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Molecular Analysis of a Gentamicin Resistance Transposonlike Element on Plasmids Isolated from North American *Staphylococcus aureus* Strains

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Plasmid-encoded resistance to the aminoglycosides gentamicin (Gm), tobramycin (Tm), and kanamycin (Km) (GmTmKm^r) in strains of *Staphylococcus aureus* isolated in Australia and North America appears to be mediated by one resistance determinant. In Australian isolates, this determinant is flanked by inverted copies of a 1.3-kb insertion sequence, IS*256*, thereby forming a composite transposon, Tn*4001*. Analysis of two conjugative plasmids and a related nonconjugative plasmid from strains of *S. aureus* isolated in North America showed that the GmTmKm^r determinant on these plasmids is also flanked by inverted repeats. In the nonconjugative plasmid, these repeats include only 425 bp of IS*256* immediately adjacent to the GmTmKm^r region and identical to that on Tn*4001*. This truncated Tn*4001* element is flanked by copies of the insertion element IS*257*, and together these elements form a truncated Tn*4001*-IS*257* hybrid transposonlike structure. A third copy of IS*257* was located 418 bp from the hybrid structure. The truncated Tn*4001* and three repeats of IS*257* were present at a conserved site on the plasmids studied. Four additional copies of IS*257* were identified on the two conjugative plasmids. These elements flank determinants for resistance to the aminoglycosides neomycin and paromomycin and to ethidium bromide and quaternary ammonium compounds, as well as the region involved in conjugative plasmid transfer.

In Australian strains of *Staphylococcus aureus*, resistance to the aminoglycosides gentamicin, tobramycin, and kanamycin (GmTmKm^r) has been shown to be encoded on a transposon, Tn*4001* (17, 18). This composite transposon consists of two inverted copies of the 1.3-kb insertion sequence IS*256* flanking a 1.9-kb unique region (5, 17, 24). The resistance *aacA-aphD* gene is contained within the unique region and codes for production of a 56.9-kDa bifunctional enzyme that specifies aminoglycoside-modifying acetyltransferase AAC(6′) and phosphotransferase APH(2″) activities (24).

Tn*4001* is commonly found on members of the pSK1 family of multiresistance plasmids (9, 18, 28). The majority of these plasmids mediate resistance to acriflavine, ethidium bromide, and quaternary ammonium compounds determined by the *qacA* gene (31, 32). In addition, pSK1-type plasmids may confer resistance to penicillin via production of a β-lactamase carried on Tn*4002* (10) and high-level resistance to trimethoprim mediated by a trimethoprim-insensitive dihydrofolate reductase encoded on the putative transposon Tn*4003* (25).

GmTmKm^r in clinical strains of staphylococci from North America is frequently mediated by a group of structurally related plasmids unrelated to the pSK1 family of plasmids found in Australian clinical strains of staphylococci. Many of these plasmids are conjugative (2, 3, 22) and, in addition to GmTmKm^r, can specify resistance to other aminoglycosides through 4′-adenyltransferase or 3′-phosphotransferase activities, and they may also mediate resistance to ethidium bromide and quaternary ammonium compounds, penicillin, and trimethoprim (1, 2, 11, 12, 14, 22).

The GmTmKm^r conjugative plasmids pSK41 and pUW3626 and the related but nonconjugative plasmid pSH6 were isolated from North American clinical strains of *S. aureus*. Previous molecular studies indicated that these plasmids contained the same GmTmKm^r determinant as found in Tn*4001* (15). Like Tn*4001*, the resistance determinants in these plasmids were shown to be flanked by inverted repeats. Although these repeats shared some homology with IS*256*, they were significantly shorter than the 1.3-kb inverted IS*256* elements on Tn*4001* (15, 18, 28). In electron micrographs of self-annealed molecules, inverted repeats of approximately 0.7 kb flanked the GmTmKm^r determinant in these plasmids. In addition, a second pair of inverted repeats, about 0.5 kb in size, was observed to the right of the GmTmKm^r region (15).

Characterization of the GmTmKm^r element on plasmid pSH6 has revealed the presence of a truncated copy of Tn*4001* flanked by inverted repeats of the staphylococcal element IS*257*. Copies of IS*257* have been found both chromosomally and on plasmids in association with a number of determinants, including those encoding resistance to mercury, methicillin, tetracycline, and trimethoprim (8, 21, 25). These elements have also been designated IS*431* (4). The IS*257* elements thus far sequenced represent a closely related group, being 788 to 790 bp in length and sharing 99% nucleotide sequence homology (4, 25, 26; P. R. Matthews, B. Inglis, and P. R. Stewart, *in* R. P. Novick, ed., *Molecular Biology of the Staphylococci*, in press). Although transposition of IS*257* has not been demonstrated, elements within this group possess features typical of insertion sequences, including 26- to 28-bp terminal inverted repeats and a single large open reading frame (ORF). This ORF potentially encodes a 224-amino-acid (aa) polypeptide, predicted to be a transposase (4, 25, 26). Furthermore, IS*257* elements share a significant degree of similarity in both their nucleotide and putative transposase aa sequences with members of the IS*15*

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TABLE 1. *S. aureus* and *E. coli* plasmids

