GENETIC AND MOLECULAR CHARACTERISATION OF RESISTANCE DETERMINANTS IN METHICILLIN- RESISTANT *STAPHYLOCOCCUS AUREUS*

F. H. KAYSER, J. WÜST AND P. SANTANAM

*Institute of Medical Microbiology, University of Zürich,
PO Box CH-8028, Zürich, Switzerland*

PLATE IX

SINCE the first use of penicillinase-resistant β -lactam antibiotics in the therapy of staphylococcal infections more than 10 years ago, methicillin-resistant staphylococci have been increasingly isolated in different countries of the world (Borowzki, Kamienska and Rutecka, 1964; Courtieu *et al.*, 1964; Barrett, McGhee and Finland, 1968; Rountree and Beard, 1968; Jessen *et al.*, 1969; O'Toole *et al.*, 1970; Parker and Hewitt, 1970; Kayser and Wüst, 1974). Epidemiological data, including similar phage-typing patterns, led us to conclude that the problem of methicillin resistance (*meth^R*) in our area resulted from the selection of one or perhaps only a few pre-formed, methicillin-resistant strains (Kayser and Mak, 1972). Further evidence of the "single clone hypothesis" is presented by the antibiotic resistance patterns. With few exceptions, the more than 600 methicillin-resistant cultures isolated between 1969 and 1975 in Zürich exhibit resistance to tetracycline (*tet^R*), streptomycin (*str^R*), erythromycin (*ery^R*) and the sulphonamides (*sul^R*), and produce penicillinase (*pen^R*). Resistance to chloramphenicol (*cm^R*) and neomycin-kanamycin (*neo^R*), on the other hand, is not regularly observed. These antibiotypes suggested "genetic stability" of the determinants of *tet^R*, *str^R*, *ery^R*, *sul^R* and *pen^R* and "instability" of the markers of *cm^R* and *neo^R*.

Spontaneous and induced loss of *neo^R*, *cm^R* and *pen^R*, as well as the absence of enhancement of transduction frequency after ultraviolet (UV) irradiation of transducing lysates, indicated a location of these markers on separate, mutually compatible plasmids (Kayser, Wüst and Corrodi, 1972). On the other hand, "replicative autonomy" (Novick, 1969) could not be demonstrated for the markers for *tet^R*, *ery^R*, *str^R* and *meth^R*, suggesting their location as part of the bacterial chromosome.

In this paper, data indicating the "physical autonomy" of the genes of *pen^R*, *cm^R* and *neo^R* are presented. The three plasmids that carry these resistance markers are characterised. Furthermore, evidence is given that the

Received 11 Apr. 1975; accepted 22 Sept. 1975.

This publication is dedicated by the authors to Professor Dr Gian Töndury on the occasion of his 70th birthday.

markers for *meth^R*, *str^R*, *tet^R*, *ery^R* and *sul^R* may be integrated into the bacterial chromosome in the Zürich strains. Absence of "physical autonomy" of the determinant or determinants of *meth^R* in strains from Australia, England, Sweden and the USA also suggest their linkage to the bacterial chromosome. A preliminary report of part of this work was presented at the meeting of the Deutsche Gesellschaft für Hygiene und Mikrobiologie at Essen in 1973.

MATERIALS AND METHODS

Organisms. *Staphylococcus aureus* strains E109 and E142 were isolated from clinical material obtained in Zürich. Strain 294 was supplied by Dr M. T. Parker (England), strain 307 by Dr Phyllis Rountree (Australia), strain DU4916 by Dr Katherine Dornbusch (Sweden) and strain C₅ by Dr S. Cohen (USA). All these strains were naturally-occurring, methicillin-resistant organisms. *S. aureus* strain 8325 (PEN524) was received from Dr R. P. Novick (USA) and its derivative, strain N8325 (PEN524), was obtained by curing it of the penicillinase plasmid PEN524 with ethidium bromide (Bouanchaud, Scavizzi and Chabbert, 1969). *S. aureus* strains RN11 and RN1305 and *Escherichia coli* strain K12 J53 (R15) were kindly provided by Dr R. P. Novick and Dr Naomi Datta (England). These latter strains were the source of reference plasmids used for determining the molecular weight of the unknown plasmids.

