Cell-wall thickness: possible mechanism of acriflavine resistance in meticillin-resistant *Staphylococcus aureus*

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Acriflavine resistance in the clinical meticillin-resistant *Staphylococcus aureus* isolate KT24 was found not to be mediated by multidrug efflux pumps encoded by *qacA/B*, *smr*, *qacE*, *qacG*, *qacH*, *qacJ* or *norA*. Early uptake and accumulation of ethidium bromide in MRSA KT24 was significantly lower than that in a susceptible strain, although the efflux rates were similar. Therefore, a permeability barrier in MRSA KT24 may be the conceivable mechanism of acriflavine resistance. Interestingly, it was found that MRSA KT24 had a significantly thickened cell wall, and that cell-wall thickness increased gradually during bacterial growth. In contrast, cell size and surface area in MRSA KT24 were not different from those in the susceptible strain. Moreover, MRSA KT24 exposure to sub-MIC concentrations of acriflavine resulted in a thicker cell wall. These results indicate that cell-wall thickness may be responsible for acriflavine resistance in *S. aureus*.

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INTRODUCTION

Meticillin-resistant *Staphylococcus aureus* (MRSA) is a clinically significant bacterial pathogen because it is resistant to a variety of antibiotics and causes nosocomial infections worldwide. The ability of *Staphylococcus* spp. to adhere and form multilayered biofilms on host tissues and other surfaces is one of the important mechanisms by which these bacteria are able to persist in some diseases (Beenken *et al.*, 2004), making the prevention of nosocomial disease dissemination difficult.

It is generally believed that bacteria rarely acquire resistance to antiseptic agents as these agents act at several target sites (McDonnell & Russell, 1999; Russell, 2003). However, MRSA has been reported to be resistant to various antiseptics, including benzalkonium chloride and chlorhexidine digluconate (Noguchi *et al.*, 1999; Suller &

Russell, 1999; Mayer *et al.*, 2001). In addition, a growing number of species have demonstrated cross-resistance not only to other antiseptic agents but also to antibiotics.

Although inactivation and target alteration are known mechanisms for resistance to antiseptic agents, they are comparatively rare. Resistance typically results from cellular changes that negatively affect the accumulation of antiseptic agents, including cell envelope changes that limit uptake or expression of efflux mechanisms (Poole, 2002; Gilbert & McBain, 2003; Russell, 2003; Piddock, 2006). In *S. aureus*, *qacA*, *qacB*, *smr*, *norA* and *mdeA*, which encode multidrug-transporter proteins, have been identified as genes that confer resistance to antiseptic agents (Rouch *et al.*, 1990; Alam *et al.*, 2003a, b; Huang *et al.*, 2004). The *norA* and *mdeA* genes are located on the chromosome of *S. aureus*, whilst the *qacA*, *qacB* and *smr* genes have been found mainly on plasmids (Piddock, 2006). The proteins encoded by *qacA/B* and *smr* belong to a major facilitator

Abbreviation: MRSA, meticillin-resistant *Staphylococcus aureus*.

superfamily and small multidrug-resistance family, respectively. Additional resistance genes, such as *qacG*, *qacH*, *qacE* and *qacJ*, have also been identified (Bjorland *et al.*, 2003).

Acriflavine has been used for a variety of biological and biochemical purposes, especially as an antiseptic agent possessing a quaternary ammonium structure. It is well known that one mechanism of acriflavine resistance is due to overexpression of efflux pump genes. However, other mechanisms are poorly understood. In this study, we examined the distribution of well-known multidrug efflux pump genes in clinical isolates and investigated the mechanism of acriflavine resistance in the MRSA isolate KT24, which does not possess efflux pump genes.

METHODS

Reagents. Benzalkonium chloride was purchased from ICN Biomedicals. Ampicillin was from Calbiochem-Novabiochem, and cefalotin, vancomycin and chloramphenicol were from Sigma-Aldrich. Sparfloxacin was synthesized at Dainippon Sumitomo Pharma. Other reagents were purchased from Wako-Pure Chemical Industries unless otherwise indicated.

