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Efflux-Related Resistance to Norfloxacin, Dyes, and Biocides in Bloodstream Isolates of *Staphylococcus aureus*▽

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Efflux is an important resistance mechanism in *Staphylococcus aureus*, but its frequency in patients with bacteremia is unknown. Nonreplicate bloodstream isolates were collected over an 8-month period, and MICs of four common efflux pump substrates, with and without the broad-spectrum efflux pump inhibitor reserpine, were determined ($n = 232$). A reserpine-associated fourfold decrease in MIC was considered indicative of efflux. Strains exhibiting efflux of at least two of the four substrates were identified ("effluxing strains" [$n = 114$]). For these strains, MICs with or without reserpine for an array of typical substrates and the expression of *mepA*, *mdeA*, *norA*, *norB*, *norC*, and *qacA/B* were determined using quantitative real-time reverse transcription-PCR (qRT-PCR). A fourfold or greater increase in gene expression was considered significant. The most commonly effluxed substrates were ethidium bromide and chlorhexidine (100 and 96% of effluxing strains, respectively). qRT-PCR identified strains overexpressing *mepA* (5 [4.4%]), *mdeA* (13 [11.4%]), *norA* (26 [22.8%]), *norB* (29 [25.4%]), and *norC* (19 [16.7%]); 23 strains overexpressed two or more genes. Mutations probably associated with increased gene expression included a MepR-inactivating substitution and *norA* promoter region insertions or deletions. Mutations possibly associated with increased expression of the other analyzed genes were also observed. Effluxing strains comprised 49% of all strains studied (114/232 strains), with nearly half of these overexpressing genes encoding MepA, MdeA, and/or NorABC (54/114 strains). Reduced susceptibility to biocides may contribute to persistence on environmental surfaces, and efflux of drugs such as fluoroquinolones may predispose strains to high-level target-based resistance.

Staphylococcus aureus is an organism of major medical importance, causing skin and soft tissue infections, bacteremia, and endocarditis (13, 16). Antimicrobial drug resistance is frequent and can result from enzymatic modification, target alteration, or efflux. Combinations of these mechanisms can lead to a multidrug resistance (MDR) phenotype. The contribution of each of these resistance mechanisms can be determined by molecular and microbiologic means, but their frequencies in a given region or an individual patient are difficult to estimate. This is especially true for efflux-related resistance because of the lack of a simple screening test. The best available screen consists of MIC determinations with and without pump inhibitors, but the contributions of other resistance mechanisms may obscure inhibitor-related MIC decreases. An example of this situation is fluoroquinolone resistance related to topoisomerase mutations in a strain overexpressing the NorA MDR pump, where the comparatively small contribution of efflux can be overshadowed by large MIC increases associated with target mutations (11, 28).

Efflux-related resistance in *S. aureus* can affect many drug classes, including biocides, tetracyclines, macrolides, and fluoroquinolones. Even low-level efflux-related MIC increases may be sufficient to allow persistence in the hospital environment or in a sequestered site of infection. The activity of drug pumps may also predispose organisms to high-level target-based resistance by reducing intracellular drug concentrations to subinhibitory levels. Support for this hypothesis is provided by showing that efflux pump inhibition in either *S. aureus* or *Streptococcus pneumoniae* can reduce the emergence of mutants resistant to fluoroquinolones (19, 20).

Analysis of genome data suggests that *S. aureus* possesses at least 20 MDR efflux pumps, predominantly major facilitator proteins (www.membranetransport.org). Only a few *S. aureus* MDR pumps have been described to date, with NorA and QacA/B being the best characterized (29, 32). Others include MepA, a multidrug and toxin extrusion family member, and the major facilitator proteins MdeA, NorB, and NorC (7, 10, 30, 31). The widespread distribution of Qac proteins, which mediate resistance to biocides, in 40% of methicillin-resistant *S. aureus* (MRSA) strains in Europe and Asia underscores their clinical relevance in those regions (1, 21, 25). However, similar studies with respect to NorA have examined only the effect of reserpine on MICs and the sequence of *norA* and its promoter (23, 24, 27, 28). None of these studies evaluated *norA* mRNA abundance. Since *norA* overexpression is correlated with MDR in *S. aureus*, such data would provide a better estimate of its contribution to drug resistance as well as the prevalence of such strains (12, 22). No work of this type has been done with respect to *mepA*, *mdeA*, *norB*, or *norC*. Thus, we used MIC testing, with and without reserpine, and gene expression analyses to arrive at an estimate of the frequency of efflux-related drug resistance in bloodstream isolates of *S. aureus*. We found that NorA- and NorB-mediated efflux is quite

