Transcriptional Induction of the Penicillin-Binding Protein 2 Gene in Staphylococcus aureus by Cell Wall-Active Antibiotics Oxacillin and Vancomycin

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Methicillin resistance in Staphylococcus aureus has been a persistent clinical problem and has risen in prevalence in many geographic locations throughout the world (16). Glycopeptides such as vancomycin and teicoplanin are often the agents of choice for treating infections caused by methicillin-resistant S. aureus (MRSA). However, vancomycin-intermediate S. aureus (VISA) clinical isolates that were obtained during prolonged, unsuccessful vancomycin therapy have been recognized in the past 5 years (reviewed in reference 18). Also, two vancomycin-resistant clinical S. aureus isolates for which vancomycin MICs are high (≥128 mg/liter) were recently identified that have proven positive for the enterococcal vanA gene (8). Given the history of ever-increasing resistance among MRSA strains, such strains are likely to become more prevalent in the future, a situation that would severely restrict treatment options for infections by this virulent pathogen.

Using complementary mechanisms, both β-lactam and glycopeptide antimicrobials inhibit cell wall biosynthesis. β-Lactams bind to and inhibit penicillin-binding proteins (PBPs), which are the enzymes involved in peptidoglycan synthesis, cell growth, and morphogenesis (47). Vancomycin interferes with the action of PBPs by binding to the D-Ala-D-Ala terminus of the peptidoglycan precursor, the substrate on which PBPs act (reviewed in reference 18). Therefore, an understanding of the factors affecting expression of PBPs might lead to the identification of novel targets for antimicrobial therapy against MRSA (and particularly VISA) isolates, for which few therapeutic alternatives exist.

Methicillin-susceptible S. aureus isolates produce five PBPs, PBP1, PBP2, PBP2B, PBP3, and PBP4, for which the genes have been cloned and sequenced (21, 28, 34, 38, 43, 51). MRSA isolates have acquired an additional PBP, termed PBP2’ or PBP2a, that has low affinity for β-lactam antibiotics and substitutes for the other PBPs in cell wall synthesis when they are inhibited by β-lactams (reviewed in reference 10). PBP2a is encoded by the mecA gene, which is carried on a large mobile genetic element (referred to as SCCmec [24, 27, 30]) that is integrated into the chromosome of MRSA strains. It has recently been revealed that the ability of PBP2a to effect cell wall synthesis in the presence of methicillin requires cooperation from the transglycosylase domain of the native PBP2 (37, 40). Also ascribed to PBP2 is a role in borderline resistance to methicillin in strains that do not contain mecA (2, 12, 50).

It has previously been demonstrated by the results of penicillin-binding assays and Western blotting that VISA strains (both clinical and laboratory-derived isolates) and a teicoplanin intermediate-resistant clinical isolate had increased PBP2 production compared with their respective related susceptible isolates (20, 33, 44). Such differences suggested that expression of the pBP2 gene was up-regulated in VISA isolates and was therefore subject to genetic control. Such an increase might mean either that PBP2 is involved in vancomycin resistance or that the gene encoding PBP2 is coregulated with vancomycin

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resistance genes. One model to explain vancomycin resistance involves thickening of the cell wall, which might require increased peptidoglycan synthesis (22). When \( \text{pbpB} \) was overexpressed from a multicopy plasmid, susceptibility to vancomycin was decreased with compared with that of the parent strain, a finding that suggested that an increased level of \( \text{PBP2} \) plays an accessory role in the vancomycin resistance mechanism (20).

Transcription of the \( \text{mecA} \) gene is induced in some isolates by \( \beta \)-lactams, and such induction is regulated by \( \text{MecI} \) and \( \text{MecR1} \), a repressor and a signal-transducing protein, respectively (10). The \( \text{mecI} \) and \( \text{mecR1} \) genes, when present, are carried beside \( \text{mecA} \) on the SCCmec element (24, 27, 30). Cross-regulation by \( \text{Blai} \) and \( \text{BlaR1} \) of \( \text{mecA} \) transcription also occurs, encoded by \( \text{blai} \) and \( \text{blaR1} \) genes carried on the \( \beta \)-lactamase plasmid along with \( \text{blaZ} \) (10). Until now, the background \( \text{PBP2} \)s in \( \text{S. aureus} \) have been assumed to be constitutively expressed.

