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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Received 25 March 1996/Accepted 13 June 1996

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, pTFI, R1162, pSC101, and pIPS01. 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 a well-conserved element 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 Tn916, 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 BglII 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,
**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Remark(s) (reference or source)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB1</td>
<td>recA&lt;sup&gt;+&lt;/sup&gt; lacP&lt;sup&gt;IZ&lt;/sup&gt;AM15</td>
<td>(this study)</td>
</tr>
<tr>
<td>JM109</td>
<td>recA&lt;sup&gt;+&lt;/sup&gt; endA&lt;sup&gt;+&lt;/sup&gt; gyr&lt;sup&gt;-96&lt;/sup&gt; thi-bsdR17&lt;sup&gt;1&lt;/sup&gt; (T&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt; m&lt;sub&gt;K&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt; supE44 relA&lt;sup&gt;-1&lt;/sup&gt; lac&lt;sup&gt;-&lt;/sup&gt; (lac-proAB) [F&lt;sup&gt;+&lt;/sup&gt; traD36 proAB lacP&lt;sup&gt;IZ&lt;/sup&gt;AM15]</td>
<td>Recombinant-deficient host</td>
</tr>
<tr>
<td><strong>S. aureus strains</strong></td>
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<td>RN450</td>
<td>Antiobiotic-susceptible host</td>
<td>ATCC 8325-4 cured of phi1, phi2, and phi3</td>
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<td>RN4220</td>
<td>Restriction deficient</td>
<td>Shuttle plasmid host (16)</td>
</tr>
<tr>
<td>RN4220SS</td>
<td>St&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Mating recipient</td>
</tr>
<tr>
<td>RN4220NR</td>
<td>N&lt;sup&gt;+&lt;/sup&gt; R&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Mating recipient (16)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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</tr>
<tr>
<td>pUC18</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 2.7 kb</td>
<td>Cloning vector</td>
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<td>pUC19</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 2.7 kb</td>
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<td>pBR322</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Te&lt;sup&gt;-&lt;/sup&gt;, 4.3 kb</td>
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<td>pOP203(A&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Te&lt;sup&gt;-&lt;/sup&gt;, 7.0 kb</td>
<td>Cloning vector (40)</td>
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<td>pRN5433</td>
<td>Cm&lt;sup&gt;-&lt;/sup&gt;, 3.0 kb</td>
<td>pSK265 with 5' HindIII site in multiple cloning site deleted (25)</td>
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<tr>
<td>pE194</td>
<td>Em&lt;sup&gt;-&lt;/sup&gt;, 3.0 kb</td>
<td>Staphylococcal replicon (12)</td>
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<tr>
<td>pGO1</td>
<td>Gm&lt;sup&gt;-&lt;/sup&gt;, Tp&lt;sup&gt;-&lt;/sup&gt; Qa&lt;sup&gt;-&lt;/sup&gt;, Neo&lt;sup&gt;-&lt;/sup&gt; Trs&lt;sup&gt;-&lt;/sup&gt;, 52 kb</td>
<td>Conjugative plasmid recovered from an S. aureus clinical isolate (32)</td>
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<td>pGO1(5B)</td>
<td>Gm&lt;sup&gt;-&lt;/sup&gt;, Trs&lt;sup&gt;-&lt;/sup&gt;, 24 kb</td>
<td>Deletion mutant of pGO1 (this study)</td>
</tr>
<tr>
<td>pGO188</td>
<td>Em&lt;sup&gt;-&lt;/sup&gt;, Gm&lt;sup&gt;-&lt;/sup&gt;, Tp&lt;sup&gt;-&lt;/sup&gt;, Neo&lt;sup&gt;-&lt;/sup&gt; Trs&lt;sup&gt;-&lt;/sup&gt;, 52 kb</td>
<td>pGO1 with Trs171lac insert in nes (this study)</td>
</tr>
<tr>
<td>pGO189</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Te&lt;sup&gt;-&lt;/sup&gt;, 7.8 kb</td>
<td>3.5-kb EcoRI-BamHI fragment of pGO188 cloned to pBR322 (21)</td>
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<td>pGO193C</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, 4.4 kb</td>
<td>1.7-kb HindIII fragment of pGO1 cloned to pUC18 with pRN5433 added (this study)</td>
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<td>pGO356</td>
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<td>1.3-kb HindIII fragment of pGO1 cloned to pUC19 (this study)</td>
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<tr>
<td>pGO374E</td>
<td>Em&lt;sup&gt;-&lt;/sup&gt;, Te&lt;sup&gt;-&lt;/sup&gt;, Trs&lt;sup&gt;-&lt;/sup&gt;, 17.4 kb</td>
<td>14.4-kb BglII fragment of pGO1 containing conjugative transfer region (trs) cloned to pBR322 at the BamHI site with pE194 added to the CiaI site (this study)</td>
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<tr>
<td>pGO400</td>
<td>Mp&lt;sup&gt;-&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, Trs&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Yale muipirinoc resistance plasmid containing BglA and B fragments and two additional BglI fragments of pGO1 (21)</td>
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<tr>
<td>pGO404C</td>
<td>Te&lt;sup&gt;-&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, 16.5 kb</td>
<td>9.5-kb EcoRI-BamHI fragment of pGO400 cloned to pOP203(A&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;+&lt;/sup&gt; with pRN5433 cloned to Srl site (this study)</td>
</tr>
<tr>
<td>pGO418C</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, 10.1 kb</td>
<td>4.4-kb EcoRI fragment of pGO1 cloned to pUC19 with pRN5433 cloned at Srl site (this study)</td>
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<td>pGO438</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, 4.6 kb</td>
<td>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 HincHI site of pUC19 (this study)</td>
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<td>pGO535C</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, 5.85 kb</td>
<td>135-bp PCR fragment of pGO1 containing oriT site cloned to Smal site of pUC19 with pRN5433 cloned to HindIII site (this study)</td>
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<td>pGO541C</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, 7.9 kb</td>
<td>2.24-kb PCR fragment of pGO1 containing oriT site, promoter region, and nes cloned to pUC19 at the Smal site with pRN5433 cloned to the BamHI site (this study)</td>
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<td>pGO542C</td>
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<td>2.18-kb PCR fragment of pGO1 containing promoter region and nes cloned to pUC19 at the BamHI-Smal site with pRN5433 cloned to the BamHI site (this study)</td>
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<td>pGO547C</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Cm&lt;sup&gt;-&lt;/sup&gt;, 6.4 kb</td>
<td>700-bp PCR fragment of pGO1 containing oriT site and first 550 bp of nes cloned to BamHI-Smal site of pUC19 with RN5453 cloned to BamHI site (this study)</td>
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</table>

