Supplemental information:

Construction of *traG* fusion strains. For strains constructed using transposon-based *lacZ* or '*phoA* insertions, *traG* was inserted into a vector for expression in *E. coli*. *traG* was PCR amplified with the primers traGup and traGdown, digested with NsiI and EcoRV, and ligated to pKH9 digested with NsiI and PmeI to make pHH36. *traG* was subcloned from pHH36 by restriction digestion with EcoRI and BamHI and ligated to pIDN3 digested with the same restriction enzymes. The *E. coli* strain harboring the resulting plasmid, pPK1005, was infected with λTn*phoA*/In or λTn*lacZ*/In as described (1). Plasmid DNA was purified from chloramphenicol resistant bacteria and used to transform *E. coli* strain CC118. Cells containing plasmids encoding *traG'-'phoA* or *traG'-lacZ* fusions were selected with chloramphenicol and screened for blue color when grown on LB agar containing 40 μg/ml 5-bromo-4-chloro-3-indolylphosphate (XP) or 40 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal) respectively. DNA sequencing of the plasmids was used to verify in-frame *traG-phoA* or *traG-lacZ* fusions. A plasmid for inserting *lacZ* into the gonococcal complementation site was created by PCR amplifying *lacZ* with the primers lacZ5'Pst and lacZ3'Mfe, digesting the resulting PCR product and pKH35 with MfeI and PstI, and ligating the digested fragments to produce plasmid pKH39. *traG'-lacZ* fusions were subcloned into pKH39 by restriction digestion with SacI and XhoI and ligation into pKH39 digested with the same restriction enzymes. *traG'-'phoA* fusions were subcloned into pKH35 by restriction digestion with XhoI and NotI and subsequent ligation to pKH35 digested with the same restriction enzymes.

We found that it was difficult to introduce *traG'-'lacZ* or *traG'-'phoA* fusions into the gonococcal chromosome, presumably because of the difficulty of introducing large regions

of heterology. To overcome this problem, we introduced *lacZ* or '*phoA* into gonococcal strain MS11 first and then subsequently introduced *traG'* fragments. To introduce the *traG-lacZ* fusions into gonococci, *lacZ* was first introduced into the complementation site of strain MS11 by transformation with pKH39. The resulting strain was designated PK180 and was transformed with plasmids containing *traG'-'lacZ* fusions. To create a plasmid to introduce *'phoA* into the gonococcal complementation site, *'phoA* was cut from pUI1156 with KpnI and XhoI and ligated to pKH37 cut with the same restriction enzymes. The resulting plasmid, pPK1031, was used to transform gonococcal strain MS11 and the resulting strain was designated PK190. Strains PK180 and PK190 were transformed with *traG'-lacZ* fusion-containing plasmids, or *traG'-'phoA* fusion-containing plasmids, respectively. Gonococcal strains were screened by PCR for the correct *traG'-'lacZ* or *traG'-'phoA* fusions.

Because several of the plasmids containing directed *traG* fusions contained segments of *traG* lacking the 5′ end of the gene, we used a strain already containing a *traG'-'lacZ* or *traG'-'phoA* fusion as the parent strain to introduce the directed *traG* fusions. Strains PK181 or PK198 were transformed with plasmids containing directed *traG-lacZ* or *traG-phoA* fusions, respectively. Gonococci were screened with PCR for the correct *traG* fusions and to verify that the 5′ end of *traG* was retained from the parent strain.

Construction of strains for DNA secretion assays. To delete *traG*, a plasmid containing *traG,* 270 bp of the downstream gene, *atlA*, and 1249 bp of the upstream gene, *traH* all from strain MS11, was constructed using pIDN1 as the parent plasmid. The resulting plasmid, pSI7, was PCR amplified with the mutagenic primers traGdelF and traGdelR. The resulting

PCR product was treated with DpnI and used to transform *E. coli.* Transformants were selected for erythromycin resistance, and plasmids of the expected size were sequenced to verify the deletion of *traG*. The resulting plasmid, pPK1023, was used to transform gonococci as described (2). Colonies were screened using PCR, and the resulting strain with a deletion of *traG* was designated PK186. For the complementation of PK186, *traG* was amplified by PCR with the primers traGup and NhetraGR, digested with NsiI and NheI, and ligated to pKH6 digested with the same restriction enzymes. The resulting plasmid, pPK1040, was used to transform strain PK186, and chloramphenicol resistant colonies were screened by PCR for insertion of the complementation construct into the desired site on the gonococcal chromosome. The resulting strain was designated PK191.

For the construction of strains HH528 and HH534, a SpeI-EcoRI fragment carrying 479 bp of the conserved region of *traG* was subcloned from pKS43 (3) and ligated to SpeI- and EcoRI-digested pKC1 (4). pKC1 contains genes conferring erythromycin resistance and streptomycin sensitivity. The resulting plasmid, pHH18 was used to transform gonococcal strains PID2059 and JC1. Isolates that were streptomycin sensitive and erythromycin resistant (at 2 μg/ml) had undergone a single crossover event, causing the plasmid to insert within the chromosomal *traG3* or *traG2*. These PID2059 and JC1 derivatives were designated HH513 and HH530 respectively. Chromosomal DNA from HH513 and HH530 was used to transform strain MS11, a naturally streptomycin resistant strain, in order to introduce the variant alleles of *traG* linked to the plasmid insertion. Streptomycin sensitive and erythromycin resistant transformants were designated HH526 (MS11 transformed with HH513 DNA) and HH533 (MS11 transformed with HH530 DNA) and screened for the presence of *traG3* or *traG2* by PCR or by PCR and restriction digestion. HH526 and HH533 were grown in GCBL without antibiotic selection for 3 h to allow for homologous

recombination to resolve out the plasmid pHH18, originally inserted in the PID2059 and JC1 genomes. We selected for the loss of the inserted plasmid using streptomycin, and screened for erythromycin sensitivity to confirm loss of pHH18 DNA. PCR and restriction digests identified the presence of *traG3* or *traG2*, and the expected construction was confirmed by DNA sequencing. The resulting MS11 derivatives containing *traG3* and *eppA* in place of *traG* and *atlA* or *traG2* in place of *traG* were designated HH534 and HH528 respectively.

Construction of strains for heterologously overexpressing *eppA*.

eppA was cloned into the overexpression vector pET28b, which codes for an N-terminal hexa-histidine tag. To generate this construct, primers EPatL5′NheI and newEP3′XhoI were used in PCR amplification of *eppA* from gonococcal strain PID2059. The PCR product was cloned into pET28b using NheI and SpeI restriction sites.

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