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Integration of pT181-Like Tetracycline Resistance Plasmids into Large Staphylococcal Plasmids Involves IS*257*

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Four large staphylococcal plasmids ranging in size from 31 to 82 kbp have been shown to mediate tetracycline resistance via an integrated copy of the *tet*(K)-encoding plasmid pT181 which was flanked by copies of the insertion element IS*257*. In two cases, IS*257* elements interrupted the *repC* reading frame of pT181 and an 8-bp sequence from within the *repC* gene was duplicated at the interrupted site. In the third plasmid, the IS*257* elements interrupted the pT181 DNA immediately upstream of the *repC* coding sequence with an 8-bp duplication. In the fourth case, the IS*257* elements flanked a pT181-like plasmid with one IS*257* in the *repC* coding sequence and the other within the recombinase (*pre*) coding sequence, so that a section of the pT181 sequence was deleted. All four integration sites detected in this study differ from those previously described for the IS*257*-mediated integration of pT181-like plasmids into large plasmids or into the chromosomal DNA.

Tetracycline resistance in various staphylococcal species is often associated with the presence of plasmid-encoded *tet*(K) genes (4,9,12). The 4.45-kbp plasmid pT181 from *Staphylococcus aureus* (4) is considered the prototype *tet*(K) plasmid. pT181 has been completely sequenced; three reading frames, encoding the Tet(K) tetracycline efflux protein, the RepC protein (involved in plasmid replication), and the Pre recombinase, were found (4). pT181-like plasmids have also been detected either integrated into large plasmids or in the chromosomal DNA (7,14). In both cases, they were flanked by directly repeated insertion sequences of the type IS*257*. IS*257* is a small mobile genetic element of 0.79 kbp which was originally detected in *S. aureus* (3,6,10).

During a study on the prevalence of different *tet* genes in staphylococci from human and animal sources, *tet*(K)-encoding plasmids were detected in epidemiologically unrelated strains of *S. haemolyticus*, *S. aureus*, *S. warneri*, and *S. epidermidis*. Since these four *tet*(K) plasmids were significantly larger than the commonly detectable *tet*(K) plasmids, they were investigated to determine if they carried complete or partial copies of small *tet*(K)-encoding plasmids as well as for the presence of elements, such as IS*257*, which might be involved in the integration of small plasmids into large plasmids.

MATERIALS AND METHODS

Bacterial isolates and antibiotic resistance testing. The four staphylococcal isolates included in this study were *S. haemolyticus* F60597A, *S. warneri* F59743E, *S. epidermidis* W69941E (all of human origin), and *S. aureus* 4257 (from a mountain goat). Species identification was achieved by using the ID32 Staph system (Biomerieux, Marcy l'Etoile, France). Antibiotic resistances were determined by the disk diffusion method (1) with disks from Unipath (Wesel, Germany) and Becton-Dickinson (Heidelberg, Germany).

Plasmid preparation and Southern blot hybridization. Plasmids were prepared by a previously described staphylococcus-specific modification of the alkaline lysis procedure and purified by affinity chromatography on Qiagen Midi columns (13). Restriction analysis, agarose gel electrophoresis, and Southern blotting were performed as previously described (12,13). The following gene probes were used: the three *Hin*dIII fragments of pT181 (4) of approximately 2.35, 1.53, and 0.56 kbp; a PCR-generated internal 0.63-kbp fragment of the IS*257*-specific transposase gene cloned into pUC18; the 0.76-kbp *Kpn*I-*Cla*I fragment of pT181, which comprised the 5′ end of the *tet*(K) gene as well as its regulatory region; the 1.5-kbp *Alu*I fragment of plasmid pSF815A (2), representing the *aacA-aphD* gene; the 0.41-kbp *Sac*I-*Bcl*I fragment of plasmid pSES6 (5), for the detection of *ermC*; the 0.9-kbp *Taq*I-*Mbo*I fragment of plasmid pSCS1 (13), for detection of the chloramphenicol acetyltransferase (*cat*) gene; and the internal 1.6-kbp *Nco*I fragment of the *mupA* gene (8) as the mupirocin resistance gene probe. All probes were labelled by using the ECL nonradioactive enhanced chemiluminescence system (Amersham-Buchler, Braunschweig, Germany). Hybridization and signal detection were performed in accordance with the manufacturer's recommendations. For identification of the resistance plasmids, the undigested plasmids of the four staphylococcal strains were probed with the resistance gene probes.

