ANTIMICROBIAL SUSCEPTIBILITY

A novel plasmid from *Staphylococcus epidermidis* specifying resistance to kanamycin, neomycin and tetracycline

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The naturally occurring plasmid pSTS7 from *Staphylococcus epidermidis* mediated resistance to tetracycline via a *tetL* gene and to kanamycin and neomycin via an *aadD* gene. Plasmid pSTS7 showed partial restriction map and sequence homology to the previously described tetracycline resistance plasmid pNS1981 from *Bacillus subtilis* and to the kanamycin/neomycin/bleomycin resistance plasmid pUB110 from *S. aureus*. Sequence analysis of the regions flanking the two resistance genes in pSTS7 led to the identification of a novel site for interplasmid recombination which could explain the derivation of pSTS7 from the incompatible pNS1981- and pUB110-like parental plasmids under tetracycline-selective pressure.

Introduction

The use of antibiotics in animals, either as feed additives or for therapeutic and prophylactic purposes, promotes the selection of resistant organisms and the spread of antibiotic resistance genes among bacteria of the same genus, and also to those of different genera [1, 2]. Previous studies have shown that pathogenic staphylococci from man and animals carry virtually the same antibiotic resistance genes on mobile genetic elements [3–5].

Tetracycline resistance (Tc^r) plasmids of staphylococci isolated from man and animals belong to a family of structurally closely related plasmids of c. 4.5 kb [5, 6]; the prototype plasmid of this family, pT181 from *Staphylococcus aureus* [7], has been sequenced completely [8, 9] and its tetracycline resistance gene has been assigned to the class K of bacterial *tet* genes [10]. A second family of Tc^r plasmids identified in staphylococci of animal origin carry a *tetL* gene [6, 11], are more variable in size, and contain additional resistance genes [6]. Plasmids that carry *tetL* genes were isolated originally from various *Bacillus* species [12–15], but can replicate and express their resistances in staphylococci [11].

Combined resistance to kanamycin and neomycin is mediated by an aminoglycoside adenyltransferase of the type AAD(4')(4''), the gene for which, designated *aadD*, is located on small plasmids of 4.2–4.5 kb or

on larger plasmids of c. 35 kb [5]. The prototype plasmid of the small *aadD*-carrying plasmids, pUB110 from *S. aureus*, has been sequenced completely [16, 17]. Since it replicates at a copy number of 30–50/cell in *B. subtilis* and expresses its *aadD* gene in this host, it is used as a cloning vector in *B. subtilis* [16].

The *tetL*-encoding plasmid pNS1981 from *B. subtilis* [13, 14] and the staphylococcal *aadD*-encoding plasmid pUB110 [16] are homologous except for the region covered by their resistance genes. The regions flanking the resistance genes contain two junctions [18] thought to be involved in the exchange of the resistance gene cassettes. This study describes a naturally occurring plasmid pSTS7 from *S. epidermidis* isolated from a pig after use of tetracyclines to control exudative dermatitis. Plasmid pSTS7 has considerable homology to the two incompatible plasmids pNS1981 and pUB110, but carries the *tetL* gene and the *aadD* gene. Sequence analyses were conducted on the regions flanking both resistance genes to determine the areas that served for co-integrate formation. A model is presented to explain how pSTS7 might have evolved from pNS1981- and pUB110-like precursors under the selective pressure of tetracycline use.

Materials and methods

Bacteria, plasmids and resistance testing

S. epidermidis strain 210 was isolated from the skin swab of a piglet suffering from exudative dermatitis that had been treated with tetracycline. *S. aureus*

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RN4220 served as recipient strain in protoplast transformation experiments. Both staphylococcal strains were grown overnight at 37°C on Blood Agar Base (Gibco) supplemented with sheep blood 7.5% w/v. For plasmid preparation *S. epidermidis* 210 was cultivated overnight in Brain Heart Infusion Broth (Merck) supplemented with either tetracycline (Tc) 30 µg/ml or kanamycin (Km) 30 µg/ml at 37°C on a rotary shaker. Antibiotic resistances were determined by the agar diffusion method [19] on DST Agar (Oxoid) with disks containing tetracycline 30 µg, kanamycin 30 µg and neomycin 30 µg. *Escherichia coli* JM107, which served as host for recombinant pBluescript vectors, was grown in Luria Bertani (LB) medium or LB solidified with agar 1.5% w/v [20]. Plasmid pBluescript II SK⁺ was from Stratagene.

