Transcriptional Analysis of the Staphylococcus aureus Penicillin Binding Protein 2 Gene

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Sequencing of the vicinity of the staphylococcal pbp2 gene and transcriptional analysis by primer extension and promoter fusions were used to show that pbp2 is part of an operon that also includes a gene with high homology to prfA of Bacillus subtilis. Two distinct promoters were identified directing transcription of pbp2 either alone or together with prfA. It was recently reported that transposon inactivation of pbp2 causes a reduction in methicillin resistance, but complementation experiments were not fully successful. We now show that introduction of the intact pbp2 gene with its two newly identified promoters into the chromosome of the transposon mutant resulted in the full recovery of high-level methicillin resistance.

All strains of Staphylococcus aureus have four penicillin-binding proteins (PBPs) which are assumed to participate in the assembly of cell wall peptidoglycan. Of this native set of PBPs, PBPa was reported to be a major peptidoglycan transpeptidase (5), since selective inhibition of this protein by cephalosporins led to the inhibition of peptidoglycan synthesis by the N-terminal half of elongation (16) and to leakage of cytoplasmic contents due to cell lysis (5). Homology between the N-terminal half of PBP2A and PBP1A, a bifunctional protein, suggests that PBP2 also has a transglycosylase domain (13, 15).

Surprisingly, PBP2 also appears to have an important role in the expression of antibiotic resistance in methicillin-resistant S. aureus (MRSA) (18). MRSA has an additional PBP—PBP2A—which has a very low affinity for β-lactam antibiotics (8, 21) and has homology to monofunctional transpeptidase PBP3 of E. coli (6, 9, 13). In current models, PBP2A is assumed to take over the biosynthetic functions of normal PBPs in the presence of inhibitory concentrations of β-lactams. According to this model, normal PBPs no longer take part in the catalysis of cell wall synthesis in the presence of the antibiotic. It was therefore surprising to find that a mutant with a transposon insertion in pbp2 (USA 130) showed a massive reduction in methicillin resistance, despite its normal production of PBP2A, indicating that intact PBP2 is essential for the optimal expression of methicillin resistance in MRSA (18). As a possible explanation, we proposed that survival and growth in the presence of the antibiotic may require functional cooperation between the penicillin-insensitive transglycosylase domain of PBP2 and the transpeptidase domain of PBP2A or that effective functioning of PBP2A may require the presence of inactivated (acylated) PBP2, which serves as structural scaffolding (18). An additional possibility that could not be excluded was that a truncated PBP2 produced by the transposon-inactivated pbp2 gene may interfere with the function of PBP2A. This hypothesis was also suggested by the fact that attempts to recover the normal—high-level of antibiotic resistance by complementation with a plasmid-born pbp2 gene were only partially successful (18).

In an attempt to clarify the reasons for the lack of success in complementation, we proceeded to do more extensive sequencing in the vicinity of the pbp2 gene and also performed transcriptional analysis. This study showed that the pbp2 gene is part of an operon and can be transcribed alone or together with a newly identified PBP-related factor (PrfA), due to the presence of two distinct promoters. Introduction of a construct that contained the entire pbp2 operon into the chromosome of the pbp2 transposon mutant resulted in the full recovery of antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are described in Table 1. S. aureus strains were grown on tryptic soy broth (TSB; Difco Laboratories) with aeration as described previously (17). E. coli strains were grown in Luria-Bertani broth (Difco) with aeration. Antibiotics were used at the following concentrations: erythromycin, 10 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml.

DNA methods. Routine DNA manipulations were performed by using standard methods (2, 22). All of the enzymes were purchased from either New England Biolabs or Boehringer Mannheim and used as recommended by the manufacturers. DNA sequencing was done at the Rockefeller University Protein DNA Technology Center with the Taq fluorescent dye terminator sequencing method by using a PE/ABI 377 automated sequencer.

Inverted PCR. COL chromosomal DNA was digested with EcoRI, purified with the Wizard DNA Clean-Up System (Promega), and ligated with T4 DNA ligase. The ligation mixture, after purification, was used as a template for a PCR with the GeneAmp PCR Reagent Kit with AmpliTaq DNA polymerase (Perkin Elmer) in accordance with the manufacturer’s instructions and by using 20 pmol each of primers PBPaP8 (CTTAGGCTGAGAAGATCCTT) and PBPaP9 (AGCTTTGCAAGAATGTCGT) and 200 μM each of dNTPs. The reaction was overlaid with an oil film and incubated for 30 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final extension step of 72°C for 5 min. The 2,862-bp fragment was sequenced by primer walking, starting with the same primers used for the PCR.