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TABLE 1. Strains and plasmids used in this study

S. aureus strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
RN4220	NCTC 8325-4 r^-; capable of stably maintaining recombinant plasmids	14
SH1000	NCTC 8325-4 with *rsbU* mutation repaired	6
SA-K3124	Clinical isolate, has mutation resulting in A103V MepR substitution	This study
SA-K2916	SH1000 *mepR*::*lacZ*	9
SA-K2916-R	SA-K2916/pK434	9
SA-K2916-R (A103V)	SA-K2916/pK519	This study
MRSA252	Strain for which genome sequence is available	NARSA[a]
Plasmids		
pALC2073	*S. aureus* vector containing a tetracycline-inducible promoter controlling expression of cloned genes; Cm^r	3
pK434	pALC2073 *mepR*	10
pK519	pALC2073 *mepR*(*A103V*)	This study

[a] Network on Antimicrobial Resistance in *Staphylococcus aureus* (www.narsa.net).

common but that strains overexpressing *mepA*, *mdeA*, and *norC* also exist in southeastern Michigan. Overexpression of multiple pumps in a single strain can also occur.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents. Bloodstream isolates (one per patient) of *S. aureus* and corresponding susceptibility data were collected from the microbiology laboratory at the Detroit Medical Center between April and November 2005 (approved by the Wayne State University Human Investigation Committee). Other strains and plasmids used are listed in Table 1. Unless otherwise noted, all reagents were of the highest grade available and were obtained from Sigma Chemical Co. (St. Louis, MO) or BD Biosciences (Sparks, MD).

S. aureus SH1000 was used for quality control purposes for agar and broth microdilution susceptibility testing and as the control strain for quantitative real-time reverse transcription-PCR (qRT-PCR) (6). pK519 was constructed as described previously for pK434, except that the ribosome binding site and *mepR* coding region from SA-K3124 were cloned into pALC2073 (3, 10). The construct was introduced into SA-K2916, producing strain SA-K2916-R (A103V). When appropriate, strains were grown in the presence of 50 ng/ml of tetracycline to induce plasmid-based *mepR* expression.

Antimicrobial susceptibility testing. MICs of chlorhexidine (CH), dequalinium (DQ), ethidium bromide (EtBr), and norfloxacin (NOR), with and without reserpine (20 μg/ml), were determined by agar dilution, and a reserpine-mediated reduction of at least fourfold was considered indicative of efflux (4). Strains with such reductions for at least three compounds, or two if they were NOR resistant, were considered effluxing strains and are referred to as such. Effluxing strains underwent microdilution MIC testing, with and without reserpine, for CH, DQ, EtBr, NOR, acriflavine (AF), benzalkonium chloride (BAC), cetrimide (CET), and tetraphenylphosphonium bromide, all of which are substrates for one or more of the known *S. aureus* MDR efflux pumps (4, 8).

qRT-PCR. Effluxing strains were grown in 50 ml of LB broth to an A_{550} of 0.4, pelleted, resuspended in Tris-EDTA buffer (pH 7.5) containing 100 μg/ml lysostaphin, and incubated for 10 min at 37°C. RNAs were recovered and purified using an RNeasy Mini kit (QIAGEN Inc., Valencia, CA) and Turbo DNase I (Ambion Inc., Austin, TX) following the directions provided by the respective manufacturers. Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA) was used to design primers and Taqman probes for *mepA*, *mdeA*, and *norABC*, based on the genome sequence of *S. aureus* 8325, which were purchased commercially (Operon Biotechnologies, Huntsville, AL). GenBank sequence X56628 was used for primer and probe design for *qacAB*. qRT-PCR was performed in triplicate using a Superscript III Platinum one-step kit (Invitrogen Inc., Carlsbad, CA) and an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA), with cycling parameters of 45°C for 30 min, 95°C for 2 min, and then 40 cycles of 1 min at 95°C and 1 min at 55°C. Negative and positive controls were included, and 16S rRNA was used as the endogenous control. The comparative cycle threshold method was used to calculate relative gene expression, and an increase of fourfold or more compared to that for *S. aureus* SH1000 was considered indicative of overexpression.

