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Mobility of Gentamicin Resistance Genes from Staphylococci Isolated in the United States: Identification of Tn*4031*, a Gentamicin Resistance Transposon from *Staphylococcus epidermidis*

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Homologous genes encoding resistance to gentamicin, tobramycin, and kanamycin through the bifunctional acetylating [AAC(6′)] and phosphorylating [APH(2″)] aminoglycoside-modifying enzyme were identified in staphylococci isolated from patients in the United States. The mobility of gentamicin resistance (Gm^r) genes found on a prototype conjugative plasmid (pGO1) was compared with that of genes cloned from chromosomal sites. Plasmid-encoded Gm^r genes and flanking sequences were introduced onto a temperature-sensitive plasmid (pRN3208) from pGO1 by homologous recombination between insertion sequence-like elements present on both replicons. Growth of *Staphylococcus aureus* strains containing the temperature-sensitive recombinant (pGO161) at the nonpermissive temperature for plasmid replication (42°C) revealed no translocation of Gm^r from its plasmid location. A transposon (Tn*551*) resident on the same replicon did translocate. Chromosomal Gm^r determinants were cloned, together with the gene for trimethoprim resistance (*dfrA*), from three geographically distinct *S. epidermidis* isolates; two were subcloned onto temperature-sensitive *Escherichia coli-S. aureus* shuttle plasmids as 7.2-kilobase *Bgl*II fragments. Growth of both recombination-deficient and -proficient *S. aureus* strains containing the cloned genes at 42°C allowed detection of transposition of Gm^r sequences and identification of insertion into random chromosomal sites. We have designated this 5-kilobase transposon from *S. epidermidis* as Tn*4031*.

Gentamicin resistance (Gm^r) among staphylococci was first described in the mid-1970s by investigators in Europe and the United States (8, 9, 18, 29, 33). Resistant isolates were soon reported to be the cause of extensive outbreaks and serious infections (7, 9, 16, 34, 40). Subsequent analysis of the biochemical basis of Gm^r disclosed that an aminoglycoside-modifying enzyme having bifunctional acetylating [AAC(6′)] and phosphorylating [APH(2″)] activities mediated resistance in the great majority of isolates and also conferred resistance to kanamycin and tobramycin (24). Genes encoding the modifying-enzyme activity were found to be homologous in all isolates examined (10, 32, 35). Resistance was initially reported to be plasmid mediated, and resistance plasmids were found in both coagulase-negative and coagulase-positive staphylococci (3, 29, 33, 41). However, while a family of structurally similar plasmids, most of which were conjugative, was found to contain the Gm^r genes in staphylococcal isolates from the United States (3, 14, 19, 31), resistance genes in isolates from Europe and Australia were located either on smaller, nonconjugative plasmids or in the chromosome (13, 32, 35, 38). Two transposons (Tn*4001* and Tn*3851*) were identified in Australian *Staphylococcus aureus* isolates that carried Gm^r genes (22, 38). The existence of these mobile elements has been offered as an explanation for the varied genetic location of Gm^r genes in European and Australian isolates (24, 35). In contrast, the similarity of Gm^r plasmids and the absence of chromosomally encoded genes among staphylococci isolated in this country suggested that these resistance genes were not transposable.

The initial goal of the following study was to assess the transposability of a Gm^r gene from a conjugative Gm^r plasmid isolated in the United States (pGO1). However, during the process of determining a detailed restriction map of the Gm^r gene and flanking sequences, a restriction pattern was discovered that was identical to one present on a chromosomal DNA fragment encoding trimethoprim resistance. Chromosomal trimethoprim resistance (Tp^r) genes had been cloned from three wild *S. epidermidis* isolates that were also resistant to gentamicin. As previously reported (11), the chromosomal Tp^r genes and flanking sequences were identical to those associated with a Tp^r gene on pGO1 except for the addition of 5 kilobases (kb) of unknown DNA. We now report that the unknown DNA contains a Gm^r gene that can transpose from its location on the clone to random chromosomal sites in both *rec*$^+$ and *rec* mutant staphylococcal hosts. We also confirm that the Gm^r genes on the conjugative plasmid, pGO1, do not transpose.

