Potential Risk of Spreading Resistance Genes within Extracellular-DNA-Dependent Biofilms of *Streptococcus mutans* in Response to Cell Envelope Stress Induced by Sub-MICs of Bacitracin

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ABSTRACT Antibiotics are used to treat or prevent some types of bacterial infection. The inappropriate use of antibiotics unnecessarily promotes antibiotic resistance and increases resistant bacteria, and controlling these bacteria is difficult. While the emergence of drug-resistant bacteria is a serious problem, the behavior of drug-resistant bacteria is not fully understood. In this study, we investigated the behavior of *Streptococcus mutans*, a major etiological agent of dental caries that is resistant to bacitracin, which is a cell wall-targeting antibiotic, and focused on biofilm formation in the presence of bacitracin. *S. mutans* UA159 most strongly induced extracellular DNA (eDNA)-dependent biofilm formation in the presence of bacitracin at 1/8× MIC. The Δ*mbrC* and Δ*mbrD* mutant strains, which lack bacitracin resistance, also formed biofilms in the presence of bacitracin at 1/2× MIC. This difference between the wild type and the mutants was caused by the induction of *atlA* expression in the mid-log phase. We also revealed that certain *rgp* genes involved in the synthesis of rhamnose-glucose polysaccharide related to cell wall synthesis were downregulated by bacitracin. In addition, glucosyltransferase-I was also involved in eDNA-dependent biofilm formation. The biofilm led to increased transformation efficiencies and promoted horizontal gene transfer. Biofilms were also induced by ampicillin and vancomycin, antibiotics targeting cell wall synthesis, suggesting that cell envelope stress triggers biofilm formation. Therefore, the expression of the *atlA* and *rgp* genes is regulated by *S. mutans*, which forms eDNA-dependent biofilms, promoting horizontal gene transfer in response to cell envelope stress induced by sub-MICs of antibiotics.

IMPORTANCE Antibiotics have been reported to induce biofilm formation in many bacteria at subinhibitory concentrations. Accordingly, it is conceivable that the MIC against drug-sensitive bacteria may promote biofilm formation of resistant bacteria. Since drug-resistant bacteria have spread, it is important to understand the behavior of resistant bacteria. *Streptococcus mutans* is bacitracin resistant, and the 1/8× MIC of bacitracin, which is a cell wall-targeted antibiotic, induced eDNA-dependent biofilm formation. The Δ*mbrC* and Δ*mbrD* strains, which are not resistant to bacitracin, also formed biofilms in the presence of bacitracin at 1/2× MIC, and biofilms of both the wild type and mutants promoted horizontal gene transfer. Another cell wall-targeted antibiotic, vancomycin, showed effects on biofilms and gene transfer similar to those of bacitracin. Thus, treatment with cell wall-targeted antibiotics may promote the spread of drug-resistant genes in biofilms. Therefore, the behavior of resis-

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tant bacteria in the presence of antibiotics at sub-MICs should be investigated when using antibiotics.

Antibiotics have contributed to human health as an effective treatment for bacterial infections. However, due to inappropriate use, bacteria have evolved resistance mechanisms to survive in the presence of antibiotics, and now, there are resistant bacteria for all antibiotics (1). Therefore, it is possible that resistant bacteria and susceptible bacteria coexist in the same bacterial flora. In this situation, if an antibiotic regulated a particular susceptible pathogenic bacterium, it would decrease the target bacterium, and resistant bacteria would become dominant; eventually, the native flora would be destroyed. In general, the destroyed flora is recovered by complex mechanisms such as bacterial interactions and bacterium-host interactions. By understanding the behavior of resistant bacteria that survive after antibiotic use, it will be possible to effectively control bacterial infections.

Streptococcus mutans, a major etiological agent of dental caries and infective endocarditis, exerts pathogenicity by forming biofilms. Biofilms are formed by polysaccharide-dependent mechanisms and polysaccharide-independent mechanisms. *S. mutans* has three glucosyltransferases (GtfB, GtfC, and GtfD, encoded by *gtfB*, *gtfC*, and *gtfD*, respectively) and a fructosyltransferase (Ftf, encoded by *sacB*) as polysaccharide syntheses, and these enzymes synthesize polysaccharides (glucan and fructan) from sucrose (2–5). In addition, we reported that Ftf synthesizes fructan from raffinose as a substrate (6). Among these enzymes, GtfB and GtfC are involved in insoluble glucan synthesis and closely related to the pathogenicity of *S. mutans*. Extracellular DNA (eDNA) is involved in polysaccharide-independent biofilm formation (7, 8). eDNA is considered to be genomic DNA (gDNA) released from dead cells and is involved in the attachment of bacteria to surfaces and adhesion between bacteria (9, 10). Accordingly, cell death is important for the production of eDNA by *S. mutans*. *atlA* encodes a major autolysin that causes autolysis (11, 12). In addition, cell death is also induced by quorum sensing via competence-stimulating peptide (CSP).

S. mutans contains bacitracin resistance genes (13). Bacitracin is an antimicrobial peptide secreted by several *Bacillus* species and has bactericidal activity mainly against Gram-positive bacteria (14, 15). Bacitracin is used in clinical settings, but systemic administration can cause anaphylactic reactions (16). In contrast, oral administration of bacitracin is effective for the control of vancomycin-resistant enterococcal infections, since bacitracin is poorly absorbed in the gastrointestinal tract (17). However, oral bacitracin has been designated an orphan drug for the treatment of pseudomembranous colitis caused by *Clostridium difficile*. Bacitracin inhibits peptidoglycan synthesis by binding to the lipid carrier C_{55}-undecaprenyl pyrophosphate (C_{55}-PP), which is involved in bacterial peptidoglycan synthesis (18, 19). *S. mutans* contains *mbrABCD* resistance genes (20). These genes are conserved in *S. mutans* and used to isolate *S. mutans* from other oral streptococci (13). *mbrAB* encodes an ABC transporter, and *mbrCD* encodes a two-component regulatory system. The deletion of these genes resulted in an approximately 100-fold increase in bacitracin sensitivity (20, 21). The bacitracin resistance genes *mbrABCD* in *S. mutans* are homologous to *bceABRS* in *Bacillus subtilis*, and the resistance mechanism has been investigated in *B. subtilis* in detail (22). *bceAB*, encoding an ABC transporter, is responsible for the removal of bacitracin from the bacitracin attack site, and *bceRS*, encoding a two-component regulatory system, induces the expression of *bceAB* in response to bacitracin (22, 23).

Rhamnose-glucose polysaccharide (RGP) is a component of the cell wall in *S. mutans* and is linked to peptidoglycan. RGP is important for fitness under stress conditions and is also involved in bacitracin resistance (20, 24). *S. mutans* contains *rgpABCDEFGHI*, genes involved in RGP synthesis. RgpG transfers *N*-acetylglucosamine to membrane-linked lipid carriers. RgpA, RgpB, and RgpF are required for the assembly of L-rhamnose

TABLE 1 MICs of bacitracin against the WT strain and the *mbr* mutants

| Strain	MIC (U/ml)	MBC (U/ml)
UA159 WT	4	64
Δ*mbrC* mutant	0.125	8
Δ*mbrD* mutant	0.125	8

on lipid carriers. Glucose side chains are formed by glucosyltransferase, RgpE, RgpH, and RgpI. Mature RGP is transferred by the ABC transporter RgpCD (24). In addition, the mutant strains lacking *rgpA*, *rgpB*, *rgpC*, and *rgpD* exhibited an approximately 5-fold increase in bacitracin sensitivity (20).

In recent years, it has been reported that many bacteria induce eDNA-dependent biofilm formation in the presence of antimicrobial substances at sub-MICs (25–27). In *S. mutans*, sub-MICs of chlorhexidine, tea polyphenols, and sodium fluoride, which are known anticaries agents, upregulated the expression of biofilm-related genes (28). We found that *S. mutans* induced biofilm formation in the presence of sub-MICs of bacitracin. The Δ*mbrC* and Δ*mbrD* strains, which were not resistant to bacitracin, could also form biofilms in the presence of sub-MICs of bacitracin. However, the biofilm formation mechanism of the mutant strains was different from that of the wild-type (WT) strain. In this study, we show that bacitracin at sub-MICs triggers the cell envelope stress response and induces eDNA-dependent biofilm formation via regulation of the expression of the *atlA* and *rgp* genes in *S. mutans*. Furthermore, we also show that the biofilm can be a potential risk for horizontal resistance gene transfer.

