GENETIC STUDIES OF A MULTI-RESISTANT STRAIN OF *STAPHYLOCOCCUS AUREUS*

R. W. LACEY AND I. CHOPRA

Department of Bacteriology, The Medical School, University of Bristol, Bristol BS8 1TD

A POLICY of limiting the use of some antibiotics in hospitals is believed to have led to a reduction in the frequency of resistance in *Staphylococcus aureus* (e.g., Bulger and Sherris, 1968). Although staphylococci have acquired resistance to several antibiotics in the past, it is not known whether this trend will continue indefinitely. Studies on the capacity of staphylococci to acquire resistance to a large number of antibiotics *in vitro* could be valuable in the formulation of an effective antibiotic strategy against this organism.

In staphylococci, resistance to two unrelated antibiotics is sometimes determined by a single plasmid (Mitsuhashi *et al.*, 1965; Annear and Grubb, 1972; Lacey and Rosdahl, 1973) although plasmids that determine resistance to more than two antibiotics are rare. The appearance and transfer of single plasmids possessing genes for resistance to many antibiotics might have serious therapeutic implications, because we would expect the resultant bacteria to maintain relatively little additional DNA and therefore to be fully virulent. This process has not occurred to any considerable extent in staphylococci, and multi-resistance is thought to result chiefly from the progressive acquisition of separate plasmids (Dyke, Parker and Richmond, 1970). The number of resistance determinants per plasmid is limited probably because plasmid size is critical for transfer between staphylococci. The DNA of one staphylococcal phage has a molecular weight (MW) of about 29×10^6 daltons (Rush *et al.*, 1969), and we have found that a plasmid of 35×10^6 daltons can be transferred only at very low frequency (Chopra, Bennett and Lacey, 1973). Thus for plasmids to become widespread in the staphylococcal population, their MW must seemingly be about 30×10^6 daltons or less. In contrast, in the *Enterobacteriaceae*, large plasmids can determine resistance to many antibiotics and be transferred *in toto* by conjugation.

Recombination between separate resistance genes is a likely explanation for the appearance of a plasmid specifying multi-resistance, because antibiotic resistance that arises by mutation occurs mainly at a chromosomal rather than a plasmid locus (e.g., Lacey and Chopra, 1972). Recombination occurs at fairly high frequency *in vitro* between pairs of staphylococcal plasmids carrying genes common to both (Richmond, 1969) and such a process might occur naturally.

In this paper, we give the results of an analysis of a constructed strain which suggest that recombination between dissimilar plasmids is rare. Maintenance of introduced plasmids in the constructed strain was also examined,

Received 10 Oct. 1973; *accepted* 7 Nov. 1973.

and our results suggest that the number of plasmids that can be maintained stably in the cell is limited. This limitation seems to be due to a restrictive process or to restrictive processes other than plasmid incompatibility.

Materials and methods

Cultures of staphylococci. Strains nos. 649, 6936, FAR1 and 8657 have been described previously (Lacey and Grinsted, 1973). Strain no. 609 was described by Grinsted and Lacey (1973*a*). Strain DU4916 was kindly supplied by Dr R. P. Novick and was described by Dornbusch, Hallander and Löfquist (1969).

General methods. Media, detection of loss of antibiotic resistance, recording of pigment, bacteriophage typing, mitomycin C-induction, transduction and determination of MIC of antibiotics were as described previously (Lacey and Grinsted, 1973). Determination of resistance to cadmium, mercuric and arsenate ions was by the method of Lacey and Rosdahl (1973).

Isolation of the covalently closed circular (CCC) DNA, and calculation of the percentage CCC in the total DNA, was by the method of Chopra *et al.* (1973). After incubation with radioactive thymidine, a proportion of each culture was retained to check that the various markers had not been lost. Loss of markers occurred at frequencies only of less than 10%. Plasmid DNA from each derivative of strain 649 was analysed on sucrose gradients to ensure that there was no significant contamination of the preparations by chromosome fragments.

Propagation of phage 88 on derivatives of strain 649. This was by the method of Williams and Rippon (1952), in three successive cycles. Resultant lysates contained between 10^8 and 10^{10} plaque-forming units (p.f.u.) per ml (on strain 649N).

Calculation of loss of antibiotic resistance per cell division. Cultures of strain 649 containing the individual plasmids were grown overnight in nutrient broth containing the respective antibiotic or heavy-metal ion as a selective agent. The overnight cultures were diluted 1 in 2000 with fresh pre-warmed nutrient broth (without added selective agent) and portions taken for viable count. The diluted cultures were then incubated at 37°C for about 3 hours and again sampled for viable count. Resultant colonies were subsequently replicated on to selective media to permit calculation of the loss of antibiotic resistance per cell division.

