

## Transcription Analysis of the *Staphylococcus aureus* Gene Encoding Penicillin-Binding Protein 4

TAMMY L. DOMANSKI,<sup>1†</sup> BOUDEWIJN L. M. DE JONGE,<sup>2‡</sup> AND KENNETH W. BAYLES<sup>3\*</sup>

*Program in Molecular and Cell Biology, University of Maryland, Baltimore County, Baltimore, Maryland 21228<sup>1</sup>;*  
*Laboratory of Microbiology, Rockefeller University, New York, New York 10021<sup>2</sup>;* and *Department*  
*of Microbiology, Molecular Biology and Biochemistry, University of Idaho,*  
*Moscow, Idaho 83844-3052<sup>3</sup>*

Received 23 July 1996/Accepted 3 February 1997

**The high level of cross-linking found in *Staphylococcus aureus* peptidoglycan is dependent on the low-molecular-weight penicillin-binding protein PBP4. Recently, the PBP4 gene, *pbpD*, was cloned and shown to be adjacent to and divergently transcribed relative to the putative ABC-type transporter gene, *abcA*. Disruption of *abcA* (in strain KB400) was previously shown to result in heightened resistance to several antibiotics known to interact with PBP4, suggesting that the regulation of *pbpD* is affected by *abcA*. In this report, this hypothesis was confirmed by use of a Northern (RNA) blot analysis which revealed increased accumulation of *pbpD*-specific transcripts in KB400 compared to that in the wild-type strain, 8325-4. By using reverse-phase high-performance liquid chromatography to examine the structure of the peptidoglycan, it was demonstrated that the increased expression of *pbpD* resulted in an increased level of peptidoglycan cross-linking in the staphylococcal cell wall. Promoter fusion studies demonstrated that the *abcA* mutation caused approximately 7-fold and 100-fold increases in *pbpD* and *abcA* promoter activities, respectively. Primer extension experiments revealed that these genes have long, untranslated leader sequences that result in a transcriptional overlap of 80 bp. Interestingly, deletion of a 26-bp region containing an inverted repeat sequence resulted in the loss of expression from both the *abcA* and the *pbpD* promoters. These data provide evidence that *abcA* and *pbpD* are under the control of a common regulatory mechanism that may involve the transport function of the *abcA* gene product.**

Methicillin-sensitive strains of *Staphylococcus aureus* produce four penicillin-binding proteins (PBPs), 1 to 4, that are involved in the assembly of the cell wall peptidoglycan. Growth studies using cefotaxime and cephalixin,  $\beta$ -lactam antibiotics that preferentially interact with PBP2 and PBP3, respectively, have provided insight into the physiological roles of these PBPs (9). Exposure of *S. aureus* cells to cefotaxime resulted in extrusion of cytoplasm and cell lysis, while exposure to cephalixin resulted in cell enlargement and the cessation of septation (9). Based on these data, it was hypothesized that PBP2 and PBP3 are essential for cell viability and function as the primary peptidoglycan transpeptidase and a septation-associated transpeptidase, respectively (9). PBP4, the only low-molecular-weight PBP produced by *S. aureus*, has been shown to be involved in secondary cross-linking. Wyke et al. (41) reported that mutants lacking PBP4 contained a hypo-cross-linked cell wall, providing evidence that this protein, although non-essential, plays an important role in the high level of cell wall cross-linking seen in *S. aureus*. In agreement with this are the studies of Kozarich and Strominger (19) demonstrating that purified PBP4 is unique among the low-molecular-weight PBPs in that it possesses transpeptidase and carboxypeptidase activities.

Methicillin-resistant *S. aureus* produces a fifth PBP, termed PBP2a, that is the primary resistance determinant (23). Several

studies have demonstrated that PBP2a has reduced binding affinity for  $\beta$ -lactams (11, 31, 40), leading to the hypothesis that PBP2a takes over the biosynthetic functions of the normal PBPs when the bacteria encounter  $\beta$ -lactam antibiotics. Although PBP2a is essential for high-level methicillin resistance, a role for PBP4 in borderline methicillin-resistant strains, which lack PBP2a, has been well documented (1, 4, 14, 39). These studies demonstrate that the overproduction or modification of PBP4 leads to increased resistance to methicillin.

Previously, the *S. aureus* 8325-4 genes encoding PBP4 (*pbpD*) and a putative ABC transporter (*abcA*) were identified and cloned (7). A mutation in *abcA* resulted in cells that were more resistant to the  $\beta$ -lactam antibiotics cefoxitin and methicillin (7). It was hypothesized that the *abcA* mutation caused increased expression of the divergently transcribed *pbpD* gene. In this report, the expression of *abcA* and *pbpD* is characterized by using a Northern (RNA) blot analysis and promoter fusion technology. This analysis demonstrates that the *abcA* mutation in KB400 causes increased *pbpD* and *abcA* promoter activity and that these promoters are subject to positive regulatory control. Furthermore, the increased expression of *pbpD* is accompanied by higher levels of peptidoglycan cross-linking in the KB400 cell wall.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *S. aureus* strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 $\alpha$  was grown in Luria-Bertani broth or agar containing 50  $\mu$ g of ampicillin per ml. *S. aureus* strains were grown in tryptic soy broth or tryptic soy agar containing 2  $\mu$ g of erythromycin per ml or 5  $\mu$ g of chloramphenicol per ml as indicated.

**DNA manipulations.** *E. coli* plasmid DNA was isolated and subsequent DNA manipulations were performed by using the methods outlined by Sambrook et al. (32). Restriction enzymes were purchased from Promega (Madison, Wis.), and other molecular reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

\* Corresponding author. Mailing address: Department of Microbiology, Molecular Biology and Biochemistry, College of Agriculture, University of Idaho, Moscow, ID 83844-3052. Phone: (208) 885-7164. Fax: (208) 885-6518. E-mail: kbayles@uidaho.edu.

