A new streptomycin-resistance plasmid from *Staphylococcus hyicus* and its structural relationship to other staphylococcal resistance plasmids

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Summary. A small plasmid of 4·4 kb encoding resistance to streptomycin (Sm^r) was detected in a multiresistant *Staphylococcus hyicus* culture from a piglet with exudative epidermitis. The plasmid-encoded properties were determined by interspecies protoplast transformation experiments. This plasmid was further characterised by restriction endonuclease analysis and a preliminary restriction map was constructed. The plasmid from *S. hyicus* that conferred streptomycin resistance was designated as pSAI-1. It showed some structural homology with the streptomycin-chloramphenicol resistance plasmid pSK68 from *S. aureus* of human origin. The MIC of streptomycin in resistance mediated by pSAI-1 was about 10 times higher than the MICs in resistance mediated by Sm^r plasmids from human *S. aureus* strains.

Introduction

Staphylococcus hyicus causes porcine exudative epidermitis, an economically important disease which preferentially strikes suckling piglets.^{1,2} Skin lesions are the predominant symptoms of this infection. However, kidneys, liver, central nervous system and joints may also be affected. Control of this infection has been attempted mainly with antibiotics.² This might have led to the relatively high incidence of antibiotic resistance amongst *S. hyicus* strains.³ Recently, we demonstrated that plasmids played an important role in the antibiotic resistance of *S. hyicus*.⁴ The 3·95-kb plasmid pSC 2, mediating chloramphenicol resistance (Cm^r),⁵ and the 4·55-kb plasmid pST 1, encoding tetracycline resistance (Tet^r),⁶ both from porcine *S. hyicus* cultures, were isolated and further characterised by restriction endonuclease analysis.

We now report the isolation and characterisation of a small *S. hyicus* plasmid encoding resistance to streptomycin (Sm^r). The Sm^r plasmid was then compared with resistance plasmids of other staphylococcal species isolated from infections of man and animals.

Material and methods

Bacterial cultures

A multiresistant *S. hyicus* strain isolated from a piglet

with exudative epidermitis was used. It was identified according to the methods of Schleifer.³

Antibiograms

The antimicrobial resistance patterns of the original *S. hyicus* strain and the pSAI-1 transformants were determined by the agar diffusion method⁷ with the following disks: ampicillin 25 µg, chloramphenicol 30 µg, clindamycin 10 µg, doxycycline 30 µg, erythromycin 15 µg, kanamycin 30 µg, streptomycin 10 µg, sulphamethoxazole 23·75 µg + trimethoprim 1·25 µg, and tetracycline 30 µg. The antibiograms were determined after incubation for 18–24 h at 37°C.⁷

Plasmid isolation and gel electrophoresis

Plasmids were isolated by a modification of the alkaline lysis procedure⁸ and subjected to electrophoresis in agarose 0·7–1·5% w/v gels.⁵ After staining the gels with ethidium bromide (Boehringer, Mannheim, FRG) 10 µg/ml, plasmid DNA was visualised by exposure to UV light and photographed (film 667; Polaroid, St Albans, Herts).

Plasmid transformation

Protoplast transformation experiments were performed according to the method of Chang and Cohen.⁸ *S. aureus* RN 4220, a derivative of *S. aureus* RN 8325–4,⁹ was chosen as the recipient because it was susceptible to all antimicrobial agents tested and did not carry any plasmid. After transformation, protoplasts were inoculated on to DM3 regeneration plates⁸ and grown for 48–72 h at 37°C. Then the cultures were replica-plated on

Trypticase Soy Agar (TSA; Oxoid) plates containing streptomycin 75 µg/ml. Clones growing on these selective TSA-plates were screened for plasmid DNA.

Restriction endonuclease digests

Plasmid DNA was treated with 5 units each of the restriction endonucleases *Bam*HI, *Bst*EII, *Cfo*I, *Cla*I, *Eco*RI, *Hpa*II, *Hind*III, *Hinf*I, *Mbo*I and *Taq*I (Boehringer).¹⁰ The plasmid fragments were analysed in agarose 0.7–2.0 w/v gels⁵ or polyacrylamide 7.5% w/v gels,¹¹ according to their sizes. The sizes of the linearised plasmids and the respective plasmid fragments were estimated on the basis of logarithmic plots against cleaved pB1/λdv I-DNA.¹²

