Modulation of virulence gene expression by cell wall active antibiotics in Staphylococcus aureus

Natalia Subrt, Lili Rosana Mesak and Julian Davies*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC V6T 123 Canada

*Corresponding author. Department of Microbiology and Immunology, Life Science Institute, University of British Columbia, 2350 Health Science Mall, Vancouver, BC V6T 123 Canada. Tel: +1-604-822-9308; Fax: +1-604-822-6041; E-mail: jed@interchange.ubc.ca

Received 1 November 2010; returned 29 November 2010; revised 23 December 2010; accepted 26 January 2011

Objectives: To investigate the effect of subinhibitory concentrations of cell wall active antibiotics on virulence gene expression and biofilm formation in *Staphylococcus aureus* Newman and in laboratory strains.

Methods: Promoter regions of *spa*, *lukE* and *agr* RNAIII were cloned upstream of a modified *luxABCDE* reporter. Using disc diffusion assays, the effects of antibiotics were observed on gene expression and quantitative real-time PCR was employed to confirm the results. Assays were performed to measure biofilm formation in wild-type *S. aureus* and respective *spa*-deficient and small colony variant mutants in the presence of subinhibitory concentrations of antibiotics.

Results: Expression of *spa* and *lukE* was stimulated by subinhibitory concentrations of penicillin and cefalotin, while *agr* RNAIII expression was not affected. Denser biofilms were formed by *S. aureus* Newman and its small colony variant in the presence of subinhibitory concentrations of cefalotin.

Conclusions: Subinhibitory concentrations of certain antibiotics have been shown to stimulate virulence gene expression in *S. aureus*; this may alter the progression of infection and thus render antimicrobial therapy unreliable. The use of appropriate combinations of antibiotics might be an approach to avoiding this situation. Promoter-*lux* reporters are sensitive tools for studying the modulation of transcription by antibiotic inhibitors, and could be used to predict novel therapeutic combinations for the treatment of infection.

Keywords: transcription modulation, biofilm formation, antibiotics, pathogenesis, promoter-*lux* reporter

Introduction

*Staphylococcus aureus* is a Gram-positive pathogen that causes community-acquired and nosocomial infections ranging from carbuncles and food poisoning to acute endocarditis and necrotizing pneumonia.\(^1\)\(^2\)\) During antimicrobial therapy bacteria may be exposed to sub-MICs of antibiotics.\(^3\) Low doses of antimicrobial compounds modulate expression of genes encoding factors essential for housekeeping and metabolic functions, and also those necessary to establish an infection.\(^4\)\(^5\)\(^6\) Some 60 years ago, *S. aureus* was susceptible to all antibiotics, but in the intervening years strains of this pathogen (like others) have developed resistance to almost every available antimicrobial agent. Repeated chemical modification of active compounds, most particularly the β-lactam class, have provided derivatives effective in the short term, but the pathogen has rapidly evolved new resistance mechanisms with each new introduction. This may be due in part to the mutagenesis-stimulating activity of sub-MIC antibiotics; such exposure also enhances virulence gene expression and concomitant aggravation of disease caused by *S. aureus*.\(^6\) Here, we report studies examining the effects of low concentrations of antimicrobial compounds on virulence gene expression in *S. aureus*.

*S. aureus* virulence is multifactorial, involving the production of a variety of cell-bound and secreted proteins as well as the formation of biofilms. These processes are under the control of global regulators, especially the accessory gene regulon (*Agr*).\(^2\) *Agr* is a two-component signal transduction quorum-sensing system that acts, at increased bacterial cell densities, to produce a regulatory RNA molecule (RNAIII), which in turn inhibits expression of cell wall associated proteins and facilitates the production of exotoxins (Figure 1).\(^7\) *Staphylococcal* protein A (*Spa*), a cell wall anchored protein, prevents opsonophagocytosis and interacts with von Willebrand factor, mediating intravascular infection.\(^8\)\(^9\)\(^10\) Production of *Spa* is down-regulated by RNAIII through inhibition of *SarS*, a DNA-binding protein that normally activates transcription of *spa*.\(^11\) RNAIII also up-regulates expression of leukotoxins E and D, two secreted proteins that act in concert to bind to polymorphonucleocytes, open Ca\(^{2+}\)
channels and allow influx of Na\(^+\) and K\(^+\) ions, thus permitting S. aureus to evade the host immune system\(^{12,13}\). S. aureus has the ability to form small colony variants that produce decreased amounts of exotoxins, thus avoiding detection by the immune system\(^{14}\). Such variants are deficient in electron transport, a property that makes them resistant to aminoglycosides and allows them to persist inside the cell; they are believed to be one of the sources of recurrent staphylococcal infections\(^{15}\).