Bacterial strains and culture conditions. A total of 38 MRSA isolates were collected at Kitazato University Hospital, Japan, between 1999 and 2000. *S. aureus* 209P, *S. aureus* ATCC 12600, *S. aureus* ATCC 29210, *S. aureus* RN4220 and *S. aureus* RN2677 were used as susceptible reference strains. The *S. aureus* strains used were cultured aerobically in soybean casein digest broth (SCDB; Nihon Pharmaceutical).

Determination of antimicrobial susceptibility. MICs were determined by a twofold serial agar dilution method using Mueller–Hinton agar (BD) as recommended by CLSI (2003) (formerly the National Committee for Clinical Laboratory Standards). To assess NorA efflux pump activity, MICs were determined in the presence of reserpine (final concentration 20 µg ml⁻¹).

Preparation of genomic DNA. *S. aureus* genomic DNA was prepared as described by Hudson & Curtiss (1990), except that 100 µg mutanolysin ml⁻¹ was replaced by 50 µg lysostaphin ml⁻¹.

PCR amplification. Amplicons corresponding to the antiseptic-resistance genes *qacA/B*, *smr*, *qacE*, *qacG*, *qacH* and *qacJ* were amplified by PCR with the primer sets shown in Table 1. PCR was performed using Ex Taq polymerase (TaKaRa). Reactions were carried out for 30 cycles of 15 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. The PCR products were analysed by agarose gel electrophoresis.

Accumulation and efflux of ethidium bromide. Ethidium bromide uptake and efflux were determined using a modified fluorescence technique (Kaatz *et al.*, 2000). Organisms were grown overnight in SCDB, and a 2% (v/v) inoculum of this culture was added to 100 ml Mueller–Hinton broth (MHB; BD). The cells were harvested at an OD₆₆₀ of 0.7, washed in ice-cold MHB and resuspended in MHB to a final OD₆₆₀ of 0.7. For the ethidium bromide uptake assay, the cell suspension was maintained at 37 °C for 2–3 min and ethidium bromide (final concentration 10 µg ml⁻¹) was added. The fluorescence of aliquots of the mixture was determined at frequent intervals (excitation wavelength 545 nm; emission wavelength 590 nm) using a luminescence spectrometer (Thermo Fisher Scientific). For the ethidium bromide efflux assay, ethidium bromide (final concentration 10 µg ml⁻¹) and carbonyl cyanide *m*-chlorophenylhydrazone (final concentration 100 µM) were added to the cell suspension. After 30 min incubation at 37 °C, the cells were pelleted and resuspended in fresh MHB. The fluorescence of aliquots of the mixture was determined at frequent intervals as above.

Electron microscope analysis. After incubation at 37 °C, bacterial cells were harvested and washed twice with Dulbecco's PBS.

For transmission electron microscopy, the cells were collected into a pellet and fixed with 2.5% glutaraldehyde, followed by 1% OsO₄. The samples were dehydrated by passing through an ethanol series and then embedded in Spurr's Epon. Ultrathin sections were obtained using an ultramicrotome with a diamond knife and examined under a JEOL JEM-2000EXII electron microscope at 80 kV.

For scanning electron microscopy, a drop of the bacterial cell suspension was mounted on a coverslip and fixed with 2.5% glutaraldehyde, followed by 1% OsO₄. The samples were stained with 1% tannic acid and fixed with 1% OsO₄. After dehydration using an ethanol series and *t*-butyl alcohol, the samples were dried and coated using platinum, and then examined using a JEOL JSM-6340F field emission scanning electron microscope at 15 kV. Cell-wall thickness was determined using photographs taken at a final magnification of ×10 000. More than ten cells that had been cut through the cell equator were chosen for each strain, and cell-wall thickness was determined at four sites for each cell.

Table 1. Primers used for PCR amplification

Target gene	Sequence (5'→3')	Reference
<i>qacA/B</i>	GCAGAAAGTGCAGAGTTCTG CCAGTCCAATCATGCCTG	Rouch <i>et al.</i> (1990)
<i>smr</i>	GCCATAAGTACTGAAGTTATTGGA GACTACGGTTGTTAAGACTAAACCT	Sasatsu <i>et al.</i> (1989)
<i>qacE</i>	GCAATAGTTGGCGAAGTAATCG GGCTGTAATTATGACGACGCC	This study
<i>qacG</i>	TCAATTGCAACAGAAATAATCGGA GATTATTGTTGTTAATACTAATCC	This study
<i>qacH</i>	CAATAGTCAGTGAAGTAATAGG AACAAATTGTTGTTAATACTAATCCT	This study
<i>qacJ</i>	GCGATTATAACTGAAATAATAGGA AACTATTGTTGTAAGAACTAGTCC	This study