Curing of antibiotic resistance with penicillins. Overnight cultures were diluted to about 10^4 cocci per ml in nutrient broth containing doubling dilutions of a penicillin and incubated statically for 20 hours at 37°C. Bacteria from the tube with the highest concentration of antibiotic that permitted visible growth were examined for loss of antibiotic or heavy-metal-ion resistance by replica plating.

Results

Properties of recipient strain number 649

Strain number 649 was chosen for study because it readily incorporated radioactive thymidine into DNA and was a suitable recipient for many plasmids. Strain 649 wild (resistant to streptomycin, but not to other antibiotics, Grinsted and Lacey, 1973*a*) when used as a recipient in transduction experiments was already resistant to cadmium, arsenate and mercuric ions, although it was not a penicillinase producer. Re-examination of the original slope yielded four types of colony (all of the same phage-typing pattern): (1) streptomycin and heavy-metal-ion sensitive (649N), (2) streptomycin sensitive, but heavy-metal-ion resistant (649*mir-r*), (3) streptomycin resistant, but heavy-metal-ion sensitive (649*str-r*), and (4) both streptomycin and heavy-metal-ion resistant (649*str-r*, *mir-r*). A plasmid was not isolated from strains 649N and 649*str-r*, but CCC

DNA isolated from strain 649*mir-r* was identical in size (determined by sucrose-gradient analysis after differential labelling) to that from strain 649*str-r*, *mir-r*. Therefore, it seems that the plasmid previously identified in strain 649 (Grinsted and Lacey, 1973*a*) determined heavy-metal-ion resistance rather than streptomycin resistance. The following evidence confirmed this view. Transduction with 649*mir-r* as donor and 649N as recipient, yields transductants containing a single plasmid identical in size with that isolated from 649*str-r*, *mir-r*. Attempts to transduce streptomycin resistance from strain 649*str-r*, *mir-r* to 649N with phage 88 also failed (transduction frequency$<5\times10^{-11}$), as had previously been found with phage 53 (Grinsted and Lacey, 1973*a*). However,

TABLE I

Loss of resistance from 649 mir-r str-r during growth in the presence of penicillins

Drug (and concentration)	Number of colonies tested	Number of colonies sensitive to	
		streptomycin	metal ions
Methicillin (0·25 μg per ml)	4,500	97	0
Benzyl penicillin (0·04 units per ml)	6,000	12	0
Ampicillin (0·35 μg per ml)	5,500	14	0
Cloxacillin (0·15 μg per ml)	3,000	305	0
Cephaloridine (0·05 μg per ml)	4,800	15	0
None	11,000	1	N.T.

N.T. = Not tested.

penicillin treatment eliminated streptomycin resistance (but not heavy-metal-ion resistance) from strain 649 wild (table I). Thus, although streptomycin resistance was not determined by a plasmid detectable as CCC DNA, it is nevertheless probably plasmid-determined because resistance is lost both on storage and after treatment with penicillins.

The molecular weight of the plasmid in strain 649 wild had been revised from 38×10^6 daltons to 35×10^6 daltons (Chopra *et al.*, 1973). The plasmid is referred to as *mir-r*.

Construction of the multi-resistant strain (649 MR)

Plasmids from other staphylococci were transferred to strain 649 by transduction; each plasmid was transduced by propagation of phage 88 on the donor strain except that determining neomycin resistance, which was transduced from a mitomycin C-induced lysate of strain 609. The properties of the derivatives of strain 649 are shown in table II. Most of these derivatives gave similar phage-typing patterns, except those that had acquired neomycin resistance, which showed a restriction in phage susceptibility. This was not due to lysogenisation with an entire prophage, because phage could not be recovered from the culture after mitomycin C treatment. A non-pigmented

derivative of strain 649N (649*pig*⁻) which also had reduced survival (Grinsted and Lacey, 1973*b*) was also studied.

