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Identification and Characterization of the Origin of Conjugative Transfer (*oriT*) and a Gene (*nes*) Encoding a Single-Stranded Endonuclease on the Staphylococcal Plasmid pGO1

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The genes mediating the conjugative transfer of the 52-kb staphylococcal plasmid pGO1 are within a 14.4-kb gene cluster designated *trs*. However, a clone containing *trs* alone cannot transfer independently and no candidate *oriT* has been found within or contiguous to *trs*. In this study, we identified a 1,987-bp open reading frame (ORF) 24 kb 3′ and 13 kb 5′ to *trs* that was essential for conjugative transfer: transposon insertions into the ORF abolished transfer and a plasmid containing the ORF could complement these transposon-inactivated pGO1 mutants for transfer. Analysis of the nucleotide sequence of this ORF revealed significant homology between the amino terminus of its predicted protein and those of several single-stranded endonucleases. In addition, a 12-bp DNA sequence located 100 bp 5′ to the ORF's translational start site was identical to the *oriT* sequences of the conjugative or mobilizable plasmids RSF1010, pTF1, R1162, pSC101, and pIP501. The ability of the ORF, designated *nes* (for nicking enzyme of staphylococci), to generate a single-stranded nick at the *oriT* was demonstrated in *Escherichia coli* by alkaline gel and DNA sequence analysis of open circular plasmid DNA. Plasmids that could be converted to the open circular form by the presence of *oriT* and *nes* could also be mobilized at high frequency into *Staphylococcus aureus* recipients with a second plasmid containing only *trs*. We propose that the 14.4 kb of *trs* and the approximately 2.2 kb of the *oriT-nes* region, coupled with an origin of replication, make up the minimal staphylococcal conjugative replicon.

Bacterial conjugation is a unique process that allows the transfer of plasmid DNA from a donor to a recipient through cell-to-cell contact (39); it has the broadest host range among the mechanisms for interbacterial genetic exchange (8). Conjugation has been observed in both gram-negative and gram-positive bacteria and even between members of the two groups (18). Classically, it is the study of gram-negative transmissible plasmids, such as F, R100, RP4, and others, that has contributed to our understanding of the genetic and molecular basis of bacterial conjugation (13). In these systems, the plasmid moves from the donor to the recipient bacterium as a single linear strand of DNA. All of the gram-negative conjugative plasmids that have been examined in sufficient detail show two well-conserved elements essential for single-strand transfer: (i) a *cis*-acting DNA segment, the origin of transfer or *oriT*, where the DNA transfer process initiates and terminates, and (ii) a site-specific endonuclease that cleaves a single strand of DNA at the *nic* site (22, 23, 37, 38, 39). These endonuclease proteins, known as relaxases, bind to the *oriT* region to form a DNA-protein complex known as the relaxosome. Binding of these proteins to the *oriT* is facilitated by the presence of directly or indirectly repeated sequences that act as recognition sites for specific DNA-binding proteins by promoting the formation of secondary structures. Moreover, the high AT content of the region flanking the *oriT* facilitates the formation of a relaxosome at this site by allowing strand separation in negatively supercoiled plasmid DNA. Once the relaxosome has formed, cleavage of a single strand of DNA occurs at the *oriT* site. Following nicking, the relaxase protein remains covalently bound to the 5′ end at the *oriT* site. Single-stranded DNA transport into the recipient cell then proceeds in the 5′ to 3′ direction. Following transfer of the single-stranded DNA, recircularization and replication of the plasmid occurs in the recipient cell.

Conjugative machineries encoded by transmissible plasmids in gram-positive bacteria have only recently been the focus of the same intensive analysis previously devoted to gram-negative conjugative plasmids (5, 14, 36). Wang and Macrina have identified an *oriT* and an *oriT*-specific endonuclease on the broad-host-range plasmid pIP501 of gram-positive origin (36). Jaworski and Clewell have also reported that the conjugative transposon Tn*916*, commonly resident in enterococci, may contain several *oriT* sequences (14). Beyond these two examples, few genetic and biochemical data concerning the mechanism of processing and transfer of plasmid DNA during conjugation in gram-positive bacteria are available.

pGO1 is a 52-kb conjugative plasmid that transfers among staphylococci broadly but is restricted in its abilities to transfer to and reside in members of this genus (32). It encodes resistance to the antimicrobial agents gentamicin, neomycin, and trimethoprim and to such disinfectants as the quaternary ammonium compounds. In addition to being self-transmissible, pGO1 can mobilize small nonconjugative plasmids for transfer (24, 33). The major conjugative transfer gene complex of pGO1 has been designated *trs* and is located on a 14.4-kb *Bgl*II fragment containing 14 open reading frames (ORFs). No candidate *oriT* sequences have been found within or flanking the *trs* region, and when *trs* is cloned on an independent replicon, this region cannot mediate self-transfer. In the following study,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Remark(s) (reference or source)
E. coli strains		
TB1	*recA*$^+$ *lacI*^{q}*ZΔM15*	
JM109	*recA1 endA1 gyr-96 thi-hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- (*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]	Recombinant-deficient host
S. aureus strains		
RN450	Antibiotic-susceptible host	ATCC 8325-4 cured of ϕ11, ϕ12, and ϕ13
RN4220	Restriction deficient	Shuttle plasmid host (16)
RN4220SS	St^r Sp^r	Mating recipient
RN4220NR	N^r R^r	Mating recipient (16)
Plasmids		
pUC18	Ap^r, 2.7 kb	Cloning vector
pUC19	Ap^r, 2.7 kb	Cloning vector (41)
pBR322	Ap^r Tc^r, 4.3 kb	Cloning vector (3)
pOP203(A_2)$^+$	Tc^r, 7.0 kb	Cloning vector (40)
pRN5543	Cm^r, 3.0 kb	pSK265 with 5′ *Hin*dIII site in multiple cloning site deleted (25)
pE194	Em^r, 3.0 kb	Staphylococcal replicon (12)
pGO1	Gm^r Tp^r Qam^r Neo^r Trs$^+$, 52 kb	Conjugative plasmid recovered from an *S. aureus* clinical isolate (32)
pGO1(5B)	Gm^r Trs$^-$, 24 kb	Deletion mutant of pGO1 (this study)
pGO188	Em^r Gm^r Qam^r Tp^r Neo^r Trs$^-$, 52 kb	pGO1 with Tn*917lac* insert in *nes* (this study)
pGO189	Ap^r Tc^r, 7.8 kb	3.5-kb *Eco*RI-*Bam*HI fragment of pGO188 cloned to pBR322 (21)
pGO193C	Ap^r Cm^r, 4.4 kb	1.7-kb *Hin*dIII fragment of pGO1 cloned to pUC18 with pRN5543 added (this study)
pGO356	Ap^r, 4.0 kb	1.3-kb *Hin*cII fragment of pGO1 cloned to pUC19 (this study)
pGO374E	Em^r Tc^r, Trs$^-$, 17.4 kb	14.4-kb *Bgl*II fragment of pGO1 containing conjugative transfer region (*trs*) cloned to pBR322 at the *Bam*HI site with pE194 added to the *Cla*I site (this study)
pGO400	Mp^r Cm^r, Trs$^+$	Yale mupirocin resistance plasmid containing *Bgl*II A and B fragments and two additional *Bgl*II fragments of pGO1 (21)
pGO404C	Tc^r Cm^r, 16.5 kb	9.5-kb *Eco*RI fragment of pGO400 cloned to pOP203(A_2)$^+$ with pRN5543 cloned to *Sst*I site (this study)
pGO418C	Ap^r Cm^r, 10.1 kb	4.4-kb *Eco*RI fragment of PGO1 cloned to pUC19 with pRN5433 cloned at *Sst*I site (this study)
pGO438	Ap^r, 4.6 kb	1.9-kb PCR fragment of pGO1 containing *oriT* site and first 550 bp of *nes* with 1.2 kb of upstream DNA cloned to *Hin*cII site of pUC19 (this study)
pGO535C	Ap^r Cm^r, 5.85 kb	135-bp PCR fragment of pGO1 containing *oriT* site cloned to *Sma*I site of pUC19 with RN5543 cloned to *Hin*dIII site (this study)
pGO541C	Ap^r Cm^r, 7.9 kb	2.24-kb PCR fragment of pGO1 containing *oriT* site, promoter region, and *nes* cloned to pUC19 at the *Sma*I site with pRN5433 cloned to the *Bam*HI site (this study)
pGO542C	Ap^r Cm^r, 7.9 kb	2.18-kb PCR fragment of pGO1 containing promoter region and *nes* cloned to pUC19 at the *Bam*HI-*Sma*I site with pRN5433 cloned to the *Bam*HI site (this study)
pGO547C	Ap^r Cm^r, 6.4 kb	700-bp PCR fragment of pGO1 containing *oriT* site and first 550 bp of *nes* cloned to *Bam*HI-*Sma*I site of pUC19 with RN5543 cloned to *Bam*HI site (this study)

