Effect of substrate exposure and other growth condition manipulations on \textit{norA} expression

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\textbf{Objectives:} Multidrug efflux is a resistance mechanism that simultaneously affects susceptibility to many structurally unrelated compounds. The regulation of \textit{norA} expression, which encodes the \textit{Staphylococcus aureus} NorA multidrug efflux pump, is not well understood but the MgrA global regulator and the \textit{arlRS} locus are involved. The expression of genes encoding proteins related to NorA, such as QacA of \textit{S. aureus} and Bmr of \textit{Bacillus subtilis}, is affected by pump substrates. In these instances, substrate interacts with regulatory proteins such that pump gene transcription is increased. The goal of this study was to identify if a similar substrate-level effect exists, or an effect of other growth condition manipulations, on the expression of \textit{norA}.

\textbf{Methods:} A transcriptional fusion between \textit{norA} and \textit{lacZ} was created in single copy on the chromosome of \textit{S. aureus} SH1000. \textit{b}-Galactosidase activity was quantified following exposure of the fusion strain to various NorA substrates, salicylate, a high salt concentration, putative soluble factors elaborated during growth, and different incubation temperatures.

\textbf{Results and conclusions:} Exposure to several substrates significantly increased \textit{norA} expression whereas salicylate and osmotic stress had no effect and no stable soluble factor affecting \textit{norA} expression was detectable. An inverse relationship between \textit{norA} expression and incubation temperature was observed and this effect was related, at least in part, to changes in \textit{norA} mRNA half-life. However, concomitant changes in translational efficiency at different temperatures could not be ruled out. We conclude that there is a substrate-level effect on \textit{norA} expression and propose that this may be mediated through substrate interaction with a regulatory protein.

\textbf{Keywords:} efflux, regulation, \textit{Staphylococcus}

\textbf{Introduction}

Efflux may be the most prevalent mechanism by which bacteria can resist the antibacterial effects of multiple structurally unrelated compounds. In Gram-negative organisms efflux-related multidrug resistance (MDR) can be mediated by single- or multi-component pumps which can interact in a cooperative manner to significantly increase resistance to common substrates.\textsuperscript{1,2} In Gram-positive bacteria, only one-component integral membrane proteins, such as the \textit{Staphylococcus aureus} NorA protein, have been found to mediate MDR. NorA, which is encoded by the naturally-occurring chromosomal \textit{norA} gene, is capable of effluxing various biocides, dyes and fluoroquinolone antimicrobial agents in a process dependent on the proton motive force (pmf).\textsuperscript{3-6}

Several years ago, we described a single-step mutant of \textit{S. aureus} selected by exposure to ciprofloxacin that exhibited inducible NorA-mediated MDR (SA-1199-3).\textsuperscript{7} Northern blot analysis indicated that exposure to either cetrimide or norfloxacin, both of which are NorA substrates, increased \textit{norA} expression in this mutant by at least six-fold. At the same time, we observed that exposure of SA-1199B, a strain that overexpresses \textit{norA} due to a promoter-region mutation, to cetrimide results in a 30\% additional increase in \textit{norA} expression. The mechanism of this substrate-level effect has not been determined.\textsuperscript{7,8}

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NorA has a high degree of homology (44%) with Bmr, a *Bacillus subtilis* MDR transporter with a substrate profile similar to that of NorA. The expression of *bmr* is regulated by the binding to its promoter of BmR, an activator protein. The crystal structure of BmR in the presence and absence of substrates has been solved and reveals that substrates interact with BmR to facilitate its binding to the *bmr* promoter; thus the expression of *bmr* can be considered inducible by its substrates. A similar mechanism of substrate-induced gene activation is thought to occur for another *B. subtilis* pmf-dependent MDR transporter, BtR, which is highly homologous with Bmr (51%) and NorA (39%).

QacA and QacB are nearly identical plasmid-encoded *S. aureus* MDR efflux pumps that confer resistance to clinically-relevant biocides. They are related to NorA by being pmf-dependent and have some overlap in substrate profile with it. All qacA/B determinants are regulated by the divergently-transcribed QacR repressor protein. Similar to BmR and perhaps BltR, QacR interacts with substrates and in their presence dissociates from its operator site resulting in augmented expression of qacA/B.

