Regulation of Expression of abcA and Its Response to Environmental Conditions

Regis A. Villet, Que Chi Truong-Bolduc, Yin Wang, Zoe Estabrooks, Heidi Medeiros, David C. Hooper

Division of Infectious Diseases and Medical Services, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

The ATP-dependent transporter gene abcA in Staphylococcus aureus confers resistance to hydrophobic β-lactams. In strain ISP794, abcA is regulated by the transcriptional regulators MgrA and NorG and shares a 420-nucleotide intercistronic region with the divergently transcribed pbp4 gene, which encodes the transpeptidase Pbp4. Exposure of exponentially growing cells to iron-limited media, oxidative stress, and acidic pH (5.5) for 0.5 to 2 h had no effect on abcA expression. In contrast, nutrient limitation produced a significant increase in abcA transcripts. We identified three additional regulators (SarA, SarZ, and Rot) that bind to the overlapping promoter region of abcA and pbp4 in strain MW2 and investigated their role in the regulation of abcA expression. Expression of abcA is decreased by 10.0-fold in vivo in a subcutaneous abscess model. In vitro, abcA expression depends on rot and sarZ regulators. Moenomycin A exposure of strain MW2 produced an increase in abcA transcripts. Relative to MW2, the MIC of moenomycin was decreased 8-fold for MW2ΔabcA and increased 10-fold for the MW2 abcA overexpresser, suggesting that moenomycin is a substrate of AbcA.

Staphylococcus aureus most commonly causes skin and soft-tissue infections but is also a major cause of bacteremia, particularly in health care settings (1, 2). In 2005, up to 20% of cases of S. aureus bloodstream infections in the United States led to death (3). This problem is further complicated by widespread multiple antibiotic resistance in methicillin-resistant strains of S. aureus (MRSA) (4). Resistance mechanisms in bacteria are based on four strategies: (i) inactivation of the antimicrobial drug, (ii) reducing access of the drug to its target, (iii) reduction of the target sensitivity, and (iv) acquisition of a new insensitive target that can perform all of the functions of the native sensitive target (5). Multidrug resistance efflux pumps with broad substrate profiles of the second group have been recognized as a common mechanism which contributes to a broad spectrum of antimicrobial resistance. These pumps are thought to have evolved from specific substrate transporters toward less substrate specificity, possibly reflecting a need for general efflux transporters to protect the cell from an array of environmental toxins, including antimicrobials (6). Multiple native transporters, namely, MepA (7), QscA/B (8), MdeA (9), NorA (10), NorB (10), NorC (11), and Tet38 (12), have been identified in the genome of S. aureus. These efflux pumps can extrude a specific class of antimicrobials, such as tetracyclines and macrolides, or can extrude structurally diverse compounds, such as quinolones, ethidium bromide, and cetrimide (13–16).

In the ABC transporter family, transporters, such as AbcA, are energized by ATP hydrolysis (17). AbcA is related to two known exporters, PepT (77% amino acid similarity) of Staphylococcus epidermidis (18) and LmrA (65% amino acid similarity) of Lactococcus lactis (19). abcA was shown to participate in cell wall autolysis (20) and resistance to β-lactam antibiotics, particularly those with lipophilic side chains (21). On the S. aureus genome, abcA shares an overlapping promoter region with the structural gene pbp4 encoding the PBP4 protein, a transpeptidase, and D,D-carboxypeptidase, which is involved in cell wall synthesis (22) (Fig. 1). Pbp4 is essential for cell growth under laboratory conditions, and its overexpression results in an increase in β-lactam resistance and in greater cross-linking of the peptidoglycan (23, 24). More recently it was reported that Pbp4 from S. aureus COL could be a β-lactamase (25). abcA and pbp4 are separated by only 420 nucleotides, but the expression of these two genes appears to be independent of each other (20). abcA expression is modulated by the agr regulatory system (20) and the global regulators MgrA (direct activator) and NorG (direct repressor) (21).

The detailed regulatory mechanisms of the expression of abcA, however, are incompletely understood. Here, we report the identification of three additional direct regulatory elements, rot, sarA, and sarZ, involved in the expression of abcA and have determined their role in the differences in abcA expression in response to specific environmental conditions. Furthermore, we identified moenomycin as a putative substrate of AbcA.

