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Identification and Cloning of the Conjugative Transfer Region of *Staphylococcus aureus* Plasmid pGO1

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The conjugative transfer (*tra*) genes of a 52-kilobase (kb) staphylococcal plasmid, pGO1, were localized by deletion analysis and transposon insertional inactivation. All transfer-defective (Tra^-) deletions and Tn*551* or Tn*917* transposon insertions occurred within a 14.5-kb *BglII* fragment. Deletions and insertions outside this fragment all left the plasmid transfer proficient (Tra^+). The *tra* region was found to be flanked by directly repeated DNA sequences, approximately 900 base pairs in length, at either end. Clones containing the 14.5-kb *BglII* fragment (pGO200) and subclones from this fragment were constructed in *Escherichia coli* on shuttle plasmids and introduced into *Staphylococcus aureus* protoplasts. Protoplasts could not be transformed with pGO200E (pGO200 on the staphylococcal replicon, pE194) or subclones containing DNA at one end of the *tra* fragment unless pGO1 or specific cloned *tra* DNA fragments were present in the recipient cell. However, once stabilized by sequences present on a second replicon, each *tra* fragment could be successfully introduced alone into other plasmid-free *S. aureus* recipients by conjugative mobilization or transduction. In this manner, two clones containing overlapping fragments comprising the entire 14.5-kb *BglII* fragment were shown to complement each other. The low-frequency transfer resulted in transconjugants containing one clone intact, deletions of that clone, and recombinants of the two clones. The resulting recombinant plasmid (pGO220), which regenerated the *tra* region intact on a single replicon, transferred at frequencies comparable to those of pGO1. Thus, all the genes necessary and sufficient for conjugative transfer of pGO1 are contained within a 14.5-kb region of DNA.

Large, 40- to 60-kilobase (kb) conjugative plasmids have been found in both *Staphylococcus aureus* and *S. epidermidis* (3, 11, 24). Conjugative transfer of these plasmids has the following characteristics: transfer occurs at a low frequency (10^{-5} to 10^{-7} progeny per donor input cell); aggregation of mating pairs is not mediated by surface structures or pheromones; mating does not take place in broth but requires a solid substrate (e.g., nitrocellulose or cellulose acetate); plasmid transfer can occur between *S. aureus* and *S. epidermidis* (2, 11); and genes required for transfer are plasmid encoded (24). Antimicrobial agent resistance genes are transferred either as part of the conjugative replicon (resistance to tobramycin, kanamycin, and gentamicin [Gm^r]; ethidium bromide and quaternary ammonium compounds [Qam^r]; trimethoprim [Tp^r]; and penicillin [Pc^r]) (3, 14); or are resident on smaller, nonconjugative plasmids that are mobilized by conjugative plasmids (resistance to chloramphenicol [Cm^r] and tetracycline [Tc^r]) (11). Conjugative staphylococcal plasmids from several geographic areas have considerable restriction fragment similarity, particularly in the putative *tra* regions (14). Outbreaks of staphylococcal infections caused by Gm^r organisms carrying conjugative plasmids have been described by several investigators, and isolates carrying these plasmids seem to have become endemic at some large teaching hospitals (2, 19).

The 52-kb conjugative plasmid pGO1, originally resident in a clinical *S. aureus* isolate at the Medical College of Virginia Hospital, has all of the transfer characteristics summarized above, encodes Gm^r, Tp^r, and Qam^r, and has both restriction fragment similarity and DNA hybridization homology to conjugative plasmids isolated from our own hospital (2) as well as to plasmids from the University of Michigan (pAM899-1) and Creighton University (pCRG1600) (G. Archer, unpublished observations). Thus, this plasmid serves as a model for the study of conjugative staphylococcal plasmid *tra* genes. As a first step toward a detailed genetic and functional analysis of the *tra* region of these plasmids, in this report we describe a determination of the physical and functional boundary of the genes encoding transfer functions on pGO1. In addition, *tra* insertion and deletion mutants and subclones of the *tra* region on *S. aureus-Escherichia coli* shuttle vectors were constructed to aid in future studies of the molecular basis for the conjugative transfer of antimicrobial resistance in these medically important bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* and *E. coli* strains and plasmids are listed in Table 1. Recombinant plasmids were constructed in *E. coli* HB101 or SK1592 (7, 22). Recombinant shuttle plasmids constructed in *E. coli* were introduced into *S. aureus* RN4220. This strain was produced by nitrosoguanidine treatment of ATCC 8325-4 (RN450) until pBR322 sequences were stably maintained (21). Nomenclature for clones containing pGO1 DNA is as follows. The initial clones selected in *E. coli* that were on *E. coli* cloning vectors were given pGO numbers. When either pE194 or pSK265 staphylococcal replicons were added to the *E. coli* vectors to produce shuttle plasmids the letter E or C was added to the end of the *E. coli* clone number (i.e., pGO200 became pGO200E when pE194 was inserted into pBR322).