Plasmid preparation and Southern blot hybridisation

A previously described modification of the alkaline lysis procedure [21] was used to prepare plasmid DNA from *S. epidermidis* 210. Restriction endonucleases (Boehringer) were used according to the manufacturer's recommendations. Transfer of the plasmid fragments from the agarose gels to nitrocellulose membranes followed the capillary blot procedure [20]. An internal 1050-bp *Bcl*I fragment of the *tet*L gene from plasmid pSTE1 [11] served as *tet*L gene probe and an internal 470-bp *Apa*I/*Bgl*II fragment of the plasmid pUB110 [16] served as *aad*D gene probe. The gene probes were labelled by the non-radioactive ECL-system (Amersham-Buchler). High stringency hybridisation and signal detection were done with the hybridisation buffer and the detection reagents provided in the ECL kit.

Transformation, cloning and sequence analyses

Polyethylene glycol-mediated protoplast transformation of the *S. epidermidis* plasmids into the recipient strain *S. aureus* RN4220 was performed as described previously [21]. The *tet*L-encoding plasmid pSTE1 [11] and the *aad*D-encoding plasmid pUB110 [16] were used as controls. Transformants were selected on DM3 regeneration plates [22] supplemented with tetracycline 15 µg/ml or kanamycin 300 µg/ml with incubation for 72 h at 37°C. The high concentration of kanamycin was necessary because aminoglycoside antibiotics were partly inactivated by other compounds in the regeneration medium. Transformation of the recombinant pBluescript II SK⁺ vectors into *E. coli* JM107 followed the CaCl₂ method [23].

Plasmid pSTS7 was cleaved with *Bcl*I/*Bgl*II and the resulting three fragments were ligated separately into the *Bam*HI site of pBluescript II SK⁺. Sequence analyses were conducted on both strands by the dideoxy chain termination method [24] with the Sequenase version 2.0 kit and α-³⁵S dATP (Amer-

sham-Buchler). Commercially available T3- and T7-primers (Stratagene) and three primers obtained from the results of these sequence analyses or derived from the published pUB110 sequence were used to analyse the regions flanking the resistance genes.

Results

Isolation and identification of plasmid pSTS7

S. epidermidis 210 was resistant to tetracycline, kanamycin and neomycin and harboured a single plasmid of 5.5 kb. Cells of *S. aureus* RN4220 transformed with plasmid DNA from *S. epidermidis* 210 contained a single plasmid of 5.5 kb and were resistant to tetracycline, kanamycin and neomycin, irrespective of which agent was used for selection. The plasmids were indistinguishable by restriction endonuclease analysis from one another and from that of *S. epidermidis* 210. This plasmid was designated pSTS7.

The restriction endonuclease map of pSTS7 was similar to the *tet*L gene region of the previously described Tc^r plasmid pNS1981 [13, 14], *tet*L-encoding plasmids from *Staphylococcus* spp. [6, 11] and *Bacillus* spp. [12, 15], and to the *aad*D gene region of the Km^r/Nm^r/Ble^r plasmid pUB110 from *S. aureus* [16] (Fig. 1). These restriction map homologies were confirmed by Southern blot hybridisation. Three fragments were obtained after *Bgl*II and *Bcl*I digestion of pSTS7: the *tet*L gene probe hybridised to a 1050-bp *Bcl*I fragment which is of the same size as the internal *Bcl*I fragment of *tet*L genes; the *aad*D gene probe hybridised to an *Apa*I/*Bgl*II fragment of c. 470 bp.

Sequence analyses of the flanking regions

The region between the aadD gene and the tetL gene. The nucleotide sequence of the 698-bp *Bgl*II/*Bcl*I fragment is shown in Fig. 2. A comparison with the corresponding sequence of pUB110 revealed identical sequences in the downstream part of the *aad*D gene, which continued for c. 125 bp downstream of the translational stop codon and was then lost. Comparison of the *tet*L gene sequence of pSTS7 with the corresponding part of plasmid pNS1981 revealed identical sequences in the upstream part of the *tet*L structural gene and the *tet*L translational attenuator. Sequence homology between the pSTS7 and pNS1981 sequences broke down c. 100 bp upstream of the translational start codon for the *tet*L leader peptide. Thus, there was substantial sequence homology between pSTS7 and pUB110 from positions 1 to 305 and between pSTS7 and pNS1981 from positions 280 to 698 (Fig. 2). The 26-bp region located between positions 280 and 305 exhibited 88.5% nucleotide

