more detail. The findings of this study revealed a lower compliance with some ICPs (protective eyewear, gloves, office infection control manuals, changing of handpieces, HBV vaccines) compared with data from other countries[7,9,10,13,15,16,22,27,29]. Lower standards of ICPs suggest that dentists need to be re-educated regarding infectious diseases and their management. In particular, groups showing poorer compliance with ICPs, i.e., older or 'general' dentists, should be a target population for such education. Moreover, it is important to motivate dentists seeing a smaller number of patients to develop more of an interest in infection control practices. The significant correlation between knowledge and ICPs suggested that increasing knowledge about infectious diseases and infection control may be of help to improve ICPs. Several studies have reported that educational intervention improved compliance with infection control practices[21] and attitudes towards HIV/AIDS of dentists[24,35,36], which supports the significance of education. One report suggested that using a questionnaire to identify deficiencies in ICPs was advantageous for

designing an education program[30]. The findings of the present study may be useful for the development of an educational program for dentists and under-graduate dental students.

Dentists with a greater awareness of providing dental health care to patients with HIV infection tended to also carry out ICP measures, but no significant association was found between them. This suggests that having such an awareness does not necessarily compel dentists to undertake ICPs. Some reports have documented that an increase in self-efficacy is necessary for dentists to provide dental treatment for patients with AIDS or HIV infection[12,37,38]. In general, different factors have been found to be associated with the health-related behavior of adults. Psychological processes related to the treatment of HIV-infected patients and of infection control practices by dentists are considered to be responsible for complex psychosocial mechanisms, and the elucidation of these mechanisms requires much more investigation. The increasing number of patients with infectious diseases highlights the need for dentists to undergo training concerning ICPs, and effective training requires more information on behavioral science concerning dentists' use of ICPs.

On the other hand, compared with a similar survey done in the same population in 2005 (unpublished data), the present study showed a marked increase in the proportion of dentists reporting the preparation of an office infection control manual (55.7 vs. 28.7, respectivery) and provision of education to clinical staff for preventing infection (83.8 vs 39.6, respectively). 'The Revision of the Medical Care Act in 2007' required the reinforcement of systems preventing nosocomial infections in medical institutions. This covers requirements for the preparation of an office infection control manual and the provision of education for clinical staff to prevent infection, and is considered to have contributed to the improvement of these two ICPs. We suggest that the government's public health policy should include requirements for improving dentists' ICPs.

Several reports have described how dentists' compliance with ICPs in other countries has improved over time[6,9,36]. Furthermore, a cohort study of Japanese dentists' attitude toward HIV-infected patients and compliance with infection control should be done. We aim to undertake a similar cohort study monitoring Japanese dentists' attitudes toward HIV-infected patients and compliance with infection control measures in the near future.

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日本人歯科医師のHIV感染症患者に対する態度，HIV/ユニバーサルプレコーションに関する知識，院内感染防止対策実施

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概要：本研究の目的は日本の歯科医師における「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」に関与する因子を探索し，さらに，「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」の相互の関連を解析することである．愛知県に開業している歯科医師3,316人に対し「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」についてのアンケート調査を行い，回答のあった人に対し統計的な解析を行った．全体として，「49歳以下」，「口腔外科専攻」，「1日36人以上の患者を診療する」はHIV感染患者への良好な態度，感染に関する高い知識，院内感染防止実施の有意な予測因子であった．49歳以下の歯科医師は50歳以上の歯科医師よりも「HIV感染患者への態度」におけるすべての項目で望ましい行動をとると回答した．1日36人以上の患者を診療する歯科医師は34人以下の歯科医師に比べ，ほとんどの院内感染防止策実施項目で実施状況が良好であった．「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」それぞれにつき，各項目の回答結果をもとにインデックス化し「院内感染防止策実施」に有意な関連をもつ因子を調べたところ「感染に関する知識」，年齢，口腔外科専攻，患者数が該当した．「49歳以下」，「口腔外科専攻」，「1日36人以上の患者を診療する」はHIV感染患者への良好な態度，感染に関する知識，院内感染防止実施の予測因子であった．「感染に関する知識」は「院内感染防止策実施」と有意な関連を示した．

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Competence-Dependent Endogenous DNA Rearrangement and Uptake of Extracellular DNA Give a Natural Variant of *Streptococcus mutans* without Biofilm Formation[∇]

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The production of water-insoluble glucan (WIG) enables *Streptococcus mutans* to survive and persist in the oral niche. WIG is produced from sucrose by glucosyltransferase encoded tandemly by the highly homologous *gtfB* and *gtfC* genes. Conversely, a single hybrid gene from the endogenous recombination of *gtfB* and *gtfC* is easily generated using RecA, resulting in *S. mutans* UA159 WIG$^-$ (rate of $\sim$1.0 $\times$ 10^{-3}). The pneumococcus *recA* gene is regulated as a late competence gene. *comX* gene mutations did not lead to the appearance of WIG$^-$ cells. The biofilm collected from the flow cell had more WIG$^-$ cells than among the planktonic cells. Among the planktonic cells, WIG$^-$ cells appeared after 16 h and increased $\sim$10-fold after 32 h of cultivation, suggesting an increase in planktonic WIG$^-$ cells after longer culture. The strain may be derived from the biofilm environment. In coculture with donor WIG$^+$ and recipient WIG$^-$ cells, the recipient cells reverted to WIG$^+$ and acquired an intact *gtfBC* region from the environment, indicating that the uptake of extracellular DNA resulted in the phenotypic change. Here we demonstrate that endogenous DNA rearrangement and uptake of extracellular DNA generate WIG$^-$ cells and that both are induced by the same signal transducer, the *com* system. Our findings may help in understanding how *S. mutans* can adapt to the oral environment and may explain the evolution of *S. mutans*.

Biofilms are defined as matrix-enclosed bacterial populations that are adherent to each other and/or to surfaces or interfaces (8). Biofilms are studied due to their significance in medical, industrial, and environmental settings. Biofilm formation consists of several phases: (i) initial adherence of planktonic cells to a surface, (ii) irreversible attachment to the surface, (iii) unstructured biofilm growth through cell division and exopolysaccharide synthesis, and (iv) maturation into a complex three-dimensional architecture of microcolonies and void spaces (13, 37). As biofilm accumulation progresses, the cells within biofilms can disperse in response to environmental changes. Biofilms are resistant to environmental stresses (e.g., nutritional, oxidative, and antibiotic stress) and to host-mediated responses (e.g., complement proteins and phagocytes) (11, 16, 33). Therefore, understanding the molecular mechanisms underlying the life cycle of biofilm-forming bacteria may assist in the development of prevention and treatment strategies.

Streptococcus mutans is an oral bacterium that depends on biofilms for survival and persistence in its natural ecosystem. Under favorable environmental conditions, *S. mutans* can rapidly produce acid from fermentable dietary carbohydrates and initiate demineralization of the tooth surface. Therefore, *S. mutans* is an important etiological agent for dental caries. *S. mutans* is capable of forming biofilms using various mechanisms, e.g., surface adhesion- and cell density-dependent gene expression (9, 18). Clinically relevant in caries development is the ability of *S. mutans* to metabolize sucrose. Sucrose is the substrate for glucosyltransferase-mediated sucrose-dependent glucan production that promotes adhesion of *S. mutans* to the tooth surface. Water-insoluble glucan (WIG) is synthesized using GtfB- and GtfC-glucosyltransferases and promotes adhesion and biofilm maturation. Conversely, reports describe the spontaneous occurrence of a naturally derived WIG$^-$ strain (1, 40). This deletion is a recombination of *gtfB* and *gtfC* (which are proximate and have a high homology) (40), resulting in the generation of the single hybrid *gtfBC* gene. This mutation decreases the synthesis of WIG and reduces its biofilm-forming ability (40). Conversely, the growth rate of the WIG$^-$ strain in media supplemented with sucrose is greater than that of the wild type (31). Additionally, the appearance of an *S. mutans* WIG$^-$ strain exerts pleiotropic effects, e.g., reduced WIG in a gnotobiotic rat model using the *gtfBC* recombinant strain showed lower cariogenicity, inactivating the glucan-binding protein and thus changing the plaque structure (15). Further, Nomura et al. (32) reported the detection of *S. mutans* in human heart valve tissues with a reduced biofilm due to *gtfB-gtfC* recombination and with a lower susceptibility to antibiotics. With this adaptive ability, the WIG$^-$ variant may play a role in survival and development of the microorganism in its environmental niche; however, its appearance and ecological significance are not understood.