Materials and media. Mueller-Hinton agar (MHA; BBL Microbiology Systems, Cockeysville, Md.) was used for culture of both *E. coli* and *S. aureus* strains; the exception was RN1030, which was propagated on brain heart infusion agar (BHIA; Difco Laboratories, Detroit, Mich.). *S. aureus*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Chromosomal genotype or phenotype	Remarks and references
E. coli		
SK1592	*hsdR4 gal endA*	Restriction-deficient host for recombinant plasmids (22)
HB101	F⁻ *hsdS20* (r_B^- m_B^-) *recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-15 mtl-1 supE44* λ⁻	Recombination-defective host (7)
S. aureus		
RN450		ATCC 8325-4
RN451		Transduction recipient (φ11 lysogen of ATCC 8325-4) (26)
RN4220		Host for shuttle plasmids (21)
GO57	Nov^r Rif^r	Recipient strain (RN4220) for conjugation (12)
GO160	Gm^r	Recipient strain (RN4220) for conjugation with Tn*4031* integrated into the chromosome (W. Thomas, submitted for publication)
RN2677	Nov^r Rif^r, restriction deficient	Lysogenized recipient strain for conjugation and transduction (26)
RN1030	*recA*	Recombination defective φ11 lysogen (26)
Plasmids		
pGO1[b]	Gm^r Tp^r Qam^r Tra$^+$, 52 kb	*S. aureus* clinical isolate from Medical College of Virginia (1)
pTVts[b]	Cm^r Em^r, 12.4 kb	Tn*917 ts* delivery vehicle (30)
pRN3208[b]	Pc^r Cd^r Em^r Mer^r, 28.2 kb	Tn*551 ts* delivery vehicle (27)
pBR322[c]	Ap^r Tc^r, 4.3 kb	Cloning vector (6)
pOP203(A2$^+$)[c]	Tc^r, 7.0 kb	Positive selection cloning vector (29)
pE194[b]	Em^r, 3.8 kb	17
pSK265[b]	Cm^r, 2.7 kb	pC194 with multiple cloning site of pUC19 (20)
pGO23[c]	Ap^r Tc^s, 6.2 kb	IS-like element associated with Tp^r from pGO1 cloned on pBR322 (12)
pGO53[c]	Tc^r, 21.5 kb	14.5-kb *Bgl*II B fragment of pGO1 cloned on pOP203 (A2$^+$) (this study)
pGO132[c]	Tc^r, 11.5 kb	4.6-kb *Eco*RI E fragment of pGO1 that contains DNA flanking the *tra* region cloned on pOP203A_2^+ (this study)
pGO200[c]	Ap^r Tc^s, 18.8 kb	*Bgl*II B fragment of pGO1 subcloned into the *Bam*HI site of pBR322 (this study)
pGO200EΔ1[b,c]	Ap^r Tc^s Em^r, 12.4 kb	Deletion derivative of pGO200E (this study)
pGO201[c]	Ap^r Tc^r, 10.4 kb	6.1-kb *Eco*RI C fragment of pGO1 cloned into the *Eco*RI site of pBR322 (this study)
pGO202[c]	Ap^r Tc^s, 10.1 kb	7.0-kb *Bgl*II-*Ava*I subclone of pGO200 produced by *Ava*I digestion of pGO200 followed by religation (this study)
pGO203[c]	Ap^r, Tc^s, 13.7 kb	9.4-kb *Hin*dIII-*Bgl*II subclone of pGO200 produced by *Hin*dIII digestion of pGO200 followed by religation (this study)
pGO210[c]	Ap^r, Tc^s, 8.8 kb	4.5-kb *Hin*dIII E fragment of pGO1 cloned on pBR322 (this study)
pGO220[b]	Em^r *tra*$^+$, 21.6 kb	Recombinant resulting from transfer of Em^r into GO57 from donor with pGO202E and pGO203C (this study)

[a] Abbreviations not used in the text: Ap, ampicillin; Mer, mercury; Nov, novobiocin; Rif, rifampin. The nomenclature for shuttle constructions was simplified by the addition of E or C to the original plasmid designation after subsequent addition of pE194 or pSK265 to the *E. coli* clones.
[b] *S. aureus* host.
[c] *E. coli* host.

was cultured for plasmid extractions in brain heart infusion broth, whereas *E. coli* was grown in Luria broth (GIBCO Diagnostics, Madison, Wis.) supplemented with 0.2% glucose. *S. aureus* was grown for protoplast transformation and transposon curing in Penassay broth (PAB; Difco). SMMP, an osmotically stabilized liquid medium for the generation of protoplasts, and DM3 agar, for the regeneration of cell wall competent cells from protoplasts, were prepared as previously described and contained the following: SMMP (1 M sucrose, 40 mM $MgCl_2$, 40 mM maleic acid, and 7% PAB); DM3 (0.5 M succinate, 20 mM $MgCl_2$, 0.5% glucose, 0.5% yeast extract [Difco], 0.5% Casamino Acids [Difco], 0.05% bovine serum albumin, and 0.8% agar) (8). Antibiotic concentrations used for selection were as follows: gentamicin (5 μg/ml) and erythromycin (20 μg/ml) in MHA for *S. aureus*; erythromycin (5 μg/ml) in DM3; erythromycin (0.5 mg/ml) in MHA for *E. coli*; chloramphenicol (20 μg/ml) in MHA for *S. aureus*; chloramphenicol (5 μg/ml) in DM3; chloramphenicol (50 μg/ml) in MHA for *E. coli*; novobiocin (5 μg/ml), rifampin (5 μg/ml), ampicillin (30 μg/ml), tetracycline (20 μg/ml), and Cd (5×10^{-5} M) in MHA and BHIA. Lysostaphin, antibiotics, succinate, and all other chemicals used in these experiments were obtained from Sigma Chemical Co., St. Louis, Mo. Organic solvents, agarose, and acrylamide reagents were obtained from International Biotechnologies, Inc., New Haven, Conn. All restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Radionuclides and labeling kits were obtained from New England Nuclear Corp., Boston, Mass. Molecular weight markers were obtained from Bethesda Research Laboratories, Bethesda, Md.

Transductions. *S. aureus* transducing phage φ11 was used to lyse RN450 containing pGO1. Phage lysates then were used to infect a φ11 lysogenized recipient, and cells were plated on either gentamicin or trimethoprim as described previously (3). Since the genome size of φ11 is approximately 45 kb, deleted versions of pGO1 (52 kb) could be selected, due to their smaller size, by their ability to be packaged. Transductants were screened for deletions by the loss of Gm^r or Tp^r as well as by electrophoresis of crude lysates of individual colonies through agarose to identify plasmids that were smaller than the parent replicon. Shuttle

plasmids in RN4220 were transduced into RN451, with selection for Em^r or Cm^r, followed by confirmation by restriction digestion and electrophoresis (see below).

Filter matings. Overnight BHIA cultures of donor and recipient strains were scraped from a plate and suspended in saline to a density of 10^8 CFU. Equal volumes (typically 1 ml) of donor and recipient suspensions were pelleted sequentially in sterile microfuge tubes. The pellets were suspended in 0.1 ml and dropped onto nitrocellulose membranes that had been placed on dry MHA plates. Matings took place on the membranes for either 6 or 18 h at 37°C, or 30°C for matings involving temperature-sensitive (*ts*) replicons. Cells were vortexed off the filters in 1 ml of saline and plated on MHA plates containing appropriate selective antibiotics.