Plasmid	Relevant characteristics[a]	Size (kb)	Reference
S. aureus			
pSK1	Gm^r Tm^r Km^r Tp^r Eb^r Qa^r	28.4	16, 31
pSH6	Gm^r Tm^r Km^r	20.8	15
pSK41	Gm^r Tm^r Km^r Nm^r Pm^r Eb^r Qa^r Tra$^+$	47.8	15
pUW3626	Gm^r Tm^r Km^r Nm^r Pm^r Eb^r Qa^r Pc^r Tra$^+$	54.4	15
E. coli			
pSK236	7.4-kb *Sal*I-*Eco*RI fragment of pSK1, encompassing IS*257*L, cloned into pBR322	11.1	8
pSK330	2.5-kb *Hin*dIII fragment of Tn*4001* cloned into pUC8	5.2	24
pSK367	1.4-kb *Pvu*II-*Hin*dIII fragment of pSK1, containing portion of IS*256* adjacent to *qacA*, cloned into pUC18	4.1	5
pSK369	2.5-kb *Hin*dIII fragment of pSH6 cloned into pUC18	5.2	This study
pSK382	1.4-kb *Dra*I fragment of pSK1, encompassing entire IS*256* element, cloned into pUC18	4.1	This study

[a] Abbreviations: Eb^r, ethidium bromide resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Nm^r, neomycin resistance; Pc^r, penicillin resistance; Pm^r, paromomycin resistance; Qa^r, quaternary ammonium compound resistance; Tm^r, tobramycin resistance; Tp^r, trimethoprim resistance; Tra$^+$, conjugative transfer.

family of IS elements from gram-negative bacteria and with ISS*1* from *Streptococcus lactis* (4, 25, 26).

In this paper, we present the results of a study detailing the structure of the GmTmKm^r element on pSH6. In addition, we have mapped the location and orientation of multiple copies of IS*257* on the conjugative plasmids pSK41 and pUW3626.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* plasmids and *Escherichia coli* recombinant plasmids used in this study and their relevant characteristics are listed in Table 1. Plasmid pSK1 was originally isolated from an Australian strain of *S. aureus* (16). pSH6, pSK41, and pUW3626 were from *S. aureus* strains isolated in North America (15). All *S. aureus* plasmids had been transferred from clinical isolates to laboratory strain SK982 or SK983 (15). The *E. coli* vector used in all initial clonings was pUC18 (36). Derivatives of bacteriophage M13, mp18 and mp19 (36), were used to generate subclones for nucleotide sequencing. These subclones were made using available restriction sites. *E. coli* host strains employed were JM101 (*supE thi* Δ[*lac-proAB*] [F′ *traD36 proAB lacI*^{q}ZΔM15]) and JM105 (*thi rpsL endA sbcB15 hspR4* Δ[*lac-proAB*] [F′ *traD36 proAB lacI*^{q}ZΔM15]) (36) and DH5α (F$^-$ *endA hsdR17 supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* φ80*dlac*ZΔM15) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

DNA manipulation procedures. The isolation of cesium chloride-purified plasmid DNA from *S. aureus* cultures, restriction endonuclease digestions, agarose gel electrophoresis, and estimation of DNA fragment sizes were performed as described previously (16). The procedures for *E. coli* cesium chloride-purified plasmid DNA isolation, small-scale whole-cell plasmid DNA isolation, and recombinant DNA techniques have been previously described (23). Specific DNA fragments to be radioactively labeled by nick translation were obtained as follows. Fragments from restriction enzyme-digested plasmid DNA were separated on an agarose gel. The relevant DNA fragment was electrophoresed onto DE81 paper (Whatman, Maidstone, Kent, United Kingdom). The DE81 paper was washed several times with 0.1 M NaCl, and DNA was eluted by using 1 M NaCl.

DNA probes and hybridization. IS*257*-specific probes were obtained from pSK236. These included the 484-bp *Acc*I-*Bgl*II fragment and the 80-bp *Bgl*II-*Alu*I fragment from the left- and right-hand sides of the *Bgl*II site of IS*257*L from Tn*4003* on pSK1, respectively (25). IS*256*-specific probes consisted of a 622-bp *Hin*dIII-*Bgl*I fragment from pSK367 and a 323-bp *Hae*III fragment derived from pSK382. DNA probes were prepared either by end labeling with [γ-^{32}P]ATP and gel purifying by the procedures of Maniatis et al. (19) or by nick translation of isolated DNA fragments using [α-^{32}P]dATP and a nick translation kit (Amersham International, Little Chalfont, Buckinghamshire, England). Southern blotting using nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) and DNA hybridizations were done using the methods of Maniatis et al. (19). Initially, all hybridizations were performed under stringent conditions at 42°C and with 50% formamide in the hybridization buffer. However, when these parameters were used the 80-bp *Bgl*II-*Alu*I IS*257* probe failed to hybridize to fragments of pSH6 carrying IS*257*/1 and IS*257*/2. This was probably due to nucleotide sequence variation within these IS*257* elements (see below). For this reason, hybridizations with IS*257*-specific probes were performed under less stringent conditions at 35°C and using 30% formamide in the hybridization buffer. Nitrocellulose filters were washed initially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2% sodium dodecyl sulfate at 20°C and exposed to autoradiograph film at −70°C, followed by a more stringent washing using 0.2× SSC and 0.2% sodium dodecyl sulfate at 65°C and reexposure of the filter.

Nucleotide sequencing. Nucleotide sequences were determined from plasmid and phage M13 clones by the dideoxy-chain termination method of Sanger et al. (27) employing Sequenase (United States Biochemical, Cleveland, Ohio) or T7 DNA polymerase (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) sequencing kits and following manufacturers' instructions. Analysis of sequence data was performed by using the program of Staden (29) as modified by A. Kyne (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia).

