**SUPPORTING INFORMATION**

**SUPPLEMENTAL MATERIALS AND METHODS**

**DNA manipulations.** MlaA lacking the signal peptide was amplified from purified FA1090 genomic DNA using primers MlaA-MBP-F, GATCCCATGGAGGATCTGTACTTTCAGAGCGAAACCCGCCCCGCCGAC, and MlaA-MBP-R, GATCAAGCTTGCTTTAGTGGTGATGGTGATGATGGGGTTGTGTTCCAGGTTGCGTTTCGG, digested with NcoI and HinDIII (New England Biolabs; recognition sites underlined), and cloned into similarly-digested pMBP-27b. Cloning resulted in the maltose binding protein (MBP) fused to the N-terminus of MlaA with two intervening TEV recognition. The MlaA C-terminus was also tagged with a 6 × Histidine tag. The resulting construct (pMBP-27b-MlaA-His) was transformed into ER2566 *E. coli* for protein production.

**Protein production and purification.** Overnight cultures of ER2566 *E. coli* harboring pMBP-27b-MlaA-His were diluted 1:100 into 1.5L of Luria-Bertani liquid media (LB) supplemented with 50 µg/mL kanamycin and cultured at 37 ºC with shaking (220 rpm). Protein production was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when cultures reached an OD600 of approximately 0.5. Induction was performed for 3 h at 37 ºC with shaking. Bacteria were collected by low-speed centrifugation (6000 × g for 10 min at 4 ºC). Pellets were subsequently suspended in lysis buffer (20 mM Tris, pH 7.4; 200 mM NaCl; 1 mM ethylenediaminetetraacetic acid [EDTA]; and a Pierce protease inhibitor tablet [ThermoFisher Scientific]), lysed by 5 passages through a French pressure cell at ~12,000 psi, and centrifuged to remove intact bacteria and cell debris. Supernatants were subjected to affinity chromatography with a MBPTrap column (GE Healthcare Life Sciences) using a Bio-Rad NGC Scout system, and protein was eluted with elution buffer (20 mM Tris, pH 7.4; 200 mM NaCl; 1 mM EDTA; 10 mM maltose). The MBP tag was cleaved by overnight incubation with a 1:40 TEV:protein (v/v) ratio during dialysis into 50 mM Tris, pH 8 and 1 mM dithiothreitol (DTT). The protein solution was subsequently applied to a Bio-Rad Bio-Scale Mini Nuvia IMAC cartridge (Bio-Rad) using the Bio-Rad NGC Scout system and eluted with nickel elution buffer (20 mM Tris, pH 8; 500 mM NaCl; 250 mM imidazole). Elutions were pooled, concentrated, and applied to a HiLoad Superdex 75 column (GE Healthcare Life Sciences) for size exclusion chromatography on the Bio-Rad NGC Scout system in column buffer (20 mM Tris, pH 8; 500 mM NaCl; 10% glycerol [v/v]). Fractions were analyzed for protein content throughout the purification process by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualization by either colloidal coomassie G-250 staining or with the SilverQuest Silver Staining Kit (ThermoFisher Scientific).

**Agar dilution assessment of antibiotic minimal inhibitory concentrations.** Non-piliated colonies cultured as in the main text were suspended in GCBL to a density of 1 × 105 CFU/mL. One hundred microliters of this suspension, corresponding to approximately 1 × 104 CFU, were spread onto GCB plates supplemented with 2-fold dilutions of antibiotics indicated in the text and 0.5 mM IPTG to induce expression of MlaA or PldA. MICs were assessed after ~22 h of incubation at 37 ºC in a 5% CO2 atmosphere. Experiments were performed on five independent occasions and majority values are reported.

**Fur induction and iron limitation growth curves.** Non-piliated colonies of WT FA1090, isogenic knockout ∆*mlaA*, and conditional knockout ∆*fur*/Plac::*fur* were suspended to an OD600 of 0.1 in GCBL supplemented with Kellogg’s supplement I and 0.042% sodium bicarbonate, but lacking Kellogg’s supplement II. Strains were cultured for an initial 3 h period at 37 ºC with shaking (220 rpm). Fur expression was induced by the addition of 100 µM IPTG during initial growth. After 3 h, ∆*fur*/Plac::*fur* cultures were centrifuged at 5000 × *g* for 5 min, the supernatant was decanted to remove IPTG, and the pellet was resuspended in medium supplemented as above but lacking IPTG. All cultures were back diluted to an OD600 of 0.1 and supplemented with either Kellogg’s supplement II (1:1,000) or 25 µM desferal. Fur expression was induced by the addition of 10, 50, or 100 µM IPTG both under standard growth conditions and iron starvation. Bacterial growth was monitored each hour by OD600 measurement. Growth curves from three independent experiments were plotted in GraphPad Prism and analyzed using the built-in two-way ANOVA to test for statistical significance at *p*<0.05 using Sidak’s multiple comparisons test.

**SUPPLEMENTAL TABLES**

**Table 1. Amino acid identity of members of the *N. gonorrhoeae* Mla operon with their *E. coli* homologs.**

|  |  |  |
| --- | --- | --- |
| **FA1090 Genetic Locus** | ***E. coli* homolog with UniProt ID** | **Amino Acid Identitya** |
| *ngo2120* | *mlaB* (P64602) | 24.3% |
| *ngo2119* | *mlaC* (P0ADV7) | 24.8% |
| *ngo2118* | mlaD (P64604) | 37.2% |
| *ngo2117* | mlaE (P64606) | 52.7% |
| *ngo2116* | mlaF (P63386) | 46.5% |

aAmino acid identity was assessed with the EMBOSS Needle online pairwise protein alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss\_needle/).

**Table 2. Agar dilution assessment of WT, ∆*mlaA*, ∆*mlaA*/Plac::*mlaA*, and ∆*mlaA*/Plac::*pldA* MICs.**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Polymyxin Ba** | **Vancomycina** | **Ampicillina** |
| **WT** | 128 | 16 | 0.25 |
| **∆*mlaA*** | 64 | 8 | 0.25 |
| **∆*mlaA*/Plac::*mlaA*** | 128 | 8 | 0.25 |
| **∆*mlaA*/Plac::*pldA*** | 32 | 8 | 0.25 |

aMIC values are presented in µg/mL