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A novel relaxase homologue is involved in chromosomal DNA processing for type IV secretion in *Neisseria gonorrhoeae*

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Abstract

The *Neisseria gonorrhoeae* type IV secretion system secretes chromosomal DNA that acts in natural transformation. To examine the mechanism of DNA processing for secretion, we made mutations in the putative relaxase gene *traI* and used nucleases to characterize the secreted DNA. The nuclease experiments demonstrated that the secreted DNA is single-stranded and blocked at the 5′ end. Mutation of *traI* identified Tyr^{93} as required for DNA secretion, while substitution of Tyr^{201} resulted in intermediate levels of DNA secretion. TraI exhibits features of relaxases, but also has features that are absent in previously characterized relaxases, including an HD phosphohydrolase domain and an N-terminal hydrophobic region. The HD domain residue Asp^{120} was required for wild-type levels of DNA secretion. Subcellular localization studies demonstrated that the TraI N-terminal region promotes membrane interaction. We propose that Tyr^{93} initiates DNA processing and Tyr^{201} is required for termination or acts in DNA binding. Disruption of an inverted-repeat sequence eliminated DNA secretion, suggesting that this sequence may serve as the origin of transfer for chromosomal DNA secretion. The TraI domain architecture, although not previously described, is present in fifty-three uncharacterized proteins, suggesting that the mechanism of TraI function is a widespread process for DNA donation.

Introduction

Neisseria gonorrhoeae encodes an unusual type IV secretion system (T4SS). T4SSs include conjugation systems (Lawley *et al.*, 2003), which transport DNA from one bacterium directly into another, and effector translocator systems, which secrete proteins into host cells that exert virulence functions (Christie, 2001). One of the best-characterized T4SSs is that of *Agrobacterium tumefaciens*, which transports proteins and plasmid DNA directly into plant cells (Christie *et al.*, 2005). The gonococcal T4SS secretes chromosomal DNA into the environment, where the secreted DNA transforms other gonococci in the population (Dillard and Seifert, 2001; Hamilton *et al.*, 2001). This unique, non-lytic mechanism for DNA donation may partly explain the high frequency of genetic exchange in *N. gonorrhoeae* and its panmictic population structure (Maynard Smith *et al.*, 1993). The high rate of transformation results in the rapid spread of antibiotic resistance genes and increased variation of surface molecules, allowing evasion of the human immune response (Reviewed in Hamilton and Dillard, 2006). Mutations in gonococcal T4SS genes result in decreased DNA in culture supernatants and in

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decreased DNA donation for natural transformation (Dillard and Seifert, 2001; Hamilton *et al.*, 2005).

The *N. gonorrhoeae* T4SS genes are encoded in the gonococcal genetic island (GGI) (Dillard and Seifert, 2001; Hamilton *et al.*, 2005). The GGI is a 57-kb horizontally-acquired genomic region present in ~80% of *N. gonorrhoeae* strains, and a few *N. meningitidis* strains, and may contribute to the virulence of these bacteria (Dillard and Seifert, 2001; Snyder *et al.*, 2005). The GGI-encoded T4SS is similar to the F-plasmid family of conjugation T4SSs in terms of gene arrangement and limited sequence similarity. Mutational analysis demonstrated that DNA secretion requires several T4SS genes predicted to encode components of the secretion apparatus, as well as a relaxase, two peptidoglycanases, a disulphide bond isomerase, and a chromosome partitioning protein (Dillard and Seifert, 2001; Hamilton *et al.*, 2001; Hamilton *et al.*, 2005; Kohler *et al.*, 2007). Because of the similarity to F-plasmid and the requirement for T4SS homologues, we predict that chromosomal DNA donation in *N. gonorrhoeae* may occur by a mechanism similar to DNA transfer from *Escherichia coli* Hfr strains, but with the DNA secreted into the surrounding milieu.

Although it was shown that gonococci secrete chromosomal DNA during growth, the characteristics of the secreted DNA have not been determined. The ability of the secreted DNA to transform other gonococci may be influenced by whether the DNA is single-stranded or double-stranded and whether it has protein bound to it or not. Previous studies using exogenously added DNA suggested that double-stranded DNA was the preferred substrate for natural transformation of *N. gonorrhoeae* (Biswas and Sparling, 1981), while one report suggested that single-stranded and double-stranded DNA transform with similar efficiency (Stein, 1991). Transformation experiments comparing DNA donation by wild-type versus a T4SS-mutant donor revealed that wild-type donors produce more transformants, even when the cultures were allowed to proceed to autolysis (Dillard and Seifert, 2001). These results suggest that the secreted DNA is more efficient at transforming recipients than DNA released by autolysis. However, the donation of DNA via a T4SS suggests that the secreted DNA is single-stranded, as is the case for conjugated DNA and DNA transferred by the *A. tumefaciens* T4SS (Llosa *et al.*, 2002). If gonococci secrete DNA by a mechanism similar to conjugation, then the DNA should be cut by a nicking enzyme or relaxase and the enzyme may remain bound to the DNA (Gomis-Ruth *et al.*, 2004).

Relaxases bind and nick supercoiled DNA in a sequence- and strand-specific manner at the origin of transfer (*oriT*) (Grinter, 1981). The tyrosine-mediated nicking reaction results in the covalent binding of the relaxase to the 5′ end of the cut DNA (Grinter, 1981; Pansegrau *et al.*, 1990). The nucleoprotein complex is recognized by the coupling protein for transport via the type IV secretion apparatus (Llosa and O'Callaghan, 2004). Therefore, relaxases function as pilot proteins for DNA transport (Grinter, 1981; Pansegrau *et al.*, 1990). Relaxases are characterized by the presence of three conserved amino acid sequence motifs (Pansegrau and Lanka, 1991). Motif I contains one or two catalytic tyrosines (Pansegrau *et al.*, 1990; Pansegrau *et al.*, 1993); motif II is thought to be involved in facilitating DNA-protein interactions at the 3′ side of the cleavage site (Pansegrau *et al.*, 1994); and motif III, also known as the three-histidine (3H) motif, has been historically used as the hallmark of relaxases and is involved in metal ion coordination (Francia *et al.*, 2004). *N. gonorrhoeae* encodes a putative relaxase, TraI, required for type IV secretion (Hamilton *et al.*, 2005). TraI exhibits features of relaxases, but also exhibits features that are absent in previously characterized relaxases, suggesting that DNA processing for secretion in *N. gonorrhoeae* may have important differences compared to DNA processing by known T4SSs. To characterize DNA secretion by *N. gonorrhoeae* and gain a better understanding of DNA donation for natural transformation, we examined the mechanism of DNA processing focusing on the putative relaxase TraI and the sensitivities of the secreted DNA to specific nucleases.

Here we show that the putative relaxase TraI has features not found in known relaxases, including an N-terminal hydrophobic region and an HD (histidine and aspartate) phosphohydrolase domain. Site-directed mutagenesis of *traI* identified two tyrosines necessary for DNA secretion, with one predicted to act in the initial cleavage of chromosomal DNA for secretion. Also a mutation in motif I of the HD domain significantly decreased DNA secretion, as did mutations affecting the unique hydrophobic region found at the N-terminus. Remarkably, this study identified fifty-three proteins with the same domain architecture as TraI. These proteins conserve an N-terminal putative relaxase domain fused to the HD domain of phosphohydrolases, all within the first three-hundred amino acids. These proteins may represent uncharacterized conjugation or DNA donation proteins. Nuclease treatment of gonococcal culture supernatants and co-culture transformation in the presence of specific nucleases revealed that the secreted DNA is only degraded by nucleases that have single-strand 3′→5′ exonuclease activity or endonuclease activity, suggesting that gonococci secrete single-stranded DNA protected at the 5′ end. An insertion disrupting an inverted repeat in a non-coding region of the GGI caused loss of DNA secretion, suggesting that this region may contain an *oriT*. Altogether, this work reveals important properties of the secreted DNA and the mechanism of DNA processing for type IV secretion in *N. gonorrhoeae*.

Results

Site-directed mutagenesis replacing Tyr^{93} and Tyr^{201} reveals their requirement for TraI function in DNA secretion

Previously we found that a strain carrying a *traI* insertion (truncating TraI at amino acid 200) is deficient in DNA secretion, indicating a requirement for TraI in type IV secretion (Hamilton *et al.*, 2005). Complementation of this strain with the wild-type copy of *traI* at a distant location on the chromosome restored DNA secretion to wild-type levels, confirming that the DNA secretion deficiency was not due to a polar effect on downstream genes (Fig. 1A). These results provide further evidence that DNA secretion in *N. gonorrhoeae* is dependent on the putative relaxase TraI. An alignment of TraI and the *Xyllela fastidiosa* putative relaxase revealed conservation of Tyr^{93}, Tyr^{201} and Tyr^{212} among the proteins (Fig. 2). These conserved tyrosines are potential catalytic amino acid residues. The F-family of relaxases conserves pairs of tyrosines reported to play different roles in DNA processing (Grandoso *et al.*, 2000). Thus, Tyr^{200} was also of interest since it was located next to Tyr^{201} forming a tyrosine pair. To determine the functional relevance of Tyr^{93}, Tyr^{200}, Tyr^{201} and Tyr^{212}, we used site-directed mutagenesis to replace these conserved tyrosines with phenylalanines. DNA secretion was eliminated in the strain carrying the Y93F mutation, while the Y212F mutation had no effect on DNA secretion (Fig.1A). Interestingly, the strain expressing a double tyrosine substitution (Y200F/Y201F) showed intermediate levels of DNA secretion (Fig. 1A). Individual tyrosine substitutions were made to determine if the intermediate DNA secretion phenotype observed in the strain carrying the Y200F/Y201F mutation could be ascribed to a particular tyrosine. The Y200F mutant showed wild-type levels of DNA secretion, whereas DNA secretion by the strain carrying the Y201F mutation resulted in the intermediate DNA secretion phenotype observed with the Y200F/Y201F substitutions (Fig. 1A). Introduction of wild-type *traI* at a distant site on the chromosome restored wild-type levels of DNA secretion in the strains carrying *traI* Y93F, Y200F/Y201F, or Y201F mutations (Fig. 1A). Analysis of native expression of $TraI_{Y93F}$ and $TraI_{Y200F/Y201F}$ by Western blot revealed that the proteins are expressed at levels similar to that of the wild-type protein (Fig.1B). These results provide evidence that Tyr^{93} and Tyr^{201} are required for the function of TraI in DNA secretion. Furthermore, the different effects of the Y93F and Y201F mutations on DNA secretion suggest non-redundant roles of these tyrosines in the activity of TraI. Tyr^{93} may be involved in the initiation of DNA processing, while Tyr^{201} may be involved either in a second cleavage for termination of DNA processing or in the stability of the DNA-protein interaction.

Single amino acid substitutions within the histidine-rich motif result in wild-type levels of DNA secretion

A histidine (H)-rich motif is found five amino acid residues from Tyr^{93} (Fig. 2). This sequence aligns well with a region from the relaxase (TraI) of *Salmonella typhi* plasmid R27, where it was referred to as the 3H motif of relaxases (Lawley *et al.*, 2002). The gonococcal TraI H-rich motif conserves Arg^{99}, His^{106}, and His^{108} (Fig. 2). Arg^{99} was of interest because it aligns well with Arg^{150} of the F-plasmid family 3H motif (data not shown), which was shown to contribute to relaxation of DNA *in vitro* (Harley *et al.*, 2002). To determine the functional relevance of Arg^{99}, His^{106}, and His^{108} in DNA secretion, we constructed strains carrying *traI* R99S, H106S, or H108S mutations. Surprisingly, these strains exhibit wild-type levels of DNA secretion (Fig. 1A). A possible explanation for this result is that single histidine substitutions or the substitution to serine may be insufficient to eliminate a possible function in metal coordination. To address this possibility, we constructed a strain expressing a double histidine substitution (H106A/H108A), where the histidines were replaced by alanines. The strain carrying *traI*H106A/H108A also showed wild-type levels of DNA secretion (Fig. 1A). These results indicate that Arg^{99}, His^{106}, and His^{108} are not required for TraI function in DNA secretion.

N. gonorrhoeae *TraI has features unlike any characterized relaxase*

TraI was identified as a putative relaxase based on N-terminal amino acid sequence homology to the putative nickase from *Pseudomonas resinovorans* and the putative relaxase from *X. fastidiosa* (Hamilton *et al.*, 2005). Strikingly, TraI conserves the five motifs characteristic of the HD domain of the metal-dependent phosphohydrolase superfamily, all within the first three-hundred amino acids (Fig. 2) (Aravind and Koonin, 1998). To determine if the presence of the HD domain was unique to *N. gonorrhoeae* (*Ng*) TraI among relaxases, we performed a database search using full-length TraI as the query. The search resulted in the identification of fifty-three proteins with a high degree of amino acid conservation to TraI (Table 1, Fig. S1). Some of the proteins in this group are annotated as putative relaxases or hydrolases, but most of them are hypothetical proteins (Table 1). These proteins come from a number of γ- and β-proteobacteria, and for a large number of them, the sequences have just recently become available. A phylogenetic tree obtained from an amino acid alignment of the full-length proteins resulted in the clustering of TraI with putative relaxases from other pathogenic bacterial species including *Vibrio cholerae*, avian pathogenic *Escherichia coli*, *Salmonella typhi*, *Proteous vulgaris*, and *Yersinia pestis* (Fig. 3). Interestingly, the large majority of these proteins are chromosomally encoded, and of these, some can be found in mobile genomic islands (e.g. integrated pKLC102) or conjugative/integrative elements (e.g. pICEhin1056). Only six of the proteins in this family are plasmid-encoded (Fig. 3).

