ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2011, p. 3261–3271 Vol. 55, No. 7
0066-4804/11/$12.00 doi:10.1128/AAC.01401-10

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

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Received 12 October 2010/Returned for modification 16 December 2010/Accepted 5 April 2011

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), cefaclore (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 inhibition 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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▽ Published ahead of print on 18 April 2011.

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

Strain, plasmid, or phage	Reference or source	Description
Strains		
8325-4	46	NCTC8325 cured of three prophages
RN4220	35	Restriction mutant of 8325-4
RN6390	49	Laboratory strain (derivative of 8325-4) that maintains its hemolytic activity on sheep erythrocyte agar
LUG855	37	RN6390 phiSLT
LUG856	This work	RN6390 *agr*::*tetM* (47) phiSLT
LUG1160	This work	RN6390 *rot*::*cat*
LUG1162	This work	LUG1160 phiSLT
LUG1677	This work	LUG1162 carrying pLUG895
LUG1053	This work	RN6390 (12) phiSLT
LUG1063	This work	ALC488 (RN6390 *sarA*::*ermC*) (12) phiSLT
LUG1619	This work	ALC488 complemented with one *sarA* copy (41), with phiSLT
LUG1436	This work	ISP479C (38) phiSLT
LUG1437	This work	ISP479C-29 *saeRS*::*kan* (26) phiSLT
LUG1477	This work	LUG855 carrying pLUG792
LUG1485	This work	LUG855 carrying pLUG795
HT20010734	23	ST1; *agr3* $mecA^+$ pvl^+
HT20020488	23	ST80; *agr3* $mecA^+$ pvl^+
HT20030203	23	ST8; *agr1* $mecA^+$ pvl^+
HT20041010	23	ST80; *agr3* $mecA^+$ pvl^+
LAC USA300	64	ST8; *agr1* $mecA^+$ pvl^+
Plasmids		
pC194	33	High-copy-number plasmid harboring *cat* gene
pMad	1	Thermosensitive origin of replication, constitutively expressed *galB* gene
pLUG641	This work	pMad derivative for deletion/replacement of *S. aureus rot* gene
pLUG792	This work	Derivative of pCN37 (11) carrying pbpA2567-2303 Cd-inducible antisense RNA and *pvl* terminator
pLUG795	This work	Derivative of pCN37 (11) carrying *pvl* terminator
pLUG895	This work	Derivative of pTCvlac (53) lacking *lacZ* and *ermB* and carrying a 1.26-kb fragment carrying *rot*
Phage		
phiSLT	45	*pvl*-containing phage isolated from strain A980470

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, cefaclore, 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, as confirmed by bacterial counts), and 50 μl of this suspension was inoculated into 5 ml CCY broth contained in glass tubes. Cultures were performed at 37°C with gyratory shaking (300 rpm) in the absence (growth control) and presence of different antibiotics at 1/4 the MIC. When needed for selection, erythromycin (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.

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).

Relative qRT-PCR. Aliquots of 1 ml of each culture were centrifuged at 13,000 × *g*, and pellets were washed with 1 ml of 10 mM Tris buffer and adjusted to an OD_{600} of 1, corresponding to approximately 1×10^9 *S. aureus* cells/ml. One milliliter of adjusted and washed bacterial suspension was centrifuged at 13,000 × *g*, and pellets were treated with lysostaphin (Sigma) at a final concentration of 200 μg/ml. 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 1 μg of total RNA was reverse transcribed using a Promega reverse transcription (RT) system with random primers as recommended by the provider. The resulting 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 (*gyrB*) as described elsewhere (37). The expression levels of investigated genes were expressed as *n*-fold differences relative to