MATERIALS AND METHODS

Bacterial strains, primers, and antimicrobials. S. aureus strain MW2 (community-acquired [CA]-MRSA; USA400 lineage) (26) was cultivated in tryptic soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C (Table 1). Primers used in this study (Tables 2 and 3) were synthesized at the MGH DNA Core Facility (Boston, MA). The S’-biotin-abcA primers were synthesized by IDT (San Diego, CA) (Table 3). Antimicrobials were obtained from Sigma (Saint Louis, MO) (penicillin, nafcillin, methicillin, vancomycin, teicoplanin), bioMérieux (Marcy l’Etoile, France) (daptomycin), and Theravance (San Francisco, CA) (telavancin). Moenomycin was kindly provided by Suzanne Walker, Department of Microbiology and Immunology, Harvard Medical School.

Growth conditions. To determine the response to stress conditions that could occur in an abscess environment, an overnight culture of S. aureus MW2 was inoculated 1:100 into fresh TSB and grown to early exponential phase (optical density at 600 nm [OD600] of 0.8) at 37°C. For each of the following assays, the early exponential-phase culture was divided in two aliquots and centrifuged at 5,000 × g for 15 min at room
temperature. The reference culture sample was resuspended in TSB, and
the sample cultures were resuspended for up to 2 h at 37°C in other media:
0.9% saline (nutrient limitation stress), TSB with 0.6 mM 2,2 dipyridyl
(Acros Organics, Morris Plains, NJ), an iron-chelating agent (iron limita-
tion stress), TSB with 0.5 or 10 mM hydrogen peroxide (Sigma, St. Louis,
MO) (oxidative stress), or TSB with pH adjusted to 5.5 using HCl (Fisher,
Pittsburgh, PA) (acidic conditions). The cultures were then collected and
centrifuged at 5,000 × g for 15 min at room temperature, frozen, and
stored at −20°C.

Antimicrobial exposure. Overnight cultures of S. aureus MW2 were
inoculated 1:100 into fresh TSB supplemented with penicillin, nafcillin,
methicillin, vancomycin, teicoplanin, telavancin, daptomycin, or merno-
mycin at 2 MIC and grown for 1 h at 37°C. The cultures were then
collected and centrifuged at 5,000 × g for 15 min at room temperature and
stored at −20°C.

Antimicrobial susceptibility testing. MICs were determined by broth
microdilution according to Clinical and Laboratory Standards Institute
(CLSI) guidelines (27).

Real-time PCR assays. Total S. aureus RNA was prepared by extrac-
tion from lysostaphin-treated cells grown to exponential phase at 37°C
and for cells exposed to different environmental conditions using an
RNeasy minikit (Qiagen, Valencia, CA). The concentration of RNA was
determined using NanoDrop microsample quantitation (Thermo Scien-
tific).
TABLE 3 Primers used for this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rot-BamHI</td>
<td>CATGGGATCCGATCTAGTTGTTTTAATCATTGT AGTGGTACCTAATATGT</td>
</tr>
<tr>
<td>Rot-EcoRI</td>
<td>AGGGCATGCTAATTAGCTAAGCAAGCATAATATTG GGTAAACTTATTTGTT</td>
</tr>
<tr>
<td>SarA-BamHI</td>
<td>CATGGGATCCGATCTAGTTGTTTTAATCATTGT AGTGGTACCTAATATGT</td>
</tr>
<tr>
<td>SarA-EcoRI</td>
<td>AGGGCATGCTAATTAGCTAAGCAAGCATAATATTG GGTAAACTTATTTGTT</td>
</tr>
<tr>
<td>SarZ-BamHI</td>
<td>CATGGGATCCGATCTAGTTGTTTTAATCATTGT AGTGGTACCTAATATGT</td>
</tr>
<tr>
<td>SarZ-EcoRI</td>
<td>CATGGGATCCGATCTAGTTGTTTTAATCATTGT AGTGGTACCTAATATGT</td>
</tr>
<tr>
<td>AbcA-UpF</td>
<td>AAACCTGGAATTACCCTTCTTTTCCATTTC</td>
</tr>
<tr>
<td>AbcA-R</td>
<td>AGTTTTCCTTGGTTTTATGATTATTATTT</td>
</tr>
<tr>
<td>MgrA-UpF</td>
<td>TTGTAATAGTCCGACCAACAACACA</td>
</tr>
<tr>
<td>MgrA-UpR</td>
<td>ATAGGCGATCAGCATTAAGAATTCCTGCC</td>
</tr>
<tr>
<td>MgrA-DnF</td>
<td>GTCTAGCTTATATGAAATCTCAATAATGTC</td>
</tr>
<tr>
<td>MgrA-DnR</td>
<td>CTGGATCACATAGATTGTTAGTA</td>
</tr>
<tr>
<td>AbcA-DnF</td>
<td>CTAGGAGGATTGCCATCACATGCAAAAGAT</td>
</tr>
<tr>
<td>AbcA-DnR</td>
<td>GGGAGGATCTGTTTTATGTT</td>
</tr>
<tr>
<td>AbcA-UpF</td>
<td>TTAGTTGCTGCATGTCAG</td>
</tr>
<tr>
<td>AbcA-UpR</td>
<td>TGATTGCTGTTTTATGATT</td>
</tr>
</tbody>
</table>