The only other putative relaxase from the *Ng* TraI family shown to be required for type IV secretion is TraI from the *S. typhi* R27 plasmid. R27 TraI contains the three signature motifs of relaxases (Lawley *et al.*, 2002). However, alignment of the fifty-four proteins in the *Ng* TraI family revealed that the R27-plasmid TraI motifs I and II (Fig. 2) are only conserved in pAPEC-01-R, R478 plasmid, and pHCM1 (Fig. 3 and Fig. S1). Amino acid conservation within the *Ng* TraI family starts with the motif containing *Ng* TraI Tyr^{93}, conserved in forty-three of the proteins, followed by the H-rich motif of signature h(Q/H)xhPASExHHHx_3GG(L/M)h (where x is any residue and h is a hydrophobic residue), present in all the proteins. Moreover, all the proteins conserve *Ng* TraI Tyr^{201} and the HD phosphohydrolase domain (Fig. S1, consensus sequence shown in Fig. 2). Finally, almost all the proteins conserve a domain of unknown function (DUF1528) near the C-terminus. This information suggests that TraI belongs to a newly discovered family of relaxases or relaxase-like proteins, and unlike any of the characterized relaxases, the domain architecture includes the HD domain of phosphohydrolases.

The HD domain motif I mutation D120N reduces DNA secretion

The HD phosphohydrolase domain highly conserves histidines and aspartates (Fig. 2). Therefore, it has been proposed that the primary function of this domain is in the coordination of divalent cations (Aravind and Koonin, 1998). The HD domain motifs I, II, and V were reported to be conserved in the entire HD domain superfamily (Aravind and Koonin, 1998). Therefore, we focused on His^{161} and Asp^{162}, which are the signature amino acids of the HD domain (present in motif II), and Asp^{120}, present in motif I (Fig. 2) (Aravind and Koonin, 1998). Asp^{120} was of interest because secondary-structure-based threading (LOOPP) placed it in close proximity to Tyr^{93}. There is evidence that interaction of an aspartate with an active site tyrosine is required for the activity of relaxases (Guasch *et al.*, 2003). Thus, to determine the contribution of these amino acids in the function of TraI in DNA secretion, we constructed strains carrying *traI* H161S, D162N, or D120N mutations. The *N. gonorrhoeae* strains carrying the H161S or the D162N mutation showed wild-type levels of DNA secretion (Fig. 1A). However, the strain carrying the D120N mutation exhibited significantly reduced levels of DNA in culture supernatants (Fig. 1A). Analysis of native expression of $TraI_{D120N}$ by Western blot revealed that the protein is expressed at levels similar to that of the wild-type protein (Fig. 1B). The precise role of the HD domain in DNA processing is not clear. Nevertheless, these results indicate that the HD domain contributes to the function of TraI in DNA secretion.

Unique N-terminal region in N. gonorrhoeae *TraI*

A feature that makes TraI unique among the members of its family and all characterized relaxases is the presence of an N-terminal region that is identified (by SignalP algorithm analysis) as a signal sequence (Fig. 4A). If this region were to function as a signal sequence, TraI would be transported into the periplasm via the *sec*-translocase prior to export by the T4SS. To address the functionality of the putative signal peptide in targeting TraI to the membrane and transporting it via the *sec*-translocase, we used the well-established *in vitro* translocation assay with isolated inverted inner membrane vesicles of *E. coli* (Cunningham and Wickner, 1989). ^{35}S-labeled $TraI_{\Delta 261\text{-}850}$ was used in a co-translational targeting/transport assay in which the translocation into inverted inner membrane vesicles was monitored by the accessibility of $TraI_{\Delta 261\text{-}850}$ to the externally added proteinase K. If TraI contains a functional signal sequence, the protein would be transported into the vesicles where it would be protected from degradation by proteinase K, and therefore, detectable by autoradiography following electrophoresis. However, if TraI's N-terminal region does not function as a *sec*-dependent signal sequence, it would remain accessible to proteinase K and be degraded. Protease-protected fragments of $TraI_{\Delta 261\text{-}850}$ were not observed in the co-translational targeting/ transport assay, unlike the protease-protected fragments detected in the positive control FtsQ (Fig. 5A). A post-translational transport assay of ^{35}S-labeled $TraI_{\Delta 261\text{-}850}$ also resulted in no detection of protease-protected fragments, unlike the ones detected for the positive control proOmpA (Fig. 5B). These results indicate that the N-terminal region does not function as a signal peptide to target TraI for transport into the periplasm via the Sec system.

The N-terminal region of N. gonorrhoeae *TraI mediates membrane association and is necessary for wild-type levels of DNA secretion*

A secondary structure prediction of the unique N-terminal region of *Ng* TraI indicated that the hydrophobic region lies on an α helix and is interrupted by polar residues, resulting in the prediction of an amphipathic α helix (Fig. 4B). Amphipathic α helixes are characterized by the presence of a polar side and a hydrophobic side that makes proteins capable of interacting with membranes (Elazar *et al.*, 2004). Therefore, an indication that TraI contains an amphipathic α helix would be membrane localization in *N. gonorrhoeae*. TraI was overexpressed in a wild-type gonococcal strain from an inducible construct on the chromosome. Overexpression was followed by cellular fractionation and detection of TraI by Western blot with TraI-specific

polyclonal antisera. TraI was detected in the cytoplasmic and membrane fractions, but not in the periplasmic fraction (Fig. 6A). A faster-migrating band (~35 kDa) that consistently cross-reacted with the TraI antisera was detected in the periplasmic fraction and barely detected in the cytoplasmic fraction (Fig. 6A, top panel), demonstrating proper extraction of periplasmic proteins. PilQ, a gonococcal outer membrane protein, was detected only in the membrane fraction (Fig. 6A, middle panel), and chloramphenicol acetyltransferase (CAT) was detected only in the cytoplasmic fraction (Fig. 6A, bottom panel). Lack of TraI detection in the periplasm further confirms that TraI is not transported by the Sec system. Fractionation of a ΔGGI strain overexpressing TraI also resulted in its detection only in the cytoplasmic and membrane fractions (data not shown), indicating that TraI's association with the membrane is not due to interactions with other proteins of the T4SS. Furthermore, the membrane association of TraI is not a result of aggregation due to overexpression, since TraI was also found in the membrane fraction of wild-type cells (Fig 6B, left panels). These results suggest that TraI exists in two forms, soluble in the cytoplasm and associated with the membrane. Another observation that results from these experiments is that TraI migrates anomalously. TraI is predicted to have a molecular weight of ~94 kDa, but it migrated at an apparent molecular weight of ~125 kDa. DNaseI treatment of fractions prior to SDS-PAGE had no effect on TraI migration (data not shown), indicating that the aberrant migration is not due to bound DNA. TraI's aberrant SDS-PAGE migration is more likely due to post-translational modifications, dimerization, or the protein adopting an unusual conformation.

To investigate if the predicted amphipathic nature of the N-terminal region is involved in mediating TraI's association with the membrane, we introduced charged residues within the hydrophobic side of the predicted amphipathic α helix (L6K/L12K) (Fig. 4B). For ease of detection, we introduced an *ermC* cassette with a strong promoter (Hamilton *et al.*, 2001) upstream of the *traI*L6K/L12K promoter region to drive *traI* transcription from its native site. Wild-type TraI overexpressed from its native site was detected in the membrane and cytoplasmic fractions (Fig. 6B, right panels). However, $TraI_{L6K/L12K}$ was detected only in the cytoplasmic fraction, suggesting that the N-terminal region mediates TraI's association with the membrane, possibly through the formation of an amphipathic α helix (Fig. 6B, right panels). We tested DNA secretion in the strain carrying the L6K/L12K mutation and found significantly reduced levels of DNA in the culture medium (Fig. 1A). These results suggest that TraI's association with the membrane is required for wild-type levels of DNA secretion and may represent a unique step that aids in the DNA transport process for type IV secretion.

Nuclease treatment results in degradation of secreted DNA only by nucleases that have single-strand 3′→5′ exonuclease activity or endonuclease activity

An additional approach to characterize the mechanism of DNA processing in *N. gonorrhoeae* is to test the susceptibility of the secreted DNA to specific nucleases. Two methods were used to assess the effects of nucleases on the secreted DNA: fluorometric detection of DNA in cell-free supernatants after incubation with nucleases, and transfer of an antibiotic resistance marker (*cat*) during co-culture transformation in the presence of nucleases. The nuclease treatment of supernatants was carried out under optimum conditions for each nuclease, while the medium for the co-culture assay was optimized to provide the best possible conditions that would allow enzyme activity and gonococcal growth. ExoIII is a double-strand specific exonuclease that converts double-stranded DNA to single-stranded DNA, by degrading one strand (Richardson *et al.*, 1964). The addition of ExoIII did not significantly affect the fluorescence of culture supernatants (Fig. 7A) or transfer of the *cat* marker (Fig. 8), but resulted in the degradation of added double-stranded λ-HindIII DNA in culture medium (Fig. 7C). The marker transferred in the co-culture transformation assay, *cat*, carries an internal EcoRI site. EcoRI is a restriction enzyme that cuts double-stranded DNA (Greene *et al.*, 1975). EcoRI cut plasmid DNA added to medium (data not shown), but did not affect transfer

of the *cat* marker in co-culture (Fig. 8). By contrast, the 3′→5′ single-strand specific nuclease ExoI (Lehman and Nussbaum, 1964) significantly reduced the fluorescence of culture supernatants (Fig. 7) and reduced transfer of the *cat* marker in co-culture (Fig. 8), suggesting that the secreted DNA is single-stranded with a free 3′ end. However, the 5′→3′ single-strand specific exonuclease $RecJ_f$ (Lovett and Kolodner, 1989) did not significantly affect the fluorescence of culture supernatants (Fig. 7A) or transfer of the *cat* marker (Fig. 8), but degraded single-stranded λ-HindIII DNA added to culture medium (Fig. 7B). Furthermore, the only other nucleases found to degrade secreted DNA were DNaseI and BAL-31 (Figs. 7 and 8). DNaseI is an endonuclease that acts on double- and single-stranded DNA (Vanecko and Laskowski, 1961), and BAL-31 is a double-strand specific exonuclease and single-strand specific endonuclease (Gray *et al.*, 1975). Since ExoIII had no effect on the secreted DNA, any activity exhibited by BAL-31 was predicted to be due to its single-strand endonuclease activity. Both nucleases significantly reduced the fluorescence of culture supernatants (Fig. 7) and reduced transfer of the *cat* marker (Fig. 8). Altogether, these results are consistent with the hypothesis that the secreted DNA is single-stranded and bound to TraI at the 5′ end.

Identification of a possible origin of transfer

Conjugative and mobilizable plasmids are recognized for transfer by the presence of a specific sequence known as the origin of transfer (*oriT*). Characterized *oriT* sequences contain an inverted repeat and are often found near the relaxase gene, between divergent transcriptional promoters, and in A-T rich regions (Lanka and Wilkins, 1995). We hypothesized that if a single *oriT* exists in the gonococcal chromosome, that it is likely to be located in the GGI. Therefore we searched the GGI sequence for inverted repeats that might mark the *oriT*. The only sequence meeting all the criteria characteristic of *oriTs* is the one that contains the inverted repeat found between the T4SS genes *yaf* and *ltgX* (Fig. 9A). Furthermore, the *oriT* in F-plasmid is adjacent to *geneX* (orf169) (Frost *et al.*, 1994), the homologue of the gonococcal *ltgX*, as is the predicted *oriT* for the GGI. An insertion disrupting this inverted repeat, and well separated from the predicted transcriptional promoters for the two transcripts, was found to abolish DNA secretion (Fig. 9B). Complementation with the *yaf-ltgX* non-coding sequence containing an intact inverted repeat at a distant location on the chromosome restored DNA secretion (Fig. 9B). These results suggest that this region contains the *oriT* and that there is only one *oriT* in the chromosome.

