Distinct groups of plasmids correlated with bacteriocin production in *Staphylococcus aureus*

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The genetic basis of bacteriocin (Bac) production by six strains of *Staphylococcus aureus* was examined. Gene transfer experiments (in which the plasmids were tagged with the erythromycin resistance transposon Tn551) and plasmid-elimination experiments by growth at 43 °C associated bacteriocin production with a particular plasmid in each strain. The Bac plasmids could be separated into two distinct groups: the first comprised plasmids larger than 40 kb, which did not specify immunity to bacteriocins; the second comprised small plasmids (8.0–10.4 kb) which also specified immunity to bacteriocins. The sequence relations among the small plasmids (pRJ6, pRJ9, pRJ10 and pRJ11) were investigated by comparing restriction enzyme digest patterns and by hybridization. Plasmids pRJ10 and pRJ11 were indistinguishable and very closely related to plasmid pRJ9. Plasmid pRJ6, although different from the others, shared regions of sequence homology with them. No homology was found between plasmids pRJ6 or pRJ9 and the large Bac plasmids.

Introduction

The ability of various micro-organisms to inhibit the growth of other micro-organisms has long been recognized and sometimes attributed to metabolic waste products, lytic enzymes, antibiotics, bacteriophages or bacteriocins (Tagg *et al.*, 1976). Bacteriocins are proteins, or proteins complexed with lipids and carbohydrates, which are lethal to other bacteria; their effect is generally limited to closely related species possessing specific receptor sites. Initial studies on bacteriocins were performed in Gram-negative bacteria, mainly in *Escherichia coli* and related species, and much knowledge has been accumulated concerning their chemistry, genetics and mode of action (Reeves, 1972; Hardy, 1975). In these organisms, bacteriocin production has been shown to be controlled by bacteriocinogenic (Bac) plasmids that often specify immunity to the bacteriocin produced. Production of bacteriocins and bacteriocin-like substances has been observed in Gram-positive bacteria, including staphylococci, and there have been various reports of the isolation, purification and biochemical properties of staphylococcal bacteriocins (Dajani & Wannamaker, 1969; Gagliano & Hinsdill, 1970; Jetten *et al.*, 1972; Jetten & Vogels, 1973; Dajani &

Taube, 1974; Nakamura *et al.*, 1983; Ersfeld-Dressen *et al.*, 1984). In *Staphylococcus aureus*, bacteriocin production was correlated with the presence of a plasmid usually involved in type B exfoliative toxin production (Warren *et al.*, 1974; O'Reilly *et al.*, 1981; Masterson *et al.*, 1983). These 42 kb plasmids were generally isolated from phage group II epidermolytic strains (Warren *et al.*, 1975; Warren, 1980; O'Reilly *et al.*, 1981). However, no detailed genetic and molecular characterization of bacteriocinogenic plasmids of *S. aureus* has been reported.

Here we describe genetic studies on the basis of bacteriocin production by six strains of *S. aureus*. Gene transfer experiments and plasmid-elimination experiments associated bacteriocin production with a particular plasmid in each strain. The plasmids involved in the production of these substances could be separated into two distinct groups: plasmids larger than 40 kb; and small plasmids (8.0–10.4 kb). The sequence relations among the small Bac plasmids were investigated by comparing restriction enzyme digest patterns and by hybridization.

Methods

Bacterial strains and culture conditions. The strains used are listed in Table 1. Strains designated H were isolated from skin lesions, the noses or throats of outpatients of two children's hospitals in Rio de Janeiro.

