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Unstable drug resistance in *Staphylococcus aureus* M4

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SUMMARY

Staphylococcus aureus M4 has chromosomal resistance to streptomycin, plasmid-borne resistance to penicillin and tetracycline and probably chromosomal inducible erythromycin resistance. It also has constitutive erythromycin resistance which is unstable and linked to kanamycin and lincomycin resistance and the ability to produce a diffusible pigment. Variants have been isolated which have stable kanamycin, lincomycin and constitutive erythromycin resistance and these do not produce the diffusible pigment. In transduction experiments kanamycin, lincomycin and constitutive erythromycin resistance were always co-transduced together with streptomycin resistance. The transduction frequencies were approximately 100 times higher with the stable variants compared with the parent. The transductants, irrespective of the donor used, all had stable resistance and did not produce the diffusible pigment. Although transduction with UV-irradiated transducing lysates was characteristic of a plasmid, no corresponding plasmid DNA has been detected.

1. INTRODUCTION

It was suggested by Annear and Grubb (1969*a*) that, in the M4 strain of *Staphylococcus aureus*, Km^R, Nm^R, Fm^R, Em^R, Spm^R and Lm^R‡ are determined by the same plasmid. The evidence for this was the concomitant loss of resistance to all these antibiotics at a high frequency. It was found during 50 serial subcultures of resistant colonies that from 4 % to 60 % of the progeny were sensitive to all the antibiotics after overnight incubation in broth at 37 °C. Lost together with resistance was the ability to produce an intense orange pigment (DiP‡). This

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‡ Abbreviations: Km = kanamycin; Nm = neomycin; Fm = framycetin; Em = erythromycin; Spm = spiramycin; Lm = lincomycin; Pc = penicillin (benzyl); Sm = streptomycin; Tc = tetracycline; Mc = methicillin; pase = penicillinase; DiP = diffusible pigment. Superscript R or S = resistance or sensitivity respectively. Superscript + or − = produced or not produced respectively.

pigment is different from the usual staphylococcal pigments, in that it readily diffuses through the medium (Annear & Grubb, 1969*b*).

Additional variants have now been isolated which have stable resistance to the antibiotics and are DiP^-.

The work reported here was undertaken to elucidate the nature of the resistance determinants in M4 and its variants by using ultraviolet (UV) irradiated transducing lysates (Arber, 1960) and by examining the organisms for plasmid DNA.

2. MATERIALS AND METHODS

(*a*) *Strains of* Staph. aureus.

M4 = Unstable (Km^R, Nm^R, Fm^R, Em^R, Spm^R, Lm^R), Dip^+, Tc^R, $Pase^+$, Mc^R, Sm^R, (Annear & Grubb, 1972).

WG336 = Sm^R, Mc^R, Tc^R, $Pase^+$, Em^R (inducible), DiP^-, variant derived from M4.

WG338 = Unstable (Km^R, Nm^R, Fm^R, Em^R, Spm^R, Lm^R), DiP^+, Mc^R, Sm^R, variant derived from M4.

WG374 = Stable (Km^R, Nm^R, Fm^R, Em^R, Spm^R, Lm^R), DiP^-, Tc^R, Mc^R, Sm^R, variant derived from M4.

M1932 = Clinical isolate sensitive to antibiotics.

WG208 = Clinical isolate sensitive to antibiotics.

PS53 = Propagating strain for International Typing Phage 53.

UB4008 = plasmid free strain from Bristol obtained from Professor G. G. Meynell, University of Kent.

(*b*) *Media.* MIB (meat infusion broth, Grubb, O'Reilly & May, 1972) and the following commercial media from BBL (Division of BioQuest, Cockeysville, Maryland, U.S.A.) were used: TSB, Trypticase Soy Broth; BHIAC, Brain Heart Infusion Agar containing 0·5% sodium citrate; BHIBC, Brain Heart Infusion Broth containing 0·5% sodium citrate.

