The Genetics of Tetracycline Resistance in *Staphylococcus aureus*

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SUMMARY

Eighty-one strains of *Staphylococcus aureus* that appeared to be tetracycline resistant on the basis of a preliminary disc-diffusion test were examined for resistance to tetracycline and to the semi-synthetic tetracycline, minocycline. Minimum inhibitory concentration (m.i.c.) values for both drugs were determined after induction of the strains by growth for 2 h in sub-inhibitory concentrations of tetracycline. Forty-seven strains (58 %) had m.i.c. values for minocycline of 12.5 µg/ml or greater, and were considered to be minocycline resistant. An additional ten strains had m.i.c. values for minocycline of 3.125 to 6.25 µg/ml and were classified as low-level resistant strains. It appears, therefore, that a fairly high proportion of tetracycline-resistant strains isolated at the present time are resistant to concentrations of minocycline unattainable *in vivo* with the recommended dosage for this antibiotic (Frisk & Tunevall, 1969).

Transductional analysis of the genetic determinants for tetracycline resistance revealed the existence of two types of resistance to high concentrations of tetracycline. Strains in the first category (A) were inducibly resistant to tetracycline but sensitive to minocycline; in these strains the resistance determinant was plasmid-borne. Strains in the second category (B) were resistant to both tetracycline and minocycline and had low induction ratios for tetracycline resistance; the genetic determinant for resistance in these strains was chromosomal. In addition, certain strains in category A were found to carry a chromosomal gene controlling low-level resistance to tetracycline and minocycline. This low-level resistance to tetracycline was masked in the presence of the tetracycline plasmid but could be demonstrated after loss of the plasmid. The results suggest that more than one mechanism of resistance to tetracyclines may exist in staphylococci.

INTRODUCTION

Resistance to tetracycline is common in strains of *Staphylococcus aureus* isolated from patients in hospital. In most strains that have been examined in detail resistance is inducible, in that growth of the strain for a few hours in sub-inhibitory concentrations of tetracycline allows the strain to grow in higher concentrations of tetracycline (Sompolinsky *et al.* 1970; Inoue, Hashimoto & Mitsuhashi, 1970). Inducible tetracycline resistance has some features in common with inducible resistance to macrolide antibiotics (Weaver & Pattee, 1964); for example, induction requires protein synthesis, and the induced state is lost within 8 to 10 h growth in the absence of inducer (Inoue *et al.* 1970). Tetracycline-sensitive strains of *Escherichia coli* (Izaki & Arima, 1963; Franklin & Godfrey, 1965) and *S. aureus* (Sasaki *et al.* 1970; Inoue *et al.* 1970; Sompolinsky *et al.* 1970) accumulate high intracellular concentrations of tetracycline by what appears to be an active transport mechanism; resistance is correlated with a decreased uptake of tetracycline, but the precise mechanism remains obscure.

Several lines of evidence have established that the genetic determinants for tetracycline

resistance in some strains of *S. aureus* may be carried on a plasmid. Firstly, growth of resistant strains at high temperature causes an irreversible loss of resistance (May, Houghton & Perret, 1964; Asheshov, 1966*a*). Secondly, the kinetics of transduction of tetracycline resistance are those expected for transduction of plasmids (Asheshov, 1966*b*; Poston, 1966). Finally, covalently closed circular DNA molecules can be isolated from tetracycline-resistant strains which are missing in the otherwise isogenic tetracycline-sensitive strain (Novick & Bouanchaud, 1971; Chopra, Bennett & Lacey, 1973). However, there is also evidence that the genetic determinants for resistance may be chromosomal in some strains. Kayser, Wüst & Corrodi (1972) concluded, on the basis of stability of tetracycline resistance under a number of different experimental conditions, and of transduction kinetics, that the gene or genes controlling resistance were chromosomal in a series of multiply antibiotic-resistant strains of staphylococci.

