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Acquired Bacitracin Resistance in *Enterococcus faecalis* Is Mediated by an ABC Transporter and a Novel Regulatory Protein, BcrR

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Bacitracin resistance (bacitracin MIC, ≥256 μg ml^{-1}) has been reported in *Enterococcus faecalis*, and in the present study we report on the genetic basis for this resistance. Mutagenesis was carried out with transposon Tn*917* to select for *E. faecalis* mutants with decreased resistance to bacitracin. Two bacitracin-sensitive mutants (MICs, 32 μg ml^{-1}) were obtained and Tn*917* insertions were mapped to genes designated *bcrA* and *bcrB*. The amino acid sequences of BcrA (ATP-binding domain) and BrcB (membrane-spanning domain) are predicted to constitute a homodimeric ATP-binding cassette (ABC) transporter, the function of which is essential for bacitracin resistance in *E. faecalis*. The *bcrA* and *bcrB* genes were organized in an operon with a third gene, *bcrD*, that had homology to undecaprenol kinases. Northern analysis demonstrated that *bcrA*, *bcrB*, and *bcrD* were transcribed as a polycistronic message that was induced by increasing concentrations of bacitracin but not by other cell wall-active antimicrobials (e.g., vancomycin). Upstream of the *bcrABD* operon was a putative regulatory gene, *bcrR*. The *bcrR* gene was expressed constitutively, and deletion of *bcrR* resulted in a bacitracin-sensitive phenotype. No *bcrABD* expression was observed in a *bcrR* mutant, suggesting that BcrR is an activator of genes essential for bacitracin resistance (i.e., *bcrABD*). The bacitracin resistance genes were found to be located on a plasmid that transferred at a high frequency to *E. faecalis* strain JH2-2. This report represents the first description of genes that are essential for acquired bacitracin resistance in *E. faecalis*.

Bacitracin is an antimicrobial that comprises a mixture of high-molecular-weight polypeptides produced by the organism *Bacillus licheniformis*. Bacitracin works by binding to and sequestering the undecaprenol pyrophosphate (UPP) carrier in the bacterial cytoplasmic membrane (32). During the synthesis and transport of peptidoglycan monomer units, undecaprenol monophosphate (UP) is phosphorylated to UPP. The UPP must be converted back to UP by the membrane-bound pyrophosphatase to enable transport of further subunits (10). Binding of bacitracin prevents the recycling of UPP and therefore causes disruption of cell wall synthesis (31, 32, 33). Bacitracin is used widely in topical applications in human medicine, and its oral use for the control of vancomycin-resistant enterococci has been suggested (25). Bacitracin is also used extensively for prophylaxis and therapy in food animals, particularly in broiler chicken production.

A number of mechanisms of bacitracin resistance have been reported in bacteria (2, 4, 5, 24, 26, 28, 34). In the bacitracin-producing organism *B. licheniformis*, resistance is encoded by the *bcrABC* genes, which encode a putative heterodimeric ATP-binding cassette (ABC) transporter that has been proposed to mediate the active efflux of bacitracin (24, 28). Homologues of this transporter have been identified in *Bacillus subtilis* (26) and *Streptococcus mutans* (34). A second recognized mechanism of bacitracin resistance is the overproduction of undecaprenol kinase (4). This enzyme converts undecaprenol to UP, increasing the amount of lipid carrier present in the cell. It is proposed that up-regulation of this enzyme increases the levels of UP, thus overcoming the sequestration of UPP by bacitracin and increasing the resistance of the organism to bacitracin. Other reported mechanisms of bacitracin resistance are proposed to be mediated by a membrane-associated phospholipid phosphatase in *B. subtilis* (2, 5, 27). In *S. mutans*, it has been shown that inactivation of the *rgpA* gene, which is involved in glucose-rhamnose polysaccharide formation in the cell wall, results in increased bacitracin sensitivity (34).

In many bacterial genera, bacitracin resistance has been detected phenotypically (1, 8, 19, 21; J. M. Manson, S. Keis, J. M. B. Smith, and G. M. Cook, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-1490, 2003); however, the mechanism(s) of resistance remains unclear. With enterococci, for example, studies on bacitracin susceptibility and resistance are limited, and no set parameters have been defined to determine the breakpoint for resistant and susceptible isolates. Zinc bacitracin is the most widely used antimicrobial in poultry in New Zealand, and a survey of 382 New Zealand poultry enterococcal isolates found that bacitracin MICs were ≥256 μg ml^{-1} for 98% of the isolates (Manson et al., 43rd ICAAC). Despite the high percentage of resistance to bacitracin in some enterococcal isolates, the genes responsible for resistance in enterococci are unknown.