Previous studies have shown that recombination of *gtfB* and *gtfC* causes the formation of the WIG$^-$ strain and is RecA dependent (3). Previous studies with *Streptococcus pneumoniae*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
E. coli DH5α	Cloning host	Takara
S. mutans		
FSM-11 WIG$^+$	Clinical isolate, wild type; Amp^s Erm^s Spc^s Km^s, serotype e	29
WIG$^+$ *recA* mutant	FSM-11 WIG$^+$::pSAR; *recA* Erm^r	This study
WIG$^+$ *comC* mutant	FSM-11 WIG$^+$::pSAC; *comC* Erm^r	This study
WIG$^+$ *comD* mutant	FSM-11 WIG$^+$::pSAD; *comD* Erm^r	This study
WIG$^+$ *comE* mutant	FSM-11 WIG$^+$::pSAE; *comE* Erm^r	This study
WIG$^+$ *comX* mutant	FSM-11 WIG$^+$::pSAX; *comX* Erm^r	This study
WIG$^+$ *recA* donor strain	FSM-11 WIG$^+$::pSAR, pSABC; *recA gtfBC* Erm^r Spc^s Kan^r	This study
FSM-11 WIG$^-$	Naturally derived variant of FSM-11 WIG$^+$; Amp^s Erm^s Spc^s Kan^s; serotype e	This study
WIG$^-$ recipient strain	FSM-11 WIG$^-$::pSAL; *ldh* Erm^s Kan^s Spc^r	This study
Plasmids		
pGEM-T	PCR cloning vector; Amp^r	Promega
pUC19	PCR cloning vector; Amp^r	43
pDL276	*E. coli-Streptococcus* shuttle vector; Kan^r	10
pResEmMCS10	Streptococcal integration plasmid; Erm^r	38
pFW5	Streptococcal integration plasmid; Spc^r	35
pSAR	pUC19 containing *recA* fragment; Amp^r Erm^r	This study
pSAC	pUC19 containing *comC* fragment; Amp^r Erm^r	This study
pSAD	pUC19 containing *comC* fragment; Amp^r Erm^r	This study
pSAE	pUC19 containing comE fragment; Amp^r Erm^r	This study
pSAX	pUC19 containing *comX* fragment; Amp^r Erm^r	This study
pSABC	pUC19 containing gtfBC fragment; Amp^r Kan^r	This study
pSAL	pUC19 containing *ldh* fragment; Amp^r Spc^r	This study

found the that *recA* gene was induced at competence (23, 24), and competence-specific induction of *recA* was also demonstrated in *Bacillus subtilis* (22). The genetics and physiology of the competence cascade in *S. mutans* have been reported (9, 24). In brief, the development of competence requires transcriptional activation of the *com* regulon, which is induced when the competence-stimulating peptide (CSP) (encoded by *comC*) stimulates its receptor, the membrane-bound histidine kinase ComD. ComD then autophosphorylates its cognate response regulator ComE and then activates the expression of early *com* genes, including *comCDE* and *comX*. The latter encodes an alternative sigma factor (19). The ComDE signal transduction system is among the primary sensory-regulatory mechanisms that mediate bacterial adaptation processes in response to environmental perturbation (24). The environmental conditions encountered by *S. mutans* in dental biofilms are highly variable, including frequent shifts in pH from above 7.0 to as low as 3.0 during the ingestion of dietary carbohydrates. In addition, *S. mutans* is subject to various environmental stresses, such as temperature fluctuation, nutritional limitation, antibiotic agents, and variation in oxygen tension (6). Therefore, the appearance of the WIG$^-$ strain may result from environmental perturbation. In this study, we investigated the mechanisms underlying expansion of the WIG$^-$ strain in a clonal population and the ecological significance of this variant.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. Brain heart infusion (BHI) (Becton Dickinson) was used, or pH-buffered BHI (prepared with phosphate-buffered saline [pH 7.2]) was used for counting WIG$^-$ cells and studying natural competency (see below). The clinical isolate *S. mutans* FSM-11 (29) was cultured at 37°C with 5% CO_2. *Escherichia coli* DH5α was used for cloning and plasmid amplification and was grown in Luria-Bertani broth or 1.5% agar at 37°C. When required, sucrose (0.25% [wt/vol]) or horse serum (10% [vol/vol]) was added to the medium. Bactericidal agents tested for the appearance of WIG$^-$ cells and natural competency were used under different conditions at a 10% to 20% reduction in optical density (OD) after 20 h of culture.

Frequency of WIG$^-$ cells. *S. mutans* was precultivated to the stationary phase. To eliminate extracellular DNA in the bacterial preculture, the culture was washed twice in distilled water (D/W) using centrifugation. An aliquot of the precultured solution with a reduced cell number (~100 to 500 cells) was inoculated in pH-buffered BHI medium with or without bactericidal agents. These cultures were incubated with 5% CO_2 at 37°C statically for 20 h. The cultures were suspended in D/W, serially diluted, and spread-plated on BHI agar to 300 colonies/plate. The morphology of the samples were determined after 48 h on plates with 0.25% sucrose, and the ratio of smooth colonies to total viable cells was determined. Over 1,000 colonies were selected from at least three independent experiments.

Biofilm formation. (i) Biofilm formation in flow cells. To show the appearance of the WIG$^-$ variant, *S. mutans* was grown in continuous-culture flow cells as described previously with modifications (30). Flow cells were inoculated with reduced numbers of precultured *S. mutans* (~100 to 500). After 1 h of incubation at 37°C without flow, pH-buffered BHI with 0.25% sucrose was added at a rate of 3.0 ml/h using a peristaltic pump. The detection cell at outflow was a silicon tube 10 cm in length and 1 mm in inner diameter. After 16 h and 32 h of cultivation, the viable cells in the biofilm and the effluent were collected and plated on BHI agar with 0.25% sucrose. Over 1,000 colonies were selected from at least three independent experiments.

(ii) Microtiter plate biofilm formation. Quantification of biofilm formation was performed using cells grown in the wells of polystyrene microtiter plates (Sumitomo Bakelite, Tokyo, Japan) (29). Strains were grown to an OD at 600 nm (OD_{600}) of 0.4, diluted 1:100 in BHI with 0.25% sucrose, and added to the wells of the plates. The plates were incubated at 37°C with 5% CO_2 for 20 h. After incubation, the plates were washed two times with D/W to remove planktonic and loosely bound bacteria, and adherent cells were stained with safranin for 10 min. Biofilm formation was then quantified by measuring the OD of the solubilized stain at 492 nm.

DNA extraction. DNA from bacterial cells was extracted using the benzyl chloride method (47). The isolated DNA was used as the template for the PCR experiments described below.