As a rule, resistance to one tetracycline confers resistance to all other members of this family of antibiotics. However, the semi-synthetic tetracycline, minocycline (7-dimethyl-amino-6-deoxytetracycline), has been reported to be active against many tetracycline-resistant strains of staphylococci (Fedorko, Katz, & Allnoch, 1968; Kuck & Forbes, 1973; Leigh & Simmons, 1974). Kuck & Forbes (1973) showed that a tetracycline-resistant, minocycline-sensitive strain of *S. aureus*, with a decreased uptake of tetracycline, continued to accumulate minocycline at an undiminished rate. Schaeffler & Francois (1973) found that some tetracycline-resistant strains of staphylococci could mutate to resistance to minocycline. These mutants were able to donate tetracycline resistance and combined tetracycline and minocycline resistance by transduction, but apparently only certain recipient strains were able to be transduced to combined resistance to tetracycline and minocycline.

To investigate this further, I have examined a number of strains of tetracycline-resistant staphylococci. Minimum inhibitory concentrations for tetracycline and minocycline were determined, and transduction was used as a means of identifying the location of the genes controlling resistance.

METHODS

Strains of Staphylococcus aureus. Eighty-one strains of staphylococci, isolated from lesions in patients in five London hospitals and judged to be tetracycline resistant on the basis of a preliminary disc-diffusion test, were examined for resistance to both tetracycline and minocycline. This collection of strains had a variety of phage-typing and antibiotic-resistance patterns. Seven strains were subsequently used as donors of tetracycline resistance in transduction experiments. The tetracycline-sensitive recipients used in these experiments were as follows: PS6 (NCTC9509), the propagating strain for typing phage 6; PS80 (NCTC9789), the propagating strain for typing phage 80; PS47 (NCTC8325), the propagating strain for typing phage 47; RN981, from Dr R. W. Lacey, a recombination-defective mutant of PS47 which does not form stable transductants for chromosomal genes (Wyman, Goering & Novick, 1974); and 57, from Professor K. C. Winkler, a strain of staphylococcus that is sensitive to the majority of staphylococcal phages. Table 1 lists some properties of the seven tetracycline-resistant donors and the five tetracycline-sensitive recipients.

Media. Nutrient broth (NB) was Oxoid nutrient broth No. 2. Nutrient agar (NA) was NB solidified with Oxoid agar No. 3.

Antibiotics. Tetracycline hydrochloride (Tetracyn) was purchased from Pfizer Ltd. Minocycline hydrochloride, with a potency of 800 µg minocycline/mg, was the gift of Lederle Laboratories.

Tests for drug resistance. (i) Disc-diffusion test. NA plates were flooded with the strain

Table 1. *Properties of tetracycline-resistant strains of S. aureus used as donors, and tetracycline-sensitive strains used as recipients, in transduction experiments*

Strain	Phage-typing pattern	Resistance pattern	Minimum inhibitory concn ($\mu\text{g/ml}$)			
			Tetracycline		Minocycline	
			Uninduced	Induced	Uninduced	Induced
Donors						
1809	52/52A/80/85w	PT	50	200	0.8	0.8
3106	47/54/75/77/84/85	PSTE	150	150	25.0	25.0
3107	52/52A/80/81	PST	75	300	3.125	3.125
3110	54/75/77/84/85	PSTE	150	150	25.0	25.0
3111	80/81	PSTN	100	250	3.125	3.125
3490	85	PSTSxM	75	300	12.5	25.0
3511	47/85	PSTESx	100	150	25.0	50.0
Recipients						
PS6	6/47/53/54/75/77/83A/84/85	Sensitive	1.5	—	0.4	—
PS80	80/81	P	1.5	—	0.8	—
PS47	47/53/54/75/77/84/85	Sensitive	1.5	—	0.4	—
RN981	47/53/54/75/77/84/85	Sensitive	1.5	—	0.4	—
57	29/52/52A/80/6/42E/47/54/75/77/84/85/81/42D	Sensitive	1.5	—	0.4	—

P, penicillin; S, streptomycin; T, tetracycline; E, erythromycin; N, novobiocin; Sx, sulphamethoxazole; M, methicillin.

under test, diluted to give semi-confluent growth after 18 h at 37 °C. Discs (6 mm in diam) containing 10 μg tetracycline, were applied to the surface. Zones of inhibition were measured after 18 h at 37 °C. Strains with inhibition zones of diameter less than 14 mm were considered resistant.

