

# Proteomics-driven Antigen Discovery for Development of Vaccines Against Gonorrhea\*<sup>§</sup>

Ryszard A. Zielke<sup>‡</sup>, Igor H. Wierzbicki<sup>‡</sup>, Benjamin I. Baarda<sup>‡</sup>, Philip R. Gafken<sup>§</sup>,  
 Olusegun O. Soge<sup>¶</sup>, King K. Holmes<sup>¶||</sup>, Ann E. Jerse<sup>\*\*</sup>, Magnus Unemo<sup>‡‡</sup>,  
 and  Aleksandra E. Sikora<sup>‡§§</sup>

Expanding efforts to develop preventive gonorrhea vaccines is critical because of the dire possibility of untreatable gonococcal infections. Reverse vaccinology, which includes genome and proteome mining, has proven very successful in the discovery of vaccine candidates against many pathogenic bacteria. However, progress with this approach for a gonorrhea vaccine remains in its infancy. Accordingly, we applied a comprehensive proteomic platform—**isobaric tagging for absolute quantification coupled with two-dimensional liquid chromatography and mass spectrometry**—to identify potential gonococcal vaccine antigens. Our previous analyses focused on cell envelopes and naturally released membrane vesicles derived from four different *Neisseria gonorrhoeae* strains. Here, we extended these studies to identify cell envelope proteins of *N. gonorrhoeae* that are ubiquitously expressed and specifically induced by physiologically relevant environmental stimuli: oxygen availability, iron deprivation, and the presence of human serum. Together, these studies enabled the identification of numerous potential gonorrhea vaccine targets. Initial characterization of five novel vaccine candidate antigens that were ubiquitously expressed under these different growth conditions demonstrated that homologs of BamA (NGO1801), LptD (NGO1715), and TamA (NGO1956), and two uncharacterized proteins, NGO2054 and NGO2139, were surface

exposed, secreted via naturally released membrane vesicles, and elicited bactericidal antibodies that cross-reacted with a panel of temporally and geographically diverse isolates. In addition, analysis of polymorphisms at the nucleotide and amino acid levels showed that these vaccine candidates are highly conserved among *N. gonorrhoeae* strains. Finally, depletion of BamA caused a loss of *N. gonorrhoeae* viability, suggesting it may be an essential target. Together, our data strongly support the use of proteomics-driven discovery of potential vaccine targets as a sound approach for identifying promising gonococcal antigens. *Molecular & Cellular Proteomics* 15: 10.1074/mcp.M116.058800, 2338–2355, 2016.

Despite current prevention and management strategies, infections caused by sexually transmitted pathogens affect hundreds of millions of women and men in both resource-constrained and developed countries (1). Gonorrhea remains a leading public health burden with an estimated 78 million new cases annually worldwide (2). Major challenges in eradicating this ancient human disease include increasing multi-drug-resistance among *Neisseria gonorrhoeae* strains and the high incidence of asymptomatic infections that contributes to the spread of gonorrhea (3, 4). Gonococcal infections often have devastating sequelae in women including pelvic inflammatory disease, ectopic pregnancy, and infertility (5, 6). In addition, gonorrhea during pregnancy causes chorioamnionitis, which can be complicated further by a septic abortion, premature membrane rupture, and preterm delivery. Infants born to mothers with cervical gonorrhea have an increased risk of neonatal gonococcal conjunctivitis, which can lead to corneal scarring and blindness. In men, untreated urethritis may evolve into penile edema, urethral stricture and epididymitis (7, 8). Furthermore, gonorrhea facilitates the transmission and acquisition of HIV (9). For these reasons, it is critical to develop effective interventions against gonorrhea. Currently, strains resistant to the last effective option for empiric monotherapy—third-generation cephalosporins—are emerging and clinical treatment failures have been documented in several countries (4, 10). History indicates that the incremental development of antibiotic resistance in *N. gonorrhoeae* inevitably continues to challenge antibiotic therapy (4). The World

From the <sup>‡</sup>Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon; <sup>§</sup>Proteomics Facility, Fred Hutchinson Cancer Research Center, Seattle, Washington; <sup>¶</sup>*Neisseria* Reference Laboratory, Department of Global Health, University of Washington, Seattle, Washington; <sup>||</sup>Departments of Medicine and Global Health, University of Washington, Seattle, Washington; <sup>\*\*</sup>Department of Microbiology and Immunology, F. Edward Herbert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland; <sup>‡‡</sup>WHO Collaborating Centre for Gonorrhoea and other Sexually Transmitted Infections, National Reference Laboratory for Pathogenic *Neisseria*, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden

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Health Organization recently recognized the escalating problem of the spread of antimicrobial resistance in *N. gonorrhoeae* and highlighted the importance of novel approaches to identify alternative strategies for the treatment and prevention of *N. gonorrhoeae* infections, including a gonorrhoea vaccine (11).

