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Genes Involved in Bacitracin Resistance in *Streptococcus mutans*†

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Streptococcus mutans **is resistant to bacitracin, which is a peptide antibiotic produced by certain species of *Bacillus*. The purpose of this study was to clarify the bacitracin resistance mechanism of *S. mutans*. We cloned and sequenced two *S. mutans* loci that are involved in bacitracin resistance. The *rgp* locus, which is located downstream from *rmlD*, contains six *rgp* genes (*rgpA* to *rgpF*) that are involved in rhamnose-glucose polysaccharide (RGP) synthesis in *S. mutans*. The inactivation of RGP synthesis in *S. mutans* resulted in an approximately fivefold-higher sensitivity to bacitracin relative to that observed for the wild-type strain Xc. The second bacitracin resistance locus comprised four *mbr* genes (*mbrA*, *mbrB*, *mbrC*, and *mbrD*) and was located immediately downstream from *gtfC*, which encodes the water-insoluble glucan-synthesizing enzyme. Although the bacitracin sensitivities of mutants that had defects in flanking genes were similar to that of the parental strain Xc, mutants that were defective in *mbrA*, *mbrB*, *mbrC*, or *mbrD* were about 100 to 120 times more sensitive to bacitracin than strain Xc. In addition, a mutant that was defective in all of the *mbrABCD* genes and *rgpA* was more sensitive to bacitracin than either the RGP or Mbr mutants. We conclude that RGP synthesis is related to bacitracin resistance in *S. mutans* and that the *mbr* genes modulate resistance to bacitracin via an unknown mechanism that is independent of RGP synthesis.**

Cariogenic *Streptococcus mutans* is known to be resistant to bacitracin. This property is often exploited in the isolation of this bacterium from the highly heterogeneous oral microflora (6). Bacitracin is a cyclic polypeptide antibiotic that is produced by certain species of *Bacillus*. The primary mechanism of action of this antibiotic is thought to be the inhibition of peptidoglycan synthesis (28). During peptidoglycan synthesis, C_{55}-isoprenyl phosphate (IP) serves as a lipid carrier (24). After the translocation of sugar-peptide units to the ends of the linear peptidoglycan strands, the C_{55}-isoprenyl pyrophosphate (IPP) is detached and dephosphorylated to IP by a membrane-bound pyrophosphatase, thus recycling IP for subsequent peptidoglycan synthesis (24, 28). Bacitracin binds tightly to IPP and prevents pyrophosphatase from interacting with IPP, thus reducing the amount of IP that is available for carrying sugar-peptide units.

In *Escherichia coli*, increased phosphorylation of IP, due to elevated intracellular levels of the lipid kinase encoded by *bacA*, appeared to confer resistance to bacitracin (1). Alternatively, *E. coli* mutants lacking membrane-derived oligosaccharides had reduced sensitivity to bacitracin because of reduced IP utilization (5). Pollock et al. (20) reported that certain gram-negative bacteria that synthesized exopolysaccharides acquired resistance to bacitracin by shutting down the synthesis of exopolysaccharides. On the other hand, Podlesek et al. (19) suggested that an ABC-type efflux system, which consisted of the BcrA, BcrB, and BcrC proteins, might be involved in the resistance of *Bacillus licheniformis* to bacitracin. However, the exact mechanism by which this transporter system mediates resistance is still unknown.

The clinical use of bacitracin by oral administration is getting much attention for its ability to eradicate vancomycin-resistant enterococci (VRE) from the gastrointestinal tracts of patients (2, 15, 25). Since *S. mutans* is found in human feces, the bacitracin-resistant phenotype of *S. mutans* could presumably be transferred to VRE. Despite increasing fears that VRE might acquire tolerance to bacitracin from *S. mutans*, the mechanism of bacitracin resistance in *S. mutans* remains a mystery.

In the present study, we isolated two bacitracin-sensitive mutants of *S. mutans* using random mutagenesis of an *S. mutans* genomic library that was constructed in an integration vector. One of the mutants had an inactivated *rgpA* gene that was previously shown to be involved in glucose-rhamnose polysaccharide (RGP) formation in the cell wall. The second mutant was disrupted in an unknown gene, and we characterized the plasmid-inserted chromosomal region of this mutant. The two mutants differed in their sensitivities to bacitracin. We discussed the relationship between bacitracin resistance and RGP synthesis in *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *S. mutans* strains and plasmids used in this study are listed in Tables 1 and 2. *S. mutans* strains Xc, Xc13, Xc26R, Xc41, Xc42, Xc43, and Xc44 were selected from the stock culture collection in the Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan. Strains of *S. mutans* were grown at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). For transformants of

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† Dedicated to the memory of Toshihiko Koga, esteemed researcher and our mentor.

‡ Deceased.

TABLE 1. *S. mutans* strains used in this study

Strain	Relevant characteristics[a]	Reference
Xc	Serotype c wild-type strain	11
Xc13	Em^r; strain Xc carrying Em^r gene inserted into *gtfC*	35
Xc26R	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *rmlD*	31
Xc41	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *rgpA*	37
Xc42	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *rgpB*	37
Xc43	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *rgpC*	37
Xc44	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *rgpD*	37
XcB1	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *rgpA*	This study
XcB2	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *mbrD*	This study
Xc101	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *mbrA*	This study
Xc102	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *mbrB*	This study
Xc103	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *mbrC*	This study
Xc104	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into *mbrD*	This study
Xc105	Em^r; strain Xc carrying P15A replicon and Em^r gene both inserted into ORF5	This study
Xc106	Em^r; strain Xc carrying Em^r gene instead of *mbrA, mbrB, mbrC,* and *mbrD*	This study
Xc146	Em^r and Tet^r; strain Xc106 carrying Tet^r gene inserted into *rgpA*	This study