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<sub>3</sub>)<sup>+</sup> (40). *E. coli* and *S. aureus* shuttle vectors were constructed by adding either of two staphylococcal replications, 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 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 (5 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* by the method of the Gene Pulser (Bio-Rad, Richmond, Calif.) with the following settings: 200 V, 25 μF, and 2.5 kV for *E. coli* and 400 V, 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 primer pairs were designed to complement pGO1 nucleotide sequences (Oligo Etc., Willows, Ore.) and 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 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′ BamHI site; 5′-CGGGATCCTAGTTAAATCCCG -3′ [see Fig. 3, nucleotides 87 to 104]) and primer 268 (includes a 5′ GGAATTCTCGAG -3′ site; 5′-CGGATACGAAACAAATTGTGTTGG -3′ [see Fig. 3, nucleotides 130 to 146]) and primer 268. The 135-bp fragment containing the oriT site was generated with primers 245, 5′-CA GATTCGTTTGAAGGTTAC-3′ (see Fig. 3, nucleotides 61 to 79), and primer 241, 5′-CGGAATTCGAAATGGTG-3′ (see Fig. 3, nucleotides 193 to 210). The PCR fragment of pGO438 was generated with primer 153, 5′-GGAATTCTGTTGGTGTCACCCG-3′, and primer 217, 5′-GGACGAAATCTCAGAGC-3′ (see Fig. 3, nucleotides 788 to 775). The 700-bp fragment of pGO574 was generated with primer 201 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 the nes gene. A 10-μl filter lysate containing the donor cells was spotted onto a 13-mm-diameter, 0.45-μm-pore-size nitrocellulose filter. After overnight incubation on BHI agar, 100-μl samples were removed from the center of the filter with a sterile loop and spread into a 10-ml BHI culture of RN4220 NR, followed by addition of 10 ml of ice-cold 48,000 × g supernatant of the BHI culture of RN4220 NR. Colonies that failed to yield transconjugants were picked with erythromycin to identify plasmids containing transposon insertions that did not affect conjugation. Colonies that failed to form transconjugants by the cetyltrimethylammonium bromide lysis method of *S. aureus* were also localized. The orientations of *Trn*9lac fusions were assessed by plating cells on media containing β-galactosidase and chloramphenicol. Colonies that turned blue were considered to have the correct orientation of the nes 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 insertions. The precise location of *Trn*9lac insertions was further determined by sequencing the DNA at the insertion site with one primer that represented one end of *Trn*9lac (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 Trn9lac 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 (United States Biochemical Corp., Cleveland, Ohio) as well as by automated sequencing with a T7 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 lysing buffer (0.05 M Tris-HCl [pH 8.0], 25% sucrose, 1.5 mg of lysosome 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 × 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 × 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 ng/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 bands corresponding to the open circular (CC) 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 isolated with appropriate restriction enzymes as defined in a previous publication (31) andasecondprimerderivedfromcomplementaryDNAfragments. 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 × 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 ng/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 bands corresponding to the open circular (CC) 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 isolated with appropriate restriction enzymes as defined in a previous publication (31) andasecondprimerderivedfromcomplementaryDNAfragments.
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 EcoRI 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 HindIII fragment. A third inactivating transposon insertion was located just beyond the HindIII site in a HindIII-EcoRI 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. 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.