Cloning and sequence analysis. The large plasmids were digested with *Bgl*II; the fragments were then cloned into the *Bam*HI site of pBluescript II SK+ (Stratagene) and transformed into *Escherichia coli* JM107 by the $CaCl_2$ method (13). The *Bgl*II fragment of the *S. haemolyticus* plasmid pSTS20 was redigested with *Hin*dIII, and the fragments of approximately 650 bp and 400 bp which were believed to carry the junctions between IS*257* and pT181 were inserted into the *Hin*dIII site of pBluescript II SK+. The oligonucleotide primers 5′-TAG TTCATACAGAAGACACC-3′ and 5′-CAGATCTACGGATTTTCGCC-3′, complementary to the nucleotide sequences specifying the N terminus and the C terminus of the IS*257*-specific transposase, respectively, were used to determine the sequences at the sites of integration of IS*257* into pT181. Sequence analyses were performed by the dideoxy chain termination method (11) with the Sequenase version 2.0 kit and [α-^{35}S]dATP.

RESULTS AND DISCUSSION

Identification of the large *tet*(K)-encoding plasmids. The original staphylococcal strains and their resistance plasmids are shown in Table 1. Each of the large plasmids pSTS20 to pSTS23 carried a *tet*(K) gene. Separate hybridization experiments using *Hin*dIII digests of plasmids pSTS20 to pSTS23 as target DNA and the three *Hin*dIII fragments of pT181 as probes confirmed that all four plasmids harbored *Hin*dIII fragments of 2.35 kbp as well as 0.56 kbp. However, by using the 1.53-kbp *Hin*dIII fragment of pT181 as a probe, a hybridizing band of 650 bp was observed in plasmid pSTS20; two hybridizing bands, of 550 and 1,500 bp, were detected in plasmids pSTS21 and pSTS22; and an apparently single hybridizing band of 1,000 bp, which comprised two fragments of approximately the same size, was seen in plasmid pSTS23. This indicated that all four plasmids carried integrated copies of pT181

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TABLE 1. Strains and plasmids investigated in this study

Strain	Antibiotic resistance[a]	Sizes (kbp) of plasmids present[b]	Plasmid designation
S. haemolyticus F60597A	Tc, Gm, Km, Em, Cc, Cm	67 [Gm and Km via *aacA-aphD*, Tc via *tet*(K)]	pSTS20
		4.5 (Cm via *cat*)	None
		2.5 (none)	None
S. aureus 4257	Tc	82 [Tc via *tet*(K)]	pSTS21
		6.2 (none)	None
S. warneri F59743E	Tc	31 [Tc via *tet*(K)]	pSTS22
		5.1 (none)	None
S. epidermidis W69941E	Tc, Mu, Em, Cc	49 [Tc via *tet*(K), Mu via *mupA*]	pSTS23
		2.3 (Em and Cc via *ermC*)	None

[a] Tc, tetracycline; Gm, gentamicin; Km, kanamycin; Em, erythromycin; Cc, clindamycin; Cm, chloramphenicol; Mu, mupirocin.
[b] Antibiotic resistances associated with plasmids are given in parentheses. The sizes of the plasmids were calculated from the sums of the fragments obtained after single digestions with *Hin*dIII or *Bgl*II.

and that the integration sites were probably located in the 1.53-kbp *Hin*dIII fragments.