Construction of mutants. Insertion-inactivation of the gene in *S. mutans* FSM-11 was performed with insertion of erythromycin (Erm), kanamycin (Kan), and/or spectinomycin (Spc) resistance genes using homologous recombination. Sequence information was obtained from the *S. mutans* UA159 genome (http:

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TABLE 2. Amplicons and oligonucleotide primers used in this study

Primer	Sequence (5′→3′)[a]	Amplicon
Long-range PCR		
GtfB186F	TTGGAGGTTCCTAATGGAC	*gtfBC*
GtfC9269R	GAAATTTACAGCTCAGACTTC	
Mutants		
RecA2F	GAGTTGCAGGTCCGGATAGC	*recA*
RecA2R	GCAATGGAGAGCCTTAGCATAGCGG	
ComE1F	CCTGAAAAGGGCAATCACCAG	*comE*
ComE1R	GCGATGGCACTGAAAAAGTCTC	
ComCuF	cccccgaattcAAATCTGAACAAGCAGGGG	*comC1*
ComCuR	cccccggtaccGATAGTGTTTTTTTCATTTTATATCTCC	
ComCdF	ccccctctagaGCCTATCAACATTTTTCCGGC	*comC2*
ComCdR	cccccaagcttCCACTAAAGGCTCCAATCGC	
ComDuF	cccccgaattcCCATTCATCTGAAACTCAGT	*comD1*
ComDuR	cccccggtaccAACAGGCAGCAGACCATAA	
ComDdF	ccccctctagaGGCGGGCAATCATATTCTT	*comD2*
ComDdR	cccccaagcttTCCTGCAATTGATGTCCTG	
ComX2uF	cccccgaattcATTGGGCGTGTTCGACATAC	*comX1*
ComX2uR	cccccggtaccTGACTTTCTTGCTGACGC	
ComX2dF	ccccctctagaTTGGTAGCAGGAGAGCAC	*comX2*
ComX2dR	cccccaagcttGAGATATGGTATCTCCTC	
LdhuF	cccccgaattcATCTGGAAGAGCCCGAGCAAC	*ldh1*
LdhuR	cccccggtaccGGTGAAGTAAAAGCAAGTGC	
LdhdF	ccccctctagaGGTATCTTCCTCGTTGCTGC	*ldh2*
LdhdR	cccccaagcttGGAATATTTACTGGGCGAAC	
GtfuF	cccccgaattcTGAGTGGTGTATGGCGTCAC	*gtfB*
GtfuR	cccccggtaccGACCGTTAATGGTTCTGGC	
GtfdF	ccccctctagaAAACTCTGACTGCTACTGATAC	*gtfC*
GtfdR	cccccaagcttGAGCAAAGCTGTTAGTGTTATCA	

[a] Sequences homologous to the *S. mutans* genome sequences are in uppercase; synthetic restriction sites added for cloning purposes are in lowercase.

//www.ncbi.nlm.nih.gov/genomes). The method for homologous recombination was described previously (29). The *recA* and *comE* gene fragments from chromosomal DNA of *S. mutans* FSM-11 were ligated into the pGEM-T Easy vector (Promega, Madison, WI). The constructed pSAR and pSAE, harboring the *recA* and *comE* genes, respectively, were disrupted using digestion with BamHI and ligation with the Erm fragment from pResEmMCS10 (38). To construct *comC*, *comD*, and *comX* mutant strains and to insert an antibiotic resistance marker in the *ldh* and *gtf* genes, a PCR fragment from the upstream region of each gene from the chromosomal DNA of FSM-11 was amplified using PCR. The amplified fragment was inserted into the respective sites of cloning vector pUC19 (43). A PCR fragment from the downstream region was ligated to pUC19 from the upstream region. The resultant plasmid was digested with BamHI, and either an Erm fragment from pResEmMCS10 (38), a Kan fragment from pDL276 (10), or a Spc fragment from pFW5 (35) was inserted. Each strain was transformed with the resultant plasmids pSAC, pSAD, and pSAX for disruption and with *comC*, *comD*, and *comX* for insertion of the antibiotic resistance marker in the *ldh* gene. Further, each strain was transformed with the resultant plasmids pSEL and pSEBC to insert the antibiotic resistance marker in the *gtf* gene. Erm-resistant strains, Kan-resistant strains, and Spc-resistant strains were selected on agar containing 10 μg/ml Erm, 800 μg/ml Kan, and 800 μg/ml Spc, respectively. The mutations were confirmed using PCR.

Long-range PCR. Amplification of the *gtfB* and *gtfC* region was achieved with the primers GtfB186F and GtfC9269R (Table 2) with LA polymerase (Takara). Amplification conditions were 30 cycles of 30 s at 95°C, 1 min at 56°C, and 9 min at 70°C. PCR products were visualized after gel electrophoresis in 0.8% agarose using ethidium bromide staining.

Transformation assay. After being washed in D/W, *S. mutans* cultures were diluted 1:20 in BHI with horse serum. To determine the transformation frequency under stress conditions of a 10% to 20% OD reduction, *S. mutans* was grown to an OD_{600} of 0.5 without horse serum. Aliquots (0.2 ml) of culture were incubated at 37°C in equal volumes of fresh medium with 100 μg purified *ldh* (~2 kb) containing the Spc^r gene. After 2 h of incubation, transformants and total cells were plated on BHI agar with and without spectinomycin, respectively. The transformation efficiency was determined after 48 h of incubation and was expressed as the percentage of transformants compared to the total number of viable recipient cells.

Coculture experiments. WIG^+ (Kan^r and Erm^r) and WIG^- (Spc^r) strains were grown separately overnight in BHI. The cultures were centrifuged and washed two times to eliminate released extracellular DNA. The cells were resuspended in fresh BHI to an OD_{600} of 0.1. Both cultures were allowed to grow to an OD_{600} of 0.3 and mixed at a 1:4 ratio of donor to recipient strains. An aliquot (500 μl) of the mixed sample was centrifuged and the supernatant discarded. The sample was resuspended and incubated at 37°C for 8 h in 1 ml of fresh BHI medium with 10% horse serum. The culture was plated on BHI with 0.25% sucrose containing various antibiotics. Transformation efficiency was determined after 48 h of incubation and was expressed as the percentage of Spc- and Kan-resistant cells.

Bacteriocin production assay. To detect bacteriocin produced by *S. mutans* FSM-11 WIG^+ and the WIG^- variant, 5 μl of an overnight culture of each strain adjusted to an OD_{600} of 0.4 was inoculated on BHI plates. After overnight culture, 5 μl each of the indicator *Streptococcus* species, *S. gordonii*, *S. mitis*, and *S. sanguinis*, at the same concentration was inoculated near the early colonies and further incubated anaerobically at 37°C. Bacteriocin production was determined by measuring the diameter of the zone inhibited compared to the indicator strains.

TABLE 3. Appearance of WIG^- cells among various *com* gene mutant cells

Genotype	No. of colonies	WIG^- cells	
		No. of colonies	Frequency ($\times 10^{-3}$)[a]
Wild type	3,468	23	6.63
recA	4,530	ND[b]	ND
comC	2,953	ND	ND
comD	3,087	ND	ND
comE	2,839	1	0.34
comX	3,513	ND	ND

[a] The WIG^- frequency was expressed as the ratio of cells with smooth colony morphology to total viable cells on a sucrose-containing BHI plate. More than 1,000 colonies were selected from at least five independent experiments.

[b] ND, not detected.

RESULTS

WIG^- strains require a complete competence cascade. Colonies with the parental WIG^+ phenotype show a hard and rough colony morphology on sucrose agar medium, whereas the naturally derived WIG^- colonies show a smooth morphology. We used colony morphology as a criterion for the WIG^- phenotype. Using reduced inocula (~100 to 500 cells) decreased the number of naturally derived variants in the bacterial preculture. After 20 h of culture in pH buffer (pH 7.2), the cultures were plated on BHI agar plates containing sucrose. Laboratory strains (MT8148, UA159, and GS5) and 16 clinical isolates (29) all showed WIG^- cells at a rate of $\sim 1.0 \times 10^{-3}$ to 1.0×10^{-2}. Interestingly, several clinical isolates (FSC-1, FSM-2, and FSM-11) produced 10-fold more WIG^- cells than the laboratory strains (31). Thus, we used the FSM-11 isolate to determine the mechanisms underlying the appearance of the WIG^- variant.

