Effects of subinhibitory concentrations of antibiotics on virulence factor expression by community-acquired methicillin-resistant Staphylococcus aureus

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Objectives: To examine the effect of subinhibitory concentrations (sub-MICs) of antistaphylococcal drugs on Panton–Valentine leucocidin (PVL), α-haemolysin (Hla) and protein A (SpA) expression by community-acquired methicillin-resistant Staphylococcus aureus (CA-MRSA).

Methods: Five clinical isolates representing the main worldwide CA-MRSA clones were grown with sub-MICs (1/8, 1/4 and 1/2 MIC) of five antibiotics (clindamycin, daptomycin, linezolid, tigecycline and vancomycin). After 4 and 6 h of incubation, culture pellets were used for relative quantitative RT–PCR with primers specific for pvl, hla, spa and gyrB. The PVL, Hla and SpA concentrations were measured in the supernatant (for PVL and Hla) and in the cell pellet (for SpA) using specific ELISAs.

Results: For all strains tested, clindamycin and linezolid dramatically reduced mRNA levels of PVL and SpA. Tigecycline also decreased the PVL and SpA mRNA levels of 3/5 and 4/5 strains tested, respectively, whereas daptomycin and vancomycin had no significant effect. PVL and SpA quantification confirmed the concentration-dependent inhibition of PVL and SpA production by clindamycin and, to a lesser extent, by linezolid and tigecycline. Only clindamycin decreased Hla mRNA expression, whereas linezolid, tigecycline and daptomycin showed heterogeneous strain-dependent results, and vancomycin had no significant effect. Analysis of the Hla level revealed a stronger concentration-dependent inhibition of Hla release by clindamycin than by linezolid.

Conclusions: The effect of sub-MICs on virulence expression depended on the antibiotic and the virulence factor. Clindamycin and linezolid consistently suppressed the expression of different virulence factors by CA-MRSA, whereas tigecycline specifically suppressed PVL expression. Daptomycin and vancomycin seem to have no significant effects at these concentrations.

Keywords: protein A, α-haemolysin, leucocidin, PVL

Introduction

Staphylococcus aureus is an important Gram-positive human pathogen that expresses a variety of virulence factors. Among these factors, protein A (SpA) plays an important role in interfering with host defences, whereas exoproteins such as α-haemolysin (Hla) and Panton–Valentine leucocidin (PVL) are pore-forming toxins that possess cytolytic properties. Hla and SpA are expressed by most S. aureus strains, and they have the ability to interact with host immune signalling, thus contributing to staphylococcal pathogenesis. PVL has been linked to specific types of human S. aureus infections, including primary skin and soft tissue infections and severe diseases such as necrotizing pneumonia, which has a high mortality rate, and recurrent complicated osteomyelitis. Moreover, PVL is frequently detected in clinical practice because it is produced by many community-acquired methicillin-resistant S. aureus (CA-MRSA) clones that are spreading throughout the world.

It has been shown previously that subinhibitory concentrations of some antibiotics influence the expression of virulence factors by S. aureus, which may affect the pathogenesis of infection. Based on several series of in vitro data, recent guidelines
recommend the use of antibiotics that inhibit the expression of virulence factors for the treatment of severe infections due to PVL-producing S. aureus.\textsuperscript{12} Therefore, the management of S. aureus infections has been hampered not only because of the increasing resistance to currently available antibiotics but also because of the need to modulate bacterial virulence to reach clinical efficacy.

In this work, we examined the in vitro effects of five antibiotics, including recently introduced anti-MRSA agents, on the expression of PVL, Hla and SpA by five clinical isolates representing the main PVL-producing CA-MRSA clones. We showed that the effects of subinhibitory concentrations on virulence factor expression depended on the antibiotic and the virulence factor. Clindamycin and linezolid consistently suppressed the expression of different virulence factors by CA-MRSA, whereas tigecycline specifically suppressed PVL expression of PVL, Hla and SpA by five clinical isolates representing the main PVL-producing CA-MRSA clones. We recommend the use of antibiotics that inhibit the expression of virulence factors for the treatment of severe infections due to PVL-producing S. aureus.\textsuperscript{12}

### Materials and methods

#### Bacterial strains

Five clinical isolates representing the main PVL-producing community-acquired methicillin-resistant S. aureus (CA-MRSA) clones were chosen for use in this study (Table 1).

