Implication of the NorB Efflux Pump in the Adaptation of Staphylococcus aureus to Growth at Acid pH and in Resistance to Moxifloxacin

Que Chi Truong-Bolduc,1 Gilles R. Bolduc,2 Ryo Okumura,1† Brian Celino,2 Jennifer Bevis,2 Chun-Hsing Liao,1 and David C. Hooper1*

Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114,1 and Massasoit Community College, One Massasoit Blvd., Brockton, Massachusetts 023022

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Staphylococcus aureus is an important pathogen that adapts and survives in low-pH environments. One component of this adaptation involves the regulation of genes encoding bacterial transporters that could affect response to antibiotics under these conditions. We previously demonstrated that the transcriptional regulator MgrA in its phosphorylated form (MgrA-P) represses the expression of norB, encoding the NorB multidrug resistance efflux pump. In this study, we focused on changes in the expression of mgrA at the transcriptional and posttranslational levels, following a shift from pH 7.0 to pH 4.5. We then correlated those changes with modifications in transcript levels of norB and to resistance to moxifloxacin, a substrate of NorB. At pH 4.5, S. aureus MgrA increased 2-fold and MgrA-P decreased 4-fold, associated with an 8-fold increase in norB transcripts and a 6-fold reduction in bacterial killing by moxifloxacin, and the phenomenon was dependent on intact mgrA. Taken together, these new data showed that phosphoregulation of MgrA at low pH reverses its repression of norB expression, conferring resistance to moxifloxacin.

Staphylococcus aureus is a major human pathogen, capable of causing diverse types of infections ranging in severity from skin infections to pneumonia and endocarditis (13, 19). S. aureus is also carried by healthy humans as part of the normal flora of the nose, throat, and skin. This pathogen can adapt to various unfavorable environmental conditions or stresses caused by variation in pH, temperature, or nutritive sources by activating several defense mechanisms, including the use of proton pumps to maintain the cellular pH at an acceptable level, or shifting the growth energy to adaptation mechanisms (1, 2, 28). These conditions may also serve as signals to the organism that are important for pathogenesis and fitness at specific sites of infection or colonization of the host. For example, low-pH conditions are found on the surface of the skin, within an abscess, and in certain cellular compartments (7).

pH is known to have broad effects on gene expression in S. aureus (2, 28), but little is known about the role of bacterial efflux transporters in response to pH. Expression of genes encoding certain multidrug resistance (MDR) transporters, such as norA, encoding the NorA efflux pump, and bcr, encoding the bicyclomycin resistance phenotype, are downregulated under low-pH conditions. In contrast, under the same pH conditions, other genes encoding drug efflux pumps, such as embBqacA and norC, exhibit substantial increases (12-fold to 15-fold, respectively) in transcript levels (2, 5). Our previous studies showed that the MDR efflux pump NorB, which confers resistance to certain quinolones, such as moxifloxacin, contributes to bacterial survival when it is selectively overexpressed in subcutaneous abscesses (8). Since the pH in the abscess environment can be as low as 5.5 or 6, it is possible that this signal may be responsible in part for the changes in norB expression seen in abscesses.

Regulation of norB expression involves the transcriptional regulator MgrA, which also affects expression of norA. The phosphorylation state of MgrA affects its binding to the norA and norB promoters and their gene transcript levels (23, 26). Transcriptional profiling data indicated that the level of expression of the Ser/Thr kinase encoded by pknB decreased at least 5-fold at acid pH. Since MgrA is a substrate of the PknB kinase, the posttranslational phosphorylation of MgrA would also be affected, which in turn modified its regulation of norA and norB efflux pump gene expression (2). Furthermore, the two-component regulatory system arlRS was also negatively affected by a low pH, as indicated by a 3-fold decrease in its transcript level (2). ArlRS was previously shown to decrease norA expression and increase norB expression, acting upstream of MgrA in the regulatory cascade (12, 18, 20).

In this study, we have correlated changes in the phosphorylation state of MgrA with increased levels of expression of norB and decreased levels of expression of norA and with a decrease in bacterial killing by moxifloxacin, a selective NorB substrate, in response to low pH, highlighting the role of posttranslational modifications of MgrA in mediating responses to environmental signals.

