Studies on Recently Isolated Cultures of Methicillin-resistant *Staphylococcus aureus*

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Of 19 recently isolated cultures of methicillin-resistant *Staphylococcus aureus*, 18 showed inducible low-level resistance to minocycline, 15 showed high-level resistance to streptomycin, and 4 showed resistance to low levels of streptomycin. Two cultures produced yellow pigment and may have been derived *in vivo* by loss of a gene(s) determining orange pigment.

Treatment of three cultures with serial exposures to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine resulted in a widening of phage typing pattern that included all reactions in group I, the great majority in group III, but none in group II. The widening in phage lysis was possibly due to the elimination of defective prophages. Transfer of tetracycline resistance occurred from 12 out of the 19 cultures to a recipient in mixed culture; this transfer required either Ca^{2+} or Mg^{2+} , was abolished by citrate, and enhanced by high cell density. It was probably mediated by defective bacteriophages.

No evidence was obtained for the occurrence of recombination within the methicillin-resistant clone in nature. Eleven methicillin-resistant cultures stored for at least 5 years on agar slopes at 20 °C had all lost this resistance at high frequency.

INTRODUCTION

During the last 25 years, epidemics of sepsis in hospitals have been caused by strains of *Staphylococcus aureus* varying in many properties, notably antibiotic sensitivity, bacteriophage typing pattern, production of pigment, carriage of prophages and extracellular products.

Epidemic strains within a particular phage group may harbour new prophages; the effect of this carriage is to narrow, or occasionally to widen, the range of lysis by typing phages. There is considerable evidence for the occurrence of lysogenization in nature within phage group I (Asheshov & Rippon, 1959; Rountree, 1959; Rountree & Asheshov, 1961; Asheshov & Winkler, 1966) and phage group III (Jevons & Parker, 1964; Jevons *et al.*, 1966; Rosendal & Bülow, 1971; Parker *et al.*, 1974).

Phage group II strains are distinct both epidemiologically and genetically from the other major phage groups; this is illustrated in the possession by group II strains of restriction endonucleases that give them immunity to the typing phages of groups I and III. These enzymes can be inactivated by heating; hence the spectrum of lysis of phage group II staphylococci by typing phages increases after heating (Asheshov & Jevons, 1963; Ralston & Baer, 1964; Stobberingh & Winkler, 1976). A further type of phage 'immunity' is the failure of typing phage 187 to adsorb to the great majority of staphylococcal strains (Odeing, 1974). Thus, although there is good evidence for the occurrence of a number of recent evolutionary events within phage groups I or III in nature, it is not clear whether there is close affinity between these groups. 'Methicillin-resistant' staphylococci, whilst frequently giving

reactions in group III, may also be lysed by phages 29 or 79 in group I (Parker *et al.*, 1974). The study of these cultures should give some insight into the relationships between phage groups I and III; such study of methicillin-resistant cultures is facilitated by their possession of some stable properties that permit identification of derivatives independently from phage typing reactions. This paper describes these experiments.

METHODS

Methicillin-resistant staphylococci. Strains M1 to M19 were isolated from laboratories in the U.K. between 1976 and 1978 (Table 1). Thrice-purified clones were stored without subculture on nutrient agar slopes at 20 °C. Before use, each slope was subcultured to a 'master' plate stored at 4 °C before further subculture. In addition, 11 methicillin-resistant cultures described previously (13136, 13137, 9254, 2273, 9463, 11164, B109, 8657, FAR1, FAR2, B262; Lacey & Grinsted, 1973) were used.

Strains 6936 and 609 were also used (Lacey, 1971). A rifampicin-resistant (minimum inhibitory concentration > 100 µg ml⁻¹) mutant of strain 6936 was used as recipient in transfer experiments.

Media. Nutrient broth was Oxoid no. 2 and nutrient agar was mast blood agar base (DM100). Pigmentation was recorded after incubation of individual colonies on milk agar (Lacey, 1971).

Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine). Static overnight cultures containing about 5 × 10⁸ viable cocci ml⁻¹ were added to a freshly prepared solution of nitrosoguanidine (from Sigma) in distilled water at a final concentration of 100 µg ml⁻¹. The mixture was held at 37 °C statically for between 10 and 60 min, then diluted in physiological saline and spread over the surface of milk agar plates. After incubation for 24 h at 37 °C, followed by 24 h incubation at room temperature, colonies were subcultured from plates containing about 1% surviving cocci. Such colonies appeared pleomorphic in pigment and size, many minute colonies being evident. Colonies comparable in size to those of the wild strain were selected at random and subcultured to milk agar. They were then tested for antibiotic sensitivity, including the possession of heterogeneous resistance to methicillin and high-level resistance to streptomycin. They were subsequently phage typed and then some were re-checked for antibiotic sensitivity. Some were stored on agar slopes and checked for stability of phage reactions on two subsequent occasions.

Mitomycin C and ultraviolet induction. These were attempted by the method of Lacey (1971). Mitomycin C (from Sigma) was added at concentrations of 0.1, 0.5, 2.5, 5.0 and 20.0 µg ml⁻¹. A culture (strain 609) known to undergo induction with either mitomycin C or ultraviolet light was included in each set of experiments. Cultures were incubated for 4 to 6 h at 37 °C following exposure to mitomycin C or ultraviolet irradiation and then examined for lysis. If no lytic activity was observed, cultures were held at room temperature for up to 48 h and examined for subsequent lysis.

Transduction and phage propagation. The method of Lacey (1971) was used. Possible transductants were selected on media containing either 0.1 mM-CdCl₂, 2.5 µg tetracycline ml⁻¹ or 5 µg erythromycin ml⁻¹. For the latter, 0.5 µg erythromycin ml⁻¹ was added with the sodium citrate in order to ensure induction of erythromycin resistance. Irradiation with ultraviolet light was as previously described (Lacey, 1971).

Transfer of antibiotic resistance between staphylococci in mixed cultures. This was done by the method of Lacey (1971) except that the counterselecting agent was 10 µg rifampicin ml⁻¹ and either 0.1 mM-CdCl₂, 2.5 µg erythromycin ml⁻¹, 2.5 µg tetracycline ml⁻¹, 25 µg methicillin ml⁻¹, 10 µg streptomycin ml⁻¹, 2.5 µg neomycin ml⁻¹ or 2.5 µg gentamicin ml⁻¹ incorporated into nutrient agar.

Attempt at transfer of pigment between strains. Potential donor and recipient cultures were inoculated into nutrient broth containing 10 mM-CaCl₂ or human blood to a final density of 10⁶ colony-forming units (c.f.u.) ml⁻¹ of each. After 24 h incubation at 37 °C with agitation, the mixtures were diluted and applied to the surface of milk agar plates containing an agent to suppress the growth of the donor. These were incubated for 2 d at 37 °C. It was possible to detect an altered pigment type from an inoculum of about 10⁸ c.f.u. per plate.

Loss of methicillin resistance from stored cultures. Agar slopes of 11 strains previously described (Lacey & Grinsted, 1973) had been stored without subculture in the dark at room temperature for between 5 and 6 years. Nutrient broth (0.1 ml) was added to each slope and then pipetted into 10 ml nutrient broth and incubated statically overnight at 37 °C. Master plates containing 5 µg tetracycline ml⁻¹ were prepared from dilutions of these cultures and subsequently replica-plated to nutrient agar plates containing 10 or 25 µg methicillin ml⁻¹. After 20 h incubation at 30 °C, six of the resultant methicillin-sensitive variants from each strain were subcultured and examined for general properties including phage typing. (The use of tetracycline in the master plates was a precaution against possible contamination occurring during prolonged storage.)

Bacteriophage typing. This was performed by a conventional procedure at routine test dilution with typing phages 29, 52, 52A, 79, 80, 3A, 3C, 55, 71, 95, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85, 81, 94, 96 and 88.

