EVOLUTION OF AUSTRALIAN ISOLATES OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*: A PROBLEM OF PLASMID INCOMPATIBILITY?

D. E. TOWNSEND, N. ASHDOWN AND W. B. GRUBB*

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

SUMMARY. Methicillin-resistant *Staphylococcus aureus* (MRSA), currently causing problems in Australian hospitals, have chromosomal penicillinase and carry a new family of incompatibility group I (InCI) plasmids that encode resistance to nucleic acid-binding compounds (NAB). These plasmids may carry additional determinants for penicillinase production and resistance to gentamicin and trimethoprim. By comparison, earlier MRSA isolates from Australia were NAB-sensitive and the penicillinase determinants were carried on InCI plasmids. The possibility that these newer MRSA isolates have the same 'clonal' origin as other MRSA isolates has been investigated. Forcible maintenance of InCI penicillinase plasmids and NAC-resistance plasmids in the same cells resulted in various recombination events. Similar recombination events to those generated in the laboratory have been found in MRSA isolates.

INTRODUCTION

It has been suggested that methicillin-resistant *Staphylococcus aureus* (MRSA) have arisen from a single clone (Lacey, 1972; Lacey and Grinsted, 1973). This hypothesis was based on the fact that MRSA characteristically have heterogeneous resistance to methicillin, orange pigmentation, similar survival rates on glass, high-level chromosomal resistance to streptomycin (Sm) and two plasmids—a stable plasmid of mol. wt 2.9×10^6 coding for tetracycline (Tc) resistance and a larger plasmid coding for penicillinase (pase) production and heavy-metal (Cd and Hg) resistance. MRSA strains which differed from the original clone could be explained on the basis of mutation or the loss or acquisition of plasmids (Lacey and Grinsted, 1973).

There has been an epidemic spread of MRSA in Australian hospitals (Pavillard *et al.*, 1982) leading to the suggestion that these strains are different from other strains of MRSA (McDonald, 1982). Using several of the characteristics mentioned above, Gedney and Lacey (1982) analysed recent isolates of MRSA from Australia and concluded that they were closely related to MRSA strains from other countries and

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* Reprint requests should be sent to Dr W. B. Grubb.

thus were derived from the original clone. However, other studies, based principally on a genetic analysis, have shown that the Australian strains differ markedly in many respects. They have chromosomal determinants for *pase*, and resistance to Cd, Hg, Tc, erythromycin (Em), and low levels of Sm (Grubb *et al.*, 1983; Townsend *et al.*, 1983*b*). In addition, characteristically they carry cryptic plasmids of mol. wt $(1.0-1.9) \times 10^6$, a plasmid of mol. wt $(2.8-3.0) \times 10^6$ when resistant to chloramphenicol (Cm) and a plasmid of mol. wt $(14.6-22) \times 10^6$ (Lyon *et al.*, 1982; Grubb *et al.*, 1983; Lyon *et al.*, 1983; Townsend *et al.*, 1983*b*, and *c*, and 1984*c*; Lyon *et al.*, 1984). The $(14.6-22) \times 10^6$ mol. wt plasmids determine resistance to quaternary ammonium compounds (Townsend *et al.*, 1983*a* and 1984*a*; Grubb *et al.*, 1983), propamidine isethionate (Townsend *et al.*, 1984*b*), ethidium bromide and a range of compounds (Emslie *et al.*, 1985, and in press) which share a common property of being nucleic acid-binding compounds (NAB). Consequently, resistance to these agents will be referred to as NAB resistance. The NAB-resistance plasmids also carry one or more of the determinants for *pase*, gentamicin (Gm) resistance or trimethoprim (Tp) resistance (Townsend *et al.*, 1983*a*, 1984*a* and 1984*b*).

There is also evidence which indicates that the MRSA strains currently being isolated in Australian hospitals have only emerged in recent years. Strains isolated from an Australian hospital before the present outbreak are genetically different from the recent isolates (Townsend *et al.*, 1984*c*). However, they are similar to the strains studied by Lacey and Grinsted (1973) on the basis of which they established their concept of the clonal origin of MRSA strains. Consequently, if the recently isolated Australian strains of MRSA originated from the same 'clone' as other MRSA strains, they must have undergone considerable rearrangement in the location of their resistance genes.