In this communication, we report on the isolation and characterization of acquired genes encoding high-level bacitracin resistance in *Enterococcus faecalis* AR01/DGVS. We propose that bacitracin resistance in this strain is mediated by a homodimeric ABC transporter that actively pumps bacitracin from the cell. The expression of this transporter is under the control of a novel regulatory protein, BcrR.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
E. coli DH10B	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara*, *leu*)*7697 araD139 galU galK nupG rspL* λ$^-$	12
E. faecalis		
JH2-2	Type strain; Rf^r Fs^r	13
AR01/DG	Strain isolated from a dog with mastitis; harbors pJM01 (Tc^r Bc^r) and pJM02 (Vm^r Em^r)	20
AR01/DGVS	AR01/DG cured of pJM02; Tc^r Bc^r	This study
DGM2	AR01/DGVS *bcrB*::Tn*917*; Em^r Bc^s Tc^r	This study
DGM4	AR01/DGVS *bcrA*::Tn*917*; Em^r Bc^s Tc^r	This study
AR01/DGVS/pTV1-OK	AR01/DGVS harbouring pTV1-OK	This study
DGM2/pAMBcr1	DGM2 harboring pAMBcr1	This study
DGM4/pAMBcr1	DGM4 harboring pAMBcr1	This study
JH2-2/pAMBcr1	JH2-2 harboring pAMBcr1	This study
JH2-2/pAMBcr2	JH2-2 harboring pAMBcr2	This study
JH2-2/pAMBcr3	JH2-2 harboring pAMBcr3	This study
JH2-2/pMGSBcr4	JH2-2 harboring pMGSBcr4	This study
JH2-Bcr	JH2-2 harboring pJM01; Tc^r Bc^r	This study
Plasmids		
pUC8	Cloning vector; Ap^r	36
p2H7	pUC8 harboring 4.7-kb EcoRI fragment from AR01/DGVS containing *bcrR*, *bcrA*, *bcrB*; and *bcrD*	This study
pTV1-OK	*repA*(Ts)-pWV01Ts *aphA3* Tn*917*; Km^r Em^r	11
pAM401	*E. coli-E. faecalis* shuttle vector; Cm^r Tc^r	35
pMGS100	*E. coli-E. faecalis* shuttle expression vector; Cm^r	9
pAMBcr1	pAM401 harboring 4.7-kb EcoRI fragment from p2H7 containing *bcrR*, *bcrA*, *bcrB*, and *bcrD*	This study
pAMBcr2	pAM401 harboring 3.6-kb EcoRV-SspI fragment from p2H7 containing *bcrA*, *bcrB*, and *bcrD*	This study
pAMBcr3	pAM401 harboring 3.2-kb EcoRI-NdeI fragment from p2H7 containing *bcrR*, *bcrA*, and *bcrB*	This study
pMGSBcr4	pMGS100 harboring *bcrD*	This study

[a] Bc^s, sensitivity to bacitracin; r, resistant; s, sensitive.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH10B was routinely grown in Luria-Bertani (LB) broth or LB agar (1.4% [wt/vol]) at 37°C or, when harboring pTV1-OK, at 28°C. *Enterococcus* strains were grown without agitation in brain heart infusion (BHI) broth or BHI agar (1.4% [wt/vol]). All *E. faecalis* strains with the exception of strains carrying pTV1-OK were incubated at 37°C; strains carrying pTV1-OK were grown at 28°C. Antibiotics supplemented in the media included ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1} for *E. coli* and 500 μg ml^{-1} for *Enterococcus*), erythromycin (10 μg ml^{-1} for *Enterococcus*), chloramphenicol (50 μg ml^{-1} for *E. coli* and 20 μg ml^{-1} for *Enterococcus*), and tetracycline (10 μg ml^{-1}).

Antibiotic sensitivity tests and plasmid curing. Isolates were assayed for resistance to bacitracin (50,000 IU g^{-1}; Aldrich Chemical Co.) in Mueller-Hinton broth by the broth microdilution technique, according to NCCLS guidelines (23). The MICs were the highest concentrations showing complete inhibition of growth. Sensitivities to tetraphenylphosphonium ion, rhodamine 123, zinc sulfate, nisin, valinomycin, and ethidium bromide were also determined by the broth microdilution method. Curing of plasmid pJM01 was carried out by subculturing an overnight culture of *E. faecalis* AR01/DG grown in BHI broth at 37°C for 5 days to obtain *E. faecalis* AR01/DG45.