Table 1. *Source of methicillin-resistant staphylococci*

Strain no.	Source	Date	Phage pattern*
M1	Wisbech	1978	77
M2	Fife	1976	77
M3	Fife	1976	77
M4	Fife	1976	79/77
M5	Fife	1976	77
M6	King's Lynn	1976	79/77
M7	King's Lynn	1976	77
M8	King's Lynn	1978	79/77
M9	Mansfield	1977	77
M10	Mansfield	1977	77
M11	Mansfield	1977	77/84
M12	Fife	1976	79/77
M13	Cambridge	1977	77
M14	Cambridge	1977	77
M15	Cambridge	1977	NT†
M16	Cambridge	1977	88
M17	Cambridge	1977	88
M18	Cambridge	1977	88
M19	King's Lynn	1978	79/77

* At routine test dilutions.

† NT, Non-typable.

The medium (defined phage agar) contained Oxoid nutrient broth no. 2 (20 g l⁻¹), NaCl (5 g l⁻¹) and Lab M agar (7.5 g l⁻¹). After autoclaving, CaCl₂·2H₂O (1.47 g l⁻¹) was added. Lytic reactions refer to major reactions (+ +, > 50 plaques). Cross-spotting for the detection of phage particles in culture filtrates was performed by applying filtered (0.45 µm pore size, Millipore) centrifuged cultures grown at 37 °C in nutrient broth to phage agar plates seeded with a prospective indicator culture as for phage typing.

Determination of antibiotic sensitivity. Preliminary disc results were confirmed by the estimation of minimal inhibitory concentrations (m.i.c.). Cultures examined for methicillin resistance were subcultured on antibiotic-free agar and inoculated into nutrient broth that was incubated overnight at 37 °C. Then undiluted cultures and cultures diluted in nutrient broth to 10⁻², 10⁻⁴ and 10⁻⁶ were applied to the surface of plates (giving 10¹ to 10⁷ c.f.u. per inoculum) containing a range of concentrations of methicillin or cloxacillin. These plates were incubated at 30 °C or 37 °C for 48 h. Cultures described here as methicillin-resistant exhibited the following characteristics: (i) a greater intrinsic resistance to methicillin than cloxacillin at 30 °C and 37 °C with each inoculum; (ii) a greater resistance to both antibiotics at 30 °C than at 37 °C for a given inoculum and time of incubation; (iii) a very marked inoculum effect for either antibiotic at 37 °C with very few cocci (about 10⁻⁵) growing on 100 µg methicillin ml⁻¹ after 24 or 48 h, but little such effect at 30 °C; (iv) any colonies surviving high levels of antibiotic at 37 °C being slow-growing and pleomorphic.

Inducible antibiotic resistance. To test for inducibility of resistance to erythromycin, minocycline, streptomycin and gentamicin, cultures were incubated in the presence of 0.5 µg ml⁻¹ of each antibiotic for 18 h, before determinations of m.i.c. Dilutions closer than doubling were used to test for inducibility.

RESULTS AND DISCUSSION

General properties of recent methicillin-resistant isolates

All 19 isolates showed characteristic heterogeneous resistance to methicillin; they were also resistant to tetracycline and to a variety of other antibiotics (Table 2). Although 15 isolates showed the non-inducible high-level resistance to streptomycin (m.i.c. > 10 mg ml⁻¹) that is characteristic of these cultures (Lacey & Grinsted, 1973), four isolates (M15, M16, M17 and M18) showed non-inducible low-level resistance to streptomycin (m.i.c. 40 µg ml⁻¹). All except one (M12) showed inducible low-level resistance to minocycline with m.i.c. values varying from 3 to 10 µg ml⁻¹ when uninduced to 5 to 25 µg ml⁻¹ after preincubation with 0.5 µg minocycline ml⁻¹. This resistance appears identical to that described by Asheshov (1975). The 11 cultures described previously (Lacey & Grinsted, 1973) were also examined for minocycline resistance; all except three showed low-level

