Transduction of Penicillinase Production and Methicillin Resistance-Enterotoxin B Production in Strains of *Staphylococcus aureus*

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SUMMARY

It is suggested that wild strain DU4916 of *Staphylococcus aureus* harbours at least two plasmids. One of them is responsible for penicillinase production and resistance to metallic ions and the other for methicillin and cephalothin resistance together with enterotoxin B production. These properties were eliminated *en bloc* with acridines as well as separately at different rates. Transductions were performed to restore the eliminated characters with typing phages 29 and 88. Recombination of the markers for penicillinase production and methicillin resistance on the transduced fragment seems advantageous for selection of methicillin resistance.

INTRODUCTION

Some hereditary characters in staphylococci are lost spontaneously at a relatively high frequency which may, however, be increased by growth in the presence of acridine dyes (Mitsuhashi, Morimura, Kono & Oshima, 1963; Hashimoto, Kono & Mitsuhashi, 1964), ethidium bromide (Bouanchaud, Scarizzi & Chabbert, 1969) or at high temperatures (May, Houghton & Perret, 1964). The elimination of genetic determinants at high frequency is often taken to imply their extrachromosomal nature.

A number of different types of plasmids have been identified in staphylococci by genetical analysis. They include plasmids conferring resistance to antibiotics (Fairbrother, Parker & Eaton, 1954; Novick, 1963; Chabbert, Baudens & Gerbaud, 1964; May *et al.* 1964; Dornbusch, Hallander & Löfquist, 1969; Novick, 1969), heavy metal ions (Richmond & John, 1964; Novick 1967), toxin and pigment production (Serwin-Massieu, 1961; Dornbusch, Hallander & Löfquist, 1969). Some strains carry more than one plasmid and strains carrying two different penicillinase plasmids have been constructed artificially (Richmond, 1968) as have strains carrying both chromosomal and extrachromosomal genes for penicillinase production (Asheshov, 1969).

In a previous report a number of strains of *Staphylococcus aureus* were described in which the genes controlling methicillin resistance and production of enterotoxin B appeared to be associated. Both characters were lost after treatment with acriflavine and later restored by transduction (Dornbusch *et al.* 1969) and it was suggested that their determinants were located on a plasmid. One of these strains was studied further to establish this plasmid location. Transduction of methicillin resistance has recently been described by Cohen & Sweeney (1970); its genetic determinant is located on the chromosome.

METHODS

Strains of Staphylococcus aureus (Table 1). Three mutants of a methicillin-resistant penicillinase-producing strain, DU4916, isolated from an infected burn wound at the University Hospital, Uppsala, Sweden (Dornbusch *et al.* 1969) were isolated after acridine treatment. Two other strains, DU3108 and DU3287, isolated locally (Dornbusch *et al.* 1969), were used as recipients in transduction experiments, as was a strain RB1 (α *pen*⁺ *Mut met-s*), kindly supplied by Professor Mark Richmond, Bristol. This methicillin-sensitive (*met-s*) strain harbours a penicillinase plasmid of α type (α *pen*⁺ *Mut*) (Richmond, 1968) which produces much less of the enzyme (5%) than wild-type.

Sensitivity tests. The disc diffusion test (Ericsson, 1960) was used for all antibiotics. Resistance to benzylpenicillin, methicillin, cephalothin and erythromycin was also determined by a plate-dilution test (Barber & Waterworth, 1964). Resistance to cadmium-sulphate and mercuric acetate was determined by plate dilution (Moore, 1960). The minimum inhibitory concentration (m.i.c.) is the lowest concentration which completely inhibits growth.

Assay of toxins. Qualitative and quantitative assays for the production of α -, β -, and δ -haemolysins, lipase, free coagulase and enterotoxin B were determined as described by Hallander (1965). DNase production was determined by growing bacteria on DNA-agar plates (Di Salvo, 1958). After 24 h at 37 °C, undigested DNA was developed by flooding the plates with 1 N-sulphuric acid.