a The underlined sequences correspond to the restriction enzyme cleavage sites.

Cloning and purification of MgrA, Rot, SarA, and SarZ proteins. mgA, rot, sarA, and sarZ genes were amplified by PCR and cloned into the vector pTcR-HisA (Invitrogen, Carlsbad, CA) for purification. Derivatives of Escherichia coli TOP10 (Invitrogen, Carlsbad, CA) harboring recombinant plasmids were grown to an OD600 of 0.6 in TSB (Difco) containing 100 μg/ml of ampicillin (Fischer, Fair Lawn, NJ). The cells were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma, St. Louis, MO) for an additional 3 h at 37°C. Bacteria were harvested by centrifugation (4,200 × g for 30 min at 4°C), resuspended in 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl (buffer B), and disrupted by sonication with a Branson sonifier 102C for 2 min with cooling. The extract was centrifuged at 13,800 × g for 30 min at 4°C, and the supernatant was mixed with an HIS-Select nickel affinity gel (Sigma, Saint Louis, MO) for affinity purification. Proteins eluted with imidazole (Sigma, Saint Louis, MO) were dialyzed against buffer B containing 100 mM NaCl. The protein concentration was determined using a NanoDrop1000 spectrophotometer. The gel mobility shift assay was carried out using the LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce, Rockford, IL) as recommended by the manufacturer. The biotin-labeled DNA was incubated with the indicated amount of cell extract or purified proteins (10 μg) from S. aureus in 20 μl of binding buffer containing 1 μg of poly(dI-dC), 200 ng of sheared herring sperm DNA, and 10% glycerol. The reaction mixture was incubated for 20 min at room temperature and analyzed by 5% nondenaturing polyacrylamide gel electrophoresis (PAGE). For the competition assays, a 100-fold excess of unlabelled specific DNA, or non-specific herring sperm DNA, was added to the reaction mixture prior to the incubation. In order to determine if there was additive binding of the different regulators, DNA mobility shift assays were performed with MgrA, SarA, SarZ, and Rot together or alone, each at a final concentration of 125 ng/μl.

