A RESISTANCE DETERMINANT TO NUCLEIC ACID-BINDING COMPOUNDS IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

K. R. EMSLIE, D. E. TOWNSEND AND W. B. GRUBB

*School of Medical Technology, Western Australian Institute of Technology, Kent Street,
Bentley, Western Australia, 6102 Australia*

SUMMARY. Recent isolates of methicillin-resistant *Staphylococcus aureus* from Australia and several other countries carry a plasmid coding for high levels of resistance to propamidine isethionate and low levels of resistance to cetyltrimethyl-ammonium bromide. The reasons for the acquisition and retention of such a determinant are not known. To define the properties of this resistance determinant in more detail, the minimum inhibitory concentrations of a series of cationic agents, including ethidium bromide, were determined and a cationic-resistance profile prepared for several strains of *S. aureus* and their isogenic, sensitive derivatives. The compounds for which resistance is coded by this determinant all bind to nucleic acids. Hence the determinant has been designated the NAB (nucleic acid-binding compounds)-resistance determinant.

INTRODUCTION

Our laboratory has been studying methicillin-resistant *Staphylococcus aureus* (MRSA) in an attempt to understand why there has been an epidemic spread of MRSA strains in Australian hospitals in recent years (Pavillard *et al.*, 1982). Genetic analysis of these epidemic strains of MRSA revealed the presence of a particular class of plasmids encoding gentamicin resistance (Grubb *et al.*, 1983; Townsend *et al.*, 1983a, 1984b and 1984c). The most common plasmid, of mol. wt 18×10^6 , also codes for trimethoprim resistance. Initially, it was thought that this class of plasmid was primarily maintained by the MRSA for the gentamicin-resistance determinant. Subsequently, the transposable nature of this determinant was confirmed (Townsend *et al.*, 1984c) and it now seems unlikely that gentamicin has been the selective agent for these plasmids (Grubb *et al.*, 1983). Consequently, several antiseptics were investigated as possible selective agents.

This class of plasmid was found to encode resistance to cetyltrimethyl-ammonium bromide and propamidine isethionate (Townsend *et al.*, 1984c). Although both these compounds are used in antiseptic preparations, it seemed unlikely that this is the reason for the spread of strains carrying such a resistance determinant. To characterise

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further the nature of resistance to these agents, various organic cationic compounds, including acriflavine and ethidium bromide, were examined. Resistance to acriflavine (Ericson, 1969) and ethidium bromide (Johnston and Dyke, 1969; Archer and Johnston, 1983; Foster, 1983; McDonnell *et al.*, 1983) has been reported previously in *S. aureus*. The level of resistance to these compounds was compared in propamidine-sensitive and -resistant strains that were isogenic except for the presence or absence of the propamidine-resistance determinant and other associated markers.

MATERIALS AND METHODS

Strains of S. aureus. The majority of strains of *S. aureus* studied have been described previously (Townsend *et al.*, 1983*b*, 1984*b* and 1984*c*) and are detailed in table I. Strains WG1908, WG1762 and WG2936 are propamidine-resistant isolates of MRSA from Singapore, Ireland and Japan respectively. Their propamidine-resistance determinants have been transferred to recipient strains and their location analysed by previously published methods (Grubb *et al.*, 1983; Townsend *et al.*, 1983*b* and 1984*b*). The transciipients generated were strains WG2306, WG2983 and WG3221 respectively (table I).

