Microbiology (2008), **154**, 3144–3153 DOI 10.1099/mic.0.2008/021188-0

Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple *in vitro* exposures to biocides and dyes

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Biocides and dyes are commonly employed in hospital and laboratory settings. Many of these agents are substrates for multiple-drug resistance (MDR)-conferring efflux pumps of both Gram-positive and Gram-negative organisms. Several such pumps have been identified in *Staphylococcus aureus*, and mutants overexpressing the NorA and MepA MDR pumps following exposure to fluoroquinolones have been identified. The effect of exposure to low concentrations of biocides and dyes on the expression of specific pump genes has not been evaluated. Using quantitative reverse-transcription PCR we found that exposure of clinical isolates to low concentrations of a variety of biocides and dyes in a single step, or to gradually increasing concentrations over several days, resulted in the appearance of mutants overexpressing *mepA*, *mdeA*, *norA* and *norC*, with *mepA* overexpression predominating. Overexpression was frequently associated with promoter-region or regulatory protein mutations. Mutants having significant increases in MICs of common pump substrates but no changes in expression of studied pump genes were also observed; in these cases changes in expression of as-yet-unidentified MDR pump genes may have occurred. Strains of *S. aureus* that exist in relatively protected environments and are repeatedly exposed to sublethal concentrations of biocides can develop efflux-related resistance to those agents, and acquisition of such strains poses a threat to patients treated with antimicrobial agents that are also substrates for those pumps, such as ciprofloxacin and moxifloxacin.

Received 5 June 2008
Accepted 27 June 2008

INTRODUCTION

Biocides are commonly used in the processes of cleaning hospital, laboratory and home environments, sterilization of medical equipment, and skin decontamination prior to surgery (McDonnell & Russell, 1999; Russell, 2003). Pathogenic organisms in the hospital environment may be sheltered from the effect of these compounds by the concomitant presence of materials that interfere with their action or their existence in an environmental niche that provides some protection (McBain *et al.*, 2002; McDonnell & Russell, 1999). Such organisms, which have been shown to contribute to hospital-acquired infections, may be repeatedly exposed to sublethal concentrations of biocides, increasing the possibility of the development of reduced susceptibility (Boyce, 2007; Smith *et al.*, 2008). The presence of biocide residues on the skin or inanimate objects may also provide such selective pressure.

One mechanism by which reduced biocide susceptibility can occur is the upregulation of multiple-drug resistance (MDR) efflux pumps that include these compounds within their substrate profiles. While it is agreed that increases in biocide MICs conferred by efflux pumps probably do not confer true resistance at the concentrations deployed for environmental or cutaneous disinfection, upregulation of MDR pumps as a result of biocide exposure may provide a survival advantage as well as resulting in low-level resistance to antimicrobial agents that also are substrates for those pumps. Such low-level resistance can have significant consequences (see below).

Abbreviations: AF, acriflavine; BAC, benzalkonium chloride; CET, cetrimide; CHX, chlorhexidine; CV, crystal violet; DEQ, dequalinium; EB, ethidium bromide; MDR, multiple-drug resistance; MFS, major facilitator; MUG, 4-methylumbelliferyl β-D-galactopyranoside; NOR, norfloxacin; PENT, pentamidine; PY, pyronin Y; qRT-PCR, quantitative reverse-transcription-PCR; RD, rhodamine 6G; TPP, tetraphenylphosphonium bromide.

Dyes such as acriflavine (AF), ethidium bromide (EB), rhodamine 6G (RD) and pyronin Y (PY) have significant utility in the research laboratory, including nucleic acid staining and the study of transport processes in eukaryotic and microbial cells (Horobin & Kiernan, 2002). In particular, EB is a good substrate for many bacterial efflux pumps and susceptibility to it in the presence and absence of an efflux pump inhibitor has been used as a screen for the presence of efflux-related resistance mechanisms (DeMarco *et al.*, 2007). Structural features of substrates of bacterial MDR pumps of the major facilitator (MFS), small multidrug resistance (SMR), and perhaps the multidrug and toxin extrusion (MATE) families include hydrophobic moieties and a positive charge; many dyes and biocides share these characteristics and both groups of compounds are commonly transported by an individual MDR pump (Hassan *et al.*, 2007; Poole, 2005).

Examination of the *Staphylococcus aureus* genome reveals numerous potential MDR efflux-pump-encoding genes (see www.membranetransport.org). Some of those that have been studied in detail include QacA and QacB, highly similar MFS pumps that are encoded on plasmids, NorA and MdeA, both chromosomally encoded MFS pumps, and MepA, a MATE-family MDR pump that also is chromosomally encoded (Kaatz *et al.*, 2005a; Paulsen *et al.*, 1996b). More recently described are the NorB, NorC and SdrM MFS pumps, the genes for which are also chromosomal (Truong-Bolduc *et al.*, 2005, 2006; Yamada *et al.*, 2006). Of considerable interest is the *sepA* gene, positioned immediately downstream of *sdrM*, which encodes a structurally unique transporter that has some similarity to the SMR family of proteins (Narui *et al.*, 2002). Unlike all other *S. aureus* SMR family pumps described to date, SepA is encoded on the chromosome.