Pioneered by Rappuoli and colleagues, reverse vaccinology—which initially included genome and later proteome mining—has proven to be very successful in the discovery of vaccine candidates against many pathogenic bacteria (12–15). In particular, these methodologies paved the way for the newly developed group B meningococcal vaccine, which was a formidable effort for many years. Among the 28 antigens that were discovered and that elicited bactericidal antibodies against group B meningococci *in vitro* were the Neisserial heparin-binding antigen (NHBA), factor H-binding protein (fHbp), and the Neisserial adhesin A (NadA). These three proteins are formulated as part of the Bexsero meningococcal group B vaccine approved by the European commission in 2013 and the United States in February of 2015 (12, 15, 16). Unfortunately, *N. gonorrhoeae* homologs of these proteins are not suitable vaccine targets (17). Further, in contrast to meningococcal vaccines, progress on gonococcal vaccines has been hampered primarily by the absence of a vaccine-targetable surface capsule, exceptional variability of several surface antigens, a poor understanding of protective responses, and until relatively recently, the lack of a small laboratory animal model to systematically test potential vaccine candidates (16). However, recent potential breakthroughs—such as the availability of transgenic mice to alleviate some host restrictions, new insights into immunosuppression mechanisms used by *N. gonorrhoeae*, and growing evidence that induction of Th1 response may be critical for vaccine efficacy—justify re-visiting gonorrhoea vaccine development and initiating a significant focus in this area (16, 18–20).

Currently, ~12 potential gonorrhoea vaccine antigens are being pursued (16). However, this is a very limited repertoire considering that, during the development of the Bexsero vaccine, out of nearly 600 candidates selected by *in silico* analysis, 350 recombinant meningococcal proteins were successfully expressed in *Escherichia coli* and evaluated for their surface exposure and ability to induce bactericidal antibodies (12). Thus, a comprehensive antigen discovery program would be exceedingly valuable toward making a gonorrhoea vaccine a reality. We are approaching this goal by applying proteomics-driven reverse vaccinology. In our previous study, a quantitative proteome analysis of cell envelopes and naturally released membrane vesicles derived from well-recognized *N. gonorrhoeae* laboratory strains FA1090, F62, MS11, and 1291 revealed a myriad of novel proteins, including 21 predicted outer membrane proteins (21). To extend these studies, herein we report high-throughput profiling of the *N. gonorrhoeae* cell envelope to identify ubiquitously and differentially expressed proteins in response to environmental cues

relevant to infection including different oxygen tensions (aerobic, anaerobic), iron deprivation, and the presence of normal human serum. In addition, a subset of five identified proteins was evaluated for the capacity to induce antibodies that recognize a collection of diverse clinical isolates and have bactericidal activity against serum resistant and serum sensitive *N. gonorrhoeae* strains.

#### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions**—The following *N. gonorrhoeae* strains were used in this study: FA1090 (22), MS11 (23), 1291 (24), F62 (25), FA19 (26), the clinical isolates LGB1, LG14, LG20, and LG26, which were collected from two public health clinics in Baltimore from 1991 to 1994 and differ in *porB* variable region type and pulsed gel electrophoresis patterns (21, 27), 13 isolates from patients attending the Public Health-Seattle & King County Sexually Transmitted Disease clinic from 2011 to 2013 (supplemental Table S1), and the WHO 2015 reference strains (10, 28, 29).

*N. gonorrhoeae* isolates were cultured from frozen stocks stored at  $-80^{\circ}\text{C}$  onto gonococcal base agar solid medium (GCB<sup>1</sup>, Difco, Sparks, MD) containing Kellogg's supplements I (1:100) and II (1:1000) (30). After incubation for 18–20 h at  $37^{\circ}\text{C}$  in a humid, 5%  $\text{CO}_2$  atmosphere, transparent colonies with piliated or nonpiliated colony morphologies were sub-cultured onto GCB plates. Piliated *N. gonorrhoeae* variants were used for transformation, whereas nonpiliated bacteria were used in all other experiments. Following ~18 h of incubation as described above, bacteria were harvested from the solid media using a polyester-tipped applicator (Puritan, Guilford, ME), and suspended to a final  $\text{OD}_{600}$  of 0.1 in gonococcal base liquid (GCBL) medium containing Kellogg's supplements I and II and sodium bicarbonate (at a final concentration of 0.042%) (30, 31). Subsequently, gonococci were propagated as stated in the text: in GCBL at  $37^{\circ}\text{C}$  or aerobically on GCB; GCB with Kellogg's supplement I and with deferroxamine mesylate salt (Desferral, Sigma, St. Louis, MO) at  $5\ \mu\text{M}$  final concentration [iron limited conditions (32)]; GCB with the addition of 7.5% normal human serum [NHS (33)]; and anaerobically on GCB with 1.2 mM nitrite as a terminal electron acceptor (34).

*E. coli* NEB5 $\alpha$  and BL21(DE3) were used for genetic manipulations and as a host for heterologous protein expression, respectively. The *E. coli* strains were grown in Luria-Bertani (LB) medium (Difco) or maintained on LB agar plates at  $37^{\circ}\text{C}$  supplemented with appropriate antibiotics. Antibiotics were used at the following concentrations: *N. gonorrhoeae* kanamycin (40  $\mu\text{g}/\text{ml}$ ) and erythromycin (0.5  $\mu\text{g}/\text{ml}$ ); *E. coli* kanamycin (50  $\mu\text{g}/\text{ml}$ ).