In a survey of over 300 MRSA strains recently isolated from hospitals in Australia, no strains were found which simultaneously carried a *pase* and heavy-metal-resistance plasmid and a NAB-resistance plasmid (Grubb *et al.*, 1983). These observations suggested that the NAB-resistance plasmids may belong to the same incompatibility (Inc) group as the *pase* and heavy-metal-resistance plasmids. If this is the case, the acquisition of a NAB-resistance plasmid by a MRSA of the type reported by Lacey and Grinsted (1973) would require the relocation of the resistance determinants on the *pase* and heavy-metal-resistance plasmid. This paper demonstrates that the NAB-resistance plasmids and the *pase* and heavy-metal-resistance plasmids belong to IncI and examines the consequences of forcing a strain to carry the two incompatible plasmids. These results are discussed in relation to the possible evolution of Australian MRSA strains from a single clone.

MATERIALS AND METHODS

Strains of S. aureus. See table I; Drs J. Pearman and D. I. Annear provided the strains of MRSA isolated from Royal Perth Hospital, Western Australia and Dr. Tay Leng, Department of Pathology, Singapore, supplied the MRSA strains from Singapore hospitals.

Media and reagents. Mueller-Hinton Agar (MHA), Brain-Heart Infusion Broth (BHIB) and Brain-Heart Infusion Agar (BHIA) were supplied by Gibco Diagnostics (Madison, Wisconsin, USA). BHIBC and BHIAC contained sodium citrate 5 g/L.

Antibacterial sensitivity testing. Propamidine isethionate (Pi) was used to detect NAB-resistance (Townsend *et al.*, 1984*b*). Sensitivity testing was performed either by multipoint replication (Annear and Grubb, 1972) or with impregnated 6-mm disks. Multipoint replication

TABLE I
Strains of *S. aureus*

Strain no.	Properties* and plasmid content	Reference
WG247	RN27, phage 80 α lysogen	Novick, 1963
WG248	RN450, plasmid free, non-lysogenic, sensitive to all antibacterial agents used	Townsend <i>et al.</i> , 1983b; Novick & Bouanchaud, 1971.
WG541	WG248 + chromosomal Fa ^R , Rf ^R	Townsend <i>et al.</i> , 1983b.
WG822	WG541, phage 95 lysogen	Townsend <i>et al.</i> , 1983b.
WG879	WG541, phage 85 lysogen	Townsend <i>et al.</i> , 1983b.
WG1876	WG541, phage J lysogen	Townsend <i>et al.</i> , 1984b.
WG516	WG248 + pI258 (pase Cd ^R Hg ^R Asa ^R Em ^R , mol. wt 18.4 $\times$ 10 ⁶)	Novick <i>et al.</i> , 1979.
WG2000	WG1876 + pWG115 (Pi ^R Tp ^R , mol. wt c.14.6 $\times$ 10 ⁶)	Townsend <i>et al.</i> , 1984b.
WG1320	WG248 + pWG53 (Pi ^R Gm ^R Tp ^R , mol. wt c.18.0 $\times$ 10 ⁶)	Townsend <i>et al.</i> , 1984b.
WG1348	WG822 + pWG51 (Pi ^R pase Gm ^R , mol. wt c.19.6 $\times$ 10 ⁶)	Townsend <i>et al.</i> , 1984a.
WG1451	WG822 + pWG54 (Pi ^R Gm ^R , mol. wt c.15.3 $\times$ 10 ⁶)	Townsend <i>et al.</i> , 1984a.
WG881	WG879 + pWG50 (pase Cd ^R Hg ^R Gm ^R , mol. wt c.28.5 $\times$ 10 ⁶)	Townsend <i>et al.</i> , 1983b.
WG1433	MRSA, isolated at Royal Perth Hospital, 1982, carries pWG405 (pase Cd ^R Hg ^R Asa ^R , mol. wt c.20 $\times$ 10 ⁶)	
WG1547	WG822 + pWG405 (transferred by mixed culture from WG1433)	
WG1437 -	MRSA isolated at Royal Perth Hospital, 1969-1973. Strain	
WG1443	WG1439 carries pWG404 (pase Cd ^R Hg ^R Asa ^R , mol. wt c.20 $\times$ 10 ⁶)	
WG1552	WG822 + pWG404 (transferred by mixed culture from strain WG1439)	
WG939	Methicillin-sensitive isolate from Royal Perth Hospital, carries pWG80 (pase Cd ^R Hg ^R Asa ^R , mol. wt c.15.1 $\times$ 10 ⁶)	
WG1119	WG248 + pWG80 (transferred by phage 80 from strain WG939)	
WG1983	MRSA, isolated in Singapore, carries pWG278 (Pi ^R Cd ^R Hg ^R , mol. wt c.16.9 $\times$ 10 ⁶)	
WG3192	WG1876 + pWG278 (transferred by mixed culture from strain WG1983)	
WG1988	MRSA, isolated in Singapore, carries pWG202 (Pi ^R pase Cd ^R Hg ^R , mol. wt c.19.5 $\times$ 10 ⁶)	
WG2306	WG1876 + pWG202 (transferred by mixed culture from strain WG1988)	
WG1986	MRSA, isolated in Singapore, carries pWG200 (Pi ^R pase Cd ^R Hg ^R , mol. wt c.18.4 $\times$ 10 ⁶)	
WG2312	WG1876 + pWG200 (transferred by mixed culture from strain WG1986).	

