SHORT COMMUNICATION

Loss of Extrachromosomal Resistances in *Staphylococcus aureus* by Treatment with Guanidine Hydrochloride

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The addition of subinhibitory concentrations (optimal 1.8 mg ml^{-1}) of guanidine hydrochloride to broth cultures of three strains of *Staphylococcus aureus* was followed by loss of penicillin and cadmium resistance. The proportion of sensitive variants, although variable, was higher than in untreated cultures. The 'penicillinase plasmid' was absent from two cultures sensitive to penicillin and cadmium. The genes for penicillinase production in one culture (13136) were apparently carried by a plasmid of 105×10^6 daltons.

INTRODUCTION

Most of the genetic determinants of antibiotic resistance in *Staphylococcus aureus* are located in extrachromosomal elements (Lacey, 1975). One of the most extensively studied plasmids determines penicillinase production and may also code for resistance to mercury, cadmium, arsenate, arsenite, bismuth, lead and zinc (Novick, 1967; Novick & Roth, 1968; Dyke *et al.*, 1970).

Various substances, such as acridine dyes (Hashimoto *et al.*, 1964; Mitsunashi *et al.*, 1963; Chabbert *et al.*, 1964), ethidium bromide (Bouanchaud *et al.*, 1969; Rubin & Rosenblum, 1971), sodium dodecyl sulphate and urea (Sonstein & Baldwin, 1972*a, b*), rifampicin (Johnston & Richmond, 1970) and cloxacillin (Lacey *et al.*, 1973) eliminate antibiotic resistance in *S. aureus*.

In this paper, treatment of three strains of *S. aureus* with guanidine hydrochloride (GuHCl) is shown to select for penicillin-sensitive colonies.

METHODS

Strains and culture media. Strains 4 and 3A6 were isolated from clinical material in São Paulo, Brazil. Strain 4 is resistant to penicillin (Pc); strain 3A6 is resistant to Pc, cadmium (Cd), mercury (Hg) and sulphonamide (Su). Strain 13136 was kindly provided by R. W. Lacey and is resistant to Pc, Cd, Hg, Su, arsenate (Asa), tetracycline (Tc), streptomycin (Sm) and methicillin (Mc). Culture media were nutrient broth and tryptic soy broth (TSB, Difco).

Loss of penicillin and cadmium resistance. About 10^4 bacteria ml^{-1} from an overnight culture were inoculated in tubes containing nutrient broth with GuHCl at subinhibitory concentrations (from 1.3 to 2.0 mg ml^{-1}). After incubation at 37°C for 15 to 18 h with shaking ($120 \text{ rev. min}^{-1}$), the suspensions were diluted and plated on nutrient agar, and incubated for 24 h at 37°C . Loss of resistance was determined by replica-plating on to nutrient agar plates containing Pc ($10 \mu\text{g ml}^{-1}$) or Cd (10^{-4} M).

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Reversion of sensitive cultures to penicillin and cadmium resistance. Sensitive bacteria, obtained after treatment with GuHCl , were treated again with GuHCl as above. After growth, the suspensions were concentrated to about 10^9 bacteria ml^{-1} , inoculated on plates containing Pc ($10 \mu\text{g ml}^{-1}$) or Cd (10^{-4} M) and incubated for 24 h at 37°C . Resistant revertant colonies should grow on these plates.

Effect of GuHCl on growth of resistant and sensitive strains. About 10^4 bacteria ml^{-1} from overnight cultures of strains 3A6 Pc^RCd^R , 3A6 Pc^SCd^R , 13136 Pc^RCd^R and 13136 Pc^SCd^R were inoculated in tubes containing nutrient broth with GuHCl (1.5 and 2.0 mg ml^{-1}). After incubation at 37°C for 15 h with shaking, the suspensions were diluted and plated on nutrient agar, incubated for 24 h at 37°C and the titres of the cultures were calculated.

Isolation of plasmid DNA. Cultures were grown in 50 ml TSB overnight at 37°C with shaking and then centrifuged in a Sorvall RC2-B centrifuge at $5090 g$ for 20 min. The bacteria were resuspended in 20 ml TES buffer (Tris 0.05 M , NaCl 0.05 M , EDTA 0.005 M , pH 8.0), recentrifuged as above and again resuspended in 2.0 ml TES buffer, with a final NaCl concentration of 2.5 M . Lysostaphin (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) was added to a final concentration of $200 \mu\text{g ml}^{-1}$, followed by gentle shaking for 15 min at 37°C . Then $200 \mu\text{l}$ EDTA (0.25 M , pH 8.0) was added and the preparations were held at 22°C for 10 min. Subsequently, 0.1 ml sodium dodecyl sulphate solution [20% (w/v) prepared in TES buffer, pH 8.5] and TES buffer were added to give a final NaCl concentration of 1.25 M . The preparations were left on ice for about 2 h and then centrifuged for 1 h at $39100 g$. The supernatants were collected in tubes containing $4 \mu\text{l}$ RNAase solution (5 mg ml^{-1} ; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and held for 1 h at 37°C . TES buffer-saturated phenol (5 ml) was then added and the tubes were gently mixed by about 10 inversions and centrifuged for 15 min at $3020 g$ at 15°C . The supernatants were collected and TES-saturated phenol (5 ml) was added. Centrifugation was then repeated for 15 min at $3020 g$ at 15°C . Residual phenol was extracted with water-saturated diethyl ether. Potassium acetate (0.1 ml , 3 M) was added to a final concentration of 0.3 M , and the DNA was precipitated by addition of 2 vol. ethanol at -20°C . After overnight storage at -20°C the tubes were centrifuged for 20 min at $14500 g$ to sediment the DNA, which was subsequently resuspended in $50 \mu\text{l}$ of Tris/HCl (0.01 M , pH 8.9). The preparations, ready for electrophoresis, were stored at -20°C .