Determination of MIC of streptomycin

Single colonies of the original *S. hyicus* culture, a pSAI-1 *S. aureus* transformant and the untransformed *S. aureus* RN 4220 were grown overnight in 2 ml of Brain Heart Infusion Broth (BHI; Merck, Darmstadt, FRG) and 50 µl (approximately 5×10^7 cfu) of each of these cultures were inoculated into 2-ml volumes of BHI containing serial concentrations of streptomycin (10-mg/L steps from 0 to 200 mg/L; 50 mg/L steps from 200 mg/L to 2 g/L) and grown for 18 h on a rotary shaker. Growth of the staphylococci was determined also by photometric measurements of the turbidity at OD₆₀₀. The MIC was recorded as the concentration of streptomycin in the first tube in the series that inhibited visible growth.¹³

Results

The *S. hyicus* strain was resistant to chloramphenicol, clindamycin, erythromycin, streptomycin and tetracycline. It contained four small plasmids up to 4.55 kb. Of these, one had been identified previously to encode resistance to chloramphenicol (Cm^r)⁵ and another to encode resistance to tetracyclines (Tet^r).⁶ This study focussed on a third plasmid that specified streptomycin resistance (Sm^r). This plasmid function was confirmed by interspecific protoplast transformation of the entire plasmid content of the multiresistant *S. hyicus* strain into a streptomycin-sensitive and plasmid-free *S. aureus* recipient strain. The resulting Sm^r clones carried a small Sm^r plasmid, which was designated pSAI-1. The pSAI-1 plasmid was further characterised by restriction endonuclease analysis with the enzymes *Bst*EII, *Cfo*I, *Cla*I, *Eco*RI, *Hind*III, *Hinf*I, *Hpa*II, *Mbo*I and *Taq*I. Digests with *Cfo*I, *Cla*I, *Eco*RI and *Hind*III linearised this plasmid (fig. 1, lane 2) and allowed an estimation of its size to be made—c. 4400 bp. *Hpa*II cleaved pSAI-1 in two fragments of 2600 and 1800 bp (fig. 1, lane 1), whereas *Mbo*I digests resulted in three

Fig. 1. Agarose gel electrophoresis of pSAI-1 digested with the restriction endonucleases *Hpa*II (lane 1) and *Eco*RI (lane 2); M, marker DNA.

fragments of about 1800, 1430, and 1180 bp. Digestion with *Hinf*I revealed seven plasmid fragments of 1170, 980, 810, 700, 540, 160 and 80 bp. In *Taq*I digests, 10 pSAI-1 fragments of about 1280, 870, 780, 430, 420, 290, 140, 80, 70 and 60 bp were detected. *Bst*EII did not cleave pSAI-1. The determination of the sizes of the pSAI-1-fragments generated by double restriction endonuclease analyses enabled the localisation of the cleavage sites for the above mentioned restriction enzymes. Thus, a preliminary restriction map was constructed for pSAI-1 (fig. 2). The MIC of streptomycin mediated by pSAI-1 was c. 1000 mg/L. The untransformed *S. aureus* recipient strain did not show visible growth in broth cultures containing streptomycin concentrations > 40 mg/L. Thus, pSAI-1 conferred on the *S. aureus* transformants a 25-fold increase in streptomycin resistance.

Discussion

Streptomycin resistance in staphylococci may be encoded either chromosomally or by plasmids.¹⁴ Chromosomal resistance occurs more frequently, being associated with mutations in the genes encoding ribosomal proteins.¹⁴ Binding of streptomycin to these altered ribosomal proteins is decreased. Carriage of chromosomal Sm^r is associated with high MICs (> 10 g/L). In contrast, plasmid-mediated Sm^r appears to be of a low-level resistance type with MICs < 100 mg/L. In *S. aureus*, three different classes of Sm^r plasmids were distinguished (table). The first was represented by large plasmids of 17–51 kb which encoded not only Sm^r, but also

Fig. 2. Circular restriction map of the Sm^r plasmid pSAI-1 from *S. hyicus*.

resistance to erythromycin, fusidic acid, kanamycin, lincomycin, neomycin or spectinomycin.^{15,16} The second class included small plasmids of 4.6–7.2 kb which carried the determinants for Sm^r and Cm^r.^{17–19} Representatives of the third class of Sm^r plasmids were small plasmids, 4.2–4.5 kb, encoding only Sm^r.^{18–20}

First attempts to identify Sm^r plasmids in *S. hyicus* were by Noble *et al.*²¹ On the basis of curing experiments they considered a plasmid of 2.6 Mda (c. 3.9 kb) to be responsible for Sm^r in eight of 10 streptomycin-resistant *S. hyicus* isolates from pigs. However, no further analysis was reported. The Sm^r plasmid from *S. hyicus* used in our studies was identified by interspecies protoplast transforma-

tion. This experimental approach produced clones that carried only the pSAI-1 Sm^r plasmid and allowed more detailed analyses. The pSAI-1 plasmid from *S. hyicus* conferred on the transformants Sm^r with a significantly higher streptomycin MIC than that conferred by the Sm^r plasmids from *S. aureus*. Plasmid pSAI-1 was also characterised by restriction endonuclease digests. Single digests were used to determine the numbers of cleavage sites for the different restriction enzymes and the sizes of the resultant plasmid fragments. Double digests with restriction endonucleases led to the construction of a restriction map of pSAI-1. Although detailed restriction maps of other staphylococcal Sm^r plasmids were not available, certain unex-