* Rf = rifampicin; Fa = fusidic acid; pase = penicillinase; Asa = arsenate; Em = erythromycin; Pi = pro-pamidine isethionate; Tp = trimethoprim; Gm = gentamicin; superscript R = resistance.

was on BHIA containing one of the following: Pi (May and Baker, Melbourne, Australia) 100 mg/L; 1×10^{-4} M Cd(NO₃)₂; benzyl penicillin (Pc; CSL, Melbourne, Australia) 150 u/L; Em (Em lactobionate, Abbott Laboratories, Kurnell, Australia) 2 mg/L; Gm (Schering Corp., Baulkham Hills, Australia) 2 mg/L; or on MHA containing Tp (Wellcome Australia Ltd) 5 mg/L. For disk sensitivity tests, MHA plates were flooded with an overnight broth culture diluted 100-fold in physiological saline. The excess fluid was removed and disks were applied to the dried plates. The disks contained the following quantities of antibacterial reagents: Pi, 100 μ g; Cd, 0.2 μ mol of Cd (NO₃)₂; Pc, 1.5 u; Em, 10 μ g; Gm, 10 μ g; Tp, 5 μ g; Hg, 0.4 μ mol of HgCl₂; Asa, 0.2 μ mol of NaHASO₄. Strain WG248 was used as a sensitive control and strains WG1320 and WG516 as resistant controls.

Pase production was detected by the PNCB method (Novick and Richmond, 1965) or with Nitrocefin (Oxoid, Melbourne, Australia), a chromogenic cephalosporin.

Bacteriophage propagation. Bacteriophage 80 α , a derivative of international typing bacteriophage 80 (Novick, 1963), produces high-titre lysates on strain WG248. This phage was induced from strain WG247 by ultra-violet (UV) irradiation followed by semi-solid propagation (Grubb

and O'Reilly, 1971) at 30°C on strains WG248, WG516 and WG1119. All phage lysates were membrane-filtered, checked that they were free of viable bacteria and stored at 4°C.

Transduction. Recipient organisms were grown on BHIA containing an antibacterial agent that would select cells containing a particular plasmid. The cells were harvested and 5×10^9 cells suspended in 9 ml of BHIB containing 0.01M CaCl_2 at 37°C. Phage 80 α was added at a multiplicity of infection of 0.1–0.2 and the mixture gently agitated for 35 min at 37°C. Ca^{++} was sequestered by the addition of 100 μl of sterile sodium citrate (500 g/L). The cells were washed twice with BHIBC and then resuspended in 1.0 ml of BHIBC. After standing for 2 h at 37°C, 100–200- μl volumes were spread on to pre-dried plates which were either single-selection plates of BHIAC + 1×10^{-4} M Cd or double-selection plates of BHIAC + 5×10^{-5} M Cd and either Gm 2 mg/L or Pi 100 mg/L. As a control, recipients without added phage were treated in the same manner. Transductants were purified on media of the same composition as used for their selection.

Mixed-culture transfer. One ml of an overnight culture of the recipient was placed in a petri dish which was slowly rotated while exposed for 30 s. to a 15 W germicidal lamp at a distance of 450 mm. Equal volumes (50 μl) of the irradiated recipient and an overnight culture of the donor were added to 5.0 ml of BHIB containing 0.01M CaCl_2 . After gentle shaking for 18 h at 37°C, the cultures were centrifuged and the pelleted cells were spread on to selective media and incubated for 48 h at 37°C. Single colonies were purified on plates of the same composition as the selection plates. Separate donor and recipient cultures were always treated in parallel as controls. All transipients were confirmed by comparing their antibiogram with that of the donor and recipient and by plasmid analysis.