RNA slot blotting. RNAs from selected strains exhibiting overexpression of pump genes (*mepA*, 5 strains; *mdeA*, 6 strains; *norA*, 8 strains; *norB*, 10 strains; and *norC*, 4 strains) were immobilized on a nylon membrane by using a Bio-Rad Bio-Dot SF apparatus (Bio-Rad Laboratories, Hercules, CA) and methods exactly as described previously (2). A PCR-generated fragment of the appropriate gene served as a probe, and labeling of the probe, hybridization, and detection were performed using BrightStar psoralen-biotin labeling, NorthernMax, and BrightStar BioDetect kits according to the manufacturer's directions (Ambion). 16S rRNA was used as the endogenous control, and data generated for *S. aureus* SH1000 were the standards to which other data were compared.

Sequencing. Promoter and coding regions of appropriate genes were amplified by PCR from strains overexpressing pump genes. Products were sequenced in both directions, using an automated method, by the Applied Genomics Technology Center, Wayne State University (26). For strains overexpressing *norA*, *-B*, or *-C*, the sequence of *mgrA* and its promoter was determined, as MgrA is known to repress the expression of *norA* and *-C* and to upregulate that of *norB* (17). Strains negative for the six genes by qRT-PCR were used as sequencing controls (*norA*, 11 strains; and *mepRA*, *mdeA*, *norB*, *norC*, and *mgrA*, 5 strains each). DS Gene 1.5 (Accelrys, Inc., San Diego, CA) was used for nucleotide sequence analyses, and genome sequence data for *S. aureus* NCTC 8325 (www.ncbi.nlm.nih.gov/genomes/lproks.cgi) were the standards to which experimental data were compared.

β-Galactosidase assay. Expression of *mepR* in SA-K2916-R and SA-K2916-R (A103V) was determined using a fluorescent β-galactosidase assay as described previously (9). Experiments were performed in duplicate, and gene expression in the presence and absence of 50 ng/ml of tetracycline was quantitated by integrating the area beneath the expression curves with SigmaPlot 10.0 (Systat Software, Inc., Point Richmond, CA).

RESULTS AND DISCUSSION

Susceptibility data. Of the 232 strains of *S. aureus* collected, 114 (49%) were identified as effluxing strains. MRSA strains made up 72% (82/114 strains) of these, whereas they comprised 59% of noneffluxing strains. MIC data, including the median reserpine effect and the proportion of effluxing strains demonstrating a fourfold or greater reserpine-mediated reduction for each substrate, are given in Table 2. CH and EtBr were each effluxed by virtually all strains, and the other tested compounds were effluxed by more than half of the strains. The number of substrates effluxed ranged from two to eight, with 91 and 46% of strains effluxing at least four and at least six, respectively. The higher proportion of MRSA strains among effluxing than noneffluxing strains suggests that such strains are more likely to possess efflux-related resistance mechanisms.

For comparative purposes, MICs were determined for 33 noneffluxing strains. Zero to six substrates were effluxed, with 45 and 3% of strains effluxing at least four and at least six, respectively (data not shown). CH and EtBr were effluxed by

TABLE 2. Microdilution susceptibility data for effluxing strains (n = 114)

Substrate	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)	Reserpine effect (median [range] reduction [fold] in MIC)	No. (%) of isolates with ≥4-fold reduction by reserpine
CH	0.31	0.63	0.16–1.25	8 (0–16)	110 (96)
DQ	1.25	5.0	0.31–10	4 (0–8)	64 (56)
EtBr	6.25	25	3.13–50	8 (4–16)	114 (100)
NOR	12.5	>100	0.39–>100	4 (0–16)	65 (57)
AF	12.5	50	6.25–50	4 (0–8)	70 (61)
BAC	2.5	5.0	0.63–5.0	4 (0–16)	67 (59)
CET	0.63	1.25	0.16–2.5	4 (0–16)	66 (58)
TPP[a]	25	50	12.5–100	4 (0–8)	61 (54)

[a] Tetraphenylphosphonium bromide.

