Plasmid Involvement in Production of and Immunity to the Staphylococcin-like Peptide Pep 5

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The staphylococcin-like peptide Pep 5 is produced by the penicillin resistant strain *Staphylococcus epidermidis* 5. This strain is immune to the peptide. Plasmid analysis of *S. epidermidis* 5 by agarose gel electrophoresis and electron microscopy demonstrated five plasmids with molecular weights ranging from 5.8×10^6 to 29×10^6 . Variants of *S. epidermidis* 5 not producing Pep 5 or which had become penicillin sensitive were induced by various curing treatments. Strains lacking the 13.9×10^6 mol. wt plasmid (pED502) had lost penicillin resistance, and those lacking the 12.3×10^6 mol. wt plasmid (pED503) failed to produce Pep 5. pED503 is also responsible for the immunity of the producer cell to Pep 5. Plasmid pED502 could be transformed into *S. aureus* RN 981 which then became resistant to penicillin. pED503 could not be transformed into *S. aureus* RN 981, but could be transformed into *S. epidermidis* 5 variants previously cured of this plasmid; the transformants then regained the properties of Pep 5 production and immunity.

INTRODUCTION

Although there are numerous reports on bacteriocins of Gram-positive organisms (Tagg *et al.*, 1976), little is known about the genetic elements controlling their production. Bernhard *et al.* (1978) demonstrated that a plasmid of molecular weight 45×10^6 was responsible for bacteriocin production in *Bacillus cereus*, and Rostas *et al.* (1980) identified a 30.9×10^6 mol. wt plasmid encoding for megacin A. Plasmids involved in the production of *Staphylococcus aureus* staphylococcins have been reported by Gagliano & Hinsdill (1970), Jetten & Vogels (1973), Dajani & Taube (1974), Rogolsky *et al.* (1974) and Warren *et al.* (1974). However, nothing is known about the genetic control of staphylococcin production in *S. epidermidis*, although staphylococcin 1580, isolated and characterized by Jetten & Vogels (1972) and Jetten *et al.* (1972), is the most thoroughly studied bacteriocin produced by a staphylococcal strain.

Pep 5, a staphylococcin-like substance produced by *S. epidermidis* 5, is different from the above-mentioned staphylococcins because of its narrow activity spectrum (only staphylococci and micrococci are sensitive) and its low molecular weight of 6000 (Sahl & Brandis, 1981). Furthermore, it is a strongly basic peptide causing a rapid efflux of low molecular weight compounds from the cytoplasm of sensitive bacteria (Sahl & Brandis, 1982, 1983). Recently, Nakamura *et al.* (1983) isolated a *S. aureus* bacteriocin which is similar to Pep 5 in its molecular weight (5000) and its isoelectric point (10.0); however, in contrast to Pep 5 it is active against many Gram-positive bacteria. The genetic determinants of this staphylococcin have not yet been elucidated.

In the present report, we demonstrate by curing studies and transformation results that a 12.3×10^6 mol. wt plasmid is involved in the production of Pep 5 and in the immunity of the producer strain to this substance.

Table 1. *Bacterial strains*

Bacterial strains	Properties	Source or reference
<i>Staphylococcus cohnii</i> 22	Clinical isolate, indicator for Pep 5 production.	Sahl & Brandis (1981)
<i>S. epidermidis</i> 5	Clinical isolate, wild-type, Pep 5 producer (Pep 5 ⁺), penicillin resistant (Pen ⁺).	Sahl & Brandis (1981)
<i>S. epidermidis</i> 5 Pep 5 ⁻	Derivative of <i>S. epidermidis</i> 5; Pep 5 ⁻ , Pen ⁺ .	This paper
<i>S. epidermidis</i> 5 Pen ⁻	Derivative of <i>S. epidermidis</i> 5; Pep 5 ⁺ , Pen ⁻ .	This paper
<i>S. aureus</i> RN 450 = 8325-4	Nonlysogenic derivative of <i>S. aureus</i> NCTC 8325.	Novick (1967)
<i>S. aureus</i> RN 981	Derivative of strain 8325-4, <i>recA1</i> .	Wyman <i>et al.</i> (1974)