Naturally occurring plasmids which carry integrated copies of pT181-like plasmids are rare. The only example has been the 34.2-kbp Mu^r Tc^r plasmid pJ3358 from *S. aureus* (7), in which the integrated copy of pT181 was flanked by two directly repeated IS*257* elements. This observation corresponded closely to the situation seen in the *mec* region of the chromo-

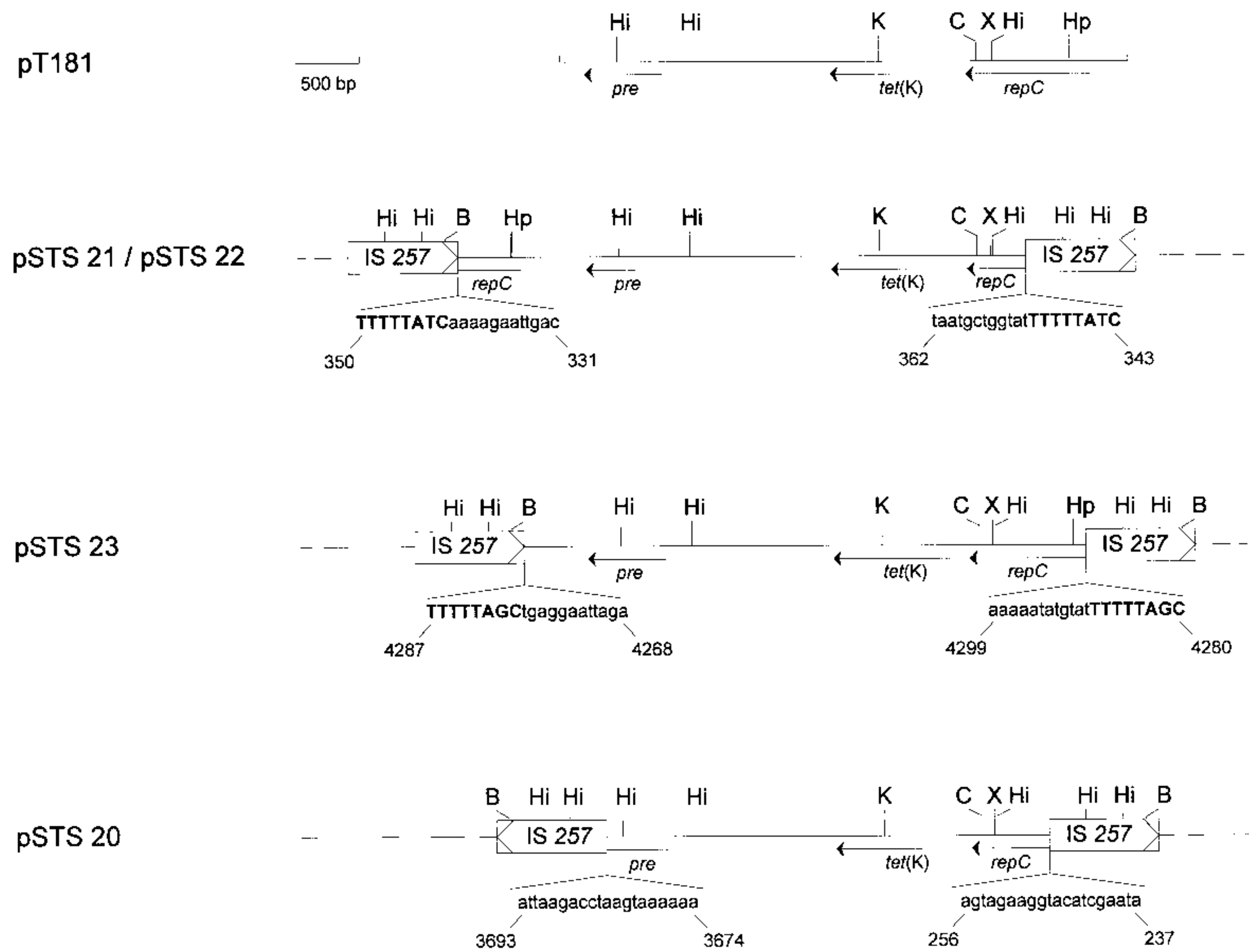

FIG. 1. Schematic presentation of pT181 and the IS*257*-flanked pT181 integrates within plasmids pSTS20 to pSTS23. Restriction endonuclease cleavage sites are abbreviated as follows: B, *Bgl*II; C, *Cla*I; Hi, *Hin*dIII; Hp, *Hpa*II; K, *Kpn*I; and X, *Xba*I. The IS*257* elements are boxed, and the arrowheads within these boxes indicate their orientations. The sequence of pT181 (displayed as the noncoding strand) at the junction to IS*257* is indicated with the sequence duplications in capital letters and the nonduplicated pT181 sequence in lowercase letters. The numbering refers to the pT181 sequence (GenBank accession numbers J01764 and J01765).

some of *S. aureus* ANS46, in which an integrated copy of pT181 was also found to be flanked by two directly repeated IS*257* elements (14). Moreover, IS*257* elements have been detected in close proximity to other resistance genes, such as the trimethoprim resistance gene *dfrA* and the mercury resistance genes *merA* and *merB* (3).

Integration of pT181-like plasmids into large plasmids involves IS*257*. A previous in vitro study showed that two plasmids can undergo IS*257*-mediated cointegration even if only one of them carries a functionally active IS*257* element (6). Since pT181 does not carry a copy of IS*257*, it is suggested that IS*257* transposes to the pT181 plasmid; subsequently, the IS*257*-carrying pT181 and the large plasmid which still carries IS*257* copies may interact to form a pT181 integrate with the IS*257* copies as direct repeats at the junctions (6). This model corresponds closely to the arrangements seen in the three plasmids pSTS21, pSTS22, and pSTS23.