(ii) Minimum inhibitory concentration (m.i.c.). NA plates containing varying concentrations of either tetracycline or minocycline were inoculated with broth cultures containing about 10^7 viable units/ml by means of a multiple applicator which delivered approximately 0.01 ml inoculum. Readings were made after incubation for 24 h at 37 °C. The m.i.c. was taken as the lowest concentration that reduced growth to a few isolated colonies or less.

Induction of tetracycline resistance. A single colony of the strain under test was inoculated into 2 ml NB in a tube and grown at 37 °C for 3 h. A sample (0.9 ml) was removed to a second tube and 0.1 ml of a solution containing 50 μg tetracycline/ml added. Both uninduced and induced cultures were incubated for a further 2 h at 37 °C before determining the m.i.c.

Transduction of tetracycline resistance. The method used was essentially the same as that of Asheshov (1969). Both typing phage 80 (NCTC9788) and phage 85 (NCTC10456) were used as transducing phages. They were grown in lytic cycle on the various donor strains. Three hours were allowed for expression of transduced genes before selecting on NA plates containing, unless otherwise stated, 5 μg tetracycline/ml and 10 mM-sodium citrate. Transductants were counted after 48 h at 37 °C. In the majority of experiments the transducing phage was given varying doses of u.v. light before being used to transduce. This allowed curves to be constructed which gave some indication of the probable location of the transduced genes. In each experiment a reasonable number of transductants were purified, their identity confirmed by phage-typing, and their m.i.c. for tetracycline and minocycline was determined.

Loss of tetracycline resistance during growth at 43.5 °C. The method was described pre-

Table 2. *Stability of tetracycline resistance in strains grown at 43.5 °C*

Strain	No. colonies screened	No. sensitive to tetracycline
1809	8600	1 (m.i.c. tetracycline: 1.5 µg/ml; m.i.c. minocycline: 0.4 µg/ml)
3106	2058	0
3107	1600	1 (m.i.c. tetracycline: 6.25 µg/ml; m.i.c. minocycline: 3.125 µg/ml)
3110	2244	0
3111	2185	0
3490	2600	0
3511	1440	0

viously (Asheshov, 1966*a*), the only difference being that incubation at this temperature was for 48 h before screening for tetracycline-sensitive mutants.

RESULTS

Inducibility of tetracycline resistance and its relation to minocycline resistance

Minimum inhibitory concentrations of tetracycline for the 81 strains varied from 25 to 300 µg/ml with uninduced cultures, and from 25 to 400 µg/ml with induced cultures. Induction ratios, obtained by dividing the m.i.c. of the induced culture by the m.i.c. of the uninduced culture, varied from 1 to 4. Twenty-four strains (29.6 %) had m.i.c. values for minocycline of less than 1 µg/ml and were classified as sensitive; 10 strains (12.3 %) had m.i.c. values of 3.125 or 6.25 µg/ml and were classified as low-level resistant strains; 47 strains (58 %) had m.i.c. values for minocycline of 12.5 µg/ml or greater and were classified as resistant. Induction with tetracycline usually doubled the m.i.c. value for minocycline in minocycline-resistant strains.

There was a distinct correlation between the induction ratio for tetracycline and minocycline resistance. Thus 26 of 29 strains with an induction ratio of less than 2 were minocycline resistant, as were 18 of 22 strains with an induction ratio of 2, and 3 of 30 strains with an induction ratio greater than 2.

Strains that were resistant to both tetracycline and minocycline usually belonged to phage-group III or were miscellaneous I–III strains, and the majority were multiply drug resistant. However, multiple drug resistance was not invariably associated with minocycline resistance.