Staphylococcus aureus bacteriophages of the International Series for Phage Typing were supplied by Silvana Corp., Millburn, NJ, USA. Bacteriophage typing was performed by the method of Blair and Williams (1961).

Determination of drug resistance. Drug resistance was determined either by the standardised single-disk test recommended by the Department of Health, Education and Welfare, Food and Drug Administration (1972), or in an agar-dilution test with the use of an inoculum of c. 10⁴ cells, which were applied to the agar with the help of a simplified Steers replicator. Resistance to erythromycin was usually examined in cultures induced with erythromycin 0.1 µg per ml for 2 h at 30°C (Kono, Hashimoto and Mitsuhashi, 1966). Resistance to inorganic ions was determined by the method of Novick and Roth (1968).

Transduction procedures. Transduction was performed with phage 80 of the International Series. This phage exhibited a low efficiency of plating on most donor strains and therefore was adapted to the donors by serial single-plaque passages. It is not known whether phage 80 grown on these donors was mutant or host-modified. Transduction of antibiotic resistance from donors to strain N8325 (PEN524) was performed as described by Kayser *et al.* (1972).

Elimination procedures. Spontaneous or induced loss of antibiotic resistance was examined as described by Kayser *et al.* (1972). The most efficient method of increasing the frequency of antibiotic-susceptible variants in a culture was storage of broth cultures at 20°C for several weeks.

Dye-CsCl density-gradients. The method of Novick and Bouanchaud (1971) was used to determine the presence of plasmid DNA. The bacteria under study were grown in Brain Heart Infusion (BHI; Difco) broth until the culture reached an optical density of about 1.0 (measured at 550 nm with a Bausch and Lomb Spectronic 20 spectrophotometer) and were labelled for 1 h with methyl-³H-thymidine 1 µCi per ml (specific activity: 19 Ci per m.mole) and control cultures were labelled with ¹⁴C-thymidine 0.1 µCi per ml (specific activity: 62 mCi per m.mole). Control strains were either the host strains of reference plasmids (nos. RN11 and RN1305) or, when antibiotic-susceptible variants were tested, the corresponding parent strain. After refrigeration for 0.5 h at 4°C, the culture under study and the control were combined and lysed. In some experiments cultures were grown in BHI broth with the addition of deoxyadenosine 250 µg per ml (Budman and Pardee, 1967) and labelled with 10 µCi of methyl-³H-thymidine and 1 µCi of ¹⁴C-thymidine. After lysis, lysates were centrifuged in ethidium bromide 500 µg per ml of the lysate-CsCl mixture instead of the 100 µg described by Novick and Bouanchaud (1971). Under these conditions, a much

better incorporation of radioactivity into the DNA, as well as a better separation of plasmid molecules from chromosomal DNA, were observed.

Approximately 8.5 ml of the lysate-dye-CsCl mixture were then centrifuged in a Beckman 50Ti fixed-angle rotor at 42 000 r.p.m. for 60 h at 20°C. Three-drop fractions were collected in tubes and 10 µl from each tube were spotted on Whatman GF/B glass-fibre filters, which were then dried, washed in trichloroacetic acid 5% (w/v) and in 96% ethanol, dried again and counted to determine the location of the plasmid peak. The appropriate fractions were pooled and dialysed for 24–48 h at 4°C against three changes of 0.01M ethylenediamine tetraacetic acid (EDTA). When *E. coli* strain K12 J53 (R15) served as source of the reference DNA, the methods of Clewell and Helinski (1970) were closely followed.

Sucrose gradients. Five to 20% neutral sucrose gradients were formed according to the method of Guerry, LeBlanc and Falkow (1973). One-tenth ml of plasmid-containing solution was then layered on to the gradients and centrifuged at 35 000 r.p.m. in a Beckman SW50 rotor at 20°C for 1.5–2 h. One-tenth ml fractions were collected directly on Whatman filters with the recovery system described by Tan (1972), and radioactivity was counted.