RESULTS

Biofilm formation in the presence of sub-MICs of bacitracin. To conduct the biofilm formation assay in the presence of bacitracin at sub-MICs, we identified the MIC of bacitracin against the *S. mutans* UA159 WT strain by preparing a 2-fold dilution series of bacitracin in brain heart infusion (BHI) broth. In accordance with other reports (20), the MIC of bacitracin was determined to be 4 U/ml against the WT strain (Table 1). Thus, we conducted a biofilm formation assay in the presence of bacitracin at concentrations less than 4 U/ml. Since the biofilm mechanisms in *S. mutans* is generally divided into polysaccharide-dependent and polysaccharide-independent biofilm formation, we first examined the effect of sub-MICs of bacitracin on polysaccharide-dependent biofilm formation. In tryptic soy broth without dextrose but with sucrose (TSBs), which are conditions capable of causing polysaccharide-dependent biofilm formation, the biofilm formation levels in the presence of bacitracin concentrations from 1/32× to 1/2× MIC and in the absence of bacitracin did not differ (see Fig. S1 in the supplemental material). Since 1× MIC of bacitracin inhibited cell growth, biofilm formation was also decreased at this concentration (Fig. S1). This result showed that biofilm formation depended on whether growth was possible and that sub-MIC levels of bacitracin did not remarkably affect the biofilm. In turn, we examined the effect of bacitracin at sub-MICs on polysaccharide-independent biofilm formation in TSB with glucose (TSBg), which does not allow enough polysaccharide to be synthesized for biofilm formation, although TSB contains small amounts of substrates (sucrose and oligosaccharide) for polysaccharide synthesis (6). *S. mutans* could not induce biofilm formation in TSBg without bacitracin, but biofilm formation was significantly increased at 1/32× MIC to 1/2× MIC of bacitracin, and the biomass reached a maximum at 1/8× MIC of bacitracin (Fig. 1A). Additionally, no significant growth inhibition occurred in the presence of the concentrations that induced biofilm formation (Fig. 1B). It was suggested that *S. mutans* actively induced biofilm formation in the presence of bacitracin at concentrations that did not affect growth. Thus, in agreement with many reports on drug-sensitive bacteria (25–27), this result indicated that biofilm formation could be induced even in drug-resistant bacteria by the presence of sub-MICs of antibiotics.

A previous report indicated that a subinhibitory concentration of *β*-defensin-3, which is a small defense peptide secreted by salivary glands and gingival and mucosal
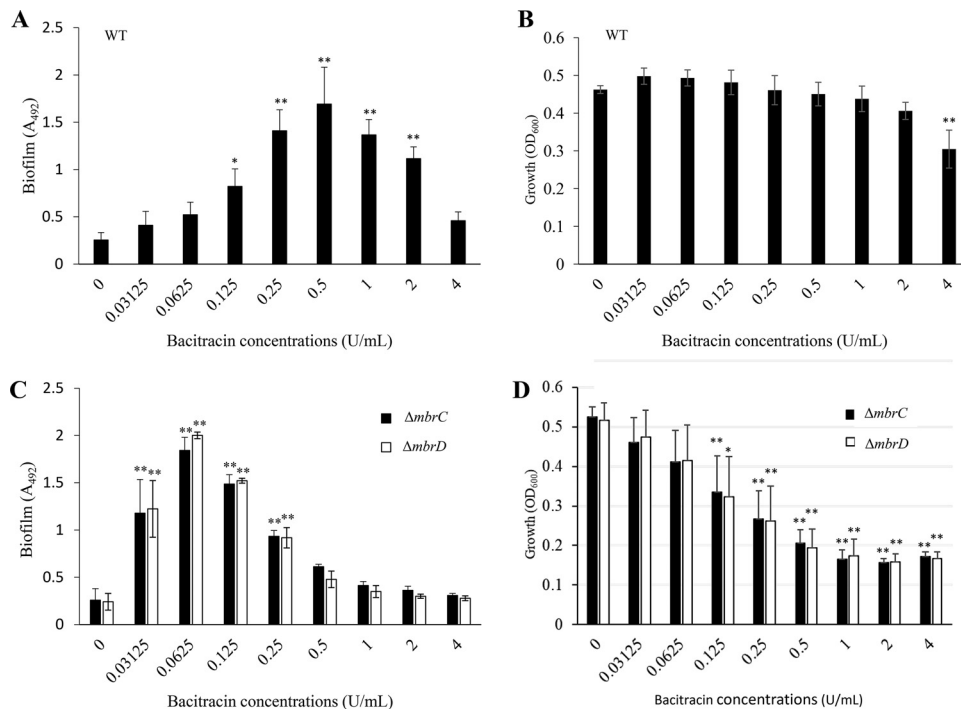
FIG 1 Biofilm formation assay in TSBg with various concentrations of bacitracin. (A and C) Effect of bacitracin on 5-h biofilms formed by the WT (A) and *mbr* mutant strains (C). To evaluate the effect of bacitracin, we performed a biofilm formation assay using TSBg medium, which is a medium that does not induce biofilm formation. (B and D) Effect of bacitracin on 5-h growth in the WT (B) and *mbr* mutant strains (D). To investigate the relationship between biofilm induction and growth, the optical density (OD_{600}) was measured when samples were cultured under the same conditions as biofilm formation. The data are the mean $\pm$ standard deviation (SD) of three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA) and a Bonferroni's posttest. The asterisks indicate a significant difference (**, $P < 0.01$; *, $P < 0.05$).

epithelia, induced biofilm formation in the WT strain but not in any of the *bceABRS* (*mbrABCD*) mutants that are not resistant to bacitracin (29). We considered that biofilm induction in the presence of bacitracin may be triggered by a bacitracin-specific response. We then constructed and analyzed bacitracin-sensitive mutant strains that were no longer responsive to bacitracin. *mbrCD* encodes a two-component regulatory system for bacitracin-specific responses. These genes are essential for bacitracin resistance, and the MIC of bacitracin decreased in strains lacking either of these genes (20, 30). To clarify whether *mbrCD* is involved in inducing biofilm, we conducted a biofilm formation assay using mutant strains with deletions of *mbrC* (Δ*mbrC*) and *mbrD* (Δ*mbrD*). First, we determined the MIC of bacitracin against the Δ*mbrC* and Δ*mbrD* mutants. The MIC of bacitracin against these mutants was decreased to 0.125 U/ml compared to the MIC against the WT strain (Table 1). Biofilm formation in the Δ*mbrC* and Δ*mbrD* mutant strains was induced in the presence of 1/4× MIC to 2× MIC of bacitracin, and the level of biofilm formation reached a maximum at 1/2× MIC of bacitracin (Fig. 1C). There was a difference in the biofilm formation of the strains: the WT strain most strongly induced biofilm formation at 1/8× MIC of bacitracin, whereas the Δ*mbrC* and Δ*mbrD* mutants most strongly induced biofilm formation at 1/2× MIC of bacitracin. Additionally, biofilm formation in the *mbr* mutant strains was induced even with bacitracin at concentrations over the MIC, although growth was significantly inhibited (Fig. 1D). This result suggested that there were some differences in the biofilm induction mechanism in the WT strain and the *mbr* mutant strains, not merely a shift in the biofilm-inducing concentrations due to a change in the MIC.

Analysis of the biofilm-inducing mechanism. To understand the mechanism of biofilm induction in TSBg, we elucidated the biofilm formation mechanism of the WT strain in the presence of 1/8× MIC of bacitracin and the *mbr* mutant strains in the
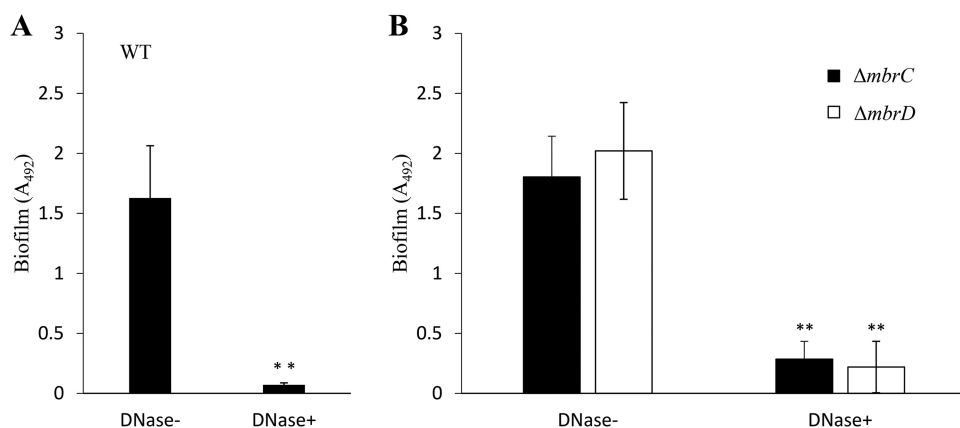
FIG 2 Inhibitory effect of DNase I on the biofilms. DNase I inhibition of biofilm formation by the WT strain (A) and *mbrC* and *mbrD* strains (B) is shown. To evaluate the contribution of eDNA to biofilm formation, we determined the inhibitory effect by adding 50 U/ml DNase I before incubation (DNase+) on the biofilm formations of the WT strain and *mbrC* and *mbrD* mutants induced by 0.5 U/ml (1/8× MIC for the WT) and 0.0625 U/ml (1/2× MIC for the *mbr* mutants) of bacitracin, respectively, and these biofilms were compared with biofilms formed without DNase I (DNase−). These data are the mean ± SD of three independent experiments. The asterisks indicate a significant difference between two groups (Student's *t* test; **, $P < 0.01$, versus DNase−).

