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1 **Beta-lactams interfering with PBP1 induce Panton-Valentine leukocidin**
2 **expression by triggering *Sar*A and *rot* global regulators of *Staphylococcus***
3 ***aureus***

4

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17

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19 imipenem, rot, sarA

20 Running head: Beta-lactams effect on leukocidin expression

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1 **Abstract**

2 Previous articles reported that beta-lactam antibiotics increase the expression of *S.*
3 *aureus* Panton-Valentine leukocidin (PVL), by activating its transcription. We
4 investigated the mechanisms underlying the inductor effect of beta-lactams on PVL
5 expression by determining targets and regulatory pathways possibly implicated.

6 We measured PVL production in presence of: oxacillin (non-selective), imipenem
7 (PBP1), cefotaxime (PBP2), cefaclore (PBP3) and cefoxitin (PBP4)-selective beta-
8 lactams. *In vitro*, we observed increased PVL production consistent with the *luk*-PV-
9 mRNA level 20 to 25 times higher for CA-MRSA cultures treated with PBP1-binding
10 oxacillin and imipenem when compared to cultures treated with others beta-lactams
11 or no antibiotic at all. This effect was also observed *in vivo* with an increased PVL
12 mRNA levels in lung tissues from CA-MRSA mouse pneumonia treated with
13 imipenem but not cefoxitin. To confirm the involvement of PBP-1 inhibition in this
14 pathway, PBP1 depletion by an inducible *pbp*1 antisense-RNA showed dose-
15 dependent relation between the level of *pbp*1 antisense-RNA and *luk*-PV-mRNA
16 level.

17 Upon imipenem treatment of exponential growing cultures, we observed *sar*A
18 increased mRNA level after 30 minutes incubation followed by *rot* decreased mRNA
19 level after 1 to 4h incubation. Unlike *agr* and *sae*RS positive regulators non-essential
20 for PVL induction by beta-lactams, *sar*A (positive) and *rot* (negative) PVL regulators,
21 were necessary for PVL induction by imipenem.

22 Our results suggest that antibiotics binding to PBP1 increase PVL expression by
23 modulating *sar*A and *rot* which are essential mediators of the inductor effect of beta-
24 lactams on PVL expression.

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Introduction

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 in a rabbit pneumonia model, which is more susceptible to PVL, confirm the important role of this toxin in pathogenesis (20). Although PVL producing strains are mainly isolated from primary skin abscesses regardless to their severity (3, 18), strong epidemiological link exits 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 *agr* (47), autolysis-related locus *arl*RS (24), *S. aureus* exoprotein expression *sae*RS (31) and staphylococcal respiratory response *srr*AB (69). DNA-binding proteins, such as SarA (13) and the recently identified SarA homologues (SarR (40), Rot (43), SarS (14, 59), SarT (56), SarU (41)), also regulate virulence factor expression. The

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multiple pathways generated by these factors allow the bacterium to adapt rapidly and promote growth in a large variety of environmental conditions, which may also include antibiotic presence.

It was recently shown that subinhibitory concentrations of beta-lactams increase PVL production *in vitro* via transcriptional activation (23, 57) while agents like clindamycin and linezolid that inhibit protein synthesis reduced *S. aureus* release of toxins, including PVL (4, 19, 23, 57). In spite of the lack of *in vivo* data but giving that beta-lactam antibiotics may lead to increased PVL production, some clinicians now recommend 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 we investigated the targets and the regulatory pathways possibly implicated in PVL modulation by beta-lactam antibiotics.

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1 **Material and methods**

2

3 ***Bacterial strains***

4 Bacterial strains, plasmids and phages used in this study are summarized in table 1.

5

6 ***Antibitiotics and MIC determination***

7 Antibiotics used in this study were: oxacillin, cefaclore and cefoxitin purchased from
8 Sigma-Aldrich (L'Isle d'Abeau, France), imipenem purchased from MSD Laboratories
9 (Paris, France) and cefotaxime purchased from Dakota Pharm (Creteil, France).
10 Minimal inhibitory concentrations were determined by broth microdilution assay as
11 recommended by CLSI standards(15).

12

13 ***Bacterial cultures***

14 Strains were cultured on trypticase blood agar plates and incubated overnight at
15 37℃. Isolated colonies were resuspended in distillated water and adjusted to 0.5
16 McFarland optical density (corresponding to 10^8 CFU/ml as confirmed by bacterial
17 count) and 50 µl of this suspension were inoculated in 5 ml CCY broth contained in
18 glass tubes. Cultures were performed at 37℃ with gyratory shaking (300 rpm) in the
19 absence (the growth control) and in presence of different antibiotics at the
20 concentrations of 1/4 of the MIC. When needed for selection, erythromycin (Sigma)
21 was used at the final concentration of 5 µg / ml, chloramphenicol (Sigma) and
22 kanamycin (Sigma) at 20 µg / ml.

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1 For expression studies of various regulators, cultures without antibiotics were
2 performed as described above and monitored for OD; when the OD600 reached 0.5,
3 imipenem was added to the culture at a concentration corresponding to ½ of the MIC
4 and cultures were re-incubated at 37°C with gyratory shaking (300 rpm). Aliquots
5 were taken after 30 minutes and hourly and cellular pellets were prepared as
6 described below for total RNA extraction.

7

8 ***PVL quantification***

9 At different times, aliquots of cultures were taken and adjusted to an optical density
10 OD600 of 1 and then centrifuged at 13,000 g for 10 minutes. PVL was quantified in
11 the supernatant by a specific ELISA assay (BioMerieux R&D department, Marcy-
12 l'Etoile, France) as described elsewhere (2).

13

14 ***Relative quantitative RT-PCR***

15 Aliquots of 1 ml of each culture were centrifuged at 13,000 g and pellets were
16 washed with 1 ml of 10 mM Tris buffer and adjusted to an optical density OD600 of 1
17 corresponding to approximately 1 × 10^9 *S. aureus* cells/ml. One ml of adjusted and
18 washed bacterial suspensions was centrifuged at 13,000 g and pellets were treated
19 with lysostaphin (Sigma®) at a final concentration of 200µg/ml. The total RNA of the
20 pellets was then purified using the Qiagen® RNeasy Plus Mini Kit according to the
21 manufacturer's instructions. The RNA yielded was assessed with a NanoDrop®
22 Spectrophotometer and 1 microgram of total RNA was reverse transcribed using the
23 Promega® Reverse Transcription System with random primers as recommended by

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1 the provider. The resulting cDNA was used as template for real time amplification
2 (LightCycler 2.0 Roche®) using specific primers in table 2. The relative amounts of
3 amplicons specific for each gene were determined by quantitative PCR relative to an
4 internal standard (*gyr*B) as described elsewhere (37). The expression levels of
5 investigated genes were expressed as n-fold difference relative to the calibrator
6 calculated with RealQuant software (Roche Diagnostics). Change in mRNA level was
7 interpreted as significant if superior to 2-fold variation.
8

9 ***Quantitative PCR***

10 Aliquots of 1 ml of LUG855 cultured with and without beta-lactams at ¼ of the MIC
11 were centrifuged at 13,000 g and pellets were washed with 1 ml of 10 mM Tris buffer.
12 Genomic DNA was isolated from the pellets by using Qiagen® QIAmp DNA Mini Kit
13 as recommended by the provider. A decimal dilution of the DNA yielded was used as
14 template for real time *pvl* and *gyr*B PCR using specific primers (table 2). Relative
15 quantification of *pvl* gene copy number was assessed after normalization of *gyrB*
16 copy number. Results are expressed as n-fold variation of the *pvl/gyrB* copy number
17 in presence of antibiotics relative to the *pvl/gyrB* copy number of the growth control.
18 Quantification of the *att*P insertion site of phiSLT phage was performed by
19 quantitative real time PCR with specific primers ISPVL1 and ISPVL2 (table 2) as
20 described in Wirtz et al (67).
21

22 ***Pbp1 antisense construction***

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1 We amplified a 265pb fragment of *pbp1* gene by using the primers pbpA2303
2 AT**GGATCC**TTAACTATGCCTGACATG containing a *Bam*HI restriction site and
3 pbpA2567 ACT**AGGCCT**TGTCAGTTTTACTGTC containing a *Stu*I restriction site.
4 The fragment was digested with *Bam*HI and *Stu*I restriction enzymes and ligated with
5 the *luk*-PV transcriptional terminator (amplified using primers phi2648 and phi2815
6 (60)). The ligation product was then used as template for PCR amplification using
7 pbpA2567 and phi2815 primers. After being verified by sequencing, the fragment
8 was ligated into pGEM®-T Easy Vector System (Promega®). The resulting plasmid,
9 pLUG789, was digested with *Eco*RI and *Stu*I and the digestion product was ligated
10 with pCN37 (11) digested with *EcoR*I and *Sma*I. The ligation product, pLUG792
11 (figure 4A), was then transformed and cloned in *Escherichia coli* DH5α before
12 successive electroporation (Bio-Rad gene pulser) into *S. aureus* RN4220 and
13 LUG855. The expression of *pbp*1 antisense was assayed by RT-PCR with primers
14 pbp1R and pbp1phi (table 2), the latter was designed in order to encompass the 3'
15 end of the pbp1 antisense and the 5' end of the *luk*-PV transcriptional terminator. The
16 control plasmid pLUG795, identical to pLUG792 but lacking the *pbp*1 2567-2303
17 fragment, was obtained by successive *Bam*HI digestion and ligation of pLUG792.
18