MATERIALS AND METHODS

Bacterial strains and plasmids. Shuttle plasmids containing staphylococcal DNA were constructed in *Escherichia coli* SK1592 (21). These plasmids were then introduced into *S. aureus* RN4220 by protoplast transformation. RN4220 was generated by nitrosoguanidine treatment of *S. aureus* RN450 (ATCC 8325-3) until pBR322 sequences were stably maintained (20). Curing experiments to demonstrate transposition were performed in RN4220, the restriction-deficient *S. aureus* RN2677, or the recombination-deficient *S. aureus* RN1030 (27). RN1030 is lysogenized with phage φ11 to allow plasmid introduction via transduction. Transductions were also performed by φ11 lysis of RN450 or RN4220 carrying plasmids of interest into restriction-defective *S. aureus* RN2677 (27).

Plasmid pGO1 was initially obtained from a clinical isolate of *S. aureus* from the Medical College of Virginia. pGO1 is

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conjugative and encodes resistance to gentamicin (Gm^r), trimethoprim (Tp^r), and quaternary ammonium compounds (Qam^r) (36). Plasmid pRN3208 is plasmid pI258 made temperature sensitive for replication. It confers resistance to cadmium (Cd^r), mercury (Mer^r), and penicillin (Pc^r) and carries a copy of Tn*551* (26). pRN3208 was used as a temperature-sensitive delivery vehicle to test for the transposition of Gm^r genes derived from pGO1. A temperature-sensitive *E. coli-S. aureus* shuttle plasmid was created by the ligation of *E. coli* plasmid pBR322 (5) to pEI94ts (28) in the *Cla*I restriction site. The resulting shuttle plasmid (pGO51) expressed resistance to ampicillin (Ap^r) at 37°C in *E. coli* and to erythromycin (Em^r) at 30°C in *S. aureus*. As previously reported (11), *S. epidermidis* chromosomal fragments were originally cloned from each of three *S. epidermidis* isolates in *E. coli* on the positive selection vector pOP203($A2^+$) by selection on trimethoprim. Two of these cloned fragments were then added to pGO51 (using trimethoprim selection in *E. coli*), creating temperature-sensitive delivery vehicles (pGO221 and pGO222) to test for transposition of chromosomal Gm^r determinants. The probe used to detect Gm^r present on the chromosome of cured strains was pGO137, created by cloning the 2.5-kb *Hin*dIII H fragment of pGO1 into the single *Hin*dIII site of pBR322 as previously reported (10). Tp^r determinants were detected by probing Southern blots with pGO18, a plasmid that contains a 500-base-pair, trimethoprim-specific, *Eco*RI-*Hin*dIII fragment isolated from pGO1 cloned onto pBR322 (1).

Media and reagents. Mueller-Hinton agar (BBL Laboratories, Cockeysville, Md.) was used for culture of both SK1592 and RN4220; brain heart infusion agar (Difco Laboratories, Detroit, Mich.) was used for culture of RN1030. *S. aureus* was cultured for plasmid extractions in brain heart infusion broth (Difco), whereas Luria broth (GIBCO Laboratories, Madison, Wis.) was used for *E. coli* plasmid extractions and transformations. Mueller-Hinton broth (BBL Laboratories) was used for susceptibility testing. *S. aureus* was grown for protoplast transformation and transposon curing in Penassay broth (Difco). SMMP, an osmotically stabilized medium for the generation of protoplasts, and DM3, a medium for the generation of wall-competent cells from protoplasts, were prepared as previously described (36). Antibiotic concentrations used were as follows: gentamicin, 5 μg/ml; erythromycin, 20 μg/ml (in Mueller-Hinton agar) and 5 μg/ml (in DM3); trimethoprim, 20 μg/ml; ampicillin, 25 μg/ml; and cadmium, 5×10^{-5} M. Lysostaphin, antibiotics, succinate, and other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Organic solvents and agarose came from International Biotechnologies, Inc., New Haven, Conn. Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Radionuclides and nick translation kits were purchased from DuPont, NEN Research Products, Boston, Mass.