Discussion

DNA donation in *N. gonorrhoeae* contributes to the high frequency of genetic exchange that results in the rapid spread of antibiotic resistance genes and in greater genetic diversity of alleles of surface molecules for evasion of the human immune response. To characterize DNA donation by *N. gonorrhoeae*, we examined the mechanism of DNA processing focusing on the putative relaxase TraI and the sensitivities of the secreted DNA to specific nucleases. Mutational analysis of TraI is consistent with its function as a relaxase, and the nuclease data indicate that the DNA is single-stranded with a free 3′ end. Therefore, we propose that *N. gonorrhoeae* secretes a nucleoprotein complex of single-stranded DNA protected at the 5′ end, presumably by TraI. Other proteins may also bind to the secreted DNA to aid in transformation. Conjugation systems have been found to transfer the relaxase as well as RecA along with plasmid DNA (Heinemann, 1999; Luo and Isberg, 2004), and *A. tumefaciens* transfers the single-stranded DNA binding protein VirE2 into host cells to aid in the establishment of the Ti DNA (Vergunst *et al.*, 2000). Similarly, *N. gonorrhoeae* may secrete proteins that bind the secreted DNA facilitating transformation and/or enhancing DNA uptake or recombination in recipient cells.

In relaxases, the active-site amino acid residue is a tyrosine, where the hydroxyl group attacks a phosphate on the DNA backbone (Pansegrau *et al.*, 1994). We searched for conserved tyrosines in the *Ng* TraI family and found that forty-two of the proteins conserve a tyrosine at position 93 (in *Ng* TraI), with the rest of the proteins conserving a tyrosine at positions -8, -5, or +3 from Tyr^{93} (Fig. S1). Substitution of Tyr^{93} with phenylalanine abolished DNA secretion, while substitution of Tyr^{201}, conserved in all the proteins, resulted in intermediate levels of DNA secretion. The requirement of Tyr^{93} for the function of TraI in DNA secretion supports the hypothesis that TraI is a relaxase, and suggests a model where Tyr^{93} is involved in the initial cleavage of supercoiled DNA. The intermediate DNA secretion phenotype of the strain carrying the Y201F mutation suggests that Tyr^{201} may be required for a second cleavage for termination of DNA processing, analogous to the TrwC mechanism of DNA processing (Garcillan-Barcia *et al.*, 2007; Gonzalez-Perez *et al.*, 2007; Grandoso *et al.*, 2000). Without termination, DNA might be secreted but remain attached to the cells. In our assay for DNA secretion, the cells are removed before the supernatants are assayed for fluorescence. Therefore, DNA in the supernatants might be reduced to the amount that is sheared from the cells. Tyrosines are not only involved in DNA cleavage reactions but are also known to contribute to non-specific binding interactions with the phosphodiester backbone for many enzymes involved in nucleic acid metabolism, including DNaseI and DNA polymerase beta (Mundle et al., 2004). Hence, it is possible that Tyr201 is required instead for the binding affinity of TraI to the DNA backbone.

The presence of the HD domain suggests an important difference in the mechanism of TraI function when compared to classical relaxases. The HD domain has been shown to be required for the metal-dependent phosphohydrolase activity of the *E. coli* dGTPase (Huber *et al.*, 1988), the ppGpp(p)-hydrolase SpoT (Gentry and Cashel, 1996), and nucleotidyltransferases (Yakunin *et al.*, 2004), providing evidence that the HD domain functions in nucleic acid metabolism (Aravind and Koonin, 1998). Furthermore, conservation of multiple histidine and aspartate residues in the HD domain (Fig. 2) indicates that one of the primary functions of this domain may be the coordination of divalent cations (Aravind and Koonin, 1998). If the HD domain of TraI performs this function, then the 3H motif of classical relaxases would be unnecessary, thus explaining the wild-type phenotypes of the *traI* H106S, H108S, and H106A/H108A mutants in DNA secretion. The H-rich motif sequence PASExHHH is a hallmark of the proteins in the *Ng* TraI family (Fig. 2 and Fig. S1). Therefore, the dispensability of the histidine residues in this motif was an unexpected result. The HD domain mutations H161S and D162N (HD motif II) also did not significantly reduce DNA secretion. However, Asp^{120} (HD motif I) was found to be required for wild-type levels of DNA secretion, providing evidence that the HD domain contributes to TraI function. Asp^{81} of F-plasmid TraI and Asp^{85} of TrwC have been shown to interact directly with tyrosines, presumably for activation of the catalytic tyrosine or for structural maintenance of the active site (Guasch *et al.*, 2003; Larkin *et al.*, 2005). Asp^{120} of the HD domain could have similar roles. Alternatively, Asp^{120} could function in metal coordination by a mechanism similar to Glu^{104} of the MbeA HEN motif (Varsaki *et al.*, 2003). MbeA is the relaxase encoded by plasmid ColE1 that contains a variant of the canonical 3H motif, where His^{97}, Glu^{104} and Asn^{106} of the HEN motif are required for the activity of the protein (Varsaki *et al.*, 2003).

In addition to the presence of the HD domain, another unusual feature of TraI is a hydrophobic region at the N-terminus. This region was initially identified as a possible signal sequence, suggesting a two-step secretion mechanism for TraI, as described for pertussis toxin (Weiss *et al.*, 1993) and some proteins of *A. tumefaciens* (Pantoja *et al.*, 2002). However, the data does not support this hypothesis. The predicted signal peptide was not functional in transporting TraI into inverted *E. coli* inner membrane vesicles (Fig. 5), and TraI was not detected in the periplasm of *N. gonorrhoeae* (Fig. 6A). Further examination of the N-terminal region revealed that, while highly hydrophobic, it contains several polar residues resulting in the secondary

structure prediction of an amphipathic α helix. Disruption of the amphipathic nature of the region (by substitution of two hydrophobic residues with charged residues, L6K/L12K) indicated that the N-terminal region is necessary not only for membrane association but also for wild-type levels of DNA secretion. Secondary structure prediction analysis of the N-terminal region of proteins in the *Ng* TraI family revealed that almost all of these proteins contain predicted amiphathic α helices (Fig. 3, Fig. S1). Therefore, association with the membrane may be a general property of relaxases in the *Ng* TraI family. The advantage of this association may be to facilitate interaction with the T4SS apparatus. Interaction with the coupling protein or other parts of the T4SS apparatus might still occur without membrane association, but might occur less often or be less stable. This idea is consistent with the reduced DNA secretion seen in the *traI* L6K/L12K mutant (Fig 1A). Alternatively, membrane association of TraI could be more important for the transformation steps after DNA secretion, possibly binding secreted DNA to the outer membrane or outer membrane vesicles of the donor for presentation to recipient cells or aiding in binding DNA to the outer membrane of recipients for DNA uptake.

DNA processing for type IV secretion is extremely specific and occurs only at the *oriT* (Lanka and Wilkins, 1995). All of the known genes for type IV secretion in *N. gonorrhoeae* are found in a 57-kb region of the chromosome called the GGI (Dillard and Seifert, 2001; Hamilton et al., 2005). We hypothesized that if a single *oriT* is present in the gonococcal chromosome that it would be present in this region. We found a sequence in the *yaf-ltgX* non-coding region of the GGI that has many of the characteristics of *oriT*s (Lanka and Wilkins, 1995). This sequence contains an inverted repeat, is located between divergently transcribed genes in an A-T rich region, and is close to *traI*. An insertion in the inverted repeat abolished DNA secretion, suggesting that this sequence is required for DNA processing for type IV secretion. Furthermore, complementation with the *yaf-ltgX* non-coding region at a distant location on the chromosome restored DNA secretion. Although it is possible that this region might encode a very small protein or a small RNA that we have not detected, we favor the hypothesis that this region is the *oriT* and may be cut by TraI to initiate DNA transfer.

To date, relaxases have been identified that contain an N-terminal relaxase domain or an N-terminal relaxase domain with a C-terminal helicase or primase domain (Francia *et al.*, 2004). The TraI domain architecture, an N-terminal putative relaxase domain fused to the HD domain of phosphohydrolases (all within the first three-hundred amino acids), has not been previously described. However, the TraI N-terminal domain architecture is not unique to TraI; it is present in at least fifty-three uncharacterized proteins. These proteins conserve (i) *Ng* TraI Tyr^{93} and Tyr^{201}, (ii) an H-rich motif of signature h(Q/H)xhPASExHHHx_3GG(L/M)h (where x is any residue and h is a hydrophobic residue), (iii) the five motifs characteristic of the HD domain of phosphohydrolases, (iv) a predicted amphipathic α helix at the N-terminus proximal region, and (v) a conserved domain of unknown function (DUF1528) near the C-terminus. Therefore, we propose that these proteins are part of a previously undescribed family of relaxases or relaxase-like proteins and that their mechanism of DNA processing may be similar to TraI. The large majority of the proteins in the *Ng* TraI family are in transfer regions that are chromosomally encoded and most of these species also encode type IV pilus components, suggesting that they may be naturally transformable. Thus, it is likely that these relaxase homologues act in processing chromosomal DNA, and it is possible that some of them may export DNA by the same mechanism as *N. gonorrhoeae*. Regardless of whether DNA donation occurs by conjugation or direct secretion, the mechanism of TraI function appears to be a widespread process for chromosomal DNA donation.

Experimental procedures

Bacterial strains and growth conditions

The *N. gonorrhoeae* strain MS11 was used as the wild-type strain and for the construction of the gonococcal strains described in Table 2. Growth of gonococci on GCB (Difco) solid medium was performed as previously described (Dillard, 2006). *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth (Sambrook *et al.*, 1989). Graver-Wade (GW) medium (pH 6.8) was used for the growth of gonococci in liquid culture (Wade and Graver, 2007). When required, chloramphenicol (Cm) was used at a concentration of 10μg ml^{-1} (Cm10) or 25μg ml^{-1} (Cm25) for gonococci and *E. coli*, respectively. Erythromycin was used at a concentration of 500μg ml^{-1} (Em500) for *E. coli* and 10μg ml^{-1} (Em10) for gonococci.

Plasmid construction and generation of gonococcal mutants

Plasmids used for mutagenesis and complementation are described in Table 2. The Bordo and Argos guidelines were used to determine which amino acids would be used for substitutions (Bordo and Argos, 1991). The *traI* mutations were introduced into gonococci by allelic exchange without selection (Dillard, 2006). Complementation was achieved by introduction of the wild-type copy of *traI* or the *yaf-ltgX* non-coding region into the gonococcal chromosome between *aspC* and *lctP* along with the *cat* marker via the pKH37 vector (Kohler *et al.*, 2007).

DNA secretion assay

This assay is a modification of the DNA secretion assay reported by Hamilton *et al.* (Hamilton *et al.*, 2005). Piliated (P^+) gonococcal colonies from an overnight culture grown on GCB agar plates were harvested with a sterile dacron swab, resuspended in GW medium, diluted to an OD_{540} 0.18 in a 3-ml culture, and grown for 2h at 37°C with aeration. After the first round of growth, the cultures were vortexed for 1min, a 600-μl volume was transferred to 2.4-ml fresh GW medium, and the cultures were grown for an additional 2.5h. Supernatants were collected at the beginning (t=0h) and end (t=2.5h) of the second round of growth in triplicate. DNA in culture supernatants was detected using the fluorescent DNA-binding dye PicoGreen (Invitrogen) and normalized to total cell protein (Bradford assay), as previously described (Hamilton *et al.*, 2005). For consistency across strains, only the results for which the final protein concentration (at t=2.5h) was within the range of 20-40μg ml^{-1} were plotted. The results are an average of at least four independent experiments. An average background fluorescence, determined by performing the secretion assay with the ΔGGI *N. gonorrhoeae* strain (ND500), was subtracted from the average result of all the strains.

In Vitro *Transcription/Translation and Transport Assay*

The full-length TraI is relatively long to synthesize in an *in vitro* transcription/translation assay, and since *sec*-dependent protein translocation is only determined by the N-terminal signal peptide and normally not affected by C-terminal truncations, a truncated version of TraI ($TraI_{\Delta 261\text{-}850}$) was constructed. To detect even small amounts of transported protein, inverted membrane vesicles derived from a strain overexpressing the SecYEG translocase were also used. SecA, SecB, and wild-type or SecYEG overexpressing inverted inner membrane vesicles (IMVs) were isolated as described (Manting *et al.*, 2000). The *in vitro* transcription and translation reaction was performed as described by van der Laan *et al.* (van der Laan *et al.*, 2004). Shortly, the RiboMax *in vitro* transcription kit (Promega) was used with plasmids pBSKftsQ (van der Laan *et al.*, 2004), pET147 (van der Wolk *et al.*, 1997) or pSJ001 to generate ^{35}S-labeled proteins. To study co-translational transport, the *in vitro* translation reactions were carried out for 30 min at 37°C in the presence or absence of wild-type or SecYEG IMVs (0.16mg ml^{-1}; (De Vrije *et al.*, 1987). Reactions were started by the addition of the ^{35}S-labeled methionine. After 30min at 37°C, a 10% synthesis control was removed and the

remainder was treated with 0.4mg ml^{-1} proteinase K for 30 min on ice in the presence or absence of 1% Triton X-100. Samples were trichloroacetic acid-precipitated and analyzed by 12% SDS-PAGE and phosphorimaging and quantified using the LumiAnalyst software from Roche Applied Science. FtsQ was used as the positive control. FtsQ is a membrane protein whose translocation into the inverted vesicles is mediated by Sec components (van der Laan *et al.*, 2004). A small domain is not transported and is accessible to degradation by proteinase K. To study post-translational transport, ^{35}S-labeled TraIΔ261-850 and proOmpA (positive control, contains a signal peptide that transports the protein into the periplasm and is processed by leader peptidase) were synthesized *in vitro*, dissolved in 6M urea, and used in post-translational translocation reactions as described (Cunningham and Wickner, 1989). Shortly, reaction mixtures contained 1.6μg of SecB, 0.5μg of SecA, and wild-type or SecYEG *E.coli* IMVs in buffer [50mM HEPES-KOH, pH 7.5, 30mM KCl, 0.5mg ml^{-1} BSA, 2mM DTT, 2mM Mg $(OAc)_2$] in an 80-μl volume. ^{35}S-proOmpA and ^{35}S-$TraI_{\Delta 261\text{-}850}$ were diluted 50-fold from a solution containing 6 M urea. Translocation reactions were performed in the presence or absence of 2mM ATP and an ATP regenerating system (10mM creatine phosphate, 0.5μg of creatine kinase) at 37°C for 30min, chilled on ice, and treated with proteinase K (0.1mg ml^{-1}) for 15 min. Transport of proteins into the *E. coli* IMVs was assayed by their accessibility to added proteinase K. Samples were analyzed by SDS-12% PAGE and autoradiography.

