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Short Communication

Bacitracin Upregulates *mbrAB* Transcription via *mbrCD* to Confer Bacitracin Resistance in *Streptococcus mutans*Yoshikazu Mikami^{1,2}, Naoto Suzuki^{2,3}, Tomihisa Takahashi^{1,2}, Kichibee Otsuka^{2,3}, and Hiromasa Tsuda^{2,3,*}¹Department of Anatomy, ²Division of Functional Morphology, Dental Research Center, ³Department of Biochemistry, Nihon University School of Dentistry, 1-8-13 Kanda Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan

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Abstract. *Streptococcus mutans* is a bacterial cause of dental caries that is resistant to bacitracin. The aim of this study was to elucidate the *mbrABCD*-related bacitracin resistance mechanism of *S. mutans*. Transcriptome data demonstrated that the expression levels of 33 genes were induced more than twofold by bacitracin. Fourteen genes were selected from the upregulated genes, and defective mutants of these genes were constructed for measurement of their sensitivity to bacitracin. Among the mutants, only the *mbrA*- or *mbrB*-deficient mutants exhibited 100- to 121-fold greater sensitivity to bacitracin when compared with the wild-type strain. Moreover, knockout of the *mbrC* and *mbrD* genes abolished the bacitracin-induced *mbrAB* upregulation. These results suggest that both *mbrC* and *mbrD* are required for *mbrAB* upregulation that confers the bacitracin-resistant phenotype on *S. mutans*.

[Supplementary Table: available only at <http://dx.doi.org/10.1254/jphs.11052SC>]**Keywords:** *Streptococcus mutans*, bacitracin, bacitracin resistance

The emergence and widespread incidence of multidrug resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), have produced a therapeutic dilemma, especially for patients with compromised immune systems. Bacitracin is a cyclic polypeptide antibiotic produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* (1, 2), which was tested as a potential application to eliminate MRSA or VRE and proved successful in some cases (3, 4). However, the possibility that multidrug-resistant bacteria will acquire bacitracin resistance in the future cannot be ignored.

Streptococcus mutans is a primary etiologic agent of dental caries that is also known to be resistant to bacitracin (5, 6). Therefore, understanding the mechanisms of bacitracin resistance in *S. mutans* is important when considering strategies for defense against multidrug-resistant bacteria.

S. mutans strains used in this study were cultured in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) at 37°C in a 5% CO₂ atmosphere. To

select and maintain the defective mutants, erythromycin (Wako, Osaka) at 10 µg/ml was added to the cultures.

At first, the transcriptome response of *S. mutans* to bacitracin was analyzed using the NimbleGen based DNA-Chip system (Nandemo Array; Gene Frontier, Kashiwa). Briefly, *S. mutans* strain UA159 culture was incubated for 30 min at 37°C in the absence or presence of 4 units (U)·ml⁻¹ bacitracin, and the bacterial cells were harvested followed by subsequent total RNA extraction. Ten micrograms of total RNA were reversely transcribed into cDNA. cDNA fragmentation, biotin labeling, and hybridization of labeled cDNA probes to arrays were performed according to the protocol provided by NimbleGen Systems (NimbleGen Systems, Madison, WI, USA). After hybridization, the arrays were scanned at 532 nm with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA), and the signal intensity data for each probe on the array was processed by Robust Multi-chip Analysis. The GEO accession number for the microarray data is GSE31502.

Bacitracin induced the expression of 33 genes by more than twofold and none of the other genes were decreased by less than one fifth. The induction levels of *mbrA* and *mbrB* were the highest (35.7 and 33.5, respectively). It was previously reported that *mbrABCD* and rhamnose-

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glucose polysaccharide (RGP) syntheses are related to bacitracin resistance in *S. mutans* (6). *mbrA* and *mbrB* genes have strong similarities with the components of other bacterial ABC transporters (6). The MbrC and MbrD proteins have high levels of identity with response regulators and histidine sensor kinases, respectively, found in the two-component regulatory systems of some bacteria (6). *S. mutans* mutants defective in *mbrABCD* exhibit 100–120 times greater bacitracin sensitivity when compared with the wild-type strain (6). On the other hand, *S. mutans* RGP consists of an α -1,2- and α -1,3-linked rhamnosyl polymer backbone structure with glucose side chains, which is localized on the surface of the *S. mutans* cell wall. *rgpA-G* is known to be required for the transport and assembly of RGP (7, 8), while *rmlA-D* is involved in the synthesis of dTDP-L-rhamnose, which is a component of the RGP-backbone (8, 9). Knockout of these genes inhibits RGP incorporation into cell walls and mutants exhibit approximately five times greater bacitracin sensitivity (6). Thus, it was expected that bacitracin treatment would increase the transcription level of *mbrABCD*, *rgps*, and *rmls*. However, microarray analysis showed that bacitracin treatment resulted in a reduction or no change in the expression of these genes, with the exception of *mbrAB*. This suggests that *mbrCD*, *rgps*, and *rmls* expression are all required for bacitracin resistance in *S. mutans* but they are not upregulated by bacitracin treatment. Thus, the expression level of these genes in the presence of bacitracin is sufficient for *S. mutans* to have a bacitracin resistance phenotype.

