International Journal of Antimicrobial Agents 45 (2015) 464–470

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Clonal relatedness is a predictor of spontaneous multidrug efflux pump gene overexpression in *Staphylococcus aureus*

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ARTICLE INFO

Article history:
Received 2 June 2014
Accepted 7 November 2014

Keywords:
Staphylococcus aureus
Efflux
spa Type
Gene overexpression
Antibiotic resistance
Ethidium bromide

ABSTRACT

Increased expression of genes encoding multidrug resistance efflux pumps (MDR-EPs) contributes to antimicrobial agent and biocide resistance in *Staphylococcus aureus*. Previously identified associations between *norA* overexpression and *spa* type t002 meticillin-resistant *S. aureus* (MRSA), and a similar yet weaker association between *mepA* overexpression and type t008 meticillin-susceptible *S. aureus* (MSSA), in clinical isolates are suggestive of clonal dissemination. It is also possible that related strains are prone to mutations resulting in overexpression of specific MDR-EP genes. Exposure of non-MDR-EP-overexpressing clinical isolates to biocides and dyes can select for MDR-EP-overexpressing mutants. *spa* types t002 and t008 isolates are predominated by multilocus sequencing typing sequence types (STs) 5 and 8, respectively. In this study, non-MDR-EP gene-overexpressing clinical isolates (MRSA and MSSA) representing ST5 and ST8 were subjected to single exposures of ethidium bromide (EtBr) to select for EtBr-resistant mutants. Measurements of active EtBr transport among mutants were used to demonstrate an efflux-proficient phenotype. Using quantitative reverse-transcription PCR, it was found that EtBr-resistant mutants of ST5 and ST8 parental strains predominantly overexpressed *mepA* (100%) and *mdeA* (83%), respectively, regardless of meticillin sensitivity. Associations between clonal lineage and MDR-EP gene overexpression differed from those previously observed and suggest the latter is due to clonal spread of efflux-proficient strains. The predilection of in vitro-selected mutants of related strains to overexpress the same MDR-EP gene indicates the presence of a consistent mutational process.

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1. Introduction

One of several contributing mechanisms to bacterial multidrug resistance is the action of membrane-based efflux proteins with broad substrate profiles, known as multidrug resistance efflux pumps (MDR-EPs). Extrusion of structurally unrelated compounds by MDR-EPs has been shown to reduce intracellular concentrations to either a non-inhibitory or borderline inhibitory level, favouring the emergence of target-based mutations and high-level resistance [1,2]. Numerous chromosomally and plasmid-encoded MDR-EPs have been identified in *Staphylococcus aureus*, a common source of community- and hospital-acquired infections. Increased expression of MDR-EP genes is common amongst clinical bloodstream isolates of *S. aureus*, contributing to limited therapeutic options and potentially enhanced survival in the environment [3–5].

Whilst not completely understood, a great deal is known about the transcriptional regulation of several MDR-EP genes in *S. aureus*. For some genes, various 'hotspots' for mutations leading to overexpression have been identified, such as promoter region and regulatory protein mutations for *norA* and *mepA*, respectively [6,7]. In contrast, little is known about MDR-EP gene overexpression patterns among genetically related strains. In a recent survey of several hundred clinical isolates collected in 2009, we found that overexpression of select MDR-EP genes was associated with *spa* type and meticillin sensitivity [3]. In particular, nearly three-quarters of *norA*-overexpressing strains were *spa* type t002, regardless of geographic origin, and 96% of type t002 *norA*-overexpressing strains were meticillin-resistant *S. aureus* (MRSA). *spa* Type t002 was rare amongst non-MDR-EP gene-overexpressing MRSA (3%). A similar but less profound association was observed among *spa* type t008 *mepA*-overexpressing meticillin-susceptible *S. aureus* (MSSA) isolates. These data suggest the possibility of widespread

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http://dx.doi.org/10.1016/j.ijantimicag.2014.11.007
0924-8579/Published by Elsevier B.V. on behalf of International Society of Chemotherapy

Table 1
Staphylococcus aureus bloodstream isolates used as parent strains in this study.

Isolate	Meticillin sensitivity	*spa* Type	ST	Origin (year)	Reference
SA-K3150	MR	t002	5	Detroit, MI (2005)	[4]
SA-K3850	MR	t002	5	Omaha, NE (2009)	[3]
SA-K4564	MS	t002	5	Omaha, NE (2009)	[3]
SA-K4639	MS	t002	5	Freiberg, Germany (2009)	[3]
SA-K3255	MR	t008	8	Detroit, MI (2005)	[4]
SA-K3946	MR	t008	8	Detroit, MI (2009)	[3]
SA-K3186	MS	t008	8	Detroit, MI (2005)	[4]
SA-K4055	MS	t008	8	Detroit, MI (2009)	[3]
SA-K3925	MR	t211	8	Boston, MA (2009)	[3]
SA-K4844	MR	t024	8	Detroit, MI (2010)	[3]

spa, Staphylococcal protein A; ST, sequence type; MR, meticillin-resistant; MS, meticillin-susceptible.

geographic dissemination of *spa* type t002 *norA*-overexpressing MRSA and *spa* type t008 *mepA*-overexpressing MSSA clones. However, a genetic predisposition among these strain types to acquire mutations resulting in *norA* and *mepA* overexpression, respectively, is also possible.