Although constitutive \( \text{pbpB} \) transcription has been studied recently (39), the factors that control or induce \( \text{pbpB} \) gene expression have not yet been explored. We characterized the \( \text{pbpB} \) gene transcription in \( \text{S. aureus} \) strains containing \( \text{mec} \) genes, when present, are carried beside \( \text{mecA} \) on the SCCmec element (24, 27, 30). Cross-regulation by \( \text{Blai} \) and \( \text{BlaR1} \) of \( \text{mecA} \) transcription also occurs, encoded by \( \text{blai} \) and \( \text{blaR1} \) genes carried on the \( \beta \)-lactamase plasmid along with \( \text{blaZ} \) (10). Until now, the background \( \text{PBP2} \)s in \( \text{S. aureus} \) have been assumed to be constitutively expressed.

**Materials and Methods**

**Isolates and growth conditions.** \( \text{S. aureus} \) cultures were routinely grown at 37°C. For long-term storage, cultures were maintained at \(-70^\circ\text{C} \) in skim milk (Difco, Detroit, Mich.) as previously described (15). The VISA derivative 52K was described previously (15), having been produced as the last of a series of isolates obtained after incubation of the glycopeptide-susceptible clinical isolate 523 in successively increasing concentrations of vancomycin. The clinical VISA isolate, IL-\( \text{F} \) (5, 7, 9), was the last isolated from a series of six clonally identical MRSA blood isolates obtained from a dialysis patient who was receiving vancomycin therapy. The broth MICs of vancomycin and teicoplanin were 12 to 16 mg/liter. However, susceptibility testing by population analysis revealed that strain IL-A was vancomycin heteroresistant, with high antimicrobial susceptibility (HAMS) and an ABI Prism sequence detection system (Applied Biosystems, Foster City, Calif.) at The University of Chicago core sequencing facility. DNA sequence comparison were performed using ClustalW alignment software (49).

**Gel retardation assay.** Whole-cell extracts were prepared essentially as described previously by Mahmood and Khan (31). Cells from \( \text{S. aureus} \) strain IL-A (80 ml) were exposed to vancomycin at the beginning of log phase and were harvested 45 min after treatment with vancomycin. The cell pellets were washed once with TEG (25 mM Tris·HCl [pH 8.0]), 5 mM EGTA and centrifuged again. The pellets were resuspended in 2 ml of TEG, quickly frozen at \(-70^\circ\text{C} \) and thawed at room temperature. After two cycles of freeze-thaw, the cell suspension was adjusted to 0.15 M KCl. Lysostaphin (Sigma) was then added to achieve a final concentration of 0.3 mg/ml, and lysate was carried out at 4°C for 1 h. The freeze-thaw process was repeated twice, and the lysates were centrifuged at 30,000 rpm for 30 min in a Beckman Ti70 rotor. The supernatant was collected, and glycerol was added to a final concentration of 20% (vol/vol). The extracts were dialyzed at 4°C overnight against 1 liter of 10 mM Tris·HCl (pH 7.5)–1 mM EDTA–1 mM dihydrothreitol–50 mM NaCl–20% glycerol and then stored at \(-70^\circ\text{C} \).

**i) DNA probes.** Five overlapping DNA probes that spanned the \( \text{prfA-pbpB} \) promoter region from (203 bp upstream through 92 bp downstream from the \( \text{P1} \) transcription start point [\( \text{tp} \)]) were produced by PCR amplification of purified chromosomal DNA (Qiagen) from strain \( \text{IL-A} \) as follows. The 141-bp fragment \( \text{Up} \) (203 through 63 bp upstream from the \( \text{P1} \) tsp) was amplified using primers \( \text{UPF} \) (5’-CACATACCTGATCCTC-3’) and \( \text{UPR} \) (5’-GTTGGAATTTCGC-3’). The 90-bp fragment \( \text{P1} \) (76 upstream through 14 bp downstream from the \( \text{P1} \) tsp) was amplified using primers \( \text{P1F} \) (5’-TGTTAAATCCACACCAAAT-3’) and \( \text{P1R} \) (5’-GCAGAATTACCAAGCAC-3’). The 168-bp fragment \( \text{P1}+\text{P1’} \) (76 upstream and 92 downstream from the \( \text{P1} \) tsp) was amplified using primers \( \text{P1F}+\text{P1’F} \) (5’TGTTAAATCCACACCAAAT-3’) and \( \text{P1R}+\text{P1’R} \) (5’-CAAGTTGTTGAGTTGCAATTGTT-3’). The 99-bp fragment \( \text{P1’} \) (7 upstream through 92 bp downstream from the \( \text{P1} \) tsp) was amplified using primers \( \text{P1’F} \) (5’-AAAGTGGTGGTATTGTTG-3’) and \( \text{P1’R} \) (5’-CAAGTTGTTGAGTTGCAATTGTT-3’). Using T4 polynucleotide kinase (Promega) as recommended by the manufacturer, the PCR fragments were gel purified and end labeled with \( \text{\textit{\textsuperscript{32}P}} \text{ATP} \) (Amersham).