METHODS

Bacterial strains and culture conditions. Bacterial strains used are listed in Table 1. Strains were maintained on Tryptone soya agar (TSA) or subcultured weekly on blood agar plates. The basic medium for growth of staphylococcal strains in liquid culture was Tryptone soya broth (TSB). TSB/YE/Gly contained additionally 0.3% yeast extract and 0.5% glycine. Pep 5 was isolated and purified as described previously (Sahl & Brandis, 1981).

Plasmid pattern of *S. epidermidis* 5. Plasmid DNA was initially prepared as a cleared lysate and subsequently banded on CsCl/ethidium bromide gradients (Clewell & Helinski, 1969; Novick & Bouanchaud, 1971). Plasmid DNA was recovered and plasmid masses were determined by electron microscopy (Kleinschmidt, 1968) using phages λ dv1 and PM2 as internal size standards.

Plasmid screening. For the rapid isolation of plasmid DNA the alkaline extraction procedure of Birnboim & Doly (1979) was slightly modified. The staphylococcal strains were grown at 35 °C with shaking in 50 ml TSB/YE/Gly to an OD₆₀₀ of 0.8 to 1.0. Cells were centrifuged (9000 g, 4 °C, 10 min), and resuspended in 1 ml of a solution containing 50 mM-glucose, 10 mM-EDTA, 25 mM-Tris/HCl (pH 8), and lysostaphin (Sigma) – 75 µg ml⁻¹ for *S. epidermidis*, and 40 µg ml⁻¹ for *S. aureus*. Further isolation steps were essentially as described by Birnboim & Doly (1979), except that ten times larger volumes were used. Volumes of 20 to 40 µl of the final DNA preparation were subjected to electrophoresis under standard conditions (0.8% horizontal gels, 50 V for 16 h at ambient temperature).

Curing. For plasmid elimination the following conditions were employed: (i) growth in liquid culture (TSB) containing acriflavine (25 µg ml⁻¹), SDS (30 µg ml⁻¹) (Sonstein & Baldwin, 1972), and either ethidium bromide (2.4 or 3.6 µg ml⁻¹) (Bouanchaud *et al.*, 1968; Warren *et al.*, 1974) or acridine orange (15 µg ml⁻¹) at 35 or 42 °C; (ii) passage of the organisms at 39 °C over 6 d in liquid culture (Laufs & Kaulfers, 1977). Control cultures were incubated at 35 °C for various periods of time. After 24 h and 48 h the cultures were diluted and plated on TSA to obtain a suitable number of colonies for replica plating. Treated cultures were examined for loss of bacteriocin activity and penicillin resistance. Colonies that failed to produce inhibition zones after 24 h of incubation at 37 °C were scored as Pep 5⁻ and those not growing after replica plating on TSA + penicillin (3 µg ml⁻¹) as Pen⁻. Pep 5 production was assayed as described by Sahl & Brandis (1981) with *S. cohnii* 22 as indicator strain. Putative cured colonies were picked from the master plates and characterized biochemically and by their plasmid profiles.

Transformation. Methods and media for preparation of protoplasts were essentially as described for *B. subtilis* by Chang & Cohen (1979), modified for staphylococci by Lindberg (1981) and Götz *et al.* (1981). Transformation of protoplasts was only performed with purified plasmid DNA. Protoplasts of *S. aureus* and *S. epidermidis* were prepared with lysostaphin at 30 µg ml⁻¹ and 60 µg ml⁻¹, respectively. Formation of protoplasts was observed by phase contrast microscopy. Controls included (i) plating the recipient culture without addition of plasmid DNA, and (ii) treatment of DNA with pancreatic deoxyribonuclease I (40 µg in 10 µl hypertonic buffer) for 1 h before the addition of protoplasts.

