Transduction and Elimination of Resistance Determinants in Methicillin-Resistant *Staphylococcus aureus*

FRITZ H. KAYSER, JÜRG WÜST, AND PETER CORRODI

Institute of Medical Microbiology, School of Medicine, University of Zurich, Zurich, Switzerland

Elimination and transduction of drug resistance was examined in methicillin-resistant strains of *Staphylococcus aureus*. Irreversible spontaneous loss and "curing" by aging of cultures and by treatment with ethidium bromide indicated that the determinants for penicillinase production and chloramphenicol resistance, and probably also for neomycin resistance, were located extrachromosomally. On the other hand, the determinants of resistance to erythromycin, streptomycin, tetracycline, and methicillin could not be eliminated by acridines, ethidium bromide, rifampin, sodium dodecyl sulfate, ultraviolet (UV) irradiation, growth at 43.5 C, aging of cultures, or combinations of these treatments. The stimulation of transduction frequency by UV irradiation of phage in the case of the stable markers, but not in the case of the unstable ones, supported further the hypothesis of chromosomal location of the markers of methicillin, erythromycin, tetracycline, and streptomycin resistance and extrachromosomal location of the determinants for penicillinase production and chloramphenicol resistance. Neomycin resistance could not be transduced. Joint elimination and co-transduction of the determinants for penicillinase production and resistance to chloramphenicol and neomycin were not observed, indicating the location of these markers on separate, mutually compatible plasmids. Co-transduction of chromosomal resistance determinants was usually less than 1%, which makes the location of these genes in a circumscribed area of the chromosome improbable.

In the past decade, it has become more and more evident that bacteria may possess, besides the chromosome, additional linkage groups of genes which are referred to as extrachromosomal genetic elements or plasmids (28). Plasmids often carry determinants of resistance against chemotherapeutic agents. They have been found mainly among members of the family *Enterobacteriaceae* and the genus *Staphylococcus*. In *Staphylococcus*, the determinants for production and regulation of the enzyme penicillinase have been found to be contained in the penicillinase plasmid (27). In some strains, the determinants of resistance against tetracycline (*tet;* 22), chloramphenicol (*cml;* 9), erythromycin (*ery;* 23), neomycin (*neo;* 21), and probably also against streptomycin (*str;* 25), fusidic acid (13), and bacitracin (8) may be located extrachromosomally.

Since the introduction of penicillinase-resistant β-lactam antibiotics into therapy almost 10 years ago, methicillin-resistant (Mth^R) staphylococci have been increasingly isolated in different countries of the world (3, 11, 16, 31, 32). In the Zurich area, approximately 20% of staphylococci isolated from hospitalized patients now exhibit Mth^R, and most of the isolated organisms show similar phage-typing patterns. Multiple antibiotic resistance in these bacteria is the rule; however, chloramphenicol resistance (Cml^R) and neomycin resistance (Neo^R) are not consistently observed. Epidemiological data led us to conclude that the increase of Mth^R in staphylococci might be due to the selection of only a few preformed, multiply resistant hospital strains. The absence of Cml^R and Neo^R could then be the result of spontaneous loss of these unstable genetic markers. Therefore, a study of the genetics of Cml^R and Neo^R in Mth^R *S. aureus* strains was begun, and the investigation was extended to other drug-resistance markers.

MATERIALS AND METHODS

Organisms. Mth^R *S. aureus* strains, indicated by "E," were isolated from clinical sources in Zurich.

