SHORT COMMUNICATION

Constitutive Erythromycin Resistance Plasmid in *Staphylococcus aureus*

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A plasmid with a molecular mass of 1.4×10^6 daltons has been identified in *Staphylococcus aureus*. This plasmid determines constitutive resistance to erythromycin and lincomycin and is the smallest naturally occurring element coding for antibiotic resistance in this species.

INTRODUCTION

Erythromycin resistance in *Staphylococcus aureus* is often inducible (Bronson & Pattee, 1972; Ono *et al.*, 1975; Lacey, 1977) but may be constitutive (Garrod, 1957). Inducible resistance can be determined by plasmid genes (Novick & Bouanchaud, 1971; Dornbusch & Dahlstrom, 1973; Lindberg & Rudin, 1975). Iordanescu (1976) has described such a plasmid with a molecular mass of 2.4×10^6 daltons. Constitutive macrolide resistance may be determined by a penicillinase plasmid (Mitsuhashi *et al.*, 1965; Novick, 1967). Integration of genes coding for constitutive resistance into the chromosome can occur (Novick, 1967; Lindberg & Rudin, 1975).

We describe here constitutive macrolide resistance coded for by a small plasmid (pRJ5) of only 1.4×10^6 daltons.

METHODS

Strains. The *Staphylococcus aureus* strains used were isolated from patients in Brazil; all were coagulase-positive and DNAase-positive. They were purified in Chapman (BBL) medium and stored on nutrient agar slants at room temperature. Strain A-443, which is resistant to penicillin (Pc), tetracycline (Tc), erythromycin (Em) and lincomycin (Lm), was used as the donor of drug resistance. Strains SCC-5, SCC-8 and SCC-10, which are sensitive to Tc, Em and Lm and marked chromosomally with resistance to rifampicin (Rif), were used as recipients.

Media. Nutrient broth, nutrient agar (Difco) and phage buffer (Novick, 1963) were used. CaCl_2 (final concentration 0.01 M) was added for the phage work.

Determination of drug resistance. The minimal inhibitory concentration (m.i.c.) was determined by an agar plate dilution method (Lacey & Grinstead, 1973).

Preparation of phage lysates. Ultraviolet-induced lysates were prepared by the method of Schwesinger & Novick (1975) by exposure to a GE-germicidal lamp (giving a dose of $0.93 \text{ J m}^{-2} \text{ s}^{-1}$) for 20 s. The titre of lysates was determined by the method of Adams (1959).

Transduction. A suspension of recipient cells, grown overnight statically in nutrient broth at 37°C , was mixed with an equal volume of lysate supplemented with CaCl_2 in a sterile centrifuge tube. A low multiplicity of infection was used (from 0.002 to 0.005). After incubation at 37°C for 30 min, sodium citrate (final

concentration 0.03 M) was added and the mixture was centrifuged for 15 min at 12000 g. The cells were resuspended in 4.5 ml nutrient broth supplemented with sodium citrate (final concentration 0.01 M) and incubated statically at 37 °C for 2 h. The cells were again centrifuged and resuspended in nutrient broth to concentrate them 5 to 20 times. Then, 0.1 ml was seeded on nutrient agar supplemented with Tc (10 µg ml⁻¹), Em (10 µg ml⁻¹) or Lm (10 µg ml⁻¹). The plates were incubated at 37 °C for 48 h to detect transductants. Simultaneous transfer of two or more drug resistance markers was analysed by replica-plating (Lederberg & Lederberg, 1952).

Loss of antibiotic resistance. About 10⁸ colony-forming units from a broth overnight culture of strain A-443 were inoculated into two flasks containing 10 ml nutrient broth. One culture was grown at 43 °C and the other at 37 °C, both with the same vigorous shaking for 6 h. Dilutions of each culture were then plated on nutrient agar, incubated for 48 h at 37 °C and replica-plated to nutrient-antibiotic agar; loss of antibiotic resistance was recorded after 24 h at 37 °C.

Isolation of plasmid DNA. The method of Meyers *et al.* (1976) was used with modifications for *S. aureus* strains. Cells grown overnight in 40 ml Tryptic Soy Broth were washed and resuspended in 1.5 ml TE buffer (10 mM-Tris, 1 mM-EDTA; pH 8.0). Lysostaphin was added to a final concentration of 500 µg ml⁻¹ and the mixture was held for 15 min at 37 °C; 200 µl EDTA (250 mM; pH 8.0) and 200 µl SDS (10%, w/v, in 50 mM-Tris, 5 mM-EDTA; pH 8.5) were then added and the mixture was kept on ice for 3 h. Cleared lysates were centrifuged for 40 min at 39100 g. After RNA digestion, phenol extraction and centrifugation were done to obtain a clear aqueous phase. The aqueous phase was brought to 0.3 M-potassium acetate (final concentration). Ethanol-precipitated DNA was resuspended in 80 µl TEB buffer (89 mM-Tris, 2.5 mM-EDTA, 8.9 mM-H₃BO₃; pH 8.2) and subjected to electrophoresis in 0.8% (w/v) agarose (Bio-Rad) in TEB buffer. To each DNA sample (10 µl), 15 µl of a solution containing 0.025% (w/v) bromophenol blue and 5% Ficoll 400000 in TEB buffer was added. The electrophoresis was performed in a vertical chamber at 10 mA for 15 min, and then at 45 mA until the dye reached the bottom of the gel. The gel was stained for 15 min in a solution of ethidium bromide in TEB buffer. The DNA bands were visualized using a short-wavelength u.v. lamp.

