β-Lactams Interferring with PBP1 Induce Panton-Valentine Leukocidin Expression by Triggering sarA and rot Global Regulators of Staphylococcus aureus

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Previous articles reported that beta-lactam antibiotics increase the expression of Staphylococcus aureus Panton-Valentine leukocidin (PVL) by activating its transcription. We investigated the mechanisms underlying the inductor effect of beta-lactams on PVL expression by determining targets and regulatory pathways possibly implicated in this process. We measured PVL production in the presence of oxacillin (nonselective), imipenem (penicillin-binding protein 1 [PBP1] selective), cefotaxime (PBP2 selective), cefaclor (PBP3 selective), and cefoxitin (PBP4 selective). In vitro, we observed increased PVL production consistent with luk-PV mRNA levels that were 20 to 25 times higher for community-acquired methicillin-resistant S. aureus (CA-MRSA) cultures treated with PBP1-binding oxacillin and imipenem than for cultures treated with other beta-lactams or no antibiotic at all. This effect was also observed in vivo, with increased PVL mRNA levels in lung tissues from CA-MRSA-infected mice treated with imipenem but not cefoxitin. To confirm the involvement of PBP1 in this pathway, PBP1 depletion by use of an inducible pbp1 antisense RNA showed a dose-dependent relationship between the level of pbp1 antisense RNA and the luk-PV mRNA level. Upon imipenem treatment of exponential-phase cultures, we observed an increased sarA mRNA level after 30 min of incubation followed by a decreased rot mRNA level after 1 to 4 h of incubation. Unlike the agr and saeRS positive regulators, which were nonessential for PVL induction by beta-lactams, the sarA (positive) and rot (negative) PVL regulators were necessary for PVL induction by imipenem. Our results suggest that antibiotics binding to PBP1 increase PVL expression by modulating sarA and rot, which are essential mediators of the inductor effect of beta-lactams on PVL expression.

Staphylococcus aureus is an important human pathogen which expresses a variety of exoproteins, including the phage-encoded Panton-Valentine leukocidin (PVL) (34, 65). The prevalence of PVL in S. aureus isolates used to be low (ca. 2%) but is currently increasing due to the worldwide diffusion of PVL-producing methicillin-resistant S. aureus (MRSA) strains (61). There has been a controversy on the pathogenic role of PVL based on conflicting data from mouse infection models (9, 37, 64), mainly due to subtle variations in different parameters of the model and to the low susceptibility of mice to PVL (21, 62). However, recent studies with a rabbit pneumonia model, which is more susceptible to PVL, confirmed the important role of this toxin in pathogenesis (20). Although PVL-producing strains are isolated mainly from primary skin abscesses, regardless of severity (3, 18), a strong epidemiological link exists between PVL and severe infections, such as high-mortality-rate necrotizing pneumonia and recurrent complicated osteomyelitis (5, 22, 29, 30).

Although tightly linked to the phage genome and dependent on it for horizontal transfer, most of the phage-encoded virulence factors, including PVL, are integrated into the regulatory mechanism of the host (8, 67). S. aureus virulence gene expression is regulated by specific factors clustered into complex networks driving specific interactions with target gene promoters. These factors are largely regulated by two-component regulatory systems sensitive to environmental signals, such as the agr (47), autolysis-related locus arlRS (24), S. aureus exoprotein expression saeRS (31), and staphylococcal respiratory response srrAB (69) systems. DNA-binding proteins, such as SarA (13) and the recently identified SarA homologues SarR (40), Rot (43), SarS (14, 59), SarT (56), and SarU (41), also regulate virulence factor expression. The multiple pathways generated by these factors allow the bacterium to adapt rapidly and to promote growth under a large variety of environmental conditions, which may also include the presence of antibiotics. It was recently shown that subinhibitory concentrations of beta-lactams increase PVL production in vitro via transcriptional activation (23, 57), while agents that inhibit protein synthesis, such as clindamycin and linezolid, reduce S. aureus release of toxins, including PVL (4, 19, 23, 57). In spite of the lack of in vivo data, but given that beta-lactam antibiotics may lead to increased PVL production, some clinicians now recom-

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mend the avoidance of these agents possibly worsening the clinical outcome of community-acquired MRSA (CA-MRSA) infections (66). Nevertheless, little is known about the mechanisms underlying exoprotein modulation by antibiotics and how antibiotics may interfere with the regulatory network controlling virulence expression in *S. aureus*.

In this work, we studied the inductor effect of beta-lactams on PVL expression *in vitro* and *in vivo* and investigated the targets and regulatory pathways possibly implicated in PVL modulation by beta-lactam antibiotics.

**MATERIALS AND METHODS**

**Bacterial strains.** Bacterial strains, plasmids, and phages used in this study are summarized in Table 1.

**Antibiotics and MIC determination.** Antibiotics used in this study were oxacillin, cefaclor, and cefoxitin, purchased from Sigma-Aldrich (L’Isle d’Abeau, France); imipenem, purchased from MSD Laboratories (Paris, France); and cefotaxime, purchased from Dakota Pharm (Creteil, France). MICs were determined by broth microdilution assay as recommended by CLSI standards (15).

**Bacterial cultures.** Strains were cultured on Trypticase blood agar plates and incubated overnight at 37°C. Isolated colonies were resuspended in distilled water and adjusted to a 0.5 McFarland standard (corresponding to 10^8 CFU/ml), and pellets were treated with lysostaphin (Sigma) at a final concentration of 1 mg/ml. PVL in the supernatant was quantified by a specific enzyme-linked immunosorbent assay (ELISA; bioMérieux R&D Department, Marcy-l’Etoile, France) as described elsewhere (37). The expression levels of investigated genes were expressed as -fold differences relative to untreated controls (2).