Generation of TraI-specific antibodies

Strain WSP34, an *E. coli* strain harboring a plasmid encoding *traI*FLAG/His10, was grown in a 30-ml LB liquid culture (Cm25) supplemented with 1M sorbitol at 37°C, from OD_{600} 0.2 to 0.5, induced with 1mM IPTG, switched to 18°C, and grown to an OD_{600} 1. Soluble protein was obtained by sonication and centrifugation (15,000 x g for 1min) of total cell extracts at 4° C. $TraI_{FLAG/His10}$ was purified over a nickel column, according to the manufacturer's instructions (Sigma), and verified by Western blot with an anti-FLAG M2 antibody (Sigma). TraI-specific polyclonal antiserum was generated in rabbits (Chemicon International).

Gonococcal growth for subcellular localization studies and detection of mutant proteins

Growth of strains that overexpress (OE) TraI from an inducible construct at a distant site on the chromosome: WSP36 ($traI^{+}_{\text{OE ectopic}}$) and WSP536 (ΔGGI + $traI_{\text{OE ectopic}}$) were grown in 3-ml liquid cultures (Cm10) with 1mM IPTG from an OD_{600} 0.1 for 4h at 37°C with aeration. Growth of strains that overexpress TraI from its native site on the chromosome: WSP38 ($traI^{+}_{\text{OE native}}$) and WSP3854 (*traI*L6K/L12$K_{\text{OE native}}$) were grown in 15-ml liquid cultures (Em10) from an OD_{600} 0.1 for 4h at 37°C with aeration. Growth of strains for detection of TraI without overexpression: ND500 (ΔGGI), MS11 (wild type), WSP5 (*traI*Y93F), WSP4 (*traI*Y200F/Y201F), and WSP52 (*traI*D120N) were grown in 30-ml liquid cultures from an OD_{600} 0.1 for 4h at 37°C with aeration. Approximately 0.5mg of total cell extracts were electrophoresed by SDS-PAGE in a 10% polyacrylamide gel.

Gonococcal fractionation

The Judd and Porcella protocol, specific for *N. gonorrhoeae*, was followed for the extraction of gonococcal periplasm (Judd and Porcella, 1993), and the membrane and soluble fractionations were performed as described (Gauthier *et al.*, 2003). Briefly, for extraction of periplasmic proteins, 20-μl volume of chloroform was added to bacterial pellets collected from 3-ml log-phase cultures, vortexed, and incubated for 15min at room temperature. Periplasmic proteins were recovered by the addition of 100-μl volume of 0.01M Tris-HCl (pH 7.0), centrifugation (15,000 x g for 1min), and collection of the aqueous fraction. The remaining cell pellet was washed with cold PBS buffer, resuspended in 500-μl volume of cold 0.01M Tris-HCl, sonicated on ice, centrifuged at 4°C to remove unbroken cells, and cleared lysates ultracentrifuged for 1h at 50,000 x g. The cytoplasmic proteins were concentrated to 100-μl

volume in a Nanosep centrifugal device (Pall, Ann Arbor, Michigan). The membrane pellet was washed with PBS and resuspended in 100-μl volume of 0.01M Tris-HCl (pH 7.0) with 0.5% N-lauroylsacosine (Sigma). Cellular fractions were electrophoresed by SDS-PAGE in 10% polyacrylamide gels. In addition to recognizing TraI, the TraI antiserum consistently identified a band of ~35 KDa on Western blots. The band was found in the periplasmic fraction from gonococcal strains that produce TraI (WT) or strains that lack the *traI* gene (ND500), indicating that this cross-reactive band does not represent TraI. Detection of this band was used to identify periplasmic material.

Western blotting

Electrophoresed proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, blocked for 1h with 3% nonfat dry milk in Tris-buffered saline (TBS), incubated for 1h with protein-specific rabbit antisera, washed for 10min, incubated for 1h with anti-rabbit peroxidase-conjugated antibody (Santa Cruz Biotechnology, 1:10,000 dilution), and washed three times for 10 min before addition of the peroxidase substrate (Immune Star, BioRad). The working dilutions of the rabbit antisera used are as follows: anti-TraI (1:10), anti-PilQ (1:10,000), and anti-CAT-1 (1:1000, Sigma). Anti-PilQ serum was provided by Dr. H. S. Seifert.

Nuclease treatment

Wild-type *N. gonorrhoeae* was grown in liquid culture, essentially as described for the DNA secretion assay. However, a 600-μl volume of the initial culture was centrifuged (15,000 x g for 1min), the pellet resuspended in fresh medium, and transferred to a 3-ml culture for a second round of growth. Cultures were grown for 2h and bacterial cells removed by centrifugation (3,000 x g for 5 min). Culture supernatants were collected, filter sterilized (0.22m filter), and stored at -20°C. DNaseI (4U ml^{-1}), Exonuclease III (400U ml^{-1}), Exonuclease I (80U ml^{-1}), $RecJ_f$ (60U ml^{-1}), or BAL-31 (4U ml^{-1}) purchased from New England Biolabs were used to treat culture supernatants in a 250-μl reaction, with their respective buffers, at 37°C for 12h. GW medium alone or culture supernatants collected from the wild-type strain MS11 or the DNA-secretion deficient strain WSP5 supplemented with double- or single-stranded λ-HindIII DNA (0.625μg ml^{-1}, NE Biolabs) were used to test for activity of the enzymes. This concentration of λ-HindIII DNA is ten-times the concentration of secreted gonococcal DNA in culture supernatants. Single-stranded λ-HindIII DNA was generated by boiling the DNA for 15min and quick cooling in an ice-water bath for 10 min. All the enzymes showed activity against their specific DNA substrate in GW medium alone (data not shown), WSP5 (*traI*Y93F) conditioned medium (data not shown), or MS11 conditioned medium (Fig. 7B, C).

Co-culture transformation assay

We used a modification of the method of Dillard and Seifert to measure transformation in co-culture (Dillard and Seifert, 2001). Briefly, a RecA-deficient, chloramphenicol (Cm) resistant donor strain (JD1545) was grown together with a spectinomycin (Sp) resistant recipient strain (MS11 Sp) in a 2-ml culture (GW medium) in the presence or absence of nucleases, and the frequency of Cm^RSp^R transformants after 2h of co-culture was determined. Co-culture growth conditions: DNaseI (1U ml^{-1}), Exonuclease III (100U ml^{-1}), and EcoRI (40U ml^{-1}), pH 6.8 and 7mM $MgSO_4$; Exonuclease I (20U ml^{-1}), pH 7.4 and 6.7mM $MgSO_4$; $RecJ_f$ (15U ml^{-1}), pH 7.4 and 10mM $MgSO_4$; BAL-31 (4U ml^{-1}), pH 7.4, 12mM $MgSO_4$, and 12mM $CaCl_2$. ExoI was reported to exhibit 20% activity at pH 7.5 (Lehman, 1960), which is approximately the expected activity of this nuclease in co-culture where the pH is 7.4. All the enzymes showed activity against their specific DNA substrate.

The LOOPP (Learning, Observing and Outputting Protein Patterns) server

Using the full-length TraI amino acid sequence, secondary-structure-based threading detected secondary structure similarities between TraI (amino acids 86 to 606, 61.53% of the sequence) and the endo/exocellulose E4 domain from *Thermomonospora fusca* (DOI 10.2210/pdb1tf4/pdb). Threading of TraI to the *T. fusca* endo/exocellulose E4 domain positioned Asp^{120} on an α-helix adjacent to the Tyr^{93} α-helix, where Asp^{120} and Tyr^{93} are facing each other.

Web-based programs used

NCBI PSI BLAST (http://www.ncbi.nlm.nih.gov/BLAST), score value of P=0.0001 as a threshold

CLUSTAL W (http://www.ebi.ac.uk/clustalw/index.html) (Thompson *et al.*, 1994)

SignalP 3.0 (http://www.cbs.dtu.dk/services/) (Bendtsen *et al.*, 2004)

Helical Wheel Custom Images (http://kael.net/helical.htm)

The LOOPP server (http://cbsuapps.tc.cornell.edu/loopp.aspx)

PROF - Secondary Structure Prediction System (http://www.aber.ac.uk/~phiwww/prof/)

Simple Interactive Statistical Analysis (SISA) *t*-test (http://home.clara.net/sisa/)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Aravind L, Koonin EV. The HD domain defines a new superfamily of metal-dependent phosphohydrolases. Trends Biochem Sci 1998;23:469–472. [PubMed: 9868367]

Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 2004;340:783–795. [PubMed: 15223320]

Biswas GD, Sparling PF. Entry of double-stranded deoxyribonucleic acid during transformation of *Neisseria gonorrhoeae*. J Bacteriol 1981;145:638–640. [PubMed: 6780524]

Bordo D, Argos P. Suggestions for “safe” residue substitutions in site-directed mutagenesis. J Mol Biol 1991;217:721–729. [PubMed: 2005621]

Christie PJ. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. Mol Microbiol 2001;40:294–305. [PubMed: 11309113]

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol 2005;59:451–485. [PubMed: 16153176]

Cunningham K, Wickner W. Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of *Escherichia coli*. Proc Natl Acad Sci U S A 1989;86:8630–8634. [PubMed: 2554321]

De Vrije T, Tommassen J, De Kruijff B. Optimal posttranslational translocation of the precursor of PhoE protein across *Escherichia coli* membrane vesicles requires both ATP and the protonmotive force. Biochim Biophys Acta 1987;900:63–72. [PubMed: 3036223]

Dillard JP, Seifert HS. 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 2001;41:263–277. [PubMed: 11454218]

Dillard, JP. Genetic manipulation of *Neisseria gonorrhoeae*. In: Coico, R.; Kowalik, T.; Quarles, JM.; Stevenson, B.; Taylor, RK.; Simon, AE., editors. Current Protocols in Microbiology. John Wiley and Sons; 2006. p. 4A.2.1-4A.2.19.

Elazar M, Liu P, Rice CM, Glenn JS. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. J Virol 2004;78:11393–11400. [PubMed: 15452261]

Francia MV, Varsaki A, Garcillan-Barcia MP, Latorre A, Drainas C, de la Cruz F. A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol Rev 2004;28:79–100. [PubMed: 14975531]

Frost LS, Ippen-Ihler K, Skurray RA. Analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol Rev 1994;58:162–210. [PubMed: 7915817]

Garcillan-Barcia MP, Jurado P, Gonzalez-Perez B, Moncalian G, Fernandez LA, de la Cruz F. Conjugative transfer can be inhibited by blocking relaxase activity within recipient cells with intrabodies. Mol Microbiol 2007;63:404–416. [PubMed: 17163977]

Gauthier A, Puente JL, Finlay BB. Secretin of the enteropathogenic *Escherichia coli* type III secretion system requires components of the type III apparatus for assembly and localization. Infect Immun 2003;71:3310–3319. [PubMed: 12761113]

Gentry DR, Cashel M. Mutational analysis of the *Escherichia coli* spoT gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. Mol Microbiol 1996;19:1373–1384. [PubMed: 8730877]

Gomis-Ruth FX, Sola M, de la Cruz F, Coll M. Coupling factors in macromolecular type-IV secretion machineries. Curr Pharm Des 2004;10:1551–1565. [PubMed: 15134575]

Gonzalez-Perez B, Lucas M, Cooke LA, Vyle JS, de la Cruz F, Moncalian G. Analysis of DNA processing reactions in bacterial conjugation by using suicide oligonucleotides. Embo J 2007;26:3847–3857. [PubMed: 17660746]

Grandoso G, Avila P, Cayon A, Hernando MA, Llosa M, de la Cruz F. Two active-site tyrosyl residues of protein TrwC act sequentially at the origin of transfer during plasmid R388 conjugation. J Mol Biol 2000;295:1163–1172. [PubMed: 10653694]