With the exception of SdrM all the transport proteins just described are highly conserved among strains for which genome sequence data are available (n=13; 95–100 % homology; see http://http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). SdrM is conserved in 10 of 13 sequenced strains (98–100 % homology), but less conservation is present in MRSA252 (87 %) and in Mu3 and Mu50, both of which have a deletion of residues 206–291. Each of the above-described transporters has various biocides and dyes as substrates and some also efflux hydrophilic fluoroquinolone antimicrobial agents such as norfloxacin, ciprofloxacin and moxifloxacin. Susceptibility to these clinically relevant agents may be sufficiently reduced by MDR pumps such that the emergence of target-based high-level resistance is favoured (Markham & Neyfakh, 1996).

In this study we exposed bloodstream isolates of *S. aureus* to low to moderate concentrations of several biocides and dyes and examined the resultant mutants for upregulation of several MDR efflux pumps, mainly those of chromosomal origin. We found that single- and multiple-step exposure led to the appearance of *mepA*, *norA*, *norC* and *mdeA*-overexpressing mutants, with *mepA* overexpression predominating. Overexpression of *mepA* most often was associated with the appearance of *mepR* point mutations that inactivated this negative regulator. MDR efflux pump overexpression in *S. aureus* thus is a relatively common consequence of biocide and dye exposure.

METHODS

Bacterial strains, plasmids, media and reagents. *S. aureus* SH1000, which is a derivative of *S. aureus* NCTC 8325-4 in which the *rsbU* mutation has been repaired, was used as a control strain (Horsburgh *et al.*, 2002). Eight clinical strains (three meticillin-susceptible and five meticillin-resistant) from a collection of unique bloodstream isolates of *S. aureus* used in a study of the frequency of baseline MDR efflux pump overexpression (DeMarco *et al.*, 2007), each overexpressing no more than one MDR efflux pump, were selected at random and employed as test strains (Table 1). Strains and plasmids used for functional analyses of *mepR* mutants are listed in Table 2. Reagents were the highest grade available and along with media were obtained from Sigma or BD Biosciences. Unless otherwise noted, the incubation temperature for all experiments was 35 °C.

Antimicrobial susceptibility testing. MICs were determined in duplicate using a microdilution procedure with and without reserpine (20 μg ml^{-1}) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006). Compounds selected for evaluation included AF, benzalkonium chloride (BAC), cetrimide (CET), chlorhexidine (CHX), crystal violet (CV), dequalinium (DEQ), EB, norfloxacin (NOR), pentamidine (PENT), PY, RD, and tetraphenylphosphonium bromide (TPP). One or more of these compounds have been shown to be substrates for many of the known *S. aureus* MDR efflux pumps (Hassan *et al.*, 2007). A reserpine-mediated MIC reduction of at least fourfold was considered indicative of efflux of that compound and hereafter will be referred to as a significant reserpine effect. For production of mutants by the single-exposure approach, MICs were also determined by agar dilution in order to incorporate accurate drug concentrations in selection plates. Compounds used for single- and multiple-exposure mutant production were chosen from those showing no significant reserpine effect in microdilution MIC testing. This approach allowed for simple identification of efflux-related phenotypes by the appearance of a significant reserpine effect for one or more common pump substrates.

Mutant production. Test organisms were exposed to low to moderate concentrations of various biocides and dyes to determine if such exposure resulted in the appearance of mutants overexpressing one or more MDR efflux pumps. For single-exposure mutant production, organisms were grown overnight in Mueller–Hinton broth (MHB) and then diluted 100-fold into fresh, pre-warmed MHB. This culture was incubated with shaking to an OD_{660} of 0.7, as previous experiments had shown that this represented the late exponential growth phase (data not shown). Cells were recovered by centrifugation and resuspended in 1 ml fresh MHB. Serial dilution and plating techniques were employed to determine the $\log_{10}$ (c.f.u. ml^{-1}) of this suspension, and aliquots of the undiluted and serially diluted suspension were plated onto Mueller–Hinton agar (MHA) plates containing two and four times the respective agar dilution MIC of the appropriate compound. Compounds used in this procedure included DEQ (strains K3043, K3166, K3221 and K3231), CET (strain K3250), BAC (strains SH1000, K3055, K3155, K3221 and K3250), EB (strains SH1000, K3043, K3155, K3166, K3225 and K3231) and RD (strains K3055, K3231 and K3250). Plates were examined for growth after 48 h incubation.