Abbreviations: AMS, antimicrobial substance(s); ETA, ETB, exfoliative toxin type A, B.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Size (kb)	Phenotype associated with plasmid*	Source or reference
A53	pRJ9	10.4	Bac ⁺ Imm ⁺	This study
A65	pRJ10	10.4	Bac ⁺ Imm ⁺	
	pRJ15	27.0	Pc ^r	
	pRJ16	4.4	Tc ^r	
	pRJ17	1.2	?	
A70	pRJ6	8.0	Bac ⁺ Imm ⁺	
A82	pRJ11	10.4	Bac ⁺ Imm ⁺	
	pRJ18	27.0	Pc ^r	
	pRJ19	4.5	Cm ^r	
	pRJ20	1.2	?	
H55	pRJ12	>40.0	Bac ⁺	Warren (1980) O'Reilly <i>et al.</i> (1981) Novick (1967) Pattee (1981)
H56	pRJ13	>40.0	Bac ⁺	
UT0101	pRW0001	42.0	Bac ⁺ Tox ⁺	
TC146	pTC146	41.0	Bac ⁺ Tox ⁺	
RN451	-	-	-	
ISP479	pRN3032	27.5	Cd ^r Em ^r Tsr	

* Bac, bacteriocin; Imm, immunity; Pc, penicillin; Tc, tetracycline; Cm, chloramphenicol; Tox, exfoliative toxin; Cd, cadmium; Em, erythromycin; Tsr, thermosensitive for replication.

Strains designated A were isolated from commercial food. The bacteria were grown in CY medium (Novick, 1967) and stored at -70 °C until needed. Subcultures were made in CNE broth [1.0% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3% (w/v) Casamino acids, 0.2% (w/v) meat extract, 0.6% (w/v) NaCl, pH 7.2].

Corynebacterium fimi (NCTC 7547), rather than *S. aureus*, was used as an indicator strain for production of antimicrobial substance(s) (AMS), because of its greater sensitivity to staphylococcal AMS (Parker & Simmons, 1959).

Assay for AMS production. The method described by Anthony *et al.* (1972) was used, with minor modifications. The strains were inoculated as a spot on the surface of a brain-heart infusion (BHI; Difco) agar plate. After 24 h at 37 °C, the bacteria were killed by exposure to chloroform vapour for 30 min and the plate sprayed with the indicator strain (*C. fimi*). AMS production was indicated by zones of inhibition around the colony after further incubation at 37 °C for 18 h. Strain UT0101 (Bac⁺) was used as a positive control.

To determine the inhibitory spectrum of each AMS, strains representative of different species of Gram-positive and Gram-negative bacteria were tested as indicators. In these experiments the producer strains were grown on both BHI and CNE plates.

Characterization of AMS. The effects of heat and enzymes were determined by the methods of Gross & Vidaver (1978). Plates with colonies of AMS producers were exposed to chloroform, incubated at 50, 65 or 75 °C for 60 min, cooled to room temperature, and sprayed with the indicator strain (*C. fimi*).

The following enzymes were examined for their effect on AMS activity: trypsin (Sigma), pronase (Calbiochem), proteinase K (Boehringer Mannheim Biochemicals, BMB) and protease (type XXIII, from *Aspergillus oryzae*, Sigma) (1 mg ml⁻¹, prepared in 0.05 M-Tris pH 8.0, 0.01 M-CaCl₂); RNAase A (Sigma) and DNAase I (BMB) (1 mg ml⁻¹, prepared in 0.05 M-Tris pH 8.0, 10 mM-MgSO₄); and phospholipase C (Sigma) (1 mg ml⁻¹, prepared in 0.1 M-phosphate buffer pH 7.0). The enzymes (40 µl) were applied around the producer colonies after chloroform treatment. The plates were incubated at 37 °C for a further 4 h and sprayed with the indicator strain (*C. fimi*).

The *M_r* values of AMS were estimated by the method of Joseph *et al.* (1983). The producer strains were grown on washed sterile dialysis membranes (12000 to 14000, 6000 to 8000, and 3500 *M_r* cutoff) on BHI agar. The producers were killed with chloroform, the membranes were removed, and the agar was sprayed with the indicator strain (*C. fimi*).

