Isolation and characterisation of a family of small plasmids encoding resistance to nucleic acid-binding compounds in *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

Summary. A family of small plasmids encoding resistance to nucleic acid-binding (NAB) compounds has recently been identified in strains of *Staphylococcus aureus* isolated in Italy, Texas and Western Australia. The mol. wts of the NAB-resistance plasmids are in the range $(1.5\text{--}1.9) \times 10^6$ and all but one encode resistance to acridine yellow, ethidium bromide and quaternary ammonium compounds. The largest of the plasmids, pWG1773, differed in that it did not confer resistance to ethidium bromide. Restriction enzyme analysis of these plasmids revealed four distinct patterns corresponding to plasmids of four different mol. wts and physical maps were constructed based on the restriction patterns. Two plasmid types of molecular sizes approximately 2440 and 2240 base pairs had a 610-base pair region in common. Physical maps of the other two plasmid types were not related. The presence of a family of small NAB-resistance plasmids which carry no other known phenotypic markers provides further evidence for the strong selective advantage associated with maintenance of this determinant in clinical isolates of *S. aureus*.

Introduction

Plasmid-borne resistance to a range of nucleic acid-binding (NAB) compounds is a common feature of methicillin-resistant *Staphylococcus aureus* strains isolated in several countries (Townsend *et al.*, 1985c). The NAB-resistance determinant is usually found on large plasmids in association with other phenotypic markers, the most common being gentamicin resistance (Townsend *et al.*, 1985c).

Two NAB-resistance phenotypes have been recognised (Emslie *et al.*, 1985a and b) which, for convenience, are designated types I and II. The most distinguishing feature of type-I NAB resistance is the expression of resistance to diamidino compounds such as propamidinium isethionate in addition to other NAB-compounds such as ethidium bromide, acridine yellow and quaternary ammonium compounds. Type-I NAB resistance is a characteristic feature of methicillin-resistant *S. aureus* in Eastern Australia (Emslie *et al.*, 1985b). Type-II NAB resistance does not include resistance to

diamidino compounds as exemplified by the NAB-resistance determinant located on conjugative gentamicin-resistance plasmids (Emslie *et al.*, 1985a).

Both Type-I and Type-II NAB-resistance determinants confer a 4–8-fold increase in resistance to cetrizide and benzalkonium chloride (Emslie *et al.*, 1985a and b). Whether this small increase in resistance to antiseptic compounds is sufficient to provide the selective pressure for maintenance of the determinant or whether the resistance determinant contributes some other selective advantage has not been elucidated. Nevertheless, the prevalence of NAB resistance amongst current isolates of methicillin-resistant *S. aureus*, particularly those associated with recent outbreaks in Australia (Grubb *et al.*, 1983; Townsend *et al.*, 1983a, 1984a and b), supports the suggestion that NAB resistance is an important attribute of clinical isolates of *S. aureus* (Townsend *et al.*, 1985b).

We have recently identified a family of small NAB-resistance plasmids with mol. wts in the range $(1.5\text{--}1.9) \times 10^6$. This paper describes their phenotypic characteristics and compares their preliminary physical maps based on restriction enzyme analysis.

Materials and methods

Strains of *S. aureus*

Clinical isolates of *S. aureus* were provided as follows: WG339, WG365, WG383 and WG910, from Dr D. Annear, were isolated at the Royal Perth Hospital between 1969 and 1981; WG2125 and WG2126 were isolated in 1982 by Dr H. Brown, Houston Medical Center, Texas; and WG3775, WG3778, WG3779 and WG3780 were isolated before 1984 by Dr G. Orefici, Istituto Superiore di Sanita, Rome, Italy. The characteristics of the NAB-resistance plasmids from these strains are detailed in table 1.

