GENETIC CONTROL IN METHICILLIN-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

R. W. LACEY

Department of Bacteriology, University of Bristol

METHICILLIN-RESISTANT strains of *Staphylococcus aureus* were first detected in 1960 (Jevons, 1961; Knox, 1961) and at that time comprised about 0·06 per cent. of all isolates. The frequency of methicillin resistance has gradually increased, so that by 1969 about 4 per cent. of British "hospital" strains were resistant (Parker and Hewitt, 1970). Since the use of all the penicillins and cephalosporins should favour the proliferation of these organisms, it is surprising that methicillin resistance has not become more common. Strains of *Staph. aureus* that are resistant to penicillin by β -lactamase production now outnumber sensitive strains both in hospital and outside (Goldie, Alder and Gillespie, 1971).

The inter-strain spread of genes may contribute to the high proportion of staphylococci resistant to other antibiotics (e.g., Novick and Morse, 1967; Richmond, 1969; Lacey, 1971*a* and *b*). Almost all methicillin-resistant strains are also resistant to streptomycin and tetracycline, and produce lactamase (Eriksen and Erichsen, 1964; Dyke, 1969; Ridley *et al.*, 1970; Parker and Hewitt). If the gene or genes determining methicillin resistance had spread between strains, the antibiotic sensitivity of methicillin-resistant strains should be more variable than it is.

This paper attempts to account for the failure of methicillin resistance to appear in sensitive staphylococci. Although the gene or genes responsible for methicillin resistance were found to be plasmid-borne, they were transduced from lysates of the strains only at low frequency and to a narrow range of recipients. Transfer of methicillin resistance did not occur in mixed cultures. But other plasmid genes were transferred from these donors to the same recipients at medium to high frequencies.

MATERIALS AND METHODS

Staphylococci. Freeze-dried cultures of six isolates of *S. aureus* representative of methicillin-resistant types prevalent in Britain were obtained from the Cross-Infection Reference Laboratory, Colindale, in February 1969 (table I). Single colonies from each ampoule were checked for antibiotic sensitivity, bacteriophage-typed and seeded on to agar slopes (Oxoid Blood Agar Base no. 2). After overnight incubation at 37°C, the cap of each slope was screwed down tightly and the cultures were stored at room temperature in subdued light until July 1971 (30 mth) without subculture.

Media. Nutrient broth was double-strength Bacto (Difco) with the addition of 0·5 per cent. (w/v) NaCl. Nutrient agar was prepared from Oxoid Blood Agar Base no. 2. Additional

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NaCl to total 5 per cent. (w/v) was added to the basic medium to detect methicillin-resistant staphylococci.

Loss of antibiotic resistance in the cultures. Preliminary subcultures indicated that substantial numbers of organisms had survived on all the slopes. Nutrient broth (0.1 ml) was then added to each slope, and the organisms were emulsified in the broth and transferred to 9 ml of broth and incubated for 20 hr at 37°C. Each culture was then diluted and spread over the surface of nutrient agar plates. After incubation overnight at 37°C, the colonies were replicated on to nutrient agar containing 25 µg per ml methicillin, or 25 µg per ml streptomycin and 20 µg per ml tetracycline, and in some instances containing 20 µg per ml erythromycin or 3 µg per ml novobiocin. The media containing methicillin were incubated for 48 hr at 30°C and the others for 24 hr at 37°C. Selection of penicillinase-negative variants was by the method of Dyke, Jevons and Parker (1966).

TABLE I
Methicillin-resistant strains studied

Colindale number	Year of initial isolation	Antibiotic resistance*	Phage-typing pattern
13136	1960	MPST	7/47/53/54/75/77/84/88 (RTD)
13137	1960	MPST	7/47/53/54/75/77/84/85/88 (RTD)
9254	1964	MPSTE	29/88/ group III inhibition (RTD × 1000)
2273	1965	MPSTE	88 (RTD)
9463	1967	MPSTE	29/88/ group III inhibition (RTD × 1000)
11164	1967	MSTENeNo	84/85/88 (RTD)

* Resistance to methicillin (M), penicillinase producer (P), resistance to streptomycin (S), tetracycline (T), erythromycin (E), neomycin (Ne), and novobiocin (No).

RTD = Routine test dilution.

