Transcriptional Regulation by TrsN of Conjugative Transfer Genes on Staphylococcal Plasmid pGO1

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The major conjugative transfer gene cluster of staphylococcal plasmid pGO1 (trs) consists of 13 open reading frames (trsA to trsM) transcribed from one DNA strand and a single 189-bp open reading frame (trsN) within the first 348 bp of trs that is transcribed divergently. Promoter regions for trsN and trsA partially overlap. TrsN, a 7,181-bp protein, was purified as a fusion to glutathione S-transferase and found to have DNA-binding activity. Increasing concentrations of the fusion protein progressively retarded the gel migration of PCR-generated DNA fragments containing predicted promoters 5' to trsL, trsA, and trsN. The target sequences contained areas of identity, including regions of dyad symmetry, that were protected in D EcoI footprinting studies. The binding of TrsN to its trsL target was required for this target DNA to be stably introduced into Staphylococcus aureus on a high-copy-number vector. Provision of excess TrsN from this high-copy-number vector in S. aureus decreased β-galactosidase activity from a trsL-lacZ transcriptional fusion and decreased pGO1 conjugation frequency. Conversely, both transcription and conjugation increased in the presence of excess trsL target. We propose that TrsN negates the transcription of genes essential for conjugative transfer by binding to regions 5' to their translational start sites.

Negative regulation of genes involved in conjugative transfer is a common theme among plasmids from both gram-negative and gram-positive bacteria. On plasmids of the IncF incompatibility group resident in Escherichia coli, the fimO gene product encoded at the 3' end of the F tra operon acts in concert with the fimP product, an RNA regulatory molecule, to repress trsA, a positive regulator of traYZ transcription (12, 29). Similarly, genes in the Tra2 region of IncP plasmids, found among a broad range of gram-negative bacterial hosts, seem to be negatively regulated by products of the korA and korB genes present on the same plasmid (16, 17). Regulation of genes associated with conjugative plasmid transfer in gram-positive bacteria has also been demonstrated. Production of plasmid-encoded aggregation substance by enterococci in response to pheromones is under both negative and positive control, and conjugative transfer genes in Streptomyces species are negatively regulated (7, 10, 23). However, genes regulating conjugative transfer of staphylococcal plasmids have not been identified.

Staphylococci carry a group of related conjugative plasmids encoding resistance to antimicrobial agents. These plasmids are widely prevalent in clinical staphylococcal isolates and can transfer between different staphylococcal species (2). A representative conjugative plasmid, pGO1, is 52 kb in size and encodes resistance to aminoglycosides, trimethoprim, and quaternary ammonium compounds (2, 18). It contains nine copies of an insertion sequence (IS257) that bounds antibiotic resistance and conjugative transfer genes (5, 18, 20). The major cluster of transfer-associated genes on pGO1 has been localized, and the nucleotide sequence has been determined (20). This region, designated trs, is 13,612 bp in length and contains 14 open reading frames (ORFs) (trsA to trsN). trsA to trsM are transcribed in the same direction on one DNA strand, whereas trsN, a 189-bp ORF, is transcribed divergently and has a promoter region partially overlapping that of trsA. We had earlier observed that certain trs subclones could not be introduced into Staphylococcus aureus by protoplast transformation on a high-copy-number shuttle replicon after initial cloning in E. coli (26). This unstable or untransformable DNA was located within a 4.6-kb HindIII fragment (pGO210) containing the 3' end of trs. However, this DNA could be stably transformed into cells that contained either the parent replicon (pGO1) or a deletion derivative of the trs complex composed of a fusion of the 5' 2.3 kb with the 3' 1.3 kb of trs cloned on an E. coli-S. aureus shuttle vector. The following study arose from the characterization of the trans-acting factor that stabilized target trs DNA for transformation and led to the discovery that trsN encodes a DNA-binding protein that negatively regulates trs transcription.

MATERIALS AND METHODS

Bacterial strains and plasmid constructs. DNA from pGO1 was cloned on one of the three E. coli vectors pBR322 (4), pUC19 (30), or pBluescript (Stratagene, La Jolla, Calif.) (25) and introduced into E. coli HB101 (5) (pBR322), TB1 (30) (pUC19) or XL1-Blue (25) (pBluescript). E. coli-S. aureus shuttle vectors were constructed by adding either of two staphylococcal replicons, pSK265 (13), encoding chloramphenicol resistance (Cm'), or pE194 (11), encoding erythromycin resistance (Em'), at an appropriate restriction site on the E. coli replicon. The construct was confirmed by expression of either Cm' or Em' in E. coli before introduction into S. aureus RN4220 by electroporation. The letter C or E after the numbered plasmid designation indicates a shuttle construct containing either pSK265 or pE194, respectively. RN4220 is a restriction-deficient derivative of RN450 (14, 21). After growth in RN4220, plasmids could then be introduced into other staphylococcal strains with intact restriction-modification sys-
TABLE 1. Plasmids constructed for this study