RESULTS AND DISCUSSION

In this study, we examined susceptibility to acriflavine and the distribution of antiseptic-resistance genes in clinically isolated MRSA. MIC values of antiseptic agents for the susceptible strains *S. aureus* ATCC 12600, *S. aureus* ATCC 29210, *S. aureus* RN4220, *S. aureus* RN2677 and *S. aureus* 209P were equal within an error range. Thus we decided to use *S. aureus* 209P as a representative susceptible strain. The acriflavine MIC value for *S. aureus* 209P was 1 µg ml⁻¹, whilst those for all of the MRSA clinical isolates were four or more times higher, ranging from 4 to >128 µg ml⁻¹. Based on the acriflavine MIC value for *S. aureus* 209P, MRSA isolates were classified into three groups: a 'high-level resistant group' (MIC >64 µg ml⁻¹), an 'intermediate-level resistant group' (MIC 16–32 µg ml⁻¹) and a 'low-level resistant group' (MIC 4–8 µg ml⁻¹). The frequency as detected by PCR of the *qacA/B* and *smr* genes, which are the most common plasmid-mediated resistance genes, is summarized in Table 2. *S. aureus* 209P was not found to possess the *qacA/B* or *smr* gene by PCR. However, overall, *qacA/B* and *smr* were detected in 55 % (21/38) and 21 % (8/38), respectively, of all of the MRSA isolates examined. MRSA isolates carrying both the *qacA/B* and *smr* genes represented 13 % (5/38) and neither *qacA/B* nor *smr* was detected in 11 % (4/38) of all isolates. Thus more than 80 % of the MRSA isolates possessed plasmid-borne resistance genes. The *qacA/B* gene in the high-level and intermediate-level resistant groups was detected at a significantly higher rate than in the low-level resistant group. In contrast, the acriflavine MIC value for MRSA isolates carrying both the *qacA/B* and *smr* genes was not higher than that for isolates carrying the *qacA/B* or *smr* gene. These findings, which are in agreement with those of a previous study (Mayer *et al.*, 2001), suggest that the *qacA/B* gene is widely distributed in MRSA.

As previous studies have indicated that reserpine is capable of inhibiting the NorA pump (Aeschlimann *et al.*, 1999; Gibbons *et al.*, 2003), NorA expression might account for the fourfold reduction in acriflavine MIC in the presence of reserpine. After the addition of reserpine, acriflavine MICs decreased by 42 % (16/38) overall in the MRSA isolates.

Therefore, the existence of a plural multidrug efflux system did not increase susceptibility to acriflavine in MRSA.

Among the 38 MRSA clinical isolates, MRSA isolate KT24 was found to exhibit high-level resistance to acriflavine (MIC 128 µg ml⁻¹) without possessing the *qacA/B* and *smr* genes. Additional resistance-related genes, such as *qacE*, *qacG*, *qacH* and *qacJ* (Paulsen *et al.*, 1996; Alam *et al.*, 2003a, b), were also not detected by PCR in MRSA KT24 (data not shown). Moreover, susceptibility determination with reserpine revealed no NorA expression in MRSA KT24. Therefore, the high-level resistance of MRSA KT24 to acriflavine may be caused by an unknown mechanism.

The MICs of a series of antiseptic agents for MRSA KT24 and the susceptible strain *S. aureus* 209P are shown in Table 3. The MIC values of some antiseptic agents, such as acriflavine, acrinol and ethidium bromide, for MRSA KT24 were significantly higher than those for the susceptible strain *S. aureus* 209P. Acriflavine, benzalkonium chloride, benzethonium chloride, hexadecyltrimethylammonium bromide and ethidium bromide all possess a quaternary ammonium structure. However, MRSA KT24 showed high-level resistance to acriflavine (MIC 128 µg ml⁻¹) but low-level resistance to benzalkonium chloride (MIC 4 µg ml⁻¹). For other compounds with different structures, MRSA KT24 susceptibility varied greatly, with, for example, MIC values of >128 µg ml⁻¹ for acrinol and 2 µg ml⁻¹ for chlorhexidine gluconate. However, MRSA KT24 was susceptible to triclosan, which contains bisphenols.