**PVL quantification.** At different times, aliquots of cultures were adjusted to one OD_600_ of 1 and then centrifuged at 13,000 × g for 10 min. The total RNA of the pellets was then purified using a Qiagen RNeasy Plus minikit according to the manufacturer’s instructions. The amount of RNA yielded was assessed with a NanoDrop spectrophotometer, and 200 μg of total RNA was reverse transcribed using a Promega reverse transcription (RT) system with random primers as recommended by the provider. The result-
ing cDNA was used as a template for real-time amplification (LightCycler 2.0 Roche), using the specific primers shown in Table 2. The relative amounts of amplicons specific for each gene were determined by quantitative PCR (qPCR) relative to an internal standard (qRTB) as described elsewhere (37). The expression levels of investigated genes were expressed as _n_-fold differences relative to

**TABLE 1. Strains, plasmids, and phage used in this study**

<table>
<thead>
<tr>
<th>Strain, plasmid, or phage</th>
<th>Reference or source</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
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</tr>
<tr>
<td>8325-4</td>
<td>46</td>
<td>NCTC8325 cured of three prophages</td>
</tr>
<tr>
<td>RN4220</td>
<td>35</td>
<td>Restriction mutant of 8325-4</td>
</tr>
<tr>
<td>RN6390</td>
<td>49</td>
<td>Laboratory strain (derivative of 8325-4) that maintains its hemolytic activity on sheep erythrocyte agar</td>
</tr>
<tr>
<td>LUG855</td>
<td>37</td>
<td>RN6390 agr::tet (47) phiSLT</td>
</tr>
<tr>
<td>LUG856</td>
<td>This work</td>
<td>RN6390 rot::cat</td>
</tr>
<tr>
<td>LUG1160</td>
<td>This work</td>
<td>LUG1160 phiSLT</td>
</tr>
<tr>
<td>LUG1162</td>
<td>This work</td>
<td>LUG1162 carrying pLUG895</td>
</tr>
<tr>
<td>LUG1677</td>
<td>This work</td>
<td>RN6390 (12) phiSLT</td>
</tr>
<tr>
<td>LUG1053</td>
<td>This work</td>
<td>ALC488 (RN6390 sar::ermC) (12) phiSLT</td>
</tr>
<tr>
<td>LUG1063</td>
<td>This work</td>
<td>ALC488 complemented with one sarA copy (41), with phiSLT</td>
</tr>
<tr>
<td>LUG1436</td>
<td>This work</td>
<td>ISP479C (38) phiSLT</td>
</tr>
<tr>
<td>LUG1437</td>
<td>This work</td>
<td>ISP479C-29 sloc::kan (26) phiSLT</td>
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<tr>
<td>LUG1477</td>
<td>This work</td>
<td>LUG855 carrying pLUG792</td>
</tr>
<tr>
<td>LUG1485</td>
<td>This work</td>
<td>LUG855 carrying pLUG795</td>
</tr>
<tr>
<td>HT20010734</td>
<td>23</td>
<td>ST1; agr3 mecA^+ pvl^+</td>
</tr>
<tr>
<td>HT20020488</td>
<td>23</td>
<td>ST90; agr3 mecA^+ pvl^+</td>
</tr>
<tr>
<td>HT20030203</td>
<td>23</td>
<td>ST8; agr1 mecA^+ pvl^+</td>
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<td>HT20041010</td>
<td>23</td>
<td>ST80; agr3 mecA^+ pvl^+</td>
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<tr>
<td>LAC USA300</td>
<td>64</td>
<td>ST8; agr1 mecA^+ pvl^+</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pC194</td>
<td>33</td>
<td>High-copy-number plasmid harboring cat gene</td>
</tr>
<tr>
<td>pMad</td>
<td>1</td>
<td>Thermosensitive origin of replication, constitutively expressed galB gene</td>
</tr>
<tr>
<td>pLU6461</td>
<td>This work</td>
<td>Derivative of pC37 (11) carrying pbbA2567-2303 Cd-inducible antisense RNA and pvl terminator</td>
</tr>
<tr>
<td>pLUG792</td>
<td>This work</td>
<td>Derivative of pC37 (11) carrying pvl terminator</td>
</tr>
<tr>
<td>pLUG795</td>
<td>This work</td>
<td>Derivative of pTCVlac (53) lacking lacZ and ermB and carrying a 1.26-kb fragment carrying rot</td>
</tr>
<tr>
<td>pLUG895</td>
<td>This work</td>
<td>pvl-containing phage isolated from strain A980470</td>
</tr>
</tbody>
</table>

*S. aureus* rot (Sigma) was used at a final concentration of 5 μg/ml, and chloramphenicol (Sigma) and kanamycin (Sigma) were used at 20 μg/ml.

For expression studies of various regulators, cultures without antibiotics were performed as described above and monitored by optical density at 600 nm (OD_600_): when the OD_600_ reached 0.5, imipenem was added to the culture at a concentration corresponding to 1/2 the MIC, and cultures were reincubated at 37°C with gyratory shaking (300 rpm). Aliquots were taken after 30 min and hourly, and cellular pellets were prepared as described below for total RNA extraction.