[a] Em^r, erythromycin resistance; Tet^r, tetracycline resistance.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pResEmBBN	Em^r; P15A replicon	23
pBluescriptII SK(+)	Ap^r; phagemid cloning vector	Stratagene
pResYT10	Em^r; P15A replicon with the unique *Pvu*II site immediately downstream of erythromycin resistance gene	23
pHT1	Em^r; marker rescue plasmid of 7.0-kb *Pst*I fragment of XcB1 chromosomal DNA which includes downstream region of *rmlD* gene and four intact *rgp* genes	This study
pHT2	Em^r; marker rescue plasmid of 9.8-kb *Hin*dIII fragment of XcB2 chromosomal DNA which includes downstream region of *gtfC* gene and four intact *mbr* genes	This study
pBSHT1	pBluescriptII SK$^+$ containing 4.3-kb *Pst*I fragment which carries of Xc *rgp* genes	This study
pBSHT2	pBluescriptII SK$^+$ containing 7.7-kb *Hin*dIII fragment which carries of Xc *mbr* genes	This study

S. mutans, erythromycin or tetracycline was added at a final concentration of 10 or 5 μg per ml, respectively.

DNA manipulation. Transformation of *S. mutans* and *E. coli* was carried out as previously described (34). Chromosomal DNA of *S. mutans* strains was prepared by the method of Perry et al. (17). Standard DNA recombinant procedures such as DNA isolation, endonuclease restriction, and ligation were carried out according to the methods of Sambrook et al. (22).

Isolation of bacitracin-sensitive transformants of *S. mutans*. A complete *Sau*3AI digest of the chromosomal DNA of *S. mutans* strain Xc was ligated to *Bam*HI- and *Bgl*II-digested pResEmBBN. The pResEmBBN integration plasmid, whose erythromycin resistant gene is able to work in *S. mutans*, cannot duplicate in *S. mutans*. This plasmid was produced by Shiroza and Kuramitsu in the process of constructing pResEmMCS11 (23) and is equivalent to pResEmMCS11 except that it lacks the restriction sites between *Xba*I and *Not*I in the multicloning site. The *S. mutans* strain Xc was randomly mutated by transformation with the *S. mutans* genomic library in pResEmBBN, as described previously (33). The insert fragment of the integration plasmid should be located within the target gene to disrupt the gene by Campbell-type recombination. Transformants were selected on BHI agar plates that contained 10 μg of erythromycin per ml. About 12,000 transformants were transferred to both BHI agar plates with or without 1 unit of bacitracin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) per ml. Bacitracin sensitivity was confirmed on bacitracin-containing (1 U/ml) plates, and then the corresponding transformants on plates containing no drug were picked up.

DNA sequence analysis. DNA sequencing of insertion fragments in pBluescript SKII(+) was performed by the primer-walking strategy (22), using the −21 M13 primer (5′-TGTAAAACGACGGCAGT-3′) or the M13RP primer (5′-CAGGAAACAGCTATGACC-3′) as an initiation primer, a BigDye Terminator cycle sequencing kit (PE Biosystems, Urayasu, Japan), and an ABI PRISM 310 genetic analyzer automated sequencer (PE Biosystems). To identify the pResEmBBN insertion site in the *S. mutans* transformants, the DNA sequences of marker-rescued plasmids were determined with the BBN-B primer (5′-GTTACACGTTACTAAAGGGA-3′) for the region downstream from the erythromycin resistance gene and with the BBN-N primer (5′-GATTTGAGCGTCAGATTTCG-3′) for the region upstream from the p15A replicon. The nucleotide sequences were assembled using the DNASIS sequence analysis program (Hitachi Software Engineering Co., Yokohama, Japan). Database searching was performed with the FASTA program of the DDBJ server at the National Institute of Genetics, Mishima, Japan. Multiple alignments of the amino acid sequences were generated with the CLUSTAL W program (29).

Nonpolar insertions in open reading frames (ORFs) flanking the plasmid integration site. To study the potential involvement of specific ORFs in bacitracin resistance, each ORF was insertionally inactivated with pResYT10. When this plasmid was introduced into the target gene, the inactivated gene and the erythromycin resistance gene were oriented in the same direction, so that the likelihood of polar effects on downstream gene transcription was avoided. It was previously shown that the promoter of the erythromycin resistance gene could direct the transcription of a gene that was located downstream of an inactivated gene in *S. mutans* (37). Constructions of *mbr*-inactivated mutants were performed as follows. Briefly, PCR fragments including the four *mbr* genes, which were amplified using the primer set of 5′-GTAAGCTACGATTCTTTAAG-3′ and 5′-CTTTAGCGGATGATTACGCA-3′, were cloned into pGEM-T PCR-cloning vector (Promega, Madison, Wis.). Four *mbr* genes on the resultant plasmid were interrupted by linearized pResYT10 at the respective restriction sites indicated (see Fig. 2). The plasmids whose erythromycin resistance gene and *mbr* gene were oriented in the same direction were selected, digested both sides of inserted site with the appropriate restriction enzymes, and introduced into the chromosome of *S. mutans* Xc by a double crossover recombination.

TABLE 3. Primers used for PCR and RT-PCR

Primer	Sequence (5′ to 3′)	Positions[a]
PAF	CTTGCAAGACGTTGACTTCA	2120–2139
PAR	GGTAGCCAAACCTAGAGCAT	2960–2979
PBF	GACTCCTCTATGGCGATGATGAT	4346–4368
PBR	GCCCTAAAGTTATCAACGC	4945–4963
PCF	GGTTGAAGATGATACAACC	4876–4895
PCR	CAAGATCACTTGGTGCAG	5763–5780
P5F	AGGCATACGCCGTGATACTA	7570–7589
P5R	CAATGACTGATGCTTGATC	6970–6988
RT1	GTAACATAAACCTGTGTTCCTTG	6437–6459
RT2	ACCAGTCACCAGACTTGTTGGTTCCTC	6876–6892

[a] The numbers give the positions on a 7.7-kb *Hin*dIII fragment sequenced in this study (EMBL/GenBank/DDBJ data bank accession no. AB078507).