In the second part of this study, we selected 12 gene candidates that were considered responsible for bacitracin resistance from among genes that were induced more than twofold. The 12 genes included 6 genes that were induced by bacitracin more than 4 times and 6 genes involved in transport systems, gene transcription, and cell cycle. We then constructed the mutants defective in these genes and measured their bacitracin sensitivities. Deletion mutants for each gene were constructed in *S. mutans* wild-type strain Xc using a rapid PCR ligation mutagenesis strategy, which involved PCR, restriction, and ligation, followed by allelic replacement, as described previously (10). The primers used in transformant construction are listed in Supplementary Table 1 (available in the online version only) and the strains defective in each gene are listed in the Table 1. A *gtfB*-deficient strain, Xc12, which lacked glucosyltransferase I, was used as an erythromycin-resistant negative control (Table 1). The minimum inhibitory concentration (MIC) of bacitracin for *S. mutans* strains was determined as described previously (6). Briefly, mid-log phase *S. mutans* cultures were adjusted to 5×10^6 CFU·ml⁻¹, and 100 μ l of this suspen-

Table 1. MICs of bacitracin for *S. mutans* strains used in this study

Strain ^a	Inactivated gene	MICs (U/ml) ^b
Xc		4.0 \pm 0.0
Xc12	<i>gtfB</i>	4.0 \pm 0.0
Xc101	<i>mbrA</i>	0.040 \pm 0.000 ^c
Xc102	<i>mbrB</i>	0.033 \pm 0.009 ^c
Xc103	<i>mbrC</i>	0.033 \pm 0.009 ^c
Xc104	<i>mbrD</i>	0.040 \pm 0.000 ^c
Xc151	SMU.1856c	4.0 \pm 0.0
Xc152	SMU.863	4.0 \pm 0.0
Xc153	SMU.862	4.0 \pm 0.0
Xc154	SMU.864	4.0 \pm 0.0
Xc155	SMU.1479	4.0 \pm 0.0
Xc156	SMU.302	4.0 \pm 0.0
Xc157	SMU.589	4.0 \pm 0.0
Xc158	SMU.412c	4.0 \pm 0.0
Xc159	SMU.934	4.0 \pm 0.0
Xc160	SMU.930c	4.0 \pm 0.0
Xc161	SMU.933	4.0 \pm 0.0
Xc162	SMU.2057c	4.0 \pm 0.0

^aFor all strains except for Xc, the genes were inactivated.

^bMICs are expressed in international units per milliliter. Each value represents the mean \pm S.D. for assays performed three times. ^cDifferences from strain Xc ($P < 0.05$).

sion was inoculated into 2.9 ml of BHI broth containing different concentrations of bacitracin (Wako). After incubation for 20 h at 37°C, the OD₅₅₀ of test cultures was measured. In the absence of bacitracin, cultures of all strains reached the stationary phase within 20 h at 37°C. Relative cell densities were calculated as the percentages of the OD₅₅₀ for the culture in the presence of different concentrations of bacitracin, relative to a control culture without bacitracin. The MIC was determined as the minimum bacitracin concentration needed to ensure that the culture did not grow by more than 10% of the relative cell density. Each assay was repeated three times. Mutant strains defective in *mbrA* (Xc101) and *mbrB* (Xc102) exhibited 100- to 120-fold higher sensitivity to bacitracin when compared with the wild type (Table 1). In contrast, the other mutant strains showed the same sensitivity to bacitracin as the wild-type strain Xc (Table 1). On the basis of these results, the induction of *mbrAB* genes may be a critical determinant of bacitracin resistance in *S. mutans*.