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Neo, neomycin and paromomycin; Mp, mupirocin; N, novobiocin; Qam, quaternary ammonium compounds; R, rifampin; Sp, spectinomycin; St, streptomycin; Tc, tetracycline; Tp, trimethoprim; Trs, staphylococcal conjugative transfer.

we identify and characterize the pGO1 origin of transfer and the gene encoding a single-stranded nicking enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmid construction. The *Staphylococcus aureus* and *Escherichia coli* strains used in this study are listed in Table 1. Recombinant plasmids were generated in *E. coli* TB1 or JM109 (41). The *E. coli* cloning vectors used included pUC18, pUC19 (41), pBR322 (3), and pOP203(A_2)$^+$ (40). *E. coli-S. aureus* shuttle vectors were constructed by adding either of two staphylococcal replicons, pRN5433 (25), which encodes chloramphenicol resistance, or pE194 (12), which encodes erythromycin resistance, to *E. coli* vectors. The letters C and E following a numbered plasmid indicate a shuttle construct containing pRN5433 or pE194, respectively. Plasmid constructs with no letter following their designation indicate plasmids resident in *E. coli*, with the exception of the native staphylococcal plasmids pGO1, pGO1(5B), and pGO188.

Materials and media. Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) was used for culture of both *E. coli* and *S. aureus*. Lennox L base broth (GIBCO-Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used for culture of *E. coli* strains, while brain heart infusion (BHI; Difco, Detroit, Mich.) broth or Trypticase soy broth (BBL Microbiology Systems) was used for culture of *S. aureus* strains. The antibiotics and concentrations used were as follows: ampicillin (50 μg/ml), tetracycline (3 μg/ml), or chloramphenicol (10 μg/ml) for the initial selection of *E. coli* strains following electroporation; erythromycin (10 μg/ml), chloramphenicol (10 μg/ml), and gentamicin (5 μg/ml) for the initial selection of *S. aureus* strains following electroporation or transduction; and streptomycin (50 μg/ml), spectinomycin (50 μg/ml), novobiocin (5 μg/ml), gentamicin (5 μg/ml), or rifampin (5 μg/ml) for the selection of transconjugants following mating studies. *S. aureus* transformants were maintained on media containing chloramphenicol (40 μg/ml) or erythromycin (20 μg/ml). All chemicals and antibiotics were supplied by Sigma Chemical Co. (St. Louis, Mo.). Lysostaphin was obtained from Applied Microbiology Inc. (Clarksville, United Kingdom). Restriction endonucleases and other enzymes used in DNA manipulation were obtained from Bethesda Research Laboratories.

DNA isolation and manipulation. *E. coli* plasmid DNA was isolated by the mini-lysis technique described by Sambrook et al. (26). *S. aureus* DNA was prepared by either the cetyltrimethylammonium bromide lysis method of Townsend et al. (35) or the rapid boiling method of Holmes and Quigley (11) as modified by Goering and Ruff (10). Recombinant plasmids were introduced into *E. coli* and *S. aureus* by electroporation with a Gene Pulser (Bio-Rad, Richmond, Calif.) with the following settings: 200 Ω, 25 μF, and 2.5 kV for *E. coli* and 400 Ω, 25 μF, and 2.5 kV for *S. aureus*. Electrocompetent cells of *E. coli* were prepared by the method described in the Gene Pulser manual, and *S. aureus* cells were prepared by the method of Schenk and Laddaga (27). Plasmid DNA was purified from *E. coli* for sequence analysis by the Midi-prep procedure (Qiagen, Chatsworth, Calif.).