Treatment with acridines. This was performed as described by Dornbusch *et al.* (1969).

Detection of penicillinase production. The starch agar method of Dyke, Jevons & Parker (1966) was used.

Phage typing. This was carried out by the method of Blair & Williams (1961) using the standard set of typing phages and the phage 88.

Propagation of phage for transduction. Typing phages 29 and 88 were propagated on donor strains by the soft-agar overlay method of Swanstrom & Adams (1951). Lysates were sterilized by Millipore filtration (0.45 μ m pore size) and phages titre determined as plaque-forming units (p.f.u.)/ml; they ranged from 1.4×10^7 to 2.7×10^9 .

Transduction. The method of Asheshov (1966) was used. The plates in transducing the *met-r* marker were incubated at 30 or 37 °C (Annear, 1968). In transduction of penicillinase production or cadmium resistance, all plates were incubated at 37 °C. Controls, including sterility tests of the phage lysate and tests for back mutation in the absence of transducing phage, were performed with each experiment.

Media. (i) For selection of penicillin or Cd²⁺-resistant transductants: nutrient agar (Difco) with 0.1 or 10 μ g benzylpenicillin/ml; or with 5.0×10^{-5} M or 7.5×10^{-5} M-CdSO₄. (ii) For selection of methicillin-resistant transductants (salt agar): 0.8% (w/v) nutrient broth (Difco, Detroit, Michigan, U.S.A.); 1.1% (w/v) Davis Agar (Davis Gelatine Ltd, Warwick); 5% (w/v) sodium chloride; 25 μ g methicillin/ml. (iii) Nutrient broth (Difco) was used for bacterial broth cultures.

Lysogeny. A strain was considered to be lysogenic if it was immune to the lysogenizing phage and produced a phage that lysed its non-lysogenic parent. For induction of lysogenic strains the method of Kjems (1955) was used.

Efficiency of detection of methicillin resistance. This was to test whether the presence of methicillin-resistant bacteria in an otherwise sensitive population could inactivate the methicillin in the salt agar plates and was necessary to establish that all methicillin-resistant colonies resulted from transduction and were not satellite colonies. Sensitive bacteria

Table 1. Phenotypes of the four variants of strain DU4916, the wild-type strains DU3108 and DU3287 and the mutant RB1 of *Staphylococcus aureus*

Strain	Phage type	Minimum inhibitory concentration ($\mu\text{g/ml}$) for						Production of							<i>Transduction in S. aureus</i>
		Benzyl- penicillin	Methicillin	Cephalo- thin	Erythro- mycin	Hg^{2+}	Cd^{2+} ($\text{M} \times 10^{-5}$)	Entero- toxin B (titre)	Haemolysins (titres)			Coagu- lase	DN- ase	Lip- ase	
									α	β	δ				
DU 4916 <i>pen</i> ⁺ <i>met-r</i>	29/88; Group III inhibitions	> 200	> 400	> 100	< 2	> 20	> 14	40	64	32	128	+	+	—	
<i>pen</i> [—] <i>met-r</i>	29/88; Group III inhibitions	10	> 400	> 100	< 2	10	1.4	40	128	64	128	+	+	—	
<i>pen</i> ⁺ <i>met-s</i>	29/88; Group III inhibitions	> 200	< 12.5	10	> 200	10	7.5	< 1	128	4	< 4	+	+	—	
<i>pen</i> [—] <i>met-s</i>	29/42E/47/53/54/ 75/78	< 0.1	< 12.5	< 1	> 200	< 1	1.4	< 1	128	4	< 4	+	+	—	
DU 3108 <i>pen</i> [—] <i>met-s</i>	29	< 0.1	< 12.5	< 1	< 10	10	> 14	< 1	4	32	< 2	+	+	+	
DU 3287 <i>pen</i> [—] <i>met-s</i>	29/79/42E/77/ 42D	< 0.1	< 12.5	< 1	< 10	10	> 14	< 1	32	32	32	+	+	+	
RB1 α <i>pen</i> ⁺ <i>Mut</i> <i>met-s</i>	29/52/52A/79/85/ 55/47/53/88	50	< 12.5	< 1	< 2	> 20	< 1.4	< 1	1024	2	256	+	+	+	
<i>pen</i> ⁺ , producer of penicillinase; <i>pen</i> [—] , non-producer of penicillinase; <i>met-r</i> , methicillin-resistant; <i>met-s</i> , methicillin-sensitive; <i>Mut</i> , mutant; Hg^{2+} , mercuric ion; Cd^{2+} , cadmium ion.															