**Isolation of Cell Envelopes**—For proteomic analysis of the *N. gonorrhoeae* cell envelope composition, *N. gonorrhoeae* FA1090 was first cultured on GCB, bacteria were collected from plates, suspended in GCBL to an  $\text{OD}_{600}$  of 0.1, and 100  $\mu\text{l}$  aliquots were spread on solid media for simultaneous growth under aerobic (standard GCB, iron limited, and in the presence of human serum) and anaerobic (in the presence of nitrite) conditions, as described above. Bacteria were harvested when the colonies reached approximately similar size (after 18 and 36 h for aerobic and anaerobic conditions, respectively), and

<sup>1</sup> The abbreviations used are: GCB, gonococcal base agar solid medium; C, cytoplasmic; CE, cell envelopes; CFU, colony-forming units; COG, Clusters of Orthologous Groups; GCBL, gonococcal base liquid medium; IPTG, isopropyl- $\beta$ -D-galactopyranoside; iTRAQ, isobaric tagging for relative and absolute quantification; MVs, membrane vesicles; NHS, normal human serum; 2D-LC, two-dimensional liquid chromatography; SCX, strong cation exchange; SGC, standard growth conditions; SS, soluble fractions of supernatants.

the cell envelope fractions were isolated as described previously (21, 35). Protein concentrations were measured using 2D Quant Kit (GE Healthcare, Piscataway, NJ).

**Two-dimensional Liquid Chromatography and Mass Spectrometry (2D LC-MS/MS)**—After isolation, the cell envelope-associated proteins were precipitated, trypsinized and labeled with iTRAQ reagents (AB Sciex, Waltham, MA) according to the procedures reported by Zielke *et al.* (21, 35). The following iTRAQ tags were used to label peptides in the cell envelope fractions derived from *N. gonorrhoeae* cultured on GCB under four growth conditions: 114 for standard aerobic; 115 for growth in the presence of 7.5% normal human serum; 116 for iron-limited; and 117 for anaerobic. iTRAQ-labeled peptides were separated using strong cation exchange (SCX) chromatography (21, 35). Desalted SCX fractions were analyzed by LC/ESI MS/MS with a ThermoScientific Easy-nLC II (Thermo Scientific) nano HPLC coupled to a hybrid Orbitrap Elite ETD (Thermo Scientific) mass spectrometer using an instrument configuration as described in (36). In-line desalting was accomplished using a reversed-phase trap column (100  $\mu\text{m} \times 20\text{ mm}$ ) packed with Magic C<sub>18</sub>AQ (5- $\mu\text{m}$ , 200 Å resin; Michrom Bioresources, Auburn, CA), followed by peptide separations on a reversed-phase column (75  $\mu\text{m} \times 250\text{ mm}$ ) packed with Magic C<sub>18</sub>AQ (5- $\mu\text{m}$ , 100 Å resin; Michrom Bioresources) directly mounted on the electrospray ion source. A 90-min gradient from 7% to 35% acetonitrile in 0.1% formic acid at a flow rate of 400 nL/min was used for chromatographic separations. The heated capillary temperature was set to 300 °C and a spray voltage of 2250 V was applied to the electrospray tip. The Orbitrap Elite instrument was operated in the data-dependent mode, switching automatically between MS survey scans in the Orbitrap (AGC target value 1,000,000; resolution 60,000; and injection time 250 msec) and MS/MS scans in the OrbiTrap (AGC target value of 50,000; 15,000 resolution; and injection time 250 msec). The 15 most intense ions from the survey scan were selected for fragmentation by higher energy collisionally activated dissociation (HCD) with normalized collision energy of 40%. Selected ions were dynamically excluded for 45 s with a list size of 500 and exclusion mass-by-mass width  $\pm 0.5$ .

**Proteomic Data Analysis**—Data analysis was performed using Proteome Discoverer 1.4 (Thermo Scientific). The data were searched against a SwissProt *N. gonorrhoeae* FA1090 database with 1963 protein entries (downloaded on January, 17, 2012) that included common contaminants [the common Repository of Adventitious Proteins (cRAP)]. Trypsin was set as the enzyme with maximum missed cleavages set to 2. The precursor ion tolerance and the fragment ion tolerance were set to 10 ppm and 0.8 Da, respectively. Variable modifications included iTRAQ4Plex (+144.102 Da) on any N-Terminus, oxidation on methionine (+15.995 Da), methyl methanethiosulfonate on cysteine (+46.988 Da), and iTRAQ4Plex on lysine (+144.102 Da). Data were searched using Sequest HT. All search results were run through Percolator for scoring. Quantification was performed using the iTRAQ 4plex method built into Proteome Discoverer. The mass spectrometry data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository ProteomeXchange with the data set identifier PXD001944. Additionally, **supplemental Table S2** lists all identified proteins with their accession number, a brief description, calculated score, protein coverage, number of unique and total peptides, iTRAQ ratios, molecular mass, and calculated pI. All statistical analyses were performed as we described previously (21, 35, 37). Briefly, only proteins identified with 1% false discovery rate (FDR) based on at least one unique peptide with  $\geq 95\%$  confidence were recorded. The default bias-correction was used and all quantitative variables were analyzed by the Proteome Discoverer 1.4. Subsequently, average ratios and standard deviations were calculated for proteins identified in three independent experiments. Pro-

teins were considered ubiquitously expressed if the iTRAQ ratios were between 0.5 and 2, and were designated as differentially expressed when the values were below 0.5 or above 2 with the corresponding *p* values  $< 0.05$ . The relative protein abundance heat maps were created using MultiExperiment Viewer (version 4.9) software (<http://www.tm4.org/mev.html>) (38).

