ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2001, p. 1480–1486 Vol. 45, No. 5
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.5.1480–1486.2001

Penicillin-Binding Protein 5 and Expression of Ampicillin Resistance in *Enterococcus faecium*

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Received 6 September 2000/Returned for modification 2 November 2000/Accepted 30 January 2001

We report a structural and transcriptional analysis of the *pbp5* region of *Enterococcus faecium* C68. *pbp5* exists within a larger operon that includes upstream open reading frames (ORFs) corresponding to previously reported *psr* (penicillin-binding protein synthesis repressor) and *ftsW* (whose product is a transmembrane protein that interacts with PBP3 in *Escherichia coli* septum formation) genes. Hybridization of mRNA from C68, CV133, and four ampicillin-resistant CV133 mutants revealed four distinct transcripts from this region, consisting of (i) *E. faecium ftsW* (*ftsW*$_{Efm}$) alone; (ii) *psr* and *pbp5*; (iii) *pbp5* alone; and (iv) *ftsW*$_{Efm}$, *psr*, and *pbp5*. Quantities of the different transcripts varied between strains and did not always correlate with quantities of PBP5 or levels of ampicillin resistance. Since the *psr* of C68 is presumably nonfunctional due to an insertion of an extra nucleotide in the codon for the 44th amino acid, the region extending from the *ftsW*$_{Efm}$ promoter through the *pbp5* gene of C68 was cloned in *E. coli* to facilitate mutagenesis. The *psr* ORF was regenerated using site-directed mutagenesis and introduced into *E. faecium* D344-SRF on conjugative shuttle vector pTCV-lac (pCWR558 [*psr* ORF interrupted]; pCWR583 [*psr* ORF intact]). Ampicillin MICs for both D344-SRF-(pCWR558) and D344-SRF(pCWR583) were 64 μg/ml. Quantities of *pbp5* transcript and protein were similar in strains containing either construct regardless of whether they were grown in the presence or absence of ampicillin, arguing against a role for PSR as a repressor of *pbp5* transcription. However, quantities of *psr* transcript were increased in D344-SRF(pCWR583) compared to D344-SRF(pCWR558), especially after growth in ampicillin; suggesting that PSR acts in some manner to activate its own transcription.

Penicillin resistance in *Enterococcus faecium* is associated with production of low-affinity penicillin-binding protein PBP5. The presence of this penicillin-binding protein (PBP) in virtually all clinical *E. faecium* strains that have been investigated (including those susceptible to clinically achievable levels of penicillin [L. B. Rice, unpublished data]) suggests that it is intrinsic to this species, rather than an acquired gene. Supportive evidence for the role of PBP5 in penicillin resistance is derived from experiments indicating that PBP5-expressing cells replicate when incubated with penicillin at concentrations sufficient to saturate all of the other PBPs, as well as from studies demonstrating that *E. faecium* strains lacking PBP5 are highly susceptible to penicillin (10–12, 25, 26).

Early studies on *Enterococcus hirae* 9790 (which until 1985 was considered to be a type strain for *Enterococus faecalis* [8, 16]) reported that elevated levels of penicillin resistance (to ca. 64 μg/ml) were associated with increased quantities of detectable PBP5. Increased PBP5 production in one resistant mutant (R40) was associated with deletion of the N-terminal portion and some upstream DNA of an open reading frame (ORF) located ca. 1 kb upstream of the *pbp5* start codon. Because of its presumed negative impact on PBP5 expression, this upstream ORF was designated *psr* (penicillin-binding protein synthesis repressor) (16). The presence of intact *psr* upstream of *pbp5* was associated with decreased quantities of PBP5 in *E. hirae* and when these genes were cloned into *E. coli*. Importantly, transcriptional studies supporting a repressor role for *psr* have not been published. More recent publications have described penicillin-resistant mutants of *E. hirae* that lack detectable deletions in *psr* (19). Moreover, changes in cell wall carbohydrate concentrations and lytic response in *psr*-deficient *E. hirae* suggest that PSR may be a global regulator of cell wall synthesis genes (19). It has been noted previously that *psr* resembles *lytR* from *Bacillus subtilis*, hypothesized to be an attenuator of the *N*-acetylmuramoyl-L-alanine amidase structural genes (19).

High-level penicillin and ampicillin resistance (128 to 512 μg/ml) in *E. faecium* has become a grave clinical problem over the past decade, especially since many ampicillin-resistant strains are resistant to vancomycin, which until recently was the only other reliable therapeutic alternative for the treatment of *E. faecium* infections (24). Studies of clinical isolates expressing high levels of ampicillin resistance suggest that increased production of PBP5 is not the most common mechanism for increased resistance but rather have noted that highly resistant strains encode PBP5 with alterations that result in a still lower affinity for penicillin (17, 23, 28). Since techniques for genetic manipulation are often not readily applicable to *E. faecium*, virtually all of the information available on ampicillin resistance in this species is based on the analysis of clinical isolates, raising questions about whether unknown differences between strains are also impacting the expression of resistance.