The molecular weights and numbers of copies per cell of strain 649 for most of the plasmids described here have already been determined (Chopra

TABLE II

Construction of a multi-resistant strain (649 MR) from strain 649 wild in vitro by transduction

Marker*	Phage-typing pattern (RTD)	Source of plasmid
649*pig*$^-$	6/47/53/54/75/85/81(88)	...
649*pig*$^+$(649N)	6/47/53/54/75/85(88)	Naturally occurring in strain 649
649*pig*$^+$,*mir-r*	6/42E/47/53/54/75/85(88)	Naturally occurring in strain 649
649*pig*$^+$,*mir-r*,*str-r*(649 wild)	6/47/53/54/75/85/81(88)	Naturally occurring in strain 649
649*pig*$^+$,*mir-r*,*str-r*,*tet-r*	6/47/53/54/75/81(88)	Strain 13136
649*pig*$^+$,*mir-r*,*str-r*,*tet-r*,*pen*$^+$	6/47/53/54/55(88)	Strain 8325
649*pig*$^+$,*mir-r*,*str-r*,*tet-r*,*pen*$^+$,*chm-r*	6/42E/47/53/54/75/85(88)	Strain 8657
649*pig*$^+$,*mir-r*,*str-r*,*tet-r*,*pen*$^+$,*chm-r*, *neo-r*	6/75(88)	Strain 609
649*pig*$^+$,*mir-r*,*str-r*,*tet-r*,*pen*$^+$,*chm-r*, *neo-r*,*mtc-r*(649MR)	6/75(88)	Strain DU4916

* *pig*$^-$ = unpigmented; *pig*$^+$ = pigmented; *mir-r* = resistant to cadmium, arsenate and mercuric ions; *str-r* = to streptomycin; *tet-r* = to tetracycline; *pen*$^+$ = producer of penicillinase and resistant to erythromycin; *chm-r* = resistant to chloramphenicol; *neo-r* = to neomycin; *mtc-r* = to methicillin.
(88) = Experimental phage.

TABLE III

Frequency of transduction of four plasmids to strain 649N and derivatives of strain 649 containing several plasmids

Plasmid transduced	Recipient	Frequency of transduction*
tet-r	649N	$3{\cdot}5 \times 10^{-6}$
tet-r	649*mir-r*,*str-r*	$4{\cdot}0 \times 10^{-6}$
pen$^+$	649N	$6{\cdot}2 \times 10^{-7}$
pen$^+$	649*mir-r*,*str-r*,*tet-r*	$4{\cdot}1 \times 10^{-7}$
chm-r	649N	$3{\cdot}0 \times 10^{-8}$
chm-r	649*mir-r*,*str-r*,*tet-r*,*pen*$^+$	$4{\cdot}3 \times 10^{-8}$
neo-r	649N	$8{\cdot}6 \times 10^{-6}$
neo-r	649*mir-r*,*str-r*,*tet-r*,*pen*$^+$,*chm-r*	$6{\cdot}6 \times 10^{-6}$

See table II for abbreviations.
* Number of transductants per plaque-forming unit of phage.

et al., 1973). In addition, as we shall show later in this paper, the plasmid determining chloramphenicol resistance (derived from strain 8657) has a molecular weight of approximately $2{\cdot}9 \times 10^6$ daltons and exists as about 23 copies per 649 cell.

The frequency of transduction of single plasmids that determine resistance to tetracycline, neomycin, chloramphenicol, or penicillin and erythromycin was similar irrespective of whether or not the recipient already possessed other plasmids (table III). Thus, there is no evidence of plasmid incompatibility in the multi-resistant strain, which contains five plasmids existing as CCC DNA when they are present individually in strain 649N. These comprise the plasmids determining resistance to tetracycline (*tet-r*), neomycin (*neo-r*), chloramphenicol (*chm-r*), penicillin and erythromycin (pen^+) and metal ions (*mir-r*). In addition, strain 649MR possesses three other plasmids that do not exist as CCC DNA. These comprise the plasmids determining streptomycin resistance (*str-r*), methicillin resistance (*mtc-r*) and pigment and survival (pig^+).

The resistance of strain 649MR to several antibiotics was identical to that of derivatives of strain 649 harbouring the individual plasmids. Thus the MICs of chloramphenicol, neomycin, tetracycline, erythromycin, and streptomycin were respectively 200, 100, 200, 1000 and 100 μg per ml for the multi-resistant strain or respective derivatives.