<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>Description</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGO210</td>
<td>4.6-kb HindIII subclone of pGO200 on pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pGO305C</td>
<td>pBR322 ligated to pSK265 at the EcoRI site; shuttle vector</td>
<td>This study</td>
</tr>
<tr>
<td>pGO344</td>
<td>1.3-kb HindIII-SphI subclone of pGO210 on pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pGO345</td>
<td>3.3-kb SphI-HindIII subclone of pGO210 on pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pGO357C</td>
<td>pGO210 derivative with interruption of trsL at NcoI site created by cloning pSK265 shuttle replicon into NcoI site</td>
<td>This study</td>
</tr>
<tr>
<td>pGO358C</td>
<td>pGO210 derivative with interruption of trsM at XbaI site created by cloning pSK265 shuttle replicon into XbaI site</td>
<td>This study</td>
</tr>
<tr>
<td>pGO200Δ1E</td>
<td>12.4-kb deletion derivative of pGO200E created when shuttle plasmid was introduced into S. aureus RN4220 by protoplast transformation; contains pBR322 (4.4-kb), pEI194 (3.7-kb) and 4.3 kb of the 14.5-kb BglII fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pGO352</td>
<td>329-bp PCR fragment containing the trsN ORF and 81 bp of 5' sequences cloned into pUC19 Smal site</td>
<td>This study</td>
</tr>
<tr>
<td>pGO187</td>
<td>772-bp EcoRI-BglII subclone of pGO200 cloned on Bluescript KS+; contains trsN ORF plus 161 bp of 3' sequences, 172 bp of 5' sequences, and 246 bp of the divergently transcribed trsA ORF</td>
<td>This study</td>
</tr>
<tr>
<td>pGO364</td>
<td>352-bp PCR fragment including 46 bp of the 3' end of trsK, the 76-bp region between trsK and lrsL, and the 5' 230 bp of the trsL ORF, cloned on pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pGO172</td>
<td>6,254-bp EcoRI trs fragment containing the 3' 726 bp of trsA, trsB-trsF, and the 5' 712 bp of trsG, cloned on pBluescript KS+</td>
<td>This study</td>
</tr>
<tr>
<td>pGO1-4A</td>
<td>pGO1: Tn917lac(trsL); oriented in the direction of lacZ transcription</td>
<td>20</td>
</tr>
<tr>
<td>pGO1-2P</td>
<td>pGO1: Tn917lac(trsL); oriented in the direction of lacZ transcription</td>
<td>20</td>
</tr>
<tr>
<td>pGO1-43</td>
<td>pGO1: Tn917lac(trsK); oriented in the direction of lacZ transcription</td>
<td>20</td>
</tr>
<tr>
<td>pGO1-62</td>
<td>pGO1: Tn917lac(trsO); oriented in the direction of lacZ transcription</td>
<td>20</td>
</tr>
<tr>
<td>pGO1-233</td>
<td>pGO1: Tn917lac(ORF U); oriented in the direction of lacZ transcription</td>
<td>20</td>
</tr>
<tr>
<td>pGO463</td>
<td>250-bp PCR fragment containing the trsN ORF (no promoter) cloned into the BamHI site of pGEX-3X</td>
<td>This study</td>
</tr>
<tr>
<td>pGO472Ets</td>
<td>pUC19-5E194 temperature-sensitive delivery vehicle containing trsN interrupted by tetM</td>
<td>This study</td>
</tr>
</tbody>
</table>

The plasmids constructed for this study are listed in Table 1.

Selective media. Clones were selected in E. coli cultured on medium containing ampicillin (50 µg/ml), and shuttle constructs were confirmed in E. coli cultured on medium containing chloramphenicol (40 µg/ml) or erythromycin (500 µg/ml). Colonies containing DNA introduced into S. aureus by electroporation were selected on medium containing chloramphenicol (10 µg/ml) or erythromycin (10 µg/ml). Selective agar for mating contained combinations of gentamicin, novobiocin, and rifampin (all 5 µg/ml). Tn917lac transposition was confirmed by culture on agar containing chloramphenicol (40 µg/ml) or erythromycin (10 µg/ml). All agar containing antibiotics was Mueller-Hinton agar (BBL, Cockeysville, Md.); agar for incubation of donors, recipients, and filters containing mating pairs was brain heart infusion (Difco, Detroit, Mich.); and broth for overnight incubation was brain heart infusion for staphylococci or Luria broth (Gibco-BRL, Gaithersburg, Md.) for E. coli.

DNA manipulation. DNA fragments were cloned into E. coli vectors by techniques described by Sambrook et al. (24). Blunt-end ligations were performed after filling in 5' overhangs with the Klenow fragment of DNA polymerase III (Gibco-BRL). Clones were introduced into either E. coli or S. aureus by electroporation by previously described methods (20). DNA sequence was determined by the Sanger technique from a double-stranded template with progress by using oligonucleotide primers, as previously described (20).

Conjugation. The techniques for mating have been described previously (20). Donors were usually selected on gentamicin, recipients were selected on novobiocin and rifampin, and transconjugants were selected on all three. Transfer frequency was determined by enumerating donors and transconjugants and determining the transconjugant-to-donor ratio.

Transposon insertion mutagenesis. Tn917 insertions were generated in the recombination-deficient strain RN1030 and were described in a previous publication (26). β-Galactosidase fusions were generated by using Tn917lac carried on the temperature-sensitive plasmid pTV32ts, a replicon designed for creating β-galactosidase transcriptional fusions in Bacillus subtilis (32). This transposon has been used for creating useful fusions in both streptococci (28) and staphylococci (20). The technique for creating and identifying appropriate inserts into the trs region of pGO1 in S. aureus has been described previously (20).