We recently reported the conjugal transfer of *pbp5* (along with the vancomycin resistance transposon Tn*5382*) from clinical isolate *E. faecium* C68 to ampicillin-susceptible, *pbp5*-lack-

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TABLE 1. Bacterial strains and plasmids used in these studies

Strain or plasmid designation	Resistance trait(s)	Purpose (reference[s])
Strains		
E. faecium C68	Ap^r Em^r Gm^r Sm^r Tc^r Vm^r	Clinical isolate (3)
E. faecium GE-1	Fus^r Rif^r Tc^r	*E. faecium* recipient; lacks *pbp5* due to a deletion of the entire region (4, 7)
E. faecium D344-SRF	Fus^r Rif^r Em^r	*E. faecium* recipient strain; lacks *pbp5* due to a deletion of the entire region (18; this study)
E. faecium CV133	Fus^r Rif^r Tc^r Ap^r Vm^r	Transconjugant from C68 × GE-1 mating (3)
E. faecium A1	Fus^r Rif^r Tc^r Ap^r Vm^r	CV133 mutant with increased AMP MIC
E. faecium A2	Fus^r Rif^r Tc^r Ap^r Vm^r	CV133 mutant with increased AMP MIC
E. faecium A3	Fus^r Rif^r Tc^r Ap^r Vm^r	CV133 mutant with increased AMP MIC
E. faecium A4	Fus^r Rif^r Tc^r Ap^r Vm^r	CV133 mutant with increased AMP MIC
Plasmids		
pUC18	Ap^r	*E. coli* cloning vector (BRL)
pTCV-lac	Em^r Km^r	Conjugative *E. coli-E. faecium* shuttle plasmid with β-galactosidase reporter construct (21)
pRK24	Ap^r	Mobilizing plasmid for transfer of pTCV-lac constructs into *E. faecium* D344-S (29)
pCWR558	Em^r Km^r Ap^r	$ftsW_{Efm}$ (two-promoter)-*psr-pbp5* region from C68 cloned into pTCV-lac (this study)
pCWR561	Em^r Km^r Ap^r	*pbp5* from C68 with its promoter cloned into pTCV-lac (this study)
pCWR583	Em^r Km^r Ap^r	$ftsW_{Efm}$ (two-promoter)-*psr-pbp5* region from C68 cloned into pTCV-lac. In this construct, the intact *psr* ORF has been regenerated by site-directed mutagenesis (this study).
pCWR564	Km^r Ap^r	$ftsW_{Efm}$ (two-promoter)-*psr-pbp5* region from C68 cloned into pCR-XL-TOPO (this study)

ing *E. faecium* recipient strain GE-1 (4). In the present paper, we present the structure of the *pbp5* region within C68, a description of gene transcription within that region, and an analysis of the impact of PSR expression on ampicillin resistance.

MATERIALS AND METHODS

Strains and plasmids. Relevant bacterial strains, cloning vectors, and plasmids are listed in Table 1. *E. faecium* GE-1 (4, 7) and D344-S (18) are devoid of *pbp5*, and the MICs of ampicillin for these strains are <0.5 μg/ml. GE-1 is resistant to rifampin and fusidic acid. D344-S was selected for resistance rifampin and fusidic acid for these experiments by plating large inocula serially on fusidic acid (25 μg/ml) and then rifampin (100 μg/ml). C68 is an *E. faecium* strain isolated from the feces of a patient hospitalized in northeast Ohio (4). It is resistant to high levels of both ampicillin and vancomycin and represents the predominant (more than 50% of area isolates) vancomycin-resistant enterococcal clone in the region (5). pTCV-lac is a gram-negative–gram-positive shuttle vector that can be mobilized from *E. coli* into gram-positive species by conjugal plasmid pRK24 (21). pCWR558 was constructed by PCR amplification of the region extending from upstream of *E. faecium ftsW*($ftsW_{Efm}$) (containing both identified promoters) to the stop codon of *pbp5* (excluding the transcription terminator downstream of *pbp5*). The amplification product was originally cloned into the multiple-cloning site of pCR-XL-TOPO (Invitrogen) and then subsequently directionally cloned (using *Sma*I and *Bam*HI) into the multiple-cloning site of pTCV-lac. pCWR583 resulted from performing site-directed mutagenesis on the insert of pCWR558 to regenerate the *psr* ORF. pCWR561 contains a PCR-generated insert containing only *pbp5* with its promoter cloned into pTCV-lac.

Antimicrobial susceptibility testing. MICs were determined by a broth macrodilution technique in brain heart infusion broth (20).

Hybridization experiments. Genomic DNA was extracted as described previously (4) and digested with restriction enzymes for 1 to 2 h at 37°C according to the specifications of the manufacturer (Promega, Madison, Wis.). Digested DNA was separated on 0.7 to 1% agarose gels overnight. Separated DNA was denatured, neutralized, and transferred to nylon filters using a negative-pressure transfer apparatus (Pharmacia, LKB, Uppsala, Sweden) and baked at 80°C for 1 to 2 h to fix the DNA to the filter. Filters were prehybridized, and this was followed by hybridization with digoxigenin-labeled probes overnight at 68°C and washing under conditions of high stringency according to the specifications of the manufacturer (Boehringer Mannheim, Indianapolis, Ind.).

Cloning of genomic DNA fragments. Once fragments of interest were identified by hybridization, they were removed from agarose gels and purified using the Qiaquick gel extraction kit (Qiagen, Valencia, Calif.). These fragments were then ligated to like-digested pACYC184 or pUC18 and transformed into *E. coli* DH5α (13) or *E. coli* DH10B (Bethesda Research Laboratories, Gaithersburg, Md.) by electroporation (Bio-Rad, Hercules, Calif.). Transformed preparations were inoculated onto plates containing antimicrobial agents selective for the cloning vectors, and colonies with the appropriate inserts were identified by colony hybridization techniques as previously described (4). These colonies were purified and subcloned as necessary for further sequencing.