RESULTS

Characterization of the GmTmKm^r determinant on pSH6. The 2.5-kb *Hin*dIII fragment of Tn*4001*, containing 298 bp of each IS*256* plus the 1.9-kb central region, has been cloned into *E. coli* vector pUC8 to create recombinant plasmid pSK330 (24). To compare the resistance determinant carried on plasmids such as pSH6, pSK41, and pUW3626, from

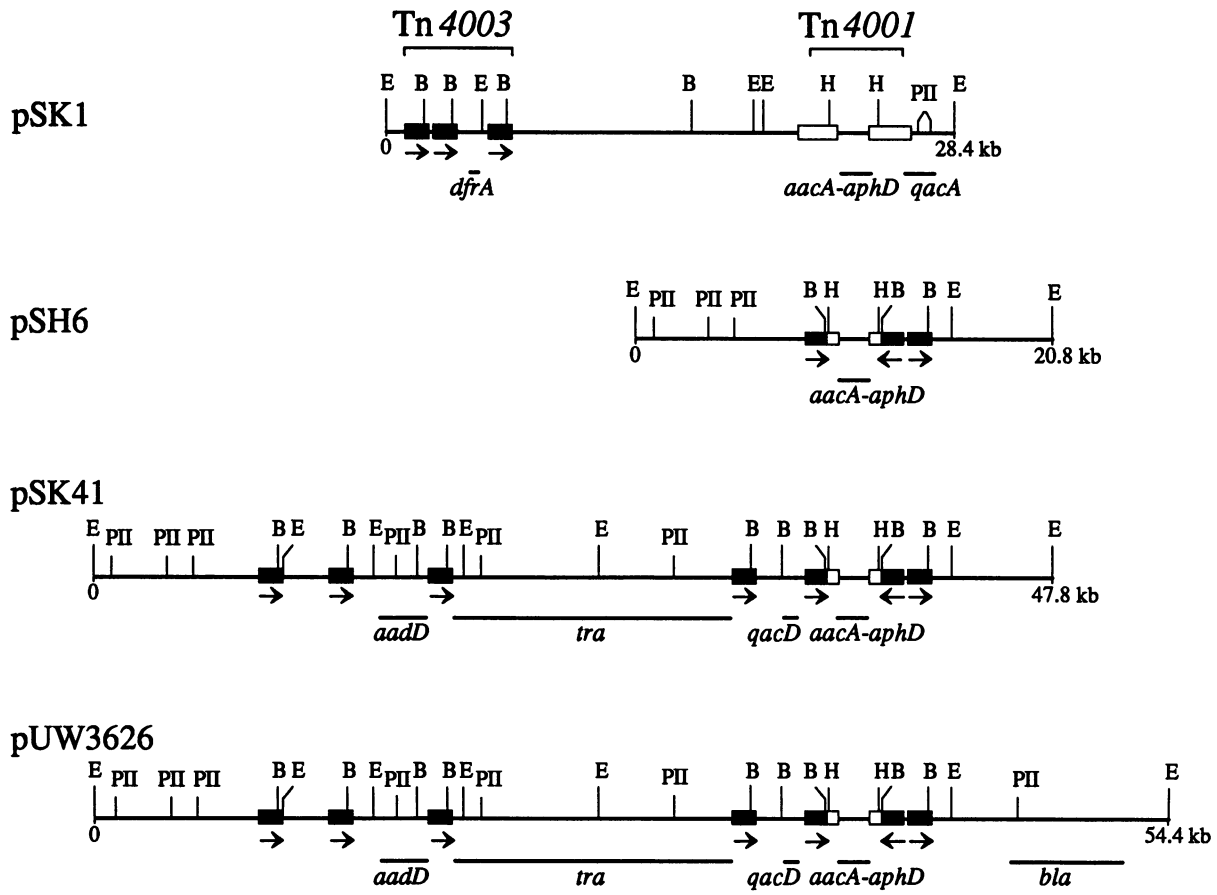

FIG. 1. Structural maps of staphylococcal plasmids pSK1, pSH6, pSK41, and pUW3626. The regions which encode aminoglycoside 6′-acetyltransferase and 2″-phosphotransferase (*aacA-aphD*) and 4′-adenyltransferase (*aadD*) activities, trimethoprim resistance (*dfrA*), quaternary ammonium resistance (*qacA* and *qacD*), β-lactamase production (*bla*), and transfer functions (*tra*) are indicated. Inverted repeats of IS*256* sequences are represented as open boxes. IS*257* elements are shown as filled boxes, and the arrows below these boxes denote the relative orientations of each IS*257*. The map of pSK1 has been redrawn to orient the GmTmKm^r elements as shown in Fig. 2. As such, on the map of pSK1, IS*257* repeats flanking the trimethoprim *dfrA* resistance determinant on Tn*4003* are organized from left to right as IS*257*R2, IS*257*R1, and IS*257*L in reverse orientation to that shown in Rouch et al. (25). Restriction endonuclease sites are indicated by B (*Bgl*II), E (*Eco*RI), H (*Hin*dIII), and PII (*Pvu*II).

North American isolates, with that of Tn*4001* from pSK1 (Fig. 1), an equivalent and homologous 2.5-kb *Hin*dIII fragment from pSH6 was cloned into pUC18 to form recombinant plasmid pSK369.

Both pSK330 and pSK369 were digested with a total of nine restriction endonucleases known to contain recognition sequences within the 2.5-kb *Hin*dIII fragment of Tn*4001*. Restriction fragments resulting from digestion of pSK369 were identical in size to those obtained from digestion of pSK330 (data not shown). This indicated that GmTmKm^r mediated by pSH6 is encoded by an *aacA-aphD* gene largely or entirely identical to that carried on Tn*4001* and that this resistance determinant is contained within a 1.9-kb region flanked by portions of IS*256* (Fig. 2).