MATERIALS AND METHODS

Bacterial strains and materials. Staphylococcus aureus RN6390 and its isogenic mutants QT1a (mgrA::cat) and QT5 (norB::cat) were previously characterized (15, 24, 27). Bacteria were cultivated in Trypticase soy broth (TSB) (Difco, Sparks, MD) or Trypticase soy agar (TSA) (Difco, Sparks, MD) at 37°C, unless otherwise stated.

† Present address: Biological Research Laboratories, Daiichi Sankyo Co., Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan.
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otherwise stated. The pH of the medium was adjusted to 4.5 using 10 M HCl. Lysozyme was obtained from Sigma Chemical Co. (St. Louis, MO). The anti-MgrA antibody was produced by GenScript (GenScript, Piscataway, NJ) using a synthetic 14-amino-acid peptide sequence (19Q to 32K) of the MgrA protein. Anti-serine phosphate antibody was purchased from Qiagen (Valencia, CA).

**Growth conditions.** To determine the S. aureus response to acid pH, overnight cultures of S. aureus RN6390 were inoculated 1:100 into fresh TSB and grown to early exponential phase (optical density at 600 nm [OD$_{600}$] = 0.4) at 37°C. The cultures were then collected and centrifuged at 5,000 x g for 15 min at room temperature and resuspended in fresh TSB or without adjustment to pH 4.5 with 10 M HCl. Growth under different pH conditions and after exposure to moroxydin was monitored by measurement of OD$_{600}$ prior to cell counts using the microfluidic flow cytometer Fishman-R (see below). pH was monitored using color pH indicator strips at pH 0 to 14 (EM Science, Gibbstown, NJ) and a pH meter (Fisher Scientific, Pittsburgh, PA). Moroxydin was used at a 16-fold MIC to measure the effect of drug on bacterial growth and viability immediately following change in pH. After 30 min in new medium with different pHs, bacteria were collected every 10 min up to 120 min. These experiments and those described below were repeated three times to ensure the reproducibility of the data obtained.

**Cell enumeration using agar plating or LIVE/DEAD cell counts by Fishman-R flow cytometry.** Viable cell counts were determined by two different methods, agar plating and LIVE/DEAD cell counts using the microfluidic flow cytometer Fishman-R (On-Chip Biotechnologies Co., Ltd., Tokyo, Japan). For agar plating, 50 µl of cells was collected every 10 min for a total of 2 h after acid induction. Cells were diluted in normal saline (0.9%), plated on TSA, and incubated overnight at 37°C, and the number of viable CFU per µl was counted. For the cell counts using the LIVE/DEAD technique coupled with the microfluidic flow cytometer Fishman-R, 50 µl of cells was collected every 10 min for a total of 2 h (4). Cells were washed twice in 1× phosphate-buffered saline (PBS) and resuspended in 50 µl of 1× PBS, with a mixture of two dyes, propidium iodide (PI) and Syto9 (Invitrogen, Carlsbad, CA) (4.5 µg each in 1 ml of 1× PBS) and centrifuged for 20 min at room temperature. Ten microliters of the dye and 10 µl of cell suspension in PBS were mixed together and incubated in the dark for 20 min before application to the flow cytometer for live-cell counting. The live-cell impermeant dye (PI) stained the dead bacteria, and the nucleic acid stain (Syto9) stained the DNA/RNA of live and dead cells. When combined, the two stains provided a distinction between live bacteria with intact membranes and dead bacteria with nonfunctional membrane properties.

**Real-time qRT-PCR assays.** Real-time quantitative reverse transcription-PCR (qRT-PCR) assays were carried out as previously described (23, 26). Total S. aureus RNA was prepared by extraction from lysozyme-treated cells collected at different time points, using an RNeasy midi kit (Qiagen, Valencia, CA). cDNAs were synthesized using a Verso cDNA synthesis kit (Thermo Scientific, ABgene, Epsom, Surrey, United Kingdom), followed by real-time qRT-PCR assays using the EvaGreen dye and the CFX96 real-time system (Bio-Rad, Hercules, CA).