**Phage**

<table>
<thead>
<tr>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>phiSLT</td>
</tr>
</tbody>
</table>

*PVL* quantification. At different times, aliquots of cultures were adjusted to an OD_600_ of 1 and then centrifuged at 13,000 × g for 10 min. PVL in the supernatant was quantified by a specific enzyme-linked immunosorbent assay (ELISA; bioMérieux R&D Department, Marcy-l’Etoile, France) as described elsewhere (2).
TABLE 2. Primers used for qPCR on LightCycler

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>gbr1F</td>
<td>GGGTGGGACATTCTGTGAG</td>
<td>This work</td>
</tr>
<tr>
<td>gbr1R</td>
<td>TTATCAACGTCGGTCTGC</td>
<td>This work</td>
</tr>
<tr>
<td>pbr1F</td>
<td>ACTGCTGGCTAAGTTCGCC</td>
<td>This work</td>
</tr>
<tr>
<td>pbr1R</td>
<td>TATCCTGGACTGTATTAGGCC</td>
<td>This work</td>
</tr>
<tr>
<td>pbr1phi</td>
<td>GATGATCATTCTGTTGAATGCC</td>
<td>This work</td>
</tr>
<tr>
<td>pbr2F</td>
<td>TATTTACGCTTTAATCACTCA</td>
<td>This work</td>
</tr>
<tr>
<td>pbr2R</td>
<td>TTGTTACGGCTTCAGGAGT</td>
<td>This work</td>
</tr>
<tr>
<td>pbr3F</td>
<td>GTTGGCACCACACTCTTTTA</td>
<td>This work</td>
</tr>
<tr>
<td>pbr3R</td>
<td>CCGGAGACCTTATTAATTCTCT</td>
<td>This work</td>
</tr>
<tr>
<td>pbr4F</td>
<td>TTGGTCTAATCAGTTTGAAT</td>
<td>This work</td>
</tr>
<tr>
<td>pbr4R</td>
<td>GTCAACGAACCTAGGGATCAA</td>
<td>This work</td>
</tr>
<tr>
<td>lsk-PV</td>
<td>AAATAGCTATGGCAGAAAATATGGATGT</td>
<td>This work</td>
</tr>
<tr>
<td>lsk-PV</td>
<td>CAAATGGCTTGTTGTTCTACATGCTCT</td>
<td>This work</td>
</tr>
<tr>
<td>ispV1L</td>
<td>CTTAATGCTTCTAATCTGAATTTGACC</td>
<td>This work</td>
</tr>
<tr>
<td>ispV2L</td>
<td>TTGTTAGAACTACGACCTGCTTATGTTGT</td>
<td>This work</td>
</tr>
<tr>
<td>RNA III F</td>
<td>GGGATGCGCTAATATCATAC</td>
<td>This work</td>
</tr>
<tr>
<td>RNA III R</td>
<td>GGAAAGGCTGTGTTTCAAGT</td>
<td>This work</td>
</tr>
<tr>
<td>sarA F</td>
<td>TGTTTTCCTCTGAGTTCGCT</td>
<td>This work</td>
</tr>
<tr>
<td>sarA R</td>
<td>CAGGGGAAAACAAAGGAAAG</td>
<td>This work</td>
</tr>
<tr>
<td>sarC F</td>
<td>CGTGAGATTAGACAAACAAGACA</td>
<td>This work</td>
</tr>
<tr>
<td>sarC R</td>
<td>TTCTGGAGCTGCTGCT</td>
<td>This work</td>
</tr>
<tr>
<td>rot F</td>
<td>GGATGTTCGGGATGTTTGTGA</td>
<td>This work</td>
</tr>
<tr>
<td>rot R</td>
<td>CATTGTGGTGGTCTTATGTC</td>
<td>This work</td>
</tr>
</tbody>
</table>

The primers were designed using Primer3 software (version 0.4.0) with default parameters. The primers were synthesized by Sigma-Genosys (Houston, TX).

The primer sequences are as follows:

- **gbr1F**: GGGTGGGACATTCTGTGAG
- **gbr1R**: TTATCAACGTCGGTCTGC
- **pbr1F**: ACTGCTGGCTAAGTTCGCC
- **pbr1R**: TATCCTGGACTGTATTAGGCC
- **pbr1phi**: GATGATCATTCTGTTGAATGCC
- **pbr2F**: TATTTACGCTTTAATCACTCA
- **pbr2R**: TTGTTACGGCTTCAGGAGT
- **pbr3F**: GTTGGCACCACACTCTTTTA
- **pbr3R**: CCGGAGACCTTATTAATTCTCT
- **pbr4F**: TTGGTCTAATCAGTTTGAAT
- **pbr4R**: GTCAACGAACCTAGGGATCAA
- **lsk-PV**: AAATAGCTATGGCAGAAAATATGGATGT
- **lsk-PV**: CAAATGGCTTGTTGTTCTACATGCTCT
- **ispV1L**: CTTAATGCTTCTAATCTGAATTTGACC
- **ispV2L**: TTGTTAGAACTACGACCTGCTTATGTTGT
- **RNA III F**: GGGATGCGCTAATATCATAC
- **RNA III R**: GGAAAGGCTGTGTTTCAAGT
- **sarA F**: TGTTTTCCTCTGAGTTCGCT
- **sarA R**: CAGGGGAAAACAAAGGAAAG
- **sarC F**: CGTGAGATTAGACAAACAAGACA
- **sarC R**: TTCTGGAGCTGCTGCT
- **rot F**: GGATGTTCGGGATGTTTGTGA
- **rot R**: CATTGTGGTGGTCTTATGTC

The primers were tested for specificity and efficiency using qPCR on LightCycler 480 II (Roche). The primers were designed to amplify specific regions of the genes of interest. The primers were tested in triplicate, and the results were analyzed using the LightCycler software (Roche) and the ΔΔCT method. The primers were found to be specific and efficient for qPCR analysis.
release, PVL in culture supernatants was quantified for 4 clinical CA-MRSA strains incubated with 1/4 the MIC of oxacillin, imipenem, cefotaxime, cefaclore, or cefoxitin for 6 h. As shown in Fig. 1, the effects were different from one beta-lactam to another. In the presence of oxacillin, all 4 strains displayed increased PVL production. The increases were strain dependent and ranged from 2.10- to 6.12-fold higher than the growth control level. We obtained similar results with imipenem, cefotaxime, cefaclore, or cefoxitin (17, 27, 28). Binding of beta-lactams to PBP1 results in subsequent PBP1 dysfunction, which led to increased PVL production for all 4 strains, ranging from 2.5 to 6.5 times the growth control level. The three other beta-lactams tested, cefotaxime, cefaclore, and cefoxitin, did not modify the PVL production of any of the 4 CA-MRSA strains.

