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New Complementation Constructs for Inducible and Constitutive Gene Expression in *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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We have created new complementation constructs for use in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The constructs contain regions of homology with the chromosome and direct the insertion of a gene of interest into the intergenic region between the genes *iga* and *trpB*. In order to increase the available options for gene expression in *Neisseria*, we designed the constructs to contain one of three different promoters. One of the constructs contains the isopropyl-β-D-thiogalactopyranoside-inducible *lac* promoter, which has been widely used in *Neisseria*. We also designed a construct that contains the strong, constitutive promoter from the gonococcal *opaB* gene. The third construct contains a tetracycline-inducible promoter, a novel use of this promoter in *Neisseria*. We demonstrate that anhydrotetracycline can be used to induce gene expression in the pathogenic *Neisseria* at very low concentrations and without negatively affecting the growth of the organisms. We use these constructs to complement an arginine auxotrophy in *N. gonorrhoeae* as well as to express a translational fusion of alkaline phosphatase with TraW. TraW is a component of the gonococcal type IV secretion system, and we demonstrate that TraW localizes to the periplasm.

Neisseria gonorrhoeae and *Neisseria meningitidis* are naturally transformable and do not regulate DNA uptake (2, 9, 18, 59). As a result, a variety of genetic tools for making mutations via transformation are available, and it is relatively easy to delete specific genes, construct point mutations, and perform insertional mutagenesis (17, 20, 47, 55). However, the tools that are available for performing complementation in the pathogenic *Neisseria* are limited. Prophages have been identified in the gonococcal and meningococcal chromosomes (14, 50), but no transducing phages have been isolated (16). Several plasmids are available for complementation in *N. gonorrhoeae* and *N. meningitidis*, including the Hermes vectors, pLES2, and pEN11 (7, 29, 62), but complementation using replicating plasmids in *Neisseria* can be problematic since engineered replicating plasmids are often unstable. Furthermore, since plasmids are nicked and taken up as a single strand during natural transformation, introduction of a replicating plasmid follows two-hit kinetics and thus occurs at a low frequency if the plasmid does not have homology to itself or the gonococcal chromosome (6, 18).

Instead, complementation in gonococci is usually performed by inserting the gene of interest into a site on the gonococcal chromosome. Several chromosomal loci have been characterized for use in gene complementation, including *proB*, *iga*, and the porin pseudogene (60, 61, 67). The intergenic region between *lctP* and *aspC* has also been used extensively for complementation, and a variety of constructs have been engineered to direct gene insertion at this site (19, 39, 64). Collectively, the existing complementation constructs have significant limitations. With the *iga* and *proB* constructs, gene insertion occurs within an open reading frame (61, 67), introducing the possibility that the growth or pathogenesis of the organism might be altered. Additionally, the available promoters for controlling gene expression are limited. In most cases, the gene of interest is either cloned along with its native promoter or expressed from the promoter of an upstream antibiotic resistance marker. To date, the *lac* promoter is the only inducible promoter that has been described for use in *Neisseria* (12, 54, 66). Additional tools for gene complementation in the pathogenic *Neisseria* are needed.

In this study, we describe a new set of complementation constructs that contain regions of homology with *iga* and *trpB* and direct gene insertion to the *iga-trpB* intergenic region following double-crossover recombination with the chromosome. These constructs were designed to contain one of three different promoters: the well-characterized *lac* promoter, a strong constitutive promoter, or a tetracycline-inducible promoter. This is the first report of tetracycline-inducible gene expression in *Neisseria*. We demonstrate that these constructs can be used to complement an arginine auxotrophy in gonococcal strain DGI2. We also use these constructs to express TraW, a required component of the gonococcal type IV secretion system (T4SS) (46), and investigate its localization within the cell. TraW, along with the other proteins required for type IV secretion, is encoded on the 57-kb gonococcal genetic island (GGI). In gonococci, the T4SS secretes single-stranded DNA into the extracellular environment (13, 18, 52), but little is known about the function of TraW in the process of secretion. Using alkaline phosphatase (AP) fusions in one of our new complementation constructs, we show that TraW localizes to the periplasm.

MATERIALS AND METHODS

Bacteria and growth conditions. Bacterial strains used in this study are described in Table 1. *Escherichia coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth at 37°C (53). Gonococcal and meningococcal strains were grown either on gonococcal base (GCB) agar plates (Difco) containing Kellogg's supplements (25) or in GCB liquid (GCBL)

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TABLE 1 Bacterial strains and plasmids

Plasmid or strain	Description	Reference or source
Plasmids		
pCK1	PCR of MS11 *traW* with traWF-XmaI and traWR-SpeI into pKH116 XmaI and SpeI sites ($TraW_{FL}$::′PhoA)	This work
pGCC6del	Derivative of pGCC6 that underwent a spontaneous deletion of portions of $lacI^q$ and downstream DNA	39
pHSS6	Backbone for complementation constructs; Km^r	56
pIDN1	Cloning vector, source of *ermC*	20
pIDN3	Cloning vector, source of *ermC*	20
pKH21	BamHI/blunt HpaI fragment from pRKO2 cloned into BamHI/blunt BstBI sites of a pGCC6del derivative modified to contain an improved polylinker	This work
pKH22	BamHI/blunt HpaI fragment from pRKO2 cloned into BamHI/blunt BstBI sites of a pGCC6del derivative modified to contain an improved polylinker	This work
pKH35[a]	*lctP-aspC* complementation construct, source of *lac* regulatory elements in pMR39	19
pKH37[a]	*lctP-aspC* complementation construct, source of *lac* regulatory elements in pMR33	27
pKH39	PCR of *lacZ* from JD1504 with lacZ5′-PstI and lacZ3′-MfeI into pKH35 PstI and MfeI sites	This work
pKH116	′*phoA* on blunt ApaI/KpnI fragment from pUI1158 cloned into pIDN1 Ecl136 and KpnI sites	This work
pMR18	PCR of *trpB-iga* from MS11 with trpBF-NotI and igaR-NotI cloned into pHSS6 NotI site	This work
pMR19	PCR around pMR18 with pMR18F and pMR18R-KpnI, digested with BsaI, and self-ligated	This work
pMR24	PCR from pKH37 with pKH37F and pKH37R-KpnI cloned into pMR19 KpnI site	This work
pMR28	PsiI/PacI fragment of PCR from FA1090 with OpaPro-PsiI and OpaPro-PacI cloned into PacI/blunt BseRI sites of pMR24	This work
pMR30	PCR around pMR18 with pMR18F and pMR18R-SacI, digested with BsaI, and self-ligated	This work
pMR32[b]	*iga-trpB* complementation construct (P_{opaB}), polylinker sites in same orientation as pKH37	This work
pMR33[b]	*iga-trpB* complementation construct (*lacPO*), polylinker sites in same orientation as pKH37	This work
pMR35	PCR from pKH35 with pKH37F and pKH37R-SacI cloned into pMR30 SacI site	This work
pMR38	PsiI/PacI fragment of PCR from FA1090 with OpaPro-PsiI and OpaPro-PacI cloned into PacI/blunt BseRI sites of pMR35	This work
pMR39[b]	*iga-trpB* complementation construct (*lacPO*), polylinker sites in same orientation as pKH35	This work
pMR41[b]	*iga-trpB* complementation construct (P_{opaB}), polylinker sites in same orientation as pKH35	This work
pMR47	PCR around pCK1 with deltraWF and deltraWR, digested with BamHI, and self-ligated ($TraW_{SS}$::′PhoA)	This work
pMR49	Blunted XhoI/FspI fragment of pKH21 cloned into blunted pMR19 KpnI site	This work
pMR50	Blunted SacI/FspI fragment of pKH22 cloned into blunted pMR19 KpnI site	This work
pMR53	PCR of pMR47 with pMR47F-SacII and pMR47R-XhoI into pMR33 SacII and XhoI sites	This work
pMR57	PCR of pMR47 with pMR47F-SacII and pMR47R-XhoI into pMR32 SacII and XhoI sites	This work
pMR58	PCR of pMR47 with pMR47F-SacII and pMR47R-XhoI into pKH37 SacII and XhoI sites	This work
pMR61	PCR of pCK1 with pMR47F-SacII and pMR47R-XhoI into pMR33 SacII and XhoI sites	This work
pMR68[b]	*iga-trpB* complementation construct (P_{tet}), polylinker sites in same orientation as pKH37	This work
pMR69[b]	*iga-trpB* complementation construct (P_{tet}), polylinker sites in same orientation as pKH35	This work
pMR70	PCR of pMR47 with pMR47F-SacII and pMR47R-SpeI into pMR68 SacII and SpeI sites	This work
pMR71	PCR of pMR47 with pMR47F-XhoI and pMR47R-SacII into pMR69 XhoI and SacI sites	This work
pMR90	PCR of *argJ* from MS11 with argJF-SacI and argJR-SpeI into pMR68 SacI and SpeI sites	This work
pRKO2	Original source of tetracycline repressor and p57opt promoter	63
pUI1158	Original source of ′*phoA* and polylinker	45
SM44	pWC147 containing mTnCm-3 insertion 44 downstream of *cnp1*	57
N. gonorrhoeae		
DGI2	AHU *N. gonorrhoeae*	44
MS11	WT *N. gonorrhoeae*	65
FA1090	WT *N. gonorrhoeae*	H. S. Seifert
JD1504	MS11 *pilE2*::mTnCmLac	31
ND500	MS11 ΔGGI	19
MR544	MS11 transformed by pMR53, *lacPO* $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR546	MS11 transformed by pMR57, P_{opaB} $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR547	MS11 transformed by pMR58, *lacPO* $TraW_{SS}$::′PhoA at *lctP-aspC*	This work
MR553	MS11 transformed by pMR61, *lacPO* $TraW_{FL}$::′PhoA at *iga-trpB*	This work
MR554	MS11 transformed by pMR71, P_{tet} $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR555	MR544 transformed by SM44, *lacPO* $TraW_{SS}$::′PhoA at *iga-trpB*, Cm^r	This work
MR556	MR553 transformed by SM44, *lacPO* $TraW_{FL}$::′PhoA at *iga-trpB*, Cm^r	This work
MR557	MS11 transformed by pMR70, P_{tet} $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR558	PK180 transformed by pMR70	This work
MR559	ND500 transformed by pMR53, *lacPO* $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR560	ND500 transformed by pMR61, *lacPO* $TraW_{FL}$::′PhoA at *iga-trpB*	This work
MR561	MR559 transformed by SM44, *lacPO* $TraW_{SS}$::′PhoA at *iga-trpB*, Cm^r	This work
MR562	MR560 transformed by SM44, *lacPO* $TraW_{FL}$::′PhoA at *iga-trpB*, Cm^r	This work
MR574	DGI2 transformed by pMR90	This work
PK180	MS11 transformed by pKH39	P.L. Kohler
N. meningitidis		
ATCC 13102		ATCC, 13
MR1000	ATCC 13102 transformed by pMR57, P_{opaB} $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR1002	ATCC 13102 transformed by pMR33, *lac* regulatory elements at *iga-trpB*	This work
MR1003	ATCC 13102 transformed by pMR68, *tet* regulatory elements at *iga-trpB*	This work
MR1004	MR1002 transformed by pMR53, *lacPO* $TraW_{SS}$::′PhoA at *iga-trpB*	This work
MR1005	MR1003 transformed by pMR70, P_{tet} $TraW_{SS}$::′PhoA at *iga-trpB*	This work