All primers used in this study were synthesized at the Tufts University Core Facility, Boston, MA, and are listed in Table 1. The housekeeping gene gmk was used as an internal control. All samples were analyzed in triplicate and normalized against gmk gene expression.

**Preparation of cell extracts.** S. aureus RN6390 cells were collected at various time points at pH 4.5 and 7.0 in accordance with the technique of Ballal and Manna (3), with some modifications. Cells were grown in 5 ml of Trypticase soy broth (TSB) at pH 7.0 or adjusted to pH 4.5 with 10 M HCl. After centrifugation at 5,000 x g at 10°C, cells were resuspended in 1 ml buffer A containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, and 100 µg/ml lysozyme. Lysozyme-treated cells were incubated at 42°C for 5 min, followed by centrifugation at 10,000 x g for 45 min to remove cell debris. The concentration of protein in the clear lysate was determined by using a Bradford protein detection kit, with bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA).

**Western blotting with anti-MgrA and anti-serine phosphate antibodies.** Equal amounts of total cell proteins were separated on 12% SDS-PAGE gel and transferred onto nitrocellulose membranes as previously described (26). After washing steps were performed at room temperature. The membrane was washed twice for 10 min with TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5), followed by incubation for 1 h in blocking buffer (5% BSA, 0.1% Tween 20 in TBS buffer). Membranes were washed twice for 10 min in TBS-Tween-Triton buffer (20 mM Tris-HCl, 300 mM NaCl, 0.05% Tween 20, 0.2% Triton X-100, pH 7.5) and once with TBS buffer. Membranes were then incubated with either anti-phosphoserine (Qiagen, Valencia, CA) or anti-MgrA (GenScript, Piscataway, NJ) antibody solutions (1/100 dilution of antibody in TBS-Tween 20 buffer) at 4°C overnight and then washed twice for 10 min in TBS-Tween-Triton buffer and once in TBS buffer. Membranes were incubated with secondary antibodies (goat anti-rabbit IgG-horseradish peroxidase [HRP] for MgrA antibody and rabbit anti-mouse IgG (Genscript [GenScript, Piscataway, NJ] and Jackson ImmunoResearch, West Grove, PA, respectively) (1/10,000 dilution) for 1 h at room temperature and washed 4 times for 10 min in TBS-Tween-Triton. The chemiluminescence detection reaction was performed, and the membranes were exposed to X-ray film according to the manufacturer’s recommendations.

**RESULTS**

**Effect of acid pH on growth.** To determine the effect of acid pH on cell growth and viability, we resuspended cells growing in early exponential phase at pH 7.0 in medium at pH 4.5 and also in medium at pH 7.0 for comparison. A shift to pH 4.5 resulted in a reduced growth rate relative to that obtained at pH 7.0 (Fig. 1) but had little effect on cell viability (Table 2). After 90 min at pH 4.5, cell growth ceased.

**pH effects on transcript levels of norA, norB, mgra, and norG.** Quantitative real-time RT-PCR assays showed that the transcript levels of several genes encoding efflux pumps were affected in opposite directions. After a shift to pH 4.5, norA transcripts showed decreases of 2-fold at 10 min and 10-fold at 20 min. In contrast, norB transcript levels increased 4-fold and 6-fold after 10 and 20 min, respectively. We also measured the
transcript levels of \textit{mgrA} and \textit{norG}, which encode two known transcriptional regulators of \textit{norA} and \textit{norB}. \textit{norG} transcripts remained unchanged throughout the 2-h exposure to pH 4.5, but we found a 3-fold increase in \textit{mgrA} transcripts at 30 min (Table 3).

**pH effects on the amounts and phosphorylation of MgrA.** To assess changes in the amount and phosphorylation status of MgrA protein following shift to pH 4.5, we collected cells at time points 10, 20, and 30 min after the shift and carried out Western blotting using anti-MgrA and anti-serine phosphate antibodies. Western blot analysis of the crude cell extracts with anti-MgrA antibody showed that MgrA was present at both pH 7.0 and pH 4.5, but the amount of MgrA increased approximately 2-fold at 20 to 30 min after shift to pH 4.5. There was no change in MgrA levels at pH 7.0 during the 30-min period (Fig. 2A). Similar Western blot analysis using anti-serine phosphate antibody showed a substantial reduction (approximately 4-fold) in the overall phosphorylation states of several proteins in the cell extract, including MgrA. This reduction occurred after 10 min of exposure to pH 4.5 (Fig. 2B).