Fig. 1. Comparison of the restriction maps of the plasmids pNS1981 from *B. subtilis*, pUB110 from *S. aureus* and pSTS7 from *S. epidermidis*. Restriction endonuclease cleavage sites are abbreviated as follows: Ac (*AccI*), Ap (*ApaI*), B (*BclI*), Ba (*BamHI*), Bg (*BglII*), C (*ClaI*), Cf (*CfoI*), E (*EcoRI*), Hp (*HpaII*), Pv (*PvuII*), and X (*XbaI*). A distance scale in kb is shown below each map; the arrows indicate the extent and the direction of transcription of the *tetL*, *aadD*, *repU*, *pre* and *ble* reading frames. The location of the recombination site A (RSA) is shown in all three restriction maps. Important areas with respect to recombination processes (Fig. 5) are numbered as [1]–[8].

sequence identity to the corresponding pUB110 section and 92.3% nucleotide sequence identity to the corresponding region of pNS1981.

The regions downstream of the *repU* genes. The plasmids pUB110, pSTS7 and pNS1981 exhibited identical sequences in the downstream part of their *repU* genes. Although the sequences downstream of the *repU* coding region were indistinguishable in pUB110 and pSTS7, the sequence identity stopped in pNS1981 16 bp downstream of the translational stop codon of the *repU* gene (Fig. 3).

The regions downstream of the *tetL* gene and the *ble* gene. The *tetL* gene of pSTS7 showed no sequence homology to any part of the pUB110 sequence, but perfect sequence homology with pNS1981. Homology between pSTS7 and the part of the pUB110 sequence downstream of the bleomycin resistance gene *ble* started *c.* 84 bp downstream of the *tetL* coding region (Fig. 4).

Discussion

The presence of structurally related antibiotic resistance plasmids in a wide variety of staphylococcal species and in bacilli indicated the occurrence of interspecies and intergenus transfer events [6, 11, 25].

The exchange of antibiotic resistance plasmids might be furthered by the presence of the antibiotics used for therapeutic or prophylactic purposes [11].

When transferred into new bacterial hosts, resistance plasmids, such as the *aadD*-encoding Km^r/Nm^r/Ble^r plasmid pUB110 or the *tetL*-encoding Tc^r plasmid pNS1981, have to face host-specific restriction systems that might protect the host cell from foreign DNA. Other problems that might arise are a general inability to replicate in the new host or the incompatibility of the new plasmid with plasmids already present. These problems can be circumvented by integration of all or part of the plasmid into the chromosomal DNA of the host cell, or by interplasmid recombination of the new resistance plasmid with other plasmids of the new host.

Chromosomal integration has been described for plasmid pUB110 and for *tetL*-encoding plasmids. In the case of the Km^r/Nm^r/Ble^r plasmid pUB110, integration into the *mec* region of the chromosome of methicillin-resistant *S. aureus* is mediated by the insertion element IS257 [26]. In contrast, little is known about the way a *tetL* gene almost identical to that of plasmid pNS1981 got into the *B. subtilis* chromosome [27].

Several small *S. aureus* plasmids undergo site-specific

*Bgl*III/*Mbo*I

pUB110 : D L P S G Y D H L C Q F V M S G Q L S D S E K
AGATCTTCCTTCAGGTTATGACCATCTGTGCCAGTTCGTAATGTCTGGTCAACTTTCCGACTCTGAGAAA
pSTS7 : AGATCTTCCTTCAGGTTATGACCATCTGTGCCAGTTCGTAATGTCTGGTCAACTTTCCGACTCTGAGAAA - 70
D L P S G Y D H L C Q F V M S G Q L S D S E K

pUB110 : L L E S L E N F W N G I Q E W T E R H G Y I V
CTTCTGGAATCGCTAGAGAATTTCTGGAATGGGATTCAGGAGTGGACAGAACGACACGGATATATAGTG
pSTS7 : CTTCTGGAATCGCTAGAGAATTTCTGGAATGGGATTCAGGAGTGGACAGAACGACACGGATATATAGTG - 139
L L E S L E N F W N G I Q E W T E R H G Y I V

pUB110 : D V S K R I P F *
GATGTGTCAAACGCATACCATTTTGAACGATGACCTCTAATAATTGTTAATCATGTTGGTTACGTATTTA
pSTS7 : GATGTGTCAAACGCATACCATTTTGAACGATGACCTCTAATAATTGTTAATCATGTTGGTTACGTATTTA - 210
D V S K R I P F *
end aadD