Previous studies demonstrated that the recombination of *gtfB* and *gtfC* causes the WIG^- phenotype and is RecA dependent (3). We found the *S. mutans* FSM-11 *recA* mutant did not produce WIG^- cells (Table 3). The *recA* gene in pneumococci regulates a late competence gene (24). The *comX* gene encodes an alternative sigma factor that recognizes a *com* box (TACGAATA) that is conserved in the putative promoter regions of the late *com* genes (19). The *recA* gene in *S. mutans* is located immediately upstream of the *cinA* gene, the location of the putative promoter *com* box. Additionally, using microarray analysis, Perry et al. (34) showed that *recA* gene expression was regulated by ComX. We therefore determined the appearance of the WIG^- variant using various *com* gene mutations.

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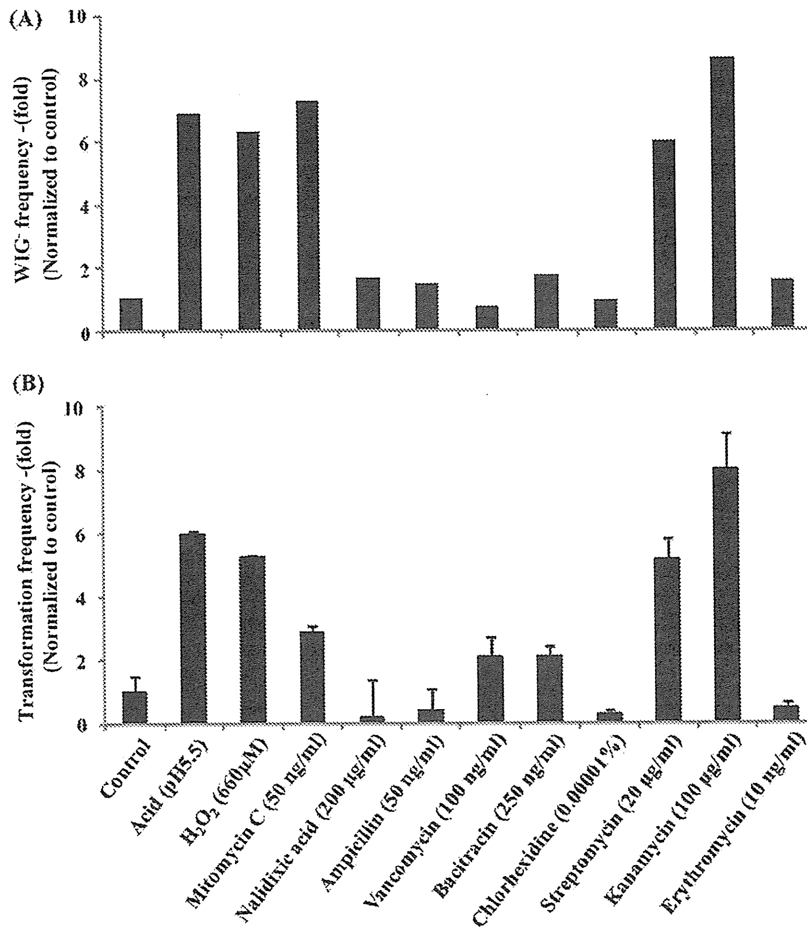

FIG. 1. Effect of environmental stresses on the appearance of WIG⁻ cells and the transformation frequency. (A) Appearance of WIG⁻ cells. More than 1,000 colonies were selected from at least three independent experiments. (B) Transformation frequency. Transformation efficiency was determined after 48 h of incubation and is expressed as the percentage of transformants among the total viable recipient cells. Data are averages from three independent experiments. Bactericidal agents tested for the appearance of WIG⁻ cells and transformation frequency were used under different conditions at a 10% to 20% OD reduction after 20 h of cultivation. Values are expressed as percentages of the control values.

comX, *comC*, and *comD* mutations completely inhibited WIG⁻ strain formation, and the inactivation of *comE* decreased the appearance of WIG⁻ cells compared to that with the wild type (Table 3). This indicates that the generation of WIG⁻ cells is regulated by the *com* system.

Environmental stress induces the WIG⁻ phenotype. Environmental stress induces the competence cascade (36); we therefore determined the effect of various environmental stresses on the appearance of the WIG⁻ variant. Acid stress is an important factor in the oral environment. A pH of 5.5 induced a 10% to 20% OD reduction where the final pH values were pH 5.8 and pH 4.6, respectively. WIG⁻ cells were present under acidic conditions at ~7-fold-higher levels than at neutral pH (Fig. 1A). Oxidative stress and bactericidal agents were tested because a number of antibiotics are linked to the development of natural competence (34, 36). The presence of 660 μM H_2O_2 in the pH-buffered medium induced the appearance of WIG⁻ cells at an increased rate. Cell wall synthesis inhibitors (ampicillin, vancomycin, bacitracin, and chlorhexidine) had no effect. The protein synthesis inhibitors kanamycin and streptomycin (not erythromycin) induced the appearance of WIG⁻ cells. The DNA synthesis inhibitor mitomycin C induced the appearance of WIG⁻ cells, whereas nalidixic acid did not. Acidic conditions and sublethal concentrations of H_2O_2, mitomycin C, streptomycin, and kanamycin induced the transformation frequency >7- to 9-fold compared to that in the culture at neutral pH (Fig. 1B), and these compounds induced the WIG⁻ strain, indicating that the activation of natural competence is related to the appearance of the WIG⁻ phenotype.

The biofilm environment induces the WIG⁻ phenotype. The biofilm has a higher natural competence than planktonic cells (21). To show the appearance of WIG⁻ biofilm cells, we introduced a diluted inoculum into the flow cell (see Materials and Methods). After 16 h and 32 h of cultivation, the biofilm was directly collected from the flow cell and plated on sucrose agar medium. We also plated the outflow at each time point (planktonic cells). The biofilm biomasses after 16 h and 32 h were 3.78×10^8 CFU/ml and 7.76×10^8 CFU/ml, respectively. The biofilm cells at 16 h and 32 h included greater numbers of WIG⁻ cells than in the planktonic cells (Fig. 2). Among plank-

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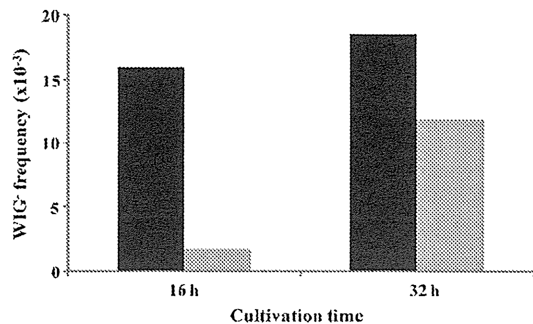

FIG. 2. Presence of WIG⁻ cells in the biofilm and among planktonic cells. Frequency is expressed as the ratio of the WIG⁻ colony number to the total colony number. The presence of WIG⁻ cells in the biofilm (black bars) and among planktonic cells (gray bars) is expressed as a percentage of biofilm cells at 16 h. More than 1,000 colonies were selected from at least three independent experiments.

tonic cells, the presence of WIG⁻ cells was slightly induced at of 16 h culture and increased after 32 h, suggesting that the increase in the numbers of WIG⁻ cells among planktonic cells after the longer cultivation period was derived from the biofilm.