† Present address: Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ 85721.

‡ Present address: Astra Research Center Boston, Inc., Cambridge, MA 02139.

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference
<i>S. aureus</i>		
8325-4	Wild-type strain cured of prophage	26
RN4220	NCTC 8325-4 r <sup>-</sup>	25
KB400	<i>abcA</i> ::pER64 integrant	7
Plasmids		
pER64	Ap <sup>r</sup> Ery <sup>r</sup> ; pER924 containing 195-bp internal <i>abcA</i> fragment	7
pLC4	Ap <sup>r</sup> Cm <sup>r</sup> ; promoterless <i>xyIE</i> gene	29
pTD420	pLC4 containing 415-bp promoter region of <i>pbpD</i>	This study
pTD430	pLC4 containing 467-bp promoter region of <i>abcA</i>	This study
pTD420Δ26	pTD420 with a 26-bp inverted repeat deletion	This study
pTD430Δ26	pTD430 with a 26-bp inverted repeat deletion	This study

**RNA isolation and Northern analysis.** *S. aureus* RNA (30 μg) was isolated as described by Hart et al. (10) and separated in a 1% agarose-formaldehyde gel. The gel was stained with ethidium bromide and washed four times in diethylpyrocarbonate-treated water to remove excess formaldehyde and ethidium bromide. RNA was transferred by capillary blotting (32) to nitrocellulose paper in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight, and the filter was dried at 80°C for 90 min. The filter was incubated overnight at 65°C in hybridization buffer [6× SSC, 2× Denhardt's reagent, 50 mM Na(PO<sub>4</sub>), 0.1% sodium dodecyl sulfate (SDS)] containing 10<sup>6</sup> cpm of *pbpD*-specific probe per ml labeled (32) with [α-<sup>32</sup>P]dATP (New England Biolabs). The probe consisted of a 1.3-kb PCR product encompassing the entire *pbpD* open reading frame (nucleotides [nt] 1 to 1302; accession number U29454). The blot was washed at 65°C for 20 min each in 2× SSC–0.1% SDS, 1× SSC–0.1% SDS, and 0.5× SSC–0.1% SDS and then exposed to Kodak XAR autoradiographic film overnight at –70°C.

**Primer extension analysis.** Primer extension analysis was performed with [<sup>32</sup>P]ATP end-labeled oligonucleotide primers that were specific for *pbpD* (nt 242 to 277 [Fig. 1]) and *abcA* (nt 387 to 422 [Fig. 1]) transcripts. Radioactively labeled primers (10<sup>6</sup> cpm) were mixed with 30 μg of RNA and ethanol precipi-

```

A A Q V P T V D S N T A Q A Y
TGCTGCTTGTACAGGGTTCAGTCACTGTTAGTAGCTTGTGCATA 45
P T M I S L T L C L I I I S
TGGTGTCAATAACTTAATGTTAAACATAAAATGATGATAATAGA 90
I L N K M PBP4
TATTAAATTTTTCATAAAGCGTTAATCTTCCCTTTTCCAATTCCT 135
AAATATTCCTTAAAGCAATGGTTATTCCTACTTACGGAAATCAT 180
TGCTAATTCACCTTACCCTTAATTAATTTGTTGAAAATAAAGTTTT 225
CTGCAGTTAATTTGAAAAATAATGCAAATATATACCGTGTGTAGC 270
TAAAGGTGTTATAATGTTTGTACGAAGAGCAAACTTACTCAAAG 315
CGATTAATTTTCATGTTTAAATAAAGACTTTGAGAAGTTATTA 360
CAAAAAATGCAATAGAAATATTCATATCATATAAATGTTATGAGCG 405
GTATTTGGGGCAACACTTTATTTGATTTTTAAAGTTTGTGGG 450
AGAAAGTATATGATAGAAATGCATGTATCTATCTAAATGAATTAA 495
CTATAAATTTCAAACAGAAGAGGTTAAAACATGAAACGAGAAAAT 540
P L F F L F K K L S W P V G L
CCATTGTTTTCTTATTTAAAAAATATCATGGCCAGTGGGTCTT 585

```

FIG. 1. Nucleotide sequence of the region separating *pbpD* and *abcA*, including portions of each open reading frame. The site of transcription initiation for each gene is indicated by a +1, and the translational start codon is shown in bold type. Putative promoter sequences are underlined, and a 10-bp inverted repeat is indicated by arrows. The bold sequence below the inverted repeat was deleted in plasmids pTD420Δ26 and pTD430Δ26.

itated. After centrifugation, the pellet was resuspended in 25 μl of buffer containing 40 mM Tris-HCl (pH 8.3) and 25 mM NaCl. Following a 5-min incubation at 70°C, the sample was incubated with 28 U of RNasin at 42°C for 1 h. Then, 21 μl of fresh hybridization buffer (100 mM Tris-HCl [pH 8.3], 100 mM KCl, 2 mM each deoxynucleoside triphosphate, 8 mM dithiothreitol, 16 mM MgCl<sub>2</sub>, 50 mM NaCl, 160 mg of bovine serum albumin per ml, 0.2 mg of actinomycin D per ml), 28 U of RNasin, and 20 U of avian myeloblastosis virus reverse transcriptase (Promega) were added. The reaction mixture was incubated for 45 min at 42°C and stopped by adding 1 μl of 0.5 M EDTA. RNase A (1 μg) was then added, and the reaction mixture was incubated for 30 min at 37°C. Following the addition of 0.15 ml of TEN (Tris-EDTA [pH 7.6] containing 0.1 M NaCl) and phenol-chloroform-isoamyl alcohol (25:24:1) extraction, the nucleic acid was ethanol precipitated by adding 0.5 ml of 100% ethanol. The pellet was washed with 75% ethanol, air dried, and resuspended in 4 μl of formamide loading buffer (80% formamide, 10 mM EDTA, 0.1 mg of bromophenol blue per ml, 0.1 mg of xylene per ml of cyanol). The mixture was boiled for 5 min and cooled on ice before loading the sample on an 8% polyacrylamide gel next to a sequencing ladder generated with the same primer. After electrophoresis, the polyacrylamide gel was dried at 80°C and exposed overnight to Kodak XAR film.