**ii) DNA-protein binding reactions.** The reaction mixtures consisted of a solution containing 10 mM Tris·HCl (pH 7.5), 1 mM dihydrothreitol, 50 mM NaCl, 5 mM MgCl\(_2\), 50 mg of cold glycerol (5 µg of cold glycerol), 1 µg of poly(dI-dC), \textit{\textsuperscript{32}P} 5 µg of whole-cell protein extract in a final volume of 15 µl. The reaction mixtures were incubated at room temperature for 15 min, and the
RESULTS

Differential expression of the \textit{pbpB} gene between genetically related vancomycin-susceptible and -resistant isolates. To determine whether the increased PBP2 production previously observed in strain 523k, the laboratory-derived VISA strain, was due to increased transcription of the \textit{pbpB} gene, the abundance levels of \textit{pbpB}-specific transcripts in strain 523k and in strain 523, the isogenic-susceptible parent strain, were compared by Northern blotting (see probe b in the map in Fig. 1). As shown by the Northern blot in Fig. 2, the abundance of the \textit{pbpB} transcripts was at least twofold greater in isolate 523k. The \textit{pbpB} probe detected 3-kb and 2.4-kb transcripts in strain 523 (the latter band was determined by comigration in the gel beside the 2.4-kb band in the RNA standard); these two transcripts likely correspond to the 2.9- and 2.1-kb \textit{pbpB} major transcripts, respectively, referred to previously by Pinho et al. (39). Interestingly, an additional, 2-kb transcript was detected in the 523k VISA isolate. This transcript migrated slightly beneath the 3.0-kb band such that the signals from the 3.0- and 2.9-kb bands often merged into one. Thus, the combined signal of these two bands was referred to as the 3.0-kb doublet. When the exposure time of the autoradiograph was increased or higher concentrations of RNA were applied to the agarose gel, the 2.9-kb band also became more apparent in strain 523 (Fig. 2B). Thus, the 2.9-kb band of the 3.0-kb doublet was expressed to a lesser extent in strain 523 than it was in strain 523k.

Growth-phase variation of \textit{pbpB} transcript abundance. The abundance of the transcripts detected by the \textit{pbpB} probe was highest for both strains 523 and 523k during the mid-logarithmic growth phase and decreased substantially in the stationary phase (Fig. 2). Interestingly, the smaller band of the 3.0-kb doublet was no longer detected by the \textit{pbpB} probe in the post-exponential phase of growth in either strain 523 or 523k. With the drastic decrease in \textit{pbpB} transcript abundance in the post-exponential phase, there was no longer a marked difference in transcript abundance between strains 523 and 523k.

Increased expression of \textit{pbpB} in clinical VISA isolate IL-F. To determine whether the increased \textit{pbpB} expression observed in strain 523k also occurred in clinical VISA strains selected in vivo during antimicrobial therapy, expression of \textit{pbpB} was compared between two genetically related strains, strain IL-A (vancomycin-heteroresistant MRSA) and strain IL-F (VISA), that were obtained from the same patient but that differed in vancomycin susceptibility. The abundance of the PBP2 protein was about twofold greater in the clinical VISA strain IL-F than that detected in the related heteroresistant strain, IL-A, as determined by Western blotting (Fig. 3). Interestingly, the Western blot clearly shows that strain IL-F produced the additional proteolytic cleavage product of PBP2 described previously (11, 12, 33) whereas the more susceptible isolate did not. As shown in the Northern blot (Fig. 4, lanes 1) and the densitometry measurements (Table 1), the \textit{pbpB} transcript abundance was twofold greater in the VISA strain IL-F than in strain IL-A. As occurred for strains 523 and 523k, the abundance of the \textit{pbpB} transcript also decreased during the stationary phase in strains IL-A and IL-F (data not shown).

