Insertion-Duplication Mutagenesis of *Neisseria*: Use in Characterization of DNA Transfer Genes in the Gonococcal Genetic Island

HOLLY L. HAMILTON, KEVIN J. SCHWARTZ, AND JOSEPH P. DILLARD*

*Department of Medical Microbiology and Immunology, University of Wisconsin—
Madison Medical School, Madison, Wisconsin 53706*

We created plasmids for use in insertion-duplication mutagenesis (IDM) of *Neisseria gonorrhoeae*. This mutagenesis method has the advantage that it requires only a single cloning step prior to transformation into gonococci. Chromosomal DNA cloned into the plasmid directs insertion into the chromosome at the site of homology by a single-crossover (Campbell-type) recombination event. Two of the vectors contain an erythromycin resistance gene, *ermC*, with a strong promoter and in an orientation such that transcription will proceed into the cloned insert. Thus, these plasmids can be used to create insertions that are effectively nonpolar on the transcription of downstream genes. In addition to the improved *ermC*, the vector contains two copies of the neisserial DNA uptake sequence to facilitate high-frequency DNA uptake during transformation. Using various chromosomal DNA insert sizes, we have determined that even small inserts can target insertion mutation by this method and that the insertions are stably maintained in the gonococcal chromosome. We have used IDM to create knockouts in two genes in the gonococcal genetic island (GGI) and to clone additional regions of the GGI by a chromosome-walking procedure. Phenotypic characterization of *traG* and *traH* mutants suggests a role for the encoded proteins in DNA secretion by a novel type IV secretion system.

Because many standard genetic tools do not function in *Neisseria gonorrhoeae*, the construction of special tools for manipulating its genome is a necessity. *N. gonorrhoeae* is highly competent for natural transformation (13, 37). Therefore several mechanisms for mutagenesis of *N. gonorrhoeae* that rely on transformation have been developed previously, including shuttle mutagenesis (34), direct cloning of antibiotic resistance markers (46), and direct transformation of constructed mutations without selectable markers (14). Shuttle mutagenesis is highly useful in that it offers a method for transposon mutagenesis in an organism that otherwise cannot be transposon mutagenized (34, 35). The procedure, however, requires several steps. The gene of interest must be cloned into an *Escherichia coli* plasmid, and the plasmid is then subjected to transposon mutagenesis in *E. coli*. Screening to identify mutants takes place, and finally the vector is transferred to the organism of interest, where homologous recombination results in the incorporation of the mutation into the chromosome. For efficient transformation of gonococci, the transposon must be flanked by a significant amount of gonococcal DNA (~500 bp). Therefore, the original target must generally contain more than a kilobase of cloned DNA. The method of direct transformation of constructed mutants in gonococcal genes has similar difficulties and requires extensive screening to find a mutant of interest.

In this paper we describe the development and use of insertion-duplication mutagenesis (IDM) plasmids for producing targeted mutations in *N. gonorrhoeae*. IDM utilizes chromosomal fragments cloned into plasmids to target insertion into

the recipient chromosome by homologous recombination, resulting in the insertion of the vector between duplicated target sequences (25) (see Fig. 1). This method of mutagenesis has been used for *Streptococcus pneumoniae* for more than 20 years (19), and similar methods have been developed for *Streptococcus mutans* (41), *Bacillus subtilis* (9, 24, 27), and *Lactobacillus* species (20, 21). Additionally, plasmid insertion methods are required for the identification of in vivo-induced genes by the in vivo expression technology (IVET) and differential fluorescence induction (DFI) techniques and have been used for the signature-tagged mutagenesis method in bacteria in which transposons are not functional (22, 29, 42). Wolfgang et al. have reported the use of plasmid insertion in gonococci for purposes of in situ cloning of *comP* and flanking sequences (45), but the vector was not optimized for this purpose and the mutagenesis process was not characterized. IDM has several advantages over other methods of mutagenesis utilized for *N. gonorrhoeae*. IDM requires only one cloning step for use in mutagenesis, and every clone of chromosomal DNA can be used to make a mutation. Because the mutation is made from an *E. coli* plasmid, it is easy to recover the construct that generated a mutation of interest by simply transforming DNA from the mutant into *E. coli*. Thus, this method has been used to identify plasmid clones capable of restoring spontaneous mutants to the wild-type phenotype (8). In addition, with the IDM vectors that we have created for use in *N. gonorrhoeae*, it is possible to make both transcriptionally polar and nonpolar mutations.

We have recently discovered the presence of a 60- to 70-kb genetic island in *N. gonorrhoeae*. The gonococcal genetic island (GGI) is variable, and certain versions of the GGI are found significantly more often in *N. gonorrhoeae* strains isolated from patients with disseminated gonococcal infections (7). Within

---

* Corresponding author. Mailing address: 1300 University Ave., 471A MSC, Madison, WI 53706. Phone: (608) 265-2837. Fax: (608) 262-8418. E-mail: jpdillard@facstaff.wisc.edu.

the GGI, there are multiple genes with homology to those of type IV secretion systems; the greatest similarity is that to the *E. coli* F-plasmid transfer genes. Type IV secretion systems are characterized by their unique ability to transfer both DNA and protein molecules. Well-studied examples of these systems include the DNA transfer systems of conjugative plasmids, the agrobacterial T-DNA transfer system, and the pertussis toxin secretion system encoded by *Bordetella pertussis* (4, 18).

Gonococci are naturally competent for transformation during all phases of growth. Although the fine points of gonococcal transformation are not fully understood, it is known that the recipients must produce pilin, PilT, and ComP for transformation in *N. gonorrhoeae* (2, 37, 45). These data suggest a role for pili and twitching motility in transformation, but their exact role remains unclear. Gonococcal transformation is dependent on RecA for homologous recombination of transforming DNA in the gonococcal chromosome (17). Both single-stranded and double-stranded DNA, as well as supercoiled and linear plasmid DNA, have been shown to transform *N. gonorrhoeae* (38). However, transforming DNA must contain the 10-bp neisserial DNA uptake sequence (DUS) for efficient uptake into a DNase-resistant state (13). Although significant information is known about the requirements of the recipient in transformation, little is known about mechanism of DNA donation. We have recently shown that gonococci secrete DNA into the medium during growth and that a mutation in a peptidoglycan hydrolase gene in the GGI (*atlA*) prevents this secretion (7). We have also shown that the GGI contains genes similar to those involved in DNA transfer during conjugation (7). Here we demonstrate that IDM mutations in these genes eliminate DNA secretion by growing gonococci. These data suggest that the GGI encodes a novel type IV secretion system involved in the donation of DNA for natural transformation.

MATERIALS AND METHODS

Bacteria and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown on Luria agar plates or in Luria broth (31) at 37°C. *N. gonorrhoeae* strains were grown either in GCBL (1.5% Proteose Peptone no. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl) plus Kellogg's supplements (16) and 0.042% $NaHCO_3$ (26) at 37°C with aeration or on GCB agar (Difco) plates under 5% CO_2 at 37°C. Erythromycin was used at 10 μg/ml for gonococci and 500 μg/ml for *E. coli* (except where otherwise indicated) to select for expression of *ermC*. Kanamycin was used at 40 μg/ml for *E. coli* carrying pHSS6 derivatives (35). Chloramphenicol was used at 10 μg/ml for gonococci, and spectinomycin was used at 75 μg/ml alone and at 50 μg/ml when in combination with chloramphenicol.