Assay for exfoliative toxin (ET) production. This test was done using culture supernatants which had been concentrated 20-fold using Carbowax 6000. Double diffusion in Ouchterlony gels was employed (Arbuthnott & Billcliffe, 1976), using type-specific anti-ET sera kindly provided by Dr Y. Piémont (Université Louis Pasteur, Strasbourg, France). Strain UT0101 was used as a positive control.

Screening for resistance determinants. This was done as described previously (Bastos & Penido, 1981). The following antibiotic-containing discs (Difco) were used: chloramphenicol (Cm, 30 µg), erythromycin (Em, 15 µg), penicillin G (Pc, 5 U), tetracycline (Tc, 30 µg), gentamicin (Gm, 10 µg), kanamycin (Km, 30 µg) and streptomycin (Sm, 10 µg).

Phage typing. The phage typing was carried out by Dr A. M. Uthida-Tanaka from the *Staphylococcus aureus* Phage Typing Laboratory, University of Ribeirão Preto, SP, Brazil, using the international basic set of typing phages.

Curing experiments. Growth of broth cultures at high temperature (43 °C) or with sodium dodecyl sulphate (SDS, 2 µg ml⁻¹) was used to cure staphylococci of AMS production or drug resistance, as described previously (Bastos & Penido, 1981).

Prophage and AMS induction. Induction of prophage and AMS production by ultraviolet (UV) irradiation was performed as described previously (Bastos *et al.*, 1980).

Transduction. Transduction with phage φ11 was done as described previously (Bastos *et al.*, 1980). Phage lysates were prepared either by UV induction of lysogenic strains or by infection of susceptible strains with wild-type φ11.

Plasmid DNA isolation. Cultures were grown in 2 ml BHI broth. The cells were harvested and washed once with TE buffer (10 mM-Tris pH 7.8, 1 mM-EDTA pH 7.5). The washed cells were resuspended in 900 µl lysis buffer [25% (w/v) sucrose, 50 mM-Tris pH 8.8, 40 mM-EDTA pH 7.5, 50 mM-NaCl]. Lysostaphin (Sigma) (2 µl of a 5 mg ml⁻¹ solution prepared in TE buffer) was added and the cells were incubated at 37 °C for 30 min. Then 100 µl pronase (10 mg ml⁻¹) was added. Lysis was completed by addition of 100 µl SDS (20%, w/v). Following incubation at 37 °C for 10 min, the samples were heated at 65 °C for 5 min, then KCl was added to 0.4 M and the samples were transferred to ice for a few minutes. The lysates were cleared by centrifugation at 12000 g for 10 min. Nucleic acids were precipitated from the supernatant by adding 2 vols absolute ethanol followed by incubation at -20 °C for 18 h. The DNA was pelleted by centrifugation at 12000 g for 10 min. The DNA pellet was dried and dissolved in 100 µl TE buffer.

Plasmid DNA for restriction endonuclease analysis and for nick-translation reactions was prepared by caesium chloride/ethidium bromide density-gradient centrifugation of cleared lysates prepared as described by Novick *et al.* (1979b).

Restriction endonuclease analysis. The restriction endonucleases were purchased from Boehringer Mannheim Biochemicals and used as specified by the manufacturer.

Gel electrophoresis. Plasmids and restriction endonuclease digests of plasmid DNA were analysed by electrophoresis in 0.7-1.0% (w/v) agarose horizontal slab gels and/or in 5.0% (w/v) polyacrylamide vertical slab gels (Maniatis *et al.*, 1982). In both cases the gel buffer and running buffer was 89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA. Size

standards for supercoiled plasmid DNA were plasmids from *E. coli* or *S. aureus*. Size standards for linear DNA fragments were bacteriophage λ DNA cleaved with *Hind*III or with *Hind*III and *Eco*RI.