**Susceptibility of \textit{S. aureus} to moxifloxacin at pH 4.5 and pH 7.0.** Increased expression of \textit{norB}, encoding the NorB efflux pump, causes reduced susceptibility to moxifloxacin in vitro and contributes to bacterial fitness in a subcutaneous abscess model (8). Since \textit{norB} expression was increased shortly after a shift to pH 4.5, we wished to determine whether susceptibility to moxifloxacin, a NorB substrate (24), was also affected by a shift to pH 4.5. Cell viability was measured in response to moxifloxacin at 30 min after a shift to pH 4.5 or pH 7.0, using rapid LIVE/DEAD cell staining with a Fishman-R cytometer (4). At 90 min after addition of moxifloxacin, there was an approximately 6-fold-greater reduction of live

\begin{table}[h]
\centering
\caption{Difference in the growth rate of \textit{S. aureus} RN6390 after the medium was adjusted to pH 4.5}
\begin{tabular}{lcccc}
\hline
Time (min) & \multicolumn{2}{c}{Mean ± SD growth rate ($10^7$ CFU/ml)} & \multicolumn{2}{c}{Ratio (pH 7.0 value \hspace{1em} pH 4.5 value)} \\
\hline & pH 7.0 & pH 4.5 & pH 7.0 value & pH 4.5 value \\
10 & 1.10 ± 0.05 & 1.08 ± 0.05 & 1.02 \\
20 & 1.65 ± 0.06 & 1.60 ± 0.05 & 1.03 \\
30 & 5.00 ± 0.06 & 2.10 ± 0.06 & 2.38 \\
40 & 8.00 ± 0.05 & 3.20 ± 0.06 & 2.50 \\
50 & 14.00 ± 0.01 & 6.50 ± 0.06 & 2.15 \\
60 & 28.00 ± 0.05 & 8.00 ± 0.01 & 3.50 \\
80 & 36.50 ± 0.01 & 8.30 ± 0.06 & 4.39 \\
90 & 40.00 ± 0.02 & 8.50 ± 0.07 & 4.71 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Time zero was considered as the moment the pH adjustment was made. 
\textsuperscript{b} SD, standard deviation. Data represent the average values for three experiments.

\begin{table}[h]
\centering
\caption{\textit{S. aureus} RN6390 gene transcripts up- and downregulated at pH 4.5}
\begin{tabular}{lccccc}
\hline
Gene & Function & Ratio\textsuperscript{a} & Fold change (+) or decrease (−) after 30 min \\
\hline
\textit{norA} & MDR efflux pump & 1 & 0.5 & 0.1 & 0.1 & 10 \\
\textit{norB} & MDR efflux pump & 1 & 6 & 8 & 8 & +8 \\
\textit{mgrA} & Global regulator & 1 & 2 & 3 & 3 & +3 \\
\textit{norG} & Global regulator & 1 & 1 & 1 & Unchanged \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Changes in gene expression are given as the ratio of sample (pH 4.5) versus control (pH 7.0) for samples collected at 10, 20, and 30 min after the medium was adjusted to pH 4.5. Time zero was considered the moment the pH adjustment was made. The final fold change was determined after 30 min. The housekeeping gene \textit{gmk} was used as the internal control. All assays were done in triplicate.
cells at pH 7.0 than at pH 4.5 (4.0 \times 10^6 CFU/ml versus 6.6 \times 10^7 CFU/ml) (Table 4), indicating a reduction in bacterial killing at pH 4.5.