PCR amplification. Amplification of genomic DNA was performed on a Perkin-Elmer Cetus 9600 thermal cycler using *Taq* DNA polymerase according to standard protocols as recommended by the manufacturer (Perkin-Elmer Cetus, Roche Molecular Systems, Branchburg, N.J.). Variations were introduced into each individual protocol depending on the expected size of the amplification product and the specifics of the primers used. A 10-μl aliquot of a total 50-μl PCR mixture was loaded on a 0.7 to 1.2% agarose gel for analysis. PCR products to be cloned were ligated to pCR-XL-TOPO (Invitrogen) and introduced into *E. coli* DH10B by electroporation. Inserts were removed from pCR-XL-TOPO by restriction digestion and ligated to pTCV-lac using T4 DNA ligase. pCWR558, pCWR561, and pCWR583 were transformed into *E. coli* HB101 containing pRK24 (mobilizing plasmid) (21) with selection on Luria-Bertani agar plates containing kanamycin (20 μg/ml). Recombinant plasmids were then introduced into *E. faecium* D344-SRF by conjugation, with selection on kanamycin (1,000 to 1,500 μg/ml) and rifampin (100 μg/ml).

DNA sequence analysis. DNA sequencing was performed from cloned DNA on double stranded templates using the A.L.F. Automated sequencing kit and fluorescein- or Cy5 indodicarbocyanine dye-labeled forward and reverse primers, or with similarly labeled primers derived from previously determined sequences. DNA was purified for sequencing using the Wizard plus minipreps DNA purification system (Qiagen). Sequence was determined using the ALFExpress automated sequencer (Pharmacia LKB). Sequences were compared for homology with those entered into the GenBank, EMBL, DDBJ, and PDB databases using Blastn and Blastx local alignment search tools (1) and further analyzed using the DNAsis (version 2.0; Hitachi, Ltd.) sequence analysis program.

RNA extraction. Cells were grown overnight, either with or without antibiotic at various concentrations. In the morning, overnight cultures were freshly inoculated into the same medium at a 1:10 dilution and grown without agitation to an OD_{600} of 0.7 (approximately 4 to 8 h). At this point cells were centrifuged, the supernatant was discarded, and the pellets were frozen at −80°C until RNA isolation was begun. Total cellular RNA extraction was accomplished using the Rneasy kit (Qiagen). The only deviation from the manufacturer's protocol was that a lysozyme concentration of 10 mg/ml in Tris-EDTA buffer was used. RNA concentration was measured by spectrophotometer prior to precipitation with 100% ethanol and sodium acetate (pH 4.0) and frozen at −80°C until use.

Northern hybridization. A volume calculated to contain 5 μg of RNA was centrifuged and resuspended in distilled water, and this was followed by dilution with sample buffer (per 300 μl: 180 μl of deionized formamide, 75 μl of deionized formaldehyde, 45 μl of 10× MOPS [morpholinepropanesulfonic acid] buffer). Samples were heated at 65°C for 10 min, chilled on ice and loaded onto 1.2% agarose gels with formaldehyde after addition of ethidium bromide and tracker dye to the sample. RNA was separated overnight at 25 V at 4°C. Gels were washed with sterile water for 15 min four times, which was followed by equilibration (four times) with 20× SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min each. RNA was transferred to nylon membranes by passive

capillary action for 48 h. Nylon membranes were then baked for 2 h at 80°C to fix the RNA. Membranes were prehybridized and hybridized at 50°C as detailed by the manufacturer (Boehringer Mannheim). Probes were labeled with digoxigenin either by a random primer method or by PCR as recommended by the manufacturer (Boehringer Mannheim). Hybridized RNA was detected using an antidigoxigenin antibody and a chemiluminescence detection system (Boehringer Mannheim). Densitometry values (in optical density units) were determined using Scion Image, a Windows-compatible version of NIH Image.

Primer extension analysis. Primer extension analysis used Cy5-labeled primers designed to direct synthesis of the complementary strand upstream of the start of the *ftsW*$_{Efm}$, *psr*, and *pbp5* ORFs (Table 1). The primers used were as follows: for *pbp5*, 5′-TTCTTGATAGTGCTGGTAG-3′; for *psr*, 5′-TCCATGGATAAACT CAA-3′; and for *ftsW*$_{Efm}$, 5′-TAAGAACTGGCACTGTAT-3′. The technique employed high temperatures to minimize complications resulting from secondary structure formation, essentially as described by Yamada et al. (27). In brief, total RNA from 0.1 to 10 μg and 1 pmol of Cy5-labeled primer was added to 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 5 mM $MgCl_2$, 1 mM concentrations of the deoxynucleoside triphosphates, and 20 U of RNase inhibitor in a 0.2 ml microcentrifuge tube, resulting in a final volume of 20 μl. The primer-extension reaction was started with the addition of 5 U of avian myeloblastosis virus reverse transcriptase. The reaction was carried out at 50°C, and the solution was subjected to phenol extraction and ethanol precipitation. The solution was resuspended and the fragment was detected by running the sample on an ALFExpress automated sequencer (Pharmacia) in parallel with a standard sequencing reaction using a plasmid containing the ORF and upstream sequence as a template.