Nucleotide sequence analysis of repeats flanking the GmTmKm^r determinant on pSH6. To more precisely define the repeats flanking the GmTmKm^r determinant on pSH6, nucleotide sequences extending 1.35 kb to the left (EMBL accession number X53952) and 2.5 kb to the right (EMBL accession number X53951) of the 1.9-kb GmTmKm^r region were obtained. Features of this region of pSH6 and comparison with Tn*4001* are shown in Fig. 2. As predicted from DNA hybridization (15) and restriction mapping, the 2.5-kb *Hin*dIII fragment of pSH6 carries, at each end, portions of IS*256* identical to the IS*256* sequences in the 2.5-kb *Hin*dIII fragment of Tn*4001*. Contiguous with the 2.5-kb *Hin*dIII fragment of pSH6 is a further 127 bp of each IS*256*. In the region sequenced, no other homology with IS*256* was found. Thus, the resistance determinant of pSH6 is flanked by a total of 425 bp of IS*256* identical to the IS*256* sequence of Tn*4001* (Fig. 2). This corresponds to the region encoding the C terminus of the putative IS*256* transposase (5).

To determine whether any other portions of IS*256* were present elsewhere on the GmTmKm^r plasmids studied, *Bgl*II digests of pSH6, pSK41, and pUW3626 were probed with a 323-bp *Hae*III fragment and a 622-bp *Hin*dIII-*Bgl*I fragment derived from the outside and middle regions of IS*256*, respectively (Fig. 2). In pSH6, the GmTmKm^r determinant and flanking 425 bp of IS*256* map within a 3.0-kb *Bgl*II fragment. As expected, the 3.0-kb *Bgl*II fragment of pSH6 hybridized weakly with only the *Hin*dIII-*Bgl*I probe, as did equivalent 3.0-kb *Bgl*II fragments of pSK41 and pUW3626 (data not shown). Both probes failed to hybridize with any other fragments, indicating that the 425 bp of IS*256* flanking the GmTmKm^r determinant is the only IS*256* sequence on these plasmids.

Analysis of the nucleotide sequence surrounding the GmTmKm^r determinant and flanking portions of IS*256* in

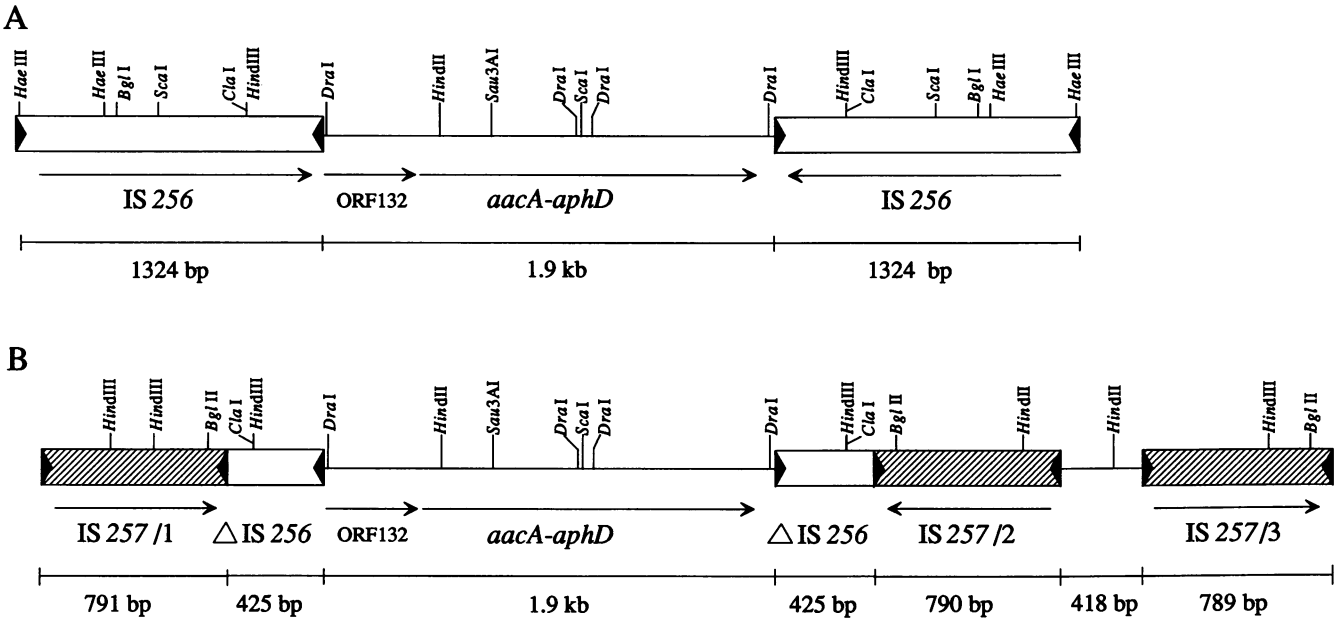

FIG. 2. Comparison of GmTmKm^r transposon Tn*4001* and GmTmKm^r region on pSH6. (A) Structural map of Tn*4001* from pSK1 showing the relative locations of the *aacA-aphD* resistance gene and adjacent upstream ORF (ORF132), which are flanked by inverted copies of IS*256*, indicated as open boxes. (B) Organization of the GmTmKm^r region of pSH6 shown aligned with that of Tn*4001* shown in panel A. Restriction enzyme digestion analysis has indicated that, like Tn*4001*, pSH6 carries the *aacA-aphD* resistance gene and ORF132. Maps of the flanking repeat elements were derived from nucleotide sequence data. The 425-bp truncated IS*256* elements (ΔIS*256*) are shown as open boxes. Three IS*257* elements, IS*257*/1 (791 bp), IS*257*/2 (790 bp), and IS*257*/3 (789 bp), are indicated as shaded boxes. Arrowheads within the insertion sequence regions represent terminal inverted repeats. Arrows below insertion elements represent the location and orientation of potential transposase genes. For clarity, only select *Hin*dII and *Sau*3AI sites are shown. This figure is based on nucleotide sequences deposited at EMBL; accession numbers are X53952 and X53951 for sequences to the left- and right-hand sides, respectively, of the 1.9-kb GmTmKm^r region of pSH6. The nucleotide sequence of Tn*4001* can be obtained from GenBank, accession number M18086.