These data with proteins related to NorA suggest that the increase in *norA* expression observed for both SA-1199B and SA-1199-3 following substrate exposure may be mediated by a regulatory protein. Recently, it has been shown that an 18 kDa protein specifically binds upstream of the −35 motif of the *norA* promoter and augments *norA* expression in the presence on the chromosome of a transposon insertion into *arlS*. The *arlR-arlS* locus encodes a two-component regulatory system involved in the adhesion, autolysis and extracellular proteolytic activity of *S. aureus*. The mechanism(s) by which disruption of *arlS* modifies *norA* expression has not been established, but the binding of the 18 kDa protein to the promoter is modified in the presence of this mutation. This protein, initially named NorR, has been purified and studied further. The binding of NorR to the *norA* promoter is modified in an *arlS* strain such that increased *norA* expression is observed; however, overexpression of *norR* in an *arlS* background also results in increased *norA* expression. In fact, only when *norR* is overexpressed is an effect on *norA* transcription observed in wild-type strains. It is significant that highly fluoroquinolone-resistant strains of *S. aureus* have been described in which *norA* expression is enhanced in the absence of any *arlR-arlS* modification or change in *norR* expression [including SA-1199B (unpublished data)], indicating that loci other than *arlR-arlS* and *norR* must be involved in the regulation of *norA* expression. Subsequent work has revealed that NorR is not a specific regulator of *norA* expression but rather is a global regulator that, in addition to affecting *norA* transcription when overexpressed, affects the transcription of several known autolytic regulators including positively regulating the expression of *arlS*. This protein, which has also been independently named Rat and MrGA, acts at the transcriptional level to enhance the expression of numerous genes, the products of which, by themselves, negatively impact the expression of murein hydrolases. In addition, NorR/Rat/MrGA (hereafter referred to as MrGA for the sake of simplicity) enhances the production of nuclease but represses α-toxin, coagulase and protein A. Whether or not a specific regulator of *norA* expression akin to BmR, BltR, or QacR exists is not known at this time.

In an effort to better verify the existence of, and characterize, a substrate-level effect on *norA* expression, we created a transcriptional fusion between *norA* and *lacZ* in single copy on the chromosome. Such a fusion affords the opportunity for a qualitative comparison of the dynamics of gene expression at different stages of growth. In addition, gene expression throughout the growth cycle can be quantified. These data are superior to those obtained by northern analysis, where in general, only a single point in time is studied.

**Materials and methods**

**Bacterial strains, media and reagents**

The strains of *S. aureus* used in this study included *S. aureus* RN4220 and SH1000. All derivatives of *S. aureus* NCTC 8325-4, including *S. aureus* RN4220, are functionally SigB deficient because of an *rsbU* mutation. Possible confounding effects of this mutation were eliminated by use of *S. aureus* SH1000, which is *S. aureus* NCTC 8325-4 *rsbU*<sup>+</sup>, as the final host strain for the *norA::lacZ* fusion (see below). Unless otherwise noted, all reagents were the highest grade available and were obtained from Sigma Chemical Co., St. Louis, MO, USA. Growth media [Brain–Heart Infusion broth (BHB) and Tryptic Soy broth (TSB)] were obtained from BD Biosciences, Inc. (Sparks, MD, USA).

**Determination of antimicrobial susceptibilities**

Antimicrobial susceptibilities were determined in triplicate using a microdilution technique according to NCCLS guidelines, except that BHB was used as the growth medium.

**Construction of a norA-lacZ fusion**

An internal fragment of the *S′* portion of *norA* was amplified by PCR followed by cloning of the product into pAZ106, a plasmid conferring resistance to ampicillin (*Escherichia coli*), erythromycin (*S. aureus*), and containing a multiple cloning site upstream of a promoterless *lacZ* gene. pAZ106 replicates only in *E. coli*, and when introduced into *S. aureus* RN4220 and selected for by erythromycin, it will integrate into the chromosome at any site of homology, in this case the *norA* gene. The integration disrupts native *norA* and creates, in a single copy on the chromosome, a transcriptional fusion between *norA* and *lacZ*. DNA sequencing and Southern blot analyses verified the proper construct, and phage 85 was used to transduce the fusion into *S. aureus* SH1000, producing strain SA-K2124.

Disruption of a chromosomal gene may have a polar effect on a downstream gene(s) that are co-transcribed with it. Existing data indicate that *norA* transcripts are the proper size for *norA* alone and that it is not part of an operon, making it highly unlikely that the *norA::lacZ* fusion has a polar effect.

