Role of PknB Kinase in Antibiotic Resistance and Virulence in Community-Acquired Methicillin-Resistant *Staphylococcus aureus* Strain USA300

Sanddeep Tamber, Joseph Schwartzman, and Ambrose L. Cheung*

Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, New Hampshire 03755

Received 24 March 2010/Returned for modification 4 May 2010/Accepted 30 May 2010

The regulation of cellular processes by eukaryote-like serine/threonine kinases is widespread in bacteria. In the last 2 years, several studies have examined the role of serine/threonine kinases in *Staphylococcus aureus* on cell wall metabolism, autolysis, and virulence, mostly in *S. aureus* laboratory isolates in the 8325-4 lineage. In this study, we showed that the *pknB* gene (also called *stk1*) of methicillin-resistant *S. aureus* (MRSA) strain COL and the community-acquired MRSA (CA-MRSA) strain USA300 is involved in cell wall metabolism, with the *pknB* mutant exhibiting enhanced sensitivity to β-lactam antibiotics but not to other classes of antibiotics, including aminoglycosides, ciprofloxacin, bacitracin, and other types of cell wall-active agents (e.g., vancomycin and bacitracin). Additionally, the *pknB* mutant of USA300 was found to be more resistant to Triton X-100-induced autolysis and also to lysis by lysostaphin. We also showed that *pknB* is a positive regulator of *sigB* activity, resulting in compromise in its response to heat and oxidative stresses. In association with reduced *sigB* activity, the expression levels of RNAII and RNAIII of *agr* and the downstream effector *hla* are upregulated while *spa* expression is downmodulated in the *pknB* mutant compared to the level in the parent. Consistent with an enhanced *agr* response in *vitro*, virulence studies of the *pknB* mutant of USA300 in a murine cutaneous model of infection showed that the mutant was more virulent than the parental strain. Collectively, our results have linked the *pknB* gene in CA-MRSA to antibiotic resistance, *sigB* activity, and virulence and have highlighted important differences in *pknB* phenotypes (virulence and *sigB* activity) between laboratory isolates and the prototypic CA-MRSA strain USA300.

The increasing morbidity and mortality of *Staphylococcus aureus* infections are raising concerns (15, 18). A major reason for this increase is the emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains that can affect previously healthy individuals outside the hospital setting. This contrasts with hospital-acquired MRSA (HA-MRSA) strains, which have been predominantly associated with immunocompromised hosts in hospitals.

CA-MRSA isolates can be distinguished from their HA-MRSA counterparts by their enhanced virulence and their spectrum of resistance to antibiotics. All MRSA strains harbor chromosomal cassette elements called staphylococcal cassette chromosome (*CSC*) elements, of which there are five types. CA-MRSA strains possess type IV and occasionally type V, while the larger types (I to III) are commonly found in HA-MRSA strains. Within all *SCCmec* elements is the invariant *mecA*, which encodes PBP2a and is the principal determinant of methicillin resistance in *S. aureus*.

Many CA-MRSA strains also carry additional virulence factors, such as Panton-Valentine leukocidin, and various staphylococcal enterotoxins. However, the role of these toxins in virulence is still debated. Recent reports have suggested that the differential expression levels of virulence factors in CA-MRSA strains may also contribute to the increased pathogenicity of these strains (19, 20).

In eukaryotic cells, many cellular processes are under the control of serine/threonine kinases. Recent studies have revealed the existence of eukaryotic serine/threonine kinase orthologs in bacteria (23). Bacterial homologues of these kinases have been shown to mediate a diverse range of functions, including cellular development, regulation, and pathogenesis (2, 23). The C termini of these kinases can bear three to five extracellular PASTA (for penicillin-binding protein [PBP] and serine/threonine kinase-associated) domains, which are penicillin-binding domains analogous to those found in penicillin-binding proteins while the N termini possess an intracellular kinase domain (23). Between these two domains is a single transmembrane segment. It is believed that the extracellular domain senses and transduces environmental signals by triggering the eukaryote-like N-terminal kinase domain to autophosphorylate and hence activate downstream signaling cascades. The *S. aureus* genome carries two putative kinase genes, one of which is *pknB* (locus SA1063 in *S. aureus* N315, also referred to as *stk1*). Using both laboratory and hospital *S. aureus* isolates, several groups have evaluated this gene (5, 9, 24). Despite some strain-to-strain variations in function of *pknB*, all three reports implicated that the kinase was involved in various aspects of cell wall metabolism, such as antibiotic resistance and autolysis; however, the precise mechanisms controlling these responses remain to be defined. Given the reported differences in antibiotic sensitivities between CA- and HA-MRSA strains, we sought to determine whether the *pknB* kinase gene has a major functional role in CA-MRSA.