#### Antibiotics and MIC determination

The antibiotics used in this study were clindamycin, daptomycin, linezolid, tigecycline and vancomycin. Linezolid, clindamycin and tigecycline were provided by Pfizer (Amboise, France), daptomycin was provided by Novartis Pharma (Rueil-Malmaison, France) and vancomycin was purchased from Sigma-Aldrich (L’Isle d’Abeau, France). MICs were determined with the standard microdilution method recommended by the CLSI\textsuperscript{13} in Mueller–Hinton (MH) broth (bioMérieux) supplemented with calcium (50 mg/L) and magnesium (12.5 mg/L). The medium was inoculated with $5 \times 10^8$ cfu/mL and incubated for 24 h at 37°C without shaking.

For PVL and Hla quantification, MICs were also determined in casein hydrolysate–yeast extract medium (CCY) inoculated with $5 \times 10^8$ cfu/mL and incubated for 24 h at 37°C without shaking.

#### Bacterial cultures

Strains were cultured on Trypticase blood agar plates, which were incubated overnight at 37°C. Isolated colonies were resuspended in MH broth supplemented with calcium (50 mg/L) and magnesium (12.5 mg/L) and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard corresponding to $10^8$ cfu/mL, as confirmed by bacterial counts. Cultures were performed at 37°C with gyration shaking (300 rpm). When the optical density (OD) reached a turbidity equivalent to that of a 2 McFarland standard, concentrations of 1/2, 1/4 and 1/8 of the MIC of each antibiotic were added to the glass culture tubes. Cultures with or without antibiotics (growth control) were reincubated at 37°C with shaking. Aliquots were taken 4 and 6 h later.

### Relative quantitative RT–PCR

Aliquots of 2 mL of each culture were centrifuged at 10000 g for 10 min. The pellets were washed with 0.5 mL of Tris buffer (10 mM) and centrifuged at 10000 g for 10 min. The OD at 600 nm was then adjusted to 0.75 in Tris buffer (10 mM). A 1.5 mL aliquot of OD-adjusted and washed bacterial suspension was centrifuged at 10000 g for 10 min, and the pellets were treated with lysostaphin (Sigma) at a final concentration of 200 $\mu$g/mL. The total RNA of the pellets was then purified using the Qiagen RNeasy Plus Mini Kit according to the manufacturer’s instructions. The RNA yield was assessed with a NanoDrop spectrophotometer, and 1 $\mu$L of total RNA was reverse transcribed using the Promega reverse transcription system with random primers as recommended by the manufacturer. The resulting cDNA was used as a template for real-time amplification (LightCycler 2.0; Roche) using the specific primers shown in Table S1 (available as Supplementary data at JAC Online). The relative amounts of amplicons specific for toxin genes (hla, spa, and sarA and rot) were determined by quantitative PCR relative to an internal standard (gyrB) as described elsewhere.\textsuperscript{14}

The expression levels of the investigated genes were expressed as n-fold variations of the hla, spa, sarA, rot and gyrB as recommended by the manufacturer. The resulting cDNA was used as a template for real-time amplification (LightCycler 2.0; Roche) using the specific primers shown in Table S1 (available as Supplementary data at JAC Online). The relative amounts of amplicons specific for toxin genes (hla, spa, sarA, rot and gyrB) were determined by quantitative PCR relative to an internal standard (gyrB) as described elsewhere.\textsuperscript{15}

The concentrations of Hla in the supernatant were determined using a specific sandwich-type ELISA (kindly provided by bioMérieux R&D Department, Marcy l’Étoile, France) as described elsewhere.\textsuperscript{15} The concentration of Hla in the supernatant was determined using a specific sandwich-type ELISA (kindly provided by GlaxoSmithKline Vaccines) targeting Hla with a solid-phase GlaxoSmithKline (GSK) monoclonal antibody anti-Hla. Detection of the antibody–antigen complex was performed with a GSK polyclonal rabbit anti-Hla antibody followed by a peroxidase-conjugated goat anti-rabbit antibody. The concentration of Hla in each sample was calculated using the linear regression method using a standard curve generated using an Hla reference solution (10 ng/mL).