Agarose gel electrophoresis of DNA. DNA was electrophoresed in 0.8% (w/v) agarose (Seakem-MCI-Biomedical) melted in TEB buffer (Tris 0.089 M , EDTA 2.5 mM , H_3BO_3 0.089 M , pH 8.2). To each DNA sample ($7 \mu\text{l}$), $5 \mu\text{l}$ of a solution containing 0.025% (w/v) bromophenol blue and 5% (w/v) Ficoll 400 (Sigma) in TEB buffer was added. Electrophoresis was performed at 10 mA for 15 min, then at 45 mA for 2 h (or until the dye reached the bottom of the gel). The gel was then stained for about 15 min in a freshly prepared solution of TEB buffer containing ethidium bromide (final concentration 100 ng ml^{-1}). The bands were visualized by fluorescence using a u.v. long-wave lamp and photographed using an orange filter and Kodak Tri-X film.

RESULTS AND DISCUSSION

Loss of resistances following exposure to optimal GuHCl . Loss of penicillin resistance in strain 4 occurred on treatment with GuHCl at a final concentration of 1.8 mg ml^{-1} , and this loss varied between 8.5 and 77.5% (Table 1). Spontaneous loss in these experiments varied from 0 to 17.9% . This observed loss suggests that the genes are part of a plasmid (Novick, 1967; Bouanchaud *et al.*, 1969; Lacey, 1975). The loss induced by GuHCl occurred only with an optimal ratio of the number of bacteria (about 10^4 ml^{-1}) to the drug concentration (from 1.3 to 2.0 mg ml^{-1}): the greatest effect occurred at subinhibitory concentrations, as reported for other curing agents, such as ethidium bromide (Bouanchaud *et al.*, 1969), acridine orange (Hahn & Ciak, 1971) and sodium dodecyl sulphate (Sonstein & Baldwin, 1972a). The effect of GuHCl in the loss of resistances was variable, but the loss of plasmids seems to be an unpredictable phenomenon (Lacey, 1975). Similar variations have been seen for other curing agents (Johnston & Richmond, 1970; Zimmermann *et al.*, 1971).

In strain 3A6, simultaneous loss of Pc and Cd resistances occurred spontaneously (from 0 to 0.5%) as well as after treatment with GuHCl (from 1.3 to 11.3%) suggesting that these markers are genetically linked, as is known for strain 13136 (Lacey & Chopra, 1972; Lacey & Grinstead, 1973).

In strain 13136, GuHCl seemed to be ineffective since loss of Pc and Cd resistances was only 0.03% . In sensitive bacteria of strain 13136, loss of Mc resistance also occurred, although Tc and Sm resistances were maintained. Sensitive bacteria from strains 3A6

Table 1. *Loss of resistance to penicillin in strain 4 by treatment with GuHCl*

Expt no.	No. of inoculated bacteria ml ⁻¹	GuHCl concn (mg ml ⁻¹)	Loss of resistance (%)	Total no. of colonies
1	1.7 × 10 ⁴	0	1.6	606
		1.8	40.2	757
2	2.7 × 10 ⁴	0	0.2	795
		1.8	19.01	142
3	2.8 × 10 ⁴	0	0	626
		1.8	77.5	209
4	3.0 × 10 ⁴	0	17.9	853
		1.8	50.4	115
5	3.0 × 10 ⁴	0	0.2	1492
		1.8	8.5	2519

Statistical analysis: $t = 3.64$; degrees of freedom = 4; $0.02 < P < 0.025$.

and 13136 had the same phage typing pattern and pigmentation as the original resistant strains. Further work with strain 4 was not carried out because it lacked other suitable markers and transduction was not detected.

Reversion of sensitive cultures to penicillin and cadmium resistance. No reversion to Pc and Cd resistance occurred when sensitive bacteria from strains 3A6 and 13136 were treated again with GuHCl.