(strains DU4916 *pen*⁺ *met-s* and *pen*⁻ *met-s*) were mixed with 0.1% resistant bacteria (DU4916 *pen*⁺ *met-r*) in nutrient broth and incubated at 37 °C for 30 min. Suitable dilutions of the mixture were plated on salt agar with or without 25 µg methicillin/ml and incubated for 48 h at 30 °C. As controls the sensitive and resistant cultures were incubated and plated separately in the same way.

RESULTS

Loss of genetic characters after growth with acridine dyes. DU4916 is a methicillin-resistant strain of *Staphylococcus aureus* which is resistant to Hg²⁺ and Cd²⁺ and produces penicillinase, enterotoxin B, α-, β- and δ-haemolysins, coagulase and DNase but not lipase (Table 1, line 1). It will be referred to as DU4916 *pen*⁺ *met-r*. Methicillin-sensitive mutants could be isolated from this strain at a frequency of 2.6% after growth in acriflavine (Dornbusch *et al.* 1969). These mutants (*pen*⁺ *met-s*) lost simultaneously production of enterotoxin B and haemolytic activity for sheep and human erythrocytes (Table 1, line 3). They also differed from the parent strain in that they show cephalothin sensitivity and inducible erythromycin resistance. Following further treatment of DU4916 *pen*⁺ *met-r* with 5-amino-acridine.HCl 0.8% of the bacteria were *pen*⁻ *met-r* and 0.05% *pen*⁻ *met-s* respectively (Table 1, lines 2 and 4). Loss of penicillinase production in *pen*⁻ *met-r* was associated with reduced m.i.c. for cadmium and mercuric ions. This mutant remained resistant to low levels of penicillin in agreement with other comparable data (Richmond, Parker, Jevons & John, 1964; Dyke *et al.* 1966; Hewitt & Parker, 1968; Dyke, 1969) but still produced enterotoxin B and β-haemolysin. The *pen*⁻ *met-s* mutant showed simultaneous loss of resistance to cadmium and to mercuric ions, loss of ability to produce penicillinase, enterotoxin B or to haemolyse sheep and human red blood cells, gained resistance to erythromycin, and was greatly changed in its phage typing pattern. Revertants to methicillin resistance or penicillinase production occurred either spontaneously or after treatment with acridines at a frequency greater than 10⁻⁸.

The characteristics of the three mutants *pen*⁺ *met-s*, *pen*⁻ *met-r*, and *pen*⁻ *met-s* suggested that these markers were present in two separate genetic groups in the parent strain DU4916 *pen*⁺ *met-r*. One of these resembles the penicillinase plasmids described in other strains of staphylococci (Richmond, 1968) and appears to carry the genetic determinants for penicillinase production linked to those controlling resistance to cadmium and mercury ions. The other genetic group appears to carry the genes controlling methicillin resistance, production of enterotoxin B, β-haemolysin and a gene conferring at least partial resistance to mercuric ions. It also seems to carry some genetic factor which blocks expression of inducible erythromycin resistance (Weaver & Pattee, 1964).

Transduction experiments

To verify the suggestion that strain DU4916 *pen*⁺ *met-r* harbours at least two plasmids, transduction experiments were performed to restore the eliminated characters and to examine their genetic association. Typing phages 29 and 88 were used as transducing vectors. The frequency of reverse mutation in the absence of transducing phage was less than 10⁻¹⁰ in all controls.