Multiple-exposure mutants were produced using the gradient plate approach (Bryson & Szybalski, 1952). Initial gradients consisted of

Table 1. Baseline characteristics of study strains

Strain*	MDR pump overexpressed†	MIC (μg ml^{-1}) for‡:										
		AF‡	BAC	CET	CHX	CV	DEQ	EB	NOR	PENT	PY	RD	TPP
SH1000	None	25 (4)	1.25 (2)	0.63 (4)	0.63 (16)	0.08 (1)	1.25 (4)	6.25 (2)	1.56 (4)	25 (1)	3.13 (2)	0.63 (1)	25 (2)
K3043 (MS)	None	25 (2)	1.25 (4)	1.25 (4)	0.16 (2)	0.08 (1)	1.25 (1)	6.25 (1)	0.78 (2)	25 (1)	3.13 (1)	0.63 (1)	25 (2)
K3055 (MR)	*norA*	50 (2)	2.5 (2)	2.5 (8)	0.63 (4)	0.08 (1)	2.5 (2)	25 (4)	>100 (1)	100 (2)	6.25 (2)	1.25 (2)	100 (2)
K3155 (MR)	None	25 (4)	1.25 (2)	0.63 (4)	0.31 (8)	0.08 (1)	1.25 (2)	6.25 (2)	0.78 (4)	25 (2)	1.56 (1)	0.63 (1)	50 (2)
K3166 (MR)	None	12.5 (2)	1.25 (2)	0.63 (1)	0.08 (4)	0.08 (1)	1.25 (2)	12.5 (2)	>100 (1)	25 (1)	3.13 (2)	0.63 (1)	25 (1)
K3221 (MS)	None	50 (8)	1.25 (2)	0.63 (2)	0.04 (1)	0.08 (1)	1.25 (2)	6.25 (4)	1.56 (4)	100 (2)	3.13 (2)	0.63 (1)	25 (2)
K3225 (MR)	*norA*	50 (8)	2.5 (2)	1.25 (4)	1.25 (4)	0.08 (1)	2.5 (2)	12.5 (2)	100 (2)	100 (1)	3.13 (2)	1.25 (2)	100 (2)
K3231 (MS)	None	50 (2)	1.25 (2)	0.63 (2)	0.16 (8)	0.16 (2)	2.5 (2)	12.5 (2)	1.56 (2)	100 (2)	3.13 (2)	0.63 (1)	50 (1)
K3250 (MR)	*norA*	50 (8)	2.5 (2)	5.0 (2)	0.31 (4)	0.08 (1)	2.5 (2)	50 (8)	>100 (1)	100 (1)	6.25 (2)	1.25 (2)	100 (2)

*MS, meticillin-susceptible; MR, meticillin-resistant.

†Compared with *S. aureus* SH1000; determined by qRT-PCR.

‡AF, acriflavine; BAC, benzalkonium chloride; CET, cetrimide; CHX, chlorhexidine; CV, crystal violet; DEQ, dequalinium; EB, ethidium bromide; NOR, norfloxacin; PENT, pentamidine; PY, pyronin Y; RD, rhodamine; TPP, tetraphenylphosphonium bromide. Numbers in parentheses indicate fold reduction in MIC by reserpine (20 μg ml^{-1}). (1) indicates no MIC change.

zero to two times the agar-dilution MIC of BAC (strains K3055, K3221 and K3250), EB (strains SH1000, K3043, K3155, K3166, K3225 and K3231), or RD (all strains). After 24 h incubation organisms from the leading edge of growth were passed again until growth occurred across the entire plate. This process was repeated using gradually increasing drug gradients to a maximum of 20, 30 and 3 μg ml^{-1} for BAC, EB and RD, respectively, and generally was completed after no more than six passages.

Several colonies from single-exposure plates and the leading edges of growth from the final gradient plates were streaked onto MHA to recover single colonies of potential mutants. One colony from each exposure condition was selected at random and microdilution MICs in the absence and presence of reserpine were determined. These organisms were passed three additional times on MHA followed by repeat MIC testing to ensure maintenance of a significant reserpine effect for at least one test compound compared with the respective parental strain. Mutants meeting this condition were considered stably resistant mutants.

qRT-PCR. The expression of *mepA*, *norA-B-C*, *mdeA*, *sdrM* and *sepA* in parent and putative MDR pump-overexpressing mutants was determined using quantitative reverse-transcription PCR (qRT-PCR) as described previously (DeMarco *et al.*, 2007). Beacon Designer 7.01 (Premier Biosoft International) was used to design Taqman probes and primers based on the genome sequence of *S. aureus* 8325, which were purchased commercially (Operon Biotechnologies). qRT-PCR was performed in triplicate using the Superscript III Platinum One-Step kit (Invitrogen) and an ABI 7500 Fast Real-Time PCR system (Applied Biosystems) with parameters of 45 °C for 30 min, 95 °C for 2 min, and 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 threshold cycle method was used to calculate relative gene expression compared with that of *S. aureus* SH1000; expression of each studied gene in this strain was considered to be 1.0 (Livak & Schmittgen, 2001).

Based on earlier experience with the strain set from which test organisms used in this study were selected, *qacA/B* is very infrequent in Detroit area *S. aureus* bloodstream isolates (DeMarco *et al.*, 2007). Thus, using primers designed based on the sequence of *qacA* (GenBank sequence X56628) that also had complete homology with *qacB*, routine PCR was employed to detect the presence or absence of these genes.