Plasmid transfer

NAB-resistance plasmids were transferred to either recipient strain WG1876 or WG3355 by mixed-culture transfer (Townsend *et al.*, 1983b). Strains WG1876 and WG3355 were derived from strain RN450 (Novick and Bouanchaud, 1971) and were lysogenic for staphylococcal phage J (Townsend *et al.*, 1984a). Strain WG1876 carries chromosomal resistance to fusidic acid and rifampicin (Townsend *et al.*, 1984b) whereas strain WG3355 carries chromosomal resistance to fusidic acid and novobiocin. Transcipients were selected on Brain-Heart Infusion Agar (Gibco Diagnostics) containing fusidic acid 5 mg/L plus either ethidium bromide 48 mg/L or acridine yellow 110 mg/L and either rifampicin 25 mg/L (for transfer experiments involving the recipient WG1876) or novobiocin 5 mg/L (for transfer experiments involving the recipient WG3355). Transcipients were purified on Brain-Heart Infusion Agar containing ethidium bromide

30 mg/L or acridine yellow 28 mg/L plus fusidic acid 5 mg/L and either rifampicin 25 mg/L or novobiocin 5 mg/L. The transcipients generated are detailed in table 1.

Antibacterial sensitivity testing and determinations of minimum inhibitory concentrations (MIC)

Disk-sensitivity testing was performed as described by Townsend *et al.* (1983b and c).

The agar dilution method (Emslie *et al.*, 1985b) was used to determine MIC values for the following NAB-compounds: propamidine isethionate (May and Baker); cetyltrimethylammonium bromide (CTAB) (Ajax Chemicals); benzalkonium chloride and ethidium bromide (Sigma Chemical Company); acridine yellow (Gurr, Searle Diagnostic). Because MIC values varied slightly with different batches of medium, results are expressed as relative values only. The sensitive strains RN450, WG541, WG1876 and WG3355 and the resistant strains WG525, WG1320, WG2726 and WG2967 (Emslie *et al.*, 1985a) were included as controls in all MIC determinations.

Restriction enzyme analysis

Plasmid DNA was isolated by precipitation with CTAB (Townsend *et al.*, 1985a). Restriction endonuclease digestion of the NAB-resistance plasmids was undertaken with the enzymes *EcoRI*, *HindIII*, *HinfI*, *PstI*, *TaqI* (Bethesda Research Laboratories Inc.) and *BamHI* (Amersham). The restriction fragments were separated by electrophoresis in horizontal 2.0% agarose gels run for 5 h at 5V/cm. The mol. wt of restriction fragments was

Table 1. NAB-resistance plasmids of *S. aureus*

Plasmid no.	Clinical isolate and source	Transcipient strain	Plasmid size (Kbp)	Antibiotic-resistance Phenotype	Relative NAB/MIC levels	PI	EB	AY	BK	CB
pWG17	WG339, Perth	WG4313; WG1876 + pWG17	2.36	...	2	32	8	4	4	4
pWG32	WG365, Perth	WG4316; WG1876 + pWG32	2.36	...	2	32	8	4	4	4
pWG36	WG383, Perth	WG4361; WG1876 + pWG36	2.36	...	2	32	8	4	4	4
pWG572	WG2125, Houston	WG4367; WG1876 + pWG572	2.36	...	2	32	8	4	4	4
pWG575	WG2126, Houston	WG4320; WG1876 + pWG575	2.36	...	2	32	16	4	4	4
pWG1770	WG910, Perth	WG4425; WG3355 + pWG1770	2.36	...	1	16	16	8	8	8
pWG1771	WG3775, Rome	WG4249; WG3355 + pWG1771	2.36	...	1	8	16	8	8	8
pWG1772	WG3778, Rome	WG4363; WG1876 + pWG1772	2.24	...	2	32	8	4	4	4
pWG1773	WG3779, Rome	WG4364; WG1876 + pWG1773	2.88	...	2	1	8	4	4	4
pWG1774	WG3780, Rome	WG4132; WG1876 + pWG1774	2.44	...	2	8	8	4	4	4
pWG53	WG525, Melbourne (Townsend <i>et al.</i> , 1984b)	WG1320; RN450 + pWG53	30	Gm ^R Km ^R Tp ^R	128	64	16	8	4	4
pWG613	661, Richmond, Virginia (Archer and Johnston, 1983; Emslie <i>et al.</i> , 1985a)	WG2967; WG541 + pWG613	48	Gm ^R Km ^R Nm ^R Tp ^R	1	8	4	4	4	4