Tn*917* mutagenesis with pTV1-OK. Independent pools of Tn*917* insertions in the host genomic DNA were generated by using a temperature switch from 28 to 44°C. An overnight culture of AR01/DGVS containing pTV1-OK was grown in BHI broth plus kanamycin (500 μg ml^{-1}) at 28°C. This culture was subcultured into fresh prewarmed BHI broth containing erythromycin (0.04 μg ml^{-1}) at 44°C and incubated overnight. Tn*917* mutants were isolated by plating the overnight cultures onto BHI agar plates containing 10 μg of erythromycin ml^{-1}. Colonies that were erythromycin resistant and kanamycin sensitive were screened for bacitracin sensitivity on BHI agar containing 100 μg of bacitracin ml^{-1}. The presence of Tn*917* in the bacitracin-sensitive mutants was confirmed by probing HindIII-digested genomic DNA with radioactively labeled pTV1-OK DNA.

Mapping of transposon inserts. To map the site of the Tn*917* insertion in mutants DGM2 and DGM4, an inverse PCR with Tn*917*-derived primers ErmP2 (5′-TACAAATTCCTCGTAGGC-3′) and Sau3A1F (5′-TCCGTTCCTTTTTC ATAGTTCC-3′) (11) was performed. Total DNA from the Tn*917* mutants was digested with Sau3A1 and self-ligated. Self-ligated DGM4 DNA was subjected to inverse PCR with 0.5 U of *Taq* DNA polymerase (Roche), 1.25 μl of dimethyl sulfoxide, and the PCR program described previously (14). Inverse PCR of DGM2 DNA was performed by use of an Expand Long Template PCR system (Roche) and the conditions recommended by the manufacturer. Amplification consisted of one cycle at 94°C for 2 min and 10 cycles at 94°C for 10 s, 60°C for 30 s, and 68°C for 4 min. This was followed by 20 cycles at 94°C for 10 s, 60°C for 30 s, and 68°C for 4 min (with the elongation time increased by 10 s per cycle) and a final cycle at 68°C for 7 min. A 2.5-kb PCR product was obtained from the Sau3A1-digested DGM4 DNA, while the PCR product amplified from DGM2 DNA was 3.5 kb. The PCR products were sequenced with Tn*917*-specific primers ErmP2 and Sau3A1F (11).

Genomic DNA extraction, transformation, and genetic techniques. *E. faecalis* chromosomal DNA was obtained by a previously described method (21). Transformation of *E. faecalis* cells grown in the presence of glycine was performed as described by Shepard and Gilmore (30). Transformants of AR01/DGVS containing pTV1-OK were selected on SR agar (30) containing 500 μg of kanamycin ml^{-1} at 28°C. Other DNA manipulations were carried out by standard procedures (29). Purified plasmid DNA was prepared with a QIAprep spin miniprep kit (Qiagen) for high-copy-number plasmid extraction or a plasmid midi kit (Qiagen) for low-copy-number vectors. Gel-extracted DNA was prepared by use of a Qiaex II gel extraction kit (Qiagen). Restriction endonucleases, ligases, and polymerases were used according to the instructions of the manufacturer. PCRs were performed in accordance with the instructions of the manufacturer by using the PCR program described previously (14). The primer sequences and specificities used in this study are as follows: primers *bcrBF* (5′-AAAGAAACCGA CTGCTGATA-3′) and *bcrBR* (5′-GCTTACTTGTATAGCAGAGA-3′) for *bcrB*, primers *bcrDF* (5′-AGGATTCGGCCGAATGGCACTTGATTTTAT-3′) and *bcrDR* 5′-GTTTCTTCGCGAAATTGCCGTTATAAGTAA-3′ for *bcrD*, and primers *bcrRF* (5′-AACAAACAGGGAGCGGCCGCATGGAATTTA-3′) and *bcrRR* (5′-TGATGTTCGCGATTTCATTCCCATCTGCTT-3′) for *bcrR*. Radiolabeled PCR products and plasmids were prepared by incorporation of [α-^{32}P]dCTP-labeled deoxynucleotides (Amersham) by using Ready-To-Go DNA labeling beads (Amersham). Southern transfer and hybridization were performed as described previously (21).