**Bioinformatic Analysis**—Subcellular localization of identified proteins was assessed as described previously (21, 35, 37) using PSORTb 3.0.2 (39), SOSUIGramN (40), and CELLO 2.5 (41), and majority-votes strategy was used for protein localization assignment. In cases where these three software systems predicted different subcellular localizations for a specific protein, the protein was assigned to a group of “unknown subcellular localization.” The predicted amino acid sequences of identified proteins were analyzed for the presence of a signal peptide using the SignalP v.4.1 server (42). Phylogenetic classification of identified proteins to the clusters of orthologous groups (COGs) functional categories was achieved using COGNitor.

**Subcellular Fractionation Procedures**—Nonpilated, transparent *N. gonorrhoeae* colonies of wild type strain FA1090 were harvested from solid media, suspended in 500 ml of GCBL to an OD<sub>600</sub> of 0.1 and cultured at 37 °C with aeration (220 rpm) to OD<sub>600</sub> of 0.6–0.8. Cells were separated from culture supernatants by centrifugation (20 min, 6000  $\times g$ ), and the cell envelopes were purified from whole-cell lysates (21, 35). The naturally secreted membrane vesicles (MVs) were harvested from the supernatants as described previously (21). The isolated soluble supernatant fractions (SS; containing secreted proteins and proteins originating from cell lysis) were stored at  $-20\text{ }^\circ\text{C}$  until used, whereas insoluble material containing MVs was reconstituted in PBS containing 0.4% SDS. The total protein amount in each isolated subproteome (cytoplasmic, cell envelopes, MVs, and SS) was measured using a DC Protein Assay Kit (BioRad, Hercules, CA).

**Conservation of Candidate Vaccine Antigens in a Panel of *N. gonorrhoeae* Isolates**—The conservation of BamA, LptD, TamA, NGO2054, and NGO2139 was assessed at both nucleotide and predicted amino acid levels between FA1090, NCCP11945 (<http://www.ncbi.nlm.nih.gov/>), the draft genome sequences of 14 different GC strains (downloaded from <http://www.broadinstitute.org/>), and the 14 WHO 2015 reference strains (10, 28, 29) using ClustalOmega (<http://www.ebi.ac.uk/>).

**Genetic Manipulations**—All genetic manipulations used to engineer different *N. gonorrhoeae* mutants utilized in this study are described in Supplemental Experimental Procedures.

**Cloning and Purification of Recombinant Protein Variants**—The genes *lptD*, *bamA*, *tamA*, *ngo2054*, *ngo2139*, *ngo0994* (*laz*), and *ngo0277* (*bamD*) were individually amplified using appropriate primers (**supplemental Table S3**). The subsequent PCR products were digested with NcoI and EcoRI or NcoI and HindIII, as indicated, and ligated into similarly cut pET28a(+) to create C-terminal-6 $\times$ His-tagged fusions. All constructed plasmids were moved to *E. coli* BL21(DE3).

For the overproduction of individual proteins, overnight cultures of *E. coli* BL21(DE3) carrying pET-LptD, pET-BamA, pET-TamA, pET-NGO2054, pET-NGO2139, pET-Laz or pET-BamD were used to inoculate 1 L of LB supplemented with kanamycin. Protein overproduction was induced by the addition of 1 mM IPTG when the cultures reached an OD<sub>600</sub> of about 0.5. After 3 h of growth at 37 °C, bacterial cells were pelleted by centrifugation and cell pellets were stored at  $-80\text{ }^\circ\text{C}$  until used. Recombinant NGO2054, NGO2139, Laz and BamD proteins were purified under native conditions whereas LptD, BamA and TamA were purified under denaturing conditions. All proteins were purified using the NGC Scout Chromatography system (Bio-Rad). For native conditions, bacterial cells were resuspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole) and

lysed by five passages through a French pressure cell at 12,000 p.s.i. Cell lysates were clarified by centrifugation and loaded onto Bio-Scale Mini Profinity IMAC cartridges (Bio-Rad). Loosely bound proteins were removed with 10 column volumes of wash buffer (20 mM Tris pH 8.0, 500 mM NaCl, 40 mM imidazole) and proteins were eluted with 40–250 mM imidazole gradient.

