Substrates and Inhibitors of Antiseptic Resistance in *Staphylococcus aureus*

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The range of substrates of antiseptic resistance associated with the *abr* gene is very wide and includes antiseptics, DNA-intercalating drugs and preservatives which have no quaternary ammonium group in methicillin-resistant *Staphylococcus aureus*. Antiseptic resistance was inhibited by calcium channel blockers and calmodulin inhibitors similarly to the resistance to multiple antitumor agents encoded by the *mdr* gene in human cells. These results show that the common properties of the *mdr* gene and the *abr* gene may be shared by the mechanisms that are involved in the removal of toxic substances from the cell.

Keywords MRSA; antiseptic resistance; substrate profile; calcium channel blocker; calmodulin inhibitor

It has been reported that a determinant for resistance to quaternary ammonium antiseptics is located on a transferable plasmid in methicillin-resistant *Staphylococcus aureus* (MRSA).^{1,2)} The complete nucleotide sequence of the *abr* gene, which is responsible for the resistance of *S. aureus* to antiseptics, has been determined.³⁾ Subsequently, a gene responsible for resistance to antiseptics was isolated from a transferable plasmid in a high-level antiseptic-resistant strain of *S. aureus*, and the complete nucleotide sequence of this gene was determined. The results demonstrated that the high-level resistant strain had duplicated *abr* genes.⁴⁾

Recently, a multidrug-resistant gene of *Bacillus subtilis* has been reported.⁵⁾ This multidrug-resistant gene has been demonstrated to have properties similar to those associated with the *mdr* gene. The *mdr* gene has been found to be present in various eukaryotic cells, including human cells, and encodes resistance to multiple antitumor agents.^{6,7)} The genes are both associated with resistance to a wide range of agents. The mechanism of resistance is an agent-efflux system in both cases. The resistance is inhibited by calcium channel blockers and calmodulin inhibitors. Also, high-level resistance can occur through amplification of the genes.

The mechanism of antiseptic-resistance encoded by the *abr* gene was also an efflux-system,⁴⁾ and the high-level resistant strain had duplicated *abr* genes.

In this study, we investigated the range of substrates and the sensitivity to inhibitors of antiseptic-resistance encoded by the *abr* gene in order to compare the properties of the *abr* gene with those of the multidrug-resistant gene of *Bacillus subtilis* and the *mdr* gene.

MATERIALS AND METHODS

Strains Five strains of *S. aureus* were used in the experiments. Low-level antiseptic-resistant strain L20A²⁾ and high-level resistant strain N20⁴⁾ were clinical isolates with transferable plasmid pTZ20 and pTZ22, respectively. Strain RN2677 is antiseptic-sensitive and was also used as a recipient strain in the plasmid-transfer experiments.⁴⁾ Transconjugants RN2677 (pTZ20) and RN2677 (pTZ22) were obtained by transfer to strain RN2677 of plasmids pTZ20 and pTZ22, respectively.

Drugs The antiseptics used were acriflavine (AF), acrinol (6,9-diamino-2-ethoxyacridine lactate: AN), alkylpolyaminoethylglycine hydrochloride (AP), benzalkonium chloride (BK), benzethonium chloride (BT), chlorhexidine gluconate (CH), cetyltrimethylammonium bromide (CT) and glutaraldehyde (GA). The DNA-intercalating dyes used were acridine orange (AO), crystal violet (CV), ethidium bromide (EB), rhodamin 123 (RD), pyronine Y (PY) and safranin O (SO). The preservatives used were butylparaben (BP) and methylparaben (MP). AF, AN, BK, BT, EB, RD and PY were purchased from Sigma Chemical Co. (St. Louis, MO). CH, CT, GA, AO, CV, SO, BP and MP were obtained from Wako Pure Chemical Industries, Ltd. (Osaka), and AP from Nippon Shoji Co. (Osaka).

Nifedipine, verapamil, prenylamine and trifluoperazine were used to examine the inhibition of resistance to an antiseptic. All of these drugs were obtained from Sigma Chemical Co.