Comparison of plasmid stabilities in the multi-resistant and single-plasmid derivatives of strain 649 grown at 42°C

Preliminary experiments indicated that resistance to neomycin, chloramphenicol, and erythromycin was unstable in strain 649MR. Accordingly, strain

TABLE IV

Plasmid loss from strain 649MR and from derivatives of strain 649N containing single plasmids after growth at 42°C

Plasmid	Host	Number of colonies tested	Number (and percentage) of colonies sensitive
neo-r	649*neo-r*	572	138 (24)
neo-r	649MR	561	85 (15)
pen^+	649pen^+	530	166 (32)
pen^+	649MR	535	202 (38)
tet-r	649*tet-r*	2,500	0
tet-r	649MR	3,250	0
mtc-r	N.T.	N.T.	N.T.
mtc-r	649MR	3,000	0
str-r	649*str-r*	2,000	0
str-r	649MR	1,850	0
chm-r	649*chm-r*	2,000	5 (0·25)
chm-r	649MR	1,250	226 (17·8)
pig^+	649N	2,300	7 (3)
pig^+	649MR	1,970	47 (24)

See table II for abbreviations.
N.T. = Not tested.

649MR was maintained at 4°C on nutrient agar containing 5 μg neomycin, 25 μg chloramphenicol, and 25 μg erythromycin per ml to select for these plasmids. Strains 649*neo-r*, 649*chm-r* and 649pen^+ were also maintained on media containing cadmium ions or the corresponding antibiotic. Strains of both 649MR and of 649 containing individual plasmids (in addition to that

determining pigment) were grown in antibiotic-free nutrient broth to a density of about 10^8 cocci per ml and then diluted to about 2×10^5 per ml in fresh pre-warmed broth before incubation overnight at 42°C. Each culture was then diluted and examined for loss of antibiotic resistance or pigment.

The frequency of loss of each marker from strain 649MR and the 649 derivatives containing single plasmids was similar except that chloramphenicol resistance and production of pigment were lost at higher frequencies from strain 649MR than from cultures of 649 *chm-r* and 649N respectively (table IV). Similar experiments at 37°C confirmed the instability of chloramphenicol resistance (table V).

TABLE V

Loss of chloramphenicol resistance from strains 649chm-r and 649MR after growth at 37°C (duplicate experiments)

Strain	Number of colonies tested	Number (and percentage) of colonies sensitive to chloramphenicol
(a) 649*chm-r*	204	4 (2)
(b) 649*chm-r*	403	3 (0·7)
(a) 649MR	106	35 (33)
(b) 649MR	221	60 (27)

See table II for abbreviations.

Transduction of markers from strain 649MR

Phage 88 was propagated on strain 649MR and then added to the recipient, strain 649 *pig*⁻ which contains no known plasmid. Transductants were selected on media containing single antibiotics and then replica plated on to a series of plates containing other drugs.

At least 800 transductants selected initially on media containing tetracycline, erythromycin, or neomycin showed no acquisition of other markers, including resistance to erythromycin, streptomycin, methicillin, cadmium ions, neomycin, tetracycline, and chloramphenicol, or production of pigment. These findings imply that (1) recombination between the dissimilar plasmids in strain 649MR is rare, and (2) simultaneous transduction of two separate plasmids is rare, though this was deliberately minimised by using a phage-to-cell ratio of 0·05.

The frequency of transduction of erythromycin, neomycin, and tetracycline resistance from strain 649MR was similar to that from strains carrying the plasmids individually, but the frequency of transduction of chloramphenicol resistance from strain 649MR was very low compared with the frequency from 649*chm-r* (table VI). This finding cannot be explained by loss of chloramphenicol resistance from strain 649MR during propagation of phage 88, because a low transduction frequency ($<10^{-10}$) was also obtained after phage 88 had been propagated on strain 649MR grown in the presence of 25 μg chloramphenicol per ml.

Thus, although strain 649MR shows full phenotypic resistance to chloramphenicol (see above), the plasmid specifying this resistance is less stable and less readily transduced from the multi-resistant cell than from cells containing this plasmid alone. These changes might have resulted from incompatibility between the " chloramphenicol plasmid " and one of the other plasmids present in the multi-resistant cell. To test this possibility, derivatives of strain 649 containing the " chloramphenicol plasmid " and one other plasmid were constructed and then examined for the stability of chloramphenicol resistance

TABLE VI

Frequency of transduction of markers from strain 649MR and 649N containing single plasmids to recipient strain 649pig⁻

Marker	Donor strain	Frequency of transduction*
str-r	649*str-r*	$<10^{-10}$
str-r	649MR	$<10^{-10}$
tet-r	649*tet-r*	$2{\cdot}0\times10^{-7}$
tet-r	649MR	$3{\cdot}5\times10^{-7}$
pen^{+}	649*pen*$^{+}$	$8{\cdot}0\times10^{-7}$
pen^{+}	649MR	$2{\cdot}2\times10^{-6}$
chm-r	649*chm-r*	$9{\cdot}2\times10^{-7}$
chm-r	649MR	$<10^{-10}$
neo-r	649*neo-r*	$4{\cdot}5\times10^{-7}$
neo-r	649MR	$4{\cdot}5\times10^{-7}$
mtc-r	N.T.	N.T.
mtc-r	649MR	$<10^{-10}$