PBP analysis. PBPs were isolated as described by Williamson et al. (26). In brief, strains were grown in Todd Hewitt broth until they reached an optical density at 600 nm of 0.5. After centrifugation and resuspension in phosphate buffer, cells were exposed to saturating concentrations of [^{3}H]penicillin. After lysis with lysozyme and muramidase 1 samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Relative amounts of PBP5, which binds penicillin with low affinity, were confirmed from samples of the same cultures after separation of PBPs under the same conditions by performing Western blot analysis using specific polyclonal antibodies raised to PBP5 (23). Relative quantities of PBPs were estimated after densitometry scanning of the gels or Western blots using the GEL ANALYST perfect image master 4.01 (Clara vision, France) and recorded to a Kaiser camera (HP Marketing Corporation, N.J.).

Site-directed mutagenesis. Site-directed mutagenesis to regenerate the *psr* ORF was performed using the plasmid pCWR564 as a template. Two commercially manufactured oligonucleotides were used (psr1: 5′-CGAAAAGTGTCCC TACCACACTAAATG-3′; psr2: 5′-CATTTAGTGTGGTAGGGACACTTTTC G-3′). Mutagenesis was performed using the Quikchange site-directed mutagenesis kit (Stratagene). Reaction mixtures consisted of 6 ng of pCWR564, 125 ng of primer psr1, 125 ng of primer psr2, 5 μl of 10X reaction buffer, 1 μl of deoxynucleoside triphosphate mix, and water to 50 μl. *Pfu* Turbo polymerase (2.5 U) was added, and the mixture was incubated for one cycle of 30 s at 95°C and 14 cycles of 95°C (30 s), 55°C (1 min), and 68°C (15 min). After amplification, the preparations were cooled on ice. One microliter of restriction enzyme *Dpn*I was then added, and the mixture was incubated at 37°C for 1 h. The mixture was then electroporated into competent *E. coli* DH10B with selection for transformants on Luria-Bertani agar plates containing kanamycin (50 μg/ml). Regeneration of the *psr* ORF was confirmed by dideoxy sequencing reactions (see above) before the insert was removed by digestion with *Bam*HI and *Sma*I and ligated to pTCV-lac (forming pCWR583).

GenBank accession number. The sequence of the 5,148-bp region extending from upstream of the *ftsW*$_{Efm}$ ORF to the left terminus of Tn*5382* is entered in the GenBank database under accession number AF11760.

RESULTS

MICs for donors and transconjugants. Several ampicillin- and vancomycin-resistant transconjugants were obtained from matings between C68 and GE-1 (4). The ampicillin MICs for these transconjugants varied from 8 to 128 μg/ml but never were equivalent to MICs observed in C68 (256 μg/ml). We chose one transconjugant (CV133) (MIC = 16 to 32 μg/ml) for further study because of its low ampicillin MIC relative to that of C68.

Mutants of CV133 expressing greater resistance to ampicillin were readily obtained at a rate of ca. 10^{-6}/CFU after plating high inocula of this transconjugant onto brain heart infusion (BHI) agar plates containing ampicillin (200 μg/ml). Mutants appeared as pinpoint colonies approximately 48 to 72 h after inoculation of the plates. This rate of mutation to resistance is higher than would be anticipated to result from point mutations within *pbp5* (attempts to derive point mutations in a susceptible *E. faecium pbp5* in a single step were never successful, suggesting a rate of less than 10^{-10}/CFU [Laurent Gutmann, unpublished data]). Four mutants (A1 to A4) were chosen for further study. Broth dilution ampicillin MICs for the resistant mutants were 128 μg/ml (for A1, A3, and A4) or 256 μg/ml (for A2). Ampicillin MICs for GE-1 and D344-S are ≤0.5 μg/ml.

PBP studies. PBP studies using radiolabeled penicillin showed, using an internal standard, a 1.8-fold increase in the amounts of PBP5 in CV133 mutant A2, but not in any other strains (Fig. 1B). These data were confirmed using an anti-PBP5 antibody in a Western blot (Fig. 1A). With the exception of A2, there was no correlation between the ampicillin MIC and the quantities of detectable PBP5 in any of the strains. The Western blot also confirmed the absence of PBP5 from recipient strains GE-1 and D344-S (Fig. 1A), which correlated with the absence of the gene visible on Southern analysis and with the absence of transcript visible on Northern hybridizations (data not shown).

Structure of the region upstream of *pbp5*. The nucleotide sequence of the 5,148-bp region extending from ca. 3 kb upstream of the *pbp5* gene into Tn*5382* downstream of the gene was determined. The structure of the region is depicted in Fig. 2. The region contained a *pbp5* gene predicting 1-amino-acid difference (F→G at amino acid 497) in comparison to the previously described *pbp5* sequence from high-level-ampicillin-resistant *E. faecium* H80721 (28). This F497G change has not been described in previous *pbp5* genes and is of unclear significance. An ORF with homologies to previously described *psr* genes was observed upstream of the *pbp5* ORF (Fig. 2). An added nucleotide in the C68 *psr* ORF resulted in a frame shift at amino acid 43 and a stop codon at amino acid 103, changes that would be expected to render PSR nonfunctional.