(*c*) *Testing for resistance.* Colonies were tested for antibiotic resistance by multipoint replication to antibiotic agar (Annear & Grubb, 1972). Em at a concentration of 7·5 μg/ml was used in testing for macrolide resistance and Km at a concentration of 25 μg/ml was used when screening for resistance to the aminoglycosides Km, Nm and Fm. When the organisms being screened were Mc^R, Pc was used at a concentration of 10 μg/ml (Annear & Grubb, 1972). On all other occasions it was used at a concentration of 0·09 μg/ml. Lm, Sm and Tc were used at concentrations of 2·4, 25 and 5 μg/ml respectively. For Minimum Inhibitory Concentration (MIC) determinations doubling dilutions of antibiotic in BHIB were inoculated with a 500-fold diluted overnight broth culture.

(*d*) *Phage propagation.* International Typing Phage 53 was propagated according to the recommendations of Blair & Williams (1961). Generally, titres of *c.* 10^{10} plaque-forming units (p.f.u.) per ml were obtained by shaking gently for 5·25 h at 37 °C 0·2 ml of an overnight culture of the propagating strain and 0·1 ml of 10^9 p.f.u./ml of

phage 53 in a 100 ml Erlenmeyer flask containing 20 ml of TSB and 0·004 M-$CaCl_2$. The phage was harvested and titrated as previously described (Grubb & O'Reilly, 1971).

(*e*) *Transduction*. The method of Grubb & O'Reilly (1971) was used. Selection was on BHIAC containing antibiotics at the concentrations used for screening, except in the case of Km and Sm which were used at concentrations of 75 and 100 μg/ml respectively. Selection for inducible Em^R (Weisblum *et al.* 1971) was by the overlay technique of Pattee & Baldwin (1962). Before being used in further experiments, transductants were subcultured onto agar containing the same type of antibiotic as the selection plates and isolated colonies picked for testing. Transduction frequencies are expressed as the number of transductants per p.f.u. of phage.

(*f*) *UV irradiation*. Transducing lysates were irradiated as previously described (Grubb & O'Reilly, 1971) with 280 μW/cm^2 UV in the 230–270 nm range.

(*g*) *Curing experiments*. Growth at elevated temperature (May, Houghton & Perret, 1964) was carried out by inoculating 20 ml of MIB in a 100 ml flask with *c.* 10^3 cells in the logarithmic growth phase and growing with shaking for 8 h. For curing with acriflavine (Felton, Grimwade and Bickford Ltd., Perth, Western Australia) dilutions were made in Oxoid Nutrient Broth No. 1 at pH 7·6 and an inoculum of *c.* 10^3 cells grown for 24 h at 37 °C. Curing with ethidium bromide was by the method of Bouanchaud, Scavizzi & Chabbert (1969).

(*h*) *Detection of plasmid DNA*. Both sucrose and dye buoyant-density gradients were used. For the neutral sucrose gradients (Hughes & Meynell, 1977) the cells were lysed by the method of Novick & Bouanchaud (1971). For dye buoyant-density gradients, cells from 1 l of culture were resuspended in 10 ml of TES 4 (Hughes & Meynell, 1977), incubated for 15 min in 100 μg/ml of lysostaphin and lysed with an equal volume of 2 % sarkosyl. The lysate was cleared at 192000 ***g*** and 4·84 ml mixed with 4·6 g of caesium chloride and 0·16 ml of 1·5 % ethidium bromide. After centrifuging for 40–60 h at 40000 rev./min. at 15 °C in an MSE superspeed 65 centrifuge using a 43127–115 rotor the tubes were examined with a UV lamp (Hughes & Meynell, 1977).

(*i*) *Detection of DiP*. Pigment production was tested for as previously described (Annear & Grubb, 1969*b*).