Gray HB Jr. Ostrander DA, Hodnett JL, Legerski RJ, Robberson DL. Extracellular nucleases of *Pseudomonas* BAL 31. I. Characterization of single strand-specific deoxyriboendonuclease and double-strand deoxyriboexonuclease activities. Nucleic Acids Res 1975;2:1459–1492. [PubMed: 1178526]

Greene PH, Poonian MS, Nussbaum AL, Tobias L, Garfin DE, Boyer HW, Goodman HM. Restriction and modification of a self-complementary octanucleotide containing the EcoRI substrate. J Mol Biol 1975;99:237–261. [PubMed: 173854]

Grinter NJ. Analysis of chromosome mobilization using hybrids between plasmid RP4 and a fragment of bacteriophage lambda carrying IS1. Plasmid 1981;5:267–276. [PubMed: 6267631]

Guasch A, Lucas M, Moncalian G, Cabezas M, Perez-Luque R, Gomis-Ruth FX, de la Cruz F, Coll M. Recognition and processing of the origin of transfer DNA by conjugative relaxase TrwC. Nat Struct Biol 2003;10:1002–1010. [PubMed: 14625590]

Hamilton HL, Schwartz KJ, Dillard JP. Insertion-duplication mutagenesis of *Neisseria*: use in characterization of DNA transfer genes in the gonococcal genetic island. J Bacteriol 2001;183:4718–4726. [PubMed: 11466274]

Hamilton HL, Dominguez NM, Schwartz KJ, Hackett KT, Dillard JP. *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. Mol Microbiol 2005;55:1704–1721. [PubMed: 15752195]

Hamilton HL, Dillard JP. Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. Mol Microbiol 2006;59:376–385. [PubMed: 16390436]

Harley MJ, Toptygin D, Troxler T, Schildbach JF. R150A mutant of F TraI relaxase domain: reduced affinity and specificity for single-stranded DNA and altered fluorescence anisotropy of a bound labeled oligonucleotide. Biochemistry 2002;41:6460–6468. [PubMed: 12009909]

Heinemann JA. Genetic evidence of protein transfer during bacterial conjugation. Plasmid 1999;41:240–247. [PubMed: 10366529]

Huber HE, Beauchamp BB, Richardson CC. *Escherichia coli* dGTP triphosphohydrolase is inhibited by gene 1.2 protein of bacteriophage T7. J Biol Chem 1988;263:13549–13556. [PubMed: 2843524]

Judd RC, Porcella SF. Isolation of the periplasm of *Neisseria gonorrhoeae*. Mol Microbiol 1993;10:567–574. [PubMed: 7968534]

Kohler PL, Hamilton HL, Cloud-Hansen K, Dillard JP. AtlA functions as a peptidoglycan lytic transglycosylase in the *Neisseria gonorrhoeae* type IV secretion system. J Bacteriol 2007;189:5421–5428. [PubMed: 17526702]

Lanka E, Wilkins BM. DNA processing reactions in bacterial conjugation. Annu Rev Biochem 1995;64:141–169. [PubMed: 7574478]

Larkin C, Datta S, Harley MJ, Anderson BJ, Ebie A, Hargreaves V, Schildbach JF. Inter- and intramolecular determinants of the specificity of single-stranded DNA binding and cleavage by the F factor relaxase. Structure 2005;13:1533–1544. [PubMed: 16216584]

Lawley TD, Gilmour MW, Gunton JE, Standeven LJ, Taylor DE. Functional and mutational analysis of conjugative transfer region 1 (Tra1) from the IncHI1 plasmid R27. J Bacteriol 2002;184:2173–2180. [PubMed: 11914349]

Lawley TD, Klimke WA, Gubbins MJ, Frost LS. F factor conjugation is a true type IV secretion system. FEMS Microbiol Lett 2003;224:1–15. [PubMed: 12855161]

Lehman IR. The deoxyribonucleases of Escherichia coli. I. Purification and properties of a phosphodiesterase. J Biol Chem 1960;235:1479–1487. [PubMed: 14415352]

Lehman IR, Nussbaum AL. The Deoxyribonucleases of *Escherichia coli* V. On the Specificity of Exonuclease I (Phosphodiesterase). J Biol Chem 1964;239:2628–2636. [PubMed: 14235546]

Llosa M, Gomis-Ruth FX, Coll M, de la Cruz Fd F. Bacterial conjugation: a two-step mechanism for DNA transport. Mol Microbiol 2002;45:1–8. [PubMed: 12100543]

Llosa M, O'Callaghan D. Euroconference on the Biology of Type IV Secretion Processes: bacterial gates into the outer world. Mol Microbiol 2004;53:1–8. [PubMed: 15225298]

Lovett ST, Kolodner RD. Identification and purification of a single-stranded-DNA-specific exonuclease encoded by the *recJ* gene of *Escherichia coli*. Proc Natl Acad Sci U S A 1989;86:2627–2631. [PubMed: 2649886]

Luo ZQ, Isberg RR. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. Proc Natl Acad Sci U S A 2004;101:841–846. [PubMed: 14715899]

Manting EH, van Der Does C, Remigy H, Engel A, Driessen AJ. SecYEG assembles into a tetramer to form the active protein translocation channel. Embo J 2000;19:852–861. [PubMed: 10698927]

Maynard Smith JM, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria? Proc Natl Acad Sci U S A 1993;90:4384–4388. [PubMed: 8506277]

Mundle ST, Fattal MH, Melo LF, Coriolan JD, O'Regan NE, Strauss PR. Novel role of tyrosine in catalysis by human AP endonuclease 1. DNA Repair (Amst) 2004;3:1447–1455. [PubMed: 15380100]

Pansegrau W, Ziegelin G, Lanka E. Covalent association of the *traI* gene product of plasmid RP4 with the 5′-terminal nucleotide at the relaxation nick site. J Biol Chem 1990;265:10637–10644. [PubMed: 2191955]

Pansegrau W, Lanka E. Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. Nucleic Acids Res 1991;19:3455. [PubMed: 1648208]

Pansegrau W, Schroder W, Lanka E. Relaxase (TraI) of IncP alpha plasmid RP4 catalyzes a site-specific cleaving-joining reaction of single-stranded DNA. Proc Natl Acad Sci U S A 1993;90:2925–2929. [PubMed: 8385350]

Pansegrau W, Schroder W, Lanka E. Concerted action of three distinct domains in the DNA cleaving-joining reaction catalyzed by relaxase (TraI) of conjugative plasmid RP4. J Biol Chem 1994;269:2782–2789. [PubMed: 8300611]

Pantoja M, Chen L, Chen Y, Nester EW. *Agrobacterium* type IV secretion is a two-step process in which export substrates associate with the virulence protein VirJ in the periplasm. Mol Microbiol 2002;45:1325–1335. [PubMed: 12207700]

Richardson CC, Lehman IR, Kornberg A. A Deoxyribonucleic Acid Phosphatase-Exonuclease from *Escherichia coli* Ii. Characterization of the Exonuclease Activity. J Biol Chem 1964;239:251–258. [PubMed: 14114851]

Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1989.

Snyder LA, Jarvis SA, Saunders NJ. Complete and variant forms of the 'gonococcal genetic island' in *Neisseria meningitidis*. Microbiology 2005;151:4005–4013. [PubMed: 16339945]

Stein DC. Transformation of *Neisseria gonorrhoeae*: physical requirements of the transforming DNA. Can J Microbiol 1991;37:345–349. [PubMed: 1908744]

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–4680. [PubMed: 7984417]

van der Laan M, Nouwen N, Driessen AJ. SecYEG proteoliposomes catalyze the Deltaphi-dependent membrane insertion of FtsQ. J Biol Chem 2004;279:1659–1664. [PubMed: 14578344]

van der Wolk JP, de Wit JG, Driessen AJ. The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events. Embo J 1997;16:7297–7304. [PubMed: 9405359]

Vanecko S, Laskowski M Sr. Studies of the specificity of deoxyribonuclease I. III. Hydrolysis of chains carrying a monoesterified phosphate on carbon 5′. J Biol Chem 1961;236:3312–3316. [PubMed: 13924728]

Varsaki A, Lucas M, Afendra AS, Drainas C, de la Cruz F. Genetic and biochemical characterization of MbeA, the relaxase involved in plasmid ColE1 conjugative mobilization. Mol Microbiol 2003;48:481–493. [PubMed: 12675806]

Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CM, Regensburg-Tuink TJ, Hooykaas PJ. VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science 2000;290:979–982. [PubMed: 11062129]

Wade JJ, Graver MA. A fully defined, clear and protein-free liquid medium permitting dense growth of *Neisseria gonorrhoeae* from very low inocula. FEMS Microbiol Lett 2007;273:35–37. [PubMed: 17559396]

Weiss AA, Johnson FD, Burns DL. Molecular characterization of an operon required for pertussis toxin secretion. Proc Natl Acad Sci U S A 1993;90:2970–2974. [PubMed: 8464913]

Yakunin AF, Proudfoot M, Kuznetsova E, Savchenko A, Brown G, Arrowsmith CH, Edwards AM. The HD domain of the *Escherichia coli* tRNA nucleotidyltransferase has 2′,3′-cyclic phosphodiesterase, 2′-nucleotidase, and phosphatase activities. J Biol Chem 2004;279:36819–36827. [PubMed: 15210699]

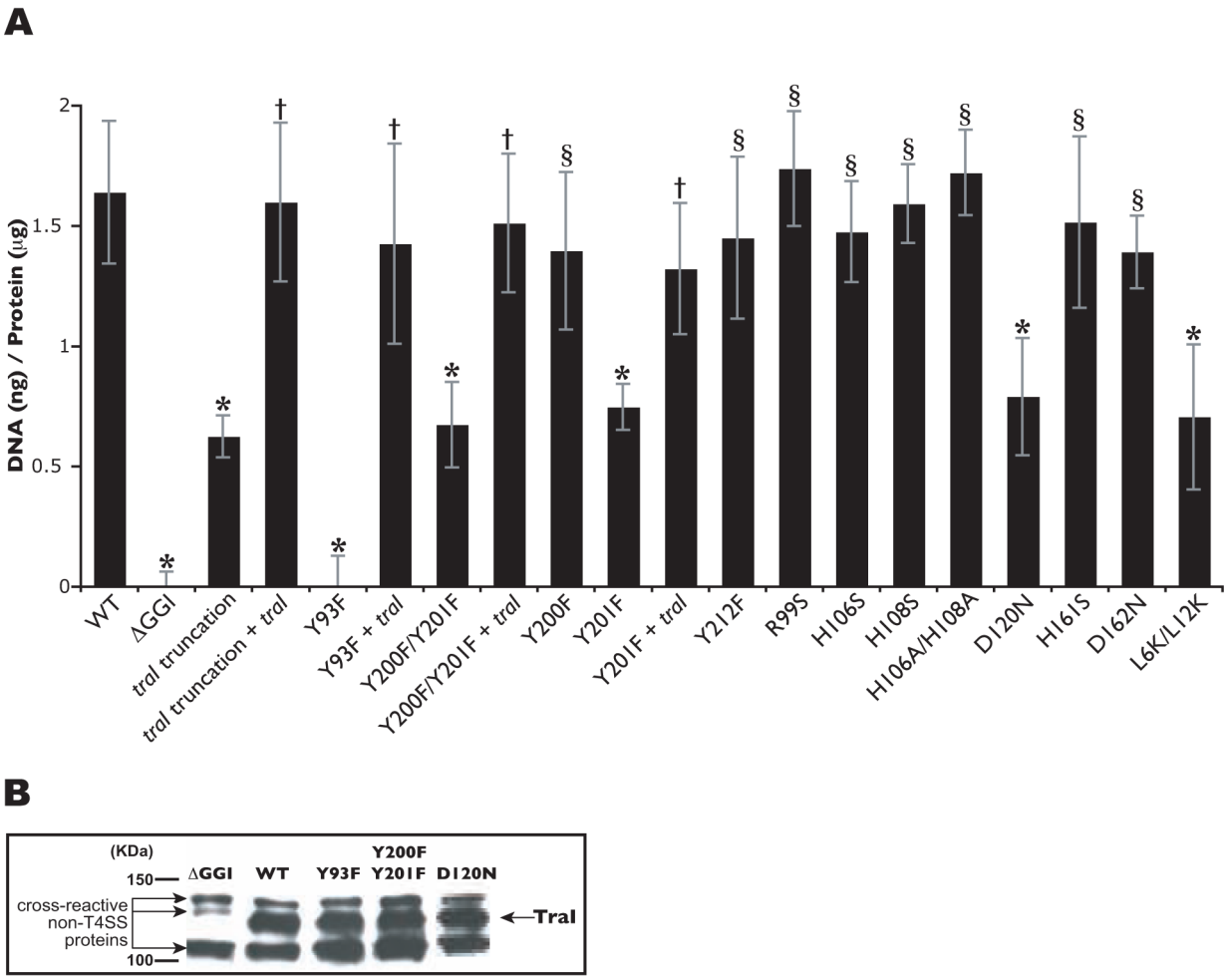

Fig. 1.