When selecting for the transfer of an individual plasmid, single-selection plates were used, consisting of BHIA containing rifampicin (Rf; Lepetit, Milan, Italy) 50 mg/L, fusidic acid (Fa; Leo Laboratories, Ballerup, Denmark) 5 mg/L and either Pi 200 mg/L or 1×10^{-4} M Cd. When a plasmid was being transferred into a strain with a resident plasmid, both plasmids were selected on double-selection plates containing Rf 25 mg/L, Fa 5 mg/L and two of the following: 5×10^{-5} M Cd, Pi 100 mg/L, Em 5 mg/L, Pc 150 u/L and Gm 2 mg/L.

Plasmid stability. All plasmids were tested for their stability in the incubation conditions used for incompatibility testing. Cultures carrying a known plasmid were grown overnight in BHIB containing an appropriate selective agent for cells carrying the plasmid. Fifty μl of overnight culture was transferred to 5.0 ml of BHIB, incubated at 30°C or 37°C for 18 h, plated out for single colonies and 100 colonies were replicated to Cd or Pi plates.

Segregation tests for plasmid incompatibility. Strains carrying plasmid pairs were established on the appropriate, double-selection plates. Five single colonies from each selection were purified on media of the same composition and a single colony from each plate was grown for 18 h in 5.0 ml of BHIB at 37°C or 30°C. Single colonies were obtained in BHIA at 37°C and 100 colonies were picked and replicated to BHIA and BHIA plates containing, separately, the antibacterial agents used to establish the plasmid pair. Any single colony that retained resistance to both agents was re-tested for segregation of the plasmid-borne resistance markers.

Plasmid pI258 was used as the reference plasmid for IncI (Novick and Richmond, 1965).

Plasmid loss at elevated temperature. A loopful of a broth culture was streaked on to a BHIA plate and incubated at 43.5°C in a sealed chamber. After incubation for 18 h, a sample of growth was plated on to BHIA for single colonies which were screened for resistance.

Plasmid DNA analysis. Methods for the isolation of plasmid DNA, analysis by agarose-gel electrophoresis and measurement of plasmid mol. wt were as described by Townsend *et al.* (1984b). Mol. wts were calculated by means of a computer programme (Duggleby *et al.*, 1981).

The location of all the transferable resistance determinants was confirmed by plasmid analysis of donor and transipient strains. If the donor carried a plasmid of identical mol. wt to that in the transipient, the resistance determinants transferred were assigned to this plasmid.

RESULTS

Plasmid stability

The results are summarised in table II.

TABLE II
Plasmid stability at 30°C and 37°C

Strain no.	Plasmid	Plasmid markers*	Resistance screened	Percentage of colonies resistant after incubation at	
				30°C	37°C
WG2000	pWG115	Pi ^R Tp ^R	Pi	86	70
WG1320	pWG53	Pi ^R Gm ^R Tp ^R	Pi	100	96
WG1348	pWG51	Pi ^R Gm ^R pase	Pi	96	93
WG1451	pWG54	Pi ^R Gm ^R	Pi	100	94
WG516	pI258	pase Cd ^R Hg ^R Asa ^R Em ^R	Cd	100	100
WG1119	pWG80	pase Cd ^R Hg ^R Asa ^R	Cd	100	100
WG1547	pWG405	pase Cd ^R Hg ^R Asa ^R	Cd	100	100
WG1552	pWG404	pase Cd ^R Hg ^R Asa ^R	Cd	100	100
WG881	pWG50	pase Cd ^R Hg ^R Gm ^R	Cd	100	100

* See footnote to table I for abbreviations.

Displacement of resident plasmid

Strain WG1320 carries the resident plasmid, pWG53, and was harvested from Pi plates to ensure all recipient cells carried this plasmid. Plasmid pI258 was transduced from strain WG516 to strain WG1320 by a Cd selection. Fifty transductants were replicated and all were resistant to Cd but sensitive to Pi.

Apparently, the resident plasmid, pWG53, was being displaced by the incoming plasmid, pI258, indicating that both may belong to the same incompatibility group (IncI). To confirm this, the segregation of the plasmids in the daughter cells was studied.

Incompatibility grouping of plasmids determining resistance to NAB

Mixed-culture transfer or transduction was used to transfer plasmid pI258 to strains carrying one of the representative, NAB-resistance plasmids—pWG53, pWG115, pWG51 or pWG54. To prevent displacement of the resident plasmid by the incoming plasmid during transfer, selection was made for both plasmids on double-selection plates. Strains carrying both plasmid markers were then tested for the segregation of these markers (table III).

