Evidence for Mutation to Streptomycin Resistance in Clinical Strains of *Staphylococcus aureus*

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SUMMARY

In three clinical strains of *Staphylococcus aureus* and one laboratory mutant (609 *str-r*) the gene determining streptomycin resistance was probably at a single chromosomal locus. Cell-free systems from these, and an additional clinical strain, showed little inhibition of protein synthesis in the presence of streptomycin, whereas systems from sensitive staphylococci were markedly affected by streptomycin.

INTRODUCTION

Streptomycin is an inhibitor of bacterial protein synthesis and streptomycin-resistant mutants obtained *in vitro* have abnormal ribosomes (Spotts & Stanier, 1961; Weisblum & Davies, 1968). The gene determining this type of resistance has a chromosomal location (Poston, 1966; Novick & Morse, 1967; Lacey, 1972).

However, in clinical isolates of *Escherichia coli* streptomycin-resistance occurs by enzymatic inactivation – either adenylation or phosphorylation (Davies, Brzezinska & Benviste, 1971; Smith, 1971), the relevant genes being carried by R factors. In these strains there is no evidence of modified ribosomes.

In a few clinical strains of *Staphylococcus aureus* there is some evidence for a plasmid locus or gene(s) determining streptomycin resistance (Ayliffe, 1970; Grubb & O'Reilly, 1971). The mechanism of resistance is uncertain. We have studied the genetical and biochemical basis of streptomycin resistance in four clinical strains of *Staphylococcus aureus*. The nature of the resistance in these strains seems identical to that of mutants selected *in vitro*.

METHODS

Strains of Staphylococcus aureus. Strains 13136, 13137, 11164 and 2273 are multi-resistant strains isolated in 1960, 1960, 1964 and 1967 respectively from clinical material. They were obtained, freeze-dried, from the Cross Infection Laboratory, Colindale, London, N.W.9. The minimum inhibitory concentration (MIC) of streptomycin was about 2000 µg/ml for each. Strain 609 *str-r* (MIC 2000 µg/ml) is a one-step streptomycin-resistant mutant of strain 609 (MIC 0.5 µg/ml) selected *in vitro*; the gene for streptomycin resistance in strain 609 *str-r* is chromosomal (Lacey, 1972). Strain 8325 (MIC of streptomycin 0.5 µg/ml) is the propagating strain (ps) of phage 47 in the International Basic Set. The recipient strain in transduction experiments was ps54.

Media, antibiotic sensitivity, mitomycin C induction, and transduction were as described previously (Lacey, 1971).

Curing. Attempts to cure streptomycin resistance were performed with rifampicin (Johnson & Richmond, 1970), acriflavine (Dornbush, Hallander & Löfqvist, 1969) and growth for 18 h at 43 °C.

Streptomycin assay was by the diffusion method of Grove & Randall (1955) using a *Micrococcus* strain (MIC 0.1 µg/ml) as indicator.

Material for cell-free system. Uniformly ^{14}C -labelled L-amino acid mixture (54 mCi/mAtom) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Ribonuclease A five times crystallized, deoxyribonuclease I electrophoretically pure, ATP and GTP were obtained from Sigma Chemical Co., London. Lysostaphin was obtained from Schwarz-Mann Laboratories, Orangeburg, New Jersey, U.S.A., puromycin from Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A., and chloramphenicol from Parke-Davis Co., Hounslow, Middlesex.

Preparation of cell-free extracts. Overnight broth cultures were diluted 1 in 25 into fresh warmed broth and grown at 37 °C with aeration to cell densities equivalent to 0.35 mg dry wt/ml (about 2.5 h). Cells from a litre of culture were then harvested by centrifugation at 4 °C, washed twice in 0.05 M-tris-hydrochloric acid buffer, pH 7.6, containing: ammonium acetate, 50 mM; magnesium chloride, 20 mM; potassium chloride, 50 mM; and dithiothreitol, 0.2 mM (TAMKSH buffer).