The minimum numbers of IS*257* copies located on plasmids pSTS20 to pSTS23 were five for pSTS20 and pSTS21, three for pSTS22, and four for pSTS23, as determined from the number of hybridizing bands seen after hybridization of *Bgl*II-digested plasmid DNA with the IS*257*-specific gene probe. The observation that IS*257*-carrying *Bgl*II fragments ranging in size from 4.9 to 5.2 kbp also hybridized with the *tet*(K) gene probe strongly suggested that IS*257* was involved in the integration of pT181 into these large plasmids.

Sequence analysis of the terminal regions of the *tet*(K)-carrying *Bgl*II fragments of plasmids pSTS21 and pSTS22 revealed that each had an integrated pT181 plasmid flanked by two IS*257* copies in a direct repeat. Duplication of the sequence TTTTTATC was detected at the site of integration of IS*257* into pT181. This integration site was located within the *repC* coding sequence and was not accompanied by loss of any of the pT181 sequence (Fig. 1). The pT181 integrate in plasmid pSTS23 was also flanked by two IS*257* copies in the same orientation; however, the integration site of IS*257* in pT181 was located immediately upstream of the *repC* coding sequence. A duplication of the sequence TTTTTAGC was detected at this integration site (Fig. 1).

The *Bgl*II fragment of plasmid pSTS20 was smaller than those of the other three large plasmids. Two IS*257* copies flanked the integrated pT181 plasmid; however, the IS*257* copies were in opposite orientations. One of the IS*257* copies was present in the *repC* coding sequence of pT181 about 100 bp upstream of the integration site seen in plasmids pSTS21 and pSTS22. The other IS*257* copy was integrated into the *pre* reading frame about 120 bp downstream of the *Hin*dIII site. The section which is located in the original pT181 plasmid between the two integration sites was not detected in plasmid pSTS20 and therefore must have been deleted. No significant homology was detected between the pT181 sequences at the junctions to the IS*257* elements; these junctions were IS*257*-ATTAAGAC-*pre* and *repC*-ATCGAATA-IS*257* (Fig. 1).