Strain E142, phage-type 7/47/54/75, was used in most of the experiments. This strain is resistant to penicillin, streptomycin, tetracycline, erythromycin, chloramphenicol, neomycin, the sulfonamides, and methicillin. Similar group III patterns of lysis and antibiotic-resistance patterns are found in most of the Mth^R strains in the Zurich area. Strains E142Pen^S, E142-Pen^{S}Neo^S, and E142Pen^{S}Neo^{S}Cml^S are susceptible variants which probably have lost the extrachromosomally located resistance markers. Strain 294 was supplied by M. T. Parker (England), strain 307 by P. Rountree (Australia), strain 4916 by K. Dornbusch (Sweden), and strain C$_5$ by S. Cohen (USA). Strains 8325(PEN$_{524}$) and N8325(PEN$_{524}$) were supplied by R. P. Novick. Strain 8325(PEN$_{524}$) was prepared by introducing the penicillinase plasmid (PEN) of 524 into 8325, the propagating strain of phage 47. N8325(PEN$_{524}$) is its plasmid-cured derivative. Strain 545 is a penicillinase-producing, cadmium-resistant, wild strain of *S. aureus* isolated in Zurich.

Staphylococcal bacteriophages of the International Series were supplied by the Swiss National Centre for Staphylococcus Phage-Typing. Bacteriophage typing was performed according to Blair and Williams (6). Transductions were carried out with phage 80 of the International Typing Series. Phage 80, which exhibited a low efficiency of plating on strain E142, was adapted to E142 by serial single-plaque passages. It is not known whether phage 80 grown on E142 was mutant or host modified.

Determination of drug resistance. Drug resistance was determined either with the standardized single-disc test of Bauer et al. (4) or in an agar dilution test with the use of an inoculum of approximately 10^4 cells which were applied to the agar with the help of a simplified Steers replicator. Resistance to erythromycin was examined in uninduced cultures and in cultures induced with 0.1 µg of erythromycin/ml for 2 hr at 37 C (19). Resistance to inorganic ions was determined according to Novick and Roth (30).

Examination of genetic stability. Spontaneous loss of antibiotic resistance was examined in cultures grown overnight at 37 C in Brain Heart Infusion (BHI) broth. Cultures treated with acridine dyes (14), ethidium bromide (7), rifampin (17), sodium dodecyl sulfate (34), ultraviolet (UV) irradiation (23), growth at 43.5 C (22), or storage at 20 C for 20 days were subjected to short, controlled exposure to ultrasound, appropriately diluted, and spread onto BHI agar plates, containing 0.01 µg of erythromycin/ml. Master plates with 100 to 200 colonies were replicated to BHI agar containing the following amounts of drugs per milliliter of medium: streptomycin, 25 µg; tetracycline, 12.5 µg; neomycin, 12.5 µg; chloramphenicol, 12.5 µg; erythromycin, 5 or 10 µg; and 6 × 10^{-4} M cadmium acetate. Methicillin or oxacillin was used in doses which were twice the concentration of the "basic resistance" (5) of the strains under study (usually 6.25 to 12.5 µg/ml). In some experiments, penicillinase-negative (Pen^S) colonies were detected directly on the master plates according to Perret's slightly modified iodide method (18). Loss of drug resistance in colonies that failed to replicate was confirmed by streaking suspected clones on new drug-containing agar. Joint elimination of other characters including Pen^R (18), as well as resistance to inorganic salts, was examined subsequently.

Kinetics of loss of resistance markers. Strains under study were grown for 24 hr on BHI agar containing penicillin (1 unit/ml), neomycin (12.5 µg/ml), or chloramphenicol (12.5 µg/ml). The cells were harvested and washed with saline, subjected to short ultrasound treatment, and inoculated into BHI broth at an initial absorbance of 0.02 to 0.05 at 550 nm (Spectronic-20 colorimeter). The cultures were incubated with shaking at 37 C, and at appropriate time intervals samples were withdrawn, cooled to 4 C, and subjected to controlled exposure to ultrasound. The absorbance was measured at 550 nm, and viable counts were performed on BHI agar. The percentage of antibiotic-susceptible cells in the samples was determined by plating 100 to 200 cells onto 30 to 50 BHI master plates. After growth at 37 C, the colonies were replicated on drug-containing agar and retested for loss of resistance as described above.