Washed cells were resuspended in 10 ml of TAMKSH buffer, lysostaphin added to 50 µg/ml and incubated for 10 min at 37 °C. Then deoxyribonuclease (5 µg/ml) was added and incubation continued for 30 s. The resultant lysates were cooled to 4 °C and centrifuged at 10000 g for 30 min to remove residual whole cells. The supernatants were retained for the following assay.

Assay for protein synthesis. Protein synthesis, directed by endogenous mRNA was estimated by the incorporation of ^{14}C from labelled amino acids into hot trichloroacetic acid (TCA) precipitable material.

Cell-free extract (0.1 ml) was added to 0.1 ml of solution containing: ATP, 7.5 mM; GTP, 1 mM; and ^{14}C -labelled amino acids in TAMKSH buffer, 10 µCi/ml. This mixture, 'the complete system', was incubated at 37 °C for 40 min and then precipitated by the addition of TCA (final concentration 5%, w/v), followed by heating at 90 °C for 20 min. Precipitates were collected, washed and dried on glass-fibre filters (Whatman GF/C), and radioactivity estimated by liquid scintillation using butyl-PBD-toluene (4 g/l) as scintillant. All experiments were done in duplicate and averaged.

RESULTS

Location of genes determining streptomycin resistance in strains 13136, 11164 and 2273. Following incubation at 43 °C, in the presence of 0.0075 µg rifampicin/ml, or 5 µg acriflavine/ml at least 3000 colonies from each strain were examined for loss of streptomycin resistance by replica plating. No loss of resistance was detected. This stability of resistance can be accounted for by either a chromosomal genetic locus or a stable plasmid. Further evidence for a chromosomal locus was obtained by studying the transduction kinetics from a mitomycin C lysate prepared from each strain. The transductants, selected on nutrient agar containing 100 µg streptomycin/ml, were counted after 48 h of incubation at 37 °C. Before plating, the recipient was incubated at 37 °C for 2 h to allow phenotypic expression. For each strain, the transduction frequency was stimulated more than 20-fold by low doses of u.v. irradiation (Fig. 1).

Although in one instance the transduction frequency of a plasmid gene is stimulated fivefold by u.v. light (Rubin & Rosenblum, 1971), a 20-fold or greater degree of stimulation is indicative of chromosomally located genes (Arber, 1960; Asheshov, 1966; Dyke, Parker & Richmond, 1970).

Fig. 1. Effect of u.v. exposure on the transduction frequency of streptomycin resistance from mitomycin C-induced lysates from three cultures. The frequency is expressed as the number of transductants/unirradiated phage titre. ■—■, Culture 13136 as donor; ●—●, culture 11164 as donor; □—□, culture 2273 as donor. For comparison ○—○ denotes the transduction of a plasmid gene, penicillinase, from strain 13136. Strain ps54 was the recipient for all.

In a series of experiments transductants from lysates of strains 13136, 11164 and 609 *str-r* were selected on media containing 10 µg streptomycin/ml. Transductants were replicated on to media containing 500 µg streptomycin/ml. About 50 colonies obtained from unirradiated lysates and 200 from lysates u.v. irradiated for 2 min were thus examined for each strain. No colony that grew at 10 µg streptomycin/ml failed to grow at 500 µg/ml. This finding is consistent with the existence of a single genetic locus in strains 13136, 11164 and 2273. Strain 609 *str-r* is a one-step high-level mutant and therefore probably has a single locus for streptomycin resistance. This is confirmed by these results.

Failure to demonstrate streptomycin inactivation. No antibiotic inactivation was observed when streptomycin (50 µg/ml) was incubated with growing cultures of the resistant strains or with cell-free extracts.

Characteristics of cell-free system. Assay systems using extracts from all strains investigated exhibited properties expected of cell-free protein synthesizing systems. As an example an assay system using an extract from one of the strains is shown in Table 1. The incorporation of radioactivity into protein was greatly reduced in the absence of ATP and GTP, or in the presence of ribonuclease, puromycin or chloramphenicol. The incorporation of radioactivity into protein was optimal at 20 mM-magnesium; this level is similar to that found for another staphylococcal cell-free system directed by endogenous mRNA (Mao, 1967).