DNA techniques. Gel purification of digested DNA fragments was performed using GeneClean (Bio 101). Following all ligations, transformation was performed in chemically competent TOP10 *E. coli* according to the protocol of the supplier (Invitrogen).

Southern blotting to confirm pIDN insertions was performed according to standard procedures (31). Chromosomal DNA from *N. gonorrhoeae* was prepared as described by Boyle-Vavra and Seifert (3). DNA digests were separated by gel electrophoresis in a 0.8% agarose Tris-borate-EDTA gel. DNA was transferred to a Stratagene Duralon-UV membrane using a vacuum blotter. Following UV cross-linking, the chromosomal DNA was probed with digoxigenin-labeled pIDN4. Blots were washed at high stringency, and chemiluminescent detection was performed as suggested by the manufacturer (Boehringer Mannheim).

Plasmid screening was performed by whole-cell lysis using the lysis solution of Kado and Liu (3% sodium dodecyl sulfate in 50 mM Tris [pH 12.6]) (15). Patched *E. coli* colonies were lysed in 100 μl of lysis solution, and 20 μl of the lysate was separated by agarose gel electrophoresis and subsequently stained with ethidium bromide. Following whole-cell lysis, possible positive plasmids

were isolated and screened further by restriction enzyme digestion. Plasmid purification was performed as described by Birnboim and Doly (1).

Plasmid constructions. pUP1 was linearized with *Hin*dIII, blunted with T4 DNA polymerase, and ligated to the blunted *Eco*RI-*Xba*I fragment from pJD1145 containing the erythromycin resistance gene. Two plasmids carrying kanamycin and erythromycin resistance resulted due to insertion of the *ermC* fragment in pUP1 in both orientations (pNH5, forward orientation; pNH5-1, reverse orientation). To remove the kanamycin resistance marker, the plasmids were digested with *Bam*HI and *Pvu*II, T4 polymerase blunted, and religated. The product with the forward orientation of *ermC* was named pNH9, and the product with the reverse orientation was named pNH9-1.

A polylinker for the pIDN vectors was obtained via PCR of pMOB with pMOB-polF and pMOB-polR (T_m, 56°C; extension time, 20 s) (Table 2). The blunted, polynucleotide kinase (PNK)-treated PCR product was ligated to *Not*I/*Cla*I-digested, T4 polymerase-blunted pNH9 and pNH9-1 to yield four new plasmids containing all possible orientations of erythromycin and the polylinker: pNH9-6, pNH9-7, pNH9-8, and pNH9-9. To make the plasmids smaller, ~200 bp of sequence was removed from pNH9-8 and pNH9-9 by PCR with pORI-F and pGCU-R to amplify the desired section of each plasmid (T_m, 55°C; extension time, 90 s). PCR products were blunted, phosphorylated with PNK, and ligated to themselves to form pIDN3 and pIDN4. *Eco*RI-*Sph*I fragments from pNH9-6 and pNH9-7 containing the polylinker and the *ermC* gene were cloned into the *Eco*RI and *Sph*I sites of pNH9-8 and pNH9-9, respectively, to create pIDN1 and pIDN2. The DNA sequence of pIDN4 was determined and was deposited in GenBank under accession number AY034154.

pNH10-1M was created by PCR amplification of MS11A chromosomal DNA using primers 23F and EndL6R (7), digestion with *Age*I and *Nsi*I, and cloning into the *Pst*I and *Xma*I sites of pNH9-9. Subclones of pNH10-1M were created by deletions of portions of the 890-bp *traG* insert and religation of the plasmids. pHH1 was created by deleting the 0.6-kb *Hin*dIII fragment. pHH2 was created in a similar manner by deleting the *Sca*I-*Eco*RV fragment. pHH3 was created by deletion of the *Sca*I-*Ecl*136II fragment. pHH1, pHH2, and pHH3 contain 290-, 540-, and 350-bp fragments of the original *traG* fragment in pNH10-1M, respectively.

pHH15 was constructed in the following manner. The PCR product of TraH-up (containing an *Nsi*I site) and TraH-down (containing a *Eco*RV site) (T_m, 66°C; extension time, 60 s) was cut with *Nsi*I and *Eco*RV and cloned into the *Nsi*I and *Pme*I sites of pGCC6. This plasmid was linearized with *Not*I and transformed into KS16 (*traH* mutant) for complementation. pGCC6 targets the inserted gene to a chromosomal location between gonococcal genes *lctP* and *aspC*.

Gonococcal transformation. Spot transformations of gonococci were performed as follows: 1 pmol of each plasmid in a 20-μl volume was spotted in three places on a prewarmed (37°C) GCB agar plate. The spots were allowed to soak into the plate. A P^+ colony of MS11A was then streaked across the plate through the DNA spots. After overnight growth at 37°C, colonies were swabbed from the spots with a Dacron swab and resuspended in 600 μl of GCB. This suspension of cells was then diluted and plated on both GCB alone and GCB plus erythromycin, and CFU were counted. Transformation frequencies were determined as the number of Em^r CFU per total CFU.

Spot transformation was also used to make IDM strains. After overnight growth of the colonies, the spots containing gonococcal transformants were swabbed onto a GCB-plus-erythromycin plate. These plates were grown for 1 to 2 days, and individual transformants were restreaked.

DNA release assay. For DNA release experiments, P^- transparent gonococcal strains were grown overnight on GCB agar plates. Strains were then inoculated with a sterile Dacron swab into 3 ml of GCBL medium with Kellogg's supplements (16) and 0.042% $NaHCO_3$ (26), with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) added where necessary. These cultures were grown for ~2 to 2.5 h. Cultures were then diluted to an optical density at 540 nm of 0.2 ($t = 0 h$) in 3 ml of Cellgro Complete tissue culture medium (Mediatech) with the following supplements: 0.35 mM cysteine, 0.15 mM cystine, 17.9 mM pyruvate, 0.1% soluble cornstarch, 0.042% NaHCO3, Kellogg's supplements, and 1 mM IPTG where necessary. These cultures were grown for an additional 5 h. Culture supernatants were collected at 0 and 5 h and were assayed for DNA release in the following manner. One hundred microliters of a 1:200 dilution of PicoGreen fluorescent dye (Molecular Probes) was added to 100 μl of culture supernatant. DNA release was immediately measured in a fluorometer at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. DNA release per milliliter of culture over 5 h of growth was then calculated.