RT-PCR analysis. Total RNA was prepared from *S. mutans* strains by using the FastPrep device (Savant Instruments, Holbrook, N.Y.) in combination with FastRNA kit-BLUE (Bio 101, Vista, Calf.) with minor modifications of the manufacturer's protocol. Briefly, bacterial cells that were grown to middle log phase in 15 ml of BHI broth were harvested and suspended in 300 μl of diethylpyrocarbonate-treated distilled water. The suspension was mixed with 900 μl of ISOGEN-LS (Nippon Gene Inc., Tokyo, Japan) and transferred to a FastPrep BLUE tube. The tube was processed for 40 s at speed level 6 in the FastPrep device, 200 μl of chloroform was added, and the mixture was shaken. Total RNA in the resultant supernatant was precipitated with 1 volume of isopropanol and washed with 500 μl of 75% ethanol. The dried pellet was suspended in an appropriate amount of diethylpyrocarbonate-treated distilled water. Before the solution was used for reverse transcriptase PCR (RT-PCR), contaminating DNA was eliminated by digestion with RNase-free DNase (DNase I; Gibco BRL Life Technologies, Grand Island, N.Y.). Reverse transcription was performed with SuperScript II RNase H^- reverse transcriptase (Gibco BRL). The relevant primers for RT-PCR are shown in Table 3. Primers RT1 and RT2 were used to synthesize cDNA in the *mbr* and ORF5 regions, respectively (Table 3). The reaction was carried out at 42°C for 50 min. The cDNA was used directly for PCR amplification with the sets of primers listed in Table 3. The PCR procedure performed with Ex *Taq* DNA polymerase (TAKARA BIO Inc., Otsu, Japan) consisted of a denaturation step at 94°C for 2 min followed by 25 cycles that comprised the following steps: denaturation at 94°C for 30 s, annealing at an appropriate temperature for 30 s, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 10 min. Negative control reactions were performed that left out the first reverse transcriptase treatment.

Antibiotics sensitivity tests. On the broth assay, *S. mutans* mid-log-phase cultures were adjusted to 5×10^6 CFU/ml. One hundred microliters of CFU-adjusted bacterial suspensions were inoculated in 2.9 ml of BHI broth containing various concentrations of antibiotics. After 20 h of incubation at 37°C, the cultures were sonicated, and the optical densities at 550 nm (OD_{550}) of the test cultures were measured. Cultures of all strains without antimicrobial agents could attain stationary phase within the 20 h at 37°C. The relative cell densities were calculated as (OD_{550} of culture in the presence of each concentration of antibiotics)/(OD_{550} of culture in the absence of antibiotics) × 100. The MIC was determined as the minimum antibiotic needed to ensure that culture did not grow to over 10% of the relative cell density.

In the plating assay, the tested strains were grown to mid-log phase in BHI broth. Approximately 50 CFU of bacterial cells were plated on the BHI agar plates containing various concentrations of antibiotics. After 5 days of incubation at 37°C, colonies were counted. MICs were determined from the highest concentration showing complete inhibition of the tested strains.

Immunological analysis of cell wall sugar components. Lyophilized *S. mutans* cells were resuspended in phosphate-buffered saline (pH 7.3; 100 mg/ml) and autoclaved at 121°C for 30 min. The suspensions were then centrifuged at 10,000 ×*g* for 20 min, and the supernatants were collected and used as autoclaved extracts. Immunodiffusion was performed in 1% (wt/vol) Noble agar in phosphate-buffered saline (pH 7.3) (16) with rabbit antisera to whole cells of *S. mutans* strains MT8148 (serotype c) and Xc31, whose cell wall polysaccharides contain only rhamnan backbones.

Chemical analysis of cell wall sugars. The sugar compositions of the cell wall preparations from *S. mutans* strains were analyzed by high-performance liquid chromatography, as described by Tsukioka et al. (32).

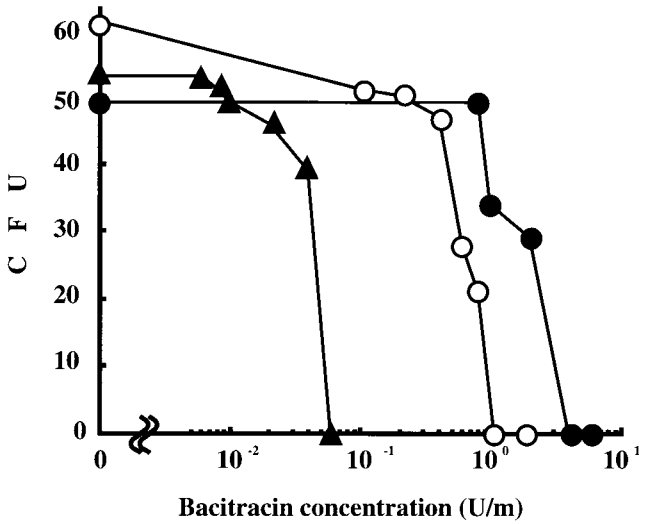

FIG. 1. Bacitracin sensitivities of *S. mutans* strain Xc and its derivatives. Approximately 50 CFU of mid-log-phase bacterial cells were plated on the BHI agar plates containing various concentrations of antibiotics. After 5 days incubation at 37°C, colonies were counted. The experiments were performed three times, and similar results were obtained in each experiment. Symbols: ●, strain Xc; ○, strain XcB1; ▲, strain XcB2.

Nucleotide sequence accession number. The 7,733-bp nucleotide sequence presented in this paper has been submitted to the EMBL/GenBank/DDBJ data bank under accession number AB078507.

RESULTS

Cloning and nucleotide sequencing of the *S. mutans* genes involved in resistance to bacitracin. Among the 12,000 transformants, only two were sensitive to bacitracin, and these were designated as strains XcB1 and XcB2. The dose dependent bacitracin sensitivities of strains XcB1 and XcB2 are displayed in Fig. 1. Neither of the mutants grew in the presence of 1 U of bacitracin per ml, and both were clearly sensitive to bacitracin compared to the wild-type strain Xc. In addition, strain XcB2 was distinctly more sensitive to bacitracin than strain XcB1. Southern blotting with a digoxigenin (DIG)-labeled PCR probe that was specific for the erythromycin resistance gene revealed that the probe hybridized with a 7.0-kb *Pst*I fragment from strain XcB1 and a 9.8-kb *Hin*dIII fragment from strain XcB2 but did not hybridize with any fragments from the wild-type strain Xc (data not shown). Using the marker rescue strategy (14), fragments that hybridized with the probe were recovered from *Pst*I-digested XcB1 and *Hin*dIII-digested XcB2 chromosomal DNA. The plasmids recovered from strains XcB1 and XcB2 were designated pHT1 and pHT2, respectively. The 4.3-kb *Pst*I fragment of the strain Xc chromosome hybridized with pHT1 that had been labeled with DIG-dUTP via random primer labeling. In contrast, the 7.7-kb *Hin*dIII fragment of the strain Xc chromosome hybridized with DIG-labeled pHT2. Both fragments were cloned in pBluescript SKII(+) by colony hybridization using DIG-labeled pHT1 and pHT2 as probes. The recombinant plasmids hybridized with pHT1 and pHT2 and were designated pBSHT1 and pBSHT2, respectively. The fragments inserted in these plasmids were