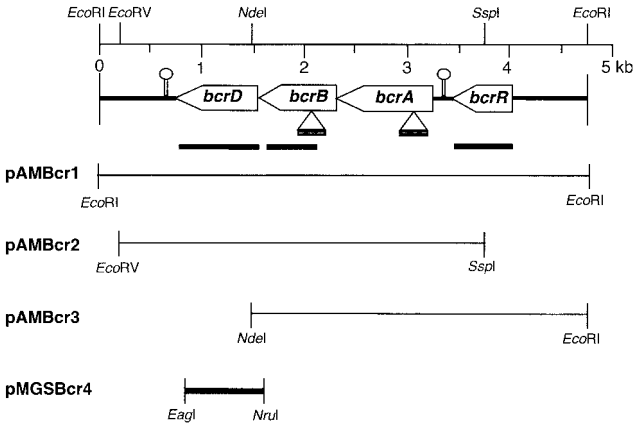

FIG. 1. Organization and restriction map of the *bcr* region in *E. faecalis* AR01/DGVS. Open arrows indicate the positions of ORFs. The restriction sites and fragments used to generate further constructs are shown. PCR products and probes are indicated by thick black lines, and transcriptional terminators are indicated by circles on stems. The Tn*917* insertion sites in bacitracin-sensitive mutants DGM2 and DGM4 are represented by triangles.

Nucleotide sequencing and sequence analysis. PCR products and plasmids were sequenced directly. Sequencing reactions were carried out with a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom) and a model ABI377 automated DNA sequencer (Applied Biosystems). The nucleotide sequences were assembled by using the Seqman program (DNASTAR, Inc.). Sequence analyses were carried out with Editseq software (DNASTAR, Inc.) for Apple Macintosh computers and the programs BLASTN, BLASTP, and BLASTX (National Center for Biotechnology Information, Los Alamos, N. Mex.), available via the Internet. The GeneMark and GeneMark.hmm software programs were used to predict the locations of gene boundaries (3, 18).

Cloning of *bcr* genes and plasmid construction. The *bcr* genes of *E. faecalis* AR01/DGVS were cloned as a 4.7-kb EcoRI fragment into pUC8, creating plasmid p2H7. The resulting insert in p2H7 was sequenced. To enable complementation, plasmid pAMBcr1 was constructed by ligating the 4.7-kb EcoRI fragment from p2H7 into the shuttle vector pAM401. To determine the role of *bcrR*, plasmid pAMBcr2 was constructed by digesting p2H7 with SspI and EcoRV (Fig. 1) and ligating the resulting 3.6-kb fragment with a truncated *bcrR* gene into pAM401. To examine the importance of *bcrD* in bacitracin resistance, plasmid pAMBcr3 was created by digesting p2H7 with EcoRI and NdeI, thereby excising the majority of the *bcrD* gene. This 3.2-kb fragment was ligated into pAM401. To express the *bcrD* gene separately from the other bacitracin resistance genes, the entire coding sequence of *bcrD* was amplified by PCR with primers *bcrDF* and *bcrDR* (see above). The amplified fragment was digested with EagI and NruI and inserted between the corresponding sites of the pMGS100 plasmid to obtain pMGSBcr4.

Isolation of RNA and Northern hybridization. Total RNA was isolated from cells grown to an optical density at 600 nm (OD_{600}) of 0.6 in BHI broth, with or without antibiotics, following the instructions of the manufacturer of the RNeasy kit (Qiagen). To analyze the effect of BcrR on *bcrABD* expression in JH2-2 cells containing either pAMBcr1 (*bcrR* and *bcrABD*) or pAMBcr2 (*bcrABD*), cells were grown to an OD_{600} of 0.6 in BHI broth containing 20 μg of chloramphenicol ml^{-1} and total RNA was extracted (under noninducing conditions). Cells were also grown in BHI broth containing 20 μg of chloramphenicol ml^{-1} to an OD_{600} of 0.6 and then challenged by the addition of 256 μg of bacitracin ml^{-1} with further incubation at 37°C for 1 h. For experiments involving $ZnSO_4$, cells (strain AR01/DGVS) were grown in medium containing 6 mM $ZnSO_4$ to an OD_{600} of 0.6 and total RNA was extracted. For vancomycin, cells were also grown in the absence of antimicrobials to an OD_{600} of 0.6, harvested, resuspended in broth containing either no vancomycin or 256 μg of vancomycin ml^{-1}, and incubated at 37°C for 1 h before RNA extraction. Purified RNA samples were either used immediately or frozen at −80°C. The RNA was quantified with a NanoDrop spectrophotometer. Northern blot analysis was carried out as described by Keis et al. (15). A 0.24- to 9.5-kb RNA ladder (Gibco BRL) was used as a standard.