EB efflux assay. All parent and each stably resistant single- and multiple-exposure mutant underwent a determination of EB efflux in real time to demonstrate efflux pump function. The procedure was performed using a fluorometric approach as described previously (Kaatz *et al.*, 2000). Experiments were performed in duplicate and results were expressed as mean total efflux over a 5 min time-course. Results for mutant strains were divided by those for the respective parent to normalize the data, which for mutants then represented an *n*-fold change compared with the parent. An increase of twofold or greater was considered significant. The effect of reserpine (20 μg ml^{-1}) was also determined and expressed as percentage reduction of efflux.

RNA slot blotting. Northern analyses were performed to verify qRT-PCR results. RNA from strains exhibiting overexpression of pump genes was immobilized on a nylon membrane using a Bio-Rad Bio-Dot SF apparatus (Bio-Rad) and methods exactly as described previously (Ausubel *et al.*, 2005). A PCR-generated fragment of the appropriate gene served as a probe, and labelling of the probe, hybridization and detection were performed using the BrightStar Psoralen-Biotin labelling, NorthernMax and BrightStar BioDetect kits according to the manufacturer's directions (Ambion). 16S rRNA was used as the endogenous control and data generated for the

Table 2. Strains and plasmids used for MepR functional analyses

Strain or plasmid	Relevant characteristic(s)*	Source or reference
***S. aureus* strains**		
SA-K2916	SH1000 *mepR*::*lacZ*	Kaatz *et al.* (2006)
SA-K2916-R	SA-K2916/pK434	Kaatz *et al.* (2006)
SA-K2916-R (Q18P)	SA-K2916/pK580	This study
SA-K2916-R (G97E)	SA-K2916/pK582	This study
Plasmids		
pALC2073	*S. aureus* vector containing a tetracycline-inducible promoter controlling expression of cloned genes; Cm^r	Bateman *et al.* (2001)
pK434	pALC2073 *mepR* wild-type	Kaatz *et al.* (2005a)
pK580	pALC2073 *mepR* (Q18P)	This study
pK582	pALC2073 *mepR* (G97E)	This study

*Cm^r, confers chloramphenicol resistance.

appropriate parent strain were used as the standard to which mutant data were compared.

Sequencing. Promoter and coding regions of MDR pump genes having increased expression relative to parent strains were amplified by PCR and then sequenced in both directions using an automated method by the Applied Genomics and Technology Center, Wayne State University (Sanger *et al.*, 1977). For strains overexpressing *norA-B-C* the sequence of *mgrA* and its promoter was determined as MgrA is known to affect the expression of each of these genes (Luong *et al.*, 2006). In addition, for strains overexpressing *mepA* the sequence of *mepR*, which encodes the repressor of *mepA* expression and is encoded immediately upstream of *mepA*, was determined also. DS Gene 1.5 (Accelrys) was used for nucleotide sequence analyses. The sequence of the respective parental strain was the standard with which experimental data were compared.

MepR functional analyses. Point mutations in *mepR* resulting in MepR amino acid substitutions were observed in two *mepA*-overexpressing mutants (see Table 4). The *mepR* coding region from each of these mutants was amplified using PCR and cloned into pALC2073, producing pK580 and pK582 (Table 2). Strains containing pACL2073-based constructs were grown in the absence and presence of 50 ng tetracycline ml^{-1} to assess the effect of induction of plasmid-based *mepR* expression.

MepR is a repressor of both *mepR* and *mepA* expression. This property was exploited to assess the functional integrity of mutant MepR proteins using a fluorescent β-galactosidase assay as described previously, employing 4-methylumbelliferyl β-D-galactopyranoside (MUG) as a substrate (Kaatz *et al.*, 2006). β-Galactosidase activity was expressed in MUG units; 1 MUG unit=1 pmol MUG cleaved per minute per OD_{600} unit. Experiments were performed in triplicate, and the expression of chromosomal *mepR* in SA-K2916-R, SA-K2916-R (Q18P), and SA-K2916-R (G97E) was quantified by integrating the area beneath the expression curves with SigmaPlot 10.0 (Systat Software).

RESULTS

Susceptibility data, parent strains

MICs for parent strains with and without reserpine are given in Table 1. Fourfold or greater decreases in MICs were observed for as few as one (CHX for both K3166 and K3231) to as many as five tested substrates (SH1000). For those compounds chosen for use in mutant production, agar-dilution MICs were within twofold of those determined from the microdilution results (data not shown). The presence of a significant reserpine effect for at least one compound in all parent strains indicated that some efflux pump activity was probably present at baseline.

Single- and multiple-exposure mutants

With the exception of K3225 all parent strains were exposed to at least two single-exposure selecting agents, and putative mutants were recovered in all cases except for two- and fourfold MIC BAC exposures for strain K3250 and fourfold MIC RD exposures for all strains. Single-exposure mutants appeared at frequencies ranging from 1 in 10^6 to 1 in 10^9 c.f.u. at twofold MIC exposures and 1 in 10^7 to 1 in 10^{10} c.f.u. at fourfold MIC exposures. Serial passage of putative mutants on drug-free media identified instability of the resistance phenotype for 9 of 17 single-exposure derivatives. Substrates having stable MIC increases in the remaining eight mutants are listed in Table 3. MIC increases ranged from as little as twofold to as high as 32-fold. Interestingly, fourfold or greater MIC increases were infrequently observed for the selecting agent (two out of eight mutants). For example, exposure of strains K3043, K3166 and K3221 to DEQ resulted in fourfold or greater MIC increases for eight, five and seven substrates, respectively, but these substrates did not include DEQ.