#### PVL, Hla and SpA quantification

Strains were cultured in microplates with CCY broth at 37°C in the absence or presence of clindamycin, linezolid or tigecycline at 1/2 or 1/4 MIC. Aliquots of each culture were taken after 24 h and centrifuged at 10000 g for 10 min. The PVL level in the supernatant was quantified using a specific ELISA (kindly provided by bioMérieux R&D Department, Marcy l’Étoile, France) as described elsewhere.\textsuperscript{15} The concentration of Hla in the supernatant was determined using a specific sandwich-type ELISA (kindly provided by GlaxoSmithKline Vaccines) targeting Hla with a solid-phase GlaxoSmithKline (GSK) monoclonal antibody anti-Hla. Detection of the antibody–antigen complex was performed with a GSK polyclonal rabbit anti-Hla antibody followed by a peroxidase-conjugated goat anti-rabbit antibody. The concentration of Hla in each sample was calculated using the linear regression method using a standard curve generated using an Hla reference solution (10 ng/mL).

For the SpA assay, strains were grown to the exponential phase in MH broth supplemented with calcium (50 mg/L) and magnesium (12.5 mg/L) and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard corresponding to $10^8$ cfu/mL, as confirmed by bacterial counts. Cultures were performed at 37°C with gyration shaking (300 rpm). When the optical density (OD) reached a turbidity equivalent to that of a 2 McFarland standard, concentrations of 1/2, 1/4 and 1/8 of the MIC of each antibiotic were added to the glass culture tubes. Cultures with or without antibiotics (growth control) were reincubated at 37°C with shaking. Aliquots were taken 4 and 6 h later.

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Description</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2</td>
<td>27</td>
<td>ST1; agr3 mecA&lt;sup&gt;+&lt;/sup&gt; PVL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>USA400</td>
</tr>
<tr>
<td>SF8300</td>
<td>28</td>
<td>ST8; agr1 mecA&lt;sup&gt;+&lt;/sup&gt; PVL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>USA300</td>
</tr>
<tr>
<td>LUG1799</td>
<td>this study</td>
<td>ST10; agr3 mecA&lt;sup&gt;+&lt;/sup&gt; PVL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>European</td>
</tr>
<tr>
<td>ST20101282</td>
<td>this study</td>
<td>ST30; agr3 mecA&lt;sup&gt;+&lt;/sup&gt; PVL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>South-West Pacific</td>
</tr>
<tr>
<td>ST20101656</td>
<td>this study</td>
<td>ST39b; agr1 mecA&lt;sup&gt;+&lt;/sup&gt; PVL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>livestock-associated MRSA</td>
</tr>
</tbody>
</table>
Statistical analyses were performed using 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.

**Results**

**MICs**

The MICs of clindamycin, daptomycin, linezolid, tigecycline and vancomycin were determined following the CLSI recommendations. The results obtained for the CA-MRSA isolates are summarized in Table 2. The MICs obtained with the two media were of the same order of magnitude for all antibiotics except daptomycin, for which the MIC in CCY broth was 8–16 times higher than the MIC obtained in Ca/Mg-supplemented MH broth. Therefore, daptomycin was not included in the experiments assessing PVL and Hla production in CCY medium. All strains were susceptible to all antibiotics tested except for ST20101656, which was found to be resistant to clindamycin.

**Effects of antibiotics on virulence factor expression and release by CA-MRSA strains**

To examine the influence of subinhibitory concentrations (1/2, 1/4 and 1/8 MIC) of clindamycin, daptomycin, linezolid, tigecycline and vancomycin on PVL, Hla and SpA expression, we performed relative quantitative RT–PCR to assess the pvl/gyrB, hla/gyrB and spa/gyrB ratios in the cultures of five clinical CA-MRSA isolates grown with and without antibiotics for 4 and 6 h. Along with the antibiotics’ effects on mRNA expression, we also examined the influence of sub-MICs (1/2 and 1/4 MIC) of antibiotics on the overnight production of PVL and Hla in the supernatant and SpA in the bacterial pellet.

**Effects of antibiotics on PVL expression and release by CA-MRSA strains**

As shown in Figure 1 and Figure S1 (available as Supplementary data at JAC Online), the effects differed among antibiotics. Clindamycin, tested on the four susceptible strains, induced a strong concentration-dependent decrease in the PVL mRNA level from 1/8 MIC to 1/4 MIC and 1/2 MIC after both 4 and 6 h of incubation (from 2.20-fold to 11.76-fold depending on the strain).