For proteins purified under denaturing conditions, cell pellets were reconstituted in denaturing lysis buffer (6 M urea, 300 mM KCl, 50 mM  $\text{KH}_2\text{PO}_4$ , 5 mM imidazole, pH 8) and lysed as described above. Lysates were cleared from cell debris by centrifugation at  $6000 \times g$  for 20 min at 4 °C and applied to Bio-Scale Mini Profinity IMAC cartridges (Bio-Rad) equilibrated with denaturing lysis buffer. Columns were washed with ten column volumes of denaturing wash buffer (6 M urea, 300 mM KCl, 50 mM  $\text{KH}_2\text{PO}_4$ , 10 mM imidazole, pH 8) and finally proteins were eluted with five column volumes of denaturing elution buffer (6 M urea, 300 mM KCl, 50 mM  $\text{KH}_2\text{PO}_4$ , 250 mM imidazole, pH 8).

Fractions containing the purified recombinant protein of interest were dialyzed against 20 mM Tris pH 8.0, 500 mM NaCl, and 10% glycerol for 24 h with three consecutive changes of the buffer. Proteins were stored at –80 °C. The *N. gonorrhoeae* Obg protein, Obg<sub>GC</sub>, used in this study as a cytoplasmic protein marker, was purified as described (43).

**Antisera Preparations**—Rabbit polyclonal antisera against the individual proteins were prepared by Pacific Immunology (Ramona, CA) using about 2 mg of the purified recombinant versions of BamA, LptD, TamA, NGO2054, NGO2139, Laz and BamD and a 13-week antibody production protocol was approved by IACUC Animal Protocol #1, in a certified animal facility (USDA 93-R-283), as well as the National Institute of Health Animal Welfare Assurance Program (#A4182–01). The polyclonal anti-Obg<sub>GC</sub> antiserum was obtained previously (43).

**Bactericidal Assays**—Non-piliated colonies of *N. gonorrhoeae* FA1090 and MS11 were inoculated into 5 ml of GCBL broth to an OD<sub>600</sub> of 0.1. Cultures were incubated with aeration at 37 °C until the mid-logarithmic phase of growth (OD<sub>600</sub> ≈ 0.6) was reached. The immune and respective preimmune sera were heat inactivated by incubation for 30 min at 56 °C and diluted in GCBL medium. Bacterial cells [ $2 \times 10^4$  colony forming units (CFUs/ml)] in 40 μl were mixed with diluted antisera or preimmune sera and incubated for 15 min at 37 °C. Following incubation, 10 μl of NHS (Cellgro, Manassas, VA) were added to each tube. As a control 10 μl of heat-inactivated NHS (HI-NHS) was used. Samples were incubated for additional 30 min at 37 °C. Finally, 50 μl of each suspension were spread onto GCB plates and the CFUs were determined after 18–20 h incubation. Controls included bacteria incubated with: test sera with HI-NHS, pre-immune sera with NHS, and pre-immune sera with HI-NHS. The average percent killing was determined from at least four independent experiments and was calculated as the number of CFUs in samples incubated with antigen specific antisera and NHS to the number of CFUs recovered from samples treated with rabbit postimmune sera and HI-NHS (44). *N. gonorrhoeae* viability was not affected in any of the control samples.

**Dot Blotting**—Non-piliated colonies of *N. gonorrhoeae* FA1090 were suspended to an OD<sub>600</sub> of 0.1 in GCBL pre-warmed to 37 °C and incubated for 3 h with shaking at 220 rpm at 37 °C. Bacterial cells were harvested and suspended in GCBL to an OD<sub>600</sub> of 2.0. To obtain whole-cell lysates, bacteria were incubated at 100 °C for 5 min followed by sonication (30 s, amplitude 50). Suspensions (5 μl) of intact and lysed cells were spotted onto a nitrocellulose membrane. The membrane was allowed to dry for 30 min at room temperature, blocked, and probed with respective polyclonal antibodies, as described below.

**Protease Treatment of Intact *N. gonorrhoeae* Cells**—The surface accessibility studies were performed using modified protocols de-