Effect of GuHCl on growth of resistant and sensitive strains. Strain 3A6 Pc^RCd^R was less inhibited by GuHCl than was strain 3A6 Pc^SCd^S indicating that the Pc plasmid does not determine enhanced GuHCl sensitivity. Strain 13136 Pc^RCd^R was more inhibited by the drug than was strain 13136 Pc^SCd^S. We cannot, therefore, exclude the possibility of selection of pre-existing sensitive bacteria by GuHCl.

Identification and characterization of plasmid DNA. Plasmids were isolated from the following strains of *S. aureus*: 13136 Pc^RCd^R, 13136 Pc^SCd^S (following exposure to GuHCl), 3A6 Pc^RCd^R and 3A6 Pc^SCd^S (following exposure to GuHCl). DNA from the control strain of *Escherichia coli* K12 711 F⁻ (which does not carry extrachromosomal elements) showed only one band corresponding to the rest of the chromosomal material (Fig. 1, column 1). In the other columns, besides this chromosomal material, additional bands appeared corresponding to intact plasmids (Meyers *et al.*, 1976; Santos, personal communication). The bands obtained with DNA isolated from strain 13136 Pc^RCd^R (Fig. 1, column 2) correspond, from top to bottom, to a plasmid of 105×10^6 daltons, chromosomal material, and plasmids of 5.0×10^6 , 2.9×10^6 and 1.15×10^6 daltons, respectively. With DNA from strain 13136 Pc^SCd^S (Fig. 1, column 3) the band corresponding to the plasmid of 105×10^6 daltons is absent. With DNA from strain 3A6 Pc^RCd^R (Fig. 1, column 4), a band corresponding to a plasmid of 14.5×10^6 daltons was observed, while with DNA from strain 3A6 Pc^SCd^S (Fig. 1, column 5), no plasmid was observed. The size of this plasmid is similar to that of the penicillinase plasmids (Novick, 1967; Novick & Bouanchaud, 1971; Lacey & Chopra, 1975; Rush *et al.*, 1975). In strain 13136, previously studied by Lacey & Grinsted (1973), a plasmid of 2.9×10^6 daltons determined tetracycline resistance (Lacey & Richmond, 1974). The plasmids of 1.15×10^6 and 5.0×10^6 daltons are apparently cryptic, as there are no known phenotypic characteristics attributable to them. On the other hand, the band corresponding to the penicillinase plasmid of 20×10^6 daltons (Lacey & Grinsted, 1973) is missing, but a large plasmid of about 105×10^6 daltons appears, which may have resulted from a recombination of the penicillinase plasmid and another large plasmid during storage for 2 years in the laboratory.

Dornbusch *et al.* (1969) and Lacey (1972) suggested that the determinant of methicillin

Fig. 1. Electrophoresis of DNA in agarose gel (0.8%, w/v) was performed in a vertical chamber; the gel was stained with ethidium bromide and bands were visualized by fluorescence as described in Methods. 1, *Escherichia coli* K12 711 F⁻ with chromosomal material. 2, *Staphylococcus aureus* 13136 Pc^RCd^R: 1st band (from top), plasmid coding for Pc Cd Mc (105×10^6 daltons); 2nd band, chromosomal material; 3rd band, cryptic plasmid (5.0×10^6 daltons); 4th band, plasmid coding for Tc (2.9×10^6 daltons); 5th band, cryptic plasmid (1.15×10^6 daltons). 3, *Staphylococcus aureus* 13136 Pc^SCd^S (following GuHCl treatment): bands identical to sample 2 except the 1st band is absent. 4, *Staphylococcus aureus* 3A6 Pc^RCd^R: 1st band, plasmid coding for Pc Cd (14.5×10^6 daltons); 2nd band, chromosomal material. 5, *Staphylococcus aureus* 3A6 Pc^SCd^S (following GuHCl treatment): the 1st band is absent. 6, *Escherichia coli* K12 Ent P (307) (54×10^6 daltons). 7, *Escherichia coli* K12 J53 R1 (52×10^6 daltons). 8, *Escherichia coli* K12 J53 W-Sa (23×10^6 daltons). 9, *Escherichia coli* K12 JC411 ColE1 (4.2×10^6 daltons). 10, *Escherichia coli* K12 HB101 PBR322 (2.6×10^6 daltons).

resistance is located on a plasmid, apparently of high molecular weight (Grinsted & Lacey, 1973), although no circular DNA to which this resistance could be attributable has yet been isolated (Lacey & Grinsted, 1973; Stiffler *et al.*, 1973; Shalita *et al.*, 1977). The failure to detect such a plasmid might be due to its fragmentation during prolonged centrifugation in caesium chloride. It is relevant that some pleomorphic high molecular weight material has been seen in extracts from strain 13136 (Lacey & Grinsted, 1973) and that Dornbusch *et al.* (1969) described apparent co-elimination of penicillinase production with methicillin resistance in other strains. Strain 13136 Pc^SCd^S (treated with GuHCl) shows sensitivity to methicillin, while the original strain 13136 Pc^RCd^R is methicillin-resistant. It is possible that recombination occurred between the penicillinase and methicillin plasmids.

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