### Role of the NorB efflux pump in the adaptation to acid shock and resistance to moxifloxacin

From the data above, we observed a lesser bactericidal activity of moxifloxacin after a shift to pH 4.5. To determine if the overexpression of \( \text{norB} \) in response to a shift to pH 4.5 contributed to the reduced susceptibility to moxifloxacin, we compared the effects of low pH and moxifloxacin susceptibility on isogenic mutants of RN6390, with mutations in the \( \text{norB} \) gene itself (QT5) or in \( \text{mgrA} \), which increases \( \text{norB} \) expression in the RN6390 background (24). Reductions in cell viability on exposure to moxifloxacin increased, as expected for the \( \text{norB} \) mutant QT5, relative to the level for the parental RN6390 but differed in magnitude at pH 4.5 and pH 7.0 (Table 5). Notably, at pH 7.0, the difference in magnitude of viable-count reduction between the two strains was 9.7-fold, but at pH 4.5, the difference was 100-fold, indicating that \( \text{norB} \) contributed relatively more to survival on exposure to moxifloxacin at pH 4.5 than at pH 7.0, a finding consistent with its observed increased expression at pH 4.5 (Table 5).

Because we demonstrated that levels of MgrA and its phosphorylated form (MgrA-P) changed on a shift to pH 4.5 (Fig. 2A and B), and MgrA-P bound to and repressed the expression of \( \text{norB} \) as was shown previously (26), we wanted to determine the dependence of pH-mediated changes in susceptibility to moxifloxacin on intact \( \text{mgrA} \). Comparing the pH differences in cell viability on exposure to moxifloxacin between strains, we found a difference of 50-fold (410-fold at pH 7.0 versus 8.2-fold at pH 4.5) (Table 5) in the magnitude of loss of viability between the two pHs for RN6390. In contrast, for the \( \text{mgrA} \) mutant QT1a, there was a lesser loss of viability (0.34-fold) between the two pH conditions (1.37-fold at pH 7.0 versus 4-fold at pH 4.5). Thus, pH-induced differences in cell viability on exposure to moxifloxacin appear to be dependent on intact \( \text{mgrA} \), suggesting that the differences in the levels and phosphorylation state of MgrA, which can modulate \( \text{norB} \) expression (23, 26), are also important for the acid-induced increased expression of \( \text{norB} \) and its consequent reduced susceptibility to moxifloxacin.

### TABLE 4. Antimicrobial activity of moxifloxacin on the growth of \( S. \text{aureus} \) RN6390 at pH 7.0 and pH 4.5

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>( \text{Mean} \pm \text{SD} ) RN6390 level (( 10^7 ) CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>pH 4.5</td>
</tr>
<tr>
<td>10 1.65 ± 0.07</td>
<td>1.65 ± 0.07</td>
</tr>
<tr>
<td>30 8.00 ± 0.05</td>
<td>4.50 ± 0.07</td>
</tr>
<tr>
<td>60 28.0 ± 0.12</td>
<td>1.40 ± 0.02</td>
</tr>
<tr>
<td>90 40.1 ± 0.16</td>
<td>0.10 ± 0.03</td>
</tr>
</tbody>
</table>

* Time zero was considered the moment moxifloxacin (0.5 \( \mu \)g/ml) was added to the medium at pH 7.0 or pH 4.5.
  * \( \delta \) represents the difference in the number of bacteria in CFU/ml (\( 10^7 \)) after addition of moxifloxacin (Moxi) at pH 7.0 and at pH 4.5. Data represent the average values for three experiments ± standard deviations (SD).
both MgrA and NorG (23, 25, 26). In our previous studies, 8-fold upon a shift from pH 7.0 to pH 4.5. NorB is regulated by the NorB efflux pump, or other regulators of the Nor pumps.

Proton export from the bacterial cell (1, 2, 5, 6, 28). A decrease in the activity of the NADH dehydrogenase, which can increase internal cell pH by assisting proton export from the bacterial cell (1, 2, 5, 6, 28). The decrease in proton export from the bacterial cell is due to the decrease in the activity of the NADH dehydrogenase, which can increase internal cell pH by assisting proton export from the bacterial cell (1, 2, 5, 6, 28).