DNA release was normalized to the total amount of protein in the culture, quantified by the Bio-Rad protein assay. A 500-μl portion of each culture was centrifuged, and the pellet was resuspended in 1 ml of H_2O. Cells were sonicated

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Properties	Source or reference
Plasmids		
pUP1	Template for pIDN plasmids; contains 10-bp neisserial DUS in polylinker; Km^r	10
pMOB	Source of polylinker for pIDN plasmids; pUC origin; Ap^r; multicloning site (MCS); T3 and T7 promoters	39
pHSS23	Source of original *ermC* gene	44
pJD1145	Source of improved *ermC* gene for pIDN construction	This work
pNH5	Forward orientation of *ermC* from pJD1145 in pUP1; Em^r Km^r	This work
pNH5-1	Reverse orientation of *ermC* from pJD1145 in pUP1; Em^r Km^r	This work
pNH9	*Bam*HI-*Pvu*II deletion of Km^r in pNH5	This work
pNH9-1	*Bam*HI-*Pvu*II deletion of Km^r in pNH5-1	This work
pNH9-6	pMOB polylinker in forward orientation in pNH9 (Em^r)	This work
pNH9-7	pMOB polylinker in reverse orientation in pNH9 (Em^r)	This work
pNH9-8	pMOB polylinker in forward orientation in pNH9-1 (Em^r)	This work
pNH9-9	pMOB polylinker in reverse orientation in pNH9-1 (Em^r)	This work
pIDN1	IDM vector: *ermC* in forward orientation, polylinker in forward orientation	This work
pIDN2	IDM vector: *ermC* in forward orientation, polylinker in reverse orientation	This work
pIDN3	IDM vector: *ermC* in reverse orientation, polylinker in forward orientation	This work
pIDN4	IDM vector: *ermC* in reverse orientation, polylinker in reverse orientation	This work
pJD1175	642-bp *Eco*RV-*Sau*3AI fragment of *traH* from MS11A cloned into pIDN1	This work
pJD1177	642-bp *Eco*RV-*Sau*3AI fragment of *traH* from MS11A cloned into pIDN3	This work
pKS43	479-bp *Sau*3AI fragment of *traG* from MS11A cloned into pIDN1	This work
pKS53	479-bp *Sau*3AI fragment of *traG* from MS11A cloned into pIDN3	This work
pNH10-1M	*Age*I-*Nsi*I fragment of *traG* from PCR of MS11A with 23F and EndL6R in *Xma*I and *Pst*I sites of pNH9-9	This work
pHH1	*Hin*dIII deletion of pNH10-1M	This work
pHH2	*Sca*I-*Eco*RV deletion of pNH10-1M	This work
pHH3	*Sca*I-*Ecl*136II deletion of pNH10-1M	This work
pNH11	Blunted PCR product of MS11A with 27F and TraH2R in *Eco*RV site of pNH9-9; contains 5′ end of *traH* and upstream sequence	This work
pGCC6	Plasmid for complementation in the gonococcal chromosome; *lac* promoter/operator; *lacI* Cm^r Km^r	23
pHH15	PCR product of MS11A with TraH-up and TraH-down, cut with *Nsi*I and *Eco*RV and cloned into the *Nsi*I and *Pme*Isites of pGCC6	This work
N. gonorrhoeae		
MS11A	Wild-type *N. gonorrhoeae*	32
KS1	MS11A transformed by IDM with pJD1175, polar mutant of *traH* (Em^r)	This work
KS58	MS11A transformed by IDM with pKS43, polar mutant of *traG* (Em^r)	This work
KS16	MS11A transformed by IDM with pJD1177, nonpolar mutant of *traH* (Em^r)	This work
KS59	MS11A transformed by IDM with pKS53, nonpolar mutant of *traG* (Em^r)	This work
HH500	MS11A transformed by IDM with pNH10-1M (Em^r)	This work
HH501	MS11A transformed by IDM with pHH1 (Em^r)	This work
HH502	MS11A transformed by IDM with pHH2 (Em^r)	This work
HH503	MS11A transformed by IDM with pHH3 (Em^r)	This work
JD1510	MS11A *atlA*::*ermC* (insertion-deletion mutation)	6
JD1603	MS11A transformed by IDM with pNH11 (Em^r)	This work
JD1545	MS11A *cnp*::*cat recA6* (Cm^r)	7
MS11-Spc	MS11A Sp^r	33
HH507	JD1545 transformed by IDM with pNH10-1M (*traG recA6* Cm^r Em^r)	This work
HH508	JD1545 transformed by IDM with pNH11 (*recA6* Cm^r Em^r)	This work
HH518	KS16 transformed with pHH15; *traH* expression in *trans* (Cm^r Em^r)	This work

three times for 10 s each time. One milliliter of a 1:5 dilution of Bio-Rad Dye Reagent Concentrate was added to 20 μl of the sonicated cell solution (in duplicate). Only gonococcal cultures that had grown ~1 log unit in 5 h of growth were used for analysis of DNA release.

Coculture transformation assay. Coculture transformation assays were performed using the P$^+$ gonococcal strain HH507 or HH508 and MS11-Spc that had been grown on GCB agar overnight. Cells were swabbed into GCBL plus Kellogg's supplements and NaHCO$_3$ and grown for ~2.5 h at 37°C with aeration. One milliliter of each culture was then centrifuged and resuspended in 4 ml of prewarmed, fresh GCBL plus supplements for inoculation (~10^7 CFU/ml). The following cultures were inoculated into 3 ml of prewarmed GCBL plus supplements: 1 ml of donor cells (HH507 or HH508), 1 ml of recipient cells (MS11-Spc), 0.5 ml of donor cells plus 0.5 ml of recipient cells, and 0.5 ml of donor cells plus 0.5 ml of recipient cells plus 25 μg of DNase/ml. These four cultures were diluted and plated for CFU per milliliter (*t* = 0 h) on GCB, GCB-chloramphenicol, GCB-spectinomycin, and GCB-chloramphenicol-spectinomycin plates. Cul-

tures were then grown at 37°C with aeration for 4 h, after which they were again diluted and plated for CFU per milliliter on plates containing the same antibiotics as above.

Nucleotide sequence accession number. The DNA sequence of pIDN4 has been deposited in GenBank under accession number AY034154.

RESULTS

Construction of IDM plasmids. To create an IDM system in gonococci, we considered several criteria, both of other IDM systems and of *N. gonorrhoeae*. The ideal vector for IDM in gonococci should be nonreplicating in *N. gonorrhoeae* but should replicate in *E. coli* for cloning purposes. The vector should also have a high transformation rate; efficient DNA

TABLE 2. PCR primers

Primer name	Sequence
pMOB-polF	5′-GTA AAA CGA CGG CCA GTG AGC GCG-3′
pMOB-polR	5′-AAC AGC TAT GAC CAT GAT TAC GCC AAG-3′
pORI-F	5′-GAA GCT AGC TTA TGC GGT GTG AAA TAC-3′
pGCU-R	5′-AAC GTT GTT GCC ATT GCT GC-3′
27F	5′-CGG TTG ACA ACG CGG ATA TTT CAG G-3′
TraH2R	5′-GCC AGG TCA AAG GCT ACA CC-3′
23F	5′-GAT TCG TTC TGC TGC TGA GGC C-3′
TraH-up	5′-CGA ATG CAT CTG CTA TAA CCG CTT CAT GG-3′
TraH-down	5′-CGA GAT ATC GTA CAG CTT TCT CCG TTA CC-3′

uptake in transformation of *N. gonorrhoeae* requires the presence of the 10-bp DUS, which is commonly found in gonococcal and meningicoccal DNA (13). The vector should be small to facilitate incorporation into the chromosome, since incorporation of heterologous DNA by gonococci is greatly affected by the length of the heterology (3). In addition, the IDM vector system should have good selection both in gonococci and in *E. coli*, a useful polylinker for cloning, and the capability to produce both polar and nonpolar insertion-duplication (ID) mutations.

Plasmid pUP1 was used as the starting point for the construction of an insertion-duplication plasmid because it is a relatively small plasmid containing the DUS (10), and like most *E. coli* plasmids, it does not replicate in gonococci. Additionally, unlike many *E. coli* cloning vectors, it has no Tn*3* resolvase site, and thus DNA cloned into it could be mutagenized with the mini-Tn*3* transposons developed for shuttle mutagenesis of gonococci (3, 34). Several factors, however, made pUP1 itself undesirable as an insertional vector for gonococci. The kanamycin resistance marker is not ideal for selection in gonococci due to the inherent kanamycin resistance of nonpiliated variants (12). Also, pUP1 has few sites in the polylinker, and the neisserial DUS is located within the polylinker, making it possible to lose the uptake sequence during cloning.