**Lysostaphin sensitivity assays.** Ten-milliliter samples of KB400 and 8325-4 were grown in tryptic soy broth to an optical density at 578 nm (OD<sub>578</sub>) of 0.5, and their relative sensitivities to lysostaphin were determined as described by Maidhof et al. (22). Briefly, 1-ml cell samples were boiled for 15 min to inactivate the bacterial murein hydrolases. After centrifugation, the cells were resuspended in 2 ml of 0.2 M Tris-HCl (pH 8.0). Lysostaphin (Applied Microbiology) was then added to a final concentration of 2.5 μg/ml, and the samples were incubated at 37°C. The decline in OD<sub>578</sub> was measured at 30-s intervals for 10 min.

**Peptidoglycan analysis.** *S. aureus* peptidoglycan was prepared, digested into muropeptides, and analyzed by reverse-phase high-performance liquid chromatography (HPLC) as described by de Jonge et al. (6).

**Construction of *pbpD* and *abcA* promoter fusions.** The *pbpD* and *abcA* promoter regions were ligated into the unique *EcoRI* and *HindIII* sites within the *xyIE* transcriptional fusion vector, pLC4 (Table 1). Each promoter was PCR amplified with oligodeoxyribonucleotide primers containing *EcoRI* and *HindIII* restriction sites which allowed for directional cloning in front of the *xyIE* reporter gene. This procedure resulted in the amplification of 415-bp (nt 103 to 517 [Fig. 1]) and 467-bp (nt 40 to 506 [Fig. 1]) fragments from the 8325-4 chromosome that were used to generate *pbpD* and *abcA* transcriptional fusions, respectively, in pLC4. The recombinant plasmids (pTD420 and pTD430) were then transformed into DH5α cells (17) and spread on Luria-Bertani agar containing 50 μg of ampicillin per ml. The integrity of the cloned DNA fragments was confirmed by double-stranded sequencing (33) of each insert with a Sequenase DNA sequencing kit (United States Biochemical). The recombinant plasmids were transformed by electroporation into *S. aureus* RN4220 (20) and then transferred by bacteriophage-mediated transduction into 8325-4 (35), selecting for resistance on tryptic soy agar containing 5 μg of chloramphenicol per ml. A 26-bp region (nt 301 to 326 [Fig. 1]) spanning an inverted repeat sequence located between *pbpD* and *abcA* was removed by a PCR-based method described by Chen and Przybyla (5), generating plasmids pTD420Δ26 and pTD430Δ26. As determined by nucleotide sequencing of these inserts, these plasmids were identical to pTD420 and pTD430, respectively, except for the 26-bp deletion.

**Catechol 2,3-dioxygenase assays.** *S. aureus* cells were grown to the transition between the exponential and stationary phases of growth (OD<sub>620</sub> = 5.0), and quantitative assays were performed basically as described by Scovill et al. (34). Assays contained 500 μl of extract, and the reactions were conducted for 15 min with OD<sub>375</sub> readings taken at 30-s intervals. One milliunit is equivalent to the formation, at 37°C, of 1.0 nmol of 2-hydroxymuconic semialdehyde per min. Specific activity is defined as milliunits per milligram of protein.

## RESULTS

**Analysis of *pbpD* expression.** Since *S. aureus* KB400 (*abcA*) exhibited increased resistance to several antibiotics known to interact with PBP4 (cefotaxime and methicillin), it was hypothesized that the *abcA* gene product (AbcA) is involved in the regulation of *pbpD* (7). In agreement with this hypothesis was the finding that KB400 also exhibited increased resistance to imipenem, another antibiotic known to interact with PBP4 (37), but not to cefotaxime, which does not interact with PBP4 (data not shown). Therefore, to examine the levels of *pbpD*-specific RNA production, 8325-4 and KB400 RNAs were isolated and subjected to Northern blot analysis. As shown in Fig. 2, significantly more *pbpD*-specific RNA was observed in KB400 cells isolated during the transition from the exponential to the stationary phase (lane 3) than in 8325-4 cells (lane 1), whose *pbpD*-specific RNA was detectable only after extended

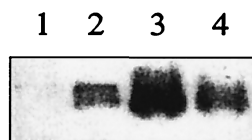


FIG. 2. Northern blot analysis. Equal amounts of 8325-4 (lanes 1 and 2) or KB400 (lanes 3 and 4) RNA were loaded onto an agarose-formaldehyde gel, transferred to nitrocellulose paper, and hybridized to a *pbpD*-specific probe. RNAs in lanes 1 and 3 were isolated from cells growing in the transition from the exponential to the stationary phase of growth ( $OD_{620} = 5.5$ ), while lanes 2 and 4 contain RNA isolated from the early stationary phase cells ( $OD_{620} = 6.0$ ).

exposure of the autoradiogram (data not shown). However, the effect of the *abcA* mutation on *pbpD* expression consistently diminished in stationary-phase cells (Fig. 2, lanes 2 and 4).