Transduction, filter mating, and protoplast transformation. Once temperature-sensitive delivery vehicles were constructed in *E. coli*, they were introduced into *S. aureus* by a protoplast transformation procedure modified from that of Chang and Cohen (6). Briefly, protoplasts were generated by digestion of logarithmically growing RN4220 with lysostaphin in SMMP to remove cell walls, and then plasmid DNA was added in the presence of polyethylene glycol. After a 2- to 3-h expression time, protoplasts were plated on DM3 agar containing erythromycin (5 μg/ml) to regenerate cell walls. Em^r transformants were shown to have the appropriate introduced plasmid by using the cetyltrimethylammonium bromide extraction method of Townsend et al. (37) followed by restriction endonuclease digestion and agarose gel electrophoresis.

Transduction was performed as previously described (3), using ϕ11 to generate a lysate from susceptible cells with subsequent infection of lysogenized recipients (RN1030 or RN2677) followed by selection on plates containing the proper antimicrobial agents. Plasmid content was again confirmed by cetyltrimethylammonium bromide lysis followed by electrophoresis.

Filter matings were performed as follows. Portions (1 ml) of overnight donor and recipient cultures containing 10^9 CFU were sequentially pelleted, suspended, and placed on nitrocellulose filter membranes on dry brain heart infusion agar plates for 18 h at 30°C. Bacteria were suspended in sterile brain heart infusion broth and plated on Mueller-Hinton agar containing suitable antibiotics as described previously (36).

Plasmid curing. Strains harboring plasmids that were temperature sensitive for replication were grown overnight at 30°C on Mueller-Hinton agar plates containing antimicrobial agents to select for the temperature-sensitive replicon (erythromycin or cadmium). Areas of confluent growth were suspended in sterile 0.9% NaCl to a concentration of 10^8 by using McFarland standards. Suspensions were diluted 10^{-3} in brain heart infusion broth and cultured overnight at 42°C. Dilutions were performed to obtain isolated colonies on Mueller-Hinton agar containing gentamicin. Gm^r colonies were screened for susceptibility to erythromycin or cadmium and then subjected to genomic DNA isolation and hybridization studies.

Cloning and DNA manipulations. All restriction endonuclease digestions, electrophoresis, generation of clones in *E. coli*, *E. coli* plasmid extraction, Southern blotting, labeling of probes, and hybridization experiments were performed by the methods of Maniatis et al. (25), by methods given in directions provided by the manufacturers, or as previously described (11). Southern blots were probed at 42°C under high-stringency conditions (11). *S. aureus* chromosomal DNA was prepared by a modification of the Marmur isolation technique (11). Staphylococcal plasmids were isolated for small-scale restriction digestion by the cetyltrimethylammonium bromide extraction method, while large-scale plasmid isolation was performed by using dye-buoyant density gradient centrifugation (1).

Susceptibility testing. Susceptibilities of *E. coli* and *S. aureus* to gentamicin and tobramycin were assessed by twofold broth microdilution with Mueller-Hinton broth as described previously (3). Susceptibility testing was performed at both 30 and 37°C because the temperature-sensitive shuttle clones (pGO221 and pGO222) replicated optimally at 30°C, whereas *E. coli* constructs and chromosomal transposon insertions could be tested at the standard incubation temperature (37°C).

RESULTS

Translocation of Gm^r genes from pGO1 to pRN3208. In order to investigate the mobility of the Gm^r gene from its location on a conjugative plasmid, it was necessary to transfer the gene and flanking sequences to a plasmid that was temperature sensitive for replication. pGO1 is a representative conjugative staphylococcal plasmid which has been shown by restriction endonuclease mapping and DNA hybridization to be similar to other conjugative staphylococcal plasmids isolated in the United States (2, 14, 19). This similarity is particularly apparent in the region of the plasmid