For phenotypic expression the protoplasts were incubated in HBM at 30 °C and 37 °C for 4 h and then plated on DM3 regeneration medium.

When transformants were selected for penicillin resistance, they were plated on DM3 plates containing penicillin (3 µg ml⁻¹) and incubated at 37 °C for 3 d. For detection of Pep 5⁺ transformants, samples of appropriate dilutions were plated on DM3 and incubated at 37 °C for 2 d. Pep 5 producing strains were identified after replica plating by means of the deferred antagonism test, using *S. cohnii* 22 as indicator (Sahl & Brandis, 1981).

RESULTS AND DISCUSSION

Plasmid elimination from S. epidermidis 5

The electron microscopic examination of plasmid DNA isolated from wild-type *S. epidermidis* 5 revealed five plasmids. The molecular weights were determined as 5.8×10^6 ,

Table 2. Size estimates of plasmids in *S. epidermidis* 5

Plasmid	10 ⁻⁶ × Molecular weight ± SD	
	Agarose gel analysis*	Electron microscopy†
pED501	28.5 ± 0.8	29.0 ± 0.5 (96)
pED502	14.2 ± 0.6	13.9 ± 0.2 (88)
pED503	12.7 ± 0.6	12.3 ± 0.2 (132)
pED504	8.9 ± 0.4	8.8 ± 0.3 (92)
pED505	5.4 ± 0.2	5.8 ± 0.2 (64)

* Based on the results of 13 independent analyses using the plasmids of *Escherichia coli* V517 (Macrina *et al.*, 1978) as reference markers.

† Size estimates made from open circular contour lengths using the phages λ dv1 and PM2 as an internal reference (four independent preparations). The numbers in parentheses are the numbers of molecules measured.

8.8 × 10⁶, 12.3 × 10⁶, 13.9 × 10⁶ and 29 × 10⁶, based on their contour lengths, using phage λ dv1 and phage PM2 as internal standards (Table 2).

The plasmid pattern of wild-type *S. epidermidis* 5 in agarose gels is shown in Fig. 1(a). Five plasmid bands, in addition to a faint chromosomal band, could be detected, with molecular weights ranging from 5.4 × 10⁶ to 28.5 × 10⁶ (Table 2). Spontaneous loss of the ability to produce Pep 5 could not be detected by repeated subculturing of *S. epidermidis* 5. Curing experiments using acriflavine and SDS were unsuccessful in isolating Pep 5 negative or penicillin sensitive variants of strain 5.

A 6 d passage at 39 °C, however, produced Pep 5 negative (*S. epidermidis* 5 Pep 5⁻) as well as penicillin sensitive variants (*S. epidermidis* 5 Pen⁻) with percentage ranges of 2.8% and 3.4%, respectively. Curing with ethidium bromide at 35 °C and 42 °C for 24 h and 48 h resulted only in Pep 5 negative variants at a frequency of 2.1% to 4.4%. In contrast, acridine orange (15 µg ml⁻¹, 42 °C, 48 h) eliminated penicillin resistance with a curing rate of 3.4%. Jetten & Vogels (1973), Dajani & Taube (1974) and Rogolsky *et al.* (1974) have also described an extrachromosomal mechanism of bacteriocin production in *S. aureus* by using different curing agents. None of our curing treatments yielded such high curing rates as were obtained for the *S. aureus* staphylococci. Acridine orange, acriflavine and SDS were not effective in curing Pep 5 production, whereas SDS was an excellent curing agent (100%) in *S. aureus* 89 (Jetten & Vogels, 1973).

After repeated subculturing, no reversion to the parent phenotype of Pep 5 production and penicillin resistance could be demonstrated in cured strains. To rule out the possibility that these 'cured' variants might be contaminants, they were subjected to a number of taxonomic and biochemical tests. In all tests, *S. epidermidis* 5 wild-type and its Pep 5 negative and penicillin sensitive variants reacted identically.