Transduction of methicillin resistance with phage 29. Strain DU4916 *pen*⁺ *met-r* was used as donor and the mutants *pen*⁻ *met-s* and *pen*⁺ *met-s* as recipients with phage at multiplicities of infection of 0.5 to 1.0. Selection was made on salt agar medium containing 25 µg methicillin/ml. Methicillin-resistant transductants were obtained in both crosses (Table 2) and the frequencies of transduction were 7.0 × 10⁻⁷ and 5.0 × 10⁻⁷ respectively. About 100 transductants from each cross were examined. All were resistant to cephalothin, produced

Table 2. Transduction of methicillin resistance with phages 29 and 88

Donor strain of DU4916	Recipient strain of DU4916	Phage	Frequency of transduction	No. of transductants	Minimum inhibitory concentration of the transductants ($\mu\text{g/ml}$) for					Production by the transductants of		
					Methicillin	Cephalothin	Erythromycin	Hg ²⁺	Cd ²⁺ ($\text{M} \times 10^{-5}$)	Penicillinase	Enterotoxin B (titre)	Haemolytic activity on sheep erythrocytes (titre)
<i>pen</i> ⁺ <i>met-r</i>	<i>pen</i> ⁻ <i>met-s</i>	29	7.0×10^{-7}	338	> 200	> 100	< 2 (94 %) > 200 (6 %)	> 40	> 14	+	40	32 (60 %) < 4 (40 %)
	<i>pen</i> ⁺ <i>met-s</i>		5.0×10^{-7}	235	> 200	> 100	< 2	> 40	> 14	+	40	32 (30 %) > 4 (70 %)
<i>pen</i> ⁺ <i>met-r</i>	<i>pen</i> ⁻ <i>met-s</i>	88	1.0×10^{-8}	5	> 200	> 100	> 2 (60 %) > 200 (40 %)	> 40 (60 %) 10 (40 %)	> 14 (60 %) 1.4 (40 %)	+(60 %) -(40 %)	40 (60 %) < 1 (40 %)	8
	<i>pen</i> ⁺ <i>met-s</i>		1.0×10^{-8}	5	> 200	> 100	< 2	> 40	> 14	+	40	8

Abbreviations as in Table 1.

Table 3. Transduction of penicillinase production with phage 29

Donor strains of DU4916	Recipient	Frequency of transduction	Minimum inhibitory concentration of the transductants ($\mu\text{g/ml}$) for					Production by the transductants of	
			Methicillin	Cephalothin	Erythromycin	Hg ²⁺	Cd ²⁺ ($\text{M} \times 10^{-5}$)	Enterotoxin B (titre)	Haemolytic activity for sheep erythrocytes (titre)
<i>pen</i> ⁺ <i>met-r</i>	DU4916 <i>pen</i> ⁻ <i>met-s</i>	3.3×10^{-5}	< 12.5	< 1	> 200	> 20	> 14	< 1	< 4
	DU4916 <i>pen</i> ⁻ <i>met-r</i>	1.4×10^{-5}				> 20	> 14		
	DU3108 <i>pen</i> ⁻ <i>met-s</i>	7.4×10^{-7}	> 400 (8/92) < 12.5 (84/92)	> 100 (8/92) < 1 (84/92)	< 10	> 20		40 (8/92) < 1 (84/92)	
	DU3287 <i>pen</i> ⁻ <i>met-s</i>	6.3×10^{-6}	> 400 (7/87) < 12.5 (80/87)	> 400 (7/87) < 12.5 (80/87)	< 10	> 20		40 (7/87) < 1 (80/87)	
<i>pen</i> ⁺ <i>met-s</i>	DU4916 <i>pen</i> ⁻ <i>met-s</i>	4.0×10^{-7}			> 200	10	> 5-14 <		
	DU4916 <i>pen</i> ⁻ <i>met-r</i>	8.0×10^{-6}			< 10	10	> 5-14 <		
	DU3108 <i>pen</i> ⁻ <i>met-s</i>	8.0×10^{-10}			< 10	10			
	DU3287 <i>pen</i> ⁻ <i>met-s</i>	1.5×10^{-9}			< 10	10			