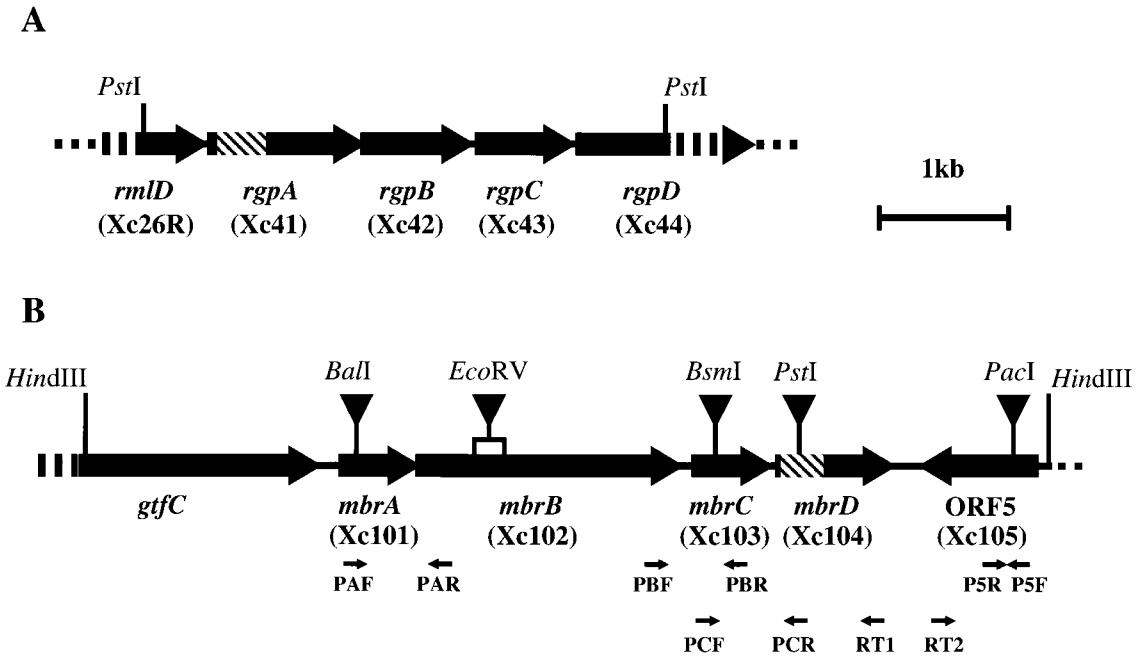

FIG. 2. Restriction maps of the 4.3-kb *Pst*I fragment (A) in pBSHT1 and the 7.7-kb *Hin*dIII fragment (B) in pBSHT2. Arrows indicate ORF locations. Hatched bars indicate the regions corresponding to a single crossover event in strains XcB1 and XcB2. Names of *S. mutans* mutants whose *rml*, *rgp*, or *mbr* gene was inactivated are indicated in the parentheses below the respective genes' name. Xc26R, Xc41, Xc42, Xc43, and Xc44 were previously constructed as described in Table 1. The pResYT10 insertion sites for inactivation of the *mbr* genes and ORF5 are indicated by the inverted closed triangles. The construction of mutants of the *mbr* gene and ORF5 was carried out as described in the text. Primers used in the RT-PCR are indicated as small arrows.

sequenced. Restriction maps of the two cloned fragments are shown in Fig. 2.

Nucleotide sequence analysis revealed the presence of three complete and two truncated ORFs in the 4.3-kb *Pst*I fragment of pBSHT1 (Fig. 2A). The sequences of these ORFs are identical to previously characterized ORFs of the *rmlD*, *rgpA*, *rgpB*, *rgpC*, and *rgpD* genes, which are involved in RGP synthesis (37). Sequencing of the region flanking the pResEmBBN insertion in pTH1 revealed that *rgpA* was insertionally inactivated by pResEmBBN in strain XcB1 (Fig. 2A).

Six ORFs were found in the 7.7-kb *Hin*dIII fragment of pBSHT2 (Fig. 2B). The first ORF represents a truncated form of the *gtfC* gene, which codes for a glucosyltransferase-SI and which is responsible for the synthesis of water-insoluble glucan (8). With the exception of the last ORF, the other five ORFs are in the same orientation. Since inactivation of the second to fifth ORFs in *S. mutans* resulted in bacitracin sensitivity, these four ORFs were designated as mutans bacitracin resistance (*mbr*) genes, i.e., *mbrA*, *mbrB*, *mbrC*, and *mbrD*, respectively (Fig. 2B). The remaining ORF (ORF5) was not involved in bacitracin resistance (Table 4). The *mbrA* gene is located 273 bp downstream from *gtfC*. In contrast, the intergenic regions between *mbrA* and *mbrB*, *mbrB* and *mbrC*, and *mbrC* and *mbrD* are only −8, 41, and −4 bp, respectively. Potential transcription terminators, in the form of stem-loop structures followed by a poly(T) sequence (positions 1847 to 1866 and 6522 to 6542) are present in the regions between *gtfC* and *mbrA* and downstream of *mbrD*. Putative Shine-Dalgarno ribosome binding sequences lie just upstream of the potential initiation codons in all of the *mbr* genes and in ORF5.