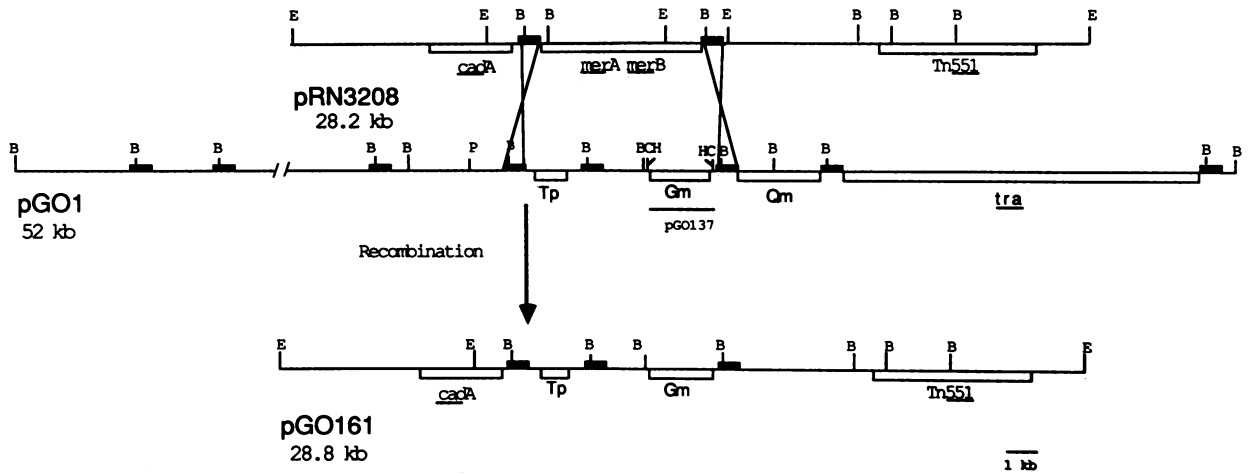

FIG. 1. Recombination between pGO1 and pRN3208 to generate pGO161. Solid lines designate the probable double crossover between IS-like elements (■) that resulted in the pGO161 recombinant. □, Specific plasmid genes. Abbreviations for plasmid determinants are as follows: Gm, gentamicin; Tp, trimethoprim; Mer, mercury; Cd, cadmium; tra, conjugative transfer. Abbreviations for restriction endonuclease cleavage sites are as follows: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. pGO1 has additional *Cla*I and *Hin*dIII cleavage sites that are not shown in the figure.

associated with the Gm^r gene. Transfer of the Gm^r determinant of pGO1 to pRN3208 made use of the presence of homologous, directly repeated insertion sequence (IS)-like elements on both plasmids. Homologous recombination between these elements would be expected to transfer fragments from pGO1 of various sizes, including the Gm^r gene, depending on the site of recombination and resistance selection. The linear map in Fig. 1 shows the locations of these IS-like elements surrounding the gentamicin gene of pGO1 and those repeats present on pRN3208 in relation to known resistance determinants. In addition, pRN3208 contains Tn*551*, an Em^r transposon. Detection of transposition of this element in cells grown at temperatures nonpermissive for plasmid replication serves as a positive control for transposition of a potentially mobile genetic element from the same plasmid.

pGO1 was introduced into *S. aureus* RN450 containing pRN3208 by filter mating at 30°C. A transducing lysate was then made from the strain containing both plasmids by using φ11, and transduction was performed into φ11-lysogenized recipient strain RN2677 with selection for cadmium resistance (Cd^r), a pRN3208 marker, and Gm^r. Since pRN3208 is smaller (28.2 kb) than pGO1 (52 kb), any Gm^r Cd^r cotransductants would be more likely to contain pRN3208 plus pGO1 DNA containing the Gm^r determinant than they would to contain pGO1 plus pRN3208 DNA or both plasmids together. The multiplicity of infection was adjusted so that each recipient cell would be infected by a single phage particle. Two Gm^r Cd^r cotransductants were identified, and the recombinant plasmids were mapped by restriction endonuclease digestion. Both were found to be a result of identical recombination events, as depicted in Fig. 1. The recombinant plasmid was designated pGO161. A 6.6-kb fragment of pRN3208 containing the genes for mercury resistance bounded by directly repeated copies of IS*257* was deleted and replaced by a 7.2-kb fragment of pGO1, also bounded by homologous directly repeated IS-like elements, containing the genes for Gm^r and Tp^r.

Transposition of resistance genes from pGO161. In order to demonstrate the potential mobility of the Gm^r determinant of pGO1, *S. aureus* RN2677 harboring pGO161 was grown at the nonpermissive temperature for plasmid replication (42°C) and plated on agar containing gentamicin or erythromycin. Of 200 Gm^r colonies, none had lost other plasmid markers (Em^r, Tp^r, or Cd^r). In contrast, from 50 to 80% of Em^r colonies had lost all other plasmid markers, indicating transposition of Tn*551*. Selection for each plasmid marker except Em^r always yielded colonies that were Gm^r Cd^r Tp^r Em^r, indicating the failure of any other marker to transpose at a detectable frequency. Examination by gel electrophoresis of cured colonies that still retained resistance to all four antimicrobial agents revealed that almost all were devoid of plasmid DNA. This suggested that the plasmid was still effectively temperature sensitive for replication but that the entire plasmid had apparently integrated into the chromosome. Thus, within the limits of this system there was no evidence that either the Gm^r or Tp^r determinant from pGO1 could move as an individual transposable element.