3. RESULTS

Antibiotic resistance was transduced from M4 into M1932, WG208, PS53 and UB4008. The transduction frequencies for Km^R, Em^R and Lm^R were all around $1{\cdot}5 \times 10^{-8}$ and the frequencies for Sm^R, Pc^R and Tc^R were $5{\cdot}5 \times 10^{-8}$, $1{\cdot}0 \times 10^{-7}$ and 2×10^{-7} respectively. Transductants selected on Km or Lm were always resistant to Sm, Km, Em and Lm, whereas transductants selected on Sm or Em were either resistant to Sm or Em only or resistant to Sm, Km, Em and Lm. Although the co-transduction of Km^R, Em^R and Lm^R confirmed the linkage of these markers

in M4, on no occasion was DiP production co-transduced. Also the markers were stable in the transductants and did not show the high frequency of loss as found in M4.

The transductants which were Em^R only were found to have inducible Em^R, whereas the $Sm^RKm^REm^RLm^R$ transductants had constitutive Em^R. When the transductions were repeated using the overlay method, the transduction frequency for inducible Em^R was *c.* 7×10^{-6}.

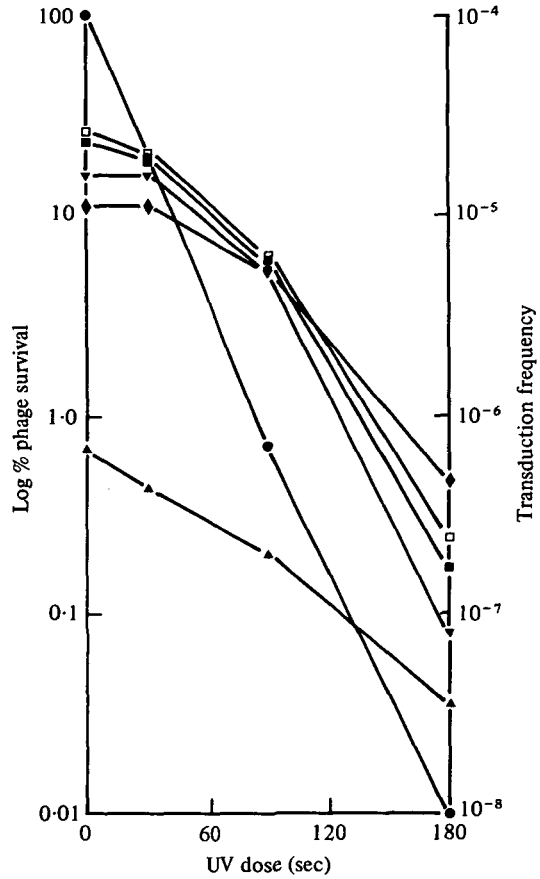

Fig. 1. Effect of UV irradiation on transduction of drug resistance from WG374 to M1932 with phage 53. □, Lm^R; ▲, Tc^R; ●, phage survival; ▼, Km^R; ■, Em^R; ◆, Sm^R.

The MIC of Sm was *c.* 400 μg/ml for those transductants which were Sm^RKm^R-Em^RLm^R and greater than 3200 μg/ml for those which were resistant only to Sm.

UV irradiation of M4 transducing lysates did not increase the transduction frequency for the linked markers $Sm^RKm^REm^RLm^R$ but increased the transduction frequencies for Sm^R only and inducible Em^R, 100- and 2-fold respectively.

WG374 is an example of a class of variants isolated from M4 which are DiP^- and have stable $Sm^RKm^REm^R$ and Lm^R. Whereas overnight incubation of a

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resistant clone of M4 resulted in up to 50 % of the population being $Km^{S}Em^{S}Lm^{S}$-DiP^{-}, no sensitive variants of WG374 were detected. Similarly, M4 but not WG374 could be cured of Km^{R}, Em^{R} and Lm^{R}: of 300 clones screened after growth at 44·5 °C, or in the presence of acriflavine or ethidium bromide, WG374 produced no sensitive variants, whereas M4 was completely sensitive. The only occasion in which $Km^{S}Em^{S}Lm^{S}$ variants of WG374 have been detected was in a culture aged for eight months at room temperature and this produced six sensitive variants out of 432 colonies tested.