Table 2. *General properties of methicillin-resistant staphylococci*

Strain no.	Strepto- mycin (m.i.c., mg ml ⁻¹)	Tetra- cycline (m.i.c., µg ml ⁻¹)	Penicil- linase production	Cd ²⁺ *	Erythro- mycin	Lincomycin	Mino- cycline	Neomycin	Gentamicin
M1	100	150	+	R	R(I)	S	R	S	S
M2	100	75	+	R	R(I)	S	R	S	S
M3	100	75	+	R	R(I)	S	R	S	S
M4	100	75	+	R	R(I)	S	R	S	S
M5	100	20	+	R	R(I)	S	R	R	S
M6	100	150	-	S	R(I)	S	R	S	S
M7	100	150	+	R	R(I)	S	R	R	S
M8	100	150	+	R	R(I)	S	R	S	S
M9	100	150	-	S	R(I)	S	R	S	S
M10	100	150	-	S	R(I)	S	R	S	S
M11	100	40	+	R	S	S	R	S	R
M12	100	150	+	R	R(C)	R(C)	S	S	S
M13	100	150	+	R	R(I)	S	R	R	R
M14	100	150	+	R	R(I)	S	R	R	R
M15	0.04	150	+	R	R(I)	S	R	R	R
M16	0.04	150	+	R	R(C)	R(C)	R	R	R
M17	0.04	150	+	R	R(C)	R(C)	R	S	S
M18	0.04	150	+	R	R(C)	R(C)	R	S	S
M19	100	150	+	R	R(I)	S	R	S	S

R, Resistance; R(I), inducible resistance; R(C), constitutive resistance; S, sensitivity.

* Resistance to Cd²⁺ denotes growth on 0.1 mM-CdCl₂.

Table 3. *Widening of phage typing pattern (routine test dilution) following four successive treatments of M2 and M17 with nitrosoguanidine*

Wild strain (typing pattern)	M2 (77)	M17 (88)
No. of derivatives examined	483	370
Phage patterns of mutants	54/77	88/80/83A/85
	79/77	Non-typable
	29/54/77	88/29/52/52A/80/95/42E/83A
	29/79/54/77	88/80
	29/79/54/75/77/85	88/29/52/52A/80/95/83A
	47/54/77	88/52/52A/80
	29/79/6/42E/47/54/75/77/85/81	88/83A
	47/54/77/81	88/52/80
	29/79/47/54/77	88/52/52A/80/95
	79/54/77	88/29/80
	29/42E/47/54	88/52/52A/80/83A
	79/47/54	88/80/54
	47/54	80
	47/54/77/85	88/85
	29/47/54	88/52A/80/83A
	29/77	
	54	
	29/79/77	
	29/79/42E/47/54/75/77/85	
	47/54/75/77/85/81	
	Non-typable	

inducible resistance to minocycline. Thus, from a total of 30 methicillin-resistant cultures isolated between 1960 and 1978, 26 showed inducible low-level resistance to minocycline.

Seventeen cultures produced orange pigment on milk agar and two (M9 and M10) produced yellow pigment.

Alterations in bacteriophage typing patterns of three methicillin-resistant cultures following nitrosoguanidine treatment

Strains M2, M14 and M17 are typical recent methicillin-resistant isolates (see Tables 1 and 2). Phage typing of these isolates on different occasions showed that their typing reactions were stable. On one occasion, 50 different colonies from four independent platings of strain M17 were typed and all gave a major reaction to phage 88 and no other reaction. It was inferred, therefore, that there was little spontaneous variation in these patterns, so that the variation following treatment with nitrosoguanidine was a result of such treatment. Derivatives obtained after successive treatments of strain M2 are shown in Table 3. The following changes were seen: (i) a widening of reactions in groups I and III, the only reactions not detected being to phages 83A and 84, but no reactions to group II phages; (ii) loss of some reactions, so that some clones gave reactions exclusive to group I or III or were non-typable. Resistance to methicillin, streptomycin and tetracycline was retained by all the derivatives. Several of the clones having patterns widely different from the wild strain were typed in replicate on subsequent occasions and showed little alteration in typing pattern. Further treatment of derivatives of strain M2 with nitrosoguanidine gave similar patterns, but after five successive treatments, the resultant organisms grew poorly, most being non-typable.

Mutagenesis of strain M17 also increased its phage susceptibility, with nine new major lytic reactions (Table 3). Mutagenesis of strain M14 (typing pattern 77) gave widening reactions in group III, so that clones typing 47/54/77/81 and 6/42E/47/54/75/77/81 were found but no group I reactions were seen (results not shown).

Fig. 1. Pathways of alteration of pigment in *Staphylococcus aureus* following treatment with nitrosoguanidine.