*Cfo*I

pUB110 : TTAACCTTCTCCTAGTATTAGTAATTATCATGGCTGTCATGGCGCATTAACGGAATAAAGGGTGTGC-TT
pSTS7 : TTAACCTTCTCCTAGTATTAGTAATTATCATGGCTGTCATGGCGCATTAACGGAATAAAGGGAGTGCTTT - 279
TAATTTTATTAAAGAGTAGTTCAAC

pNS1981: TAATTTTATTAAAGAGTAGTTCAAC

pUB110 : AAATCGGGCCAT-TTTGC-GTAATAAGAAAAAGGATTAATTATGAGCGAATTGAATTAATAATAAGGTAAT
pSTS7 : AAATCGGGCCATATTGTTGTA-TAAGTGATGAAATACTGAATTTAAAACTTAGTTTATATGTGGTAAAAAT - 349
pNS1981: AAA-CGGGCCATATT-GTTGTA-TAAGTGATGAAATACTGAATTTAAAACTTAGTTTATATGTGGTAAAAAT

pSTS7 : M K C N E C N R V Q L K E G
GTTTAAATCAAGTTTAGGAGGAATTAATTATGAAGTGAATGAATGAACAGGGTTCAATTAAAAGAGGGA - 420
pNS1981: GTTTAAATCAAGTTTAGGAGGAATTAATTATGAAGTGAATGAATGAACAGGGTTCAATTAAAAGAGGGA
M K C N E C N R V Q L K E G
start tet(L) leader

pSTS7 : S V S L T L * V N T S Y S
AGCGTATCATTAACCTTATAAACTACGTCTGCCCTCATTATTGGAGGGTGAAATGTGAATACATCCTATTCA - 492
pNS1981: AGCGTATCATTAACCTTATAAACTACGTCTGCCCTCATTATTGGAGGGTGAAATGTGAATACATCCTATTCA
S V S L T L * V N T S Y S
start tet(L)

pSTS7 : Q S N L R H N Q I L I W L C I L S F F S V L N E
CAATCGAATTTACGACACAACCAAATTTAATTTGGCTTTGCATTTTATCTTTTATAGCGTATTAAATGAA - 564
pNS1981: CAATCGAATTTACGACACAACCAAATTTAATTTGGCTTTGCATTTTATCTTTTATAGCGTATTAAATGAA
Q S N L R H N Q I L I W L C I L S F F S V L N E

pSTS7 : M V L N V S L P D I A N D F N K P P A S T N W V
ATGGTTTTGAACGTCTCATTACCTGATATTGCAAATGATTTTAATAAACACCTGCGAGTACAACTGGGTG - 636
pNS1981: ATGGTTTTGAACGTCTCATTACCTGATATTGCAAATGATTTTAATAAACACCTGCGAGTACAACTGGGTG
M V L N V S L P D I A N D F N K P P A S T N W V

*Bcl*I/*Mbo*I

pSTS7 : N T A F M L T F S I G T A V Y G K L S D
AACACAGCCTTTATGTTAACCTTTTCCATTGGAACAGCTGTATATGGAAAGCTATCTGATCA - 698
pNS1981: AACACAGCCTTTATGTTAACCTTTTCCATTGGAACAGCTGTATATGGAAAGCTATCTGATCA
N T A F M L T F S I G T A V Y G K L S D

Fig. 2. Nucleotide sequence of the 698 bp *Bgl*III/*Bcl*I fragment of plasmid pSTS7 (region [6]) which comprises the region between the end of the *aadD* gene and the start of the *tetL* gene. The vertical bars (|) indicate identical bases in comparison to the corresponding sequences of the *aadD* gene of plasmid pUB110 (region [4]) and the *tetL* gene of plasmid pNS1981 (region [1]). The amino-acid sequences of the *aadD*, *tetL* leader and *tetL* reading frames are displayed in the single letter code. Asterisks (*) mark translational stop codons. Important restriction endonuclease cleavage sites with respect to Fig. 1 are indicated. The region that is thought to be involved in co-integrate formation is boxed.