Antibiotic sensitivity was determined by the use of antibiotic disks (Lacey, 1971a) and also by measuring the minimum inhibitory concentration (MIC) of the antibiotic incorporated in agar in plates. Additional Mast disks used contained lincomycin 2 µg, oleandomycin 5 µg, spiramycin 5 µg, novobiocin 5 µg, or methicillin 10 µg. Resistance to mercury and cadmium ions was determined by the disk method of Dyke, Parker and Richmond (1970). Inocula for sensitivity tests were prepared by overnight incubation at 37°C, after preliminary experiments at 30° and 37°C had shown that the temperature of pre-incubation did not influence methicillin sensitivity. In tests with antibiotics other than methicillin the inoculated plates were also incubated at 37°C. In establishing the MIC of methicillin for cultures, the methicillin-containing plates were incubated either at 30° or 37°C and some at either temperature contained 5 per cent. NaCl. The MIC was taken as the concentration of antibiotic that inhibited the growth of 80 per cent. or more of the inoculum (about 10⁵ organisms in 0.025 ml broth) after 48 hours' incubation, unless otherwise stated. The occasional colony surviving higher levels of the drug was discounted.

Haemolysin production. Alpha- and delta-haemolysins were assayed by a semi-quantitative method. Spot inocula of the cultures were made on to agar containing 5 per cent. (v/v) rabbit or human erythrocytes and zones of haemolysis measured after 48 hours' incubation at 37°C. Anti alpha-toxin was incorporated into control plates. Beta-lysin was detected by observing the appearance of "hot-cold" lysis.

Lipase production was detected on Tween 80 medium (Gonzalez and Sierra, 1961). *Pigmentation* was recorded after incubation of strains at 37°C for 72 hr on 40 per cent. milk agar (Willis and Turner, 1963).

Bacteriophage typing was done by the method of Blair and Williams (1961) with the addition of four new typing phages—no. 83A, 84 and 85 (Report, 1967) and no. 88, an experimental phage lytic for some methicillin-resistant staphylococci provided by Dr M. T. Parker, Cross-Infection Reference Laboratory, Colindale.

Mitomycin C induction was by the method of Lacey (1971b).

Propagation and harvesting of phages was by the freeze-thawing method of Williams and Rippon (1952).

Transduction of antibiotic resistance. The following procedure was generally used; variations are described where they apply. Phage preparations before each experiment were checked for sterility by plating 1 ml on to the surface of nutrient agar in plates, which were incubated at 37°C for 20 hr. Volumes of the phage preparation were added, at a multiplicity of about 0.3 phage to 1 cell, to 5-ml volumes of overnight broth cultures of the recipient in 25-ml screw-capped bottles. Calcium chloride was added to 3 mM and the mixtures were kept at 37°C for 30 min. Sodium citrate (to 12 mM) and 18 ml of fresh pre-warmed nutrient broth were added and, after mixing, the cultures were incubated statically for a further 60 min. at 37°C. The cultures were then centrifuged at 3000g for 10 min. and the cells transferred to the surface of appropriate media. The recovery medium for methicillin-resistant recipients contained 12.5 µg methicillin per ml and 5 per cent (w/v) NaCl and was incubated for 5 days at 30°C. That for streptomycin resistance contained 50 µg of the drug per ml, for tetracycline and erythromycin resistance 20 µg of each per ml, for cadmium resistance 10⁻⁴M cadmium acetate. Apart from the transduction of methicillin resistance, all the plates were incubated at 37°C for 48 hr. Controls, in which no phage preparations were added, were included in each experiment.

Irradiation with ultraviolet (UV) light. Lysates of cultures, after induction with mitomycin C, were exposed at a distance of 25 cm from a "Hanovia" lamp to a dose of c. 0.28 ergs per mm² per s, for 15 s, 30 s, 1, 2 or 5 min.

Transfer of antibiotic resistance between staphylococci in nutrient broth. The method used was similar to that for neomycin resistance (Lacey, 1971a); as controls the donor and recipient were incubated singly in each experiment. The recipients used were laboratory-selected mutants resistant to both novobiocin (5 µg per ml) and rifampicin (50 µg per ml). Transfer of tetracycline, erythromycin and streptomycin resistance was detected by plating the mixed culture on media containing either 20 µg per ml tetracycline, 20 µg per ml erythromycin, or 50 µg per ml streptomycin, together with 20 µg per ml rifampicin. Transfer of penicillinase was detected by selecting for cadmium resistance (10⁻⁴M cadmium acetate) and rifampicin resistance (20 µg per ml). Attempts to detect the transfer of methicillin resistance were made by means of recovery media containing 12.5 µg methicillin per ml and 5 per cent. (w/v) NaCl with either 5 µg novobiocin per ml or 20 µg rifampicin per ml; this medium was incubated at 30°C for 5 days. Other recovery media were incubated at 37°C for 48 hr. The frequency of transfer was expressed as the proportion of recipients, if any, that had acquired the antibiotic resistance after 18 hours' incubation with the donor.