PI = propamidine isethionate, EB = ethidium bromide, AY = acridine yellow, BK = benzalkonium chloride, CB = cetyltrimethyl-ammonium bromide, Gm = gentamicin, Tp = trimethoprim, Km = Kanamycin, Nm = neomycin, superscript R = resistance, Kbp = Kilo base pairs.

* The relative NAB MIC levels for each NAB plasmid was calculated by dividing the MIC of the transcipient strain with that of the recipient strain.

estimated by computer analysis (Duggleby *et al.*, 1981) with *Pst*I digests of phage lambda DNA (Daniels *et al.*, 1983) and *Hinf*I digests of plasmid pBR322 (Maniatis *et al.*, 1982) as mol.-wt standards. Double and partial digests with the above enzymes were made when necessary in order to construct physical maps of the NAB-resistance plasmids.

Results

Ten strains of *S. aureus* isolated in Perth, Texas and Italy carried small plasmids range with mol. wts in the $(1.5-1.9) \times 10^6$. After mixed-culture transfer with selection for resistance to either ethidium bromide or acridine yellow, all transipients examined carried a small plasmid identical in mol. wt to that carried by the donor clinical isolate. The transipients generated were resistant to the NAB-compounds, cetrимide, benzalkonium chloride and acridine yellow but, like the donor strains, they were sensitive to the NAB-compound propamidine isethionate (table 1). All the transipients were resistant to ethidium bromide with the exception of strain WG4364 which had a MIC value for ethidium bromide identical to that of the plasmid-free recipient, WG1876.

The NAB-resistance plasmids did not confer resistance to methicillin, penicillin, gentamicin, kanamycin, neomycin, streptomycin, erythromycin, lincomycin, chloramphenicol, tetracycline, trimethoprim, spectinomycin, cadmium nitrate, mercuric chloride, phenylmercuric acetate or sodium arsenate.

Restriction enzyme analysis of the ten NAB-resistance plasmids revealed four distinct restriction patterns (table 2). Plasmids pWG1774 and pWG1772 both had a single *Eco*RI restriction site. Neither of these two plasmids had restriction sites for *Hind*III, *Pst*I or *Bam*HI. Plasmids pWG17, pWG32, pWG36, pWG572, pWG575, pWG1770 and pWG1771 had single restriction sites for *Pst*I and *Hind*III but no restriction sites for *Eco*RI or *Bam*HI. These plasmids are represented by pWG32 (table 2). Plasmid pWG1773 which did not confer resistance to ethidium bromide, had no restriction sites for *Eco*RI, *Hind*III and *Pst*I but had a single restriction site for the enzyme *Bam*HI.

A more accurate estimate of the plasmid mol. wts was produced by electrophoresis of the linear form of the plasmids after digestion by the enzymes with a single restriction site. The molecular sizes of the four representative plasmids pWG1774, pWG1772, pWG32 and pWG1773 were estimated as 2440, 2240, 2360 and 2880 base pairs respectively (table 2).

Table 2. Estimated restriction fragment sizes of four representative NAB-resistance plasmids

Restriction Endonuclease	Fragments obtained from plasmids*			
	pWG1774	pWG1772	pWG32	pWG1773
<i>Eco</i> RI	2440	2240	—	—
<i>Hind</i> III	—	—	2360	—
<i>Pst</i> I	—	—	2360	—
<i>Bam</i> HI	—	—	—	2880
<i>Taq</i> I	810	770 ^a	910	1600 ^d
	760 ^a	740	720 ^b	660
	690	550	500 ^c	310
	180	180	230	310
<i>Hinf</i> I	1780 ^a	1650 ^a	1410 ^b	1030
	660	590	640 ^c	590
			230	570
			80	460 ^d
				230

* Restriction fragment sizes are expressed in base pairs. a = restriction fragment cut after double digestion with *Eco*RI and either *Taq*I or *Hinf*I; b = restriction fragment cut after double digestion with *Pst*I and either *Taq*I or *Hinf*I; c = restriction fragment cut after double digestion with *Hind*III and either *Taq*I or *Hinf*I; d = restriction fragment cut after double digestion with *Bam*HI and either *Taq*I or *Hinf*I. The 80-base pair *Hinf*I fragment of pWG32 was not detected in the electrophoresis conditions used.