19 ***RN6390 rot::cat construction***

20 The deletion/replacement *rot::cat* mutant of *S. aureus* RN6390 (LUG 1160) was
21 obtained by using pMAD, a thermosensitive plasmid which contains a constitutively
22 expressed β-galactosidase gene that allows positive selection of double cross-over
23 by screening the β-galactosidase activity on X-gal agar plate (1). A 1036 bp DNA
24 fragment corresponding to the chloramphenicol-acetyl-transferase gene was

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1 amplified from pC194 plasmid (33) and cloned in pMAD between two DNA fragments
2 corresponding to the 640 pb 5' flanking region of *rot* gene generated using primers
3 rot824 CAA**GTCGAC**TATATCGTGACTTAGTTGAAG containing *Sal*I restriction site
4 and rot1464 CTT**ATCGAT**AAAACTACAAGTGTAAATAAACTTGC containing *Cla*I
5 restriction site and a 581 pb 3' flanking region of *rot* gene generated using primers
6 rot1951 GTG**AAGCTT**TAATAGCATAAAAAGAGGTTTTC containing *Hind*III
7 restriction site and rot2532 ATT**GATATC**CGAACAAGTACCAGAA containing *EcoR*V.
8 The resulting plasmid, pLUG641, was successively electroporated into RN4220 and
9 RN6390. Transformants were grown at the non-permissive temperature (37°C) to
10 select for cells in which the plasmid had been integrated into the chromosome by
11 homologous recombination. To favor the second recombination event, a single
12 colony was grown at 30°C for 10 generations and plated at 37°C overnight. Cells,
13 which have lost the plasmid vector through a double crossing-over event, were
14 detected on X-gal agar plates. PCR amplifications were used to confirm the loss of
15 *rot* gene, which was replaced by the *cat* gene.

16 Complementation of the mutated strain was performed with pLUG895 plasmid
17 containing a 1.26 kb fragment encoding rot which was obtained after *EcoR*I digestion
18 of the fragment generated by the amplification of *rot* gene with *EcoR*I restriction site
19 containing primers TT**GAATTC**GTATATCACATTTTATACACATTTG and
20 CGA**GAATTC**AAGCTATTAATTCATTGCTATC.

21

22 ***Visualization of the pbp1 antisense effect on cell structure***

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1 The localization of new cell wall synthesis in strains LUG1477 and LUG1485 was
2 done as previously described (52). Briefly, cells were grown for 18h in BHI with 5 µM
3 of CdCl2 and 0.125 M of D-serine, to promote the incorporation of this amino acid
4 into the cell wall. The culture was diluted 1/250 into the same medium, grown to an
5 OD600 of 0.6, washed, and resuspended in the same volume of BHI with 5 µM
6 CdCl2 and without D-serine. The cells were then incubated for 15 min at room
7 temperature, to allow the incorporation of D-alanine into the cell wall and then labeled
8 with a mixture of equal amounts of vancomycin (Sigma) and a BODIPY FL conjugate
9 of vancomycin (Van-FL, Molecular Probes) at the final concentration of 1 µg/ml, for 5
10 min at room temperature with agitation. Cells were then analyzed by fluorescence
11 microscopy using a Zeiss Axiovert inverted microscope.

12

13 ***Mouse lung infection***

14 For mouse lung infection *S. aureus* strain LAC (USA300) was cultured aerobically on
15 blood agar at 37°C overnight, and then in 5 mL of CCY medium.
16 Animal experiments were carried out with 6-week-old female Balb/c mice (Harlan,
17 Indianapolis, IN, USA), in accordance with the National Institute of Health guidelines,
18 and were approved by the Institutional Animal Care Use Committee at the Texas
19 A&M HSC Institute of Biosciences and Technology. Lung infections were induced
20 with $5x10^7$ *S. aureus* cells in a volume of 20 µl intranasally as previously described
21 (37). Six hours after infection, mice were treated intramuscularly with either imipenem
22 at 25 mg/kg, cefoxitin at 150 mg/kg or PBS. Injections were repeated every 12 hours
23 and mice were sacrificed after 3 days. Lung samples were homogenized in Trizol
24 (Sigma Aldrich®) and processed in the MagNA Lyser system (Roche®), then total

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1 RNA was extracted using the MagNA Pure LC RNA Isolation Kit (Roche®) as
2 recommended by the provider. Total RNA yield was measured by a NanoDrop®
3 Spectrophotometer and 1 microgram of total RNA was reverse transcribed using the
4 Promega® Reverse Transcription System with specific *gyr*B R and *pvl* R primers
5 (table 2). The resulting cDNA was used as template for real time amplification
6 (LightCycler 2.0 Roche®) with *gyr*B and *pvl* primers. Furthermore *pvl* specific
7 transcripts were normalized with respect to *gyrB* transcription level and *pvl*
8 expression levels in mice treated with either imipenem or cefoxitin were expressed as
9 n-fold difference relative to *pvl* expression level in animals treated with PBS. RT-PCR
10 experiments were conducted as triplicates on lung samples from five different mice
11 treated with imipenem, cefoxitin or PBS respectively.

12

13 ***Statistical analysis***

14 The statistical analyses were based on the use of one way ANOVA followed by the *a*
15 *posteriori* Dunnett's test. The level of statistical significance was set at 0.05.The tests
16 were carried out with SPSS for Windows version 12.0 software.

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1 **Results**

2

3 ***MICs of beta-lactams***

4 MICs for several beta-lactams, oxacillin, imipenem, cefotaxime, cefaclore and
5 cefoxitin, were performed following CLSI recommendations. Results obtained on the
6 CA-MRSA isolates and on LUG855 are summarized in table 3. All derivative of
7 LUG855 shown in table 1 displayed similar susceptibility to imipenem with MICs of
8 0.03 µg/ml (not shown).

9

10 ***Beta-lactams effect on PVL production by the CA-MRSA strains***

11 To examine the influence of beta-lactams on PVL release, PVL was quantified in the
12 culture supernatant of 4 clinical CA-MRSA strains incubated with ¼ of the MIC of
13 oxacillin, imipenem, cefotaxime, cefaclore and cefoxitin for 6 h. As shown in figure 1,
14 the effects were different from one beta-lactam to another. In presence of oxacillin, all
15 4 strains displayed increased PVL production. The increase was strain dependent
16 and ranged from 2.10 to 6.12 fold when compared to the growth control level. We
17 obtained similar results with imipenem, which lead to PVL increased production of all
18 4 strains ranging from 2.5 to 6.5 times the growth control level. The three other beta-
19 lactams tested, cefotaxime, cefaclore and cefoxitin, did not modify the PVL
20 production of any of the 4 CA-MRSA strains.

21

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1 ***Beta-lactams effect on PVL expression by LUG855***

2 We used the laboratory strain LUG855 in order to further examine the effects of
3 subinhibitory concentrations of beta-lactams (¼ of the MIC) on PVL expression. We
4 performed PVL-specific ELISA quantification on the supernatants of 6h cultures and
5 we observed that oxacillin and imipenem increased PVL expression by 2.25 and 3.4
6 fold when compared to the growth control level, while cefotaxime, cefaclore and
7 cefoxitin did not increase PVL level compared to the no antibiotic control (figure 1).
8 PVL relative qRT-PCR performed on the cellular pellets yielded similar results:
9 cultures treated with oxacillin and imipenem displayed increased PVLmRNA levels
10 17.3 and 22.5 fold respectively, while cefotaxime, cefaclore and cefoxitin did not
11 modify the PVL mRNA level. To rule out a possible excision of the phage in response
12 to stress-induced antibiotics, the copy number of the *pvl* gene was analysed by qPCR
13 and showed no variation regardless of the beta-lactams added (figure 2). This result
14 was confirmed by the absence of detectable newly formed *att*P insertion sites of
15 phiPVL phage as measured by specific qPCR (results not shown).