TABLE 2. Primers used for qPCR on LightCycler

Primer	Sequence (5′–3′)	Reference
gyrB F	GGTGGCGACTTTGATCTAGC	37
gyrB R	TTATACAACGGTGGCTGTGC	37
pbp1 F	AGGTAGCGGTTTTGTGTCC	This work
pbp1 R	TATCCTTGTCAGTTTTACTGTC	This work
pbp1phi	GATTATCTATCTGTTTAGGATCC	This work
pbp2 F	TATTTAGCCGGTTTACCTCA	This work
pbp2 R	TTTTGACGTTCTTCAGGAGT	This work
pbp3 F	GTGGACCAACCTCATCTTTA	This work
pbp3 R	CGGGAGACCCTTATTATTCT	This work
pbp4 F	TGGTGCTAACTGCTTTGTAA	This work
pbp4 R	GCTAAAGCTATCGGAATGAA	This work
lukS-PV F	AATAACGTATGGCAGAAATATGGATGT	This work
lukS-PV R	CAAATGCGTTGTGTATTCTAGATCCT	This work
ISPVL1	CTAATTGTTCTTTAACTTGAATTAAGTTTGACC	This work
ISPVL2	TGTTAAGAACATGAAGCCTTTGATTGT	This work
RNA III F	GGGATGGCTTAATAACTCATAC	This work
RNA III R	GGAAGGAGTGATTTCAATGG	This work
sarA F	TGTTTGCTTCAGTGATTCGT	This work
sarA R	CAGCGAAAACAAAGAGAAAG	This work
saeR F	CGTGGATGATGAACAAGACA	This work
saeR R	TTCACGGTATTAGCATCTTCG	This work
rot F	GGGATTGTTGGGATGTTTGTTA	This work
rot R	CATTGCTGTTGCTCTACTTGC	This work

the calibrator, calculated with RealQuant software (Roche Diagnostics). Changes in mRNA levels were interpreted as significant if the variation was >2-fold.

qPCR. Aliquots of 1 ml of strain LUG855 cultured with and without beta-lactams at 1/4 the MIC were centrifuged at 13,000 × *g*, and pellets were washed with 1 ml of 10 mM Tris buffer. Genomic DNA was isolated from the pellets by using a Qiagen QIAmp DNA minikit as recommended by the provider. A decimal dilution of the DNA yielded 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 in the presence of antibiotics relative to the *pvl*/*gyrB* copy number of the growth control.

Quantification of the *attP* insertion site of phiSLT phage was performed by quantitative real-time PCR with specific primers ISPVL1 and ISPVL2 (Table 2) as described by Wirtz et al. (67).

Pbp1 antisense sequence construction. We amplified a 265-bp fragment of the *pbp1* gene by using the primers pbpA2303 (AT**GGATCC**TTAACTATGCCTG ACATG), containing a BamHI restriction site (bold and underlined), and pbpA2567 (ACT**AGGCCT**TGTCAGTTTTACTGTC), containing a StuI restriction site. The fragment was digested with the BamHI and StuI restriction enzymes and ligated with the *luk*-PV transcriptional terminator (amplified using primers phi2648 and phi2815 [60]). The ligation product was then used as a template for PCR amplification using the pbpA2567 and phi2815 primers. After being verified by sequencing, the fragment was ligated into the pGEM-T Easy vector system (Promega). The resulting plasmid, pLUG789, was digested with EcoRI and StuI, and the digestion product was ligated with pCN37 (11) digested with EcoRI and SmaI. The ligation product, pLUG792 (see Fig. 4A), was then transformed and cloned into *Escherichia coli* DH5α before successive electroporation (Bio-Rad gene pulser) into *S. aureus* RN4220 and LUG855. The expression of the *pbp1* antisense sequence was assayed by RT-PCR with primers pbp1R and pbp1phi (Table 2), the latter of which was designed to encompass the 3′ end of the *pbp1* antisense sequence and the 5′ end of the *luk*-PV transcriptional terminator. The control plasmid pLUG795, identical to pLUG792 but lacking the *pbp1* 2567-2303 fragment, was obtained by successive BamHI digestion and ligation of pLUG792.

RN6390 *rot*::*cat* construction. The *rot*::*cat* deletion/replacement mutant of *S. aureus* RN6390 (LUG 1160) was obtained by using pMAD, a thermosensitive plasmid which contains a constitutively expressed β-galactosidase gene that allows for positive selection of double-crossover mutants by screening the β-galactosidase activity on an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) agar plate (1). A 1,036-bp DNA fragment corresponding to the chloramphenicol acetyltransferase gene was amplified from pC194 plasmid (33) and cloned into pMAD between two DNA fragments, corresponding to the 640-bp 5′-flanking region of the *rot* gene generated using primers rot824 (CAA **GTCGAC**TATATCGTGACTTAGTTGAAG [containing a SalI restriction site]) and rot1464 (CTT**ATCGAT**AAAACTACAAGTGTAAATAAACTTGC [containing a ClaI restriction site]) and the 581-bp 3′-flanking region of the *rot* gene generated using primers rot1951 (GTG**AAGCTT**TAATAGCATAAAAAGAGG TTTTC [containing a HindIII restriction site]) and rot2532 (ATT**GATATC**CG AACAAGTACCAGAA [containing an EcoRV restriction site]). The resulting plasmid, pLUG641, was successively electroporated into RN4220 and RN6390. Transformants were grown at the nonpermissive temperature (37°C) to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination. To favor the second recombination event, a single colony was grown at 30°C for 10 generations and plated at 37°C overnight. Cells which had lost the plasmid vector through a double-crossover event were detected on X-Gal agar plates. PCR amplifications were used to confirm the loss of the *rot* gene, which was replaced by the *cat* gene.