The deduced amino acid sequences encoded by *mbrA* and

TABLE 4. MICs of bacitracin for *S. mutans* strains used in this study

Strain[a]	MIC[b]
Xc	4.0 ± 0.0[d,f]
Xc13 (*gtfC*)	4.0 ± 0.0[d,f]
Xc26R (*rmlD*)	0.80 ± 0.00[c,f]
Xc41 (*rgpA*)	0.73 ± 0.09[c,f]
Xc42 (*rgpB*)	0.73 ± 0.09[c,f]
Xc43 (*rgpC*)	0.80 ± 0.00[c,f]
Xc44 (*rgpD*)	0.73 ± 0.09[c,f]
XcB1 (*rgpA*)	0.73 ± 0.09[c,f]
XcB2 (*mbrD*)	0.033 ± 0.009[c,d]
Xc101 (*mbrA*)	0.033 ± 0.009[c,d]
Xc102 (*mbrB*)	0.033 ± 0.009[c,d]
Xc103 (*mbrC*)	0.040 ± 0.000[c,d]
Xc104 (*mbrD*)	0.040 ± 0.000[c,d]
Xc105 (ORF5)	3.3 ± 0.9[e,g]
Xc106 (*mbrA, mbrB, mbrC,* and *mbrD*)	0.033 ± 0.009[c,d]
Xc146 (*mbrA, mbrB, mbrC, mbrD,* and *rgpA*)	0.020 ± 0.000[c,d]

[a] For all strains besides Xc, the genes were inactivated.
[b] Results are expressed in international units per milliliter. Each value represents the mean ± standard deviation for assays performed three times.
[c] Differences from strain Xc ($P < 0.05$ [Welch's *t* test]).
[d] Differences from strain Xc41 ($P < 0.01$ [Welch's *t* test]).
[e] Differences from strain Xc41 ($P < 0.05$ [Welch's *t* test]).
[f] Differences from strain Xc106 ($P < 0.01$ [Welch's *t* test]).
[g] Differences from strain Xc106 ($P < 0.05$ [Welch's *t* test]).

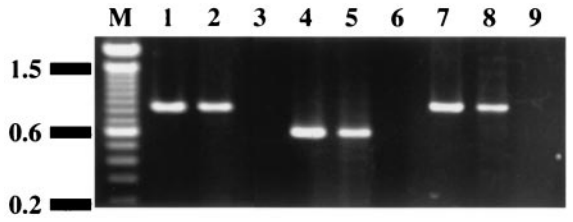

FIG. 3. RT-PCR analysis of mRNA from the *mbr* operon. Lanes 1, 4, and 7, PCR using the chromosomal DNA of strain Xc as the template (positive controls); lanes 2, 5, and 8, RT-PCR using total RNA from strain Xc as the template; lanes 3, 6, and 9, PCR using total RNA from strain Xc as the template (negative controls). The RT1 primer was used for reverse transcription. PCR amplification was performed with the following primer sets: PAF and PAR (lanes 1, 2, and 3), PBF and PBR (lanes 4, 5, and 6), and PCF and PCR (lanes 7, 8, and 9).

mbrB genes show strong similarities to the components of other bacterial ABC transporters. The *mbrA*-encoded protein contains four highly conserved motifs that are characteristic of ATP-binding proteins: a Walker A site at amino acid positions 41 to 48, a Walker B site at positions 165 to 169, a linker peptide at positions 145 to 152, and a switch region at the positions 193 to 202 (9, 12). Secondary structure predictions for the MbrB protein (performed with the SOSUI program; http://sosui.proteome.bio.tuat.ac.jp/welcomeE.html) revealed 10 putative transmembrane helices, suggesting that this protein may be membrane localized.

The MbrC and MbrD proteins exhibit high levels of identity with the response regulators and the histidine sensor kinases, respectively, of two-component regulatory systems in certain bacteria. MbrC protein contains a region that is characteristic of the helix-turn-helix motif (at amino acid positions 36 to 55), which is associated with DNA binding. The N terminus of MbrC is highly conserved and includes several aspartate residues (at amino acid positions 12, 13, 49, 54, 98, and 99) that may form the phosphorylation site of the protein. The histidine kinase domain of MbrD contains four highly conserved amino acid sequences, termed the H, N, D/F, and G boxes (27). These motifs presumably form a nucleotide-binding surface within the active site. Moreover, secondary structure prediction for the MbrD protein using the SOSUI program revealed that this protein possesses two putative transmembrane helices (at amino acid positions 12 to 34 and 38 to 60) (10). The flanking region of the pResEmBBN inserted in pTH2 was sequenced and we confirmed that *mbrD* was insertionally inactivated by pResEmBBN in XcB2 (Fig. 2B).

Transcription of the *mbr* genes in *S. mutans*. The *mbr* genes are located close to each other, suggesting polycistronic transcription of these genes. RT-PCR analysis of total RNA from the wild-type strain Xc was performed to demonstrate that all of the *mbr* genes were included in a single polycistronic transcript. RT-PCR using the following primer sets: PAF and PAR; PBF and PBR; and PCF and PCR (Fig. 2), produced 0.9-, 0.6-, and 0.9-kb fragments, respectively (Fig. 3). Total RNA preparations that had not undergone reverse transcription did not give amplified fragments, suggesting that the RT-PCR products were derived from mRNA and not from contaminating chromosomal DNA (Fig. 3).