42 and 58% of strains, respectively, and DQ was effluxed by none. The median reserpine effect reached fourfold only for EtBr, AF, and BAC, whereas in effluxing strains this was achieved for all substrates (Table 2). MICs for *S. aureus* SH1000 were less than or equal to half the MIC_{90} values shown in Table 2; this strain would not have met our effluxing strain definition in that only its CET MIC was reduced fourfold by reserpine (data not shown). These data support our criteria for identification of effluxing strains, but we also appreciate the fact that some true effluxing strains may have been missed because reserpine-insensitive resistance mechanisms can mask the contribution of efflux.

Gene expression. qRT-PCR revealed the overexpression of at least one pump gene in 54/114 effluxing strains, establishing a frequency of 47.4% (23.3% among all 232 studied strains) (Table 3). Slot blotting verified the qRT-PCR data for each strain for which it was performed (data not shown). *norA* and *norB* overexpression predominated (26 and 29 strains, respectively), but strains having increased expression of *mepA*, *mdeA*, and *norC* were also observed. No isolate overexpressing *qacA/B* was identified; PCR using primers designed to detect the gene revealed it to be absent from all effluxing strains (data not shown).

Increased expression of one pump gene was observed in 31 effluxing strains and that of two or more genes was observed in 23 effluxing strains, with 14, 3, and 6 strains overexpressing two, three, and four genes, respectively. Overexpression of one gene was most common in strains overexpressing *norA* (15/26 strains [57.7%]) or *mepA* (3/5 strains [60%]). When two or more genes were overexpressed concomitantly, *norBC* predominated (15 strains). The number of substrates effluxed was not predictive of increased expression of a particular pump or pump combination. For example, strains overexpressing four genes effluxed four to six substrates, compared to two to eight for those having increased expression of only one pump. MRSA comprised 76% (41/54 strains) of strains overexpressing any pump gene. Interestingly, four of five *mepA*-overexpressing strains were methicillin susceptible. Identification of additional *mepA*-overexpressing strains will be necessary to determine if this trend is maintained.

Increased expression of pump genes is common in bloodstream isolates of *S. aureus* in the Detroit area. Overexpression was slightly more common among MRSA strains (41/82 strains [50%]) than among methicillin-susceptible *S. aureus* strains (13/32 strains [41%]). Our data represent low-end estimates based on our screening procedures and the possibility of false-negative qRT-PCR results. The probes and primers employed were based on the genome sequence of *S. aureus* 8325, and it is possible that sequence variations in clinical strains could result in poor or no amplification of or hybridization with targets.

The frequency of *norA* overexpression in our strains underscores the clinical relevance of this MDR pump. However, our data also reveal that other pumps, especially that encoded by *norB*, can contribute to reduced susceptibility to dyes, biocides, and NOR. Simultaneous overexpression of two or more pumps may result in an enhanced effect for common substrates, which has been documented for *Escherichia coli* and *Pseudomonas aeruginosa* (15). Existing data for NorA, NorB, and NorC indicate that all three transport selected fluoroquinolones, and further work overexpressing them singly and in combination is required to determine if enhanced effects on biocide and fluoroquinolone resistance occur in *S. aureus* (30–32).

QacA and -B are highly similar plasmid-encoded MDR pumps that are common in some parts of the world but rare or absent in southeastern Michigan *S. aureus* isolates (1, 21, 25). However, some substrates transported by QacA/B are also transported by NorA, NorB, and NorC. Biocides such as quaternary ammonium compounds and CH are employed for environmental decontamination and skin cleansing, respectively, including preoperative use (18). Efflux-related MIC increases for these compounds may allow an organism to survive in relatively protected or sequestered environments, including the skin surface, potentially increasing the likelihood of subsequent infection. The transport of clinically important antimicrobial agents, such as fluoroquinolones, by these pumps further increases concern.