Determination of minimum inhibitory concentrations (MIC) of cationic agents. For MIC determinations, plates containing serial two-fold dilutions of each compound were prepared in

TABLE I
Plasmids and strains of Staphylococcus aureus

Plasmid no.	Phenotype	Mol.wt(10 ⁶)
pWG115	Pi ^R Tp ^R	c.14.6
pWG53	Pi ^R Gm ^R Tp ^R	c.18.0
pWG54	Pi ^R Gm ^R	c.15.3
pWG401	Pi ^R Nm ^R	c.18.0
pWG202	Pi ^R Cd ^R Hg ^R Pase	c.20.0
Strain no.	Characteristics	Reference
WG335	Oxford strain, NCTC 6571	...
RN450	Plasmid-free, non-lysogenic, drug-sensitive	Novick and Bouanchaud, 1971
WG541	RN450 mutated to Fa ^R Rf ^R	Townsend <i>et al.</i> , 1983 <i>b</i> , 1984 <i>c</i>
WG822	WG541, phage 95 lysogen	Townsend <i>et al.</i> , 1983 <i>b</i> , 1984 <i>b</i>
WG1876	WG541, phage J lysogen	Townsend <i>et al.</i> , 1984 <i>c</i>
WG1437	MRSA isolated at Royal Perth Hospital before 1973	Townsend <i>et al.</i> , 1984 <i>a</i>
WG588	MRSA isolated at Royal Prince Alfred Hospital since 1980 carrying pWG115	Townsend <i>et al.</i> , 1984 <i>c</i>
WG2000	WG1876 + pWG115	Townsend <i>et al.</i> , 1984 <i>c</i>
WG935	WG588 - pWG115	...
WG525	MRSA isolated at Royal Melbourne Hospital since 1980 carrying pWG53	Townsend <i>et al.</i> , 1983 <i>b</i> , 1984 <i>c</i>
WG1320	RN450 + pWG53	Townsend <i>et al.</i> , 1984 <i>c</i>
WG974	WG822 + pWG53	...
WG686	WG525 - pWG53	Townsend <i>et al.</i> , 1983 <i>b</i>
WG1451	WG822 + pWG54	Townsend <i>et al.</i> , 1984 <i>b</i>
WG2983	WG822 + pWG401	...
WG2306	WG1876 + pWG202	...
WG3221	WG1876 + chromosomal Pi ^R	...

Pi = propamidine isethionate, Tp = trimethoprim, Gm = gentamicin, Nm = neomycin, Pase = penicillinase production, Fa = fusidic acid, Rf = rifampicin, superscript R = resistance.

15 ml of Mueller-Hinton Agar (Gibco Diagnostics). Strains were grown overnight in Brain Heart Infusion Broth (Gibco), diluted 1 in 500 in 1% peptone water and then inoculated on to the test plates by the multipoint method. Plates were incubated overnight at 37°C and the lowest concentration of compound that prevented the appearance of visible growth was taken as the MIC.

For each strain of *S. aureus*, the MIC of the following compounds was determined: ethidium bromide, methylglyoxal bis(guanyldihydrazone), spermine hydrochloride and spermidine trihydrochloride (Sigma); propamidine isethionate and amicarbalide isethionate (May and Baker); benzoflavin and coriphosphine O (K & K Laboratories Inc.); pyronin Y, acridine yellow and crystal violet (Gurr, Searle Diagnostic); imidocarb dipropionate (Wellcome Australia Ltd); acriflavine (BDH Chemicals Ltd); cetyltrimethyl-ammonium bromide (Ajax Chemicals); acridine orange and safranin O (Chroma-Gesellschaft Schmid and Co.); chlorhexidine digluconate (Imperial Chemical Industries).

RESULTS

All strains of *S. aureus* carrying a resistance determinant to propamidine isethionate were also found to have increased resistance to several other cationic agents (table II). The level of resistance to these compounds was independent of other markers on the same plasmid. However, the MICs of these agents for strain WG3221, which carries the resistance determinant on the chromosome, were reduced by 2- to 8-fold in comparison with strains carrying plasmid-borne resistance.

