Integration of pT181-Like Tetracycline Resistance Plasmids into Large Staphylococcal Plasmids Involves IS257

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Received 30 April 1996/Returned for modification 2 August 1996/ Accepted 10 September 1996

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 IS257. In two cases, IS257 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 IS257 elements interrupted the pT181 DNA immediately upstream of the repC coding sequence with an 8-bp duplication. In the fourth case, the IS257 elements flanked a pT181-like plasmid with one IS257 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 IS257-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 IS257. IS257 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 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 IS257, 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 F60857A, S. warneri F59743E, S. epidermidis W69941E (all of human origin), and S. aureus F60597A (from a mountain goat). Species identification was achieved by using the ID32 Staph system (Biomerieux, Marcy l’Etiole, 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 HindIII fragments of pT181 (4) of approximately 2.35, 1.53, and 0.56 kbp; a PCR-generated internal 0.63-kbp fragment of the IS257-specific transposase gene cloned into pUC18; the 0.76-kbp KpnI-ClaI fragment of pT181, which comprised the 5’ end of the tet(K) gene as well as its regulatory region; the 1.5-kbp HgiII1 fragment of plasmid pST215 (2), representing the mupA-mupD gene; the 0.41-kbp SacI-BclI fragment of plasmid pSES6 (5), for the detection of ermC; the 0.9-kbp TaqI-MboI fragment of plasmid pSCS1 (13), for detection of the chloramphenicol acetyltransferase (cat) gene; and the internal 1.6-kbp NcoI fragment of the mupA gene (8) as the mupirocin resistance gene probe. All probes were labelled 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 BglII, the fragments were then cloned into the BamHI site of pBluescript II SK+ (Stratagene) and transformed into Escherichia coli JM107 by the CaCl₂ method (13). The BglII fragment of the S. haemolyticus plasmid pSTS20 was redigested with HindIII, and the fragments of approximately 650 bp and 400 bp which were believed to carry the junctions between IS257 and pT181 were inserted into the HindIII site of pBluescript II SK+. The oligonucleotide primers 5’-TAG TTCATAAGAAGACACCC-3’ and 5’-CAGATCTACGAGGTTCGCC-3’, complementary to the nucleotide sequences specifying the N terminus and the C terminus of the IS257-specific transposase, respectively, were used to determine the sequences at the sites of integration of IS257 into pT181. Sequence analyses were performed by the dideoxy chain termination method (11) with the Sequenase version 2.0 kit and [γ-³²P]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 HindIII digests of plasmids pSTS20 to pSTS23 as target DNA and the three HindIII fragments of pT181 as probes confirmed that all four plasmids harbored HindIII fragments of 2.35 kbp as well as 0.56 kbp. However, by using the 1.53-kbp HindIII 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.
and that the integration sites were probably located in the 1.53-kbp HindIII fragments.

Naturally occurring plasmids which carry integrated copies of pT181-like plasmids are rare. The only example has been the 34.2-kbp Mu' Tc' plasmid pJ3358 from S. aureus (7), in which the integrated copy of pT181 was flanked by two directly repeated IS257 elements. This observation corresponded closely to the situation seen in the mec region of the chromo-

![Diagram of pT181 and IS257-flanked pT181](http://aac.asm.org/)

**FIG. 1.** Schematic presentation of pT181 and the IS257-flanked pT181 integrates within plasmids pSTS20 to pSTS23. Restriction endonuclease cleavage sites are abbreviated as follows: B, BglII; C, Clal; H, HindIII; H, HpaII; K, KpnI; and X, XbaI. The IS257 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 IS257 is indicated with the sequence duplications in capital letters and the nonreplicated 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 IS257 elements (14). Moreover, IS257 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 IS257. A previous in vitro study showed that two plasmids can undergo IS257-mediated co-integration even if only one of them carries a functionally active IS257 element (6). Since pT181 does not carry a copy of IS257, it is suggested that IS257 transposes to the pT181 plasmid; subsequently, the IS257-carrying pT181 and the large plasmid which still carries IS257 copies may interact to form a pT181 integrate with the IS257 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 IS257 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 BglII-digested plasmid DNA with the IS257-specific gene probe. The observation that IS257-carrying BglII fragments ranging in size from 4.9 to 5.2 kbp also hybridized with the tet(K) gene probe strongly suggested that IS257 was involved in the integration of pT181 into these large plasmids.

Sequence analysis of the terminal regions of the tet(K)-carrying BglII fragments of plasmids pSTS21 and pSTS22 revealed that each had an integrated pT181 plasmid flanked by two IS257 copies in a direct repeat. Duplication of the sequence TTTTTATC was detected at the site of integration of IS257 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 IS257 copies in the same orientation; however, the integration site of IS257 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 BglII fragment of plasmid pSTS20 was smaller than those of the other three large plasmids. Two IS257 copies flanked the integrated pT181 plasmid; however, the IS257 copies were in opposite orientations. One of the IS257 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 IS257 copy was integrated into the pre-reading frame about 120 bp downstream of the HindIII 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 IS257 elements; these junctions were IS257-ATTAAGAC-pre and repC-ATCGAATA-IS257 (Fig. 1).

In contrast to the two previously reported cases (7,14), IS257 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 IS257 insertion. In plasmid pSTS23, IS257 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 IS257 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 IS257-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.

ACKNOWLEDGMENTS

This study was supported by the Bundesministerium für Ernährung, Landwirtschaft und Forsten and the United States Department of Agriculture (USDA/FAS/ICD.RSEDMH17). C-W. is the recipient of a fellowship of the Graduiertenkolleg Zell- und Molekularbiologie in der Medizin (GRK 158/2-96) funded by the Deutsche Forschungsgemeinschaft.

We thank J. Davies and M. Coley for staphylococcal strains, K. G. Dyke for many helpful comments and the mupA gene probe, N. El Sohli for providing the IS257 gene probe, and E. Nullebeck for excellent technical assistance.

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