Erythromycin resistance was chosen as the selectable marker for the IDM plasmids. Erythromycin is preferred over kanamycin because it is a cleaner selection agent in gonococci. However, *E. coli* is inherently resistant to high levels of erythromycin. To increase our ability to select for transformants containing *ermC*-carrying plasmids in *E. coli*, we constructed an improved promoter for the *ermC* gene. The *ermC* gene in pHSS23 has a *Sac*I site between the −35 and −10 hexamers, and the vector contained a perfect −35 sequence in the polylinker region. A deletion was made in pHSS23 between the *Cla*I site in the vector and the *Sac*I site in the *ermC* promoter to generate a smaller *ermC* gene with a consensus sigma-70 promoter that has a spacing of 16 bp between the −10 and −35 sequences. Thus, except for the effects of intervening or surrounding sequence, the new *ermC* promoter should have a strength similar to that of the *tac* promoter, which also has a perfect consensus sequence and a spacing of 16 bp. This new

ermC promoter is strong enough to read through the *ermC* transcriptional terminator, and for this reason this marker was used by Mehr et al. to make nonpolar mutations upstream of essential genes in *N. gonorrhoeae* (23). When the enhanced *ermC* was tested for erythromycin resistance, it was found that *E. coli* cells carrying the construct grew well in media containing 200 to more than 1,000 μg of erythromycin/ml.

The improved *ermC* was cloned into pUP1 in both orientations. By creating a plasmid in which transcription will read into the cloned insert, we will be able to create insertions in the gonococcal chromosome that are effectively nonpolar on downstream genes (Fig. 1). The opposite orientation of *ermC* would be more desirable for creating a polar mutation or when transcription of the insert might result in the production of a detrimental product. Kanamycin resistance was then removed to make the vectors smaller, resulting in plasmids pNH9 and pNH9-1.

To complete the construction, we inserted a polylinker and decreased the size of the plasmid by removing unnecessary sequence. A 224-bp region containing a polylinker was obtained by PCR amplification of the small sequencing and mutagenesis plasmid pMOB (39). The polylinker was cloned in both orientations to facilitate cloning of fragments in both orientations. This region contains a polylinker with 16 unique sites, as well as binding sites for universal sequencing primers. The region also contains T7 and T3 promoters and could be used for controlled transcription in certain *E. coli* strains or for in vitro transcription. The final step in the constructions was to eliminate approximately 200 bp of unnecessary DNA between the DUS and the origin. This was accomplished by PCR of the plasmid and religation. This resulted in the final plasmid constructs, which we have named pIDN, for plasmid for IDM of *Neisseria* (Fig. 2).

Testing IDM in *N. gonorrhoeae* and generation of GGI mutants. Before the completion of the IDM vectors, we tested our idea that IDM could work efficiently as a method of mutagenesis in *N. gonorrhoeae*. There are several reasons why IDM
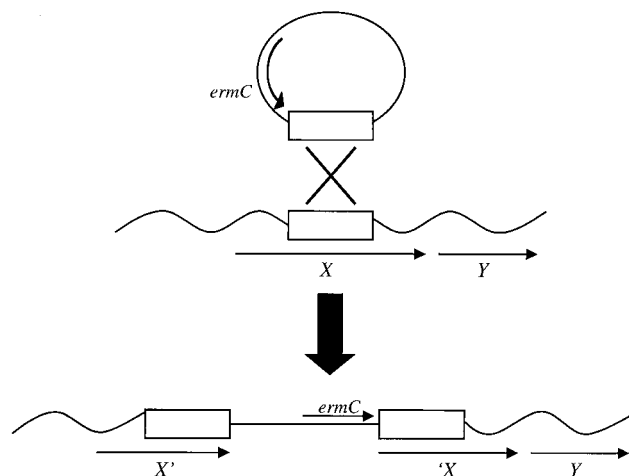

FIG. 1. Generation of a nonpolar insertion mutation. IDM results in a single-crossover recombination event, insertion of the plasmid into the chromosome, and duplication of homologous sequences. The strong *ermC* promoter drives the transcription of downstream genes.
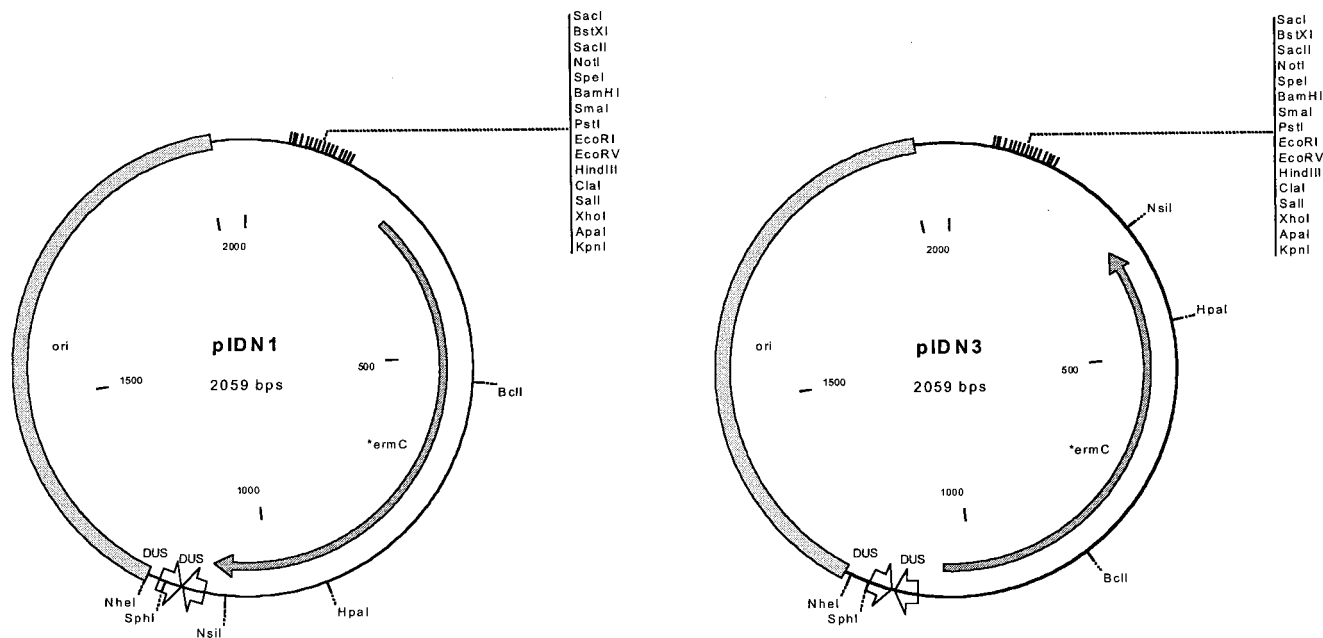
FIG. 2. Plasmid maps of pIDN1 and pIDN3. Plasmids consist only of the *ermC* gene, a polylinker, an *E. coli* origin of replication, and two copies of the neisserial DUS. pIDN2 and pIDN4 (not shown) are the same as pIDN1 and pIDN3, respectively, differing only in the orientation of the polylinker.

might not work well in gonococci. Gonococci appear to undergo a significant degree of illegitimate recombination, which can be seen in attempts to knock out essential genes by insertion of an antibiotic resistance marker. These markers are often incorporated, but the essential gene is duplicated in the transformants, presumably by an illegitimate recombination event (23, 40). Thus, IDM plasmids might be incorporated into arbitrary locations in the chromosome. In addition, *N. gonorrhoeae* does not efficiently incorporate large regions of heterologous DNA (3). Thus, plasmid insertion frequencies might be too low for IDM to be useful. Gonococci are also known to undergo very high levels of recombination (12, 36). If recombination between the homologous segments of the insertions in the chromosome were to occur at a high frequency, IDM mutations would be lost and the method would not be useful for mutagenesis of *N. gonorrhoeae*.

