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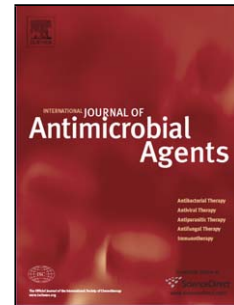
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**Identification of the plasmid-encoded *qacA* efflux pump gene in  
meticillin-resistant *Staphylococcus aureus* (MRSA) strain  
HPV107, a representative of the MRSA Iberian clone**

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## ABSTRACT

Meticillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial bacterium for which prevention and control measures consist mainly of the application of biocides with antiseptic and disinfectant activity. In this study, we demonstrated the presence of the plasmid-located efflux pump gene *qacA* in MRSA strain HPV107, a clinical isolate representative of the MRSA Iberian clone. The existence of efflux activity in strain HPV107 due to the QacA pump was also established and this QacA efflux activity was linked with a phenotype of reduced susceptibility towards several biocide compounds. No association could be made with antibiotic resistance. This work emphasises the potential of QacA pump activity in the maintenance and dissemination of important MRSA strains in the hospital setting and, increasingly, in the community.

## 1. Introduction

Treatment, prevention and control of infectious diseases have undergone remarkable changes during the last century. Use of antibiotics to eliminate pathogenic bacteria, combined with the application of chemical compounds for disinfectant and/or antiseptic purposes (also known as biocides), has allowed the management of several pathogenic bacteria known to cause life-threatening conditions [1]. However, the selective pressure derived from increasing usage of these antimicrobial compounds combined with the adaptability of bacteria has resulted in the emergence of bacterial strains resistant to representatives of one or more antibiotic classes as well as to biocides [1]. Bacteria that possess a multidrug-resistant (MDR) phenotype are of foremost concern in healthcare settings inasmuch as they are problematic for therapy.

Amongst the known mechanisms of resistance to antibiotics, the activity of multidrug efflux pumps is a major contributor to the development of MDR phenotypes [2]. In the pathogenic bacterium *Staphylococcus aureus*, several multidrug efflux pumps have been described and were found to be encoded by chromosomal genes (*norA*, *norB*, *norC*, *mepA*, *mdeA*, *sepA* and *sdrM*) as well as genes located on plasmids (e.g. *qacA/B*, *smr*, *qacG*, *qacJ* and *qacH*) [2]. Usually, chromosomally encoded multidrug efflux pumps can confer reduced susceptibility to either antibiotics or biocides, whilst the plasmid-encoded pumps can only confer reduced susceptibility to biocides [2]. Two such examples are the multidrug efflux pump QacA and the closely related QacB that extrude from *S. aureus* a wide variety of monovalent (both QacA and QacB) and divalent (only QacA) cationic compounds using the proton-motive force [3].

This study reports the finding of the multidrug efflux pump QacA in a clinical methicillin-resistant *S. aureus* (MRSA) strain HPV107, representative of the so-called MRSA Iberian clone that is currently disseminated throughout several European countries and the USA [4].

## 2. Materials and methods

### 2.1. Strains

The strain HPV107, representative of the MRSA Iberian clone, was isolated at a Portuguese hospital in 1992 and is characterised by resistance to several classes of antibiotics, particularly  $\beta$ -lactams, aminoglycosides, fluoroquinolones, macrolides, rifampicin and tetracycline [5–7]. Strain HPV107<sub>cured</sub> corresponds to MRSA strain HPV107 cured of plasmids. *Staphylococcus aureus* ATCC 25923, a clinical isolate collected in Seattle, WA, in 1945 and fully susceptible to antibiotics, was used as a reference strain. Strains were grown in tryptic soy broth or tryptic soy agar (TSA) (Oxoid Ltd., Basingstoke, UK) at 37 °C. For minimum inhibitory concentration (MIC) determination, cultures were grown in Mueller–Hinton broth (Oxoid Ltd.) at 37 °C.

### 2.2. Compounds

All antibiotics, biocides, dyes and efflux pump inhibitors (EPIs) were purchased from Sigma-Aldrich (St Louis, MO) or Fluka Chemie GmbH (Buchs, Switzerland).