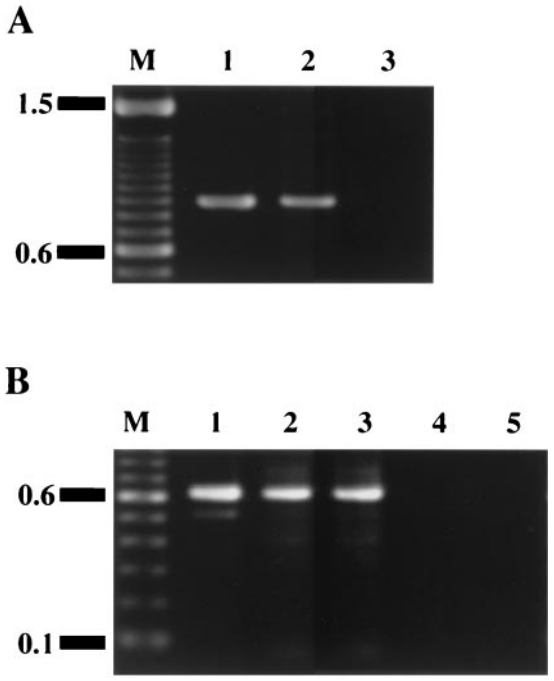

FIG. 4. RT-PCR analysis of the transcription of the region located downstream of the *mbrC* gene and upstream of the *mbrD* gene in strain Xc102 (A), and of ORF5 transcription in strains Xc102 and Xc104 (B). (A) Lane 1, PCR using the chromosomal DNA of strain Xc as the template (positive control); lane 2, RT-PCR using total RNA from strain Xc102 as the template; lane 3, PCR using total RNA from strain Xc102 as the template (negative control). The RT1 primer was used for reverse transcription. PCR amplification was performed with primers PCF and PCR. (B) Lane 1, PCR using the chromosomal DNA of strain Xc as the template (positive control); lanes 2 and 3, RT-PCR using total RNA from strains Xc102 and Xc104, respectively, as the templates; lanes 4 and 5, PCR using total RNA from strains Xc102 and Xc104, respectively, as the templates (negative controls). The primer RT2 was used for reverse transcription. PCR amplification was performed with primers P5F and P5R.

The effect of insertional inactivation of *mbr* genes on downstream genes. We used pResYT10 to insertionally inactivate each *mbr* gene in order to reduce the likelihood of a polar effect on the transcription of the downstream genes. RT-PCR was performed to confirm *mbrD* gene transcription in strain Xc102 (an *mbrB*-defective mutant) and ORF5 transcription in strains Xc102 and Xc104 (an *mbrD*-defective mutant). A 0.9-kb RT-PCR fragment was amplified from the total RNA of strain Xc102 (Fig. 4A), whereas 0.6-kb RT-PCR products were obtained from the total RNA of strains Xc102 and Xc104 (Fig. 4B). Total RNA preparations that were not first reverse transcribed give no PCR products (Fig. 4). We thus concluded that insertional inactivation of the *mbr* genes with pResYT10 did not interrupt the transcription of downstream genes.

Bacitracin sensitivity tests. To determine which genes were responsible for resistance to bacitracin, *S. mutans* mutants that were defective in *mbrA*, *mbrB*, *mbrC*, *mbrD*, or ORF5 were constructed by insertional inactivation with pResYT10, as described in the Materials and Methods section (Table 1 and Fig. 2B). The strains in which *mbrA*, *mbrB*, *mbrC*, *mbrD*, and ORF5

TABLE 5. Sugar composition of cell wall preparations

Strain	Sugar content[a] (μg/mg)		
	Glucose	Rhamnose	*N*-Acetylglucosamine
Xc	161	357	163
Xc106	126	253	121

[a] Values are micrograms per 1 mg (dry weight) of the purified cell wall preparation.

were inactivated were designated Xc101, Xc102, Xc103, Xc104, and Xc105, respectively. *S. mutans* mutants that were defective in each of the *rgp* genes or in *rmlD* were constructed as described previously (31, 37). The previously constructed strain Xc13 (35) was used as the *gtfC* mutant. The bacitracin sensitivity of *gtfC*-deficient strain Xc13 was the same as that of the wild-type strain Xc (Table 4). Similarly, insertional inactivation of ORF5 (strain Xc105) did not significantly change the bacitracin resistance of *S. mutans* (Table 4). On the other hand, strains Xc101, Xc102, Xc103, and Xc104 exhibited about 100- to 120-fold-higher sensitivity to bacitracin than did strain Xc; the enhanced sensitivities to bacitracin were not significantly different from that of strain XcB2 (Table 4). In addition, we constructed an *S. mutans* mutant in which chromosomal region corresponding to all four *mbr* genes was replaced by erythromycin resistance gene (strain Xc106). The bacitracin resistance of strain Xc106 was not significantly different from that of strains Xc101, Xc102, Xc103, and Xc104 (Table 4).

On the other hand, strains Xc26R, Xc41, Xc42, Xc43, and Xc44, which are defective in *rmlD*, *rgpA*, *rgpB*, *rgpC*, and *rgpD*, respectively, were about fivefold more sensitive to bacitracin than strain Xc (Table 4). Strain XcB1 was similar in sensitivity to bacitracin as strains Xc26R, Xc41, Xc42, Xc43, and Xc44 (Table 4). In addition, we constructed a double mutant that was defective in all of the *mbr* genes and the *rgpA* gene (strain Xc146), to examine the relationship between *mbr* genes and *rgp* genes functions in bacitracin resistance of *S. mutans*. Strain Xc146 was more sensitive to bacitracin than strains XcB1 and XcB2 and exhibited an approximately 200-fold-higher sensitivity to bacitracin than strain Xc (Table 4). Similar results were obtained from the experiments by plating assay (Fig. 1).

Analysis of cell wall polysaccharides of the *mbr* mutant. To examine whether the inactivation of *mbr* genes influenced RGP synthesis, cell wall polysaccharides of strain Xc106 were analyzed immunologically and chemically. Immunodiffusion analysis was carried out with both serotype c-RGP-specific antiserum and rhamnan-backbone-specific rabbit antiserum (36). The serotype c-specific antiserum formed a single precipitin line between the antiserum and the autoclaved extracts of strains Xc or Xc106, while the rhamnan-specific antiserum did not react with autoclaved extracts of either strain Xc or Xc106 (data not shown). In addition, the sugar composition of the cell wall preparations of strains Xc and Xc106 were analyzed by high-performance liquid chromatography. The amounts of rhamnose, glucose, and *N*-acetylglucosamine per milligram (dry weight) of cell wall preparation of strain Xc106 did not differ from the corresponding values for strain Xc (Table 5).