---

* Corresponding author. Mailing address: Department of Microbiology, Dartmouth Medical School, Hanover, NH 03755. Phone: (603) 650-1340. Fax: (603) 650-1362. E-mail: ambrose.cheung@dartmouth.edu.

† Published ahead of print on 14 June 2010.
In this report, we demonstrate that pknB of CA-MRSA strain USA300 is also involved in cell wall metabolism including antibiotic resistance. In particular, the pknB mutant was more sensitive to β-lactam antibiotics and became more resistant to the endopeptidase lysostaphin and also to Triton X-100-induced autolysis. In searching for the mechanism by which pknB affected antibiotic resistance, we discovered that the pknB gene was positively involved in controlling the activity of the alternative sigma factor SigB. Consequently, the pknB mutant was compromised in its response to heat and oxidative stresses. As the toxin-expressing phenotype related to agr (e.g., alpha toxin) has been shown to be enhanced in a sigB mutant (7), we also found that the expression levels of regulatory genes (e.g., sarA and agr) and selected target genes (e.g., hla) were upregulated in the pknB mutant compared with those in the parent USA300. The enhanced virulence of the mutant was also confirmed in a murine cutaneous model of infection. Together, our data suggest that the pknB kinase gene has an interesting phenotype of enhancing antibiotic resistance and SigB activity and downregulation of virulence in the most common CA-MRSA strain, USA300.

**MATERIALS AND METHODS**

**Reagents, bacterial strains, and growth conditions.** All reagents were obtained from either Fisher or Sigma unless specified otherwise. The bacterial strains and plasmids used in this study are listed in Table 1. S. aureus strains were cultured on either tryptic soy broth/agar (TSB/A) or Mueller-Hinton broth/agar. *S. aureus* strains were cultured on Luria-Bertani medium (LB). Bacterial growth was monitored at 650 nm, using 18-mm borosilicate glass tubes on a Spectronic 20D+ spectrophotometer (Spectronic Analytical Instruments, Garforth, England). Antibiotic selection and plasmid maintenance were provided at the following concentrations: for *S. aureus*, 2.5 μg/ml erythromycin, 10 μg/ml chloramphenicol, and 75 μg/ml kanamycin, and for *E. coli*, 100 μg/ml ampicillin and carbenicillin and at 34 μg/ml chloramphenicol. Xylose was provided at a concentration of 0.2% (wt/vol) for gene induction from the heterologous promoter of pEPSA5 as needed.

**DNA manipulations.** A list of the oligonucleotides used for strain construction is available from the authors upon request. Routine cloning procedures were carried out according to standard laboratory protocols.

A markerless, in-frame deletion of pknB in strains USA300 and COL was constructed in accordance with a method described previously (23). Briefly, 1-kb regions flanking the gene of interest were amplified by PCR and joined by overlap extension. The resulting spliced product was inserted into either pMAD (COL) or pMAD-Kn (USA300) and transformed first into *E. coli* DH5α, then into RN4220 for proper methylation, and finally into either COL or USA300. Successive temperature shifts from 30°C to 42°C were used to promote the recombination of the pMAD constructs into the chromosome, resulting in the replacement of the native gene with the spliced allele. Correct clones were identified by colony PCR and confirmed by sequencing.

The pknB mutant strains were complemented by amplifying the native genes from either USA300 or COL genomic DNA and inserting them downstream of the xylose-inducible promoter of the *S. aureus* expression vector pEPSA5, followed by sequence confirmation.