In addition to the antiseptic agents mentioned above, a number of antimicrobial agents with different targets and mechanisms of action, such as inhibitors of cell-wall synthesis, protein synthesis and DNA synthesis, were also evaluated. Although MRSA KT24 was resistant to some antimicrobial agents, such as erythromycin, norfloxacin and tetracycline, it was susceptible to vancomycin, chloramphenicol, rifampicin and novobiocin. In general, MRSA KT24 showed resistance to various kinds of antiseptic and antimicrobial agents with different structures and mechanisms of action. For the purpose of this study, we examined further the mechanism underlying MRSA KT24 resistance to acriflavine.

Table 2. Distribution of the antiseptic drug efflux genes *qacA/B* and *smr*, and NorA expression, in 38 isolates of MRSA

Results are shown as numbers of isolates, with the percentage detection rate for each group shown in parentheses.

Acriflavine susceptibility group*	MIC of acriflavine (µg ml ⁻¹)	No. of isolates	Gene(s)				NorA expression
			<i>qacA/B</i>	<i>smr</i>	<i>qacA/B</i> + <i>smr</i>	ND†	
I	>64	15	12 (80)	0 (0)	0 (0)	3 (20)	3 (20)
II	16–32	16	8 (50)	5 (31)	3 (19)	0 (0)	10 (63)
III	4–8	7	1 (14)	3 (43)	2 (29)	1 (14)	3 (43)
Total	–	38	21 (55)	8 (21)	5 (13)	4 (11)	16 (42)

*Group I, high-level resistance group; group II, intermediate-level resistance group; group III, low-level resistance group.

†ND, Neither *qacA/B* nor *smr* was detected.

Table 3. Antimicrobial susceptibility of MRSA KT24 and the susceptible strain *S. aureus* 209P

Agent	MIC ($\mu\text{g ml}^{-1}$)	
	KT24	209P
Acriflavine	128	1
Acrinol	>128	8
Benzalkonium chloride	4	1
Benzethonium chloride	16	1
Chlorhexidine gluconate	2	0.5
Ethidium bromide	64	0.5
Hexadecyltrimethylammonium bromide	8	0.5
Rhodamine 6G	2	0.25
Triclosan	0.063	0.063
Ampicillin	0.25	0.063
Cefalotin	0.5	0.125
Chloramphenicol	4	2
Erythromycin	>128	0.125
Gentamicin	16	0.063
Norfloxacin	64	0.125
Novobiocin	<0.063	<0.063
Rifampicin	<0.063	<0.063
Sparfloxacin	8	0.063
Tetracycline	32	0.125
Vancomycin	1	0.5

To assess bacterial efflux activity and cell permeability, we examined ethidium bromide accumulation in MRSA KT24 and *S. aureus* 209P. In *S. aureus* 209P cells, ethidium bromide uptake was rapid, with effective accumulation; in contrast, early uptake and accumulation of ethidium bromide in MRSA KT24 cells was significantly lower (Fig. 1a). However, the efflux rate of ethidium bromide in MRSA KT24 cells was similar to that in *S. aureus* 209P (Fig. 1b). It has been reported that active efflux pumps play a major role in micro-organism resistance to antiseptic agents (Gilbert & McBain, 2003). However, our results showed that uptake and accumulation of ethidium bromide in MRSA KT24 cells, rather than efflux activity, resulted in reduced susceptibility to the antiseptic agent. It is therefore possible that a permeability barrier is one of the mechanisms underlying MRSA KT24 resistance to acriflavine.