See table II for abbreviations.
N.T. = Not tested.
* Number of transductants per plaque-forming unit of phage.

under conditions selective for the other plasmid. Chloramphenicol resistance was not unusually unstable in strains carrying any of the other plasmids (table VII). Although it has been impossible to construct a cell harbouring only the plasmids for chloramphenicol and methicillin resistance, incompatibility between these is unlikely because chloramphenicol resistance is relatively stable in another methicillin-resistant strain, no. 8657 (Lacey and Grinsted, 1973). In addition, chloramphenicol resistance was transduced at " normal " frequencies from each of the derivatives containing pairs of plasmids (table VII). Thus the instability and low transduction frequencies of chloramphenicol resistance in strain 649MR seem to be due to a non-specific effect.

Plasmid (CCC) DNA content of strain 649MR and other derivatives of strain 649

In the following it is assumed that plasmids that are not in a CCC form when present singly in strain 649 are also not present in this form in strain 649MR.

Assuming that the content of those plasmids detectable as CCC DNA was simply additive in strain 649MR, we would expect a value of 16–17% CCC plasmid to total DNA. This is based on data obtained for the individual

TABLE VII

Stability at 42°C of chloramphenicol resistance in derivatives of strain 649 harbouring the plasmid for chloramphenicol resistance and one other plasmid. The transduction frequencies of chloramphenicol resistance from these derivatives to strain 649 pig⁻ are also shown

Culture phenotype	Number of colonies examined	Number sensitive to chloramphenicol	Transduction frequency of chloramphenicol resistance
649*chm-r*	292	6	$1{\cdot}2\times10^{-7}$
649*mir-r,chm-r*	250	1	$9{\cdot}7\times10^{-8}$
649*str-r,chm-r*	308	2	$5{\cdot}1\times10^{-8}$
649*tet-r,chm-r*	260	7	$3{\cdot}6\times10^{-8}$
649*pen⁺,chm-r*	320	9	$8{\cdot}3\times10^{-8}$
649*neo-r,chm-r*	295	13	$8{\cdot}9\times10^{-8}$

See table II for abbreviations.

plasmids in strain 649 (table VIII, and Chopra *et al.*, 1973). However, the percentage of CCC plasmid to total DNA in strain 649MR was only 9·7 (table VIII).

TABLE VIII

Proportion of plasmid (CCC) DNA to total DNA in derivatives of strain 649

Culture phenotype	Plasmid DNA as percentage of total DNA (number of estimations)
649MR	9·7±0·8 (6)
649*mir-r,tet-r*	10·0±1·2 (4)
649*neo-r,chm-r*	3·0±1·3 (4)
649*pen⁺,mir-r*	5·3±1·6 (12)
649*mir-r,chm-r*	8·7±0·9 (3)
649*chm-r*	1·7±0·3 (4)

See table II for abbreviations.

To test the possibility that the reduction in plasmid DNA was due to interaction or interactions between specific plasmids, derivatives of strain 649 that contained two plasmids (present as CCC DNA) were examined for CCC DNA content. For most of the derivatives the plasmid DNA content was similar to that predicted (table VIII). However, strain 649*mir-r, pen⁺* contained less CCC DNA than expected—5·3% instead of about 10%. Although these 649*mir-r, pen⁺* derivatives were constructed by transduction of the *pen⁺* plasmid into strain 649*mir-r* in the presence of 10^{-4}M cadmium acetate, it was possible that the incoming *pen⁺* plasmid (which also carries the genes for

metal-ion resistance) had displaced the resident *mir-r* plasmid. Sedimentation of plasmid DNA from 649*mir-r*, *pen*$^+$ derivatives in sucrose gradients showed two major peaks, one of which co-sedimented with plasmid DNA from strain 649*mir-r*. This indicates that the *mir-r* plasmid was not eliminated by the incoming *pen*$^+$ plasmid and that the 649*mir-r*, *pen*$^+$ derivatives contained both plasmids, the phenotypic markers of penicillinase production and erythromycin resistance demonstrating the presence of the *pen*$^+$ plasmid. However, it was impossible to calculate the relative proportions of each plasmid from the sucrose-gradient profiles, because the open circular form of the *mir-r* plasmid co-sedimented with the CCC form of the *pen*$^+$ plasmid.