### *2.3. Minimum inhibitory concentration determination*

MICs of the study strains towards several antibiotics (oxacillin, ciprofloxacin, norfloxacin, levofloxacin, sparfloxacin, gentamicin, trimethoprim, kanamycin, chloramphenicol, tetracycline and erythromycin), biocides [tetraphenylphosphonium bromide, dequalinium chloride, benzalkonium chloride, hexadecyltrimethylammonium bromide, cetrimide, chlorhexidine (CHX) and pentamidine isethionate salt] and dyes [ethidium bromide (EtBr), berberine, acriflavine and crystal violet] were determined by the two-fold microdilution broth method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [8], in the absence and presence of EPIs, namely thioridazine, chlorpromazine (CPZ), verapamil and reserpine.

### *2.4. Plasmid DNA isolation and plasmid curing*

Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), with an additional incubation step with lysostaphin (35 mg/L) (Sigma-Aldrich) at 37 °C for 90 min prior to cell lysis. HPV107 was cured of plasmids by successive passage in medium containing increasing subinhibitory concentrations of CPZ (from 2 mg/L to 22 mg/L) [9] or novobiocin (NOV) (2 µg/L to 125 µg/L) [10]. The efflux capacity of each subculture was evaluated by plating overnight cultures on TSA media containing the broad-range efflux pump substrate EtBr. Cells with lower efflux activity will not be able to extrude the fluorochrome EtBr substrate, which accumulates within the cell producing pink fluorescence under ultraviolet light. On the other hand, cells with higher efflux



activity will be able to extrude EtBr, requiring higher EtBr concentrations to fluoresce. Thus, by inoculating each of the subcultures obtained on TSA plates containing 2.5 mg/L EtBr, it was possible to select non-fluorescent and fluorescent colonies (potentially lacking the plasmid) [10].

### *2.5. Screening of efflux pump genes by polymerase chain reaction (PCR) and enzymatic restriction analysis*

Plasmid-mediated efflux pump genes were screened by PCR using specific primers for *qacA/B* and *smr* [11]. The reaction mixture (50  $\mu$ L) contained 2.5 U of *Taq* polymerase (Fermentas Inc., Ontario, Canada), 1 $\times$  *Taq* buffer (Fermentas Inc.), 25 pmol of each primer, 0.2 mM of dNTP and 1.75 mM of  $MgCl_2$ . PCR reactions were conducted in a Mastercycler<sup>®</sup> Personal 5332 thermocycler (Eppendorf AG, Hamburg, Germany) under the following conditions. DNA was denatured at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 40 °C (*qacA/B*) or 48 °C (*smr*) for 1 min and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Amplification products were visualised by 1% agarose gel electrophoresis. The resulting *qacA/B* fragment was purified and sequenced on both strands with the same set of primers. Sequence alignment and analysis were conducted with the aid of programs ClustalW and BioEdit v.7.0.8.0, respectively.

The *qacA/B* internal fragment was digested with the restriction enzyme *AluI* (Fermentas Inc.) at 37 °C for 2 h. Restriction products were analysed by 3.5% Metaphor agarose gel (Lonza Rockland Inc., Rockland, ME) electrophoresis.

### 2.6. Macrorestriction analysis

HPV107 and HPV107<sub>cured</sub> were typed by pulsed-field gel electrophoresis (PFGE) using well established protocols. Briefly, agarose disks containing intact chromosomal DNA were prepared as previously described [11] and were restricted with *SmaI* according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA). Restriction fragments were then resolved by PFGE, which was carried out in a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRIII; Bio-Rad, Hercules, CA) as previously described [8].

### 2.7. Semi-automated fluorometric method

Efflux activity of the study strains was evaluated using a previously described semi-automated fluorometric method [11,12]. This method, with the aid of the Rotor-Gene 3000<sup>TM</sup> thermocycler and real-time analysis software (Corbett Research, Sydney, Australia), allows real-time detection of accumulation and efflux of the fluorescent efflux pump substrate EtBr.