TABLE 3. Gene expression analysis by qRT-PCR

Gene	No. (%) of effluxing strains with increased expression	Fold increase in expression (mean ± SD [range])	No. of substrates effluxed	Unique promoter region mutations found in effluxing strains (no. of strains with mutations)
mepA	5 (4.4)	9.3 ± 2.5 (6.5–11.8)	5–7	C→T at +3 (1) and A→G at +4 (2) of *mepR*[a]
norA	26 (22.8)	15.8 ± 10.5 (4.8–50.6)	2–6	AAT or CAAT insertion (17), 15-bp deletion (2)[b]
norB	29 (25.4)	13.7 ± 14.7 (5.1–80.2)	3–8	C→T 25 bp 3′ of −10 motif (4)
norC	19 (16.7)	10.0 ± 6.4 (5.1–30.4)	3–7	T→C 3 bp 3′ of −35 motif (1)
mdeA	13 (11.4)	10.5 ± 5.3 (5.0–21.5)	3–6	G→T 8 bp 3′ of −35 motif (3)

[a] Relative to the *mepR* transcription start site.
[b] Insertions start immediately 3′ and deletions start 9 bp 3′ of the −10 motif.

Sequencing. No *mgrA* mutations were identified in strains overexpressing *norA*, *norB*, or *norC*, eliminating MgrA substitutions as etiologic. A variety of point mutations, some within established or putative promoter regions, were observed in strains overexpressing pump genes but were also found in control strains not overexpressing any pump gene (data not shown). It is unlikely that such mutations are significant. However, mutations unique to effluxing strains were observed and could play a role in gene overexpression (Table 3). An analysis of available complete genome data for *S. aureus* (nine strains [www.ncbi.nlm.nih.gov/genomes/lproks.cgi]) identified the G→T *mdeA* transversion in two strains (MRSA252 and *S. aureus* RF122), whereas the *norB*, *norC*, and *mepR* mutations were not observed. The *mepR* upstream mutations lie within the established MepR footprint and may affect *mepR* autoregulation (9).

The *norA* promoter region mutations identified occurred either immediately or 9 bp 3′ of the −10 motif (AAT and CAAT insertions and 15-bp deletion, respectively). These mutations were remarkable in that they were present in 19 of 26 *norA*-overexpressing strains. MRSA252 possesses an ATCAAT insertion immediately 3′ of the −10 motif, and in this strain, *norA* expression was increased 23-fold (data not shown). This finding supports the conclusion that insertions such as those found at this position result in augmented *norA* expression. The 15-bp deletion is within the 5′-untranslated region of *norA* mRNA and conceivably could alter the mRNA half-life (5). The introduction of these and previously described promoter region mutations into the proper host strain, followed by a determination of gene expression and mRNA half-life, where appropriate, is required to accurately assess their effects.

A number of mutations within structural genes resulting in amino acid substitutions were also observed (data not shown). Other than the A103V substitution in MepR (see below), such substitutions are not important with respect to gene expression but could alter protein function. Some of these substitutions are found among the nine strains for which genome sequences are available. The effects of these substitutions on the functions of the respective pumps require further study.

MepR is a repressor of *mepR* and *mepA* expression (9). The A103V MepR substitution identified in one *mepA*-overexpressing strain is novel in that it was not found in any control strain or strain for which a whole genome sequence is available. The induction of wild-type *mepR* expression in SA-K2916-R resulted in a 90% reduction in chromosomal *mepR* expression, whereas this effect was <10% in SA-K2916-R (A103V) (data not shown). These data indicate that the A103V substitution severely impairs the function of MepR and is the likely reason for *mepA* overexpression in the strain in which it was found.

Concluding remarks. In the presence of antimicrobial substrates, efflux pump activity increases the chances for organism survival and the possible emergence of high-level target-based resistance. Overexpression of MDR efflux pump genes is common in *S. aureus* in southeastern Michigan and supports the notion that such pumps have clinical relevance. Previous work has indicated that combinations of substrates and efflux pump inhibitors reduce the emergence of target-based resistance mechanisms (19, 20). Because such an approach may provide therapeutic benefits, further work toward the development of *S. aureus* efflux pump inhibitors is warranted.

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