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Abstract

BcrR has been identified as a novel regulatory protein of high level bacitracin resistance encoded by the bcrABD operon in Enterococcus faecalis. The N-terminal domain of BcrR has similarity to the helix-turn-helix motif of DNA-binding proteins, and topological modeling predicts that the C-terminal domain contains four transmembrane alpha-helices. These data have led to the hypothesis that BcrR functions as both a membrane-bound sensor and transducer of bacitracin availability to regulate bcrABD expression. To characterize the bcrABD promoter and identify the promoter elements to which BcrR binds, a series of bcrA-lacZ fusions were constructed. A 69-bp region was identified that was essential for bacitracin-dependent bcrA-lacZ expression. Mutations that targeted this region were used to identify two inverted repeat sequences, each with the sequence 5'-GACA(N)(7)TGTC-3', on the bcrABD promoter that were required for bcrA-lacZ expression. To study BcrR binding to this region, we over-produced BcrR with a C-terminal hexa-histidine tag in Escherichia coli membranes, extracted the protein with n-dodecyl-beta-d-maltoside, and subsequently purified it via Ni(2+)-nitrilotriacetic acid and gel filtration chromatography to apparent homogeneity. Purified BcrR was reconstituted into liposomes, and BcrR binding to bcrABD promoter DNA was analyzed using electrophoretic mobility shift assays. Both inverted repeat sequences were required for BcrR binding, both in the presence and absence of bacitracin. These data demonstrate that membrane-bound BcrR binds specifically to the bcrABD promoter, irrespective of bacitracin concentration. We therefore propose that bacitracin-dependent induction of bcrABD expression by BcrR occurs after DNA binding.
MOLECULAR ANALYSIS OF BCRR: A MEMBRANE-BOUND BACITRACIN SENSOR AND DNA-BINDING PROTEIN FROM ENTEROCoccus FAECALIS

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BcrR has been identified as a novel regulatory protein of high-level bacitracin resistance encoded by the bcrABD operon in Enterococcus faecalis. The N-terminal domain of BcrR has similarity to the helix-turn-helix motif of DNA-binding proteins, and topological modeling predicts that the C-terminal domain contains four transmembrane α-helices. These data have led to the hypothesis that BcrR functions as both a membrane-bound sensor and transducer of bacitracin availability to regulate bcrABD expression. To characterize the bcrABD promoter and identify the promoter elements to which BcrR binds, a series of bcrA-lacZ fusions were constructed. A 69-bp region was identified that was essential for bacitracin-dependent bcrA-lacZ expression. Mutations that targeted this region were used to identify two inverted repeat sequences, each with the sequence 5′-GACA(N)7TGTC-3′, on the bcrABD promoter that were required for bcrA-lacZ expression. To study BcrR binding to this region, we overproduced BcrR with a C-terminal hexa-histidine tag in Escherichia coli membranes, extracted the protein with n-dodecyl-β-D-maltoside, and subsequently purified it via Ni²⁺-NTA and gel filtration chromatography to apparent homogeneity. Purified BcrR was reconstituted into liposomes, and BcrR binding to bcrABD promoter DNA was analyzed using electrophoretic mobility shift assays. Both inverted repeat sequences were required for BcrR binding, both in the presence and absence of bacitracin. These data demonstrate that membrane-bound BcrR binds specifically to the bcrABD promoter, irrespective of bacitracin concentration. We therefore propose that bacitracin-dependent induction of bcrABD expression by BcrR occurs after DNA binding.

Bacitracin is a polypeptide antibiotic that functions by binding to and sequestering undecaprenyl pyrophosphate (UPP) (1). As UPP acts as a carrier of peptidoglycan monomeric units across the cell membrane (2), the antibacterial nature of bacitracin results from its ability to block cell wall synthesis. High-level bacitracin resistance in Enterococcus faecalis is encoded by the bcrABD operon that is under the control of a putative membrane-bound DNA-binding protein, BcrR (3). In the presence of bacitracin, transcription of the bcrABD operon leads to the production of BcrA and BcrB, which are proposed to act as an ABC exporter of bacitracin, thus conferring high-level bacitracin resistance (MIC ≥ 256 μg/ml) to the cell (3). BcrD is proposed to function as an undecaprenyl pyrophosphate phosphatase that functions to increase the amount of UP available to the cell (3). It has been demonstrated that bcrR, which is transcribed constitutively, is essential for high-level bacitracin resistance in E. faecalis and that transcription of bcrABD is abolished in the absence of bcrR (3). The expression of bcrABD was found to be inducible with
increasing concentrations of bacitracin (3). The N-terminal domain (amino acid residues 5-61) of BcrR has homology to the Xre family of helix-turn-helix (HTH) DNA-binding proteins and, based on topological modeling, the C-terminal domain is predicted to contain four membrane-spanning α-helices. These data combined have led to the proposal that BcrR is a transmembrane regulatory protein that functions both as a sensor and signal transducer of bacitracin availability in the environment (3).

Regulation of bacitracin resistance in other bacterial genera has been exclusively shown to be via two-component regulatory systems. These two-component systems regulate genes that encode putative ABC transporters, which, although not yet experimentally demonstrated, are hypothesized to function by pumping bacitracin from the cell. In the bacitracin producer, Bacillus licheniformis, regulation of the ABC transporter (BcrABC) is via the BacRS two-component system (4). Similar systems exist in Bacillus subtilis (5) and in Streptococcus mutans (6). It has recently been demonstrated that the presence of both the putative bacitracin transporter, BceAB, and the two-component signal transduction pathway, BceRS, are required for activation of bceAB expression in the presence of bacitracin in B. subtilis (7). These results indicate that the BceAB transporter interacts with the BceRS two-component signal transduction system in some manner that is necessary for bceAB expression (7).

A recent survey of prokaryotic genomes has revealed that one-component signal transduction systems, in which an input and output domain are fused in a single protein molecule, are more numerous than classical two-component systems (8). This is the case in the genome of E. faecalis V583 that encodes 17 two-component systems and 158 one-component systems (9). One such system is that of PrkC, which contains a cytoplasmic kinase output domain fused to an extracellular input domain via a transmembrane region. This protein has been shown to modulate antimicrobial resistance and intestinal persistence, presumably in response to the perturbation of the cell wall by bile salts as detected by the extracellular domain (9). The majority of one-component systems are thought to be cytoplasmic, as 97% of those containing a HTH output domain lack transmembrane regions. It has been hypothesized that, although membrane-bound DNA-binding proteins may represent a more simplistic method of signal transduction than the classical two-component system, their use may be limited by restrictions in the ability of the protein to locate its target DNA (8).

Examples of transmembrane regulators are rare and include the ToxR family in Vibrio cholerae (10) and close relatives (11). ToxR contains a single transmembrane α-helix separating a cytoplasmic DNA-binding domain from a short C-terminal periplasmic domain (10). A role in signal transduction by ToxR may be likely in the presence of bile salts, where it regulates cholera toxin production (12) and expression of the outer membrane porins OmpU and OmpT (13).