## 3. Results and discussion

As expected, the susceptibility profiles of strain HPV107 and the reference strain *S. aureus* ATCC 25923 towards several antibiotics, biocides and dyes

showed a fully resistant profile of HPV107 to most of the antibiotics tested together with reduced susceptibility to all biocides compared with the reference strain (Table 1). However, in the presence of EPIs, in particular thioridazine, a general decrease in the MICs for biocides and dyes was observed (Table 1). This observation suggests that HPV107 has an efflux activity that correlates with decreased susceptibility to biocides and dyes. Chromosomally encoded efflux pumps usually possess a range of substrates that include not only biocides and dyes, but also antibiotics such as fluoroquinolones. MICs for HPV107 of fluoroquinolones also decreased with some EPIs, although to a lesser extent than that observed for the biocides and dyes. Thus, the phenotype presented by HPV107 towards biocides and dyes probably results mainly from a multidrug efflux pump gene carried on a plasmid. To confirm this hypothesis, the presence of the plasmid-located efflux pump genes *qacA/B* and *smr* was screened by PCR in the *S. aureus* study strains. An internal fragment of the *qacA/B* gene was amplified by PCR (data not shown). To distinguish between the genes *qacA* and *qacB*, which only differ by seven nucleotides, the *qacA/B* internal fragment was digested with the restriction enzyme *AluI*, which recognises an additional restriction site within *qacB* [13]. Thus, when the *qacA* amplification product is restricted with *AluI*, several fragments are produced, the larger of which has 200 bp, whilst those obtained from the *qacB* gene are of a significantly smaller size. Since a 200-bp *AluI* fragment was detected (Fig. 1-A), we could infer that the plasmid present in HPV107 carries the *qacA* gene and not *qacB*. Sequencing of the *qacA/B* fragment corroborated the identification of the *qacA* gene. These conclusions were supported by the decrease in MIC

values determined for divalent cationic biocides in the presence of EPIs, which are substrates for QacA only (Table 1).

To confirm the location of the *qacA* gene in HPV107, the strain was cured of plasmids by exposure to CPZ or NOV. The resulting cultures were then screened for the presence or absence of plasmids using EtBr as an indicator of efflux activity. The resulting fluorescent colonies presented morphology similar to the non-fluorescent colonies, albeit slightly smaller. Plasmid DNA extraction was carried out both from fluorescent and non-fluorescent colonies. No plasmids could be obtained from the fluorescent colonies in contrast to the non-fluorescent colonies that were found to carry a single 32-kb plasmid. These results indicate that the *qacA* gene was carried on a 32-kb plasmid in strain HPV107 and were supported by PCR amplification for the *qacA/B* internal fragment, which was positive for the non-fluorescent colonies and negative for the fluorescent colonies (Fig. 1-B). One of the fluorescent colonies obtained after successive passage with CPZ was selected for further testing and was designated HPV107<sub>cured</sub>. PFGE patterns showed that no contamination occurred during the plasmid curing procedure (data not shown). A single difference was detected among the PFGE patterns of HPV107 and HPV107<sub>cured</sub>, which corresponded to a fragment of ca. 30 kb, which was detectable for HPV107 only and may correspond to the *qacA*-carrying plasmid. MICs of HPV107<sub>cured</sub> for the antibiotics, biocides and dyes previously tested were determined and it was found that HPV107<sub>cured</sub> maintained the antibiotic-resistant profile, but it became more susceptible to the biocides and dyes

tested, with the exception of CHX (Table1). Analysis of other fluorescent and non-fluorescent colonies revealed similar phenotypes.

To confirm further that the activity of the QacA pump accounted for the reduced susceptibility to a diverse panel of biocides and dyes in HPV107, the efflux activities of HPV107, HPV107<sub>cured</sub> and *S. aureus* ATCC 25923 were characterised by a semi-automated fluorometric method as previously described [11,12]. Data obtained showed that HPV107 had a higher efflux activity in comparison with that presented by *S. aureus* ATCC 25923 (Fig. 2-B) and, consequently, a lower retention of EtBr (Fig. 2-A). Moreover, strain HPV107<sub>cured</sub>, without the *qacA* gene, showed a behaviour similar to the fully antibiotic-susceptible strain *S. aureus* ATCC 25923, with residual efflux activity and concomitantly a higher accumulation of EtBr (Fig. 2). The residual efflux activity detected in HPV107<sub>cured</sub> could be the result of the basal activity of chromosomally encoded efflux pumps. These results confirmed that the activity of the efflux pump QacA was the main contributor to reduced susceptibility of the HPV107 to biocides and dyes.