presence of 1/2× MIC of bacitracin, because these concentrations strongly induced biofilm formation. Since the main carbon source in TSBg is glucose, which is not available for polysaccharide synthesis, we predicted that substances other than polysaccharides are important in biofilm formation and considered the possibility that eDNA is a major component of the biofilm. We then indirectly assessed the contribution of eDNA to biofilm formation by examining the inhibition of biofilm formation when DNase I was added to the medium. When DNase I was added to the medium at 50 U/ml, the WT strain did not form biofilms (Fig. 2A). Additionally, the amount of eDNA was significantly higher in the presence of bacitracin than in the absence of bacitracin (Fig. S2). Biofilms formed by the Δ*mbrC* and Δ*mbrD* strains were also markedly reduced by the addition of DNase I (Fig. 2B). These results indicated that biofilm formation in both the WT and *mbr* mutant strains largely depended on eDNA. eDNA is generally considered genomic DNA released from dead cells (6, 10). Thus, it was predicted that cell lysis and release of DNA frequently occur under biofilm-inducing conditions. Confocal laser scanning microscopy (CLSM) analysis of the biofilm cells stained by the Live/Dead BacLight bacterial viability kit revealed that the WT and Δ*mbrC* biofilms both contained dead cells (Fig. 3A). The WT strain formed a biofilm in which the ratio of live cells to dead cells was high (Fig. 3B), and many aggregates and biofilm pieces were widely observed (Fig. 3C). On the other hand, the biofilm formed by the mutant strains had a lower ratio of live cells to dead cells than the WT strain, the cells were scattered in the biofilm, and no aggregates of cells were observed. These results showed that the WT strain actively forms a three-dimensional (3D) biofilm that is rich in live bacteria, while the *mbr* mutant strains form sediment-like biofilms that do not have the distinguishable 3D structure characteristics that the wild type has and are rich in dead cells.

To clarify the cause of lysis, we assessed whether bacitracin had direct bactericidal activity against the WT, Δ*mbrC*, and Δ*mbrD* strains at biofilm-inducing concentrations. The number of live cells was calculated by comparing the number of CFU at 5 h of incubation with bacitracin to that without bacitracin (Table 2). There was no difference in number of live cells, even with bacitracin at the concentration at which biofilm formation was most strongly induced. The minimum bactericidal concentrations (MBCs) of bacitracin against the WT and *mbr* mutant strains were determined to be 64 U/ml and 8 U/ml, respectively (Table 1). These concentrations were much higher than the concentration with the greatest biofilm induction (1/8× MIC for the WT strain and 1/2× MIC for the *mbr* mutant strains). Thus, we reasoned that biofilm formation at these concentrations was not associated with the direct bactericidal activity of bacitracin.
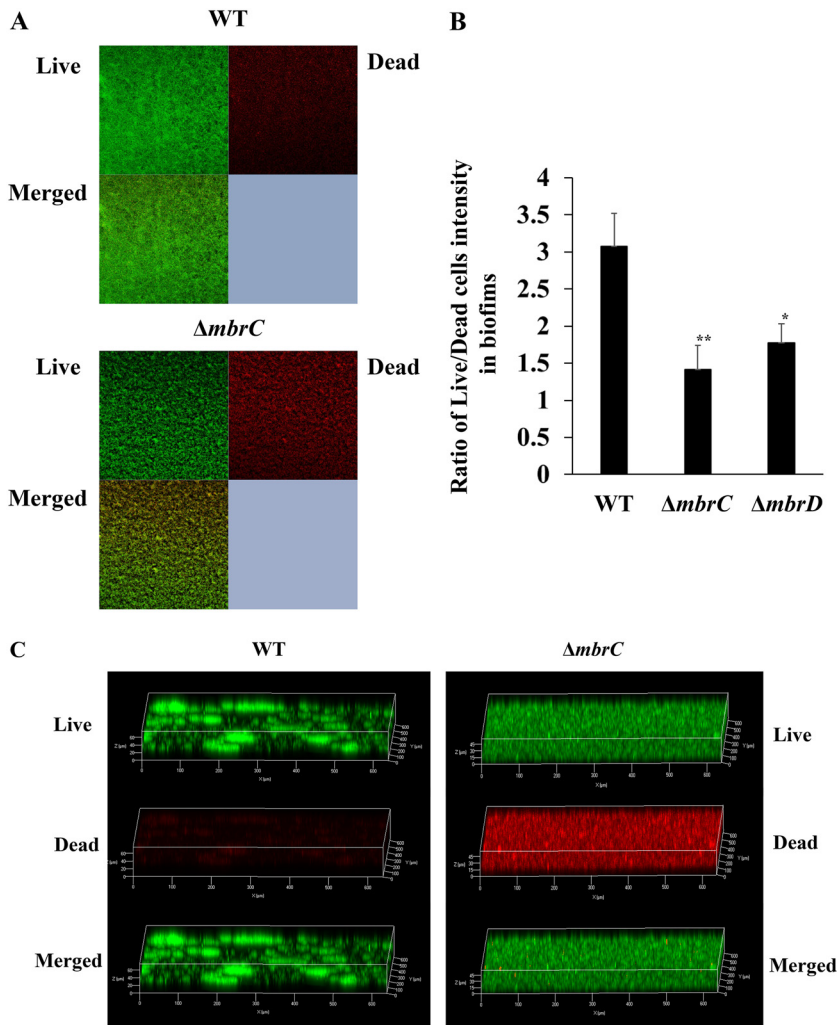
FIG 3 Observation of live and dead cells in the biofilm. (A and C) Two-dimensional (A) and three-dimensional (C) images of biofilm formed by the WT strain in TSBg with 0.5 U/ml of bacitracin (1/8× MIC for the WT) and by the Δ*mbrC* mutant strain in TSBg with 0.0625 U/ml of bacitracin (1/2× MIC for the *mbr* mutants) are shown. The biofilm cells were stained with the Live/Dead BacLight bacterial viability kit for 30 min and observed with CLSM. A representative image from three independent experiments is shown. (B) Ratios of Live/Dead staining intensities were also observed in biofilms from WT, Δ*mbrC*, and Δ*mbrD* strains. These data are the mean ± SD of three independent experiments. The asterisks indicate a significant difference between WT and Δ*mbrC* or Δ*mbrD* strains (Student's *t* test; **, $P < 0.01$; *, $P < 0.05$; versus WT).

In turn, we focused on autolysis as a mechanism that led to the production of eDNA in biofilms. AtlA is the major autolysin in *S. mutans* (11). We predicted that this autolysin contributed to biofilm formation. We quantified the expression of the *atlA* gene in the early and mid-log phases by reverse transcription- quantitative PCR (RT-qPCR). In the early log phase, the expression levels of *atlA* in the WT strain were the same in the presence and absence of bacitracin (Fig. 4A). Meanwhile, the expression of *atlA* in

TABLE 2 Changes in the number of the live cells by bacitracin treatment[a]

| Strain	Change in no. of live cells	
	bac−	bac+
UA159 WT	$(5.53 \pm 2.79) \times 10^8$	$(5.58 \pm 3.58) \times 10^8$
Δ*mbrC* mutant	$(5.91 \pm 2.39) \times 10^8$	$(6.21 \pm 0.7) \times 10^8$
Δ*mbrD* mutant	$(5.38 \pm 0.87) \times 10^8$	$(5.27 \pm 2.04) \times 10^8$