Although transmembrane regulators are rare, the Xre family of transcriptional regulators is a large family, comprised to date of 6002 proteins across all branches of life (SMART database http://smart.embl.de/) (14). The Xre family has been incorrectly annotated as the ‘xenobiotic response element family of transcriptional regulators’ by the conserved domain database (15) in NCBI. Rather, the Xre-like family of HTH proteins is named after the family member Xre, a repressor in the Bacillus subtilis prophage PBSX (16). Although most members of the family remain uncharacterized, a few have been well characterized, including the phage 434 (17) and lambda phage (18) repressors. Two members of the family in E. faecalis are CylR2, which functions as a repressor of cytolysin exotoxin production (19), and PrgX, which regulates conjugation in response to a pheromone signal (20).

The aim of this study was to study the interaction of membrane-bound BcrR with the bacitracin resistance operon bcrABD in E. faecalis using an in vitro system. We show that membrane-bound BcrR binds to two inverted repeats, each with the sequence 5’-GACA(N)3-TGTC-3’, on the bcrABD promoter. Both repeats are required for bacitracin-dependent bcrA-lacZ expression.

EXPERIMENTAL PROCEDURES
Bacterial strains, plasmids, and growth conditions—The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* MC1061 (21) or DH10B (22) were used for cloning and *E. coli* C41(DE3) (23) was used to produce BcrR containing a C-terminal hexa-histidine tag for purification by Ni²⁺-nitrilotriacetic acid (NTA) affinity chromatography. *E. faecalis* strains AR01/DGVS (3) and JH2-2 (24) were used for bacitracin challenge and β-galactosidase assays. Plasmids used were pTrc99A (25) for overexpression of histidine-tagged BcrR, pTCVlac (26) as a transcriptional reporter and pAM401 (27) for the introduction of bcr genes into *E. faecalis*. For both cloning and protein expression, *E. coli* strains were grown at 37 °C in 2x YT broth or on Luria-Bertani (LB) agar (28). For routine growth and β-galactosidase assays, *E. faecalis* strains were grown at 37 °C without agitation in brain heart infusion (BHI) broth or on BHI agar. *E. coli* transformants were selected on LB agar containing either ampicillin at 100 μg/ml (pTrc99A), kanamycin at 50 μg/ml (pTCVlac) or 10 μg/ml tetracycline (pAM401). *E. faecalis* transformants were selected on SR agar (29) containing erythromycin at 10 μg/ml (pTCVlac) or 20 μg/ml chloramphenicol (pAM401).

General molecular techniques—All molecular biology techniques were carried out according to standard procedures (28). Restriction or DNA modifying enzymes and other molecular biology reagents were obtained from Roche Diagnostics or New England Biolabs. Genomic DNA of *E. faecalis* was isolated as previously described (30). Transformation of *E. faecalis* cells grown in the presence of glycine was performed as described by Shepard and Gilmore (29). All PCR were carried out with the Phusion™ high-fidelity PCR kit (Finnzymes) according to the manufacturer’s instructions.

Construction of vectors for complementation—To introduce wild-type and hexa-histidine tagged bcrR into JH2-2, the constructs pAMBCrR and pAMBCrRHis were constructed, respectively. To construct plasmid pAMBCrR, containing bcrR under the control of its own promoter, primers BcrRpAMf (5′-AAATTTCCATGGTCACAGAATTTC TGCAC-3′) and BcrRpAMr (5′-AAATTTGAATTCTATTCCATTCATCCATC TGCTTT-3′) containing NcoI and EcoRI restriction sites (underlined), respectively, were used to amplify bcrR by PCR using AR01/DGVS genomic DNA as a template. The amplicon was digested with NcoI and EcoRI and cloned into pAM401 digested with the same restriction enzymes. To construct plasmid pAMBCrRHis, containing C-terminal hexa-histidine tagged bcrR under the control of its own promoter, primers BcrRpAMf and BcrRpAMHisr (5′-AAATTTGAATTCTTAATGATGATGATG TAGATGTTTTCATCCATCTGCTTT-3′) containing NcoI and EcoRI restriction sites (underlined), respectively, and histidine codons (italicized) were used to amplify bcrR by PCR using AR01/DGVS genomic DNA as a template. The amplicon was digested with NcoI and EcoRI and cloned into pAM401 digested with the same restriction enzymes. These constructs were introduced into *E. faecalis* strain JH2-2 to create JH2-2pAMBCrR and JH2-2pAMBCrRHis, respectively.

Construction of bcrA-lacZ transcriptional fusions—To construct a series of bcrA-lacZ reporter fusions, primers listed in Table 2 were used to amplify bcrABD promoter DNA using plasmid pAMbcR1, unless otherwise stated, as a template. Amplified DNA was digested with the corresponding enzymes as indicated in Table 2 and ligated into pTCVlac digested with the same enzymes. All promoter regions amplified by PCR were confirmed by DNA sequencing. To map the bcrABD promoter, a series of promoter-lacZ fusions were created as shown in Figure 1. To create plasmids pTCVA, pTVCB, pTVCV, pTCVD, and pTCVE the forward primers EfbcrAP2F, EfbcrAP7F, EfbcrAP6F, EfbcrAP5F, and EfbcrAP3F were used, respectively, with the reverse primer EfbcrAP2R.

In order to identify DNA motifs important for BcrR binding in the bcrABD promoter, a series of nucleotide substitutions were introduced by PCR overlap extension (31) into the same 389-bp bcrABD promoter sequence used to create plasmid pTCVA. The mutated PCR product was then cloned into pTCVlac and sequenced to ensure fidelity of the PCR reaction. To create pTCVA1, two pairs of primers (viz. EfbcrAP2EcoF with BcrAarea4R and
BcrAarea4F with EfbcRAP2R) and pAMbcR1 template were used to generate mutated DNA fragments. These overlapping fragments were then used for overlap extension PCR with the external primers EfbcRAP2EcoF and EfbcRAP2R (Table 2). To create pTCV2A, pTCV3A, pTCV4A, pTCV1A+3, and pTCV2A+4 the same procedure as above was followed using the internal primers given in Table 2, with the exception of pTCV1A+3 and pTCV2A+4 where plasmids pTCV3A and pTCV2A served as template, respectively.

**Bacitracin challenge and β-galactosidase assays** - E. faecalis containing bcrABD-promoter reporter constructs in pTCVlac were grown to an OD600 of approximately 0.5. Cells were separated into 10 ml aliquots and 256 µg/ml of bacitracin added unless otherwise stated. Following 1 h incubation at 37 °C, cells were harvested, rapidly frozen, and stored at -20 °C. The β-galactosidase activity of cells was determined as described previously (32).