A. Fluorometric detection of secreted DNA. Piliated gonococcal strains were grown for 2.5h in liquid culture. Cell-free culture supernatants were collected, and DNA was detected with the fluorescent DNA-binding dye PicoGreen and normalized to total cell protein. MS11 was used as the wild-type (WT) strain and ND500 (ΔGGI in MS11) as the negative control. The results are an average of at least four independent experiments. *p-value<0.003 when compared to wild-type. †p-value<0.003 when compared to the respective mutant. §Not significantly different than wild type. B. Detection of TraI by Western blot. TraI was produced by wild-type (wt) and mutant strains at approximately the same levels. TraI is not detected in strain ND500, a strain in which the GGI was deleted.

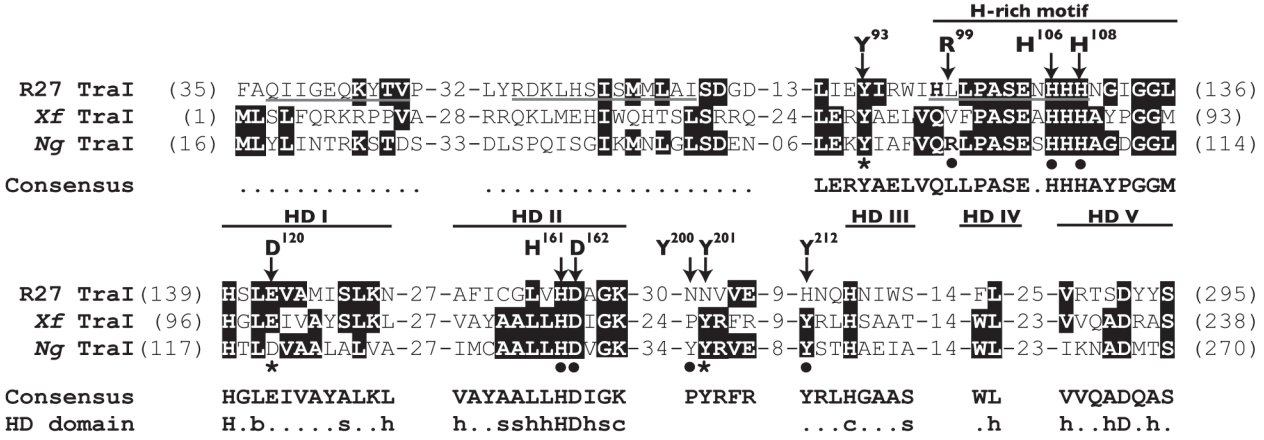

Fig. 2.

Amino acid sequence alignment of predicted relaxases from plasmid R27 (R27 TraI), *X. fastidiosa* (*Xf* TraI), and *N. gonorrhoeae* (*Ng* TraI). Identical amino acids are highlighted. Underlined sequences in R27 TraI were reported to correspond to the three motifs of classical relaxases. However, the first two motifs are not conserved in the *Ng* TraI family. The hallmarks of the *Ng* TraI family are a conserved tyrosine (Tyr^{93} in *Ng* TraI) closely followed by a histidine (H)-rich motif, and the HD phosphohydrolase domain. The signature sequence of the H-rich motif is h(Q/H)xhPASExHHHx_3GG(L/M)h, where h is a hydrophobic residue and x is any residue. The HD domain motifs are labeled HD I to V (where HD II is the signature motif). The consensus amino acid sequence for the fifty-four proteins in the *Ng* TraI family of predicted relaxases is shown (the dotted lines represent no consensus). The consensus amino acid sequence for the HD domain is also shown [(b) big, (s) small, (h) hydrophobic, (c) charged, and the capital letters represent invariant amino acids]. The arrows indicate the amino acids in *Ng* TraI that were the targets for site-directed mutagenesis in this study. Mutations that decrease DNA secretion are indicated with a star. Mutations that have no effect on DNA secretion are indicated with a solid circle.

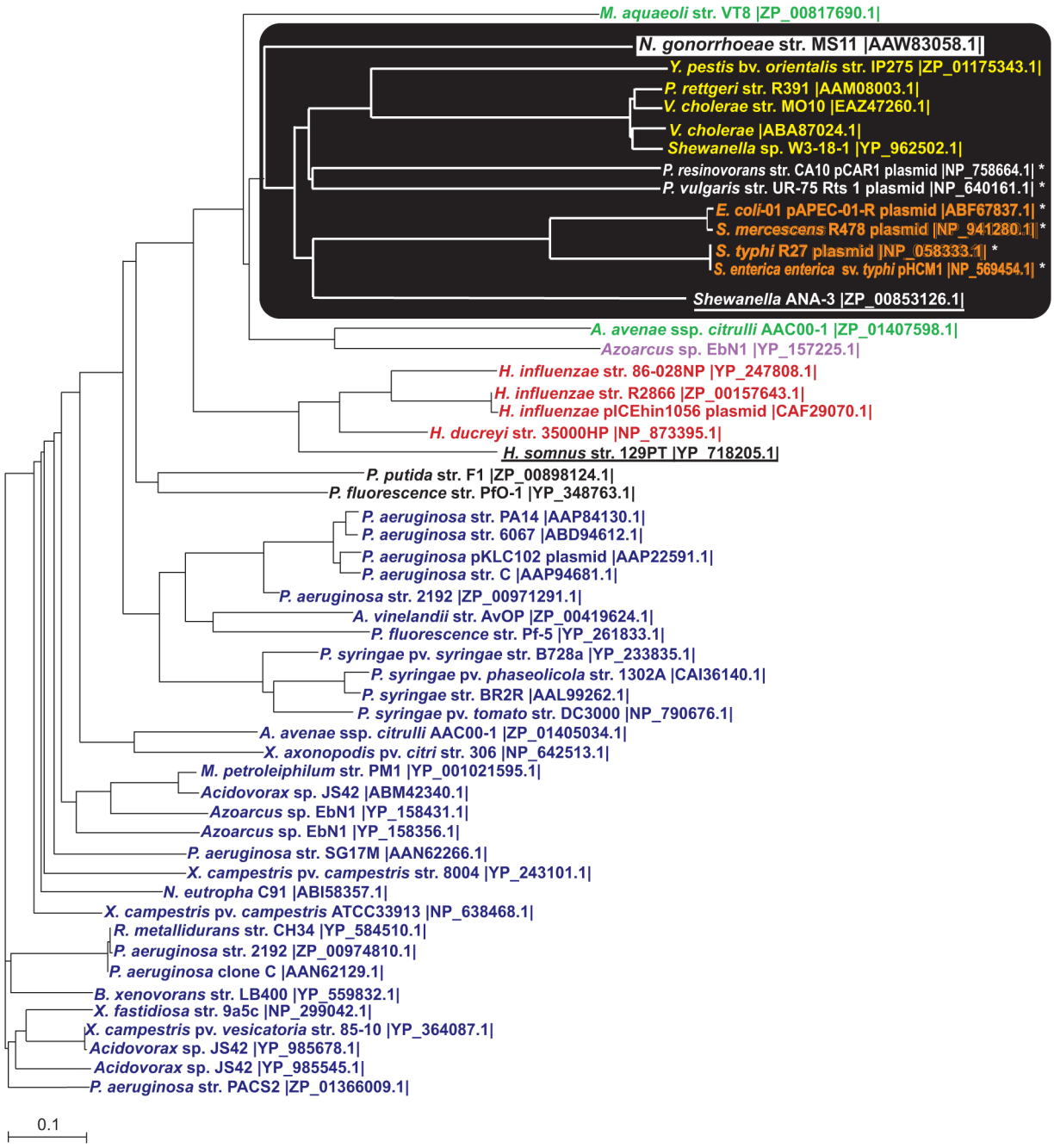

Fig. 3.

Phylogenetic tree of proteins with the same domain architecture as *Ng* TraI. The phylogenetic tree was generated from the CLUSTAL W alignment of the full-length proteins. A black box highlights the cluster containing *Ng* TraI. The different font colors represent proteins with similar predicted amphipathic α helices at the N-terminus proximal region: yellow, orange, red, and navy blue. Black font and white font indicate proteins with unique amphipathic α helices. *Ng* TraI, black font (white background), contains an N-terminal amphipathic α helix. *Azoarcus* sp. EbN1 (purple font) contains a predicted N-terminal transmembrane domain in addition to the N-terminus proximal amphipathic α helix. Green font indicates proteins that contain two predicted N-terminus proximal amphipathic α helices. Underlined proteins do not

contain predicted amphipathic α helices. *H. somnus* contains a charged reside on the hydrophobic side, and the *Shewanella* ANA-3 sequence available is truncated. Orange font indicates proteins that conserve motif I and II of relaxases. *Plasmid-encoded proteins.

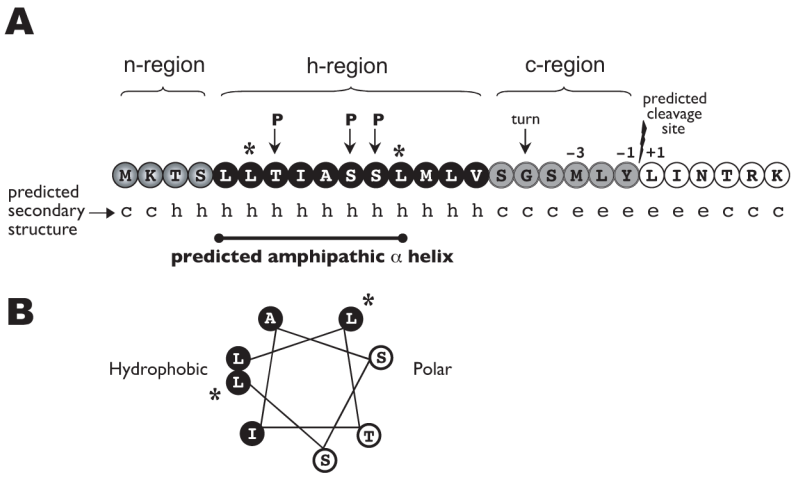

Fig. 4.

Map of the TraI N-terminal region.

A. A SignalP 3.0 algorithm analysis of the *Ng* TraI amino acid sequence predicted the presence of a signal sequence (0.923 probability) with a cleavage site between Tyr^{21} and Leu^{22} (0.5 probability). The hydrophobic (h) region is interrupted by polar residues (P), which results in the secondary structure prediction of an amphipathic α helix (c, coil; h, helix; e, sheet).

B. Helical wheel of amino acids 5 to 12. Black font, white background represents amino acids in the polar side of the helix. White font, black background represents amino acids in the hydrophobic side. *Charged residues were introduced into the hydrophobic side of the predicted amphipathic α helix at positions 6 and 12 (L6K/L12K).

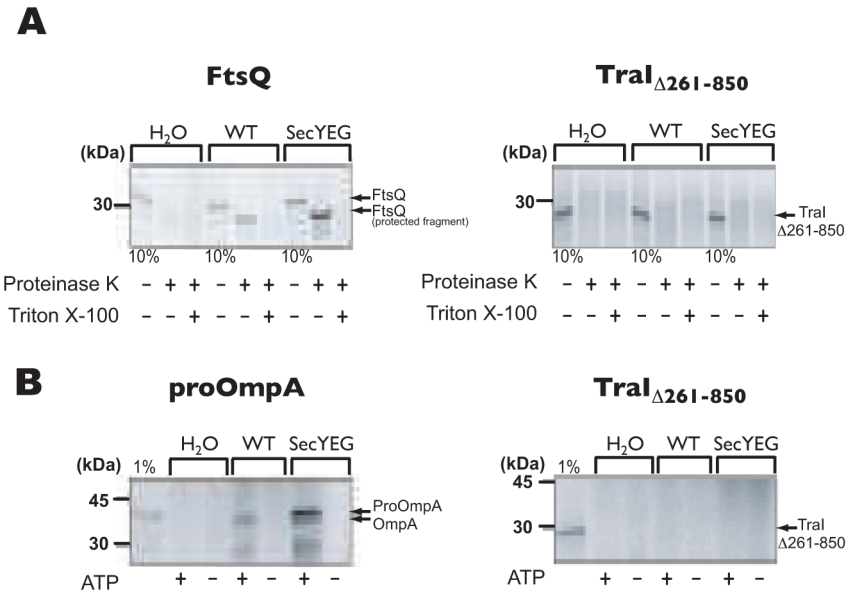

Fig. 5.

In vitro translocation assay with isolated inverted inner membrane vesicles of *E. coli*.

A. FtsQ and $TraI_{\Delta 261\text{-}850}$ were used in a co-translational targeting/transport assay in which the translocation into inverted inner membrane vesicles was monitored by the accessibility of the transported protein to externally added proteinase K, in the presence or absence of Triton X-100. To detect even small amounts of transported protein, inverted membrane vesicles derived from a strain overexpressing the SecYEG translocase were also used. Ten (10) % of the reaction mixture before addition of proteinase K was loaded as a synthesis control. FtsQ is a membrane protein whose insertion into the vesicles is mediated by Sec proteins. Only a small domain of FtsQ is exposed on the outside of the vesicles and is accessible to degradation by proteinase K.