**β-Galactosidase assay**

SA-K2124 was grown overnight in TSB, washed with phosphate-buffered saline (pH 7.0), and then diluted 1:200 in 100 mL of pre-warmed BHB and grown at 37°C with agitation. β-Galactosidase activity was measured employing 4-Methylumbelliferone (MB) as a substrate as described previously. Briefly, 0.5 mL culture aliquots were obtained at intervals during growth for measurement of OD<sub>600</sub>. The cells in a second 0.5 mL aliquot were harvested by centrifugation and the pellets snap-frozen at −70°C while awaiting assay. Cells were thawed, resuspended in 0.5 mL of ABT (100 mM NaCl, 60 mM K<sub>2</sub>HPO<sub>4</sub>, 40 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1% Triton X-100), incubated at 37°C for 15 min, and then 50 μL...
of MUG (4 mg/mL stock) was added followed by incubation at room temperature for 1 h. The reaction was stopped by the addition of 0.5 mL of 0.4 M Na₂CO₃. Samples were serially diluted in a 1:1 (v/v) mixture of ABT and Na₂CO₃ in 96-well white polystyrene microtiter plates (Corning Inc., Corning, NY, USA). A range of concentrations of 4-methylumbelliferone were used to generate a standard curve, and β-galactosidase activity (expressed in MUG units; 1 unit = 1 pmol MUG cleaved per min per OD₆₀₀) was determined by fluorescence using a Bio-Tek FLX800 plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Cumulative norA expression over the course of the experiment (10 h) was determined by integrating the area beneath expression curves using SigmaPlot 8.0 (Systat Software, Inc., Point Richmond, CA, USA).

Effect of substrate exposure on norA expression

norA expression studies were carried out using SA-K2124 grown overnight in drug-free TSB and then diluted into fresh BHIB containing or lacking subinhibitory (1/4 MIC) concentrations of various NorA substrates including benzalkonium chloride (BAC), cetrimide, ethidium bromide (EtBr; also tested at 1/8 and 1/16× the MIC), norfloxacin, and tetraphenylphosphonium bromide (TPP). The effects of tetracycline and acridine orange, which are not NorA substrates, were also determined.

Other chemical and environmental manipulations

Salicylate is known to induce antibiotic resistance in E. coli by augmenting marA expression, which leads to reduced production of selected outer membrane porins and induction of at least three MDR efflux pump operons including acrAB. The products of these genes, plus that of tolC, form the tripartite AcrAB-TolC MDR efflux pump. Exposure of S. aureus to salicylate also increases resistance to various antimicrobial agents, some of which are known substrates for MDR efflux pumps (including NorA). Employing a norA-deleted strain, we have shown previously that NorA is not required for the salicylate effect in S. aureus; however, it remained possible that salicylate had an indirect effect on norA expression. To test this, SA-K2124 was grown in BHIB supplemented with 2 mM salicylate and the effect on norA expression was determined.

The possible elaboration of a soluble factor affecting norA transcription was investigated by growing SA-K2124 overnight in BHIB. Cells were pelleted and the growth supernatant was recovered, filter-sterilized, and diluted 1:1 with fresh BHIB. SA-K2124 was inoculated into this medium and norA expression was determined. The effect of osmolar stress on norA expression was investigated using BHIB supplemented with 1 M NaCl as the growth medium, and the effect of high and low temperature was studied by growing SA-K2124 at 30, 37 and 42°C.

Determination of norA mRNA half-life and quantification of mRNA

SH1000 and SA-K2124 were grown at 30, 37 and 42°C to OD₆₀₀ = 1 in Luria–Bertani broth, and at T = 0, a 1 mL aliquot was removed, 0.5 mL of NaCl₃ was added, and then cells were pelleted and snap-frozen at −70°C. Rifampicin (150 μg/mL) was added to the remaining culture to stop new RNA synthesis and additional aliquots were removed at frequent intervals and treated as described above. Cell pellets were thawed on ice, lysed and RNA was isolated using the RNeasy Kit (Qiagen Inc., Valencia, CA, USA). The Primer Extension System-AMV Reverse Transcriptase kit (Promega Corp., Madison, WI, USA) was used to specifically label norA mRNA. An oligonucleotide complimentary to the 5′ region of norA was end-labelled with [γ-³²P]ATP (3000 Ci/mmol; Perkin–Elmer Life and Analytical Sciences, Boston, MA, USA) according to procedures recommended by the manufacturer of the Primer Extension System. Primer extension (run-off) products were digitally quantified using a phosphorimaging system (Storm 860, Molecular Dynamics, Sunnyvale, CA, USA) and Phoretix 1D Advanced software (version 5.20, Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). The half-life of norA mRNA was determined by calculating the rate of reduction of band intensities of run-off transcripts following rifampicin addition.