The compounds exhibiting the greatest difference in MIC (up to a 128-fold increase) between resistant strains and their isogenic, sensitive derivatives were ethidium bromide, acriflavine, safranin O, benzoflavin and propamidine isethionate (table II). Other compounds with an appreciable difference in MIC were pyronin Y, acridine yellow and imidocarb dipropionate. The resistance determinant conferred only a small increase in resistance to crystal violet (gentian violet), cetyltrimethylammonium bromide, amicarbalide isethionate, and methylglyoxal bis(guanyldihydrazone). There was no significant difference between propamidine-resistant and -sensitive strains in their resistance to chlorhexidine digluconate, coriphosphine O, acridine orange, spermine or spermidine.

DISCUSSION

Strains of *S. aureus* that were resistant to propamidine isethionate and cetyltrimethylammonium bromide showed various degrees of cross-resistance to a broad range of cationic agents. An examination of the chemical structure of these compounds revealed several common features. Where the increase in MIC for a compound was more than 16-fold, the compound was symmetrical and contained two or more basic aromatic groups with terminal primary amine, tertiary amine, amidino or imidazole rings.

Despite the wide spectrum of compounds against which the resistance determinant was directed, relatively minor modifications in the chemical structure of the agents sometimes reduced significantly the degree of resistance. Thus, di-methyl substitution of one or both amine groups of acriflavine to form coriphosphine O or acridine orange respectively negated the effect of the resistance determinant. In fact, strains of *S. aureus* carrying the cationic-resistance determinant tended to be more sensitive than their isogenic derivatives to acridine orange.

Because of their basic nature, all the compounds whose resistance is coded for by

TABLE II
MIC values of various cationic agents for S. aureus

Strain no.	Characteristic	MIC* of agent																
		CV	CH	EB	SO	AF	PY	AY	PI	BF	ID	CO	AI	CB	AO	MG	SM	SD
WG335	"Oxford" "old" MRSA	0.5	0.9	3.8	7.5	7.5	15	7.5	7.5	31	31	31	62	15	125	2000	4000	8000
WG1437		0.5	0.9	3.8	15	31	31	15	15	62	62	62	125	15	125	2000	1000	4000
WG935		0.5	0.9	7.5	15	31	31	15	15	62	62	62	31	125	31	125	4000	2000
WG588	MRSA (pWG115)	1.9	3.8	125	500	250	500	500	500	2000	2000	125	1000	125	125	64000	1000	8000
WG686		0.5	1.9	7.5	15	62	31	15	15	62	62	62	125	31	250	4000	1000	8000
WG525		0.9	3.8	125	1000	250	1000	500	1000	2000	4000	125	2000	62	250	16000	1000	4000
RN450	plasmid-free RN450 + pWG53	0.5	0.9	7.5	7.5	31	15	15	15	62	125	62	125	15	250	4000	2000	4000
WG1320		1.9	3.8	250	500	500	500	500	1000	2000	2000	125	1000	125	125	64000	1000	4000
WG822		0.3	0.9	3.8	7.5	7.5	15	15	15	15	125	31	250	15	125	4000	500	4000
WG974	WG822 + pWG53 WG822 + pWG401 WG822 + pWG54	1.9	1.9	250	250	250	500	500	2000	2000	2000	62	2000	125	62	32000	250	4000
WG2983		0.9	1.9	250	250	250	500	500	2000	1000	2000	62	1000	125	31	32000	250	4000
WG1451		1.9	3.8	250	250	500	500	500	1000	2000	4000	62	2000	125	125	64000	500	8000
WG1876	plasmid-free recipient	0.3	0.9	3.8	1.9	7.5	15	15	15	62	125	31	250	15	250	4000	500	4000
WG2000		1.9	1.9	500	250	1000	500	500	2000	2000	4000	62	2000	125	62	32000	250	8000
WG2306		0.9	1.9	125	250	250	250	250	500	2000	4000	62	1000	62	62	32000	500	4000
WG3221	WG1876 + chromo- somal resistance	0.5	1.9	62	125	250	250	250	500	1000	4000	62	1000	125	125	32000	1000	4000