16

17 ***Beta-lactams effect on PBP1 expression by LUG855***

18 PBP1 is supposed to be the common target of oxacillin and imipenem (68), but not of
19 cefotaxime, cefaclore and cefoxitin (17, 27, 28). Binding of beta-lactams to PBP1
20 results in subsequent PBP1 dysfunction leading to mechanically weak peptidoglycan,
21 which bacteria may tend to compensate by enhanced *pbp*1 gene transcription.

22 We performed qRT-PCR with PBP1 specific primers (table 2) on the cellular pellets
23 and detected significant increased PBP1 mRNA level of 4.5 fold when cultures were

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1 treated with imipenem and slight increased PBP1 mRNA level of 2.3 fold when
2 cultures were treated with oxacillin (figure 3). We also performed qRT-PCR with
3 PBP2-4 specific primers (table 2) in order to study the effect on PBP2-4 expression
4 upon exposure to imipenem and oxacillin (figure 3). As expected, imipenem (high
5 affinity ligand of PBP1 (68)) only modified PBP1 mRNA level, while oxacillin (non-
6 selective PBPs ligand) increased PBP1 and PBP2 and, in a lesser extent, PBP3
7 mRNA levels. Therefore, we concluded that the common bacterial target of oxacillin
8 and imipenem was PBP1. We did not detect increased PBP1 mRNA levels in
9 cultures treated with cefotaxime, cefaclore or cefoxitin (results not shown).

10

11 ***The effect of the antisense RNA depletion of pbp1 on PVL expression***

12 As oxacillin and imipenem are the two beta-lactams increasing PVL expression and
13 also interfering with PBP1, we hypothesized that PBP1dysfunction could somewhat
14 lead to augmentation of PVL. We investigated this hypothesis by reducing *pbp1*
15 expression by a cadmium inducible cassette that expresses antisense *pbp*1-RNA
16 (figure 4A). As PBP1 was shown to be an essential enzyme involved in septum
17 formation (51), we checked for the cadmium-induced PBP1 depletion by observing
18 fluorescence microscopy images of LUG1477 and LUG1485 (negative control)
19 stained with Van-FL specifically binding to the nascent division septum (figure 4B).
20 LUG1485 cadmium treated cultures displayed complete septa at normal equatorial
21 position resulting in tetrads and octets of normal sized daughter-cells. LUG1477
22 cadmium treated cultures displayed abnormally septa with asymmetric polar
23 localization leading to tetrads and octets of cocci accumulating with disrespect of the
24 normal alternating perpendicular planes.

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1 Strains LUG1477 harboring pLUG792 and its control LUG1485 harboring pLUG795
2 (derived from pLUG792 but lacking *pbp1* antisense fragment) were cultured for 6h in
3 the presence of different cadmium concentrations (ranging from 0 to 5 µM). qRT-
4 PCR was performed on the cellular pellets for PVL expression and for antisense
5 *pbp*1-RNA expression (with primers pbp1R and pbp1phi from table 2). As shown in
6 figure 5, we observed dose-dependent antisense *pbp*1-RNA and *luk*-PV-mRNA
7 levels induction with CdCl2 from 0 to 5 µM. Thus, at 2.5 µM CdCl2, antisense *pbp*1-
8 RNA increased 120 fold while PVL mRNA increased 5.4 fold. The biggest increase
9 was observed with 5 µM CdCl2: 166 fold for antisense *pbp*1-RNA and 18.5 for PVL-
10 mRNA. Higher levels of CdCl2 were inhibitory to growth. Bacterial count of viable
11 cells was performed for LUG1477 cultures by dilution and plating which yielded 5×10^8
12 CFU/ml for LUG1477 cultured without Cd and 1.8×10^8 CFU/ml for LUG1477 cultured
13 with 5 µM CdCl2.

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

17

18 ***Effect of major S. aureus regulators on PVL expression and on PVL induction***
19 ***by imipenem***

20 PVL expression is known to be modulated by several global virulence regulators of
21 *S. aureus* (7, 67). We therefore investigated the impact of *agr*, *sae*RS, *rot* and s*arA*
22 deletions on PVL induction by imipenem to find out whether one of these regulators
23 were potential mediators of the inductive effect of beta-lactams on PVL expression.

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1 We used isogenic *S. aureus* strains belonging to the same genetic background
2 (RN6390) and deleted for one of each of the major regulators investigated. Isogenic
3 pairs (parental strain and mutant of interest) were cultured for 6 h with ¼ of the MIC
4 of imipenem as described above and PVL production was measured in the
5 supernatants while cellular pellets were assayed for *luk*-PV-mRNA expression by
6 qRT-PCR.

7 As shown in figures 6A, 6B, 7A and 7B, *agr* and *sae* are powerful positive regulators
8 of PVL expression as their deletion leads to dramatically decreased PVL expression:
9 48 fold decrease of *luk*-PV-mRNA level in Δ*agr* and 18.7 fold decrease of *luk*-PV-
10 mRNA level in Δ*sae*RS (figure 7 A and B). PVL quantification in the supernatant
11 yielded 10 fold decrease of PVL production in Δ*agr* and 15 fold decrease of PVL
12 production in Δ*sae*RS strain (figure 7 A and B). Nevertheless, when cultured with
13 imipenem, both *agr* and *sae*RS deleted strains displayed increased PVL expression
14 although the range of increased expression was reduced in comparison with the
15 range obtained in the parental strains.

16 Rot deletion resulted in slightly increased PVL production of 1.5 fold along with an
17 8.4 increased *luk*-PV-mRNA level. As shown in the figures 6C and 7C, imipenem
18 incubation of the Δ*rot* strain yielded neither increased PVL production nor enhanced
19 *luk*-PV-mRNA level, suggesting that *rot* deletion interferes with the inducing effect of
20 imipenem on PVL expression. The inductive effect of imipenem was restored by a
21 low copy number plasmid *rot* complementation. When cultured with imipenem, the *rot*
22 complemented strain showed a 2.2 fold increase of the PVL production and a 8.8 fold
23 enhancement of *luk*-PV-mRNA level (figure 6C and 7C).

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1 Contrarily to *rot*, *sar*A deletion resulted in decreased PVL production of 3.6 fold along
2 with a 7.2 decreased *luk*-PV-mRNA level. As shown in the figures 6D and 7D,
3 imipenem incubation of the Δ*sar*A strain yielded neither increased PVL production
4 nor enhanced *luk*-PV-mRNA level, suggesting that the absence of SarA also
5 interferes with the inductive effect of imipenem on PVL expression. To confirm this
6 hypothesis, the inducing effect of imipenem was restored by the chromosomal *sar*A
7 complementation. When cultured with imipenem, the *sar*A complemented strain
8 showed 2.1 fold increase of the PVL production and 4 fold enhanced *luk*-PV-mRNA
9 level.
10

11 ***The modulator effect of imipenem on the major S. aureus regulators***
12 ***expression***

13 By using a laboratory strain and three clinical strains belonging to three major CA-
14 MRSA lineages (ST1, ST8 and ST80), we investigated for a possible modulation of
15 virulence regulators of *S. aureus* by subinhibitory concentrations of imipenem. As our
16 results suggest that *rot* and *sarA* are global regulators involved in the induction of
17 PVL expression by imipenen, we assayed by qRT-PCR for a direct modulation of *rot*
18 and *sarA* expression in *S. aureus* cultured with imipenem. Accordingly, qRT-PCR
19 was performed with specific primers for RNAIII (the major transcript of *agr* operon),
20 *saeR*, *sarA* and *rot* (table 2) on aliquots removed after 30 minutes and hourly from
21 LUG855, HT2003 0203, HT2002 0488 and HT2001 0734, cultured with and without
22 imipenem at ½ of the MIC. The mRNA level of the 4 regulators observed in the
23 presence of antibiotic was compared to the mRNA level expressed without
24 antibiotics. As shown in figure 8, after 30 minutes of incubation with imipenem, we

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1 observed increased SarA mRNA level for all the strains tested, ranging from 4.6 to
2 8.9 fold when compared to the growth control levels. As assessed by qRT-PCR, the
3 expressions of RNAIII, *sae*R and *rot* were not modified after 30 minutes incubation
4 with imipemem for any strain tested. Furthermore, after 1 hour incubation with
5 imipenem, we observed *sarA* mRNA increased levels for LUG 855 (4.5 fold) and
6 HT2002 0488 (2.9 fold) and decreased *rot* mRNA levels for HT2003 0203 (5.9 fold)
7 and HT2001 0734 (12 fold). Significant decrease in *rot* mRNA level was detected for
8 all strains, ranging from 7.7 to 25 fold and from 4.5 to 27 fold after 2 and 3 hours
9 respectively when compared to the growth control level. After 4 hours incubation with
10 imipenem, we only detected decreased *rot* mRNA level in the three clinical strains: 12
11 fold decrease in HT2003 0203, 12.4 fold decrease in HT2002 0488 and 2.7 fold
12 decrease in HT2001 0734. After 5 hours of incubation with imipenem, no variation in
13 the expression of any of the 4 regulators investigated was detected whatever the
14 strain. Nevertheless, starting with 4 hours incubation, we observed significant
15 increase of the PVL mRNA level in presence of imipenem for all strains tested
16 ranging from 2.4 to 6.6 fold increase at 4 hours and from 6.5 to 11.5 fold increase at
17 5 hours. The same experiment was performed with another beta-lactam as a control
18 (cefoxitin at ½ of the MIC). We observed no variation in the expression of any of the 4
19 regulators investigated, neither we detected PVL increased mRNA expression after
20 cefoxitin treatment (results not shown).