Staphylococcal cell wall functions are the major targets for antimicrobial therapy. In this study we investigated transcription modulation of spa, lukE and agr RNAIII in the presence of sub-MICs of a number of cell wall active antibiotics using the promoter–reporter fusions spa-lux, lukE-lux and agrP3-lux, respectively, in S. aureus hosts Newman, 8325-4, RN4220, ALC488 and ALC1927. We examined combinations of antimicrobials that might influence the production of virulence factors during antibiotic therapy and investigated the effects of antibiotics on biofilm formation in S. aureus Newman on its hemB-deficient mutant, which mimics the small colony variant phenotype, and also a spa-deficient mutant.

### Materials and methods

**Strains, plasmids and growth conditions used in this study**

S. aureus strains and E. coli DH10B were grown at 37°C in NYE [1% casein hydrolysate (Sigma), 0.5% yeast extract (Difco) and 0.5% NaCl (Fisher Scientific)] and Luria–Bertani (LB) [1% tryptone (Difco), 0.5% yeast extract (Difco) and 0.5% NaCl (Fisher Scientific)] media, respectively. S. aureus hosts for promoter-lux reporter fusions are listed in Table 1. Antibiotics were obtained from Sigma and antibiotic discs from Becton Dickinson (BD) and Difco.

**Construction of plasmids carrying the promoter-lux reporter fusions**

The nucleotide sequences for agrP3 and lukE were retrieved from the S. aureus NCTC 8325 genome database and oligonucleotide primers were prepared (Integrated DNA Technologies) to amplify selected promoters of interest (Table 2). PCR conditions were as follows: 94°C for 5 min (initial denaturation); 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 45 s; and 72°C for 5 min. The PCR products were digested with BamHI (New England Biolabs) and ligated into BamHI-digested and phosphatase-treated pAmilux\(^{16}\) using T4 DNA ligase (New England Biolabs). The ligation mix was transformed into E. coli DH10B and transformants selected on LB agar supplemented with 100 mg/L of ampicillin. The insert orientation was confirmed by sequencing using primers as shown in Table 2 and plasmid DNA, isolated from E. coli DH10B and S. aureus RN4220, was transformed into S. aureus RN4220 and other S. aureus strains, respectively, using standard procedures\(^{17}\). The S. aureus transformants were selected on NYE supplemented with 10 mg/L of chloramphenicol (NYEC).

**Disc diffusion assay**

A single colony of S. aureus on NYEC agar was resuspended in 50 μL sterile water and samples were mixed with 7 mL of 0.7% agar and poured as overlays on NYEC agar plates. Paper discs containing antibiotics were placed on the overlay and the plates were incubated at 37°C.

![Figure 1](image-url) Virulence gene regulation. (a) Agr system, transcribed as from agrP2 and agrP3 promoters. Transcription is stimulated by SarA and is also autoactivated by AgrA via quorum-sensing system; (b) Agr RNAIII down-regulates SarS, which results in down-regulation of spa transcription, and Agr RNAIII stimulates expression of lukED. SarS may also activate expression of lukED, which is indicated by the truncated arrow.
### Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-luxA</td>
<td>AGTCCGCTTCTCAGGAAGT</td>
<td>16</td>
</tr>
<tr>
<td>R-luxA</td>
<td>CAAAGCCGAGCAGCTCTACA</td>
<td>16</td>
</tr>
<tr>
<td>L-aqrB</td>
<td>ATGCTCCGCAAGCAACTAA</td>
<td>this study</td>
</tr>
<tr>
<td>R-aqrB</td>
<td>TTGAATGAAATTGGGCAATGT</td>
<td>this study</td>
</tr>
<tr>
<td>L-spa</td>
<td>TAACGAGGGGAACTGACTAC</td>
<td>this study</td>
</tr>
<tr>
<td>R-spa</td>
<td>TTATCATGTCTGATCCTGGTTT</td>
<td>this study</td>
</tr>
<tr>
<td>L-pykg</td>
<td>TGAGGTCTACGGGAAATAGT</td>
<td>this study</td>
</tr>
<tr>
<td>R-pykg</td>
<td>TCGGTTGACACATACGCTT</td>
<td>this study</td>
</tr>
</tbody>
</table>