Complementation of the mutated strain was performed with the pLUG895 plasmid containing a 1.26-kb fragment carrying *rot* which was obtained after EcoRI digestion of the fragment generated by the amplification of the *rot* gene with the EcoRI restriction site-containing primers TT**GAATTC**GTATATCACATTTTATA CACATTTG and CGA**GAATTC**AAGCTATTAATTCATTGCTATC.

Visualization of the effect of *pbp1* antisense sequence on cell structure. The localization of new cell wall synthesis in strains LUG1477 and LUG1485 was done as previously described (52). Briefly, cells were grown for 18 h in brain heart infusion (BHI) with 5 μM $CdCl_2$ and 0.125 M D-serine to promote the incorporation of this amino acid into the cell wall. The culture was diluted 1/250 in the same medium, grown to an OD_{600} of 0.6, washed, and resuspended in the same volume of BHI with 5 μM $CdCl_2$ and without D-serine. The cells were then incubated for 15 min at room temperature to allow the incorporation of D-alanine into the cell wall and then labeled with a mixture of equal amounts of vancomycin (Sigma) and a Bodipy FL conjugate of vancomycin (Van-FL; Molecular Probes) at a final concentration of 1 μg/ml for 5 min at room temperature with agitation. Cells were then analyzed by fluorescence microscopy using a Zeiss Axiovert inverted microscope.

Mouse lung infection. For mouse lung infection, *S. aureus* strain LAC (USA300) was cultured aerobically on blood agar at 37°C overnight and then in 5 ml of CCY medium.

Animal experiments were carried out with 6-week-old female BALB/c mice (Harlan, Indianapolis, IN), in accordance with National Institutes of Health guidelines, and were approved by the Institutional Animal Care and Use Committee at the Texas A&M HSC Institute of Biosciences and Technology. Lung infections were induced with 5×10^7 *S. aureus* cells in a volume of 20 μl administered intranasally as previously described (37). Six hours after infection, mice were treated intramuscularly with either imipenem at 25 mg/kg of body weight, cefoxitin at 150 mg/kg, or phosphate-buffered saline (PBS). Injections were repeated every 12 h, and mice were sacrificed after 3 days. Lung samples were homogenized in Trizol (Sigma-Aldrich) and processed in a MagNA Lyser system (Roche), and then total RNA was extracted using a MagNA Pure LC RNA isolation kit (Roche) as recommended by the provider. Total RNA yield was measured by a NanoDrop spectrophotometer, and 1 μg of total RNA was reverse transcribed using a Promega reverse transcription system with specific *gyrB* and *pvl* reverse primers (Table 2). The resulting cDNA was used as a template for real-time amplification (LightCycler 2.0 Roche) with *gyrB* and *pvl* primers. Furthermore, *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 *pvl* expression levels in animals treated with PBS. RT-PCR experiments were conducted in triplicate on lung samples from five different mice treated with imipenem, cefoxitin, or PBS.

Statistical analysis. Statistical analyses were based on the use of one-way analysis of variance (ANOVA) followed by *a posteriori* Dunnett's test. The level of statistical significance was set at 0.05. The tests were carried out with SPSS for Windows, version 12.0, software.

RESULTS

MICs of beta-lactams. MICs of several beta-lactams, i.e., oxacillin, imipenem, cefotaxime, cefaclore, and cefoxitin, were determined following CLSI recommendations. Results obtained with CA-MRSA isolates and with LUG855 are summarized in Table 3. All derivatives of LUG855 shown in Table 1 displayed similar susceptibilities to imipenem, with a MIC of 0.03 μg/ml (not shown).

Effects of beta-lactams on PVL production by CA-MRSA strains. To examine the influence of beta-lactams on PVL

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

Strain	MIC (μg/ml)				
	Oxacillin	Imipenem	Cefotaxime	Cefaclore	Cefoxitin
HT20010734	16	8	4	16	256
HT20020488	8	8	2	16	128
HT20030203	16	8	8	64	256
HT20041010	16	2	2	64	128
LUG855	0.06	0.03	0.5	2	0.5

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, 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,