Induction of \textit{prfA-pbpB} transcripts by vancomycin. Since PBP2 transcript abundance was increased in VISA strains compared with related susceptible isolates, we hypothesized that vancomycin can induce expression of \textit{pbpB}. A dose-response study of the inducing effect of vancomycin on \textit{prfA-pbpB} transcript abundance was performed by incubating strains IL-A and IL-F in BHI broth containing 0, 2.5, 4, or 8 mg of vancomycin/liter and harvesting RNA from the cells during the mid-logarithmic and late logarithmic growth phases. As seen in the Northern blots probed with the \textit{pbpB} probe (Fig. 4, lanes 2, 3, and 4), when vancomycin was present in the medium, the abundance of the 3.0-, 2.9-, and 2.4-kb \textit{pbpB} transcripts increased in both strains compared with that produced when each isolate was grown in the absence of vancomycin (lanes 1, baseline). However, quantification of the signal intensities from the 3.0-kb doublet revealed that, surprisingly, the kinetics of induction differed for the two strains (Table 1). In strain IL-A, a 3-fold induction over baseline was observed when 2.5 mg of vancomycin/liter was added to the medium and a further
over, at 8 mg of vancomycin/liter, the higher concentrations of up to 8 mg of vancomycin/liter. More-with 2.5 mg of vancomycin/liter but a plateau was reached with a 5.5-fold induction resulting when 8 mg of vancomycin/liter was used. In strain IL-F, a 1.7-fold induction occurred with each increase in the drug concentration, a 2.9- and 3.0-kb bands but not the 2.4-kb transcript (data not shown). Thus, both bands that comprise the upper 3.0-kb doublet contained both the prfA and pbpB genes and the 2.4-kb transcript only contains pbpB.

Characterization of transcripts. As illustrated in the map in Fig. 1, immediately upstream of the pbpB open reading frame (ORF) and encoded within the same operon is a gene called prfA (PBP-related factor A), which has homologues in other gram-positive species (36, 39). Using a primer internal to prfA and another internal to pbpB, a fragment was amplified previously from cDNA by reverse transcriptase PCR that indicated that the two ORFs can be transcribed together (39). However, direct evidence by Northern blotting with prfA or pbpB probes was lacking, and with the discovery that there are two transcripts in the 3.0-kb doublet, we wanted to confirm that both transcripts present in the 3.0-kb doublet contained both prfA and pbpB genes. Thus, Northern blotting was performed using a prfA gene probe that did not overlap with the 2.4-kb transcript (probe a; Fig. 1). The prfA probe hybridized to both the 2.9- and 3.0-kb bands but not the 2.4-kb transcript (data not shown). Thus, both bands that comprise the upper 3.0-kb doublet contained both the prfA and pbpB genes and the 2.4-kb transcript only contains pbpB.

DNA sequence of the pbpB gene of strains IL-A and IL-F. The prfA and pbpB gene sequences of strains IL-A and IL-F were determined to assess whether changes in DNA sequence in the promoter region accounted for the observed increases in prfA-pbpB transcript abundance. The sequences of the entire pbpB operons were identical in strains IL-A (accession number AF508980) and IL-F (accession number AF508981), including those of the three promoter regions (P1, P1’, and P2) (39). The DNA sequence of the pbpB operon from the IL strains was also compared with those from strains Mu50 (AP003133), N315 (AP003362), and COL (Y17795), which were available in the GenBank. Whereas nine nucleotide mismatches were found between the IL isolates and strain COL, only two mismatches were present between the IL isolates and strains Mu50 and N315. Although all of the interstrain nucleotide differences were contained in the pbpB ORF, only one resulted in a single amino acid difference (a leucine in strains IL-A and IL-F instead of serine in strains Mu50, N315, and COL) in residue 707 of the translated protein (relative to the sequence in the database at accession number CAA76853).

increase occurred with each increase in the drug concentration, with a 5.5-fold induction resulting when 8 mg of vancomycin/liter was used. In strain IL-F, a 1.7-fold induction occurred with 2.5 mg of vancomycin/liter but a plateau was reached with higher concentrations of up to 8 mg of vancomycin/liter. Moreover, at 8 mg of vancomycin/liter, the pbpB transcript abundance in strain IL-A became greater than that of strain IL-F (Table 1). Thus, the vancomycin-inducing potential of pbpB was actually lower in the VISA strain. No further increases occurred in either strain when 16 mg of vancomycin/liter was used in the growth medium (data not shown). The 2.4-kb pbpB transcript appeared in much lower abundance than the 3.0-kb doublet, but induction of this transcript followed a dose response similar to that of the 3.0-kb prfA-pbpB doublet.