Mutagenesis of pigmentation in M2, M9, M10 and M17

Two cultures producing orange pigment (M2 and M17) were treated with nitrosoguanidine and yielded about 2% colonies that gave yellow pigment and 4% that were non-pigmented (white). Further treatment of these yellow-pigmented or white-pigmented cultures with nitrosoguanidine did not result in any orange colonies from about 10000 colonies screened. The yellow variants, however, yielded white colonies at the same frequency as above, but treatment of white colonies produced no further variants.

Treatment of the two yellow-pigmented cultures (M9 and M10) gave no orange colonies, and about 5% were non-pigmented. Further mutagenesis of the latter with nitrosoguanidine did not reverse this.

During these experiments, it was noted that variants were common (about 20% of total colonies) with pigmentation either more or less intense than the wild strains. Subculture of the more intensely coloured colonies caused this additional pigmentation to be lost, but the pigment in the less intensely pigmented colonies appeared stable. These events are summarized in Fig. 1.

Since, in general, most strains of *Staphylococcus aureus*, including methicillin-resistant cultures, produce orange pigment rather than either yellow or white, and the change from orange to yellow appears irreversible, it is likely that the capacity to produce orange pigment gives the cell a selective advantage over other pigment phenotypes. Indeed, in mixed cultures comprising one each of all three pigment types derived from M2, clones producing orange pigment overgrew the other types both in nutrient broth and in blood, and also survived better on glass (results not shown). However, because it has not been possible to construct strains isogenic apart from variation in pigment production, it is not possible to ascribe such survival differences to pigmentation *per se*. As before (Grinsted & Lacey, 1973), in mixed culture experiments, we were unable to transfer the capacity to synthesize orange or yellow pigment to non-pigmented cultures.

Thus, the yellow-pigmented cultures M9 and M10 have features characteristic of other methicillin-resistant staphylococci and are probably derived from the predominant orange cultures. Yellow-pigmented methicillin-sensitive strains (e.g. Willis *et al.*, 1966) might be derived similarly.

Table 4. *Incidence of methicillin-sensitive bacteria in methicillin-resistant staphylococci stored for 5 to 6 years on agar slopes*

Strain no.	No. of colonies tested	No. (%) methicillin-sensitive
13136	286	6 (2)
13137	495	40 (8)
9254	846	64 (7)
2273	221	13 (6)
9463	68	7 (10)
11164	168	22 (13)
B109	343	24 (7)
8657	506	10 (2)
FAR1	488	215 (43)
FAR2	191	7 (4)
B262	168	8 (5)

Loss of methicillin resistance from stored cultures

Although there is still uncertainty as to the precise cellular location of the genes determining methicillin resistance, the genetic features of this resistance are fairly uniform, although there have been conflicting reports over the stability of the resistance. Annear & Grubb (1976) have reported reliable loss of the resistance in ageing broth cultures, although others consider the resistance to be stable.

Accordingly, 11 methicillin-resistant cultures (see Lacey & Grinsted, 1973) stored for between 5 and 6 years on agar slopes without subculture were examined for the loss of the resistance by replica-plating. A recently isolated strain (M17) was also studied and from about 5000 colonies examined none sensitive to methicillin was detected. However, each of the methicillin-resistant cultures stored for at least 5 years yielded methicillin-sensitive derivatives (Table 4).

Six methicillin-sensitive variants (together with resistant cells) of each culture were examined for general sensitivity to antibiotics and were phage typed. No major changes in typing patterns were seen, and the only associated loss of resistance was that in three cultures a minority of the methicillin-sensitive cells were penicillinase-negative and sensitive to Cd^{2+} – presumably due to the coincidental loss of the penicillinase plasmid. Thus, these findings support those of Annear & Grubb (1976), that instability of methicillin resistance on storage seems to be a general property of these strains.

Evidence for recombination/transposons in methicillin-resistant cultures

The properties of these methicillin-resistant cultures are in many ways similar to those previously described (Lacey, 1972; Lacey & Grinsted, 1973). The cultures isolated recently are probably derived from these earlier strains. Thus, any changes detected in the recently isolated strains may be interpreted as being due to natural events within this cell line.