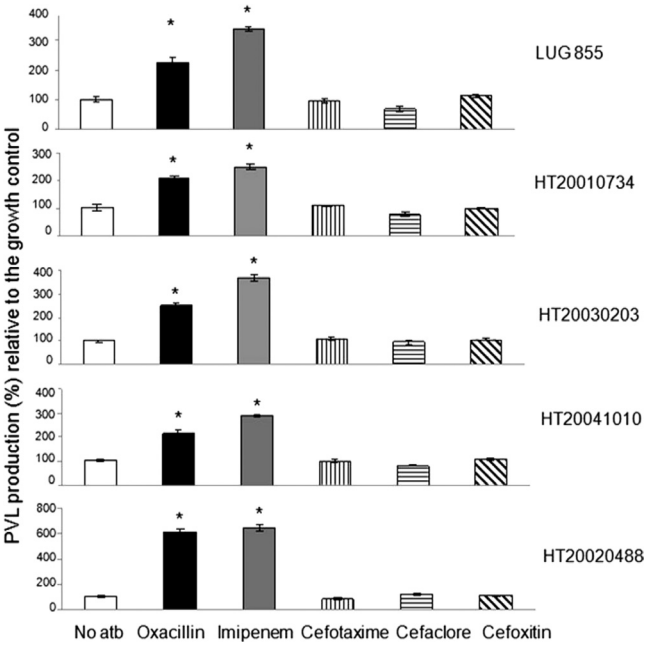

FIG. 1. Effects of beta-lactams on PVL expression. *S. aureus* strains HT20010734, HT20030203, HT20041010, HT20020488, and LUG855 were grown in CCY medium at 37°C with gyratory shaking (300 rpm) in the absence (growth control) and presence of different antibiotics at 1/4 the MIC. After 6 h of incubation, aliquots of cultures were adjusted to an OD_{600} of 1 and then centrifuged at 13,000 × *g* for 10 min. PVL in supernatants was quantified by ELISA. Results are ratios of μg of PVL/ml of adjusted culture incubated with the indicated antibiotic to means of μg of PVL/ml of adjusted culture incubated without antibiotic and are expressed as percentages. 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.

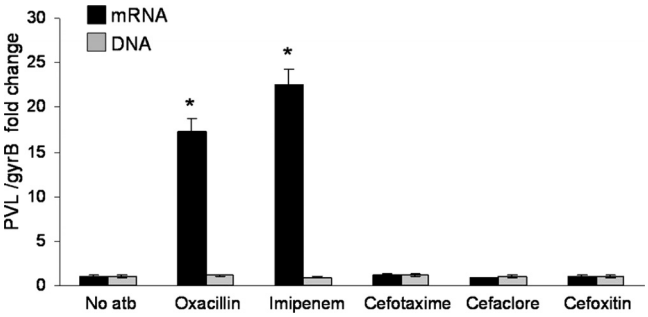

FIG. 2. Effects of beta-lactams on PVL expression and *pvl* gene copy number. *S. aureus* LUG855 cultures were performed as described in the legend to Fig. 1. After 6 h of incubation, aliquots of the same cultures were used for either total RNA or genomic DNA extraction as described in Materials and 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 *luk*-PV (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).

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 *pbp1* gene transcription.

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

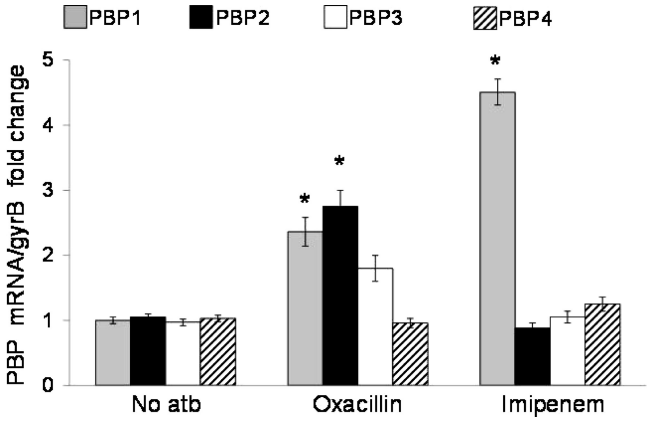

FIG. 3. Effects of oxacillin and imipenem on PBP1 to -4 expression. *S. aureus* LUG855 cultures with either imipenem or oxacillin were performed as described in the legend to Fig. 1. After 6 h of incubation, aliquots were used for total RNA extraction and subsequent reverse transcription with random primers, as described in the text, and the cDNA obtained was used as a template for LightCycler PCRs with *pbp1*- to -*4*-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 *pbp1* (gray bars), *pbp2* (black bars), *pbp3* (white bars), and *pbp4* (hatched bars) mRNA levels detected in the presence of different antibiotics relative to PBP1 to -4 mRNA expression of the strain grown without antibiotics. Values are means ± standard deviations (three different experiments). Changes in mRNA levels were interpreted as significant if they were >2-fold.