Table 2. MICs of selected antibiotics for CA-MRSA isolates in MH broth and CCY broth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clindamycin</th>
<th>Daptomycin</th>
<th>Linezolid</th>
<th>Tigecycline</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW2</td>
<td>0.125/0.125</td>
<td>0.5/4</td>
<td>2/1</td>
<td>0.25/0.125</td>
<td>1/1</td>
</tr>
<tr>
<td>SF8300</td>
<td>0.125/0.125</td>
<td>0.5/4</td>
<td>2/1</td>
<td>0.125/0.0625</td>
<td>1/2</td>
</tr>
<tr>
<td>LUG1799</td>
<td>0.125/0.125</td>
<td>0.5/8</td>
<td>2/1</td>
<td>0.125/0.125</td>
<td>1/1</td>
</tr>
<tr>
<td>ST20101282</td>
<td>0.25/0.125</td>
<td>0.25/4</td>
<td>2/1</td>
<td>0.25/0.125</td>
<td>1/1</td>
</tr>
<tr>
<td>ST20101656</td>
<td>&gt;128/&gt;128</td>
<td>0.5/8</td>
<td>2/1</td>
<td>0.5/0.125</td>
<td>1/2</td>
</tr>
</tbody>
</table>

Linezolid also induced a concentration-dependent decrease in the PVL mRNA level after both 4 and 6 h of incubation (from 2.00-fold to 11.26-fold depending on the strain). However, the decrease was consistently observed only for concentrations of 1/2 MIC. When compared with clindamycin, linezolid was less effective at low concentrations, in particular against the LUG1799 and ST20101656 strains.

Tigecycline induced a decrease in the PVL mRNA levels for three of the five strains tested as follows: a 2.02- to 15.38-fold decrease after 4 h of incubation for the ST20101282 and ST20101656 strains and a 2.20-fold decrease after 6 h of incubation for the MW2 strain. By contrast, a slight increase in the PVL mRNA level was observed with tigecycline for LUG1799 strain after 6 h of incubation.

Daptomycin and vancomycin induced isolated significant effects (3.7- and 5.8-fold increase for MW2 strain with 1/2 MIC and 1/4 MIC of vancomycin), but as a whole these two antibiotics had no relevant effects on the PVL mRNA level.

Concerning PVL release (Figure 2 and Figure S2 (available as Supplementary data at JAC Online)), clindamycin, linezolid and tigecycline induced a concentration-dependent decrease in PVL production from 1/4 MIC to 1/2 MIC. Considering all strains together, significant effects were observed as follows: clindamycin reduced PVL release by 67% at 1/4 MIC and by 81% at 1/2 MIC, linezolid reduced PVL release by 46% at 1/4 MIC and by 57% at 1/2 MIC, and tigecycline reduced PVL release by 61% at 1/2 MIC. The decrease induced by tigecycline, though concentration-dependent, was only significant at the highest concentration tested (1/2 MIC).

**Effects of antibiotics on Hla expression and release by CA-MRSA strains**

As shown in Figure 3 and Figure S3 (available as Supplementary data at JAC Online), the effects differed among antibiotics, and the effects on the LUG1799 strain were different from the effects on the other strains.

Except for the LUG1799 strain, clindamycin induced a decrease in the Hla mRNA level after 4 and 6 h of incubation (from 2.38-fold to 20.00-fold) in susceptible strains.

Linezolid induced a significant decrease in the Hla mRNA level in the SF8300 and ST20101282 strains after both 4 and 6 h of incubation (from 2.12-fold to 7.41-fold). A strong concentration-dependent decrease in the Hla mRNA level was observed with tigecycline for LUG1799 strain after 6 h of incubation.

Concerning PVL release [Figure 2 and Figure S2 (available as Supplementary data at JAC Online)], clindamycin, linezolid and tigecycline induced a concentration-dependent decrease in PVL production from 1/4 MIC to 1/2 MIC. Considering all strains together, significant effects were observed as follows: clindamycin reduced PVL release by 67% at 1/4 MIC and by 81% at 1/2 MIC, linezolid reduced PVL release by 46% at 1/4 MIC and by 57% at 1/2 MIC, and tigecycline reduced PVL release by 61% at 1/2 MIC. The decrease induced by tigecycline, though concentration-dependent, was only significant at the highest concentration tested (1/2 MIC).
Tigecycline induced a decrease in the Hla mRNA levels for only the MW2 and ST20101282 strains at 1/8 MIC and 1/4 MIC after 4 and 6 h of incubation (from 2.13-fold to 8.70-fold depending on the strain). By contrast, tigecycline had no significant effect on the Hla mRNA levels for the SF8300 and ST20101656 strains.