Hybridization. Southern blot hybridization was done as described previously (Bastos & Murphy, 1988), except that the denatured DNA was transferred to Nytran membranes (Schleicher & Schuell). Probe DNA was labelled by nick-translation as described by Maniatis *et al.* (1982).

Results and Discussion

Production of antimicrobial substances

Among 137 strains of *S. aureus* tested for AMS production, 15 showed inhibitory activity against *C. fimi*. The zones of inhibition produced by the AMS⁺ strains isolated from food were consistently larger than those produced by the AMS⁺ strains isolated from patients.

Characterization of AMS⁺ strains

The AMS⁺ strains belonged to phage group II (5 strains), phage group III (5 strains) and to phage groups I and III (2 strains). Three strains were not typable. Most strains carried resistance to more than one drug. None of these strains produced ETB, and only one strain (H30) expressed ETA.

All the AMS⁺ strains harboured at least one plasmid (data not shown) and most carried more than one. Strains A53, A70, H55 and H56 carried a single plasmid.

Elimination of AMS production by growth at high temperature

Plasmid elimination experiments were performed in order to assign the AMS markers to plasmids. Ten of the AMS⁺ strains did not lose their ability to produce AMS after growth at 43 °C. Elimination of AMS⁺ was observed only with strains A65, A70, A82, H55 and H56.

Characterization of the AMS⁻ derivatives

The phage type and the susceptibility of the AMS⁻ derivatives to antibiotics were the same as those of their parental AMS⁺ strains.

To obtain further evidence that AMS production was plasmid-determined, five cured variants from each of strains A65, A70, A82, H55 and H56 were screened for plasmids by agarose gel electrophoresis. Representative gels are shown in Fig. 1. In all cases, the loss of AMS production was accompanied by the loss of a plasmid. Thus strains A65 AMS⁻, A70 AMS⁻ and A82 AMS⁻ had lost plasmids of 10, 7.5 and 10 kb, respectively (Fig. 1a, lanes B, D and F). The other plasmids found in strains

A65 AMS⁻ and A82 AMS⁻ specify drug resistance (see below). Strains H55 AMS⁻ and H56 AMS⁻ had each lost a >40 kb plasmid and were devoid of other plasmids (Fig. 1b, lanes B and D). These findings suggest that the production of AMS by strains A65, A70, A82, H55 and H56 is controlled by plasmids.

Sensitivity of AMS⁺ and AMS⁻ strains to AMS

None of the AMS⁺ strains was sensitive to the AMS that they produced (Table 2). The AMS produced by strain A70 inhibited the growth of all other AMS⁺ strains and their AMS⁻ derivatives. The AMS produced by strains A53, A65 and A82 showed a similar spectrum of activity, being able to inhibit the growth of strains A70, H55 and H56 and their AMS⁻ derivatives.

In addition to loss of AMS production, the AMS⁻ derivatives of strains A65, A70 and A82 were susceptible to inhibition by the AMS produced by the parental strain, suggesting that the plasmids determined both the production of AMS and immunity to its action. On the other hand, cured derivatives of strains H55 and H56 retained resistance to the H55 and H56 AMS. Similar results have been reported for other strains of *S. aureus* (Gagliano & Hinsdill, 1970; Jetten & Vogels, 1973; Dajani & Taube, 1974).

Properties of AMS from strains A53, A65, A70, A82, H55 and H56

Strain A53 was included in these studies because it contains a single plasmid. Although this strain could not be cured of AMS production, other experiments (see below) showed that this plasmid carries the genetic information for AMS production and immunity.

The inhibitory activity of these strains was not inducible by UV irradiation, nor could bacteriophages be detected in excised zones of inhibition when placed on lawns of sensitive staphylococci.