Susceptibility to lysostaphin. Cultures were grown at 30°C in nutrient broth without methicillin for 48 hr, centrifuged twice at 3000g for 10 min. and resuspended in a tris buffer at pH 7.4 (0.05M tris and 0.145M NaCl). Lysostaphin (Mead Johnson Research Laboratories, Evansville, Indiana, USA) was diluted in this buffer and added in doubling dilutions to molten nutrient agar containing 5 per cent. (w/v) NaCl at 45°C. The solidified medium was inoculated with 0.025-ml drops of the neat bacterial suspension and with cells diluted in the buffer to give about 20 colony-forming units. The inoculated plates were incubated for 48 hr at 30°C. The MIC was measured as the smallest amount of lysostaphin that (1) produced a greater than 50 per cent. reduction in the count from the diluted suspension and (2) that reduced confluent growth to discrete colonies from the neat inoculum.

In other experiments, 2.5 µg lysostaphin per ml was added to the neat suspensions of cells in buffer at 30°C and reduction in optical density (at 620 nm) on a Unicam SP600 spectrophotometer was followed for 30 min.

Acriflavine treatment was by the method of Dornbusch, Hallander and Löfquist (1969).

RESULTS

Loss of antibiotic resistance in six strains of Staphylococcus aureus after storage for 30 mth at room temperature

About 5000 colonies of each strain were examined by replica plating for loss of penicillinase production (except strain no. 11164 which is not a producer), and of resistance to methicillin, streptomycin and tetracycline. Loss of erythromycin resistance was investigated in the four strains that were resistant, and loss of novobiocin resistance was investigated in strain no. 11164. In five strains, penicillinase-negative segregants comprised from about 1 to 12 per

TABLE II
Loss of antibiotic resistance in six strains of Staphylococcus aureus stored for 30 mth at room temperature

Strain number	Number of colonies					
	screened	that showed loss of resistance to				
		penicillin	methicillin	streptomycin	tetracycline	erythromycin
9254	4812	607	0	0	0	0
13136	5772	52	8	0	4	...†
13137	4964	61	8	0	2	...†
11164	5759	...*	10	0	0	1
2273	5644	547	3	0	7	21
9463	4764	501	45	0	3	0

* Not a penicillinase producer.

† Original strain sensitive to erythromycin.

cent. of the colonies (table II). No streptomycin-sensitive colonies were found, but four out of the six strains gave rise to tetracycline-sensitive derivatives. Novobiocin resistance was stable in strain no. 11164. Resistance to cadmium and mercury ions was always lost with penicillinase, and resistance to lincomycin, spiramycin and oleandomycin was lost with resistance to erythromycin in strains no. 2273 and 11164. There was no other linked loss.

Properties of methicillin-sensitive segregants

Methicillin-sensitive colonies were obtained from five of the six strains. All the sensitive colonies were tested further. The sensitive segregants (denoted mtc⁻) resembled the corresponding wild strains in susceptibility to other antibiotics and cadmium and mercury ions, Tween 80 reaction, haemolysin production, pigment and phage-typing pattern. All the wild strains exhibited the heterogenous type of methicillin resistance (Dyke; Parker and Hewitt) characterised by low-level resistance to methicillin of the majority of the cells when grown at 37°C with infrequent (about 1 in 10⁻⁵) resistant colonies. Reduction in temperature or the incorporation of 5 per cent. NaCl (w/v) in the

medium produced a uniformly resistant cell population. All the cocci in each of the cultures from the sensitive segregants were sensitive to methicillin under these conditions ($\text{MIC} < 5 \mu\text{g}$).

No reversions were obtained from plate cultures (about 10^{12} viable cocci) of one sensitive segregant of each of the five strains after plating on to nutrient agar containing $25 \mu\text{g}$ methicillin per ml and 5 per cent. NaCl and incubation for 5 days at 30°C .