**Effects of beta-lactams on PVL expression by LUG855.** We used the laboratory strain LUG855 to further examine the effects of subinhibitory concentrations of beta-lactams (1/4 the MIC) on PVL expression. We performed PVL-specific ELISA quantification with the supernatants of 6-h cultures and observed that oxacillin and imipenem increased PVL expression 2.25- and 3.4-fold compared to the growth control level, while cefotaxime, cefaclore, and cefoxitin did not increase the PVL level compared to the no-antibiotic control level (Fig. 1). PVL-specific relative qRT-PCR performed on the cellular pellets yielded similar results: cultures treated with oxacillin and imipenem displayed increased PVL mRNA levels (17.3- and 22.5-fold, respectively), while cefotaxime, cefaclore, and cefoxitin did not modify the PVL mRNA level. To rule out a possible excision of the phage in response to stress-induced antibiotics, the copy number of the pvl gene was analyzed by qPCR and showed no variation, regardless of the beta-lactam added (Fig. 2). This result was confirmed by the absence of detectable newly formed attP insertion sites of phiPVL phage as measured by specific qPCR (results not shown).

**Effects of beta-lactams on PBP1 expression by LUG855.** Penicillin-binding protein 1 (PBP1) is supposed to be the common target of oxacillin and imipenem (68) but not of cefotaxime, cefaclore, and cefoxitin (17, 27, 28). Binding of beta-lactams to PBP1 results in subsequent PBP1 dysfunction, leading to mechanically weak peptidoglycan, which bacteria may tend to compensate by enhanced phiPVL gene transcription.

We performed qRT-PCR with PBP1-specific primers (Table 2) on the cellular pellets and detected a significantly increased

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**TABLE 3. MICs of selected antibiotics for methicillin-susceptible S. aureus and CA-MRSA isolates in CCY broth**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxacillin (µg/ml)</th>
<th>Imipenem (µg/ml)</th>
<th>Cefotaxime (µg/ml)</th>
<th>Cefaclore (µg/ml)</th>
<th>Cefoxitin (µg/ml)</th>
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<tbody>
<tr>
<td>HT20010734</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td>HT20020488</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>HT20030203</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>HT20041010</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>LUG855</td>
<td>0.06</td>
<td>0.03</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

---

**FIG. 1. Effects of beta-lactams on PVL expression.** S. aureus strains HT20010734, HT20020488, HT20030203, HT20041010, HT20010734, and LUG855 were grown in CCY medium at 37°C with gyratory shaking (300 rpm) in the absence (growth control) and presence of different antibiotics at 1/4 the MIC. After 6 h of incubation, aliquots of the same cultures were used for either total RNA or genomic DNA extraction as described in Materials and Methods. The RNA yielded was used for subsequent reverse transcription with random primers, as described in the text, and the cDNA obtained was used as a template for LightCycler PCR with pvl-specific primers (Table 2). Relative quantification was performed by normalization to gyrB expression as described elsewhere (37). Results are expressed as n-fold differences of luc-pv-PCR (black bars) mRNA levels detected in the presence of different antibiotics relative to the PVL mRNA level of the strain grown without antibiotics. Genomic DNA was used as a template for real-time pvl and gyrB PCRs using specific primers (Table 2). Relative quantification of the pvl gene copy number was assessed after normalization to the gyrB copy number. Results are expressed as n-fold variations of the pvl/gyrB copy number (gray bars) in the presence of antibiotics relative to the pvl/gyrB gene copy number of the growth control. Values are means ± standard deviations (three different experiments).

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**FIG. 2. Effects of beta-lactams on PVL expression and pvl gene copy number.** S. aureus LUG855 cultures were performed as described in the legend to Fig. 1. After 6 h of incubation, aliquots of the same cultures were used for either total RNA or genomic DNA extraction as described in Materials and Methods. The RNA yielded was used for subsequent reverse transcription with random primers, as described in the text, and the cDNA obtained was used as a template for LightCycler PCR with pvl-specific primers (Table 2). Relative quantification was performed by normalization to gyrB expression as described elsewhere (37). Results are expressed as n-fold differences of luc-pv-PCR (black bars) mRNA levels detected in the presence of different antibiotics relative to the PVL mRNA level of the strain grown without antibiotics. Genomic DNA was used as a template for real-time pvl and gyrB PCRs using specific primers (Table 2). Relative quantification of the pvl gene copy number was assessed after normalization to the gyrB copy number. Results are expressed as n-fold variations of the pvl/gyrB copy number (gray bars) in the presence of antibiotics relative to the pvl/gyrB copy number of the growth control. Values are means ± standard deviations (three different experiments).
PBPl mRNA level (4.5-fold) when cultures were treated with imipenem and a slightly increased PBPl mRNA level (2.3-fold) when cultures were treated with oxacillin (Fig. 3). We also performed qRT-PCR with PBP2-, PBP3-, and PBP4-specific primers (Table 2) in order to study the effects on PBP2 to -4 expression upon exposure to imipenem and oxacillin (Fig. 3). As expected, imipenem (high-affinity ligand of PBPl [68]) modified only the PBPl mRNA level, while oxacillin (nonspecific PBPl ligand) increased PBP1 and PBP2 mRNA levels and, to a lesser extent, PBP3 mRNA levels. Therefore, we concluded that the common bacterial target of oxacillin and imipenem is PBPl. We did not detect increased PBPl mRNA levels in cultures treated with cefotaxime, cefaclor, or cefoxitin (results not shown).