Daptomycin induced only a slight decrease in the Hla mRNA level for the ST20101282 and SF8300 strains (ranging from 2.35-fold to 3.85-fold) and had no significant effect on the Hla mRNA level for the MW2 and ST20101656 strains. With vancomycin, no relevant modification in the HLA mRNA level was observed for the MW2, SF8300, ST20101656 and ST20101282 strains.

In the LUG1799 strain, daptomycin, linezolid, tigecycline and vancomycin induced a strong increase in the Hla mRNA level at 1/8 MIC after 6 h of incubation (from 3.15-fold to 9.74-fold depending on the antibiotic). With clindamycin, we only observed one significant variation: a 2.45-fold decrease in Hla mRNA level after 6 h of incubation with 1/2 MIC. Concerning Hla release [Figure 4 and Figure S4 (available as Supplementary data at JAC Online)], strains behaved more homogeneously. Clindamycin induced a strong decrease in Hla production by the four susceptible strains tested: considering all strains together, Hla release was lowered by 62% at 1/4 MIC and by 82% at 1/2 MIC. Linezolid also significantly decreased the Hla production of all tested strains at 1/2 MIC: Hla release was reduced by 51% for the five strains when considered together. By contrast, both linezolid and tigecycline at 1/4 MIC did not modify Hla release by the tested isolates, whereas...
tigecycline at 1/2 MIC decreased the Hla production of the SF8300 and ST20101282 strains (2.22- and 1.36-fold, respectively). Considering all strains together, tigecycline did not modify Hla release.

Effects of antibiotics on SpA expression and production by CA-MRSA strains

As shown in Figure 5, Figure S5 (available as Supplementary data at JAC Online), Figure 6 and Figure S6 (available as Supplementary data at JAC Online), the effects differed among the antibiotics.

Clindamycin, tested on the four susceptible strains, induced a strong concentration-dependent decrease in the SpA mRNA levels from 1/8 MIC to 1/4 MIC and 1/2 MIC after both 4 and 6 h of incubation (from 2.11-fold to 50.00-fold). At the protein level, SpA production was decreased from 2.15- to 10-fold depending on the strain. SpA production was lowered by an average of 69.8% by the four strains (Figure 6).

Linezolid also induced a concentration- and time-dependent decrease in the SpA mRNA level, mainly after 6 h of incubation (from 2.00-fold to 5.71-fold depending on the strain). As assessed by ELISA in linezolid-treated cultures, SpA production was decreased from 2- to 9.57-fold depending on the strain. On average for the five strains, SpA production was reduced by 25.2% (Figure 6).

Concerning tigecycline treatment, we observed variations among the tested isolates. A significant inhibitory effect of tigecycline on SpA mRNA expression was only observed for the ST20121656 strain after 4 h incubation with 1/2 MIC and 1/4 MIC.
The SpA levels in the bacterial pellets showed that SpA production was reduced not only for the ST20101656 strain (6-fold decrease) but also for the LUG1799, MW2 and ST20101282 strains (1.53-, 5- and 3.84-fold, respectively) (Figure S6, available as Supplementary data at JAC Online). However, when considering all strains together, tigecycline did not significantly decrease the SpA level in the bacterial pellets.

After daptomycin or vancomycin treatment, both the SpA mRNA and SpA protein levels were unmodified, regardless of the strain and the concentration tested (Figure 5, Figure S5 (available as Supplementary data at JAC Online), Figure 6 and Figure S6 (available as Supplementary data at JAC Online)).