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DNA sequence of the pbpB gene of strains IL-A and IL-F. The prfA and pbpB gene sequences of strains IL-A and IL-F were determined to assess whether changes in DNA sequence in the promoter region accounted for the observed increases in prfA-pbpB transcript abundance. The sequences of the entire pbpB operons were identical in strains IL-A (accession number AF508980) and IL-F (accession number AF508981), including those of the three promoter regions (P1, P1’, and P2) (39). The DNA sequence of the pbpB operon from the IL strains was also compared with those from strains Mu50 (AP003133), N315 (AP003362), and COL (Y17795), which were available in the GenBank. Whereas nine nucleotide mismatches were found between the IL isolates and strain COL, only two mismatches were present between the IL isolates and strains Mu50 and N315. Although all of the interstrain nucleotide differences were contained in the pbpB ORF, only one resulted in an amino acid difference between the IL strains and the others. Compared with strains Mu50, N315, and COL, both IL-A and IL-F contained a nucleotide mismatch (a T instead of C at nucleotide 3473 of the COL sequence [Y17795]) that resulted in a single amino acid difference (a leucine in strains IL-A and IL-F instead of serine in strains Mu50, N315, and COL) in residue 707 of the translated protein (relative to the sequence in the database at accession number CAA76853).
This polymorphism lies at the carboxyl terminus of the translated protein and outside the known penicillin-binding motifs and the transpeptidase and transglycosylase domains. This unique polymorphism in strains IL-A and IL-F and the phylogeny trees produced from the sequence alignments (data not shown) reinforce the previous conclusion that strain IL-F is derived from strain IL-A. Also, overall, the \( pbpB \) sequences from strains N315 and Mu50 were more closely related to each other than to those from either of the IL isolates.

**Induction of \( prfA \) and \( pbpB \) transcripts by oxacillin.** Since PBP2 is a cofactor required for the synthesis of peptidoglycan in the presence of oxacillin, and since oxacillin is known to induce transcription of the methicillin resistance gene, mecA, we investigated whether oxacillin can also induce \( pbpB \) expression. When 4 mg of oxacillin/liter was added to cultures of strains IL-A and IL-F and cells were allowed to grow for an additional hour, the abundance of the 3.0-kb \( pbpB \) doublet increased 4- and 3.2-fold for strains IL-A and IL-F, respectively (Fig. 5). As expected, the mecA gene transcript was also induced by oxacillin under these conditions (data not shown).

**Effect of inactivation of \( agr \) on \( pbpB \) growth phase-regulated gene expression and induction by vancomycin.** *S. aureus* controls the expression of many extracellular gene products by using a growth-regulated, quorum-sensing system encoded by the \( agr \) locus (26, 35). To investigate whether \( agr \) also plays a role in the growth phase-regulated expression of the \( pbpB \) gene, we tested whether inactivation of \( agr \) alters \( pbpB \) transcript abundance in mid-log and stationary growth phases. To this end, Northern blotting of RNA from strain RN6911, which has the entire \( agr \) locus replaced by a tetracycline resistance gene, was performed using a \( pbpB \) gene probe. As was the case for expression of the gene in strains 523, 523k, IL-A, and IL-F, \( pbpB \) gene expression in strain RN6911 was high during the mid-log phase and dramatically lower in stationary phase (Fig. 6). Also, \( pbpB \) transcript abundance from strain RN6911 increased when grown under vancomycin-inducing conditions (Fig. 6). Such induction was observed 1 and 3 h after vancomycin was added to the culture medium. Similarly, the inactivation of \( agr \) did not alter the ability of oxacillin to induce \( pbpB \) transcription (data not shown). These data demonstrate that although \( agr \) regulates expression of many extracellular proteins in a growth phase-dependent manner, it is not responsible for growth-phase control or induction of \( pbpB \) gene expression in the presence or absence of vancomycin or oxacillin.