[a] Constructs for complementation at the *lctP-aspC* site.
[b] Constructs for complementation at the *iga-trpB* site.

TABLE 2 PCR primers

Primer name	Sequence (5′ → 3′)[a]
argJF-SacI	GGTGGTGAGCTCCGCAAGGAGAACCGTTATGG
argJR-SpeI	GGTGGTACTAGTGTGGAGGTCATGGTATGTTGTC
deltraWF	GGTGGTGGATCCGTTCTGGAAAACCGGGCTGCTCAGG
deltraWR	GGTGGTGGATCCTTCCGCTATTGGGTATGTGG
igaR-NotI	GGTGCGGCCGCTATGTGGCCGGCGATATTGG
lacZ5′-PstI	GCGACTGCAGCACACAGGAAACAGCTATGA
lacZ3′-MfeI	GAGCCAATTGGGCCTGCCCGGTTATTATTA
OpaPro-PsiI	CGACGGTTATAAGGAATGACGGCGGAAAGATG
OpaPro-PacI	CGTTAATTAAGGGCGGATTATATCGGGTTC
pKH37F	CCATCCGTTCTGCTCTATAC
pKH37R-KpnI	GGTGGTACCTTGGTCATGGCCAGCTTATC
pKH37R-SacI	GGTGAGCTCTTGGTCATGGCCAGCTTATC
pMR18F	GCCGGGTCTCCCAGTTTATGCATCCCTTAAATTTAGT ATTTTAGAAACGAATCT
pMR18R-KpnI	GCCGGGTCTCCACTGCATCCCTTAAGGGGGTACCTCA TAGCAAAATAAAATGCCGTCT
pMR18R-SacI	GCCGGGTCTCCACTGCATCCCTTAAGGGGAGCTCTCA TAGCAAAATAAAATGCCGTCT
pMR47F-SacII	GGTGGTCCGCGGGCCGGCTTAATCCATTCAGG
pMR47F-XhoI	GGTGGTCTCGAGGCCGGCTTAATCCATTCAGG
pMR47R-SacII	GGTGGTCCGCGGCCGTGATCTGCCATTAAGTC
pMR47R-SpeI	GGTGGTACTAGTCCGTGATCTGCCATTAAGTC
pMR47R-XhoI	GGTGGTCTCGAGCCGTGATCTGCCATTAAGTC
traWF-XmaI	GGTCCCGGGCCGGCTTAATCCATTCAGGGC
traWR-SpeI	GGTACTAGTCGGTTTCATGACCTCTACACG
trpBF-NotI	GGTGCGGCCGCAAAGCGCAGATGCAGGAAGC

[a] Restriction sites are underlined.

medium containing Kellogg's supplements and 0.042% $NaHCO_3$ (41) as described previously (12). Erythromycin was used at 10 μg/ml for meningococci, 2 μg/ml for gonococcal strain MR574, 10 μg/ml for all other gonococcal strains, and 500 μg/ml for *E. coli*. Kanamycin was used at 40 μg/ml for *E. coli*. Chloramphenicol was used at 10 μg/ml for gonococci and 25 μg/ml for *E. coli*. Gene expression in gonococci was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) or 2 ng/ml anhydrotetracycline (ATc), except where otherwise noted. To observe alkaline phosphatase activity, meningococcal strains were grown on GCB-Tris agar plates containing 5-bromo-4-chloro-3-indolylphosphate (XP) (8) with or without 1 mM IPTG or 20 ng/ml anhydrotetracycline. To measure growth of the AHU (arginine, hypoxanthine, and uracil) auxotrophic strain DGI2 and the complemented strain MR574, the strains were grown in liquid gonococcal genetic medium (GGM) (30) without soluble starch in the presence or absence of L-arginine, and the optical density at 540 nm (OD_{540}) was recorded.

DNA techniques. Plasmid DNA was isolated using a QIAPrep miniprep kit (Qiagen). Gel purification of digested DNA was performed using a QIAQuick gel extraction kit (Qiagen). Blunting of digested DNA ends was performed using T4 DNA polymerase (New England BioLabs). After ligation, DNA was transformed into chemically competent RapidTrans TAM1 *E. coli* cells (Active Motif). Plasmid screening was conducted by generating whole-cell lysates using the lysis solution of Kado and Liu and analyzing the lysates by agarose gel electrophoresis (24). Further screening of possible transformants was conducted by restriction enzyme digest and PCR.