[a]bac−, without bacitracin; bac+, with bacitracin.
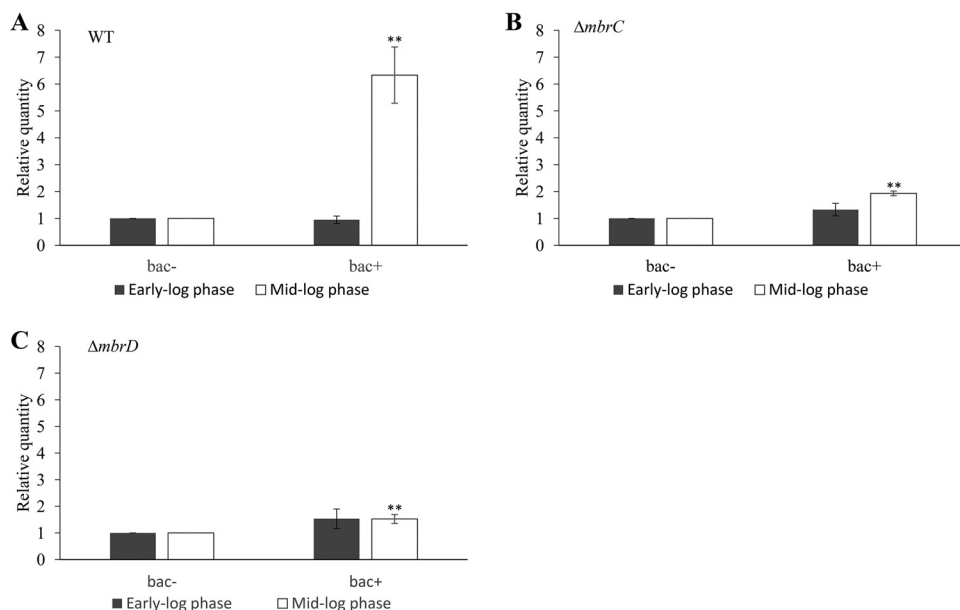
FIG 4 Gene expression of *atlA* in the presence of sub-MIC of bacitracin. We determined the gene expression of *atlA* at an OD_{600} of 0.3 (early log phase) and an OD_{600} of 0.5 (mid-log phase). (A) The WT strain was cultivated in TSBg with 0.5 U/ml of bacitracin (1/8× MIC for the WT) (bac+) and without bacitracin (bac−). (B and C) The ΔmbrC (B) and ΔmbrD (C) mutant strains were cultivated in TSBg with 0.0625 U/ml of bacitracin (bac+) and without bacitracin (bac−). These figures indicate the relative expression levels of *atlA* compared with the expression in TSBg cultivated without bacitracin. These data were normalized to the expression of an endogenous control (lactate dehydrogenase, *ldh*). These data are the mean ± SD of three independent experiments. The asterisks indicate a significant difference between two groups (Student's *t* test; **, $P < 0.01$, versus bac−).

the mid-log phase was higher in the presence of bacitracin than in the absence of bacitracin (Fig. 4A). These results suggest that autolysis caused by AtlA might be induced in response to bacitracin at the mid-log phase. On the other hand, the expression pattern of the *atlA* gene in the ΔmbrC and ΔmbrD strains was different from that in the WT strain. An increase in *atlA* expression was also observed in the presence of bacitracin even in the ΔmbrC and ΔmbrD mutant strains, but the increase in expression in these strains was less than that in the WT strain (Fig. 4B and C). These results showed that the induction of *atlA* expression was affected by the loss of responsiveness to bacitracin. In addition, this suggests that the difference in *atlA* expression may be responsible for the difference in the inducing concentrations and the morphology of the biofilm between the WT and *mbr* mutant strains. We then examined the role of *atlA* in biofilm formation using the *atlA* deletion mutant (Δ*atlA*). We first observed the cell morphology by phase-contrast microscopy and confirmed that in comparison with the WT strain, the Δ*atlA* strain formed a long chain (Fig. 5A and B). This result is in agreement with other reports (11). Biofilm formation induced by sub-MICs of bacitracin was not observed in the Δ*atlA* mutant (Fig. 5C and D). Therefore, the biofilm formation induced by sub-MICs of bacitracin was considered to be dependent on autolysis and cell separation after cell division by *atlA*, which is induced in the mid-log phase.

To confirm the involvement of polysaccharides, we performed a biofilm formation assay using deletion mutants of *gtfB* (Δ*gtfB*) (which encodes GtfB, an enzyme involved in the synthesis of an insoluble glucan), *gtfC* (Δ*gtfC*) (which encodes GtfC, an enzyme involved in the synthesis of an insoluble and soluble glucan), *gtfBC* (Δ*gtfBC*), *gtfD* (Δ*gtfD*) (which encodes GtfD, an enzyme involved in the synthesis of a soluble glucan), and *sacB* (Δ*sacB*) (which encodes Ftf, an enzyme involved in the synthesis of a soluble fructan). These strains deficient in polysaccharide synthesis did not form biofilms in the absence of bacitracin or in the presence of 1/2× MIC of bacitracin (Fig. 6A and B). The biofilm formation of the Δ*gtfC*, Δ*gtfD*, and Δ*sacB* strains was equivalent to that of
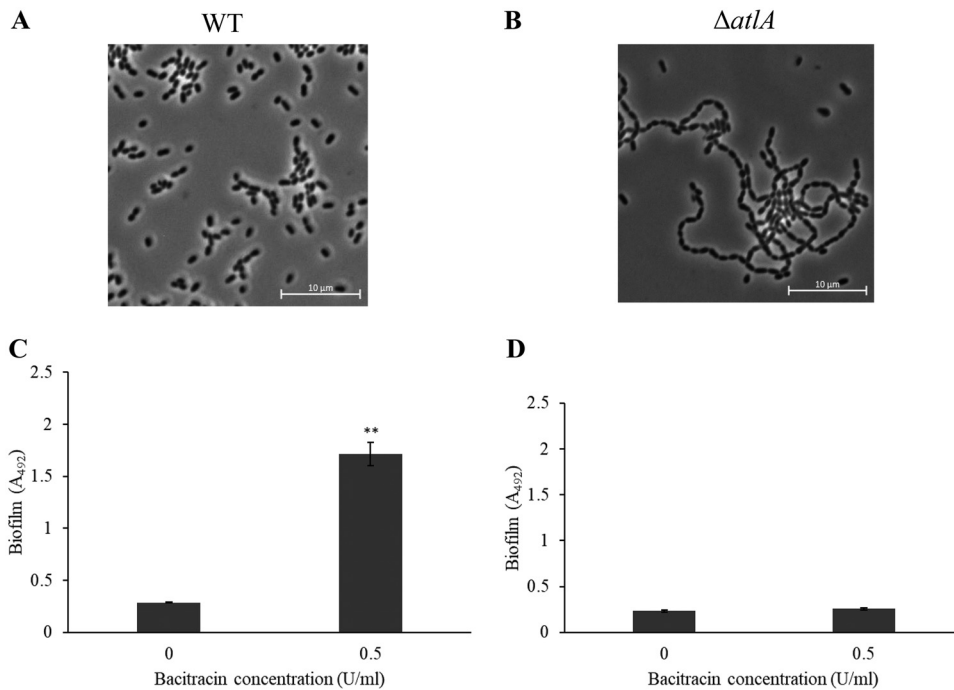
FIG 5 Cell morphology and biofilm formation of the ΔatlA strain. The WT (A) and ΔatlA (B) strains cultivated in TSBg were observed by phase-contrast microscopy. A biofilm formation assay was performed in TSBg with 0.5 U/ml of bacitracin (1/8× MIC for the WT) using the WT (C) and the ΔatlA (D) strains. The data are the mean ± SD of three independent experiments. The asterisks indicate a significant difference between two groups (Student's t test; **, $P < 0.01$, versus bac−).

the WT strain in the presence of 1/8× MIC of bacitracin, but biofilm formation was not induced in the ΔgtfB and ΔgtfBC strains (Fig. 6C). From this result, it was predicted that insoluble glucan synthesized by GtfB from sucrose was involved in inducing biofilm formation in the presence of sub-MICs of bacitracin, and TSB medium contained a small amount of sucrose. We then changed the biofilm formation medium from TSBg to semidefined medium plus 0.25% glucose (SDMg), a sucrose-free medium, and conducted the biofilm formation assay. Biofilm formation was not induced in the WT strain at any bacitracin concentration (Fig. S3). Therefore, it was suggested that polysaccharide synthesis by GtfB from sucrose, the abundance of which was low in TSB, is necessary for biofilm formation. To examine whether GtfB contributes to biofilm formation, we performed Live/Dead staining of the WT and ΔgtfB strains cultured in sucrose-free medium and observed cells by CLSM. The WT strain showed more aggregates formed by complexes of live and dead cells in the presence of bacitracin than in the absence of bacitracin (Fig. 7). However, the numbers of aggregates in the ΔgtfB mutant did not change with or without bacitracin (Fig. 7). ΔgtfC and ΔgtfD mutants showed results similar to those of the WT (data not shown). These results suggested that the physical presence of GtfB promoted the aggregation of cells, and the synthesis of insoluble glucan from the small amount of sucrose in TSB supported biofilm formation induced by sub-MICs of bacitracin.