The multiple-exposure, or gradient plate, process resulted in the recovery of putative mutants in all cases in which this procedure was applied. As for single-exposure mutants, not all strains maintained their resistance profiles after serial passage in drug-free media. However, the proportion that did was greater than observed for single-exposure organisms (14 out of 18 versus 8 out of 17). Instability was observed for SH1000 (EB exposure), K3055

Table 3. Susceptibility changes for single- and multiple-exposure mutants (compared with parent strain)

Parent	Mutant*	No. of substrates with stable MIC increases (range of fold increase)	Substrates with ⩾4× MIC increase
SH1000			
	SE-EB	4 (2–4)	TPP
	ME-RD	6 (2–8)	EB, RD, TPP
K3043			
	SE-DEQ	11 (2–16)	AF, CHX, EB, NOR, PENT, PY, RD, TPP
	ME-EB	8 (2–8)	CHX, EB, PENT, RD
	ME-RD	5 (2–16)	CHX, CV, RD
K3055			
	SE-RD	7 (2–4)	EB
	ME-RD	8 (2–4)	CV, RD
K3155			
	SE-BAC	10 (2–16)	EB, CV, PENT, PY, RD
	SE-EB	7 (2–8)	EB, CV, PENT, PY
	ME-EB	11 (2–16)	AF, CET, EB, NOR, PENT, PY, RD
K3166			
	SE-DEQ	11 (2–16)	CET, CHX, CV, PENT, PY
	ME-EB	11 (2–16)	CET, CHX, CV, EB, PENT, PY
	ME-RD	11 (2–16)	CET, CHX, EB, PENT, TPP
K3221			
	SE-DEQ	11 (2–32)	CET, CHX, CV, EB, NOR, PY, RD
	ME-BAC	9 (2–32)	CET, CHX, EB, TPP
	ME-RD	9 (2–8)	CET, CHX, CV, DEQ, EB, PY, RD, TPP
K3225			
	SE-EB	6 (2–8)	EB, PY, RD
	ME-EB	6 (2–8)	CET, EB, PY, RD
	ME-RD	8 (2–8)	CET, CV, EB, PY, RD
K3231			
	ME-EB	9 (2–16)	BAC, CET, CHX, EB, NOR, PY, RD
	ME-RD	7 (2–8)	CHX, NOR, RD
K3250			
	ME-RD	5 (2–4)	CHX

*Selection conditions and compound. SE and ME, single- and multiple-exposure. See Table 1 footnote for test compound abbreviations.

and K3250 (BAC exposure), and K3155 (RD exposure). Fourfold or greater MIC increases for selecting agents were not always observed but were more common than in single-exposure mutants (11 out of 14; 79 %).

Gene expression analyses and EB efflux

All parental strains and stable mutants were analysed for their expression levels of *mepA*, *norA-B-C*, *mdeA*, *sepA* and *sdrM* compared with that of SH1000. Data for mutants then were normalized to those of the appropriate parent, and a fourfold or greater increase in expression compared with the parent was considered significant (Table 4). qRT-PCR results for genes demonstrating significantly increased expression were verified by slot-blot analyses (data not shown). *qacA/B* were found to be absent from this strain set using a PCR-based screening approach (data not shown). None of the parental strains demonstrated increased expression of any pump gene with the exception of K3055, K3225 and K3250, which had elevated *norA* expression at baseline compared with SH1000 (6.6-, 7.1-, and 4-fold, respectively).

Increased expression of *mepA* predominated (8 out of 22 mutants, or 36 %), resulting most commonly after multiple exposure [BAC (1), EB (3), RD (2)] but also occurring with single exposure [DEQ (1) and EB (1)]. Increased *norA* expression was observed in three mutants (14 %) and followed single exposure to DEQ (1) and multiple exposure to EB (2). Increased *mdeA* and *norC* expression were observed only after multiple-exposure to RD (one mutant each). One multiple-exposure mutant overexpressed two pump genes (*mepA* and *norA*; K3155 ME-EB). No increased expression of *norB*, *sepA* or *sdrM* was observed. The magnitude of increased pump gene expression ranged from as little as 7-fold to more than 450-fold. There also