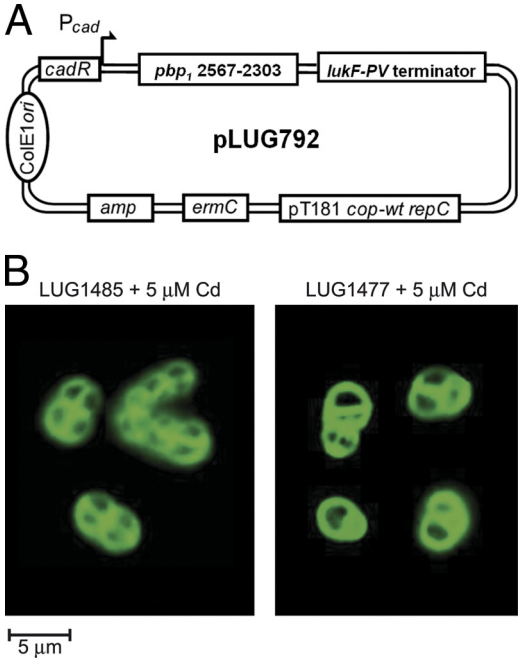

FIG. 4. (A) Map of pLUG792 plasmid. pLUG792 is a derivative of the *S. aureus* expression vector pCN37 which employs a cadmium-inducible promoter (P*cad*) with the cadmium regulator (*cadR*) to express a specific 265-nucleotide PBP1 antisense RNA cloned upstream of the *pvl* terminator. The plasmid contains a staphylococcal pT181 replicon and a colE1 replicon, as well as erythromycin resistance (*ermC*) and ampicillin resistance (*ampR*) genes. (B) Fluorescence microscopy images of LUG1485 and LUG1477 stained with Van-FL specifically binding to the nascent division septum. Cd-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.

PBP1 mRNA level (4.5-fold) when cultures were treated with imipenem and a slightly increased PBP1 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 PBP1 [68]) modified only the PBP1 mRNA level, while oxacillin (nonselective PBP 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 PBP1. We did not detect increased PBP1 mRNA levels in cultures treated with cefotaxime, cefaclore, or cefoxitin (results not shown).

Effect of antisense RNA depletion of *pbp1* on PVL expression. Since oxacillin and imipenem are the two beta-lactams increasing PVL expression and also interfering with PBP1, we hypothesized that PBP1 dysfunction could somewhat lead to augmentation of PVL. We investigated this hypothesis by reducing *pbp1* expression by use of a cadmium-inducible cassette that expresses antisense *pbp1* RNA (Fig. 4A). Since PBP1 was shown to be an essential enzyme involved in septum formation (51), we checked for cadmium-induced PBP1 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. Cadmium-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 *pbp1* 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 *pbp1* RNA expression (with primers pbp1R and pbp1phi [Table 2]). As shown in Fig. 5, we observed dose-dependent inductions of antisense *pbp1* RNA and *luk*-PV mRNA with $CdCl_2$ at 0 to 5 μM. Thus, at 2.5 μM $CdCl_2$, antisense *pbp1* RNA increased 120-fold, while PVL mRNA increased 5.4-fold. The biggest increases were observed with 5 μM $CdCl_2$, with increases of 166-fold for antisense *pbp1* RNA and 18.5-fold for PVL mRNA. Higher levels of $CdCl_2$ were inhibitory to growth. Bacterial counts of viable cells were performed for LUG1477 cultures by dilution and plating, which yielded 5×10^8 CFU/ml for LUG1477 cultured without Cd and 1.8×10^8 CFU/ml for LUG1477 cultured with 5 μM $CdCl_2$.