Plasmid construction. See Table 1 for a description of the plasmids used in this study. The sequences of all PCR primers are provided in Table 2. Briefly, to make the complementation constructs for IPTG-inducible gene expression, the *lac* regulatory elements, chloramphenicol acetyltransferase (CAT) gene (*cat*), and a polylinker were PCR amplified from pKH37 (27) using the primers pKH37F and pKH37R-KpnI. The PCR product was digested with KpnI and ligated into the KpnI site of pMR19 to generate pMR24. To make the same plasmid as pMR24 but with the polylinker sites in the reverse orientation, the primers pKH37F and pKH37R-SacI were used to generate a PCR product from pKH35 (19). The PCR product was digested with SacI and ligated into the SacI site of pMR30 to make pMR35. The *cat* markers in these plasmids were replaced with *ermC* by ligating the blunt NsiI/SphI fragment from pIDN3 (20) into the blunt Bpu10I/BseRI fragment of pMR24 and pMR35 to form pMR33 and pMR39, respectively.

To make the complementation constructs for constitutive gene expression, the *opaB* promoter (P_{opaB}) was PCR amplified from chromosomal DNA of gonococcal strain FA1090 using the primers OpaPro-PsiI and OpaPro-PacI. The PCR product was digested with PsiI and PacI and ligated into the PacI/blunted BseRI fragment of pMR24 and pMR35 to generate pMR28 and pMR38, respectively. The *cat* markers in these plasmids were replaced with *ermC* by ligating the blunted NsiI/SphI fragment from pIDN3 (20) into the blunted Bpu10I/BseRI fragment from pMR28 and pMR38 to form pMR32 and pMR41, respectively.

To make the complementation constructs for tetracycline-inducible gene expression, the *tet* regulatory elements were cloned from pRKO2 (63) into pGCC6del. This plasmid was further modified, and new polylinkers were generated to make plasmids pKH21 and pKH22. The *tet* regulatory elements, the polylinker, and the *cat* marker contained on the blunted XhoI/FspI fragment from pKH21 and the blunted SacI/FspI fragment from pKH22 were cloned into the blunted KpnI site of pMR19 to form pMR49 and pMR50, respectively. To replace the *cat* markers in these plasmids with *ermC* and to add additional DNA uptake sequences (DUS), the blunted KpnI/NheI fragment from pIDN1 (20) was cloned into the blunted AflII/SacI-HF fragments from pMR49 and pMR50 to form pMR68 and pMR69, respectively.

Transformation of gonococci and meningococci. Spot transformations were used to transform gonococci and meningococci with complementation construct plasmid DNA. Plasmids smaller than 8,000 bp were linearized by digestion with NheI or PciI. Between 1 and 10 μg plasmid DNA in 20 μl water was spotted onto a GCB agar plate and allowed to dry. Several piliated colonies of *N. gonorrhoeae* strain MS11, ND500, or DGI2 or *N. meningitidis* strain ATCC 13102 were streaked over the spots, and the plate was incubated overnight at 37°C with 5% CO_2. A Dacron swab was used to transfer colonies from the spots onto GCB agar plates containing 10 μg/ml erythromycin (2 μg/ml for DGI2) or 10 μg/ml chloramphenicol. Individual transformants were restreaked and screened by PCR.

Immunoblot analysis. Proteins were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad) either for 1 h at 100 V or overnight at 30 V. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.5% Tween 20 (TTBS) either for 1 h or overnight. Membranes were incubated with primary antibody in TTBS for 1 h, washed with TTBS for 15 min, and incubated with the secondary antibody in TTBS for 1 h. Blots were then washed with TTBS for 30 min, developed using an Immun-Star horseradish peroxidase substrate kit (Bio-Rad), and exposed to film. For the primary antibodies, anti-PhoA (Chemicon) was used at 1:1,000, anti-CAT (Sigma) was used at 1:7,000, and anti-PilQ (H. S. Seifert) was used at 1:10,000. The appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at a 1:10,000 dilution. Densitometry analysis of immunoblots was performed using ImageJ software (1).

β-Galactosidase and alkaline phosphatase assays. Gonococcal strains were grown overnight at 37°C on GCB agar plates. Strains were inoculated into 3 ml of GCBL medium containing Kellogg's supplements and 0.042% $NaHCO_3$ (41) at an OD_{540} of 0.25 and grown for 3 h. Cultures were vortexed, and 1 ml of cells was subcultured into 2 ml fresh medium at an OD_{540} of approximately 0.15. Cultures were induced with 0.5 mM IPTG or 2 ng/ml ATc and grown for an additional 2 h.

Assays for alkaline phosphatase activity were conducted as described previously (36). Cells from a 0.5-ml volume of culture were harvested by centrifugation at 13,600 × *g* for 30 s, washed once in 10 mM $MgSO_4$ in 10 mM Tris-HCl, pH 8.0, and resuspended in 0.5 ml cold 1 M Tris-HCl, pH

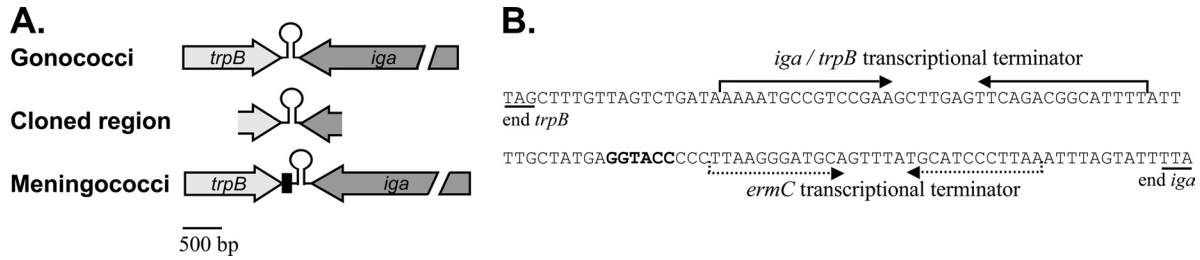

FIG 1 Analysis of the intergenic region between the genes *iga* and *trpB*. (A) Schematic of *iga-trpB* locus in gonococci and meningococci. The 1,134-bp region of genomic DNA from *N. gonorrhoeae* strain MS11 that was cloned into the complementation constructs is also represented. The transcriptional terminator shared by *iga* and *trpB* is indicated by a stem-loop structure. Meningococcal strains contain a 103-bp Correia element (black rectangle) inserted between the stop codon of *trpB* and the transcriptional terminator. (B) Changes made to the *iga-trpB* intergenic region in the complementation constructs. The stop codons of *trpB* and *iga* are underlined. A second transcriptional terminator from the erythromycin resistance gene *ermC* was inserted downstream of *iga*, and a restriction site was added between the two terminators for cloning purposes (bold type). All gene insertion is directed into this restriction site, such that both *iga* and *trpB* maintain transcriptional terminators.

8.0. To this suspension was added 0.5 ml 0.1 mM $ZnCl_2$ in 1 M Tris-HCl, pH 8.0, followed by 50 μl chloroform and 50 μl 0.1% SDS. Cells were vortexed, incubated for 5 min at 37°C, and then incubated for 5 min on ice. A volume of 100 μl of 4 mg/ml *p*-nitrophenylphosphate in 1 M Tris-HCl, pH 8.0, was added, and the solution was incubated at 37°C until it turned pale yellow, usually within 25 min. The reaction was terminated by the addition of 120 μl of 83 mM EDTA, 166 mM KH_2PO_4. Tubes were placed on ice, and the OD_{550} and OD_{420} were measured.

β-Galactosidase assays were conducted as previously described (40). Briefly, cells from a 0.5-ml volume of the culture were harvested by centrifugation at 13,600 × *g* for 30 s. The pellet was washed once in 10 mM $MgSO_4$ in 10 mM Tris-HCl, pH 8.0, and resuspended in cold 1 M Tris-HCl, pH 8.0. To this cell suspension was added a 0.5-ml volume of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $Na_2HPO_4 \cdot H_2O$, 10 mM KCl, 50 mM β-mercaptoethanol, pH adjusted to 7.0), followed by 50 μl chloroform and 50 μl 0.1% SDS. Cells were vortexed and incubated for 5 min at 37°C, followed by 5 min on ice. A volume of 200 μl 4 mg/ml *o*-nitrophenyl-β-galactopyranoside in Z buffer was added, and the reaction mixture was incubated at 28°C until a pale yellow color resulted. The time was noted, and the reaction was terminated by the addition of 120 μl 1 M Na_2CO_3. The OD_{420} and OD_{550} were measured.