**Effect of *abcA* mutation on the cell wall.** Based on the high levels of *pbpD* expression seen in KB400, it was predicted that this strain would have an increased level of peptidoglycan cross-linking compared to that of 8325-4. As shown in Fig. 3, 8325-4 was more sensitive than KB400 to lysostaphin (an enzyme that cleaves the pentaglycine cross-bridge [30]), exhibiting a 75% greater reduction in culture turbidity in 5 min than KB400 when incubated in the presence of this enzyme. These results are consistent with the hypothesis that KB400 peptidoglycan has an increased level of cross-linking, although other structural changes that affect the sensitivity to lysostaphin could have a similar effect on the cell wall. For example, a mutation in *femA*, an auxiliary methicillin resistance gene which affects the glycine content of the peptidoglycan cross-linkers, also results in decreased lysostaphin sensitivity (22). Therefore, as a direct measure of cell wall cross-linking, peptidoglycan was isolated from 8325-4 and KB400 and analyzed by reverse-phase HPLC. The results of this analysis (Fig. 4) confirm that KB400 contains a higher level of peptidoglycan cross-linking than strain 8325-4 does and demonstrate that this effect is growth phase dependent. In cells isolated in the transition between the exponential and stationary phases of growth, 42% of the KB400 muropeptides (expressed as a percentage of total UV absorption) were found in a highly cross-linked form

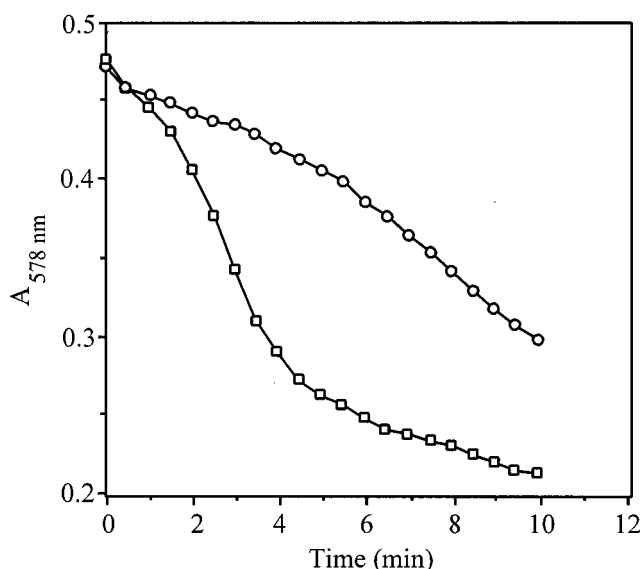


FIG. 3. Lysostaphin sensitivity assays. Heat-killed 8325-4 (squares) and KB400 (circles) were incubated at 37°C in the presence of lysostaphin (2.5 mg/ml), and the decline in OD was measured at 15-s intervals.

(containing multimers larger than octamers), compared to 29% in strain 8325-4. For early exponential-phase cells, these values were 40 and 37%, respectively.

**Transcription initiation site determination.** As noted in Fig. 1, the *abcA* and *pbpD* genes are divergently transcribed and their open reading frames are separated by 421 bp. To determine the transcription start site for each gene, a primer extension analysis was performed with primers that were specific for the *pbpD* and *abcA* transcripts. Although initial attempts to map the transcription start sites failed when primers that hybridized within the *pbpD* and *abcA* coding sequences were used, the use of primers that hybridized upstream relative to the coding sequences was successful. Based on this analysis (Fig. 5), it was determined that the *abcA* transcript initiates at an adenine residue located 234 bp 5' to the *abcA* start codon while *pbpD* transcription initiates at an adenine that is located 268 bp upstream of the *pbpD* start codon (Fig. 1). The start site for *pbpD* in 8325-4 was found to be identical to that found in KB400 (data not shown). Interestingly, these data indicate that *pbpD* and *abcA* transcription overlaps by 80 nucleotides. Furthermore, examination of this overlap revealed a 10-bp imperfect inverted repeat sequence that is a potential regulatory site for both genes.

**Transcriptional fusion studies.** To investigate transcription directed by the *abcA* and *pbpD* promoters, the intervening region between these genes was ligated next to the *xylE* gene in the transcriptional fusion vector pLC4, such that *abcA* and *pbpD* transcriptional fusions were generated (Table 1). The *xylE* gene encodes catechol 2,3-dioxygenase, an enzyme which converts catechol to 2-hydroxymuconic semialdehyde, producing a yellow color which can be measured spectrophotometrically. The *pbpD* promoter in the plasmid pTD420 directs approximately sevenfold-higher levels of catechol 2,3-dioxygenase activity in strain KB400 than in strain 8325-4 (Table 2). These results are consistent with the increased levels of *pbpD*-specific RNA found in KB400 compared to those in 8325-4 and confirm the effect of the *abcA* mutation on *pbpD* transcription shown in Fig. 2. Furthermore, the *abcA* promoter in pTD430 directs approximately 100-fold-higher levels of catechol 2,3-dioxygenase activity in strain KB400 than in strain 8325-4 (Table 2).

**Inverted repeat deletions.** To investigate the role of the 10-bp inverted repeat sequence located downstream relative to the *pbpD* and *abcA* transcription start sites (Fig. 1), deletions were generated in pTD420 and pTD430 by using a PCR-based method (see Materials and Methods). The resulting plasmids, pTD420 $\Delta$ 26 and pTD430 $\Delta$ 26, were identical to their parental constructs except for the absence of the 26-bp region spanning the inverted repeat. Interestingly, substantial decreases in *pbpD* and *abcA* promoter activity in the absence of the inverted repeat in both 8325-4 and KB400 were observed. As shown in Table 2, catechol 2,3-dioxygenase specific activities resulting from the mutant *pbpD* and *abcA* promoter constructs were nearly undetectable in 8325-4 and KB400, indicating that this sequence may play an important role in the expression of *pbpD* and *abcA*.