WG374 also differs from M4 in that the transduction frequencies for Sm^{R}, Km^{R}, Em^{R} and Lm^{R} are 100-fold higher. This would appear to be a characteristic of the markers rather than the variant because the transduction frequency for Tc^{R} is similar for both organisms. Also other variants of the WG374 type gave similar results.

Transductants, whether from M4 or WG374 and irrespective of the recipients used, were always DiP^{-} and had stable resistance. This was also the case when WG336, a cured derivative of M4, was used as a recipient. Transductants, when used as donors, also gave high transduction frequencies similar to WG374.

Transduction with UV-irradiated lysates of WG374 gave characteristic plasmid kinetics for $Sm^{R}Km^{R}Em^{R}$ and Lm^{R} (Fig. 1).

In spite of the typical plasmid characteristics of the $Sm^{R}Km^{R}Em^{R}$ and Lm^{R} determinants, no corresponding plasmid DNA was detected on either neutral sucrose or caesium chloride gradients of M4, WG338, WG374 or their transductants.

4. DISCUSSION

Before the advent of physical means for identifying plasmids, the Arber (1960) experiment was regarded as the most definitive criterion for determining the location of a gene in the staphylococci (Richmond, 1972). However, Rubin & Rosenblum (1971) have subsequently demonstrated that small increases in the transduction frequency can also occur when a transduced plasmid recombines with a plasmid in the recipient. Nevertheless the evidence to date still indicates that large increases in the transduction frequency following UV irradiation are indicative of chromosomal determinants.

The results for the inducible Em^{R} are equivocal. Although UV irradiation produced a small increase (*c.* 2-fold) in the transduction frequency, the frequencies with unirradiated lysates were much higher than usually encountered for typical chromosomal determinants. Also there is no evidence that the inducible Em^{R} is plasmid-borne in the parent strain or that it combines with a plasmid in the recipient. It could be significant that the results obtained in the Arber experiment are similar to those obtained by Richmond & Johnston (1969) for the integrated constitutive Em^{R} of pI258 which has now been found to be transposon Tn551 (Novick *et al.*, 1979).

In accord with the work of Grinsted & Lacey (1973) there are two kinds of Sm^{R}: high-level resistance, which is transduced with typical chromosomal kinetics with

UV-irradiated lysates, and low-level resistance, which is transduced with plasmid kinetics.

The rate at which $Km^REm^RLm^R$ and DiP^+ are concomitantly lost would indicate a plasmid linkage for the markers (Richmond, 1972), and the transduction results demonstrate that low level Sm^R is also linked to them. However, the failure to co-transduce DiP^+ could indicate that the complete linkage group is too large to be co-transduced. This could mean a large linkage group is involved as the genomes of staphylococcal typing bacteriophages range in size from 29 to 40×10^6 daltons (Pariza & Iandolo, 1974) and the largest plasmid reported to be transduced by typing phage 53 is 35×10^6 daltons (Lacey & Chopra, 1974).

The stability of the $Sm^RKm^REm^RLm^R$ following the loss of DiP^+ could be explained if the markers had become integrated into the chromosome. However, the results with the UV-irradiated transducing lysates do not support a chromosomal location for the determinants.

In the light of these results it is surprising that no plasmid DNA has been detected which corresponds to the $Sm^RKm^REm^RLm^R$ and DiP^+. It could be that the methods used were not detecting plasmid DNA. However, those used have demonstrated plasmid DNA corresponding to Tc^R and $pase^+$ in M4 and various other staphylococcal plasmids such as the 35×10^6 dalton plasmid of Lacey & Chopra (1974). Also similar methods have demonstrated plasmids ranging from 4·6 to 61×10^6 daltons in Gram-negative bacteria (Hughes & Meynell, 1977; Hardy, personal communication).

Other markers in staphylococci have been reported that have some plasmid characteristics although plasmid DNA could not be detected (Lacey & Chopra, 1974; Novick & Bouanchaud, 1971). As one of these has now been shown to be transposable element Tn554 (Phillips & Novick, 1979) this does afford another possible explanation for the nature of the $Km^RLm^REm^R$ determinants.

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