An ORF terminating 91 bp upstream of the *psr* translation initiation codon had significant identity (30 to 70%) with previously described *ftsW* genes from *E. hirae, E. coli*, and *B. subtilis*, among others (3, 6, 14, 15). We have designated this ORF *ftsW*$_{Efm}$.

Northern hybridization studies of donor, recipient, and resistant mutants. mRNA derived from C68, GE-1, CV133, and mutants A1 to A4 was hybridized with a probe spanning the downstream end of *psr* into *pbp5* (Fig. 3). mRNA was extracted in late log phase after growth in non-selective BHI broth or in BHI broth supplemented with ampicillin (10 μg/ml). Hybridization to a 2.3-kb transcript was observed in all strains (GE-1 had previously been shown to be devoid of sequences homologous to *pbp5* [4]). An additional 4.3-kb band detectable only in late log phase was observed in all ampicillin-resistant mutants of CV133 only after incubation with ampicillin. A smaller amount of this extra transcript was also observed in CV133, and even less was observed in C68, after exposure to ampicillin. An additional, very intense 3-kb band was observed in A2 (the mutant that expressed the highest levels of ampicillin resistance and the largest quantity of PBP5 protein) with or without

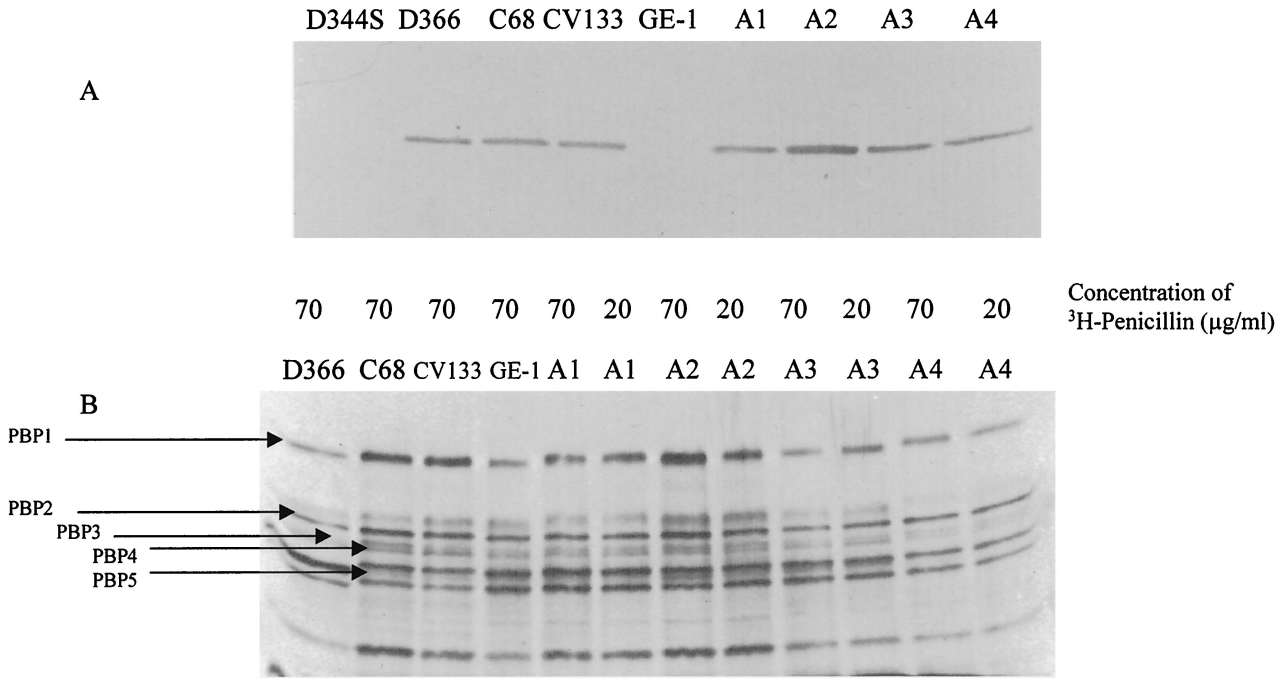

FIG. 1. PBP studies of the strains described in this paper. (A) Western blot of PBP5 using a polyclonal antibody to PBP5. Strains are indicated above the diagram and described in Table 1. D366 is a low-level-penicillin-resistant *E. faecium* strain used as a positive control. (B) PBP analysis using [^{3}H]penicillin. The concentration of [^{3}H]penicillin used for each experiment is listed above the figure.

exposure to ampicillin. A very slight increase in the *pbp5* 2.3-kb transcript was visible to the eye in all strains after exposure to ampicillin, especially for strains for which there was a marked increase in detectable 4.3-kb transcript.

Using internal probes for the region upstream of $ftsW_{Efm}$, the $ftsW_{Efm}$ ORF, the *psr* ORF, the *pbp5* ORF, and the terminus of Tn*5382*, we were able to determine the genetic regions represented by the transcripts. Results are summarized in Fig. 2. The 2.3-kb transcript contains *pbp5* alone. The 3.0-kb transcript visible only in A2 hybridized strongly only with internal *psr* and *pbp5* probes. These data suggest that this transcript originates in the region between the $ftsW_{Efm}$ and *psr* ORFs. Its absence in the other strains suggested that a mutation had occurred in A2 that either creates or uncovers a powerful promoter of *psr* transcription, and that no effective transcriptional terminator was present after the *psr* ORF. The region upstream of *psr* was cloned, and its sequence was compared to the corresponding region of CV133. The regions were found to be identical (data not shown), excluding the presence of a point mutation or an IS element as the cause of the appearance of the 3-kb transcript in A2.