**Mapping of the transcriptional start site** (TSS) of bcrABD - The TSS of bcrABD was mapped by 5'-RACE (rapid amplification of cDNA ends) using the components of the 3'/5'-RACE kit (Roche) according to the manufacturer’s instruction. RNA was isolated from E. faecalis AR01/DGVS grown in the presence of 256 µg/ml bacitracin. First strand cDNA was synthesised from 4.8 µg total RNA with the bcrA-specific primer bcrA-RACE1 (5'-GAGTAATCGGAAAAGACCT-3'). The resulting cDNA was purified and dA-tailed following the kit instructions. Purified dA-tailed cDNA was then used as a template for PCR using the Oligo dT-anchor primer and bcrA-specific primer bcrA-RACE2 (5'-CTACTCCACTTTAGATAC-3'). The resulting PCR product was gel purified and used as template for a second PCR using the PCR anchor primer and nested bcrA-specific primer bcrA-RACE3 (5’-CTCATACCTGTAAATTACTG-3’). This PCR product was then sequenced using primer bcrA-RACE3.

**Construction of vectors for over-expression of BcrR** - Two constructs for the expression of recombinant BcrR were created using the expression vector pTrc99A. To construct plasmid pTrcBcrR, for the expression of BcrR, primers BcrRFwd (5’-AAATTTCATGGAATTTAATGAAAGCTTACAA-3’) and BcrRRev (5’-AAATTGGTCTGACTTTATTTCCATTCCCATGTC-3’) containing NcoI and SalI restriction sites (underlined), respectively, were used to amplify bcrR by PCR using AR01/DGV/S genomic DNA as a template. The amplicon was digested with NcoI and SalI and cloned into pTrc99A digested with the same restriction enzymes. To construct plasmid pTrcBcrRHis, for the expression of BcrR containing a hexa-histidine tag at the C-terminus (corresponding to italicized nucleotides), primers BcrRFwd and HisBcrRRev (5’-AATTGGTCTGACTTTAGTGGTGGTGTTGTTAATTTACCTCCATTCCATGTC-3’), containing a SalI site (underlined), were used to amplify bcrR by PCR. The amplicon was digested with NcoI and SalI and cloned into pTrc99A digested with the same restriction enzymes. All regions amplified by PCR were verified by DNA sequencing.

**Expression and purification of hexa-histidine tagged BcrR-Plasmid pTrcBcrRHiss** was electroporated into E. coli C41(DE3) prior to expression trials. Following inoculation with an overnight culture (0.1%), a 15-l culture was grown in 2 × YT medium supplemented with 100 µg/ml ampicillin in a fermenter (Bioflo 410, New Brunswick Scientific) with agitation (200 rpm) and aeration (12 l/min) at 37 °C. At an OD600 of 0.7, the culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for a further 2 h. Cells were harvested by centrifugation at 9,220 × g for 10 min. Cells were washed once in 20 mM sodium phosphate buffer (pH 7.4), resuspended in 20 mM sodium phosphate buffer (pH 7.4) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by three passages through a French pressure cell (American Instrument Company) at 20,000 psi. DNaseI was added (0.1 mg/ml) and the suspension incubated on ice for 1 h. Unbroken cells were removed by centrifugation at 8,000 × g for 10 min and the membranes collected from the cell-free supernatant by ultracentrifugation at 180,000 × g for 50 min. The membranes were washed once in 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM PMSF and 5 mM DL-dithiothreitol (DTT),
and stored at -70 °C until use. Prior to solubilization, membranes were washed in buffer A (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 0.1 mM PMSF, 5 mM DTT, 10% glycerol) containing 1% sodium cholate. The membranes were then solubilized in buffer A containing 20 mM imidazole and 1% n-dodecyl-β-D-maltoside (DDM) with gentle stirring at room temperature for 1 h at a membrane protein concentration of approximately 5 mg/ml. The mixture was ultracentrifuged at 180,000 × g for 50 min. The supernatant (solubilized BcrR) was then loaded in two batches onto a 1-ml HisTrap™ HP Ni²⁺-NTA-sepharose column (Amersham Biosciences) pre-equilibrated with buffer A containing 20 mM imidazole and 0.05% DDM. The protein was eluted with a step-wise gradient of buffer B (buffer A with 500 mM imidazole and 0.05% DDM). Fractions containing BcrR were pooled and concentrated using an Amicon® centrifugal filter with a 10,000 molecular weight cutoff. Concentrated protein (approx. 2 mg) was loaded in 0.5 ml fractions onto a Superose 6 (10/300) GL column (Amersham Biosciences) for further purification by gel filtration and eluted in buffer C (20 mM HEPES, pH 7.0, 150 mM NaCl, 0.1 mM PMSF, 5 mM DTT, 10% glycerol, 0.025% DDM) at a flow rate of 0.5 ml/min.

**General biochemical techniques**—Protein concentrations of membrane fractions were determined by the BCA method (Pierce) using bovine serum albumin (BSA) as a standard. Protein concentrations of purified BcrR and proteoliposomes were estimated by comparison to BSA standards separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or by Bradford assay (33). Protein samples (15-20 µg, unless otherwise stated) were routinely separated by 14% SDS-PAGE using the buffer system described by Laemmli (34). Protein was visualized by staining with Simply Blue Safe Stain (Invitrogen).

**Western Blotting**—Samples separated by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane by electrophoretic transfer to a polyvinylidene fluoride (PVDF) membrane by electrophoretic transfer using a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad) according to the manufacturer’s instructions. Histidine-tagged BcrR was detected by incubation with Anti-His HRP conjugate (Qiagen) according to the manufacturer’s instructions. The antibody-specific bands were visualized using the SuperSignal® West Pico chemiluminescence system (Pierce).

**Reconstitution of purified BcrR into liposomes**—A 50 mg/ml suspension of either phosphatidylcholine or E. coli total lipid extract (Avanti Polar Lipids, Inc.) was produced by vortexing the lipid in proteoliposome buffer (10 mM HEPES, pH 7.0, 1 mM MgCl₂, 5 mM DTT). To prepare the lipids for reconstitution, the suspension was sonicated twice for 30 s on ice with a microtip sonicator at an output of 40 W and cooled on ice for 1 h. Purified BcrR was added to the suspension to obtain a lipid-to-protein ratio of 50:1 (w/w). In some instances Zn²⁺-bacitracin was also added at a ten-fold molar excess to BcrR (0.33 mM). Triton X-100 was added to a final concentration of 0.3% (w/v) and the mixture was incubated at room temperature with gentle stirring for 20 min. Aliquots of 50 mg, 50 mg, and 80 mg Bio-Beads® (Bio-Rad) were added consecutively, each time removing the exhausted Bio-Beads®, and incubated at room temperature with gentle stirring for 45 min each. The proteoliposomes were collected by ultracentrifugation at 180,000 × g for 50 min and the pellet resuspended in proteoliposome buffer. BcrR incorporation into liposomes was estimated by SDS-PAGE analysis to be 90% by comparing supernatant and pellet fractions. Proteoliposomes were either used immediately or stored at 4 °C for not more than 48 h prior to use in electrophoretic mobility shift assays.