Isolation of RNA and Northern blot hybridization. Overnight cultures of S. aureus were diluted 1:50 in TSB and grown to mid-log phase (optical density at 620 nm [OD620] 0.7). The cells were pelleted and processed as an RNaseasy Mini Kit (Qiagen) or with a FastRNA Blue isolation kit (Bio 101, Inc.) in combination with FastPrep FP120 (Bio 101 Savant) in accordance with the manufacturer’s recommendations. RNA (5 μg) was electrophoresed through a 1.2% agarose-0.66 M formaldehyde gel in morpholinepropanesulfonic acid running buffer (Sigma). Blotting of RNA onto Hybond N+ membranes (Amersham) was performed with the Turboblottor alkaline transfer system (Schleicher & Schuell). For the detection of specific transcripts, RNA probes were labelled by using the Gene Images random prime labelling module (Amersham Life Science) and hybridized under high-stringency conditions. The blots were subsequently washed and autoradiographed.

RT-PCR. Reverse transcription (RT)-PCR was performed by using the GeneAmp RNA PCR kit (Perkin Elmer). COL RNA treated with DNase was used as the template. Random hexamers were used for the reverse transcriptase reaction.
tion, and a primer internal to prfA—prfD1 (ATGCACTATCTATCGGCGG G) — and a primer internal to phy2—IP6 (TCTTAGCTATCCCATCGT)— were used in the PCR. The following conditions were used: 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 1.75 min, and 72°C for 5 min; and one final extension step of 72°C for 5 min.

**Constructions of promoter fusions.** Three fragments encompassing the region upstream of phy2 were amplified by high-fidelity PCR with the GeneAmp XL PCR kit (Perkin Elmer), which includes Tth DNA polymerase XL. To further decrease the probability of errors during the PCR, a hot start and the following conditions were used: 94°C for 2 min, 20 cycles of 94°C for 30 s, and 55°C for 1.75 min; and one final extension step of 55°C for 5 min. Primer pairs pro5-pro9, pro5-pro8, and pro9-pro10 (Fig. 1) were used to amplify the three fragments that were subsequently cloned into pLC4.

**Enzyme assays.** Catechol 2,3-dioxygenase assays were performed essentially as previously described (23), except for the lysis of bacteria, which was done by using glass beads and FastPrep 120 (Bio 101 Savant) in 100 mM phosphate buffer (pH 7.5) containing 10% acetone. Volumes of 20 to 30 ml corresponding to different OD620 values were serially removed from cultures growing in TSB. Assays contained 100 μl of extract, and the reactions were conducted at room temperature for 5 min with OD620 readings taken at 30-s intervals. One milliunit corresponds to the formation at room temperature of 1 nmol of 2-hydroxymuconic semialdehyde per min. Specific activity is reported in milliunits per milliliter of extract.

**Primer extension analysis.** Primer extension analysis was performed by using primers PE2 and PE3 (Fig. 1), which were end labelled with [γ-32P]ATP and purified with Sephadex G-25 spin columns (Boehringer Mannheim). RNA from COL (50°C) or RN4220 (10 μg) was hybridized with the appropriate primer at 65°C for 90 min and slowly cooled to room temperature. The reaction product was incubated with RNase H (3 U) at 37°C for 30 min, ethanol precipitated, resuspended in 10 μl of Sequenase stop solution, denatured, and applied to a 6% sequencing gel. Sequencing reaction mixtures prepared by using SuperScript RT (Gibco BRL) at 42°C for 90 min, and the primer at 65°C for 90 min and slowly cooled to room temperature. RT was carried out by using SuperScript RT (Gibco BRL) at 42°C for 90 min, and the primer at 65°C for 90 min and slowly cooled to room temperature. RT was carried out by using SuperScript RT (Gibco BRL) at 42°C for 90 min, and the primer at 65°C for 90 min and slowly cooled to room temperature.