21

22 ***Beta-lactams effect on PVL mRNA expression in mouse lung infection***

23 To examine the influence of beta-lactams at subinhibitory concentrations on PVL
24 expression *in vivo*, mice were challenged intranasally with *S. aureus* CA-MRSA strain

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1 LAC (USA300). Six hours after infection, mice were treated twice daily with either
2 imipenem, cefoxitin or no antibiotic (10 – 12 mice per group), then sacrificed at day 3.
3 PVL expression in lung tissues was only detected in tissues were CFU counts of
4 recovered bacteria were superior to 5 decimal logs per gram of tissue, corresponding
5 to 5 animals per group. In these samples, *pvl* specific transcripts were normalized
6 with respect to *gyrB* transcription level and *pvl* expression levels in mice treated with
7 either imipenem or cefoxitin were expressed as n-fold difference relative to *pvl*
8 expression level in untreated animals. As shown in figure 9, PVL mRNA levels
9 detected in imipenem treated mice were 6.7 times higher than PVL mRNA levels
10 observed in PBS treated mice. There was no modification of *pvl* transcripts in mice
11 treated with cefoxitin when compared to the untreated mice.

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1 **Discussion**

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

18 By using a mouse model of CA-MRSA pneumonia we provide for the first time *in vivo*
19 data supporting that some beta-lactams such as imipenem may promote PVL mRNA
20 expression in infected tissues. However, we were unable to measure higher amounts
21 of PVL in the lungs of imipenem treated mice as our ELISA assay was not sensitive
22 enough in this setting (results not shown). Nevertheless, the increased expression of
23 PVL in our mouse model was not followed by increased morbidity as assessed by the
24 weight loss data (results not shown). Recently it has been emphasized that mouse

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PMNs are relatively resistant to PVL whilst 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 whether higher amounts of PVL achieved during beta-lactam treatment would impact the outcome.

Beta-lactam antibiotics initiate bacterial cell death subsequently to their interaction with transmembrane penicillin binding proteins (PBPs). In wild type *S. aureus*, four penicillin-binding proteins (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 presence of subinhibitory concentrations of 5 carefully selected beta-lactams: oxacillin, a non-selective beta-lactam, imipenem (PBP1 selective (68)), cefotaxime (PBP2 selective (27)), cefaclore (PBP3 selective (28)) and cefoxitin (PBP4 selective (17)). When cultures where treated with either oxacillin or imipenem (PBP1 selective) but not with cefotaxime, cefaclore or cefoxitin, PVL production in the culture supernatant and *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 we observed dose-dependent relation between the *pbp*1 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 for the formation of the division septum and for 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, orthologue of the *S.*

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1 *aureus* PBP1, also involved in peptidoglycan synthesis during cell division, induced
2 SOS-promoting *recA* and *lexA* genes (44). Recently, it has been shown that beta-
3 lactams induce SOS response in *S. aureus* and promote genetic mobile elements
4 replication and transfer in *rec*A and *lex*A dependent manner, potentially resulting in
5 the dissemination of virulence factors harbored by temperate phages (39). Wirtz *et al*
6 obtained *rec*A-dependent PVL-encoding phage induction by using mitomycin C. They
7 observed increased *luk*-PV-mRNA levels mirroring the increased phage copy number
8 due to phage replication. One hypothesis explaining PBP1-mediated PVL induction in
9 *S. aureus* would thus be related to PVL-encoding phage induction promoted by a
10 SOS response. Nevertheless, quantitative PCR assays of PBP1 depleted *S. aureus*
11 by both antibiotics and antisense *pbp*1 RNA did not indicate PVL-phage replication
12 (figure 2), thus arguing against the SOS-based hypothesis.
13

14 Furthermore, we investigated the possible implication of major virulence regulators of
15 *S. aureus* in PVL modulation by PBP1-interfering beta-lactams such as imipenem.
16 Positive regulation of PVL transcription by the *agr, sae*RS and *sar*A locus have
17 already been reported (8, 67). Another SarA homologue, rot "repressor of toxin"
18 down-regulates *hla* and the gamma-hemolysin gene (*hlg*B and *hlg*C) (55) by
19 interacting with the promoter regions of target genes during the exponential growth
20 phase. During the post-exponential growth phase, the level of *rot*-mRNA is repressed
21 by *agr*-RNAIII by an antisense mechanism (6).

22 By using isogenic *S. aureus* strains deleted for each one of the *agr*, *sae*RS, *rot* and
23 *sar*A loci, we investigated the impact of the absence of these regulators on PVL
24 induction by subinhibitory concentrations of imipenem. To determine whether one of

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1 these regulators was essential and therefore would be a potential mediator of the
2 inductor effect of beta-lactams on PVL expression. We confirmed several previous
3 reports assessing that *agr* and *sae*RS are powerful positive regulators of PVL
4 expression (8, 67). However, *agr* and *sae* were not considered essential for PVL
5 induction by beta-lactams as PVL induction by imipenem still occurred in their
6 absence. This observation seems contradictory to a recent report from Kuroda *et al*
7 showing that subinhibitory concentrations of beta-lactams induce *hla* expression in *S.*
8 *aureus* through the *sae*RS system (36). This difference in observation could be
9 attributed to strain variations. Nevertheless, as in this work, the inductor used was not
10 imipenem, but cefoxitin which is a PBP4 selective beta-lactam (17), we may
11 hypothesize that a different signaling pathway was triggered to explain the different
12 modulator profile.

13 On the contrary, SarA and its homologue, rot, are probably essential for PVL
14 induction by imipenem (Fig. 5 and 6). PVL expression in *sar*A and *rot* inactivated
15 strains was not modified by imipenem whilst the inducing effect of imipenem on PVL
16 expression was restored in the complemented strains. The positive regulatory effect
17 of SarA on PVL expression was already reported (8); here we report for the first time
18 the negative regulator role of *rot* on PVL expression, consistent with several previous
19 observations of *rot* as down-regulator of exoprotein expression (55).

20 Kinetic measures of the regulators transcription level during CA-MRSA incubation
21 with sub-inhibitory concentrations of imipenem suggest that firstly, *sar*A expression is
22 triggered by a so far unknown signal and then, *rot* is down-regulated, both
23 modifications resulting in enhanced PVL expression. Our results do not clarify
24 whether down-regulation of *rot* occurs independently or whether it is a subsequent

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1 effect of SarA over-expression. Although the mechanism is still not clearly
2 established, Manna and Ray (42) reported experiments indicating that SarA is
3 involved in negative regulation of *rot* transcription, suggesting that *rot* down-
4 regulation during imipenem treatment may be a consequence of SarA enhanced
5 expression. Utaida *et al* reported neither *sar*A nor *rot* modulation when performing
6 the genome-wide transcriptional profile of *S. aureus* 8325-4 strain upon oxacillin
7 treatment (63). However, the authors assayed after 1 hour culture in presence of 5 to
8 10 times the MIC of oxacillin, whilst we assayed after 30 minutes to 5 hours culture
9 with ½ of the MIC of imipenem. Therefore, these different observations may not be
10 held as contradictory.

11 We were surprised to observe a 3 hours delay between the activation of *sar*A and
12 *luk*SF-PV induction. Stevens *et al* reported the delayed inductor effect of nafcillin sub-
13 inhibitory concentrations on PVL expression (57). They detected increased PVL
14 mRNA levels starting with 4 hours and up to 34 hours after nafcillin treatment.
15 Altogether these data suggest that beta-lactam inductor effect of PVL and the
16 mechanisms involved may be dependent of the growth phase and promoted during
17 the post-exponential phase. This hypothesis is also consistent with the fact that SarA
18 is involved in negative regulation of *rot* transcription manly during the post-
19 exponential phase of growth (42).