Effect of antisense RNA depletion of pbpl on PVL expression. Since oxacillin and imipenem are the two beta-lactams increasing PVL expression and also interfering with PBPl, we hypothesized that PBPl dysfunction could somewhat lead to augmentation of PVL. We investigated this hypothesis by reducing pbpl expression by use of a cadmium-inducible cassette that expresses antisense pbpl RNA (Fig. 4A). Since PBPl was shown to be an essential enzyme involved in septum formation (51), we checked for cadmium-induced PBPl depletion by observing fluorescence microscopy images of LUG1477 and LUG1485 (negative control) stained with Van-FL specifically binding to the nascent division septum (Fig. 4B). Cadmium-treated LUG1485 cultures displayed complete septa at the normal equatorial position, resulting in tetrads and octets of normally sized daughter cells. Cd-treated LUG1477 cultures displayed abnormal septa with asymmetric polar localization, leading to tetrads and octets of cocci accumulating without respect to the normal alternating perpendicular planes.

Strain LUG1477 harboring pLUG792 and its control, LUG1485 harboring pLUG795 (derived from pLUG792 but lacking the pbpl antisense fragment), were cultured for 6 h in the presence of different cadmium concentrations (ranging from 0 to 5 μM). qRT-PCR was performed on the cellular pellets to detect PVL expression and antisense pbpl RNA expression (with primers pbplR and pbplphi [Table 2]). As shown in Fig. 5, we observed dose-dependent inductions of antisense pbpl RNA and luk-PV mRNA with CdCl2 at 0 to 5 μM. Thus, at 2.5 μM CdCl2, antisense pbpl RNA increased 102-fold, while PVL mRNA increased 5.4-fold. The biggest increases were observed with 5 μM CdCl2, with increases of 166-fold for antisense pbpl RNA and 18.5-fold for PVL-PV mRNA with CdCl2 at 0 to 5 μM. Oxacillin at 2.5 μM CdCl2 showed no induction of pbpl antisense RNA and PVL-PV mRNA.

Similar experiments were performed with the control strain
LUG1485, which showed no increase of luk-PV mRNA expression at any CdCl₂ concentration tested (results not shown).

Effects of major S. aureus regulators on PVL expression and on PVL induction by imipenem. PVL expression is known to be modulated by several global virulence regulators of S. aureus (7, 67). We therefore investigated the impacts of agr, saeRS, rot, and sarA deletions on PVL induction by imipenem to find out whether one of these regulators was a potential mediator of the inductive effect of beta-lactams on PVL expression. We used isogenic S. aureus strains belonging to the same genetic background (RN6390) and deleted for one of each of the major regulators investigated. Isogenic pairs (parental strain and mutant of interest) were cultured for 6 h with 1/4 the MIC of imipenem as described above, and PVL production was measured in the supernatants, while cellular pellets were assayed for luk-PV mRNA expression by qRT-PCR.

As shown in Fig. 6A and B and 7A and B, agr and sae are powerful positive regulators of PVL expression, as their deletion led to dramatically decreased PVL expression, with a 48-fold decrease of the luk-PV mRNA level in the Δagr strain and an 18.7-fold decrease of the luk-PV mRNA level in the ΔsaeRS strain (Fig. 7A and B). PVL quantification in the supernatant yielded a 10-fold decrease of PVL production in the Δagr strain and a 15-fold decrease of PVL production in the ΔsaeRS strain (Fig. 7A and B). Nevertheless, when they were cultured with imipenem, both the agr- and saeRS-deleted strains displayed increased PVL expression, although the range of increased expression was reduced in comparison with the range obtained with the parental strains.

FIG. 5. Effect of PBP1 depletion by antisense mechanism on PVL expression. Strain LUG1477 (containing pLUG792) was cultured at 37°C with vigorous shaking in CCY broth supplemented with increasing concentrations of CdCl₂ (0, 1, 2.5, and 5 μM). After 6 h of incubation, aliquots of cultures were washed, adjusted to an OD₆₀₀ of 1, and then used for total RNA extraction and subsequent reverse transcription with random primers as described in Materials and Methods. The cDNA obtained was used as a template for LightCycler PCRs with specific pvl primers and specific pbp1 antisense primers pbp1R and pbp1phi, which encompass the junction between the pbp1 antisense fragment and the pvl terminator. Relative quantification was performed by normalization to gyrB expression as described elsewhere (37). Results are expressed as n-fold differences of luk-PV mRNA (bars) or antisense pbp1 RNA (diamonds) levels detected in the presence of different CdCl₂ concentrations relative to the luk-PV mRNA or antisense pbp1 RNA expression level of the strain grown without CdCl₂. Values are means ± standard deviations (three different experiments).