β-Galactosidase assay. A modification of the method described by Miller (19) was used to assay β-galactosidase activity. An overnight culture was diluted in 50 ml of pre-warmed Mueller-Hinton broth containing the appropriate antibiotic(s) and grown to an A600 of 0.6 to 0.8. Cells were collected by centrifugation, resuspended in half the original volume of Z-buffer (60 mM Na2HPO4·7H2O, 40 mM NaH2PO4·H2O, 10 mM KCl, 1 mM MgSO4·7H2O, 50 mM β-mercaptoethanol [pH 7.0]), and centrifuged again. The cell pellet was resuspended in 1 ml of Z-buffer containing 2.5 g of 0.1-mm-diameter zirconium beads. Cells were disrupted by agitating the tube at the top speed for 3 min in a mini-bead beater (BioSpec Products, Bartlesville, Okla.). Cell debris was removed by centrifugation in a microcentrifuge at 14,000 × g for 5 min, and 0.2 ml of the supernatant was added to 0.8 ml of Z-buffer. After equilibration of the cell supernatant at 30°C for 5 min, 0.2 ml of ONPG (o-nitrophenyl-β-d-galactopyranoside), prepared at 4 mg/ml in A-buffer [60 mM K2HPO4, 3.3 mM KH2PO4, 7.5 mM (NH4)2SO4, 0.17 mM sodium citrate], was added, and incubation was continued for 20 min at 30°C. The reaction was stopped by adding 0.5 ml of 1 M Na2CO3, and the A550 and A420 of the samples was measured. β-Galactosidase produced was expressed as units per milligram of protein.

PCR amplification. Amplification of DNA sequences by PCR was performed with primers synthesized to match 5' and 3' sequences under conditions described previously (20). The resultant fragments were blunt-end ligated into the Smal site of pUC19.

Production of a glutathione S-transferase (GST)-TrsN fusion protein. A 250-bp fragment containing the trsN coding...
sequence was generated from pGO1 by PCR amplification, resolved on a 3% agarose gel, and recovered by electroelution (International Biotechnologies, Inc., New Haven, Conn.) and ethanol precipitation. This fragment was cloned into the expression vector, pGEX-3X (Pharmacia LKB Biotechnology, Piscataway, N.J.), and the appropriate translational fusion between the 3′ end of the gene encoding GST and the 5′ end of trsN was confirmed by DNA sequencing. This plasmid was designated pGO463. A 50-ml sample of an overnight culture of *E. coli* TB1 containing either pGEX-3X or pGO463 was added to 1 liter of Luria-Bertani broth supplemented with 50 μg of ampicillin per ml, and the cells were grown for 2 h with shaking at 37°C. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM to induce transcription from the tac promoter, and the culture was incubated for another 3 h. The cells were then harvested, frozen at −70°C, thawed, resuspended in 10 ml of MTPBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄ [pH 7.3]) and passed once through a French press. Cellular debris was removed by centrifugation at 14,000 × g for 10 min, and the supernatant was mixed at room temperature with 2 ml of 50% glutathione-agarose beads to which 1 ml of MTPBS was added. After absorption for 2 min, the beads were collected by centrifugation at 500 × g for 2 min and washed three times with 50 ml of MTPBS. The fusion protein was eluted by competition with glutathione, with several washes with 1 bead volume of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione (Sigma Chemical Co., St. Louis, Mo.). The purity of eluted material was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% polyacrylamide). The concentration of purified fusion protein was determined by the Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif.).

**Generation of antibody against the fusion protein.** New Zealand White rabbits were immunized subcutaneously with 0.5 mg of GST-TrsN fusion protein in complete Freund's adjuvant and given a booster dose 2 weeks later with protein in incomplete Freund's adjuvant. Animals were bled 3 weeks after the booster dose, and the sera were extensively absorbed. First, 0.5 ml of serum was added to an equal volume of phosphate-buffered saline containing 500 μg of GST and incubated for 2 h at room temperature. Next, 0.6 ml of a whole-cell lysate of *S. aureus* RN450, created by homogenizing cells in a mini-bead-beater, was added to an equal volume of GST-absorbed serum, incubated for 48 h at 4°C, and centrifuged at 4,000 × g to remove cell debris. Finally, a homogenate of *E. coli* TB1 was added to the supernatant of *S. aureus* RN450-absorbed cells and processed in the same manner. Antibody titers to the GST-TrsN protein were first determined by enzyme-linked immunosorbent assay (ELISA) with both GST-absorbed and unabsorbed sera. The presence of specific antibody to TrsN was confirmed by Western immunoblot (see below) with proteins from *E. coli* TB1 containing pGO352 (trsN cloned on pUC19) as a target.

**Western immunoblot analysis.** *S. aureus* RN450 cells containing pGO1, pGO352C, or pGO1 plus pGO352C were grown overnight at 37°C in brain heart infusion broth. Cells were pelleted, resuspended in 1 ml of Z-buffer, and disrupted in a bead beater. The cell debris was removed by centrifugation, and the supernatant, containing about 100 μg of protein, was resolved on an SDS–15% polyacrylamide slab gel. The gel was blotted to a nitrocellulose membrane. The membrane was rinsed briefly in water and blocked with 5% gelatin in TTBS (20 mM Tris·HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.4) for 20 min. The membrane was incubated with gentle shaking for 16 h in a 1:75 dilution of absorbed rabbit polyclonal antiserum (see above for the absorption protocol). The membrane was rinsed briefly in water and washed in 3% gelatin in TTBS for 20 min to remove any nonspecifically bound antibodies. The membrane was then incubated for 60 min in a 1:3,000 dilution of a goat antirabbit secondary antibody conjugated with alkaline phosphatase, and the antibody-antigen complexes were visualized by using a Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate indicator system (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.). Once the bands appeared, the membrane was rinsed in water for 15 min and air dried.