TABLE III

Susceptibility to lysostaphin of methicillin-resistant strains of Staphylococcus aureus and their methicillin-sensitive derivatives

Strain number	Sensitivity to methicillin	Minimum inhibitory concentration of lysostaphin (μg per ml) for the stated strain, in tests with	
		small inoculum (20 organisms)	large inoculum (c. 10^7 organisms)
13136 wild	R	0.05	1.0
13136 (mtc ⁻)	S	0.05	1.0
13137 wild	R	0.025	1.0
13137 (mtc ⁻)	S	0.05	1.0
11164 wild	R	0.05	1.0
11164 (mtc ⁻)	S	0.05	1.0
2273 wild	R	0.05	0.5
2273 (mtc ⁻)	S	0.05	1.0
9463 wild	R	0.05	1.0
9463 (mtc ⁻)	S	0.05	1.0
6936 wild	S	0.05	1.0
609 wild	S	0.1	2.0

R = Resistant; S = sensitive.

Five methicillin-resistant wild strains and the sensitive segregants derived from them, and two methicillin-sensitive wild strains, were tested for susceptibility to lysostaphin. The MIC of lysostaphin for all the cultures was similar both at high and low inoculum density (table III). The MIC with the higher inoculum was about 20 times that at the lower. Similarly, the rate of lysis after adding lysostaphin at $5 \mu\text{g}$ per ml to cells suspended in tris-saline buffer was similar for all the strains whether methicillin-sensitive or resistant.

Effect of acriflavine treatment on loss of methicillin resistance

Acriflavine was added to broth cultures of strains no. 11164 ($5 \mu\text{g}$ per ml) and no. 9463 ($10 \mu\text{g}$ per ml). At higher concentrations acriflavine was inhibitory. About 0.2 per cent. of the cocci of each culture had become methicillin-sensitive after acriflavine treatment but none from about 3000 cocci of control cultures grown at 37° or 42°C . These sensitive colonies resembled the wild strains in

sensitivity to cadmium and mercury, haemolysin and penicillinase production and phage type. The sensitivity to methicillin was much more variable than that shown by the segregants occurring on storage. The MIC (at 30°C, no added salt) for those from strain no. 11164 varied from 12.5 to 1.5, and those from strain no. 9463 from 25 to 3 µg per ml.

Transduction of methicillin resistance

Transduction of methicillin resistance was attempted by propagating phages on the donor cultures and also by the use of lysates of them induced with mitomycin C. Typing phages no. 53 of the International Basic Set and no. 88 were propagated on strains no. 13136 and 11164 respectively. The resultant phage preparations were added to the corresponding *mtc*⁻ derivatives and also to propagating strain (PS) 54 of the International Basic Set. No transductants were isolated from about 3×10^{10} plaque-forming units (p.f.u.) of each preparation.

Lysates induced with mitomycin C were obtained from strains no. 13136, 13137, 11164 and 2273, but not from no. 9254 and 9463. Single-colony isolates obtained from the stored cultures were generally used for induction. Transduction was attempted from each of the four lysates to each of the five *mtc*⁻ derivatives, to PS 6, 42E, 47, 53, 54, 75, 77 and 83A, and to strain no. 6936, making 56 combinations in all. From about 5×10^{10} p.f.u. of each lysate added at a multiplicity of phage to cell of 0.3, transduction was achieved in three crosses only and at low frequency: from strain no. 2273 to no. 9463 (*mtc*⁻), from no. 13136 to no. 11164 (*mtc*⁻) and from no. 11164 to no. 11164 (*mtc*⁻) at frequencies of 1.3×10^{-9} to 6×10^{10} . The failure to transduce methicillin resistance to any of the propagating strains is surprising, since tetracycline resistance was transduced to most of these from the same lysates under the same conditions. Lysates from strains no. 13136 and 13137 transduced tetracycline resistance to six of the strains at frequencies from 10^{-5} to 10^{-7} , and those from no. 11164 and 2273 to all except PS 42E (10^{-6} to 10^{-8}).