DNA sequencing and PCR generation of pGO1 fragments. Oligonucleotide primers complementary to pGO1 nucleotide sequences (Oligos Etc., Wilsonville, Oreg.) were synthesized for use in amplification of DNA by PCR. In several cases, restriction enzyme sites were added to primers to facilitate ligation to cloning vectors. $Vent_R$ polymerase (New England Biolabs, Beverly, Mass.) was used to extend the primer template, and thermocycling conditions were as recommended by the manufacturer's guidelines. The primers used and their nucleotide locations as seen in Fig. 3 are as follows. PCR amplification of the 2.24-kb pGO1 fragment containing the *oriT* site and 1,987-bp *nes* gene used primer 271 (the sequence includes a 5′ *Bam*HI site; 5′-CGGGATCCTAGTTAAATCCCG TAAG-3′ [see Fig. 3, nucleotides 87 to 104]) and primer 268 (includes a 5′ *Eco*RI site; 5′-GATGAACAGAATAAAGAAATTG-3′ [see Fig. 3, nucleotides 3010 to 2289]). The 2.18-kb fragment containing the promoter region and *nes* was generated with primer 256 (includes a 5′ *Bam*HI site; 5′-CGGGATCCGCGTGCT AAATTCGTG-3′ [see Fig. 3, nucleotides 130 to 146]) and primer 268. The 135-bp fragment containing the *oriT* site was generated with primer 240, 5′-CA GATTCAAGTTTTACCG-3′ (see Fig. 3, nucleotides 61 to 79), and primer 241, 5′-CAAATATTGAGCGAAG-3′ (see Fig. 3, nucleotides 193 to 178). The 1.9-kb PCR fragment of pGO438 was generated with primer 153, 5′-GGAACTTTGT GTTCCCACCC-3′, and primer 217, 5′-GCAGAAACTCTAACAG-3′ (see Fig. 3, nucleotides 788 to 773). The 700-bp fragment of pGO547 was generated with primer 271 and primer 217.

Mating studies. Two types of filter mating were used in this study. The first, spot mating, was less sensitive and was used as an initial screen in order to identify those colonies containing plasmids with transposon insertions that had inactivated transfer. An erythromycin-resistant donor colony was mixed in a microcentrifuge tube with a BHI culture of the recipient strain (RN4220NR), and 100 μl of the donor-recipient mixture was spotted onto a 13-mm-diameter, 0.45-μm-pore-size nitrocellulose filter. After overnight incubation on BHI agar at 37°C, each filter was vortexed and plated on agar containing novobiocin, rifampin, and gentamicin to select transconjugants. Transconjugant colonies from matings were picked with erythromycin to identify plasmids containing transposon insertions that did not affect conjugation. Colonies that failed to produce transconjugants were examined by the more sensitive syringe mating technique as previously described (20). Colonies that failed to yield transconjugants by either method were deemed transfer deficient.

All mating studies to analyze complementation and mobilization were performed by the syringe method. Transconjugants were plated on media containing chloramphenicol, streptomycin, and spectinomycin when *S. aureus* RN4220SS was used as the recipient and on medium containing chloramphenicol, novobiocin, and rifampin when *S. aureus* RN4220NR was used as the recipient. Transconjugants were confirmed by cell lysis and electrophoresis. Transfer frequencies were determined by dividing the number of transconjugants by the number of donor cells.

Mobilization studies. Mobilization studies used the *E. coli-S. aureus* shuttle plasmid pGO374E (Table 1) constructed from the 14.4-kb *trs* region, pBR322, and the staphylococcal replicon pE194. pGO374E lacks the ability to undergo self-transfer but can mobilize small nonconjugative plasmids. Various fragments of pGO1 could thus be tested for mobilization with *trs* if they were on *E. coli-S. aureus* shuttle plasmids containing a pE194-compatible staphylococcal replicon (pRN5543) (Table 1).

Transduction. *S. aureus*-transducing phage 80α was used to lyse *S. aureus* RN4220 containing plasmids of interest by a modification (15) of the technique of Thompson and Patee (34). Recipient cells were grown on a BHI agar slant to a density of 1×10^{10} to 5×10^{10} cells/ml and resuspended in 1 ml of Trypticase soy broth containing 5×10^{-3} $CaCl_2$. One-half milliliter of the cellular suspension and 0.5 ml of the phage 80α-transducing lysate (1×10^{10} to 5×10^{10} PFU/ml) were added to a sterile 45-ml tube which contained 1.0 ml of Trypticase soy broth supplemented with 5×10^{-3} M $CaCl_2$. The tube was shaken vigorously (100 rpm) at 37°C for 20 min, at which time the tube received 1.0 ml of ice-cold 0.05 M sodium citrate. The cells were pelleted by centrifugation, resuspended in 1.0 ml of ice-cold 0.05 M sodium citrate, and then plated on BHI agar containing 500 mg of sodium citrate per ml and antibiotics appropriate for the selection of transductants.

Transposon insertion mutagenesis and complementation. Transposon insertions and β-galactosidase fusions were generated with Tn*917lac* delivered by pTV32ts as described previously (20). pTV32ts is a temperature-sensitive replicon encoding chloramphenicol resistance on the delivery vehicle and erythromycin resistance on the transposon. The transposon also contains the *E. coli lacZ* gene with a *Bacillus subtilis* ribosomal binding site and no promoter in order to generate transcriptional fusions in target genes (42). All transposon insertions were generated in RN450 (Table 1). Curing of the temperature-sensitive delivery vehicle was accomplished by two rounds of growth at 42°C, as previously described (20). Colonies resistant to erythromycin and susceptible to chloramphenicol were analyzed by the spot and syringe mating procedures, as described above, for those colonies that contained pGO1 derivatives incapable of conjugative transfer. The locations of Tn*917lac* insertions inactivating transfer were then determined by restriction endonuclease digestion analysis of plasmid DNA. Those insertions located outside the previously identified *trs* region were saved for further analysis. Transposon insertions into pGO1 that did not affect transfer were also localized. The orientations of Tn*917lac* fusions were assessed by plating cells on media containing β-D-galactopyranoside. Colonies that turned blue were considered to have the *lac* gene inserted in the direction that allows the *lac* gene's transcription from the promoter of the inactivated gene on pGO1. This was subsequently confirmed by restriction enzyme mapping of pGO1, which contains these transposon inserts. The precise location of Tn*917lac* insertions was further determined by sequencing the DNA at the insertion site with one primer that represented one end of Tn*917lac* (31) and a second primer derived from sequences in pGO1 DNA near the insertion site.

For complementation studies, cloned pGO1 fragments were introduced into RN450 containing pGO1 with a Tn*917lac* insertion that had inactivated transfer. Complementation was assessed by mating studies in which transconjugants were sought on media containing gentamicin and erythromycin.

DNA sequencing and analysis. Sequence analysis was performed by the Sanger dideoxy chain termination method with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) as well as by automated sequencing with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) on an ABI 373A sequencer. DNA sequences were analyzed for ORFs, motifs, and encoded amino acids with programs of the Genetics Computer Group.