Transposon mutagenesis. The temperature-sensitive plasmids pRN3208 and pTV1ts were used to deliver the transposons Tn*551* and Tn*917*, respectively, to pGO1 (27, 30). Tn*551* mutagenesis of pGO1 was done in *S. aureus* RN450, whereas Tn*917* insertions were generated in recombination-deficient *S. aureus* RN1030 (26). pRN3208 was introduced into RN450 by protoplast transformation, whereas pTV1ts was transduced into φ11-lysogenized RN1030 by φ11 transduction. pGO1 was introduced into both of these strains by filter mating. Overnight colonies propagated at 30°C were suspended in saline to a density of 10^8 CFU, diluted 1:1,000 in PAB, and incubated at 42°C for 16 h. Dilutions were made such that isolated colonies could be obtained on plates containing erythromycin. Cured cultures also were plated on cadmium or chloramphenicol to test for the loss of the pRN3208 or pTV1Ts delivery vehicle, respectively. Successful curings yielded 10^2 more CFU on plates containing erythromycin than on plates containing cadmium or chloramphenicol. Individual Em^r colonies that were sensitive to the vehicle markers were used as donors in filter matings with strain GO57 as the recipient. Cured colonies (Em^rCd^s and Em^rCm^s) were used as donors directly without plasmid extraction and transformation to enrich for plasmid insertions.

Cloning, transformation, and DNA manipulation. All restriction endonuclease digestions, electrophoresis, generation of clones in *E. coli*, *E. coli* plasmid extraction, Southern blotting, and hybridization experiments were performed as described by Maniatis et al. (23) or according to directions provided by the manufacturers. Southern blots were probed at 42°C under the following stringency conditions: low stringency, 25% formamide and 0.9 M NaCl; high stringency, 50% formamide and 0.9 M NaCl. Crude lysates for the small-scale production of *S. aureus* plasmids were produced by low-salt lysis followed by centrifugation as previously described (4). Staphylococcal plasmids were isolated for small-scale restriction digestion by the cetyltrimethylammonium bromide extraction method of Townsend et al. (28), and large-scale plasmid isolation was performed by using dye-buoyant density gradient centrifugation as previously described (12). Recombinant shuttle plasmids were introduced into *S. aureus* RN4220 by a modified version of the protoplast transformation procedure of Chang and Cohen (8). Briefly, protoplasts were formed by the gentle digestion of log-phase cells for 5 h with lysostaphin (0.4 mg/ml) in SMMP broth. Protoplasts were harvested by centrifugation at 15,000 × *g* and suspended in fresh SMMP, and plasmid DNA was added. Polyethylene glycol was added for not more than 2 min and then diluted with SMMP. Protoplasts were harvested again by 15,000 × *g* centrifugation, suspended in SMMP, and allowed to express at 32°C for 3 to 4 h before they were plated on DM3 agar containing erythromycin and/or chloramphenicol.

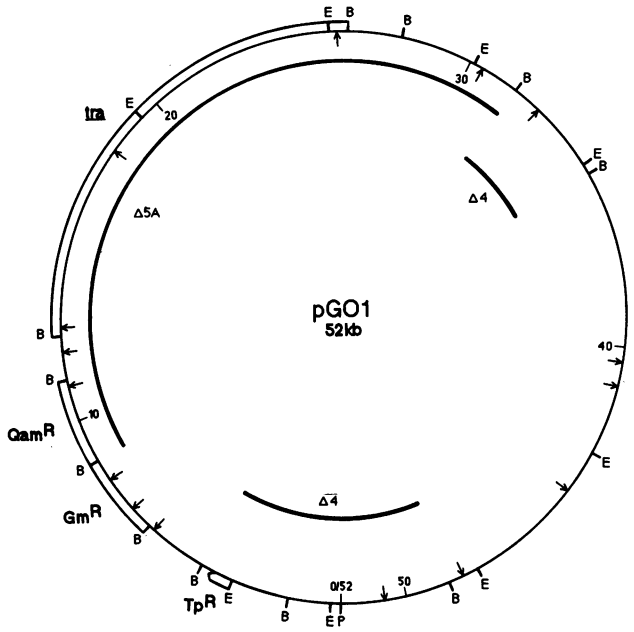

FIG. 1. Physical and functional map of plasmid pGO1. Map coordinates in kilobases are assigned by using the single *Pst*I site (P) as the origin. Restriction endonuclease cleavage sites for *Eco*RI (E) and *Bgl*II (B) are also shown. Phenotypic designations (depicted by open boxes) are as described in the text. Sequences lost from pGO1 in the transduction deletion derivatives pGO1Δ4 and pGO1Δ5A are shown by bold arcs. Two deletion events apparently took place to produce pGO1Δ4 (innermost arcs), a 41-kb plasmid that is Tp^s but transfer proficient. pGO1Δ5A is 31 kb in size and confers Tp^r and Gm^r but is Qam^s and transfer negative. The mapped sites of Tn*551* insertions that had no significant effect on the conjugative transfer of pGO1 are shown as arrows. Note that three of these insertions are internal to the *tra* region (area of 25 *tra* insertion sites).

RESULTS

Deletion mutants. The localization of sequences encoding conjugative transfer genes on pGO1 was first attempted by the production of deletion mutants by using transduction with phage φ11. The size of pGO1 (52 kb) was greater than that of φ11 (~45 kb); therefore, transductants that had deletions were readily generated. Deletion was apparently nonrandom; several major classes of deletions were repeatedly isolated in independent transduction experiments (Fig. 1). One of these isolates, pGO1Δ4, lost 11 kb of DNA, rendering the plasmid Tp^s but transfer proficient. This deletion ruled out 23% of the plasmid DNA as being involved in conjugation. A second deletion derivative, pGO1Δ5A, which was still Gm^r and Tp^r but transfer defective, involved a deletion of 20 kb. This was the most common deletion isolated, comprising 30% of derivatives, and was not helpful in precise localization of *tra* genes because of the large amount of missing DNA. Because of the small number of different classes of deletions and the large *tra* deletion produced, transposon mutagenesis was used to more precisely identify genes necessary for transfer.