Subsequently, we examined whether the Com regulation system, known as a cell death-inducing mechanism, was involved in biofilm formation and performed a biofilm formation assay using com gene mutant strains (ΔcomC, ΔcomD, ΔcomE, and ΔcomX) (31). comC, comD, comE, and comX encode a CSP precursor, a histidine kinase for CSP recognition, a response regulator for CSP response, and an alternative sigma factor for induction of a ComX regulon, respectively. Similar to the WT strain, the com mutant strains did not form biofilms in the absence of bacitracin or in the presence of 0.0625 U/ml of bacitracin (1/2× MIC for mbr mutants), but these mutants could form biofilms in the presence of 0.5 U/ml of bacitracin (1/8× MIC for the WT) (Fig. 6). Therefore, cell
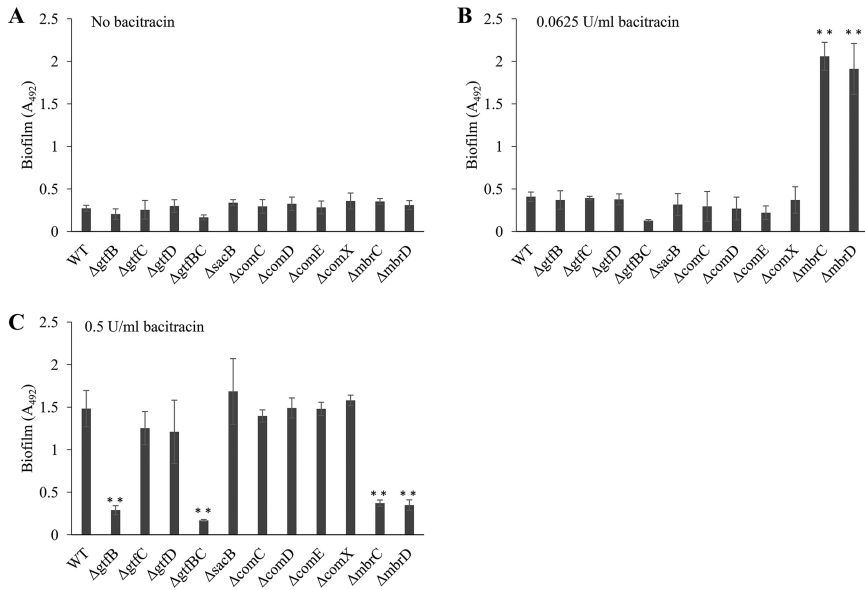
FIG 6 Effects of polysaccharides and Com regulation system on biofilm formation. (A to C) A biofilm formation assay was performed in TSBg without bacitracin (A), with 0.0625 U/ml of bacitracin (1/2× MIC for the *mbr* mutants) (B), and with 0.5 U/ml of bacitracin (C). To assess the involvement of polysaccharides and Com-dependent quorum sensing in biofilm formation, we examined the 5-h biofilm formed by the polysaccharide synthase and *com* gene mutants. The data are the mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA and a Bonferroni's posttest. The asterisks indicate a significant difference (**, $P < 0.01$).

death controlled by the Com regulation system was not involved in the eDNA-dependent biofilm formation induced by sub-MICs of bacitracin.

Cell envelope stress involvement in biofilm induction. If the biofilm formation induced by sub-MICs of bacitracin is associated with bacitracin-specific recognition by *mbrCD*, biofilm formation may be affected by the lack of responsiveness to bacitracin
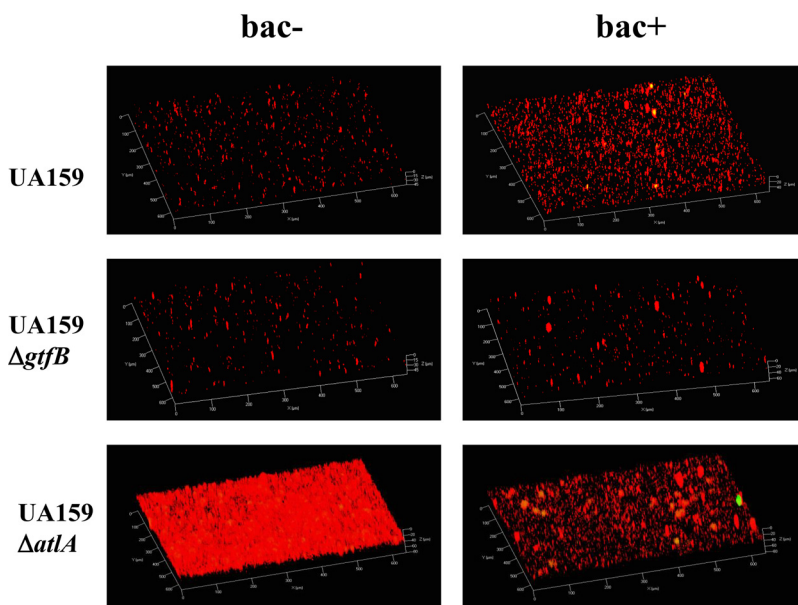
FIG 7 Observation of dead and live cells in the aggregates. CLSM analysis of aggregates was performed for the WT, Δ*gtfB*, and Δ*atlA* strains cultivated in BHI broth (bac−) or in BHI broth with 0.5 U/ml of bacitracin (1/8× MIC for the WT) (bac+). The aggregates were stained using the Live/Dead BacLight bacterial viability kit for 30 min and observed with CLSM. Live cells and dead cells are shown in green and red, respectively. A representative image from three independent experiments is shown.
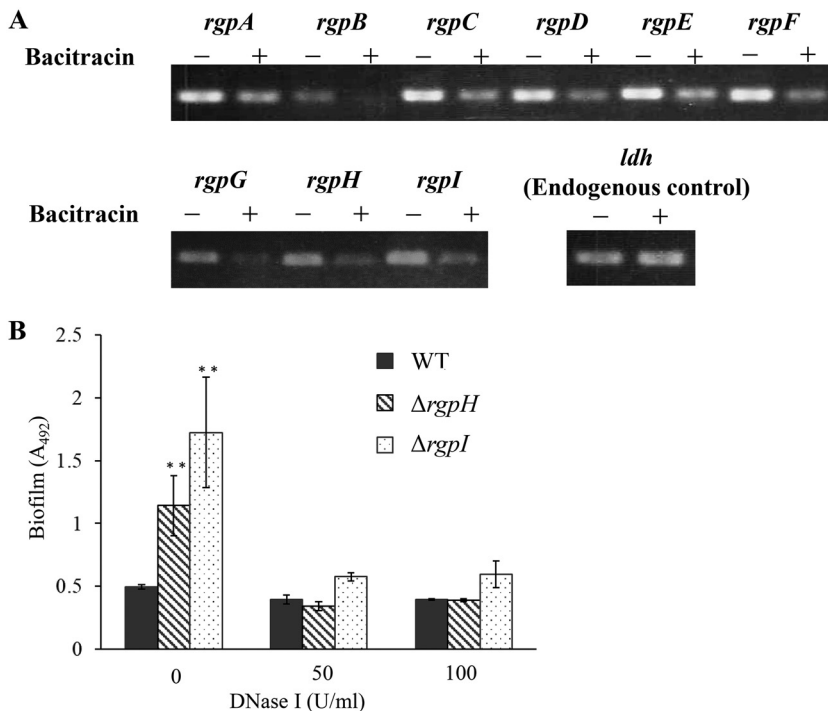
FIG 8 Expressions of *rgp* genes in biofilm stimulated with bacitracin. (A) The WT was cultured in TSBg or TSBg with 0.5 U/ml of bacitracin (1/8× MIC for the WT) until the early log phase (OD$_{600}$ = 0.3), and RNA was extracted from the cells. RT-PCR was performed using the RNA, and aliquots of PCR products were electrophoresed on a 2% agarose gel. The housekeeping gene *ldh* was used as an endogenous control. (B) To evaluate the effect of RGP deficiency on eDNA-dependent biofilm formation, a biofilm formation assay was performed in TSBg with or without DNase I using the Δ*rgpH* and Δ*rgpI* strains. The data are the mean ± SD of three independent experiments. The data were analyzed with one-way ANOVA and a Bonferroni's posttest. The asterisks indicate a significant difference (**, $P < 0.01$).

in the Δ*mbrC* and Δ*mbrD* strains. Nonetheless, the Δ*mbrC* and Δ*mbrD* strains retained the ability to form biofilms. This result suggested that biofilm formation was induced by a mechanism other than a bacitracin-specific response via the *mbr* system. We then hypothesized that *S. mutans* induced biofilm formation in response to cell envelope stress by bacitracin. *S. mutans* contains a gene, *brpA*, that participates in the regulation of the cell envelope stress response (32). We constructed a deletion mutant of *brpA* (Δ*brpA*) and conducted a biofilm formation assay using the mutant. When the Δ*brpA* strain was cultured in TSBg medium supplemented with various concentrations of bacitracin, the mutant did not form biofilms at any bacitracin concentration (Fig. S4). In contrast, biofilm formation in the Δ*brpA* strain was induced at levels comparable to those in the WT strain when the mutant strain was cultivated in TSB medium, supporting that polysaccharide was synthesized (Fig. S5). We confirmed that the Δ*brpA* strain displayed a long-chain phenotype, which was observed in another report (33) (Fig. S6). Therefore, the direct stimulation of biofilm formation induced by sub-MICs of bacitracin is dependent on cell envelope stress, and it has been suggested that the Δ*brpA* strain failed to respond to the stress caused by sub-MICs of bacitracin, exhibiting a long-chain phenotype that adversely affects biofilm formation (34), and could not induce eDNA-dependent biofilm formation.