To determine molecular weights of unknown plasmids, sedimentation constants and molecular weights of the unknowns were compared with reference plasmids and calculated by the methods of Hudson, Clayton and Vinograd (1968) and Clowes (1972). The molecular form of the DNA peaks—covalently closed circular (CCC) or open circular (OC)—were determined by one of two methods. The first was by ageing the plasmid solutions at 4°C (Clowes, 1972), which results in spontaneous nicking of the circular molecules, followed by sedimentation analysis in a neutral sucrose gradient. In the second, plasmid DNA was identified by sedimentation of the material in a 5–20% alkaline sucrose gradient, made up in 0.3M NaOH and 0.01M EDTA, for 60 min. at 35 000 r.p.m. and 20°C (Falkow *et al.*, 1971). In some experiments the form of the DNA-peak molecules was determined by electron microscopy.

Electron microscopy. Plasmid-containing fractions were collected in tubes from CsCl density-gradients. After dialysis against 0.01M EDTA, pH 7.5, the DNA solutions were examined immediately or after storage for several weeks at 4°C (Clowes, 1972) to allow CCC molecules of plasmid DNA to become converted to OC forms. Copper grids were prepared according to the spreading procedure of Kleinschmidt (1968) and examined with a Phillips E201 electron microscope, at a magnification of 15 000. Photographs of OC molecules were taken, and contour lengths were calculated from the known magnification determined with a calibration grid. Molecular weights were estimated on the assumption that 1.0 µm corresponds to 2.07×10^6 daltons of DNA (Lang, 1970).

RESULTS

Isolation of plasmid-specific DNA

To be able to demonstrate plasmid linkage of antibiotic-resistance determinants in strains E109 and E142, resistance markers were transferred with phage 80 to strain N8325 (PEN524), which is known to be free of any detectable plasmid. Transduction could be easily carried out in case of *tet*^R, *cml*^R and *pen*^R. *Ery*^R-transductants were selected after treatment of receptor cells with erythromycin 0.1 µg per ml for 2 h at 37°C. Selection of transductants resistant to streptomycin was withheld for some time after the end of the transduction process to overcome the so-called “phenotypic lag phenomenon”. Recipient cells resistant to methicillin could be selected only after UV irradiation of transducing lysates. We were unable to transfer the marker for *neo*^R with phage 80, with UV-induced lysates of donor strains or by mixed cultivation (Lacey, 1971) of donor and recipient (for further details see Kayser *et al.*, 1972).

Log-phase cultures of resistant recipients, as well as reference-plasmid-containing strains, were labelled with radioactive thymidine and lysed. Appropriate volumes of the lysate were centrifuged to equilibrium in dye-buoyant-density gradients giving rise to the density profile shown in fig. 1*a*. A satellite component in the plasmid-positive control culture no. 8325 (PEN524) is seen at a position of higher density than the chromosomal DNA. Such a satellite component, which is not observed in the plasmid-negative recipient strain N8325 (PEN524) is characteristic of CCC DNA. Figure 1*b* demonstrates the presence of plasmid-specific DNA in strain N8325 (PEN524) *cml*^R, which had received the determinant of chloramphenicol resistance from wild strain E142. Similarly the presence or absence of CCC DNA in antibiotic-resistant transductants were examined. The results of these experiments are summarised in table I. From the data it can be concluded that, in representative isolates from Zürich, *pen*^R and *cml*^R are plasmid-located. The markers for *mtb*^R, *str*^R, *ery*^R and *tet*^R, however, are not part of demonstrable plasmid-specific DNA, and there is evidence that the determinant or determinants of *mtb*^R in strains from Australia, England, Sweden and the USA are linked to the bacterial chromosome.

To exclude the possibility that plasmid-connected genes in wild strains become integrated into the recipient chromosome after transfer, variants of strains that had lost unstable resistance markers were examined. From table II it can be concluded that the markers of *cml*^R, *neo*^R and *pen*^R in strains E109 and E142 from Switzerland as well as *pen*^R in the strain from the USA are

FIG. 1.—Dye-CsCl buoyant-density-gradient centrifugation of cleared lysates of *Staphylococcus aureus* (density increases from right to left): 1*a*: ●—● = strain 8325 (PEN524); ○-----○ = strain N8325 (PEN524); 1*b*: ●—● = strain N8325 (PEN524) *cml*^R; ○-----○ = strain N8325 (PEN524).

plasmid-bound. The markers of *ery*^R, *str*^R, *sul*^R, *tet*^R and *mth*^R, however, are not linked to demonstrable covalently closed circular DNA in the Zürich cultures. In strains from Australia, England and Sweden, only *pen*^R could be eliminated. The resulting variants still contained plasmid DNA. It is at present not known whether these variants still contain resistance plasmids that could not be eliminated or genetically "silent" elements the somatic functions of which have not yet been identified. In strain 294, an increase in transduction frequency of *tet*^R after UV irradiation of lysates could not be demonstrated, indicating an extrachromosomal location of the *tet*^R determinant.