Transfer experiments and PFGE. Transfer experiments were performed in broth, as described by Christie et al. (7), with *E. faecalis* JH2-2 (13) as the recipient strain and *E. faecalis* AR01/DGVS as the donor strain. Transconjugants were selected on BHI agar containing bacitracin (100 μg ml^{-1}), rifampin (50 μg ml^{-1}), and fusidic acid (25 μg ml^{-1}). Genomic DNA embedded in agarose was prepared and digested with I-CeuI and SmaI, as described previously (21). Pulsed-field gel electrophoresis (PFGE) was performed by contour-clamped homogeneous electric field electrophoresis with a CHEF-DRIII system (Bio-Rad Laboratories). Gels were run at 6 V/cm and 14°C at an included angle of 120° on a 1.2% agarose gel (Amersham), with pulse times of 5 to 25 s for 22 h.

Nucleotide sequence accession number. The DNA sequence of the 4,702-bp EcoRI fragment encoding the bacitracin resistance genes *bcrA*, *bcrB*, *bcrD*, and *bcrR* has been deposited in GenBank under accession number AY496968.

RESULTS AND DISCUSSION

Identification and characterization of bacitracin resistance genes in *E. faecalis*. In order to elucidate the genetic basis for bacitracin resistance in enterococci, we chose to study a previously described bacitracin-resistant (MIC, ≥256 μg ml^{-1}) *E. faecalis* isolate, AR01/DG (20). To enable the use of pTV1-OK (which carries an erythromycin resistance gene) as a mutagenesis vector, strain AR01/DG was subcultured in the absence of antibiotics to cure the strain of plasmid pJM02 (20). This endogenous plasmid contains the *vanA* and *ermB* genes, responsible for vancomycin and erythromycin resistance, respectively, in this isolate (20). Importantly, curing of plasmid pJM02 did not affect the bacitracin MIC for this strain. The resulting strain, designated AR01/DGVS (bacitracin MIC, ≥256 μg ml^{-1}), was transformed with pTV1-OK, and transposon mutagenesis was carried out to identify Tn*917* insertional mutants with a bacitracin-sensitive phenotype. For the purpose of this communication, bacitracin sensitivity is defined as an MIC ≤32 μg ml^{-1}.

In total, 8,000 Tn*917* insertion mutants of strain AR01/DGVS were screened for their ability to grow on BHI agar containing 100 μg of bacitracin ml^{-1}. This concentration of bacitracin was chosen as it was approximately threefold greater than the intrinsic bacitracin MIC of 32 μg ml^{-1} noted for JH2-2. Two bacitracin-sensitive mutants (MICs, 32 μg ml^{-1}) were isolated and designated DGM2 and DGM4, and Southern hybridization confirmed that the Tn*917* insertions were at different genetic loci (data not shown). Inverse PCR was used to determine the sequence flanking the left variable arm of Tn*917*, and the DNA sequence obtained from DGM2 was found to have homology to BcrB from *B. licheniformis*, which encodes a membrane-bound permease which is proposed to be involved in the efflux of bacitracin from the cell (28). Translated DNA from the PCR product amplified from DGM4 was found to have homology to BcrA from *B. licheniformis*, which encodes the bacitracin efflux ABC transporter BcrABC (28).

To further characterize the bacitracin-sensitive mutants and examine the specificity of the ABC transporter, the profiles of their susceptibilities to several cationic inhibitors were tested. No differences in susceptibilities to tetraphenylphosphonium ion, zinc sulfate, rhodamine 123, valinomycin, nisin, or ethidium bromide were observed between bacitracin-resistant parent strain AR01/DGVS and bacitracin-sensitive mutants DGM2 and DGM4 (data not shown).

Southern hybridization analysis of EcoRI-digested DNA of AR01/DGVS showed that *bcrA* is present on a 4.7-kb EcoRI fragment. This DNA fragment was cloned into pUC8 to create

plasmid p2H7 and was sequenced by using synthetic oligonucleotide primers. By using GeneMark software, it was found that the 4,702-bp insert in p2H7 had four identifiable open reading frames (ORFs) (Fig. 1). The four ORFs were oriented in the same direction and were designated *bcrR* (615 bp), *bcrA* (927 bp), *bcrB* (750 bp), and *bcrD* (831 bp). Tn*917* insertions were mapped to position 2049 within *bcrB* in mutant DGM2 and position 3038 within *bcrA* in mutant DGM4 (Fig. 1). The *bcrR* gene was located 166 bp upstream from the *bcrA* gene, while the intergenic regions between *bcrA* and *bcrB* and between *bcrB* and *bcrD* were −7 and 0 bp, respectively, and appear to comprise an operon (Fig. 1).