To confirm that the production of Pep 5 and penicillin resistance were plasmid determined, the plasmid compositions of the cured variants were analysed by agarose gel electrophoresis as well as by electron microscopy. In both cases it could be demonstrated that all Pep 5⁻ strains of *S. epidermidis* 5 had lost the 12.3 × 10⁶ mol. wt plasmid (pED503) and all Pen⁻ strains of *S. epidermidis* 5 the 13.9 × 10⁶ mol. wt plasmid (pED502). Between 10 and 15 strains of each cured variant were tested. Electrophoresis patterns of wild-type *S. epidermidis* 5 and samples of its cured variants are shown in Fig. 1(a). The functions of the other three plasmids were not established. The small 5.8 × 10⁶ mol. wt plasmid was eliminated at the same time as pED502 or pED503, or lost spontaneously in a few cases. Furthermore, no positive correlation was observed when representative strains cured for either function were tested for the simultaneous elimination of the other function. These findings indicate that the production of Pep 5 and penicillin resistance by strain 5 are controlled by two distinct plasmids.

In addition to loss of Pep 5 production, *S. epidermidis* 5 Pep 5⁻ variants were susceptible to the bactericidal action of the peptide. This could be demonstrated by using overnight cultures of *S. epidermidis* 5 wild-type, *S. epidermidis* 5 Pep 5⁻, *S. epidermidis* 5 Pen⁻, and for control *S. cohnii* 22 as indicator strain for Pep 5 production. With *S. epidermidis* 5 Pep 5⁻ and *S. cohnii* 22

Fig. 1. Agarose gel electrophoresis of plasmid DNA obtained from *Staphylococcus epidermidis* 5 and its cured variants, and from Pep 5 producing and penicillin resistant transformants. (a) *Staphylococcus epidermidis* 5 Pep 5⁻ (lanes 1 and 2); *S. epidermidis* 5 (lane 3); *S. epidermidis* 5 Pen⁻ (lanes 4 and 5); *Escherichia coli* V517 (lane 6) as molecular weight standard (Macrina *et al.*, 1978). (b) *Staphylococcus aureus* RN 981 transformed for penicillin resistance with pED502 (lane 1); *S. epidermidis* 5 (lane 2); *S. epidermidis* 5 Pep 5⁻ transformed for Pep 5 production with pED503 (lanes 3 to 5). Numbers in parentheses are $10^{-6} \times$ mol. wt. The DNA was prepared by the screening method as described in the text. A 25 μ l sample of DNA was mixed with 10 μ l dye solution and subjected to electrophoresis (50 V, 16 h).

as indicators, large zones of inhibition were observed, whereas in the cases of *S. epidermidis* 5 wild-type and *S. epidermidis* 5 Pen⁻, no inhibition of growth was visible. To confirm these results, quantitative sensitivity tests with purified Pep 5 (Sahl & Brandis, 1981) were carried out with the four above-mentioned strains. By comparison with *S. cohnii* 22, which by definition is sensitive at a concentration of 1 arbitrary unit (a.u.) Pep 5 ml⁻¹ (Sahl & Brandis, 1981), the strain *S. epidermidis* 5 Pep 5⁻ was inhibited by 4 a.u. The other two strains tested were not inhibited by the highest concentration obtainable in aqueous solution (40 000 a.u. ml⁻¹).

Immunity of the producing strain to its own substance has also been reported for *S. epidermidis* 1580 (Jetten & Vogels, 1973) and *S. aureus* 414 (Gagliano & Hinsdill, 1970),

whereas *S. aureus* C55 was shown to be resistant, i.e. unable to adsorb staphylococcin C55 (Dajani & Taube, 1974). Bacteriocin cured cells of *S. aureus* strain C55 adsorb the staphylococcin and become sensitive. The mechanism of the immunity of *S. epidermidis* 5 at the molecular level is not yet clear. It can only be stated that the presence of the bacteriocin plasmid renders the cells immune to the action of Pep 5.