S. mutans produces RGPs surrounding the surface of the cells. The loss of RGPs resulted in an increased susceptibility to certain cell wall-targeting antibiotics, while a recent study suggested that RGP may influence biofilm formation (35). We performed reverse transcription-PCR (RT-PCR) to examine whether the presence of bacitracin affected the expression of the RGP genes. The expression of all *rgp* genes decreased in the presence of sub-MICs of bacitracin (Fig. 8A). We confirmed that RT-PCR worked properly by checking the expression of a housekeeping gene, *ldh*. Additionally, we
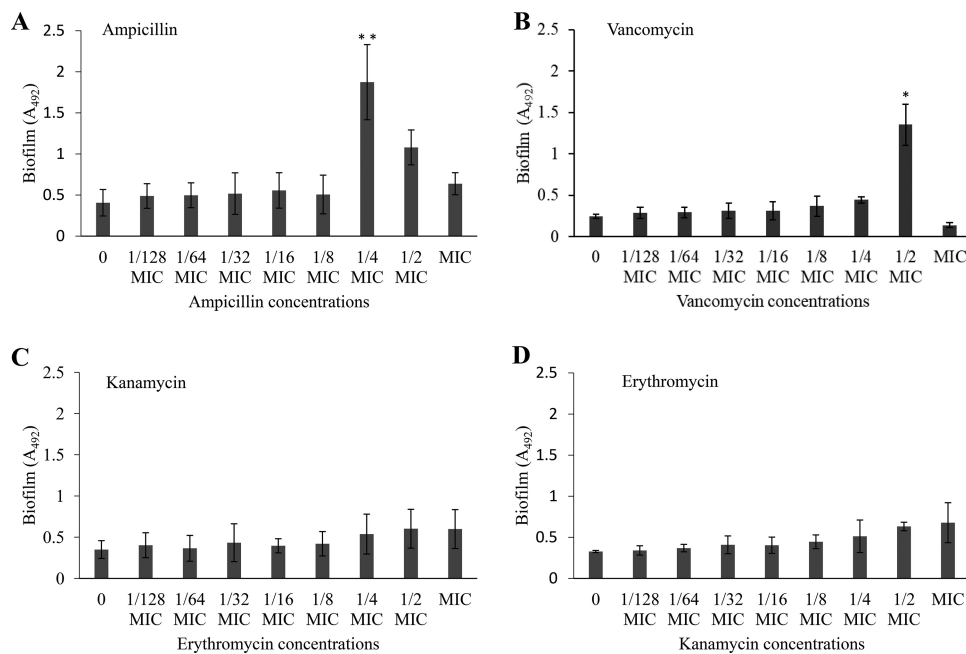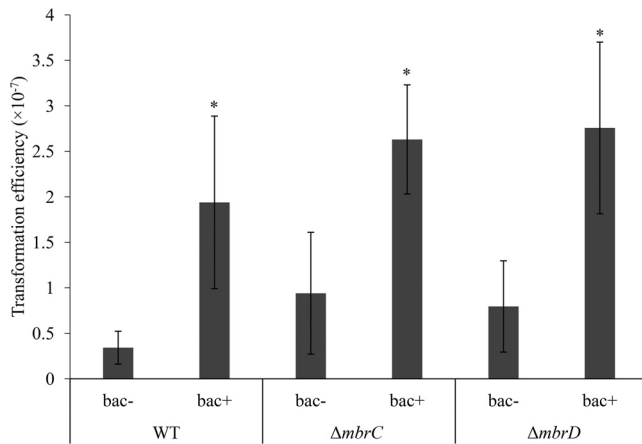
FIG 9 Biofilm formation assay in the presence of other antibiotics. To investigate whether antibiotics other than bacitracin induced biofilm formation, biofilm formation assays were conducted in the presence of antibiotics with different mechanisms of action: inhibition of peptidoglycan synthesis (ampicillin [MIC, 0.05 μg/ml] [A], vancomycin [1 μg/ml] [B]) and inhibition of protein synthesis (kanamycin [240 μg/ml] [C] and erythromycin [0.1 μg/ml] [D]). The data are the mean $\pm$ standard deviation (SD) of three independent experiments. The data were analyzed with one-way ANOVA and a Bonferroni's posttest. The asterisks indicate a significant difference (**, $P < 0.01$; *, $P < 0.05$).

constructed mutant strains with deletions in *rgpH* ($\Delta rgpH$) and *rgpI* ($\Delta rgpI$) and cultured these strains in the absence of bacitracin. The $\Delta rgpH$ and $\Delta rgpI$ strains formed biofilms, but the WT strain did not form biofilms (Fig. 8B). Biofilm formation by the $\Delta rgpH$ and $\Delta rgpI$ strains was inhibited by the addition of DNase I (Fig. 8B). Therefore, the reduced expression of *rgp* genes in the WT strain was thought to result in an increase in eDNA-dependent biofilm formation in the presence of sub-MICs of bacitracin.

If cell envelope stress by bacitracin induces eDNA-dependent biofilm formation, biofilm formation should also be induced by antibiotics other than bacitracin, which stresses the bacterial cell envelope. Biofilm formation assays were then conducted in the presence of antibiotics with different mechanisms of action. Biofilm formation was also induced in the presence of sub-MICs of ampicillin and vancomycin, antibiotics that inhibit peptidoglycan synthesis (Fig. 9A and B). However, biofilm formation was not induced in the presence of sub-MICs of erythromycin and kanamycin, which inhibit protein synthesis (Fig. 9C and D). From these results, it seemed that antibiotics that inhibit peptidoglycan synthesis caused cell envelope stress in bacterial cells and induced eDNA-dependent biofilm formation. In contrast, sub-MICs of other types of antibiotics did not induce signals for biofilm formation.

Increased risk of drug resistance by biofilm induction. It has been pointed out that biofilms can contribute to horizontal gene transfer (36). We examined the possibility that *S. mutans* may facilitate the acquisition of new drug resistance genes by inducing biofilm formation. Transformation assays using the pDL278 plasmid carrying a spectinomycin resistance gene showed that transformation efficiencies were increased in both the WT and *mbr* mutant strains in the presence of sub-MICs of bacitracin (Fig. 10). The transformation efficiency was also increased by sub-MICs of vancomycin (Fig. S7). Therefore, it was shown that the induction of biofilm formation in response to cell envelope stress by sub-MICs of bacitracin and vancomycin promotes horizontal gene transfer.

FIG 10 Transformation assay in the absence and presence of bacitracin. *S. mutans* was cultured in TSBg containing the pDL278 plasmid carrying a spectinomycin resistance gene. Bacitracin was added at the concentration at which each strain most strongly induced biofilm formation (bac+, 0.5 U/ml [1/8× MIC for the WT strain] and 0.0625 U/ml [1/2× MIC for the *mbr* mutant strains]). After culture for 5 h, the cells were cultured on a mitis salivarius agar plate with or without spectinomycin for 36 h. We measured the number of CFU and determined the transformation efficiency. The data are the mean ± SD of three independent experiments. The asterisks indicate a significant difference between two groups (Student's *t* test; *, $P < 0.05$, versus bac−).

DISCUSSION

In this study, we revealed that eDNA-dependent biofilm formation was induced at bacitracin concentrations below the MIC regardless of whether bacitracin resistance was present. Since the concentration that most strongly induced biofilm formation in the WT strain, 0.5 U/ml, was above the MIC of other bacitracin-sensitive and non-biofilm-forming bacteria in commensal oral flora, such as *Streptococcus salivarius* (37), the bacitracin concentration not only induces biofilm formation of *S. mutans* but may also change the balance of resistant and sensitive bacteria. Because the addition of antibiotics changed the prevalence and composition of the antibiotic-resistant microflora in dental plaque containing lactobacilli, streptococci, and *Actinomyces* spp. (38), it is important to understand the behavior of drug-resistant bacteria, especially those resistant to cell wall synthesis inhibitors, and take countermeasures against these bacteria. Data on drug-resistant bacteria will help to develop better bacterial control strategies.

The expression of *atlA* was increased in the *mbr* mutant strain at mid-log phase but not to a level as high as that of the WT strain. This suggests that WT strains more actively induce biofilm formation by changes in gene expression, whereas the induction mechanism of *mbr* mutant strains may be incomplete. In fact, based on CLSM, there were relatively many live cells in the biofilm formed by the WT strain, and the biofilm exhibited a microcolony-like morphology, which is one of the characteristics of the biofilm of *S. mutans* (6); however, the biofilm formed by the *mbr* mutants had relatively few live cells, and no prominent structures were observed. Since it was reported that the balance between lysed cells and living cells was important for drug-induced biofilm formation in *Enterococcus faecalis* (26), we examined cell death induction via the Com regulation system and the direct bactericidal effect of bacitracin, but these appeared to have little effect. Accordingly, it was predicted that the balance between live and dead cells due to the difference in the induction of *atlA* expression at mid-log phase resulted in the difference in the concentration that induced biofilm formation.