The complete amino acid sequences of BcrA (ATP-binding domain) and BrcB (membrane-spanning domain) are predicted to constitute a homodimeric ABC transporter. ABC transporters have previously been reported to be responsible for bacitracin resistance in *B. licheniformis*, *B. subtilis*, and *S. mutans* (26, 28, 34). In these three systems it has been proposed that bacitracin is pumped from the cell via an efflux mechanism, but this has yet to be proven experimentally. BcrD was found to have 62% identity and 81% similarity to a putative undecaprenol kinase in *Clostridium thermocellum*. Undecaprenol kinases have previously been postulated to be involved in bacitracin resistance (4, 6, 16).

The BcrR protein exhibited homology to the xenobiotic response element (XRE) family of transcriptional regulators, with a conserved domain spanning positions 5 to 61. This family of regulatory proteins is poorly characterized, with only a hypothetical function assigned to most members. However, some regulators of this family have been found to regulate stress responses in bacteria (17). In addition to the helix-turn-helix N-terminal domain of BcrR, analysis of the secondary structure of BcrR revealed four probable transmembrane helices (positions 82 to 104, 127 to 149, 155 to 177, and 180 to 202) in the C-terminal domain of the protein, which could indicate that BcrR is anchored in the cytoplasmic membrane. These data suggest that BcrR may act as a sensor and a transducer of bacitracin availability. A precedent for such a mechanism has been documented for other membrane-localized activators, like ToxR, that bind to DNA and activate transcription (22). In contrast to ToxR, BcrR is predicted to be anchored in the membrane by four membrane-spanning helices, whereas ToxR has a single transmembrane domain (22). No sequence for an apparent sensor type of protein was present upstream or downstream of the *bcrR* gene, and no regulatory protein-binding signatures were discernible (data not shown). In all other bacitracin efflux ABC transporters characterized to date, regulation of the transporter is controlled by a two-component regulatory system comprising a sensor kinase and a response regulator (24, 26, 34).

Complementation of bacitracin-sensitive mutants. The 4,702-bp EcoRI fragment containing *bcrR*, *bcrA*, *bcrB*, and *bcrD* was cloned into the *E. coli-E. faecalis* shuttle vector pAM401 for complementation studies. The resulting plasmid, pAMBcr1 (Fig. 1), was electroporated into DGM2 (*bcrB*::Tn*917*) and DGM4 (*bcrA*::Tn*917*), and in both the transformants, the wild-type bacitracin-resistant phenotype was restored (MIC, ≥256 μg ml^{-1}). To determine if pAMBcr1 contained all the genes necessary for bacitracin resistance, pAMBcr1 was electroporated into JH2-2 (bacitracin MIC, 32 μg ml^{-1}). The bacitracin MIC for the resulting strain (JH2-2/pAMBcr1) was ≥256 μg ml^{-1}, suggesting that only *bcrR*, *bcrA*, *bcrB*, and *bcrD* are necessary to convey bacitracin resistance to a susceptible host. To examine the function of *bcrR* and its putative role in regulation, a plasmid construct that contained a deletion of the 5′ end of the *bcrR* gene (pAMBcr2) was made (Fig. 1). Plasmid p2H7 was digested with SspI and EcoRV, and the resulting 3,578-bp fragment was ligated into pAM401. Electroporation of this construct into JH2-2 resulted in a bacitracin MIC of 32 μg ml^{-1}, indicating that *bcrR* is essential for the bacitracin resistance phenotype. To examine the role of the putative undecaprenol kinase (*bcrD*) in bacitracin resistance, two constructs were made. First, p2H7 was digested with EcoRI and NdeI, and the resulting 3.2-kb fragment was blunt ended and ligated into pAM401. This plasmid (pAMBcr3; Fig. 1) contained *bcrR*, *bcrA*, *bcrB*, and truncated *bcrD*. The effect of truncation of *bcrD* on bacitracin resistance was tested by transformation of pAMBcr3 into JH2-2. The bacitracin MIC for strain JH2-2/pAMBcr3 was ≥256 μg ml^{-1}, suggesting that *bcrD* is not required for high-level bacitracin resistance, but we cannot rule out the possibility that a chromosomal copy or homologue of *bcrD* may exist in JH2-2 and may rescue the phenotype. Interestingly, the genome sequence of *E. faecalis* V583 reveals a homologue of this enzyme on the chromosome, and this may explain the low level of intrinsic resistance (MIC, 32 μg ml^{-1}) seen in JH2-2 if indeed an undecaprenol kinase is present on the chromosome of this bacterium. To further examine the role of *bcrD*, the gene was amplified by PCR and ligated in frame into the expression vector pMGS100. This plasmid, pMGSBcr4, was electroporated into JH2-2, and the bacitracin MIC was found to be 32 μg ml^{-1}, demonstrating that *bcrD* alone has no significant effect on bacitracin resistance levels in JH2-2.