Transformation of recipient strains to Pep 5 production and penicillin resistance

Polyethylene-glycol-mediated protoplast transformation was used to confirm the results of curing studies and the association of Pep 5 production and penicillin resistance with specific plasmids. Strains *S. epidermidis* 5 Pep 5⁻, *S. epidermidis* 5 Pen⁻, *S. aureus* RN 450, and *S. aureus* RN 981 were used as recipients. Transformation of Pep 5 production occurred only in the previously cured strain *S. epidermidis* 5 Pep 5⁻ with a transformation frequency of 3.4×10^{-2} . No Pep 5⁺ transformant could be detected among 7×10^4 regenerants of *S. aureus* RN 981 and *S. aureus* RN 450. In contrast, penicillin resistant transformants could be isolated from strain *S. aureus* RN 981, with frequencies of about 1×10^{-3} , but not from *S. aureus* strain RN 450 nor from *S. epidermidis* 5 Pen⁻.

The failure of transformation of Pep 5 production into the recipient *S. aureus* RN 981 contrasted with the ability of this host to acquire and express the penicillin resistance plasmid. This could be explained as follows.

(i) Either the Pep 5 plasmid could not be transferred into *S. aureus* RN 981 or it could not express its function if present in this strain.

(ii) There are two or more genetic loci necessary for the production of active Pep 5. In this case, plasmid pED503 could encode for an inactive precursor of Pep 5, which has to be modified enzymically for activity. The genetic information for the enzyme(s) could be located on the chromosome or one of the other plasmids (pED501 or pED504).

The latter explanation is likely, because Pep 5 contains lanthionine (unpublished results). This non-protein amino acid, a thioether derived from serine and cysteine, is also present in the peptide antibiotics nisin and subtilin. Ingram (1969) showed that lanthionine is post-translationally formed in nisin precursor peptides, thereby creating intramolecular ring structures in those peptides. Assuming a similar synthesis of lanthionine in Pep 5, it cannot be produced in a recipient lacking the modifying enzyme.

Fifteen of the penicillin resistant and fifteen Pep 5 producing transformants were screened for their plasmid DNA content (Fig. 1*b*). All penicillin resistant transformants of *S. aureus* RN 981 contained the 13.9×10^6 mol. wt plasmid, which is in the molecular weight range of previously reported penicillin resistance plasmids (10×10^6 to 25×10^6) of *S. epidermidis* (Totten *et al.*, 1981) and *S. aureus* (Novick *et al.*, 1979).

All Pep 5 producing transformants of *S. epidermidis* 5 Pep 5⁻ had regained the 12.3×10^6 mol. wt plasmid; six clones lost the small 5.8×10^6 mol. wt phenotypically cryptic plasmid. Furthermore it was observed that 14 *S. epidermidis* 5 Pep 5⁻ transformants lost the 13.9×10^6 mol. wt plasmid upon protoplast formation, so that most *S. epidermidis* 5 Pep 5⁻ transformants showing Pep 5 production lacked the 13.9×10^6 mol. wt plasmid and were sensitive to penicillin (Fig. 1*b*). The fact that protoplast formation eliminates the 13.9×10^6 mol. wt plasmid effectively in *S. epidermidis* 5 Pep 5⁻ may also explain the observation that this plasmid could not be transferred back into clones previously cured of penicillin resistance.

The results obtained by transformation supported the curing studies showing that Pep 5 production and immunity to this peptide are encoded on the 12.3×10^6 mol. wt plasmid. This is similar to the situation described for certain colicins like E1, K, Ia, etc. The immunity to colicin Ia depends on a 14000 to 14500 Dal polypeptide (Weaver *et al.*, 1981), which is located in the cytoplasmic membrane and encoded on the ColIa plasmid. The immunity is thought to be brought about by a stoichiometric complex of immunity protein and colicin. Thus, an immunity breakdown was observed with colicins Ia and Ib when the concentrations of the colicins that were applied were higher than the amount of immunity protein in the inner membrane (Konisky, 1982). This was not observed with *S. epidermidis* 5, which remained immune at the highest Pep 5 concentration obtainable in aqueous solution.

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