The microarray analysis showed that bacitracin treatment hardly increased *mbrCD* transcription. Thus, the effect of bacitracin treatment on the transcription of

mbrABCD was confirmed using real-time reverse transcription (RT)-PCR. RT reaction and quantitative real-time PCR were performed as described previously (6). In brief, 500 μ l aliquots of an overnight culture of the *S. mutans* wild-type strain Xc or the mutants were inoculated into 4.5 ml of BHI broth and incubated at 37°C. The total RNA was purified after culture using a FastPure RNA Kit (TaKaRa, Otsu) with DNase treatment, according to the manufacturer's protocol. cDNAs were synthesized using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka), according to the manufacturer's protocol. Real-time PCR was performed using a Thermal Cycler Dice Real Time System (TaKaRa) and Thunderbird SYBR qPCR Mix (Toyobo). The oligonucleotide primers used for real-time PCR are listed in Supplementary Table 1. 16S rRNA was used as an internal reference gene. Data were calculated as the relative expression levels of mRNA compared with the control (data from strain Xc in the absence of bacitracin) after normalization with endogenous housekeeping *16S rRNA* expression levels. The unpaired Welch's *t*-test was used in statistical analyses. The real-time RT-PCR results were consistent with the microarray analysis (Fig. 1): bacitracin treatment induced the level of *mbrAB* genes in the wild-type strain Xc but those of *mbrC* or *mbrD* transcription was not significantly induced by bacitracin treatment (Fig. 1).

Deduced amino acid sequences encoded by *mbrCD* exhibit high levels of identity with the response regulators and the histidine sensor kinases, respectively, of two-component regulatory systems in certain bacteria (6). In addition, they have some characteristic motifs found in each component of the two-component system

(6). Therefore, it was hypothesized that *mbrCD* controls the induction of *mbrAB* transcription in the presence of bacitracin. To evaluate the hypothesis, we compared the expressions of *mbrAB* between wild type and mutants defective in *mbrCD*. Significant *mbrAB* expression was induced in the wild-type strain by bacitracin treatment. On the other hand, the *mbrC*- or *mbrD*-deficient mutants failed to induce *mbrAB* transcription (Fig. 2), and the mutants exhibited 100–120 times more sensitivity to bacitracin than the wild type (Table 1).

The present study is the first to show that bacitracin induces *mbrAB* transcription via *mbrCD* without upregulating *mbrCD* transcription, which results in bacitracin resistance. Although bacitracin treatment seemed to slightly increase the *mbrC* transcription level, no statistically significant difference was observed between bacitracin-treated cells and untreated control cells (Fig. 1). Therefore, it is suggested that the increase in the *mbrC* transcription level was not a significant factor contributing to the important increase in *mbrAB* transcription after bacitracin treatment. We next considered that a post-transcriptional modification of MbrCD, such as phosphorylation, might play an important role in the mechanism by which bacitracin induces *mbrAB* transcription in *S. mutans*. Indeed, Kitagawa et al. (11) reported that the aspartate residue at position 54 of MbrC is a promising candidate for phosphorylation in a bacitracin-sensing system and is indispensable for the bacitracin resistance phenotype of *S. mutans*. In addition, they demonstrated that MbrC binds to the promoter region of *mbrA*. On the basis of these reports, Kitagawa et al. (11) concluded that MbrC phosphorylation up-regulates *mbrA* transcription,