Similar experiments were performed with the control strain

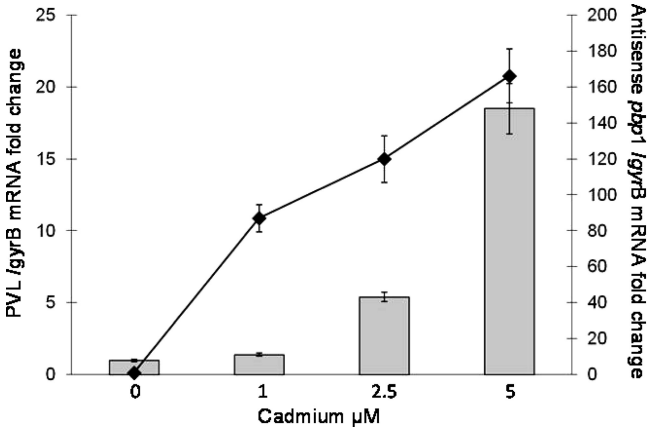

FIG. 5. Effect of PBP1 depletion by antisense mechanism on PVL expression. Strain LUG1477 (containing pLUG792) was cultured at 37°C with vigorous shaking in CCY broth supplemented with increasing concentrations of $CdCl_2$ (0, 1, 2.5, and 5 μM). After 6 h of incubation, aliquots of cultures were washed, adjusted to an OD_{600} 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 $CdCl_2$ concentrations relative to the *luk*-PV mRNA or antisense *pbp1* RNA expression level of the strain grown without $CdCl_2$. Values are means ± standard deviations (three different experiments).

LUG1485, which showed no increase of *luk*-PV mRNA expression at any $CdCl_2$ 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 Δ*agr* strain and an 18.7-fold decrease of the *luk*-PV mRNA level in the Δ*saeRS* strain (Fig. 7A and B). PVL quantification in the supernatant yielded a 10-fold decrease of PVL production in the Δ*agr* strain and a 15-fold decrease of PVL production in the Δ*saeRS* 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.

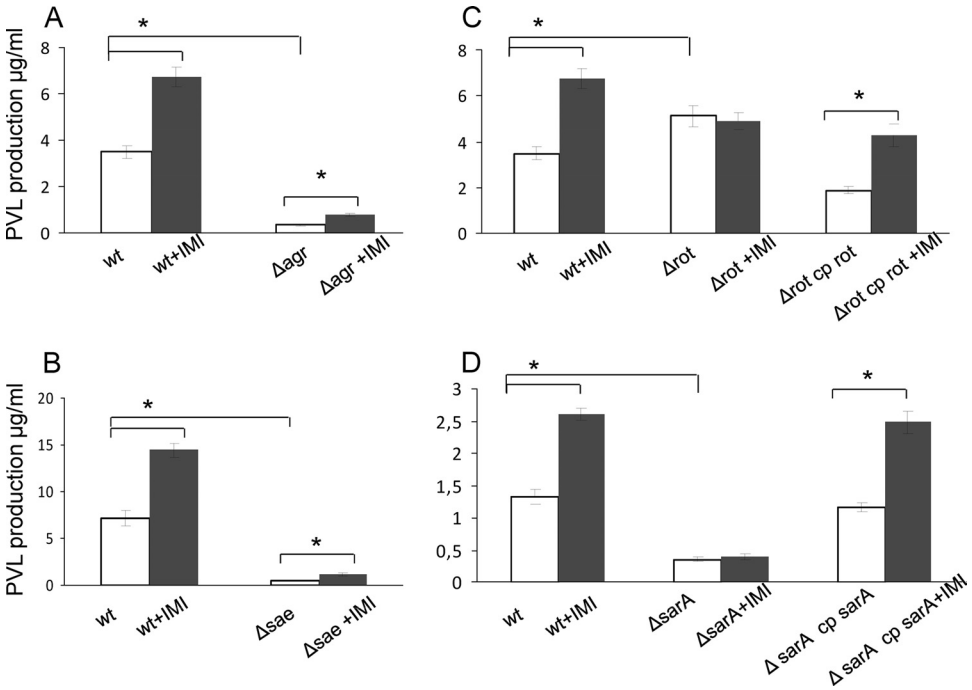

FIG. 6. Effects of imipenem (IMI) on PVL production by isogenic strains deleted for *agr* (A), *saeRS* (B), *rot* (C), and *sarA* (D). Cultures were performed in CCY medium at 37°C with gyratory shaking (300 rpm) in the absence (growth control; white bars) and presence of imipenem at 1/4 the MIC (gray bars). After 6 h of incubation, aliquots of cultures were adjusted to an OD_{600} 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.