TABLE I

Demonstration of plasmid-specific DNA after transduction of resistance determinants into strain N8325 (PEN524)

Wild strain no. (and country of origin)	Marker: resistance to*	Plasmid-specific DNA
E109 (Switzerland)	<i>pen</i>	demonstrable
E142 (Switzerland)	<i>cml</i>	demonstrable
	<i>ery</i>	absent
	<i>mth</i>	absent
	<i>str</i>	absent
	<i>tet</i>	absent
307 (Australia)	<i>mth</i>	absent
294 (England)	<i>mth</i>	absent
DU4916 (Sweden)	<i>mth</i>	absent
C ₅ (USA)	<i>mth</i>	absent

* *pen* = Penicillin G; *cml* = chloramphenicol; *ery* = erythromycin; *mth* = methicillin; *str* = streptomycin; *tet* = tetracycline.

TABLE II

Demonstration of plasmid-specific DNA in multiply resistant staphylococci before and after elimination of various unstable resistance determinants.

Wild strain no. (and country of origin)	Antibiotic resistance* patterns of wild strain and segregants	Plasmid-specific DNA
E109 (Switzerland)	<i>cml—neo—pen—ery—str—sul—tet—mth</i>	demonstrable
E142 (Switzerland)	<i>ery—str—sul—tet—mth</i>	absent
	<i>cml—ery—str—sul—tet—mth</i>	demonstrable
	<i>neo—ery—str—sul—tet—mth</i>	demonstrable
	<i>pen—ery—str—sul—tet—mth</i>	demonstrable
C ₅ (USA)	<i>pen—str—sul—mth</i>	demonstrable
	<i>str—sul—mth</i>	absent
307 (Australia)	<i>pen—ery—str—sul—tet—mth</i>	demonstrable
294 (England)	<i>ery—str—sul—tet—mth</i>	demonstrable
DU4916 (Sweden)	<i>pen—ery—str—sul—mth</i>	demonstrable
	<i>ery—str—sul—mth</i>	demonstrable

* See footnote to table I.

*Identification and molecular weight determination of
plasmid DNA species*

Plasmid DNA of strain E142, and of segregants of this strain susceptible to penicillin and chloramphenicol, neomycin and chloramphenicol, or penicillin and neomycin, was isolated and analysed by sedimentation through 5–20% neutral sucrose gradients (fig. 2). Identification of the molecular form of the

FIG. 2.—Neutral sucrose-gradient analysis of plasmid DNA of three segregants of *S. aureus* strain E142. Sedimentation was carried out for 90–100 min. at 20°C in a 5–20% gradient (from right to left).

Fig. 2a: ○-----○ = 40×10^6 -dalton reference plasmid of *Escherichia coli* strain K12 J53 (R15) as marker ($S_{ccc} = 69.8$); ●——● = neomycin-resistance plasmid of strain E142; the slower sedimenting peaks on the right represent open circular molecules.

Fig. 2b: ○-----○ = 18.6×10^6 -dalton reference plasmid of *S. aureus* strain RN11 as marker ($S_{ccc} = 47.4$); ●——● = penicillinase plasmid of strain E142.

Fig. 2c: ○-----○ = 3.1×10^6 -dalton reference plasmid of *S. aureus* strain RN1305 ($S_{ccc} = 21.5$); ●——● = chloramphenicol-resistance plasmid of strain E142.

sedimenting DNA was usually done by simultaneous sedimentation of the material in an alkaline sucrose gradient. Under these conditions, covalently closed molecules will sediment 3–4 times faster than an open circle or a single-strand filament (fig. 3). In a limited number of experiments, the molecular form of the DNA was identified either by electron microscopy or by ageing of the samples, which results in a progressive decrease in the size of a CCC peak and a corresponding increase in size of a slower-sedimenting band of OC molecules. Identification of plasmid DNA was especially necessary in the case of the neomycin plasmid, because this extremely labile plasmid tended to be converted to OC molecules soon after the end of the CsCl run. The single peak in sucrose-gradient centrifugation, which was often observed, could then be mistaken for CCC DNA.