Cloning chromosomal Gm^r genes. As reported earlier (11), three Tp^r clones were obtained in *E. coli* on the positive selection vector, pOP203(A2$^+$). The DNA had been cloned from the chromosomes of three clinical Tp^r Gm^r (gentamicin MIC, >6.3 μg/ml for all three isolates) *S. epidermidis* strains by selection on trimethoprim. All three isolates contained a 7.2-kb *Bgl*II fragment with restriction sites in locations similar to those of the 2.2-kb Tp^r determinant and flanking sequences found on pGO1. However, they also contained approximately 5 kb of unknown additional DNA. Two clones were identical, while the third contained the 5 kb of unknown DNA in a different location relative to the DNA associated with Tp^r (11) (Fig. 2). All three contained *Cla*I-*Hin*dIII restriction sites within the unknown DNA that were in the same position as those seen around the Gm^r gene of pGO1. A 2.5-kb *Hin*dIII probe derived from the Gm^r gene of pGO1 hybridized with a similarly sized *Hin*dIII fragment contained within the unknown 5 kb of DNA on each clone (data not shown). We therefore concluded that the undefined DNA contained genes homologous to those mediating Gm^r on pGO1.

The 7.2-kb *Bgl*II fragments (Fig. 2) representing the two unique insertions of the unidentified 5 kb of DNA were subcloned into the single *Bam*HI site of pGO51 (*E. coli-S. aureus* shuttle plasmid temperature sensitive for replication in *S. aureus*) and transformed into *E. coli* by selection for

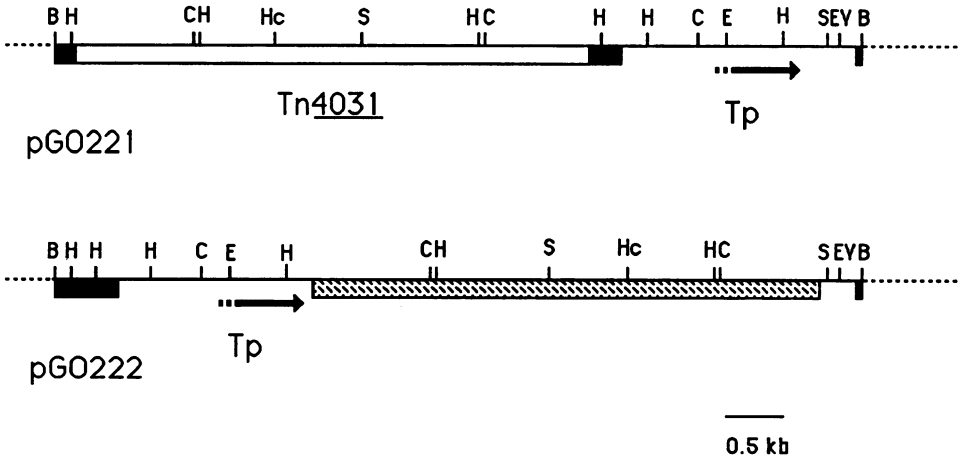

FIG. 2. Physical and genetic maps of pGO221 and pGO222. Open bar, Tn*4031* DNA; hatched bar, Tn*4031*-like DNA; solid bars, sequences of IS-like elements homologous to IS*431* (4). The Tp^r determinant and its direction of transcription are indicated by the arrow. Dotted lines flanking the 7.2-kb *Bgl*II fragments indicate vector (pGO51) sequences. Abbreviations are the same as for Fig. 1 except for the following restriction endonuclease sites: EV, *Eco*RV; Hc, *Hinc*II; S, *Sca*I.

Tp^r. The resulting shuttle plasmids (pGO221 and pGO222) were introduced into RN4220 by protoplast transformation at 30°C, using selection for Em^r. These transformants were also resistant to trimethoprim and gentamicin. The Gm^r phenotype in both *E. coli* and *S. aureus* was confirmed by susceptibility testing (see below).