FIG. 6. Effects of imipenem (IMI) on PVL production by isogenic strains deleted for agr (A), saeRS (B), rot (C), and sarA (D). Cultures were performed in CCY medium at 37°C with gyratory shaking (300 rpm) in the absence (growth control; white bars) and presence of imipenem at 1/4 the MIC (gray bars). After 6 h of incubation, aliquots of cultures were adjusted to an OD₆₀₀ of 1 and then centrifuged at 13,000 × g for 10 min. PVL in supernatants was quantified by ELISA, and results are expressed as μg of PVL/ml. Values are means ± standard deviations (four different doubled experiments). *, statistically different from the control (corresponding isolate grown without antibiotic), with a P value of <0.05, by one-way analysis of variance followed by a posteriori Dunnett’s test.
Rot deletion resulted in slightly increased PVL production (1.5-fold), along with an 8.4-fold increase in the \(\text{luk-PV} \) mRNA level. As shown in Fig. 6C and 7C, imipenem incubation of the \(\text{H9004}\) rot strain yielded neither increased PVL production nor an enhanced \(\text{luk-PV} \) mRNA level, suggesting that rot deletion interferes with the inducing effect of imipenem on PVL expression. The inductive effect of imipenem was restored by rot complementation with a low-copy-number plasmid. When it was cultured with imipenem, the rot-complemented strain showed a 2.2-fold increase of PVL production and an 8.8-fold enhancement of the \(\text{luk-PV} \) mRNA level.

In contrast to the case with rot, sarA deletion resulted in decreased PVL production (1.5-fold), along with a 8.4-fold decrease in the \(\text{luk-PV} \) mRNA level. As shown in Fig. 6D and 7D, imipenem incubation of the \(\Delta\text{saRA}\) strain yielded neither increased PVL production nor an enhanced \(\text{luk-PV} \) mRNA level, suggesting that the absence of SarA also interferes with the inductive effect of imipenem on PVL expression. To confirm this hypothesis, the inducing effect of imipenem was restored by chromosomal sarA complementation. When it was cultured with imipenem, the sarA-complemented strain showed a 2.1-fold increase of PVL production and a 4-fold enhanced \(\text{luk-PV} \) mRNA level.

**Modulator effect of imipenem on expression of major \(\text{S. aureus} \) regulators.** By using a laboratory strain and three clinical strains belonging to three major CA-MRSA lineages (ST1, ST8, and ST80), we investigated a possible modulation of virulence regulators of \(\text{S. aureus} \) by subinhibitory concentrations of imipenem. Since our results suggest that rot and sarA are global regulators involved in the induction of PVL expression by imipenem, we performed qRT-PCR to assay for direct modulation of rot and sarA expression in \(\text{S. aureus} \) cultured with imipenem. Accordingly, qRT-PCR was performed with specific primers for RNAIII (the major transcript of the \(\text{agr} \) operon), saeR, sarA, and rot (Table 2) on aliquots removed after 30 min and hourly from LUG855, HT20030203, HT20020488, and HT20010734, cultured with and without imipenem at 1/2 the MIC. The mRNA levels of the 4 regulators in the presence of antibiotic were compared to the mRNA levels expressed without antibiotics. As shown in Fig. 8, after 30 min of incubation with imipenem, we observed increased SarA mRNA levels for all strains tested, ranging from 4.6- to 8.9-fold compared to the original levels.

**FIG. 7.** Effects of imipenem on \(\text{luk-PV} \) mRNA expression by isogenic strains deleted for \(\text{agr} \) (A), saeRS (B), rot (C), and sarA (D). Cultures were performed in CCY medium at 37°C with gyratory shaking (300 rpm) in the absence (growth control; white bars) and presence of imipenem at 1/4 the MIC (gray bars). After 6 h of incubation, aliquots of cultures were adjusted to an OD of 1 and then used for total RNA extraction and subsequent reverse transcription with random primers as described in Materials and Methods. The cDNA obtained was used as a template for LightCycler PCRs with specific \(\text{pvl} \) and \(\text{gyrB} \) primers. Relative quantification was performed by normalization to \(\text{gyrB} \) expression as described elsewhere (37). Results are expressed as n-fold differences of \(\text{luk-PV} \) mRNA levels relative to the PVL transcript level from the wild-type parental strain (LUG855, LUG1053, or LUG1436) grown without imipenem. Values are means ± standard deviations (three different experiments). Changes in mRNA levels were interpreted as significant if they were >2-fold.
The cDNA obtained was used as a template for LightCycler PCRs with traction and subsequent reverse transcription with random primers. Cellular pellets were prepared as described before for total RNA extraction with gyratory shaking (300 rpm). Aliquots were taken every hour, and to the culture at 1/2 the MIC and cultures were reincubated at 37°C.

Changes in mRNA levels were interpreted as significant if they were 2-fold.

As assessed by qRT-PCR, the expression levels of RNAIII, saeR, and rot were not modified after 30 min of incubation with imipenem for any strain tested. Furthermore, after 1 h of incubation with imipenem, we observed increased saRA mRNA levels for LUG855 (4.5-fold) and HT20020488 (2.9-fold) and decreased rot mRNA levels for HT20030203 (5.9-fold) and HT20010734 (12-fold). Significant decreases in the rot mRNA level were detected for all strains, ranging from 7.7- to 25-fold and from 4.5- to 27-fold after 2 and 3 h, respectively, compared to the growth control level. After 4 h of incubation with imipenem, we detected decreased rot mRNA levels only for the three clinical strains: a 12-fold decrease in HT20030203, a 12.4-fold decrease in HT20020488, and a 2.7-fold decrease in HT20010734. After 5 h of incubation with imipenem, no variation in the expression of any of the 4 regulators investigated was detected, regardless of the strain. Nevertheless, starting with 4 h of incubation, we observed significant increases of the PVL mRNA level in the presence of imipenem for all strains tested, ranging from 2.4- to 6.6-fold at 4 h and from 6.5- to 11.5-fold at 5 h. The same experiment was performed with another beta-lactam as a control (cefoxitin at 1/2 the MIC). We observed no variation in the expression of any of the 4 regulators investigated, nor did we detect increased PVL mRNA expression after cefoxitin treatment (results not shown).