Biocides are heavily used in healthcare settings for the management of nosocomial infections as well as in domestic and food industry settings [1]. Consequently, nosocomial bacteria, and increasingly community bacteria, are constantly being exposed to these daily-use compounds, raising concerns about the role of biocides as a selective pressure for the appearance and maintenance of antibiotic-resistant bacteria. Inasmuch as bacteria with reduced susceptibility to biocides could be more prone to survive in such environments,

they can also create the opportunity for bacteria to develop an antibiotic resistant-phenotype, or even a MDR phenotype. Reduced susceptibility is mainly conferred by efflux pumps that have the capacity to expel from the cell interior a wide range of chemically dissimilar compounds, including one or several classes of antibiotics, and may render the cell with a MDR phenotype [2].

In the particular case of *S. aureus*, prevention of nosocomial infections is mainly conducted by the use of antiseptics and disinfectants [14]. Hence, the emergence of biocide-resistant, and possibly antibiotic-resistant, bacteria can be of great importance. One such example of possible cross-resistance is the existence of plasmids that can harbour efflux pump genes that confer resistance to biocides as well as resistance determinants to antibiotics and heavy metals [2]. Previous studies have shown that the plasmid-encoded *qacA/B* gene is the main efflux pump gene responsible for reduced susceptibility to biocides in *S. aureus* isolates and that it is widespread in Europe [15]. Although some data do not support a link between increased biocide resistance and antibiotic resistance, other studies highlight the potential biocide/antibiotic cross-resistance in *S. aureus*, particularly among hospital-acquired MRSA strains, and potentially community-acquired MRSA strains [14], owing to the common linkage of *qacA/B* and *smr* genes with  $\beta$ -lactamase transposons as well as to resistance determinants to gentamicin and trimethoprim, among others [16]. This was not the case for MRSA HPV107, since both HPV107 and HPV107<sub>cured</sub> showed identical levels of resistance to gentamicin, kanamycin and

trimethoprim (Table 1), thus excluding the presence of transposons on the plasmid carrying the *qacA* determinant.

However, conjugation of a highly successful genetic background, such as that of strain HPV107, a representative of the multiresistant international MRSA Iberian clone, with a plasmid carrying the structural gene for an efflux pump that confers resistance to antiseptic and disinfectant compounds usually employed in healthcare settings suggests that *S. aureus* strains carrying this determinant may be more prone to survive in such environments, thereby increasing their capacity to disseminate widely in a hospital setting.

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### **Competing interests**

None declared.

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**Fig. 1.** (A) Restriction products of the *qacA/B* internal fragment digested with *AluI*, resolved in 3.5% Metaphor agarose gel. (B) Amplification products with *qacA/B* primers (628 bp) from colonies selected after plasmid curing with either chlorpromazine (CPZ) (lane 2, fluorescent colony; and lanes 3 and 4, non-fluorescent colonies) or novobiocin (NOV) (lanes 5 and 6, fluorescent colony; and lane 7, non-fluorescent colony). Lane 1, 100-bp Gene Ruler DNA ladder (Fermentas Inc.); and lane 8, negative control for *qacA/B* amplification. Fluorescence is an indicator of efflux capacity towards the broad-range efflux pump substrate ethidium bromide (EtBr). Cells with lower efflux activity will not be able to extrude the fluorochrome EtBr substrate, which accumulates within the cell producing pink fluorescence under ultraviolet light. On the other hand, cells with higher efflux activity will be able to extrude EtBr and will be non-fluorescent.

**Fig. 2.** Analysis of ethidium bromide (EtBr) accumulation and efflux by a semi-automated fluorometric method. (A) Accumulation assays of *Staphylococcus aureus* ATCC 25923, HPV107 and its plasmid-cured derivative (HPV107<sub>cured</sub>) in the presence of increasing EtBr concentrations (mg/L). (B) Efflux assays of the strains *S. aureus* ATCC 25923, HPV107 and HPV107<sub>cured</sub> in the absence and presence of glucose 0.4% (p/v), washed after a period of incubation of 60 min at 25 °C in the presence of EtBr at 0.25 mg/L (*S. aureus* ATCC 25923), 2 mg/L (HPV107) and 0.5 mg/L (HPV107<sub>cured</sub>) and of the efflux pump inhibitor verapamil at 200 mg/L (subinhibitory concentration). Controls containing solely the bacterial cultures or EtBr showed no or residual fluorescence (data not shown).