DNA uptake affects the appearance of WIG⁻ cells. Activation of the *com* cascade allows endogenous chromosomal DNA to be transported and allows incorporation of exogenous DNA (9). We therefore considered that incorporation of extracellular DNA may induce the presence of WIG. This was difficult to evaluate quantitatively because both exogenous DNA and endogenous DNA affect the appearance of WIG⁻ recombination. WIG⁻ cells were capable of transformation (data not shown). Therefore, the appearance of the revertant WIG⁻ to WIG⁺ with an intact *gtfBC* may indicate exogenous transformation. To test this, we used a change of phenotype in a coculture experiment. To monitor DNA transfer, a WIG⁺ strain with a Kan^r marker in the intergenic region between *gtfB* and *gtfC* gene was constructed as the donor cell. The WIG⁺ strain with Kan^r also was disrupted in the *recA* gene to eliminate exogenous DNA integration into its chromosomal DNA. The constructed WIG⁺ strain (Kan^r and Erm^r) lost the integration of exogenous DNA into the genome (Table 4). Additionally, the WIG⁺ cells (Kan^r and Erm^r) showed a rough morphology on plates including sucrose (Fig. 3A, panel b); however they showed a decrease in safranin-stained biofilm biomass compared to that for the parental WIG⁺ strain in the microtiter plate biofilm assay with sucrose (Fig. 3C). We also inserted a spectinomycin resistance gene into the *ldh* gene of the WIG⁻ strain. The constructed WIG⁻ (Spc^r) strain maintained a natural competency as did the wild type (Table 4), with a stable smooth colony morphology (Fig. 3A, panel c); no revertants were detected after several passages (data not shown). Both the WIG⁺ (Kan^r and Erm^r) and WIG⁻ (Spc^r) strains showed strong inhibitory activity against *S. gordonii* (data not shown). We prepared a mixed 1:4 inoculum containing the donor WIG⁺ and the recipient WIG⁻ strains and inoculated them into BHI with horse serum. After 8 h of coculture, we plated on Kan-Spc to select revertants from the recipient WIG⁻ cells and on Spc to detect the nontransformed recipient cells. After 2 days of culture, Kan-Spc-resistant cells with a rough morphology were found at a level of 8.45×10^{-6} using medium with sucrose (Table 4). The *gtfBC* region of the revertants was determined using long-range PCR. PCR products of ~9.0 kb were amplified from the chromosomal DNAs of the parent WIG⁺ cells (Fig. 3B, lane 2) and the donor WIG⁺ cells (Fig. 3B, lane 3), whereas a 5.0-kb product was amplified from the chromosomal DNA of the recipient WIG⁻ cells (Fig. 3B, lane 4). A randomly selected rough-morphology colony on the Kan-Spc-sucrose plate was used for replacement with a 9.0-kb gene fragment (Fig. 3B, lane 5). In connection with its gene replacement, biofilm formation of the revertant was restored to the level of the donor WIG⁺ strain (Fig. 3C).

DISCUSSION

The spontaneous generation of genetic variation in bacteria can be induced using three natural methods (2): small local changes in the nucleotide sequence of the genome, intragenomic rearrangement of genomic sequences, and acquisition of DNA sequences from the environment. Previous studies showed that the generation of the WIG⁻ variant was dependent on endogenous DNA rearrangement of *gtfB* and *gtfC* using homologous recombination (40). Here we found that in mixed cultures of WIG⁺ and WIG⁻ cells, the reversion from WIG⁻ to WIG⁺ was enabled with exogenous DNA uptake, indicating that extracellular DNA recombination contributes to the expansion of mutants in a population. In general, natural genetic transformation in bacteria uses active uptake of extracellular DNA and heritable incorporation of this genetic information (9). Most of the natural transformations in strepto-

TABLE 4. Transformation efficiencies of WIG⁺ and WIG⁻ strains in single- and two-species cultures

DNA recipient strain	Donor DNA[a]	Target transformant gene	Transformant frequency[b]
WIG⁺	*ldh* (Spc) gene	Spc^r	$3.87 \times 10^{-4} \pm 1.45 \times 10^{-4}$
WIG⁺ (*gtfBC* and *recA*)	*ldh* (Spc) gene	Spc^r	ND[c]
WIG⁺ (*gtfBC* and *recA*)	WIG⁻ (Spc^r) strain	Spc^r	ND
WIG⁻	*ldh* (Spc) gene	Spc^r	$3.34 \times 10^{4} \pm 2.48 \times 10^{4}$
WIG⁻ (*ldh*)	WIG⁺ (Kan^r and Erm^r) strain	Kan^r	$8.45 \times 10^{-6} \pm 3.81 \times 10^{-6}$

[a] The *ldh* gene with Spc^r in the donor DNA was amplified from the *S. mutans* FSM-11 *ldh* mutant. An aliquot (100 ng) of purified PCR product was added to the BHI medium with horse serum. The WIG⁻ and WIG⁺ donor DNA strains were cocultured with the recipient strain in BHI with horse serum.
[b] Transformation efficiency in single cultures was determined and was expressed as the percentage of Spc-resistant cells compared to total viable cells after 2 days of culture. With coculture, the number of recipient WIG⁺ cells was expressed as the percentage of Spc-Erm-resistant cells compared to Erm-resistant cells, and the number of recipient WIG⁻ cells was expressed as the percentage of Spc-Kan-resistant cells compared to Spc-resistant cells. The data are the averages and standard deviations from three independent experiments.
[c] ND, not detected.

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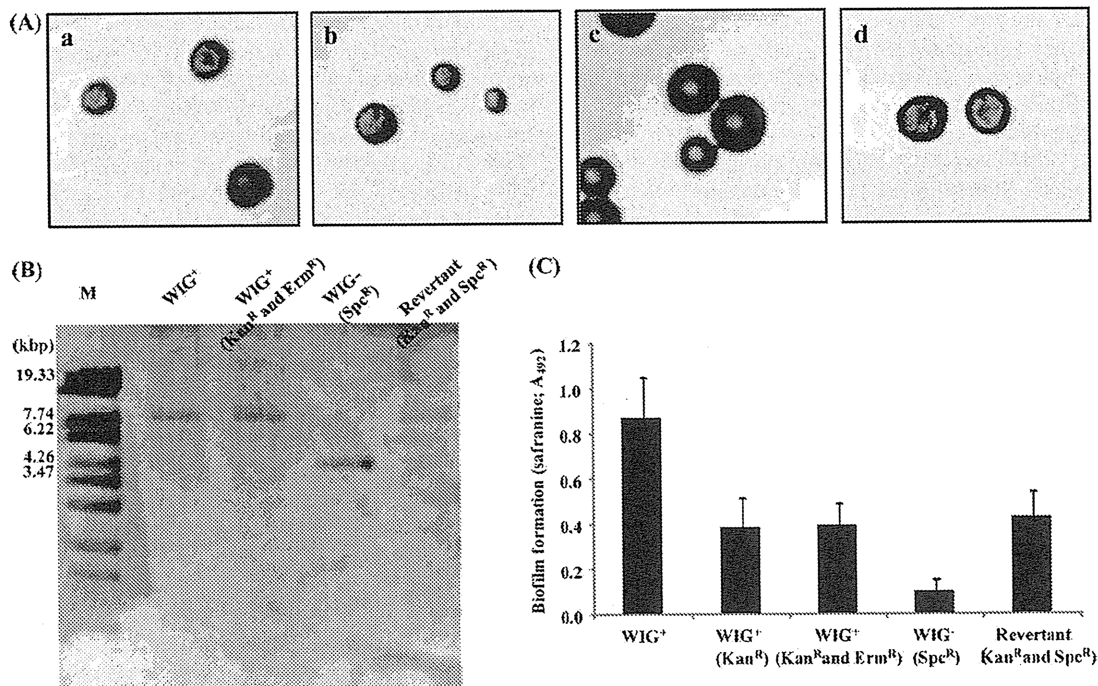

FIG. 3. Characterization of parental WIG$^+$ cells, donor WIG$^+$ cells, recipient WIG$^-$ cells, and WIG$^+$ revertant cells. (A) Colony morphologies of WIG$^+$ (a), WIG$^+$ donor (Kan^r and Erm^r) (b), WIG$^-$ recipient (Spc^r) (c), and revertant (Kan^r Spc^r) (d) cells on BHI agar plates with 0.25% sucrose. Colonies were observed using a stereomicroscope (Nikon). (B) Amplification of the *gtfB* and *gtfC* regions. Lane M, StyI-digested lambda DNA; lane 2, WIG$^+$ cells; lane 3, donor WIG$^+$ (Kan^r and Erm^r) cells; lane 4, recipient WIG$^-$ (Spc^r) cells; lane 5, revertant (Kan^r Spc^r) cells. (C) Biofilm formation assay on a microtiter plate. Cells were grown in BHI medium with 0.25% sucrose at 37°C. Quantification of the biofilm after 20 h of cultivation was performed using the safranin staining method (see Materials and Methods). The data are expressed as means ± standard deviations for three independent experiments.