RESULTS AND DISCUSSION

The resistances to Tc and Em-Lm of donor strain A-443 were transduced at high frequencies (from 1.4×10^{-4} to 7.4×10^{-4}) to all three recipient strains (SCC-5, SCC-8, SCC-10). There was 100% cotransduction of Em and Lm resistance; this was expected because strain A-443 showed constitutive resistance to the macrolide antibiotics (data not shown).

When the transductants were selected by Em or Lm, 16.9 to 19.1% of the tested clones were also Tc^R, but when selected by Tc, only 1.3 to 2.4% of the colonies were also Em^R-Lm^R. The pigmentation and phage type of the transductant strains were identical to those of the recipients and their resistance was similar to that of the donor strain A-443.

Following growth at 37 °C, Em resistance was lost from 12.1 to 31.0% of the colonies and at 43 °C, from 12.0 to 52.6%. Resistance to Pc or Tc was lost only occasionally. In rare cases, there was simultaneous loss of resistance to Pc and Em (0.48 to 1.60%), to Tc and Em (0.06 to 0.27%) or to Pc and Tc (0.06 to 0.22%).

The above results indicate that in strain A-443 the genes coding for Pc^R, Tc^R and Em^R-Lm^R are probably carried by different extrachromosomal elements.

The results of plasmid isolation are shown in Fig. 1. In column A, the lower band (diffuse) corresponds to residual chromosomal matter and the upper band to plasmid DNA (62×10^6 daltons); although the sample is *Escherichia coli* strain A-252 F⁻ Lac⁻, it may indicate a plasmid other than F. Column B shows the bands obtained with DNA extracted from strain A-443; these correspond, from top to bottom, to chromosomal matter and to plasmids of 5.8×10^6 , 2.8×10^6 , 2.4×10^6 and 1.4×10^6 daltons, respectively. In column C, which represents DNA from recipient strain SCC-8 Rif^RPc^R, no plasmid bands are visible. In column D, representing DNA from transductant T121 (SCC-8 Rif^RPc^RTc^R), there are two bands corresponding to plasmids of 5.8×10^6 and 2.8×10^6 daltons. Column E, representing DNA from cotransductant T124 (SCC-8 Rif^RPc^RTc^REm^R-Lm^R), shows the same four plasmid bands as in column B. In column F, representing DNA from

Fig. 1. Agarose gel electrophoresis of DNA. Strains: A, *Escherichia coli* A-252 F⁻ Lac⁻; B, *Staphylococcus aureus* A-443 Pc^RTc^REm^R-Lm^R; C, *S. aureus* SCC-8 Rif^RPc^R; D, *S. aureus* SCC-8 Rif^RPc^RTc^R; E, *S. aureus* SCC-8 Rif^RPc^RTc^REm^R-Lm^R; F, *S. aureus* SCC-8 Rif^RPc^REm^R-Lm^R; G, *S. aureus* SCC-8 Rif^RPc^RTc^REm^R-Lm^R; H, *S. aureus* 13136 Pc^RTc^R (penicillinase plasmid, 20×10^6 daltons; tetracycline plasmid, 2.9×10^6 daltons); I, *E. coli* K12 HB101 pBR322 (2.6×10^6 daltons); J, *E. coli* K12 J53 RP4 (34×10^6 daltons); K, *E. coli* K12 711 F⁻ Ent P307 (54×10^6 daltons); L, *E. coli* K12 pSF2124 (6.5×10^6 daltons).

transductant E129 (SCC-8 Rif^RPc^REm^R-Lm^R), two bands corresponding to plasmids of 2.4×10^6 and 1.4×10^6 daltons are visible. Column G, showing DNA from cotransductant E119 (SCC-8 Rif^RPc^RTc^REm^R-Lm^R), again has the same four plasmid bands as in column B. Columns H to L contain reference plasmids.

Thus, strain A-443 harbours at least two different plasmids, one of 2.8×10^6 daltons and the other of 1.4×10^6 daltons. Like other small plasmids of *S. aureus* (Grubb *et al.*, 1972; Kono & Sasatsu, 1976; Iordanescu, 1977), they can be cotransduced. However, they segregate separately in the cotransductant strains.

As the plasmids of 5.8×10^6 and 2.8×10^6 daltons were also present in the extracts of transductant T121 (Tc^R) and the cotransductants T124 and E119 (both Tc^R Em^R-Lm^R), we conclude that these plasmids correspond, respectively, to the open circular and covalently closed circular forms of the Tc plasmid. The size of this plasmid is in accordance with literature data (Novick & Bouanchaud, 1971; Chopra *et al.*, 1973; Stiffler *et al.*, 1974).