Further evidence for interaction between the *mir-r* and *pen*$^+$ plasmids was obtained by examination of segregants that had lost erythromycin resistance after growth at 42°C. Ten colonies from each of ten different 649*mir-r*, *pen*$^+$ clones that had lost erythromycin resistance were tested. All 100 were penicillinase negative, but eight were sensitive to metal ions; two of the derivatives that had retained metal-ion resistance were examined further. Each contained a single plasmid of about 25×10^6 daltons which comprised about 3% of the total DNA. It seems, therefore, that this plasmid has probably arisen from recombination between the *pen*$^+$ and *mir-r* plasmids, the *mir-r* plasmid having a MW of 35×10^6 daltons and the *pen*$^+$ 20×10^6 daltons. The high incidence of co-ordinate loss of the two plasmids from the cell also indicates some type of interaction.

Thus the presence of the *mir-r* and *pen*$^+$ plasmid within the same cell results in (1) a reduction in total plasmid DNA, (2) probable recombination between the plasmids, (3) their simultaneous loss. However, the molecular events underlying these changes are not known.

The apparent deficit (6–7%) in the quantity of plasmid DNA in strain 649MR can therefore be accounted for by (1) interaction between the *mir-r* and *pen*$^+$ plasmids resulting in loss of 4–5% plasmid DNA, (2) another 1·5–2% can probably be accounted for by a reduction in the numbers of the chloramphenicol plasmid. This is based on the observations that (*a*) chloramphenicol resistance is unstable in 649MR and (*b*) that it has a low frequency of transduction from this strain. However, it seems impossible to prove that the " chloramphenicol plasmid " exists only in a few copies per cell because of its similarity in size to the " tetracycline plasmid ".

Significance of plasmids present in multiple copies

Two suggestions can be made to explain the presence of plasmids in multiple copies per cell, largely from study of plasmids in the Enterobacteriaceae: that it serves (1) to ensure distribution to daughter cells on cell division, or (2) to provide a system permitting survival in the presence of high antibiotic concentrations, because it seems that each plasmid copy is expressed (Kontomichalou, Mitani and Clowes, 1970; Rownd, Kasamatsu and Mickel, 1971).

Although the instability of chloramphenicol resistance in strain 649MR could be explained by a reduction in the number of plasmid copies per cell,

i.e., a concept in line with assertion (1) above, this cannot be the only explanation for multiple plasmid copies, because there was no correlation between the number of plasmid copies per cell and marker stability (table IX). Lack of correlation is most striking for those plasmids coding for neomycin and chloramphenicol resistance (present in about 15 and 20 copies per cell respectively).

We investigated possibility (2), that the number of plasmid copies might increase in the presence of antibiotic, by incubating strain 649*mir-r*, *tet-r* with and without 10 μg tetracycline per ml in the presence of radioactive thymidine. The plasmid DNA was then isolated and analysed on sucrose gradients and

TABLE IX

Plasmid loss from derivates of strain 649 containing single plasmids after growth for 10 generations at 37°C. The number of copies per cell for each plasmid is also shown

Plasmid	Loss per cell generation	Plasmid copies per cell*(range)
649*mir-r*	$1{\cdot}3\times10^{-4}$	4–7
649*pen*$^+$	$2{\cdot}0\times10^{-4}$	6–15
649*neo-r*	25×10^{-4}	9–16
649*chm-r*	$7{\cdot}5\times10^{-4}$	19–27
649*tet-r*	$<10^{-6}$	31–47

See table II for abbreviations.

* Except for data on the chloramphenicol plasmid these figures are from Chopra, Bennett and Lacey (1973).

the proportion of the " *tet-r* plasmid " to the " *mir-r* plasmid " calculated; because these plasmids are very different in size they can easily be resolved on sucrose gradients. There was no difference in the proportion of each plasmid whether or not the culture was grown in the presence of tetracycline. We were also unable to isolate stable mutants that showed a significantly increased resistance to tetracycline, neomycin or chloramphenicol by plating derivatives of strain 649 on media containing amounts of antibiotic just higher than the MIC. There is thus no evidence that the presence of these plasmids in multiple copies provides a " flexible " form of resistance to the antibiotic in question.