Table 5. Antimicrobial activity of moxifloxacin on the growth of S. aureus RN6390 and its isogenic mutant at pH 7.0 and pH 4.5

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Mean ± SD cell count (10^7 CFU/ml) after 90 min in new medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 7.0</td>
</tr>
<tr>
<td></td>
<td>+ Moxi</td>
<td>− Moxi</td>
</tr>
<tr>
<td>RN6390</td>
<td>Wild type</td>
<td>410 ± 0.07</td>
</tr>
<tr>
<td>QT5</td>
<td>norB::cat</td>
<td>40.0 ± 0.15</td>
</tr>
<tr>
<td>QT1a</td>
<td>mgra::cat</td>
<td>41.0 ± 0.15</td>
</tr>
</tbody>
</table>

*Δ represents the ratio of viable-bacterium counts at pH 7.0 and at pH 4.5 after 90 min of growth. A higher value indicates that more bacteria were killed by moxifloxacin (Moxi). Data represent the average values for three experiments ± SD.

**DISCUSSION**

**S. aureus** adapts to and survives many environmental conditions, including body sites such as skin and abscesses at which pH is low. These adaptations are multifaceted, involving changes in expression of many genes with various functions, but the specific mechanisms are not well studied. The acid stress response has been shown to include increased transcript levels of genes involved in utilization of urease (ureA, ureB, ureC, and ureF) and of _nuoF_, which encodes an NADH dehydrogenase, which can increase internal cell pH by assisting proton export from the bacterial cell (1, 2, 5, 6, 28). A decrease in transcripts encoding the _F_0F_1_ ATPase was also observed, which was somewhat surprising since this _F_0F_1_ ATPase is considered an important proton export mechanism in several Gram-positive bacterial species, such as _Streptococcus mutans_ (17). Although acid stress responses do not include increases in major virulence factors or biofilm formation, there were noteworthy changes in several global regulators that control the expression of virulence genes and drug efflux transporters (5), such as the SaeRS and ArlRS, two-component regulators, the Ser/Thr kinase PknB, and its phosphatase Stp1. These global regulators have been shown to be important in modulating virulence factor and drug transporter expression (9, 11, 22, 23, 26). Other regulators exhibit increased expression at pH 4.5, including the transcriptional regulator of the iron regulator Fur family (SACOL1541), the histidine kinases PhoP and KdpD, and the VraS protein of the cell wall stimulus response (10, 14, 16). Regarding MDR transporters, the most noticeable change in expression was that of _norA_, the drug transport gene SACOL2460, and the bicyclic resistance gene _ber_, all of which decreased at least 3-fold after 30 min at pH 4.5. In contrast, the genes encoding the NorC efflux pump and the EmrB/OacA drug transporter showed increases in their transcript titers of at least 10-fold after 20 min at pH 4.5 (2, 5). These data for _norA_ are consistent with our findings, but there are no prior data on acid stress response of _norB_, encoding the NorB efflux pump, or _mgra_ and _norG_, encoding transcriptional regulators of the Nor pumps.

In this study, we found that _norB_ expression was increased 8-fold upon a shift from pH 7.0 to pH 4.5. NorB is regulated by both MgrA and NorG (23, 25, 26). In our previous studies, overexpression of _norG_ from a plasmid increased _norB_ expression (25), but we found no difference in _norG_ transcript levels at pH 4.5 and pH 7.0, suggesting that NorG is not part of the acid stress response and specifically that it is not directly responsible for increased _norB_ expression at acid pH. In contrast, we found increases in the amount of MgrA and a decrease in its phosphorylation state in response to acid stress. We have shown previously that MgrA in its phosphorylated form (MgrA-P) binds to the _norB_ promoter and appears to function as a repressor. We also demonstrated that the Ser/Thr kinase PknB and the Ser/Thr phosphatase RsbU can affect the level of MgrA phosphorylation _in vitro_ (23, 26). Strain RN6390, used in this study, is of the RN6390 background, which lacks a functional _rsbU_ gene (thus, baseline levels of MgrA-P may be increased) (23), and the 4-fold reduction in MgrA-P that we found upon a shift to pH 4.5 was not due to the RsbU phosphatase. Notably, expression of _pknB_ has been shown to be decreased 6-fold at pH 4.5 (5). Thus, the reduced levels of PknB under acid stress may be responsible for the decreased level of MgrA-P under these conditions. In our model, reduced MgrA-P would thereby result in reduced repression and increased expression of NorB.