Transduction procedure. Transduction and propagation of phages were carried out in a nutrient broth or agar consisting of the following ingredients per liter: nutrient broth (BBL), 20 g; glucose, 1 g; NaCl, 8.5 g; and CaCl$_2$·2H$_2$O, 0.588 g; 1.5% agar for plates and 0.7% agar for the soft-agar technique were added to the broth when necessary. Propagation of transducing phage was performed according to the soft-agar technique (33). Transducing lysates were usually UV-irradiated until the number of plaque-forming units was reduced to 90%. Recipient organisms were selected from 50 strains differing in phage type and antibiotic-resistance pattern. Most competent recipients belonged to phage group III. Sometimes drug-susceptible variants of the donor strains were used as receptors. Recipient organisms were grown with shaking until the end of logarithmic growth. Amounts of 1 ml of the recipient culture and 1 ml of phage were mixed and incubated with gentle shaking at 37 C. Under these conditions, 80% of the phages will usually be absorbed after 10 min of incubation. In most experiments, the phage to bacteria ratio was from 0.5 to 1, because transduction frequency (expressed as transductants per input phage particle in the unirradiated sample) was highest in this range. After 30 min of incubation, further absorption of phage in the mixture was halted by adding 0.5% sodium citrate, followed by centrifuging at 4 C and two washings with BHI broth plus 0.5% sodium citrate (BHI/citrate). The pellet was resuspended in 0.5 ml of BHI/citrate broth, mixed undiluted or in appropriate dilutions with 2.5 ml of BHI soft agar, and plated onto BHI agar containing the following amounts of drugs per milliliter: tetracycline, 12.5 µg; chloramphenicol, 12.5 µg; and 6 × 10^{-4} M cadmium acetate. In the case of selection of Ery^R transductants, the resuspended pellet was subjected to subinhibitory concentrations of erythromycin (0.01 µg per ml of BHI/citrate broth) for 30 min at 37 C before selection was carried out on a plate containing 10 µg of erythromycin. In selecting Str^R transductants, the pellet was resuspended in saline plus sodium citrate; 2.5 ml of soft agar in saline was added, and the mixture was

plated on Y-agar (20) containing 25 μg of streptomycin/ml. The plates were incubated at 37 C for 48 hr. Transfer of resistance in developing colonies was confirmed by restreaking onto drug-containing agar and by determination of phage type. Co-transduction of other drug-resistance markers was examined by replicating transductant colonies onto drug-containing agar plates.

In transduction of Mth^R, the method of Cohen and Sweeney (10) was strictly followed.

In experiments in which phenotypic lag was examined, the washed bacteria of the transduction mixture were reincubated in 7 ml of BHI/citrate broth at 37 C for different periods of time. After centrifugation, selection was carried out as described above. An increase in frequency of transduction by a factor of 5 or more was considered as "phenotypic lag."

RESULTS

Elimination of resistance markers. Table 1 summarizes the stability of antibiotic resistance in two strains of the most frequently occurring types of Mth^R *S. aureus* in the Zurich area. Of 5,000 ($<0.02\%$) to 20,000 ($<0.005\%$) colonies examined loss of resistance to methicillin, erythromycin, streptomycin, and tetracycline was not observed. In the case of Mth^R, five additional strains, two of which were Pen^S variants, were examined. Spontaneous or induced instability was not found when 5,000 to 20,000 colonies were examined. Pen^R was shown to be due to penicillinase production. The determinant for this resistance, as well as those for Cml^R and Neo^R, on the other hand, proved to be spontaneously unstable (Table 2). Storage of broth cultures at room temperature was the only treatment which significantly increased the frequency of loss of all three markers. Growth at 43.5 C did not have any effect, and treatment with ethidium bromide increased only the loss of penicillinase.

TABLE 1. *Stability of antibiotic resistance in Mth^R S. aureus strains E142 and E420*

Treatment	Frequency of loss of resistance markers[a]
None	<0.01
Acridines	<0.01
Ethidium bromide	<0.005
Rifampin	<0.02
Sodium dodecyl sulfate	<0.02
UV irradiation	<0.02
Growth at 43.5 C	<0.005
Storage (20 days, 20 C)	<0.005
Ethidium bromide + growth at 43.5 C	<0.005

[a] Percent loss per 18-hr culture of the markers for Mth^R, Ery^R, Str^R, and Tet^R. For loss of the Mth^R marker, the additional strains E208, E8, 9997P$^-$ (Munich, Germany), W$_1$P$^-$ (England), and 4916 (Sweden) were examined. Strains 9997P$^-$ and W$_1$P$^-$ were Pen^S variants of Mth^R strains.