Transposition of gentamicin resistance from pGO221 and pGO222. Once the Gm^r phenotype of the 7.2-kb cloned fragments had been demonstrated in *S. aureus*, experiments were undertaken to investigate the mobility of the Gm^r determinant. Preliminary curing experiments were performed in the recombination-proficient host, RN4220. Independent sites of insertion were identified in eight strains (four cured of pGO221 and four cured of pGO222) by restriction analysis and hybridization studies. After demonstration of transposition into unique chromosomal locations in RN4220, pGO221 and pGO222 were introduced into the recombination-defective host, RN1030, by transduction. Chromosomal DNA was prepared from two independently cured Gm^r and Em^s colonies that originally contained pGO221 and from four Gm^r- and Em^s-cured colonies that contained pGO222. These chromosomal DNA samples were then digested with restriction endonuclease *Eco*RI, electrophoresed in 1.0% agarose gels, and transferred to nitrocellulose paper by Southern transfer. The blot was then probed with ^{32}P-labeled pGO137, the Gm^r probe obtained from pGO1 (Fig. 1). Since there are no *Eco*RI restriction sites within the Gm^r element, independent insertion should generate *Eco*RI fragments of different size. The results of autoradiography performed after probing with ^{32}P-labeled pGO137 are shown in Fig. 3. The presence of hybridization signals of different size in each lane containing chromosomal DNA indicated the insertion of Gm^r transposons into independent sites of the chromosomes of these isolates. Independent insertion sites were confirmed in a similar fashion by using restriction endonucleases *Bgl*II and *Xba*I to digest chromosomal DNA from the same strains. To confirm that Gm^r transposed in the absence of Tp^r mobility, the Southern blot shown in Fig. 3 was boiled to remove the Gm^r probe and rehybridized with ^{32}P-labeled pGO18, a plasmid containing the Tp^r structural gene (1). Cured isolates did not demonstrate hybridization (data not shown). These data indicate that Gm^r genes transposed from plasmid loci to independent chromosomal locations.

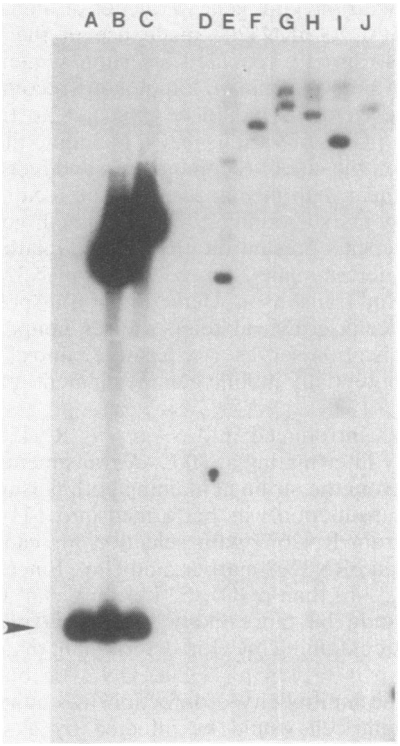

FIG. 3. Demonstration of Tn*4031* transposition. Plasmid and chromosomal DNA was digested with restriction endonuclease *Hin*dIII or *Eco*RI, respectively, electrophoresed in 1.0% agarose gels, transferred to nitrocellulose, probed with ^{32}P-labeled pGO137, and subjected to autoradiography. Lanes A, B, and C contain pGO1, pGO221, and pGO222 plasmid DNA, respectively, digested with *Hin*dIII as positive controls. The presence of 2.5-kb hybridization signals (arrowhead) demonstrated the homologous Gm^r genes. Lane D contains chromosomal DNA from RN1030 as a negative control. The remaining six lanes contain chromosomal DNA prepared from RN1030 cured of pGO221 (lanes E and F) or pGO222 (lanes G, H, I, and J) and digested with restriction endonuclease *Eco*RI. Single hybridization signals of various sizes indicated Tn*4031* insertion into unique chromosomal locations. Fainter hybridization signals in chromosomal lanes were due to incomplete restriction digestion.