During the process of construction of the pIDN vectors, an intermediate plasmid that contained *ermC* in the reverse orientation and the polylinker in the reverse orientation was created. This plasmid, pNH9-9, was used to test IDM in gonococci. An 890-bp fragment of the GGI gene, *traG*, from *N. gonorrhoeae* strain MS11A was cloned into this intermediate plasmid, and the resulting plasmid was named pNH10-1M. Transformation of gonococci with this plasmid resulted in 2,500 Em^r transformants/ml. When transformed into gonococci, pNH10-1M inserted into the chromosome, resulting in the *traG* mutant HH500. Southern analysis confirmed that this mutant contained the expected insertion of the construct interrupting *traG*.

In order for IDM to be a useful method of mutagenesis in gonococci, it must work with small fragments of genes. It has been found in IDM systems in other organisms that the size of the chromosomal fragment within the IDM vector affects

transformation frequency (19). We made deletions in pNH10.1-M, resulting in three plasmids with smaller target regions of DNA: 290-, 540-, and 350-bp fragments of *traG*. We found that the length of *traG* sequence affected transformation frequency (Table 3). Frequencies ranged from 1.23×10^{-7} to 4.39×10^{-6} transformants/total CFU (7.21×10^{-8} to 2.09×10^{-6} transformants/μg of plasmid DNA). Plasmids containing smaller fragments resulted in lower frequencies of transformation. However, even the smallest target tested (pHH1, with 290 bp) still attained a transformation frequency higher than 10^{-7}, well within an acceptable range for mutagenesis.

The gonococcal strains HH500, HH501, HH502, and HH503, along with two other IDM strains, KS1 and KS58, were characterized by Southern blotting to confirm their insertion locations (Fig. 3). The blot was probed with pIDN4. The further down the chromosome the plasmid is inserted, the larger the *Mfe*I-*Nhe*I fragment is. Except in the case of KS1, which contains a duplication of the *Mfe*I sites in *traH*, as the insert is moved down the chromosome, the *Nhe*I-*Mfe*I fragment grows smaller. In this blot, as well as other Southern blots of these strains, the mutants were found to have correctly incorporated insertion plasmids.

TABLE 3. Transformation frequencies of plasmid insertion into the gonococcal chromosome

Plasmid	Insert size (bp)	Transformation frequency[a]
pNH10-1M	890	$4.39 \times 10^{-6} \pm 9.9 \times 10^{-7}$
pHH1	290	$1.23 \times 10^{-7} \pm 3.4 \times 10^{-8}$
pHH2	540	$8.51 \times 10^{-7} \pm 4.2 \times 10^{-8}$
pHH3	350	$3.52 \times 10^{-7} \pm 1.6 \times 10^{-8}$

[a] Reported as the number of Em^r transformants per total CFU. All values are significantly different; $P < 0.005$ by Student's *t* test.
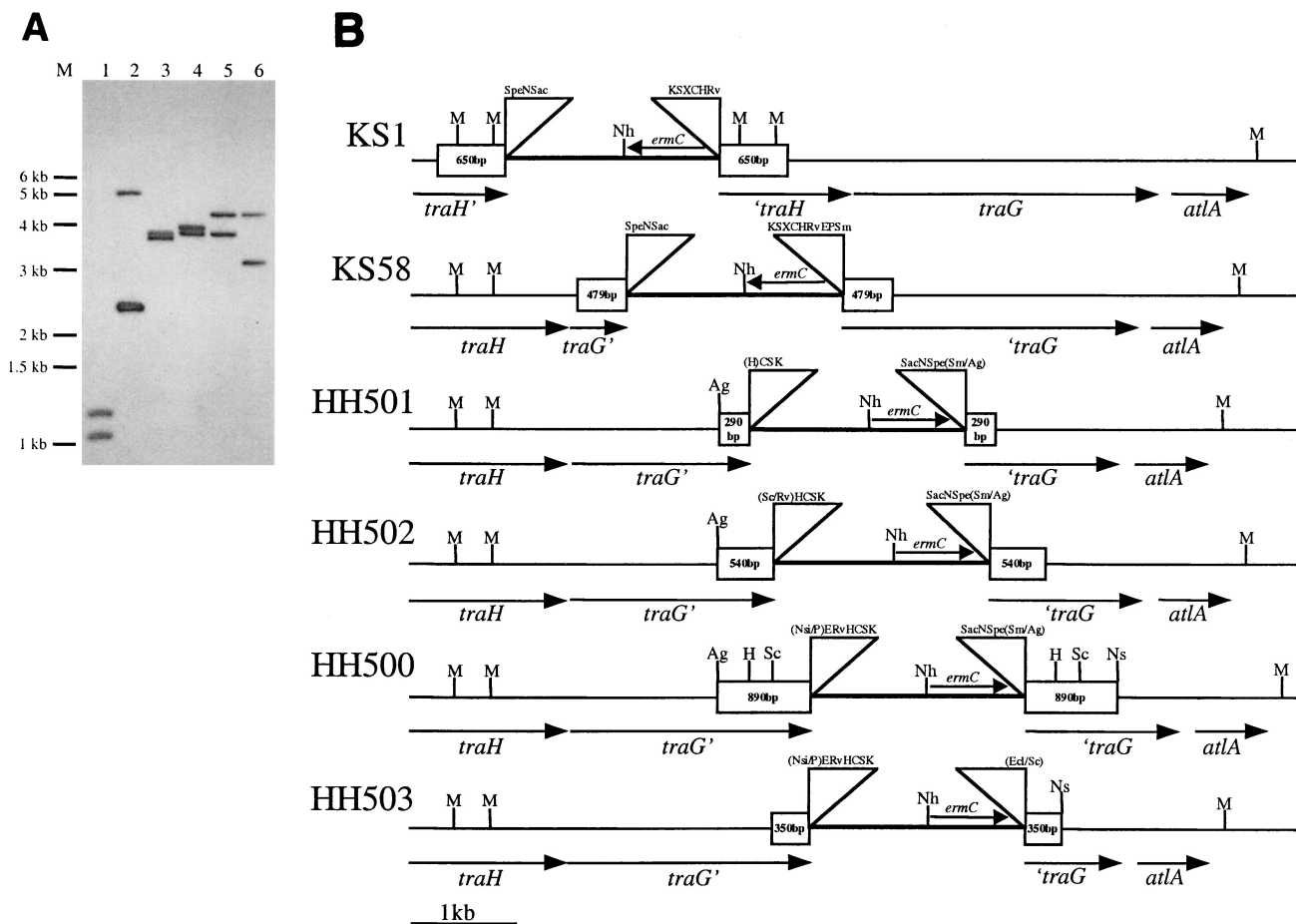
FIG. 3. (A) Southern blot of IDM strains KS1, KS58, HH501, HH502, HH500, and HH503 (lanes 1 through 6, respectively) digested with *Mfe*I and *Nhe*I. This blot was probed with pIDN4, which binds to the plasmid inserted within the *N. gonorrhoeae* chromosome. (B) Schematic of *N. gonorrhoeae* IDM insertions.