Transposon insertions. Insertional inactivation of transfer-associated genes was accomplished by using both Tn*551* and Tn*917*. Both transposons encode erythromycin resistance (*ermB*) and are virtually identical, differing only in their

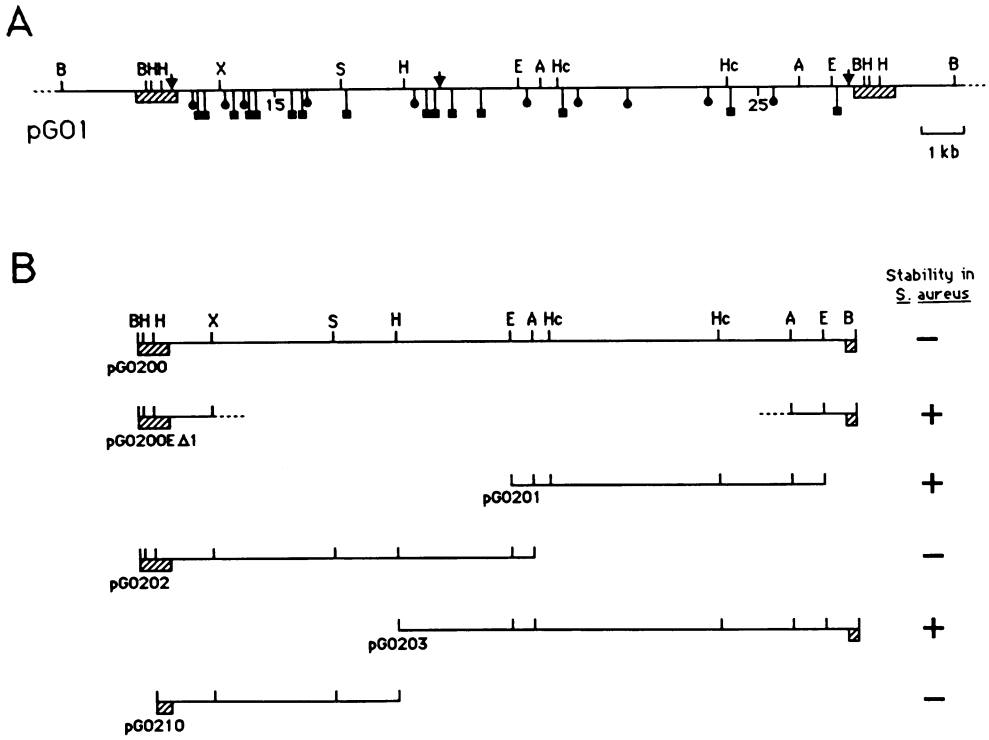

FIG. 2. (A) Detailed restriction map of the conjugative transfer region of pGO1. All 15 Tn*551* (■) and 10 Tn*917* (●) sites of insertion that abolished transfer mapped to within this region. The sites of several Tn*551* insertions into this region that had no significant effect on conjugation are shown by arrows (▼). The hatched areas (▨) mark directly repeated sequences flanking these insertion sites. Map coordinates of pGO1 are given below the insertion map of the *tra* region. (B) Subclones of the *tra* region, obtained by using pBR322, and their plasmid designations. The ability of each of the subclones to be stably introduced into *S. aureus* RN4220 by protoplast transformation is also shown. Abbreviations for restriction sites are as in Fig. 1, plus the following: A, *Ava*I; H, *Hin*dIII; Hc, *Hin*cII; S, *Sph*I; and X, *Xba*I.

inverted repeats and the inducibility of Em^r (25). Tn*917* has been shown to effectively insert into *S. aureus* chromosomal loci not previously targeted by Tn*551* (30). Tn*551* insertions into pGO1 were generated in a *S. aureus* 450 background, whereas Tn*917* insertions were produced in the *recA* stain, RN1030, to facilitate future complementation experiments. After curing, 200 Cd^s Em^r colonies were screened for Tn*551* insertions and 200 Cm^s Em^r colonies were analyzed for Tn*917* insertions. In both cases, more than 60% of the transposon insertions were into plasmid rather than chromosomal DNA targets. This was determined by mating each of the 400 colonies and identifying Gm^r Em^r transconjugants in addition to identifying colonies producing no transconjugants. Transconjugants contained a transposon insertion into plasmid DNA not essential for conjugation (Tra^+), whereas colonies that yielded no transconjugants (Tra^-) were assumed to have insertions into DNA essential for transfer. There were 15 independent Tra^- Tn*551* insertions and 10 independent Tra^- Tn*917* insertions as determined by detailed restriction endonuclease mapping of purified plasmid DNA; all mapped within the 14.5-kb *Bgl*II B fragment (Fig. 2). Mapping of Tn*551* and Tn*917* Tra^+ insertions showed all but three to fall outside the *Bgl*II B fragment and to be distributed throughout the rest of the plasmid replicon. Of the three Tra^+ insertions within *Bgl*II B, two were at each end of the fragment, outside all Tra^- insertions, further delimiting a contiguous *tra* region. The third was in the middle of the *Bgl*II fragment. Because of the low and variable transfer frequency of staphylococcal plasmids, it was not possible to assign gradations of transfer phenotype to different insertions. Thus, all insertion mutants were classified as either transfer proficient or unable to transfer at a detectable frequency ($<10^{-9}$ progeny per donor cell).

Clones of *tra* region fragments. Although both transposon insertion and transduction deletion mutants identified sequences required for transfer, they did not rule out other areas of pGO1 involved in transfer that may have been missed given the nonrandom character of transposon insertion and the site-specific nature of transduction deletions. To address this problem, we attempted to subclone the *Bgl*II B fragment onto *S. aureus-E. coli* shuttle plasmids in *E. coli* and to reintroduce the constructions back into *S. aureus*. The *Bgl*II B fragment was initially isolated by cloning it into the single *Bgl*II site of the positive selection vector pOP203($A2^+$) (29). pGO200 was created by subcloning the *Bgl*II fragment into the *Bam*HI site of pBR322. The subsequent addition of pE194 at the single *Cla*I site created the shuttle pGO200E. When pGO200E was introduced by protoplast transformation into RN4220, only a Tra^- deletion derivative (pGO200EΔ1) was obtained. To determine whether the deletion was due to the size of pGO200E or to gene products encoded by specific sequences lost in the deletion, subclones of the region on shuttle plasmids were produced and tested for their ability to transform *S. aureus*. The subclones (Fig. 2) were constructed as follows: pGO202E was produced by cleaving pGO200E with *Ava*I followed by religation. All other subclones were either a cleavage and religation of pGO200 or a direct subclone of the appropriate fragment onto pBR322. Shuttle plasmids were then made by adding either staphylococcal replicon pE194 or pSK265 back to the *E. coli* clone by direct selection in *E. coli* (15). pSK265 was made by adding the multiple cloning site of