In the case of the acridine dyes, a significant increase in the frequency of loss of the determinants for Cml^R and Neo^R seemed to be observed. A careful analysis of this effect, however, demonstrated that acriflavine acted merely as a selective agent for susceptible variants. The kinetics of growth and occurrence of Neo^S cells under the influence of 4 μg of acriflavine/ml in strain E142 is shown in Fig. 1. After an initial increase in the number of viable cells, extensive killing takes place, whereas Neo^S variants continue to multiply. After 13 hr of incubation, the percentage of Neo^S cells has increased from 0.2% at the beginning of growth to 9%. One would expect that at this time the viable population would be comprised of 100% Neo^S cells. The reason that this is not so is because of acriflavine-resistant cells of E142, which are observed after 7

TABLE 2. *Spontaneous and induced loss of antibiotic resistance in Mth^R S. aureus strain E142*

Treatment	Frequency of loss[a] of the markers for		
	Pen^R	Cml^R	Neo^R
None	0.11	0.05–0.07	0.7–2.1
Acridines	0.03	0.27[b]	5.9–98.4[c]
Ethidium bromide	10.8–55.8[d]	0.08	1.9–2.1
Growth at 43.5 C	0.06	0.05	1.5–2.1
Storage (20 days, 20 C)	1.4–3.8[e]	0.3–0.6[f]	18.3–37.5[g]

[a] Percent per 18-hr culture.
[b] $X^2 = 4.4$; $P = 0.05$.
[c] $X^2 = 17.1$; $P < 0.001$.
[d] $X^2 = 370.1$; $P < 0.001$.
[e] $X^2 = 40.5$; $P < 0.001$.
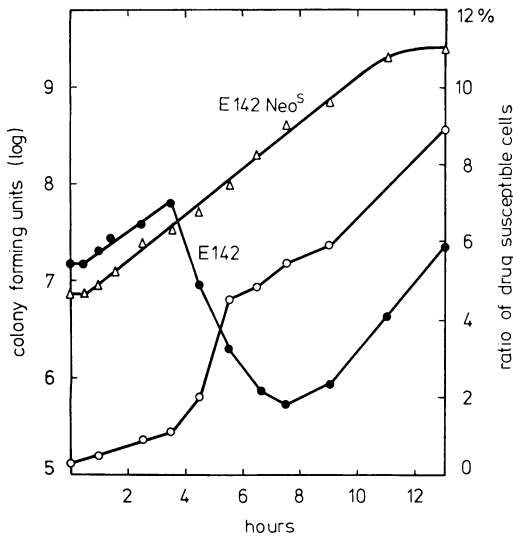[f] $X^2 = 8.5$; $P = 0.01$.
[g] $X^2 = 79.1$; $P < 0.001$.

FIG. 1. *Kinetics of growth and appearance of Neo*[S] *cells under the influence of acriflavine (4 μg/ml; BHI broth). Symbols:* △, *viable counts, E142Neo*[S] *(log);* ●, *viable counts, E142 (log);* ○, *ratio of neomycin-susceptible cells (%).*

hr of incubation and which multiply at a rather fast rate in the acriflavine broth. Probably these are acriflavine-resistant mutants of E142. At lower concentrations of acriflavine (1, 2, or 3 μg/ml), the parent is not killed but grows at a slower rate than susceptible clones (Fig. 2). This small selective effect can produce the apparent phenomenon of an increased frequency of loss under the influence of acridine dyes.