Resistance phenotype of Tn*4031*. MICs of gentamicin and tobramycin were determined for *E. coli* and *S. aureus* strains harboring Tn*4031* in both plasmid and chromosomal locations. The Gm^r genes on pGO1 have been shown to mediate resistance to gentamicin, tobramycin, and kanamycin with a substrate profile suggesting that the gene encodes the AAC(6′)/APH(2″) bifunctional enzyme (2). The Gm^r determinants of pGO221 and pGO222 expressed resistance in *E. coli* (MIC of gentamicin, 6.3 μg/ml [both plasmids]; MICs of tobramycin, 6.3 μg/ml [pGO221] and 25 μg/ml [pGO222]). There was no difference in expression of pGO221 versus pGO222 when either was present in RN4220 (MICs of gentamicin and tobramycin, 25 μg/ml). Tn*4031* conferred resistance to gentamicin and tobramycin (MICs, 25 μg/ml) in seven of eight *S. aureus* chromosomal insertions. Interestingly, one of the strains with an independent insertion of Tn*4031* into an RN4220 chromosomal locus was significantly more resistant to gentamicin (MIC, 100 μg/ml) and tobramycin (MIC, 125 μg/ml) than were the other seven strains with chromosomal insertions.

DISCUSSION

In the United States, gentamicin-resistant staphylococci have been isolated both during outbreak investigations and as a result of surveillance studies. Virtually all isolates were resistant to gentamicin, tobramycin, and kanamycin by virtue of a gene that encoded a bifunctional AAC(6′)/APH(2″) aminoglycoside-modifying enzyme (2, 7, 15, 19, 40). This resistance was plasmid mediated, and the plasmids were usually large conjugative replicons that were closely related by restriction enzyme mapping and DNA hybridization (2, 3, 14, 19, 31). In contrast, *S. aureus* isolates identified in studies from Australia, Ireland, and France contained Gm^r genes that encoded a variety of different aminoglycoside-modifying enzymes, were found in both plasmid and chromosomal locations, and were not associated with conjugative replicons (13, 32, 35). In addition, Gm^r *S. aureus* strains isolated in both Australia and Ireland were found to contain genes encoding the bifunctional AAC(6′)/APH(2″) enzyme carried on a transposable element (30, 35). Genes on conjugative plasmids in staphylococcal isolates from multiple geographic locations in the United States were thought not to be on transposons, however, because they were found in the same relative genetic locations, with identical flanking sequences. Furthermore, electron microscopy of homoduplexes showed the stem of the stem-loop structure from the Australian transposable element (Tn*4001*) to be longer than the stem formed by DNA flanking the Gm^r gene on representative plasmids from the United States (22). This suggested that the inverted repeats of the latter elements were truncated or degenerate. Restriction endonuclease cleavage site mapping of pGO1 revealed the presence of a copy of a repeated sequence element homologous (by Southern hybridization) to IS*431* in close proximity to the Gm^r gene of pGO1 (Fig. 1). This repeated element is located within 250 base pairs of the *Hin*dIII site defining the right end of the gentamicin gene of pGO1, while the Tn*4031* element extends nearly 1 kb beyond this site. Perhaps the insertion of the IS-like element so close to the Gm^r gene of pGO1 interrupted sequences crucial to transposition which remain intact on Tn*4031*. The data presented here offer additional functional genetic evidence that the Gm^r gene on a representative conjugative staphylococcal plasmid, pGO1, is not transposable at a detectable frequency.

Although the Gm^r gene on pGO1 did not appear to be mobile, we identified a transposable element containing a Gm^r gene homologous with that on pGO1 in the chromosomes of three *S. epidermidis* isolates. The three isolates were distinct as judged by plasmid pattern analysis, origin (two of the three were isolated in different cities), and chronology (each was isolated in a different year) (11). However, the elements all appeared to be indistinguishable on the basis of size and restriction endonuclease cleavage sites. The element present on pGO221 has been designated Tn*4031*, while the other two elements should be considered to be Tn*4031*-like. These elements appear to be similar to the well-characterized gentamicin resistance transposon in Australian *S. aureus* isolates, Tn*4001*. Both Tn*4001* and Tn*4031* are similar in size (4.7 to 5 kb), both encode the bifunctional AAC(6′)/APH(2″) enzyme, and both contain a 2.5-kb internal *Hin*dIII fragment as well as *Sca*I and *Hin*cII sites within the Gm^r gene (30). Since the *Hin*dIII sites in Tn*4001* are in IS-like elements (IS*256*) that bound the *aacA-aphD* gene, forming a composite transposon, sequences similar to IS*256* may bound Tn*4031* as well (23). Tn*4031* contains *Cla*I restriction sites very close to the *Hin*dIII sites that have not been noted in Tn*4001*, and the species of origin was *S. epidermidis* for all Tn*4031*-like transposons, as opposed to the *S. aureus* origin for all Tn*4001*-like elements. Definitive similarities and differences between the two transposons can probably not be determined until sequence comparisons are made.