Stability of tetracycline resistance

The 7 tetracycline-resistant strains listed in Table 1 were examined for loss of resistance after growth for 48 h at 43.5 °C. Tetracycline-sensitive variants were isolated from two strains, 1809 and 3107, with a low frequency (Table 2). Although loss of resistance suggests that the genetic determinants for resistance are carried on a plasmid, failure to detect loss in the other five strains cannot be regarded as evidence of a chromosomal location of the resistance genes, since it is known that the tetracycline plasmid is unusually stable (see Lacey, 1973).

The tetracycline-sensitive variant of strain 1809 had m.i.c. values for both tetracycline and minocycline which were characteristic of fully sensitive strains. However, the sensitive variant isolated from strain 3107 retained a low-level resistance to tetracycline (m.i.c. 6.25 µg/ml) and the low-level resistance to minocycline characteristic of the parent strain

Fig. 1

Fig. 1. Frequency of transduction of tetracycline resistance plotted as a function of u.v. dose given to the transducing phage, phage 80. ○, donor 3111, recipient ps80; ■, donor 3107; recipient ps80; ●, donor 1809, recipient 57.

Fig. 2

Fig. 2. Frequency of transduction of tetracycline resistance to ps6, plotted as a function of u.v. dose given to the transducing phage, phage 85. ○, donor 3106; ■, donor 3110; ●, donor 3511.

(m.i.c. 3.125 µg/ml). Assuming that loss of high-level resistance to tetracycline in strain 3107 represents loss of a tetracycline plasmid, it would seem that this strain carries a second locus controlling low-level resistance to both tetracycline and minocycline.

Transductional analysis of tetracycline resistance

Kinetics of transduction of tetracycline resistance from strains resistant to tetracycline but sensitive to minocycline. Strains 1809, 3107 and 3111 were used as the donors of tetracycline resistance to several recipient strains. The transducing phage, phage 80, was given varying doses of u.v. light before being used to transduce. Figure 1 shows the curves obtained when transduction frequency was plotted against u.v. dose. Although there was a considerable difference in transduction frequency with the three donors, all of the curves showed an exponential decrease in frequency with increasing u.v. doses, the response expected for the transduction of plasmids (Arber, 1960). Transductants had m.i.c. values for tetracycline identical to those of the donor strains. However, the transductants of strains 3107 and 3111 failed to inherit the low-level resistance to minocycline characteristic of these two donor strains, supporting the conclusion that the genetic determinant for this resistance is unlinked to the tetracycline plasmid.

Kinetics of transduction of low-level resistance to tetracycline and minocycline. The tetracycline-sensitive variant of strain 3107, isolated after growth at 43.5 °C (Table 2), was used as the donor in transduction experiments in which ps80 served as recipient. Transductants were selected on NA plates containing 1 µg tetracycline/ml and 10 mM-sodium citrate. The kinetics of transduction were typical for chromosomal genes in that frequency was low with unirradiated phage (from 3×10^{-8} to 1×10^{-7}) but was stimulated 10- to 30-fold by small u.v. doses given to the transducing phage, phage 80. Transductants had m.i.c. values identical to the donor (6.25 µg/ml for tetracycline; 3.125 µg/ml for minocycline). In each experiment a small number of colonies grew on the control plates. These appeared to be single-step

Fig. 3

Fig. 4

Fig. 3. Frequency of transduction of tetracycline resistance to ps6 and 57 from strain 3490. ○, ps6 transduced to resistance to tetracycline and minocycline; ●, ps6 transduced to resistance to tetracycline (minocycline-sensitive); ■, 57 transduced to resistance to tetracycline (minocycline-sensitive); △, 57 transduced to resistance to tetracycline and minocycline.

Fig. 4. Frequency of transduction to ps47 and RN981 from strain 3490. ○, ps47 transduced to resistance to tetracycline and minocycline; ●, ps47 transduced to resistance to tetracycline (minocycline-sensitive); ■, RN981 transduced to resistance to tetracycline (minocycline-sensitive).

mutants of ps80 and had the same m.i.c. values for tetracycline and minocycline as the transductants. The number of these spontaneous mutants was about one-tenth the number of transductants obtained with unirradiated phage. Corrections were made for these mutants in calculating transduction frequencies.