Despite the probable failure of acridines in elimination, the extrachromosomal location of the three unstable markers was suspected, owing to the above-mentioned effects of ethidium bromide and storage, and because reversions to resistance in susceptible variants did not occur. Among 5 × 10^{9} cells, no revertants to Pen[R] or Cml[R] were observed. Although neomycin-resistant mutants occurred at a frequency of 5 × 10^{−7} per cell per generation in strain E142Neo[S], the frequency was clearly lower than that of spontaneous loss of the marker in E142 (2 × 10^{−4} per cell per generation). Furthermore, the phenotypic characters of these mutants, such as colonial morphology, pigmentation, or growth rate, were markedly different from those of the wild strain E142. Joint loss of the determinants of Pen[R], Cml[R], or Neo[R] in a frequency higher than that for the spontaneous loss of the single markers was never observed. In the case of the determinants for resistance to chloramphenicol and neomycin, no other markers of resistance to chemotherapeutic agents or inorganic salts were lost. The penicillinase plasmid,

on the other hand, contained the markers for resistance to cadmium, mercury, arsenate, arsenite, and antimony. When the penicillinase trait was lost, concomitant loss of these markers was observed. Approximately 35% of penicillinase was found extracellularly.

Transduction of resistance markers. It has been observed in *Escherichia coli* (1) and in *Staphylococcus* (26) that UV irradiation of transducing lysates increases the transduction frequency of markers that are chromosomally located, whereas such a stimulation is not seen for plasmid markers (Fig. 3). Furthermore, the mechanisms of chromosomal drug resistance
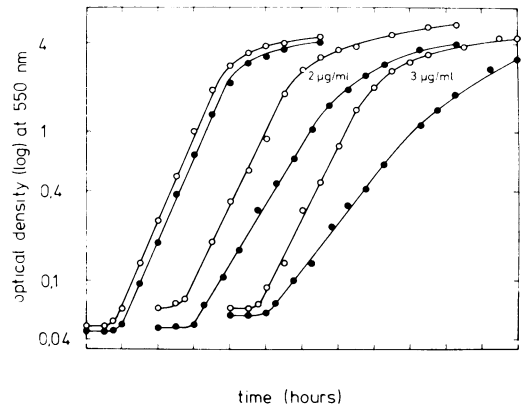

FIG. 2. *Growth curves of Staphylococcus aureus strain E142 and E142Cml*[S] *under the influence of subinhibitory concentrations of acriflavine. Symbols:* ○, *strain E142;* ●, *strain E142Cml*[S]; *first pair of curves, no acriflavine; second pair of curves, 2 μg of acriflavine per ml; third pair of curves, 3 μg of acriflavine per ml.*
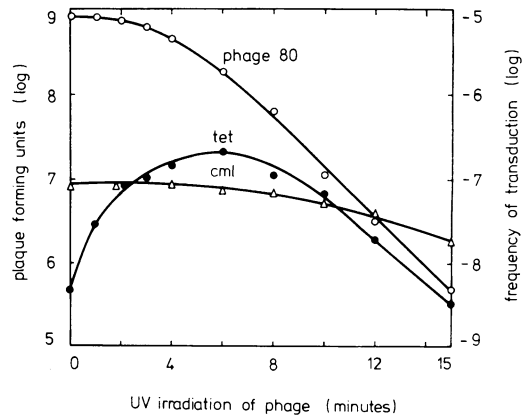

FIG. 3. *Transduction frequencies as a function of ultraviolet irradiation of transducing phage 80 lysate. Symbols:* ○, *survival of phage 80 (log);* ●, *transduction frequency of tet (log);* △, *transduction frequency of cml (log).*

often are such that a "phenotypic lag" after transfer of the resistance markers does occur. Extrachromosomal resistance, on the other hand, has always been found to be dominant over susceptibility. Under the conditions of transduction used in our experiments, a "phenotypic lag" would be indicated by an increase in frequency when selection is withheld for some time after the end of the transduction process (Fig. 4.)