Transductants were obtained from the lysates of strains M9 and M11 after mitomycin C induction, and from strain M2 after propagation of typing phage 80 on a mutant clone lysed by this phage. From each of these lysates, transductants were obtained which were resistant to either tetracycline or Cd^{2+} (the latter were also penicillinase producers). Replica-plating of these colonies on to media containing single antibiotics to which these strains were also resistant (Table 2) showed that there was no cotransduction, i.e. the genomes (presumably plasmids in view of the frequencies obtained) determining penicillinase production and tetracycline resistance are still discrete, as was the case in the 'prototype' methicillin-resistant isolate 13136 (Lacey & Grinsted, 1973). Erythromycin was transduced at high frequency (2.5×10^{-6}) from the lysate of M2 by modified phage 80 to the recipient strain 6936. Ultraviolet treatment of the lysate showed an exponential decline in frequency,

consistent with a plasmid locus for this resistance. There was no cotransduction of the other resistances, an unexpected finding since plasmid-mediated erythromycin resistance is usually linked to other resistances (see Lacey, 1975).

Each of the recipient colonies that acquired resistance to the antibiotics after mixed culture incubation (see below, Table 5) was replica-plated to media containing other relevant antibiotics added singly. Apart from one colony resistant to both minocycline and tetracycline (presumably there had been coincidental rather than linked transfer), the only instance of linked transfer was the finding in strain M7 of a transmissible element determining resistance to neomycin, streptomycin and Cd^{2+} and also production of penicillinase. Approximately 90% of recipient cocci harbouring the transferred element showed the complete set of resistance traits. The level of resistance to streptomycin in the recipient harbouring these genes was low (m.i.c. $100 \mu\text{g ml}^{-1}$). This suggests that strain M7 is diploid for streptomycin resistance, one gene determining high-level resistance (m.i.c. 100 mg ml^{-1} , Table 2), presumably chromosomal, and the other low-level resistance, presumably part of a polyfunctional plasmid.

Explanation for the widening of typing pattern after mutagenesis

The widening of the phage typing reaction following mutagenesis is likely to be due to the loss of one of the general mechanisms that protect the staphylococcal cell from bacteriophage lysis, i.e. (a) lysogenic immunity, (b) failure of phage adsorption and (c) restriction endonucleases. Each of these possibilities was investigated.

If the widening of phage typing was due to the loss of an entire temperate phage, then filtrates from the wild strains M2, M14 and M17 should contain particles lytic for the derivatives. Using a cross-spotting technique, filtrates from the wild strains were tested against 35 derivatives with a markedly widened phage typing reaction. No plaques were seen. Filtrates of cultures M2, M14 and M17 were also examined for the presence of plaque-forming activity on each of the propagating strains of the typing phages (see Methods) and on strains 609, 6936 and 1030 (strain 1030 is non-lysogenic and lysed by all the typing phages). No plaques were seen. These results suggest that M2, M14 and M17 do not harbour temperate phages, and that the widening in phage susceptibility is not associated with entire prophage loss.

If the cultures were 'normally' lysogenic, then they might be inducible with mitomycin C or ultraviolet light. Accordingly, each of the cultures M1 to M19 was treated with mitomycin C at concentrations from 0.1 to $20 \mu\text{g ml}^{-1}$ on three occasions; only two (M9 and M11) cleared. Cultures M2, M14 and M17 were also treated with varying doses of ultraviolet light. As with mitomycin C treatment, a known lysogenic culture (strain 609) lysed, whereas none of these cultures did so. Filtrates of these cultures treated with either ultraviolet light or mitomycin C did not produce any plaques on the above cultures.

The second possibility (b), that the wild strains could not adsorb typing phage particles whereas the mutants could, is unlikely because, phage 187 apart, all staphylococcal cultures adsorb typing phages whether or not they are subsequently lysed (Oeding, 1974). However, each wild strain and mutant which was lysed by typing phages 80, 81 or 83A was incubated for 30 min at 37°C with each of these phages, and then filtered. Both wild strains and the corresponding mutants adsorbed each phage similarly. Thus, widening of phage lysis is not explicable by enhanced phage adsorption.