Previous work with plasmids encoding resistance to NAB agents had demonstrated that they were unstable at elevated incubation temperatures (Townsend *et al.*, 1983a and b, and 1984b). The results shown in table II suggest that they are more stable at 30°C than 37°C. An empirical observation has also been made that more plasmid DNA can be detected in agarose gels when the cells carrying the NAB-resistance plasmids are grown at 30°C instead of 37°C. This suggests that the copy number for these plasmids was higher at the lower temperature. In view of these observations, the

TABLE III
Segregation of incompatible plasmids

Percentage of colonies exhibiting characters* at 37°C and 30°C												
Plasmid pair	Donor strain	Recipient strain	Transfer method	Selection agent*	Cd ^R Pi ^S		Cd ^S Pi ^R		Cd ^S Pi ^S		Cd ^R Pi ^R	
					37°C	30°C	37°C	30°C	37°C	30°C	37°C	30°C
pI258, pWG53	WG516	WG1320	transduction	Cd Pi	93	0	6	99	0	0	1	1
pI258, pWG115	WG516	WG2000	transduction	Cd Pi	90	48	5	48	4	1	1	3
pI258, pWG51	WG516	WG1348	mixed culture	Cd Pi Fa Rf	95	56	1	42	4	2	0	0
pI258, pWG54	WG516	WG1451	mixed culture	Cd Pi Fa Rf	46	4	53	95	0	0	1	1
pWG53, pWG405	WG1320	WG1547	mixed culture	Cd Pi Fa Rf	...	44	...	55	...	0	...	1
pWG53, pWG404	WG1320	WG1505	mixed culture	Cd Pi Fa Rf	...	46	...	52	...	0	...	2
pWG53, pWG80	WG1119	WG1320	transduction	Cd Pi	...	47	...	52	...	0	...	1
					Em ^R Gm ^S 30°C	Em ^S Gm ^R 30°C	Em ^S Gm ^R 30°C	Em ^S Gm ^S 30°C	Em ^S Gm ^S 30°C	Em ^R Gm ^R 30°C	Em ^R Gm ^R 30°C	Em ^R Gm ^R 30°C
pI258, pWG50	WG516	WG881	mixed culture	Gm Em Fa Rf	54	45	0	0	0	0	1	1
					Em ^R Pi ^S 30°C	Em ^S Pi ^R 30°C	Em ^S Pi ^S 30°C	Em ^S Pi ^R 30°C	Em ^S Pi ^S 30°C	Em ^R Pi ^R 30°C	Em ^R Pi ^R 30°C	Em ^R Pi ^R 30°C
pI258, pWG202	WG516	WG2306	mixed culture	Em Pi Fa Rf	10	83	2	2	5	5	5	5
pI258, pWG200	WG516	WG2312	mixed culture	Em Pi Fa Rf	15	80	2	2	3	3	3	3
pI258, pWG278	WG516	WG3192	mixed culture	Em Pi Fa Rf	12	81	3	3	4	4	4	4

* See footnote to table I for abbreviations.

segregation tests were repeated at 30°C and all further tests were performed at this temperature (table III).

All colonies that retained both plasmid markers showed segregation on re-testing.

Incompatibility grouping of plasmids determining penicillinase production and heavy-metal resistance

The above results established that the plasmids encoding resistance to NAB were IncI plasmids. Consequently, one of these plasmids, pWG53, was used to determine whether the pasc plasmids in other isolates belonged to IncI.

MRSA strains WG1433 and WG1437–WG1443 all carried a plasmid of mol. wt $c. 20 \times 10^6$ which transferred to WG822 in mixed culture with selection for Cd resistance. All plasmids coded for pasc and resistance to Cd, Hg and Asa. Representative plasmids, pWG404 and pWG405 (from strains WG1439 and WG1433, respectively), were tested for incompatibility with plasmid pWG53. The results are summarised in table III.

Isolate WG939 contained a plasmid, pWG80, of mol. wt $c. 15.1 \times 10^6$ which was representative of pasc and heavy-metal-resistance plasmids in several strains of methicillin-sensitive *S. aureus* isolated from Royal Perth Hospital. When plasmid pWG80 was transduced to strain WG1320, segregation tests indicated that the two plasmids belonged to the same Inc group (table III). Plasmid pWG80 had the same phenotype and EcoRI restriction fragments as pI6187 (unpublished results), which is a reference IncI plasmid isolated from several parts of the world (Shalita *et al.*, 1980).

Gene rearrangements.