Kinetics of transduction of tetracycline resistance from strains resistant to tetracycline and minocycline. Strains 3106, 3110, 3511 and 3490 were the donors in these experiments and phage 85 was the transducing phage. Transductants were selected on tetracycline agar and were replica-plated to agar containing 2.5 µg minocycline/ml to detect co-transduction of minocycline resistance. Two recipients were used, ps6 and 57, and the results showed considerable difference depending on the recipient.

Figure 2 shows the curves obtained in experiments in which the donors were strains 3106, 3110 and 3511, and the recipient was ps6. Frequency is plotted as a function of the irradiating dose given to the phage. Frequency was low with unirradiated phage but was stimulated by small u.v. doses, suggesting that the transduced genes were chromosomal (Arber, 1960). When strain 57 was the recipient, the frequency was considerably lower (approx. 10⁻⁹ with unirradiated phage) and there were too few transductants to allow any conclusions to be drawn as to the kinetics of transduction. All transductants were co-transduced to minocycline resistance and m.i.c. values for both tetracycline and minocycline were identical to those of the respective donors.

When strain 3490 was used as the donor to ps6 and 57, two sorts of transductants were obtained, those resistant to both tetracycline and minocycline and those resistant only to tetracycline. Figure 3 shows the curves obtained for each sort of transductant for the two recipients. Frequency of transduction of tetracycline alone was similar for the two recipients and showed the exponential decrease expected for the transduction of a plasmid. The curves for transduction of combined resistance to tetracycline and minocycline to ps6

Table 3. *Minimum inhibitory concentrations for tetracycline and minocycline of transductants of PS6 and 57 in experiments in which the donor was strain 3490*

Strain	Resistance	No. clones tested	Minimum inhibitory concn ($\mu\text{g/ml}$)			
			Tetracycline		Minocycline	
			Uninduced	Induced	Uninduced	Induced
3490 (donor)	TM	1	75	> 200	12.5	25
PS6	TM	6	75	100	12.5	25
PS6	T	6	75	> 200	< 1.5	< 1.5
57	TM	6	75	100	12.5	25
57	T	6	75	> 200	< 1.5	< 1.5

T, tetracycline; M, minocycline.

showed a slight stimulation in frequency with small u.v. doses, suggesting a chromosomal location. Once again, strain 57 proved a poor recipient of the genetic determinants of combined tetracycline and minocycline resistance.

In these experiments, the transduction frequency for tetracycline resistance alone, which appears to be determined by a plasmid, was lower than the frequency found for transduction of combined tetracycline and minocycline resistance, which appears to be controlled by a chromosomal gene. This is unusual in that plasmids are generally transduced with a higher frequency than chromosomal genes. However, there is some evidence that phage 85, which was used as the transducing phage in these experiments, is defective in its ability to transduce both the penicillinase plasmid and the tetracycline plasmid.

A number of transductants of each sort were purified and their m.i.c. values for tetracycline and minocycline determined (Table 3). Transductants acquiring resistance to both drugs had low induction ratios for tetracycline resistance, while those acquiring resistance only to tetracycline had induction ratios greater than 2. It therefore appears that strain 3490 has a chromosomal locus controlling resistance to tetracycline and minocycline, and a tetracycline plasmid carrying the gene for resistance to tetracycline. Strain PS6 preferentially acquires the chromosomal gene, whereas strain 57 preferentially acquires the tetracycline plasmid.

Transduction of tetracycline resistance to PS47 and RN98I. To establish more precisely the cellular location of the genes controlling tetracycline resistance, transductions were carried out in which PS47 and its recombination-defective mutant RN98I were used as recipients. It was expected that PS47 would accept both chromosomal and plasmid resistance determinants equally well. However, RN98I should not form stable transductants for chromosomally-determined tetracycline resistance. The donors in these experiments were strains 3106, 3511 and 3490.