The AMS produced by all six strains were resistant to heat, retaining activity even after incubation at 75 °C for 1 h. In addition, NaOH (0.2 M), chloroform, RNAase A and phospholipase C had no effect on AMS activity. The AMS from strains A53, A65 and A82 was not affected by proteolytic enzymes. In contrast, activity of the AMS produced by strain A70 was markedly reduced or eliminated by incubation with protease from *Aspergillus oryzae*, trypsin, proteinase K or DNAase I, while the AMS produced by strains H55 and H56 were inactivated by protease and pronase.

The AMS produced by strains H55 and H56 were not dialysable even when a dialysis membrane with an M_r cutoff of 12000–14000 was used. The AMS produced by

Fig. 1. Agarose gel electrophoresis of plasmid DNA isolated from AMS⁺ strains of *S. aureus* and from AMS⁻ derivatives. The agarose concentration was 0.7% (w/v). The lanes contained the following. (a) A, A65 AMS⁺; B, A65 AMS⁻; C, A70 AMS⁺; D, A70 AMS⁻; E, A82 AMS⁺; F, A82 AMS⁻; G, RN451; H, RN4288 (pEM9609, 13.8 kb); I, RN2448 (pSA2100, 6.8 kb); J, RN1777 (pS177, 4.0 kb). (b) A, H55 AMS⁺; B, H55 AMS⁻; C, H56 AMS⁺; D, H56 AMS⁻; E, RN451; F, TC146 (pTC146, 41.0 kb); G, RN11 (pI258, 27.5 kb); H, RN1465 (pII147, 31.3 kb); I, *E. coli* K12 711 (pEnt P307, 81.2 kb). The sizes (kb) of the standard plasmids are indicated to the right.

Table 2. Sensitivity to AMS of AMS⁺ strains and their cured AMS⁻ derivatives

These tests were repeated at least three times. ++, zones of inhibition >10 mm; +, zones of inhibition ≤10 mm; -, no inhibition.

Indicator strain	AMS from					
	A53	A65	A82	A70	H55	H56
<i>C. fimi</i>	++	++	++	++	+	+
A53 AMS ⁺	-	-	-	+	-	-
A65 AMS ⁺	-	-	-	+	-	-
A65 AMS ⁻	++	++	++	+	-	-
A82 AMS ⁺	-	-	-	+	-	-
A82 AMS ⁻	++	++	++	+	-	-
A70 AMS ⁺	+	+	+	-	-	-
A70 AMS ⁻	+	+	+	+	-	-
H55 AMS ⁺	++	++	++	+	-	-
H55 AMS ⁻	++	++	++	+	-	-
H56 AMS ⁺	++	++	++	+	-	-
H56 AMS ⁻	++	++	++	+	-	-

strains A53, A65, A70 and A82 were dialysable when a dialysis membrane with an M_r cutoff of 12000–14000 was used, being retained by membranes with a 6000–8000 M_r cutoff.

These preliminary results showed that these AMS are a heterogeneous group of substances with properties similar to AMS characterized by other authors (Gagliano & Hinsdill, 1970; Rogolsky & Wiley, 1977; Nakamura *et al.*, 1983). The AMS produced by strains A70, H55 and H56 were inactivated by proteolytic enzymes and are typical bacteriocins (Tagg *et al.*, 1976). The bacteriocin produced by strain A70 is a protein of M_r 6000–14000 and is sensitive to DNAase I, like a bacteriocin produced by a strain of *Rhizobium trifolii* (Schwinghamer, 1975). The M_r of the bacteriocin produced by strains H55 and H56 is higher than 14000. The AMS produced by strains A53, A65 and A82 also have M_r values in the range 6000–14000. However, they are resistant to proteolytic