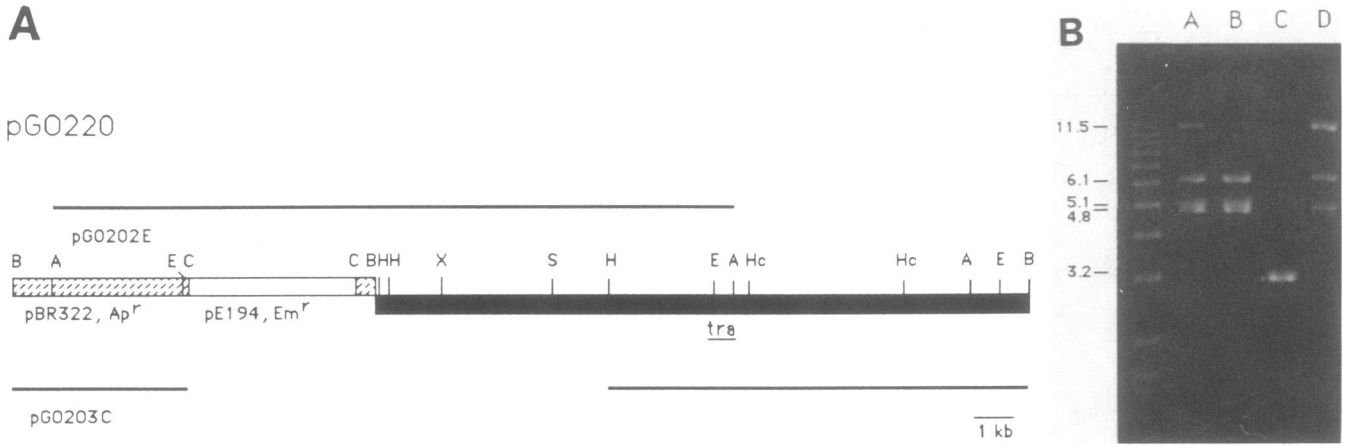

FIG. 3. Restriction endonuclease map of pGO220 and plasmid DNA from transconjugants of complementing clones. (A) Locations of restriction endonuclease cleavage sites of pGO220. Sequences of pBR322 (▨) and pE194 (□) present in pGO220 are indicated, as is DNA involved in conjugative transfer (■). Relative contribution of DNA sequences to pGO220 by each of the plasmids present in the donor strain (pGO202E and pGO203C) is shown by lines labeled accordingly. (B) Plasmid DNA was isolated and purified by dye-buoyant density gradient centrifugation and subjected to digestion with *Eco*RI before electrophoresis in a 0.7% agarose gel and then ethidium bromide staining. The numbers to the left indicate the sizes of the *Eco*RI restriction fragments of pGO202E (11.5 and 3.2 kb) and pGO203C (6.1, 5.1, and 4.8 kb). Lanes: A, pGO203C and pGO202E (donor strain); B, Cm^r transconjugant (pGO203C); C, second class of Cm^r transconjugant (deletion); D, Em^r transconjugant (pGO220). The recombinant transconjugant pGO220 contains the large (11.5-kb) *Eco*RI fragment of pGO202E as well as the 6.1- and 5.1-kb fragments of pGO203C. Abbreviations are as in Fig. 1 and 2, plus the following C, *Cla*I.

pUC19 to pC194, creating unique restriction sites that facilitated shuttle construction (20). The *E. coli* shuttle clones then were transformed into *S. aureus* RN4220. The stabilities of individual shuttle subclones are indicated in Fig. 2. Some of the subclones that could not be stably maintained in *S. aureus* were transformed on first one staphylococcal replicon and then the other. In addition, the staphylococcal plasmids were added back at more than one pBR322 restriction site. Neither maneuver resulted in stable transformants. Thus, the instability was not felt to be due to the shuttle plasmid construction. The large size of some of the stably transformed plasmids (i.e., pGO203C) also ruled against size being a major factor in the failure to isolate stable transformants. However, the 4.5-kb *Hin*dIII fragment, subcloned as pGO210, was contained within all of the unstable shuttle plasmids and was itself unstable. We hypothesized that a factor or factors produced by genes within this fragment were responsible for instability.

This hypothesis was strengthened by the finding that unstable shuttle plasmids (pGO210E, pGO210C, and pGO202E) could be stably transformed into recipients containing pGO1; transformed plasmids replicated autonomously without detectable integration into the resident plasmid. Since pGO1Δ5A could not stabilize these plasmids, sequences encoding stabilizing factors were lost in this deletion. The region containing *trans*-stabilizing factor(s) was further localized by the finding that pGO210C could be stably transformed into recipient strains containing pGO200EΔ1, the deletion derivative of pGO200E that appeared in pGO200E transformants. This deletion contained at least 1.0 kb of the *Hin*dIII fragment at one end of pGO200 and at least 1.1 kb at the opposite end of the cloned *Bgl*II B fragment but was missing the middle 10.3 kb of this fragment (Fig. 2). Since pGO203C could not stabilize pGO210E and pGO203C carried all of the *tra* region sequences except those present on pGO210, we assumed that the 4.5-kb *Hin*dIII fragment contained genes for production of factors causing instability as well as sequences encoding stabilizing factor(s).