Strains 3106 and 3511 transduced tetracycline resistance to PS47 but failed to transduce RN98I. The kinetics of transduction to PS47 were typically chromosomal and all transductants were co-transduced to minocycline resistance. Strain 3490 transduced tetracycline resistance to both PS47 and RN98I. On replication to minocycline agar, PS47 transductants proved to be of two sorts, those resistant to tetracycline but sensitive to minocycline, and those resistant to both drugs. However, all transductants obtained with RN98I were minocycline sensitive. Co-transduction of tetracycline and minocycline resistance to PS47 was stimulated with small u.v. doses (Fig. 4), whereas transduction of tetracycline alone decreased exponentially with increasing irradiation. In the majority of experiments the curve

obtained for transduction of tetracycline resistance to RN981 showed a slight stimulation (approx. 1.5-fold) with small u.v. doses. The reason for this is not clear. The stimulation observed with irradiation of a transducing phage when chromosomal genes are transduced is believed to be due to an enhanced recombination of the transduced genes. However, RN981 is defective in recombination and should therefore not respond to irradiated DNA. A possible explanation, that transductants obtained with unirradiated DNA were lost through lysis, would seem unlikely, since the multiplicity of infection was low (0.2 to 0.5), and superinfection was prevented by the addition of sodium citrate both to the suspending medium and to the agar used for selection.

A number of transductants were isolated and their m.i.c. values and induction ratios determined. Transductants of PS47 that were resistant to tetracycline but sensitive to minocycline had induction ratios greater than 2, and those resistant to both drugs had induction ratios less than 2; RN981 transductants, all of which were resistant to tetracycline but sensitive to minocycline, had induction ratios greater than 2.

DISCUSSION

The genetic determinants for tetracycline resistance may have a plasmid or chromosomal location in *S. aureus* (see Lacey, 1973). It appears, however, that resistance controlled by the plasmid gene is qualitatively different from that controlled by the chromosomal locus. The plasmid gene confers inducible resistance to tetracycline but not to minocycline, whereas the chromosomal gene controls resistance to both drugs which is, on the whole, non-inducible. Recipients acquiring chromosomal tetracycline resistance were always co-transduced to minocycline resistance, indicating either that a single genetic locus determines both resistances or that the two resistances are determined by closely linked loci.

Resistance to tetracyclines in *S. aureus* has been shown to be correlated with a decreased uptake of tetracycline (Sompolinsky *et al.* 1970; Kuck & Forbes, 1973). The strains studied by these two groups were invariably those in which resistance was determined by plasmid-borne genes. It seems probable that a second mechanism of resistance to tetracyclines exists in staphylococci, controlled by the chromosomal locus.

In addition to chromosomally-determined high-level resistance to tetracycline and minocycline, a chromosomal gene conferring low-level resistance to these two drugs was found in strain 3107 after loss of the tetracycline plasmid. A similar level of resistance could apparently be acquired by spontaneous mutation in PS80.

Schaeffer & Francois (1973) showed that tetracycline-resistant strains of phage group III could mutate to minocycline resistance. It is not clear from their results whether these strains owed their initial resistance to tetracycline to the presence of a tetracycline plasmid, and their subsequent resistance to minocycline to a chromosomal mutation. However, the results they describe for the transduction of tetracycline resistance from these strains are not inconsistent with such an interpretation. They found that only certain recipients could be co-transduced to resistance to tetracycline and minocycline, although all of their recipients could be transduced to tetracycline resistance. These results are similar to those described here for transduction from strain 3490 to the two recipients, PS6 and 57. Strain PS6 could accept the chromosomal gene conferring resistance to both tetracycline and minocycline, and, with a slightly lower frequency, the tetracycline plasmid carried by strain 3490. Strain 57, on the other hand, rarely acquired the chromosomal gene but was a fairly efficient recipient of the tetracycline plasmid. The reason for this difference in recipient ability is not clear, but may indicate a lack of sufficient genetic homology between the donor and recipient.

It should be noted, however, that strain 57 was able to form stable transductants for chromosomally-determined streptomycin resistance from strains 3106, 3110 and 3490 at normal frequencies. It would appear, therefore, that there is some peculiarity about the region of the chromosome carrying the tetracycline resistance determinant that prevents its recombination into the chromosome of certain recipient strains.

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