In a few instances we have identified ID mutants that did not contain the expected construction. In the process of making nearly 80 plasmid insertion mutations in the gonococcal island, we have screened 45 gonococcal ID mutants by Southern analysis. Among these we have obtained only two mutants that did not give the expected restriction map when analyzed by Southern blotting, and in neither case was the unusual insertion the only or the predominant type of insertion. Since the pIDN plasmids contain two copies of the DUS, a sequence repeated many times in the gonococcal chromosome, these unusual plasmid insertions may have resulted from recombination at the DUS. We have also found mutants that contain a duplication of the plasmid insertion. These double insertions appeared as an additional band the size of the plasmid on the Southern blot and may result from insertion of a plasmid dimer formed in *E. coli* or from a duplication event in the gonococcal chromosome. Dimers have appeared only twice in all of the mutants screened.

To estimate the frequency of excision of IDM plasmids from the gonococcal chromosome, a strain carrying the 890-bp IDM insert in *traG* was tested for loss of the plasmid insertion in two separate experiments. The strain was grown in broth culture for 20 h without erythromycin and then plated onto GCB

plates at various dilutions. After growth overnight, the colonies were replica plated to plates containing erythromycin and to plates containing no antibiotic. The pattern of colonies produced on the plates was recorded using a photodocumentation system, and the images were superimposed. We were unable to detect a single revertant among approximately 10^5 CFU.

IDM-generated GGI mutants are deficient in DNA release. The conventional wisdom is that gonococci donate DNA for transformation by cell death and autolysis (28, 33); however, our data suggest that the putative type IV secretion system in the GGI contributes to DNA donation via specific transport, before autolysis occurs in culture. Strains with mutations in *atlA*, a peptidoglycan hydrolase gene in the GGI, were found to be deficient in DNA donation. AtlA function may be necessary for assembly of the type IV secretion apparatus (7). Type IV secretion systems characteristically secrete both DNA and protein (4). Therefore, assaying for DNA released into the extracellular medium can be used to evaluate the importance of GGI genes in type IV secretion. Nonpolar ID mutants of the GGI genes, *traG* and *traH* (KS59 and KS16, respectively), were tested for the ability to release DNA into the extracellular medium. Both KS16 and KS59 were analyzed by Southern blotting and were found to contain the proper plasmid inser-
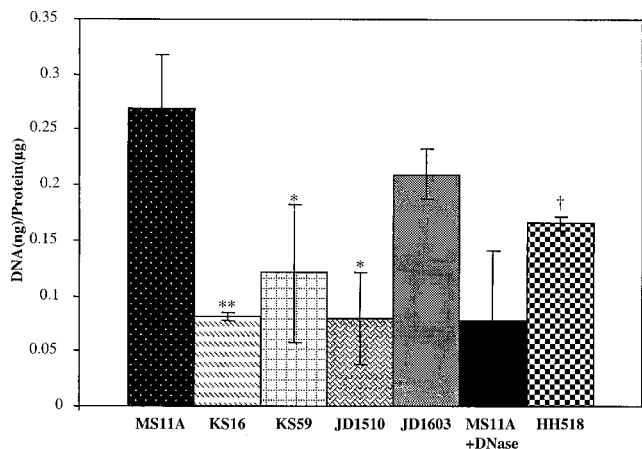
FIG. 4. DNA released into the medium by gonococcal strains MS11A (wild type), KS16 (*traH* mutant), KS59 (*traG* mutant), JD1510 (*atlA* mutant), JD1603 (an intergenic ID mutant), and HH518 (with *traH* complementation) during 5 h of growth. For comparison, the supernatant of MS11A was treated for 30 min with DNase. DNA release was normalized to the total amount of protein. ∗, $P < 0.01$; ∗∗, $P < 0.05$; †, $P < 0.01$ compared to KS16 (Student's t test).

tions (data not shown). In addition to these strains, wild-type, *atlA* mutant, and plasmid control gonococci were tested. JD1603, our positive control, contains a plasmid insert creating a duplication of the beginning of *traH* (containing the promoter), resulting in a full copy of the gene. This mutation was designed to control for any nonspecific effects of having a pIDN insertion. The *traG* and *traH* mutants (KS16 and KS59) exhibited greatly reduced DNA release, with values similar to those of a DNase-treated control (Fig. 4).

traH was complemented in *trans* using a plasmid construct that targeted the inducible complementing gene to an irrelevant location on the chromosome of strain KS16. In this construct *traH* is under the control of the *lac* promoter-operator and can be induced using IPTG. Upon induction, the ability of the complemented *traH* mutant HH518, to release DNA is restored (Fig. 4). Not only does this result demonstrate a requirement for *traH* in DNA release; it also shows that the original *traH* mutant is effectively nonpolar as we predicted, i.e., it is not greatly affected with regard to expression of the downstream genes *traG* and *atlA*.

Coculture transformation assays. We examined gonococcal strains HH507 and HH508 for the ability to donate DNA for natural transformation by a coculture transformation assay. In this assay, donor strains have been made incapable of DNA recombination by a mutation in *recA*. Donor strains (Cm^r) were mixed 1:1 with the recipient strain (Sp^r), and after 4 h of growth, cultures were plated for selection of transformants expressing both antibiotic resistances. The transformation frequency obtained by using HH508 as the donor is ≥50-fold greater than that with HH507 (*traG* mutant) (Table 4). The addition of DNase abolishes transformation of the recipient strain by HH508. These results support the findings (Fig. 4) that the material released from *N. gonorrhoeae* is DNA and demonstrate the importance of the type IV secretion of DNA for transformation.

Use of IDM for chromosome walking. In addition to providing a convenient method for creating mutations, IDM also facilitates the cloning of unknown regions of the bacterial chromosome. Morrison et al. have described its use for this purpose in cloning transformation genes of *S. pneumoniae* (25). More recently, plasmids inserted in the chromosome of *Salmonella enterica* serovar Typhimurium were used to clone the genes found to be induced during infection in a DFI screen (42). We have used our insertions in the GGI to clone unknown GGI DNA. To clone the region upstream of *traH*, a strain containing an insertion at *traH* was mapped by Southern blotting to find a reasonably sized fragment that would contain the plasmid and upstream flanking region. The *Mfe*I fragment was excised from the gel, purified, and ligated. Transformation of *E. coli* generated 43 transformants. Screening of 18 of the transformants showed that 17 contained a plasmid of the expected size (data not shown). One of the 18 transformants contained a plasmid identical in size to the plasmid used to make the original insertion. This result suggests that at a low frequency the plasmid is excising from the chromosome of *N. gonorrhoeae* or that *E. coli* can recombine incompletely digested chromosomal DNA to generate the original plasmid.

DNA sequencing of the region cloned from upstream of *traH* revealed the presence of two open reading frames. The first showed no significant similarity to any sequence in the databases. The second open reading frame was significantly similar to that encoding TraF of the *E. coli* F plasmid. The gonococcal TraF homologue showed 45% similarity and 29% identity with the *E. coli* TraF, with similarity extending over nearly the entire length of the proteins. In *E. coli*, TraF is a periplasmic protein necessary for F-pilus assembly and conjugative transfer (11).