The $ftsW_{Efm}$ probe hybridized to a 1.4-kb transcript that did not hybridize to any of the other probes. This 1.4-kb transcript is present most abundantly in early log phase, trailing off to nearly undetectable levels as the organisms approach stationary phase (data not shown). The large, 4.3-kb transcript that appears most abundantly in the mutants expressing higher levels of resistance to ampicillin hybridized to $ftsW_{Efm}$, *psr*, and *pbp5* probes. These data suggest that the 4.3-kb transcript results from transcription originating immediately upstream of $ftsW_{Efm}$ and continuing through both *psr* and *pbp5*, terminating at the transcriptional terminator downstream of the *pbp5* gene.

Transcription initiation sites. We used primer extension analysis to identify precisely the transcriptional start sites for the three ORFs in this operon. Three potential transcription initiation sites were identified upstream of $ftsW_{Efm}$ (shown in boldface type below). The furthest upstream was located 351 bp 5′ of the $ftsW_{Efm}$ start codon (GAACT**C**GTAAA). This was preceded by credible −10 (TATCAT) and −35 (TTGCCT) regions spaced 17 bp apart. The second was located 277 bp upstream of the start codon (CGTCG**A**AAGTC) and was not preceded by sequences resembling the sigma 70 consensus promoters. The third potential start was located 132 bp upstream of the start codon (GACCT**T**TTCTTT). It was not preceded by consensus promoter sequences and in fact fell at the downstream end of an inverted repeat. This site may represent not a transcriptional start but rather a fall-off of the avian myeloblastosis virus reverse transcriptase due to secondary structure created by interaction between the two arms of

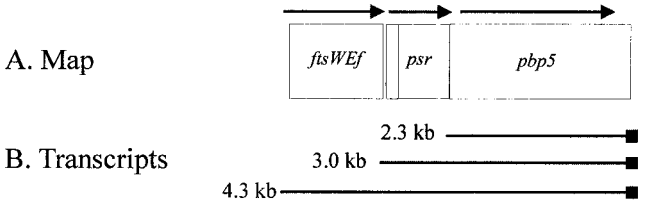

FIG. 2. Map of genomic area of *E. faecium* C68. (A) Physical structure. Each boxed area represents an ORF. The *psr* ORF is split into two parts because it is interrupted in C68 by the nonsense mutation (see text). (B) Composition of transcripts observed in Northern hybridization experiments. The 3.0-kb transcript was seen only in CV133 mutant A2.

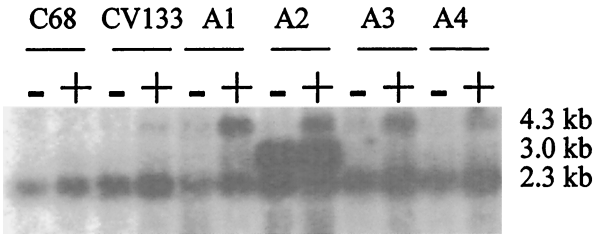

FIG. 3. Northern hybridization of mRNA derived from strains described in this study. See Table 1 for description of strains. mRNA was extracted from strains grown in the presence (+) or absence (−) of ampicillin (10 μg/ml). The sizes of the different transcripts (see Fig. 2 for description of their contents) are listed to the right of the figure.

the inverted repeat. The transcriptional start for the 3.0-kb *psr-pbp5* message seen only in strain A2 was located 88 bp upstream of the *psr* start codon (TAAGA**G**AAACTG). This start site was preceded by credible-10 (AACAAT) and −35 (TTGCCG) regions that were spaced 16 bp apart. The transcription initiation site for the 2.3-kb *pbp5* message was located 39 bp upstream of the *pbp5* start codon (TAAACA**G**GTATA AA) and was preceded by possible-10 (TAGAAT) and -35 (TGGATT) sequences spaced 17 bp apart.

Nucleotide sequence of the region between $ftsW_{Efm}$ and *psr*. Examination of the sequence between $ftsW_{Efm}$ and *psr* in both CV133 and A2 reveals several interesting features (Fig. 4). The distance between the stop codon of $ftsW_{Efm}$ and the start codon of *psr* is 91 nucleotides. Seven base pairs downstream of the $ftsW_{Efm}$ stop codon lies a perfect 6-bp inverted repeat (inverted repeat 1 [IR1]) followed by three T's. If the last three T's are included within the inverted repeat, it is then a perfect repeat of 9 bp. This sequence could serve as a *rho*-independent terminator of $ftsW_{Efm}$ transcription. Beginning 2 bp downstream of the $ftsW_{Efm}$ stop codon and terminating at the first base pair of the downstream portion of IR1 lies a perfect 8-bp direct repeat (DR). Similar DRs have been shown in other instances to be sites for binding by proteins (22). There is also a larger imperfect (17 of 22) inverted repeat sequence (IR2) that involves most of IR1 for the upstream arm, and the ribosome binding site and *psr* start codon for the downstream arm. Interaction between the two arms of IR2 and/or IR1 could obscure any intervening promoter sequences. It is noteworthy that the 3.0-kb *psr-pbp5* transcript originates within the upstream arm of the DR and immediately upstream of the two inverted repeats. In summary, this relatively short DNA segment is rich in sequences that could be important in the regulation of transcription initiation or termination.

