J. Hyg., Camb. (1984), 93, 1–8
Printed in Great Britain

Occurrence of a nonplasmid-located determinant for gentamicin resistance in strains of *Staphylococcus aureus*

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(*Received* 27 *October* 1983; *accepted* 4 *January* 1984)

SUMMARY

The occurrence of resistance to gentamicin in strains of *Staphylococcus aureus* with different phage patterns in two hospitals is described. The data suggest transfer of gentamicin resistance between different strains. In the strains investigated the determinant for resistance to gentamicin (parallel resistance to kanamycin and tobramycin) is located on the chromosome. The transfer of this resistance determinant in a mixed culture of donor and recipient cells *in vitro* is mediated by transduction because it is dependent upon lysogenicity of the donor for a serogroup-B phage and because it can be inhibited by γ-globulin. The transfer is always associated with lysogenization of the recipient cells by this phage.

INTRODUCTION

Gentamicin resistance in *Staphylococcus aureus* was first observed in the GDR in 1978 (Witte & Dünnhaupt, 1980). From 1978 to 1979 the gentamicin-resistant strains were rather uniform (phage pattern 77/84/85+; chromosomal gene for resistance to oxytetracycline and to minocycline), and distribution of gentamicin resistance as a result of spread of a multiresistant strain-clone was likely (Witte & Dünnhaupt, 1982; Tschäpe, Witte & Rische, 1983). But from 1980 to the present, gentamicin resistance has appeared in a variety of strains with different phage patterns (Witte, Hummel & Rische, 1983). This observation has also been reported from other countries (Warren & Roberts, 1976; Bint *et al.* 1977; Naidoo & Noble, 1978; Buckwold *et al.* 1979; Greenhood *et al.* 1979; Rosendal, Bang & Rosdahl, 1981).

In this paper we present epidemiological data on the occurrence of gentamicin resistance in different *S. aureus* from two hospitals and we report experiments on the genetic characterization of the gentamicin-resistance determinant and on the *in vitro* transductional transfer of this determinant in mixed cultures.

MATERIAL AND METHODS

Staphylococcal strains. Wild strains were sent to the Institut für Experimentelle Epidemiologie for typing. All further investigations started from cultures on sheep blood agar. The strains used in transfer experiments are listed in Table 1.

Table 1. S. aureus *strains used in genetic experiments*

8325-4	Prophage and plasmid-free (Novick, 1967)
RN 981	Recombination-deficient mutant of strain 8325 (Wyman, Goering & Novick, 1974)
8325-4, fus-r, nov-r	Double mutant derived from strain 8325-4 by independent selection for resistance to fusidic acid (5·0 μg/ml) and novobiocin (5·0 μg/ml) and used as recipient strain for transfer in mixed cultures
8325-4, fus-r, nov-r (Ø 1152)	Recipient strain lysogenic for phage 1152
1152/82	Gentamicin-resistant wild strain belonging to group 1 (Table 2)
8325-4, gen_{1152}	Gentamicin-resistant transductant, transduced from strain 1152/82
8325-4, gen_{1152} (Ø 1152)	Gentamicin-resistant transductant lysogenic for phage 1152

Nutrient media (produced by Staatliches Institut für Immunpräparate und Nährmedien, Berlin-Weissensee, GDR). Nutrient agar I with the addition of sheep blood (3%) was used for cultivation of strains; nutrient agar I with the addition of calcium chloride (10^{-3} M) was used for phage typing; nutrient agar L_4 poor in antagonists was used for determination of drug resistance; nutrient medium I for broth cultures.

Phage typing was carried out according to the methods of Blair & Williams (1961), and determination of phage-serogroup as described by Rountree (1949).

Demonstration of lysogenicity and isolation of phages from lysogenic strains. The strain studied was incubated in nutrient medium I for 12 h at 37 °C and the culture supernatant inoculated after sterile filtration on to nutrient agar medium flooded with a log phase culture of strain 8325-4. Plaques can be detected after incubation at 30 °C for 12–18 h. Lysogenic derivatives of strain 8325-4 can be isolated after further incubation of these plates for 12 h at 37 °C.

Resistance determinations. The agar diffusion test was used (Witte *et al.* 1980); MICs were determined by agar dilution test (Witte & Reissbrodt, 1983).

Transduction of plasmids and chromosomal resistance determinants. This was achieved using phage 80 as described by Witte (1976). The influence of u.v. irradiation on the transduction frequency was studied by irradiating the transducing lysate with a u.v. dose which reduces the efficiency of plating to 10%.