DISCUSSION

We have characterized the successful use of IDM in *N. gonorrhoeae*. Using small vectors that we constructed containing as little as 290 bp of homologous DNA, we observed transformation at frequencies that are well within the range for successful mutagenesis and resulted in stable insertion mutations. Using four different-sized fragments of the *traG* gene, we observed that smaller inserts gave lower gonococcal transformation frequencies. These data are consistent with IDM work performed with *S. pneumoniae*, where a strong correlation was found between the length of the homologous sequence and the

TABLE 4. Transformation frequencies as determined by coculture assay

Donor strain	Transformation frequency[a]
HH508 (plasmid control) + MS11-Spc	2.32×10^{-5}
HH508 + MS11-Spc + DNase	$<1.06 \times 10^{-7b}$
HH507 (*traG* mutant) + MS11-Spc	$<4.70 \times 10^{-7b}$
HH507 + MS11-Spc + DNase	$<3.62 \times 10^{-7b}$
HH508	$<4.50 \times 10^{-8b}$
HH507	$<1.04 \times 10^{-7b}$
MS11-Spc	$<1.62 \times 10^{-8b}$

[a] Calculated as Cm^r Sp^r CFU/total CFU for HH508, HH507, and MS11-Spc and as Cm^r Sp^r CFU/total recipient (Sp^r) CFU for mixed cultures. Frequencies are expressed as geometric means from three different experiments.

[b] No transformants were obtained. Values expressed are limits of detection.

transformation frequency (19). Although there have been a few reports of insertional mutagenesis in *Neisseria* (30, 45), this is the first report of plasmids created specifically for IDM of *Neisseria* and the first in-depth analysis of the process.

The pIDN plasmids constructed in this study are not only useful in targeted mutagenesis; they are also useful in chromosome walking, as shown here for the cloning of *traF*. Additionally, as part of an ongoing GGI cloning and sequencing project, we have used the pIDN plasmids to clone and map approximately 50 kb of the GGI. There is also potential to use IDM for random insertional mutagenesis in *N. gonorrhoeae*. Lee et al. have shown that by constructing a library of IDM vectors containing random 300-bp fragments of *S. pneumoniae* chromosomal DNA, it is possible to use IDM in a random mutagenesis method (19). A similar method could be employed for *N. gonorrhoeae*.

The IDM mutations in *traG* and *traH* have provided insight into the functions of these genes in *N. gonorrhoeae*. *traG* and *traH* mutants were shown to release less DNA into the medium than the wild-type parent during growth. Furthermore, a *traG* mutant was shown to be deficient in the ability to donate chromosomal DNA for natural transformation. This suggests that these genes play a role in a type IV secretion system encoded within the genetic island. *E. coli* TraH and TraG are necessary for conjugation of the F plasmid. *E. coli* TraG is involved in mating pair stabilization, and both TraG and TraH function in conjugative pilus assembly (11). However, our DNA transfer in coculture is sensitive to DNase, indicating that the transfer is not occurring by conjugation (Table 4). Furthermore, the DNA can be detected in cell-free medium. Thus, these proteins are not functioning in conjugation. These results raise the question of what the likely roles of these proteins are in gonococci. F-plasmid TraG is an inner membrane protein with multiple membrane-spanning segments. It is essential for DNA transfer and may aid in stabilizing mating contacts at an early stage of the interaction between donor and recipient cell surfaces. It has been speculated that TraG may form part of the channel for DNA transfer during conjugation in *E. coli* (11). Sequence analysis shows that the GGI *traG* has 22% identity and 40% similarity with the *E. coli* F-plasmid *traG*. The gonococcal TraG may also have a role in the formation of a channel through the membranes by which DNA is exported into the extracellular medium, composing part of the apparatus of a type IV secretion system. TraH of the F plasmid is predicted to be a periplasmic protein involved in F-pilus assembly. It contains an ATP-binding motif (Walker box), suggesting that it may be involved in the energetics of assembly or DNA transfer during conjugation. The GGI TraH also contains a Walker box and has 24% identity and 42% similarity to its F-plasmid homologue. The gonococcal TraH may be performing a similar function, i.e., assembly of a pilus-like apparatus involved in DNA release.

Many questions regarding DNA donation and transformation by *N. gonorrhoeae* are still unanswered. Do the DNA-donating cells die? We have previously shown that DNA release is observed before autolysis can be detected, in the early stages of growth, when gonococci are growing healthily (7). The great advantage of a DNA donation method would seem to be the ability to release DNA without having to undergo autolysis and death. The DNA donation system described here

might operate similar to that of an *E. coli* Hfr, i.e., the gonococcal chromosome may be nicked or cut and the entire chromosome exported. Alternatively, there may be specific genes or regions of the chromosome that are transferred. We do not yet know the size of the DNA being exported or if the exported DNA molecules are coupled with proteins. Future studies of these questions will enhance the comprehension of the mechanisms and functions of gonococcal DNA donation and transformation.

Currently, type IV secretion systems are known to export three types of substrates: (i)DNA conjugation intermediates (DNA-protein complexes), (ii) the multisubunit pertussis toxin, and (iii) monomeric proteins (5). *Agrobacterium tumefaciens*, the type IV secretion prototype, transfers T-DNA directly into plant cells and transfers the proteins VirE2 (a single-stranded binding protein) and VirF (a virulence factor) as well. The *Legionella pneumophila dot/icm* system, also a type IV secretion system, is known to conjugate RSF1010-related plasmids (IncQ) and presumably secretes effector molecules to promote survival within macrophages and free-living amoebae (43). The type IV secretion system of *B. pertussis* (composed of *ptl* gene products) is known only to secrete a protein substrate (pertussis toxin). Type IV secretion systems have also been identified in *Helicobacter pylori*, *Rickettsia prowazekii*, *Brucella* spp., and a number of nonpathogenic bacteria (5). With the evidence of both DNA and protein transfer in other type IV secretion systems, further studies of the *N. gonorrhoeae* GGI and its secretion system may reveal similar findings of effector molecule or virulence factor secretion.

REFERENCES

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
2. **Biswas, G. D., T. Sox, E. Blackman, and P. F. Sparling.** 1977. Factors affecting genetic transformation of *Neisseria gonorrhoeae*. J. Bacteriol. **129:** 983–992.
3. **Boyle-Vavra, S., and H. S. Seifert.** 1993. Shuttle mutagenesis: two mini-transposons for gene mapping and for *lacZ* transcriptional fusions in *Neisseria gonorrhoeae*. Gene **129:**51–57.
4. **Burns, D. L.** 1999. Biochemistry of type IV secretion. Curr. Opin. Microbiol. **2:**25–29.
5. **Christie, P. J., and J. P. Vogel.** 2000. Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. Trends Microbiol. **8:**354–360.
6. **Dillard, J. P., and H. S. Seifert.** 1997. A peptidoglycan hydrolase similar to bacteriophage endolysins acts as an autolysin in *Neisseria gonorrhoeae*. Mol. Microbiol. **25:**893–901.
7. **Dillard, J. P., and H. S. Seifert.** A variable genetic island specific for *Neisseria gonorrhoeae* is involved in providing DNA for natural transformation and is found more often in disseminated infection isolates. Mol. Microbiol., in press.
8. **Dillard, J. P., and J. Yother.** 1994. Genetic and molecular characterization of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 3. Mol. Microbiol. **12:**959–972.
9. **Duncan, C. H., G. A. Wilson, and F. E. Young.** 1978. Mechanism of integrating foreign DNA during transformation of *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **75:**3665–3668.
10. **Elkins, C., C. E. Thomas, H. S. Seifert, and P. F. Sparling.** 1991. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J. Bacteriol. **173:**3911–3913.
11. **Frost, L. S., K. Ippen-Jhler, and R. A. Skurray.** 1994. Analysis of the se-

quence and gene products of the transfer region of the F sex factor. Microbiol. Rev. **58:**162–210.