Isolation and analysis of OC plasmid DNA. Open circular (OC) plasmid DNA was isolated by the protocol of Murphy and Malamy (22) with modifications. In brief, 250 ml of an overnight culture of *E. coli* containing either pGO541 or pGO542 (Table 1) was harvested by centrifugation, resuspended in 5 ml of lysis buffer (0.05 M Tris-HCl [pH 8.0], 25% sucrose, 1.5 mg of lysozyme per ml), and incubated on ice for 30 min. Following the addition of 1% Triton X-100 prepared in 0.06 M EDTA, the lysate was kept on ice for 20 min and then centrifuged at $48,000 \times g$ to pellet chromosomal DNA and other cellular debris. Release of the nicked OC DNA from the relaxation complex was facilitated by treating the lysate with sodium dodecyl sulfate (SDS) and proteinase K to a final concentration of 2.5% and 30 μg/ml, respectively, for 15 min at 37°C. SDS was precipitated in the presence of 1 M potassium phosphate and cleared by centrifugation at $5,000 \times g$ for 15 min. The supernatant was extracted once with equal volumes of phenol-chloroform, and the resultant aqueous phase was treated with RNase (20 μg/ml) at 37°C for 30 min, after which the supernatant was precipitated with ethanol in the presence of 2 M ammonium acetate. The pellet was dissolved in 5 ml of TE (0.01 M Tris-HCl [pH 8.0], 0.001 M EDTA) and dialyzed extensively in the same buffer to remove potassium phosphate. The dialysate was subjected to cesium chloride-ethidium bromide density gradient centrifugation (26). The two DNA bands corresponding to the covalently closed circular (CCC) and OC forms of plasmid DNA were visualized by UV illumination, aspirated, extracted with *n*-butanol, and dialyzed against TE to remove ethidium bromide and cesium chloride. Following purification, CCC and OC forms of plasmid DNA were linearized with appropriate restriction enzymes and analyzed on a 0.8% alkaline-agarose gel to determine the number and sizes of single-stranded DNA fragments (22, 23).

Localization of the nick site. In order to localize the nick site more precisely, DNA sequencing of OC forms of plasmid DNA was performed. Gradient-purified, OC plasmid DNA was electrophoresed through a 0.7% agarose gel, cut from the gel, and subjected to electrolution (International Biotechnologies, Inc., New Haven, Conn.) to separate it from small amounts of copurified CCC plasmid DNA. Approximately 10 μg of electroluted DNA was sequenced by the dideoxy chain termination method with primers annealing to complementary strands of DNA located on both sides of the putative nick site. In order to minimize the formation of secondary structures in the region of the nick site, dGTP labeling mixture was replaced with 7-deazaGTP.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank with the accession number U50629.

RESULTS

Identification and nucleotide sequence of a second locus of pGO1 essential for conjugative transfer. Transposition mutagenesis experiments have previously shown that there is one major locus responsible for the conjugative transfer of pGO1. The locus designated *trs*, a 14.4-kb *Bgl*II fragment containing 14 ORFs, has been described in detail in a previous publication (20). No *oriT*-like sequences were noted within *trs* or within contiguous flanking DNA (19). Extension of the original transposon mutagenesis studies identified a second locus, distant