For both assays, a 1.5-ml volume of bacterial culture was harvested by centrifugation at 13,600 × *g* for 30 s, resuspended in 0.5 ml 1 M Tris-HCl, pH 8.0, and lysed by sonication, and a Bradford assay was performed to measure total protein. Phosphatase activity and β-galactosidase activity were calculated using the following equation: $1{,}000 \times (OD_{420} - [1.75 \times OD_{550}])/(\text{time in minutes} \times \text{mg of protein} \times \text{volume in ml})$.

Subcellular fractionation. Gonococcal strains were grown overnight at 37°C on GCB agar plates. For each strain, two 4-ml cultures were inoculated in GCBL medium containing Kellogg's supplements and 0.042% $NaHCO_3$ (41) at an OD_{540} of 0.25. The cultures were induced with 0.5 mM IPTG and grown for 3 h. The cells were harvested by centrifugation at 11,952 × *g* for 5 min and washed once in ice-cold 0.01 M Tris-HCl, pH 7.0. The cell pellet was resuspended in 4 ml 0.01 M Tris-HCl, pH 7.0, and sonicated for 30 s at 45% amplitude with a 1-s on-off pulse using a Branson digital sonifier. The sonicate was centrifuged at 15,557 × *g* in an F-20/Micro rotor for 10 min to remove any unlysed cells, and the supernatant was passed through a 0.2-μm-pore-size filter. The supernatant was centrifuged at 126,000 × *g* in a TLA110 rotor for 1.5 h to separate soluble from membrane fractions. The membrane fraction was washed with 750 μl 0.01 M Tris-HCl, pH 7.0, and centrifuged at 127,000 × *g* in a TLA120.2 rotor for 1.5 h. The pellet was resuspended in 50 μl 0.1 M Tris-HCl buffer, pH 7.0. A Bradford assay was used to normalize the amount of protein electrophoresed on SDS-polyacrylamide gels.

RESULTS

Design of complementation constructs. In order to identify a chromosomal region for complementation, we reviewed sites that had successfully been used in the past and chose the *iga* locus (67). This locus encodes immunoglobulin A1 (IgA1) protease, a secreted protease produced only by the pathogenic *Neisseria* that cleaves the hinge region of IgA (28, 42) and also cleaves LAMP1 in epithelial cells (32). The *iga* locus was also attractive since it was shown that insertions within *iga* do not affect infection in human male volunteers (22). We improved upon the existing constructs for complementation at *iga* by designing our constructs so that gene insertion occurs in the intergenic region between *iga* and the neighboring gene, *trpB*, thereby leaving the *iga* open reading frame intact. Plasmid pHSS6 was used as the starting point to construct the complementation constructs because of its small size and that fact that it replicates in *E. coli* but not *N. gonorrhoeae* (56).

The *iga* and *trpB* genes share a transcriptional terminator (Fig. 1A) (23). A 1,134-bp region of chromosomal DNA from gonococcal strain MS11 containing portions of the *iga* and *trpB* genes as well as the intergenic region was cloned into pHSS6 (Fig. 1A). The *iga-trpB* region is conserved between gonococci and meningococci. The sequence of the *iga-trpB* locus from MS11 is between 89 and 92% identical to the sequence from several sequenced meningococcal genomes (GenBank accession no. CP000381.1, AE002098.2, AM421808.1, AL157959.1, and AM889136.1), and the 1,134-bp sequence cloned into pHSS6 is even more highly conserved (94 to 95% identical). Sequence analysis of the *iga-trpB* region revealed that meningococci differ from gonococci by the presence of a 103-bp Correia element inserted between the stop codon of *trpB* and the shared transcriptional terminator (Fig. 1A) (10, 11, 58). Given the high overall conservation within the *iga* and *trpB* open reading frames, however, we predicted that these constructs would be useful for complementation of both gonococci and meningococci.

To ensure that both *trpB* and *iga* would maintain transcriptional terminators following gene insertion in the intergenic region, we cloned the transcriptional terminator from the erythromycin resistance gene (*ermC*) downstream of *iga* in the complementation constructs (Fig. 1B). We also added a restriction site between the two terminators, and this site served as the insertion site for the promoters, polylinkers, and selectable markers (Fig. 1B). In order to make the *iga-trpB* complementation constructs useful in combination with the existing constructs that direct gene insertion at the *lctP-aspC* site (pKH35 and pKH37) (19, 27), we designed the *iga-trpB* constructs to contain a different selectable marker (erythromycin resistance, *ermC*) (Fig. 2).

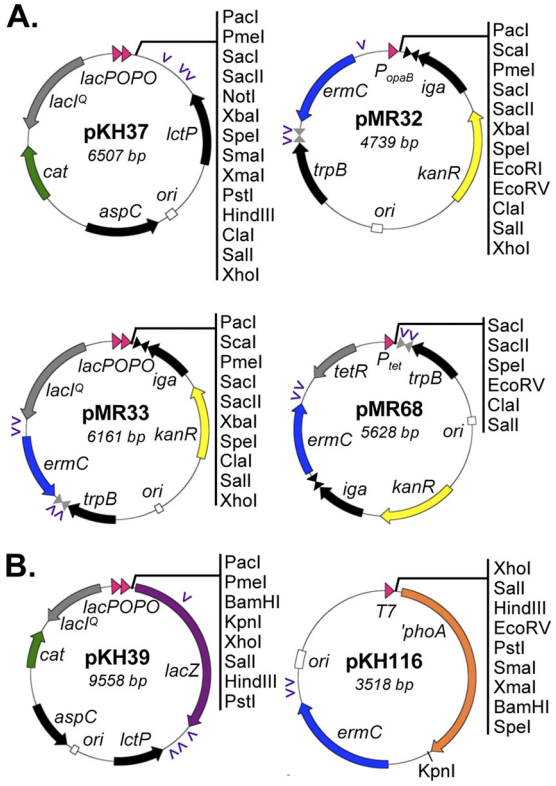

FIG 2 Description of complementation constructs and plasmids for constructing *lacZ* and *phoA* fusions. (A) Plasmid maps of complementation constructs. Regions of homology with the gonococcal and meningococcal chromosomes are shown in black. Plasmid pKH37 directs gene insertion between *lctP* and *aspC* and contains tandem copies of the *lac* promoter and operator (*lacPOPO*), the *lac* repressor (*lacI*^{q}), and the selectable marker chloramphenicol acetyltransferase (*cat*). Plasmids pMR32, pMR33, and pMR68 all direct gene insertion between *trpB* and *iga* and encode an erythromycin resistance marker (*ermC*). Plasmid pMR33 contains *lacPOPO* and *lacI*^{q}, pMR32 contains the constitutive promoter from the *opaB* gene of *N. gonorrhoeae* strain FA1090 (P_{opaB}), and pMR68 contains the tetracycline-inducible promoter (P_{tet}) and *tet* repressor (*tetR*). The transcriptional terminator found in the chromosome between *iga* and *trpB* is indicated by gray inverted triangles. The added transcriptional terminator from the *ermC* gene is indicated by black inverted triangles. Unique restriction sites in the polylinkers are shown. DNA uptake sequences are indicated by the purple arrowheads. Plasmids pKH35, pMR41, pMR39, and pMR69 (data not shown) are identical to pKH37, pMR32, pMR33, and pMR68, respectively, except that the orientation of the sites in the polylinker is reversed to facilitate cloning. (B) Plasmids for creating *lacZ* and *phoA* fusions in *N. gonorrhoeae*. Plasmid pKH39 contains *lacZ* with a ribosome binding site in pKH35, while pKH116 encodes *phoA* without its signal sequence (*'phoA*).