**Gel retardation assay.** To determine whether vancomycin induces the expression of a transcription factor that directly binds to the \( pbpB \) promoter and to determine which portion of the \( pbpB \) promoter is important for binding, gel retardation assays were performed. This assay relies on the fact that proteins that bind to a DNA fragment retard the mobility of the fragment in a gel during electrophoresis. Five overlapping fragments (illustrated in Fig. 7) that spanned the entire \( prfA-pbpB \) promoter region were radiolabeled and incubated with extracts from vancomycin-induced and uninduced cells in the presence of excess cold competitor DNA. When the fragment (P1) that contained only the P1 promoter (−76 through +14 bp relative to the P1 tsp) was used, extracts from both induced and uninduced cells produced two major retarded species that were absent from the control lane containing the radiolabeled probe not exposed to protein extracts (free probe). The extracts from the vancomycin-induced cultures produced a third minor band of retarded mobility in the gel (Fig. 7, arrow a). Moreover, the signal intensity was greater in the fragments retarded by proteins from vancomycin-induced cells than in those by retarded by proteins from uninduced cells. Thus, the induced cultures contained either a greater abundance of protein capable of binding to the P1 promoter or a protein with a greater affinity for the P1 promoter.

A second fragment (P1+P1′) was also tested. This fragment contained an additional 78 bp downstream of the P1 promoter (−76 through +92 bp relative to the P1 tsp) and included both the P1 and P1′ promoters. Compared with the P1 fragment, a greater amount of the P1+P1′ probe was bound by protein from either extract, as judged by the signal intensities of the retarded species in the respective autoradiographs. Also, protein extracts from vancomycin-induced cells produced a larger number of retarded species of the P1+P1′ fragment with increased intensity than did the extracts from uninduced cultures or free probe. For example, a single fragment of retarded mobility present in the lane containing free probe (Fig. 7, arrow b) was shifted slightly higher in the gel and became more intense upon exposure to either protein extract, but the intensity of this fragment increased in the vancomycin-induced extracts. Both the fragments with deletions of 69 bp (fragment P1′) and those with deletions of 100 bp (fragment P1′S) from the 5′ end of the P1+P1′ fragment abolished the differences observed between the uninduced and induced extracts, al-
though retarded fragments were still produced by either extract. This finding suggests that the region between −76 and −7 bp upstream from the P1 tsp contains binding sites for a vancomycin-inducible transcription factor. These data also support the notion that vancomycin induces an increased amount of the putative transcription factor in the cytosol of vancomycin-induced cells. The increased binding characteristics of the P1+P1' fragment compared with those of the P1 fragment suggests that cooperative binding occurred in the region that spans the P1 and P1' promoters (−76 through +92 bp relative to the P1 tsp).

**MecI, MecR1, BlaI, and BlaR1 are not involved in induction of the *pbpB* gene by vancomycin and oxacillin.** MecI-MecR1 and BlaI-BlaR1 regulatory complexes control β-lactam-dependent induction of *mecA* and *blaZ* genes, respectively. To examine whether these regulatory complexes also played a role in the induction of *pbpB* transcription by vancomycin and oxacillin, a methicillin-susceptible, β-lactamase-negative clinical strain (strain 1715), which therefore likely lacks these regulatory genes, was tested for the ability of these agents to induce *pbpB* transcription. This strain was fully capable of having its *pbpB* gene induced by these agents (data not shown). Thus, these regulatory proteins are not required for induction of *pbpB* transcription by these antimicrobials.

**DISCUSSION**

This report demonstrates that the transcription of *pbpB* and *prfA* transcripts in *S. aureus* is modulated in response to the growth phase and is induced by the cell wall-active antibiotics.

![Diagram of gel retardation assay](http://aac.asm.org/)
vancomycin and oxacillin. Additionally, this is the first demonstration that PBP2 production increased in a clinical VISA isolate (IL-F) compared with that in a genetically identical, more susceptible isolate (IL-A) obtained from the same patient during vancomycin therapy. These data extend the results of previous studies that demonstrated greater PBP2 production in VISA clinical isolates than in epidemiologically unrelated susceptible strains or in laboratory-derived VISA isolates (20, 33) and teicoplanin intermediate-resistant clinical isolates (32, 44). This report extends these previous observations by demonstrating that the increased production of PBP2 in the strains we tested was due to increased transcription of the pbpB gene.

An increase in pbpB transcript abundance in response to antibiotic-induced stress has not been previously reported. This is likely due to the fact that pbpB transcriptional activity has only recently been explored and that the optimal conditions for detecting such induction had not been known until now. Furthermore, although horizontally acquired genes that confer vancomycin or β-lactam resistance have been shown to be induced by their cognate antibiotics, antibiotic induction of housekeeping genes involved in cell wall biosynthesis has not been previously reported. Such induction makes sense for bacterial survival; an increased abundance of prfA-pbpB transcripts provides S. aureus with a rapid means to respond to the damaging effects of cell wall antibiotics by accelerating cell wall synthesis.