Table. Representative members of the three classes of staphylococcal Sm^r plasmids

Class	Plasmid	Resistance markers	Size (kb)	Reference
I	pWA 1	Sm, Nm, Km, Sp, Fa	17.55	15
	pWG 14	Sm, Nm, Km, Sp, Em	45–51	16
II	pSK 68	Sm, Cm	4.60	17
	pSC 194	Sm, Cm	7.20	18
	pSA 2100	Sm, Cm	7.05	19
III	pS 177	Sm	4.35	20
	pS 194	Sm	4.5	18
	pSA 0501	Sm	4.2	19
	pSAI-1	Sm	4.4	This paper

Cm, chloramphenicol; Em, erythromycin; Fa, fusidic acid; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin.

Fig. 3. Restriction maps of the plasmids pSC 2 from porcine *S. hyicus*, pSK 68 from human *S. aureus* and pSAI-1 from porcine *S. hyicus*. Restriction endonuclease cleavage sites are: Ac (*AccI*), Bs (*BstEII*), C (*ClaI*), Cf (*CfoI*), E (*EcoRI*), H (*HindIII*), Hp (*HpaII*), M (*MboI*), T (*TaqI*), Xh (*XhoI*). The regions encoding replication functions (REP) and chloramphenicol acetyltransferase (CAT) are indicated below the map of pSK 68; the arrows describe the directions of transcription of the REP and CAT genes. For the purpose of better comparison the previously mapped *HinfI* cleavage sites are deleted in the linear restriction map of pSAI-1; there are no corresponding data concerning *HinfI* cleavage sites in pSC 2 or pSK 68.

pected similarities were apparent between pSAI-1, pSC 2 (a Cm^r plasmid from *S. hyicus*)⁵ and the $\text{Cm}^r\text{-Sm}^r$ plasmid pSK 68 isolated from human *S. aureus* strains in Australia (fig. 3). The pSK 68 plasmid belonged to the class II Sm^r plasmids and had a replication region (REP) similar to those of pSAI-1 and pSC 2, both from porcine *S. hyicus* strains isolated in Germany. Furthermore, the CAT region in pSK 68, containing the genetic information for one monomer of the enzyme chloramphenicol acetyltransferase, was also homologous with the respective region in pSC 2. On the basis of their REP/CAT regions, both plasmids had been classified as belonging to the pC 221 family of Cm^r plasmids.^{5,22} Plasmids pSAI-1 and pSK 68 also shared a region of about 1200 bp, probably involved in streptomycin resistance. In pSAI-1 this region differed from the REP region. In pSK 68 it was obviously involved in neither replication functions nor chloramphenicol resistance. The region, characterised by *MboI*, *EcoRI* and *HindIII* cleavage sites, also corresponded well to those mapped in the $\text{Cm}^r\text{-Sm}^r$ plasmid pSA 2100¹⁹ and in the Sm^r plasmid pSA 0501.¹⁹ Moreover, this region was not

detectable in the restriction maps of other small plasmids encoding resistance to chloramphenicol,²² tetracyclines^{6,23} or macrolides/lincosamides.^{24,25}

The restriction maps of plasmids pSAI-1, pSC 2 and pSK 68 suggested that $\text{Cm}^r\text{-Sm}^r$ plasmids, such as pSK 68, were co-integrates between small Sm^r and small Cm^r plasmids.¹⁴ In this connection a recombination site upstream of the REP gene as well as the CAT terminator were found to be involved in recombination events between the pC 221 Cm^r plasmids and other staphylococcal plasmids.²² This recombination site and the CAT terminator possibly also played a role in the formation of $\text{Cm}^r\text{-Sm}^r$ plasmids.

Our findings revealed surprising structural similarities between plasmids encoding the same phenotypic markers in different staphylococcal species from different host species in different parts of the world. These results strongly suggest an exchange of resistance determinants. The transfer of small plasmids between different staphylococcal species by transducing phages has already been demonstrated for the *S. aureus* Tet^r plasmid pT 181.¹⁴ Structural similarities between small Cm^r and Tet^r

plasmids from porcine *S. hyicus* and their respective counterparts from human *S. aureus* strains have also been shown previously^{5,6} and further suggest that there has been a much higher frequency of plasmid exchange between staphylococci from man

and animals *in vivo* than predicted from purely *in vitro* experimental results.²⁶

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