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN4220</td>
<td>Heavily mutagenized strain that accepts foreign DNA</td>
<td>This work</td>
</tr>
<tr>
<td>USA300</td>
<td>Community-acquired MRSA strain</td>
<td>This work</td>
</tr>
<tr>
<td>COL</td>
<td>Hospital-acquired MRSA strain</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6630</td>
<td>USA300 pknB</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6634</td>
<td>USA300 pknB with pEPSA5</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6632</td>
<td>USA300 pknB complemented with pknB&lt;sub&gt;USA300&lt;/sub&gt; in pEPSA5</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6625</td>
<td>COL pknB</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6629</td>
<td>COL pknB with pEPSA5</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6627</td>
<td>COL pknB complemented with pknB&lt;sub&gt;COL&lt;/sub&gt; in pEPSA5</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6633</td>
<td>USA300 pknB complemented with pknB&lt;sub&gt;COL&lt;/sub&gt; in pEPSA5</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6628</td>
<td>COL pknB complemented with pknB&lt;sub&gt;USA300&lt;/sub&gt; in pEPSA5</td>
<td>This work</td>
</tr>
<tr>
<td>ALC6635</td>
<td>USA300 pknB with plasmid pALC2109</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XLI-Blue</td>
<td>General cloning strain</td>
<td></td>
</tr>
<tr>
<td>BL21-DE3</td>
<td>Strain used to express genes driven by the T7 promoter in the pET vector</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMAD</td>
<td><em>E. coli-S. aureus</em> shuttle vector containing a thermosensitive origin of replication, bgfR; Em' Ap'</td>
<td>1</td>
</tr>
<tr>
<td>pMAD-Kn</td>
<td>pMAD with Kn'</td>
<td>This work</td>
</tr>
<tr>
<td>pEPSA5</td>
<td><em>E. coli-S. aureus</em> shuttle vector containing a xylose inducible promoter; Ap' Cm'</td>
<td>13</td>
</tr>
<tr>
<td>pALC2109</td>
<td>pSK236 containing the tetR repressor and the xyl-tetO promoter driving sigB; Cm'</td>
<td>4</td>
</tr>
<tr>
<td>pET14b</td>
<td><em>E. coli</em> expression vector to create N-terminal hexahistidine tags, containing an IPTG inducible T7 promoter; Ap' Cm'</td>
<td></td>
</tr>
<tr>
<td>pALC6640</td>
<td>pET14b with pknB&lt;sub&gt;USA300&lt;/sub&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pALC6641</td>
<td>pET14b with pknB&lt;sub&gt;COL&lt;/sub&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>
expression was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Equivalent samples of the cultures were taken at the 0-, 2-, and 4-h time points, resuspended in 1× SDS-PAGE sample buffer (containing 62.5 mM Tris-HCl [pH 6.8], 2% SDS, 0.01 mg/ml bromophenol blue, 10% glycerol, 10 mM diithiothreitol [DTT]), boiled for 10 min, resolved on 10% polyacrylamide SDS-PAGE gels, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore), and analyzed according to standard Western blotting procedures. The primary antibody was mouse anti-His tag antibody (Novagen) used at 1:5,000 dilution, and the secondary antibody was donkey anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:10,000 dilution. The membrane was detected using the enhanced chemiluminescence (ECL) Western Blot system from GE Healthcare (Piscataway, NJ).

**Autolysis, bacteriolysis, and lysostaphin sensitivity assays.** Static autolysis assays were performed using heat-killed cells as substrates and bacterial supernatants as the source of secreted autolysins. Heat-killed cells were prepared in phosphate-buffered saline (PBS) by heating the bacterial suspension at 60°C for 2 hours. Supernatants were prepared by an OD650 of 5.0. Secreted autolysins in the culture supernatants were prepared from overnight cultures grown in TSB, normalized to cells at an OD650 of 10, and sterilized by passage through a 0.2-μm filter. Bacteriolytic activity was assessed by mixing 1 ml of heat-killed cells with 5 ml of supernatant (or TSB as a control) and incubating them at 37°C with shaking at 250 rpm, and the OD650 monitored. For some experiments, bacteria were cultured overnight in TSB plus 1 mM NaCl, harvested, washed twice with ice-cold, sterile water, resuspended in 50 mM Tris-HCl (pH 7.5) plus 0.05% Triton X-100, and incubated at 30°C with shaking at 250 rpm, and the optical densities were serially monitored.

Bacteriolytic assays were performed using heat-killed cells as substrates and bacterial supernatants as the source of secreted autolysins. Heat-killed cells were prepared in phosphate-buffered saline (PBS) by heating the bacterial suspension at 60°C for 2 hours. Supernatants were prepared by an OD650 of 5.0. Secreted autolysins in the culture supernatants were prepared from overnight cultures grown in TSB, normalized to cells at an OD650 of 10, and sterilized by passage through a 0.2-μm filter. Bacteriolytic activity was assessed by mixing 1 ml of heat-killed cells with 5 ml of supernatant (or TSB as a control) and incubating them at 37°C with shaking at 250 rpm, and the OD650 monitored. For some experiments, bacteria were cultured overnight in TSB plus 1 mM phenylmethylsulfonyl fluoride (PMSF) to inactivate serine proteases in the supernatant.

Lysostaphin sensitivity was assessed by adding lysostaphin (AMBI Products, LLC, Lawrence, NY) at a final concentration of 1.7 μg/ml (for USA300) or 2.5 μg/ml (for COL) to late-exponential-phase cells (OD650 = 1), followed by incubation at 37°C with shaking at 250 rpm. The optical densities of the cultures were then serially measured.