Depending on the growth or Northern blotting conditions, we found up to three pbpB-hybridizing transcripts (3.0, 2.9, and 2.4 kb) that were large enough to accommodate the pbpB ORF. The 2.9-kb transcript was not detected previously in Northern blots and was only recognized as a faint tsp, as detected by a primer extension analysis (39). This was likely due to the fact that the 2.9-kb transcript was usually less easily detectable in vancomycin-susceptible strains than in VISA strains. In contrast, in the VISA strains, this 2.9-kb band was consistently detected without the need for autoradiographic enhancement during the mid-log phase, although it was less readily detected during stationary phase.

In Streptococcus pyogenes and Bacillus subtilis, expression or production of PBPs has been shown to decrease in the post-exponential phase (42, 48). We provide evidence (by Northern blotting) that all three pbpB transcripts decrease during stationary phase in S. aureus, a result that extends the findings that the detection of tss from two of the promoters (P1 and P2) decreased at the end of stationary phase (39). Thus, growth-regulated expression of PBP genes might be a general strategy used by bacteria. However, the genetic basis for this phenomenon has remained uncharacterized. Discovering the basis for this could lead to the identification of a novel target for antimicrobial therapy.

Growth phase-variable expression of many secreted and cell wall-associated virulence factors in S. aureus is controlled by the agr quorum-sensing system and the SarA global regulator (13, 35). Piriz Duran et al. reported that neither agr nor sar gene inactivation influenced PBP2 activity (41); however, that study focused on PBP activity during mid-log phase and did not investigate whether the effect of such inactivation influenced the decrease of PBP activity in stationary phase. Thus, our data now rule out a role for the agr locus in modulating growth phase-variable expression of the prfA-pbpB operon and for induction by antibiotics as well. In addition, with the finding that pbpB gene induction can be influenced by vancomycin in strain RN6911, a derivative of a natural rsbU mutant shown to lack SigB (an alternate sigma factor) activity (19), these data also indicate that an active form of SigB is not required for vancomycin-mediated induction of pbpB. It remains to be determined whether SarA is involved in antibiotic-mediated induction of pbpB expression. However, overexpression of the sarA gene on a multicopy plasmid in strain 523k did not alter expression of any of the pbpB transcripts in the absence of vancomycin or β-lactams (our unpublished data).

The role of pbpB overexpression in vancomycin resistance is not yet clear. However, several observations regarding VISA isolates support the idea that increased PBP production plays an accessory role in the resistance phenotype. A strategy for overcoming the inhibitory effect of vancomycin on cell wall synthesis might include maximizing the cell wall metabolic machinery. Accordingly, increased production of PBP2, increased cell wall thickness, accelerated cell wall metabolism in VISA isolates (as evidenced by increased incorporation of [14C]N-acetylglucosamine), release of this compound into liquid medium, and an increase in the murein monomer precursor pool might all aid in this regard (20). However, it is clear that the role of PBP2 in resistance to vancomycin also depends on other factors, since overproduction from a multicopy plasmid does not raise the vancomycin MIC to an intermediate level (≥8 mg/liter) (20). Of particular interest is the paradoxical finding that the vancomycin induction potential of pbpB expression became lower in VISA strain IL-F as the vancomycin concentration increased. Thus, although constitutive levels of pbpB transcription were slightly higher in strain IL-F, the absolute transcript abundance induced by vancomycin was lower in strain IL-F than in strain IL-A at concentrations higher than 4 mg of vancomycin/liter. The significance of this finding is that less PBP2 is expressed in the resistant strain than in the susceptible strain at concentrations of vancomycin between 4 and 8 mg/liter, a concentration range that is within that of the trough serum level during vancomycin therapy (5 to 15 mg/liter). This, of course, confounds our understanding of how PBP2 affects vancomycin resistance. However, if pbpB is not involved in vancomycin resistance, it is possible that the putative regulator of pbpB gene induction coregulates other genes involved in vancomycin resistance. Nevertheless, these data indicate that the putative factors that control vancomycin induction of the pbpB gene are altered in strain IL-F. Also, the fact that induction in strain IL-F was merely hampered, not abolished, suggests that at least two factors cooperate in vancomycin induction of pbpB gene expression and that one of these factors has been altered in some way in strain IL-F. The results from the gel retardation assay, in which the presence of both the P1 and P1’ promoters was required for enhanced binding of vancomycin-induced proteins to the pbpB promoter, is further evidence that the induction involves cooperative binding. Whether any of the regulatory genes that have been implicated in glycopeptide resistance are also involved in pbpB expression remains to be addressed (4, 29).