With the ^{14}C -labelled chloramphenicol plasmid of strain RN1305 and the penicillinase plasmid of strain RN11 (PEN258) (Novick and Bouanchaud, 1971) as sedimentation references, average sedimentation-constant values ($S_{20,w}$) of the CCC form of the plasmids were calculated to be 21.5 for the chloramphenicol and 48.5 for the penicillinase plasmid. The sedimentation behaviour of the neomycin plasmid of strain E142 was compared with that of the *E. coli* reference plasmid J53 (R15) (Falkow *et al.*, 1974). The sedimentation-constant value of this plasmid was found to be 66.5 for the CCC form. According to these values, the approximative molecular weights of the three plasmids were calculated to be respectively 3.1×10^6 , 19.6×10^6 and 36.5×10^6 daltons (table III).

FIG. 3.—Sedimentation of the neomycin-resistance plasmid DNA of *S. aureus* strain E142 in a 5–20% (from right to left) alkaline sucrose gradient for 60 min. at 20°C.

Electron microscopy

Fig. 4 illustrates the size of the OC form of the three different plasmids. Contour-length measurements of these open circular molecules gave molecular-weight values that closely resembled those obtained from the sedimentation experiments (see table III).

DISCUSSION

The data presented here clearly reveal the presence of three resistance plasmids in representative strains of methicillin resistant *S. aureus* isolated in Zürich. The criteria of replicative autonomy, which we presented in a previous paper (Kayser *et al.*, 1972), as well as the demonstration of "physical autonomy", which is reported here, have been accepted as proof that antibiotic resistances are plasmid borne (Novick, 1969). Thus, the absence of *cml*^R and *neo*^R in some of the more than 600 methicillin-resistant strains isolated in Zürich between 1965 and 1975 seems to be the result of the genetic instability of these markers. On the other hand, resistance to tetracycline, streptomycin, erythromycin and sulphonamides is regularly found in our strains. Absence of replicative and physical autonomy in the case of these markers indicates their integration into the bacterial chromosome. Thus, these data give further evidence of the "single clone hypothesis".

Lately some discussion has arisen on the location of the determinants of methicillin resistance within the staphylococcal cell. The absence of CCC DNA in the strains examined, as well as absence of replicative autonomy,

TABLE III
Physical properties of plasmid-DNA molecules isolated from strain E142

Strain no.	Plasmid	Number of determinations	Sedimentation constant of CCC form (median)	Mol. wt. $\times 10^6$ based on S_{ccc}^*	Number of molecules measured	Contour length (μm) $\pm$ standard deviation	Mol. wt. $\times 10^6$ based on contour length ($\pm$ standard deviation) [†]
<i>S. aureus</i> E142	<i>cml</i> ^R	4	21.5	3.1	14	1.435 $\pm$ 0.046	2.97 $\pm$ 0.09
<i>S. aureus</i> E142	<i>pen</i> ^R	4	48.5	19.5	10	9.703 $\pm$ 0.105	20.08 $\pm$ 0.22
<i>S. aureus</i> E142	<i>neo</i> ^R	10	66.5	36.5	11	17.900 $\pm$ 0.322	37.05 $\pm$ 0.66
<i>S. aureus</i> RN1305	CML22.1	...	21.5	3.1	...	...	...
<i>S. aureus</i> RN11	PEN258	...	47.4	18.6	...	...	...
<i>Escherichia coli</i> K12 J53(R15)	R15	...	69.8	40	...	...	...

* The equation $S_{ccc} = 7.44 + 0.00243 \cdot M^{0.58}$ was used to estimate molecular weight (Hudson *et al.*, 1968).

[†] The equation $1 \mu m$ of DNA equals 2.07×10^6 daltons (Lang, 1970) was used for converting contour length into molecular weight.

METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

FIG 4.—Electron micrographs of open-circle plasmid DNA of segregants of *S. aureus* strain E142. 4A: neomycin-resistance plasmid; 4B: penicillinase plasmid; 4C: chloramphenicol-resistance plasmid.

suggests their chromosomal location. These results are in accordance with data published by Stiffler, Sweeney and Cohen (1973). On the other hand, it was suggested—mainly on the basis of elimination experiments—that methicillin resistance in some strains could be plasmid borne (Al Sahily and James, 1972; Grubb and Annear, 1972; Lacey, 1972; Dornbusch and Hallander, 1973). Lacey and Richmond (1974) tried to find a synthesis between these different experimental data. They suggested that the marker of *meth^R* is part of a huge plasmid that cannot be detected by conventional biophysical methods. Such a plasmid, however, would be expected to be of great instability. The inability to eliminate *meth^R* in the strains we examined is against such an explanation.

The discrepancies between the results of different authors may also be explained by the suggestion that *meth^R* is chromosomal in some strains but part of a plasmid in others. Such a situation has been found for genes determining penicillinase production (Novick, 1963; Asheshov, 1966), resistance to tetracycline (May, Houghton and Perret, 1964), erythromycin (Mitsushashi *et al.*, 1965) and streptomycin (Morimura *et al.*, 1970). The possibility, however, of an atypical plasmid cannot be excluded. It has to be emphasised that the structure of the staphylococcal genome is still unknown, and that the chromosomal location of a genetic marker can therefore be demonstrated only by indirect methods.

Sedimentation analysis as well as electron microscopy of the DNA from the dye-CsCl satellite component have established three size classes of molecules. Contour-length measurements of the OC forms of these plasmids and calculation of molecular weights from these give values in agreement with those based on the sedimentation rates in sucrose-density gradients. The sizes of the chloramphenicol and the penicillinase plasmid in strain E142 are similar to those of other plasmids of staphylococci. The size of the *neo^R* plasmid, on the other hand, is unusual for these bacteria. To our knowledge no plasmids in *S. aureus* and *S. epidermidis* larger than 21×10^6 daltons carrying antibiotic resistance markers have been detected. Extrachromosomal resistance to aminoglycosides in *S. aureus* (Chopra, Bennett and Lacey, 1973; Stiffler *et al.*, 1974) and *S. epidermidis* (Rosendorf and Kayser, 1974) were shown to be associated with small plasmids. Large plasmids presumably would be of limited benefit to the species, and in the absence of strong selection pressure would soon be irreversibly lost. Indeed, in the case of strain E142, a high degree of instability of *neo^R* was observed *in vitro* (Kayser *et al.*, 1972). The large size of the *neo^R* plasmid is probably the explanation for the unsuccessful transduction experiments with phage 80. The genome of this phage exhibits a molecular weight of 31×10^6 daltons (Pariza and Iandolo, 1974). It is reasonable to assume therefore that a DNA molecule of larger size will not fit into the phage head and hence not be transferred *in toto* with bacteriophage.

SUMMARY

A genetic analysis of resistance to antibiotics in methicillin-resistant *Staphylococcus aureus* was performed. Demonstration of plasmid-specific

DNA either in transductants that had received antibiotic-resistance markers from multiply-resistant strains, or in segregants of methicillin-resistant strains that had lost unstable determinants except the one under study, indicated that markers of resistance to penicillin, chloramphenicol and neomycin are present on separate, mutually compatible plasmids. Absence of covalently closed circular DNA was demonstrated in transductants that were resistant to methicillin, tetracycline, erythromycin and streptomycin, as well as in segregants that had lost the penicillinase, chloramphenicol and neomycin plasmid, but were still resistant to methicillin, tetracycline, erythromycin, streptomycin and the sulphonamides. Analysis of plasmid DNA either in a 5–20% neutral sucrose gradient or by electron microscopy revealed the presence of three readily distinguishable plasmids. The molecular weights of these plasmids were estimated by comparing the sedimentation rate constants with those of known reference plasmids and by contour-length measurements. The molecular weight of the penicillinase plasmid was estimated to be 20×10^6 daltons, that of the chloramphenicol plasmid 3×10^6 daltons and that of the plasmid carrying the neomycin resistance marker 37×10^6 daltons.

The authors wish to thank Mrs E. Huf for technical assistance and Mrs L. Ruegg for help in preparing this manuscript. We are grateful to Dr A. Vogel and Dr T. Koller for help and advice in preparing the electron micrographs.