**Gel retardation (electrophoretic mobility shift) assay.** The assay used was based on the method of Chodosh (6). DNA target fragments were amplified from pGO1 by PCR, gel purified, and cloned into pUC19. PCR fragments corresponded to the following bases in the published *trs* sequence (20) and are shown in Fig. 6: bases 262 to 428 (166 bp), 347 to 671 (324 bp) and 12069 to 12403 (334 bp). These fragments were liberated from pUC19 by using EcoRI and HindIII. The HindIII site was labeled with [32P]dCTP by filling in the recessed end with the Klenow fragment. The end-labeled fragments were purified from unincorporated [32P]dCTP and other reaction components by reaction column chromatography (Worthington Biochemical Corp., Freehold, N.J.). The DNA-binding assay was carried out in a 15-μl volume containing about 7,500 cpm of labeled fragment with various amounts of GST-TrsN fusion protein. Some of the reaction tubes contained a 50-fold excess of unlabeled target sequence as a specific competitor or nontarget *trs* DNA as a nonspecific competitor. The reaction mixture also contained 12 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9), 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 10 μg of bovine serum albumin, 2 μg of salmon sperm DNA, and 12% glycerol. The DNA fragments were then analyzed on a 4% nondenaturing polyacrylamide gel.

**DNase I footprint analysis.** Approximately 160,000 cpm of 32P-end-labeled DNA fragments with gel mobility retarded by GST-TrsN as described above was incubated with increasing concentrations of GST-TrsN fusion protein in a DNA-binding assay. After a 30-min incubation, the magnesium and calcium ion concentrations were adjusted to 5 mM each and 2 μl of DNase I (United States Biochemical Corp., Cleveland, Ohio) containing 0.002 U of activity was added. The reaction mixture was then incubated at 30°C for 2 min and extracted with phenol-chloroform. The DNA was recovered by ethanol precipitation and resuspended in 10 μl of DNA sequencing buffer, and 5 μl of sample was analyzed on an 8% sequencing gel. The DNA region protected by TrsN was identified by running in parallel a sequence ladder generated with the same fragment used in DNase I footprinting. The fragments generated by PCR were cloned so that the TrsN target sites were closest to the HindIII site. Thus, labeled fragments containing bound TrsN would be small enough to be easily seen on a sequencing gel.

**Inactivation of trsN by allelic exchange.** Plasmid pGO473EtS was constructed as follows. A 166-base portion of the 5′ end of trsN including the promoter was amplified by PCR from pGO1 and cloned into pUC19. The tetM gene with its own promoter (3 kb) was then cloned in tandem followed by a PCR fragment containing 118 bases of the 3′ end of trsN sequence extending 16 bases beyond the translational stop. A temperature-sensitive staphylococcal replicon (pE194) unable to replicate at 42°C was then cloned downstream of the trsN construct into the pUC19 PstI site. pGO473EtS was electroporated into *S. aureus* RN4220 containing pGO1, and the resultant transfor-
FIG. 1. Ability of cloned trs DNA to be transformed into \textit{S. aureus} RN4220 by electroporation. (A) Restriction map of the 13,612-kb trs region of pGO1. Abbreviations: B, BglII; H, HindIII; X, XbaI; Nc, NcoI; Sp, SphI; E, EcoRI; Hc, HincII; A, Avai. Arrows indicate the direction of transcription of lettered ORFs. (B) The 4.6-kb HindIII fragment cloned on pBR322 to produce pGO210. The first column on the right gives the clone designation; C indicates the addition of pSK265 to each clone to produce a staphylococcal replicon. The second column on the right indicates the ability of clones to be electroporated into RN4220. The dark boxes at the ends of fragments are portions of the IS-like element, IS257. (C) Localization of trs DNA that allows pGO210C to be electroporated into \textit{S. aureus}. The column on the left lists plasmids containing indicated subclones of the trs region of pGO1, and the column on the right shows the ability of these plasmids to stabilize pGO210C in \textit{S. aureus}.

RESULTS

Localization of trs DNA that could not be transformed into \textit{S. aureus}. Previous studies identified a 4.6-kb HindIII fragment from the trs region cloned on a pBR322/pSK265 \textit{E. coli}-\textit{S. aureus} shuttle vector (pGO210C) that could not be introduced into \textit{S. aureus} RN4220 by protoplast transformation; all other trs subclones could be readily transformed (26). We reconfirmed our inability to transform protoplasts of pGO210C into RN4220, even when the HindIII fragment and staphylococcal replicon were cloned in all possible orientations, and observed that the lack of transformability of DNA was also seen with electroporation. Because of its ease, electroporation was subsequently used as the assay method for transformability of clones.

As shown in Fig. 1A and B, the transformation instability of pGO210C was localized to a 329-bp PCR fragment containing sequences between the end of trsK and the beginning of trsL, including the putative trsL promoter and a 17-bp region of dyad symmetry. Inactivation of each intact ORF on the pGO210 clone (trsJ-trsM) by deletion or insertion did not serve to stabilize the clone for transformation, suggesting that no ORF, by itself, was responsible for this instability.

Sequence analysis. DNA sequence was analyzed for ORFs, motifs, encoded amino acids, and conformation of predicted proteins by using programs of the Genetics Computer Group, Madison, Wis.

Statistics. Conjugation frequencies were compared by one-way analysis of variance. Post hoc comparisons were made by the Student-Newman-Keuls test. Differences were considered significant if $P < 0.05$. 

mam containing both of these plasmids was grown at 30°C overnight in Mueller-Hinton broth. This culture was diluted 1:250 in fresh broth, incubated with shaking at 42°C for 24 h, diluted again in fresh broth, and grown at 42°C for another 24 h. Various dilutions of this culture were plated on Mueller-Hinton agar in the absence or presence of tetracycline at 10 $\mu$g. The ratio of tetracycline-resistant to total cells provided an estimate of the efficiency of curing. Tetracycline-resistant colonies were then picked to agar containing 10 $\mu$g of erythromycin per ml, and both erythromycin-susceptible and erythromycin-resistant colonies were analyzed to assess recombination between pGO473Ets and pGO1. The site of the construct integration into pGO1 was determined by restriction endonuclease digestion of plasmid DNA. The precise sequence rearrangements in recombinants and deletion derivatives was not determined.