The antibiotics used in this study were 10 μg cloxacillin (Sigma), 10 μg bacitracin (BD), 5 μg methicillin (BD), 0.15 μg penicillin G (BD), 0.75 μg nafcillin (Sigma), 0.75 μg oxacillin (Sigma), 0.5 μg imipenem (Sigma), 30 μg vancomycin (BD), 2.5 μg ceftazidime (Sigma), 1.5 μg cefalotin (Sigma) and 30 μg daptomycin (BD). After 20 h, inhibition zones were measured and luminescence responses were detected using a LuminoMax 190 microplate spectrophotometer (Molecular Devices).

### Results and discussion

β-lactam antibiotics are the drugs of choice for treating staphylococcal infections, either alone or in combination with other antimicrobial agents. We investigated the ability of cell wall active compounds to modulate the transcription of spa, lukE and agr RNAIII in S. aureus. A selection of strains carrying promoter-lux reporter constructs was employed to monitor changes in transcription and qRT–PCR was used to confirm the results.

### Antibiotics differentially modulate the transcription of S. aureus genes

It has been shown previously that sub-MIC antibiotics enhance virulence in staphylococcal infections. We found that expression of spa and lukE was strongly stimulated by sub-MICs of penicillin and cefalotin in S. aureus Newman, suggesting that such compounds may enhance bacterial pathogenesis (Figure 2a). Induction levels varied between closely related antibiotics of the same class (cloxacillin and oxacillin; cefalotin and ceftazidime) (Figure 2a). Cloxacillin and oxacillin differ only in a chlorine substitution on the aromatic ring, while cefalotin and ceftazidime are group I and II cephalosporins, respectively. The variations in stimulatory effects within these β-lactams suggest that the transcriptional differences we observed may reflect subtle alterations in the interaction of the inhibitors with their cellular targets, such as altering binding efficiency. qRT–PCR was used to confirm that modulation of gene expression in S. aureus Newman took place at the transcription level in cultures exposed to sub-MICs of active antibiotics. Expression of spa was examined when cells were exposed to sub-MICs of nafcillin, cefalotin or vancomycin (see Table S1 available as Supplementary data at JAC Online).

### Antibiotic interactions

We investigated the effects of combinations of gentamicin plus penicillin G as well as gentamicin plus ciprofloxacin, alone and in combination (Figure 2b) on transcription of the virulence genes. Antagonistic interactions were observed in the cases of
spa, *luk*E, and agr RNAIII between gentamicin (GEN) and penicillin (PEN) or ciprofloxacin (CIP) using *S. aureus* Newman constructs. The level of induction of PEN and CIP is decreased in the presence of GEN and arrows indicate antagonism observed between the two antibiotics. Decreased induction of virulence genes by penicillin G in the presence of gentamicin was observed (Figure 2b). Combinations of antibiotics are frequently used to treat severe staphylococcal infections. However, because of differences in antibiotic diffusion rates and distribution, not all bacteria are exposed to the required lethal concentrations of bactericidal agents and therefore are likely subjected to sub-MIC antibiotic effects. We employed promoter-lux reporter constructs to analyse antibiotic interactions that might occur under treatment conditions. Three representative *S. aureus* promoter-lux reporter constructs were studied for their responses to antibiotic combinations. Discs containing antibiotics were placed at specific distances to ensure the inhibition zones were not overlapping, and synergies and antagonisms of antibiotics in the transcription level (light production) were observed. We tested 26 different antibiotic combinations (see Table S2 available as Supplementary data at JAC Online). Representative results of antibiotic interactions are shown in Figure 2c. These findings illustrate an advantage of using lux reporters for studying antibiotic interactions at the transcription level.
Modulation of virulence gene expression