Abbreviations as in Table 1.

penicillinase, enterotoxin B and were resistant to Cd^{2+} and Hg^{2+} and some possessed haemolytic activities for sheep and human erythrocytes. Ninety-four out of 100 transductants from the first cross and all from the second differed from the recipient in that they were sensitive to erythromycin. Transduction to methicillin resistance also resulted in change in the phage typing pattern. The transductants were non-typable, but some were weakly lysed by phage 88. Resistance to typing phage 29, to which the recipient was sensitive, suggested that the transductants might have become lysogenized by the transducing phage (Rountree, 1959). However, only three out of 30 transductants from the first cross were inducible. In the second cross all transductants were immune to the transducing phage, and were non-inducible.

Attempts were also made to transduce methicillin resistance from the mutant *pen⁻ met-r*, selecting on either methicillin-salt agar (25 µg/ml) or benzylpenicillin agar (10 µg/ml). No methicillin-resistant transductants were obtained from the crosses *pen⁺ met-s* × phage 29/*pen⁻ met-r* or *pen⁻ met-s* × phage 29/*pen⁻ met-r*.

Transduction of methicillin resistance with phage 88. Methicillin-resistant transductants were obtained from the crosses *pen⁻ met-s* × phage 88/*pen⁺ met-r* and *pen⁺ met-s* × phage 88/*pen⁺ met-r* at a frequency of 1×10^{-8} (Table 2). All transductants were resistant to cephalothin as well. Three out of the five transductants from the first cross and all five from the second produced penicillinase and enterotoxin B and were resistant to Cd^{2+} and Hg^{2+} . They differed from the recipient in that they were sensitive to erythromycin. The two remaining methicillin-resistant transductants from the first cross did not produce penicillinase or enterotoxin B and were sensitive to Cd^{2+} and Hg^{2+} and showed inducible erythromycin resistance. Transduction to methicillin resistance in these two crosses did not result in an alteration of the phage typing pattern.

Attempts to transduce methicillin resistance from strain *pen⁻ met-r* into the *pen⁻ met-s* or *pen⁺ met-s* strains were unsuccessful.

Plating efficiency of methicillin resistance. There was no stimulation of growth of methicillin-sensitive cells when a mixture of sensitive and resistant cells were plated on methicillin-salt agar plates. Since the proportion of resistant and sensitive bacteria was maintained after plating, the resistant bacteria did not apparently eliminate methicillin from the agar.

Transduction of penicillinase production or cadmium resistance with typing phage 29. In order to detect the presence of a penicillinase plasmid, the strains DU4916 *pen⁺ met-r* and *pen⁺ met-s* were used as donors and the penicillinase-negative variants of the same strain and two penicillinase-negative, methicillin-sensitive wild strains of *Staphylococcus aureus* DU3108 *pen⁻ met-s* and DU3287 *pen⁻ met-s* as recipients. Selection was made on nutrient agar containing benzylpenicillin or cadmium sulphate.

The frequencies of penicillinase producing transductants were in the range of 3.3×10^{-5} to 8×10^{-10} (Table 3). All the transductants were both Cd^{2+} and Hg^{2+} resistant. In the crosses DU3108 *pen⁻ met-s* × phage 29/*pen⁺ met-r* and DU3287 *pen⁻ met-s* × phage 29/*pen⁺ met-r* 8% co-transduction of methicillin resistance and toxin production was obtained. The *pen⁺ met-r* transductants were lysed by phages 29 and 88 as was the donor, whereas all the *pen⁺ met-s* transductants showed the same typing patterns as the respective recipients. Co-transduction of methicillin resistance and penicillinase production on methicillin-salt agar was not obtained with DU3108 *pen⁻ met-s* and DU3287 *pen⁻ met-s* as recipients. Cd^{2+} -resistant transductants were obtained from these crosses with selection on cadmium agar. The frequencies of transduction were between 3.4×10^{-5} and 1.0×10^{-7} . All transductants produced penicillinase and were resistant to Hg^{2+} .

