Effect of Antibiotics on *Staphylococcus aureus* Producing Panton-Valentine Leukocidin

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We examined the capacity of *Staphylococcus aureus* strains to release Panton-Valentine leukocidin (PVL) in the presence of antibiotics. No PVL was detected when *S. aureus* was incubated at inhibitory concentrations, while subinhibitory concentrations of oxacillin enhanced the PVL level; clindamycin, linezolid, and fusidic acid were inhibitory; and vancomycin had roughly no effect.

*Staphylococcus aureus* is an important human pathogen. It expresses a variety of exoproteins, including Panton-Valentine leukocidin (PVL) (31). While Voyich et al. could not establish clear differences in virulence between isogenic pairs of PVL-positive/negative strains (29), Labandeira-Rey et al. clearly demonstrated the role of PVL as a major determinant of virulence in an acute pneumonia mouse model using other sets of isogenic strains for PVL (13) and thus confirmed the results of the princi ples experiments showing that PVL is a virulence factor (15). The apparent discrepancy between these studies basically comes from the choice of the experimental models and the choice of the strains.

PVL is now frequently detected in clinical practice, as it is produced by community-acquired methicillin-resistant *S. aureus* (CA-MRSA) clones currently spreading throughout the world (27). PVL has been linked to specific human *S. aureus* infections such as primary skin and soft tissue disease and severe necrotizing pneumonia, where the mortality rate is about 75% (10, 14). Several lines of evidence incriminate PVL in necrotizing pneumonia pathogenesis, including the strong epidemiological link with PVL-producing *S. aureus* isolates (10) and the immunodetection of PVL in the lung (9) and that solely PVL-producing *S. aureus* isolates are able to reproduce necrotizing pneumonia in experimental models (13). Antibiotics that inhibit PVL production may be more appropriate for the treatment of severe necrotizing pneumonia, by analogy with their use in streptococcal toxic shock syndrome (8, 25, 26).

We examined the effect of antibiotics on PVL release by methicillin-sensitive *S. aureus* and CA-MRSA strains in vitro. We chose a reference strain lysogenized by phage phiSLT (encoding luk-PV) and five isolates representing the main PVL-producing CA-MRSA clones (Table 1) (23, 24, 27, 30). We intended to use experimental procedures as close as possible to Clinical Laboratory Standards Institute (CLSI) recommendations for MIC determinations in terms of the culture medium, bacterial inoculum, and growth conditions in order to be able to extrapolate our results to the clinical setting (20). The PVL concentration was determined in culture supernatants by using a specific solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (Agro-Bio; bioMérieux). Unfortunately, when using Mueller-Hinton (MH) medium and CLSI conditions, the PVL level was close to the detection limit in the absence of antibiotics (data not shown). MH medium was thus replaced by casein hydrolysate and yeast extract (CCY) medium, which increases PVL levels (32). The PVL level was 50 times higher in CCY than in MH medium (data not shown), while the MICs obtained with the two media were of the same order (Table 2), except with gentamicin, which dramatically increased (632-fold). CCY medium was therefore used in the rest of the study, and gentamicin was excluded.

### TABLE 1. Strains, plasmid, and phage

<table>
<thead>
<tr>
<th>Strain, plasmid, or phage</th>
<th>Reference or source</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><em>S. aureus</em> strains</td>
<td></td>
<td></td>
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<tr>
<td>RN6390</td>
<td>22</td>
<td>Laboratory strain that maintains its hemolytic activity when propagated on sheep erythrocyte agar (parental strain)</td>
</tr>
<tr>
<td>LUG855</td>
<td>This study</td>
<td>RN6390 phiSLT</td>
</tr>
<tr>
<td>LUG1124</td>
<td>This study</td>
<td>plUG547</td>
</tr>
<tr>
<td>HT200010734</td>
<td>18</td>
<td>ST1; agr3 mecA (-) lukS-PV lukF-PV(+)</td>
</tr>
<tr>
<td>HT200020488</td>
<td>27</td>
<td>ST80; agr1 mecA (-) lukS-PV lukF-PV(+)</td>
</tr>
<tr>
<td>HT200030203</td>
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<td>ST8; agr1 mecA (-) lukS-PV lukF-PV(+)</td>
</tr>
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<td>HT200040332</td>
<td>30</td>
<td>ST9; agr1 mecA (-) lukS-PV lukF-PV(+)</td>
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<tr>
<td>HT200410101</td>
<td>23</td>
<td>ST80; agr3 mecA (-) lukS-PV lukF-PV(+)</td>
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<tr>
<td>Plasmid</td>
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<td></td>
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<tr>
<td>pLUG547</td>
<td>This study</td>
<td>Derivative of pTCV-lac containing the lukS lukF-PV promoter (nucleotide (\sim 480) to the start codon) fused to lacZ</td>
</tr>
<tr>
<td>Phage phiSLT</td>
<td>19</td>
<td>lukS lukF-PV(+)-containing phage isolated from A980470</td>
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</table>

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To examine the influence of antibiotics on PVL release, PVL was quantified in the culture supernatant of *S. aureus* LUG855 incubated with various concentrations of oxacillin, vancomycin, clindamycin, and linezolid for 24 h. As shown in Fig. 1A, no PVL was detected when bacteria were incubated with inhibitory concentrations of oxacillin, clindamycin, fusidic acid, linezolid, or vancomycin. This could be explained by the fact that PVL production requires bacterial growth (3).