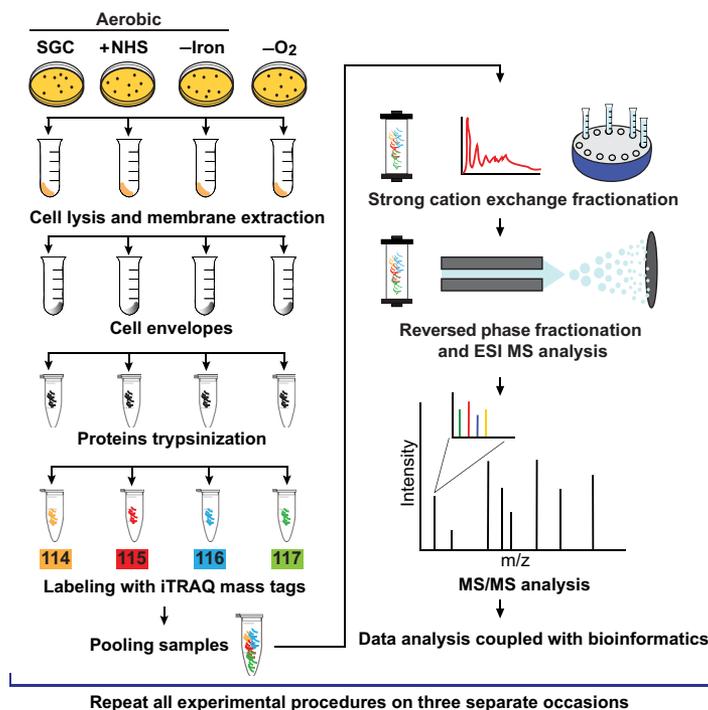
scribed previously (45, 46). After 3 h of growth in GCBL, *N. gonorrhoeae* FA1090 cells were harvested by centrifugation for 10 min at  $2000 \times g$  and 4 °C. The pellet was suspended in PBS (pH 8.0) to an OD<sub>600</sub> of 2.5 and 500 μl of this solution were incubated with 0, 40, or 80 μg/ml of Trypsin-Ultra™ [L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin, NEB, Ipswich, MA] for 1 h at 37 °C. The reactions were stopped by the addition of 10 μl of 50 mM phenylmethanesulfonyl fluoride (PMSF) and cells were washed with PBS. Finally, the *N. gonorrhoeae* cells were suspended in PBS and the OD<sub>600</sub> of the suspension was measured. The cells were pelleted again, lysed in SDS sample buffer and subjected to SDS-PAGE and immunoblotting analysis, as outlined below.

**SDS-PAGE and Immunoblotting Analysis**—Whole-cell lysates were obtained from *N. gonorrhoeae* grown in GCBL with aeration and on GCB plates cultured under conditions specified in the text. Bacteria were harvested from liquid media either at different time points of growth or at desired OD<sub>600</sub> values, as indicated. Different *N. gonorrhoeae* clinical isolates were collected from GCB plates, followed by suspension in prewarmed GCBL, and measurement of the cell density at OD<sub>600</sub>. Fractions containing either cytoplasmic, cell envelope, MVs, or secreted proteins (the same amount of total protein loaded per well), or whole-cell lysates matched by equivalent OD<sub>600</sub> units, were prepared in SDS sample buffer in the presence of 50 mM dithiothreitol and separated in either 10–20% Criterion Tris-Tricine TGX (BioRad) or 4–20% Mini-PROTEAN TGX precast gels (Bio-Rad). The proteins were transferred onto 0.2 μm nitrocellulose membrane (Bio-Rad) using a Trans-blot Turbo (Bio-Rad). A solution of 5% milk in phosphate buffered saline pH 7.0 (PBS, Li-Core, Lincoln, NE) supplemented with 0.1% Tween 20 (PBST) was used for blocking. Following 1 h of incubation, polyclonal rabbit antisera against LptD (1:5,000), BamA (1:10,000), TamA (1:5,000), NGO2054 (1:10,000), NGO2139 (1:20,000), Obg<sub>GC</sub> [1:5,000, (43)], polyclonal anti-AniA antibodies [1:10,000;(21)], monoclonal mouse anti-MtrE antisera (1:10,000), monoclonal mouse anti-Ng-MIP antibodies (1:10,000; a gift of Mariagrazia Pizza, Novartis Vaccines, Italy), and polyclonal rabbit anti-TbpB antisera (1:1,000; a gift of Cynthia Cornelissen, Virginia Commonwealth University, Richmond) diluted in PBST as indicated in parenthesis were added to the membranes. Horseradish peroxidase conjugate of goat anti-rabbit IgG antiserum (BioRad) or goat anti-mouse IgG antibody (ThermoFisher Scientific), correspondingly, was utilized as the secondary antibody at a 1:10,000 dilution. The reactions were developed using Clarity Western ECL-Substrate (BioRad) and a Chemi-Doc™ MP System (BioRad) was used for Western blot imaging.

**Experimental Design and Statistical Rationale**—All studies to identify potential gonorrhoea vaccine antigens including iTRAQ LC-MS/MS experiments were performed on three separate occasions. Statistical analysis was performed as described above. All other experiments were conducted at least in biological triplicates and mean values with corresponding S.E. were presented.

## RESULTS AND DISCUSSION

**Experimental Strategy**—*N. gonorrhoeae* is an exclusive human pathogen that primarily infects the lower genitourinary tract. Ascending infections are also common in women, as are pharyngeal and rectal infections in both men and women. Conjunctival infections can also occur (5). During colonization within these microecological niches, *N. gonorrhoeae* encounters areas of different oxygen tension (aerobic and anaerobic sites of infection), iron deprivation and, in the event of disseminated infection, exposure to human serum (5, 47, 48). An effective vaccine with a broad spectrum of protection would preferentially consist of antigens that are not only highly con-