**Preparation of DNA probes for electrophoretic mobility shift assay**—DNA probes for electrophoretic mobility shift assay were generated by PCR. A 116-bp PCR product, encompassing the putative BcrR DNA-binding sites, designated Bind1, was generated using primers EbcrbAP7F (5’-AACATCGAATTTCCCTGAAAATAGGCTCTGAC-3’) and EbcrbAP7R (5’-CACATAGGATTCGCCAAGAAACCTACCGTCAC-3’), respectively, and plasmid pAMbcr1 as template. A 184-bp PCR product, encompassing the mutated BcrR DNA-binding sites, designated Bind2, was generated using primers EbcrAP2EcoF, and EbcrAP7R with plasmid pTCVA1+3 as template. A PCR product, encompassing 116 bp of the bcrA coding region, designated bcrA116 was generated using primers
BcrA116F (5’-TGAACCGATTAACGGGCTTG-3’) and BcrA116R (5’-TCGCCTTAAACATGGCTTG-3’) and plasmid pAMbcr1 as template. In instances where the PCR product was radioactively-labeled, PCR was conducted in the presence of 5 mM dNTPs and 0.13 µM (20 µCi) [α-32P]dCTP (Amersham Biosciences). PCR products were purified using a High Pure PCR Product Purification kit (Roche). The concentration of unlabeled PCR products was determined by spectrophotometry and the concentration of labeled PCR products was determined by gel electrophoresis with unlabeled DNA of a known concentration.

**Electrophoretic mobility shift assay**

Binding reactions were performed in a total volume of 10 µl of binding buffer (10 mM HEPES, pH 7.5, 4% glycerol, 0.5 mM EDTA, 1 mM MgCl2, 0.5 mM DTT, 50 mM NaCl, 0.5 µg/ml bacitracin (ie. < 1 Miller unit), nor was induction of *bcrA-lacZ* expression achieved at 256 µg/ml bacitracin. No induction of *bcrA-lacZ* was observed in the absence of bacitracin (ie. < 1 Miller unit). These results show an identical pattern of bacitracin-dependent *bcrABD* expression to that reported for *bcrABD* expression obtained by Northern blot analysis (3). Importantly, the data demonstrate that the promoter region used for the *bcrA-lacZ* construct in pTCVA contains the critical regulatory sequences necessary for bacitracin-dependent expression.

To investigate if *bcrAB* had a role in the activation of *bcrA-lacZ* expression by bacitracin in a similar manner to that described for *bceAB* in *B. subtilis* (7), a two-plasmid system was established in the bacitracin-sensitive *E. faecalis* strain, JH2-2. The transcriptional reporter, pTCVA was introduced into JH2-2 along with either *bcrR* alone, as harbored on pAMBcrR, or with both *bcrR* and *bcrAB*, as harbored on pAMBcr3 (3). When cells were challenged with bacitracin, expression of *bcrA-lacZ* increased with increasing bacitracin concentration (Figures 1B and C) in a similar pattern to that observed previously (Fig 1A). However, the sensitivity of the system was strongly influenced by the presence of *bcrAB*. Maximal levels of *bcrA-lacZ* expression were achieved at 256 µg/ml bacitracin in the presence of *bcrAB* (Fig. 1B) and at only ≥ 0.5 µg/ml bacitracin in the absence of *bcrAB* (Fig. 1C). In all instances, no induction of *bcrA-lacZ* was observed in the absence of bacitracin (ie. < 1 Miller unit), nor was induction of *bcrA-lacZ* expression observed in JH2-2 in the absence of *bcrR* at any of the bacitracin concentrations tested (data not shown).

As we observed *bcrA-lacZ* expression in either the presence or absence of BcrAB, we conclude that BcrAB is not required for activation of transcription of *bcrABD* in *E. faecalis*. Although this observation differs from that in *B. subtilis*, where *bceAB* is
required for bceAB expression (7), it is unsurprising, as the putative transporters BcrAB and BceAB of *E. faecalis* and *B. subtilis*, respectively, belong to different families within the ABC transporter class (7). We propose that the activation of *bcrA-lacZ* expression at lower bacitracin concentrations in the absence of *bcrAB* (0.5 µg/ml compared to 256 µg/ml) is due to the absence of the function of the BcrAB transporter. In the absence of BcrAB, the bacitracin-related stimulus is not removed from BcrR and the system is saturated at lower bacitracin concentrations. The observation that *bcrA-lacZ* expression can be activated by as little as 0.5 µg/ml bacitracin indicates that activation of *bcrA-lacZ* expression is not due to the antibacterial activity of bacitracin, as at this concentration bacitracin is likely to have no effect on this strain with an MIC to bacitracin of 32 µg/ml. Rather, we propose that BcrR is highly sensitive to bacitracin either directly, or via an intermediate such as UPP. In accordance with this proposal, challenge of AR01/DGVS/pTCVA with a range of other cell wall-active antimicrobial compounds was unable to induce *bcrA-lacZ* expression, indicating that BcrR does not sense cell wall stress or a build up of cell wall precursors (data not shown).

**Mapping of the bcrABD promoter** To elucidate the interaction of BcrR with the *bcrABD* promoter region, we set out to identify the regulatory DNA elements required for BcrR binding and activation of *bcrABD* transcription. A series of *bcrA-lacZ* fusions that progressively truncated the intergenic region between *bcrR* and *bcrABD* was constructed (Fig. 2). *E. faecalis* strain AR01/DGVS was transformed with these constructs and the induction of *bcrA-lacZ* expression in response to bacitracin studied (Fig. 2). As the promoter of *bcrABD* was progressively truncated, we observed no significant change in the induction of *bcrA-lacZ* by bacitracin until we reached 133 bp upstream of the *bcrA* start codon (Fig. 2). We determined that a 69-bp region (-152 to -83) was essential for maximum induction of *bcrA-lacZ* expression. The ability of BcrR to induce *bcrA-lacZ* expression was impaired in constructs containing only a partial segment of this region (i.e. pTCVC and pTCVD) and was abolished in a construct (i.e. pTCVE) completely lacking this region (Fig. 2).

DNA sequence analysis of the 69-bp region revealed two inverted repeat regions separated by 15 nucleotides with the sequence 5’-GACA(N)CTGTC-3’ (Fig. 3). To determine the significance of the inverted repeats within the *bcrABD* promoter, PCR overlap extension was used to introduce mutations in the repeats using the full-length promoter construct pTCVA (Fig. 3). These mutated *bcrA-lacZ* promoter constructs were electroporated into strain AR01/DGVS and the induction of *bcrA-lacZ* expression in response to bacitracin was studied (Fig. 3). Mutation of 5’-CTGACA-3’ to 5’-CCCCCA-3’ in regions R1 and R3 (Fig. 3) resulted in induction of *bcrA-lacZ* expression by bacitracin at approximately only 13% and 20% of wild-type activity, respectively. Mutation of 5’-GTGTC-3’ to 5’-GAAAAA-3’ in regions R2 and R4 resulted in only 6% and 61% of wild-type *bcrA-lacZ* expression levels, respectively. To determine the effect of mutating both inverted repeats, mutations were introduced in regions R1 and R3 simultaneously and also regions R2 and R4 simultaneously. Both of these mutated regions completely abolished expression of *bcrA-lacZ* (Fig. 3), indicating that the presence of the inverted repeats was essential for induction of *bcrA-lacZ* expression by BcrR in the presence of bacitracin. No significant *bcrA-lacZ* expression was observed in any of the mutant constructs in the absence of bacitracin (data not shown). Mutations made within the 69-bp region that fell outside of the inverted repeat sequences had no effect on the induction of *bcrA-lacZ* expression by bacitracin (data not shown).