Effects of cell wall active antibiotics on virulence gene expression in S. aureus SarA and SarS mutants

SarA controls transcription of a variety of functions by either modulating expression of agr by binding to the intergenic region between agrP2 and agrP3 promoter or by binding directly to the promoter regions of sarP-dependent genes (Figure 1). RNAIII down-regulates expression of SarS, a DNA-binding protein that stimulates expression of spa. Furthermore, plasmids carrying spa-lux, lukE-lux and agrP3-lux were transformed into S. aureus RN6390 mutants deficient in SarA (ALC488) or SarS (ALC1927) regulators and antibiotic-induced transcription responses examined.

Subsequent analysis showed that expression of spa and lukE was strongly stimulated by certain β-lactam antibiotics in S. aureus ALC488, but were unaffected in ALC1927 (Figure 2d). The greater sensitivity of the lux assays could explain the discrepancy with the earlier results of Doss et al., who found that methicillin did not affect protein A production. The expression of agr RNAIII was stimulated by sub-MIC β-lactam antibiotics in both S. aureus ALC488 and ALC1927 (Figure 2d); however, the light response profiles in ALC488 differed from those in S. aureus ALC1927. The different genetic backgrounds of S. aureus (pathogenic strain Newman and laboratory strain RN6390) and the virulence regulators (SarA and SarS) clearly influence the expression of the spa, lukE and agr RNAIII genes in the presence of sub-MIC β-lactam antibiotics (Figure 2a and d). The reasons for these differences are not apparent.

Effects of β-lactam antibiotics on biofilm formation

S. aureus is one of the leading causes of implant-associated infections in hospitals. Numerous exogenous and endogenous factors, including the charge and hydrophobicity of the target matrix, adhesins, host proteins, and production of α-toxin facilitate biofilm formation by the pathogen. We tested several β-lactam antibiotics (methicillin, nafcillin, cefalotin and cefoperazone) on S. aureus Newman and its spa and hemB mutants for their effects on biofilm formation. S. aureus Newman and its hemB mutant formed up to 4- and 3-fold denser biofilms, respectively, at 1/3 MIC and 1/4 MIC of cefalotin, while the S. aureus spa knockout mutant formed up to 3-fold denser biofilms at 1/4 MIC of cefalotin (P<0.05). Other antibiotics (methicillin, nafcillin and cefoperazone) had no effect on biofilm formation in the S. aureus strains listed (Figure 3).

Biofilm formation is one phenotypic measure of virulence expression. Biofilm assay results were different from those of the disc diffusion assays with respect to the effect of sub-MIC cell wall active antibiotics. However, both methods portray strong modulatory effects of sub-MIC cefalotin, which induced virulence genes (spa, lukE and agr RNAIII) in S. aureus Newman (Figure 2a) and induced biofilm formation in the wild-type, spa mutant and hemB mutant (Figure 3). These results are in agreement with earlier findings by Haddadin et al., showing that ceftalexin, a first-generation cephalosporin similar to cefalotin, stimulates denser biofilm formation over a wide range of antibiotic concentrations. The hemB mutant is a small colony variant known to be resistant to aminoglycosides and β-lactam antibiotics and to produce decreased amounts of exotoxins.

The demonstrated stimulation of S. aureus virulence gene expression by sub-MICs of antibiotics could influence the course of disease and compromise antimicrobial therapy. Promoter-lux reporters provide simple and practical tools to study the activities of sub-MICs of antibiotics and their combinations on the expression of different virulence genes. These tools are now available for both Gram-positive and Gram-negative organisms; their use provides novel insights into antibiotic modes of action and they can be employed to reveal favourable therapeutic combinations for the treatment of bacterial infections.

Acknowledgements

We thank C. von Eiff, T. J. Foster and A. L. Cheung for the generous gifts of S. aureus strains.

Funding

This work was supported by CIHR (Canadian Institutes of Health Research).

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References