**TABLE 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td>S. aureus COL</td>
<td>Homogeneous Mcr (MIC, 1,600 μg/ml)</td>
<td>RU collection</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>Mutant strain of 8325-4 that is r</td>
<td>R. Novick</td>
</tr>
<tr>
<td>S. aureus RUSA130</td>
<td>COL1703 (phy2::Tn551) Em' heterogeneous Mcr (MIC, 12 μg/ml)</td>
<td>4</td>
</tr>
<tr>
<td>S. aureus RUSA130/pMGP19</td>
<td>RUSA130 with pMGP19 plasmid containing prfA and phy2 genes and promoters</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>supE44ΔlacU169 (Δ800lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA</td>
<td>Bethesda Research Laboratories</td>
</tr>
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**FIG. 1.** Nucleotide sequence of the region upstream of phy2. Putative promoter regions are highlighted by boxed sequences and labelled +10 and +35. The promoters are designated P1 and P2. P1 corresponds to a weaker signal in the primer extension analysis. Putative ribosome-binding sites are underlined and labelled SD. The 5' end of the RNA determined by primer extension is labelled +1. Start codons are in boldface and double underlined. The prfA stop codon is indicated by an asterisk. Primers are indicated by arrows. The deduced amino acid sequence of prfA is aligned under the DNA sequence.
allowed to migrate in parallel tracks across the agar surface to the opposite side of each plate, and the plates were incubated at 30°C for 48 h (11).

Nucleotide sequence accession number. The complete nucleotide sequence determined in this study is available in the EMBL and GenBank databases under accession no. Y17795.

RESULTS

Sequencing of the region upstream of the pbp2 gene. A fragment of 2,862 bp, which included the region upstream of the pbp2 gene of strain COL, was obtained by inverted PCR and sequenced by primer walking. The sequence of the pbp2 gene from COL was obtained by using primers based on the published sequence of S. aureus SRM705 (15) (accession no. X62288). The fragment upstream of pbp2 is presented in Fig. 1.

Computer analysis identified an open reading frame (ORF) of 627 bp upstream of pbp2. In fact, the last four nucleotides of this ORF overlap the pbp2 coding sequence. The deduced amino acid sequence of this ORF was compared to sequences of known polypeptides in the EMBL and GenBank databases by using the Gapped Blast algorithm (1). Significant homology (52% identity) was found with the protein encoded by gene prfA (PBP-related factor A) from Bacillus subtilis (19), which is located upstream of the gene that encodes PBP1 (the B. subtilis homologue of S. aureus PBP2). This protein has also been identified in Streptococcus mitis and Streptococcus pneumoniae (12).

Analysis of the transcription of pbp2. To determine if the prfA and pbp2 genes were transcribed together, a Northern blot of total RNA of COL was hybridized with a probe internal to pbp2 (nucleotides 330 to 716 of the pbp2 sequence). The appearance of two bands (data not shown), one with a molecular size of 2.1 kb, corresponding to the size expected for the pbp2 transcript, and another with a molecular size of 2.9 kb, corresponding to the size of a transcript with the two ORFs, suggests that the pbp2 gene can be transcribed either alone or together with prfA.

A third band, corresponding to a molecular size of 1.6 kb and located just above the rRNA band, was occasionally observed in some of the Northern blot hybridizations. The size of this band is smaller than that of the pbp2 gene, and it probably corresponds to degradation products retained in this region.

RT-PCR was performed by using one primer internal to prfA and another internal to pbp2. The successful amplification of a 1.2-kb fragment clearly indicates that the two ORFs can be transcribed together.

These results suggested the existence of two promoters, one upstream of prfA (promoter 1) that would direct the transcription of the 2.9-kb transcript and another upstream of pbp2 (promoter 2) that would direct the transcription of the 2.1-kb transcript.

We amplified, by high-fidelity PCR, three fragments: one containing 678 bp (from nucleotide 133 to nucleotide 802 in Fig. 1) and including the region where we expected promoter 1 to be; a second, 311-bp fragment (from nucleotide 777 to nucleotide 1088 in Fig. 1) including the region of promoter 2; and a third, 967-bp fragment (from nucleotide 133 to nucleotide 1088 in Fig. 1) including both promoter regions. The fragments were cloned into pLC4, and the inserts were sequenced. This plasmid has a promoterless xylE gene, and production of catechol 2,3-dioxygenase in S. aureus is dependent on the introduction of a promoter, upstream of xylE, that is functional in the gram-positive host (26).

Determination of the specific activity of catechol 2,3-dioxygenase (Fig. 2) indicated that promoter 1 generates higher levels of catechol 2,3-dioxygenase activity than promoter 2 and
that the activity of both promoters was found to decrease with the end of the exponential growth phase.

**Determination of transcription initiation sites.** Primer extension analysis was performed to determine the transcription start site corresponding to each promoter by using primers specific for the prfA and pbp2 transcripts. RNAs prepared from both COL (data not shown) and RN4220pro9/10 (Fig. 3) (i.e., the strain containing a plasmid with an insert encompassing both promoter regions) were used as templates for the RT reaction.