The third possibility (c), that treatment with nitrosoguanidine eliminated some of the genes determining restriction endonucleases, was examined as follows. If the wild strain did possess such enzymes, then it might be possible to inactivate them by heating the culture before application of phages (Stobberingh & Winkler, 1976). Small volumes (2.0 ml) were therefore incubated in a 56°C water bath for 0, 1.5, 2 and 2.5 min before phage typing in the usual way, except that after application of phage, a duplicate set of plates was incubated at 37°C . No widening of bacteriophage typing pattern was seen. Thus, no evidence for the

Table 5. *Transfer of resistances from strains M1 to M19 to recipient culture 6936*

Results are expressed as the number of resistant recipients ml⁻¹ after incubation of the mixed culture for 24 h.

Donor strain	Cd ²⁺ resistance and			
	Tetracycline resistance	penicillinase production	Erythromycin resistance	Neomycin resistance
M1	2.7 × 10 ⁸	5.0 × 10 ⁰	ND	—
M2	4.1 × 10 ⁸	1.1 × 10 ⁸	1.0 × 10 ¹	—
M3	9.0 × 10 ⁸	ND	ND	—
M4	5.0 × 10 ⁴	5.0 × 10 ⁰	ND	—
M5	ND	ND	ND	5.0 × 10 ⁰
M6	1.4 × 10 ⁸	—	ND	—
M7	6.5 × 10 ⁸	3.5 × 10 ¹	ND	3.5 × 10 ¹
M8	2.6 × 10 ⁸	ND	ND	—
M9	1.05 × 10 ⁸	—	ND	—
M10	1.1 × 10 ⁸	—	ND	—
M11	ND	ND	—	—
M12	ND	ND	ND	—
M13	2.4 × 10 ⁸	8.9 × 10 ⁸	5.0 × 10 ⁰	ND
M14	2.3 × 10 ⁴	2.4 × 10 ⁴	ND	ND
M15	ND	ND	ND	ND
M16	ND	ND	ND	—
M17	ND	ND	ND	—
M18	ND	ND	ND	—
M19	5.0 × 10 ⁸	ND	ND	—

ND, No transfer detected; —, organism sensitive to antibiotic.

presence of restriction endonucleases in the wild strains was obtained. Such enzymes seem to be typical of phage group II rather than other groups (Stobberingh & Winkler, 1976). However, the presence of endonucleases indifferent to the effect of heat has not been excluded; such enzymes might be phage-associated.

Examination for defective prophage carriage in cultures M2, M14 and M17

Since there is good evidence for epidemic spread of prophages in staphylococci (see Introduction) and that some previously isolated methicillin-resistant organisms harbour such phages (Lacey, 1972), and since cultures M9 and M11 each contain an inducible prophage (see above), it is possible that cultures M2, M14 and M17 contain remnants of previously acquired temperate prophages.

Such defective prophages might transfer determinants from these cultures to a recipient in mixed culture experiments, since the transfer vehicle in mixed culture mating is probably a defective prophage (Lacey, 1975). Accordingly, transfer of antibiotic resistance from each of the cultures M1 to M19 to 6936 was attempted. Tetracycline resistance was transferred to the recipient from 12 out of the 19 donors, Cd²⁺ resistance and penicillinase production from 6 out of 16, erythromycin resistance from 2 out of 18, and neomycin resistance from 2 out of 5 donors. No transfer at all was detected from 6 strains (Table 5). Resistance to streptomycin (from 19), to methicillin (from 19) and to gentamicin (from 5) was non-transferable. Not only was the highest incidence that of transfer of tetracycline resistance amongst the potential donors, but the frequency obtained was also higher than the other resistances. These findings are characteristic of transferable resistance in *Staph. aureus* (Lacey, 1975) and are consistent with the proposal that the transfer vector is a defective bacteriophage.

Further evidence for this was obtained by showing that environmental conditions favourable for transfer from M2 and M4 were also optimal for the transfer of resistance from strain 609, a known lysogenic donor (Lacey, 1971). Thus, the presence of Ca²⁺ or Mg²⁺ promoted transfer and citrate abolished it; high cell density was associated with a

Table 6. *Effects of ions on the transfer of tetracycline resistance from strains M2, M14 and 609 (control) to recipient culture 6936*

Results are expressed as the number of resistant recipients ml⁻¹ after incubation of the mixed culture for 24 h.