Transcriptional analysis of the *bcr* genes. From the DNA sequence analysis, it appeared that *bcrA*, *bcrB*, and *bcrD* comprise an operon. Northern blot analysis was performed to determine whether these genes are cotranscribed. The results obtained by using RNA isolated from AR01/DGVS cells grown in the presence of various concentrations (10 to 256 μg ml^{-1}) of bacitracin revealed a 2.7-kb hybridizing band with probes specific for *bcrA* and *bcrD* (Fig. 2A and B), indicating that *bcrABD* is transcribed as a polycistronic message. The levels of the *bcrA* and *bcrD* transcripts increased significantly as the concentration of bacitracin in which the cells were grown increased, suggesting that *bcrABD* is inducible (Fig. 2A and B). Northern analysis with a probe specific for *bcrR* revealed a 0.7-kb transcript which was expressed constitutively in either the presence or the absence of bacitracin (Fig. 2C). No *bcrABD* transcript was detected in bacitracin-sensitive mutants DGM2 and DGM4; however, the *bcrR* transcript was present in the two strains at the same levels as in the wild-type strain, demonstrating that mutation in either *bcrA* or *bcrB* has no effect on *bcrR* transcription (data not shown). To determine the effect of BcrR on *bcrABD* expression, strain JH2-2 containing either pAMBcr1 (*bcrR* and *bcrABD*) or pAMBcr2 (*bcrABD*) was grown to mid-log phase and then challenged with 256 μg of bacitracin ml^{-1}. The 2.7-kb *bcrABD* transcript was observed only in JH2-2 containing *bcrR*, demonstrating that BcrR is required for activation of *bcrABD* transcription (Fig. 2D).

To determine whether induction of the *bcr* genes was due to

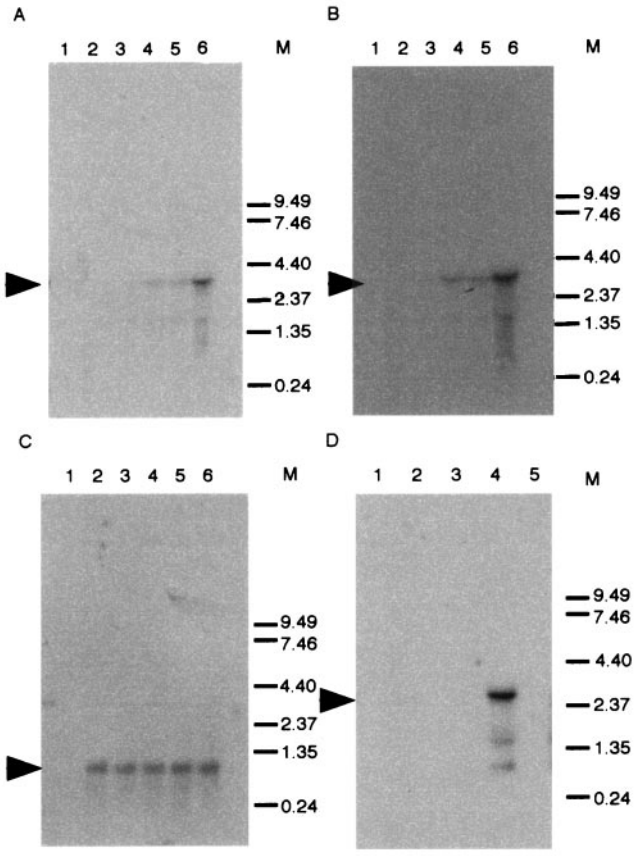

FIG. 2. Northern blot analysis of total RNA from JH2-2 and AR01/DGVS probed with *bcrA* (A), *bcrD* (B), and *bcrR* (C). Lanes 1, JH2-2 (negative control); lanes 2, AR01/DGVS; lanes 3 to 6, AR01/DGVS grown with bacitracin at 10, 50, 100, and 256 μg ml^{-1}, respectively; lanes M, molecular size standard (in kilobases; Gibco BRL). Arrowheads indicate the major transcript for each gene, sized at 2.7 kb for *bcrA* and *bcrD* and 0.7 kb for *bcrR*. Fainter nonspecific hybridization to 16S rRNA can be observed in panels A and B. (D) Northern analysis of total RNA from JH2-2 probed with *bcrD*. Lane 1, JH2-2 (negative control); lane 2, JH2-2/pAMBcr1; lane 3, JH2-2/pAMBcr2; lane 4, JH2-2/pAMBcr1 after 1 h of exposure to 256 μg of bacitracin ml^{-1}; lane 5, JH2-2/pAMBcr2 after 1 h of exposure to 256 μg of bacitracin ml^{-1}. Isolation of total RNA and Northern hybridization were carried out as described in Materials and Methods, with 10 μg of RNA loaded per lane.