Table 4. Gene expression, EB efflux, and sequence analyses

Parent*	Mutant†	Expression increased‡	Magnitude§	EB efflux§	Sequence‖
SH1000				1.0	
	SE-EB	*mepA*	6.9	5.9	Premature *mepR* stop after six codons
	ME-RD	*mdeA*	394	5.4	GTGCTA→TTGCTA (−35 motif); G→T at +2 of *mdeA* mRNA
K3043				1.0	
	SE-DEQ	*norA*	39.3	11.5	*mgrA* and *norA* same as parent
	ME-EB	*mepA*	39.9	11.9	Premature *mepR* stop after 10 codons; A302S MepA
	ME-RD	None	–	1.0	ND
K3055 (↑ *norA*)				1.0	AAT insertion 3′ to −10 motif
	SE-RD	None	–	1.3	ND
	ME-RD	None	–	1.0	ND
K3155				1.0	
	SE-BAC	None	–	3.6	ND
	SE-EB	None	–	3.9	ND
	ME-EB	*mepA*	8.6	3.3	*mepRA* same as parent
		norA	9.7		Possible *norA* promoter up-mutation
K3166				1.0	
	SE-DEQ	*mepA*	26.5	3.4	G97E MepR
	ME-EB	*mepA*	68.4	3.9	Premature *mepR* stop after one codon
	ME-RD	*mepA*	198	4.1	Q18P MepR
K3221				1.0	
	SE-DEQ	None	–	1.7	ND
	ME-BAC	*mepA*	11.8	5.2	*mepRA* same as parent
	ME-RD	*mepA*	457	6.3	Premature *mepR* stop after four codons
K3225 (↑ *norA*)				1.0	AAT insertion 3′ to −10 motif
	SE-EB	None	–	1.7	ND
	ME-EB	None	–	1.2	ND
	ME-RD	*norC*	9.2	1.1	*norC* same as parent
K3231				1.0	
	ME-EB	*norA*	358	3.6	Possible *norA* promoter up-mutation
	ME-RD	None	–	1.0	ND
K3250 (↑ *norA*)				1.0	AAT insertion 3′ to −10 motif
	ME-RD	None	–	1.0	ND

*Strains with increased *norA* expression at baseline are indicated (↑ *norA*).
†Selection conditions and compound. SE and ME, single- and multiple-exposure. See Table 1 footnote for test compound abbreviations.
‡Compared with parent strain.
§Normalized to parent strain.
‖Only differences from parent strain are shown. ND, Not determined.

were five single- and five multiple-exposure mutants in which no change in pump gene expression was identified.

Like the qRT-PCR data, EB efflux data for mutants were normalized to the respective parent strain (Table 4). Interestingly, one mutant having a ninefold increase in *norC* expression had no change in EB efflux (K3225 ME-RD). Similar behaviour with respect to EB efflux was evident in 8 of the 10 mutants for which no increased expression of the genes studied here was identified. Where increased EB efflux was observed, reserpine generally was effective in reducing the efflux by at least 50 % (data not shown).

Sequencing

The 10 mutants not overexpressing any studied pump gene were omitted from this analysis. For the remaining 12 mutants sequence data are provided in Table 4; only differences from the respective parent strain are shown. For strains overexpressing *mepA* a variety of mutations were observed in *mepR*, including four strains having changes resulting in the creation of premature stop codons very early in the reading frame and two others with point mutations resulting in amino acid substitutions in MepR. The *mepRA* sequence of the remaining two *mepA*-overexpressing strains was unchanged from that of the

respective parent. A novel point mutation resulting in an A302S MepA substitution was observed for one mutant.

The single *mdeA*-overexpressing mutant was found to have a point mutation that changed the −35 motif from **G**TGCTA to **T**TGCTA as well as a G⟶T transversion at the +2 position of *mdeA* mRNA (Huang *et al.*, 2004). The −35 motif change results in a closer match to the consensus sequence of TTGACA.

Three parental strains overexpressed *norA* and in each case a previously described mutation associated with increased *norA* expression, consisting of an insertion of AAT immediately 3′ to the −10 promoter motif, was observed (DeMarco *et al.*, 2007). Overexpression of *norA* in two mutants (K3155 ME-EB and K3231 ME-EB) was associated with a novel potential promoter up-mutation, consisting of an A⟶G transition between the −35 and −10 motifs. This alteration disturbs an inverted repeat that has been shown to be important for the full repressive effect of MgrA (Kaatz *et al.*, 2005b). The sequence of *mgrA* for both of these strains was wild-type. The sequences of *norA* and *mgrA* were wild-type in the remaining *norA*-overexpressing mutant (K3043 SE-DEQ). Likewise, the sequences of *mgrA* and *norC* were unchanged from those of the parent strain for the *norC*-overexpressing mutant (K3225 ME-RD).

Mutant MepR proteins

The induction of wild-type *mepR* expression in SA-K2916-R resulted in an 83 % reduction in chromosomal *mepR* expression, whereas minimal to no effect was observed for strains SA-K2916-R (Q18P) and SA-K2916-R (G97E), respectively (Fig. 1).

DISCUSSION

MDR efflux pumps are an important mechanism by which bacteria can evade the effect(s) of antimicrobial agents. This resistance mechanism has received considerable attention in recent years, and there are ongoing efforts to develop inhibitors of MDR pumps. Effective inhibitors would expand the antibacterial armamentarium to include pump substrates and reduce the likelihood of the emergence of high-level target-based resistance mechanisms. Unfortunately, inhibitors evaluated to date have not been broad-spectrum in their activity. Inhibitors of MFS pumps are not active against resistance-nodulation-division (RND) family pumps such as AcrB of *Escherichia coli* or MexB of *Pseudomonas aeruginosa*, organisms of considerable medical importance. Likewise, RND inhibitors do not have activity against MFS pumps (Kaatz, 2005). Nevertheless, safe and effective inhibitors of the major classes of bacterial MDR pumps, even if separate compounds are required for MFS and RND inhibition, would be a welcome addition to antibacterial chemotherapy.