Impact of PSR on *pbp5* and *psr* transcription and quantities of PBP5. In order to examine the effect of an intact *psr* ORF on the expression of ampicillin resistance and transcription of the *psr* and *pbp5* genes, we used site-directed mutagenesis to remove the additional nucleotide in the pCWR558 *psr* (Table 1), thereby regenerating the *psr* ORF. The regions extending from $ftsW_{Efm}$ through *pbp5* with (pCWR583) or without (pCWR558) the intact *psr* ORF were conjugated into *E. faecium* D344-SRF since these constructs were less stable in GE-1. As an mRNA quantity control, we measured the amount of *aph-A3* transcript (from the gene conferring kanamycin resistance in pTCV-lac) as well. The ampicillin MIC was the same for both constructs (64 μg/ml). Consistent with this observation, the amount of *pbp5* transcript was similar in the two constructs relative to the amount of *aph-A3* transcript present (Table 2). These data suggest that PSR does not serve as a repressor of *pbp5* transcription. The presence of a *psr* ORF also did not seem to have an impact on the quantity of the major 2.3-kb *pbp5* transcript present after growth in ampicillin (Table 2). In contrast, the *psr-pbp5* message (corresponding to the 3.0-kb message seen in A2) was detectable in both strains only after prolonged exposure of the X-ray film (overnight). There was an increase in the quantity of *psr* transcript in D344-SRF(pCWR583) after exposure to ampicillin in comparison to D344-SRF(pCWR558), suggesting that PSR may serve to amplify its own transcription (and therefore that of *pbp5*) after exposure to ampicillin.

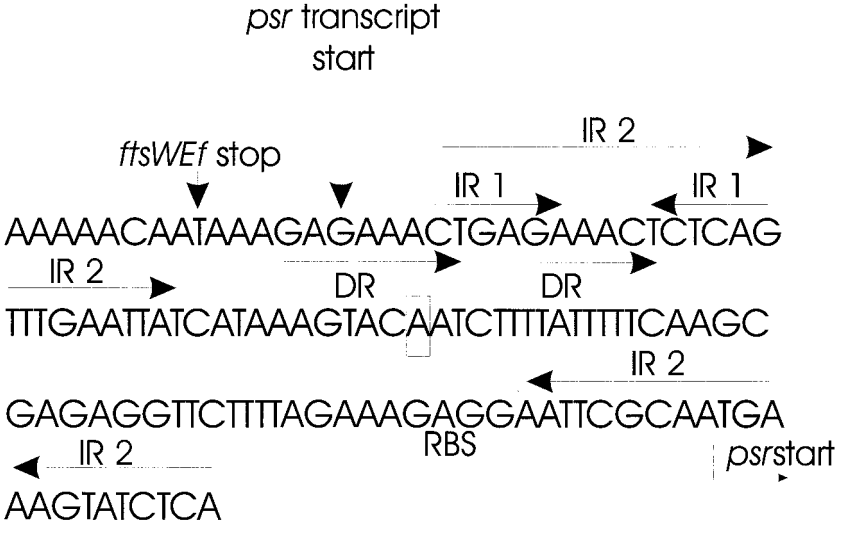

FIG. 4. Sequence extending from the $ftsW_{Efm}$ stop codon through the *psr* start codon. The $ftsW_{Efm}$ stop (translation) and *psr* starts (transcription and translation) are indicated, as in the *psr* ribosome binding site (RBS). The two inverted repeats and the DRs are also indicated. The nucleotide corresponding to the 5′ end of the deletion previously reported PBP5 hyper-producer *E. hirae* R40 is boxed. See text for details.

TABLE 2. Densitometry measurements of specific mRNA in strains containing plasmid constructs

Plasmid[a]	Densitometry measurement[b] (density units) for indicated message								
	pbp5			*psr*			*aph-A3*		
	No AMP	AMP[c]	AMP[c]/no AMP	No AMP	AMP	AMP/no AMP	No AMP	AMP	AMP/no AMP
pCWR558	11,722	11,520	0.98	1,846	2,106	1.14	30,330	29,162	0.96
pCWR583	14,930	16,321	1.09	1,660	5,902	3.55	27,648	32,312	1.17

[a] Plasmids present in *E. faecium* D344-SRF.
[b] Absolute number cannot be used to compare the quantities of transcript between blots, since the strengths of the probes and the development time may have differed. Therefore, relative amounts of only those messages included on the same blot and hybridized with the same probe can be compared.
[c] Ampicillin was used at a concentration of 10 μg/ml.

The same strains underwent quantitation of PBP5 by Western blotting. Quantities of PBP5 were equivalent in strains D344-SRF(pCWR583) and D344-SRF(pCWR558) after growth in the presence or absence of ampicillin, consistent with the results of Northern hybridization and MICs and suggesting that the quantities of *psr-pbp5* message generated by the two constructs were insufficient to impact overall PBP5 quantities. Quantities of PBP5 detectable from D344-SRF(pCWR561), which lacks the $ftsW_{Efm}$ and *psr* upstream of *pbp5*, were less than those observed with the other two constructs (data not shown), consistent with the lower MICs of ampicillin (8 μg/ml) observed for this construct.