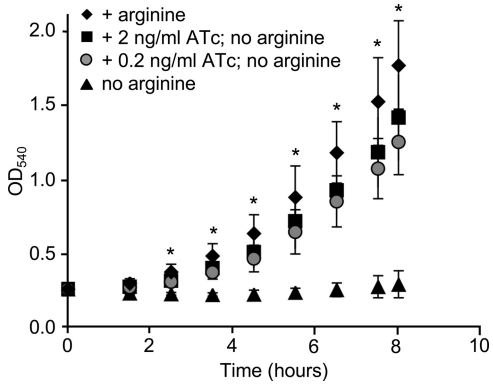

FIG 3 Complementation of *argJ* in strain DGI2, which requires arginine, hypoxanthine, and uracil for growth. DGI2 was transformed with pMR90 to generate strain MR574, which expresses the wild-type *argJ* under the control of P_{tet} at the *iga-trpB* locus. Strain MR574 was grown in liquid gonococcal genetic medium (GGM) containing arginine or in medium without added arginine. ArgJ production was induced during growth in GGM lacking arginine by the addition of 0.2 ng/ml or 2 ng/ml ATc. The mean ± standard deviation of three independent experiments is shown. *, growth of MR574 in the presence of ATc inducer was not significantly different from growth in the presence of arginine (Student's two-tailed *t* test, $P > 0.05$), but growth under both conditions was significantly different from growth in the absence of both arginine and ATc (Student's two-tailed *t* test, $P < 0.05$).

We designed the *iga-trpB* constructs to contain one of three different promoters (Fig. 2A). Plasmid pMR32 contains the strong constitutive *opaB* promoter from *N. gonorrhoeae* strain FA1090 (P_{opaB}). P_{opaB} differs by only 1 bp in the −35 hexamer (TTGAAA) from the sigma-70 consensus promoter sequence (5). Plasmid pMR33 contains the tandem *lac* promoter/operator (*lacPOPO*) and *lac* repressor (*lacI*^{q}) from pKH37 (27). Plasmid pMR68 contains the tetracycline-inducible P57opt promoter/operator (P_{tet}) and tetracycline repressor (*tetR*) from pRKO2 (63). The P57opt promoter has been optimized so that the −35 hexamer matches the sigma-70 consensus sequence, resulting in increased promoter activity (63). The tetracycline-regulated promoter has been used in a variety of prokaryotic and eukaryotic expression systems (4, 63), but this is the first description of its use in *Neisseria*. We also constructed variants of pMR32, pMR33, and pMR68 in which the orientation of the polylinker sites is reversed to facilitate cloning (pMR41, pMR39, and pMR69, respectively) (Fig. 2A).

Functional complementation of an arginine auxotrophy. To demonstrate that the constructs that we describe can be used for functional complementation, we used pMR68 to complement an arginine auxotrophy in gonococcal strain DGI2, which is part of a group of strains requiring arginine, hypoxanthine, and uracil for growth (AHU auxotrophs) (44). The requirement for arginine in AHU strains is due to a mutation in the *argJ* gene, encoding ornithine acetyltransferase (37, 38, 49). Strain DGI2 is an *argJ* mutant (GenBank accession no. ACIG00000000.1) and does not grow in GGM lacking arginine (data not shown). We transformed DGI2 with pMR90, which contains the wild-type (WT) *argJ* gene from strain MS11, and measured growth of the resulting strain, MR574, in the presence or absence of arginine and the anhydrotetracycline (ATc) inducer. Strain MR574 grew in the presence but not the absence of arginine, suggesting that appreciable amounts of ArgJ are not produced from P_{tet} in the absence of inducer (Fig. 3). The addition of 0.2 or 2 ng/ml ATc enabled growth of MR574 in the absence of arginine (Fig. 3).

Characterization of gene expression from complementation constructs. In order to better quantify expression from the different complementation constructs, we made use of two plasmids that allow the construction of *lacZ* or *phoA* fusions. Plasmid pKH39 is an *lctP-aspC* complementation construct containing *lacZ*, while pKH116 encodes *phoA* without its signal sequence (*'phoA*) (Fig. 2B). We decided to characterize the relative expression from the *lacPO*, P_{opaB}, and P_{tet} constructs by measuring the activity of an alkaline phosphatase (PhoA) fusion to TraW. TraW is a component of the gonococcal type IV secretion system and contains a predicted signal sequence (SignalP, version 4.0, server) (48), suggesting that TraW may localize to the periplasm. We

constructed two different PhoA fusions containing either the full-length TraW open reading frame ($TraW_{FL}$::′PhoA) or the predicted TraW signal sequence ($TraW_{SS}$::′PhoA). Expression of both PhoA fusions in *E. coli* resulted in blue colonies when the bacteria were grown on XP indicator plates, indicating that both fusions localize to the periplasm in *E. coli* (data not shown).

The $TraW_{SS}$::′PhoA fusion was cloned into pKH37, pMR32 (P_{opaB}), pMR33 (*lacPO*), and pMR68 (P_{tet}). The resulting plasmids were introduced into gonococci by natural transformation to generate strains MR547, MR546, MR544, and MR557, respectively. We measured the growth of these strains in GCBL medium and confirmed that gene insertion at the *iga-trpB* site did not alter their growth rate (data not shown). We then conducted alkaline phosphatase assays to assess production of $TraW_{SS}$::′PhoA in these strains (Fig. 4A). Wild-type *N. gonorrhoeae* does not express AP activity (Fig. 4A) (8). AP activity was similar in strains expressing $TraW_{SS}$::′PhoA under the control of *lacPO* at either the *lctP-aspC* site (MR547) or the *iga-trpB* site (MR544), although AP activity was slightly lower in the strain transformed with the *iga-trpB* construct (Fig. 4A). In both cases, AP activity was observed only in the presence of IPTG, and growth in the absence of the inducer for 2 h resulted in background levels of AP activity (Fig. 4A). Compared to MR544, a strain expressing $TraW_{SS}$::′PhoA from P_{opaB} at the *iga-trpB* site (MR546) produced almost three times as much AP activity, confirming that the P_{opaB} construct provides strong, constitutive gene expression (Fig. 4A). In a strain expressing $TraW_{SS}$::′PhoA from the tetracycline-inducible promoter (MR557), the levels of AP activity in the presence of 2 ng/ml ATc were similar to the levels of AP activity produced by MR544. AP activity was at background levels in the absence of the ATc after 2 h of growth (Fig. 4A). An immunoblot detected levels of PhoA correlating with the activity measurements. These results demonstrate that the new complementation constructs can be used to produce substantial amounts of a protein of interest from the very strong constitutive *opaB* promoter or from the regulatable *lac* or *tet* promoters. Furthermore, they establish P_{tet} as the second inducible promoter available for gene expression in *N. gonorrhoeae*.

Complementation in *N. meningitidis*. Due to the similarity of the *iga-trpB* region in meningococci and gonococci, we expected that these constructs would also be useful for complementation in meningococci. We transformed meningococcal strain ATCC 13102 with pMR53 (*lacPO*), pMR57 (P_{opaB}), and pMR70 (P_{tet}), which all encode $traW_{SS}$::′*phoA*. We were able to transform strain ATCC 13102 with pMR57, and the resulting strain, MR1000, produced blue colonies when grown on XP indicator plates (data not shown). We were unable to transform ATCC 13102 with pMR53 or pMR70 after several attempts, likely because the size of the nonhomologous insert in these plasmids was significantly larger than that in pMR57. However, we were able to transform these constructs into ATCC 13102 in two steps. We first transformed ATCC 13102 with the empty complementation constructs pMR33 and pMR68. The size of the nonhomologous insert is smaller in these constructs since they do not contain the $traW_{SS}$::′*phoA* fusion, and we were able to obtain erythromycin-resistant colonies that carried the *lac* or *tet* regulatory elements on the chromosome. We then transformed these strains with pMR53 and pMR70 to introduce $traW_{SS}$::′*phoA* and screened for blue $PhoA^+$ colonies on XP indicator plates, generating strains MR1004 and MR1005, respectively.