Complementation of pGO203C and pGO202E. Difficulties encountered in introducing pGO200E and pGO202E by transformation led to attempts to construct complementing strains by mobilization or transduction. pGO202E could be mobilized by pGO1, occasionally without the cotransfer of pGO1. pGO202E could also be introduced into lysogenized recipients by φ11 transduction. Thus, pGO202E could be introduced into plasmid-free strains by conjugative mobilization or transduction but not by protoplast transformation, suggesting that factors produced by this *tra* region subclone affected protoplasts but not cells with cell walls.

pGO202E was mobilized into RN4220 containing pGO203C. Cm^r Em^r Gm^s transconjugants were lysed to confirm the plasmid composition. With this strain as a donor, either Cm^r or Em^r transconjugants were found at low but detectable frequencies after mating into strain GO57. Cm^r transconjugants contained pGO203C or deleted versions of the plasmid, whereas Em^r transconjugants all contained a single recombinant of the two plasmids. The recombinant contained the entire *Bgl*II B fragment of pGO1 intact on a single replicon (Fig. 3). The plasmid had a restriction endonuclease digestion pattern identical to that of pGO200E and transferred into GO160 at a frequency of 10^{-6} to 10^{-7} transconjugants per donor cell input, a frequency of transfer identical to that of pGO1. Table 2 shows the conjugation

TABLE 2. Transfer frequency of pGO1 and subclones[a]

Donor plasmid	Transfer frequency
pGO1	1.4×10^{-7}
pGO202E + pGO203C	4.5×10^{-10}
pGO202E	$<9.0 \times 10^{-9}$
pGO203C	$<7.0 \times 10^{-9}$
pGO220 (mini-*tra*)	2.8×10^{-6}

[a] Filter matings were performed as described in Materials and Methods with GO57 as a recipient in all mating experiments. Transfer frequency was calculated as the number of transconjugants per donor cell present on the filters after 18 h.

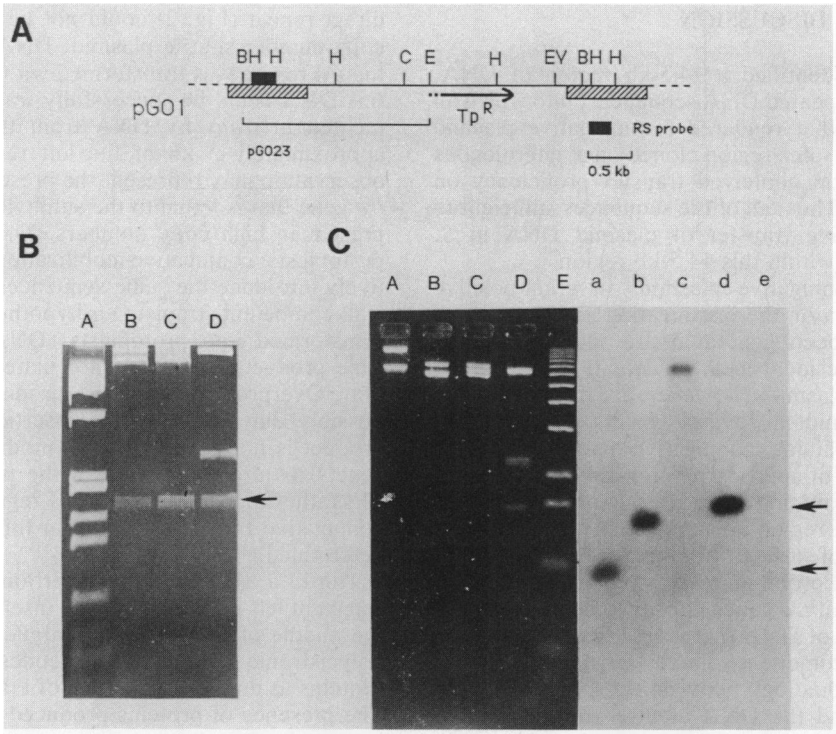

FIG. 4. Identification of directly repeated DNA sequences flanking the *tra* region of pGO1. (A) Previously reported location of directly repeated sequences on pGO1 surrounding the gene for trimethoprim resistance (12). A *Bgl*II-*Hin*dIII-*Hin*dIII restriction site pattern is associated with the repeat. The presence of a 250-bp *Hin*dIII fragment on acrylamide gels is also characteristic of this repeat. (B) A 10% acrylamide gel of *Hin*dIII digestions of the following (lanes): A, pGO53; B, pGO132; C, pGO23. Lanes A through C display a 250-bp fragment characteristic of the repeated sequence. The precise location and orientation of the repeated sequences were determined by hybridization of clones of the region with the 250-bp fragment obtained from pGO23. Digestion with *Bgl*II allowed orientation to be determined, since the probe hybridized only to one side of the *Bgl*II site within the repeat. Hybridization to fragments produced by digestion with *Xba*I or *Eco*RI together with *Bgl*II (Fig. 2) allowed the repeats to be localized to positions flanking the *tra* region (panel C). (C) Lanes: A through C, pGO53 digested with *Bgl*II and *Xba*I (A), *Eco*RI and *Xba*I (B), and *Bgl*II and *Eco*RI (C); D, pGO132 digested with *Bgl*II and *Eco*RI. The corresponding lowercase letters designate the same DNA transferred to nitrocellulose and probed with the 250-bp *Hin*dIII fragment, internal to the repeat, labeled with ^{32}P. The probe was purified from a 10% polyacrylamide gel and labeled by hexamer priming. The autoradiograph reveals location of the repeats to the left end (lanes: a [lower arrow], 1.1-kb *Xba*I-*Bgl*II fragment; b, 1.5-kb *Xba*I-*Eco*RI fragment; c, 7.2-kb *Eco*RI-*Bgl*II fragment) and the right end (lane d, 1.6-kb *Bgl*II fragment) but not to sequences central to the *Bgl*II fragment (lane b, no hybridization signal to 6.1- and 6.2- kb internal fragments of pGO53).

frequency of plasmid constructions, and Fig. 3 is an agarose gel showing plasmid DNA in transconjugants after the mating of the donor strain containing pGO202E and pGO203C.