Based on this analysis, it was determined that the transcript that includes prfA initiates at a thymine residue (start point of promoter P1 in Fig. 1) located 104 bp upstream of the prfA start codon. Nevertheless, a fainter band was consistently observed at a guanine residue 74 bp downstream (start point of promoter P1' in Fig. 1). The pbp2 transcript initiates at an adenine residue located 73 bp upstream of the pbp2 start codon and therefore in the prfA coding sequence.

**Recovery of methicillin resistance in mutant RUSA130.** A 3.2-kb fragment containing the complete sequences of prfA and pbp2, as well as the promoter regions, was amplified by high-fidelity PCR and cloned into pSPT181. Plasmid pMGP19 was first introduced into RN4220 by electroporation and afterwards into RUSA130 by transduction. This plasmid is incompatible with the tetracycline resistance-encoding plasmid present in COL and in the RUSA130 mutant. Therefore, strain RUSA130 with pMGP19 had the plasmid integrated into the chromosome by a Campbell-type mechanism and also retained the transposon-inactivated copy of pbp2—as confirmed by Southern blotting, followed by hybridization with a probe specific for pbp2 (data not shown). Figure 4 shows that RUSA130/pMGP19 fully recovered the high-level methicillin resistance of parental strain COL.

**DISCUSSION**

In previous studies of pbp2, it was suggested that the −10 region of the pbp2 promoter (annotated in the GenBank database, accession no. L25426 [7]) was located in the region corresponding to nucleotides 1027 to 1032 of Fig. 1. Our data shows that this is unlikely and that, in fact, not one but two promoters direct the transcription of pbp2 (promoters 1 and 2 in Fig. 1), neither one of which coincides with the previously suggested promoter.

The lack of success in recovering high-level antibiotic resistance in the complementation experiments described before (18) may have been caused by the use of pbp2 without a correct promoter. By using the pbp2 operon as defined by the results described here, it was possible to recover the high, parental level of methicillin resistance in pbp2 transposon mutant RUSA130. Construct RUSA130/pMGP19 contained single copies of both the truncated and normal forms of the pbp2 gene on the chromosome, each preceded by native promoters.

The possibility could not be previously excluded that the truncated allele of PBP2 present in RUSA130 might interfere with the function of PBP2A and thus cause the reduction of methicillin resistance in strain RUSA130 (18). However, the recovery of high methicillin resistance in RUSA130/pMGP19 makes it unlikely that the truncated allele could have a dominant negative effect on the activity of PBP2A. The reappearance of parental-level methicillin resistance in this construct also provides final proof of the importance of functional PBP2 in the expression of resistance to methicillin.

The results described here indicate that transcription of pbp2 can occur together with that of prfA, a gene located immediately upstream of pbp2 with an overlap of four nucleotides, and the two genes therefore constitute an operon. However, pbp2 can also be transcribed alone. It is conceivable that changes in the preferential use of the two promoters may occur under specific physiological conditions, for instance, in the presence of antibiotics. However, analysis of the activities of the two promoters through the growth cycle did not show any striking change of promoter usage: promoter 1 activity was
always higher, and the expression of \textit{pbp2} from both promoters declined as the bacteria entered stationary phase.

The function of the protein encoded by \textit{prfA} is unknown. In \textit{B. subtilis}, the combined effects of mutations in the \textit{PBP1 \textit{(ponA)}} and \textit{pbp4} genes on the bacterial growth rate were more dramatic than the effect of either one of the individual mutations, suggesting involvement of \textit{PrfA} with the function of \textit{PBP1} (19), a homologue of \textit{S. aureus PBP2}. The existence of two promoters in \textit{B. subtilis} has also been suggested, although attempts to determine their positions by primer extension were inconclusive (19). The fact that the two genes are part of an operon in at least two different organisms reinforces the hypothesis that their functions may be related. \textit{PrfA} may modulate the activity of the staphylococcal \textit{pbp2} promoter, although the presence of \textit{prfA} in a multicopy plasmid (pro9/10) did not significantly affect the transcription directed by the \textit{pbp2} promoters in the catechol 2,3-deoxygenase assay. It is possible that this protein interacts with \textit{PBP2}, as a part of a multienzyme complex responsible for the catalysis of cell wall synthesis in a manner similar to the one proposed for \textit{E. coli} (3). Studies are in progress to test this possibility.

Overexpression of \textit{PBP2} was observed in vancomycin- and teicoplanin-resistant \textit{S. aureus} (14, 24). The identification of \textit{pbp2} promoter regions, as well as the characterization of the \textit{PrfA} protein, may be relevant to the understanding of the mechanisms of resistance to both \textit{\beta}-lactams and glycopeptides.

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\textbf{REFERENCES}