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Identification and characterization of a gene that stabilized trs sequences for transformation in trans. If either pGO1 or a deletion derivative of the entire cloned trs region on a pE194-based shuttle vector (pGO200ΔE [Fig. 1C]) were present in RN4220, pGO210C could be transformed by protoplast transformation (26) or electroporation. An 881-bp subclone of pGO200ΔE (pGO187E), consisting of only the first 769 bp of the trs complex, contained this trans-stabilizing activity (Fig. 1C). This fragment contains a single complete ORF, the 189-bp trsN, and the first 245 bp of the 971-bp ORF, trsA.

The sequence of the trsN ORF and 5′ DNA, encoded on the opposite strand from the other trs ORFs, has been previously published (20). The predicted trsN ORF and 81 bp of 5′ DNA containing potential promoter sequences were amplified from pGO1 by PCR and cloned into pUC19 (to produce pGO352), and the fidelity of amplification was confirmed by DNA sequence analysis. Following addition of pE194 to construct a shuttle vector, the plasmid, pGO352E, was introduced into RN4220 and shown to stabilize pGO210C for transformation to the same degree as pGO187E. In all cases, stable electroporation resulted in 100 to 200 colonies growing on selective agar. Without trans-stabilization, no colonies were seen.

DNA-binding activity of purified TrsN. The trsN ORF, obtained from pGO1 by PCR amplification, was cloned into pGEX-3X, and the appropriate fusion to GST was confirmed by DNA sequence analysis. The purified fusion protein was shown to be of the appropriate size by PAGE. As shown in Fig. 2, the fusion construct produced a mixture that was only half the fusion protein with the rest GST alone. This GST-TrsN fusion protein–GST mixture was used, without cleavage of TrsN from the enzyme, in DNA-binding assays. PCR-amplified DNA fragments were used as targets for binding of the GST-TrsN fusion protein in gel retardation assays. For the nucleotide sequences of target fragments, see Fig. 6. In addition to the trsK-trsL sequence identified as DNA unstable for transformation, the sequence between trsN and trsA was included as a possible TrsN target because of the similarity between the trsK-trsL and trsN-trsA sequences and the presence in both of identical 22-bp palindromes. A comparison of these sequences has been reported previously (20). Finally, because the −35 region of the predicted trsN promoter overlaps the extremity of one of the arms of the palindrome, a fragment containing just the predicted trsN promoter and initial coding sequence was evaluated for TrsN binding. As a control, a fragment containing the initial coding sequence of trsD and 5′ DNA, identified by complementation studies as a possible promoter (20), was also evaluated both for direct TrsN binding and for competitive inhibition of TrsN binding to other target sequences. Figures 3 and 4 show the results of gel retardation studies. The GST-TrsN fusion retarded the migration of trsK-trsL, trsN-trsA, and trsN sequences (Fig. 3). There was no effect of GST alone, and trsC-trsD sequences neither competitively inhibited TrsN binding (Fig. 3) nor retarded the migration of the PCR fragment itself (data not shown). Retardation increased with increasing concentration of protein, reaching saturation at 12 μg (a ratio of 12 μg of protein to 0.025 μg of target DNA) for both the trsL and the trsA target sites (Fig. 4). Thus, the binding of TrsN was progressive and specific to DNA fragments containing trs promoter-like sequences.

Binding sites within target sequences were determined by DNase I footprinting, and results are shown in Fig. 5. Concentrations of the GST-TrsN fusion protein mixture ranged from 0.2 to 12 μg (with the protein-to-DNA ratio ranging from 0.01 to 0.6 μg of protein per 0.025 μg of DNA) and protected progressively more DNA as the concentration increased. As shown in Fig. 5A and 6A, TrsN protected only the 3′ end of the trsK coding sequence at the lowest protein concentrations (protected area 1 in Fig. 5A and 6A), whereas the palindrome, promoter, and Shine-Dalgarno sequences of trsL (protected area 2 in Fig. 5A and 6A) were protected at 6 μg of sequence protein. The protein-to-DNA ratio resulting in the protection of sequence 1 is equal to that causing the least fragment retardation in Fig. 3A, lane 3; the ratio resulting in the protection of sequence 2 is roughly equal to that causing the degree of retardation shown in Fig. 4A, lane 4. Figures 5B and 6B and C show the extensive region of intervening sequence between trsA and trsN protected by increasing concentrations of TrsN fusion protein. At the lowest protein concentrations, comparable to the amount producing the least fragment retardation in Fig. 3B, lane 3, protected sequences included the Shine-Dalgarno sequence and −10 and −35 sequences of
**FIG. 4.** Mobility shift assay showing the progressive binding of TrsN to trsL-trsK (A) and trsA-trsN (B) PCR fragments. Approximately 7,500 cpm of the ³²P-5'-end-labeled fragment was used in each assay. Lanes: 1, no protein; 2, 12 µg of GST; 3 to 9, 0.2, 0.6, 2, 6, 12, 24, and 36 µg, respectively, of the GST-TrsN fusion–GST protein mixture. F and B indicate free and bound probe.

trsN and the −35 region of one of the two predicted tandem trsA promoters (protected area 1). At higher protein concentrations, comparable to those producing the degree of fragment retardation seen in Fig. 4B, lanes 3 and 4, −35 and −10 regions of the second trsA promoter and the 3′ half of a region of dyad symmetry homologous to the palindrome between trsK and trsL were protected (protected area 2). For all fragments, the amount of protein causing extended DNase I protection of target sequences (protected area 2) was always smaller than the amount causing maximal fragment retardation. The specific binding of TrsN to promoter-like sequences suggested that the protein not only stabilized these sequences for transactivation but also was likely to regulate transcription of downstream genes.