It is interesting that in all three cases the Gm^r determinants cloned from *S. epidermidis* were associated with a 2.3-kb region of DNA identical to a region on pGO1 by restriction mapping and DNA hybridization (11). This DNA contained the gene for Tp^r, DNA sequences flanking the Tp^r gene, and two directly repeated IS-like elements that are probably the same as elements designated either IS*431* or IS*257* (4, 12). This association was not an artifact of selection, since the DNA was originally obtained by cloning the gene for Tp^r; the Gm^r gene and transposon were discovered fortuitously as unidentified DNA within the Tp^r gene complex. Plasmid-borne copies of Tn*4001* in Australian isolates (24) and the nontransposable Gm^r gene on pGO1 (Fig. 1) also map adjacent to the Tp^r gene complex and the IS-like sequences. The preferred insertion of Tn*4031* into the Tp^r complex and IS-like DNA could be because of the presence of hot spots for transposition in this area. Hot spots for transposon insertion have been described for *E. coli* (17).

An evolutionary scheme for the mobility of AAC(6′)/APH(2″) Gm^r genes can be offered on the basis of current data. The original resistance gene may have become mobile when the fortuitous insertion of flanking IS elements produced a composite transposon, as proposed by Lyon et al. (23). In those countries in which conjugative plasmids have not become widespread, mobility of Gm^r genes continues to be by transposition to plasmids that are transferred inefficiently from cell to cell by mechanisms that probably involve phages (39). However, in countries like the United States, in which conjugative plasmids have become widespread in staphylococci of all species, the presence of Gm^r genes on an easily transmissible replicon may substantially lower the selection pressure for further dissemination through transposition. Thus, Gm^r genes on conjugative plasmids that no longer needed to transpose to spread to other cells evolved. Nevertheless, it is likely that transposable Gm^r elements have always been present in Gm^r staphylococci in the United States, particularly in coagulase-negative staphylococci, but their presence has been obscured by the widespread dissemination of conjugative plasmids carrying the nontransposable gene. This hypothesis is supported by our recent examina-

tion of 10 randomly chosen Gm^r *S. epidermidis* and 10 Gm^r *S. haemolyticus* isolates from our clinical microbiology laboratory. All of the 20 isolates hybridized with the *aacA-aphD* gene probe from pGO1; 8 of them (5 *S. epidermidis* and 3 *S. haemolyticus*) carried the gene in a chromosomal location (10).

IS-like elements may also play a role in translocation of antimicrobial resistance genes in staphylococci apart from their presence in composite transposons. In this study, we demonstrated that these elements could serve as areas of homology for the recombinational translocation of genes encoding Gm^r and Tp^r from pGO1 to pRN3208 while effecting the deletion of resident pRN3208 genes encoding mercury resistance (Fig. 1). Other staphylococcal antimicrobial resistance genes shown to be associated with similar IS-like elements include those encoding resistance to methicillin, tetracycline, and quaternary ammonium compounds (4, 12). Since there is no evidence that these IS-like elements retain a capacity for independent mobility (4), their major current role may be to provide points for homologous recombination. The presence of eight copies of IS-like sequences on pGO1 suggests that many of the sequences on conjugative plasmids, including genes for conjugative transfer themselves, have been assembled by recombination between various disparate replicons.

The identification of a transposon encoding Gm^r able to insert into targets in coagulase-positive staphylococci should offer another tool for transposon mutagenesis in this species. However, well-described elements that are effective for mutagenesis in *S. aureus* (i.e., Tn*551*, Tn*917*, and Tn*916*) may not transpose and insert randomly in coagulase-negative staphylococci. Since Tn*4031* originated in *S. epidermidis*, it would be an attractive candidate for use in mutagenesis studies in this species, providing preferential insertional sites do not limit its range. Future studies should answer this question.

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LITERATURE CITED

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