All data from the autolysis experiments described above are reported as percentages of the initial turbidity (at the zero time point) and are representative of three independent experiments.

**Zymography.** Autolysis activity was also visualized on 8% SDS-polyacrylamide gels containing either heat-killed RN4220 or lyophilized Micrococcus lysodeikticus as described previously (27). Autolysins were extracted from exponential-phase cells (10 ml at an OD650 of 0.7) with 100 μl of 4% SDS, quantitated using a Pierce bichinonic acid (BCA) protein assay kit, applied to the gels, and resolved electrophoretically. After electrophoresis, the gels were washed extensively with 2× SDS, transferred to a Hybond XL membrane as described previously (16) and stained with 0.1% methylene blue. Zones of cell lysis were visualized as clear bands against a blue background. Data shown are representative of three independent experiments.

**Transcriptional analyses.** RNA was extracted from approximately 1.2 × 10^11 cells grown to the indicated growth phases using Trizol and a reciprocating shaker as described previously (26). For quantitative real-time PCR (qRT-PCR) analysis, the RNA was treated with a DNA-free kit (Ambion) to remove residual DNA, followed by reverse transcription using a Roche Transcripter first-strand synthesis kit. qRT-PCR was performed with Maxima SYBR green qPCR master mix (Fermentas) in a Roche LightCycler 1.5 system. Absolute and relative quantification of the resulting amplicons was carried out using LightCycler software version 4.0. Data reported are normalized to the 16S rRNA levels.

For Northern blot analysis, RNAs were separated on a 1.5% agarose-0.66 M formaldehyde gel in 20 mM morpholinepropanesulfonic acid, 10 mM sodium acetate, 2 mM EDTA (pH 7) and transferred to a Hybond XL membrane as described previously (23). PCR-generated probes were labeled with [-32P]dCTP (GE Life Sciences) using a random primed DNA labeling kit (Roche). Following prehybridization, hybridization with the probe was conducted at 65°C overnight. The membranes were washed under high-stringency conditions and visualized by autoradiography. Bands were quantitated with ImageJ as needed. Images shown are representative of three independent experiments.

**RESULTS**

**Antibiotic susceptibility of a pknB mutant.** The pknB kinase gene is predicted to encode three extracellular penicillin-bind- ing domains, called the PASTA domain. We thus speculated that this protein might be involved in methicillin resistance in MRSA isolates. To test this hypothesis, a markerless in-frame deletion of pknB was made in both COL (HA-MRSA) and USA300 (CA-MRSA) and the antibiotic susceptibilities of each mutant were compared to those of the parent and complemented mutants (Tables 2 and 3). In both strain backgrounds, a pknB mutation led to increased susceptibilities to many β-lactams, including the carbapenem imipenem, with the USA300 pknB mutant being more sensitive to a wider range of β-lactams than the pknB mutant of COL. Provision of pknB in trans with the plasmid pEPSA5 to both mutant strains resulted in either a full or a partial restoration of the MIC to wild-type levels.

Both sets of mutants displayed equivalent susceptibilities to

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Wild type</th>
<th>pknB</th>
<th>pknB/pEPSA5</th>
<th>pknB/pknB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>32</td>
<td>1</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>1</td>
<td>0.125</td>
<td>0.25</td>
<td>2</td>
</tr>
<tr>
<td>Penicillin</td>
<td>16</td>
<td>0.25</td>
<td>0.25</td>
<td>16</td>
</tr>
<tr>
<td>Carbencill</td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>32</td>
<td>1</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Cefalothin</td>
<td>32</td>
<td>0.5</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td>Imipenem</td>
<td>22</td>
<td>37</td>
<td>40</td>
<td>27</td>
</tr>
</tbody>
</table>

All MICs, with the exception of those for imipenem, are presented in μg/ml. Imipenem sensitivity was measured as zones of clearing in mm.
representative drugs from other antibiotic classes, including aminoglycosides, ciprofloxacin, trimethoprim-sulfamethoxazole, and other cell wall-active agents, such as cycloserine, vancomycin, and bacitracin, compared with their respective parents, indicating that the antibiotic sensitivity of the \( \text{pknB} \) mutants was limited mostly to the \( \beta \)-lactam class of antibiotics.

**Role of the PASTA domain in antibiotic sensing.** Examination of the \( \text{pknB} \) genes of USA300 and COL revealed that both sequences were identical, with the exception of a G-to-T transversion at position 1165 in COL. This point mutation translates into a stop codon, prematurely truncating the kinase protein in COL (Fig. 1). This region of COL was subsequently resequenced to confirm the transversion.