Biofilm formation in the presence of bacitracin was dramatically reduced with DNase I treatment, but biofilm formation was not completely polysaccharide independent because it was not induced in the Δ*gtfB* and Δ*gtfBC* strains. TSB contains a small amount of sucrose, which is a substrate for the synthesis of glucan (6). When cultured

in sucrose-free medium, the WT strain aggregated, while the Δ*gtfB* mutant did not aggregate. These results suggested that Gtf-I encoded by *gtfB* is involved in the aggregation of cells at the protein level, not in the enzymatic activity, because the substrate (sucrose) is absent. We previously reported that Gtf-I is essential for eDNA-dependent biofilm formation (6). Therefore, it was suggested that Gtf-I physically and chemically contributes to eDNA-dependent biofilm formation in TSBg with bacitracin medium.

We also revealed that the stress response of the cell envelope and RGP, which is part of the cell wall, were necessary for biofilm induction. The deletion of *brpA*, which plays pivotal roles in the cell envelope stress response, leads to various phenotypes (32, 33). The Δ*brpA* strain showed a long-chain phenotype consistent with other reports (33). It is known that abnormal chaining in streptococci results in a biofilm-defective phenotype (30). Our results also indicated that the Δ*brpA* strain was unable to form biofilms in the presence of sub-MICs of bacitracin. In addition, it was considered that the decreased expression of the RGP synthetic genes in the presence of sub-MICs of bacitracin contributed to biofilm induction. In streptococci, cells not encapsulated by polysaccharides showed increased attachment to surfaces and formed aggregates and biofilms (39–43). In addition, deletion of *rgpI* decreased glucan and increased eDNA levels (44). We confirmed that the Δ*rgpH* and Δ*rgpI* strains can form biofilms without bacitracin. Therefore, it was shown that *S. mutans* induces biofilm formation by regulating the structure of the cell surface and responding to cell envelope stress. In support of the notion that stresses on the cell envelope trigger biofilm formation, we showed that the cell wall-inhibiting antibiotics ampicillin and vancomycin also induce biofilm formation at sub-MICs. In Gram-positive bacteria, including *S. mutans*, it has recently been reported that biofilm formation was induced by antimicrobial agents targeting the cell envelope, including β-lactams (25–28). Therefore, it is necessary to elucidate the common mechanisms that may underlie biofilm formation.

The accumulation of microorganisms as mono- or polymicrobial aggregates is commonly referred to as a biofilm, which can consist of a diverse community of bacteria (45). The formation of these sessile communities and their inherent resistance to antibiotics are at the root of persistent and chronic bacterial infections. If resistant bacteria or highly tolerant bacteria form a multispecies biofilm with susceptible bacteria, there is a potential risk that various genes, including drug resistance genes, will spread within the community due to horizontal gene transfer. Therefore, understanding the behavior of preexisting drug-resistant and biofilm-forming bacteria and the role of cell wall inhibitory antibiotics in horizontal gene transfer is important for treating infectious diseases.

In conclusion, we analyzed the response of bacitracin-resistant bacteria such as *S. mutans* to bacitracin and focused mainly on biofilm formation. We found that regulation of the expression of the *atlA* and *rgp* genes, which are involved in cell surface properties, contributes to biofilm formation induced by sub-MICs of bacitracin. GtfB, which is highly bound to the cell surface, was also essential for biofilm formation. Our results suggested that *S. mutans* induced eDNA-dependent biofilm formation in response to the cell envelope stress caused by sub-MICs of bacitracin and other cell wall inhibitory antibiotics. In addition, drug resistance genes may spread in biofilms, since horizontal gene transfer occurs more frequently in biofilm cells than in planktonic cells and biofilms contain a large amount of eDNA. Therefore, when using cell wall-targeted antibiotics at sub-MICs, attention may need to be paid to the behavior of resistant bacteria in bacterial infections.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 3. *S. mutans* UA159 and isogenic mutants were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C in an anaerobic atmosphere containing 5% CO_2 (AnaeroPack-CO_2; Mitsubishi Gas Chemical Company, Tokyo, Japan). These prepared cells were used in this study.

Construction of mutants. Primers used to construct mutants are listed in Table 4. Sequences of the target genes were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database

TABLE 3 Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
S. mutans strains		
UA159	Wild type; Erm[s] Kan[s] Spc[s]	53
Δ*mbrC* mutant	UA159 *mbrC* Erm[r]	This study
Δ*mbrD* mutant	UA159 *mbrD* Erm[r]	This study
Δ*gtfB* mutant	UA159 *gtfB* Erm[r]	6, 54
Δ*gtfC* mutant	UA159 *gtfC* Kan[r]	6, 54
Δ*gtfD* mutant	UA159 *gtfD* Kan[r]	6
Δ*sacB* mutant	UA159 *sacB* Erm[r]	6
Δ*gtfBC* mutant	UA159 *gtfB* Erm[r] *gtfC* Kan[r]	6, 54
Δ*brpA* mutant	UA159 *brpA* Erm[r]	This study
Δ*atlA* mutant	UA159 *atlA* Erm[r]	N. Obana, University of Tsukuba, unpublished
Δ*rgpH* mutant	UA159 *rgpH* Erm[r]	This study
Δ*rgpI* mutant	UA159 *rgpI* Erm[r]	This study
Plasmids		
pUC19+Em	pUC19 containing Erm[r]	6
pResEmMCS10	Streptococcal integration plasmid; Erm[r]	30
pDL278	*E. coli-Streptococcus* shuttle vector; Spc[r]	35

[a]Erm[s], erythromycin susceptibility; Kan[s], kanamycin susceptibility; Spc[s], spectinomycin susceptibility; Erm[r], erythromycin resistance; Kan[r], kanamycin resistance; Spc[r], spectinomycin resistance.

(http://www.genome.jp/kegg/). Deletion mutants were generated by inserting an antibacterial resistance gene as described previously (6, 46). DNA fragments for generating Δ*mbrC*, Δ*mbrD*, Δ*rgpH*, and Δ*rgpI* deletion mutants were created with PCR and ligation (41). The upstream and downstream sequences of the target genes and the erythromycin resistance gene were amplified from *S. mutans* UA159 gDNA and pUC19+Em, which is a plasmid with the erythromycin resistance gene from pResEmMCS10 inserted in the multicloning site, respectively (47). The amplicons were digested with BamHI or BamHI and HindIII, and the resulting fragments were ligated. These fragments were introduced into *S. mutans* UA159 competent cells. The DNA fragment for generating the Δ*brpA* mutant was constructed by overlap extension PCR. The upstream and downstream sequences of *brpA* were amplified from gDNA of *S. mutans* UA159. The erythromycin resistance gene was amplified from pUC19+Em. The amplicons were ligated with overlap extension PCR, and the resultant fragment was introduced into *S. mutans* UA159. The transformants were selected on mitis salivarius agar (Difco Laboratories, Detroit, MI) plates supple-

TABLE 4 Primers for mutant construction

Primer	Sequence[a]
mbrC uF	cccccgaattcCTTCCTGCCTTTACTTATGGC
mbrC uR	cccccggtaccTGGGCATGGTCATGCTCTTTC
mbrC dF	ccccctctagaAAGAGACTATCCAATTGACTCC
mbrC dR	cccccaagcttTGTCAGACTGTTCCTGCAGC
mbrD uF	cccccgaattcGTGCTAACGAGTTCACTAAGC
mbrD uR	cccccggtaccCCGCCAGTATAAAATGAGAC
mbrD dF	ccccctctagaACTGCTGCAGGAACAGTCTGAC
mbrD dR	cccccaagcttCTTAATGGTGATCTGCCCAC
brpA uF	GTTGGTACTCTCATTGGTACAGGAG
brpA uR	cccatgcgtttgggcccCTCCTAAAGCTACCAAGC
brpA dF	cccaaggagatggccggCAGATTACAGTAGCAGCG
brpA dR	CCATGGCTATCATCTCCAAGTGCTC
ermR F	gggcccaaacgcatgggAATACGCAAACCGCCTCT
ermR R	ccggccatctccttgggGTGCCACCTGACGTCTAA
832 uF	GGCAAGGGCTATACAAAA
832 uR-Bam	ccggatccGGCGGTAATGATTCTTAATAAAA
832 dF-Bam	ccggatccCGTTGATTATTTATACGG
832 dR	AGCGTCCATAACCGTAAT
833 uF	AGAATTATTTTTGTAACCGCAGA
833 uR-Bam	ccggatccTGGTTTCTTCCTCATTATAACAA
833 dF-Bam	ccggatccGGAAACCAAGAAAAGACCA
833 dR	GTCGTACTAAATTATTGCCGAA

[a]Additional sequences which do not correspond to the sequences of the template DNA are indicated by lowercase letters.

mented with erythromycin (10 μg/ml). Mutations in the target genes of the genomic DNA were confirmed by PCR.