The finding that strain 649MR probably contained only a few copies of the chloramphenicol plasmid, yet was phenotypically fully resistant to chloramphenicol, suggested that only one or a very few plasmid copies may determine resistance. Further evidence for this was found by showing that several plasmids were expressed immediately after transduction into a recipient. Phage 88 was propagated on strain 649 derivatives, sterilised, and then added to the recipient, strain 6936, at 37°C at a multiplicity of infection of 0·05/1. After 1 min., sodium citrate (to 0·03M) was added. The cultures were immediately cooled to 0°C, the bacteria harvested by centrifugation and then plated on agar containing 15 μg neomycin per ml, 25 μg chloramphenicol per ml, or

10^{-4}M cadmium acetate. Controls included recipients treated as above, but incubated for 2 hours to allow for phenotypic expression before plating.

For the *pen*$^{+}$, *neo-r* and *chm-r* plasmids, the number of transductants obtained from immediate plating was between 12 and 18% of the number after 2 hours. The size of the *pen*$^{+}$ plasmid (20×10^{6} daltons) seems to exclude the possibility of multiple-copy transduction.

These findings suggest that one or a very few copies of each of these plasmids can express high-level resistance. If this is so, the presence of staphylococcal plasmids as multiple copies is not necessary for full phenotypic expression of resistance. It seems more likely that the multiple-plasmid copies are related to transduction frequency; the plasmid coding for tetracycline resistance is present in the largest number of copies and can be transferred at highest frequency (Lacey, 1971; Chopra *et al.*, 1973). Thus the frequency of plasmid transfer seems to be related to (1) the nature of the phage in the donor, (2) the size of the plasmid in the donor (Chopra *et al.*, 1973), (3) the number of copies of that plasmid, and (4) the recipient.

Discussion

An artificially constructed strain was chosen for study because (*a*) certain antibiotic-resistant genes are difficult to cure from naturally occurring multi-resistant strains and (*b*) use could be made of plasmids that had already been characterised.

The strain constructed *in vitro* probably contained eight naturally occurring plasmids. Although the multi-resistant cell could maintain these plasmids simultaneously, the plasmids that determined resistance to chloramphenicol and the production of pigment were less stable in the presence of the other plasmids than when in the host singly. Loss of pigment under natural conditions would result in sensitivity to desiccation (Grinsted and Lacey, 1973*b*) and this might put the multi-resistant isolates at a disadvantage in comparison with sensitive organisms. The instability of chloramphenicol resistance in the multi-resistant strain, if it occurred under natural conditions and was due to a decrease in the number of plasmid copies, might not only result in daughter cells lacking chloramphenicol resistance, but might prevent spread of the resistance to other cells by transduction. An interaction between the penicillinase-and-erythromycin plasmid and the plasmid determining heavy-metal-ion resistance also occurred, resulting in a decrease in the number of plasmid copies per cell. These findings suggest that the amount of extrachromosomal DNA that a cell can maintain is limited, even though specific pairs of plasmids may be otherwise compatible. This must give some hope that strains will not acquire new plasmids in the future *ad infinitum*.

Significant co-transduction between dissimilar plasmids could not be demonstrated, suggesting that recombination may be infrequent, except between plasmids containing common markers. The potential for recombination under natural conditions is therefore probably confined to the " penicillinase plasmids ". Because these plasmids may also harbour the genes for

resistance to erythromycin (Mitsuhashi, *et al.*, 1965) or fusidic acid (Lacey and Rosdahl, 1973), however, there is a danger that a single plasmid might evolve that harbours all three resistance genes.

Few analyses of naturally occurring multi-resistant staphylococci have been made. However, in one strain (FAR1; Lacey and Grinsted, 1973), there are probably six distinct plasmids coding for (1) penicillinase production and fusidic-acid resistance, (2) resistance to methicillin, (3) to neomycin, (4) to tetracycline, (5) to erythromycin, and (6) production of pigment (R. W. Lacey, unpublished observations). Antibiograms of other multi-resistant strains suggest that few harbour more than six plasmids. This supports our finding that a constructed strain cannot maintain limitless amounts of extrachromosomal DNA.

SUMMARY

A multi-resistant strain of *Staphylococcus aureus* (no. 649MR) containing eight plasmids was constructed *in vitro*. Chloramphenicol resistance and pigment production were less stable in strain 649MR than when present singly in strain 649. Chloramphenicol resistance could not be transduced from strain 649MR to other strains. Recombination occurred between only two of the plasmids in strain 649MR; these determined (1) metal-ion resistance only (*mir-r*) and (2) resistance to erythromycin and production of penicillinase in addition to metal-ion resistance (*pen*$^+$).