B. proOmpA and $TraI_{\Delta 261\text{-}850}$ were used in a post-translation protein transport assay in the presence or absence of inner membrane vesicles, as described above, with or without ATP. One (1) % of synthesized protein before addition of proteinase K was loaded as a control. proOmpA contains a signal peptide for transport into the periplasm and is processed by leader peptidase.

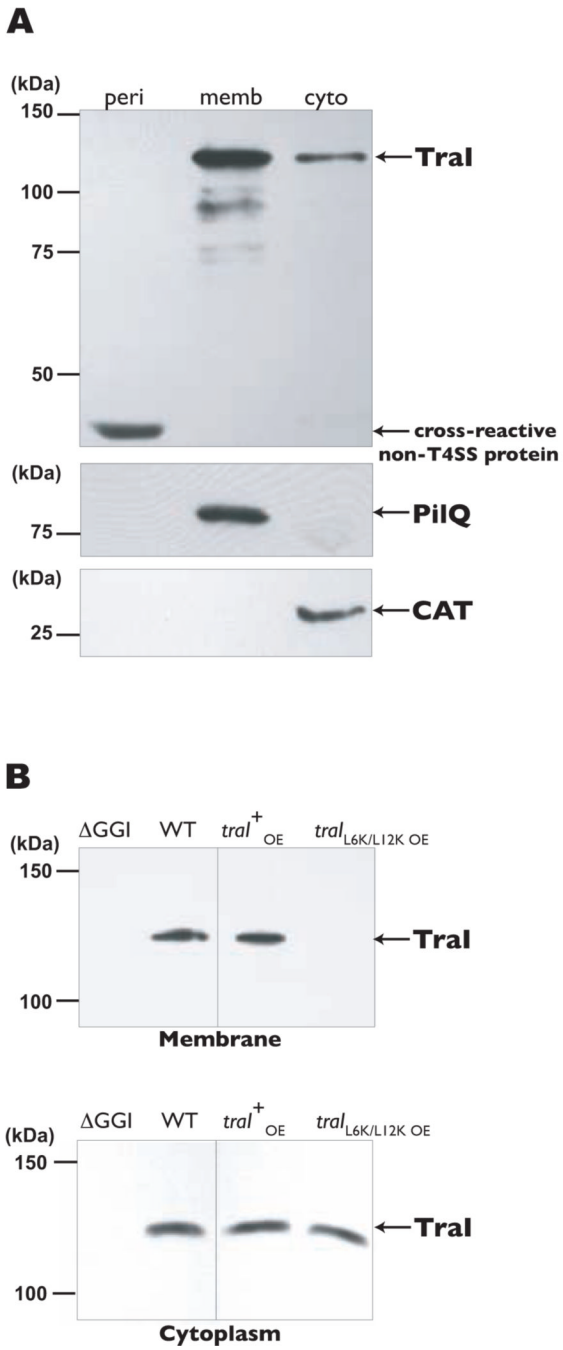

Fig. 6.

Subcellular localization of TraI.

A. Full-length TraI overexpressed in a wild-type strain from an inducible construct on the chromosome. TraI was detected by Western blot with TraI-specific polyclonal antiserum. Periplasmic (peri), membrane (memb), and cytoplasmic (cyto) fractions are indicated. A cross-reactive protein (~35kDa) was found in the periplasmic fractions from strains with or without the T4SS. Antiserum to the gonococcal outer membrane protein PilQ was used to detect membrane material. Chloramphenicol acetyltransferase (CAT) was detected to identify cytoplasmic material.

B. Top Panels. Membrane fractions from wild-type (WT) and ΔGGI strains (left panel) and strains overexpressing (oe) *traI* or *traI*L6K/L12K from the native site (right panel). Bottom Panels. Cytoplasmic fractions from wild-type (WT) and ΔGGI strains (left panel) and strains overexpressing (oe) *traI* or *traI*L6K/L12K from the native site (right panel). TraI was detected by Western blot with TraI-specific polyclonal antiserum.

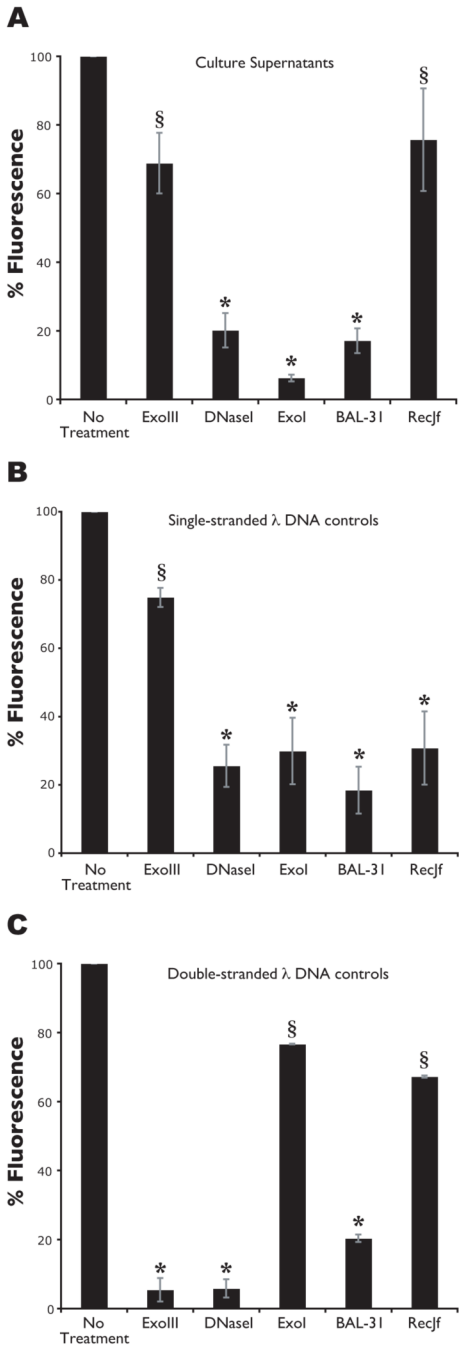

Fig. 7.

Nuclease treatment of culture supernatants. Fluorometric detection of DNA in cell-free supernatants after overnight incubation with nucleases. ExoIII is a double-strand specific exonuclease that converts double-stranded DNA to single-stranded DNA, by degrading one strand. ExoI is a 3′→5′ single-strand specific exonuclease. DNaseI and BAL-31 have endonuclease activity. BAL-31 also has double-strand specific exonuclease activity. $RecJ_f$ is a 5′→3′ single-strand specific exonuclease. Culture supernatants collected from a wild-type strain (A), culture supernatants supplemented with HindIII-digested, single-stranded λ DNA (B) or with double-stranded λ DNA (C). *p-value<0.05. §Not significantly different from no treatment.

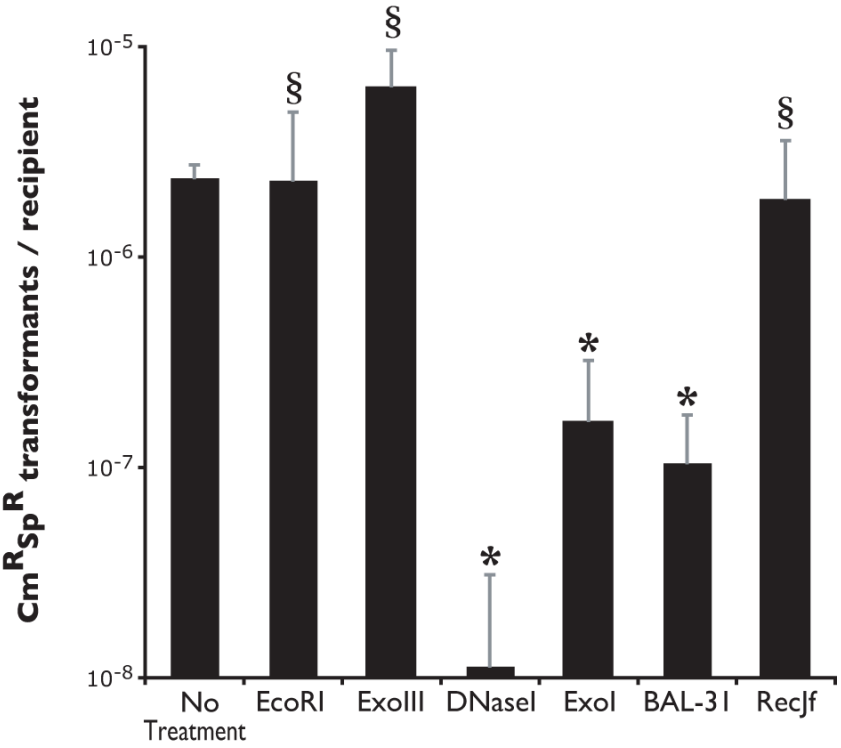

Fig. 8.

Co-culture transformation in the presence of nucleases. Transfer of an antibiotic resistance marker during co-culture transformation in the presence of nucleases. The transferred marker, *cat*, carries an internal EcoRI site. EcoRI is a restriction enzyme that cuts double-stranded DNA. ExoIII is a double-strand specific exonuclease. ExoI is a 3′→5′ single-strand specific exonuclease. $RecJ_f$ is a 5′→3′ single-strand specific exonuclease. DNaseI has double- and single-strand endonuclease activities; BAL-31 has single-strand specific endonuclease and double-strand specific exonuclease activities. *p-value<0.05. §Not significantly different from no treatment.

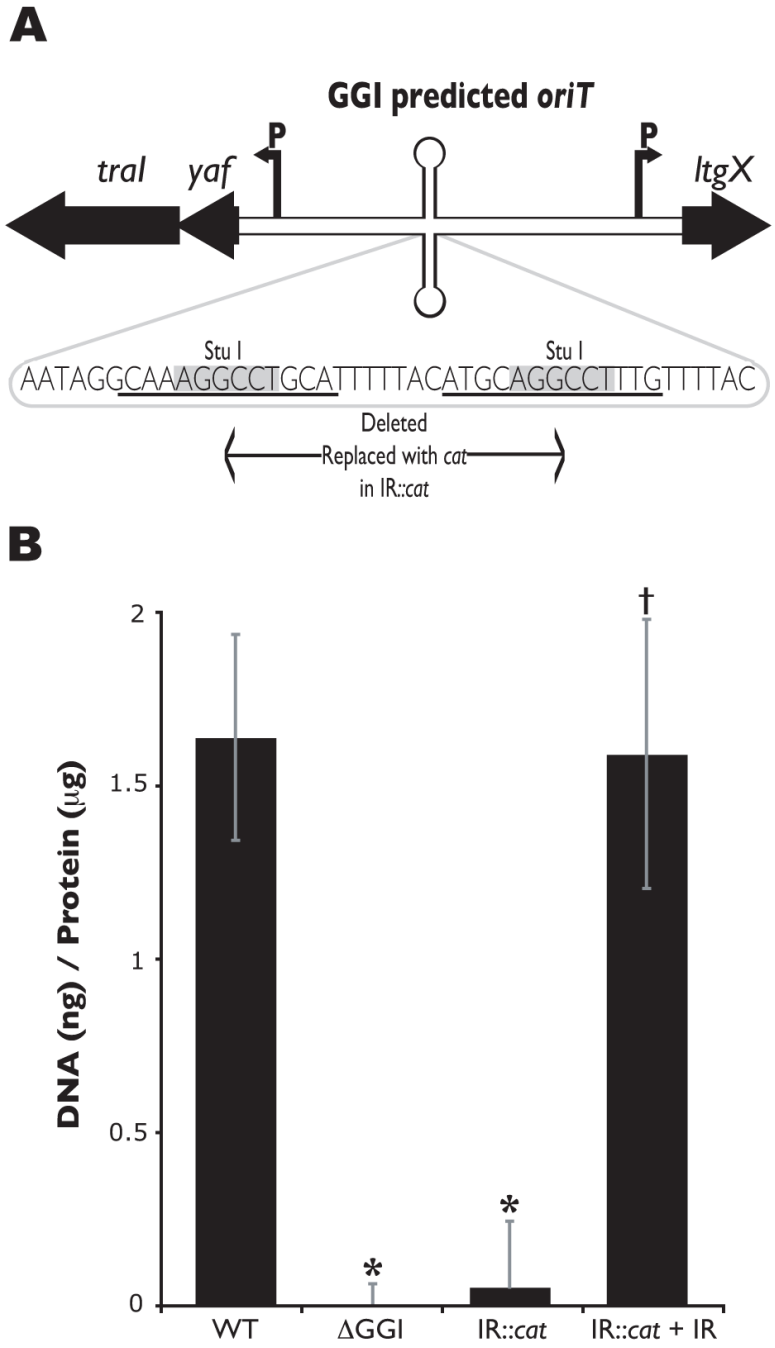

Fig. 9.

Predicted *oriT* sequence located in the GGI.