**TrsN characterization.** The protein encoded by trsN was predicted to contain the following 62 amino acids: MNNNEENS VFFGKKKVKVSLHLVDPMKDEIIKYAQEKDFDNVSQA GREILKKGEOQKSNK. The peptide had a predicted molecular mass of 7,181 Da and an isoelectric point of 9.09. Programs generated by the Genetics Computer Group program predicted the protein to contain two α helices, but the classic helix-turn-helix motif of well-characterized DNA-binding proteins that serve as transcriptional regulators was not evident (22). No significant amino acid or nucleic acid homology with other sequences in the data base was found.

**Effect of TrsN on trsL transcription and pGO1 transfer.** To assess the regulatory effect of TrsN on trsN gene transcription, we first wanted to evaluate the possibility of inactivating trsN by allelic replacement mutagenesis. We did not think that inactivation of trsN would be possible. Our hypothesis was that, in the absence of TrsN, pGO1 would no longer be stable. We introduced the trsN construct, on which trsN sequences were interrupted by tetM, on a temperature-sensitive delivery vehicle (pGO472Ets) into *S. aureus RN4220* containing pGO1 and sought tetracycline-resistant (Tet⁺) colonies after growth of cells at the nonpermissive temperature for plasmid replication. Efficient plasmid curing was believed to have occurred on the basis of a ratio of Tet⁺ to Tet⁻ cells of 10³:10⁰. By replica plating we were able to find 14 colonies, of 179 screened, that were Tet⁻ Erm⁺. In each case, the trsN-tetM construct, together with a portion of the vector, had integrated into one of the nine copies of an IS-like element present on pGO1 (5, 18). The copy of trsN on pGO1 was intact, and the plasmid transferred at wild-type frequencies. An additional 30 Tet⁺ Erm⁻ colonies
FIG. 6. The DNA sequence of trsL-trsK (A), trsA-trsN (B), and trsN (C) target fragments used in mobility shift and DNase I footprinting assays. Protected sequences are bracketed and numbered. The numbers correspond to those in Fig. 5. S.D., Shine-Dalgarno sequence. Underlined sequences designate predicted promoter regions. Palindromic sequences are overlined by arrows.

were analyzed by gel electrophoresis. Twenty-two were found to contain pGO472Es as a separate, unintegrated replicon, whereas in eight the entire plasmid was integrated into IS-like sequences on pGO1. There was no integration into trsN. Our experience with a similar construct to inactivate other trs genes was that replacement mutagenesis occurred readily (1). Therefore, we concluded that we could not identify trsN allelic replacement mutants either because copies of pGO1 with inactivated trsN were unstable or because unbound target sequences affected the viability of the host cell.

We next sought to demonstrate a TrsN regulatory effect by providing gene product in excess. The progressive nature of binding with increasing TrsN in gel retardation assays suggested that we could get an increased regulatory effect with increased gene product in the intact cell. We cloned trsN on a staphylococcal vector (pSK265, a pC194 derivative) that has a copy number of approximately 100 chromosomal equivalents (21), compared with a copy number of approximately 10 for pGO1 (3). To quantitate the difference in the amount of TrsN produced from the clone (pGO352C) versus the amount produced from pGO1, we generated polyclonal antibody to TrsN. Since we were unable to cleave TrsN from GST in sufficient quantity for immunization, we immunized rabbits with the entire fusion. The presence of anti-TrsN antibody was confirmed by ELISA and immunoblotting. In ELISA, antibody titers against the fusion protein fell by only 50% following absorption with GST while absorption with the fusion reduced titers to that seen with no antigen. Immunoblotting was performed by using GST-absorbed antibody reacting with target proteins from total-cell extracts of E. coli containing pGO3522 (cloned trsN). A strongly reactive protein of 6.8 to 7.0 kDa, corresponding to the predicted size of TrsN, was seen (Fig. 7). To assess the relative abundance of TrsN in S. aureus cells containing the low-copy-number replicon (pGO1), the high-copy-number replicon (pGO352C), or both plasmids, we immunoblotted staphylococcal protein extracts with GST-absorbed anti-TrsN antibody. As shown in Fig. 7, the concentration of TrsN was related to the copy number of the trsN-containing clone (pGO352C + pGO1 > pGO352C > pGO1). Thus, TrsN was shown to be produced from pGO352C in excess in comparison with the amount produced from pGO1.

To assess transcriptional regulation of trsL, we made a Tn917lac transcriptional fusion in trsL (pGO1-4A). The effect of excess TrsN (trsN on pGO352C) on β-galactosidase production is shown in Table 2. There was a 50% decrease in β-galactosidase production from pGO1-4A in the presence of trsN carried on pGO352C. Controls consisting of the shuttle vector alone or other cloned trs fragments had no effect on β-galactosidase production.

In addition, pGO352C was added to strains containing pGO1 with Tn917lac transcriptional fusions into trsl (pGO1-2P), trsg (pGO1-63), and trsk (pGO1-43). These ORFs are encoded within a long 10-kb transcript immediately preceding the trsL-M transcript (Fig. 1) (20). In the presence of excess TrsN, each of the fusions into these genes also exhibited some reduction in β-galactosidase activity. In contrast, a Tn917lac-
generated β-galactosidase fusion into a non-trs gene on pGO1 (pGO1-233), which produced 10 times as much enzyme as the trsL fusion did, was unaffected by trsN. The pGO1-233 insert is into a pGO1 ORF of unknown function. Thus, TrsN affects the transcription of trs genes that are not directly under the control of promoters within the trsL-trsM or trsA-trsN targets.