Evaluation of Sensitivity to an Antiseptic The sensitivity of each tested strain to antiseptics was expressed in terms of the minimum inhibitory concentration (MIC). MIC was determined by the standard doubling-dilution method on agar plates, as proposed by the Japan Society of Chemotherapy.⁸⁾ An overnight culture of each strain in Mueller-Hinton (MH) broth (Difco, Detroit, MI) at 37 °C was diluted 1:100 with the same broth (to about 10⁶ colony forming unit/ml), and 5 µl of the diluted suspension was inoculated, with an MIT-P microplanter (Sakuma Seisakusho Ltd., Tokyo), into MH agar medium that contained one of the antimicrobial agents to be tested. After incubation at 37 °C for 18–24 h, bacterial growth was evaluated. The lowest concentration of each antiseptic exhibiting a growth-inhibitory effect was taken as the MIC.

Assessment of the Inhibitory Effects of Calcium Channel Blockers and Calmodulin Inhibitors on Resistance to an Antiseptic The effect of calcium channel blockers and calmodulin inhibitors on the resistance to antiseptics was evaluated by determining the 50% inhibitory dose (ID₅₀) of each antimicrobial agent in the presence or absence of these compounds. Cells of each strain, after overnight culture in penassay broth (PEN; antibiotic medium 3; Difco) at 37 °C, were diluted 1:20 with the same broth,

and the diluted culture was incubated with shaking at 37°C until bacterial growth reached the logarithmic phase. ID₅₀ was determined by plotting the growth rate during the logarithmic phase (percentage of the control) versus the concentration of the antimicrobial agent, as described by Treffers.⁹⁾

RESULTS AND DISCUSSION

Sensitivity to Antiseptic of Strains *S. aureus* and Their Transconjugants Table I shows the sensitivity of strains RN2677 (antiseptic-sensitive), L20A (MRSA), N20 (MRSA), RN2677 (pTZ20) and RN2677 (pTZ22) to various antimicrobial agents. The MIC values for the high-level resistant strain N20 were the same as those for the low-level resistant strain L20A for BP, CH and CT, respectively. The other antiseptics and two preservatives had a 2-fold higher MIC value for strain N20 than for strain L20A. The dyes tested had about 2- to 16-fold higher MIC values for strain N20 than for strain L20A. MIC values for the two transconjugants were the same as

those for the corresponding donor strains with all the antimicrobial agents tested.

The present results show that the range of antiseptic resistance associated with the *abr* gene is very wide and includes antiseptics, DNA-intercalating dyes and preservatives. The antiseptic-resistance of *S. aureus* has been also called "quaternary ammonium" resistance because *S. aureus* exhibits resistance to quaternary ammonium compounds. Cross-resistance to agents without a quaternary ammonium group was apparent in our antiseptic-resistant strains. This result suggests that the antiseptic-efflux protein encoded by the *abr* gene does not recognize the quaternary ammonium group specifically.

Inhibitory Effects of Calcium Channel Blockers and Calmodulin Inhibitors on Resistance to Antiseptics In an attempt to characterize an antiseptic-efflux protein, we examined whether calcium channel blockers and the calmodulin inhibitors could inhibit the action of this protein. The inhibitory effects of calcium channel blockers and calmodulin inhibitors on resistance to an antiseptic were evaluated by determining the ID₅₀ of antimicrobial

TABLE I. Resistance of Strains N20, L20A, and Their Transconjugants to Various Agents

Antimicrobial agents		MIC (μg/ml)				
		N20	RN2677 (pTZ20)	L20A	RN2677 (pTZ22)	RN2677
Antiseptics	AF	200	200	25	25	6.25
	AN	100	100	50	50	25
	AP	50	50	25	25	6.25
	BK	6.25	12.5	3.13	6.25	1.56
	BT	12.5	12.5	3.13	6.25	0.78
	CH	3.13	3.13	3.13	1.56	0.78
	CT	12.5	12.5	12.5	12.5	3.13
	GA	1600	1600	800	800	400
Dyes	AO	100	100	50	50	25
	CV	3.13	1.56	0.2	0.2	0.1
	EB	400	400	50	50	3.13
	RD	200	400	50	50	25
	PY	100	100	6.25	12.5	1.56
	SO	200	200	25	25	6.25
	BP	200	200	200	200	100
Preservatives	MP	≥ 3200	≥ 3200	1600	1600	800