Mouse subcutaneous abscess model. Swiss Webster male mice that were 4 to 6 weeks old were used for the subcutaneous abscess model (29, 30). Exponential-phase cultures were prepared by diluting overnight cultures 1:100 into TSB and incubating the culture at 37°C with agitation until the OD600 was 0.8. MW2 cells were washed and diluted 1:20 in phosphate-buffered saline (PBS). The cell suspension was then mixed with an equal volume of autoclaved Cytodex-1 beads (131 to 220 μm; Sigma, St. Louis, MO) in PBS. Each 200 μl of cell and bead mixture was injected subcutaneously into each shaved flank of a mouse. The inoculum for each abscess typically contained 1 × 10^9 to 3 × 10^8 CFU. After 48 h, mice were euthanized, and the abscesses were removed aseptically and homogenized in 5 ml PBS. The exact cell input was estimated by colony counts. The total numbers of bacteria recovered from the abscess were determined by plating the abscess homogenate on tryptic soy agar (TSA) (Fisher, Pittsburgh, PA). Total MW2 RNA collected from abscesses was isolated as previously described (12).
were electroporated into S. aureus RN4220, isolated, and then electroporated into S. aureus MW2. Allelic exchange using plasmids pΔabcA and pΔmgrA was performed as previously described (35). The sequence analysis of PCR product for the mutant loci for MW2 abcA and MW2 mgrA confirmed the in-frame deletion. These two strains were constructed and kindly provided by Yanpeng Ding.

Construction of sarZ and rot mutants of MW2. The S. aureus strains MW2ΔsarZ and MW2Δrot were constructed using a phage transduction procedure, with some modifications. S. aureus MW2 was used as a recipient, and phage lysates were constructed on strains ALC6374 and ALC4908 (kindly provided by Ambrose Cheung), which were in-frame deletion mutants of sarZ and rot, respectively, in S. aureus RN6390. The MICs of tetracycline of ALC6374 and ALC4908 mutants were 1.0 μg/ml and 0.5 μg/ml, respectively. The MICs of tetracycline for strains MW2 and RN6390 were 0.1 μg/ml. Transductions were carried out as previously described (33), and transductants were selected on TSA plates containing tetracycline (0.25 μg/ml). Positive colonies were verified by PCR and sequencing for the absence of sarZ and rot.

RESULTS
Effect of abcA on susceptibility to cell envelope-active antimicrobials. abcA has been shown to affect cell envelope processes (autolysis) and susceptibility to β-lactams in the methicillin-susceptible S. aureus (MSSA) background but has not been studied for its effects in an MRSA strain background or on other cell envelope-active agents.

Thus, we constructed an abcA mutant and overexpresser in MW2, an MRSA strain, and tested for effects on susceptibility. In MW2, the susceptibility to β-lactam antibiotics penicillin, nafcillin, and methicillin did not differ between the abcA mutant and the parental strain (Table 4). In addition, susceptibility to other cell envelope-active antibiotics not directly affected by methicillin resistance, including vancomycin, teicoplanin, telavancin, and daptomycin, did not differ in the abcA mutant. A notable exception was moenomycin, for which there was 8-fold increased susceptibility in the abcA mutant.

We previously reported that abcA overexpression in the methicillin-susceptible S. aureus strain ISP794 (37) conferred resistance to β-lactam antibiotics, with increasing effects seen for those β-lactams with higher hydrophobicity (21). abcA overexpression from a plasmid in MRSA strain MW2 produced a diverse pattern of changes in susceptibility to cell envelope-active antimicrobials. For β-lactams, overexpression led to an increase in the MIC for nafcillin (4-fold) but no change for penicillin and methicillin. For other cell envelope-active agents, the MIC of vancomycin was not affected by abcA overexpression in either MW2 (2-fold) or ISP794, a methicillin-susceptible strain. For both MW2 and ISP794, overexpression of abcA results in decreases in the MICs of teicoplanin (12.5- and 8.3-fold, respectively) and telavancin (3.1- and 3.0-fold, respectively). In contrast, abcA overexpression led to an increase in the MIC of daptomycin for MW2 (4-fold) but no change for ISP794. Susceptibility to moenomycin appears to be the exception. Overexpression of abcA results in an increase in MIC in both MW2 (10-fold) and ISP794 (100-fold), and as noted above, an abcA mutant is 8-fold more susceptible.

Expression of abcA in response to environmental conditions. Because other efflux pumps, such as NorA (38) and NorB (39), are expressed differently in response to environmental conditions that could affect the activity of the antimicrobials that are their substrates, we evaluated a range of conditions that have been shown to affect expression of other pumps for their effects on abcA expression. Expression data for response to different conditions included abscess and exposure to antimicrobials (cell envelope stress).