The β-galactosidase fusions that produced less enzyme in the presence of excess TrsN each abolished pGO1 conjugative transfer, indicating that the genes encoded essential transfer functions or had a polar effect on essential downstream genes. Therefore, we surmised that constitutive repression by overproduction of TrsN might decrease pGO1 conjugative transfer. The mean transfer frequencies of seven separate mating experiments, comparing pGO1 containing only the shuttle vector with pGO1 plus pGO352C, are shown in Table 3. In each mating experiment, pGO352C always transferred the target plasmid, indicating that constitutive transfer is abolished by pGO352C. When pGO352C was transferred by pGO1, the conjugative transfer frequency ranged from 7.3- to 66.7-fold in transfer frequency for pGO352C when compared with pGO1 alone.

We also postulated that if the DNA sequence 5′-to trsL that was stabilized in the presence of trsN was a target for TrsN transcriptional repression, it should be possible to titrate out the repressor by providing the target in excess. This experiment was performed by introducing the trsL target (pGO364C [Fig. 1]) into RN4220 containing either pGO1 or pGO1-4A. In the presence of excess trsN target sequences, β-galactosidase activity from pGO1-4A decreased from 1,823 ± 387 to 2,356 ± 410 Miller units per mg of protein per h (a 23% increase) and pGO1 transfer frequency increased eightfold (Table 3). A trs clone containing no TrsN target sequences (pGO172C) had no effect on conjugation (Table 3) or β-galactosidase activity (data not shown).

### DISCUSSION

We identified a gene (trsN) encoding a small protein (TrsN, 62 amino acids) that bound to two specific DNA segments in the conjugative transfer gene cluster (trs) of the staphylococcal plasmid, pGO1. The target sites were previously shown to contain predicted promoter sequences that preceded each of two short trs transcription units (20). The first target appeared to be extensive on the basis of the number of nucleotides (129 bases) protected by TrsN in a DNase I footprinting analysis. It contained seven bases (5′) to the initial gene in the trs complex (trsA) and 5′ to trsN itself, transcribed from the opposite DNA strand. Previous studies involving Northern (RNA) blot analysis of transcript size and complementation of transposon inserts that abolished transfer suggested that trsA, trsB, and trsC were transcribed by a predicted promoters within this first target for TrsN binding (20). The bases protected from DNase I included the trsN promoter and each of two predicted tandem promoters for trsA, one of which contained a perfect 8-base palindromic sequence at the −35 region (Fig. 6). The second region bound by TrsN, sequences 5′-to trsL, contained a more extensive 17-base palindromic, the internal 8 bases and central core of which were identical to those preceding trsA. The 17-base palindromic also included the −35 region of the trsL promoter. It is interesting, however, that the DNA 5′-to trsL protected at the lowest TrsN concentrations involved only the 3′ end of the trsK coding sequence and none of the trsK-trsL intergenic region. What effect binding of TrsN to this region might have on trsL and trsM transcription or on the stability of these sequences is not clear.

The nature of TrsN binding was suggested by gel retardation studies. The addition of increasing quantities of purified TrsN caused progressive retardation of each of the target DNA fragments until binding sites were saturated. A single retarded band was seen at each protein concentration, suggesting that

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**TABLE 2. Effect of clone overproducing TrsN on expression of trs::lacZ fusions**

<table>
<thead>
<tr>
<th>pGO1 derivative containing lac fusion (target gene)</th>
<th>β-Galactosidase activity in the presence of pGO352C (trsN)</th>
<th>% decrease in β-galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>96.43 ± 25.62 (n = 8)</td>
<td>92.04 ± 15.28 (n = 3)</td>
</tr>
<tr>
<td>pGO1-4A (trsL)</td>
<td>1,823 ± 386.90 (n = 7)</td>
<td>938 ± 99.64 (n = 4)</td>
</tr>
<tr>
<td>pGO1-2P (trsL)</td>
<td>1,088 ± 163.16 (n = 7)</td>
<td>540.4 ± 44.40 (n = 3)</td>
</tr>
<tr>
<td>pGO1-43 (trsK)</td>
<td>555.20 ± 127.76 (n = 4)</td>
<td>417.04 ± 119.07 (n = 3)</td>
</tr>
<tr>
<td>pGO1-62 (trsG)</td>
<td>554.58 ± 36.90 (n = 4)</td>
<td>343.50 ± 82.99 (n = 3)</td>
</tr>
<tr>
<td>pGO1-233 (pGO1 ORFU)</td>
<td>13,540.91 ± 4,676 (n = 4)</td>
<td>13,000 ± 4,733 (n = 3)</td>
</tr>
</tbody>
</table>

* Miller units per milligram of protein per hour; results are means of experiments indicated in parentheses ± standard deviation.

* There was no difference in baseline values with or without the shuttle vector alone.