*Taq*I restriction enzyme digestion of each of the four representative NAB-resistance plasmids generated four fragments (figs. 1–3). Plasmids pWG1774 and pWG1772 had a common *Taq*I fragment of molecular size 180 base pairs (table 2, fig. 1). However, there were no other *Taq*I fragments in common amongst the four plasmids. *Hinf*I restriction enzyme digestion of the four representative plasmids produced from two to five restriction fragments (figs. 1–3). Plasmids pWG1772 and pWG1773 both had a 590-base pair *Hinf*I fragment. However, a *Hinf*I-*Taq*I double digest of those two plasmids demonstrated that these two fragments were not identical (figs. 1 and 3).

Orientation and ordering of the *Taq*I and *Hinf*I restriction fragments for each plasmid was achieved with partial *Hinf*I, partial *Taq*I and *Hinf*I-*Taq*I digests together with partial and complete double-digests of the single cut restriction enzymes with both *Hinf*I and *Taq*I (table 2, figs. 1–3). The physical maps produced by these restriction patterns revealed that plasmids pWG1774 and pWG1772 had a 610 base pair region in common which was bordered by a *Taq*I restriction site and the single *Eco*RI restriction site and encompassed one *Hinf*I and one *Taq*I restriction site (fig. 4). The physical maps of pWG32 and pWG1773 were quite distinct

Fig. 1. Agarose gel electrophoresis of plasmids pWG1774 and pWG1772 after restriction endonuclease digestion. Lanes 1 and 10, *Pst*I digests of phage lambda DNA; lanes 2-5, plasmid pWG1774 digested with *Taq*I, *Eco*RI-*Taq*I, *Hinf*I-*Taq*I and *Hinf*I respectively; lanes 6-9, plasmid pWG1772 digested with *Taq*I, *Eco*RI-*Taq*I, *Hinf*I-*Taq*I and *Hinf*I respectively.

Fig. 2. Agarose gel electrophoresis of plasmid pWG32 after restriction endonuclease digestion. Lanes 1 and 10, *Pst*I digests of phage lambda DNA; lanes 2-9, plasmid pWG32 digested with: lane 2, *Taq*I; lane 3, *Pst*I-*Taq*I; lane 4, *Hind*III-*Taq*I; lane 5, *Hinf*I-*Taq*I; lane 6, *Hinf*I; lane 7, *Hind*III-*Hinf*I; lane 8, *Pst*I-*Hinf*I; lane 9, *Hind*III-*Pst*I.

Fig. 3. Agarose gel electrophoresis of plasmid pWG1773 after restriction endonuclease digestion. Lanes 1 and 8, *Pst*I digests of phage lambda DNA; lanes 2–6, plasmid pWG1773 digested with: lane 2, *Taq*I; lane 3, *Bam*HI-*Taq*I; lane 4, *Hinf*I-*Taq*I; lane 5, *Hinf*I; lane 6 *Bam*HI-*Hinf*I; lane 7, plasmid pBR322 digested with *Hinf*I.

Fig. 4. Preliminary restriction maps of the four representative NAB-resistance plasmids, pWG1774, pWG1772, pWG32 and pWG1773. Restriction endonuclease sites are indicated by B (*Bam*HI), E (*Eco*RI), Hd (*Hind*III), Hf (*Hinf*I), P (*Pst*I) and T (*Taq*I).

and had no features in common with either pWG1774 or pWG1772 (fig. 4).