Based on the finding that the inverted repeats are essential for the induction of *bcrA-lacZ* expression in response to bacitracin, we propose that they may constitute the BcrR recognition sequence for BcrR binding to the *bcrABD* promoter. To test this hypothesis, we conducted electrophoretic mobility shift assays with *bcrABD* promoter DNA, as described below.

To determine the start site for *bcrABD* transcription, the 5’-end of the *bcrABD* transcript from AR01/DGVS grown in 256 µg/ml bacitracin was identified by 5’-RACE analysis (Fig. 4). Putative -10 and -35 elements were also identified. The inverted repeats proposed to function as BcrR-binding sites were located upstream of these elements.
Expression of BcrR in E. faecalis and E. coli-To study membrane-bound DNA-binding proteins, DNA binding assays have been either performed with inverted membrane vesicles that may contain other potentially contaminating proteins (10, 35), soluble variants of the protein (36, 37), in the presence of detergent (38), or by reconstitution of purified protein into liposomes (39). The level of bcrR expression in native membranes of E. faecalis was too low for electrophoretic mobility shift assays with bcrABD promoter DNA (data not shown). Therefore, we developed a recombinant method to overproduce BcrR in E. coli. Initial experiments utilized the expression vector pTrc99A harboring bcrR with no affinity tags (i.e. pTrcBcrR). IPTG induction of E. coli C41(DE3) containing plasmid pTrcBcrR inhibited E. coli growth when compared to the uninduced control or E. coli containing pTrc99A, suggesting that bcrR expression in E. coli was toxic (data not shown). SDS-PAGE analysis of the membrane fraction of this strain revealed the presence of an additional protein band at a molecular weight of approximately 20 kDa (Fig. 5A, lane 3) that was absent in the empty vector control (Fig. 5A, lane 2). N-terminal sequencing by Edman degradation of the overproduced protein confirmed that the first ten N-terminal amino acids were identical to the predicted BcrR N-terminal protein sequence (MEFNEKLQQL). No BcrR was found in the soluble fraction, confirming the predicted membrane localization of BcrR, even in E. coli (data not shown). Electrophoretic mobility shift assays conducted with E. coli inverted membrane vesicles in which BcrR had been overproduced, yielded inconsistent results (data not shown).

Purification of BcrR-To further facilitate electrophoretic mobility shift assays, we purified BcrR using affinity chromatography. A hexa-histidine tag was introduced at the C-terminus of BcrR, to create plasmid pTrcBcrRHis. The addition of the histidine tag had no significant effect on the function of BcrR, as we observed no significant difference in the activation of bcrA-lacZ expression by bacitracin in JH2-2pTCVA in either the presence of wild-type (pAMBcrR), or hexa-histidine tagged BcrR (pAMBcrRH). When pTrcBcrRH was expressed in E. coli C41(DE3), a dominant membrane associated band, with an approximate molecular weight of 21 kDa, became apparent by SDS-PAGE analysis (Fig. 5A, lane 4). Probing His-tagged proteins in the membrane fraction with an anti-His antibody by Western blot analysis demonstrated that this overproduced band contained a histidine tag and was indeed His-tagged BcrR (BcrRHis) (data not shown).

BcrRHis was extracted from inverted membrane vesicles of E. coli using 1% DDM (Fig. 5B, lane 4). Other detergents such as Triton X-100, n-octylglucoside, or sodium dodecylsulfate were less effective in extracting BcrRHis from E. coli membranes (data not shown). A 1% sodium cholate wash of the inverted membrane vesicles was included prior to extraction with DDM, as it was found to remove contaminating proteins, but did not extract BcrRHis (Fig. 5B, lane 6). Solubilized BcrRHis was bound to a Ni2+-NTA column and eluted in 300 mM imidazole (Fig. 5C, lane 2). BcrRHis was further purified by gel filtration (Fig. 5C, lane 3). Gel-filtration resulted in the elution of three peaks possibly corresponding to monomer, dimer and aggregated states of the protein, with the monomer predominating (data not shown). The purity was estimated to be greater than 95% by SDS-PAGE (Fig. 5C). BcrRHis displayed an intrinsic ability to dimerize, as a second fainter band that also associated with the anti-His antibody in Western Blot analysis was consistently observed running at approximately twice the molecular weight of BcrR in SDS-PAGE (data not shown).

BcrR binds to the bcrABD promoter and recognizes inverted repeats-In order to study the DNA-binding function of BcrR, we attempted to use BcrRHis in DDM and BcrRHis reconstituted into liposomes (DDM removed) for electrophoretic mobility shift assays with the bcrABD promoter region. BcrRHis was reconstituted using Triton X-100 treated liposomes and removal of the detergent with Bio-Beads®. After harvesting the proteoliposomes by high-speed centrifugation, >90% of BcrRHis had been reconstituted into the liposomes (Fig. 5D, lane 3), and only trace amounts of BcrRHis were found in the supernatant fraction (non-incorporated) (Fig. 5D, lane 2). The protocol used here is known to favor insertion of
proteins with the hydrophilic portion protruding outwards (40), but some BcrRHis molecules will be in the opposite orientation, unable to bind DNA.

A 116-bp [32P]-labeled DNA fragment, designated Bind1, encompassing the 69-bp region required for expression of bcrA-lacZ activity and containing the BcrR-binding sites was used as a DNA probe for BcrRHis binding. In addition, a 184-bp [32P]-labeled DNA fragment, designated Bind2, in which the inverted repeats had been mutated in regions R1 and R3, was also obtained. Despite numerous attempts, no specific retardation of bcrABD promoter DNA could be observed with BcrRHis in DDM (data not shown). In the presence of proteoliposomes containing BcrRHis, Bind1 displayed a mobility shift at approximate molar ratios of 2000:1 protein:DNA and above (Fig. 6A, lanes 2-5). We hypothesize that a large excess of protein is required to induce a mobility-shift due to a number of factors. These include a low ratio of protein-to-lipid in the proteoliposomes, BcrRHis orientated in the opposite direction with the DNA-binding domain on the inside of the proteoliposomes, and potential clumping of proteoliposomes preventing BcrRHis from accessing target DNA. Previously described electrophoretic mobility shift analyses with proteoliposomes have also required large quantities of protein to DNA for similar reasons (39).