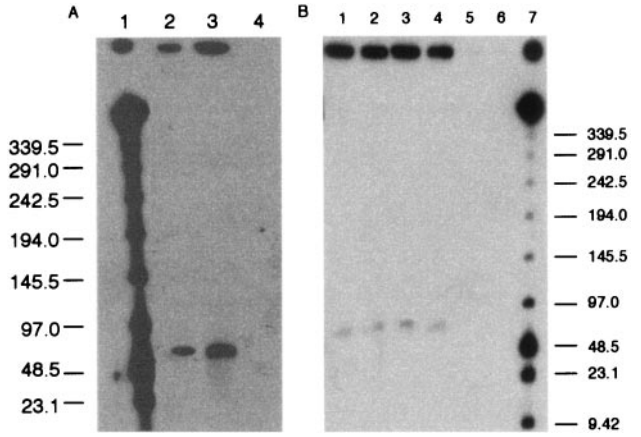

FIG. 3. Southern blot analysis of strains involved in conjugative transfer of bacitracin resistance. (A) SmaI-digested genomic DNA from donor, recipient, and transconjugant strains was probed with a *bcrB* gene probe. Lane 1, bacteriophage lambda DNA ladder standard; lane 2, donor strain AR01/DGVS; lane 3, transconjugant strain JH2-Bcr; lane 4, recipient strain JH2-2. (B) Hybridization with a *bcrB* gene probe to I-CeuI-digested genomic DNA. Two lanes of genomic DNA from each isolate are shown. The first DNA in each pair was not digested with I-CeuI, while the second DNA was incubated with the enzyme. Lanes 1 and 2, AR01/DGVS; lanes 3 and 4, JH2-Bcr; lanes 5 and 6, JH2-2; lane 7, bacteriophage lambda DNA ladder standard. Sizes (in kilobases) are indicated on the left and right.

the presence of bacitracin or to another downstream effect of bacitracin activity (e.g., the buildup of cell wall precursors), we tested the effect of vancomycin on *bcrABD* expression. No induction of the *bcrABD* operon was seen by Northern blot analysis (data not shown), suggesting that the buildup of cell wall precursors is not involved in the induction of *bcrABD* expression. As bacitracin is present as a zinc salt, the possible role of zinc in the induction of the *bcr* genes was investigated. Again, no induction of the *bcrB* or *bcrD* gene was noted (data not shown).

Bacitracin resistance is transferable and plasmid borne in *E. faecalis*. To examine if bacitracin resistance was transferable, broth matings were carried out with strain JH2-2 as the recipient (MIC, 32 μg ml^{-1}). High-level bacitracin resistance (MIC, ≥256 μg ml^{-1}) was found to be transferred at a frequency of conjugation of 7.28×10^{-3}. Transfer of the resistance genes from the donor strain to the transconjugants was confirmed by PFGE and hybridization with a *bcrB* gene probe. The *bcrB* probe hybridized to a SmaI fragment of approximately 72 kb in both the donor and the transconjugant, illustrating the presence of the *bcr* operon (Fig. 3A, lanes 2 and 3). I-CeuI digestion of total genomic DNA was used to further investigate the location of the *bcrB* gene (Fig. 3B). I-CeuI is a restriction enzyme that recognizes a specific site in the 23S rRNA operon and thus cleaves only chromosomal DNA. Hybridization of I-CeuI-digested and nondigested DNA can therefore determine whether a gene is present on the chromosome or is plasmid borne. The *bcrB* probe hybridized to a band of approximately 72 kb in both I-CeuI-digested and nondigested DNA from AR01/DGVS, demonstrating that the *bcr* operon is plasmid borne in this isolate (Fig. 3B). In other bacterial genera, bacitracin resistance genes are chromosomally encoded (26, 28, 34).

Zinc bacitracin is used in New Zealand poultry production at very high levels, and the use of this compound has selected for enterococcal strains harboring bacitracin resistance genes. The fact that transferable bacitracin resistance has been detected in a vancomycin-resistant *E. faecalis* isolate potentially negates the use of bacitracin to eliminate colonization with vancomycin-resistant enterococci, as has been suggested previously (25). Further investigation is needed to determine how widespread these genes are in enterococcal strains and species from different environments.

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