We grew strains MR1004 (producing $TraW_{SS}$::′PhoA under

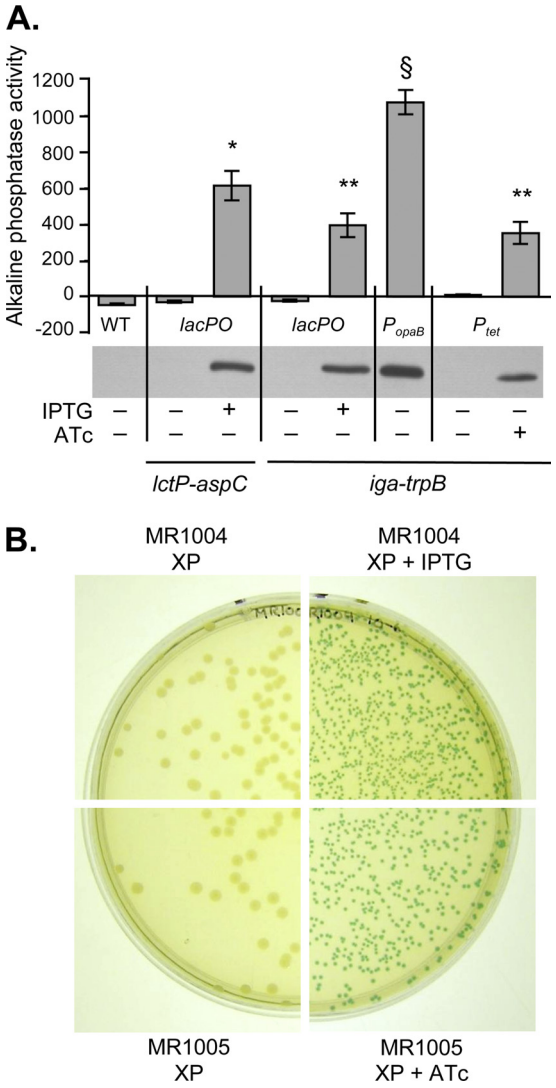

FIG 4 Characterization of gene expression from the complementation constructs. (A) Alkaline phosphatase activity and immunoblot analysis of $TraW_{SS}$::′PhoA expression in gonococcal strain MS11 (WT) and in gonococcal strains transformed with the complementation constructs. Expression of $TraW_{SS}$::′PhoA from the *lctP-aspC* site was driven by the *lac* promoter (*lacPO*) in strain MR547. Expression of $TraW_{SS}$::′PhoA from the *iga-trpB* site was driven by one of three promoters: *lacPO* in strain MR544, the constitutive *opaB* promoter (P_{opaB}) in strain MR546, or the tetracycline-inducible promoter (P_{tet}) in strain MR557. Production of $TraW_{SS}$::′PhoA was induced with either 0.5 mM IPTG or 2 ng/ml ATc. Alkaline phosphatase activity was normalized to mg of protein. The amount of $TraW_{SS}$::′PhoA fusion protein was detected by immunoblotting with anti-PhoA (α-PhoA) antibodies. The mean ± standard deviation of at least three independent experiments is shown. *, alkaline phosphatase activity from the strain with *lacPO* at the *lctP-aspC* site in the presence of inducer was significantly different from that of the uninduced control ($P < 0.005$) and also different from the activities of strains with *lacPO*, P_{opaB}, and P_{tet} at the *iga-trpB* site ($P \leq 0.01$) by Student's two-tailed *t* test; **, alkaline phosphatase activity from the strains with *lacPO* or P_{tet} at the *iga-trpB* site in the presence of inducer was significantly different from that of the respective uninduced controls ($P \leq 0.001$) but was not significantly different between strains ($P > 0.05$) by Student's two-tailed *t* test; §, alkaline phosphatase activity from the strain with P_{opaB} at the *iga-trpB* site was significantly different from the activities of all other inducible strains ($P < 0.001$) by Student's two-tailed *t* test. (B) Use of the *iga-trpB* complementation constructs in *N. meningitidis*. Strains MR1004 (producing $TraW_{SS}$::′PhoA under the control of *lacPO*) and MR1005 (producing $TraW_{SS}$::′PhoA under the control of P_{tet}) were grown on GCB-Tris-XP agar plates containing either the XP substrate alone, XP with 1 mM IPTG, or XP with 20 ng/ml ATc.

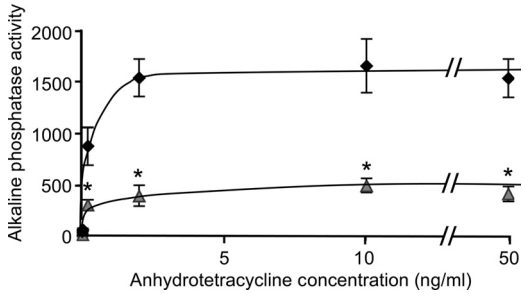

FIG 5 Alkaline phosphatase activity of gonococcal strains expressing $TraW_{SS}$::′PhoA under the control of the tetracycline-inducible promoter in the presence of different concentrations of ATc. Black diamonds, MR554 (MS11 transformed with pMR71); gray triangles, MR557 (MS11 transformed with pMR70). Alkaline phosphatase activity was normalized to mg of protein. The mean ± standard deviation of three independent experiments is shown. *, $P \leq 0.002$ compared to MR554, Student's two-tailed *t* test.

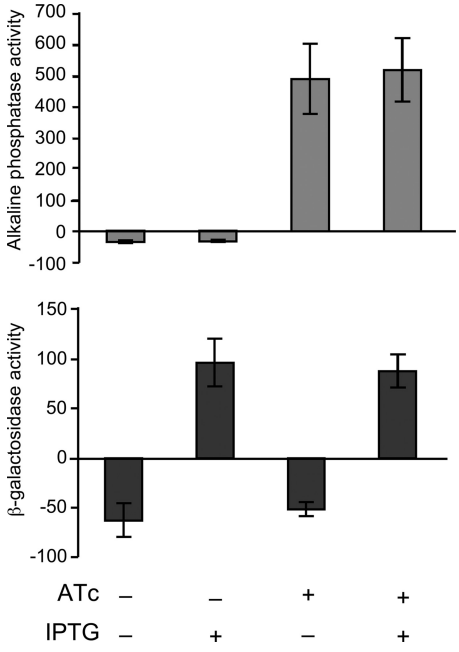

FIG 6 Independent and simultaneous induction of the *lac* promoter and tetracycline-inducible promoter (P_{tet}) within the same gonococcal cell. We constructed a strain (MR558) that expresses *lacZ* under the control of the *lac* promoter at the *lctP-aspC* site and $traW_{SS}$::′*phoA* under the control of P_{tet} at the *iga-trpB* site. We then measured alkaline phosphatase activity and β-galactosidase activity in the presence or absence of 0.5 mM IPTG and/or 2 ng/ml ATc. Alkaline phosphatase activity and β-galactosidase activity were normalized to mg protein. The mean ± standard deviation of three independent experiments is shown.

the control of *lacPO*) and MR1005 (producing $TraW_{SS}$::′PhoA under the control of P_{tet}) on XP indicator plates with or without the appropriate inducer. Both strains produced white colonies on plates containing the XP substrate without inducer (Fig. 4B). On plates that contained both XP and either the IPTG or ATc inducer, both strains produced colonies that appeared light blue by 24 h of growth and were dark blue after 40 h of growth (Fig. 4B). The *lac* promoter has been used before in meningococci (66), but this is the first report of tetracycline-inducible gene expression in meningococci.

Optimal induction of P_{tet} promoter. Since this is the first description of tetracycline-inducible gene expression in *Neisseria*, we sought to better characterize the induction of the P_{tet} promoter with ATc. In our initial induction experiments in gonococci, ATc concentrations of both 2 ng/ml and 20 ng/ml were sufficient to generate high levels of AP activity from $TraW_{SS}$::′PhoA. To determine optimal induction conditions, we then tested a range of ATc concentrations. Maximum levels of AP activity were detected with ATc concentrations as low as 2 ng/ml, and increasing the ATc concentration to as high as 50 ng/ml did not result in any additional increases in AP activity (Fig. 5). Importantly, ATc did not negatively affect cell growth, even at relatively high concentrations. Concentrations of ATc lower than 2 ng/ml (for example, 0.2 and 0.02 ng/ml) produced intermediate levels of AP activity, making it possible to modulate promoter strength by changing the concentration of the inducer (Fig. 5).