## DISCUSSION

Low-molecular-weight PBPs are D-carboxypeptidases that function to remove the C-terminal D-alanine of the pentapeptide side chain in peptidoglycan (12). They are typically non-essential proteins that play secondary roles in peptidoglycan metabolism. For example, the low-molecular-weight PBPs of *E. coli* can all be mutated with virtually no effect on cell viability (12), indicating that they probably have minor functions

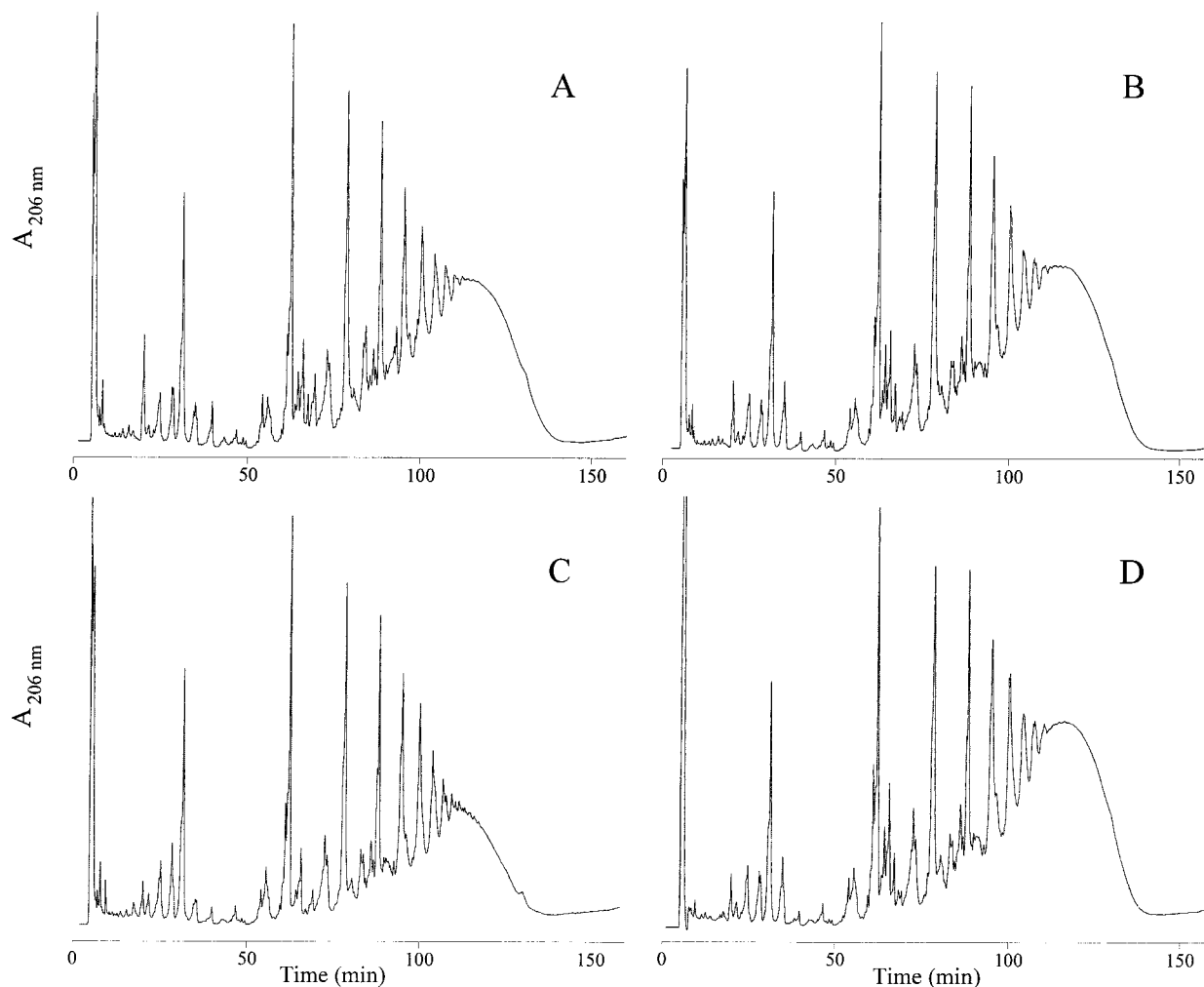


FIG. 4. *S. aureus* cell wall analysis. Peptidoglycan was obtained from *S. aureus* 8325-4 (A and C) and KB400 (B and D) cells that were growing in the early exponential phase of growth ( $OD_{620} = 0.4$ ) (A and B) and in the transition from the exponential to the stationary phase of growth ( $OD_{620} = 5.5$ ) (C and D). The peptidoglycan was digested with muramidase, and the resulting muropeptides were separated by reverse-phase HPLC.

in maintaining the normal physiology of the cell wall under standard laboratory conditions. In *Bacillus subtilis*, the sporulation-specific PBP, PBP5\*, is nonessential in vegetative cells but is required for full heat resistance in spores (3, 28). Cells containing a mutation in the PBP5\*-encoding gene, *dacB*, produce spores that have a 99.9% reduction in viability when incubated at 90°C for 10 min; this is in contrast to the parental strain, which exhibits only a 50% reduction in spore viability under these conditions (3). Analysis of the spore peptidoglycan from this strain revealed that it contained an 18% increase in cross-linking compared to that of the wild type (27). In *S. aureus*, PBP4 has been shown to catalyze both transpeptidase and carboxypeptidase reactions (19). Consistent with the presence of a transpeptidase activity are studies suggesting that PBP4 is required for the high level of cross-linking within *S. aureus* peptidoglycan (15, 41). Our results are in agreement with this since increased expression of *pbpD* (Fig. 2 and Table 2) corresponds with increased levels of peptidoglycan cross-linking (Fig. 4). Unfortunately, the biological consequence of this increased cross-linking, and for that matter the role of the high level of peptidoglycan cross-linking in *S. aureus*, remains unknown. Although nonessential in standard culture condi-