We examined the hydrophobicity of MRSA KT24 following the assay described previously by Rosenberg & Rosenberg (1981) and compared it with that of *S. aureus* 209P. Although about 80% of *S. aureus* 209P cells remained in the aqueous phase after mixing with *n*-octane, only approximately 30% of MRSA KT24 cells remained in the aqueous phase (data not shown). Based on the criteria for distinguishing between hydrophilic and hydrophobic properties described by Yamada & Matsumoto (1990), MRSA KT24 was found to have a hydrophobic surface, whilst *S. aureus* 209P cells were hydrophilic. Dominant hydrophobicity is probably due to proteins and protein-associated molecules localizing at the surface of the

Fig. 1. Uptake and accumulation (a) and efflux (b) of ethidium bromide. ■, *S. aureus* 209P; ▲, MRSA KT24.

organism and is believed to arise from a lack of teichoic acid, protein A or coagulase production (Reifsteck *et al.*, 1987). Therefore, the surface proteins of MRSA KT24 may be different from those of *S. aureus* 209P. Wadström (1990) reported that cell-surface hydrophobicity may affect bacterial susceptibility to antimicrobial and antiseptic agents.

To clarify additional potential resistance mechanisms, the morphological characteristics of the cells were observed by electron microscopy. Stationary-phase cells of MRSA KT24 were compared with those of *S. aureus* 209P based on scanning electron microscopy analysis (Fig. 2a). No differences in individual cell size and cell surface were observed between *S. aureus* 209P and MRSA KT24. In addition, transmission electron microscopy observations of MRSA KT24 and *S. aureus* 209P cells at different growth phases (early exponential phase, late exponential phase and stationary phase) revealed that both *S. aureus* 209P and MRSA KT24 cells at early exponential phase possessed a rough outer surface and that cell-wall thickness increased gradually on growth. However, the cell wall of MRSA KT24 was clearly thicker at each phase than that of *S. aureus* 209P (Fig. 2b). Cell-wall thickness in MRSA KT24 was approximately 1.9-, 1.4- and 1.2-fold that of *S. aureus* 209P at early exponential phase, late exponential phase and stationary phase, respectively (Fig. 2c). Moreover, no difference in cell-wall thickness was observed in *S. aureus* ATCC 12600, ATCC 29213 and RN4220 compared with *S.*

Fig. 2. Morphological analysis of MRSA KT24. Scanning electron microscopy (a) and transmission electron microscopy (b) of *S. aureus* 209P (i) and MRSA KT24 (ii) and measurements of cell-wall thickness (c) at the early exponential (EE), late exponential (LE) and stationary (S) growth phases. (c) Open bars, *S. aureus* 209P; filled bars, MRSA KT24.

aureus 209P. The cell wall of MRSA KMP9, a multidrug-resistant strain that overexpresses the NorA efflux pump, was not thickened (data not shown).

Next, we examined the morphological changes in MRSA KT24 cells exposed to sub-MIC concentrations of acriflavine. Fig. 3 shows transmission electron micrographs of representative cells cultured in SCDB with acriflavine for 4 h. The thickened cell wall in MRSA KT24 was significantly thicker following exposure to $16 \mu\text{g acriflavine ml}^{-1}$ (0.125 MIC) (Fig. 3b). From the results of ethidium bromide accumulation and electron microscopic observation, the mechanism of acriflavine resistance in MRSA KT24 seems to involve a permeability barrier that affects cell-wall thickness.

The first paper describing the mode of cell-wall thickening in *S. aureus* was published by Nishino (1975), who reported that treatment with erythromycin resulted in both cell-wall thickening and a slight surface swelling in *S. aureus*. Recent studies of MRSA have shown that vancomycin is affinity trapped inside the peptidoglycan layers by false targets, serving as a physical barrier against the penetration of vancomycin molecules and resulting in vancomycin resistance (Sasatsu *et al.*, 1989; Cui *et al.*, 2000, 2003; Sieradzki & Tomasz, 2003). In addition, it was shown that a thickened cell wall is responsible for vancomycin resistance in *S. aureus* (Cui *et al.*, 2003). We found that MRSA KT24 had a thickened cell wall, but was susceptible to vancomycin, although we examined this using population analysis profiles (Hiramatsu *et al.*, 1997). Therefore, cell-wall thickening in MRSA KT24 may be due to a mechanism different from that of vancomycin resistance. Further studies of the correlation between cell-wall thickness and acriflavine resistance are necessary to clarify the physiological role of cell-wall thickness in *S. aureus*.

Fig. 3. Thin-section micrographs of *S. aureus* KT24 cells with and without exposure to acriflavine. (a) Normal KT24 cells; (b) KT24 cells after exposure to $16 \mu\text{g acriflavine ml}^{-1}$ (0.125 MIC) for 4 h.

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