Table 4. Transduction of methicillin resistance or penicillinase production with mixed phage lysates

Donor strains of DU4916	Recipient strain of DU4916	Phage	Selective marker	Frequency of transductants	No. of transductants	Minimum inhibitory concentration of the transductants ($\mu\text{g/ml}$) for					Transductant extracellular markers		Haemolytic activities for erythrocytes (titre)	
						Methicillin	Cephalothin	Erythromycin	Hg ²⁺	Cd ²⁺ ($\text{M} \times 10^{-5}$)	Penicillinase	Enterotoxin B (titre)	Sheep	Homologous
<i>pen</i> ⁻ <i>met-r</i> <i>pen</i> ⁺ <i>met-s</i>	<i>pen</i> ⁺ <i>met-s</i>	29	<i>met-r</i>	—	5	> 200	> 100	> 200			< 1	8	8	128
		88	<i>met-r</i>	0.8×10^{-9}										
<i>pen</i> ⁻ <i>met-r</i> <i>pen</i> ⁺ <i>met-s</i>	<i>pen</i> ⁻ <i>met-s</i>	29	<i>met-r</i>	—	47	< 12.5	< 10	> 200	> 40	> 14	+	< 1		
			<i>pen</i> ⁺	3×10^{-8}										
		88	<i>met-r</i>	1.1×10^{-8}	7	> 200	> 100	< 2	10	1.4	—	40	16	128
			<i>pen</i> ⁺	—										

Abbreviations as in Table 1.

Table 5. Transductions of methicillin resistance with phage 29 for compatibility test

Donor strain of DU4916	Recipient strain of DU4916	Frequency of transduction	No. of transductants	Minimum inhibitory concentration of the transductants ($\mu\text{g/ml}$) for					Production by the transductants of		
				Methicillin	Cephalothin	Erythromycin	Hg ²⁺	Cd ²⁺ ($\text{M} \times 10^{-5}$)	Penicillinase of	Enterotoxin B	Haemolytic activity on sheep erythrocytes (titre)
<i>pen</i> ⁺ <i>met-r</i>	<i>pen</i> ⁺ <i>Mut met-s</i>	1.9×10^{-7}	97	30	10	> 200	> 40	< 1.4	Mutant type	< 1	< 2

Abbreviations as in Table 1.

Transduction of methicillin resistance and penicillinase production using mixed phage lysates. As methicillin resistance could only be transduced from penicillinase-producing donors, co-transfer was investigated further. Typing phage 29 was propagated on strains DU4916 *pen*⁻ *met-r* and DU4916 *pen*⁺ *met-s* separately to a titre of 10^9 p.f.u./ml. The sterile lysates were mixed and used to transduce the strains DU4916 *pen*⁺ *met-s* and DU4916 *pen*⁻ *met-s*. Selection was made on methicillin-salt or penicillin agar. No methicillin-resistant transductants were obtained from the crosses *pen*⁺ *met-s* × phage 29/*pen*⁻ *met-r*/*pen*⁺ *met-s* or *pen*⁻ *met-s* × phage 29/*pen*⁻ *met-r*/*pen*⁺ *met-s*. Penicillinase-producing transductants resistant to both Cd²⁺ and Hg²⁺ were, however, found at the low frequency of 3×10^{-8} in the cross *pen*⁻ *met-s* × phage 29/*pen*⁻ *met-r*/*pen*⁺ *met-s* with no linked transfer of *met-r* (Table 4).