AF, acriflavine; AN, acrinol; AP, alkylpolyaminoethylglycine hydrochloride; BK, benzalkonium chloride; BT, benzethonium chloride; CH, chlorhexidine gluconate; CT, cetyltrimethylammonium bromide; GA, glutaraldehyde; AO, acridine orange; CV, crystal violet; EB, ethidium bromide; RD, rhodamine 123; PY, pyronin Y; SO, safranin O; BP, butylparaben; MP, methylparaben.

TABLE II. Effects of Calcium Channel Blockers and Inhibitors of Calmodulin on Resistance to Antiseptics of Strain L20A^{a)}

Calcium channel blocker or calmodulin inhibitor (μg/ml)		ID ₅₀ (μg/ml)					
		AP		BT		EB	
		L20A	RN2677	L20A	RN2677	L20A	RN2677
None		3.15 ± 0.26	1.83 ± 0.12	1.29 ± 0.08	0.33 ± 0.04	3.50 ± 0.44	0.60 ± 0.06
Nifedipine	(10)	1.73 ± 0.56	1.81 ± 0.25	0.81 ± 0.18	0.30 ± 0.04	1.12 ± 0.29	0.65 ± 0.05
	(20)	1.25 ± 0.35	2.02 ± 0.63	0.66 ± 0.14	0.35 ± 0.12	0.53 ± 0.30	0.62 ± 0.01
Verapamil	(50)	1.88 ± 0.71	1.92 ± 0.36	0.78 ± 0.19	0.33 ± 0.02	1.87 ± 0.05	0.58 ± 0.02
	(100)	1.59 ± 0.71	1.80 ± 0.50	0.48 ± 0.06	0.40 ± 0.12	1.32 ± 0.33	0.71 ± 0.08
Prenylamine	(1.0)	2.13 ± 0.10	2.11 ± 0.56	1.00 ± 0.10	0.38 ± 0.10	2.07 ± 0.28	0.70 ± 0.10
	(2.5)	1.92 ± 0.23	1.98 ± 0.26	0.87 ± 0.14	0.33 ± 0.06	1.49 ± 0.24	0.68 ± 0.09
Trifluoperazine	(1.0)	1.81 ± 0.14	1.88 ± 0.56	0.83 ± 0.10	0.34 ± 0.10	1.31 ± 0.53	0.75 ± 0.12
	(2.5)	1.78 ± 0.12	1.82 ± 0.70	0.60 ± 0.06	0.33 ± 0.01	0.95 ± 0.31	0.61 ± 0.08

a) Mean ± S.D. of 3 determinations.

agents in the presence and absence of these blockers and inhibitors (Table II). The ID₅₀ values of AP, BT and EB for strain L20A decreased in the presence of calcium channel blockers (nifedipine and verapamil) and calmodulin inhibitors (prenylamine and trifluoperazine), as compared with the ID₅₀ values in the absence of these drugs, indicating that these compounds inhibit resistance to an antiseptic. But the ID₅₀ values of AP, BT and EB for strain RN2677 did not decrease in the presence of calcium channel blockers and calmodulin inhibitors.

These results show that the range of antiseptic resistance associated with the *ebr* gene is very wide and the resistance is inhibited by calcium channel blockers and calmodulin inhibitors. Calcium channel blockers and calmodulin inhibitors have been reported to inhibit drug resistance associated with the *mdr* gene^{6,7)} and with the multidrug-resistant gene of *Bacillus subtilis*.⁵⁾ The *mdr* gene and the *ebr* gene are not homologous with respect to nucleotide and amino acid sequences. Rather, the common properties of the *mdr* gene, multidrug-resistant

gene of *Bacillus subtilis*, and the *ebr* gene may be shared by the mechanisms that are involved in the removal of toxic substances from the cell.

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