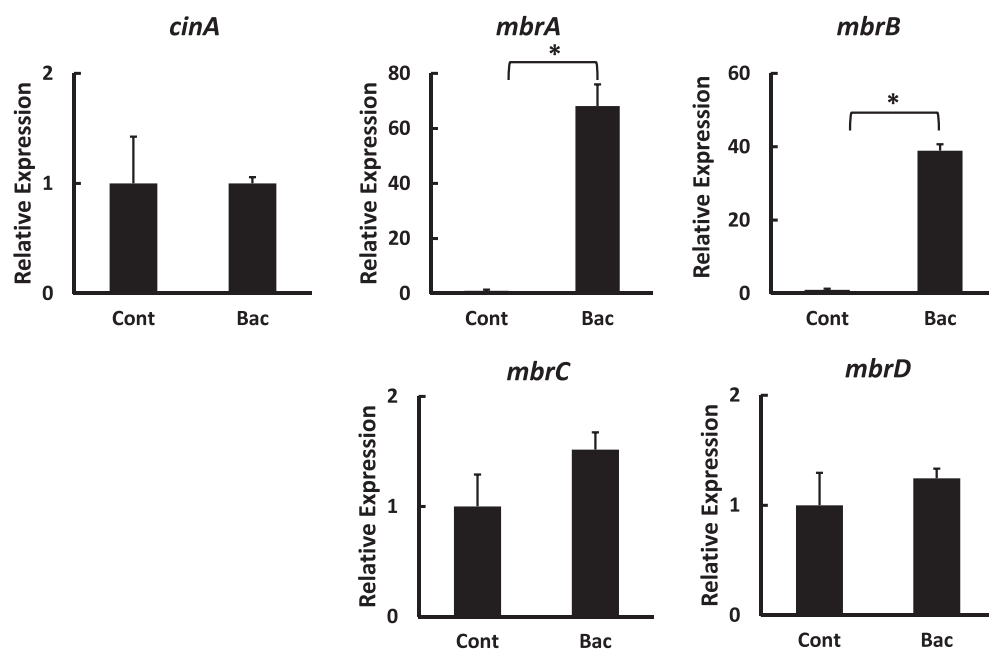


Fig. 1. Bacitracin treatment up-regulates *mbrAB* but not *mbrCD*. Effects of bacitracin treatment on *mbrABCD* expression. The expression levels of *mbrA*, *mbrB*, *mbrC*, and *mbrD* were measured using real-time RT-PCR of total RNA from wild-type strain Xc treated with or without 4 U·ml⁻¹ bacitracin at 37°C for 30 min. Data were normalized using endogenous housekeeping *16S rRNA* expression levels (n = 3, *P < 0.01). *cinA* expression was used as the negative control for bacitracin treatment.

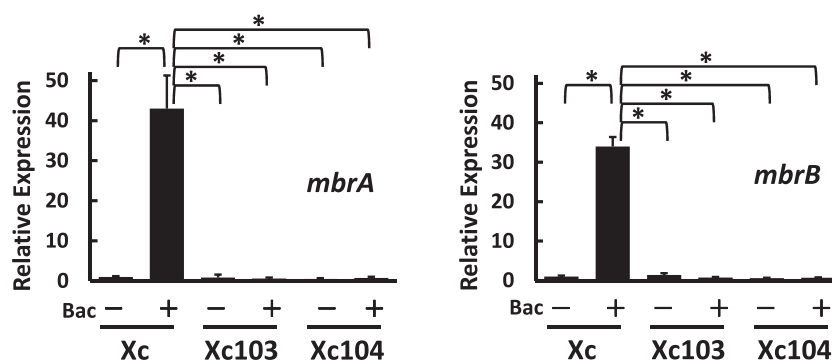


Fig. 2. Bacitracin treatment failed to upregulate *mbrAB* in *mbrCD* mutants. Effects of the inactivation of *mbrC* or *mbrD* on bacitracin-induced *mbrA* and *mbrB* upregulation. The expression level of *mbrA* and *mbrB* were measured using real-time RT-PCR with total RNA from wild-type strain Xc, *mbrC* mutant (Xc103), and *mbrD* mutant (Xc104) treated with or without 4 U·mL⁻¹ bacitracin at 37°C for 30 min. Relative expression of *mbrA* or *mbrB* was compared with strain Xc without bacitracin treatment, as shown. Data were normalized using endogenous housekeeping *16S rRNA* expression levels (n = 3, *P < 0.01).

resulting in the bacitracin resistance of *S. mutans* strain UA159. In their report, however, no evidence indicating the importance of MbrD for *mbrAB* upregulation and the bacitracin resistant phenotype was presented. By contrast, our data showed that *mbrAB* upregulation and the bacitracin resistant phenotype of *S. mutans* strains Xc is required for not only *mbrC* but also *mbrD* expression (Fig. 1). MbrD proteins exhibit high levels of identity with histidine sensor kinases of two-component regulatory systems. The histidine sensor kinase of two-component systems typically autophosphorylate their own histidine residues and transfer the phosphate groups to aspartate residues of the response regulator (12). Therefore, the *mbrD* gene product may work as a histidine kinase of two-component systems, which phosphorylates aspartate residues of MbrC and transcriptionally upregulate *mbrAB* genes. Although there is no evidence demonstrating that MbrD is a histidine kinase of the two-component system, our data imply that bacitracin-induced post-transcriptional modification of MbrD may play an important role in bacitracin-induced *mbrAB* gene upregulation and the bacitracin resistance mechanism of *S. mutans*.

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