Hybridization of the *tra* region with defined DNA. pGO200 was used to probe Southern blots containing other gram-positive conjugative elements as targets. The plasmids pIP501 (16) and pAMB1 (9a) were chosen as two examples of broad-host-range plasmids isolated from *Streptococcus faecalis*. A representative pheromone response plasmid pCF-10, which contains a copy of the conjugative transposon Tn*925*, was also used for hybridization studies (9). When the plasmids pIP501, pAMβ1, and pCF-10 were probed under conditions of high and low stringency, no hybridization was detectable. However, unexpected signals were seen in the lane containing control DNA (pGO1 digested with *Bgl*II) when hybridization was performed under conditions of high stringency. The hybridization pattern, in which 8 of the 10 *Bgl*II fragments gave positive signals, was identical to that seen when sequences surrounding the trimethoprim resistance gene (Fig. 4) were used as a probe. This repeated sequence was identified by its *Bgl*II-*Hin*dIII restriction site pattern. Digestion with *Hin*dIII released a characteristic 250-base-pair (bp) fragment detectable by polyacrylamide gel electrophoresis (Fig. 4). Polyacrylamide gels of *Hin*dIII digests of pGO200 as well as a clone of DNA flanking the right end of the *Bgl*II B fragment (pGO132) both demonstrated this 250-bp fragment. The internal 250-bp *Hin*dIII fragment from a copy of this repeated sequence 5′ to the Tp^r gene (pGO23 [12]) was used as probe against the pGO1 *Bgl*II B fragment double digested with *Bgl*II-*Xba*I, *Bgl*II-*Eco*RI, and *Xba*I-*Eco*RI to release the left end, the right end, and the central portion of the *tra* region, respectively (Fig. 4). pGO132, the *Eco*RI fragment flanking the right end of *Bgl*II-B, was also probed after double digestion with *Eco*RI-*Bgl*II. The repeated sequence probe hybridized with the 1.2-kb *Bgl*II-*Xba*I fragment corresponding to the left end of pGO200 and to the 1.5-kb *Bgl*II K fragment in pGO132 that is contiguous with the right end of the *Bgl*II B fragment. This information, together with restriction site mapping and polyacrylamide gel electrophoresis, allowed us to conclude that the *tra* region was flanked by directly repeated sequences. Thus, sequences apparently not involved in transfer and repeated at different locations on pGO1 were localized to within 1 kb of insertions that abolished conjugation, further defining the limits of the *tra* region.

DISCUSSION

In this study, we identified a 14.5-kb region of DNA, bounded by directly repeated DNA sequences, into which all transposons inserted that rendered a conjugative plasmid transfer deficient. The same region cloned on a heterologous nonconjugative replicon conferred transfer proficiency on the resulting plasmid. Thus, all of the sequences sufficient to mediate the conjugative transfer of plasmid DNA in *S. aureus* were included within this 14.5-kb region.

The only other conjugative plasmids in gram-positive bacteria for which data on the organization and function of the *tra* genes have been generated are the pheromone-responsive plasmids resident in *S. faecalis* (9, 10). Staphylococcal conjugative plasmids, however, are dissimilar from these *S. faecalis* plasmids in several aspects. First, staphylococcal donors containing conjugative plasmids do not clump in the presence of appropriate recipients, and macroscopic donor-recipient mating aggregates do not form. Thus, the staphylococcal *tra* region appears to encode a different and less efficient type of surface structure for forming mating aggregates than do pheromone-responsive plasmids. Second, the staphylococcal *tra* genes appear to be contained in one contiguous region of DNA that is less than one-half the size required for pheromone-mediated transfer (9, 10). Finally, there was no homology between the 14.5-kb staphylococcal *tra* region and the DNA of pCF-10, a typical *S. faecalis* pheromone-responsive plasmid, even under conditions of low stringency. Staphylococcal *tra* genes may be functionally more similar to those found on the broad-host-range gram-positive conjugative elements resident in various streptococcal species (9, 16). However, staphylococcal conjugative plasmids are confined to a relatively narrow host range, and the *tra* region DNA from pGO1 also showed no homology with pIP501, pAMβ1, or Tn*925*, representatives of broad-host-range streptococcal elements. Although lack of homology does not rule out a similarity between *tra* region genes in organization and function, it makes it unlikely that these gram-positive conjugative transfer systems evolved directly from one another. Precise relationships between gram-positive conjugative transfer genes and the similarity of these systems to the well-characterized gram-negative conjugative plasmids await further genetic and functional analysis.

The genetic analysis of the *tra* genes of pGO1 revealed several interesting results. First, the directly repeated sequences that were found at either end of the *tra* region have restriction-site similarity to insertion sequence (IS)-like elements described by Gillespie et al. (IS257 [13]) and Barberis-Maino et al. (IS431 [5]). Homologous sequences in directly repeated orientation have also been found in six additional locations on pGO1, including one copy at each end of the gene encoding resistance to trimethoprim (12). These IS-like elements have also been identified at either end of the genes encoding mercury resistance on staphylococcal penicillinase plasmids and at one end of the staphylococcal chromosomal gene(s) responsible for methicillin resistance (5). The association of these IS-like elements with multiple resistance determinants in both plasmid and chromosomal loci implies an involvement of these elements in the movement of genes in staphylococci, including those for conjugative transfer. Thus, these sequences, either by virtue of independent mobility or by serving as recombination sites, may be important in the construction and evolution of large conjugative antimicrobial resistance plasmids in staphylococci.

Second, the 4.5 kb of *tra* region DNA adjacent to the left direct repeat (Fig. 2) could not be transformed on a high-copy-number shuttle plasmid; DNA comprising the rest of the *tra* region was transformed without difficulty. However, this DNA could be successfully transformed when complemented in *trans* by DNA from the same region, within approximately 3 kb of the left repeat. We feel that this observation may represent the presence of a *trans*-regulated *tra* gene that is lethal to the staphylococcal protoplast when present in high copy numbers. This effect is only seen in protoplasts; conjugative mobilization and transduction effectively introduce the same sequences into plasmid-free, cell-wall-competent strains. Our hypothesis is that when initially transformed into protoplasts, DNA containing the lethal gene product(s) may be unregulated due to zygotic induction. Overproduction of the product is lethal to the cell, possibly due to membrane insertion. However, the gene product is not produced or is modified when the regulator gene(s) is already present in the transformation recipient. Thus, this appears to be a *trans*-regulated factor involved in conjugative transfer. Its role in this process has yet to be determined.

Third, a single Tn*551* insertion into the *Bgl*II B *tra* fragment left pGO1 Tra^+. This insertion, almost precisely in the middle of the *tra* region, argues against a single major polycistronic transcript that encodes all of the *tra*-associated proteins as do the *tra* operons of F and F-like plasmids (18). The presence of proteins produced by *tra* region subclones in *E. coli* minicells, complementation of Tra^- Tn*551* insertions by pGO210C (W. Thomas, unpublished observations), and the complementation for transfer of overlapping *tra* fragments further suggest that several promoters are likely to exist and function in the staphylococcal *tra* region. However, these results could also be explained by the transcription of *tra* genes by transposon or vector promoters, and the transfer of one of the overlapping *tra* fragment clones could be explained by mobilization after the recombinational rescue of the intact *tra* region in the same cell. The genetic organization of the *tra* region awaits the results of complementation analysis and the identification of individual promoters.