pSH6 revealed the presence of three repeat sequences between 789 and 791 bp in length. Each of these repeats showed extensive homology with insertion element IS*257*. Inverted copies of the repeated sequence, designated IS*257*/1 and IS*257*/2, flank the GmTmKm^r region of pSH6, immediately adjacent to the truncated IS*256* elements. A third copy of the repeated element, designated IS*257*/3, was found 418 bp to the right of IS*257*/2 and in the same orientation as IS*257*/1 (Fig. 2).

Examination of sequences immediately adjacent to all three IS*257* elements on pSH6 for the presence of duplicated sequences, which are produced in the target DNA by most transposable elements, showed no direct repeats (data not shown).

Analysis of IS*257* elements on pSH6. The IS*257* elements on pSH6 are 789 to 791 bp in length, with terminal inverted repeats of 27 to 29 bp (Fig. 3). The single ORF in both IS*257*/1 and IS*257*/3 potentially encodes a 224-aa polypeptide, while the predicted polypeptide from the ORF in IS*257*/2 is only 221 aa in length (Fig. 4). Like the other sequenced IS*257* elements, both the nucleotide sequences and the predicted polypeptide sequences of the IS*257* elements on pSH6 show significant homology with IS elements and their respective putative transposases from other bacteria (data not shown). These IS elements include members of the IS*15* family, such as IS*15*, IS*15*-Δ, IS*26*, IS*46*, and IS*140*, from gram-negative bacteria, ISS*1* from *S. lactis* (4, 25, 26), IS*240* from *Bacillus thuringiensis* (6), and IS*6100* from *Mycobacterium fortuitum* (20).

Interestingly, the IS*257* elements on pSH6 show a greater sequence variation relative to those elements found elsewhere. For comparison, the nucleotide sequences of IS*257*L from the putative trimethoprim resistance transposon Tn*4003*, carried on pSK1, and IS*257*/1, 2, and 3 from pSH6 are shown aligned in Fig. 3. As noted in the legend to Fig. 1, the map of pSK1 has been redrawn such that IS*257*L is on the right-hand side of Tn*4003* on this map.

At the nucleotide level, IS*257*/1 and IS*257*/3 are 97% homologous to IS*257*L. Most of the differences between IS*257*/1 and IS*257*L, including 16 of the 24 nucleotide substitutions, the 3 insertions, and 1 deletion, are clustered in the region of 610 to 775 bp. Likewise, 21 of the 26 nucleotide substitutions found in IS*257*/3, relative to IS*257*L, are concentrated within a 120-bp region, from nucleotide 220 to 345. A single ORF, potentially encoding a 224-aa protein, is maintained in both elements. Many of the nucleotide sequence differences between IS*257*L, IS*257*/1, and IS*257*/3 do not affect the predicted polypeptide sequence (Fig. 4). This evolutionary trend, in which the predicted polypeptide sequence is maintained in spite of nucleotide sequence variation, lends further support to the proposal that the single large ORF of IS*257*L encodes a functional product, suggested previously to be a transposase (25). If this is the case, IS*257*/1 and IS*257*/3 on pSH6 may also code for functional transposases.

In contrast, IS*257*/2 differs from IS*257*L by more than 11% at the nucleotide level, with a total of 90 nucleotide substitutions, 2 deletions, and 3 insertions relative to IS*257*L (Fig. 3). A single-base-pair deletion at nucleotide 662 creates a shift in the ORF of IS*257*/2 relative to that of IS*257*L, IS*257*/1, and IS*257*/3 (Fig. 4), a result which suggests that IS*257*/2 does not code for a functional product.