Demonstration of plasmid-DNA by agarose-gel electrophoresis. The cells were lysed by lysostaphin (Witte & Dünnhaupt, 1982), chromosomal DNA was precipitated by alkaline treatment (Birnboim & Doly, 1979) and incubation at 60 °C (Kado & Lui, 1981). Agarose-gel electrophoresis was carried out according to Meyers *et al.* (1976); in this method chromosomal DNA is mostly removed, so that there is no masking of plasmid-DNA bands.

Transfer of resistance in mixed cultures. This was done using techniques described by Witte (1977). After incubation as mixed culture the cells were plated on L_4-agar containing rifampicin (5 μg/ml) and gentamicin (2 μg/ml). After incubation at 37 °C for 48 h, colonies were picked from these plates and the recipient character was confirmed by checking for resistance to fusidic acid on L_4-agar plates containing 5 μg/ml of this antibiotic. Spontaneous rifampicin-resistant mutants of the donor cells were isolated with a frequency between 5×10^{-10} and 1×10^{-9}.

Selection of gentamicin-resistant spontaneous mutants. A quantity of 0·1 ml from a culture in nutrient broth, shaken for 3 h at 37 °C, was inoculated on an L_4-agar plate containing 2 μg/ml of gentamicin. After incubation at 37 °C for 48 h gentamicin-resistant mutants could be picked from these plates.

RESULTS

Frequency of resistance to gentamicin in S. aureus *isolated in the GDR*

To date resistance to gentamicin has been found only in strains isolated in connection with hospitals. No gentamicin-resistant strains were observed in infections outside the hospitals or in nasal swabs of healthy carriers.

In 1978 gentamicin-resistant strains occurred with a frequency of 2 %; in 1980, 1981 and 1982 the frequency of these strains was 11–13 %. All the strains regarded as resistant to gentamicin, tobramycin and kanamycin exhibited MIC values for these antibiotics between 8 and 32 μg/ml; none was resistant to amikacin (MIC values 0·063–0·125 μg/ml) and to netilmicin (MIC 0·063 μg/ml). This resistance was not linked to definite phage patterns or antibiograms. These observations indicate a spread of the gentamicin-resistance determinant among different strains in hospitals, and this assumption was studied in more detail in the two hospitals Z and D.

Occurrence of gentamicin resistance in S. aureus *from hospitals Z and D*

In hospital Z the strains were isolated from post-operative wound infections in the surgical department, in hospital D the strains were isolated from various infections (skin, eye) in the children's clinic. In both hospitals the first gentamicin-resistant strains exhibited the phage pattern 77/84/85 at RTD. Later, gentamicin resistance also occurred in strains with other phage patterns (Tables 2 and 3). In five cases from hospital Z and in four cases from hospital D strains with the same phage pattern and with the same resistance to other antibiotics besides gentamicin were observed before the appearance of resistance to gentamicin and at the same time.

Theoretically, the appearance of this resistance could be due to a mutation to aminoglycoside resistance or to a spread of a gentamicin-resistance determinant. The first possibility is rather unlikely.

When checked *in vitro*, gentamicin-resistant mutants can be selected from a log-phase broth culture of the gentamicin-sensitive strains (groups 1, 2, 3, 5 and 6 in Table 2 and groups 8, 9, 11, 12 in Table 3), with a mutation frequency between 1 and 5×10^{-10}. The MIC of gentamicin for these mutants was between 2 and 4 μg/ml. All of these mutants (10 colonies of each strain were checked) were also resistant to streptomycin (MIC 4–8 μg/ml). In contrast the gentamicin-resistant wild strains were sensitive to streptomycin (MIC 0·063–0·125 μg/ml).

Genetic characterization of the gentamicin-resistance determinant

In strains from groups 1, 3, 4, 5, 6, 12 and 13 (Tables 2 and 3) transduction of gentamicin resistance to strain 8325-4 was possible, but it was not possible to the rec⁻-mutant of strain 8325-4 (Table 4). No plasmid-specific bands could be detected in agarose-gel electrophoresis in the DNA extracted from the transductants.