Quantification of norA mRNA recovered from SA-K2124 with or without exposure to 1/4× MIC of EtBr was carried out using the Primer Extension System as described above except that BHIB was used as the growth medium, aliquots were collected at OD₆₀₀ = 1, rifampicin was not added, and incubation was at a single temperature (37°C).

Statistical analysis

All norA expression experiments were carried out a minimum of three times, and results were compared using the Student’s t-test.

Results and discussion

MIC data are shown in Table 1. The effect of norA disruption by fusion with lacZ is evident by the reproducible two- to fourfold reductions in MICs to the known NorA substrates BAC, cetrimide, EtBr, norfloxacin and TPP. Susceptibility to the non-NorA substrates acridine orange and tetracycline was unaffected. The quantity of norA message produced, as assessed by run-off transcript analysis using SH1000 and SA-K2124, was not affected by fusion with lacZ (data not shown). The nucleotide sequence of arlS and mgrA plus its two promoters in the fusion strain was wild-type (data not shown).¹⁸,²⁶,²¹

The expression of norA in the presence and absence of selected substrates is shown in Figure 1. Without antibiotic exposure, peak expression occurred at mid- to late-exponential growth (3h) and fell off relatively rapidly as the culture approached stationary growth phase (3.5–5h). Generation times during exponential growth were 15.4, 17.4 and 35.3 min for SA-K2124 exposed to no antibiotic, BAC and EtBr, respectively. In addition to slowing exponential growth slightly, BAC exposure also delayed peak norA expression by 0.5 h. However, peak expression was higher (by 30%) and the elevated expression compared to control was maintained for at least 3 h. EtBr exposure had a more pronounced effect on exponential growth (doubling of the exponential phase generation time), but by 10h, total cell mass achieved was near that of control. Peak norA expression was delayed but was approximately equal in

<table>
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<tr>
<th>Strain</th>
<th>BAC</th>
<th>CET</th>
<th>EtBr</th>
<th>NOR</th>
<th>TPP</th>
<th>AO</th>
<th>TET</th>
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<td>SH1000</td>
<td>1.25</td>
<td>0.63</td>
<td>6.3</td>
<td>1.25</td>
<td>12.5</td>
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<tr>
<td>SA-K2124</td>
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<td>0.31</td>
<td>1.6</td>
<td>0.63</td>
<td>6.25</td>
<td>50</td>
<td>0.31</td>
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* BAC, benzalkonium chloride; CET, cetrimide; EtBr, ethidium bromide; NOR, norfloxacin; TPP, tetraphenylphosphonium bromide; AO, acridine orange; TET, tetracycline.

* In mg/mL.
Substrate and environmental effects on norA expression

Figure 1. norA expression in the presence and absence of subinhibitory concentrations of NorA substrates. A representative experiment is shown in panel (a). Open circles, growth of SA-K2124 without antibiotic; inverted open triangles, with exposure to 1/4MIC of benzalkonium chloride (BAC); open squares, with exposure to 1/4 MIC of ethidium bromide (EtBr); filled circles, norA expression (β-galactosidase activity) without antibiotic; filled inverted triangles, with exposure to 1/4 MIC of BAC; filled squares, with exposure to 1/4 MIC of EtBr. Panel (b), cumulative norA expression over the entire 10 h experiment. TPP, tetraphenylphosphonium bromide. $P$ values were determined by comparison with control.

magnitude to that of control and still occurred in the middle of exponential growth. Compared to control, higher levels of expression were maintained from 3.5–10h. Exposure to 1/8 and 1/16MICs of EtBr and 1/4MIC of cetrimide had minimal effects on growth and resulted in augmented norA expression similar to that observed in the presence of BAC; namely, a higher peak and sustained augmented similar to that observed in the presence of BAC; namely, a higher peak and sustained augmented expression (Figure 2). Low temperature slowed bacterial growth, which results in a delay in the onset of stationary phase and perhaps the associated decrease in norA expression may be a confounding factor in the interpretation of data gathered in the presence of 1/4MIC of EtBr. However, augmented norA expression was observed at EtBr concentrations that minimally affected growth. This fact, combined with the norA-stimulatory effect of BAC and cetrimide in the face of minimal growth inhibition, supports the conclusion that these compounds do stimulate norA expression.