First, to assess abcA expression during infection in vivo, we used a mouse subcutaneous abscess model, as previously described (12). Strain MW2 was chosen because it causes skin and soft-tissue infections in both humans and mice. After subcutaneous injection of 10⁶ CFU, abscesses were harvested at 48 h and demonstrated an increase of CFU to ~2 × 10⁶ CFU/abscess. Total RNA was purified from abscesses and analyzed by real-time PCR. The level of abcA transcripts decreased 10-fold relative to bacteria grown in stationary phase in TSB (Table 5).

To assess possible determinants of the change in abcA expression in the abscess model, we next tested conditions in vitro that may reflect those that exist in the abscess environment.

Growth phase. In vitro, relative to levels during exponential phase, abcA increased by 3.5-fold in early stationary phase and 4.2-fold in stationary phase (Table 5). In contrast, pfp4 did not change in early (1.1-fold) and stationary phases (1.2-fold).

Stress conditions. We have recently shown that reduced aera-
tion induces a 4-fold decrease of \( abcA \) expression (39). Exposure of exponentially growing cells to iron limitation, oxidative stress (\( H_2O_2 \)), and acidic pH (5.5), all of which may exist in abscess environments for 0.5 to 2 h, had no effect on \( abcA \) or \( pbp4 \) expression (data not shown). Nutrient limitation for 1 and 2 h, however, produced a 4.7- to 7.9-fold increase in \( abcA \) transcripts. In contrast, \( pbp4 \) transcript levels remained stable upon shifting to this condition.

**Exposure to antimicrobials.** MW2 was exposed to different concentrations of cell envelope stress antibiotics for 1 h (Table 6). For penicillin, nafcillin, methicillin, and vancomycin exposure there was minimal to no change for \( abcA \) and \( pbp4 \) transcript levels at any concentration. For teicoplanin, telavancin, daptomycin, and moenomycin there were modest increases in \( abcA \) transcripts, ranging from 2.7- to 4-fold, with the maximum seen with moenomycin exposure at 2-fold MIC. There were weak increases for \( pbp4 \) expression with the tested conditions, with the greatest increase at 2.3-fold for telavancin.

**Regulatory pathways that mediate changes in expression of \( abcA \).** To understand the regulatory pathways that mediate changes in expression of \( abcA \), we first tested the role of MgrA, a known regulator, in strain MW2, which lacks NorG, and found that changes in expression on entry into stationary phase (Table 7), starvation, and moenomycin induction (Table 8) were not dependent on MgrA, indicating that other regulators were involved in \( abcA \) expression.

To identify additional potential direct regulators of \( abcA \) expression, we then used the promoter/operator region upstream of \( abcA \) as an affinity reagent to identify proteins in cell extracts that bind specifically to that region of DNA.

Proteins eluted with 0.5 M NaCl were separated by SDS-PAGE.

**Table 6** expression analyzed for MW2 exposed to cell envelope-directed antibiotics

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>( abcA )</th>
<th>( pbp4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Methicillin</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>1.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Telavancin</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Moenomycin</td>
<td>1.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>( abcA )</th>
<th>( pbp4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Nafcillin</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Methicillin</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>1.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Telavancin</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Moenomycin</td>
<td>1.0</td>
<td>2.7</td>
</tr>
</tbody>
</table>

**Table 7** Effects of growth phase on expression of \( abcA \)

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>MW2 (( \Delta mgrA ))</th>
<th>RV235 (( \Delta mgrA ))</th>
<th>RV236 (( \Delta rot ))</th>
<th>RV238 (( \Delta sarA ))</th>
<th>RV237 (( \Delta sarZ ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponential</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Early stationary</td>
<td>3.5</td>
<td>4.1</td>
<td>1.0</td>
<td>3.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Stationary</td>
<td>4.2</td>
<td>4.0</td>
<td>0.7</td>
<td>6.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**Table 8** Environmental signals affecting expression of \( abcA \)