20 Taken together, these data suggest that at sub-inhibitory concentrations, beta-
21 lactams such as imipenem and oxacillin which bind to and inactivate PBP1, trigger by
22 a so far uncharacterized pathway and increase SarA expression which subsequently
23 down-regulates rot expression, thus leading to enhanced PVL expression. Although
24 the link between PBP1 inactivation and *sar*A triggering have never been put forward,

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1 it may be related to the essential role performed by PBP1 during septum formation
2 and cell division. PBP1 inactivation results in dramatic inhibition of autolysis and rapid
3 decline in transcription of most autolytic enzymes, especially of the *atl* locus (50) thus
4 preventing bacterial death. SarA was shown to be a negative regulator of *atl*
5 expression and necessary for bacterial survival during penicillin induced killing (25);
6 therefore it may be involved in autolysis repression by beta-lactams. In this respect,
7 SarA modulation could be the consequence of an adapting mechanism allowing
8 bacterial persistence in presence of imipenem. Another argument supporting the role
9 of the autolytic signals in the beta-lactam induced regulatory pathway is that
10 proteomic analysis of *S. aureus* undergoing increased autolysis triggered by Triton X-
11 100 also resulted in increased SarA levels, coupled with a significant reduction in Rot
12 level (16).

13 We propose – as a working hypothesis – that the alterations in the composition of
14 peptidoglycan caused by PBP1 dysfunction generate a signal processed by a
15 transduction pathway that controls the expression of autolytic genes, resulting in the
16 inhibition of the activity of the autolytic system and promoting bacterial survival. The
17 existence of complex regulatory circuits coupling the autolytic system to the cell wall
18 synthesis possibly involving SarA regulator remains to be explored. It should provide
19 a link between the cell-wall turnover control and virulence regulation in *S. aureus,*
20 suggesting that enhanced virulence may be promoted during beta-lactam induced
21 bacterial persistence. Therefore, our results could explain the trend towards
22 increasing resistance and virulence of PVL producing *S. aureus* and also promote
23 appropriate treatment guidance to combat PVL-related infections.

24

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1 **Tables and figures**

2 **Table 1** Strains, plasmids, and phage

Strains	Reference or source	Description
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 when propagated 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 *sar*A copy (41) 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; *agr*3 *mecA*+ *pvl*+
HT20020488	(23)	ST80; *agr*3 *mecA*+ *pvl*+

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HT20030203	(23)	ST8; *agr*1 *mecA*+ *pvl*+
HT20041010	(23)	ST80; *agr*3 *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 β*gal*B 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 encoding rot
Phages		
phiSLT	(45)	*pvl* containing phage isolated from A980470

1

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1 **Table 2** Primers used for qPCR on LightCycle

Primer	5'3' sequence	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

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1 **Table 3** MICs of selected antibiotics for MSSA and CA-MRSA isolates in CCY broth

2

Strains	MICs (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

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1 **Figure 1** Effect of beta-lactams on PVL. *S. aureus* strains HT20010734,
2 HT20030203, HT20041010, HT20020488 and LUG855 cultures were performed in
3 CCY medium at 37℃ with gyratory shaking (300 rpm) in the absence (the growth
4 control) and in presence of different antibiotics at the concentrations of ¼ of the MIC.
5 After 6h of incubation, aliquots of cultures were taken and adjusted to an optical
6 density OD600 of 1 and then centrifuged at 13,000 g for 10 minutes. PVL was
7 quantified in supernatants by ELISA. Results are ratios of µg of PVL/ml of adjusted
8 culture incubated with the indicated antibiotic by means of µg of PVL/ml of adjusted
9 culture incubated without antibiotic and expressed as percentage values. Values are
10 means ± standard deviations (four doubled different experiments). * statistically
11 different from the control (corresponding isolate grown without antibiotic), with a p
12 value < 0.05, by one-way analysis of variance followed by *a posteriori* Dunnett's test.

13

14 **Figure 2** Effect of beta-lactams on PVL expression and *pvl* gene copy number.
15 *S. aureus* LUG855 cultures were performed as in figure 1. After 6h of incubation,
16 aliquots of same cultures were either used for total RNA or for genomic DNA
17 extraction as described in Material and Methods. The RNA yielded was used for
18 subsequent reverse transcription with random primers as described and the cDNA
19 obtained was used as template for LightCycler PCR with *pvl* specific primers (table
20 2). Relative quantification was performed by reporting to *gyr*B expression as
21 described elsewhere (37). Results are expressed as the n-fold difference of *luk*-PV
22 (dark bars) mRNA level detected in presence of different antibiotics relative to the
23 PVL mRNA level of the strain grown without antibiotics. Genomic DNA was used as
24 template for real time *pvl* and *gyrB* PCR using specific primers (table 2). Relative

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1 quantification of *pvl* gene copy number was assessed after normalization of *gyrB* 2 copy number. Results are expressed as n-fold variation of the *pvl/gyrB* copy number 3 (grey bars) in presence of antibiotics relative to the *pvl/gyrB* copy number of the 4 growth control. Values are means ± standard deviations (three different experiments).

5

6 **Figure 3** Effect of oxacillin and imipenem on PBP1-4 expression. *S. aureus* LUG855 7 cultures with either imipenem or oxacillin were performed as in figure 1. After 6h of 8 incubation, aliquots were used for total RNA extraction and subsequent reverse 9 transcription with random primers as described and the cDNA obtained was used as 10 template for LightCycler PCR with *pbp1-4* specific primers (table 2). Relative 11 quantification was performed by reporting to *gyr*B expression as described elsewhere 12 (37). Results are expressed as the n-fold difference of *pbp*1 (grey bars), *pbp*2 (black 13 bars), *pbp*3 (white bars) or *pbp*4 (hatched bars) mRNA level detected in presence of 14 different antibiotics relative to the PBP1-4 mRNA expression of the strain grown 15 without antibiotics. Values are means ± standard deviations (three different 16 experiments). Change in mRNA level was interpreted as significant if superior to 2- 17 fold variation.

18

19 **Figure 4**

20 **Figure 4A** Map of pLUG792 plasmid: pLUG792 a derivative of the *S. aureus* 21 expression vector pCN37, which employs a cadmium-inducible promoter (P*cad*) with 22 the cadmium regulator (*cad*^{R}) to express a specific 265 nt PBP1 antisense RNA 23 cloned upstream to *pvl* terminator. The plasmid contains a staphylococcal pT181

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1 replicon and a colE1 replicon, as well as erythromycin-resistance (*erm*C) and
2 ampicillin-resistance (*amp*R) genes.

3 **Figure 4B** Fluorescence microscopy images of LUG1485 and LUG1477 stained with
4 Van-FL specifically binding to the nascent division septum. LUG1485 Cd treated
5 cultures display complete septa at normal equatorial position resulting in tetrads and
6 octets of normal sized daughter-cells. LUG1477 Cd treated cultures display
7 abnormally septa with asymmetric polar localization leading to tetrads and octets of
8 cocci accumulating with disrespect of the normal alternating perpendicular planes.

9

10 **Figure 5** Effect of PBP1 depletion by antisense mechanism on PVL expression.
11 Strains LUG1477 (containing pLUG792) was cultured at 37°C with vigorous shaking
12 in CCY broth supplemented with growing concentrations of CdCl2 (0, 1, 2.5 and 5
13 µM). After 6h of incubation, aliquots of cultures were taken washed and adjusted to
14 an optical density OD600 of 1 and then used for total RNA extraction and subsequent
15 reverse transcription with random primers as described above. The cDNA obtained
16 was used as template for LightCycler PCR with specific *pvl* primers and specific *pbp1*
17 antisense primers pbp1R and pbp1phi which encompass the junction between *pbp1*
18 antisense fragment and *pvl* terminator. Relative quantification was performed by
19 reporting to *gyr*B expression as described elsewhere (37). Results are expressed as
20 the n-fold difference of *luk*-PV-mRNA (bars) or antisense *pbp*1 RNA (diamonds)
21 levels detected in presence of different CdCl2 concentrations relative to the *luk*-PV-
22 mRNA or antisense *pbp*1-RNA expression of the strain grown without CdCl2. Values
23 are means ± standard deviations (three different experiments).

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1

2 **Figure 6** Imipenem (IMI) effect on PVL production by isogenic strains deleted for *agr*
3 (A), *sae*RS (B), *rot* (C) and *sar*A (D). Cultures were performed in CCY medium at
4 37°C with gyratory shaking (300 rpm) in the absence (the growth control, represented
5 as white bars) and in presence of imipenem at ¼ of the MIC (dark bars). After 6h of
6 incubation, aliquots of cultures were taken and adjusted to an optical density OD600
7 of 1 and then centrifuged at 13,000 g for 10 minutes. PVL was quantified in
8 supernatants by ELISA and results are expressed as µg of PVL/ml. Values are
9 means ± standard deviations (four doubled different experiments). * statistically
10 different from the control (corresponding isolate grown without antibiotic), with a p
11 value < 0.05, by one-way analysis of variance followed by *a posteriori* Dunnett's test.