Discussion

A family of small, $(1.5-1.9) \times 10^6$ mol. wt. plasmids encoding resistance to NAB compounds has been isolated from several strains of *S. aureus* obtained from sources in Italy, Texas and Western Australia. Plasmid-borne resistance determinants to NAB compounds are widely distributed amongst current isolates of methicillin-resistant *S. aureus* (Townsend *et al.*, 1985c) and are characteristic features of the strains of methicillin-resistant *S. aureus* associated with recent outbreaks in Eastern Australia (Grubb *et al.*, 1983; Townsend *et al.*, 1983a, 1984a and b). However, the NAB-resistance determinant is most frequently located on the largest plasmid of these *S. aureus* strains and is usually associated with resistance to gentamicin (Townsend *et al.*, 1985c). This is the first report of a small NAB-resistance plasmid which carries no other known phenotypic markers.

After mixed-culture transfer of the NAB-resistance plasmids, nine of the 10 transipients expressed a type-II NAB-resistance phenotype. However, strain WG4364 which carried the largest plasmid, pWG1773, showed no increased resistance to ethidium bromide despite expressing full resistance to acridine yellow and quaternary ammonium compounds. This new phenotype has been classified as type-III NAB resistance.

The presence of three unique NAB-resistance phenotypes may reflect the existence of several genes encoding resistance to NAB compounds or it may arise from variations in phenotypic expression of the determinant. This second possibility is supported by the observation that MIC values for the NAB compounds were slightly different when similar plasmids (pWG32 and pWG1770) were located in the two recipient backgrounds, WG1876 and WG3355 (table 1). Furthermore, since the clinical isolate WG3778 carried plasmid pWG1772 and expressed Type-III NAB resistance, it was sensitive to ethidium bromide (unpublished observations). However, after transfer of plasmid pWG1772 to the recipient strain WG1876 (selecting for resistance to acridine yellow) the transipient, WG4363,

expressed full ethidium bromide resistance thus changing in phenotype to Type-II NAB resistance.

Restriction enzyme analysis revealed four distinct restriction-fragment patterns which corresponded to plasmids of four different mol. wts. Two related plasmids, pWG1774 and pWG1772 of molecular sizes 2440 and 2240 base pairs respectively, were carried by strains of *S. aureus* isolated in Italy. These two plasmids have a region in common of c. 610 base pairs. Plasmid pWG1773, which was also isolated from an Italian strain of *S. aureus*, conferred type-III NAB resistance. Based on the preliminary physical maps, this plasmid of 2880 base pairs appeared to be unrelated to the plasmids pWG1774 and pWG1772. The fourth unique plasmid type represented by plasmid pWG32 of 2360 base pairs was the most widespread, being carried by strains of *S. aureus* isolated in Perth, Houston and Rome between 1969 and 1982.

Whilst NAB-resistance determinants have more recently been found in association with gentamicin resistance, the identification of these small NAB-resistance plasmids in strains isolated as early as 1969 together with other early reports of NAB resistance located on a penicillinase plasmid (Ericson, 1969; Johnston and Dyke, 1969) clearly demonstrate that resistance to NAB compounds was prevalent in the *S. aureus* population well before the emergence of gentamicin resistance. Furthermore, the maintenance for some 15 years of a NAB-resistance plasmid which confers no other known resistance phenotype and the widespread distribution of strains of *S. aureus* carrying such a plasmid emphasises the selective pressure operating to maintain such a resistance mechanism. Further genetic characterisation of these small plasmids will define the gene or genes responsible for NAB resistance more precisely and will make possible the comparison of these NAB-resistance determinants with those more recently found on large gentamicin-resistance plasmids.

We thank May and Baker, Australia for donating the propamidine isethionate, Dr G. Orefici, Rome, Italy for strains WG3775, WG3778, WG3779 and WG3780 and Dr H. Brown, Houston, Texas for strains WG2125 and WG2126. KRE is the recipient of a National Health and Medical Research Council Australian Post-Doctoral Fellowship. This work was funded in part by a National Health and Medical Research Council Grant to WBG.