<table>
<thead>
<tr>
<th>Stress</th>
<th>MW2 (( \Delta mgrA )) (( \Delta rot ))</th>
<th>RV235 (( \Delta mgrA )) (( \Delta rot ))</th>
<th>RV236 (( \Delta sarA )) (( \Delta sarZ ))</th>
<th>RV237 (( \Delta sarZ )) (( \Delta sarZ ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline exposure</td>
<td>4.7</td>
<td>15.1</td>
<td>6.4</td>
<td>62.9</td>
</tr>
<tr>
<td>Moenomycin A exposure</td>
<td>4.0</td>
<td>3.5</td>
<td>1.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Two principal protein bands of \( \approx 18.0 \) and \( \approx 14.0 \) kDa were identified by Coomassie blue staining. These bands were then excised from the gel and analyzed by mass spectrometry. MgrA (17.0 kDa), Rot (18.0 kDa), SarA (17.4 kDa), and SarZ (14.7 kDa) were each identified. MgrA protein has been shown previously to bind the \( abcA \) promoter/operator region (21).

For each protein, the gene was expressed by cloning the open reading frame into \( pTrcHisA \) (His tag expression vector). After induction with isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG) and purification on a nickel affinity column, we isolated MgrA, Rot, SarA, and SarZ proteins. For each purified protein, SDS-PAGE indicated the preparations contained approximately 99% homogeneous protein (data not shown). Each purified protein, when incubated with the 420-bp \( abcA-pbp4 \) promoter fragment, showed a shift in the DNA banding pattern (Fig. 2). Band shifts were reduced in the presence of 100-fold excess unlabeled promoter/operator region DNA and remained unchanged in the presence of excess herring sperm DNA, indicating specific binding to the DNA fragment. In order to determine if the DNA-protein interactions are mutually exclusive or additive, we performed the same shift assay but included MgrA, Rot, SarA, and SarZ together at equimolar concentrations. Although the concentrations of individual proteins were lower than those shown in Fig. 2, they were sufficient alone to cause a DNA band shift. The proteins combined, however, produced a band shift that was similar to those with each protein individually (data not shown), suggesting that these proteins have the same or overlapping DNA binding sites.

**Effects of reporter DNA binding proteins on \( abcA \) expression.** We then generated \( rot, sarA, \) and \( sarZ \) mutants of MW2 and tested their effect on baseline expression of \( abcA \) in the exponential phase of growth. We then tested the role of these regulators in mediating increased expression seen in stationary phase, starvation conditions, and moenomycin exposure. Increases in \( abcA \) expression in stationary phase were dependent on both intact \( rot \) and intact \( sarZ \) (Table 7). For shifts to starvation conditions, induction of \( abcA \) was not affected in a \( rot \) mutant but was enhanced in \( mgrA \) (3-fold), \( sarA \) (13-fold), and \( sarZ \) (3.6-fold) mutants. Induction by moenomycin was, in contrast, dependent on intact \( rot \) and intact \( sarA \) but unaffected by loss of \( mgrA \) or \( sarZ \) (Table 8).

**DISCUSSION**

According to the established classification (40), AbcA is a typical type III ABC transporter with both transmembrane and nucleotide-binding domains fused into one polypeptide. The functions of the AbcA transporter of \( S. aureus \) are complex and include...
effects on cell wall autolysis and susceptibility to antimicrobials that act on the cell envelope (20, 21). This complexity is highlighted by the differences in the effects of \(abcA\) expression in different strain backgrounds and in its effects on susceptibility to various cell envelope-active agents. Notably, among the cell-envelope-active agents tested, moenomycin A was consistently affected by \(abcA\) expression, whereas \(\beta\)-lactams were variably affected in MSSA and MRSA backgrounds. Moenomycin A, which is a natural hydrophobic antibiotic that inhibits the glycosyltransferase domain of penicillin-binding proteins (PBPs) (41), is known to be a substrate of ATP-dependent transporters in other species. In \(Streptococcus\) \(ghanaensis\), the genes \(moeD5moeJ5\) and \(moeX5moeP5\), which are highly similar to \(abc\) transporter genes, are involved in the export of moenomycin A (41). We have now shown that in an MSSA background there is a 100-fold increase in the MIC of moenomycin A with overexpression of \(abcA\) and a 10-fold effect in the MRSA background. Furthermore, in a MRSA background an \(abcA\) mutant has an 8-fold decrease in MIC. These data suggest that moenomycin is a substrate of \(AbcA\). The overexpression of \(abcA\) caused a decrease in MIC to teicoplanin (12.5-fold in MW2, 8.3-fold in ISP794) and telavancin (3.1-fold in MW2, 3.0-fold in ISP794). Schrader-Fischer and Berger-Bachi showed that spontaneous whole-cell autolysis was faster in an \(abcA\) mutant (20). These data suggest that \(AbcA\) plays a direct or indirect role in the balance of membrane synthesis and autolysis.