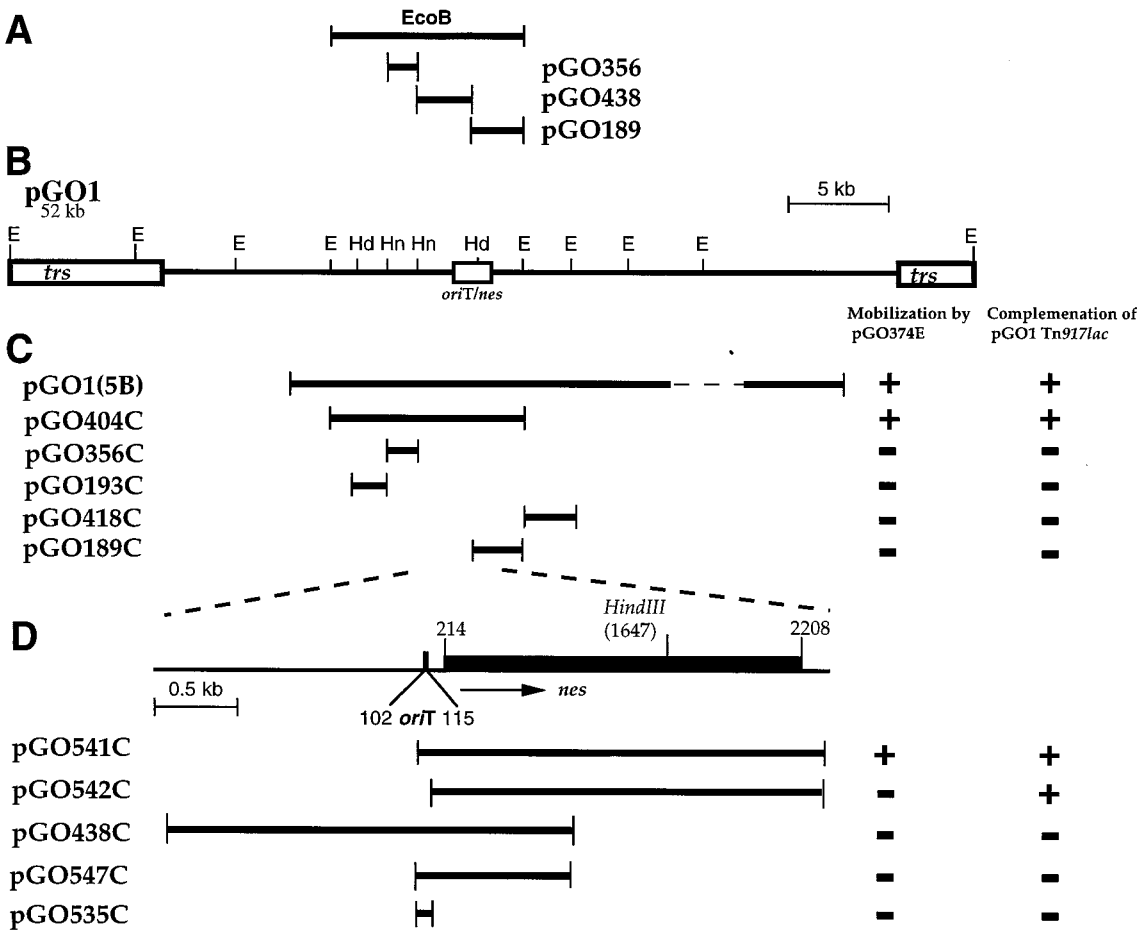

FIG. 1. Schematic representation of pGO1 with results of complementation and mobilization studies. (A) Locations of the EcoB fragment of pGO1 and the *E. coli* subclones used in sequencing to generate the composite sequence presented in Fig. 3. (B) Linear map of pGO1 showing restriction enzyme sites as well as locations of the conjugative transfer genes (*trs*). Abbreviations: E, *Eco*RI; Hn, *Hin*cII; Hd, *Hin*dIII. (C) Clones containing fragments of pGO1 tested in mobilization and complementation studies are listed in the left column. The letter C following the clone designation indicates the addition of pRN5433 to produce a staphylococcal replicon. The first column on the right gives the results of mobilization studies in which subclones were tested for the ability to be mobilized by pGO374E (see the text for a complete description). The second column on the right indicates the results of complementation studies in which subclones were tested for the ability to complement the transfer of pGO1, which had been rendered transfer deficient through Tn*917lac* insertion into *nes*. +, >100 transconjugants per mating; −, 0 to 10 transconjugants per mating. (D) Expanded view of subclones of the EcoB fragment tested in mobilization and complementation studies. The numbers above and below the linear map correspond to the nucleotide sequence presented in Fig. 3. The arrow below the map indicates the direction of transcription of *nes*. Again, the results of mobilization and complementation studies are presented in the two columns on the right.

from *trs*, containing transposon insertions that abolished the conjugative transfer of pGO1. One of these transposon inserts has been briefly described in a previous publication (30). All of the insertions inactivating pGO1 transfer located outside of the *trs* region were contained within a 9.5-kb *Eco*RI fragment designated EcoB (Fig. 1A). Two of the transposon insertions inactivating the conjugative transfer of pGO1 (inserts 249 and 206) (Fig. 2) were contained within a 5.8-kb *Hin*dIII fragment. A third inactivating transposon insert was located just beyond the *Hin*dIII site in a *Hin*dIII-*Eco*RI fragment (insert 233) (Fig. 2). In order to further characterize this area, sequencing was performed on a variety of EcoB subclones (pGO356, pGO438, and pGO189 [Fig. 1A]), generating the composite sequence presented in Fig. 3.

This sequence contains a 1,987-bp ORF, *nes* (for nicking enzyme in staphylococci; see below), that begins with an ATG translational start codon preceded by an appropriately spaced ribosomal binding site. Because of the high AT content of the DNA upstream of the ribosomal binding site, we did not attempt to identify consensus −10 and −35 promoter sequences. However, we know that a strong promoter is present as demonstrated by the production of a large quantity of β-galactosidase from a Tn*917lac* transcriptional fusion (Fig. 2, insert 233), as detailed in a previous publication (30). A putative rho-independent terminator is located immediately downstream of

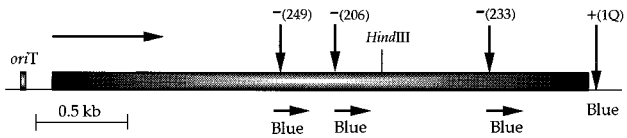

FIG. 2. Results of Tn*917lac* transposon mutagenesis of *nes*. Schematic representation of *nes*; the arrow above the bar indicates the direction of transcription. Vertical arrows above the bar indicate transposon inserts, and their designations are given in parentheses; a − indicates a transposon insertion which abolished the conjugative transfer ability of pGO1, while a + indicates a transposon insert which did not affect transfer ability. Arrows below the bar indicate the direction of transcription of transposon inserts; the color of observed colonies is noted underneath. The nucleotide locations of inserts 249, 233, and 1Q as determined by sequence analysis of PCR products are 1070, 1848, and 2223, respectively (which correspond to the nucleotide sequence given in Fig. 3). Insert 206 was mapped by restriction enzyme digestion analysis and is accurate to within 50 bp.