Fig. 2. Agarose gel electrophoresis of plasmid DNA isolated from *S. aureus* strain A70 and from transductants. The agarose concentration was 0.7% (w/v). Lanes contained: A, A70(pRJ6); B, RN451; C, Em^rCd^rBac⁺ transductant derived from A70(pRJ6/pRN3032); D, Em^rCd^rBac⁺ derivative of A70 harbouring pRJ6::Tn551; E, RN451 carrying pRJ6::Tn551; F, Em^rBac⁻ derivative of RN451; G, RN4288 (pEM9609, 13.8 kb); H, RN2448 (pSA2100, 6.8 kb); I, *E. coli* HB101 (pSA30, 10.3 kb); J, *E. coli* K12 (pGR113, 6.1 kb) (chromosomal DNA is not seen in this lane because it contained plasmid DNA purified by CsCl/ethidium bromide density-gradient centrifugation). The sizes (kb) of the standard plasmids are indicated to the right.

enzymes and can be considered to be bacteriocin-like substances.

The inhibitory effect of these bacteriocins and bacteriocin-like substances was not restricted to staphylococci and *C. fimi*: many other Gram-positive bacteria, but none of the Gram-negative bacteria tested, were sensitive (data not shown). This is a common feature of bacteriocins of Gram-positive organisms, including staphylococci (Dajani *et al.*, 1970; Gagliano & Hinsdill, 1970; Jetten & Vogels, 1972). *C. fimi* and *Bacillus megaterium* were the best indicators for the bacteriocins produced by strains A53, A65, A70 and A82, whereas *C. fimi* and *Micrococcus* sp. were more sensitive indicators for those produced by strains H55 and H56.

Gene transfer experiments

To obtain further evidence for the involvement of the small plasmids in bacteriocin production, strains A53 and A70 were used in gene transfer experiments. Strains A53 and A70 carry single plasmids, pRJ9 (10 kb) and pRJ6 (7.5 kb), respectively. Plasmid-elimination experiments suggested that pRJ6 mediates bacteriocin production. However, plasmid pRJ9 could not be eliminated from strain A53. Since neither plasmid carried a readily selectable marker, the plasmids were tagged with Tn551.

This 5.2 kb transposon specifies resistance to Em and other macrolide antibiotics in *S. aureus*; it can transpose to many different sites in the *S. aureus* chromosome and in plasmids (Novick *et al.*, 1979a; Pattee, 1981). Plasmid pRN3032, a temperature-sensitive Cd^r plasmid that carries Tn551, was transferred to strains A70 and A53 by transduction from ISP479, selecting for Em^r and Cd^r. Strains carrying pRN3032 and pRJ6 or pRJ9 were grown at 43 °C (the nonpermissive temperature for pRN3032), in the presence of Em. Em^r colonies which were Cd^s were identified. The stable maintenance of Em^r was indicative of Tn551 transposition either into the chromosome or into a plasmid. Plasmid DNA was isolated from Em^rCd^s colonies that were Bac⁺ and the pRJ6 or pRJ9 derivatives carrying Tn551 were presumptively identified by an increase in size. Plasmids pRJ6::Tn551 and pRJ9::Tn551 were transduced into the plasmid-free strain RN451, selecting for Em^r.

In experiments with A70(pRJ6), seven of 22 Em^rCd^s colonies had lost both pRJ6 and pRN3032 and carried a plasmid of about 13 kb, corresponding to the sum of pRJ6 (7.5 kb) and Tn551 (5.2 kb) (Fig. 2, lane D). Plasmid pRJ6::Tn551 was transduced into RN451, and 86 of 87 Em^r transductants were also Bac⁺Imm⁺ and contained pRJ6::Tn551 (Fig. 2, lane E). The single Em^rBac⁻ transductants had no plasmid DNA (Fig. 2, lane F). Similar results were obtained for strain A53

carrying both pRJ9 and pRN3032 (data not shown). These results confirmed the involvement of plasmids pRJ6 and pRJ9 in bacteriocin production by strains A70 and A53.

Strain RN451 carrying pRJ9::Tn551 was immune to the bacteriocin-like substances produced by strains A65(pRJ10) and A82(pRJ11), indicating that bacteriocin immunity is plasmid-controlled in strain A53. This is consistent with the observation that strains A65 and A82, but not their cured derivatives, were immune to the bacteriocin specified by plasmid pRJ9 (Table 2).