PVL is associated with intense necrosis in vivo, possibly leading to poor antibiotic diffusion and suboptimal concentrations at sites of infection (4). We therefore examined the effect of subinhibitory antibiotic concentrations on PVL. PVL levels released by LUG855 depended on the antibiotic and the concentration used (Fig. 1A). Clindamycin and linezolid induced concentration-dependent decreases in PVL levels from one-eighth the MIC, while it was significantly increased (up to threefold) at one-eighth and one-quarter the MIC with oxacillin and was unmodified using sub-MIC concentrations of vancomycin. As LUG855 is highly sensitive to fusidic acid, the latter antibiotic was not tested for its effect on PVL release.

To confirm these results, experiments were reproduced using five different CA-MRSA isolates (Table 1 and Fig. 1B). Linezolid induced a concentration-dependent decrease in the PVL level from one-eighth the MIC (four of five isolates) and from one-quarter the MIC (all isolates) to the MIC. Clindamycin, tested on the two susceptible strains, induced a strong concentration-dependent decrease in PVL levels from one-eighth the MIC to the MIC. Again, PVL release by the CA-MRSA isolates increased in the presence of all subinhibitory oxacillin concentrations, by 2- to 6.5-fold. With vancomycin, PVL levels were unmodified except with HT20020488 at one-quarter and one-eighth the MIC and HT20010734 at one-half the MIC, which decreased and increased PVL release, respectively. Fusidic acid, tested on isolates with MICs higher than

![FIG. 1. Effect of antibiotics on PVL. *S. aureus* LUG855 (A) and *S. aureus* strains HT20010734, HT20020488, HT20030203, HT20040332, and HT20041010 (B) were incubated in CCY medium with or without antibiotics (at 1, [1/2], [1/4], and [1/8] MIC), according to standard CSLI procedures, for 25 h at 37°C without shaking. Samples were taken for bacterial counting (plate counting of colonies from diluted broth) and PVL quantification by ELISA. Results are ratios of μg of PVL/log_{10} CFU of bacteria cultured with the indicated concentrations of antibiotic by means of μg of PVL/log_{10} CFU of bacteria cultured without antibiotic and expressed as percentage values. Values are means ± standard deviations (five different experiments in panel A and three different experiments in panel B). *, 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. ND, not determined.]
0.03 μg/ml, induced a concentration-dependent decrease in the PVL level.

To examine the effect of antibiotics on PVL gene transcription, S. aureus LUG1124 (containing the plasmid-borne luk-PV promoter fused to the lacZ gene described in Table 1) was cultured with or without oxacillin, vancomycin, or linezolid at one-eighth, one-quarter, and one-half the MIC and assayed for β-galactosidase activity. Samples were adjusted to an optical density at 600 nm of 1 before cell lysis with the FastPrep instrument (QBiogen). Protein concentrations and β-galactosidase activity were determined in the lysates by using the Bradford method (1) and the Beta-Glo system (Promega), respectively. As shown in Fig. 2, β-galactosidase activity was significantly enhanced, by 3- to 20-fold, by oxacillin at sub-MIC
concentrations, reflecting luk-PV promoter activation. It was higher than expected with ELISA quantification, but we used a nonlinear luminometric method to quantify β-galactosidase activity. By contrast, LacZ expression was strongly reduced by clindamycin, and fusidic acid administration could improve the outcome of severe infections due to PVL-producing S. aureus strains.

In conclusion, subinhibitory concentrations of oxacillin enhanced PVL levels by all the isolates through PVL promoter activation as previously observed for S. aureus alpha-hemolysin (21). How oxacillin enhances luk-PV transcription remains to be determined. We could hypothesize the involvement of SOS pathway stimulation by β-lactams (16) and those of response regulatory pathways engaged in peptidoglycan synthesis (7, 12). By contrast, subinhibitory concentrations of clindamycin, linezolid, and fusidic acid significantly reduced PVL release. This was not explained by the impact of these antibiotics on bacterial growth because PVL was detectable at the cell density achieved (data not shown). These antibiotics have previously been shown to reduce the production of several other toxins (2, 5, 6, 8, 25, 26, 28), possibly through their impact on bacterial protein synthesis and transcription (11, 17).

These data showing that subinhibitory antibiotic concentrations can either up-regulate or down-regulate PVL release by S. aureus may have therapeutic implications. It provides a logical basis for future studies to examine whether linezolid, clindamycin, or fusidic acid administration could improve the outcome of severe infections due to PVL-producing S. aureus strains.

References

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FIG. 2. Variation of lukS-PV lukF-PV gene transcription induced by subinhibitory concentrations of antibiotics. S. aureus LUG1124 containing a plasmid-carried luk-PV promoter-lacZ fusion was grown during 24 h at 37°C with or without one-eighth, one-quarter, and one-half the MIC of oxacillin, vancomycin, and linezolid and assayed for β-galactosidase activity. β-Galactosidase activity is expressed as a ratio of arbitrary units per milligram of bacterial protein cultured with the indicated concentration of antibiotic by arbitrary units per milligram of bacterial protein cultured without antibiotic. Values are means ± standard deviations (three different experiments). *, statistically different from control (LUG1124 grown without antibiotics), with a P value of <0.05, by one-way analysis of variance followed by a posteriori Dunnett’s test.