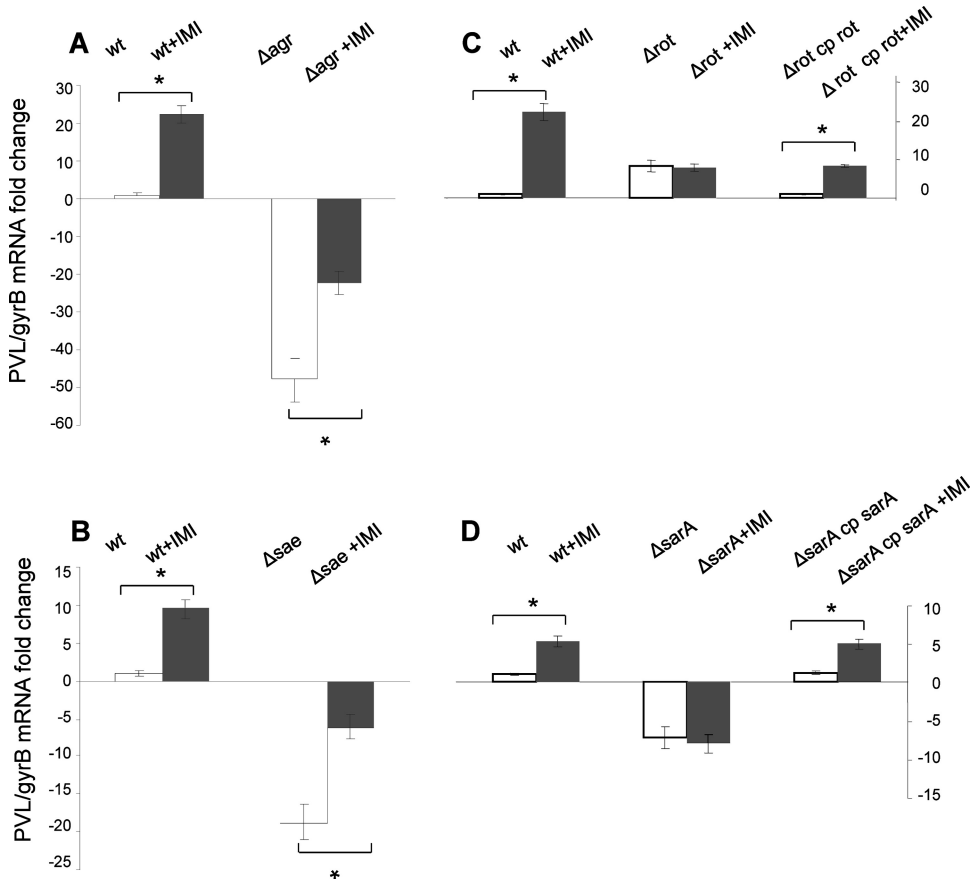

FIG. 7. Effects of imipenem on *luk*-PV mRNA expression by isogenic strains deleted for *agr* (A), *saeRS* (B), *rot* (C), and *sarA* (D). Cultures were performed in CCY medium at 37°C with gyratory shaking (300 rpm) in the absence (growth control; white bars) and presence of imipenem at 1/4 the MIC (gray bars). After 6 h of incubation, aliquots of cultures were adjusted to an OD_{600} 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* and *gyrB* primers. Relative quantification was performed by normalization to *gyrB* expression as described elsewhere (37). Results are expressed as *n*-fold differences of *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.

Rot deletion resulted in slightly increased PVL production (1.5-fold), along with an 8.4-fold increase in the *luk*-PV mRNA level. As shown in Fig. 6C and 7C, imipenem incubation of the Δ*rot* strain yielded neither increased PVL production nor an enhanced *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 *luk*-PV mRNA level (Fig. 6C and 7C).

In contrast to the case with *rot*, *sarA* deletion resulted in decreased PVL production (3.6-fold), along with a 7.2-fold decrease in the *luk*-PV mRNA level. As shown in Fig. 6D and 7D, imipenem incubation of the Δ*sarA* strain yielded neither increased PVL production nor an enhanced *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 *luk*-PV mRNA level.

Modulator effect of imipenem on expression of major *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 *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 *S. aureus* cultured with imipenem. Accordingly, qRT-PCR was performed with specific primers for RNAIII (the major transcript of the *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