Table 2. *Occurrence of gentamicin resistance in* S. aureus *isolated from infections in the surgical department of district hospital Z*

Grouping	Phage pattern	Antibiogram	Year of isolation	Number of strains isolated
1	77/84/85, RTD	Pn, Cm, Tc-Mn, Em, Lm	1977, 1978, 1979	7
	77/84/85, RTD	Pn, Cm, Tc-Mn, Em, Lm, Gm	September 1979	4
2	6, 100 × RTD	Pn, Tc-Mn, Sm, Em, Lm, Ox	1978, 1979, 1980	11
	6, 100 × RTD	Pn, Tc-Mn, Sm, Em, Lm, Ox, Gm	February 1980	1
			August 1981	2
			February 1982	1
3	52A/79/6/53/75 + 100 × RTD	Pn, Cm	1979	2
	82A/79/6/53/75 + 100 × RTD	Pn, Cm, Gm	August 1980	4
4	85, RTD	Pn, Gm	December 1981	1
5	80, RTD	Pn, Cm, Tc, Sm, Em	1979, 1980, 1981	5
	80, RTD	Pn, Cm, Tc, Sm, Em, Gm	March 1982	1
6	29/52 + RTD	Pn, Cm,	1980, 1981, 1982	7
	29/52 + RTD	Pn, Cm, Gm	August 1982	6

Abbreviations. RTD, routine test dilution; Pn, benzylpenicillin; Cm, chloramphenicol; Tc, oxytetracycline; Mn, minocycline; Em, erythromycin; Lm, lincomycin; Ox, oxacillin; Gm, gentamicin.

Table 3. *Occurrence of gentamicin resistance in* S. aureus *isolated from infections in children's clinics D*

Grouping	Phage pattern	Antibiogram	Year of isolation	Number of strains isolated
7	85+, RTD	Pn, Cm, Tc-Mn, Em, Lm, Gm	1978	2
8	A994, RTD	Pn, Cm, Tc-Mn, Sm, Em, Lm, Ox	1978–1982	37
	A994, RTD	Pn, Cm, Tc-Mn, Sm, Em, Lm, Ox, Gm	1979–1982	32
9	6/54, 100 × RTD	Pn, Tc, Sm, Em, Lm	1979–1982	8
	6/54, 100 × RTD	Pn, Tc, Sm, Em, Lm, Gm	1980–1982	9
10	29/77/83A, 100 × RTD	Pn, Tc, Gm	1980	3
11	29 RTD	Pn	1978–1982	16
	29 RTD	Pn, Gm	1980	5
12	94/96 RTD	Pn	1978–1982	19
	94/96 RTD	Pn, Gm	1981, 1982	2

Abbreviations as in Table 2.

Table 4. *Genetical characterization of gentamicin-resistance determinants*

Donor strains, grouping according to Tables 2 and 3	Donor strains, phage patterns	Frequency of transduction of gen to strain 8325-4, u.u. dose 10 s	Frequency of transduction of gen to RN 981, rec⁻ u.v. dose 10 s	Demonstration of plasmid DNA in the transductants of strain 8325-4
1	77/84/85, RTD	$1{\cdot}8 \times 10^{-9}$	$< 1 \times 10^{-10}$	No
3	52A/79/6/53/75, 100 × RTD	$2{\cdot}1 \times 10^{-9}$	$< 1 \times 10^{-10}$	No
4	85, RTD	$1{\cdot}7 \times 10^{-9}$	$< 1 \times 10^{-10}$	No
5	80, RTD	$3{\cdot}2 \times 10^{-9}$	$< 1 \times 10^{-10}$	No
6	29/52, RTD	$2{\cdot}5 \times 10^{-9}$	$< 1 \times 10^{-10}$	No
12	29, RTD	$1{\cdot}3 \times 10^{-9}$	$< 1 \times 10^{-10}$	No
13	94/96, RTD	$1{\cdot}1 \times 10^{-9}$	$< 1 \times 10^{-10}$	No

RTD = Routine test dilution.

The results strongly suggest a chromosomal location of the determinant for gentamicin resistance in the strains investigated.

Experiments on the transfer of gentamicin resistance in mixed cultures

Because the occurrence of gentamicin resistance started with multiply resistant strains exhibiting the phage pattern 77/84/85 and because we assumed that the development of resistance to gentamicin in the hospitals Z and D originated from these strains, strain 1152/83, a representative of this group, was chosen as the donor strain for the transfer experiments. As recipient we used the prophage- and plasmid-free strain 8325-4 with resistance to novobiocin as the marker for selection after transfer and resistance to fusidic acid as the marker for the confirmation of the recipient strain.

From three independent transfer experiments we checked 100 colonies of strain 8325-4 rif-r, fus-r which received the gentamicin resistance in mixed culture from strain 1152/82 and found an MIC of 16 μg/ml for gentamicin with parallel resistance to kanamycin and tobramycin. Human γ-globulin as a source of immunoglobulin G is known to inhibit phage-adsorption unspecifically (Nordström, Forsgren & Cox, 1974; Witte, 1977). As shown in Table 5, it has also an inhibiting influence on the transfer of gentamicin resistance in mixed culture. This observation indicates that the transfer of gentamicin resistance in mixed culture is mediated by transduction (phage as vehicle). The following experiment also supports this idea. Strain 1152/83 was found to be lysogenic when the supernatant of an overnight culture in nutrient medium I at 37 °C was inoculated on strain 8325-4. The phage isolated in this way was named Ø 1152; it belongs to serogroup B.