Tables 3 and 4 summarize the results of transduction experiments. Transduction of Mth^R was achieved only with irradiated phage 80 at low frequency. With phage 29 or phage 53, as well as with induced lysates of lysogenic donor strains, we did not succeed in transferring Mth^R. Phenotypic lag before expression of resistance in the case of this marker was not observed. In transduc-

tion of Ery^R (dissociated type) and resistance to streptomycin and tetracycline, the transduction frequency was increased by UV irradiation as well as by the time allowed for phenotypic expression of resistance. In the case of the penicillinase plasmid and Cml^R, a stimulation by UV light or a phenotypic lag before expression of resistance was not observed. The marker for Neo^R could not be transduced with phage 80. We also failed to transduce this latter marker with UV-induced lysates of E142. Mixed cultivation of E142, E142Cm^S, E142Cml^{S}Pen^S, and 6 additional Mth^R donor strains with E142Cm^{S}Neo^{S}Pen^S and 10 additional receptor strains differing in phage type did not result in the finding of a competent system of transfer of the *neo* marker.

DISCUSSION

In *S. aureus*, many determinants of drug and chemical resistance have been found to be located extrachromosomally. In most of the strains examined, the markers for regulation and production of penicillinase, as well as for metal ion resistance, are located on a plasmid (27), but may also be connected with the chromosome in rare cases (2). In some strains resistance to tetracycline (22), neomycin (21), chloramphenicol (9), and erythromycin (23) has been found to be plasmid-linked, and this may be true also for resistance to streptomycin (25), bacitracin (8), and fusidic acid (13).

However, extrachromosomal drug resistance in staphylococci is not encountered as frequently as in the *Enterobacteriaceae*. In Mth^R wild strains of *S. aureus* in Zurich, for instance, chromosome linkage of resistance markers is suggested in the case of Tet^R, Str^R, Ery^R, and Mth^R. For Mth^R, this seems to be true not only for strains endemic in Zurich but also for strains isolated in Australia,
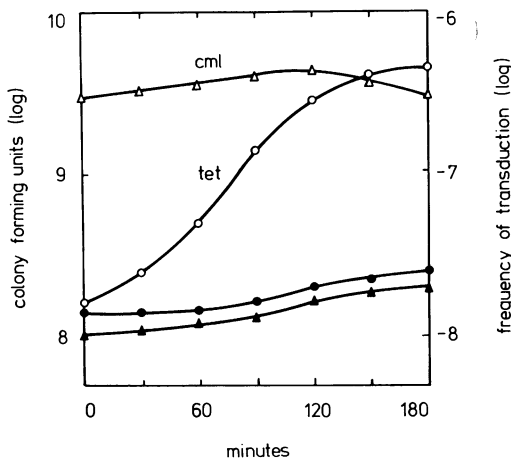

FIG. 4. *Influence of time allowed for phenotypic expression of resistance in transductants. Symbols:* ○, *transduction frequency of tet (log);* △, *transduction frequency of cml (log);* ●, ▲, *total viable counts during time allowed for expression of resistance (log).*

TABLE 3. *Transduction of Mth^R in S. aureus*

Donor	Transduction frequency[a]		Co-transduction[b]
	Before UV	After UV	
E109 (Zurich)	<4.3 × 10^{-10}	2.4 × 10^{-8}	ND[c]
E142	<5 × 10^{-10}	3.5 × 10^{-9}	0/31
E359	<7 × 10^{-10}	7 × 10^{-9}	ND
E480	<1.25 × 10^{-9}	3 × 10^{-8}	0/176
E536	<7 × 10^{-10}	2 × 10^{-9}	ND
294 (England)	<1.4 × 10^{-10}	7 × 10^{-9}	0/393
307 (Australia)	<4 × 10^{-10}	5 × 10^{-8}	0/263
C$_5$ (USA)	<7.5 × 10^{-10}	4 × 10^{-8}	ND
4916 (Sweden)	<1.5 × 10^{-9}	6.8 × 10^{-8}	ND

[a] Number of transductants per unirradiated plaque-forming unit.
[b] Co-transduction of the markers for Ery^R, Str^R, and Tet^R.
[c] Not done.