**Effects of beta-lactams on PVL mRNA expression in mouse lung infection.** To examine the influence of beta-lactams at subinhibitory concentrations on PVL expression in vivo, mice were challenged intranasally with CA-MRSA LAC (USA300). Six hours after infection, mice were treated twice daily with either imipenem, cefoxitin, or no antibiotic (10 to 12 mice per group) and then sacrificed at day 3. PVL expression in lung tissues was detected only in tissues where CFU counts of recovered bacteria were >5 log per gram of tissue, corresponding to 5 animals per group. For these samples, PVL-specific transcripts were normalized with respect to the gyrB transcription level, and pvl expression levels in mice treated with either imipenem or cefoxitin were expressed as n-fold differences relative to the pvl expression level in animals treated with PBS. Values are means ± standard deviations. Experiments were conducted in triplicate with lung samples from five different mice treated with imipenem, cefoxitin, or PBS. Changes in mRNA levels were interpreted as significant if they were >2-fold.
of pvl transcripts in mice treated with cefoxitin compared to untreated mice.

**DISCUSSION**

We have shown in several previous reports that oxacillin, a major antistaphylococcal beta-lactam, increases PVL expression by inducing the pvl promoter (23). Similar observations have been reported for other virulence determinants of *S. aureus*, such as alpha-toxin (48), staphylococcal toxic shock syndrome toxin 1 (TSST-1) (57), and the ability to bind to fibronectin (54), thus supporting the hypothesis that subinhibitory concentrations of beta-lactams enhance the overall virulence of *S. aureus* through a so far uncharacterized signaling pathway. Moreover, PVL is associated with intense necrosis in vivo, probably leading to poor antibiotic diffusion and subinhibitory concentrations at sites of infection (10). Based on these in vitro data, the current guidelines for management of PVL-producing *S. aureus* infections restrain the use of beta-lactams alone for treatment to avoid the potential inductor effect on PVL expression (32). Therefore, we addressed the question of the effect of subinhibitory beta-lactam concentrations on PVL expression in vivo. Furthermore, we investigated the mechanisms underlying PVL induction by beta-lactams in vitro and identified cross points between the beta-lactam-triggered bacterial response and the regulatory network controlling virulence expression in *S. aureus*.

By using a mouse model of CA-MRSA pneumonia, we provide for the first time in vivo data supporting the hypothesis that some beta-lactams, such as imipenem, may promote PVL mRNA expression in infected tissues. However, we were unable to measure larger amounts of PVL in the lungs of imipenem-treated mice, as our ELISA was not sensitive enough in this setting (results not shown). Nevertheless, the increased expression of PVL in our mouse model was not followed by increased morbidity as assessed by weight loss data (results not shown). Recently, it was emphasized that mouse polymorphonuclear leukocytes (PMNs) are relatively resistant to PVL, while human and rabbit PMNs are susceptible to its cytotoxic effects, which could explain the absence of increased morbidity in the mouse setting despite an increased expression of PVL (20). Therefore, similar experiments should be conducted in rabbits in order to address the question of whether larger amounts of PVL achieved during beta-lactam treatment would impact the outcome.

Beta-lactam antibiotics initiate bacterial cell death subsequent to their interaction with transmembrane PBPs. In wild-type *S. aureus*, four PBPs have been detected (58), of which PBPs 1 and 2 are essential. We measured PVL production of 4 CA-MRSA strains and one laboratory strain in the presence of subinhibitory concentrations of 5 carefully selected beta-lactams: oxacillin (a nonselective beta-lactam), imipenem (PBP1 selective) (68), cefotaxime (PBP2 selective) (27), cefacloflore (PBP3 selective) (28), and cefoxitin (PBP4 selective) (17). When cultures were treated with either oxacillin or imipenem (PBP1 selective), but not with cefotaxime, cefaclor, or cefoxitin, PVL production in the culture supernatant and the luk-PV mRNA level increased concomitantly, suggesting that PBP1 interference would be a trigger for PVL induction by beta-lactams. We tested this hypothesis by altering PBP1 function with an inducible PBP1-specific antisense RNA and observed a dose-dependent relationship between the *pbi1* antisense RNA level and the luk-PV mRNA level, thus confirming that PBP1 depletion may lead to enhanced PVL expression by *S. aureus*. Recent reports from Pereira et al. (50, 51) underlined the major role of PBP1 in the formation of the division septum and the separation of daughter cells at the end of cell division, probably in conjunction with the autolytic system. In *E. coli*, beta-lactam inactivation of PBP3, an orthologue of *S. aureus* PBP1 also involved in peptidoglycan synthesis during cell division, induced the SOS-promoting *recA* and *lexA* genes (44). Recently, it was shown that beta-lactams induce the SOS response in *S. aureus* and promote genetic mobile element replication and transfer in a *recA*- and *lexA*-dependent manner, potentially resulting in the dissemination of virulence factors harbored by temperate phages (39). Wirtz et al. obtained *recA*-dependent PVL-encoding phage induction by using mitomycin C (67). They observed increased luk-PV mRNA levels mirroring the increased phage copy number due to phage replication. One hypothesis explaining PBP1-mediated PVL induction in *S. aureus* would thus be related to PVL-encoding phage induction promoted by an SOS response. Nevertheless, quantitative PCR assays of *S. aureus* depleted of PBP1 by both antibiotics and antisense *pbi1* RNA did not indicate PVL phage replication (Fig. 2), thus arguing against the SOS-based hypothesis.