* NS, no significant change.
TABLE 3. Effect of trsN on pGO1 conjugation frequency

<table>
<thead>
<tr>
<th>Donor plasmids</th>
<th>Conjugation frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGO1 + pGO305C (shuttle)</td>
<td>(1.95 ± 0.95) × 10⁻⁴</td>
</tr>
<tr>
<td>pGO1 + pGO352C (trS)</td>
<td>(2.3 ± 0.6) × 10⁻⁴</td>
</tr>
<tr>
<td>pGO1 + pGO364C (trS target)</td>
<td>(1.4 ± 0.2) × 10⁻³</td>
</tr>
<tr>
<td>pGO1 + pGO172C (trS promoter)</td>
<td>(2.4 ± 0.6) × 10⁻⁴</td>
</tr>
</tbody>
</table>

*Conjugation frequency = donor input cells/transconjugants. All donor plasmids were in RN220; the recipient was RN4220 resistant to novobiocin and rifampin (RN4220 NR). Results are expressed as Mean ± standard deviation. These numbers are the mean of seven separate matings, performed on different days. Comparisons of lines 1 and 2 and of lines 1 and 3 were significantly different at P < 0.05. There was no significant difference between lines 1 and 4.

The increased retardation at increasing protein concentration was due to binding to contiguous unoccupied sites and/or protein-protein interactions rather than to binding to multiple targets of differing affinities. The latter generally results in the appearance of multiple bands; new bands appear at increasing protein concentration as sites of lower affinity are bound (15, 27). DNAase I protection studies also suggested cooperative binding or binding to unoccupied sites with increasing TrsN concentrations. As the TrsN concentration increased, the protected segment increased both 3' and 5' from that protected at lower concentrations. We do not yet know whether TrsN forms oligomers or multimers in solution before binding. The concentration-dependent binding of TrsN to various targets, including its own promoter, suggests that modulation of the concentration of this protein may be critical to induction of the mating response and/or the timing of the production of various trs gene products.

To assess transcription from trs genes in the absence of regulatory protein, we wanted to inactivate trsN but were unable to do so. We had initially identified TrsN activity on the basis of its ability to stabilize in trans incoming trs clones containing the trsL target sequence; if trsN were not in the recipient cells, trsL clones could not be transformed. TrsN appeared to act either to prevent binding of some undefined S. aureus host factor to these sequences or to prevent the overtranscription of lethal downstream genes. Therefore, we predicted that inactivation of trsN would be lethal for pGO1 or the host cell. Our finding that the only copies of pGO1 containing integrations had insertions into regions of pGO1 removed from conjugative transfer genes appeared to confirm our hypothesis. Thus, we were forced to assess the effect of TrsN on trs transcription by overproducing the protein from a clone on a high-copy-number vector.

Excess TrsN reduced β-galactosidase production from a trsL-lacZ transcriptional fusions by 50% and decreased conjugation frequency 10-fold. Thus, TrsN seemed to be a repressor.

Although these changes were modest, they represented repression added to that already afforded by the gene on pGO1 and therefore did not give a quantitative estimate of the magnitude of TrsN repression. However, the potential regulatory range of TrsN can be estimated by noting the 100-fold difference in transfer frequency of pGO1 and the 2.5-fold change in production of β-galactosidase from trsL fusions in the presence of excess protein (maximally repressed) versus excess target (derepressed). By way of comparison, the change in transfer frequency of F when finO is inactivated is only 1000-fold (29).

We noted that β-galactosidase activity was also reduced in the presence of excess TrsN from fusions in trs genes that were not part of the trsL-trsM or trsL-trsK transcription units. β-Galactosidase activity was decreased by 25 to 48% from fusions in trsG, trsL, and trsK. These genes were previously shown to be contained within a single long (10.2-kb) complementation group transcribed by a promoter 5' to trsD (20). The effect of TrsN on transcription from these genes could be due to its action on sequences 5' to trsA, since we found no TrsN binding to sequences immediately 5' to trsD. This would suggest that although the majority of transcription from trsA includes only three genes, at certain times transcription proceeds from trsA through trsK. Alternatively, genes transcribed from either the trsA or trsL promoter could be involved in transcription of trsD through trsK, and repression decreases the production of these additional regulatory factors.

The similarity between trsN regulation and the kil-kor system of conjugative Streptomyces plasmids (e.g., pJ101) should be noted (10). In Streptomyces species, kil sequences are defined as those that cannot be cloned unless kor (kil override) genes are present in the same cell. kilA, the target of korA, is the promoter region between korA and tra, the major conjugative transfer gene. korA, transcribed divergently from tra, encodes a 25-kDa protein that both represses its own transcription and that of tra. The similarity of function and organization between korA and trsN, kilA and trsA target sequences, and tra and trsA is striking even though there is no significant DNA or amino acid homology between these staphylococcal and Streptomyces sequences. There is also a kil-kor system on broad-host-range gram-negative plasmids (e.g., RK2/RP4) that involves trans-stabilization of lethal target sequences and genes involved in both conjugal transfer and plasmid replication (9, 16, 17, 31). Thus, trsN shares a regulatory function with conjugative plasmids in both gram-positive and gram-negative bacteria.

The role of trsN in regulation of conjugal transfer between staphylococci is not yet clear. Production of TrsN and repression of target gene transcription appear to be graded responses that can be modulated rather than either turned off or turned on. Thus, rather than a switch that activates conjugation, TrsN may modulate the production of various trs gene products so that they are made at the appropriate time and in the proper quantity. This suggests that various complex feedback loops may exist and that trsN itself may be regulated. However, it is not clear if trsN responds to trs gene products alone or if there are signals external to the cell that trigger or modulate conjugal gene functions. External signals are important in conjugal plasmid transfer between enterococci (pheromones) (7) and between Agrobacterium species and plants (opines) (8), whereas for transfer of F or broad-host-range plasmids between gram-negative bacteria no specific environmental signal has been identified. The continuing investigation of trsN and the regulation of staphylococcal conjugal plasmid transfer should be able to define the signals that determine production of trs genes.

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1. Archer, G. Unpublished observations.

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