DISCUSSION

The data presented in this paper underscore the importance of PBP5 in the expression of ampicillin resistance in *E. faecium*. Lacking this PBP, *E. faecium* strains GE-1 and D344-S are highly susceptible to ampicillin. Reintroduction of the *pbp5* gene, however, either on a large transposon (as with CV133) or on a transferable plasmid, is associated with expression of increased levels of ampicillin resistance.

Work performed to date suggests that the level of ampicillin resistance expressed by *E. faecium* strains will be impacted by the quantity and the structure of the PBP5 itself (12, 16, 23). The data presented in this paper are supportive of a relationship between PBP5 quantity and resistance in that mutant strain A2 produces roughly twice the amount of PBP5 as other mutant strains and consistently demonstrated MICs that were approximately twice those for the other mutants. Published data supporting a correlation between the structure of PBP5 and the level of ampicillin resistance are based entirely on analyses of clinical strains and correlation with penicillin binding affinities. At the present time there are no data substantiating the importance of the structural alterations in the C68 PBP5, although several of the mutations present in this *pbp5* gene have been observed in other low-affinity PBP5s (28).

The transcriptional studies presented in this paper suggest that the 2.3-kb transcript that includes *pbp5* alone is the primary determinant of the quantity of PBP5 produced by most *E. faecium* strains, as evidenced by the close correlation between transcript and protein quantities among all of the strains except A2. The increased quantities of PBP5 seen in A2 are explainable by the presence of large amounts of the 3-kb *psr-pbp5* transcript. The discordance between the presence of large quantities of the $ftsW_{Efm}$-*psr-pbp5* transcript in the CV133 mutants A1, A3, and A4 is more difficult to reconcile but may be explainable by the fact that these transcripts appeared primarily in late log to stationary phase, whereas the PBP5 studies were performed at mid-log phase. Alternatively, secondary structure within the 4.3-kb transcript could impair translation of *pbp5* when it is present in this large message.

To state that the quantity and structure of PBP5 are important for the expression of ampicillin resistance in *E. faecium* is not, however, to suggest that these are the only factors at play in determining the level of resistance expressed by clinical *E. faecium* isolates. We were unable to document any substantial difference in PBP5 quantities between C68 (ampicillin MIC, 256 to 512 μg/ml); CV133 (ampicillin MIC, 16 to 32 μg/ml), and A1, A3, or A4 (ampicillin MIC, 128 μg/ml). Recent reports of studies performed with *Staphylococcus aureus* and *Streptococcus pneumoniae* (2, 9) suggest that levels of methicillin or ampicillin resistance associated with the expression of low-affinity PBPs are significantly impacted by the nature of the peptidoglycan precursors. An important future goal will be to examine peptidoglycan precursors in isogenic *E. faecium* strains expressing different levels of ampicillin resistance.

The hypothesis that PSR serves as a repressor of PBP5 production originated from experiments in which increased quantities of PBP5 were associated with a deletion of the upstream portion of this ORF in *E. hirae*. Our data suggest that PSR is neither a transcriptional repressor nor an activator of *pbp5* transcription but that it may serve to amplify its own transcription when cells are grown in the presence of ampicillin. *psr* transcription is virtually undetectable after growth in BHI broth unsupplemented by antibiotics. Even after growth in ampicillin, quantities of *psr* transcript are very small (data not shown) compared to quantities of *pbp5* transcript, which may explain why ampicillin-induced increases in *psr-pbp5* transcription in *psr*-intact strains are not associated with increases in ampicillin resistance. The observation in the prior study that increases in PBP5 quantities were associated with a deletion of the upstream portion of *psr* (16) may have been due to the elimination of secondary structure that serves to obscure the *psr* promoter. Loss of this secondary structure could "open up" the *psr* promoter, resulting in increased *pbp5* transcription. In fact, the prior publication had presumed, based on sequence analysis, that part of the promoter for the *psr* gene had been deleted. Comparing the sequence deleted from that strain with the sequence upstream of the C68 *psr*, it is clear that the promoter was not deleted in that case, since the transcriptional start site identified in these studies lies upstream of the deleted segment. We observed a variant of this scenario in A2, where dramatically increased *psr-pbp5* transcript quantities were as-

sociated with increased PBP5 and increased ampicillin MICs. Since no structural changes were observed in the region upstream of *psr* in A2, we currently hypothesize that increased *psr-pbp5* transcription in this mutant is due to the presence of an activator that interacts with the *psr* promoter region in a way that makes the promoter available to the sigma factor responsible for initiating *psr* transcription.

The overall relevance of these findings for the mechanisms of ampicillin resistance in clinical *E. faecium* isolates is not clear. While our experiments describe mechanisms by which transcription of *pbp5* could be increased, the majority of these transcriptional changes do not appear to impact the final quantities of translated product or degrees of antibiotic resistance. Despite these limitations, the present study provides important insight into processes that impact the production of PBP5, which is required for the expression of ampicillin resistance. This information will be important for the subsequent interpretation of experiments designed to examine the additional factors that contribute to levels of ampicillin resistance in *E. faecium*.

ACKNOWLEDGMENT

This work was supported by the Medical Research Service of the Department of Veterans Affairs.

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