In contrast to results obtained using Bind1, Bind2 containing the inverted repeats mutated at regions R1 and R3, displayed no mobility shift in the presence of proteoliposomes containing BcrRHis at similar molar protein:DNA ratios (Fig. 6A, lanes 7-10). This indicated that the inverted repeats within the putative BcrR binding domains are necessary for BcrR recognition and binding of bcrABD promoter DNA. These data corroborate our expression data, where the region encompassed by Bind1 is necessary for full induction of bcrA-lacZ activity and the mutations contained in Bind2 abolish bcrA-lacZ expression.

To investigate the specificity of BcrR binding to Bind1, we amplified a 116-bp fragment of the bcrA coding region by PCR to produce bcrA116. Addition of unlabeled bcrA116, and also unlabeled Bind2, in both 50 and 100-fold excess to [32P]-labeled Bind1 did not influence the ability of BcrRHis to shift Bind1 (Fig. 6B, lanes 5-6 and lanes 9-10). In contrast, addition of both 50 and 100-fold excess unlabeled Bind1 completely abolished the ability of BcrRHis to shift [32P]-labeled Bind1 (Fig. 6B, lanes 7-8). These results indicate that complex formation between BcrRHis and Bind1 is a specific interaction and that the ability of BcrR to bind DNA is dependent on the presence of functional BcrR binding sites within the DNA sequence.

As bacitracin is the inducer of BcrR, we reasoned that the addition of bacitracin in electrophoretic mobility shift assays may have an effect on the interaction of BcrR with bcrABD promoter DNA. To test this hypothesis, we conducted electrophoretic mobility shift assays with proteoliposomes containing BcrRHis that had been formed in both the presence and absence of bacitracin, at ten-fold molar excess to BcrRHis, in the reaction mixture. Under all conditions, using [32P]-labeled Bind1 as a DNA probe, we did not observe any difference in the shift or the amount of protein required to induce a shift of DNA as compared to proteoliposomes prepared in the absence of bacitracin (data not shown). We assumed that because bacitracin binds to UPP in lipid membranes, the presence of UPP may be necessary in the liposomes in order for bacitracin to influence the BcrR-DNA interaction. All experiments were therefore repeated with proteoliposomes containing total E. coli lipids. No significant difference in BcrRHis-DNA interactions were observed between proteoliposomes made with total E. coli lipids and those made with phosphatidylcholine, suggesting UPP from E. coli had no effect on BcrRHis binding to DNA (data not shown). We hypothesized that no change in BcrRHis-DNA interaction was observed in the presence of bacitracin, either because the mechanism of induction by bacitracin is more complex than this experimental system will allow, or the nature of DNA-binding is altered in a manner that is not detectable by electrophoretic mobility shift assay, such as a shift of BcrR on the DNA without a significant alteration in affinity.

It has been hypothesized that the number of membrane-bound transcriptional activator proteins must be limited due to difficulties encountered by the protein in locating a specific DNA target (8). BcrRHis
was able to specifically bind bcrABD promoter DNA in the absence of the inducer bacitracin. These data may suggest that membrane-bound BcrR overcomes the need to locate its specific DNA target in the presence of bacitracin by simply remaining bound to the DNA and undergoing a conformational shift on the bcrABD promoter upon bacitracin binding. In this regard, BcrR shows some similarities with the CylR1 and CylR2 complex of E. faecalis (19, 41). CylR1 is a predicted membrane protein with no homologues of known function that acts in concert with CylR2, a cytoplasmic protein that contains a XRE family HTH domain, to repress transcription of cytolysin genes. As both CylR1 and CylR2 are required for repression, it has been proposed that CylR1 and CylR2 form a complex (19). This complex could potentially represent a system that, like BcrR, functions to transmit a signal through the membrane without phosphorelay. The CylR2 repressor binds as a dimer to an inverted repeat within the cytolysin promoter (41). Structure modeling of CylR2 binding to this repeat predicts that the protein binds with dyad symmetry to two adjacent major grooves on the DNA. The sequence to which CylR2 is thought to bind is strikingly similar to the sequence of the inverted repeats identified for BcrR binding. The CylR2 subunit binding to the inverse DNA strand is predicted to bind to T1, G3, A4, C5 of the sequence 5’-TTGACA-3’ found on the cytolysin promoter (41). By comparison, the upstream sequence of both inverted repeats for BcrR-binding is 5’-ctGACA-3’. It has been proposed that the presence of inducer causes a shift in the footprint of CylR2 on the promoter (41). We observed that bacitracin had no effect on the mobility-shift of bcrABD promoter DNA. We propose, therefore, that BcrR binds as a dimer at each inverted repeat sequence on the bcrABD promoter irrespective of bacitracin concentration and that activation of bcrABD expression occurs post DNA binding of BcrR. Further study is required to gain additional insight into the mechanism of bcrABD transcriptional activation.

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FIGURE 1. The effect of bacitracin concentration on the expression of bcrA-lacZ. Cells of E. faecalis strains were grown to an OD_{600} of 0.5, challenged with varying concentrations of bacitracin for 1 h prior to harvesting and assayed for β-galactosidase activity, expressed in Miller units. Results are shown as the mean of two biological replicates. A, strain AR01/DGVSpTCVA (BcrR⁺, BcrAB⁺). B, strain JH2-2pAMbcr3pTCVA (BcrR⁺, BerAB⁺). C, strain JH2-2pAMBerRpTCVA (BcrR⁺, BerAB⁺).

FIGURE 2. Physical map of bcrA-lacZ fusions used for promoter mapping studies. E. faecalis strain AR01/DGVS was transformed with bcrA-lacZ fusion constructs and induction of bcrA-lacZ expression by bacitracin was studied. The region of the bcrABD promoter contained within each construct is shown by a horizontal line with numbers given in bp relative to the bcrA start codon. Cells were grown to an OD_{600} of 0.5 and challenged with 256 µg/ml bacitracin for 1 h prior to harvesting and assayed for β-galactosidase activity. The mean β-galactosidase activity for each construct is expressed in Miller units ± the standard error of the mean from three biological replicates.

FIGURE 3. Mutation of the inverted repeats within the bcrABD promoter. E. faecalis strain AR01/DGVS was transformed with mutated bcrA-lacZ fusion constructs and induction of bcrA-lacZ expression by bacitracin was studied. The location of inverted repeat sequences within the 69-bp region of the bcrA promoter as encoded in pTCVA are in boldface. Also in boldface is the location of mutations made in these repeats. Numbers given are in bp relative to the bcrA start codon. Cells were grown to an OD_{600} of 0.5 and challenged with 256 µg/ml bacitracin for 1 h prior to harvesting and assayed for β-galactosidase activity. The mean β-galactosidase activity for each construct is expressed in Miller units ± the standard error of the mean from three biological replicates.