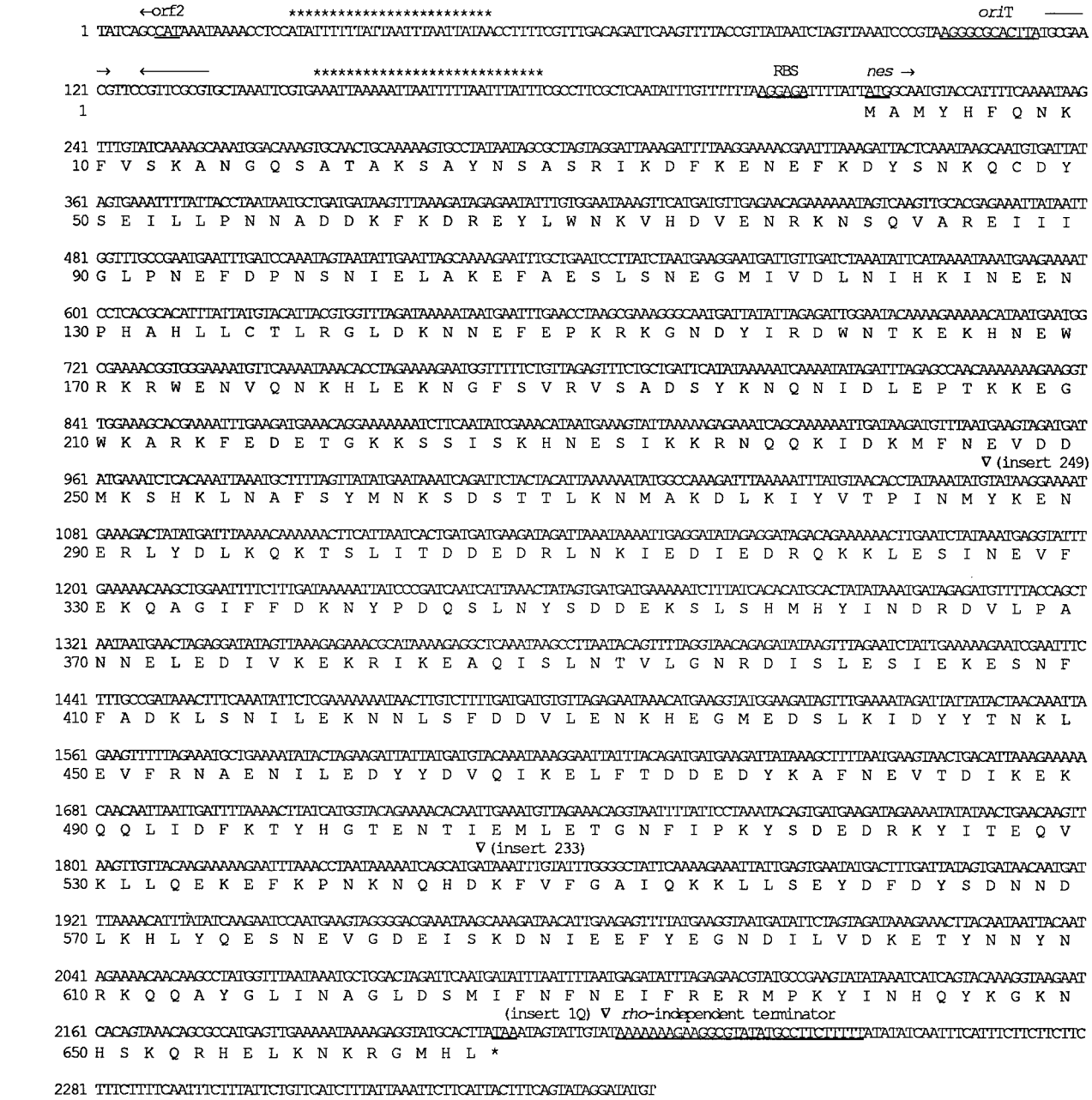

FIG. 3. Complete nucleotide sequence of *nes* (GenBank accession number U50629). The translated amino acid sequence is given below the corresponding DNA sequence. Translational start and stop codons are underlined. Asterisks above the DNA sequence indicate a region high in AT content. Arrows above the DNA sequence indicate positions of inverted repeats. The 12-bp *oriT* site is underlined and designated *oriT*. Immediately preceding the ORF, the putative ribosomal binding site (RBS) is indicated. Next to the ORF is the putative rho-independent terminator, which is underlined. Transposon insertions are indicated by open triangles above the sequence; their designations are in parentheses (Fig. 2).

nes. A minimum free energy of −12.5 kcal/mol is predicted (44). *nes* codes for a predicted 74-kDa protein of 666 amino acids. Approximately 100 bp upstream of *nes* is a 12-bp sequence that is completely homologous to the *oriT* sites of several gram-negative IncQ plasmids. Between the *oriT* site and the *nes* start site is a palindromic sequence with a 7-bp inverted repeat interrupted by the sequence GAA. This arrangement of the *oriT* site with the palindromic sequence is also a feature seen among other *oriT* sites (Fig. 4). The flanking regions upstream and downstream of the *oriT* are AT rich (25 and 28 bp, respectively). These sequences may facilitate strand separation in preparation for nicking at the *oriT* site (17, 39). Ninety base pairs upstream of the *oriT* site and *nes* is a second ORF, which is oriented in the direction opposite that of *nes* (Fig. 3). This second ORF, designated ORF2, does not appear to be essential for conjugation, as shown by mobilization experiments, and its function remains to be elucidated. Interestingly, ORF2 occupies a position similar to those of the genes

FIG. 4. Comparison of the nucleotide sequences of the *oriT* regions of the plasmids pSC101, pTiC58, R1162, pIP501, RSF1010, pTF1, and pGO1. Identical base pairs in the active *oriT* appear in boldface type. Arrows above the DNA sequence represent locations of inverted repeats, which center on the nucleotide sequence GAA in all plasmids except pTiC58. *nic* sites are indicated by the symbol ▼. The putative *nic* site of pGO1 was determined by sequencing of OC plasmid DNA and represents the actual strand which is nicked and complementary to the sequenced strand seen in Fig. 7.

for accessory mobilization protein MobS of pTF1 and MobC of RSF1010 relative to the *oriT* site (9, 29), yet it bears no nucleotide sequence homology to these two genes.

Location of transposon insertions inactivating transfer. Three transposon insertions (inserts 249, 206, and 233) (Fig. 2) that abolished the conjugative transfer ability of pGO1 were contained within the C-terminal 1,138 bp of *nes*. One transposon insert (insert Q) (Fig. 2) located 13 bp beyond the *nes* stop coding sequence did not affect conjugative transfer. This insertion was beyond the *nes* coding sequence and helped define the limits of the gene.

Complementation. The results of complementation studies are presented in Fig. 1C and D. Several subclones of the EcoB fragment were tested for their ability to restore the conjugative transfer ability of pGO1 that had been inactivated by the Tn*917lac* insertion (insert 233) (Fig. 2). Four plasmids demonstrated complementation when provided in trans: pGO1 (5B), a deletion derivative of pGO1; pGO404C; pGO541C; and pGO542C. pGO1(5B) and pGO404C contained the entire EcoB fragment. Two subclones of the EcoB fragment, pGO541C and pGO542C, contained the entire 1,987-bp *nes* gene and upstream sequences, with pGO541C differing from pGO542C only by the presence of the *oriT* site. Several clones having only portions of *nes*, such as pGO438C, pGO547C, and pGO189C, failed to demonstrate complementation, indicating that the entire *nes* is essential for conjugative transfer ability.

Mobilization. We next tested the ability of several subclones to be mobilized by pGO374E. pGO374E contains the entire *trs* region of pGO1 cloned as a 14.4-kb *Bgl*II fragment onto a staphylococcal (pE194)-*E. coli*(pBR322) shuttle plasmid. It has the ability to mobilize small plasmids but is unable to undergo self-transfer. Either deletion derivatives of pGO1 lacking *trs* [pGO1(5B)] or subclones of pGO1 on a pE194-compatible, *S. aureus-E. coli* shuttle plasmid containing the staphylococcal replicon pRN5433 (which encodes chloramphenicol resistance) were introduced into *S. aureus* RN450 containing pGO374E by transduction or electroporation. Two plasmids that contained the entire EcoB fragment, pGO1(5B) and pGO404C, were able to be mobilized by pGO374E. Of the EcoB subclones, only one plasmid (pGO541C) was able to undergo mobilization by pGO374E. The frequencies of mobilization ranged between 10^{-5} and 10^{-7} and were comparable to frequencies of pGO1 transfer (10^{-4} to 10^{-7}). pGO541C contained the *oriT* site, putative *nes* promoter region, and entire 1,987-bp gene. Several plasmids were found to undergo mobilization at a low frequency ($<10^{-8}$), but examination of these transconjugants demonstrated that mobilization occurred through either cointegrate formation or comobilization. pGO542C, which contained the entire *nes* gene but lacked the postulated *oriT* site, was unable to be mobilized by pGO374E, suggesting that the *oriT* site was essential for mobilization and that the *nes* gene product acted upon this site. Clones which contained the *oriT* site and portions of the ORF, namely, pGO438C and pGO547C, were also unable to be mobilized, confirming the requirement of having the entire gene for mobilization.