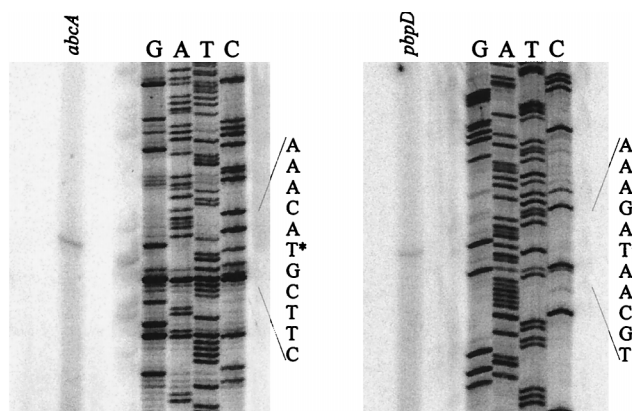


FIG. 5. Primer extension analysis of the *S. aureus* *pbpD* and *abcA* transcripts. *S. aureus* 8325-4 RNA was isolated and subjected to a primer extension analysis as described in Materials and Methods with primers that hybridized to *pbpD* or *abcA* transcripts. The sizes of the extended products were determined by comparison with DNA sequencing ladders generated with the corresponding *pbpD*- and *abcA*-specific primers.

TABLE 2. Catechol 2,3-dioxygenase assays

Plasmid	Catechol 2,3-dioxygenase activity <sup>a</sup>	
	8325-4	KB400
pTD420	0.46 ± 0.49	3.35 ± 2.10
pTD430	0.13 ± 0.10	16.59 ± 10.10
pTD420Δ26	0.04 ± 0.04	0.18 ± 0.23
pTD430Δ26	0.03 ± 0.04	0.18 ± 0.40

<sup>a</sup> Specific activity is defined as milliunits per milligram of protein. Values are means ± standard deviations.

tions, the biological function of *S. aureus* PBP4, like *B. subtilis* PBP5\*, may only be apparent when subjected to the appropriate environmental conditions.

Recent data has demonstrated that the expression of some low-molecular-weight PBPs are, in fact, under the control of complex regulatory signals. For example, the expression of *B. subtilis* PBP5\* is controlled in a mother-cell-specific fashion involving a  $\sigma^E$ -specific pathway (36). The data presented in this report suggests that the expression of *S. aureus* PBP4 is also subject to regulatory control. As shown in Fig. 2 and 5, strain 8325-4 produced very low levels of *pbpD*-specific RNA while that seen in the *abcA* mutant strain was increased sevenfold. One reason for the low level of expression observed in 8325-4 is that *pbpD* expression may be subject to positive regulatory control and that the appropriate activating signals have not yet been identified. In agreement with this is the observation that the *pbpD* promoter region identified contains a poor sigma 70 recognition sequence and potentially requires activating factors for maximal promoter activity (Fig. 1). Consistent with this is the finding of Henze and Berger-Bachi (14), who isolated an *S. aureus* mutant that overexpressed PBP4. A sequence analysis of the *pbpD* promoter region in this strain (14) revealed a 90-bp deletion (nt 381 to 470 [Fig. 1]) and an insertion of a

single nucleotide (between nt 482 and 483 [Fig. 1]). These mutations created a -10 region, with 5 of 6 bases matching the *E. coli* sigma 70 consensus -10 region, which is 17 bp downstream of a near-consensus (5 of 6 bases) -35 sequence. This promoter is presumably stronger than that of the wild type and would explain the increased level of PBP4 expression found in these cells. In the wild-type strain, however, additional activating factors may be required for maximal *pbpD* and *abcA* expression. The reduced expression of the promoter fusion constructs lacking the inverted repeat sequence located adjacent to the *pbpD* and *abcA* (Table 2) supports this hypothesis. However, whether this sequence serves as a binding site for transcriptional activation of these genes or whether it is involved in transcript stability awaits further investigation.

Recently, a model outlining the possible role of AbcA in the regulation of *pbpD* expression was described (7, 13). Briefly, it was proposed that AbcA imports free D-alanine that had been liberated from the stem peptide of peptidoglycan as a result of the transpeptidase activity of PBP4. It was speculated that this D-alanine could serve as a signal that affects *pbpD* transcription. However, recent transport assays performed in our laboratory demonstrated that 8325-4 and KB400 import D-[<sup>3</sup>H]alanine at equal rates (data not shown), indicating that AbcA is not required for the import of D-alanine. Therefore, how AbcA affects *abcA* and *pbpD* promoter activity or even whether this protein functions as an exporter or an importer remains to be elucidated.

Clues to AbcA function can be obtained by taking advantage of the wealth of information regarding the ABC transport superfamily of proteins. First, unlike ABC exporter genes, genes encoding ABC importers are typically cotranscribed along with genes encoding auxiliary transport factors (8). These auxiliary factors form a membrane complex that is required for substrate binding and subsequent transport into the cell (2). Our sequence analysis of the DNA downstream of