TABLE 4. *Transduction of antibiotic resistance in S. aureus strain E142*

Selective drug	Recipient	Transduction frequency[a]		Co-trans-duction	Phenotypic lag before expression of resistance
		Before UV	After UV		
Erythromycin	545	4×10^{-9}	7.6×10^{-8}	0/100	Observed
Streptomycin	545	1×10^{-9}	1×10^{-6}	0/200	Observed
Tetracycline	545	4.5×10^{-9}	1.9×10^{-7}	0/60	Observed
Cadmium[b]	N8325(P524)	2×10^{-7}	2.8×10^{-8}	0/100	Not observed
	E142Pen^{S}Cml^{S}Neo^S	1.5×10^{-6}	ND[c]	0/50	Not observed
Chloramphenicol	545	8.6×10^{-8}	7×10^{-8}	0/250	Not observed
	E142Pen^{S}Cml^{S}Neo^S	2.5×10^{-7}	ND	0/50	Not observed

[a] Number of transductants per unirradiated plaque-forming unit.

[b] Selection for the penicillinase plasmid was performed by selecting for resistance to cadmium acetate.

[c] Not done.

England, Germany (FRG), Sweden, and the USA (see Tables 1, 3, and 4). Our results are in contrast to the findings of Dornbusch and coworkers (12), who concluded from their experiments, that Mth^R resided on a plasmid. However, the stability of mth^R in strain 4916, as well as the stimulation of transduction by UV irradiation of phage, points to the opposite. Co-transduction of drug resistance in the strains examined was not observed. Therefore, a cluster of genes (15) concerned with resistance to tetracycline, erythromycin, streptomycin, and methicillin seems to be unlikely, but definite conclusions cannot be drawn. To date, the lack of a well-mapped chromosome and the absence of a conjugation system has prevented the genetic analysis of chromosomal drug resistance in *S. aureus*.

Although the criteria used to demonstrate the location of markers are not sufficient to prove chromosomal or extrachromosomal resistance beyond any doubt, it can be strongly suggested that resistance to chloramphenicol, neomycin, and penicillin is plasmid-linked. The penicillinase plasmid was constantly found in all of our isolates, suggesting a selective advantage of Pen^R, Mth^R staphylococci in the hospital environment. This plasmid could best be made more unstable by treatment with ethidium bromide and aging. Aging of cultures also increased the loss of Neo^R and Cml^R. A selective effect of this procedure on spontaneously occurring, plasmid-negative cells could be excluded. Spontaneous loss of Neo^R and Cml^R was connected with increased resistance to the acridine dyes. Treatment of a broth culture with acridines thus increased the number of susceptible variants, but did not increase instability. Whether a specific, plasmid-linked determinant of cellular susceptibility to acriflavine or a nongenetic variability is involved in this

phenomenon was not determined. The observation demonstrates clearly that in curing experiments much attention should be paid to possible selective effects of the agents used. Neo^R was not transducible in the strains examined with phage 80. Perhaps the plasmid is too large to be carried by this phage. Mixed cultivation of donor and receptor by the method of Lacey (21), who recently succeeded in transferring unstable Neo^R with this technique, did not result in successful transduction in our strains. However, the extreme, spontaneous instability of this marker points to an extrachromosomal location. Furthermore, laboratory-produced Neo^R chromosomal mutants differed in colonial morphology, pigmentation, and growth rate from Neo^R wild strains used in our study.

The failure to demonstrate joint elimination or co-transduction in the case of extrachromosomal drug resistance in Mth^R staphylococci indicates that the markers determining resistance to penicillin, chloramphenicol, and neomycin are located on different plasmids. These plasmids are mutually compatible and can be lost independently from each other. The observed absence of Neo^R or Cml^R, or of both resistances in some of our Mth^R staphylococcal isolates, which according to phage type and epidemiological data are supposed to belong to the same strain, thus can be explained by a rather frequent spontaneous loss of these markers under nonselective conditions.

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