In summary, we have defined the genetic and functional limits of the *tra* region of a conjugative staphylococcal plasmid and identified a *trans*-regulated *tra* function that is probably lethal to protoplasts when cloned on a high-copy-number vector. Because of the similarity of conjugative staphylococcal plasmids isolated in diverse geographic locations, data generated from studies of the pGO1 *tra* region are likely to serve as paradigms for staphylococcal conjugative transfer. Since there are no data on gram-positive conjugative plasmid transfer genes from any system other than the unique pheromone-responsive system in *S. faecalis*, a study of staphylococcal *tra* genes may provide important new insights into the evolution and dissemination of plasmids in gram-positive bacteria. Furthermore, it may be possible to improve systems for the in vitro manipulation of genes between staphylococci of the same and different species.

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LITERATURE CITED

1. **Archer, G. L., J. P. Coughter, and J. L. Johnston.** 1986. Plasmid encoded trimethoprim resistance in staphylococci. Antimicrob. Agents Chemother. **29**:733–740.
2. **Archer, G. L., D. R. Dietrick, and J. L. Johnston.** 1985. Molecular epidemiology of transmissible gentamicin resistance among coagulase-negative staphylococci in a cardiac surgery unit. J. Infect. Dis. **151**:243–251.
3. **Archer, G. L., and J. L. Johnston.** 1983. Self-transmissible plasmids in staphylococci that encode resistance to aminoglycosides. Antimicrob. Agents Chemother. **24**:70–77.
4. **Archer, G. L., and C. G. Mayhall.** 1983. Comparison of epidemiological markers used in the investigation of methicillin resistant *Staphylococcus aureus* infections. J. Clin. Microbiol. **18**:395–399.
5. **Barberis-Maino, L., B. Berger-Bachi, H. Weber, W. D. Beck, and F. H. Kayser.** 1987. IS*431*, a staphylococcal insertion sequence-like element related to IS*26* from *Proteus vulgaris*. Gene **59**:107–113.
6. **Bolivar, F., F. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Cross, and S. Falcow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459.
8. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
9. **Christie, P. J., and G. M. Dunny.** 1986. Identification of regions of the *Streptococcus faecalis* plasmid pCF-10 that encode antibiotic resistance and pheromone response functions. Plasmid **15**:230–241.

9a. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.

10. **Ehrenfeld, E. E., and D. B. Clewell.** 1987. Transfer functions of the *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. J. Bacteriol. **169**:3473–3481.
11. **Forbes, B. A., and D. R. Schaberg.** 1983. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. J. Bacteriol. **153**:627–634.
12. **Galetto, D. W., J. L. Johnston, and G. L. Archer.** 1987. Molecular epidemiology of trimethoprim resistance among coagulase negative staphylococci. Antimicrob. Agents Chemother. **31**:1683–1688.
13. **Gillespie, M. T., B. R. Lyon, L. S. L. Loo, P. R. Mathews, P. R. Stewart, and R. A. Skurry.** 1987. Homologous direct repeat sequences associated with mercury, methicillin, tetracycline, and trimethoprim resistance determinants in *Staphylococcus aureus*. FEMS Microbiol. Lett. **43**:165–171.
14. **Goering, R. V., and E. A. Ruff.** 1983. Comparative analysis of conjugative plasmids mediating gentamicin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **24**:450–452.
15. **Hardy, K., and C. Haefeli.** 1982. Expression in *Escherichia coli* of a staphylococcal gene for resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **156**: 524–526.
16. **Horodniceanu, T., D. Bouanchaud, G. Beit, and Y. Chambert.** 1976. R plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **10**:795–801.
17. **Hourinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
18. **Ippen-Ihler, K. A., and E. G. Minkley, Jr.** 1986. The conjugation system of F, the fertility factor of *Escherichia coli*. Annu. Rev. Genet. **20**:593–624.
19. **Jaffe, H. W., H. M. Sweeny, R. A. Weinstein, S. A. Kabins, C. Nathan, and S. Cohen.** 1982. Structural and phenotypic varieties of gentamicin resistance plasmids in hospital strains of *Staphylococcus aureus* and coagulase negative staphylococci. Antimicrob. Agents Chemother. **21**:773–779.
20. **Jones, C. L., and S. A. Khan.** 1986. Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J. Bacteriol. **166**:29–33.
21. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock exotoxin structural gene is not detectably transmitted by a prophage. Nature (London) **305**:709–712.
22. **Kushner, S.** 1978. An improved method for transformation of *E. coli* with *Col*E1-derived plasmids, p. 17–23. *In* H. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier Biomedical Press, Amsterdam.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McDonnell, R. W., H. M. Sweeny, and S. Cohen.** 1983. Conjugational transfer of gentamicin resistance plasmids intra- and interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. **23**:151–160.
25. **Murphy, E.** 1989. Transposable elements in gram-positive bacteria, p. 269–288. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
26. **Novick, R.** 1967. Properties of a high frequency transducing phage in *Staphylococcus aureus*. Virology **33**:155–166.
27. **Novick, R. P., I. Edelman, M. D. Schwesinger, A. D. Gruss, E. C. Swanson, and P. A. Pattee.** 1979. Genetic translocation in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **76**:400–404.
28. **Townsend, D. E., N. Ashdown, S. Bolton, and W. B. Grubb.** 1985. The use of cetyltrimethylammonium bromide for the rapid isolation from *Staphylococcus aureus* of relaxable and non-relaxable plasmid DNA suitable for *in-vitro* manipulation. Lett. Appl. Microbiol. **1**:87–94.
29. **Winter, R. B., and L. Gold.** 1983. Overproduction of bacteriophage QB maturation (A_2) protein leads to cell lysis. Cell **33**:877–885.
30. **Youngman, P.** 1985. Plasmid vectors for recovering and exploiting Tn*917* transpositions in *Bacillus* and other gram-positive bacteria, p. 79–104. *In* K. G. Hardy (ed.), Plasmids: a practical approach. IRL Press, Oxford.