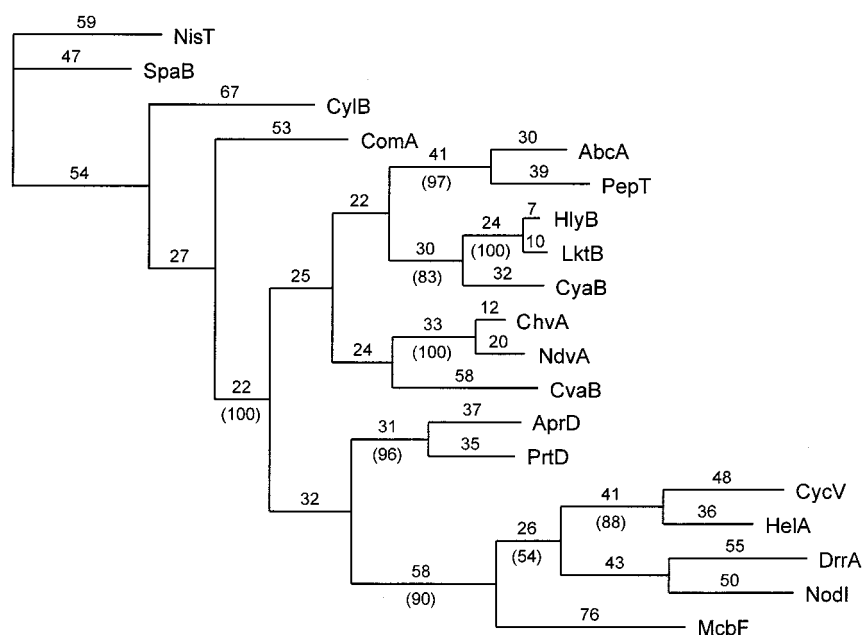


FIG. 6. Unrooted phylogenetic tree showing the evolutionary relatedness of the 190-amino-acid ABC domains found in 19 ABC-type transporters. The analysis was performed by use of the PAUP program (38), where horizontal branch lengths are proportional to evolutionary distances. The results of a bootstrap analysis (shown in parentheses) was performed with 200 replicates. The sequences used are described by Fath and Kolter (8), with the exception of those of AbcA (7) and PepT (24). The top 14 proteins in this tree are classified as group A ABC-type transporters, which are known to have only export functions, while the bottom five proteins are group B ABC-type transporters, which are believed to be involved in export and import (8).

*abcA* does not reveal any such auxiliary transport factor genes (data not shown). Second, ABC transporters can be divided into two groups, one (group A) whose members contain the membrane-spanning and ATP-binding domains on a single polypeptide, and another (group B) in which the membrane-spanning and ATP-binding domains are located on separate polypeptides (8). The transporters in group A appear to function only in export, while those in group B can facilitate import or export (8). As shown in Fig. 6, *AbcA* falls into the group A class of ABC exporters. Interestingly, *AbcA* contains 51% identical and 67% similar residues with the *Staphylococcus epidermidis* PepT protein, a putative ABC transporter that is involved in the biosynthesis of the Pep5 lantibiotic (24). In the absence of PepT, *S. epidermidis* produced approximately 10% of extracellular Pep5 compared to that produced in the presence of PepT (24), indicating that PepT was involved in Pep5 export. Because of the striking similarity of *AbcA* to PepT and other ABC exporters, we hypothesize that *AbcA* functions as an exporter and that it or its exported substrate is involved in the regulation of *bbpD* and *abcA*. A system such as this may be analogous to signaling systems found in *Bacillus subtilis*, *Streptococcus pneumoniae*, and *S. aureus*, where secreted peptides are involved in the initiation of sporulation, competent cell formation, and virulence, respectively (16, 18, 21). Small peptides that are exported by *AbcA* could serve a quorum sensing function to signal events within the cell population related to the physiology of the cell wall. Studies ongoing in our laboratory are aimed at defining these events and understanding the biological significance of this regulatory system.

#### ACKNOWLEDGMENTS

We thank Harold Schreier for his support and insightful observations and Philip Berger for his help with the phylogenetic analysis of *AbcA* and PepT.

This work was supported by USDA grant 9304112.