cocci are regulated by a ComDE two-component regulatory system and the extracellular CSP level. Here we found that endogenous and exogenous DNA recombination generates WIG$^-$ cells and that both were induced by the same signal transducer, the so-called *com* system. The RecA-induced DNA recombination in pneumococci is regulated by ComX (23, 24, 34), and ComY has been shown to be essential for binding DNA to the cell surface and is also dependent on ComX (27). We speculated that the generation of the ancestral WIG$^-$ variant and expansion of the mutant through the uptake of extracellular DNA may be temporally coincident in the population. This coordinated mechanism may be required for the release of extracellular DNA from the cell. In *S. pneumoniae* a competence-specific *recA* transcript was shown to induce *lytA*, which is located immediately downstream of *recA* and encodes an autolysin (23, 28). Conversely, the downstream sequence of *recA* in *S. mutans* is different from that in *S. pneumoniae*, where disruption of *S. mutans* SMU2083 and SMU2084, located downstream of the *recA* gene, did not affect the amount of extracellular DNA uptake (data not shown). Recent studies indicate that *S. mutans* can control the expression of many of its bacteriocins through the *com* system (14, 17, 46). The coordination of bacteriocin production and competence suggests that *S. mutans* may generate DNA for uptake using lysis of neighboring species (17). Perry et al. (34) indicate that there also is a relationship between competence-regulated bacteriocin production and cell lysis and discuss the dissemination of a fitness-enhancing gene in the oral biofilm. We propose that coordination among endogenous DNA recombination, exogenous DNA recombination, and cell lysis through the *com* system contributes to the production of heterogeneity in the clonal population, where this complex regulatory system may not be limited to *in vitro* conditions. *S. mutans* is organized on biofilms, where the cells are closely packed together and multiple species of microorganisms coexist. A previous study indicates that the *S. mutans* WIG$^-$ variant has a stronger presence within biofilm cells than among planktonic cells (3). Our data are in agreement with this (Fig. 2). Oral streptococci such as *S. gordonii* and *S. salivarius* contain WIG homologs sharing high homology with that of *S. mutans*. Future research will examine interspecies interaction through uptake of WIG.

In *S. mutans*, no binding motif for ComE is present in the promoter region of ComX, suggesting a missing link between both regulators and ComX. Recent studies reported that competence in *S. mutans* is controlled by two different quorum-sensing systems, ComDE and ComRS (20, 25). They proposed a model where the ComRS system is the proximal regulator of *comX* and ComDE is an upstream regulator that may be connected to the ComRS system. Signaling information from competence regulators such as ComDE is integrated through the control of ComRS activity, though their effects may not direct and could be relayed through one or more undiscovered components (25). The disruption of SMU61, encoding *comR*, in the FSM-11 strain completely inhibited the appearance of WIG$^-$

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cells (data not shown). This result supports at least our conclusion that the appearance of WIG$^-$ cells is regulated by competence. However, the detailed mechanisms underlying the integration of the ComRS and ComDE systems will need further investigation.

E. coli and other bacteria have a highly effective response to DNA damage, the SOS response, that minimizes the lethal and mutagenic consequences of damage (12). Cell DNA damage blocks the replication fork, generating a single-strand DNA region to which RecA binds to form a nucleoprotein filament. These complexes stimulate the self-cleavage of the LexA repressor and lead to the induction of the SOS genes, including *recA*. However, *S. pneumoniae* has a natural competence system similar to that of *S. mutans* and lacks an SOS-like induction system, instead using the competence regulatory cascade to coordinate responses to stress (36). We did not find a *lexA*-like sequence in the genome database of *S. mutans*. Additionally, acidic and oxidative stresses and mitomycin C induce the SOS response in *E. coli*, resulting in accelerated natural competency (Fig. 1B). Wen et al. (45) indicated that acid and oxidative stresses are linked to the development of natural competence. This suggests that the development of natural competence in *S. mutans* maybe a substitute for the SOS response. However, the parallel is only partial because competence was not induced by nalidixic acid and ampicillin, which induce the SOS response in *E. coli*, and kanamycin and streptomycin induce heat shock protein expression (41), suggesting that stress signals are processed differently in the two species.

The number of WIG$^-$ cell among planktonic cells increased during prolonged cultivation periods (Fig. 2), suggesting that the former were derived from the biofilm environment. The biofilm biomass contents after 16 h and 32 h of cultivation were similar. In addition, the biomass in the outflow solution also remained unchanged using both cultivation periods (data not shown). This indicates that after 16 h the biofilm had attained a steady state where the intact mature biofilm does not disperse after prolonged culture. Because WIG is associated with the biofilm architecture (15, 39), the prevalence of WIG$^-$ cells in biofilms may change the structure, resulting in the release of WIG$^-$ cells. Plaque pH can be lower than 5.0 and remain so for some time; this low pH leads to demineralization of the enamel (42). Our data show that an acidic pH promotes the presence of WIG$^-$ cells (Fig. 1A). In addition, the growth rate of WIG$^-$ cells with sucrose supplementation was greater than that of the wild type (31), suggesting that carbohydrate fermentation by the mutant contributes to an early shift to a more acidic environment. Therefore, the WIG$^-$ variant may contribute to further demineralization and development of caries. An improved understanding of the behavior of the variant *in situ* would help to determine its role in natural ecosystems.

Other than sucrose-dependent adherence, *S. mutans* is diverse in its antigenic polysaccharides that determine serotype, and it also varies in sugar fermentation, bacteriocin type, capacity to produce acid, interaction with salivary components, and release of surface proteins (44). In natural ecosystems, population heterogeneity may mitigate the impact of environmental perturbation because the presence of diverse members extends the range of conditions under which the organism can persist (7, 25). Diversity can also enhance population stability and productivity because of cooperative interactions among community members (4, 5, 7, 26). Therefore, one consequence of the appearance of the *S. mutans* WIG$^-$ variant would be to enhance fitness in the human oral environment. Our data may help in understanding how *S. mutans* can adapt to the oral environment and ultimately help explain the evolution of *S. mutans*. The mechanism of the appearance of the WIG$^-$ variant observed in this work may serve as a useful model for studying population heterogeneity among species.

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Outer Membrane Vesicles of *Porphyromonas gingivalis* Elicit a Mucosal Immune Response

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Abstract

We previously reported that mutation of *galE* in *Porphyromonas gingivalis* has pleiotropic effects, including a truncated lipopolysaccharide (LPS) O-antigen and deglycosylation of the outer membrane protein OMP85 homolog. In the present study, further analysis of the *galE* mutant revealed that it produced little or no outer membrane vesicles (OMVs). Using three mouse antisera raised against whole cells of the *P. gingivalis* wild type strain, we performed ELISAs to examine the reactivity of these antisera with whole cells of the wild type or the *galE* mutant. All three antisera had significantly lower reactivity against the *galE* mutant compared to wild type. OMVs, but not LPS, retained the immunodominant determinant of *P. gingivalis*, as determined by ELISAs (with wild type LPS or OMVs as antigen) and absorption assays. In addition, we assessed the capacity of OMVs as a vaccine antigen by intranasal immunization to BALB/c mice. Synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid [Poly (I:C)], an agonist of Toll-like receptor 3 (TLR3), was used as the mucosal adjuvant. Vaccination with OMV elicited dramatically high levels of *P. gingivalis*-specific IgA in nasal washes and saliva, as well as serum IgG and IgA. In conclusion, the OMVs of *P. gingivalis* have an important role in mucosal immunogenicity as well as in antigenicity. We propose that *P. gingivalis* OMV is an intriguing immunogen for development of a periodontal disease vaccine.