DNA sequence comparison. Comparison of the nucleotide sequence of the *oriT* locus of pGO1 to other well-characterized *oriT*s (Fig. 4) revealed a 12-bp sequence that is identical to the *oriT* sequence of gram-negative plasmids RSF1010, pTF1, R1162, and pSC101 as well as the recently published *oriT* sequence of the *Streptococcus agalactiae* plasmid pIP501 (4, 7, 9, 36). The *oriT* of pGO1 also showed a high degree of homology to the *oriT* of the *Agrobacterium tumefaciens* plasmid pTiC58, with 11 of 12 bp showing identity (6). An additional similarity was seen by the presence of inverted repeats directly adjacent to the *oriT* site. These inverted repeats are thought to confer secondary structure to the *oriT* region, allowing specific recognition sites for DNA-binding proteins (17). Although all analyzed conjugative or mobilizable plasmids showed differences in the nucleotide sequences of their inverted repeats, the palindromes were in similar locations relative to *oriT*. In addition, the inverted repeats of RSF1010, pTF1, R1162, pIP501, and pGO1 all centered on the nucleotide sequence GAA.

The amino acid sequence of *nes* was also similar to sequences of several mobilization proteins of conjugative and mobilizable plasmids. In Fig. 5 the *nes* amino terminus is compared with those of three mobilization proteins: MobL of pTF1, MobA of RSF1010, and CnjA of pIP501. The protein encoded by *nes* demonstrated 31% identity in its initial 155 amino acids to the CnjA protein of pIP501. CnjA has been shown to have endonuclease activity and is encoded by the first ORF adjacent to the *oriT* site of pIP501 (36). In similar fashion, the *nes* gene product showed significant homology to the proteins involved with nicking at the *oriT* of the gram-negative mobilizable plasmids RSF1010 and pTF1. The *nes* gene product showed 32.8% identity in the first 125 amino acid to the MobL protein of pTF1 and 25.8% identity in its first 213 amino acids to MobA of RSF1010.

Demonstration of nicking at the *oriT* site. Specific single-strand nicking mediated by the gene product of *nes* at the *oriT* site was demonstrated by analyzing pGO541, the clone containing both *nes* and *oriT*, in *E. coli*. Following gentle lysis of an *E. coli* strain containing the plasmid, OC DNA was separated from the unnicked CCC form by cesium chloride-ethidium bromide density gradient centrifugation. Both OC and CCC DNAs were digested with *Eco*RI and run on an alkaline gel to separate the two DNA strands. The OC form produced three DNA bands. The upper band of 4.9 kb was a single linear strand, corresponding in size to the only band seen with the CCC DNA. The two smaller bands, representing the two frag-

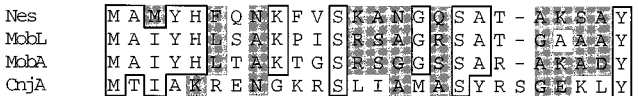

FIG. 5. Comparison of N-terminal amino acids of four mobilization proteins. The predicted amino acid sequences of pGO1 Nes, pTF1 MobL, RSF1010 MobA, and pIP501 CnjA are presented with aligned regions of similarity. Identical amino acids are boxed, while similar amino acids appear shaded.

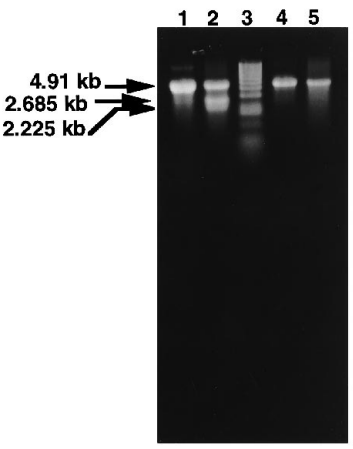

FIG. 6. Analysis of *Eco*RI-digested OC and CCC plasmid DNAs of pGO541 and pGO542 on an alkaline agarose gel. Lanes 1 and 2 contain *Eco*RI-digested CCC and OC forms of pGO541 plasmid DNA, respectively; lane 3 contains the DNA sizing ladder; and lanes 4 and 5 contain *Eco*RI-digested OC and CCC forms of pGO542 plasmid DNA, respectively. Arrows indicate single-stranded DNA fragments generated after *Eco*RI digestion. The sizes of these fragments are indicated to the left of the arrows.

ments produced from the nicked linear strand, were 2.685 and 2.225 kb, corresponding in size to the distance from the *Eco*RI site at each end of the fragment to the single-strand nick at the *oriT* site. The same analysis performed on pGO542, the clone containing *nes* without *oriT*, showed a single 4.9-kb band, demonstrating that the *nes* gene product mediates site-specific nicking of a phosphodiester bond within the *oriT* site. This is shown in Fig. 6.

The exact site of the nick was shown by sequencing the OC form of pGO541. With primers specific for sequences on complementary strands of DNA on either side of the *oriT*, it was shown that the sequencing reaction stopped precisely within the *oriT* sequence on the nicked strand of the OC plasmid DNA. The complete sequence was generated on the complementary OC strand and on both strands of CCC DNA. This demonstrates the site- and strand-specific cleavage at the pGO1 *oriT*. The site-specific cleavage sequence is shown in Fig. 7 and indicated by the triangles in Fig. 4.

DISCUSSION

In this study we identified a DNA sequence on the staphylococcal conjugative plasmid pGO1 that was identical to the sequences of *oriT* sites of the well-characterized conjugative or mobilizable plasmids pSC101, R1162, RSF1010, pTF1, and pIP501 (2, 4, 7, 9, 36). Adjacent to this *oriT* was a 1,987-bp ORF that we have designated *nes*. The following evidence confirms that the *oriT* site identified is the origin of conjugal transfer of pGO1 and that *nes* encodes an *oriT*-specific endonuclease required for conjugative transfer. First, the requirement of *nes* and its gene product for conjugative transfer was shown by localizing transposon insertions within the *nes* coding sequence that abolished pGO1 transfer and by the ability of *nes*, when provided in *trans* on a separate replicon, to restore the conjugation proficiency to these transfer-deficient derivatives of pGO1. Second, the functional significance of *nes* and the putative *oriT* site was documented by showing that the *trs* cluster of conjugation genes, incapable of self-transfer, could mobilize a plasmid containing *oriT* and *nes* but not a plasmid from which *oriT* or portions of *nes* were deleted. Finally, the site and strand-specific nature of *nes*-mediated *oriT* cleavage was demonstrated by alkaline gel analysis and DNA sequencing of OC DNA, which showed a "pileup" of labeled nucleotides at a precise location within the *oriT* site.