CV = crystal violet, CH = chlorhexidine, EB = ethidium bromide, SO = safranin O, AF = acriflavine, PY = pyronin Y, AY = acridine yellow, PI = propamidine isethionate, BF = benzoflavin, ID = imidocarb dipropionate, CO = coriphosphine O, AI = amicarbalide isethionate, CB = cetyltrimethyl-ammonium bromide, AO = acridine orange, MG = methylglyoxal, bis(guanyldihydrozone), SM = spermine hydrochloride, SD = spermidine tri-hydrochloride.
* MIC values were calculated as described in *Materials and methods* and are expressed as $\mu\text{mol/L}$.

the determinant bind to a variety of anionic compounds including nucleic acids, mast-cell heparin and mucopolysaccharides. Bis(guanylhydrazones) (Denny and Cain, 1979), quaternary ammonium compounds (Scott, 1962) and diamidine compounds such as propamidine and amicarbalide (Anne *et al.*, 1980) bind to nucleic acids, and the DNA-intercalating properties of ethidium bromide (Cain *et al.*, 1978) and the acridines (Wolfe *et al.*, 1972) have been well documented. This common property of binding to nucleic acids has prompted us to refer to the determinant encoding resistance to these compounds as the NAB (nucleic acid-binding compounds)-resistance determinant.

The antibacterial action of human semen has been attributed, at least in part, to the naturally occurring polyamines, spermine and spermidine (Razin and Rozansky, 1957) and the inhibitory action of these compounds on gram-positive cocci has been well documented (Rozansky *et al.*, 1954; Fair and Wehner, 1971; Bachrach, 1973). The NAB-resistance determinant did not confer an increased resistance to spermine hydrochloride or spermidine tri-hydrochloride. In contrast, NAB-resistant strains of *S. aureus* tended to show an increased sensitivity towards spermine hydrochloride in comparison to their NAB-sensitive isogenic derivatives (table II).

Three representative isolates of MRSA were examined for their resistance to NAB agents. Strain WG1437, isolated from Royal Perth Hospital before 1973, is representative of the earlier strains of MRSA that lacked resistance to propamidine, whereas strains WG525 and WG588 were isolated after 1980 and carry a NAB-resistance determinant (Townsend *et al.*, 1984*b*). The NAB-resistance profile of strain WG1437 was very similar to that of the Oxford strain, NCTC 6571, and other sensitive recipient strains such as RN450 and WG1876 (table II). Loss of the NAB-resistance plasmid from strains WG525 and WG588 (becoming strains WG686 and WG935 respectively) decreased their NAB resistance to that of sensitive organisms (table II).

Regardless of the source of the NAB-resistance determinant, the relative degree of resistance conferred to each compound studied was always similar. The decrease in levels of resistance displayed by strain WG3221, which carries chromosomal NAB-resistance, may reflect a gene dosage effect, because there may be more copies of the resistance determinant per cell when it is plasmid-borne than when it is located on the chromosome. The present results suggest that the NAB-resistance determinants present in the isolates of MRSA studied are similar and are distributed world-wide. They indicate that resistance to all the NAB compounds is conferred by the same determinant. Current studies will probably confirm this. Genetic determinants for resistance to acriflavine (Ericson, 1969) and ethidium bromide (Johnston and Dyke, 1969; Archer and Johnston, 1983; Foster, 1983; McDonnell *et al.*, 1983) have been reported previously in *S. aureus* and they may be similar to the NAB-resistance determinant described here.

The use of agents such as acriflavine, propamidine, cetyltrimethyl-ammonium bromide and crystal violet in topical antiseptic creams may provide an explanation for the maintenance of the NAB-resistance determinant, but the current lack of widespread use of acriflavine and propamidine and the very small increase in resistance to cetyltrimethyl-ammonium bromide and crystal violet make this interpretation unlikely. We are now investigating several alternative possibilities that may indicate why this resistance determinant is carried by the MRSA strains currently being isolated in epidemic proportions in Australia (Pavillard *et al.*, 1982).

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