12

13 **Figure 7** Imipenem effect on *luk*-PV-mRNA expression by isogenic strains deleted for
14 *agr* (A), *sae*RS (B), *rot* (C) and *sar*A (D). Cultures were performed in CCY medium at
15 37°C with gyratory shaking (300 rpm) in the absence (the growth control, represented
16 as white bars) and in presence of imipenem at ¼ of the MIC (dark bars). After 6h of
17 incubation, aliquots of cultures were taken and adjusted to an optical density OD600
18 of 1 and then used for total RNA extraction and subsequent reverse transcription with
19 random primers as described above. The cDNA obtained was used as template for
20 LightCycler PCR with specific *pvl* and *gyrB* primers. Relative quantification was
21 performed by reporting to *gyr*B expression as described elsewhere (37). Results are
22 expressed as the n-fold difference of *luk*-PV-mRNA level reported to the PVL
23 transcript level from the wt parental strains (LUG855, LUG1053 or LUG1436) grown
24 without imipenem. Values are means ± standard deviations (three different

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1 experiments). Change in mRNA level was interpreted as significant if superior to 2-
2 fold variation.

3

4 **Figure 8** Imipenem effect on *agr*, *sae*, *rot* and *sarA* mRNA expression. *S. aureus*
5 strains LUG855, HT2003 0203, HT2002 0488 and HT2001 0734 were cultured
6 without antibiotics as described above and monitored for OD; when the OD600
7 reached 0.5, imipenem was added to the culture at ½ of the MIC and cultures were
8 reincubated at 37°C with gyratory shaking (300 rpm). Aliquots were taken every hour
9 and cellular pellets were prepared as described before for total RNA extraction and
10 subsequent reverse transcription with random primers. The cDNA obtained was used
11 as template for LightCycler PCR with *gyrB, RNA III, saeR, rot, sarA* and *pvl* specific
12 primers. Relative quantification was performed by reporting to *gyr*B expression as
13 described elsewhere (37). Results are expressed as the n-fold difference of *RNAIII*
14 (black bars), *sae* (white bars), *sar*A (hatched bars), *rot* (grey bars) and *pvl* (horizontal
15 hatched bars) mRNA levels detected in presence of imipenem relative to the mRNA
16 expression of the strain grown without antibiotics different times (30 minutes to 5h).
17 Values are means ± standard deviations (three different experiments). Change in
18 mRNA level was interpreted as significant if superior to 2-fold variation.

19

20 **Figure 9** Beta-lactam effect of PVL expression *in vivo*. Six-week-old female Balb/c
21 mice were challenged intranasally with $5x10^7$ *S. aureus* CA-MRSA LAC (USA300) as
22 previously described (37). Six hours after infection, mice were treated twice daily with
23 either imipenem, cefoxitin or PBS. Mice were sacrificed after 3 days and lung

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1 samples were assessed for PVL mRNA expression as described above. PVL specific
2 transcripts were normalized with respect to *gyrB* transcription level and *pvl*
3 expression levels in mice treated with either imipenem or cefoxitin were expressed as
4 n-fold difference relative to *pvl* expression level in animals treated with PBS. Values
5 are means ± standard deviations. Experiments were conducted as triplicates on lung
6 samples from five different mice treated with imipenem, cefoxitin or PBS respectively.
7 Change in mRNA level was interpreted as significant if superior to 2-fold variation.

8

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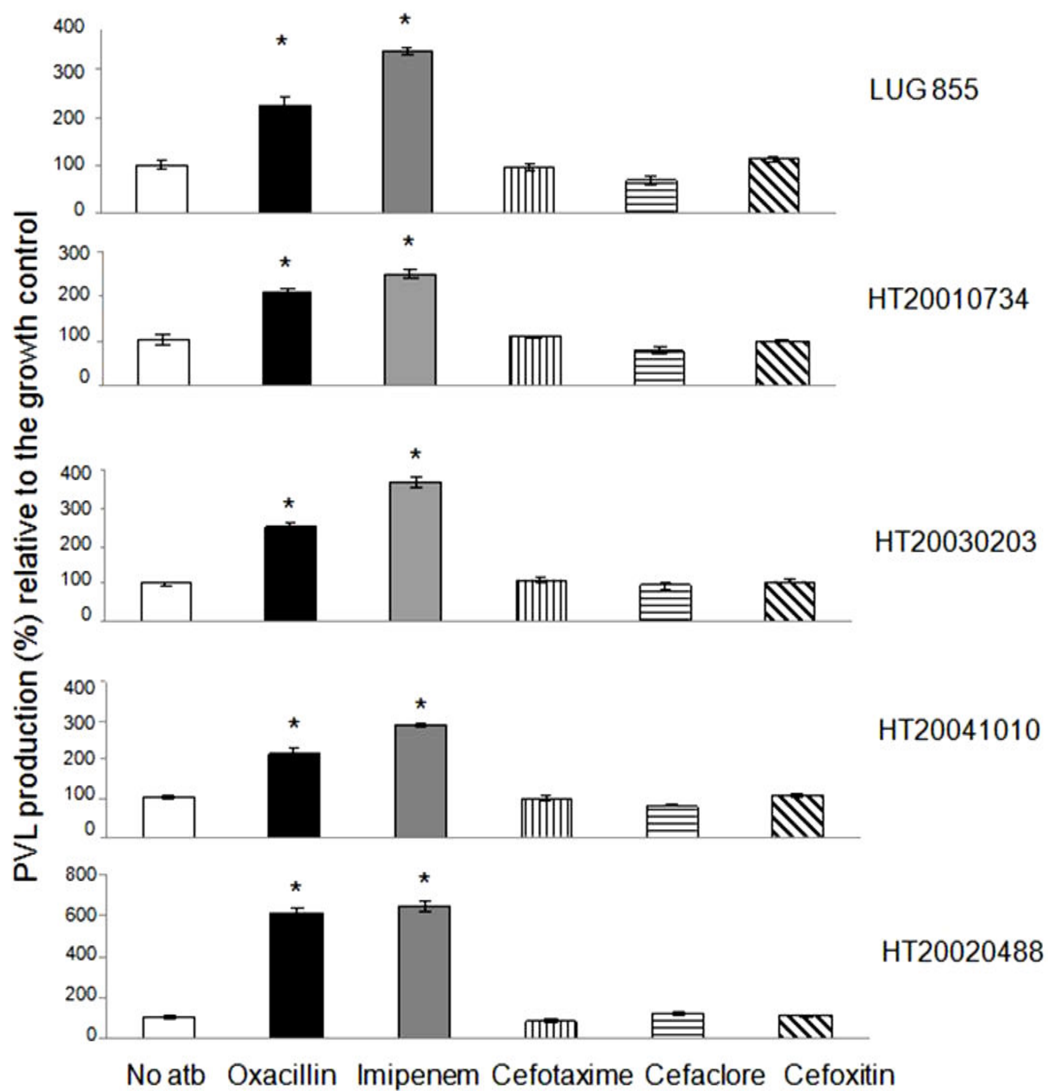
1 **Figure 1**



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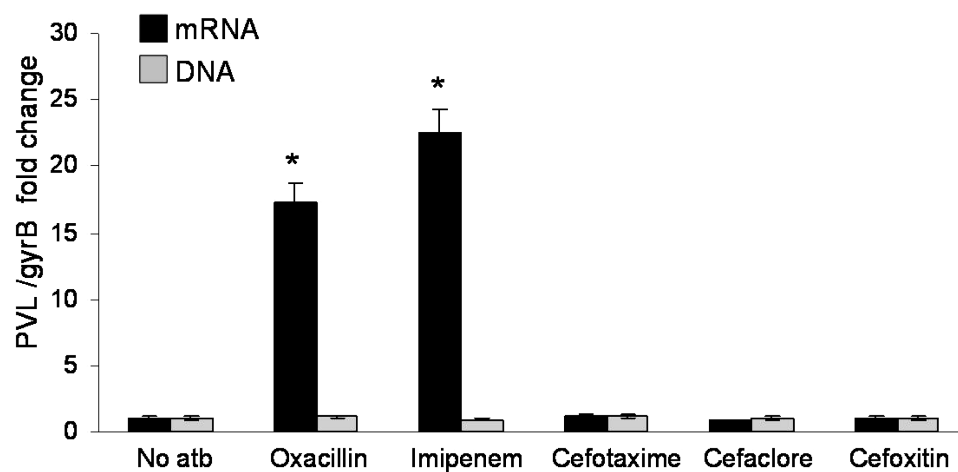
1 **Figure 2**