When the gentamicin-resistant transductant of strain 8325-4 (derived from transduction by phage 80) was lysogenized with Ø 1152 and used as donor, transfer was also possible to strain 8325-4 fus-r, nov-r. It was not possible, when the nonlysogenic transductant was used (Table 5).

The transfer of gentamicin resistance is associated with a lysogenization of the recipient cells. From 100 colonies of strain 8325-4 fus-r, nov-r which received gentamicin resistance in mixed culture from strain 1152/82 supernatants of overnight cultures in nutrient medium I were checked for phages. All of these

https://doi.org/10.1017/S0022172400060861 Published online by Cambridge University Press

Table 5. *Transfer of resistance to gentamicin in mixed cultures*

Donor	Recipient	Medium	Frequency of transfer
1152/82	8325-4, fus-r, nov-r	Nutrient broth	6×10^{-6}
1152/82	8325-4, fus-r, nov-r	Nutrient broth + γ-globulin	2×10^{-9}
8325-4, gen_{1152}	8325-4, fus-r, nov-r	Nutrient broth	$< 10^{-10}$
8325-4, gen_{1152} (Ø 1152)	8325-4, fus-r, nov-r	Nutrient broth	2×10^{-5}
8325-4, gen_{1152} (Ø 1152)	8325-4, fus-r, nov-r, (Ø 1152)	Nutrient broth	$< 10^{-10}$

colonies were found to be lysogenic for a phage which lyses strain 8325-4 but not strain 8325-4 (Ø 1152). If the transfer of the chromosomal gene for gentamicin resistance was mediated by a general transduction, lysogenization of the recipient cells should not be observed. Therefore we assume an additional function of the phage, which was also indicated by the following experiments. Phage 1152 was propagated on the transductant 8325-4, gen_{1152} and used for transduction of gentamicin-resistance to strain 8325-4. This transduction is only possible after u.v. irradiation of the lysate with a u.v. dose which leads to a 10-fold reduction of the efficiency of plating. However, u.v. irradiation of the transduced gene as a prerequisite for its integration into the recipient's chromosome is a rather severe condition, which is not fulfilled in the transfer in a mixed culture. Therefore we assumed a participation of phage 1152 in the integration of the chromosomal gene for gentamicin resistance. This assumption is supported by the observation that there is no transfer to recipient strain 8325-4 fus-r, nov-r lysogenic for phage 1152.

The role of phage in the transfer of gentamicin resistance is also indicated by the finding that all of the gentamicin-resistant strains isolated in hospital Z are lysogenic for a phage which is not able to lyse strain 8325-4 (Ø 1152) but able to lyse strain 8325-4. This superinfection immunity points to an identity of these phages with phage 1152.

DISCUSSION

Resistance to gentamicin has been found in a variety of different strains of staphylococci in the GDR; this differs from observations on other antibiotic resistances (Rische, Witte & Hummel, 1981). The non-plasmid location of the determinant for gentamicin resistance in these strains corresponds to the results which Kayser, Homberger & Devaud (1981) obtained with *S. aureus* strains from London and from Zürich. Our results on gentamicin resistance in different strains isolated from two hospitals indicate that there is a spread of this resistance determinant among different strains.

In contrast to this, other chromosomally located resistance determinants in *S. aureus* are obviously bound to definite strains such as the determinants for resistance to oxytetracycline–minocycline (Asheshov, 1975; Witte & Dünnhaupt, 1982) and for methicillin resistance (Lacey, 1975). The spread of the chromosomal determinant for resistance to gentamicin is probably due to a special mechanism of a transductional transfer.

Like Lacey & Lord (1980) we have found an active role for phage in the transfer of gentamicin resistance *in vitro* in mixed cultures; the phage obviously serves as a transducing vehicle, phage-coded functions are probably involved in the establishment of the gentamicin-resistance determinant in the recipient cell. Two possibilities for this mechanism can be imagined: an association between the phage genome and the gentamicin-resistance determinant (transient or stable) as an autotransmissible element or a cis-action of a phage-coded function which is necessary for the integration or excision of the gentamicin-resistance determinant into or from the host's chromosome. Experiments for a deeper understanding of this mechanism are in progress.

We are indebted to Mrs Christa Cuny for valuable technical assistance.

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