Nes appears to act in a fashion similar to that of the mobilization proteins seen in the gram-negative plasmids RSF1010 and pTF1 and in the gram-positive plasmid pIP501. MobA of the *E. coli* plasmid RSF1010 is the best characterized among the mobilization proteins of the IncQ plasmids (1, 28, 29). MobA is either a bifunctional protein or two independent proteins (28). The N-terminal portion contains relaxase or endonuclease activity and can both cleave and rejoin single-stranded DNA containing the *oriT* site (1, 28). The carboxy terminus exhibits primase activity and is a functional analog of RP4 TraJ, which acts by binding to the *oriT* site, the initial step in relaxosome complex formation (43). The endonuclease activity of both MobA and CnjA, the nicking enzyme of pIP501, persist in C-terminally truncated proteins. *nes* may encode a bifunctional protein as well, as the protein sequences of Nes and MobA (Fig. 5) are quite similar. Confirmation of this hypothesis awaits the construction of specific deletions of *nes.*

The functional domain of Nes also appears to be very similar to the relaxase proteins RSF1010 MobA, pTF1 MobL, and pIP501 CnjA. The relaxase activity of the MobA protein has been shown to involve a specific tyrosine in the N-terminal portion of the protein. The side chain of tyrosine 24 of this protein forms a phosphodiester link with the 5′ phosphate of the terminal nucleotide of the transfer-strand DNA (28). A structural analysis of the N-terminal amino acids shows that the relaxases of pTF1, pIP501, and pGO1 all have predicted tyrosine residues which may be involved in relaxase activity

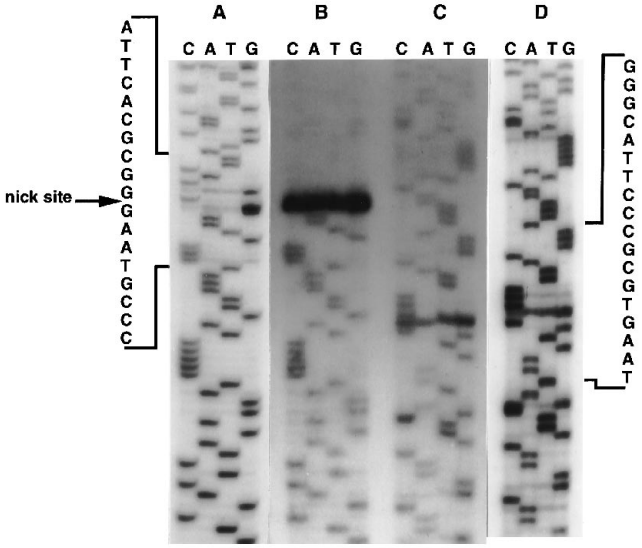

FIG. 7. Mapping of the 5′ end of the single-stranded nick at the putative *oriT* site. Gels A and D contain the sequencing ladders generated by sequencing both strands of the CCC form of pGO541 plasmid DNA. The reverse primer of pUC19 (located 40 bp upstream from the *Eco*RI site in the multiple cloning sequence) was used to generate the sequence of the noncoding strand 5′ to the nick site and a second primer (5′-CAAATATTGAGCGAAGG-3′, complementary to nucleotides 193 to 177 [Fig. 3]) to obtain the sequence of the coding strand 3′ to the nick site. Gels B and C show the DNA sequences obtained by sequencing both strands of the OC form of pGO541. The sequence of the *oriT* site is indicated (5′ to 3′, bottom to top) on the left of gel A, and its complement is indicated on the right of gel D. The arrow marks the proposed nick site and indicates the position of the nucleotide in gel B at which the DNA sequence begins to pile up because of the single-strand interruption. Because there is compression within the sequence, the actual nick site may be 1 to 2 bp beyond this nucleotide.

(Fig. 5): tyrosine 25 of CnjA (pIP501) and tyrosines 24 of both MobL (pTF1) and Nes (pGO1). All four proteins demonstrate significant homology in their N termini, suggesting that the relaxase activity of these mobilization proteins is well conserved among both gram-negative and gram-positive systems. In addition, all four proteins are encoded by the first ORF adjacent to the *oriT* site (7, 9, 36). Despite the fact that all four of these proteins demonstrate significant homology in their N-terminal portions, they demonstrate no homology in their C termini. This is consistent with a host-specific role for the C terminus-encoded portion of a bifunctional protein, a role making this function accessory to the protein's major endonuclease activity.

This study adds further evidence that pGO1 transfers from donor to recipient cells as a single strand of DNA, conforming to the model established for the conjugative transfer of gram-negative plasmids. Previously, indirect evidence supporting single-stranded transfer came from the observation that pGO1-mediated transfer of the mobilizable plasmid pC221 involved conversion of pC221 to the OC form and that one of two genes required for mobilization, *mobA*, encoded nicking activity (24). The structural similarities of *oriT* of pGO1 and the relaxase protein Nes to other *oriT* and relaxase proteins of well-characterized gram-negative plasmids suggest that the DNA transfer occurs by single-stranded transfer in all of these plasmids.

The plasmid location of the *oriT*-relaxase locus relative to the location of the conjugative transfer gene cluster on pGO1 is unique in comparison with the locations of the corresponding loci on other conjugative plasmids. In the gram-negative plasmids F and RP4, as well as in the gram-positive plasmid pIP501, the *oriT* and relaxase genes are contiguous to the other conjugative genes. In contrast, the conjugative transfer genes of pGO1 (*trs*) are located on a 14.4-kb DNA segment which is approximately 13 kb in the 5′ direction and 24 kb in the 3′ direction from *nes* and the *oriT* site. The physical separation of *trs* and *oriT* in pGO1 may be related to the cassette-like formation of staphylococcal plasmids. A recent study analyzing the construction of naturally occurring conjugative mupirocin resistance plasmids provided an illustration of the way that pGO1-like plasmids may have evolved (21). pGO1 contains eight copies of a directly repeated insertion (IS) element, IS*257*, that flanks discrete segments of DNA containing either entire integrated plasmids or antimicrobial resistance genes. Presumably, the IS elements mediated acquisition of DNA by transpositional cointegration or homologous recombination with other plasmids containing the elements. The conjugative mupirocin plasmids, however, contained only the *trs* region flanked by IS elements, the mupirocin resistance gene flanked by a third IS element, and the EcoB fragment containing *nes*, *oriT*, and, presumably, the origin of vegetative replication. Thus, without resistance genes and other DNA acquired by an IS-mediated mechanism, the minimal conjugative replicon would consist of the 14.4-kb *trs* region, the approximately 2.2-kb *oriT-nes* region, and an origin of replication. The separation of these two regions by acquisition of intervening DNA may also illustrate the evolution of independent function. As previously shown, the *trs* region is under the partial negative transcriptional regulation of a gene within *trs*, namely, *trsN*, and most of the *trs* transcripts, therefore, are low in amount (30). In contrast, *nes* is independent of *trsN* regulation and produces abundant levels of transcript and β-galactosidase from *nes-lacZ* fusions. The regulation of these two regions and the relationship between the production of their gene products during the growth and mating cycles are under active investigation.

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