Exposure of non-MDR-EP-overexpressing clinical isolates to biocides and dyes can select for the emergence of mutants that constitutively overexpress MDR-EP genes [5]. In this study, non-MDR-EP gene-overexpressing *S. aureus* clinical isolates of two clonal lineages and different meticillin sensitivities were exposed to ethidium bromide (EtBr) and the resultant mutants were examined for overexpression of several chromosomally encoded MDR-EP genes. The results indicate that in vitro-selected MDR-EP gene overexpression is associated with parental lineage, although not as expected from previous epidemiological studies.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and reagents

S. aureus bloodstream isolates collected in 2005 and 2009 from geographically distinct hospitals have been described previously [3,4]. Ten isolates (six MRSA and four MSSA) found not to overexpress chromosomally- encoded MDR-EP genes (*mdeA*, *mepA*, *norA*, *norB*, *norC*, *sdrM* and *sepA*) and that were devoid of plasmid-encoded MDR-EP genes (*qacA*, *qacB* and *qacC*) were randomly selected among multilocus sequencing typing (MLST) sequence types (STs) 5 and 8 strains (Table 1). These STs are predominated by *spa* types t002 (83%) and t008 (97%), respectively (http://spa.ridom.de). Lack of chromosomal pump gene overexpression and absence of plasmid-encoded pump genes were confirmed by quantitative reverse-transcription PCR (qRT-PCR) and PCR, respectively [3,4]. Media and reagents were obtained from Sigma-Aldrich Co. (St Louis, MO) or BD Biosciences (Sparks, MD). The incubation temperature for all experiments was 35 °C.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of EtBr were determined by agar dilution in order to incorporate accurate EtBr concentrations in selection plates for production of EtBr-resistant mutants. Microdilution and agar dilution MICs were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. Compounds selected for evaluation included chlorhexidine (CHX), EtBr and tetraphenylphosphonium bromide (TPP), which have been shown to be substrates for various *S. aureus* MDR-EPs [9].

2.3. Mutant production

Test organisms were exposed to various concentrations of EtBr to select for the appearance of mutants overexpressing one or more MDR-EPs. Single-exposure mutant production was performed as described previously with some modifications [5]. Organisms were grown overnight in cation-supplemented Mueller–Hinton broth (SMHB), were recovered by centrifugation and concentrated 100-fold in 1 mL of fresh SMHB. Various dilutions then were plated onto supplemented Mueller–Hinton agar (SMHA) plates containing two (25 μg/mL) and four (50 μg/mL) times the EtBr agar dilution MIC. These plates were examined 24 h (EtBr-free SMHA) and 48–72 h (EtBr-containing SMHA) later for growth.

Four randomly selected EtBr-resistant colonies from each selection procedure were passed three times on SMHA to assure colony purity and the stability of any resistance phenotype present. Mutants whose EtBr microdilution MICs were at least twice that of their respective parental strains were considered stably resistant. To determine whether the observed frequencies of gene overexpression among ST5 and ST8 mutants were reproducible, four parent strains (representing each combination of ST and meticillin susceptibility) were re-exposed to EtBr and new mutants were characterised.

2.4. Ethidium bromide efflux assay

EtBr efflux assays were employed to demonstrate efflux as the mechanism of EtBr resistance in stably resistant mutants. The procedure was performed using a real-time fluorometric approach essentially as described previously [6]. Mutants and their parent strains were grown overnight in SMHB and were diluted 25-fold into fresh SMHB. Cultures were incubated at 35 °C with shaking to an optical density at 600 nm (OD_{600}) of 0.7–0.8. Cells were pelleted and re-suspended at OD_{600} = 0.8 in 0.5 mL aliquots of SMHB containing EtBr plus carbonyl cyanide *m*-chlorophenyl hydrazone (final concentrations, 25 μM and 100 μM, respectively). Following gentle agitation for 20 min at room temperature, cells were pelleted and stored on ice. Pellets were warmed at room temperature for 5 min and then re-suspended in 1 mL of fresh SMHB and 200 μL aliquots were immediately transferred into the wells of opaque 96-well flat-bottom plates (Corning Inc., Corning, NY). Fluorescence was monitored continuously using a BioTek FLx800 microplate reader (BioTek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 485 nm and 645 nm, respectively, for 5 min. Experiments were performed in triplicate with two technical replicates per biological replicate. Efflux activity of parent strains was expressed as percent fluorescence decrease over a 5-min time course. Results for mutant strains were divided by those for the respective parent to normalise the data, which for mutants then represented an *n*-fold change compared with the parent.