**Table 1**

Characterisation of susceptibility profiles towards antibiotics, biocides and dyes, in the absence and presence of 0.5× MIC of efflux pump inhibitors (EPIs), for *Staphylococcus aureus* strain ATCC 25923, methicillin-resistant *S. aureus* (MRSA) strain HPV107 and its plasmid-cured derivative (HPV107<sub>cured</sub>)

Antimicrobial compound	MIC (mg/L) <sup>a</sup>						
	ATCC 25923	HPV107 <sup>b</sup>					HPV107 <sub>cured</sub>
		No EPI	+ TZ	+	+	+	
			CPZ	VER	RES		
<b>Antibiotics</b>							
CIP	0.25 (S)	16 (R)	8	8	16	16	16 (R)
NOR	0.5 (S)	64 (R)	32	32	<b>16</b>	32	64 (R)
LEV	0.125 (S)	8 (R)	8	8	8	8	8 (R)
SPFX	0.125 (S)	16 (R)	16	16	8	8	16 (R)
OXA	0.125 (S)	128 (R)	64	64	128	128	128 (R)
TET	0.25 (S)	32 (R)	16	16	16	32	16 (R)
ERY	0.5 (S)	>1024 (R)	1024	1024	>1024	>1024	>1024 (R)
GEN	0.5 (S)	256 (R)	N/D	N/D	N/D	N/D	256 (R)
KAN	1 (S)	>256 (R)	N/D	N/D	N/D	N/D	>256 (R)
TMP	1 (S)	1 (S)	N/D	N/D	N/D	N/D	1 (S)
CHL	3 (S)	4 (S)	N/D	N/D	N/D	N/D	4 (S)
<b>Biocides and dyes</b>							
<b>Monovalent</b>							

TPP	12.5	256	<b>8</b>	<b>32</b>	<b>64</b>	256	8
BAC	0.75	4	<b>1</b>	2	2	4	1
CET	2	8	<b>4</b>	4	8	8	2
CTAB	0.75	8	<b>2</b>	4	4	4	2
EtBr	6.25	256	<b>32</b>	<b>64</b>	<b>64</b>	128	1
BER	200	>1024	1024	>1024	>1024	>1024	128
ACR	16	>512	512	>512	>512	>512	16
CV	0.03	1	<b>0.125</b>	<b>0.25</b>	0.5	1	0.125
Divalent							
DC	6.25	32	<b>0.5</b>	<b>2</b>	<b>1</b>	16	4
CHX	0.375	1	0.5	0.5	0.5	1	1
Pt	50	256	<b>32</b>	128	128	256	16

MIC, minimum inhibitory concentration; TZ, thioridazine; CPZ, chlorpromazine; VER, verapamil; RES, reserpine; CIP, ciprofloxacin; NOR, norfloxacin; LEV, levofloxacin; SPFX, sparfloxacin; OXA, oxacillin; TET, tetracycline; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; TMP, trimethoprim; CHL, chloramphenicol; TPP, tetraphenylphosphonium bromide; BAC, benzalkonium chloride; CET, cetrimide; CTAB, hexadecyltrimethylammonium bromide; EtBr, ethidium bromide; BER, berberine; ACR, acriflavine; CV, crystal violet; DC, dequalinium chloride; CHX, chlorhexidine; Pt, pentamidine isethionate salt; N/D, not determined.

<sup>a</sup> Letters in parenthesis correspond to the phenotype of the isolate according to Clinical and Laboratory Standards Institute (CLSI) guidelines [8] (S, susceptible; and R, resistant). Values in bold correspond to a decrease in the original MIC by  $\geq 4\times$  in the presence of an EPI.

<sup>b</sup> The following EPI concentrations were used: TZ 6.25 mg/L; CPZ 12.5 mg/L; VER 200 mg/L; and 20 mg/L RES.