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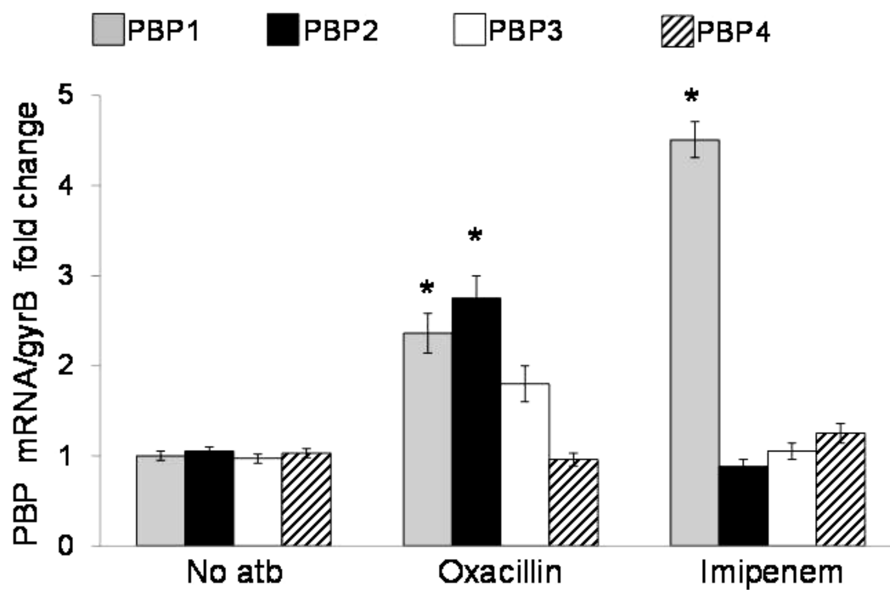
5 **Figure 3**

6



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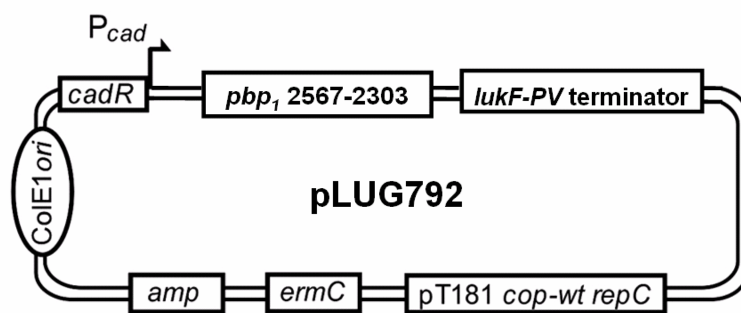
1 **Figure 4A**



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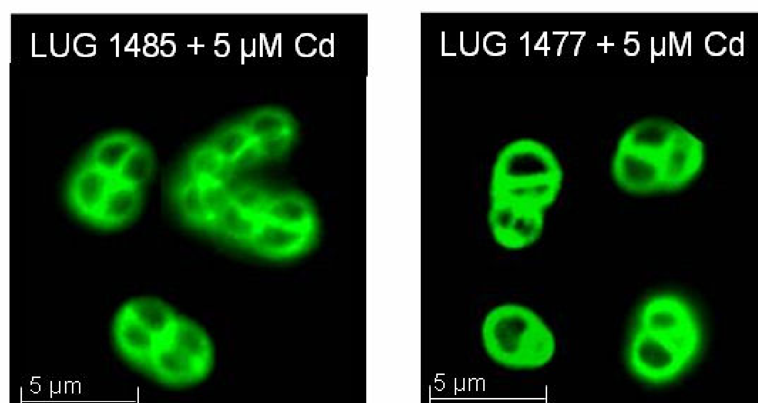
5 **Figure 4B**



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1 **Figure 5**

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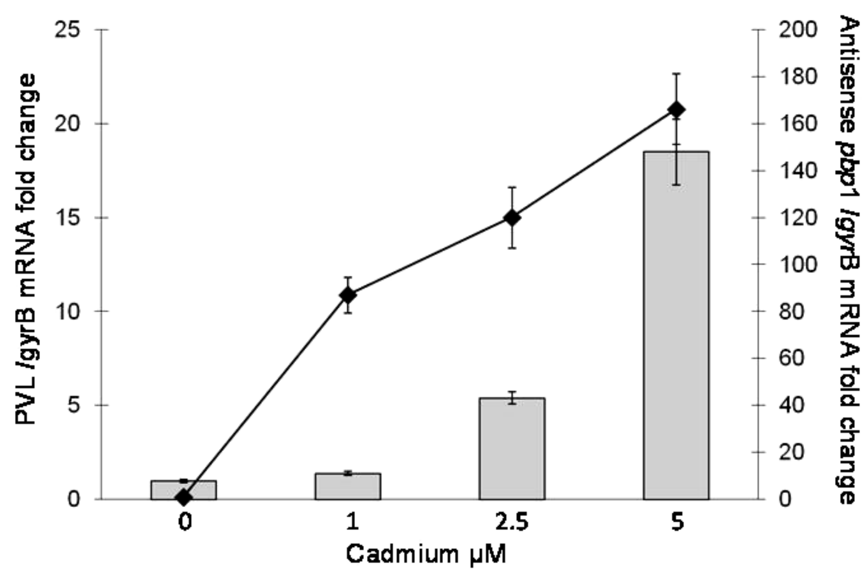

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1 **Figure 6**

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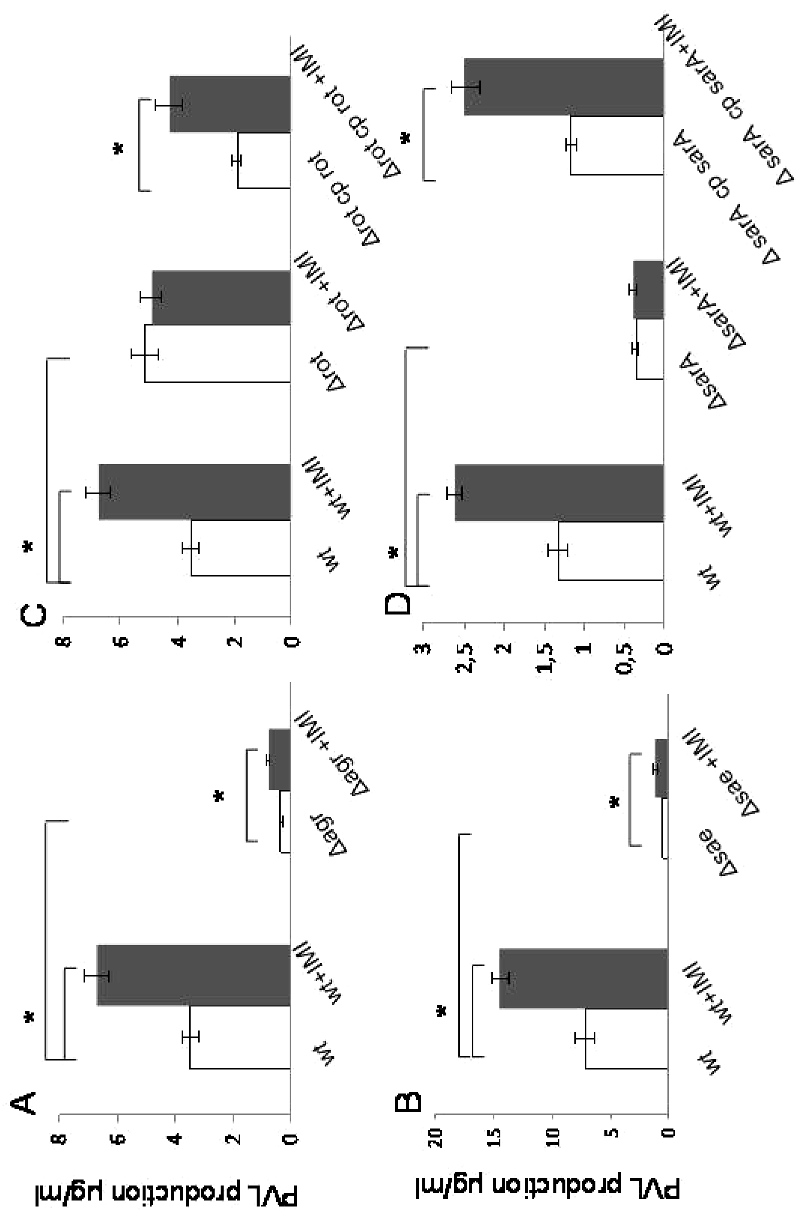