Intercalating agents such as acridine orange and EtBr, which physically interact with DNA, can alter its supercoiling and in turn may affect gene transcription.30 We felt it unlikely that the physical interaction of EtBr with DNA was responsible for the apparent increase in norA expression observed in its presence as BAC and cetrimide, which do not interact with DNA, also stimulated norA expression. However, to control for this possibility, norA expression studies were carried out in the presence of 1/4MIC of acridine orange and no effect was seen. We thus conclude that the effect of EtBr, which at 1/4MIC is more sustained than that of any other substrate, and that of BAC, cetrimide and lower concentrations of EtBr is due to increased transcription of norA. Furthermore, based on information available from related systems, it is most likely that this effect is mediated by a regulatory protein(s) that interacts with the substrate which in turn alters norA expression by a mechanism that may be similar to that of QacR or BmrR.

It is intriguing that different NorA substrates have different effects on norA expression as assessed by the method we employed. For instance, norfloxacin and TPP did not stimulate norA transcription despite both being good NorA substrates. The simplest explanation for this observation is that norfloxacin and TPP do not interact with the presumed regulatory protein(s) and thus do not augment norA expression. Alternatively, it is feasible that these compounds, in and of themselves, do augment norA transcription but their action(s) on other cellular processes may indirectly reduce transcription such that the observed gene expression is the result of the sum of stimulatory and repressive effects. Of significant interest is the fact that norfloxacin exposure has been shown to stimulate norA expression in SA-1199-3, a mutant that overexpresses norA in an inducible manner.7 The mechanism(s) of induction in this strain have not been determined, but it is possible that more than one norA regulatory protein exists. Perhaps a mutational change in one regulatory protein results in an exaggerated norA expression upon substrate exposure that any detrimental effect a compound may have on gene expression by way of its antibacterial activity is overcome.

Salicylate exposure and 1M NaCl had no effect on norA transcription in our test system (data not shown). Thus, it can be concluded that NorA does not contribute in any way to the salicylate-mediated MIC increases observed in S. aureus, and NorA is not involved in any response the organism may mount to osmotic stress. We also saw no effect on norA transcription by growing SA-K2124 in medium composed of a 1:1 mixture of overnight culture supernatant and fresh BHIB. This indicates that it is unlikely that S. aureus elaborates significant amounts of a stable soluble factor that affects norA expression. It remains possible, however, that an unstable soluble factor affecting norA expression may be produced and would not have been detected by the conditions we employed.

High and low incubation temperature resulted in significant changes in norA expression (Figure 2). Low temperature slowed
growth considerably but high temperature did not; generation times of 37.7, 19.2 and 19.9 min were observed at 30, 37 and 42°C, respectively. Regardless of temperature, peak norA expression still occurred at mid- to late-exponential growth and total cell mass achieved by 10 h was the same.

Temperature may affect the stability of mRNA. The half-life of E. coli ompA mRNA, which encodes the major outer membrane protein OmpA, falls from 13 to 9 min as the temperature is increased from 28 to 37°C. The temperature effect observed on norA expression, as measured by β-galactosidase activity, may result from true changes in gene transcription, changes in mRNA half-life, increased or decreased β-galactosidase enzyme activity, or a combination of these. Half-lives for the fusion transcript were 2.1, 1.1 and 0.7 min at 30, 37 and 42°C, respectively. The fusion had no confounding effect on mRNA half-lives as similar results were observed for intact norA transcripts of SH1000. These data indicate that the presumed temperature effect on norA expression is related, at least in part, to changes in mRNA stability. It is possible, however, that translational efficiency is enhanced or reduced with temperature shifts in order to balance the effect of increased or decreased mRNA half-life. Despite the shorter half-life of ompA mRNA at higher temperatures, translation is enhanced such that total cellular OmpA expression remains constant. Our study was not designed to address this possibility with respect to NorA, but it is worthy of further investigation. MIC determinations for norfloxacin and TPP using SH1000 incubated at 30, 37 and 42°C were not different, lending indirect evidence to the conclusion that the quantity of NorA expression still occurred at mid- to late-exponential growth and total cell mass achieved by 10 h was the same.

Figure 2. Apparent effect of temperature on norA expression. (a) Open circles, growth of SA-K2124 at 30°C; open inverted triangles, at 37°C; open squares, at 42°C; filled circles, norA expression (β-galactosidase activity) at 30°C; filled inverted triangles, at 37°C; filled squares, at 42°C. (b) Cumulative norA expression over the entire 10 h experiment at the indicated temperature. P values were determined by comparison with the 37°C data.

References


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Substrate and environmental effects on norA expression