For expression, the \( \text{pknB} \) genes from both COL and USA300 were cloned behind the N-terminal His tag of pET14b. After IPTG induction, the resulting fusion proteins were detected by Western blot analysis using an anti-His tag antibody (Fig. 1A). The USA300 \( \text{pknB} \) construct produced a 72-kDa His tag protein, in agreement with the predicted size of \( \text{pknB} \), while the His tag PknB protein of COL was only 48 kDa, corresponding to the truncated size of PknB without the PASTA domains. Therefore, it appeared that translation of the COL PknB protein terminated prematurely, as predicted from the G-to-T transversion.

To determine whether the PASTA domains of \( \text{pknB} \) made any contribution to antibiotic resistance, we performed a cross-complementation experiment. The truncated kinase gene from COL was provided in \textit{trans} to the USA300 \( \text{pknB} \) mutant and vice versa. Remarkably, both the truncated and full-length kinase genes were able to complement the \( \text{pknB} \) mutation in both strains to equivalent degrees with respect to sensitivity to \( \beta \)-lactams (Fig. 1B), suggesting that the kinase domain alone

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (( \mu \text{g/ml} )) for COL</th>
<th>Wild type</th>
<th>( \text{pknB} )</th>
<th>( \text{pknB/pEPSA} )</th>
<th>( \text{pknB/pknB}^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>256</td>
<td>64</td>
<td>64</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>512</td>
<td>2</td>
<td>2</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>128</td>
<td>32</td>
<td>32</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Nafcillin</td>
<td>256</td>
<td>64</td>
<td>64</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>1,024</td>
<td>512</td>
<td>512</td>
<td>1,024</td>
<td></td>
</tr>
<tr>
<td>Cephalothin</td>
<td>128</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 3. Effect of \( \text{pknB} \) on the MICs of \( \beta \)-lactam antibiotics against COL**

![FIG. 1. Structural differences between the PknB kinase proteins of USA300 and COL. (A) Western blot analysis of PknB from USA300 (top panel) and COL (bottom panel). The \( \text{pknB} \) gene from each strain was cloned behind an N-terminal His tag. Expression of the fusion proteins after IPTG induction at 0, 2, and 4 h was monitored by Western blot analysis using a primary antibody directed toward the hexahistidine moiety. The schematic on the right shows the probable architecture of each PknB kinase protein on the basis of the estimated sizes of each fusion protein. Each of the PASTA domains is depicted as rectangles, while the kinase domain is represented by an oval. (B) Role of the PASTA domain in antibiotic resistance. COL and USA300 \( \text{pknB} \) mutants were complemented and cross-complemented with either their own or the heterologous kinase gene. The designations \( \text{pknBu} \) and \( \text{pknBc} \) correspond to the \( \text{pkn} \) genes of USA300 and COL, respectively. The antibiotic susceptibilities of the resultant strains were then compared between the parent and mutant strains by performing MIC assays using nafcillin and cloxacillin.**
was sufficient to restore antibiotic resistance to the pknB mutants. In light of these data, we chose to pursue further work on pknB in USA300.

Cell wall phenotype of the pknB mutant. Mutants with altered expression of penicillin-binding proteins (PBPs) are frequently sensitive to a range of β-lactam antibiotics (21, 22, 24). Therefore, we speculated that the sensitivity of the pknB mutants to antibiotic may have been due to altered levels of penicillin-binding proteins. However, Northern blot and real-time PCR analyses of gene transcription revealed that the transcript levels of five genes encoding penicillin-binding proteins (mecA, pbp1, pbp2, pbp3, and pbp4) were equivalent in both the pknB mutant and the USA300 parent strain, suggesting that the antibiotic sensitivity of the pknB kinase mutant was not due to alterations in pbp transcription. Transmission electron microscopy also revealed that the cellular morphologies (i.e., size, cell wall thickness, and septum formation) of all three strains (parent, mutant, and complemented mutant) were virtually indistinguishable, supporting the notion that PBP activity was unaltered in the pknB mutant compared with that in the parent (data not shown).

We next examined the peptidoglycan cross-bridging of the pknB mutant by determining its sensitivity to the glycyl-glycine endopeptidase lysostaphin. Remarkably, the pknB mutant exhibited greater resistance to lysostaphin. After 24 min of lysostaphin exposure, only 60% of the pknB cells exhibited lysis whereas cells of the parent USA300 and complemented strains were virtually indistinguishable, supporting the notion that the antibiotic sensitivity of the pknB mutant may have been due to altered levels of penicillin-binding proteins (PBPs) are frequently sensitive to a range of β-lactam antibiotics (21, 22, 24).