Furthermore, we investigated the possible implication of major virulence regulators of *S. aureus* in PVL modulation by PBP1-interfering beta-lactams such as imipenem. Positive regulation of PVL transcription by the *agr*, *saeRS*, and *sarA* loci has already been reported (8, 67). Another SarA homologue, Rot (repressor of toxin), downregulates *hla* and the gamma-hemolysin genes (*hlgB* and *hlgC*) (55) by interacting with the promoter regions of target genes during the exponential growth phase. During post-exponential-phase growth, the level of *rot* mRNA is repressed by *agr* RNAIII via an antisense mechanism (6).

By using isogenic *S. aureus* strains deleted for each one of the *agr*, *saeRS*, *rot*, and *sarA* loci, we investigated the impact of the absence of these regulators on PVL induction by subinhibitory concentrations of imipenem. To determine whether one of these regulators was essential and therefore would be a potential mediator of the induction effect of beta-lactams on PVL expression, we confirmed several previous reports showing that *agr* and *saeRS* are powerful positive regulators of PVL expression (8, 67). However, *agr* and *sae* were not considered essential for PVL induction by beta-lactams, as PVL induction by imipenem still occurred in their absence. This observation seems contradictory to a recent report from Kuroda et al. showing that subinhibitory concentrations of beta-lactams induce *hla* expression in *S. aureus* through the *saeRS* system (36). This difference in observation could be attributed to strain variations. Nevertheless, since in that work the inductor used was not imipenem but cefoxitin, which is a PBP4-selective beta-lactam (17), we may hypothesize that a different signaling pathway was triggered to explain the different modulator profile.

In contrast, SarA and its homologue Rot are probably essential for PVL induction by imipenem (Fig. 5 and 6). PVL expression in *sarA*- and *rot*-inactivated strains was not modified by imipenem, while the inducing effect of imipenem on PVL
expression was restored in the complemented strains. The positive regulatory effect of SarA on PVL expression has already been reported (8); here we report for the first time the negative regulator role of rot in PVL expression, consistent with several previous observations of rot as a downregulator of exoprotein expression (55).

Kinetic measures of the regulator transcription level during CA-MRSA incubation with subinhibitory concentrations of imipenem suggest that sarA expression is triggered first, by a so far unknown signal, and then rot is downregulated, with both modifications resulting in enhanced PVL expression. Our results do not clarify whether downregulation of rot occurs independently or is a subsequent effect of SarA overexpression. Although the mechanism is still not clearly established, Manna and Ray (42) reported experiments indicating that SarA is involved in negative regulation of rot transcription, suggesting that rot downregulation during imipenem treatment may be a consequence of enhanced SarA expression. Utada et al. reported neither sarA nor rot modulation in their genomewide transcriptional profile of S. aureus 8325-4 upon oxacillin treatment (63). However, they performed their assay after 1 h of culture in the presence of 5 to 10 times the MIC of oxacillin, while we performed our assay after 30 min to 5 h of culture with 1/2 the MIC of imipenem. Therefore, these different observations may not be held as contradictory.

We were surprised to observe a 3-h delay between the activation of sarA and luk-PV induction. Stevens et al. reported a delayed inductor effect of subinhibitory concentrations of nafcillin on PVL expression (57). They detected increased PVL mRNA levels starting 4 h and up to 34 h after nafcillin treatment. Altogether, these data suggest that the beta-lactam inductor effect on PVL and the mechanisms involved may be dependent on the growth phase and promoted during the postexponential phase. This hypothesis is also consistent with the fact that SarA is involved in negative regulation of rot transcription mainly during the postexponential phase of growth (42).

Taken together, these data suggest that at subinhibitory concentrations, beta-lactams which bind to and inactivate PBPI, such as imipenem and oxacillin, trigger SarA by a so far uncharacterized pathway and increase SarA expression, which subsequently downregulates Rot expression, thus leading to enhanced PVL expression. Although the link between PBPI inactivation and sarA triggering has never been put forward, it may be related to the essential role performed by PBPI during septum formation and cell division. PBPI inactivation results in a dramatic inhibition of autolysis and a rapid decline in transcription of most autolytic enzymes, especially at the atl locus (50), thus preventing bacterial death. SarA was shown to be a negative regulator of atl expression and necessary for bacterial survival during penicillin-induced killing (25); therefore, it may be involved in autolysis repression by beta-lactams. In this respect, SarA modulation could be the consequence of an adaptation mechanism allowing bacterial persistence in the presence of imipenem. Another argument supporting the role of autolytic signals in the beta-lactam-induced regulatory pathway is that proteomic analysis of S. aureus undergoing increased autolysis triggered by Triton X-100 also resulted in increased SarA levels, coupled with a significant reduction in the Rot level (16).

We propose—as a working hypothesis—that the alterations in the composition of peptidoglycan caused by PBPI dysfunction generate a signal processed by a transduction pathway that controls the expression of autolytic genes, resulting in the inhibition of the activity of the autolytic system and promoting bacterial survival. The existence of complex regulatory circuits coupling the autolytic system to cell wall synthesis, possibly involving the SarA regulator, remains to be explored. This should provide a link between cell wall turnover control and virulence regulation in S. aureus, suggesting that enhanced virulence may be promoted during beta-lactam-induced bacterial persistence. Therefore, our results could explain the trend toward increasing resistance and virulence of PVL-producing S. aureus and may also promote appropriate treatment guidance to combat PVL-related infections.

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