Moxifloxacin is one of the substrates of NorB that are not substrates of NorA or NorC, as was shown in our previous study (24). Thus, we next examined the effect of pH shift on moxifloxacin activity by rapid assays of viable cells using vital dye cytometric measurements of bacteria after exposure to moxifloxacin. Decreases in the magnitude of moxifloxacin reduction in viable cells by a shift from pH 7.0 to pH 4.5 were over 50-fold. Because slower bacterial growth, as was observed at pH 4.5, can itself reduce bacterial killing by quinolones such as moxifloxacin, and to link NorB expression to the pH-mediated change in killing, we compared levels of bacterial killing at the two pHs in a _norB_ mutant. Indeed, lack of _norB_ in mutant QT5 led to an increase in killing by moxifloxacin, and notably, the effect was substantially greater at pH 4.5 than at pH 7.0, linking _norB_ expression with relative survival on exposure to moxifloxacin. Furthermore, because reduced phosphorylation of MgrA was also correlated with increased _norB_ expression at acid pH, we sought to determine the dependence of reduced moxifloxacin killing at acid pH on intact MgrA. In the _mgra_ mutant QT1a, no decrease in moxifloxacin killing (rather a slight increase, 1.36-fold versus 4-fold) (Table 5), occurred on a shift from pH 7.0 to pH 4.5, indicating that the enhanced resistance to moxifloxacin seen on a shift to acid pH is dependent on intact MgrA. These data thus link the phosphorylation state of MgrA with changes in _norB_ expression and relative resistance to a NorB substrate. It is also possible that the reductions in _pknB_ expression account for the reductions of MgrA-P on a shift to acid pH.

The antimicrobial activity of moxifloxacin has previously been shown to be reduced by acid pH (21). Although changes in bacterial growth rate and changes in the charge properties of...
moxifloxacin could contribute to reduced moxifloxacin killing at acid pH, our data indicate that adaptations by S. aureus in upregulating the NorB efflux pump at acid pH and the associated lower growth rate have a specific role in affecting moxifloxacin action. Since quinolones are synthetic antimicrobials, it is likely that increased expression of norB at acid pH represents a function of NorB other than resistance to these antimicrobial agents. NorA is known to be a proton antiporter, and it is likely that NorB is also. Such a property of NorB, however, is a repressor of norB expression. Thus, the regulation of cell-wall biosynthesis pathway in S. aureus involves a two-component system involved in adhesion autolysis, and extracellular proteolytic activity of S. aureus in infection.

In summary, acid pH is a trigger for increased expression of norB, which contributes to relative resistance to moxifloxacin. The mechanism underlying upregulation of norB expression at acid pH is dependent on MgrA and correlates with its reduced phosphorylation state, in keeping with model in which MgrA-P is a repressor of norB expression.

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REFERENCES

20. Luong, T. T., and C. Y. Lee. 2006. The arl locus positively regulates Staphy-
lococcus aureus type 5 capsule via an mraA-dependent pathway. Microbiol-
ogy 152:3123–3131.
23. Truong-Bolduc, Q. C., Y. Ding, and D. C. Hooper. 2008. Posttranslational modification influences the effects of MgrA on norA expression in Staphylo-
25. Truong-Bolduc, Q. C., Y. Ding, and D. C. Hooper. 2008. Posttranslational modification influences the effects of MgrA on norA expression in Staphylo-
26. Truong-Bolduc, Q. C., Y. Ding, and D. C. Hooper. 2008. Posttranslational modification influences the effects of MgrA on norA expression in Staphylo-
27. Truong-Bolduc, Q. C., Y. Ding, and D. C. Hooper. 2008. Posttranslational modification influences the effects of MgrA on norA expression in Staphylo-
29. Truong-Bolduc, Q. C., D. C. Hooper. 2010. Phosphorylation of MgrA and its effect on expression of the NorA and NorB efflux pumps of Staphy-