Addition to nutrient broth	Donor strain		
	M2	M14	609
None	0	0	0
1 mM-CaCl ₂	2.1×10^3	6.8×10^3	7.4×10^3
2.5 mM-CaCl ₂	1.1×10^3	2.8×10^3	2.9×10^3
10 mM-CaCl ₂	2.7×10^3	5.0×10^4	3.0×10^4
100 mM-CaCl ₂	7.5×10^3	6.2×10^3	1.2×10^3
10 mM-CaCl ₂ + 20 mM-sodium citrate	0	0	0
10 mM-MgCl ₂	5.5×10^3	1.5×10^3	4.8×10^3

Table 7. *Association of cell density with the frequency of transfer of tetracycline resistance from strains M2, M14 and 609 (control) to recipient culture 6936*

Mixed cultures in 3, 10 or 25 ml nutrient broth containing 10 mM-CaCl₂ in 25 ml Universal bottles were incubated with aeration for 24 h. The frequency of transfer of tetracycline resistance is expressed as the number of resistant recipients per inoculated donor cell. Final cell densities are indicated in parentheses.

Culture volume (ml)	Donor strain		
	M2	M14	609
3	4.0×10^{-6} (5.4×10^9)	8.0×10^{-5} (3.3×10^9)	7.1×10^{-5} (2.5×10^9)
10	2.1×10^{-7} (1.0×10^9)	4.5×10^{-5} (5.5×10^8)	1.9×10^{-5} (1.5×10^9)
25	1.3×10^{-7} (3.0×10^8)	2.0×10^{-6} (1.8×10^8)	3.4×10^{-7} (1.6×10^8)

high frequency of transfer (Tables 6 and 7). (It is not possible to ascertain whether it is the high cell density, oxygenation or other factors that are responsible for high transfer frequencies.)

Although cultures M2, M14 and M17 were not visibly inducible with ultraviolet light, this treatment could cause the release of non-lytic prophages if they were present. To test for this, cultures of M2, M14 and M17 that had been exposed to ultraviolet light for optimal induction (as shown by the lysis of strain 609) were filtered, and the filtrates were checked for sterility and then tested for their ability to transduce tetracycline resistance to strain 6936 in conventional transduction experiments. Tetracycline resistance was transduced (3.3×10^3 per ml filtrate) from the filtrate of culture M14, a finding consistent with the presence of defective phage particles in that culture. Transduction was not successful with cultures M2 or M17.

Filtrates of the methicillin-resistant cultures other than M2, M14 and M17 were examined for the presence of plaque-forming activity on each of the propagating strains and on cultures 1030, 609 and 6936. Eight of the 16 cultures yielded no plaques on any of the indicator strains from overnight cultures and also from cultures treated with either mitomycin C or ultraviolet light. It is possible that the failure to detect lysis is due to the selection of inappropriate indicator cultures. However, filtrates from all the 11 methicillin-resistant cultures examined previously (Lacey & Grinsted, 1973) produced lysis on at least one of these indicator strains. It is likely, therefore, that cultures M2, M14 and M17 contain defective prophages; it is also possible that the widening in typing pattern following nitrosoguanidine treatment is due to the elimination of such elements.

In a recent survey, Rosendal *et al.* (1977) described methicillin resistance in staphylococci in phage groups other than in group III, and including group I. The findings here suggest a close relationship between phage groups I and III, and it is possible that phage group III methicillin-resistant (and perhaps also sensitive) staphylococci have evolved from phage group I. In favour of this suggestion is the natural history of the hospital staphylococcus over the last 25 years where epidemics of the group I complex have been generally replaced by group III strains. The evolutionary sequence from phage group I to III may have occurred through a series of lysogenic events, with subsequent loss of the capacity of the temperate phages to cause lysis. Two such phages in *Staph. aureus* have recently been characterized fully (Duval-Iflah *et al.*, 1977). Further work is required to fully characterize these proposed defective phages.

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