Effect of the culture supernatants of *S. mutans* strains Xc and Xc106 on the bacitracin sensitivity of strain Xc106. Since MbrA and MbrB represent a putative ABC transporter, they may function to export specific molecules that inactivate bacitracin. In order to examine this possibility, spent culture supernatants from strains Xc and Xc106 were added, in various proportions, to cultures of strain Xc106, and the bacitracin sensitivity of strain Xc106 was measured. Briefly, *S. mutans* strains Xc and Xc106 were grown to stationary phase in BHI broth. After centrifugation, culture supernatants were collected and filtered through 0.2-μm-pore-size filters. The collected supernatants were mixed with fresh BHI broth in proportions that ranged from 1:28 to 15:14 to give a total volume of 2.9 ml for each mixture. Aliquots (100 μl) of overnight cultures of strain Xc106 were inoculated in the supernatant mixtures in BHI broth that contained bacitracin at 0.1 U per ml. The cultures were incubated at 37°C for 20 h, and the OD_{550} was measured. There were no significant differences between the culture supernatants of strains Xc and Xc106 in terms of their effects on the bacitracin sensitivity of Xc106.

Sensitivities to other antibiotics. In addition to its resistance to bacitracin, the *S. mutans* wild-type strain Xc is resistant to

TABLE 6. MICs of antibiotics for *S. mutans* strains

Antibiotic	MIC[a]			
	Xc	Xc106	Xc41	Xc146
Kanamycin	60 ± 0^{d}	53.3 ± 9.4^{e}	$20 \pm 0^{b,g}$	$16.7 \pm 4.7^{b,f}$
Spectinomycin	100 ± 0^{d}	93.3 ± 9.4	80 ± 0^{b}	$73.3 \pm 9.4^{c,h}$
Bacitracin	$4.0 \pm 0.0^{d,f}$	$0.033 \pm 0.009^{b,d}$	$0.73 \pm 0.09^{b,f}$	$0.02 \pm 0.00^{b,d}$
Ampicillin	0.06 ± 0.00^{d}	0.06 ± 0.00^{d}	$0.04 \pm 0.00^{b,f}$	$0.04 \pm 0.00^{b,f}$
Nisin	100 ± 0^{d}	100 ± 0^{d}	$0.25 \pm 0.00^{b,f}$	$0.23 \pm 0.02^{b,f}$

[a] Results are expressed in micrograms per milliliter, except for those with bacitracin, which are in international units per milliliter. Each value represents the mean ± standard deviation for assays performed three times.
[b] Differences from strain Xc ($P < 0.01$ [Welch's *t* test]).
[c] Differences from strain Xc ($P < 0.05$ [Welch's *t* test]).
[d] Differences from strain Xc41 ($P < 0.01$ [Welch's *t* test]).
[e] Differences from strain Xc41 ($P < 0.05$ [Welch's *t* test]).
[f] Differences from strain Xc106 ($P < 0.01$ [Welch's *t* test]).
[g] Differences from strain Xc106 ($P < 0.05$ [Welch's *t* test]).
[h] Differences from strain Xc106 ($P < 0.1$ [Welch's *t* test]).

kanamycin (MIC, 60 μg/ml [broth assay]) and spectinomycin (MIC, 100 μg/ml [broth assay]) (Table 6). To investigate whether the *mbr*- and RGP-mediated bacitracin resistance mechanisms participated in resistance to kanamycin or spectinomycin, we examined the sensitivities to some antibiotics of strains Xc, Xc106, Xc41, and Xc146 by the broth assay. Strain Xc106 was resistant to kanamycin, spectinomycin, and nisin to the same extent as strain Xc (Table 6). In addition, the sensitivity to ampicillin of strain Xc106 was as high as that of the strain Xc (Table 6). In contrast, strain Xc41, which is defective in RGP synthesis, was more sensitive to these antibiotics than strain Xc (Table 6). The sensitivities of strain Xc146 to kanamycin, spectinomycin, nisin, and ampicillin were not significantly different from those of strain Xc41, although they differed in their sensitivities to bacitracin (Table 6). No difference was found between the sensitivities to tetracycline and ofloxacin of strains Xc, Xc106, and Xc41 (data not shown). The double mutant Xc146 had the same sensitivity to ofloxacin of strain Xc, Xc106, and Xc41 (data not shown). As strain Xc146 has tetracycline resistance gene, this mutant had strong resistance to tetracycline (data not shown).

DISCUSSION

In this study, we identified and sequenced genes that were involved in resistance of *S. mutans* to bacitracin and examined the bacitracin sensitivity of mutants that were defective in the *rml*, *rgp*, and *mbr* genes. We found that strains Xc26R, Xc41, Xc42, Xc43, and Xc44, which are defective in *rmlD*, *rgpA*, *rgpB*, *rgpC*, and *rgpD*, respectively, were about five- to six-fold more sensitive to bacitracin than the wild-type strain Xc (Table 4). The RGPs of *S. mutans* have a backbone structure that is composed of α 1,2- and α 1,3-linked rhamnosyl polymer with glucose side chains (13, 21). We previously reported that the *rgpA*, *rgpB*, *rgpC*, and *rgpD* genes were required for the assembly of RGP from dTDP-L-rhamnose and for the export of RGP across the cytoplasmic membrane and that mutants defective in these genes did not incorporate RGP into their cell walls (37). The *rmlD* gene is involved in the synthesis of dTDP-L-rhamnose, which is an immediate precursor for RGP-backbone production, and RGP was not found in the cell wall of *rmlD* mutant Xc26R (31). All of these mutants exhibited similar sensitivities to bacitracin (Table 4). These findings suggest that the presence of RGP in the cell wall may confer resistance to bacitracin in *S. mutans*. Pollock et al. (20) reported that exopolysaccharide-synthesizing gram-negative bacteria acquired resistance to bacitracin by repressing the synthesis of exopolysaccharide. Exopolysaccharide synthesis requires the same carrier IP that is needed for the synthesis of peptidoglycan. By repressing the synthesis of exopolysaccharide, bacteria can use the excess IP for peptidoglycan synthesis and thus become resistant to bacitracin. If IP is required for RGP synthesis, mutants defective in *rmlD*, *rgpA*, *rgpB*, *rgpC*, or *rgpD* genes should be more resistant to bacitracin than the wild-type strain Xc. We previously suggested that the transfer of *N*-acetylglucosamine to a lipid carrier, such as IP, was required for the RGP synthesis (33). However, the mutants defective in *rmlD*, *rgpA*, *rgpB*, *rgpC*, or *rgpD* (strains Xc26R, Xc41, Xc42, Xc43, or Xc44, respectively) exhibited significantly enhanced sensitivity to bacitracin compared with strain Xc (Table 4). These results appear to contradict the model outlined above. The lipid carrier for *N*-acetylglucosamine transfer during RGP synthesis in *S. mutans* might not be IP. Putative mechanisms for RGP-mediated resistance of *S. mutans* to bacitracin are discussed below.