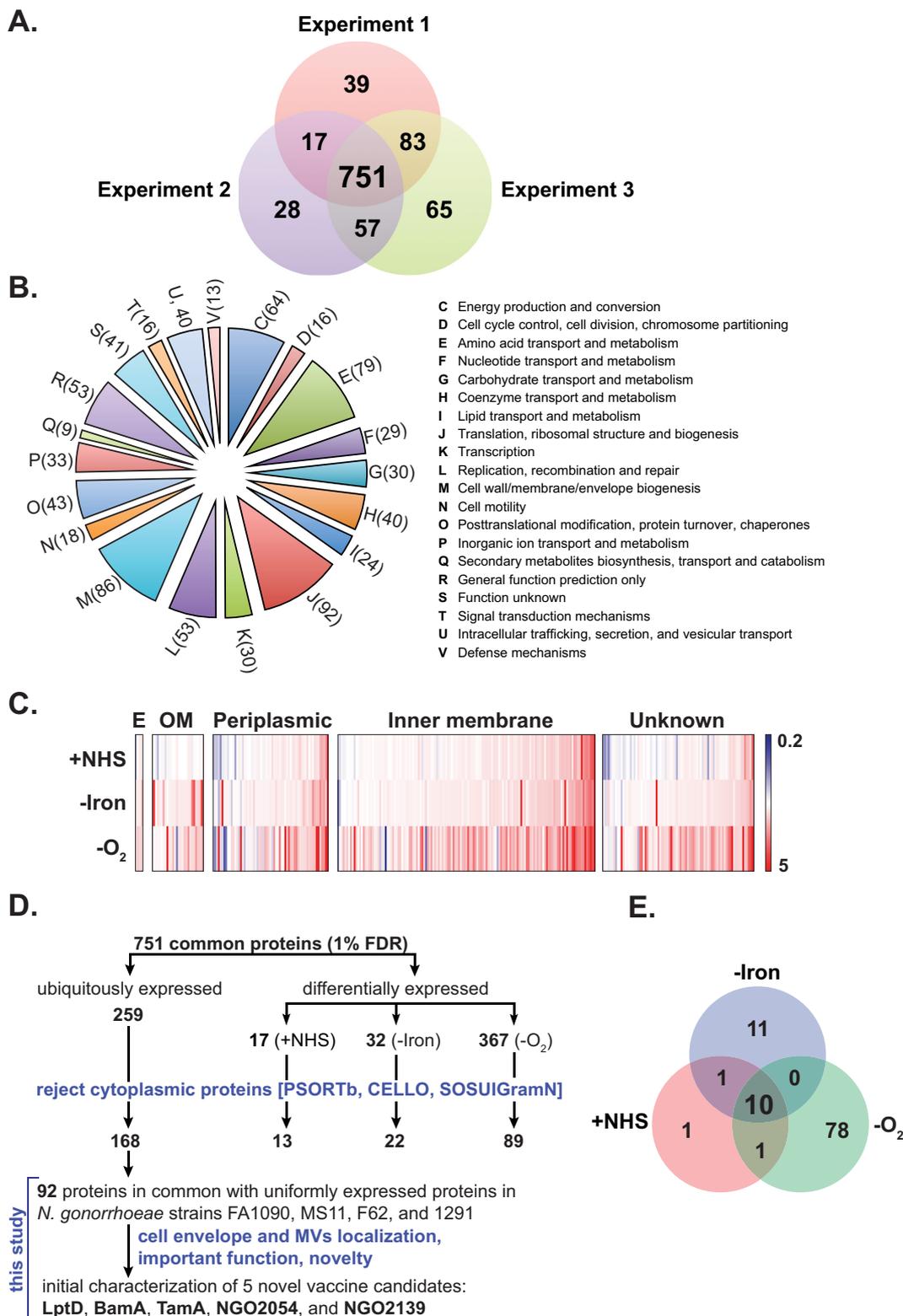
**FIG. 1. Experimental design of quantitative proteomic profiling of the *Neisseria gonorrhoeae* cell envelopes across different growth conditions.** *N. gonorrhoeae* strain FA1090 was cultured on gonococcal base agar solid medium (GCB) aerobically under standard growth conditions (SGC); on GCB with the addition of 7.5% normal human serum (+NHS); iron limited [GCB without ferric nitrate in Kellogg's Supplements and with deferoxamine mesylate salt at 5  $\mu\text{M}$  final concentration (-Iron)]; and anaerobically on GCB with nitrite as a terminal electron acceptor (-O<sub>2</sub>). Bacteria were harvested from solid media when the colonies reached approximately the same size. Following lysis, cell envelope proteins were enriched using a sodium carbonate extraction procedure and ultracentrifugation. The same amounts of proteins (100  $\mu\text{g}$ ) in each sample were subjected to trypsin digestion. Peptides in different samples were labeled with the following iTRAQ tags: 114 for SGC, 115 for NHS, 116 for -Iron, 117 for -O<sub>2</sub>. Following labeling, samples were pooled and the peptides were separated by strong cation exchange and reversed-phase chromatography. The Orbitrap Elite was used to collect mass spectra and proteins were identified and quantified using Proteome Discoverer. All experiments described above were repeated on three separate occasions.