In contrast to the two previously reported cases (7,14), IS*257* integration caused a functional deletion of the pT181 plasmid replication system in all four pT181 integrates investigated in this study. In plasmids pSTS20, pSTS21, and pSTS22, the *repC* reading frame was interrupted by the IS*257* insertion. In plasmid pSTS23, IS*257* integration occurred immediately upstream of the *repC* coding sequence, between the translational start codon of the *repC* gene and the *repC*-associated ribosomal binding site. No sequence that could replace the deleted *repC* ribosomal binding site was detected within a reasonable distance in the adjacent IS*257* sequence. Functional deletion of the pT181-specific replication system ensures that replication properties, copy number, and plasmid incompatibility properties will be specified by the original large plasmid.

These data confirm that IS*257*-mediated integration of small *tet*(K)-encoding plasmids into large plasmids has occurred in several staphylococcal species from different sources. It might contribute to the spread of these *tet* genes and further their acquisition even by new hosts in which the entire pT181 plasmid is not able to replicate efficiently.

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REFERENCES

1. **Barry, A. L., and C. Thornsberry.** 1985. Susceptibility tests: diffusion test procedures, p. 978–987. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
2. **Ferretti, J. J., K. S. Gilmore, and P. Courvalin.** 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. **167:**631–638.
3. **Gillespie, M. T., B. R. Lyon, L. S. L. Loo, P. R. Matthews, P. R. Stewart, and R. A. Skurray.** 1987. Homologous direct repeat sequences associated with mercury, methicillin, tetracycline and trimethoprim resistance determinants in *Staphylococcus aureus*. FEMS Microbiol. Lett. **43:**165–171.
4. **Khan, S. A., and R. P. Novick.** 1983. Complete nucleotide sequence of pT181, a tetracycline resistance plasmid from *Staphylococcus aureus*. Plasmid **10:**251–259.
5. **Lodder, G., S. Schwarz, P. Gregory, and K. Dyke.** 1996. Tandem duplication in *ermC* translational attenuator of the macrolide-lincosamide-streptogramin B resistance plasmid pSES6 from *Staphylococcus equorum*. Antimicrob. Agents Chemother. **40:**215–217.
6. **Needham, C., W. C. Noble, and K. G. H. Dyke.** 1995. The staphylococcal insertion sequence IS*257* is active. Plasmid **34:**198–205.
7. **Needham, C., M. Rahman, K. G. H. Dyke, and W. C. Noble.** 1994. An investigation of plasmids from *Staphylococcus aureus* that mediate resistance to mupirocin and tetracycline. Microbiology **140:**2577–2583.
8. **Rahman, M., W. C. Noble, and K. G. H. Dyke.** 1993. Probes for the study of mupirocin resistance in staphylococci. J. Med. Microbiol. **39:**446–449.
9. **Roberts, M. C.** 1994. Epidemiology of tetracycline-resistance determinants. Trends Microbiol. **2:**353–357.
10. **Rouch, D. A., and R. A. Skurray.** 1989. IS*257* from *Staphylococcus aureus*: a member of an insertion sequence superfamily prevalent among Gram-positive and Gram-negative bacteria. Gene **76:**195–205.
11. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
12. **Schwarz, S., and W. C. Noble.** 1994. Tetracycline resistance genes in staphylococci from the skin of pigs. J. Appl. Bacteriol. **76:**320–326.
13. **Schwarz, S., U. Spies, and M. Cardoso.** 1991. Cloning and sequence analysis of a plasmid-encoded chloramphenicol acetyltransferase gene from *Staphylococcus intermedius*. J. Gen. Microbiol. **137:**977–981.
14. **Stewart, P. R., D. T. Dubin, S. G. Chikramane, B. Inglis, P. R. Matthews, and S. M. Poston.** 1994. IS*257* and small plasmid insertions in the *mec* region of the chromosome of *Staphylococcus aureus*. Plasmid **31:**12–20.