Previous studies in our laboratory have shown that exposure of *S. aureus* to low concentrations of fluoroquinolone antimicrobial agents can result in upregulation of NorA and MepA, but the effect of biocide exposure on drug pump expression in this organism has not been evaluated in detail (Kaatz & Seo, 1995; Kaatz *et al.*, 2000). Others have shown previously that exposure of *E. coli* to antiseptic agents such as pine oil and triclosan results in the upregulation of the AcrB MDR pump, which includes several antimicrobial agents in its substrate profile (Levy, 2002; McMurry *et al.*, 1998; Moken *et al.*, 1997). Our data now extend this observation to *S. aureus* in that we have

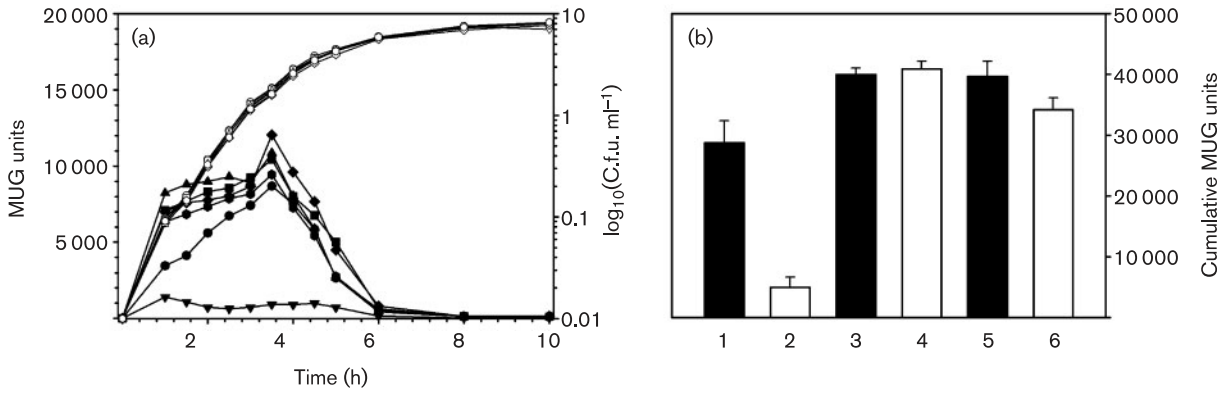

Fig. 1. Expression of chromosomal *mepR* in SA-K2916 containing pK434 (SA-K2916-R), pK580 [SA-K2916-R (Q18P)], and pK582 [SA-K2916-R (G97E)]. (a) *mepR* expression as a function of growth: ● and ▼, SA-K2916-R; ■ and ◆, SA-K2916-R (Q18P); ▲ and ⬣, SA-K2916-R (G97E). In each case data represented by the first and second symbols were obtained in the absence and presence of 50 ng tetracycline ml^{-1}, respectively. Open symbols illustrate growth of test organisms. Error bars are omitted for the sake of clarity. (b) Cumulative expression of *mepR* over the entire experiment. Black and white bars illustrate expression in the absence and presence of tetracycline, respectively. 1 and 2, SA-K2916-R; 3 and 4, SA-K2916-R (Q18P); 5 and 6, SA-K2916-R (G97E). Means ± SD are plotted.

shown that exposure of clinical isolates to low concentrations of a variety of biocides and dyes can produce mutants with increased expression of one or more MDR pumps. Even though the increases in biocide MICs observed in these mutants were moderate, such organisms are likely to have a survival advantage in environmental niches where biocide delivery is compromised.

Compounds chosen for mutant production were those showing no more than a twofold MIC reduction for parental strains in the presence of the efflux pump inhibitor reserpine. This approach resulted in variable agents being employed for individual test strains. However, the purpose of our work was not to compare one agent with another with respect to the frequency of emergence of mutants but rather to determine, in general, if biocides and dyes commonly employed in the hospital or laboratory environments could induce overexpression of MDR efflux pumps.

The absence of stably raised MICs for the selecting agent employed for some mutants was of considerable interest. This observation was more common for single-exposure than multiple-exposure mutants (6 out of 8 versus 3 out of 14, respectively). The lower incidence of this characteristic in multiple-exposure mutants most likely relates to the increased opportunities for accumulation of mutations in strains repeatedly exposed to a particular compound. Additive or synergistic effects on susceptibility are expected as the number of resistance-conferring mutations increases. However, for those mutants demonstrating instability of selecting-agent MICs, substrate affinity for the pump(s) in question may play a role. MIC changes for compounds with lower affinity are likely to be less pronounced than for those that are better substrates. Alternatively, it is conceivable that unidentified mutations may be present in these mutants affecting susceptibility to selecting agents in a direction opposite to that of the MDR pumps. It also must be recalled that we employed a definition of a stable fourfold or greater MIC increase as significant, but if twofold or greater were employed then the selecting agent would be included in all cases (data not shown).

