SHORT COMMUNICATION

Constitutive Erythromycin Resistance Plasmid in
Staphylococcus aureus

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A plasmid with a molecular mass of $1.4 \times 10^6$ 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 $2.4 \times 10^6$ 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 $1.4 \times 10^6$ 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 & Grinsted, 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 J m$^{-2}$ 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 12,000 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 10⁶ 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 39,100 g. After RNA digestion, phenol extraction and centrifugation were done to obtain a clear aqueous phase. The aqueous phase was brought to 0.8 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-H₃BO₄, 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 Tc⁵, but when selected by Tc, only 1.3 to 2.4% of the colonies were also Em⁵-Lm⁵. 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 Pc⁵, Tc⁵ and Em⁵-Lm⁵ 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 × 10⁶ 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 × 10⁸, 2.8 × 10⁶, 2.4 × 10⁶ and 1.4 × 10⁶ daltons, respectively. In column C, which represents DNA from recipient strain SCC-8 Rif⁵-Pc⁵, no plasmid bands are visible. In column D, representing DNA from transductant T121 (SCC-8 Rif⁵-Pc⁵-Tc⁵), there are two bands corresponding to plasmids of 5.8 × 10⁶ and 2.8 × 10⁶ daltons. Column E, representing DNA from cotransductant T124 (SCC-8 Rif⁵-Pc⁵-Tc⁵ Em⁵-Lm⁵), 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*- *Tc*- *Em*- *Lm*; C, *S. aureus* SCC-8 *Rif*- *Pc*; D, *S. aureus* SCC-8 *Rif*- *Pc*- *Tc*; E, *S. aureus* SCC-8 *Rif*- *Pc*- *Tc*- *Em*- *Lm*; F, *S. aureus* SCC-8 *Rif*- *Pc*- *Em*- *Lm*; G, *S. aureus* SCC-8 *Rif*- *Pc*- *Em*- *Lm*; H, *S. aureus* 13136 *Pc*- *Tc* (penicillinase plasmid, 20 x 10^6 daltons; tetracycline plasmid, 2.9 x 10^6 daltons); I, *E. coli* K12 HB101 pBR322 (2.6 x 10^6 daltons); J, *E. coli* K12 7111 F- *Pc*- R4 (2.6 x 10^6 daltons); K, *E. coli* K12 7111 F- EntP307 (5.4 x 10^6 daltons); L, *E. coli* K12 pSF2124 (6.5 x 10^6 daltons).

transductant E129 (SCC-8 *Rif*- *Pc*- *Em*- *Lm*), two bands corresponding to plasmids of 2.4 x 10^6 and 1.4 x 10^6 daltons are visible. Column G, showing DNA from cotransductant E119 (SCC-8 *Rif*- *Pc*- *Tc*- *Em*- *Lm*), 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 x 10^6 daltons and the other of 1.4 x 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 x 10^6 and 2.8 x 10^6 daltons were also present in the extracts of transductant T121 (Te^b) and the cotransductants T124 and E119 (both Te^b Em^a-Lm^b), we conclude that these plasmids correspond, respectively, to the open circular and covalently closed circular forms of the Te 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 x 10^6 and 1.4 x 10^6 daltons were always present in the DNA preparations obtained from transductant E129 (Em^a-Lm^b) and from cotransductants E119 and T124 (both Te^b Em^a-Lm^b). 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 \textit{ermB} gene (coding for constitutive Em\textsuperscript{R}) included in the penicillinase plasmid pI258 is a transposable element of \(3.5 \times 10^6\) daltons.

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

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