Role for pknB in cell stress. Since the pknB mutant did not exhibit any gross morphological defects in the cell wall that may have been the underlying cause of its hypersusceptibility toward β-lactams, we next sought alternative explanations for the observed phenotype. Recent literature has demonstrated that bactericidal antibiotics, such as β-lactams, exert their killing effects through the production of reactive oxygen species (29). Thus, we investigated the potential involvement of the
kinase in the stress response by examining the activity of its primary mediator, the alternative sigma factor SigB, in the pknB mutant. Quantitative real-time PCR analysis of the SigB-dependent alkaline shock protein gene asp23 revealed that the pknB mutant had approximately half the amount of transcript that the parent and complemented mutant had (Fig. 5A). Correspondingly, the pknB mutant was compromised when it was grown in the presence of 10 mM hydrogen peroxide or 10% (vol/vol) ethanol, compared to both the parental strain USA300 and the complemented mutant (Table 4). The most dramatic growth defect, however, was observed when the strains were grown at a temperature of 42°C, with the pknB mutant severely compromised at the elevated temperature (Fig. 5B). Remarkably, provision of sigB in trans restored the growth of the mutant to the wild-type level, confirming that the growth defect was indeed due to reduced SigB activity in the pknB mutant (Fig. 5C).

The antibiotic susceptibility of the pknB mutant cross-complemented with sigB was then tested to ascertain whether the antibiotic sensitivity of the pknB mutant could be attributed to reduced activity of SigB. The susceptibilities of pknB and its cross-complemented mutant to β-lactams were identical, suggesting that reduced SigB activity was not involved in the β-lactam sensitivity of the mutant (data not shown).

Involvement of pknB in expression of virulence determinants. A number of reports have demonstrated an inverse relationship between SigB activity and virulence (4, 6, 7, 16, 25). Therefore, we sought to determine whether reduced SigB expression and augmented protease expression in the pknB mutant could be linked to a more general virulence phenotype. Northern blot analyses of stationary-phase cells showing the transcriptions of atl and spl (carrying a serine protease operon) in the pknB mutant in comparison with the levels in the parent and the complemented strain. (B) Protease activity in the supernatants of stationary-phase cells as assessed by an Enz-Chek Bodipy fluorescence kit. AU, arbitrary units. (C) Bacteriolytic activity of supernatants taken from stationary-phase cultures of different constructs grown in the presence of 1 mM PMSF using heat-killed USA300 as the substrate.
levels for the parent and the complemented mutant strain (Fig. 6A). Additionally, the transcription of genes regulated by agr, such as hla (α hemolysin gene) and spa (protein A gene) in the mutant was altered in a manner consistent with enhanced agr expression, displaying upregulation of hla and downregulation of spa in the mutant compared to the levels for the parent and the complemented strains. Notably, the pknB mutant demonstrated a 7-fold increase in hemolytic activity compared with the parental strain (Fig. 6B); these data suggest that the pknB mutant likely possesses a hypervirulent phenotype.

Enhanced virulence of the pknB mutant in a murine cutaneous model of infection. To evaluate whether the above-mentioned regulatory and phenotypic changes bore any clinical significance, we assessed the virulence of the pknB mutant in a murine model of cutaneous infection. Twenty-two C57B6 mice were injected subcutaneously with 100 μl of PBS containing 10^7 CFU (8 mice each with the wild-type and the complement strains and 6 mice with the mutant). The weights of the mice were also determined pre- and postinfection as an indicator of their general health (Fig. 7A). The greatest weight loss was observed on the first day postinfection, with the mice infected with the pknB mutant losing on average 1.75 g and the mice in the wild-type and complement groups losing on average 1.25 g and 1 g, respectively. One day after injection, all of the mice infected with the pknB mutant developed areas of redness surrounding the injection sites while inflammation was observed only for 4 of the mice in the wild-type group. By the second day after infection, all of the mice had developed erythematous cutaneous lesions. The cutaneous lesions of the mice infected with the pknB mutant were the most severe, reaching an average diameter of ~14.5 mm on the third and final day of the experiment. The average sizes of the cutaneous lesions in the mice infected with the parent and complemented strains were 7.6 mm and 4.5 mm, respectively (Fig. 7B). A greater number of CFU per gram of tissue was recovered from the wound sites of the mice in the pknB mutant group (4.5 × 10^7) than from those infected with either the USA300 parent strain (9.9 × 10^6) or the complemented mutant (1.2 × 10^6) (Fig. 7C). Histological sections of the wound site stained with H&E stain from mice infected with the pknB mutant revealed a more...
severe form of infection than the mice from the other two groups, as indicated by the greater numbers of bacteria in the mice infected with the \textit{pknB} mutant than in those from the other two groups. In addition, there was a complete loss of the outer dermal layer and a complete lack of anatomic definition and cellular structures in the epidermal layer of the mice infected with the \textit{pknB} mutant, compared with those seen for the parent (Fig. 7D).