Usually, in the plasmid transfer experiments, when selection was made for two plasmids, both the selection and purification plates had mainly small colonies with the occasional (< 1%) large colony. It was reasoned that the small colonies resulted from the cells being forced to carry two incompatible plasmids, while in the cells of the large colonies, the plasmid genes had undergone rearrangement to produce a more stable configuration which would depend upon the selective pressure exerted and upon the original plasmid pairs. To confirm this, large colonies from different plasmid pairs and different double-selection media were picked and purified on media of the same composition. The colonies were tested for segregation of selection markers and screened by disk sensitivity tests for plasmid markers. Additionally, they were analysed for plasmid DNA. Representative examples of the results are presented in table IV. None of the large colonies showed subsequent segregation of resistance markers (results not shown).

When plasmids pWG53 and pWG80 were paired by transducing pWG80 into strain WG1320 and selection was made on Gm and Cd, a much higher proportion (up to 30%) of larger colonies was observed. Five small colonies, tested for plasmid incompatibility, showed the same pattern of segregation for this plasmid pair as shown in table III. Of 20 large colonies examined, 19 were resistant to Pc, Cd, Hg, Asa and Gm but sensitive to Pi and Tp. All 19 showed co-loss of resistance to Pc and Cd after incubation at elevated temperature but no loss of Gm resistance. Plasmid analysis

TABLE IV
Recombinants generated from pairing incompatible plasmids

Strain no.	Incoming plasmid	Resident plasmid	Double selection agents*	Antibacterial-resistance profile*	Number of plasmids	Plasmid mol. wt ($\times 10^6$)
WG2383	pWG53	pWG80	Gm Pc	Gm Pc Cd Hg Asa	1	c.17
WG2386	pWG53	pWG80	Pi Cd	Pi Gm Tp Pc Cd Hg Asa	1	c.33
WG2410	pWG53	pWG80	Pi Cd	Pi Gm Cd Hg	1	c.30
WG2422	pWG53	pWG80	Pi Pc	Pi Gm Tp Pc Cd Hg Asa	1	c.33
WG2404	pI258	pWG51	Em Pi	Pc Cd Hg Em Pi Gm	1	> 35†
WG2407	pI258	pWG54	Em Pi	Em Pi Gm	1	c.17
WG2468	pI258	pWG50	Em Gm	Pc Cd Hg Asa Em Gm	1	> 35†
WG1357	pWG80	pWG53	Cd Gm	Pc Cd Hg Asa Gm	1	c.15
WG1359	pWG80	pWG53	Cd Gm	Pc Cd Hg Pi Gm Tp	1	c.33

* See footnote to table I for abbreviations.

† A more accurate estimate of the mol. wt has not been given because the mol. wts of the plasmids fall outside the limits of the standard curve (see *Materials and Methods*).

revealed a plasmid identical in mol. wt to plasmid pWG80. The results obtained with strain WG1357 are given, as an example, in table IV.

The one remaining large colony (from strain WG1359) was resistant to Pc, Cd, Hg, Gm, Tp and Pi but sensitive to Asa. Plasmid loss at elevated temperature was at a low frequency (3%) and corresponded with co-loss of all the plasmid resistance markers. Plasmid analysis revealed a plasmid of mol. wt $c.33 \times 10^6$ (table IV).

Naturally occurring recombinant plasmids.

The results obtained with strain WG1359 indicated that one type of gene rearrangement within a strain carrying incompatible plasmids is recombination between the plasmids. Consequently, MRSA isolated from around the world were analysed for plasmids which could have arisen as the result of a similar recombination.

Of 16 MRSA strains received from Singapore hospitals, 13 were resistant to Pi and 11 transferred this resistance to strain WG1876 in mixed culture. Analysis of the transipients from each MRSA revealed that nine produced *pase* and expressed resistance to Pi, Cd and Hg; seven of these carried a plasmid of mol. wt $c.19.5 \times 10^6$, and the remaining two carried a plasmid of mol. wt $c.18.4 \times 10^6$. The other two transipients expressed resistance to Pi, Cd and Hg and carried a plasmid of mol. wt $c.16.9 \times 10^6$. In each case, the plasmid in the transipient was of the same mol. wt as the plasmid in the original MRSA (results not shown).

Plasmids pWG200, pWG202 and pWG278 were selected as representative of each plasmid type and tested for incompatibility with plasmid pI258, with Pi and Em as the selective markers for the plasmid pairs. All were shown to be IncI plasmids (table III).