The transduction crosses were repeated using the mixed phage 88 lysates from the two donor strains. Methicillin-resistant transductants were obtained at a frequency of 0.8×10^{-9} using the mutant *pen*⁺ *met-s* as recipient and at 1.1×10^{-8} using *pen*⁻ *met-s* as recipient (Table 4). The transductants from the first cross produced no enterotoxin B but possessed some haemolytic activity for sheep and human erythrocytes and were resistant to cephalothin as well. They were still inducibly erythromycin resistant and lysed by the phages 29 and 88 as was the recipient. The *met-r* transductants from the second cross produced enterotoxin B and possessed haemolytic activity but had no penicillinase. They were also sensitive to Cd²⁺ and Hg²⁺. All transductants were resistant to cephalothin but sensitive to erythromycin. Their phage type pattern was the same as the recipient. When selection was made on penicillin agar, the *pen*⁺ marker could not be transduced into the *pen*⁻ *met-s* strain.

Transduction of methicillin resistance into a strain harbouring a defined mutated penicillinase plasmid. To detect any possible association between methicillin resistance and penicillinase production in strain DU4916, another strain, RBI (α *pen*⁺ *Mut met-s*), was used for compatibility studies (Richmond, 1968). This strain harbours an α -plasmid as does the strain DU4916 *pen*⁺ *met-r*, but the former is a mutant which produces small amounts of penicillinase as compared with wild-type. This plasmid was transferred into strain DU4916 *pen*⁻ *met-s* with phage 29 at a frequency of 1.2×10^{-5} . One of the penicillinase transductants from this cross was then used as recipient in the cross DU4916 *pen*⁺ *Mut met-s* × phage 29/DU4916 *pen*⁺ *met-r* with selection on methicillin-salt agar. Methicillin-resistant transductants were obtained at a frequency of 1.9×10^{-7} (Table 5). The m.i.c. of the transductants for methicillin was, however, only 30 µg/ml as compared with more than 200 for the transductants from other crosses (Tables 2 and 4). All transductants produced penicillinase of the mutant type (i.e. like the recipient) but they did not produce enterotoxin B or possess haemolytic activities. They were all erythromycin resistant and sensitive to Cd²⁺ but resistant to Hg²⁺ and their phage type pattern was unchanged. No methicillin-resistant colonies were obtained when the same transduction crosses were repeated using phage 88.

Treatment of methicillin-resistant transductants with acriflavine. Since almost all *met-r* transductants were co-transduced to penicillinase production, a *pen*⁺ *met-r* transductant was treated with acriflavine to test if the suggested recombination of the markers was stable. Loss of *met-r* was obtained among transductants from the cross DU4916 *pen*⁻ *met-s* × phage 29/DU4916 *pen*⁺ *met-r* at a frequency of 0.14 % (3/2004) and of the *pen*⁺ marker at a frequency of 0.9 % (18/2000).

Since the *pen*⁺ *Mut met-r* transductants from the cross DU4916 *pen*⁺ *Mut met-s* phage 29/DU4916 *pen*⁺ *met-r* did not show haemolytic activities for sheep erythrocytes, *met-s* colonies could not be picked from 1 % sheep blood agar plates after acriflavine treatment (Dornbusch *et al.* 1969). However, 0.3 % (6/2000) of colonies were penicillinaseless

after acriflavine treatment by the replica plating technique with no co-loss of methicillin resistance.

DISCUSSION

The appearance of sensitive derivatives after treatment of strain DU4916 with acridines may imply the presence of two plasmids (Table 1). If so, one of them is a typical penicillinase plasmid with the genetic determinants for penicillinase production and resistance to Hg^{2+} and Cd^{2+} (Novick, 1967; Richmond, 1968) and the other a plasmid-controlling methicillin and cephalothin resistance, enterotoxin B and β -haemolysin production and maybe some factor which blocks expression of resistance to erythromycin. A deletion in a chromosomal control locus for one or both genes caused by acridines has, however, not been excluded. The loss of β -haemolysin was always accompanied by a loss in the ability to haemolyse human erythrocytes. As purified β -lysin as well as α -lysin has only a slight effect on human cells, this phenomenon may be explained by a synergistic effect or by a simultaneous loss of δ -lysin as well (Hallander & Bengtsson, 1967). Loss of methicillin resistance from strains of *Staphylococcus aureus* after growth at 43 °C has recently been reported (Al Salihi & James, 1972) as a result of changed surface properties at different temperatures. The authors suggest that the methicillin resistance genes are carried on a plasmid.