**Effects of tigecycline on the expression of regulatory genes**

To explain the discrepancies in the variations of the expression levels of the toxin (PVL and Hla) mRNAs and proteins among the different isolates upon tigecycline treatment, we studied the expression of the major virulence regulatory genes in S. aureus (agr, saeP, sarA and rot). As shown in Figure 7, 6 h of
incubation with 1/4 MIC of tigecycline induced an increase in the RNAIII level for the strains SF8300 and LUG1799 (6.11- and 4.17-fold, respectively). The saeP mRNA level also increased in these strains (4.03- and 2.48-fold, respectively). None of these regulators was modulated by tigecycline in the MW2 strain. Tigecycline did not modify rot or sarA expression in any strain.

Discussion

S. aureus produces virulence factors that play an important role in the pathogenesis of infection. Recent studies have shown that subinhibitory concentrations of antibiotics modulate the expression of virulence factors in S. aureus and therefore may impact the outcome of severe staphylococcal infections. Thus, the efficacy of antibiotic treatment for S. aureus infections might depend not only on the bacteriostatic or bactericidal effects of the antibiotic but also on its capacity to prevent the release of virulence factors.9–11,16,17

The increasing incidence of bacterial infections due to MRSA and the development of resistance to classical anti-MRSA agents (such as vancomycin) have reduced the number of therapeutic options. Therefore, several new antistaphylococcal agents, such as linezolid, daptomycin and tigecycline, have been introduced in recent years and been promoted for MRSA treatment. In our study, we investigated the effects of five antistaphylococcal drugs, including three recently introduced anti-MRSA agents (linezolid, daptomycin and tigecycline), on the expression of PVL, Hla and SpA by CA-MRSA strains. Five clinical isolates representing the main CA-MRSA clones were chosen for this study.

We intended to use experimental procedures as close as possible to the CLSI recommendations for MIC determination in terms of culture medium, bacterial inoculum and growth conditions to be able to extrapolate our results to the clinical setting. MH supplemented with Ca and Mg was used as the culture medium for all experiments in this study except for experiments involving specific ELISAs for PVL and Hla that required CCY medium, as previously described.16,17

CA-MRSA infection is associated with intense necrosis in vivo, which most likely leads to the poor diffusion of antibiotics, resulting in subinhibitory concentrations at the sites of infection. Therefore, the testing of antibiotics at subinhibitory concentrations not only allows the study of their effects on virulence factors but also provides an in vitro basis for understanding the events that occur during treatment for CA-MRSA infection.

We explored the effects of antibiotics on PVL, Hla and SpA mRNA levels, and we found that subinhibitory concentrations of clindamycin decreased the levels of the three mRNAs studied. This observation was consistent with the specific ELISA results, which showed decreased PVL, Hla and SpA production in all strains tested. As an inhibitor of protein synthesis, clindamycin is expected to decrease the production of staphylococcal exoproteins, primarily by blocking mRNA translation at the level of the ribosome. However, we also observed a decrease in the mRNA level upon clindamycin treatment, suggesting a possible inhibitory effect of clindamycin on the transcription of virulence factor genes. Our results are consistent with previous reports by Herbert et al.18 supporting the idea that clindamycin specifically interferes with one or more virulence factor regulators. Although these regulators have not yet been clearly identified, there is evidence suggesting that the sae system could be involved, as it has been shown that sub-MICs of clindamycin interfere with the promoter switch required for a full functional sae system.19

Linezolid reduced the mRNA and protein expression of PVL and SpA, though to a lesser extent than clindamycin. These results are consistent with previous reports by us and other authors.9,10,16,17 Moreover, upon linezolid treatment, Hla expression was decreased at the mRNA level for two strains, whereas Hla release was reduced for all tested strains, suggesting that in the case of linezolid the inhibition of virulence expression is primarily due to the blockage of protein translation at the ribosome. Surprisingly, linezolid-treated LUG1799 cultures exhibited an enhanced Hla mRNA level, with an increase of up to 9.74-fold. This increase can be explained by the fact that 50S ribosomal-based inhibitors such as linezolid not only block peptidyl transferase activity but also inhibit proteases and RNases, thus resulting in the accumulation of the intermediate products of the translational complex.20 Though not detected in our setting, it has been previously reported that linezolid may also increase the expression of the pvl and enterotoxin genes at 72 h in a hollow-fibre infection model.21 By contrast, the PVL concentration was lowered during linezolid exposure in the same model. Therefore, and despite the increase in the levels of these toxin gene transcripts, we believe that, overall, linezolid inhibits the production of multiple virulence factors by CA-MRSA. Interestingly, LUG1799 showed increased hla expression also upon daptomycin, tigecycline and vancomycin treatment, which may raise the question of a strain-specific pattern of Hla modulation by these antibiotics. This question should be further addressed by using a panel of additional CA-MRSA ST80 strains.