While performing these experiments, we noticed that a strain of gonococci that had been transformed with pMR71 (strain MR554) consistently produced higher levels of AP activity than a strain of gonococci that had been transformed with pMR70 (strain MR557) (Fig. 5). Plasmids pMR70 and pMR71 both contain $TraW_{SS}$::′PhoA under the control of P_{tet} and differ only in the orientation of the restriction sites in the polylinker. The reason for the observed difference in AP activity between the two strains is unknown, but DNA sequencing confirmed that it is not due to any sequence mutations in the region containing the P_{tet} promoter or the promoter for the *tet* repressor (data not shown).

Simultaneous use of two inducible promoters. Since the pKH35 and pKH37 complementation constructs contain a *cat* marker and recombine at *lctP-aspC* and all of the new constructs described in this work contain an *ermC* marker and recombine at *iga-trpB*, it should be possible to express two different gene products from the two chromosomal sites and to induce the expression of these genes either independently or simultaneously. To investigate this possibility, the WT *N. gonorrhoeae* strain MS11 was transformed with pKH39 to generate a strain that expresses *lacZ* under the control of *lacPO* at the *lctP-aspC* site (strain PK180). We then transformed this strain with pMR70, which produces $TraW_{SS}$::′PhoA under the control of P_{tet} to generate strain MR558.

We grew MR558 either without inducer, with IPTG or ATc alone, or in the presence of both inducers. We then conducted β-galactosidase and alkaline phosphatase assays on bacteria grown under the different conditions (Fig. 6). In the absence of both inducers, we did not detect activity in either assay. In the presence of IPTG, we detected only β-galactosidase activity, and in the presence of ATc, we detected only AP activity. In the presence of both inducers, we detected activity in both assays (Fig. 6). There was no noticeable growth defect when cells were grown in the presence of both IPTG and ATc, and activity levels for alkaline phosphatase and β-galactosidase were comparable whether both inducers were present or whether a single inducer was present. Thus, it is possible to use both *lacPO* and P_{tet} in the same cell and to turn them on and off independently of one another.

Subcellular localization of TraW::PhoA fusions. We used expression of the TraW::PhoA translational fusions from the new complementation constructs to examine subcellular localization of TraW. Expression of both $TraW_{SS}$::′PhoA and $TraW_{FL}$::′PhoA from the *iga-trpB* complementation site in *N. gonorrhoeae* resulted in AP activity (Fig. 7A). Since alkaline phosphatase must be transported to the periplasm in order to be active, these results

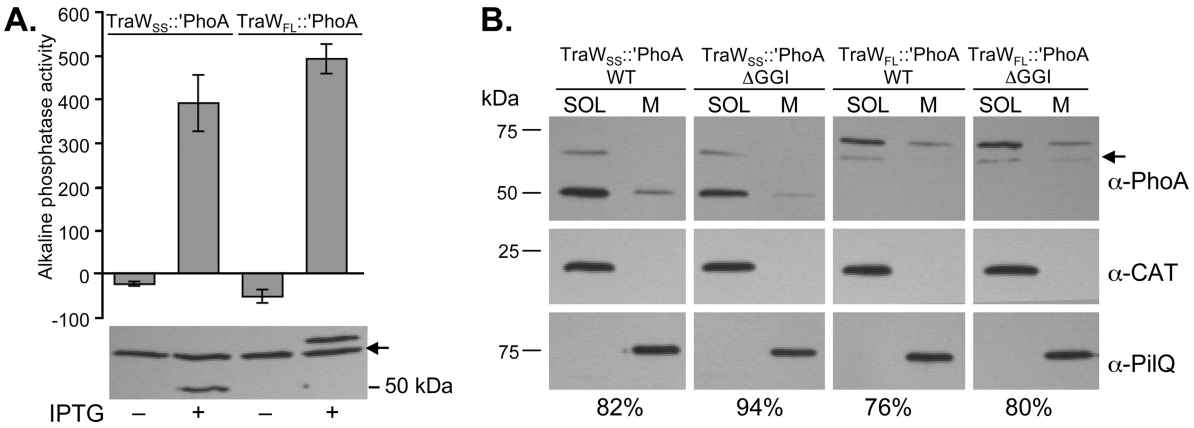

FIG 7 Activity and localization of TraW::PhoA fusions in *N. gonorrhoeae*. (A) Alkaline phosphatase activity and immunoblot analysis of *N. gonorrhoeae* strains producing TraW$_{SS}$::′PhoA (MR544) and TraW$_{FL}$::′PhoA (MR553). Alkaline phosphatase units were normalized to total mg of protein. The mean ± standard deviation of three independent experiments is shown. Arrow, a band that cross-reacts with the anti-PhoA antibody. (B) Subcellular localization of TraW::PhoA fusions in gonococcal cells. Four chloramphenicol-resistant strains were constructed: MR555 (expressing *traW$_{SS}$::′phoA* in a WT background), MR561 (expressing *traW$_{SS}$::′phoA* in a ΔGGI background), MR556 (expressing *traW$_{FL}$::′phoA* in a WT background), and MR562 (expressing *traW$_{FL}$::′phoA* in a ΔGGI background). Cells were fractionated into a soluble fraction (SOL) containing the cytoplasm and periplasm as well as a membrane fraction (M) containing the inner and outer membranes. Subcellular fractions were probed with antibodies against alkaline phosphatase (PhoA), the cytoplasmic CAT, and the outer membrane protein PilQ. Densitometry analysis was performed using ImageJ software. The percentage of total TraW::PhoA fusion protein associated with the soluble fraction for each strain is reported. Arrow, a band that cross-reacts with the anti-PhoA (α-PhoA) antibody.

confirm our hypothesis that TraW localizes to the periplasm in *Neisseria* and demonstrate that the predicted TraW signal sequence functions for protein export. The TraW homolog in F plasmid (TraW$_F$) is involved in F-pilus assembly and is part of an interaction group of six proteins (21, 35). TraW$_F$ was shown to localize to the periplasm when expressed in maxicells (35) but localized to the outer membrane when expressed in cells carrying the entire F transfer region (3).

We asked whether the TraW::PhoA fusions that we had constructed would localize differently in *N. gonorrhoeae* if expressed in the presence or absence of other T4SS proteins. We expressed TraW$_{SS}$::′PhoA and TraW$_{FL}$::′PhoA under the control of *lacPO* at the *iga-trpB* site in the WT gonococcal strain MS11 and in an isogenic strain carrying a deletion of the entire GGI (ΔGGI). The resulting strains were fractionated into a soluble fraction containing cytoplasmic and periplasmic material and a membrane fraction containing both inner and outer membranes. The proteins present in each fraction were then analyzed by immunoblotting (Fig. 7B). CAT was detected only in the soluble fraction, and the gonococcal outer membrane protein PilQ was detected only in the membrane fraction, indicating that we were able to achieve clean fractionation of soluble and membrane material. The localization of the TraW$_{SS}$::′PhoA fusion would not be expected to vary in the presence or absence of other T4SS proteins, since it contains only the signal sequence portion of TraW. Densitometry analysis indicated that 82% of the total TraW$_{SS}$::′PhoA fusion localized to the soluble fraction when expressed in a WT background, while 94% localized to the soluble fraction when expressed in a ΔGGI background (Fig. 7B). For the TraW$_{FL}$::′PhoA fusion, 76% of the protein localized to the soluble fraction when expressed in a WT background, and 80% localized to the soluble fraction when expressed in a ΔGGI background (Fig. 7B). These results are representative of several independent experiments and indicate that TraW$_{SS}$::′PhoA and TraW$_{FL}$::′PhoA primarily localize to the soluble fraction (presumably the periplasm) and that a small amount of each fusion protein is associated with the membranes. The pattern of localization for both TraW::′PhoA fusion proteins is similar regardless of whether they are produced in the presence or absence of other T4SS proteins.

