Supplemental Experimental Procedures

Genetic manipulations. The *N. gonorrhoeae* FA1090 genome sequence (NC_002946) was used as a template to design oligonucleotides using Geneious 7 software. All oligonucleotides were synthesized by Integrated DNA Technologies and are listed in Supplemental Table S2. Genomic DNA was isolated from *N. gonorrhoeae* FA1090 using the Wizard Genomic DNA Purification Kit (Promega). Plasmid DNA and PCR products were purified with the QIAprep Spin Miniprep Kit (QIAGEN). The individual PCRs were performed with the FA1090 chromosomal DNA as a template, the appropriate primers, and Q5® High-Fidelity DNA Polymerase (NEB). Constructs were introduced into piliated FA1090 by transformation (1). All FA1090 mutant derivatives were selected on GCB supplemented with kanamycin or kanamycin, erythromycin and 100 μM isopropyl-β-D-thiogalactoside (IPTG). The obtained constructs and the presence of desired mutations were verified by sequencing at the Center for Genome Research and Biocomputing at Oregon State University.

Construction of ngo1801 (bamA), ngo0277 (bamD), and ngo1955 (tamA) conditional knockouts. The conditional mutant strains were constructed by placing an additional copy of the gene of interest under the control of the IPTG-inducible promoter, P_{lac}, within an intergenic region located between *lctP* and *aspC* in the FA1090 chromosome (2), and a subsequent in-frame replacement of the respective gene in its native locus with the nonpolar kanamycin resistance cassette derived from pKD4 (3).

Specifically, FA1090 Δ*bamA* P_{lac}::*bamA*, was constructed in the following steps. First, the additional copy of *bamA* was amplified with primers bamA-up and bamA-down and the PCR product was cloned under the control of P_{lac}, carried on pGCC4 (4) yielding pGCC4-*bamA*. To replace the chromosomal *bamA* with a kanamycin cassette, plasmid pNEB193-Δ*bamA* was created using the Gibson Assembly method (5). DNA fragments containing 1 kb

upstream and 1 kb downstream from *bamA* gene, as well as the kanamycin resistance gene from pKD4 (3), were amplified using the primers listed in Supplemental Table S2. Plasmid pNEB193 (NEB) was linearized using PCR and the Gibson Assembly Master Mix (NEB) was applied to assemble all fragments according to the manufacturer's recommendation. Finally, ScaI-linearized pNEB193-Δ*bamA* was used for allelic exchange of *bamA* in strain FA1090 carrying pGCC4-P_{lac}::*bamA*.

The FA1090 Δ*tamA* P_{lac}::*tamA* was engineered by placing an additional copy of *tamA* on the chromosome using pGCC4-P_{lac}::*tamA*, which was created by the amplification of *tamA* using primers tamA_pGCC_fwd and tamA_pGCC_rev, digesting the 1926 bp PCR fragment with FseI, and cloning into the FseI/ScaI-cut pGCC4 vector. The obtained plasmid pGCC4-P_{lac}::*tamA* was used in the transformation of piliated FA1090. The constructs for *tamA* deletion via homologous recombination were created by amplification of the region upstream and downstream from *tamA* with oligonucleotides NGO1956_up_fwd and NGO1956_up_rev, and NGO1956_down_fwd and NGO1956_down_rev, respectively. The PCR product corresponding to the upstream region was treated with EcoRI and KpnI and ligated into similarly prepared pUC18K to generate pUC18K-*tamA*up. The DNA fragments encompassing the downstream region from *tamA* and pUC18K-*tamA*up were treated with BamHI and HindIII and ligated to create pUC18K-Δ*tamA*. Finally, linearized pUC18K-Δ*tamA* was used to transform FA1090 P_{lac}::*tamA* to create FA1090 Δ*tamA* P_{lac}::*tamA*.

To generate the FA1090 Δ*bamD* P_{lac}::*bamD* knockout strain, the DNA fragment containing the entire *bamD* with the native ribosomal binding site was amplified with oligonucleotides bamD_rbs_fwd and bamD_rbs_rev. The 834 bp fragment was digested with FseI and cloned into FseI/ScaI treated pGCC4, yielding pGCC4-P_{lac}::*bamD*. Wild type piliated FA1090 was transformed with the resulting plasmid to generate FA1090 carrying P_{lac}::*bamD*.

The 577 bp upstream from the *bamD* encoding region were amplified using primers bamD_up_fwd and bamD_up_rev, and the PCR product was cloned into EcoRI-KpnI-digested pUC18K (6), resulting in pUCK18K-*bamD*up. The fragment downstream of *bamD* was amplified with bamD_down_fwd and bamD_down_rev and the 565 bp DNA fragment was cloned into BamHI-HindIII-digested pUC18K-*bamD*up, yielding pUC18K-Δ*bamD*. This plasmid was subsequently used for deletion of *bamD* in *N. gonorrhoeae* FA1090 P_{lac}::*bamD* using homologous recombination.

Construction of in-frame deletion mutants Δngo2054 and Δngo0994 (lipid modified azurin protein, laz). To engineer the FA1090 strain lacking NGO2054, the upstream region of the NGO2054 gene was amplified with primers NGO2054_up_fwd and NGO2054_up_rev. The 533 bp PCR product was digested with EcoRI-KpnI and ligated into the pUC18K vector (6), generating pUC18K-*ngo2054*up. The downstream region of NGO2054 was amplified with primers NGO2054_down_fwd and NGO2054_down_rev. The 531 bp PCR product was cloned into pUC18K-*ngo2054*up after digestion with BamHI and SphI, resulting in pUC18K-Δ*ngo2054*. The pUC18K-Δ*ngo2054* linearized with NdeI was used for *N. gonorrhoeae* transformation. Colonies that arose on GCB containing kanamycin were picked for verification by PCR using primers NGO2054_ch_fwd and NGO2054_ch_rev.

The FA1090 Δ*laz* strain was engineered as follows. The chromosomal DNA fragments located upstream (585 bp) and downstream (571 bp) from NGO0994 were amplified with primers NGO0994_up_fwd and NGO0994_up_rev, and NGO0994_down_fwd and NGO0994_down_rev; respectively. The 585 bp PCR product was cloned into pUC18K digested with EcoRI and KpnI, yielding pUCK18K-*ngo0994*up. The 571 bp product was digested with XbaI and PstI and cloned into likewise cut pUCK18K-*ngo0994*up. The resulting pUC18K-Δ*ngo0994* was linearized and used in transformation of wild type FA1090.

To complement the FA1090 Δ*ngo2054* and Δ*laz*, the genes were amplified with primers NGO2054_pGCC_fwd, NGO2054_pGCC_rev and NGO0994_pGCC_fwd, NGO0994_pGCC_rev, respectively. The PCR products were digested with FseI, and cloned into FseI-ScaI-treated pGCC4. Finally, the obtained pGCC4-*ngo2054* and pGCC4-*laz* were used to transform the FA1090 ∆*ngo2054* and FA1090 ∆*laz*, respectively. The construction of the FA1090 ∆*ngo2139* strain was previously described (7) and complementation was accomplished according to the scheme described above using primers NGO2139_pGCC_fwd and NGO2139_pGCC_rev (Supplemental Table S2).

References

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