Transduction experiments (Table 3) confirm that strain DU4916 carries a penicillinase plasmid, i.e. transduction of pen^+ and Cd^{2+} resistance regardless of whether selection for penicillin or cadmium resistance is made. These markers were also easily transduced to other strains. However, there was less success in transducing the methicillin resistance marker alone. No transductants were isolated when the strain DU4916 $\text{pen}^- \text{met-r}$ was used as donor.

Most methicillin-resistant transductants were also penicillinase producers (Table 2). Apparently methicillin-resistant transductants are most easily selected when they are also transduced to penicillinase production. Using typing phages 29 or 88, transduction of methicillin resistance succeeded when both donor and recipient were pen^+ and when the donor was pen^+ and the recipient pen^- , but in the latter case, with few exceptions, met-r was co-transduced with pen^+ . The results suggest that the markers for $\text{pen}^+ \text{met-r}$ might recombine on the transduced fragment for transduction of methicillin resistance. It was also clear from the plating efficiency experiments that all met-r transductants were real transductants and not just satellite colonies due to breakdown of methicillin in the plates around a met-r colony. In two crosses with recipients which were not derived from the strain DU4916, methicillin resistance and toxin production were 8% co-transduced with penicillinase-production (Table 3). This fact further supports the suggestion that the two linkage groups can recombine.

With a mixture of phage 88 lysates propagated separately on the donors $\text{pen}^- \text{met-r}$ and $\text{pen}^+ \text{met-s}$, methicillin resistance and enterotoxin B production were, however, transduced into $\text{pen}^- \text{met-s}$ at low frequencies (Table 4) with no co-transduction of penicillinase production. When selecting on penicillin-agar no transductants could be obtained. This might be a dilution effect, since the phage lysate after propagation on $\text{pen}^+ \text{met-s}$ was mixed with that propagated on $\text{pen}^- \text{met-r}$. These facts may further support the suggestion that if the $\text{pen}^+ \text{met-r}$ markers are recombined on the transduced fragment the frequency of transduction of methicillin resistance and toxin production is stimulated. This recombination does not seem to be a stable event since acriflavine treatment of $\text{pen}^+ \text{met-r}$ transductants resulted in loss of the two markers at different rates. Methicillin resistance was, however, also separately transduced, though only partially (Table 5) when strain DU4916 $\text{pen}^+ \text{Mut met-s}$

was used as recipient. There was no co-transfer of either wild-type penicillinase or enterotoxin B production. The transductants were resistant to erythromycin like the recipient. In this case the linkage group for methicillin resistance has evidently been deleted, leaving the remaining part together with markers for toxin and enzyme production outside the phage head (Wood & Henninger, 1969).

The genetic markers for methicillin resistance and mutant penicillinase production in these transductants were shown to be located in separate linkage groups since enzyme production could be eliminated after acriflavine treatment with no co-ordinate loss of methicillin resistance. Transduction of chromosomal methicillin resistance has been described in another system (Cohen & Sweeney, 1970).

In transduction of methicillin resistance nutrient agar was unsatisfactory but transductants were obtained on hypertonic salt agar medium. Thus the nature of the expression of the *met-r* marker complicates the selection technique. Transduction of methicillin resistance was not stimulated at 30 °C (Annear, 1968; Hallander, Laurell & Dornbusch, 1969; Hewitt, Coe & Parker, 1969).

The expression of inducible erythromycin resistance among the variants of strain DU4916 will be studied further as well as the compatibility with known plasmid systems.

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