DISCUSSION

Despite the overall genetic tractability of the pathogenic *Neisseria*, the tools available for gene complementation are limited. Due to the problems associated with the use of replicating plasmids in *Neisseria*, complementation is usually achieved by expressing the gene of interest from a chromosomal locus. Relatively few loci have been developed for complementation, however, and the options for promoters to drive gene expression are similarly limited. We describe a set of new complementation constructs to address these problems. The constructs undergo double-crossover recombination with the *iga* and *trpB* genes following transformation into *Neisseria*. Gene insertion occurs in the intergenic region between the genes, ensuring that both genes are left intact. Additionally, to minimize the possibility that gene insertion might affect neighboring genes, we engineered an additional transcriptional terminator in the constructs such that both *trpB* and *iga* maintain transcriptional terminators following gene insertion.

In order to increase the number of available promoters for gene expression in *Neisseria*, we designed the *iga-trpB* complementation constructs to contain three different promoters: *lacPO*, P_{opaB}, and P_{tet}. We characterized gene expression from these constructs by measuring the activity of an alkaline phosphatase fusion. The tandem *lac* promoter/operator (*lacPOPO*) provides strong gene expression in *Neisseria*. Induction of *lacPOPO* inserted upstream of the *pilE* gene resulted in approximately 87% of wild-type *pilE* mRNA levels (34), while expression of the *traD* gene from the *lctP-aspC* site under the control of *lacPOPO* resulted in a 123-fold increase in transcript levels compared to wild-type levels (51). We show that the *iga-trpB* constructs containing *lacPOPO* generate levels of AP activity similar to the levels of these previously characterized *lctP-aspC* constructs (19, 27, 54). We also designed a construct containing the strong, constitutive promoter from the

opaB gene. As expected on the basis of the large amounts of opacity protein produced by gonococcal strains, the P_{opaB} construct was the strongest of those that we characterized in this study, providing three times as much AP activity as the *lacPO* construct. Because P_{opaB} provides strong constitutive expression, the usefulness of these constructs largely depends on whether the gene of interest is toxic to the cell when expressed at high levels. Finally, we designed a construct containing a tetracycline-inducible promoter. The tetracycline-inducible promoter has not been described for use in *Neisseria*, although it has been used in a variety of prokaryotic and eukaryotic expression systems. We demonstrated that gene expression from this promoter can be induced specifically in the presence of anhydrotetracycline. Various levels of expression from the P_{tet} construct can be achieved by adding different amounts of inducer, and maximum expression levels are similar to those achieved with the *lacPOPO* constructs.

We used a P_{tet} construct to functionally complement an arginine auxotrophy in gonococcal strain DGI2. Strain DGI2 was isolated from a patient with disseminated gonococcal infection in the 1980s and is part of a group of strains that require arginine, hypoxanthine, and uracil for growth (AHU auxotrophs) (44). These strains have been extensively studied and share a number of features in common (26), including the production of type 1 IgA1 protease (43). Most other gonococcal isolates, including MS11, produce type 2 IgA1 protease. The portion of *iga* cloned into the complementation constructs is conserved between type 1 and type 2 IgA1 proteases, however, and we were able to transform strain DGI2 with a P_{tet} construct expressing wild-type *argJ*. The complemented strain did not grow in the absence of arginine if the inducer was not present, but the addition of anhydrotetracycline allowed growth in the absence of arginine, demonstrating that the new constructs can be used to functionally complement a gonococcal mutant.

The *iga-trpB* region is also conserved between *N. gonorrhoeae* and *N. meningitidis*, and we demonstrated that the constructs that we describe function for complementation in meningococci as well as gonococci. It should be noted that the sequence of the *iga-trpB* intergenic region in meningococci differs from that in gonococci by the presence of a 103-bp Correia element between the stop codon of *trpB* and the shared transcriptional terminator. Correia elements are common repetitive elements in the *Neisseria* genomes (33). Previous work has indicated that these elements may function as promoters for transcription, transcriptional terminators, targets for RNA processing, or sites for DNA recombination (58). We were able to transform meningococci with the P_{opaB} construct containing $TraW_{SS}$::′PhoA and obtained blue colonies on XP indicator plates. We found that it was necessary to transform the *lac*- and *tet*-inducible constructs containing $TraW_{SS}$::′PhoA into meningococci in two steps, likely because of the relatively large nonhomologous inserts in these constructs. We used the resulting strains to demonstrate tetracycline-inducible gene expression, increasing the number of inducible promoters available for gene expression in meningococci.

We designed the *iga-trpB* constructs to be used in combination with previously characterized complementation constructs that direct gene insertion at the *lctP-aspC* site. Two different gene products were expressed from these two chromosomal loci either independently or simultaneously, one under the control of the IPTG-inducible *lacPO* and one under the control of the tetracycline-inducible P_{tet}. While we experienced no difficulty constructing a strain that contained both P_{tet} and *lacPO*, we had some difficulty transforming a pMR33-derived construct into a strain that already contained the *lacPO*/*lacI*^{q} elements at the *lctP-aspC* site. In this case, the incoming complementation construct has homology with the *lac* regulatory elements present at the *lctP-aspC* site, in addition to homology with the *iga-trpB* locus. We were able to obtain transformants carrying the expected inserts at both complementation sites only if we carried out selection in the presence of both chloramphenicol and erythromycin. In the presence of erythromycin alone, the resulting transformants did not carry the expected insert at the *iga-trpB* site and instead appeared to have undergone recombination between the *lacPO*/*lacI*^{q} elements, resulting in the loss of the *cat* marker from the *lctP-aspC* site.

We constructed two different alkaline phosphatase fusions, one containing the full-length TraW open reading frame and one containing only the predicted TraW signal sequence. TraW is a component of the gonococcal type IV secretion system (T4SS), which secretes single-stranded chromosomal DNA into the extracellular environment, where it is functional for the natural transformation of other gonococci in the population (19, 52). TraW is encoded in the gonococcal genetic island (GGI) along with the other genes required for type IV secretion, but little is known about its function in DNA secretion (13, 19, 46). In the *E. coli* F plasmid, a homolog of TraW ($TraW_F$) is required for F pilus assembly (15) and is likely part of the secretion apparatus. Previous studies have shown that $TraW_F$ localizes to the periplasm (35) but is associated with the membrane when expressed in the presence of the full F transfer region (3). We hypothesized that gonococcal TraW would also localize to the periplasm in gonococci and chose it as a fusion partner for ′PhoA.

In agreement with our hypothesis, production of both $TraW_{SS}$::′PhoA and $TraW_{FL}$::′PhoA in *E. coli* and in *N. gonorrhoeae* resulted in AP activity, indicating that TraW localizes to the periplasm. We hypothesized that TraW might be membrane associated in gonococci and asked whether the subcellular localization of $TraW_{SS}$::′PhoA or $TraW_{FL}$::′PhoA fusion was altered if expressed in a strain carrying a deletion of the GGI (ΔGGI). We fractionated cells into a soluble fraction and a membrane fraction. The fractionation results indicated that both $TraW_{SS}$::′PhoA and $TraW_{FL}$::′PhoA localize primarily in the soluble fraction, regardless of whether they were produced in a WT or ΔGGI background. These results indicate that TraW is a soluble, periplasmic protein in *N. gonorrhoeae*. The presence of T4SS proteins in *N. gonorrhoeae* did not cause the overexpressed TraW to associate with membrane material, which may indicate that it does not associate tightly with the T4SS apparatus or that there were insufficient amounts of other T4SS proteins to influence the localization of significant amounts of the expressed TraW fusion protein.

The complementation constructs developed in this study are a significant addition to the genetic tools available for *N. gonorrhoeae* and *N. meningitidis*. In combination with previously characterized constructs, these new constructs make it possible to complement multiple mutations in a single cell and assess the relative contributions of each gene to an overall phenotype. Additionally, these constructs allow the expression of new genes in strains that already contain the *lac* promoter or make use of existing complementation sites, allowing increasingly more complex questions to be asked regarding the basic biology, natural transformation, and pathogenesis of these significant human pathogens.

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