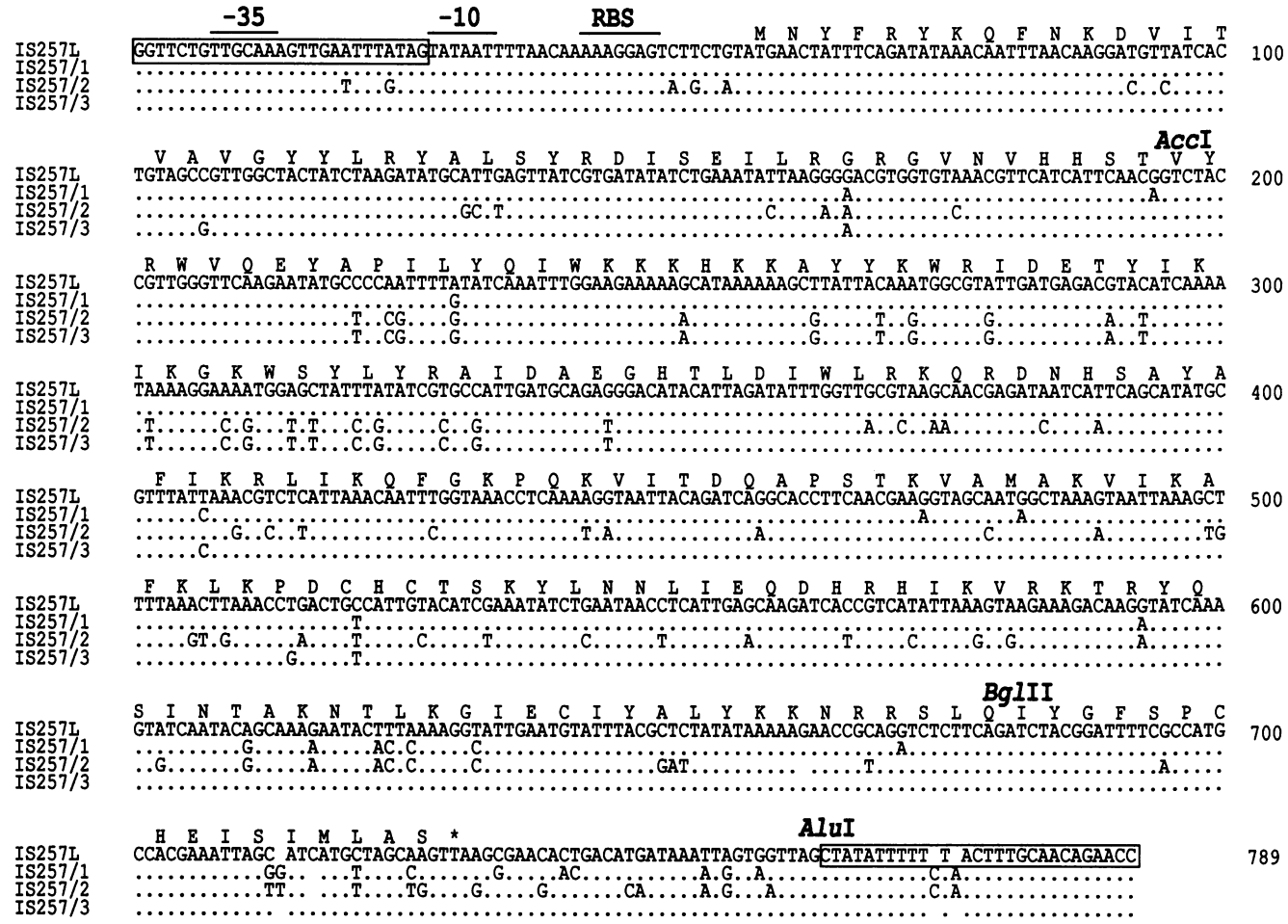

FIG. 3. Comparison of nucleotide sequences of IS*257*L from trimethoprim resistance transposon Tn*4003* from pSK1 (25) and the three IS*257* elements on pSH6. The complete nucleotide sequence of IS*257*L is shown. Above this sequence is the predicted aa sequence of the putative IS*257*L transposase. Bold lines mark the positions of the potential promoter sequences (−35 and −10) and ribosome-binding site (RBS). The 27-bp terminal inverted repeats of IS*257*L are boxed. Identity of IS*257*/1, IS*257*/2, and IS*257*/3 to this sequence is indicated by a dot, with nucleotides shown only where they differ from IS*257*L. Restriction sites used to generate IS*257*-specific probes (see the text and Fig. 5) are also indicated.

The nucleotide sequence variation observed for IS*257*/1, 2, and 3 (Fig. 3) accounts for the restriction enzyme site heterogeneity within these elements and also explains the arrangement of two differently sized adjacent stem-and-loop structures we had previously observed in electron micrographs of self-annealed plasmid DNA (15). The larger stem-and-loop structure, with a stem of approximately 0.7 kb, could form due to base pairing between sequences on the left-hand side of the GmTmKm^r region and sequences on the right-hand side of this region. These sequences would encompass IS*256* and the 3′ end of IS*257*/1 on the left pairing with IS*256* and the 3′ end of IS*257*/2 on the right (Fig. 2B) and include the regions where homology between IS*257*/1 and IS*257*/2 is greatest (Fig. 3). Since IS*257*/2 shares more homology at the 5′ end with IS*257*/3 than with IS*257*/1 (Fig. 3), preferential base pairing between the 5′ regions of IS*257*/2 and IS*257*/3 would form the second, smaller stem-and-loop structure, with a stem of about 0.5 kb.

Mapping of IS*257* elements on North American GmTmKm^r plasmids. The two conjugative plasmids, pSK41 and pUW3626, from North American isolates share some restriction map identity with pSH6 but additionally mediate resistance to neomycin and paromomycin via *aadD* and resistance to ethidium bromide and quaternary ammonium compounds, encoded by *qacD*; plasmid pUW3626 also mediates resistance to penicillin via a β-lactamase (15). Preliminary hybridization studies indicated that these two plasmids contained multiple copies of IS*257*. Furthermore, all IS*257* elements thus far characterized invariably contain an internal *Bgl*II restriction site. Thus, we investigated the possibility that some or all of the nine *Bgl*II restriction sites in pSK41 and pUW3626 were associated with the presence of IS*257*.

*Bgl*II-*Eco*RI double digests of pSK1, pSH6, pSK41, and pUW3626 (Fig. 5A) were probed in separate hybridizations with two IS*257*-specific fragments that were chosen to show both the location and orientation of IS*257* elements. The probes included the 484-bp *Acc*I-*Bgl*II fragment and the 80-bp *Bgl*II-*Alu*I fragment from the left- and right-hand sides of the *Bgl*II site of IS*257*L from Tn*4003* on pSK1, respectively (Fig. 3). For each *Bgl*II or *Bgl*II-*Eco*RI fragment hybridizing with one of the probes, an adjacent *Bgl*II or *Bgl*II-*Eco*RI fragment was predicted to hybridize with the alternate probe. If this hybridization pattern was obtained, it could be concluded that a copy of IS*257* spanned the intervening *Bgl*II site.