Antimicrobial susceptibility assays. A broth microdilution method was used with a 96-well microtiter plate to identify the MICs of the antibiotics bacitracin (Wako Pure Chemical Industries, Tokyo, Japan), ampicillin (Wako Pure Chemical Industries, Tokyo, Japan), vancomycin (Wako Pure Chemical Industries, Tokyo, Japan), kanamycin (Sigma Chemical, St. Louis, MO), and erythromycin (Sigma Chemical, St. Louis, MO) for the *S. mutans* UA159 strain. Two microliters of overnight culture was inoculated into 198 μl of BHI broth containing a 2-fold dilution series of antibiotics. The culture plates were incubated at 37°C for 24 h in an aerobic atmosphere containing 5% CO_2. After incubation, the optical density at 620 nm (OD_{620}) was measured on a plate reader (Thermo Bioanalysis Japan, Tokyo, Japan).

Minimal bactericidal concentrations (MBCs) of bacitracin were identified using MIC test plates. A total of 20 μl of culture was plated on a BHI agar plate without antibiotics, and the MBC was determined as the lowest concentration at which <5 CFU were observed after 24 h of incubation at 37°C in an aerobic atmosphere containing 5% CO_2.

Human saliva collection. Whole saliva samples from volunteers with good oral health were collected in ice-chilled sterile bottles. To remove debris, the whole saliva was centrifuged (4°C, 14,000 $\times$ g, 15 min). The supernatant was filtered with a 0.22-μm syringe filter (Millex-GP; Merck Millipore, Darmstadt, Germany) to sterilize the sample. The sterilized saliva was used immediately for biofilm formation assays.

Biofilm formation assays. We used TSB (Difco Laboratories, Detroit, MI) supplemented with 0.25% (wt/vol) TSBg or TSBs or semidefined medium (SDM) with 0.25% glucose for biofilm formation (6). Overnight cultures were inoculated into these media at a ratio of 1:5 in a human saliva-coated 96-well microtiter plate (Sumitomo Bakelite, Tokyo, Japan). The biofilm formation assay was performed at 37°C for 5 h in an aerobic atmosphere containing 5% CO_2 under static conditions. After incubation, the medium was removed, and the wells were washed two times with distilled water (DW). The plate was air dried, and the biofilm was stained with safranin (0.25% safranin and 0.5% ethanol in H_2O; Muto Pure Chemicals, Tokyo Japan) for 15 min. The wells were then washed two times with DW to remove the excess dye and air dried. Safranin was extracted from the biofilm with 100 μl of 70% (vol/vol) ethanol, and the absorbance at 492 nm (A_{492}) was measured.

To evaluate the contribution of eDNA to biofilm formation, we conducted a biofilm inhibition experiment by adding DNase I. Before the biofilm formation assay, 50 U/ml of DNase I (Roche Applied Science, Mannheim, Germany) was added to the medium to evaluate the inhibitory effect of eDNA degradation on biofilm formation. The biofilm formation assay and quantification were performed by the above-mentioned procedure.

Quantitative analysis of eDNA. Quantification of eDNA was conducted as described elsewhere (48). Briefly, 1 ml of overnight culture was mixed with 5 ml of TSBg with or without bacitracin in a saliva-coated 6-well plate (Iwaki, Tokyo, Japan). The plate was incubated at 37°C for 5 h in an aerobic atmosphere containing 5% CO_2 under static conditions. After incubation, the biofilm formed on the bottom of the culture plate was removed, and the biofilm and supernatant were treated with 5 U/ml dextranase (Sigma Chemical, St. Louis, MO) at 40°C for 30 min and proteinase K solution (Qiagen, Venlo, The Netherlands) at 56°C for 30 min to disperse the biofilm cells. The sample was filtered through a 0.22-μm syringe filter to remove the cells. The eDNA contained in the sample was isolated with the cetyltrimethylammonium bromide (CTAB) method as described elsewhere (49). The yield of eDNA was quantified by absorptiometry (NanoDrop Lite spectrophotometer; Thermo Fisher Scientific, Waltham, MA).

CLSM. Biofilm cells were stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Leiden, The Netherlands) after biofilms were formed. SYTO 9 and propidium iodide (PI) were used to stain bacteria with intact cell membranes (live cells, green fluorescence) and bacteria with damaged cell membranes (dead cells, red fluorescence), respectively. After 30 min of staining, the biofilm was washed two times with DW to remove excess dye. The biofilms were observed by confocal laser scanning microscopy (CLSM; LSM 700, Carl Zeiss, Jena, Germany), and three-dimensional (3D) images of biofilms were acquired by taking two-dimensional (2D) images with a Plan-Apochromat 10$\times$/0.45 M27 lens objective. Zen software (Zeiss) was used to analyze the biofilms.

RNA extraction and RT-qPCR. Total RNA was isolated from cells according to the method established by Cury et al. (50). To collect the cells at early and mid-log phase, 2 ml of overnight culture and 28 ml of TSBg with or without bacitracin were mixed in a 50-ml polyethylene tube and incubated at 37°C in an aerobic atmosphere containing 5% CO_2 under static conditions until the early log ($OD_{600} = 0.3$) and mid-log ($OD_{600} = 0.5$) phases were reached. The cells were collected by centrifugation (5,500 $\times$ g, 10 min) and resuspended in cold phosphate-buffered saline (PBS). To separate the cells and extracellular substances, the cells were sonicated at 2 W for 30 s. These manipulations, including resuspension and sonication, were repeated two times. The bacterial cell pellet was suspended in NAES buffer (50 mM sodium acetate buffer, 10 mM EDTA, and 1% SDS [pH 5.0]). A total of 625 μl of acidified phenol and 125 μl of chloroform was added, and the sample was transferred to a 2-ml tube containing 0.5 g of 0.1-mm-diameter glass beads; the samples were shaken on a TissueLyser (Qiagen, Venlo, The Netherlands) for 3 min. After centrifugation (4°C, 14,000 $\times$ g, 5 min), the aqueous phase of the samples was transferred into a microtube, and 625 μl of acidified phenol and 125 μl of chloroform were added. These manipulations were repeated one more time. After centrifugation (4°C, 14,000 $\times$ g, 5 min), the aqueous phase of the samples was transferred to a microtube, and 750 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the solution. The aqueous phase of the samples was transferred to a microtube after centrifugation (4°C, 14,000 $\times$ g, 5 min). The total RNA in solution was precipitated with 2-propanol and washed with 70% ethyl alcohol (EtOH) three times. cDNA was synthesized with ReverTra Ace qPCR

TABLE 5 Primers for RT-qPCR and RT-PCR

Primer	Sequence
ldh-Fw	TTGGCGACGCTCTTGATCTTAG
ldh-Rv	GTCAGCATCCGCACAGTCTTC
atlA-Fw	CAGCAAACACAGGAACAGTCTG
atlA-Rv	TCAGGAGTCTGCACAGAACTAG
rgpA-Fw	GCAGTTAACTTATCATGTGGCCTG
rgpA-Rv	CATATCATAAGCAATGACTCTGGCAG
rgpB-Fw	GCAGACTTTGCTAAGTCTGATGC
rgpB-Rv	CTGCCAAACATCATCTTGATCGC
rgpC-Fw	CGTTATCAAGGAAGCGCCATTG
rgpC-Rv	CCATACCCATCATGGTTGTTTCTTG
rgpD-Fw	GTTTGAGAACTGCCTTAGTCAATCG
rgpD-Rv	CAGAACCATTTCGGCCTACAATAC
rgpE-Fw	GAAGCTGGATTGGTGAAGCTATTG
rgpE-Rv	CTAGCTTTCTTACAAATATCGGACCAAG
rgpF-Fw	CGTTTATCAGTTGACTCAAATGAGATCC
rgpF-Rv	CCAGAATTCTGCCGTTGAATGAAG
rgpG-Fw	GTTGATAATCCAAATGCGAGACGG
rgpG-Rv	CAAAATAGGATTTTCCTCCGATTTGC
rgpH-Fw	GTTTTATTAAGAATCATTACCGCCTATTG
rgpH-Rv	GATCGTTTGATCTCCTGCATC
rgpI-Fw	GTTATAATGAGGAAGAAACCATTTACCC
rgpI-Rv	CTGTTAAAGTCTTATCACTTGAACCATC

RT master mix with gDNA remover (Toyobo, Osaka, Japan) using 500 ng of total RNA as the template for reverse transcription. RT-PCR using the primers in Table 5 was performed by Tks Gflex DNA polymerase (TaKaRa, Shiga, Japan). We used the lactate dehydrogenase gene *ldh*, which is a housekeeping gene in *S. mutans*, as an endogenous control (51).

Transformation assay. Cultures of the WT, Δ*mbrC*, and Δ*mbrD* strains were prepared by the same procedure described for the biofilm formation assay in TSBg. The concentrations of bacitracin were 0.5 U/ml (1/8× MIC) for the WT and 0.0625 U/ml (1/2× MIC) for the *mbr* mutant strains. The pDL278 plasmid was added to each medium at a final concentration of 2.5 μg/ml (52). After culture for 5 h under the same conditions as for biofilm formation, the cell suspensions were poured and cultured on mitis salivarius agar plates with or without spectinomycin (500 μg/ml) for 36 h. We counted the number of CFU and determined the transformation efficiency.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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