Of the other PBPs, a decrease in PBP4 (carboxypeptidase) (17, 45) and PBP2a (46) activity was found in VISA isolates, although the latter result has not been consistently found. For
instance, isolates IL-A and IL-F express equivalent amounts of mecA (unpublished data) and are equally resistant to methicillin (5). A decrease in PBP4 activity would result in lower carboxypeptidase activity, which was proposed to explain the decrease in peptidoglycan cross-linking found in some VISA isolates. Such decreased cross-linking would result in an increase in the number of D-Ala-D-Ala termini on peptidoglycan stem peptides and thereby increase the false vancomycin-binding sites in the preformed cell wall. According to one model of vancomycin resistance (14), increased nonspecific binding to the cell wall presumably would lower the effective concentration of vancomycin that is able to reach its vital target, the peptidoglycan precursor, which is attached to the cell membrane (18). Challenging this model are the results of a study which used high-performance liquid chromatography of cell wall muramidase digestion products that demonstrated increased peptidoglycan cross-linking in VISA strains that included strain 523k (6) and clinical isolates IL-F and PC (7). Therefore, contrary to the model, these strains would actually have fewer D-Ala-D-Ala termini and fewer false binding sites for vancomycin. We speculate that the decrease in PBP4 activity observed in other VISA isolates is a compensatory response to the increased PBP2 production that balances the total cross-linking activity in the cell. Alternately, pbpB gene expression might be coordinately up-regulated by factors that decrease the expression of the phyD gene that encodes PBP4.

Given the central role that PBP2 plays in cell wall metabolism and resistance to oxacillin and (perhaps) vancomycin and the finding that pbpB transcription is inducible by cell wall-active antibiotics, it is now of interest to identify the factors that control such pbpB gene induction. The difference in PBP2 expression between strains IL-A and IL-F was not due to changes in the cis-regulatory elements of the pbpB promoter region. Therefore, a change in transcription factor activity must be involved. The results of the gel shift assays support the view that such transcription factors are either induced by vancomycin or are in some way induced to have a greater affinity for the pbpB promoter.

It is well established that oxacillin induces the mecA gene that encodes PBP2a in MRSA isolates via the MecI repressor and the MecR1 signal-transducing protein (23) or by BlaI and BlaR1, a repressor and signal-transducing protein, respectively, that control β-lactamase gene expression (1). Increased expression of pbpB in response to oxacillin is therefore consistent with the recent discovery that PBP2 cooperates with PBP2a in peptidoglycan synthesis when oxacillin is present (37, 40). Thus, it is understandable that oxacillin simultaneously induces the pbpB and mecA genes to coordinate methicillin resistance. It is interesting that a β-lactam induces expression of a gene that encodes one of the eventual targets of this antibiotic, since increased PBP2 production can lead to borderline resistance. Future investigations may determine whether other classes of β-lactams are also capable of inducing PBP2 expression, whether other PBP2s are also induced, and whether by inhibiting the induction it is possible to increase the effectiveness of β-lactams.

Our data, showing that methicillin-susceptible (mecA-negative) strains that also lacked β-lactamase were capable of inducing pbpB gene transcription in response to the presence of vancomycin and oxacillin, demonstrate that MecR1, BlaI, MecI, and BlaI are not involved in the pbpB induction pathway.

From these data, we propose that pbpB expression is stimulated by vancomycin and oxacillin via a putative regulatory system other than MecR1/MecI or BlaR1/BlaI that does not require agr or SigB activity. The role of prfA is poorly understood. However, it does not appear to play a role in pbpB constitutive transcription (39), and its role in antibiotic induction has not yet been investigated. We speculate that since vancomycin does not enter the cell, it is possible that a signal-transducing mechanism is involved in pbpB gene induction. Alternately, since vancomycin and oxacillin are from unrelated classes of antibiotics, it is possible that the signal for pbpB gene activation results from the inhibition of cell wall synthesis, analogous to the system controlling AmpC β-lactamase gene induction in gram-negative bacteria (25).

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REFERENCES

ERRATUM

Transcriptional Induction of the Penicillin-Binding Protein 2 Gene in Staphylococcus aureus by Cell Wall-Active Antibiotics Oxacillin and Vancomycin

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