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 Tn917lac transcriptional fusion (Fig. 2, insert 233), as detailed in a previous publication (30). A putative rho-independent terminator is located immediately downstream of

![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, EcoRI; Hn, HindII; Hd, HindIII. (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 Tn917lac 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.

![FIG. 2. Results of Tn917lac 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.](http://jb.asm.org/Downloaded_from)
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
for accessory mobilization protein MobL 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 conjugal 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 conjugal 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 conjugal transfer ability of pGO1 that had been inactivated by the Tn917lac 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 containing only portions of *nes*, such as pGO438C, pGO547C, and pGO189C, failed to demonstrate complementation, indicating that the entire *nes* is essential for conjugal transfer ability.

**Mobilization.** We next tested the ability of several subclones to be mobilized by pGO374E. pGO374E contains the entire *trス* region of pGO1 cloned as a 14.4-kb BgII fragment onto a staphylococcal (pEI194)-*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 *trス* [pGO1(5B)] or subclones of pGO1 on a pEI194-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 10⁻⁷ and 10⁻⁵ 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 co-integrate 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 sequences (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 of 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 EcoRI 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-
ments produced from the nicked linear strand, were 2.685 and 2.225 kb, corresponding in size to the distance from the EcoRI 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 conjugal transfer. First, the requirement of nes and its gene product for conjugal 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.
tyrosine 25 of CjujA (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 orfT 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 conjugal 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 orfT of pGO1 and the relaxase protein Nes to other orfT 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 location of the orfT-relaxase locus relative to the location of the conjugal transfer gene cluster on pGO1 is unique in comparison with the locations of the corresponding loci on other conjugal plasmids. In the gram-negative plasmids F and RP4, as well as in the gram-positive plasmid pIP501, the orfT and relaxase genes are contiguous to other conjugal genes. In contrast, the conjugal 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 orfT site. The physical separation of trs and orfT in pGO1 may be related to the cassette-like formation of staphylococcal plasmids. A recent study analyzing the construction of naturally occurring conjugal 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, IS257, 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 the plasmid containing the elements. The conjugal transfer of 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, orfT, and, presumably, the origin of vegetative replication. Thus, without resistance genes and other DNA acquired by an IS-mediated mechanism, the minimal conjugal replicon would consist of the 14.4-kb trs region, the approximately 2.2-kb orfT-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.

ACKNOWLEDGMENT

These studies were supported, in part, by Public Health Service grant R37 AI35705 from the National Institute of Allergy and Infectious Diseases.

REFERENCES