All of the *mbr*-defective mutants were approximately 100- to 120-fold more sensitive to bacitracin than the parental strain Xc. Analysis of the deduced amino acid sequences of MbrA and MbrB strongly suggested that these proteins represented the components of an ABC transporter. Assuming that this is true, what is the target of this transporter? We suggest two possibilities: (i) the transporter exports a molecule that inactivates bacitracin, and indeed, it has been reported that some metabolites inhibit bacitracin activity at very low concentrations (18); or (ii) the transporter modulates the movement of bacitracin itself. Since Podlesek et al. could not detect any substances that suppressed the bacitracin activity of a strain of *B. subtilis* that carried cloned *bcr* genes, they assumed that the Bcr-encoded ABC transporter of *B. licheniformis* transported bacitracin itself (19). The latter hypothesis is likely to be true in *S. mutans*, because the culture supernatants of strains Xc and Xc106 had similar effects on the bacitracin sensitivity of strain Xc106. The deduced amino acid sequences of MbrC and MbrD were highly homologous to those of response regulators and histidine-sensor kinases, respectively, which are found in two-component regulatory systems of certain bacteria, and these proteins have some highly conserved motifs. However, based on current information, it is difficult to speculate on the functions of MbrC and MbrD.

The strain Xc146, which is defective in four *mbr* genes and *rgpA*, was constructed, and its bacitracin sensitivity was examined to elucidate the relationship between the two mechanisms of resistance to bacitracin. Strain Xc146 was approximately 200 and 37 times more sensitive to bacitracin than strains Xc and Xc41, respectively, and was approximately two times more sensitive to bacitracin than strain Xc106. These results suggest that the *mbr*-mediated mechanism of bacitracin resistance is independent of RGP synthesis. Indeed, immunological (data not shown) and chemical (Table 5) analysis of the cell-wall components of strain Xc106 confirmed that the *mbr* genes were not involved in RGP synthesis.

The *S. mutans* wild-type strain Xc is resistant not only to bacitracin but also to kanamycin and spectinomycin (Table 6). To clarify whether the *mbr*- and RGP-related bacitracin resistance mechanisms were specific for bacitracin, we examined the sensitivities to kanamycin and spectinomycin of *S. mutans* strains Xc, Xc106, Xc41, and Xc146. The kanamycin and spectinomycin sensitivities of the *mbr*-defective strain Xc106 were similar to those of the wild-type strain Xc. In addition, there were no significant differences in sensitivity to ampicillin, ofloxacin, tetracycline, and nisin between strains Xc and Xc 106 (Table 6 and Results section). Furthermore, the kanamycin, spectinomycin, ampicillin, ofloxacin, and nisin sensitivities of strain Xc146 were similar to those of strain Xc41 (Table 6 and Results section). These results suggest that the *mbr* genes of *S. mutans* are specific for resistance to bacitracin. On the other hand, RGP-defective strain Xc41 was more sensitive to kanamycin, spectinomycin, bacitracin, ampicillin, and nisin than strain Xc (Table 6). As well as strain Xc41, strain Xc24R

(defective in the *rmlB* gene, which is involved in dTDP-L-rhamnose synthesis) lacked RGP on the cell surface (30, 32) and exhibited a higher sensitivity to bacitracin than strain Xc (data not shown). The bacterial capsule and exopolysaccharide prevent the migration of the antibiotic to its target by prolonging the time to needed to equilibrate the difference in antibiotic concentration between the external medium and the bacterial cell surface and by providing a frictional resistance to diffusion (3, 4, 26). Indeed, electron microscopic observations of cell surface architectures indicated that the cell-wall-like layers of strain Xc24R were thinner than those of strain Xc (30). Thus, we speculate that RGP may act as a barrier that prevents some antibiotics reaching their target molecules and may partly contribute to the resistance to some antibiotics of *S. mutans*. However, sensitivities of RGP mutant to tetracycline and ofloxacin did not significantly differ from those of wild type strain (data not shown). At present, it is difficult to precisely define the function of RGP in the resistance of *S. mutans* to antibiotics.

Since the first reports in the late 1980s, VRE have become established pathogens in many hospitals, and the number of cases involving this organism has increased rapidly throughout the world (A. H. Uttley, C. H. Collins, J. Naidoo, and R. C. George, Letter, Lancet **i:**57-58, 1988). Outbreaks reported in the press have spread alarm in the community. The emergence and widespread incidence of VRE have produced a therapeutic dilemma. Some clinicians have recently suggested that oral administration of bacitracin might be a safe and effective way to eliminate VRE from the gastrointestinal tract of patients, and in this respect bacitracin is receiving a good deal of attention (2, 15, 25). On the other hand, bacitracin-resistant *S. mutans* colonizes in the oral cavities of most of human beings, and this organism has been detected in feces (7). Oral administration of bacitracin might ensure the predominance of *S. mutans* in the gastrointestinal tract, thus increasing the potential for contact between VRE and bacitracin-resistant *S. mutans*. Under these conditions, the *mbr* genes of *S. mutans* might be transferred to VRE, thereby conferring the bacitracin resistance phenotype of *S. mutans* to VRE. Therefore, it is important to understand the bacitracin resistance mechanism of *S. mutans*, in order to prevent the appearance of bacitracin-resistant VRE. At present, we can only eliminate *S. mutans* from the oral cavity of VRE-infected patients mechanically, by stringent tooth brushing. Further studies on the bacitracin resistance mechanism of *S. mutans* are needed in preparation for the emergence of bacitracin-resistant VRE.

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