REFERENCES

- AL SALIH, S. M. AND JAMES, A. M. 1972. Loss of methicillin resistance from resistant strains of *Staphylococcus aureus*. *Lancet*, **2**, 331.
- ASHESHOV, E. H. 1966. Chromosomal location of the genetic elements controlling penicillinase production in a strain of *Staphylococcus aureus*. *Nature, Lond.*, **210**, 804.
- BARRETT, F. F., MCGHEE, R. F. JR AND FINLAND, M. 1968. Methicillin-resistant *Staphylococcus aureus* at Boston City Hospital. Bacteriologic and epidemiologic observations. *New Engl. J. Med.*, **279**, 441.
- BLAIR, J. E. AND WILLIAMS, R. E. O. 1961. Phage typing of staphylococci. *Bull. Wld Hlth Org.*, **24**, 771.
- BOROWSKI, J., KAMIENSKA, K. AND RUTECKA, I. 1964. Methicillin-resistant staphylococci. *Br. med. J.*, **1**, 983.
- BOUANCHAUD, D. H., SCAVIZZI, M. R. AND CHABBERT, Y. A. 1969. Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. *J. gen. Microbiol.*, **54**, 417.
- BUDMAN, D. R. AND PARDEE, A. B. 1967. Thymidine and thymine incorporation into deoxyribonucleic acid: inhibition and repression by uridine of thymidine phosphorylase of *Eschericia coli*. *J. Bact.*, **94**, 1546.
- CHOPRA, I., BENNETT, P. M. AND LACEY, R. W. 1973. A variety of staphylococcal plasmids present as multiple copies. *J. gen. Microbiol.*, **79**, 343.
- CLEWELL, D. B. AND HELINSKI, D. R. 1970. Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. *Biochemistry, N. Y.*, **9**, 4428.
- CLOWES, R. C. 1972. Molecular structure of bacterial plasmids. *Bact. Rev.*, **36**, 361.
- COURTIEU, A. L., GUILLERMET, F. N., LONGERAY, C., MAKI, G. AND CHABBERT, Y. A. 1964. Fréquence des staphylocoques présentant une résistance hétérogène à la méthicilline et l'oxacilline en milieu hospitalier. *Ann. Inst. Pasteur, Paris*, **107**, 691.
- DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, FOOD AND DRUG ADMINISTRATION. 1972. Antibiotic susceptibility discs. *Fed. Reg.*, **37**(191), 20527.