The plasmids of 2.4×10^6 and 1.4×10^6 daltons were always present in the DNA preparations obtained from transductant E129 (Em^R-Lm^R) and from cotransductants E119 and T124 (both Tc^R Em^R-Lm^R). Thus, these plasmids represent, respectively, the open circular and covalently closed circular forms of the Em plasmid.

As far as we are aware, this is the first report of a plasmid coding only for constitutive

erythromycin resistance in *S. aureus*. Novick *et al.* (1979) showed that the *ermB* gene (coding for constitutive Em^R) included in the penicillinase plasmid pI258 is a transposable element of 3.5×10^6 daltons.

Our plasmid, designated pRJ5, differs from plasmid pE194 isolated by Iordanescu (1976) in that it is smaller and codes for constitutive resistance. Further work is required to fully characterize this plasmid.

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REFERENCES

- ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience.
- BRONSON, D. L. & PATTEE, P. A. (1972). Genetic analysis of resistance to erythromycin and oleandomycin in *Staphylococcus aureus*. *Canadian Journal of Microbiology* **18**, 429–434.
- CHOPRA, I., BENNETT, P. M. & LACEY, R. W. (1973). A variety of staphylococcal plasmids present as multiple copies. *Journal of General Microbiology* **79**, 343–345.
- DORNBUSCH, K. & DAHLSTROM, A. (1973). A genetic study of inducible erythromycin resistance in *Staphylococcus aureus*. *Acta pathologica et microbiologica scandinavica* **B81**, 497–507.
- GARROD, L. P. (1957). The erythromycin group of antibiotics. *British Medical Journal* **2**, 57–63.
- GRUBB, W. B., O'REILLY, R. J. & MAY, J. W. (1972). Segregation of co-transduced streptomycin and tetracycline resistance in *Staphylococcus aureus*. *Genetical Research* **20**, 43–50.
- IORDANESCU, S. (1976). Three distinct plasmids originating in the same *Staphylococcus aureus* strain. *Archives roumaines de pathologie expérimentale et de microbiologie* **35**, 111–118.
- IORDANESCU, S. (1977). Relationship between co-transducible plasmids in *Staphylococcus aureus*. *Journal of Bacteriology* **129**, 71–75.
- KONO, M. & SASATSU, M. (1976). Association of a penicillin resistance gene with a tetracycline resistance plasmid (P_{TP-2}) in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **9**, 706–712.
- LACEY, R. W. (1977). Lack of evidence for mutation to erythromycin resistance in clinical strains of *Staphylococcus aureus*. *Journal of Clinical Pathology* **30**, 602–605.
- LACEY, R. W. & GRINSTED, J. (1973). Genetic analysis of methicillin-resistant strains of *Staphylococcus*: evidence for their evolution from a single clone. *Journal of Medical Microbiology* **6**, 511–526.
- LEDERBERG, J. & LEDERBERG, E. M. (1952). Replicating and selection of bacterial mutants. *Journal of Bacteriology* **63**, 390–406.
- LINDBERG, M. & RUDIN, L. (1975). Thymineless bacteriophage induction in *Staphylococcus aureus*. II. Specific transduction of constitutive and inducible erythromycin resistance. *Journal of Virology* **16**, 1367–1374.
- MEYERS, J. A., SANCHEZ, D., ELWELL, L. P. & FALKOW, S. (1976). Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **127**, 1529–1537.
- MITSUHASHI, S., HASHIMOTO, H., KONO, M. & MORIMURA, M. (1965). Drug resistance of staphylococci. II. Joint elimination and joint transduction of the determinants of penicillinase production and resistance to macrolide antibiotics. *Journal of Bacteriology* **89**, 988–992.
- NOVICK, R. P. (1963). Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *Journal of General Microbiology* **33**, 121–136.
- NOVICK, R. P. (1967). Penicillinase plasmid of *Staphylococcus aureus*. *Federation Proceedings* **26**, 29–38.
- NOVICK, R. P. & BOUANCHAUD, D. (1971). Extrachromosomal nature of drug resistance in *Staphylococcus aureus*. *Annals of the New York Academy of Sciences* **182**, 279–294.
- NOVICK, R. P., EDELMAN, I., SCHWESINGER, M. D., GRUSS, A. D., SWANSON, E. C. & PATTEE, P. A. (1979). Genetic translocation in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 400–404.
- ONO, H., INOUE, M., MAO, J. C. H. & MITSUHASHI, S. (1975). Drug resistance in *Staphylococcus aureus*. Induction of macrolide resistance by erythromycin, oleandomycin and their derivatives. *Japanese Journal of Microbiology* **19**, 343–347.
- SCHWESINGER, M. R. & NOVICK, R. P. (1975). Prophage-dependent plasmid integration in *Staphylococcus aureus*. *Journal of Bacteriology* **123**, 724–738.
- STIFFLER, P. W., SWEENEY, H. M. & COHEN, S. (1974). Co-transduction of plasmid mediating resistance to tetracycline and chloramphenicol in *Staphylococcus aureus*. *Journal of Bacteriology* **120**, 934–944.