Instability of the resistance phenotype to serial passage in the absence of drug was intriguing but not necessarily surprising. Pump overexpression in the absence of substrate could confer a selective disadvantage by way of wasted resources or perturbed growth kinetics. Reversion to a wild-type phenotype in the absence of selective pressure would reverse these potential disadvantages.

The magnitude of pump gene overexpression did not necessarily correlate with increases in EB efflux observed. This discrepancy is exemplified by the BAC and RD multiple-exposure mutants of K3221, where *mepA* expression was 12- and 450-fold increased, respectively, with similar increases in EB efflux (Table 4). Transcript quantity may not correlate with translated protein. There may be a maximal amount of MDR pump tolerated and in this situation excess transcripts are degraded prior to translation. Differences in sensitivity between the qRT-PCR and EB efflux assays also may have played a role.

Ten mutants demonstrated significant MIC increases but no change in expression of any of the pumps included in our study. It is possible that these MIC changes may be the result of a non-pump-related mechanism(s). However, it is more likely that these organisms have increased expression of pumps other than those we evaluated as all had a significant reserpine effect for at least one of the tested substrates. EB efflux of 8 of these 10 strains, including the RD multiple-exposure mutants of K3043, K3055, K3231 and K3250, the RD and DEQ single-exposure mutants of K3055 and K3221, respectively, and EB single- and multiple-exposure mutants of K3225 was $\leqslant$2-fold increased from that of the appropriate parent strain (Table 4). Five of these eight mutants were derived from parental strains that overexpressed *norA*; EB efflux in those parental strains was $\geqslant$47 %. The baseline activity of NorA in mutants derived from these parents almost certainly would obscure any contribution to EB efflux from other pumps. It is also conceivable that EB may be a poor substrate or not a substrate at all for these as-yet-unidentified pumps. Baseline increased *norA* expression and the fact that EB is a relatively poor NorC substrate probably accounts for the lack of change in EB efflux by the RD multiple-exposure mutant of K3225, which overexpressed *norC* (Hassan *et al.*, 2007).

Among the genes included in our screen, overexpression of *mepA* predominated (8 out of 22 mutants). Mutations in the gene encoding the MepR repressor were responsible in six cases, including four having premature truncations of MepR and two with amino acid substitutions (Q18P and G97E) resulting in severe impairment in MepR activity (Fig. 1). With respect to the two mutants having no mutations in the *mepRA* region, one or more mutations in alternative regulatory systems may be present. Overexpression of *mep*A has been observed in another strain in which the *mepRA* sequence is wild-type (Kaatz *et al.*, 2005a). It is apparent that *mepA* expression can be regulated independently of MepR.

In addition to augmented *mepA* expression, an A302S MepA substitution was identified in K3043 ME-EB. It seems unlikely that this change contributed to the 12-fold increase in EB efflux observed for this mutant. Single amino acid differences, both within transmembrane helices and in extramembrane loops, are known to alter substrate specificity, as has been shown for QacA/B and the tetracycline efflux transporter TetA (Paulsen *et al.*, 1996a; Sapunaric & Levy, 2005). In addition, there are conserved charged residues in the extramembrane loop between transmembrane helices 2 and 3 in many MFS efflux proteins, changes in which can adversely affect protein function (Yamaguchi *et al.*, 1992). However, to the best of our knowledge, mutations resulting in increased efficiency of transport for a particular substrate have not been identified. Introduction of the A302S mutation into MepA

and a comparison of the kinetics of EB efflux with that of wild-type MepA would be required to establish or refute any role it might have.

Two of the three mutants overexpressing *norA* were found to have the same mutation in the promoter region, consisting of an A→G transition between the −35 and −10 motifs. This change disrupts an inverted repeat, but whether or not it affects *norA* expression requires further study. Likewise, the *mdeA*-overexpressing mutant had a mutation resulting in the conversion of the −35 motif into a sequence that more closely matches the preferred consensus sequence and may improve promoter strength (Szoke *et al.*, 1987). It also had a G→T transversion at the +2 position that could affect mRNA stability, but further work would be required to assess this possibility formally. Increased *norA* and *norC* expression not associated with sequence changes in known regulatory elements, such as the promoter regions or known regulatory loci such as *mgrA*, is likely to result from mutational alterations in as-yet-uncharacterized loci that affect the expression of these genes.

Exposures to MDR pump substrates, whether those substrates are clinically relevant antimicrobial agents or compounds used to disinfect the hospital environment, can result in the emergence of *S. aureus* strains adapted to the presence of these compounds. Acquisition of such strains by patients may compromise the therapy of infections caused by them. Prudent use of antimicrobial agents and the use of biocides that are not known to be MDR pump substrates may reduce the frequency at which MDR-pump-overexpressing strains are found. Alternatively, the combination of a pump inhibitor with an antimicrobial agent or biocide will reduce the emergence of such strains and their clinical impact.

ACKNOWLEDGEMENTS

This work was supported by VA Research Funds.

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Edited by: J. H. Cove