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Introduction

Periodontitis is an oral disease characterized by destruction of periodontal tissues and ultimately exfoliation of the teeth in humans [1]. In addition, recent reports from epidemiological studies [2,3,4] as well as *in vitro* and animal model experiments [5,6,7] have shown an association between periodontitis and systemic diseases such as diabetes mellitus, cardiovascular disease, and atherosclerosis. Therefore, development of a safe vaccine for periodontal diseases would be a great benefit to improving public health. Among the various Gram-negative anaerobes that reside within the subgingival pockets, *Porphyromonas gingivalis* is a major causative agent in the initiation and progression of severe forms of periodontal disease [1]. Several virulence factors of *P. gingivalis* are known, including fimbriae, gingipains, hemagglutinins, lipopolysaccharide (LPS), and outer membrane vesicles (OMVs) [1]. However, it is not known that *P. gingivalis* components are targeted by the protective humoral immune system during disease onset and progression.

Gram-negative bacteria are distinguished from other prokaryotes by an outer membrane that surrounds their peptidoglycan layer. The outer membrane contains essential molecules, such as LPS and outer membrane proteins [8]. Of particular medical interest are the miscellaneous surface-exposed molecules on intact bacteria that are recognized by the immune system and possess a powerful potential to activate the host immune system. Gram-negative bacteria release OMVs from the cell surface during bacterial growth [9]. OMVs range in size from 20 to 250 nm in diameter and contain not only components of the outer membrane, such as LPS, outer membrane proteins, and phospholipids, but also periplasmic proteins and cell wall components, i.e., peptidoglycan, given that OMVs entrap some of the underlying periplasmic proteins and small particles of the cell wall when the blebs are extruded from the cell surface. OMVs play a role in such pathogenic processes as toxin export [10,11,12] and adherence to eukaryotic cells [13]. As with OMVs of other bacteria, *P. gingivalis* OMVs contain several virulence factors, such as LPS, fimbriae, and gingipains [14,15,16,17]. Recently, *P. gingivalis* has been shown to possess a system that selectively sorts virulence factors into OMVs [18]. OMVs of *P. gingivalis* also can be internalized into host cells via a lipid-raft-dependent endocytic pathway and are subsequently routed to the early endosome followed by sorting into lysosomal compartments [19]. After lysis of the OMV, various antigens may be recognized and processed by antigen-presenting

cells such as dendritic cells and macrophages, leading to induction of adaptive immunity including pathogen-specific antibody production.

OMVs have also been recognized as a vaccine candidate for infectious diseases. In several countries, wild type OMVs of *Neisseria meningitidis* serogroup B were approved as vaccines for parenteral use with reported efficacy rates of 70% to 83% in adults and children [20]. Currently, OMV vaccines are the only formulation that have shown efficacy against serogroup B meningococcal diseases. Intranasal administration of OMVs derived from *Vibrio cholerae* has also induced protective immunity against this gastrointestinal pathogen in mice [21]. In the case of *P. gingivalis*, although parenteral administration of OMVs in mice is protective against challenge infection [22], the efficacy of OMVs as an intranasal vaccine has not yet been determined.

In the present study, we identified an OMV-negative mutant of *P. gingivalis* and investigated its antigenicity by comparative analysis with the wild type strain. Our results indicated that OMVs play a significant role in the antigenicity of *P. gingivalis*. In addition, we demonstrated that intranasal administration of *P. gingivalis* OMVs effectively elicited not only serum IgG and IgA, but also secretory IgA (s-IgA) in nasal washes and saliva that recognize *P. gingivalis*.

Materials and Methods

Bacterial strains and culture conditions

P. gingivalis ATCC 33277 and the *galE* mutant [23] were maintained in brain heart infusion (BHI) broth supplemented with hemin and menadione (HM) or on BHI-HM blood agar plates in an anaerobic chamber (miniMACS anaerobic workstation, Don Whitley Scientific Ltd., Shipley, UK) using 80% N_2, 10% H_2, and 10% CO_2.

Preparation of OMVs and LPS from *P. gingivalis*

Preparation of OMV was performed as described previously with some modifications [11]. In brief, the supernatant of a two-day culture of strain 33277 was collected by centrifugation at $3{,}410\times g$ for 15 min at 4°C, then filtered through a 0.22-μm PVDF filter and ultra-centrifuged at $100{,}000\times g$ for three hours at 4°C in a 41 Ti rotor (Beckman Instruments, Inc., USA). The resulting OMV pellet was resuspended in 20 mM Tris-Cl (pH 8.0) and the protein concentration was measured by Bradford assay [24] using bovine serum albumin as a standard. Preparation of LPS from *P. gingivalis* was performed using the hot phenol water extraction method with some modifications [23]. Finally, LPS was lyophilized, weighed and used for mitogenic assays or ELISAs.

Mitogenic assays

The mitogenic activity of LPS prepared from the wild type and the *galE* mutant was assessed by adding various concentrations of LPS (0.1–100 μg/ml) to primary splenocyyte cultures (5×10^5 cells/ml) prepared from BALB/c mice. A proliferation assay using WST-1 (Dojindo Laboratories, Kumamoto, Japan) was then performed.

Scanning electron microscopy (SEM)

To analyze cell morphology, *P. gingivalis* wild type or the *galE* mutant strain were grown on non-treated plastic sheets (Wako Chemical Ltd., Osaka, Japan) placed in 6-well polystyrene cell culture plates. Four $\times10^7$ CFU of *P. gingivalis* were grown in two ml of BHI-HM broth per well at 37°C for 12 hours under anaerobic conditions. For analysis of OMVs derived from *P. gingivalis*, the bacterial supernatant was collected at different time points, then incubated on poly L-lysine-coated cover slips for 30 min at room temperature to attach the OMVs to the glass. The attached bacteria and OMVs were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in PBS for 30 min at room temperature, followed by three washes in PBS. The samples were washed in PBS; dehydrated in 50% ethanol to absolute ethanol; immersed in isoamyl acetate; dried by critical point drying; coated with osmium vapor using an osmium plasma coater; and visualized by SEM (S-5200, HITACHI, Hitachi, Japan).

Time-course analysis of bacterial growth and LPS in the supernatant

Growth assays were performed by measuring OD_{600}. The bacterial supernatant was collected by centrifugation at $17{,}400\times g$ for two minutes at different time points. The amount of LPS in the supernatant was measured by Limulus amoebocyte lysate (LAL) reagent according to the manufacturer's instructions (Seikagaku Corporation, Tokyo, Japan).

Immunization

All animal experiments were performed in accordance with our institutional guidelines. Female BALB/c mice (Japan SLC, Inc., Hamamatsu, Japan), aged 6 to 8 weeks at the time of immunization, were used in all experiments. For the first half of the experimental plan, the mice were immunized by the conventional method to obtain *P. gingivalis*-specific antisera as follows: 1×10^8 CFU of heat-killed bacteria emulsified in TiterMax Gold (TiterMax USA; Norcross, GA) was injected into mice intraperitoneally followed by two booster injections on days 8 and 15 with 5×10^7 CFU of heat-killed bacteria in incomplete Freund's adjuvant. For the second half of the experimental plan, the mice were immunized intranasally. In brief, mice were immunized by dropping 5 μl of PBS containing 5 μg of polyriboinosinic polyribocytidylic acid [Poly (I:C)] (Sigma, St. Louis, MO) with or without 0.5 μg of immunogen (OMVs or heat-killed whole cells of *P. gingivalis* ATCC 33277) into each nostril on day 0 and week 3 under anesthesia. The protein concentration of these immunogens was determined by Bradford assay [24]. At two weeks after the second immunization, sera, nasal washes, and saliva specimens were collected and used for ELISA. Saliva samples were obtained as described previously [25].