To understand better the role of \(AbcA\) in the physiology of \(S. aureus\), we evaluated the effects of environmental conditions on expression of \(abcA\) and \(pbp4\). At 48 h after inoculation in subcutaneous abscesses, which is one of the most common types of infection caused by \(S. aureus\), we observed a downregulation of expression of both \(abcA\) and \(pbp4\) relative to their expression in stationary phase in \textit{vitro}. There was also downregulation compared to exponential growth in \textit{vitro}, but the magnitude of the effect was less for \(abcA\), since \(abcA\) expression but not \(pbp4\) expression increased upon progression from exponential to stationary phases of growth. We then evaluated in \textit{vitro} a range of conditions that occur in the abscess environment (acidic pH, oxidative stress [with \(H_2O_2\) exposure], iron limitation, nutrient limitation, and antibiotic exposure) for their effects on expression. Shift to acidic pH, \(H_2O_2\) exposure, or iron limitation had no effect on \(abcA\) and \(pbp4\) transcript levels (data not shown), suggesting that these conditions do not influence \(abcA\) and \(pbp4\) expression and are not likely determinants of \(abcA\) expression in abscesses. Under reduced aeration in RN6390, it was shown that \(abcA\) expression decrease 4.0-fold after 30 min of exposure (39). A decreased aeration environment in the abscess may have contributed to the decreased expression of \(abcA\) that we observed. Under nutrient limitation conditions, in contrast, we observed an increase in transcript levels of \(abcA\) and no change in \(pbp4\) transcript levels. These data indicate the existence of a distinct regulatory mechanism for \(abcA\) and \(pbp4\), as suggested previously (20). Exposure to several different classes of cell envelope-active antibiotics had little effect on the \(abcA\) and \(pbp4\) expression with the exception of exposure to moenomycin A, which produced an increase in \(abcA\) expression.

In order to identify regulators that directly control the expression of \(abcA\), we amplified the 420-nucleotide intercistronic region between \(abcA\) and \(pbp4\) that is known to play a role in the regulation of both genes (20) and identified four transcriptional regulators that bound specifically to this DNA region, MgrA, Rot, SarA, and SarZ, suggesting that they functioned as direct regulators of \(abcA\) and/or \(pbp4\). Direct regulation of \(abcA\) has been shown for MgrA and NorG (21), which was absent from the MRSA strain used here. Each of these regulators has also been shown to affect the expression of a variety of other genes, including genes involved in virulence, capsule synthesis, and autolysis (42–45). With regard to expression of efflux pumps, both MgrA and SarZ have also been shown to regulate the expression of \(norB\) and \(tet38\), which encode two secondary efflux transporters conferring resistance to quinolones and tetracycline, respectively (16). The interregulatory relationship between the different effectors of \(abcA\) is summarized in Fig. 3. It is well established that members of the SarA family regulate other members of their own family, as well as other regulatory systems and target genes in \(S. aureus\). \(mgrA\), \(rot\), \(sarA\), and \(sarZ\) expression were observed in all laboratory and clinical \(S. aureus\) strains tested so far (RN6390, SH1000, MW2, Newman COL, and UAMS-1) (20, 21, 42, 46). Except for \(sarA\), which has the same level of expression between the different strains, \(mgrA\), \(rot\), and \(sarZ\) show varied levels of expression from strain to strain (42, 46). Finally, the direct \(abcA\) repressor NorG is absent from MW2. These data underline the complex regulation