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1 **Figure 7**

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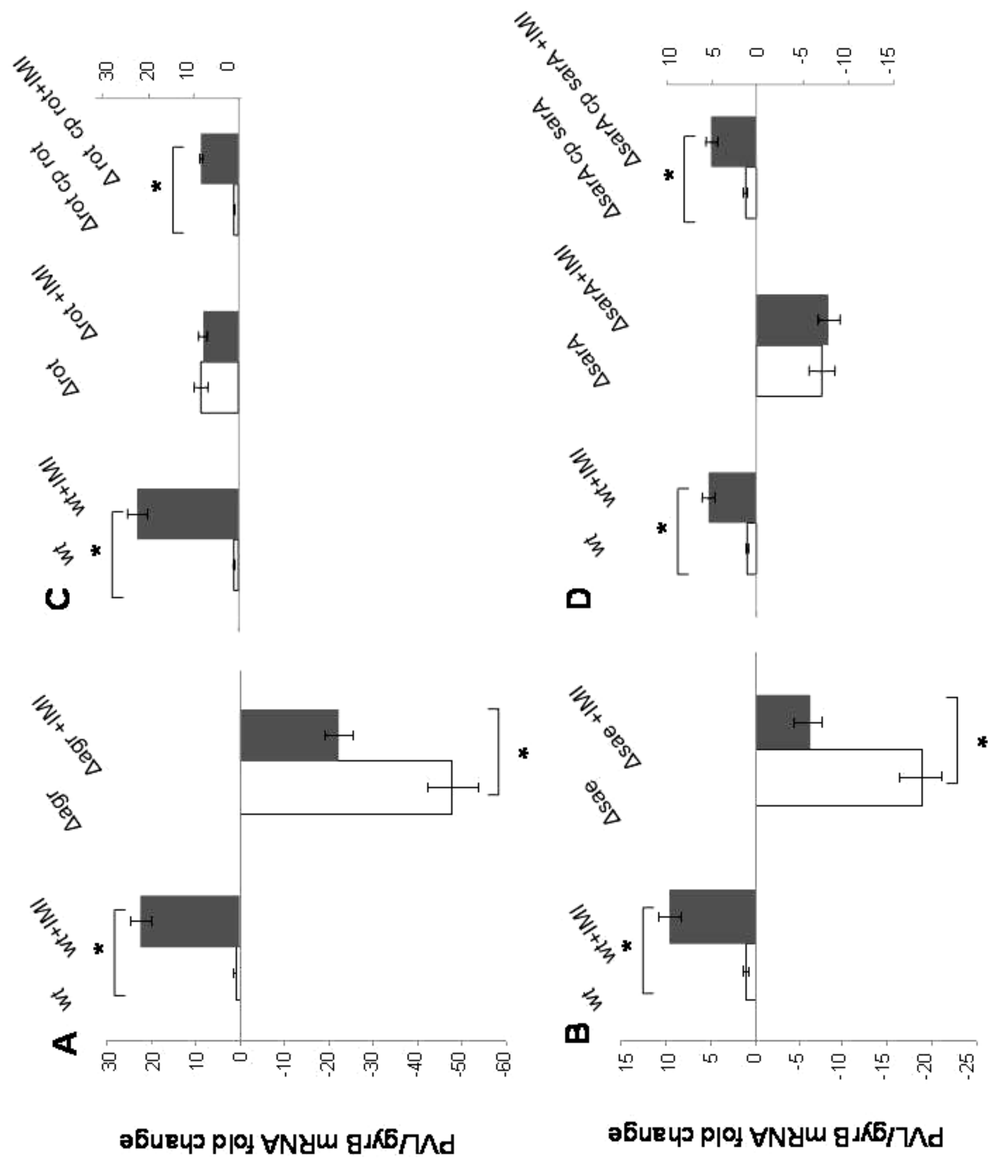

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1 **Figure 8**

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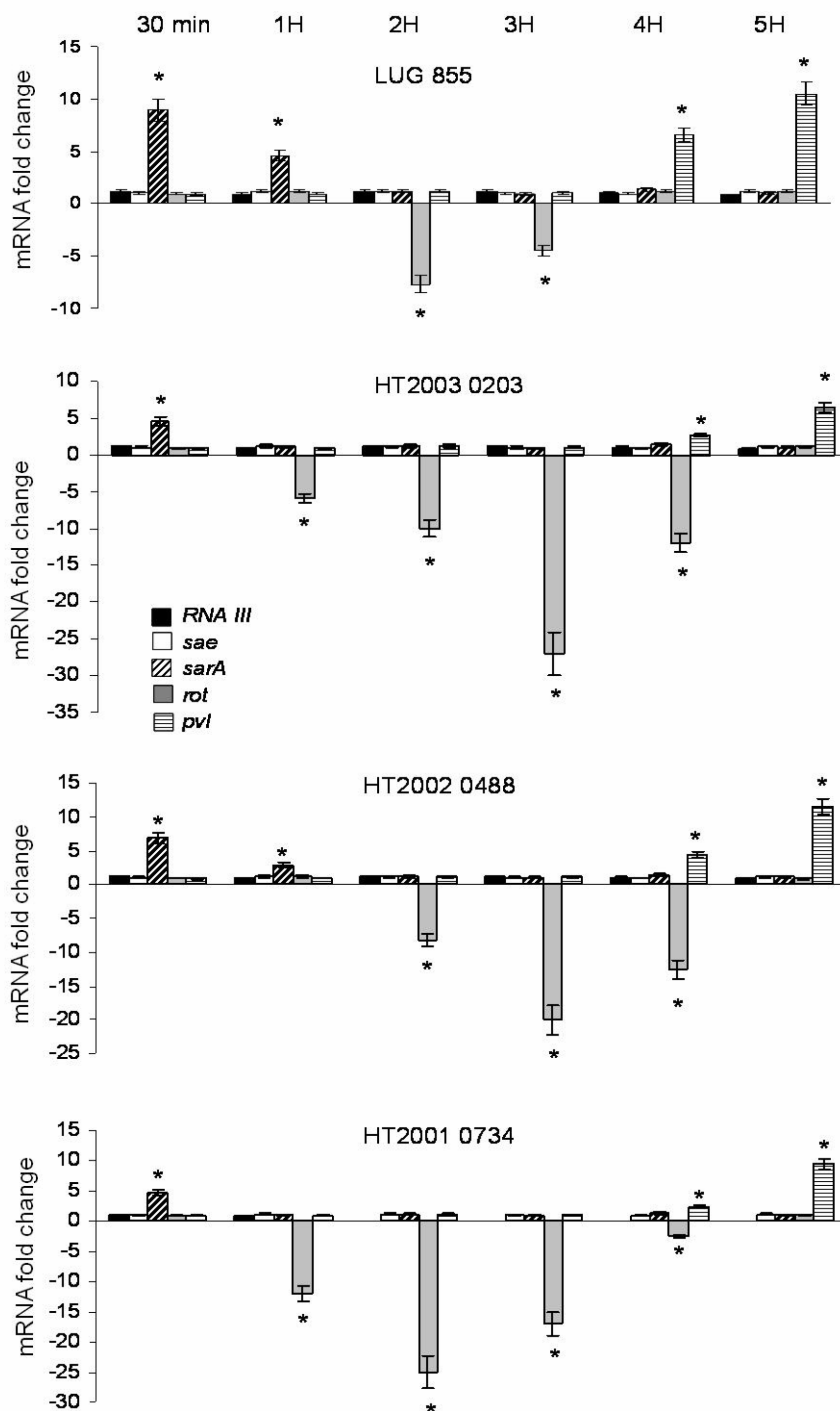

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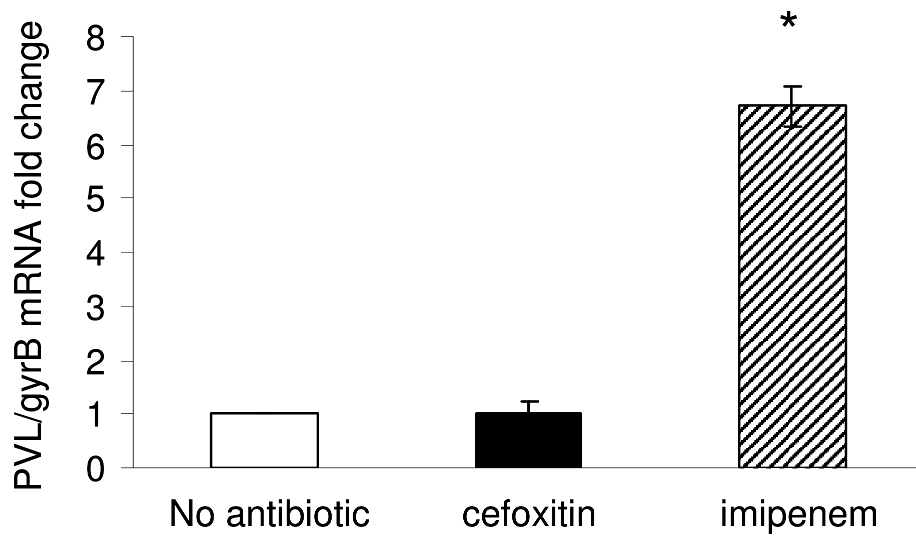
Figure 9



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