Fig. 3. Comparison of the sequences preceding the *aadD* genes in pUB110 (region [3]) and pSTS7 (region [7]) as well as the *tetL* gene in pNS1981 (region [1]). The vertical arrow (↓) indicates a previously described junction point between homologous and non-homologous regions in the plasmids pNS1981 and pUB110. The inverted repeats downstream of the *rep* genes are indicated by arrows (→←).

Fig. 4. Comparison of the nucleotide sequences downstream of the *tetL* genes in pNS1981 (region [2]) and pSTS7 (region [8]) with the corresponding sequence of pUB110 (region [5]). The inverted repeated sequences that represent the rho-independent transcriptional terminator of the *tetL* gene are indicated by arrows (→←). The amino-acid sequence of the carboxyterminal part of the TetL protein is given in the one-letter code. Important restriction endonuclease cleavage sites with respect to Fig. 1 are indicated. The vertical arrow (↓) indicates a previously described junction point between homologous and non-homologous regions in the plasmids pNS1981 and pUB110.

Fig. 5. Model to explain the derivation of plasmid pSTS7 from pNS1981- and pUB110-like precursors by RS_A -mediated co-integrate formation and subsequent intraplasmid homologous recombination.

interplasmid recombination with formation of stable co-integrates [28]. Recombination sites RS_A and RS_B are used most often [29, 30], but there are also small areas of sequence homology that serve for homologous recombination of staphylococcal plasmids mediating chloramphenicol and streptomycin resistance [31] as well as chloramphenicol and tetracycline resistance [30].

Co-integrate formation between plasmids of the pUB110 and pNS1981 type allows a possible explanation for the development of plasmid pSTS7. Previous investigations described the *aadD*-encoding $Km^r/Nm^r/Ble^r$ plasmid pUB110 [16] and the *tetL*-encoding Tc^r plasmid pNS1981 [13, 14] as homologous *repU* based replicons that harboured different resistance gene cassettes. However, an identical *rep* gene assigned the

respective plasmids to the same incompatibility group. Normally, plasmids of the same incompatibility group cannot be maintained stably together in the same bacterial cell [32], but if recombination systems are intact and the selective pressure is strong, incompatible plasmids can be accommodated by forming a hybrid plasmid consisting of parts from both individual plasmids [33]. Both plasmids, pUB110 and pNS1981, had a recombination site A (RS_A) and carried a *pre* gene that encoded a recombinase that acts on RS_A . Thus, the presence of plasmid-encoded recombination systems allowed recombination to occur independently of the host cell-specific recombination systems. Co-integrate formation via RS_A might result in a hybrid plasmid that carried two *repU* and *pre* genes, two RS_A sites and the resistance genes *tetL*, *aadD* and *ble* (Fig. 5). Since small staphylococcal plasmids replicate via a single-stranded intermediate [34], they are prone to recombination in that state of replication, provided that there are sufficient structural homologies [34]. Thus, the hybrid plasmid could undergo intraplasmid homologous recombination favoured by almost identical sequences flanking the resistance genes in pNS1981 and in pUB110. It is suggested that homologous recombination may have occurred between region [1] in pNS1981 and region [4] in pUB110 (to give rise to region [6] in pSTS7; Fig. 2, boxed area). Such a recombination event would replace the *ble* gene and its downstream sequences with the *tetL* gene and its associated regulatory sequences (Fig. 5). Since the sequences downstream of the *ble* gene in pUB110 and those downstream of the *tetL* reading frame in pNS1981 are identical, this recombination allowed the exchange of the *ble* gene by a *tetL* gene cassette.

The *S. epidermidis* strain 210 from which pSTS7 was isolated, was obtained from a pig that had received tetracyclines for therapeutic purposes. This could explain the selective pressure that favoured the exchange of Tc^r plasmids within a staphylococcal population. Since pUB110-like $Km^r/Nm^r/Ble^r$ plasmids as well as pNS1981-like Tc^r plasmids have been shown to be present in the staphylococcal gene pool and have been identified in different epidemiologically unrelated, staphylococcal isolates [5, 6], it is unlikely that a plasmid, such as pSTS7, has developed *de novo* in the *S. epidermidis* strain 210. It is more likely to have been derived from putative pUB110 and pNS1981 plasmids as a consequence of recombination events that might be favoured by the incompatibility properties of the plasmids, the presence of plasmid recombination systems, and the selective pressure of the antibiotics used.

The nucleotide sequences presented in this manuscript have been submitted to GenBank and assigned accession number U35229.

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