Curing drug resistance determinants from strains A65 and A82

Strains A65 and A82 express resistance to Pc, Sm and Tc and to Pc, Sm and Cm, respectively. Both strains harbour at least three plasmids (Fig. 1a, lanes A and E). The 10 kb plasmids are thought to be responsible for the production of the bacteriocin-like substances. To obtain strains carrying only the Bac plasmids, strains A65 and A82 were cured of Pc and Tc or Pc and Cm resistances, respectively, either by growth in broth with SDS or by growth at 43 °C. These curing experiments indicated (data not shown) that a 27 kb plasmid probably determined resistance to Pc, and that resistance to Tc or Cm was probably determined by 4.4–4.5 kb plasmids. The size of the plasmids responsible for drug resistance in these strains agrees with other reports (Novick & Bouanchaud, 1971; Chopra *et al.*, 1973).

The Pc^rTc^r derivatives of strain A65 and the Pc^rCm^r derivatives of strain A82 still contained a small cryptic plasmid (1.2 kb) (Fig. 4) not detected in the gel shown in Fig. 1.

Attempts to cure strains A65 and A82 of Sm^r were unsuccessful.

Restriction endonuclease analysis of the small Bac plasmids

The immunity experiments and characterization of bacteriocins suggested that at least two distinct antagonistic substances were produced by the strains harbouring small Bac plasmids. Plasmid pRJ6 represents the first group and plasmids pRJ9, pRJ10 and pRJ11 form the second one.

In order to compare the structures of these plasmids, purified DNA was analysed by restriction endonuclease cleavage and by gel electrophoresis. A physical map was constructed for pRJ6 (Fig. 3). pRJ6 had no sites for *Xho*I, *Bam*HI, *Hae*III, *Kpn*I, *Sma*I and *Bst*EII. There are two

Fig. 3. Restriction map of pRJ6. The reference point of the map is one of the *Eco*RV sites. The distances are given in kb.

restriction sites (one for *Pst*I and one for *Cla*I) around coordinate 0.8, but their exact position could not be determined.

Cleavage of plasmids pRJ9, pRJ10 and pRJ11 with *Eco*RV, *Cla*I, *Pst*I, *Hind*III (Fig. 4) and *Hpa*I (data not shown) resulted in the same banding patterns. The plasmids had no sites for *Xho*I, *Bam*HI, *Bst*EII and *Xba*I. However, some restriction site polymorphisms were also observed: pRJ10 and pRJ11 had a single site for *Eco*RI which was absent from pRJ9, while pRJ9 had a single site for *Bgl*/II which was absent from pRJ10 and pRJ11. These data indicate that pRJ10 and pRJ11 are probably identical, and that they are very similar to pRJ9, but different from pRJ6. This confirms the relatedness of pRJ9, pRJ10 and pRJ11 suggested by the cross-immunity data and by the properties of the bacteriocin-like substances specified by them.

The sizes of the four plasmids were estimated from the sizes of the fragments generated by digestion with restriction enzymes. Plasmid pRJ6 was 8.0 kb, while plasmids pRJ9, pRJ10 and pRJ11 were 10.4 kb, a little larger than the sizes estimated from the data in Fig. 1.

Hybridization analysis

The relationship between the Bac plasmids was also investigated by Southern hybridization experiments with total plasmid DNA from all Bac⁺ strains, including the reference strains TC146 and UT0101, using pRJ6 and pRJ9 as probes.

Fig. 4. Agarose gel electrophoresis (0.7% agarose) of purified DNA of pRJ9 (lanes B, E, H and K), pRJ10 (lanes C, F, I and L) and pRJ11 (lanes D, G, J and M) cleaved with *EcoRV* (lanes B–D), *ClaI* (lanes E–G), *PstI* (lanes H–J) or *HindIII* (lanes K–M). Lane A contains bacteriophage λ DNA cut with *HindIII*; the sizes (kb) of the standard fragments are indicated on the left. The arrow at the bottom of the gel indicates different forms of a previously undetected cryptic plasmid present in strains A65 and A82.