The plasmid-DNA content of the multi-resistant strain was only 60% of that expected from analysis of the individual plasmids in the host strain, and this could be only partially explained by interaction between the *mir-r* and *pen*$^+$ plasmids.

These findings suggest that under natural conditions the number of plasmids that a staphylococcal cell can maintain will be limited and that recombination will be confined to the " penicillinase plasmids ". The mechanism or mechanisms that limit the number of plasmids within the cell are not known, but factors additional to plasmid incompatibility must operate.

The existence of plasmids as multiple copies cannot be accounted for entirely as a means for ensuring their distribution at cell division, nor as provision of maximum levels of antibiotic resistance, but may be related to transfer between cells by transduction.

We thank Mrs Evelyn Lewis for technical assistance. Financial support was provided by a grant from the Medical Research Council to Professor M. H. Richmond.

REFERENCES

ANNEAR, D. I. AND GRUBB, W. B. 1972. Linked and unstable resistance to kanamycin and penicillin, and diffusible pigment production in an isolate of *Staphylococcus aureus*. *J. med. Microbiol.*, **5**, 109.

BULGER, R. J. AND SHERRIS, J. C. 1968. Decreased incidence of antibiotic resistance among *Staphylococcus aureus*: a study in a university hospital over a 9-year period. *Ann. intern. Med.*, **69**, 1099.

CHOPRA, I., BENNETT, P. M. AND LACEY, R. W. 1973. A variety of staphylococcal plasmids present as multiple copies. *J. gen. Microbiol.*, **79**, 343.

DORNBUSCH, K., HALLANDER, H. O. AND LÖFQUIST, F. 1969. Extrachromosomal control of methicillin resistance and toxin production in *Staphylococcus aureus*. *J. Bact.*, **98**, 351.

DYKE, K. G. H., PARKER, M. T. AND RICHMOND, M. H. 1970. Penicillinase production and metal-ion resistance in *Staphylococcus aureus* cultures isolated from hospital patients. *J. med. Microbiol.*, **3**, 125.

GRINSTED, J. AND LACEY, R. W. 1973*a*. Genetic variation of streptomycin-resistance in clinical strains of *Staphylococcus aureus*. *J. med. Microbiol.*, **6**, 351.

GRINSTED, J. AND LACEY, R. W. 1973*b*. Ecological and genetic implications of pigmentation in *Staphylococcus aureus*. *J. gen. Microbiol.*, **75**, 259.

KONTOMICHALOU, P., MITANI, M. AND CLOWES, R. C. 1970. Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. *J. Bact.*, **104**, 34.

LACEY, R. W. 1971. Transfer of tetracycline-resistance between strains of *Staphylococcus aureus* in mixed cultures. *J. gen. Microbiol.*, **69**, 229.

LACEY, R. W. AND CHOPRA, I. 1972. Evidence for mutation to streptomycin resistance in clinical strains of *Staphylococcus aureus*. *J. gen. Microbiol.*, **73**, 175.

LACEY, R. W. AND GRINSTED, J. 1973. Genetic analysis of methicillin-resistant strains of *Staphylococcus aureus*; evidence for their evolution from a single clone. *J. med. Microbiol.*, **6**, 511.

LACEY, R. W. AND ROSDAHL, V. T. 1973. An unusual " penicillinase plasmid " in *Staphylococcus aureus*: evidence for its transfer under natural conditions. *J. med. Microbiol.*, **7**, 1.

MITSUHASHI, S., HASHIMOTO, H., KONO, M. AND MORIMURA, M. 1965. Drug resistance of staphylococci. II. Joint elimination and joint transduction of the determinants of penicillinase production and resistance to macrolide antibiotics. *J. Bact.*, **89**, 988.

RICHMOND, M. H. 1969. Extrachromosomal elements and the spread of antibiotic resistance in bacteria. *Biochem. J.*, **113**, 225.

ROWND, R., KASAMATSU, H. AND MICKEL, S. 1971. The molecular nature and replication of drug resistance factors of the enterobacteriaceae. *Ann. N.Y. Acad. Sci.*, **182**, 188.

RUSH, M. G., GORDON, C. N., NOVICK, R. P. AND WARNER, R. C. 1969. Penicillinase plasmid DNA from *Staphylococcus aureus*. *Proc. natn. Acad. Sci. USA.*, **63**, 1304.

WILLIAMS, R. E. O. AND RIPPON, J. E. 1952. Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg. Camb.*, **50**, 320.