- DORNBUSCH, K. AND HALLANDER, H. O. 1973. Transduction of penicillinase production and methicillin resistance-enterotoxin B production in strains of *Staphylococcus aureus*. *J. gen. Microbiol.*, **76**, 1.
- FALKOW, S., GUERRY, P., HEDGES, R. W. AND DATTA, N. 1974. Polynucleotide sequence relationships among plasmids of the I compatibility complex. *J. gen. Microbiol.*, **85**, 65.
- FALKOW, S., TOMPKINS, L. S., SILVER, R. P., GUERRY, P. AND LEBLANC, D. J. 1971. The replication of R-factor DNA in *Escherichia coli* K-12 following conjugation. *Ann. N.Y. Acad. Sci.*, **182**, 153.
- GRUBB, W. B. AND ANNEAR, D. I. 1972. Spontaneous loss of methicillin resistance in *Staphylococcus aureus* at room-temperature. *Lancet*, **2**, 1257.
- GUERRY, P., LEBLANC, D. J. AND FALKOW, S. 1973. General method for the isolation of plasmid deoxyribonucleic acid. *J. Bact.*, **116**, 1064.
- HUDSON, B., CLAYTON, D. A. AND VINOGRAD, J. 1968. Complex mitochondrial DNA. *Cold Spring Harb. Symp. quant. Biol.*, **33**, 791.
- JESSEN, O., ROSENDAL, K., BÜLOW, P., FABER, V. AND ERIKSEN, K. R. 1969. Changing staphylococci and staphylococcal infections. *New Engl. J. Med.*, **281**, 627.
- KAYSER, F. H. AND MAK, T. M. 1972. Methicillin-resistant staphylococci. *Am. J. med. Sci.*, **264**, 197.
- KAYSER, F. H. AND WÜST, J. 1974. Resistance to methicillin in *Staphylococcus aureus* and *Staphylococcus epidermidis*: epidemiological observations. In *Progress in chemotherapy* (Proc. 8th int. Congr. Chemother.), vol. 1, p. 247.
- KAYSER, F. H., WÜST, J. AND CORRODI, P. 1972. Transduction and elimination of resistance determinants in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.*, **2**, 217.
- KLEINSCHMIDT, A. K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. In *Methods in enzymology*, vol. 12B, edited by L. Grossman and K. Moldave, New York, p. 361.
- KONO, M., HASHIMOTO, H. AND MITSUHASHI, S. 1966. Drug resistance in staphylococci. 3. Resistance to some macrolide antibiotics and inducible system. *Jap. J. Microbiol.*, **10**, 59.
- LACEY, R. W. 1971. High-frequency transfer of neomycin resistance between naturally occurring strains of *Staphylococcus aureus*. *J. med. Microbiol.*, **4**, 73.
- LACEY, R. W. 1972. Genetic control in methicillin-resistant strains of *Staphylococcus aureus*. *J. med. Microbiol.*, **5**, 497.
- LACEY, R. W. AND RICHMOND, M. H. 1974. The genetic basis of antibiotic resistance in *S. aureus*: the importance of gene transfer in the evolution of this organism in the hospital environment. *Ann. N.Y. Acad. Sci.*, **236**, 395.
- LANG, D. 1970. Molecular weights of coliphages and coliphage DNA. III. Contour length and molecular weight of DNA from bacteriophages T4, T5, and T7 and from bovine papilloma virus. *J. molec. Biol.*, **54**, 557.
- MAY, J. W., HOUGHTON, R. H. AND PERRET, C. J. 1964. The effect of growth at elevated temperatures on some heritable properties of *Staphylococcus aureus*. *J. gen. Microbiol.*, **37**, 157.
- 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.
- MORIMURA, M., WATANABE, K., MORI, H. AND MITSUHASHI, S. 1970. Lability of streptomycin resistance in *Staphylococcus aureus*. *Jap. J. Microbiol.*, **14**, 253.
- NOVICK, R. P. 1963. Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *J. gen. Microbiol.*, **33**, 121.
- NOVICK, R. P. 1969. Extrachromosomal inheritance in bacteria. *Bact. Rev.*, **33**, 210.
- NOVICK, R. P. AND BOUANCHAUD, D. 1971. Extrachromosomal nature of drug resistance in *Staphylococcus aureus*. *Ann. N.Y. Acad. Sci.*, **182**, 279.
- NOVICK, R. P. AND ROTH, C. 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bact.*, **95**, 1335.

- O'TOOLE, R. D., DREW, W. L., DAHLGREN, B. J. AND BEATY, H. N. 1970. An outbreak of methicillin-resistant *Staphylococcus aureus* infection. Observations in a hospital and nursing home. *J. Am. med. Ass.*, **213**, 257.
- PARIZA, M. W. AND IANDOLO, J. J. 1974. Determination of genome size of selected typing bacteriophages of *Staphylococcus aureus*. *Appl. Microbiol.*, **28**, 510.
- PARKER, M. T. AND HEWITT, J. H. 1970. Methicillin resistance in *Staphylococcus aureus*. *Lancet*, **1**, 800.
- ROSENDORF, L. L. AND KAYSER, F. H. 1974. Transduction and plasmid deoxyribonucleic acid analysis in a multiply antibiotic-resistant strain of *Staphylococcus epidermidis*. *J. Bact.*, **120**, 679.
- ROUNTREE, P. M. AND BEARD, M. A. 1968. Hospital strains of *Staphylococcus aureus* with particular reference to methicillin-resistant strains. *Med. J. Aust.*, **2**, 1163.
- STIFFLER, P. W., SWEENEY, H. M. AND COHEN, S. 1973. Absence of circular plasmid deoxyribonucleic acid attributable to a genetic determinant for methicillin resistance in *Staphylococcus aureus*. *J. Bact.*, **116**, 771.
- STIFFLER, P. W., SWEENEY, H. M., SCHNEIDER, M. AND COHEN, S. 1974. Isolation and characterization of a kanamycin resistance plasmid from *Staphylococcus aureus*. *Antimicrob. Agents Chemother.*, **6**, 516.
- TAN, K. B. 1972. A simple device for fractionating density gradients. *Analyt. Biochem.*, **45**, 306.