Fig. 5. DNA–DNA hybridization analysis of Bac plasmids. Undigested plasmid DNA samples were separated on a 0.7% (w/v) agarose gel, transferred to a Nytran membrane and hybridized to ^{32}P -labelled pRJ6 (b) or pRJ9 (c) DNA. (a) Photograph of ethidium bromide-stained agarose gel. Lanes contain DNA isolated from the following strains: A, A53; B, A65; C, A70; D, A82; E, UT0101; F, TC146; G, H55; H, H56.

When pRJ6 was used as probe, the distribution of radioactivity coincided with the distribution of the fluorescent bands seen in the ethidium-bromide-stained agarose gels and corresponded to the open circular (OC) and supercoiled (SC) forms of the Bac plasmids carried by strains A53, A65 and A82 (Fig. 5b, lanes A, B and D). pRJ6 also showed much weaker homology with the Cm^r

plasmid carried by strain A82 (lane D). The nature of the sequence homology between pRJ6 and the Cm^r plasmid is unknown. No hybridization was observed between pRJ6 and the large Bac plasmids carried by strains UT0101, TC146, H55 and H56 (Fig. 5b, lanes E, F, G and H), or to the penicillinase plasmids and Tc^r plasmid carried by strains A82 and/or A65 (lanes D and B).

Similar results were obtained when pRJ9 was used as probe (Fig. 5c), except that no homology was detected with the Cm^r plasmid of strain A82 (lane D). pRJ9 showed strong homology with pRJ10 and pRJ11 (lanes B and D) and weaker homology with pRJ6 (lane C). This is consistent with the restriction analysis of these plasmids.

In Fig. 5(b) and (c), lanes A, B and D, it appears that the chromosomal bands also hybridized with pRJ6 or pRJ9. However, this hybridization can be attributed to the OC forms of plasmids pRJ9, pRJ10 and pRJ11 comigrating with the chromosomal fragments.

Conclusions

The six bacteriocinogenic plasmids carried by the *S. aureus* strains we studied could be separated into two groups: (i) plasmids larger than 40 kb which code for a high- M_r bacteriocin and that do not confer immunity, and (ii) small plasmids (8.0–10.4 kb) which code for bacteriocins or bacteriocin-like substances with a low M_r and that also confer immunity.

The large plasmids we isolated do not specify either ETA or ETB, and were found in clinical isolates from phage groups I and/or III. The bacteriocins specified by these plasmids produced small zones of inhibition, limited to a few species of Gram-positive bacteria. The small plasmids were found in strains isolated from food and the bacteriocins or bacteriocin-like substances specified by them produced large zones of inhibition on a broader range of strains. The lack of detectable homology between plasmids pRJ6 or pRJ9 and the large Bac plasmids confirmed that the latter constitute a distinct group.

Two groups were differentiated among the four strains harbouring small Bac plasmids, on the basis of differences in the cross-immunity patterns, in the chemical characteristics of their bacteriocins and in the physical analysis of their Bac plasmids. The first group comprises strains A53, A65 and A82, whose bacteriocin-like substances have similar properties and are specified by closely related plasmids of 10.4 kb. Moreover, these strains are not sensitive to bacteriocins produced by strains of the same group, but become susceptible to them after elimination of the Bac plasmid. The second type is defined by strain A70, the producer of a bacteriocin with distinct chemical properties which is specified by a smaller plasmid of 8.0 kb. This plasmid, although different from the others, shares regions of sequence homology with them. Experiments to define the extent of homology between these small Bac plasmids, and to determine their incompatibility relationships and their genetic organization, are currently in progress.

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