**DISCUSSION**

Protein phosphorylation of serine and threonine residues on effector proteins is a common regulatory event in eukaryotes. Analyses of an increasing number of bacterial genomes have divulged eukaryote-like Ser/Thr protein kinase and phosphatase genes in prokaryotes (3, 23). In \textit{S. aureus}, the kinase gene \textit{pknB}, lying immediately downstream of the phosphatase gene \textit{stp1}, is transcribed together with the phosphatase gene as an operon (10). Similar to other serine/threonine kinases in Gram-positive bacteria, the PknB protein of \textit{S. aureus} has three domains, including an amino-terminal kinase domain, a transmembrane segment, and three PASTA domains at the C terminus. However, in analyzing the \textit{pknB} genes of strains USA300 and COL, we found that the PknB protein in strain USA300 is intact while its counterpart in COL is truncated due to a G-to-T transversion at position 1165, leading to a premature stop codon and hence lacking the PASTA domains (Fig. 1). Western blot analysis indeed confirmed that the His tag PknB protein of USA300 (at 72 kDa) is full-length His tag PknB protein of USA300 (at 72 kDa). As the PASTA domain has previously been linked to the binding of \( \beta \)-lactams (2, 30), we proceeded to determine the sensitivity of the \textit{pknB} mutant of USA300 to these antibiotics. Similar to what has been found with N315 (5), the \textit{pknB} mutants of strains USA300 and COL, representing CA- and HA-MRSA, respectively, were more sensitive to an assortment of \( \beta \)-lactam antibiotics, including a carbapenem (i.e., imipenem), than the respective parents. Interestingly, there were no differences in sensitivity to other cell wall-active agents, including vancomycin, cycloserine, and bacitracin, between the \textit{pknB} mutant and the corresponding parent, thus implying restricted sensitivity to common \( \beta \)-lactams and carbapenem and not to other cell wall-active agents.

Prior studies suggested that the PASTA domain may sense the presence of \( \beta \)-lactam antibiotics, leading to activation of the kinase domain (30). However, despite not having any of the PASTA domains, the \textit{pknB} gene of COL was able to restore the MICs of the \textit{pknB} mutant of USA300 for nafcillin and cloxacillin to parental levels in cross-complementation experiments (Fig. 1B). Given that PknB proteins can autophosphorylate (5), this finding implied that the sensing of \( \beta \)-lactam by the PASTA domain and the ensuing activation of the kinase domain may be more intricate than previously thought.

Despite the sensitivity of the \textit{pknB} mutant of USA300 to \( \beta \)-lactam, we did not observe any gross morphological cell wall changes in the mutant on transmission electron microscopy, nor did we discern any significant changes in cross-linking in the HPLC analysis of muropeptides in the mutant, compared
Besides resistance to lysostaphin-mediated lysis, the pentaglycine cross-bridge between peptidoglycan strands.

The effect of pknB on resistance to autolysis in strain USA300 may be 2-fold. First, the transcription of atl, the major autolysin gene, was decreased in the mutant compared to the level in the parent. This effect of pknB on atl was supported by recent microarray studies by Donat et al., demonstrating that atl and htrM expression is reduced in the pknB mutant of 8325 while transcription of htrR and lgrA, which are repressors of autolysis, is elevated (12). Second, the effect of autolysin may be decreased due to enhanced serine protease activity in the mutant (Fig. 4B). The latter observation was corroborated by the restoration of autolytic activity of the pknB mutant upon the addition of PMSF to the culture medium.

Perturbations in cell wall metabolism represent a unique form of cell stress. For this reason, we explored if pknB modulates the activity of SigB, a transcription factor intimately involved in the stress response of S. aureus. Analysis of the SigB-dependent alkaline shock protein gene asp23 revealed decreased sigB expression in the pknB mutant, but this activity was restored in the complemented mutant. Consistent with the defect in SigB activity were the slower growth at the elevated temperature (42°C) and the longer generation times of the mutant upon exposure to 10 mM hydrogen peroxide or 10% ethanol than of the parent. These findings are also in concordance with the observation by Truong-Bolduc et al. in which they proposed that additional factors related to sigB and pknB may contribute to the regulatory effect of virulence determinants (28).