![Gel mobility shift assay with purified MgrA, Rot, SarA, and SarZ proteins mixed with the biotinylated 420-bp sequence between \(abcA\) and \(pbp4\). The biotin-labeled DNA was incubated with 10 µg of purified protein. The reaction mixture was incubated for 20 min at room temperature and analyzed by 5% nondenaturing polyacrylamide gel electrophoresis (PAGE). For the competition assays, a 100-fold excess of unlabeled specific DNA or nonspecific herring sperm DNA was added to the reaction mixture prior to the incubation.](http://jb.asm.org/)

**FIG 2** Gel mobility shift assay with purified MgrA, Rot, SarA, and SarZ proteins mixed with the biotinylated 420-bp sequence between \(abcA\) and \(pbp4\). The biotin-labeled DNA was incubated with 10 µg of purified protein. The reaction mixture was incubated for 20 min at room temperature and analyzed by 5% nondenaturing polyacrylamide gel electrophoresis (PAGE). For the competition assays, a 100-fold excess of unlabeled specific DNA or nonspecific herring sperm DNA was added to the reaction mixture prior to the incubation.
of abcA and the interactions of the regulatory elements which may differ among *S. aureus* strains.

In order to determine if the any of four transcriptional regulators that were identified to bind to the *abcA*-*pbp4* intercistronic DNA mediated the changes in expression of abcA found under different growth conditions, we generated mutants and tested them for changes from the parental strain in growth in conventional medium, with nutrient limitation, and with moenomycin A exposure. With growth in TSB, *abcA* expression exhibited similar increases on entry into stationary phase in parental MW2 and its *mgrA* and *sarA* mutants. In contrast, *sarZ* and *rot* mutants showed no changes in *abcA* expression by growth phase. Thus, Rot and SarZ both appear to be necessary for growth phase differences in *abcA* expression. It was previously reported that MgrA acts as a direct activator of *abcA* expression in ISP794 (21). Deletion of *mgrA* in strain MW2, however, does not change *abcA* expression, indicating that *mgrA* is not essential for *abcA* transcription. For other conditions that induced expression of *abcA*, different patterns of regulator involvement were found. Enhanced expression of *abcA* occurred in *mgrA*, *sarZ*, and *sarA* mutants exposed to starvation conditions, but moenomycin A induction was not seen in *rot* and *sara* mutants. These findings highlight the involvement of multiple regulators in the control of *abcA* expression and the complexity of their involvement depending on the environmental conditions that trigger changes in *abcA* expression. The findings also expand the range of conditions identified to produce differential expression of *pbp4* and *abcA*. Notably, moenomycin A appears to be a substrate of AbcA which is induced by substrate exposure, raising the possibility that inhibitors of its function enhance the activity of the moenomycin family of antimicrobials as future antistaphylococcal agents.

The complexity of efflux pump regulation underlines their importance in cell physiology and adaptation to different environments. Our study provides new information about the regulation and possible functions of AbcA in *S. aureus*, but direct studies of pump function and its natural substrates will be needed to understand more fully its role in *S. aureus* physiology in addition to its ability to confer resistance to antimicrobials.

**FIG 3** General overview of the predicted direct regulatory elements of *abcA* and their relationship (including the proteins identified in this work and the regulators previously identified) under normal conditions of growth. Black lines are for interactions identified in RN6390 (20, 42). Purple lines are for interactions identified in ISP794 (21). Green lines are for interactions identified in MW2 background (this study). *, NorG is absent from MW2. Effector indicates a direct regulator that can be a repressor or an activator depending on the strain and the environment.

**ACKNOWLEDGMENTS**

This work was supported by the NIH-NIAID National Institute of Allergy and Infectious Diseases (Antimicrobial and Efflux Pumps in *S. aureus*/Harvard-wide Program on Antibiotic Resistance number 2P01AI083214-04).

We thank Yanpeng Ding (Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA) for providing the MW2ΔmgrA and MW2ΔabcA mutants. We thank Ambrose Cheung (Department of Microbiology and Immunology, Dartmouth Medical School, Hanover, NH, USA) for providing the RN6390Δrot (ALC4908) and RN6390ΔsarZ (ALC6374) mutants. We thank Suzanne Walker (Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, USA) for providing moenomycin A.

**REFERENCES**