A. Map of the GGI region predicted to contain the *oriT*. *traI* encodes the putative relaxase, *yaf* encodes an unknown protein, and *ltgX* encodes a putative lytic transglycosylase. Arrows labeled "P" mark the promoter region for the transcripts. The sequence of the *yaf-ltgX* inverted repeat is shown. An insertion containing a *cat* marker was introduced at the *Stu*I site of the inverted repeat, deleting the sequence in between the *Stu*I sites.

B. Fluorometric detection of secreted DNA. Piliated gonococcal strains were grown for 2.5h in liquid culture. Cell-free culture supernatants were collected and DNA was detected with the fluorescent DNA-binding dye PicoGreen and normalized to total protein in the cell pellet.

MS11 was used as the wild-type (WT) strain and ND500 (ΔGGI in MS11) as the negative control. The results are an average of at least four independent experiments. *p-value<0.003 when compared to wild-type. †p-value<0.003 when compared to its respective mutant.

Table 1

Proteins with similar domain architecture as *N. gonorrhoeae* TraI

Protein origin ***Neisseria gonorrhoeae*** **str. MS11** **Relaxase**	**Predicted Function** **850**	**Length (bp)**	**Identify/Range (aa)** **100%/850**	**Acc. Number** **AAW83058.1**
Acidovorax avenae ssp. *citrulli* str. AAC00-1	Phosphohydrolase	861	35%/26	YP_971114.1
Acidovorax avenae ssp. *citrulli* str. AAC00-1	Relaxase	623	47%/86	YP_968947.1
Acidovorax sp. JS42	Relaxase	627	36%/115	YP_985545.1
Acidovorax sp. JS42	Relaxase	614	36%/100	ABM42340.1
Acidovorax sp. JS42	Relaxase	610	26%/284	YP_985678.1
Methylibium petroleiphilum str. PM1	Hypothetical	612	36%/100	YP_001021595.1
Burkholderia xenovorans str. LB400	Hypothetical	615	39%/101	YP_559832.1
Ralstonia metallidurans str. CH34	Relaxase	680	37%/115	YP_584510.1
Azoarcus sp. EbN1	Hypothetical	711	54%/42	YP_157225.1
Azoarcus sp. EbN1	Hypothetical	623	46%/88	YP_158431.1
Azoarcus sp. EbN1	Hypothetical	604	46%/88	YP_158356.1
Nitrosomonas eutropha str. C91	Relaxase	621	37%/100	ABI58357.1
Haemophilus somnus str. 129PT	Hypothetical	640	37%/94	YP_718205.1
Haemophilus influenzae str. 86-028NP	Hypothetical	638	46%/77	YP_247808.1
Haemophilus influenzae plasmid pICEhin1056	Hypothetical	635	27%/393	CAF29070.1
Haemophilus influenzae str. R2866	HD hydrolase	603	27%/393	ZP_00157643.1
Haemophilus ducreyi str. 35000HP	Hypothetical	217	41%/86	NP_873395.1
Xanthomonas campestris pv. *campestris* ATCC33913	Hypothetical	340	37%/103	NP_638468.1
Xanthomonas campestris pv. *campestris* str. 8004	Hypothetical	642	44%/92	YP_243101.1
Xanthomonas campestris pv. *vesicatoria* str. 85-10	Hypothetical	610	35%/100	YP_364087.1
Xanthomonas axonopodis pv. *citri* str. 306	Hypothetical	633	38%/107	NP_642513.1
Xylella fastidiosa str. 9a5c	Hypothetical	616	34%/101	NP_299042.1
Pseudomonas syringae str. BR2R	Hypothetical	638	35%/103	AAL99262.1
Pseudomonas syringae pv. *syringae* str. B728a	Relaxase	318	35%/103	YP_233835.1
Pseudomonas syringae pv. *syringae* str. B728a	Relaxase	634	35%/103	YP_234511.1
Pseudomonas syringae pv. *phaseolicola*	HD hydrolase	578	35%/103	CAI36140.1
Pseudomonas syringae pv. *tomato* str. DC3000	Hypothetical	642	76%/17	NP_790676.1
Pseudomonas aeruginosa str. 6077	Hypothetical	643	40%/100	ABD94612.1
Pseudomonas aeruginosa clone C plasmid pKLC102	Hypothetical	639	41%/100	AAP22591.1
Pseudomonas aeruginosa clone C	Hypothetical	608	40%/100	AAP94681.1
Pseudomonas aeruginosa str. PA14	Pathogenesis-related	639	40%100	AAP84130.1
Pseudomonas aeruginosa str. SG17M	Hypothetical	600	37%/100	AAN62266.1
Pseudomonas aeruginosa clone C	Hypothetical	630	37%/115	AAN62129.1
Pseudomonas aeruginosa str. 2192	HD hydrolase	312	40%/100	ZP_00971291.1
Pseudomonas aeruginosa str. 2192	HD hydrolase	602	37%/115	ZP_00974810.1
Pseudomonas aeruginosa str. PACS2	Hypothetical	614	38%/101	ZP_01366009.1
Pseudomonas resinovorans str. CA10 plasmid pCAR1	Nickase	900	70%/27	NP_758664.1
Pseudomonas fluorescence str. PfO-1	Hypothetical	575	47%/76	YP_348763.1
Pseudomonas fluorescence str. Pf-5	Hypothetical	628	39%/100	YP_261833.1
Pseudomonas putida str. F1	Hypothetical	595	44%/97	ZP_00898124.1
Azotobacter vinelandii str. AvOP	Relaxase	627	40%/108	ZP_00419624.1
Yersinia pestis bv. orientalis str. IP275	HD hydrolase	992	66%/36	ZP_01175343.1
Providencia rettgeri str. R391	Relaxase	716	42%/87	AAM08003.1

Protein origin ***Neisseria gonorrhoeae*** **str. MS11**	**Predicted Function** **Relaxase**	**Length (bp)** **850**	**Identify/Range (aa)** **100%/850**	**Acc. Number** **AAW83058.1**
Proteus vulgaris str. UR-75 plasmid Rts 1	DNA helicase	892	69%/26	NP_640161.1
Salmonella enterica ssp. *enterica* sv. *typhi* plasmid pHCM1	Hypothetical	1011	44%/68	NP_569454.1
Salmonella typhi plasmid R27	Relaxase	1011	44%/68	NP_058333.1
Escherichia coli plasmid pAPEC-01-R sv. 01:K1	Relaxase	1050	66%/27	ABF67837.1
Serratia mercescens plasmid R478	Hypothetical	1050	66%/27	NP_941280.1
Marinobacter aquaeoli str. VT8	Relaxase	624	48%/80	YP_957144.1
Shewanella sp. W3-18-1 plasmid 1	Phosphohydrolase	716	42%/87	YP_962502.1
Shewanella sp. ANA-3	Relaxase	941	29%/164	YP_863780.1
Vibrio cholerae	Relaxase	716	42%/87	ABA87024.1
Vibrio cholerae str. MO10	Relaxase	716	42%/87	EAZ47260.1

Table 2

Bacterial plasmids and strains

Plasmid/Strain	Description	Reference
Plasmids		
pIDN1	Cloning vector (Em^R), 2 kb	Hamilton *et al.* (2001)
pIDN2	Cloning vector (Em^R), 2 kb	Hamilton *et al.* (2001)
pIDN3	Cloning vector (Em^R), 2 kb	Hamilton *et al.* (2001)
pKH37	Complementation vector (Cm^R), 6.5 kb	Kohler *et al.* (2007)
pKS94	pIDN2 containing ~7 kb of the GGI from *traI*' to *traB*', 9 kb	Hamilton *et al.* (2001)
pWSP3	'*yaf-traI*'; ~0.8 kb *Bam*HI-*Mfe*I fragment of pKS94 in pIDN2, 2.8 kb	This study
pWSP4	*traI*Y200F/Y201F (TAT to TTT); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP5	*traI*Y93F (TAT to TTT); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP14	Full-length *traI*; PCR amplified from MS11, *Cla*I and *Sac*I digested in pIDN1, 4.7 kb	This study
pWSP15	*traI* 3′ end Flag tag; PCR of pWSP14 with primers containing the Flag tag; GAC TAC AAG GAC GAC GAC AAG, 4.7 kb	This study
pWSP30	'*traI-ltgX*'; ~5.2 kb *Pvu*II-*Spe*I fragment of pKS94	This study
pWSP31	IR::*cat*; *Pme*I-*Fsp*I *cat*-containing fragment from pKH37 in *Stu*I-digested pWSP30, ~8.1 kb	This study
pWSP33	*traI* 3′ end Flag & His10 tags; PCR of pWSP15 with primers containing the His10 tag, 4.7 kb	This study
pWSP34	*traI* 3′ end Flag and His10 tags; ~2.7 kb *Cla*I-*Sac*I fragment of pWSP33 in pKH37, 9 kb	This study
pWSP36	Full-length *traI*; PCR amplified from MS11 *Cla*I- and *Sac*I-digested in pKH37, 9 kb	This study
pWSP37	*yaf-ltgX* non-coding region; PCR amplified, ~750 bp in pIDN1, 2.4 kb	This study
pWSP38	0.9 kb *EcoR*V-*Nhe*I *ermC*-containing fragment from pIDN3 in *Psi*I-digested pWSP37, 4.5 kb	This study
pWSP42	*traI*Y200F (TAT to TTC); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP43	*traI*Y201F (TAT to TTC); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP44	*traI*R99S (AGG to AGC); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP45	*traI*H106S (CAT to TCG); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP46	*traI*H108S (CAT to AGC); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP47	*traI*'; ~2.9 kb *Bse*RI-*Xho*I fragment of pWSP14	This study
pWSP48	*traI*H106A/H108A (CAT to AGC); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP49	*traI*H161S (CAC to AGT); mutagenesis PCR of pWSP47, 2.9 kb	This study
pWSP50	*traI*D162N (GAC to AAC); mutagenesis PCR of pWSP47, 2.9 kb	This study
pWSP51	*traI*Y212F (TAT to TTT); mutagenesis PCR of pWSP47, 2.9 kb	This study
pWSP52	*traI*D120N (GAC to AGT); mutagenesis PCR of pWSP47, 2.9 kb	This study
pWSP54	*traI*L6K/L12K (CTC and CTA to AAG); mutagenesis PCR of pWSP3, 2.8 kb	This study
pWSP58	*yaf-ltgX* non-coding region in pKH37, 8.2 kb	This study
pSJ001	*traI*Δ261-850; PCR amplified from MS11, *Bsp*HI and *Xho*I digested in pET15b	This study
pBSKftsQ	Plasmid encoding FtsQ	van der Laan *et al.* (2004)
pET147	Plasmid encoding proOmpA	van der Wolk *et al.* (1997)
Strains		
MS11	Wild-type *N. gonorrhoeae*	Swanson *et al.* (1971)
ND500	Gonococcal genetic island deletion in MS11	Hamilton *et al.* (2005)
WSP4	MS11 transformed with pWSP4; *traI*Y200F/Y201F	This study
WSP5	MS11 transformed with pWSP5; *traI*Y93F	This study
WSP31	MS11 transformed with pWSP31; IR::*cat*	This study
WSP34	*E. coli* overexpressing $TraI_{FLAG/His10}$ (C-terminus tags)	This study
WSP36	MS11 transformed with pWSP36; $traI^{+}_{OE}$ from an inducible construct on the chromosome	This study
WSP38	MS11 transformed with pWSP38; $traI^{+}_{OE}$ from native site	This study
WSP42	MS11 transformed with pWSP42; *traI*Y200F	This study
WSP43	MS11 transformed with pWSP43; *traI*Y201F	This study
WSP44	MS11 transformed with pWSP44; *traI*R99S	This study
WSP45	MS11 transformed with pWSP45; *traI*H106S	This study
WSP46	MS11 transformed with pWSP46; *traI*H108S	This study
WSP48	MS11 transformed with pWSP48; *traI*H106A/H108A	This study
WSP49	MS11 transformed with pWSP49; *traI*H161S	This study
WSP50	MS11 transformed with pWSP50; *traI*D162N	This study
WSP51	MS11 transformed with pWSP51; *traI*Y212F	This study
WSP52	MS11 transformed with pWSP52; *traI*D120N	This study
WSP54	MS11 transformed with pWSP54; *traI*L6K/L12K	This study
HH532	TraI C-terminus truncation in MS11; *traI*Δ349-850	Hamilton *et al.* (2005)
WSP532	HH532 transformed with pWSP36; *traI* C-terminus truncation complement	This study
WSP3604	WSP4 transformed with pWSP36; *traI*Y200F/Y201F complement	This study
WSP3605	WSP5 transformed with pWSP36; *traI*Y93F complement	This study
WSP3643	WSP43 transformed with pWSP36; *traI*Y201F complement	This study
WSP3854	WSP54 transformed with pWSP38; $traIL6K/L12K_{OE}$ from native site	This study
WSP5831	WSP31 transformed with pWSP58; IR::*cat* complement	This study
MS11Sp	Spectinomycin resistant, recipient strain (MS11 background)	Dillard and Seifert (2001)
JD1545	MS11 *recA6 cnp::cat*, donor strain (MS11 background)	Dillard and Seifert (2001)

Plasmid/Strain	Description	Reference