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                .         .         .         .         .         .         .         .
IS257L     MNYFRYKQFNKDVITVAVGYYLRYALSYRDISEILRGRGVNVHHSTVYRWVQEYAPILYQIWKKKHKKAYYKWRIDETYI   80
IS257/1    ..................................E.......................................
IS257/2    ..................................E.................V.................V.....
IS257/3    ..................................E.................V.................V.....

                .         .         .         .         .         .         .         .
IS257L     KIKGKWSYLYRAIDAEGHTLDIWLRKQRDNHSAYAFIKRLIKQFGKPQKVITDQAPSTKVAMAKVIKAFKLKPDCHCTSK  160
IS257/1    ..........................................................I..................
IS257/2    ....Q.C........D..........K..HQ.................MI..............I..V.....N......
IS257/3    ....Q.C........D..........................................................

                .         .         .         .         .         .
IS257L     YLNNLIEQDHRHIKVRKTRYQSINTAKNTLKGIECIYALYKKNRRSLQIYGFSPCHEISIMLAS                224
IS257/1    ..........................................................RD....
IS257/2    ....F.................V..............G....TVGLFRSTD.HHATKLV.C
IS257/3    ..........................................................
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FIG. 4. Comparison of predicted aa sequence of the IS*257*L putative transposase (25) with those of IS*257*/1, IS*257*/2, and IS*257*/3 from pSH6. aa are shown only when they differ from IS*257*L; identical aa are indicated by dots.

As expected, the *Acc*I-*Bgl*II and *Bgl*II-*Alu*I probes each hybridized with three fragments of pSK1 (Fig. 5B and C, lane b). These hybridizing bands correspond to the location and orientation of the three IS*257* elements of Tn*4003* on pSK1. Only two restriction fragments of pSH6 hybridized with each probe (Fig. 5B and C, lane c). These fragments could be associated with at least the presence of IS*257*/1 and IS*257*/3. The presence of IS*257*/2 was not directly apparent, since the 3.0-kb *Bgl*II fragment which hybridized with the smaller probe contains the 3′-terminal region of both IS*275*/1 and IS*257*/2. The adjacent 1.9-kb *Bgl*II fragment, which hybridized with the larger probe, encompasses the major portions of IS*257*/2 and IS*257*/3.

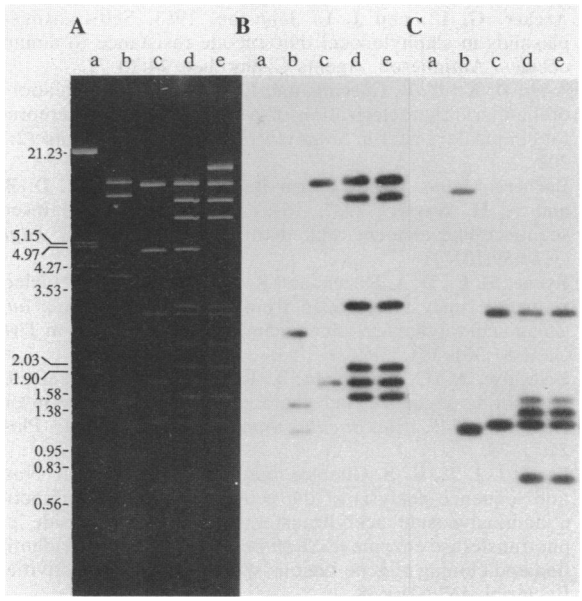

FIG. 5. Determination of the number of IS*257* elements and their relative orientations on pSH6, pSK41, and pUW3626. (A) Agarose gel (1.0% [wt/vol]) electrophoresis of purified plasmid DNA from *S. aureus* cleaved with *Bgl*II and *Eco*RI. Lanes: a, bacteriophage λ DNA cleaved with *Hin*dIII and *Eco*RI (fragment sizes [in kilobases] are shown at the left); b, pSK1; c, pSH6; d, pSK41; e, pUW3626. (B and C) DNA from gel A transferred to nitrocellulose and hybridized with a [α-^{32}P]dATP nick-translated probe consisting of the 484-bp *Acc*I-*Bgl*II fragment of IS*257*L (B) and an 80-bp *Bgl*II-*Alu*I fragment of IS*257*L end labeled with [γ-^{32}P]dATP (C). The 80-bp *Bgl*II-*Alu*I probe hybridized to three pSK1 fragments (C, lane b), one 8.2-kb fragment and two comigrating fragments of 1.2 kb (A, lane b). Hybridization of this probe to a 0.3-kb fragment in pSK41 and pUW3626 (C, lanes d and e) is weak in this figure but was clearly evident on the original autoradiograph.

pSK41 and pUW3626 showed identical hybridization patterns, with six fragments hybridizing with each probe (Fig. 5B and C, lanes d and e). Six of the nine *Bgl*II sites in each of these plasmids could thus be associated with the presence of directly repeated copies of IS*257* (Fig. 1). Another copy of IS*257*, in the opposite orientation, is predicted to flank the right-hand side of the GmTmKm^r determinant in pSK41 and pUW3626, since both of these plasmids share restriction map identity with pSH6 in this region. Restriction mapping and nucleotide sequence analysis indicated that the two remaining *Bgl*II restriction sites in pSK41 and pUW3626 are not associated with IS*257* elements (our unpublished data).