ELISA

Reactivities of mouse sera and nasal washes with whole cells of the bacterial strains were tested using an ELISA described previously with some modifications [26]. Unwashed whole cells of *P. gingivalis* were prepared from fresh bacterial culture by centrifuging only once at $3{,}410\times g$ for 15 minutes at 4°C without washing. For washed whole cells, bacteria were collected by centrifugation and resuspended in PBS by pipetting. The centrifugation and washing steps were repeated. Both the unwashed and washed bacteria were collected and freeze-dried. The dried bacteria were weighed and used as antigen for whole-cell ELISA. ELISA plates were coated with 10 μg of the freeze-dried bacteria resuspended in 100 μl of ELISA coating buffer per well. After overnight blocking at 4°C with 1% skim milk in PBS with 0.5% Tween 20 (PBS-T), each serum or nasal wash sample was serially diluted with 1% skim milk in PBS-T, added to the wells and incubated for 1 hour at 37°C. The wells were then incubated for 1 hour at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgG, IgA, and IgM (Invitrogen, Carlsbad, CA) at a dilution of 1:1000. Subsequently, *P. gingivalis*-specific antibody was detected by chromogenic development using para-nitrophenyl phosphate as the alkaline phosphatase substrate. Absorbance at OD_{405} was

measured at different time points; 15 minutes, 30 minutes, 1 hour, and 2 hours. The titer of each antiserum recognizing LPS and OMV from *P. gingivalis* 33277 was determined using the same ELISA protocol, except the microtiter wells were initially coated with antigen at 100 ng/well. In the absorption assay, each serum sample was diluted at 1:1000 in PBS and pre-incubated with 10 ng/ml of LPS or OMV purified from strain 33277 for 1 hour at 37°C. Then, the absorbed serum samples were passed through a Detoxigel™ (Thermo Fisher Scientific, Waltham, MA) column twice to remove possible contaminant LPS and LPS-associated molecules. An LAL assay was performed to confirm that the serum did not contain any detectable LPS. The purified sera were used for ELISA.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney's U-test. P-values of 0.05 or less were considered to be statistically significant.

Results

P .gingivalis galE mutant essentially lacks OMVs as well as LPS in the supernatant

We previously demonstrated that mutation of the *galE* gene in *P. gingivalis* caused pleiotropic effects associated with the metabolic pathway of galactose, resulting in autoaggregation/biofilm formation as well as deglycosylation of LPS and the outer membrane protein Omp85 homolog [23,27]. Notably, *galE* mutation also affects components of the outer membrane, therefore we hypothesized that morphological changes might occur at the bacterial surface of the *galE* mutant.

Firstly, we examined the morphology of wild type and the *galE* mutant using scanning electron microscopy (SEM). There was no difference in the size or shape of mutant cells compared to wild type (Fig. 1A). On the surface of the wild type cells, many OMVs were clearly visible as spherical structures approximately 50–70 nm in diameter (Fig. 1A). However, we could not find any OMVs on the surface of the *galE* mutant (Fig. 1A). We also analyzed by SEM the OMVs discharged into the bacterial supernatant at different time points during three days of culture (Fig. 1B). The numbers of OMVs in the supernatant of the wild type strain increased until day 3 (Fig. 1B). In contrast, we could not find any OMVs in the supernatant of the *galE* mutant throughout the three days (Fig. 1B). To test whether the *galE* mutant also releases less LPS during culture, we examined the kinetics of the LPS activity in the supernatants of the wild type and *galE* mutant by LAL assay (Fig. 1C) with their growth curves (Fig. 1D). The Limulus activity in the supernatant of the wild type increased through day 3. In contrast, the Limulus activity in the *galE* mutant supernatant increased minimally during the same time period, remaining similar to that of sterile BHI-HM liquid media (7.54 EU/ml) shown by an arrow in Fig. 1C. To evaluate LPS quality, we compared LPS prepared from the wild type and the *galE* mutant in a mitogenic assay. The mitogenic assay showed that LPS from both strains had similar activities (data not shown), suggesting that the lower Limulus activity of *galE* mutant supernatant compared to wild type (Fig. 1C) is due to less total LPS, not lower LPS activity.

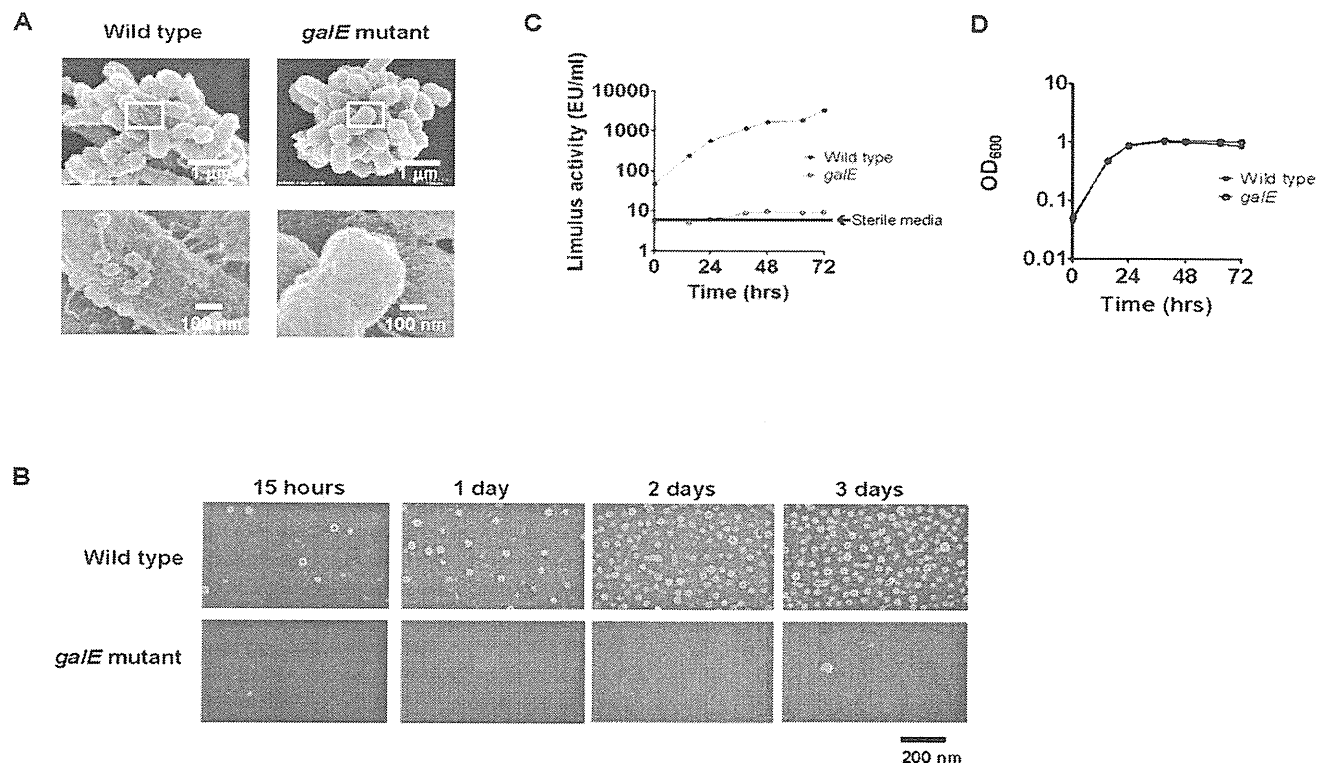

Figure 1. SEM images of *P. gingivalis* wild type and the *galE* mutant. (A) *P. gingivalis* wild type and *galE* mutant were grown on plastic sheets for 12 hours. Upper panels show the morphology of the attached cells. The lower panels, enlargements of the squares in the upper panels, show the presence and absence of OMVs on wild type and *galE* mutant cells, respectively. The scale is shown at the lower right of each electron micrograph. (B, C, and D) OMVs of wild type and the *galE* mutant were harvested at different time points during culture. (B) The OMVs were applied to plastic sheets, and visualized by SEM. The scale is shown at the lower right. The limulus activity (C) and growth (D) of each sample were recorded.
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