Transduction of methicillin resistance by means of lysates, from strain no. 11164 to no. 11164 (*mtc*⁻) and from strain no. 13136 to PS 54, was investigated under various conditions. Incubation of the donor in sub-inhibitory concentrations of methicillin (10 µg per ml) before induction or at 30°C, or incubation of the recipient before and during the addition of the lysate in 0.25 µg methicillin per ml or at 30°C failed to raise the transduction frequency. Also, incubation of the recipients for up to 6 hr after adsorption of phage had an effect no more than might be accounted for by multiplication of the organisms. Increase in the phage-cell ratio from 0.3 to 3.0 and the use of the two lysates with one recipient also had little effect.

Transduction of methicillin resistance from strain no. 11164 to no. 11164 (mtc⁻)

The transduction of methicillin resistance from strain no. 11164 to no. 11164 (*mtc*⁻) was examined to confirm the plasmid location of the resistance and to attempt to account for the low frequencies obtained. Lysates obtained from

three well-isolated colonies and from a subculture of the stored slope by treatment with mitomycin C were irradiated with UV light for varying times. With each of the lysates, an exponential decay in the transduction frequency with increasing exposure to UV light was observed. This is characteristic of most plasmid-borne genes (e.g., Arber, 1960; Asheshov, 1966, 1969). However, the initial transduction frequency from the lysate is much lower than that for most other plasmid genes (e.g., Asheshov, 1966; Dyke *et al.*, 1970).

Six colonies of no. 11164 (mtc⁻) to which methicillin resistance had been transduced, denoted as 11164 (mtc⁻→mtc⁺), were investigated further. Each of the cultures of 11164 (mtc⁻→mtc⁺) gave the same degree of heterogenous resistance to methicillin as did the wild strain no. 11164. The cultures of 11164 (mtc⁻→mtc⁺) were induced with mitomycin C and transduction was attempted from the lysates to 11164 (mtc⁻). From three cultures, no transductants were obtained and from the other three transduction occurred and its frequency (about 10⁻¹⁰) was stimulated more than 10-fold in each case by small doses of UV light. This suggests a probable chromosomal location for the methicillin-resistant genes in three of the cultures of 11164 (mtc⁻→mtc⁺). When genetic material is transduced into a cell, it can either replicate autonomously as a plasmid, be inserted into another plasmid or else be integrated into the chromosome (Richmond and Johnson, 1969). The low frequency of transduction from strain no. 11164 (wild) may therefore possibly be due to only a fragment of the methicillin-resistant plasmid being incorporated into the phage during induction. This fragment contains the gene or genes for methicillin resistance but not areas of the plasmid genome vital for its maintenance. Thus, for phenotypic expression in the recipient, the transduced plasmid fragment becomes inserted into the chromosome.

Location of the gene or genes for penicillinase production, and for streptomycin, tetracycline and erythromycin resistance in strains no. 13136, 13137, 11164 and 2273

Mitomycin C-induced lysates prepared from single-colony isolates of each of these strains after storage were exposed to UV light for various times and then transduced to a penicillinase-negative variant of PS 54. In each case the effect of UV light on the transduction frequency was typical for either a plasmid or a chromosomal marker (table IV). These results taken in conjunction with the stability of the wild strains on storage enable the cellular location of most of the genes of these strains to be made (table V).

Transfer of resistance genes from strains no. 13136, 2273 and 11164 to four recipients in mixed culture

Cadmium resistance and penicillinase production were co-transferred at a frequency of 1.1×10^{-8} to 2.2×10^{-6} from strains no. 13136 and 2273 (table VI). Resistance to tetracycline was transferred at a frequency from 4.3×10^{-5} to 1.2×10^{-8} from all three strains, and erythromycin resistance, together with

cross-resistance to lincomycin, spiramycin and oleandomycin, at from 5.6×10^{-6} to 6.2×10^{-8} from strains no. 2273 and 11164. Resistance to streptomycin

TABLE IV

Transduction of penicillinase, and of resistance to streptomycin, tetracycline and erythromycin from mitomycin C-induced lysates of strains no. 13136, 13137, 2273 and 11164 to strain no. PS 54