FIGURE 4. The bcrABD promoter. A, region shown extends from -219 to +170 relative to the bcrA start codon, corresponding with the region encompassed by pTCVA. The 69 bp sequence (-83 to -152) required for maximum bcrA-lacZ expression (Fig. 2) is shown in bold. The location and sequence of the direct repeat that constitutes the putative BcrR-binding sites (Fig. 3) are shown below their location in the wild-type sequence. The putative -35, -10 regulatory transcription elements, as determined by sequence homology, and the +1 transcriptional start, as determined by 5′-RACE (Fig. 4b), are shown underlined. The putative Shine-Dalgarno ribosome binding sequence, as determined by sequence homology, is boxed and the transcriptional start codon of bcrA and stop codon of bcrR are italicised. Amino-acids encoded by bcrA and bcrR are denoted by a single letter below the sequence. B, chromatogram obtained from sequencing the inverse cDNA strand obtained by 5′-RACE. The box indicates three nucleotides, of which one is the transcriptional start site.

FIGURE 5. Expression, solubilization, purification and reconstitution of recombinant hexa-histidine tagged BcrR. A, expression of BcrR in E. coli C41(DE3) inverted membrane vesicles. Lanes 2-4 contain 20 µg of protein. Lane 1, Protein molecular weight marker (Fermentas); lane 2, C41(DE3)pTrc99A vesicles; lane 3, C41(DE3)pTrcBcrR vesicles; lane 4, C41(DE3)pTrcBcrRHis vesicles. B, solubilization of hexa-histidine tagged BcrR. Lanes 2-6 contain 20 µg of protein. Lane 1, SeeBlue ladder (Invitrogen); lane 2, C41(DE3)pTrc99A vesicles; lane 3, C41(DE3)pTrcBcrRHis vesicles; lane 4, DDM-solubilized fraction; lane 5, DDM insoluble fraction; lane 6, sodium cholate-solubilized fraction. C, purification of DDM-solubilized protein fraction. Lane 1, protein molecular weight marker; lane 2, pooled BcrR_{His}^-containing Ni^{2+}-NTA affinity chromatography fractions (8 µg); lane 3, gel-filtration purified...
BcrR<sub>His</sub>-containing fraction (4 µg). D, reconstitution of gel-filtration purified BcrR<sub>His</sub> into phosphatidylcholine liposomes. Lane 1, pooled, concentrated BcrR<sub>His</sub>-containing gel-filtration fractions (5 µg) before the reconstitution procedure; lane 2, 20 µl supernatant fraction after the reconstitution procedure; lane 3, BcrR-containing proteoliposomes (0.5 µl reconstituted fraction).

FIGURE 6. Electrophoretic mobility shift assays of BcrR-containing lipoposomes and probe DNA. A, 1.2 ng of radioactively-labeled Bind1 (lanes 1-5), encompassing the putative BcrR-binding sites, or Bind2 (lanes 6-10), encompassing mutated BcrR binding sites, were incubated with different amounts of BcrR-containing liposomes (lanes 2-5 and 7-10) to give the following BcrR to DNA molar ratios; lanes 2 and 7, 1000:1 (380 ng and 240 ng BcrR, respectively); lanes 3 and 8, 2000:1 (760 and 480 ng BcrR, respectively); lanes 4 and 9, 3000:1 (1140 and 720 ng BcrR, respectively); lanes 5 and 10, 4000:1 (1520 and 960 ng BcrR, respectively). B, 1.2 ng radioactively-labeled Bind1 was incubated with different amounts of BcrR-containing liposomes (lane 1, 0 ng; lane 2, 380 ng, lane 3, 760 ng, lanes 4-10, 1520 ng) and different amounts of competitive unlabeled bcrA116, encompassing 116 bp of the bcrA coding region (lane 5, 60 ng; lane 6, 120 ng), Bind1, encompassing the putative BcrR binding sites (lane 7, 60 ng; lane 8, 120 ng) and Bind2, encompassing BcrR binding sites mutated in regions R1 and R4 (lane 9, 95 ng; lane 10, 191 ng).
### TABLE 1

**Bacterial strains and plasmids used in this study**

| Strain or plasmid          | Description                                                                 | Reference or source |
|----------------------------|-----------------------------------------------------------------------------|---------------------|
| **E. faecalis**            |                                                                             |                     |
| AR01/DGVS                  | AR01/DG cured of pJM02; Te\(^c\), Be\(^c\), bcr\(^R\), bcr\(^ABD\)         | (3)                 |
| AR01/DGVS/pTCVA            | AR01/DGVS harboring pTCVA                                                  | This study          |
| AR01/DGVS/pTCVB            | AR01/DGVS harboring pTCVB                                                  | This study          |
| AR01/DGVS/pTCVC            | AR01/DGVS harboring pTCVC                                                  | This study          |
| AR01/DGVS/pTCVD            | AR01/DGVS harboring pTCVD                                                  | This study          |
| AR01/DGVS/pTCVE            | AR01/DGVS harboring pTCVE                                                  | This study          |
| AR01/DGVSpTCVA1            | AR01/DGV harboring pTCVA1                                                 | This study          |
| AR01/DGVSpTCVA2            | AR01/DGV harboring pTCVA2                                                 | This study          |
| AR01/DGVSpTCVA3            | AR01/DGV harboring pTCVA3                                                 | This study          |
| AR01/DGVSpTCVA4            | AR01/DGV harboring pTCVA4                                                 | This study          |
| AR01/DGVSpTCVA1+3          | AR01/DGV harboring pTCVA1+3                                               | This study          |
| AR01/DGVSpTCVA2+4          | AR01/DGV harboring pTCVA2+4                                               | This study          |
| JH2-2                      | Type strain, R\(^R\), FS\(^R\)                                            | (24)                |
| JH2-2/pAMBcrRpTCVA         | JH2-2 harboring pAMBcrR and pTCVA                                          | This study          |
| JH2-2/pAMBcrRHispTCVA      | JH2-2 harboring pAMBcrRHis and pTCVA                                       | This study          |
| JH2-2/pAMbcr3pTCVA         | JH2-2 harboring pAMbcr and pTCVA                                           | This study          |
| **E. coli**                |                                                                             |                     |
| MC1061                     | araD139, Δ(ara, leu)7697, ΔlacX74, galU\(^-\), galK\(^-\), hsr\(^-\), hsm\(^+\), strA | (21)                |
| DH10B                      | F\(^-\) merA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 mupG | (22)                |
| C41(DE3)                   | Uncharacterized mutant derivative from BL21(DE3)                           | (23)                |
| C41(DE3)pTrec99A           | C41(DE3) harboring expression vector pTrec99A                              | This study          |
| C41(DE3)pTrecBcrR          | C41(DE3) harboring pTrecBcrR                                               | This study          |
| C41(DE3)pTrecBcrRHis       | C41(DE3) harboring pTrecBcrRHis                                            | This study          |
| **Plasmids**               |                                                                             |                     |
| pAM401                     | E. coli-E. faecalis shuttle vector; CM\(^R\) TC\(^R\)                      |                     |
| pAMbcr1                    | pAM401 harboring 4.7 kb EcoRI fragment containing bcr\(^R\) and bcr\(^ABD\) | (3)                 |
| pAMbcr3                    | pAM401 harboring 3.2-kb EcoRI-Ndel fragment from p2H7 containing bcr\(^R\), bcr\(^A\), and bcr\(^B\) | (3)                 |
| pAMBcrR                    | pAM401 harboring 1.28-kb PCR fragment containing bcr\(^R\)                 | This study          |
| pAMBcrRHis                 | pAM401 harboring 1.28-kb PCR fragment containing bcr\(^R\) with a C-terminal hexa-histidine tag | This study          |
| Vector        | Description                                                                 |
|--------------|-----------------------------------------------------------------------------|
| pTCVlac      | E. coli-gram positive shuttle transcriptional fusion vector; Erm<sup>r</sup>, Kan<sup>r</sup> |
| pTrc99A      | E. coli expression vector; trc promoter, Amp<sup>r</sup>                     |
| pTCVA        | pTCVlac harboring 389 bp of bcr<sub>ABD</sub> promoter region (position -219 to +170 relative to bcr<sub>A</sub> start codon) |
| pTCVB        | pTCVlac harboring 321 bp of bcr<sub>ABD</sub> promoter region (position -152 to +170 relative to bcr<sub>A</sub> start codon) |
| pTCVC        | pTCVlac harboring 302 bp of bcr<sub>ABD</sub> promoter region (position -133 to +170 relative to bcr<sub>A</sub> start codon) |
| pTCVD        | pTCVlac harboring 280 bp of bcr<sub>ABD</sub> promoter region (position -110 to +170 relative to bcr<sub>A</sub> start codon) |
| pTCVE        | pTCVlac harboring 252 bp of bcr<sub>ABD</sub> promoter region (position -83 to +170 relative to bcr<sub>A</sub> start codon) |
| pTCVA1       | pTCVA containing 3 bp substitution mutation (positions -135 to -133 relative to bcr<sub>A</sub> start codon) |
| pTCVA2       | pTCVA containing 4 bp substitution mutation (positions -123 to -120 relative to bcr<sub>A</sub> start codon) |
| pTCVA3       | pTCVA containing 3 bp substitution mutation (positions -103 to -101 relative to bcr<sub>A</sub> start codon) |
| pTCVA4       | pTCVA containing 3 bp substitution mutation (positions -91 to -88 relative to bcr<sub>A</sub> start codon) |
| pTCVA1+3     | pTCVA containing two 3 bp substitution mutations (positions -103 to -101 and -135 to -133 relative to bcr<sub>A</sub> start codon) |
| pTCVA2+4     | pTCVA containing two 3 bp substitution mutations (positions -123 to -120 and -91 to -88 relative to bcr<sub>A</sub> start codon) |
| pTrcBcrR     | pTrc99A harboring bcr<sub>R</sub>                                           |
| pTrcBcrRHis  | pTrc99A harboring bcr<sub>R</sub> with a C-terminal hexa-histidine tag        |