On the basis of the above observations on USA300, we have proposed a model (Fig. 8) whereby the PknB protein regulates SigB activity and also stress resistance. Activation of SigB would downregulate agr and its effector genes, including hla and sflA-sflF, leading to reduced virulence of S. aureus. Additionally, the reduced effect of protease on Atl in a pknB+ strain may promote normal autolysis in a growing S. aureus cell. Besides agr, it also appears that PknB positively regulates sarA transcription. It has been known that SarA can downregulate its own promoter (8). Moreover, recent studies by Didier et al. have described the phosphorylation of SarA by PknB (11). Whether phosphorylated SarA binds differently to the sarA promoter compared to its unphosphorylated counterpart is not known. The above-mentioned scenarios, coupled with the finding that SarA is also a positive regulator on agr, implied that the effect of PknB on target genes such as hla and spa is complex and may involve a combination of factors, including SarA and agr. The relationship among PknB, SarA, and agr with respect to virulence, autolysis, stress response, and antibiotic resistance in community-acquired strains of S. aureus is currently under investigation in our laboratory.

**ACKNOWLEDGMENTS**

This work was supported in part by NIH grants AI37142 and AI47441 to A.L.C.

**REFERENCES**


 aureus eukaryote-like serine/threonine kinase and phosphatase. Infect. Im-
mun. 77:1406–1416.
6. Bischoff, M., P. Dunman, J. Kormaneck, D. Macapagal, E. Murphy, W. Moun-
hemolysin in a sigB mutant is associated with elevated SarA expression in
190:2239–2243.
antimicrobial susceptibility tests for bacteria that grow aerobically. Approved
standard. Clinical and Laboratory Standards Institute, Wayne, PA.
10. Débarbouillé, M., S. DramsL, O. Dussurget, M. A. Nahori, E. Vaganay, G.
serine/threonine kinase involved in virulence of Staphylococcus aureus. J.
virulence regulator SarA modulates its ability to bind DNA in Staphylo-
Lalk, and K. Ohlsen. 2009. Transcriptome and functional analysis of the
eukaryotic-type serine/threonine kinase PknB in Staphylococcus aureus. J.
King, M. McCarthy, C. Malone, B. Misiner, D. Robbins, Z. Tan, Z. Y. Zhu
2002. A genome-wide strategy for the identification of essential genes in
system involved in adhesion, autolysis, and extracellular proteolytic activity
Rubinstein, G. R. Corey, D. Spelman, S. F. Bradley, B. Barsic, P. A. Pappas,
K. J. Anstrom, D. Wray, C. Q. Fortes, I. Anguera, E. Athan, P. Jones, J. T.
v van der Meer, T. S. Elliott, D. P. Levine, and A. S. Bayer. 2005. Staphy-
lococcus aureus endocarditis: a consequence of medical progress. JAMA
293:1763–1771.
resistance: characterization of a functional rsbU strain derived from Staphy-
17. Kahl, B. C., G. Belling, P. Becker, I. Chatterjee, K. Wardecki, K. Hilgert,
Staphylococcus aureus small-colony variants are associated with extensive
alterations in regulator and virulence gene expression profiles. Infect. Im-
mun. 73:4119–4126.
2007. Invasive methicillin-resistant Staphylococcus aureus infections in the
H. F. Chambers, Y. Lu, and M. Otto. 2009. Evolution of virulence in epi-
cdemic community-associated methicillin-resistant Staphylococcus aureus.
Virulence expression in human community-acquired Staphylococcus aureus
Staphylococcus aureus PBP4 is essential for beta-lactam resistance in com-
munity-acquired methicillin-resistant strains. Antimicrob. Agents Chem-
other. 52:3955–3966.
bactericidal targets for penicillin in pneumococci: autolysis-dependent and
23. Ohslen, K., and S. Donat. 2010. The impact of serine/threonine phosphory-
ation of the essential peptidoglycan transpeptidase function of penicil-
lin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphy-
virulence factors and represses biofilm formation by modulating SarA and
2009. Role of mgrA and sarA in methicillin-resistant Staphylococcus aureus
autolysis and resistance to cell wall-active antibiotics. J. Infect. Dis. 199:209–
218.
modification influences the effects of MgrA on norA expression in Staphy-
29. Yeats, C., R. D. Finn, and A. Bateman. 2002. The PASTA domain: a beta-

Editor: A. Camilli

Downloaded from http://iai.asm.org on October 14, 2017 by guest