#### REFERENCES

- Berger-Bachi, B., B. Strassle, and F. H. Kayser. 1989. Natural methicillin resistance in comparison with that selected by in-vitro drug exposure in *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **23**:179-188.
- Boos, W., and J. M. Lucht. 1996. Periplasmic binding protein-dependent ABC transporters, p. 1175-1209. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli and Salmonella: cellular and molecular biology*, vol. 1. ASM Press, Washington, D.C.
- Buchanan, C. E., and A. Gustafson. 1992. Mutagenesis and mapping of the gene for a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. *J. Bacteriol.* **174**:5430-5435.
- Chambers, H. F., M. J. Sachdeva, and C. J. Hackbarth. 1994. Kinetics of penicillin binding to penicillin-binding proteins of *Staphylococcus aureus*. *Biochem. J.* **301**:139-144.
- Chen, B., and A. E. Przybyla. 1994. An efficient site-directed mutagenesis method based on PCR. *Biotechniques* **17**:657-659.
- de Jonge, B. L., Y. S. Chang, D. Gage, and A. Tomasz. 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. *J. Biol. Chem.* **267**:11248-11254.
- Domanski, T. L., and K. W. Bayles. 1995. Analysis of *Staphylococcus aureus* genes encoding penicillin-binding protein 4 and an ABC-type transporter. *Gene* **167**:111-113.
- Fath, M. J., and R. Kolter. 1993. ABC transporters: bacterial exporters. *Microbiol. Rev.* **57**:995-1017.
- Georgopadarakou, N. H., B. A. Dix, and Y. R. Mauriz. 1986. Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **29**:333-336.
- Hart, M. E., M. S. Smeltzer, and J. J. Iandolo. 1993. The extracellular protein regulator (*xpr*) affects exoprotein and *agr* mRNA levels in *Staphylococcus aureus*. *J. Bacteriol.* **175**:7875-7879.
- Hartman, B. J., and A. Tomasz. 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J. Bacteriol.* **158**:513-516.
- Heijenoort, J. V. 1996. Murein synthesis, p. 1025-1034. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli and Salmonella: cellular and molecular biology*, vol. 1. ASM Press, Washington, D.C.
- Henze, U. U., and B. Berger-Bachi. 1996. Penicillin-binding protein 4 overproduction increases beta-lactam resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **40**:2121-2125.
- Henze, U. U., and B. Berger-Bachi. 1995. *Staphylococcus aureus* penicillin-binding protein 4 and intrinsic beta-lactam resistance. *Antimicrob. Agents Chemother.* **39**:2415-2422.
- Henze, U. U., M. Roos, and B. Berger-Bachi. 1996. Effects of penicillin-binding protein 4 overproduction in *Staphylococcus aureus*. *Microb. Drug Resist.* **2**:193-199.
- Hoch, J. 1993. *spo0* genes, the phosphorelay, and the initiation of sporulation, p. 747-755. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. ASM Press, Washington, D.C.
- Inoue, H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**:23-28.
- Ji, G., R. C. Beavis, and R. P. Novick. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl. Acad. Sci. USA* **92**:12005-12059.
- Kozarich, J. W., and J. L. Strominger. 1978. A membrane enzyme from *Staphylococcus aureus* which catalyzes transpeptidase, carboxypeptidase and penicillinase activities. *J. Biol. Chem.* **253**:1272-1278.
- Kraemer, G. R., and J. J. Iandolo. 1990. High-frequency transformation of *Staphylococcus aureus* by electroporation. *Curr. Microbiol.* **21**:373-376.
- Magnuson, R., J. Solomon, and A. D. Grossman. 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. *Cell* **77**:207-216.
- Maidhof, H., B. Reinicke, P. Blumel, B. Berger-Bachi, and H. Labischinski. 1991. *femA*, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. *J. Bacteriol.* **173**:3507-3513.
- Matsushashi, M., M. D. Song, F. Ishino, M. Wachi, M. Doi, K. Inoue, K. Ubukata, N. Yamashita, and M. Konno. 1986. Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *S. aureus*. *J. Bacteriol.* **167**:975-980.
- Meyer, C., G. Bierbaum, C. Heidrich, M. Reis, J. Suling, M. Iglesias-Wind, C. Kemper, E. Molitor, and H. Sahl. 1995. Nucleotide sequence of the lantibiotic Pep5 biosynthetic gene cluster and functional analysis for a role of PepC in thioether formation. *Eur. J. Biochem.* **232**:478-489.
- Novick, R., J. Kornblum, B. Kreiswirth, S. Projan, and H. Ross. 1990. Regulation of post-exponential-phase exoprotein synthesis in *Staphylococcus aureus*, p. 3-18. In E. M. Ayoub, G. H. Cassell, W. C. Branche, Jr., and T. J. Henry (ed.), *Microbial determinants of virulence and host response*. American Society for Microbiology, Washington, D.C.
- Novick, R. P. 1990. The staphylococcus as a molecular genetic system, p. 1-37. In R. P. Novick (ed.), *Molecular biology of the staphylococci*. VCH Publishers, New York, N.Y.
- Popham, D. L., J. Helin, C. E. Costello, and P. Setlow. 1996. Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. *J. Bacteriol.* **178**:6451-6458.
- Popham, D. L., B. Illades-Aguilar, and P. Setlow. 1995. The *Bacillus subtilis* *dacB* gene, encoding penicillin-binding protein 5\*, is part of a three-gene operon required for proper spore cortex synthesis and spore core dehydration. *J. Bacteriol.* **177**:4721-4729.
- Ray, C., R. E. Hay, H. L. Carter, and C. P. Moran, Jr. 1985. Mutations that affect utilization of a promoter in stationary-phase *Bacillus subtilis*. *J. Bacteriol.* **163**:610-614.
- Receci, P. A., A. D. Gruss, and R. P. Novick. 1987. Cloning, sequence and expression of the lysostaphin gene from *Staphylococcus simulans*. *Proc. Natl. Acad. Sci. USA* **84**:1127-1131.
- Reynolds, P. E., and C. Fuller. 1986. Methicillin-resistant strains of *Staphylococcus aureus*: presence of identical additional penicillin-binding protein in all strains examined. *FEMS Microbiol. Lett.* **33**:251-254.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
- Scovill, W. H., H. J. Schreier, and K. W. Bayles. 1996. Identification and characterization of the *pckA* gene from *Staphylococcus aureus*. *J. Bacteriol.* **178**:3362-3364.
- Shafer, M. W., and J. J. Iandolo. 1979. Genetics of staphylococcus enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. *Infect. Immun.* **25**:902-911.
- Simpson, E. B., T. W. Hancock, and C. E. Buchanan. 1994. Transcriptional control of *dacB*, which encodes a major sporulation-specific penicillin-binding protein. *J. Bacteriol.* **176**:7767-7769.
- Sumita, Y., M. Fukasawa, and T. Okuda. 1990. Affinities of sm-7338 for penicillin-binding proteins and its release from these proteins in *Staphylo-*

- coccus aureus*. Antimicrob. Agents Chemother. **34**:484–486.
38. **Swofford, D. L.** 1993. PAUP: phylogenetic analysis using parsimony, 3.1 ed. Computer program distributed by the Illinois Natural History Survey, Champaign.
39. **Tomasz, A., H. B. Drugeon, H. M. de Lancastre, D. Jabes, L. McDougal, and J. Bille.** 1989. New mechanism for methicillin resistance in *Staphylococcus aureus*: clinical isolates that lack the PBP2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob. Agents Chemother. **33**:1869–1874.
40. **Ubukata, K., N. Yamashita, and M. Konno.** 1985. Occurrence of a beta-lactam-inducible penicillin-binding protein in methicillin-resistant staphylococci. Antimicrob. Agents Chemother. **27**:851–857.
41. **Wyke, A. W., J. B. Ward, M. V. Hayes, and N. A. C. Curtis.** 1981. A role *in vivo* for penicillin-binding protein 4 of *Staphylococcus aureus*. Eur. J. Biochem. **119**:389–393.