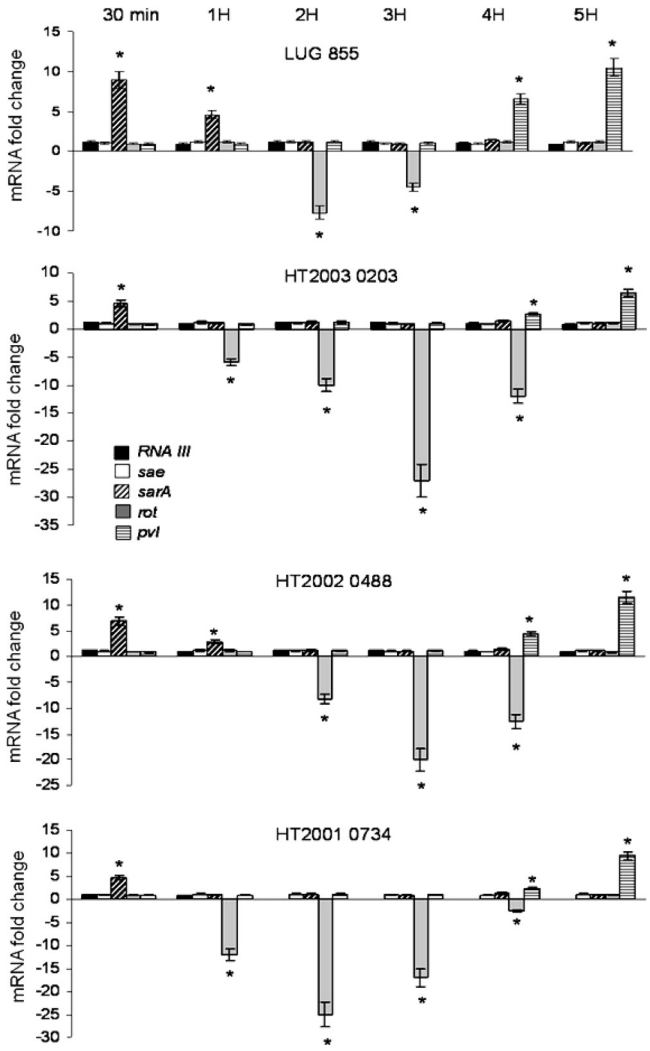

FIG. 8. Effects of imipenem on *agr*, *sae*, *rot*, and *sarA* mRNA expression. *S. aureus* strains LUG855, HT20030203, HT20020488, and HT20010734 were cultured without antibiotics as described above and monitored for OD; when the OD_{600} reached 0.5, imipenem was added to the culture at 1/2 the MIC and cultures were reincubated at 37°C with gyratory shaking (300 rpm). Aliquots were taken every hour, and cellular pellets were prepared as described before for total RNA extraction and subsequent reverse transcription with random primers. The cDNA obtained was used as a template for LightCycler PCRs with *gyrB*-, *RNA III*-, *saeR*-, *rot*-, *sarA*-, and *pvl*-specific primers. Relative quantification was performed by normalization to *gyrB* expression as described elsewhere (37). Results are expressed as *n*-fold differences of *RNAIII* (black bars), *sae* (white bars), *sarA* (hatched bars), *rot* (gray bars), and *pvl* (horizontally striped bars) mRNA levels detected in the presence of imipenem relative to the mRNA expression of the strain grown without antibiotics for different times (30 min to 5 h). Values are means ± standard deviations (three different experiments). Changes in mRNA levels were interpreted as significant if they were >2-fold.

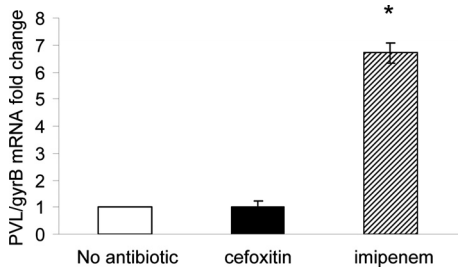

FIG. 9. Effects of beta-lactams on PVL expression *in vivo*. Six-week-old female BALB/c mice were challenged intranasally with 5×10^7 *S. aureus* CA-MRSA LAC (USA300) organisms as previously described (37). Six hours after infection, mice were treated twice daily with either imipenem, cefoxitin, or PBS. Mice were sacrificed after 3 days, and lung samples were assessed for PVL mRNA expression as described above. 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.

growth control levels. 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 strain 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 untreated animals. As shown in Fig. 9, the PVL mRNA level detected in imipenem-treated mice was 6.7 times higher than the PVL mRNA level observed in PBS-treated mice. There was no modification

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), cefaclore (PBP3 selective) (28), and cefoxitin (PBP4 selective) (17). When cultures were treated with either oxacillin or imipenem (PBP1 selective), but not with cefotaxime, cefaclore, 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 *pbp1* 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 *pbp1* 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. Utaida 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 PBP1, 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 PBP1 inactivation and *sarA* triggering has never been put forward, it may be related to the essential role performed by PBP1 during septum formation and cell division. PBP1 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 PBP1 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.

ACKNOWLEDGMENTS

We thank Richard Novick for providing RN6390 $agr^{+/-}$ isogenic strains and Martine Rougier, Annie Martra, Christine Courtier, Christine Gardon, Céline Spinelli, Caroline Bouveyron, and Florence Couzon-Vincent for their technical advice.

The laboratory has received research grants from Pfizer and Leo-Pharma.

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