Strain number	Transduction of							
	penicillinase		streptomycin resistance		tetracycline resistance		erythromycin resistance	
	Frequency* with unirradiated lysate	Maximum UV stimulation†	Frequency with unirradiated lysate	Maximum UV stimulation†	Frequency with unirradiated lysate	Maximum UV stimulation†	Frequency with unirradiated lysate	Maximum UV stimulation†
13136	3.3×10^{-6}	$\times < 1.0$	4.1×10^{-7}	$\times 26$	1.1×10^{-5}	$\times < 1.0$	...	...
13137	4.5×10^{-6}	$\times < 1.0$	7.2×10^{-7}	$\times 33$	8.7×10^{-5}	$\times < 1.0$	...	...
2273	9.5×10^{-6}	$\times < 1.0$	6.2×10^{-7}	$\times 40$	5.0×10^{-7}	$\times < 1.0$	6.6×10^{-6}	$\times < 1.0$
11164	...	...	3.8×10^{-8}	$\times 75$	4.9×10^{-7}	$\times 1.5$	4.1×10^{-8}	$\times 1.2$

... Strain sensitive to this antibiotic.

* Transduction frequency expressed per phage titre in unirradiated lysate.

† This occurred either at 15- or 30-s exposure. The reduction of phage titre by this amount of irradiation was in the range 3.5- to 14.0-fold.

and to methicillin was not transferable. There was no other linked transfer. Methicillin resistance was also found not to be transferable from strain no. 11164 (wild) to a rifampicin resistant mutant of no. 11164 (mtc⁻).

TABLE V

Probable location of genes in six methicillin-resistant strains of Staphylococcus aureus

Strain number	Methicillin resistance	Penicillinase production	Streptomycin resistance	Tetracycline resistance	Erythromycin resistance
13136	Plasmid	Plasmid	Chromosomal	Plasmid	*
13137	Plasmid	Plasmid	Chromosomal	Plasmid	*
11164	Plasmid	†	Chromosomal	Plasmid	Plasmid
2273	Plasmid	Plasmid	Chromosomal	Plasmid	?
9463	Plasmid	Plasmid	?	Plasmid	?
9254	?	Plasmid	?	?	?

* Sensitive to erythromycin.

† Does not produce penicillinase.

The failure to demonstrate transfer of methicillin and streptomycin resistance might have been due to a synergistic effect of these drugs with rifampicin. This was excluded by showing that (a) there was no transfer if selection was made with novobiocin instead of rifampicin, and (b) rifampicin was not demonstrably synergistic with streptomycin or methicillin in its action on the methicillin-resistant strains. Methicillin resistance was also not transferred when the

cultures were incubated at 30°C. Failure to demonstrate recipients resistant to methicillin or streptomycin therefore probably indicates a true lack of transfer of these genes.

DISCUSSION

The finding of sensitive segregants after storage in each of the five strains examined suggests that the gene or genes controlling methicillin resistance are

TABLE VI
Transfer of resistance from Staphylococcus aureus strains 13136, 2273 and 11164 to four recipients in mixed cultures

Donor strain number	Resistance selected for	Frequency* of transfer of the stated resistance to recipient strain number			
		609	6936	PS 54	PS 77
13136	Methicillin	0†	0	0	0
	Penicillinase	N.T.	1.1×10^{-8}	0	N.T.
	Streptomycin	0	0	0	0
	Tetracycline	2.8×10^{-6}	1.5×10^{-6}	1.2×10^{-5}	4.3×10^{-5}
2273	Methicillin	0	0	0	0
	Penicillinase	N.T.	2.2×10^{-6}	7.5×10^{-8}	0
	Streptomycin	0	0	0	0
	Tetracycline	0	8.8×10^{-7}	6.1×10^{-8}	0
	Erythromycin	0	5.6×10^{-6}	7.9×10^{-7}	6.2×10^{-8}
11164	Methicillin	0	0	0	0
	Streptomycin	0	0	0	0
	Tetracycline	0	1.2×10^{-7}	4.4×10^{-8}	0
	Erythromycin	0	8.7×10^{-6}	3.2×10^{-6}	0

* Frequency = number of resistant recipients/total count of recipient strain after 18 hours' incubation.

† 0 = less than 10^{-11} .

N.T. = Not tested.

located on a plasmid. However, the strains had been stored for 30 mth before the transduction experiment was performed, and it is possible that during this time a piece of chromosome might have become extruded to form a plasmid, as has been described for the genes controlling penicillinase production (Asheshov, 1969). If this had occurred in the methicillin-resistant genes of strain no. 11164, cocci from the 30-mth-old slope should contain a mixture of chromosomal and plasmid-located genes. But examination of a sweep and three separate colonies of this culture all gave transduction curves typical of plasmid genes. The genes for streptomycin resistance in four strains appear to be typical chromosomal determinants. If the exclusion of areas of the staphylococcal chromosome were a frequent occurrence on storage, some evidence for a plasmid location for the streptomycin resistance might be expected. It therefore seems extremely unlikely that the genes for methicillin resistance are

normally chromosomal and have produced a plasmid on storage in each of the five cultures.