REFERENCES

- Archer G L, Johnston J L 1983 Self-transmissible plasmids in staphylococci that encode resistance to aminoglycosides. *Antimicrobial Agents and Chemotherapy* **24**:70-77.
- Daniels D L, Schroeder J L, Szybalski W, Sanger F, Blattner F R 1983 A molecular map of Coliphage Lambda. In: Hendrix R W *et al.* (eds) *Lambda II*, Cold Spring Harbour Laboratory NY pp. 469-517.
- Duggleby R G, Kinns H, Rood J I 1981 A computer program for

- determining the size of DNA restriction fragments. *Analytical Biochemistry* **110**:49–55.
- Emslie K R, Townsend D E, Bolton S, Grubb W B 1985a Two distinct resistance determinants to nucleic acid-binding compounds in *Staphylococcus aureus*? *FEMS Microbiology Letters* **27**:61–64.
- Emslie K R, Townsend D E, Grubb W B 1985b A resistance determinant to nucleic acid-binding compounds in methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology* **20**:139–145.
- Ericson C 1969 Resistance to acriflavine and cadmium and changed phage reactions: markers of a new staphylococcal penicillinase plasmid? *Acta Pathologica et Microbiologica Scandinavica* **76**:333.
- Grubb W B, Townsend D E, Greed L C, Ashdown N, Momoh M 1983 Characteristics of methicillin-resistant *Staphylococcus aureus* endemic in Australian hospitals. In: Spitzky K H, Karrer K (eds) Proceedings of the 13th International Congress of Chemotherapy, Verlag H. Egermann, Vienna. Part 83:23–26.
- Johnston L H, Dyke K G H 1969 Ethidium bromide resistance, a new marker on the staphylococcal penicillinase plasmid. *Journal of Bacteriology* **100**:1413–1414.
- Maniatis T, Fritsch E F, Sambrook J 1982 Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratory, NY pp. 493–503.
- Novick R P, Bouanchaud D 1971 Extrachromosomal nature of drug resistance in *Staphylococcus aureus*. *Annals of the New York Academy of Science* **182**:279–294.
- Townsend D E, Ashdown N, Bolton S, Grubb W B 1985a. The use of cetyltrimethylammonium bromide for the rapid isolation from *Staphylococcus aureus* of relaxable and non-relaxable plasmid DNA suitable for *in vitro* manipulation. *Letters in Applied Microbiology* **1**:87–94.
- Townsend D E, Ashdown N, Greed L C, Grubb W B 1984a. Analysis of plasmids mediating gentamicin resistance in methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **13**:347–352.
- Townsend D E, Ashdown N, Greed L C, Grubb W B 1984b. Transposition of gentamicin resistance to staphylococcal plasmids encoding resistance to cationic agents. *Journal of Antimicrobial Chemotherapy* **14**:115–124.
- Townsend D E, Ashdown D, Grubb W B 1985b. Evolution of Australian isolates of methicillin-resistant *Staphylococcus aureus*—a problem of plasmid incompatibility? *Journal of Medical Microbiology* **20**:49–61.
- Townsend D E, Ashdown N, Momoh M, Grubb W B 1985c. Distribution of plasmid-borne resistance to nucleic acid binding compounds in methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **15**:417–434.
- Townsend D E, Greed L C, Ashdown N, Grubb W B 1983a. Plasmid-mediated resistance to quaternary ammonium compounds in methicillin-resistant *Staphylococcus aureus*. *Medical Journal of Australia* **2**:310.
- Townsend D E, Grubb W B, Ashdown N 1983b. Gentamicin-resistance in methicillin-resistant *Staphylococcus aureus*. *Pathology* **15**:169–174.
- Townsend D E, Grubb W B, Ashdown N 1983c. Genetics of drug resistance in methicillin-resistant *Staphylococcus aureus* from Australian Hospitals. *Journal of Hospital Infection* **4**:331–337.