12. **Gibbs, C. P., and T. F. Meyer.** 1996. Genome plasticity in *Neisseria gonorrhoeae.* FEMS Microbiol. Lett. **145:**173–179.

13. **Goodman, S. D., and J. J. Scocca.** 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae.* Proc. Natl. Acad. Sci. USA **85:**6982–6986.

14. **Gunn, J. S., and D. C. Stein.** 1996. Use of a nonselective transformation technique to construct a multiply restriction/modification-deficient mutant of *Neisseria gonorrhoeae.* Mol. Gen. Genet. **251:**509–517.

15. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.

16. **Kellogg, D. S., Jr., W. L. Peacock, W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae.* Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.

17. **Koomey, M., and S. Falkow.** 1987. Cloning of the *recA* gene of *Neisseria gonorrhoeae* and construction of gonococcal *recA* mutants. J. Bacteriol. **169:** 790–795.

18. **Krause, S., W. Pansegau, R. Lurz, F. de la Cruz, and E. Lanka.** 2000. Enzymology of type IV macromolecule secretion systems: the conjugative transfer regions of plasmids RP4 and R388 and the *cag* pathogenicity island of *Helicobacter pylori* encode structurally and functionally related nucleoside triphosphate hydrolases. J. Bacteriol. **182:**2761–2770.

19. **Lee, M. S., C. Seok, and D. A. Morrison.** 1998. Insertion-duplication mutagenesis in *Streptococcus pneumoniae*: targeting fragment length is a critical parameter in use as a random insertion tool. Appl. Environ. Microbiol. **64:**4796–4802.

20. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis.* Appl. Environ. Microbiol. **55:**394–400.

21. **Leloup, L., S. D. Ehrlich, M. Zagorec, and F. Morel-Deville.** 1997. Single-crossover integration in the *Lactobacillus sake* chromosome and insertional inactivation of the *ptsI* and *lacL* genes. Appl. Environ. Microbiol. **63:**2117–2123.

22. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.

23. **Mehr, I. J., C. D. Long, C. D. Serkin, and H. S. Seifert.** 2000. A homologue of the recombination-dependent growth gene, *rdgC*, is involved in gonococcal pilin antigenic variation. Genetics **154:**523–532.

24. **Michel, B., B. Niaudet, and S. D. Ehrlich.** 1982. Intermolecular recombination during transformation of *Bacillus subtilis* competent cells by monomeric and dimeric plasmids. Plasmid **10:**1–10.

25. **Morrison, D. A., M.-C. Trombe, M. K. Hayden, G. A. Waszak, and J.-D. Chen.** 1984. Isolation of transformation-deficient *Streptococcus pneumoniae* mutants defective in control of competence, using insertion-duplication mutagenesis with the erythromycin resistance determinant of pAMβ1. J. Bacteriol. **159:**870–876.

26. **Morse, S. A., and L. Bartenstein.** 1974. Factors affecting autolysis of *Neisseria gonorrhoeae.* Proc. Soc. Exp. Biol. Med. **145:**1418–1421.

27. **Niaudet, B., A. Goze, and S. D. Ehrlich.** 1982. Insertional mutagenesis in *Bacillus subtilis*: mechanism and use in gene cloning. Gene **19:**277–284.

28. **Norlander, L., J. Davies, A. Norqvist, and S. Normark.** 1979. Genetic basis for colonial variation in *Neisseria gonorrhoeae.* J. Bacteriol. **138:**762–769.

29. **Polissi, A., A. Pontiggia, G. Feger, M. Altieri, H. Mottl, L. Ferrari, and D. Simon.** 1998. Large-scale identification of virulence genes from *Streptococcus pneumoniae.* Infect. Immun. **66:**5620–5629.

30. **Quinn, M. L., S. J. Weyer, L. A. Lewis, D. W. Dyer, and P. M. Wagner.** 1994. Insertional inactivation of the gene for the meningococcal lactoferrin binding protein. Microb. Pathog. **17:**227–237.

31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

32. **Segal, G., J. J. Russo, and H. A. Shuman.** 1999. Relationships between a new type IV secretion system and the *icm/dot* virulence system of *Legionella pneumophila.* Mol. Microbiol. **34:**799–809.

33. **Seifert, H. S., R. S. Ajioka, C. Marchal, P. F. Sparling, and M. So.** 1988. DNA transformation leads to pilin antigenic variation in *Neisseria gonorrhoeae.* Nature **336:**392–395.

34. **Seifert, H. S., R. S. Ajioka, D. Paruchuri, F. Heffron, and M. So.** 1990. Shuttle mutagenesis of *Neisseria gonorrhoeae*: pilin null mutations lower DNA transformation competence. J. Bacteriol. **172:**40–46.

35. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA **83:**735–739.

36. **Seifert, H. S., and M. So.** 1988. Genetic mechanisms of bacterial antigenic variation. Microbiol. Rev. **52:**327–336.

37. **Sparling, P. F.** 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. **92:**1364–1371.

38. **Stein, D. C.** 1991. Transformation of *Neisseria gonorrhoeae*: physical requirements of the transforming DNA. Can. J. Microbiol. **37:**345–349.

39. **Strathmann, M., B. A. Hamilton, C. A. Mayeda, M. I. Simon, E. M. Meyerowitz, and M. J. Palazzolo.** 1991. Transposon-facilitated DNA sequencing. Proc. Natl. Acad. Sci. USA **88:**1247–1250.

40. **Taha, M. K., M. So, H. S. Seifert, E. Billyard, and C. Marchal.** 1988. Pilin expression in *Neisseria gonorrhoeae* is under both positive and negative transcriptional control. EMBO J. **7:**4367–4378.

41. **Tao, L., J. M. Tanzer, H. K. Kuramitsu, and A. Das.** 1993. Identification of several *rod* loci and cloning of the *rodD* locus of *Streptococcus mutans.* Gene **126:**123–128.

42. **Valdivia, R. H., and S. Falkow.** 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277:**2007–2011.

43. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of *Legionella pneumophila.* Science **279:**873–875.

44. **Wainwright, L. A., K. H. Pritchard, and H. S. Seifert.** 1994. A conserved DNA sequence is required for efficient gonococcal pilin antigenic variation. Mol. Microbiol. **13:**75–87.

45. **Wolfgang, M., J. P. M. van Putten, S. F. Hayes, and M. Koomey.** 1999. The *comP* locus of *Neisseria gonorrhoeae* encodes a type IV prepilin that is dispensable for pilus biogenesis but essential for natural transformation. Mol. Microbiol. **31:**1345–1357.

46. **Zhou, D., and M. A. Apicella.** 1996. Plasmids with erythromycin resistance and catechol 2,3-dioxygenase- or beta-galactosidase-encoding gene cassettes for use in *Neisseria* spp. Gene **171:**133–134.