Another plasmid, pWG50, from MRSA strain WG526, isolated at Royal Melbourne Hospital, Victoria, has previously been described as a recombinant plasmid (Townsend *et al.*, 1984a). This conclusion was based on *EcoRI* restriction analysis and on its stability at an elevated incubation temperature. Incompatibility tests indicated that this plasmid also belongs to the IncI group (table III).

DISCUSSION

The results of the segregation tests (see table III) cannot be explained in terms of plasmid instability (see table II) but clearly establish plasmids pWG51, pWG53, pWG54 and pWG115 as IncI plasmids. These plasmids are representative of a family of NAB-resistance plasmids which have been found in MRSA strains recently isolated in Australia (Townsend *et al.*, 1984*a* and *b*).

Staphylococcal plasmids have been classified into incompatibility groups and these contain plasmids of the same or similar phenotype. IncI plasmids are basically heavy-metal-resistance plasmids most of which carry a *pase* determinant and one, pI258, carries the Em-resistance determinant, Tn551 (Shalita *et al.*, 1980). All the other staphylococcal Inc groups contain plasmids with the same phenotype (Ruby and Novick, 1975; Iordanescu *et al.*, 1978; Iordanescu and Surdeanu, 1980). The discovery of NAB-resistance plasmids belonging to IncI means that this is the first group of staphylococcal plasmids to be shown to contain plasmids with unrelated phenotypes. Moreover, plasmids pWG51, pWG53, pWG54 and pWG115 do not appear to be related to *pase* plasmids when examined in an *Eco*R1-restriction-enzyme analysis (Townsend *et al.*, 1984*a* and *b*), a method which has previously been used to establish relatedness amongst IncI plasmids (Novick *et al.*, 1979).

The segregation patterns of plasmids pWG51, pWG53, pWG54 and pWG115, when paired with plasmid pI258, differed considerably depending on the plasmid pair and the incubation temperature (table III). The results suggest that the effect of temperature may have been a reflection of the copy number of the plasmids. At 30°C, Pi-resistance plasmids apparently have a higher copy number which favours their retention. This accords with a mathematical model proposed for segregation of incompatible plasmids by Novick and Hoppensteadt (1978).

Because Pi resistance is an easily selected and identifiable plasmid marker, plasmid pWG53 was used to classify pWG80, pWG404 and pWG405 as IncI plasmids (table III). Plasmid pWG80 appeared to be indistinguishable from pI6187 and, therefore, these results extend the observations of Shalita *et al.* (1980) that plasmids of this type are found in staphylococci isolated in widely distributed hospitals. Of greater interest were plasmids pWG404 and pWG405. The former plasmid is representative of the *pase* plasmids carried by MRSA strains predominant at Royal Perth Hospital before 1973 (Townsend *et al.*, 1984*c*). The latter plasmid was from a MRSA isolated at Royal Perth Hospital in 1982, which has become rare in Australia since MRSA strains carrying resistance to NAB have become prevalent (Grubb *et al.*, 1983; Townsend *et al.*, 1984*a*).

All the evidence suggests that previously reported MRSA strains characteristically carried an IncI, *pase* plasmid. Dyke (1969) reported that MRSA strains produced type-A *pase* and the determinant for this is usually carried on IncI plasmids (Peyru *et al.*, 1969). Furthermore, Dornbusch (1973) reported that the *pase* plasmid in a MRSA was displaced by an incoming IncI plasmid. Finally, the IncI plasmids pWG404 and pWG405 are phenotypically indistinguishable from *pase* plasmids previously detected in MRSA strains (Lacey and Grinsted, 1973). These results show clearly that the earlier MRSA strains would not have been able to acquire a NAB-resistance plasmid while still retaining an IncI *pase* plasmid.

MRSA strains characteristically produce *pase*. Although the reason for this has not been ascertained it has been suggested that *pase* is essential for them (Lacey, 1975).

Both the older MRSA strains and the more recent isolates from Australia produce *pase*. In the former strains, the determinants are on an *IncI* plasmid, whereas, in the latter isolates, the determinants are chromosomal. This, together with other genetic studies, suggests that the two types of MRSA are unrelated (Lyon *et al.*, 1982; Grubb *et al.*, 1983; Townsend *et al.*, 1983*b* and *c*; Lyon *et al.*, 1984; Townsend *et al.*, 1984*c*). However, Gedney and Lacey (1982) have suggested that the Australian MRSA isolates have the same clonal origin as other MRSA strains. If this is the case, the recent Australian isolates have subsequently undergone a rearrangement of their resistance determinants.