This study
| Name               | Sequence 5'-3'                     | Restriction site | Used to create mutant construct |
|--------------------|-----------------------------------|-----------------|----------------------------------|
| EfbcrAP2F          | GTACTTCCCGGTTGCTGTTAATCGGCAAGAT   | Smal            |                                  |
| EfbcrAP2Eco F      | GTACTTGAATTCCTGTGTTAATCGGCAAGAT   | EcoRI           |                                  |
| EfbcrAP2R          | ATCCGTGGATCCAGCAAGTGCAACATCTATT   | BamHI           |                                  |
| EfbcrAP3F          | TTGTGTCGCCGTTGACGGTAGCTTGGTTT     | Smal            |                                  |
| EfbcrAP5F          | GTCCAGAATTTCCTATCGACATCTATTGGTT   | EcoRI           |                                  |
| EfbcrAP6F          | ATAGCCGAATTCCAGATAATGTCGAGCTTT    | EcoRI           |                                  |
| EfbcrAP7F          | AACATCGAATTCCTTGAAAAATAGGCTCTGAC | EcoRI           |                                  |
| EfbcrAP7R          | CACATAGGAATCGCAAGAAACCTACCCTGCA  | BamHI           |                                  |
| BcrAarea1/3F       | CATCTATGGAAAAAGCATTGTGACGTTAGT    | pTEVA2, pTEVA4  |                                  |
| BcrAarea1/3R       | CATCTATGGAAAAAGCATTGTGACGTTAGT    | pTEVA2, pTEVA4  |                                  |
| BcrAarea3F         | TTTTATGACCCATCCTATTGCTAGCATTG     | pTEVA3          |                                  |
| BcrAarea3R         | CAATAGATGGGGCATGAAAAAGCCTCCGACA  | pTEVA3          |                                  |
| BcrAarea4F         | AATAGGCTCCCACGATAGTTCGAGGCTTT    | pTEVA1, pTEVA1+3 |                                |
| BcrAarea4R         | CTTATCGTGGGGGAGCCTATTTCAAGGT    | pTEVA1          |                                  |
| BcrAarea2/4F       | CACGATAAGAAAAGGACCTTTTCTGCTGAC  | pTEVA2          |                                  |
| BcrAarea2/4R       | AAAAGCTCCCTTTCTATCGTGCAGGCTAT    | pTEVA2          |                                  |
FIG. 1
| Construct | bcrA157E Expression (Miller units) | Percent activity |
|-----------|-----------------------------------|------------------|
| pTCVA     | 229.8 ± 14.4                      | 100.0%           |
| pTCVA1    | 29.6 ± 2.1                        | 13.2%            |
| pTCVA2    | 26.9 ± 1.0                        | 5.6%             |
| pTCVA3    | 46.3 ± 6.0                        | 20.2%            |
| pTCVA4    | 153.2 ± 17.4                      | 60.5%            |
| pTCVA1+3  | 86.6 ± 1.1                        | 0.3%             |
| pTCVA2+4  | 0.05 ± 0.3                        | 0.02%            |

FIG. 3
FIG. 4

A

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TTGCTGTTAATCGGCAAGATATTGTTTTGATTTAAGCAAGATGGGAATGAAAATAAACACTCATAA
LLILIKIFVWIKQMGMK

ACCTTGAAAATAGGCCTGACACGATAAGTGTCGGAGCTTTTTTCATGCTGACCTCTATTGTGTGC
GACAXXXXXXTGTC

AGCATGTGACGCTAGTTTCTTGGCTTTTGCTATGTGTGGACAGATAATGATACTTGTCAGCA
-35

LMIMEYVICTENLTK

CAATATGGGGAACTACCGTTGTGAAATAAGATAATCTCCATGTCCTCCAAAAGGCAAATCTATGGC
QYGGETTVVNKNLHVPKGKIXY

TTGCTTGCCAGAATGGGCAAGAAAAAACACCACGAATGAAATGGTTCAGATTGGCCT
LLGRNGAGKTAMKMLQAL

+170

B

CTGTTTGCTGACCA

CAAA

AAAAA

AAAAAA

AAAAAA

FIG. 4