In our setting, tigecycline only decreased the PVL mRNA levels for three of the five strains tested, with limited strain-dependent
inhibitor effects on the Hla and SpA mRNA levels. However, at the protein level tigecycline consistently reduced PVL release in all tested strains, whereas the reduced SpA production was strain-dependent. Tigecycline inhibits protein translation in bacteria by binding to the 30S ribosomal subunit and blocking the elongation of peptide chains. In this study, we showed for the first time that tigecycline specifically inhibits the expression of PVL and, to a lesser extent, SpA in CA-MRSA. Moreover, although the major mechanism involved is ribosomal translation blockage, tigecycline also inhibits PVL mRNA expression in some strains. Although tigecycline decreased PVL release by all tested strains, we observed increased PVL mRNA levels in the SF8300 and LUG1799 strains (2.35- to 3.2-fold increase). Likewise, in the LUG1799 strain the Hla mRNA level was increased up to 8.5-fold upon tigecycline treatment, but there was no overall impact on Hla production as assessed by ELISA. This result could be explained, as for linezolid, by an RNase inhibitor effect, although such an effect has never been previously reported for tigecycline. A more likely explanation could be provided by the expression levels of the major virulence regulatory genes in S. aureus (agr, saeP, sarA and rot), which differed among LUG1799, SF8300 and MW2. Indeed, upon tigecycline treatment, SF8300 and LUG1799 showed increased agr (RNAIII) and sae expression, which could account for the increased PVL and Hla mRNA levels observed in these strains. However, as the overall effect of tigecycline treatment is inhibitory, the blockage of ribosomal translation seems to overshadow the agr/sae activation in our setting. Our results are consistent with the work of Smith et al., showing that tigecycline reduces the expression of S. aureus staphylococcal toxic shock toxin TSS1-1 in an EMRSA-16 biofilm-associated model, whereas microarray analysis showed the up-regulation of several exotoxin-enhancing regulators, such as sarA and saeR.

Our results showed no significant effect of vancomycin on the PVL, Hla and SpA mRNA levels, consistent with several previous publications reporting vancomycin’s neutral effect on toxin release. Therefore, we did not further explore the effect of vancomycin on PVL and Hla release. Regarding the effect of daptomycin on toxin release, the PVL and Hla ELISAs could not be performed because of the loss of activity of daptomycin in CCY medium, which was required for this experiment. We found no published reports on the inhibitory effect of daptomycin on S. aureus toxin production. SpA measurements performed using the bacterial pellet showed no impact of daptomycin on SpA production.

In this study, we showed that subinhibitory concentrations of clindamycin or linezolid are able to inhibit the production of several virulence factors. Moreover, this inhibition most likely involves variations in the transcription of the target genes, as suggested by the modulation of the mRNA levels. This pleiotropic effect suggests a potential role of S. aureus global virulence regulators, which fine-tune virulence factor production in response to the changing environment. One of the regulators involved could be the sae system, which up-regulates the expression of exoprotein genes such as hla and pvl. Moreover, it has been suggested that the sae locus is involved in the control of spa expression, as SpA expression appears to be dramatically reduced in sae mutants. We have shown that tigecycline is able to diminish PVL production and, to a lesser extent, SpA expression by CA-MRSA. In our study, PVL was strongly and consistently inhibited by tigecycline relative to Hla or even SpA, suggesting that tigecycline may specifically inhibit PVL release. Further studies with a larger panel of isolates are necessary to confirm these findings and elucidate the mechanism involved. Whether tigecycline may be a useful adjunct therapy for the treatment of severe infections by PVL-producing strains remains to be determined. In conclusion, our observations suggest that clindamycin and linezolid can be used to target CA-MRSA virulence factors in severe infections. Tigecycline could become useful as an anti-PVL drug if our results are confirmed by future studies. Finally, vancomycin and daptomycin, both useful as anti-MRSA drugs, seem to be neutral with respect to toxin expression by CA-MRSA.

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Transparency declarations
None to declare.

Supplementary data
Table S1 and Figures S1 to S6 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References