Based on this assumption, an original clonal MRSA carrying an *IncI*, *pase* plasmid has several alternatives if it is to retain the determinants of this plasmid and is under selection pressure to acquire an *IncI*, NAB-resistance plasmid: recombination between the two plasmids, integration of one of the plasmids into the chromosome or transposition of plasmid-borne determinants to another replicon.

The first of these alternatives, recombination between plasmids, has been shown to occur in laboratory conditions (see table IV). Colonies carrying recombinant plasmids were detected by their larger size on double-selection plates. This is in keeping with the results of other studies on recombinant plasmids generated from incompatible plasmids (Novick, 1969; Novick and Brodsky, 1972). Based on their phenotype, *Inc* group and mol. wt, the NAB-resistance plasmids, pWG278, pWG202 and pWG200, transferred from Singapore MRSA strains, appear to have resulted from recombination between a *pase*, heavy-metal-resistance plasmid and a NAB-resistance plasmid. Another example of a naturally occurring recombinant plasmid is pWG50, isolated from MRSA strain WG526 (Townsend *et al.*, 1983*b*). This is also an *IncI* plasmid (Table III) and has been previously characterised as a recombinant plasmid based on phenotype and restriction-enzyme analysis (Townsend *et al.*, 1984*a*). A more detailed study is necessary before one can conjecture about the selective pressures that have influenced the emergence of these plasmids.

The second alternative, namely insertion of a plasmid into the chromosome, provides a possible explanation for Australian MRSA strains, because recombination between the *pase* plasmids and the NAB-resistance plasmids does not appear to have been the major event in these strains. Wyman and Novick (1974) have reported the complete integration of an *IncI* plasmid, accompanied by loss of incompatibility, subsequent to double selection for two *IncI* plasmids. Other integrations of this type frequently involved inactivation of the *Asa*-resistance determinant (Schwesinger and Novick, 1975). Australian strains of MRSA are also *Asa* sensitive and have chromosomal *pase* and heavy-metal-resistance, which is in keeping with the integration of a *pase* plasmid. Novick (1979) has reported that an integrated, *IncI*, *pase* plasmid still expressed the *Inc* genes. In order for the NAB-resistance plasmids to be maintained in the Australian MRSA strains, the *Inc* genes of the putative, integrated, *pase* plasmid would have to be suppressed or deleted.

The third alternative, recombination *via* transposition events, provides another route for the evolution of Australian MRSA strains. The *pase* determinants in an Australian MRSA strain have been shown to be transposable (Tn 3852; Kigbo *et al.*, in press) and the Hg-resistance operon of *IncI* plasmids is potentially a transposable element (Shalita *et al.*, 1980). Thus, the acquisition of a NAB-resistance plasmid may have been accompanied by transposition of the *pase* and heavy-metal-resistance

determinants into the chromosome. In laboratory conditions, when plasmids pWG80 and pWG53 were selected on Gm and Cd, the most common event was the insertion of the Gm-resistance determinant into the chromosome. This was ascertained from the stability of the Gm-resistance determinant and the fact that the single plasmid retained (e.g., WG1357, table IV) was identical in mol. wt to plasmid pWG80. These results are not surprising in view of the high transposition frequencies of the Gm-resistance transposon, Tn3851, which is believed to be located on plasmid pWG53 (Townsend *et al.*, 1984b). Furthermore, these results emphasise the importance of selective pressure in determining the type of recombination event likely to be detected.

These results and conjectures provide some insight into the possible molecular events which must have occurred if the Australian MRSA strains evolved from the original MRSA clone. Furthermore, incompatibility between plasmids has been used as a means of classifying plasmids into related groups (Iordanescu *et al.*, 1978). The above results suggest that incompatibility between plasmids may play an important role in shaping the molecular evolution of a particular strain.

An alternative solution is that Australian MRSA strains have arisen from a different series of events which coincidentally provided a favourable genetic background for the acquisition of the NAB-resistance plasmids. The fact that the Australian strains also differ from other MRSA strains in having, for example, chromosomal Tc resistance and low-level, chromosomal Sm resistance, tends to support this hypothesis. However, there may have been other pressures, not yet elucidated, that resulted in these changes.

Regardless of whether the Australian MRSA strains have or have not evolved from the postulated 'single clone', a study of the properties conferred by this new family of IncI, NAB-resistance plasmids may lead to an understanding of why these strains have spread so successfully in Australian hospitals.

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