The strains described here differ in some respects from those investigated by Dornbusch *et al.* (1969). None of the former showed linkage of methicillin resistance to cadmium and mercury resistance, penicillinase or haemolysin production as did the Swedish strains. It is unlikely that the latter strains had heterogenous resistance to methicillin, since transduction of methicillin resistance was achieved by incubating the recovery medium at 37°C, at which temperature only about one cell in 10⁵ is resistant. But in many other respects, for example, sensitivity to other antibiotics, phage type and Tween 80 reaction, the two sets of strains are similar.

Methicillin resistance in itself does not give rise to resistance to lysostaphin. However, Sabath *et al.* (1970) found that highly methicillin-resistant clones from heterogenously resistant strains that were incubated at 37°C in the presence of methicillin were more resistant to lysostaphin than a sensitive culture grown in the absence of methicillin. But methicillin-resistant staphylococci have reduced growth rates in the presence of methicillin (Sutherland and Rolinson, 1964) and this, rather than resistance to methicillin *per se*, might lower susceptibility to lysostaphin.

My findings may provide an explanation for the relatively slow increase in total numbers of methicillin-resistant staphylococci. Methicillin resistance was not transferred between cells in mixed cultures, and even transduction from mitomycin C-induced lysates occurred at low frequency and to very few of the recipients tested. Transfer to a staphylococcus of genes determining resistance probably gives it an important advantage in the hospital environment. Tetracycline and erythromycin resistance, and penicillin resistance due to penicillinase production, were transferred from the same donor cultures to recipients at medium-to-high frequencies. The proportion of naturally occurring staphylococci that are resistant to these antibiotics is much higher than the proportion resistant to methicillin (Jevons *et al.*, 1966; Ridley *et al.*, 1970). Yet the selection pressure exerted in some instances, e.g., by erythromycin, may well have been less than that of antibiotics believed to select for methicillin resistance.

Where the probable location of the resistance genes has been determined, the findings are constant. Resistance to streptomycin is chromosomal, but penicillinase production and resistance to cadmium and mercury ions, and resistance to tetracycline and methicillin are plasmid-borne. It seems likely that most, if not all, methicillin-resistant isolates have evolved from a single origin. Resistance to antibiotics other than methicillin, streptomycin and tetracycline, and lactamase production, may have been acquired by mutation (e.g., novobiocin resistance) or by the establishment of new plasmids (e.g., neomycin and erythromycin resistance). The variation in phage-typing patterns may result from acquisition of new prophages, as has been suggested for other group III strains (Jevons *et al.*). There is some evidence for this in the strains studied here. Strain no. 2273 carries a phage lytic for strains no. 13136 and 13137 and strain no. 11164 carries a phage lytic for strains no. 2273, 13136 and 13137. The real danger from methicillin-resistant strains may be that they will

acquire new resistances rather than that they will transfer methicillin resistance to other strains.

SUMMARY

Six methicillin-resistant strains of *Staphylococcus aureus* isolated in Britain were examined for loss of resistance after storage for 30 mth at room temperature. Resistance to methicillin was lost from five out of six, to tetracycline from four out of six, to erythromycin from two out of four, and penicillinase from all five that were producers of it. The methicillin-sensitive segregants resembled the wild strains in bacteriophage-typing pattern, haemolysin production, Tween 80 reaction, pigmentation, and resistance to lysostaphin and to other antibiotics.

Methicillin resistance was transduced from lysates of three of the strains. Although the effect of ultraviolet light was characteristic for a plasmid gene in strain no. 11164, transduction occurred at low frequency (10^{-9} – 10^{-10}) from this and from the other strains, and to a narrow range of recipients. Methicillin resistance was not transferred to recipients in mixed culture.

In contrast, plasmid genes determining lactamase production, or resistance to tetracycline or erythromycin, were transduced from lysates and transferred in mixed cultures from these strains at frequencies from 10^{-5} – 10^{-8} . These results may account for the confinement of methicillin resistance to few types of staphylococci whilst other resistance genes have become much more widespread.

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