Effect of streptomycin on protein synthesis in the cell-free system. At a streptomycin concentration of 10 µg/ml protein synthesis in extracts from two streptomycin-sensitive strains (8325 and 609) was more than 70% inhibited (Table 2). Systems from both the laboratory mutant (609 *str-r*) and the clinical strains 13136 and 1116 showed less than 15% inhibition.

Table 1. *Properties of a typical system using a cell-free extract from Staphylococcus aureus 8325 assayed for amino acid incorporation*

Conditions of system	Hot TCA precipitated radioactivity (c.p.m.)*
Complete	6403
With 25 μ g ribonuclease/ml	194
With 50 μ g chloramphenicol/ml	208
With 250 μ g puromycin/ml	106
Without ATP and GTP	336

* A time 0 background count of 183 c.p.m. has been subtracted from all values.

Table 2. *Effect of various concentrations of streptomycin on the incorporation of labelled amino acids into protein (expressed as % inhibition) in cell-free systems obtained from five strains of Staphylococcus aureus*

Strain	MIC of streptomycin (μ g/ml)	Origin of resistance	% inhibition of protein synthesis at various streptomycin levels (μ g/ml)						
			0.1	0.3	0.5	1	2.5	5	10
8325	0.5	—	0	12	53	61	76	68	73
609	0.5	—	12	16	42	63	84	83	80
609 <i>str-r</i>	2000	Laboratory mutant	0	0	0	0	0	0	0
13136	2000	Clinical strain	0	0	0	0	0	0	0
11164	2000	Clinical strain	0	0	0	4	7	8	15

Systems from strains 13137 and 2273 showed 12 and 29% inhibition respectively at this concentration.

At a lower concentration of streptomycin (0.5 μ g/ml) inhibition of amino acid incorporation in extracts from the sensitive strains was approximately 50%, with no inhibition for 609 *str-r*, 13136, 11164, 2273 and 13137.

DISCUSSION

In three strains of *Staphylococcus aureus* obtained from clinical sources, the genes determining streptomycin resistance probably have a single chromosomal locus. Evidence for this conclusion is based on the stability of the resistance under various conditions and transduction kinetics. U.v. irradiation stimulated markedly the transduction frequency, and high-level resistance was always acquired by the recipient even when selected for low-level resistance. The genetic basis of streptomycin resistance in these strains closely resembled that of the laboratory mutant 609 *str-r*.

The mechanism of streptomycin resistance in these three, and in another clinical isolate (13137), seems to be identical to that of the laboratory strain 609 *str-r*. Cell-free extracts were prepared which incorporated amino acids into protein. Those derived from the resistant strains were unaffected by streptomycin at concentrations known to inhibit systems from sensitive organisms (Luzzatto, Apirion & Schlesinger, 1968). Preparations from the streptomycin-sensitive strains (609 and 8325) were affected by as little as 0.3 μ g streptomycin/ml. Possibly resistance to streptomycin is due to a modified component of protein synthesis other than the ribosome. However, such a mechanism is not known, and it is more likely that in both clinical and laboratory strains, resistance is ribosomal. Additionally, we have eliminated the possibilities that streptomycin resistance in these strains could be mediated

by enzymatic inactivation or streptomycin impermeability. Therefore, on the basis of genetic and biochemical evidence, the clinical strains resemble the laboratory mutants and the former may well have acquired streptomycin resistance *in vivo* in a way comparable to strains *in vitro*.

Recent evidence suggests that the acquisition of multiple antibiotic resistance amongst enteric organisms is predominantly by R factors. In *Staphylococcus aureus*, genes determining resistance to a variety of antibiotics other than streptomycin are plasmid borne and transferable at medium-high frequencies between mixed cultures (e.g. Lacey, 1971); this may account for the high incidence of strains resistant to such antibiotics. However, clinical strains of *Staphylococcus aureus* may have acquired streptomycin resistance through a mutational step. Analysis of further staphylococci is needed before the significance of this type of resistance can be assessed.

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