DISCUSSION

Resistance to gentamicin and the related aminoglycosides tobramycin and kanamycin is common in nosocomial strains of staphylococci isolated in hospitals around the world. In Australian isolates, GmTmKm^r is associated with the presence of transposon Tn*4001*, which is most frequently found on pSK1 family plasmids (9, 15, 18) but can also be chromosomally located (9). Tn*4001* has been characterized on heavy-metal resistance plasmids from Australian and West German *S. aureus* isolates (18; J. Evans and K. G. H. Dyke, unpublished data) and most likely occurs on pSK1 family plasmids found in some *S. aureus* isolates from London hospitals (35; C. L. Wright, M. E. Byrne, and R. A. Skurray, unpublished data). Elements similar in size to Tn*4001* and encoding GmTmKm^r via AAC(6′) and APH(2″) aminoglycoside-modifying activities occur on chromosomes of *S. aureus* strains from Dublin (30) and *Staphylococcus epidermidis* strains isolated in North America (34). Furthermore, a gentamicin resistance transposon found on a conjugative plasmid in *Enterococcus faecalis* shares restriction map identity with Tn*4001* (13). The nucleotide sequence of another plasmid-encoded gentamicin resistance element from *E. faecalis* shares nearly complete homology with a region of Tn*4001*. This homology includes the resistance determinant and a portion of IS*256* (7, 24). Thus, it seems likely that Tn*4001* and closely related elements have contributed significantly to the spread GmTmKm^r in clinically significant gram-positive bacteria.

The GmTmKm^r element found on pSH6 consists of two inverted copies of IS*257* flanking a region that appears identical to an internal portion of Tn*4001*. This includes the central resistance determinant and 425 bp of each flanking IS*256*. Given this homology, it is most likely that the GmTmKm^r element on pSH6 and the related conjugative plasmids pSK41 and pUW3626 evolved from a copy of Tn*4001* in which insertion of IS*257* generated a truncated Tn*4001*-IS*257* hybrid structure.

Since IS*256* and IS*257* do not share any significant homology, it is unlikely that the truncated Tn*4001*-IS*257* hybrid structure was created by recombination between these elements. This hybrid structure may have resulted from transposition of IS*257* into each IS*256* element of a copy of Tn*4001*. The sites of insertion into each IS*256* appear to be identical, suggesting that the sequence in this region of IS*256* is a hot spot for IS*257* transposition. Since the outer portions of IS*256* are absent from the plasmids studied here, it is probable that this initial transposition event occurred on an alternative replicon. Transfer of the truncated Tn*4001* and flanking IS*257* elements from their original location to another replicon, possibly a precursor of the GmTmKm^r conjugative plasmids, would have dissociated the truncated Tn*4001*-IS*257* structure from the outer 900 bp of each IS*256*. The latter transfer even could have resulted from IS*257*-mediated transposition, with subsequent deletion of any target sequence duplication, or alternatively may have involved recombination with other preexisting IS*257* elements on the target replicon.

It is interesting that a complete copy of the GmTmKm^r transposon, Tn*4031*, which is found on the chromosomes of several *S. epidermidis* strains from North America (34), appears similar to Tn*4001* and possibly carries two inverted copies of IS*256*. Tn*4031* has been located within an IS*257*-related sequence of a trimethoprim resistance element (34); the latter element appears to share significant restriction map identity with Tn*4003*. Thus, there is more than one instance in which the two unrelated insertion sequences, IS*256* and IS*257*, occur in close association with each other.

The large conjugative plasmids pSK41 and pUW3626 were found to carry seven copies of IS*257*. Multiple copies of this element have also been mapped on the conjugative plasmid pGO1 (33, 34). On pSK41 and pUW3626, both the *aadD* resistance determinant and the *qacD* gene are flanked by direct repeats of IS*257*. Restriction map identity with pGO1 (33) suggests that the region involved in conjugative plasmid transfer of pSK41 and pUW3626 maps between the *aadD* and *qacD* resistance determinants and is also flanked by direct repeats of IS*257* (Fig. 1).

The multiple copies of IS*257* on these plasmids conceivably provide a number of sites for excision or insertion of resistance determinants through homologous recombination. Such recombinational events could easily serve to explain the structural relationship between pSH6 and pSK41 (Fig. 1). Indeed, gene exchange involving recombination between IS*257* elements on conjugative plasmid pGO1 and a derivative of β-lactamase plasmid pI258 has been demonstrated (34).

IS*257* appears to generate an 8-bp duplication of the target sequence during transposition. This is suggested by the following observations. On pSK1, the putative transposon Tn*4003* is flanked by 8-bp direct repeats (25). Furthermore, a copy of the tetracycline resistance plasmid, pT181, which has integrated into the chromosome of an *S. aureus* strain (8, 21), has an 8-bp duplication of pT181 sequence located adjacent to flanking IS*257* elements; it is possible that this duplication resulted from transposition of IS*257* into pT181 prior to integration of this plasmid into the chromosome (Matthews et al., in press). In contrast, examination of the sequences flanking all three IS*257* elements on plasmid pSH6 showed no flanking direct repeats; this result is in agreement with genetic experiments in which we (15) and others (34) have been unable to detect transposition of the GmTmKm^r determinant from conjugative *S. aureus* plasmids to a second site. In addition, analysis of nucleotide sequences adjacent to several IS*257* elements on pSK41 has revealed only one apparent example of target sequence duplication, viz., that associated with the neomycin and paromomycin (*aadD*) resistance determinant (M. E. Byrne, M. T. Gillespie, and R. A. Skurray, unpublished data). Taken together, these observations tend to suggest that IS*257*-mediated transposition has not played a major role in dissemination of genetic elements in *S. aureus*. Rather, IS*257* has probably been involved in homologous recombination events and in this way has most likely contributed to the evolution of multiresistance conjugative plasmids.

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