served among different *N. gonorrhoeae* strains but are also utilized by the bacteria to persist at anatomically distinct sites within its sole human host. Accordingly, herein we performed proteomic studies to identify potential vaccine antigens that are ubiquitously expressed or induced by these environmental cues. The experimental strategy is outlined in Fig. 1. *N. gonorrhoeae* FA1090 was spread on GCB plates and maintained concurrently under standard growth conditions (SGC), GCB supplemented with 7.5% normal human serum (+NHS), iron-depleted GCB (-Iron), and anaerobically in the presence of nitrite as terminal electron acceptor (-O<sub>2</sub>). The plate-grown gonococci were collected when the colonies reached approximately similar size, lysed, and the membrane proteins were isolated using sodium carbonate extraction coupled with ultracentrifugation. This methodology enables enrichment of the cell envelope proteins and yields samples compatible with downstream proteomic applications, including iTRAQ labeling and MS/MS analysis (21, 35, 49, 50). Isolated proteins from bacteria cultured under individual growth conditions were subsequently digested with trypsin, and the acquired peptides were labeled with the following iTRAQ tags: SGC - 114; NHS - 115; -Iron - 116; and -O<sub>2</sub> - 117 (Fig. 1). The labeled

samples were pooled and the peptide mixtures were fractionated in two dimensions using off-line strong-cation exchange chromatography followed by reversed-phase chromatography performed in-line with the mass spectrometer. Finally, the MS/MS analysis was accomplished using an Orbitrap Elite mass spectrometer. These experimental procedures were repeated on three separate occasions and all raw data were deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository ProteomeXchange with the data set identifier PXD001944.

**Proteomic Data Analyses**—The total number of proteins identified based on at least two unique peptides filtered to a 1% FDR was 890, 853, and 956 in the first, second, and third independent biological experiment, respectively (Fig. 2A). Further analyses were applied solely to a group of 751 common proteins identified during four growth conditions in biological triplicate experiments.

**Protein Functional Categories**—Functional classification using COG clustering identified twenty different phylogenetic protein groups (Fig. 2B), with the largest subsets in category J (translation, ribosomal structure and biogenesis; 92 pro-



**FIG. 2. Analysis of identified proteins.** A, Venn diagram illustrating the distribution of proteins identified in independent triplicate proteomic experiments. A total of 890, 853, and 956 individual protein species were identified in Experiment 1, 2, and 3; respectively. Further analyses were applied to 751 common proteins identified in all experiments. B, A phylogenetic classification of common proteins was accomplished using the Clusters of Orthologous Groups of proteins (COGs). Letters and digits displayed on the pie chart represent individual COGs and numbers of proteins assigned to each phylogenetic group. C, Heat map illustrating the changes in *N. gonorrhoeae* proteome upon exposure to the environmental cues relevant to infection. Relative abundance of proteins identified during different growth conditions (NHS, -Iron, and

TABLE I  
Differentially expressed *Neisseria gonorrhoeae* cell envelope proteins in response to normal human serum

Accession	Name	Gene	Average ratio ± S.D. <sup>a</sup>	SP <sup>b</sup>
Periplasmic				
Q5F7X1 <sup>c</sup>	Putative uncharacterized protein	NGO1044	2.2 ± 1.74	N
Q5FAC5 <sup>c</sup>	Cytochrome c4	<i>cyc4</i> , NGO0101	2.5 ± 2.06	Y
Inner membrane				
Q5F4Z5 <sup>c</sup>	ATP synthase C chain	<i>atpE</i> , NGO2145	2.1 ± 2.25	N
Q5F9V4 <sup>c</sup>	Putative uncharacterized protein	NGO0284	2.8 ± 2.69	N
Q5F9N2 <sup>c</sup>	Putative HemY protein	NGO0361	2.9 ± 1.19	N
Q5F6Y2 <sup>c</sup>	Putative uncharacterized protein	NGO1411	3 ± 4.04	N
Q5F767	Putative paraquat-inducible protein A	<i>pqiA</i> , NGO1319	3.5 ± 1.77	N
Q5F6V9 <sup>c</sup>	Putative cadmium resistance protein	<i>cadD</i> , NGO1435	3.7 ± 2.29	N
Q5F6K9	Penicillin-binding protein 2	<i>penA</i> , NGO1542	5.3 ± 4.53	N
Q5FA49 <sup>c</sup>	Sec-independent protein translocase protein TatA	<i>tatA</i> , NGO0183	5.8 ± 7.8	N
Q5F6M1 <sup>c,d</sup>	Cell division protein FtsQ	<i>ftsQ</i> , NGO1530	2.8 ± 1.77	N
Unknown localization				
Q5F9C2 <sup>c</sup>	Putative uncharacterized protein	NGO0477	3.5 ± 3.65	N
Q5FAD1 <sup>c</sup>	Pilin assembly protein	<i>pilP</i> , NGO0095	5.1 ± 7.36	Y

<sup>a</sup> Average ratios and standard deviations were calculated for proteins identified in three independent experiments.

<sup>b</sup> Predictions of signal peptide cleavage site using SignalP (42).

<sup>c</sup> iTRAQ ratios were 0.5 < proteins < 2 in one of the biological triplicate experiments.

<sup>d</sup> The inner membrane localization of FtsQ was verified experimentally (103).

teins), category M (cell wall/membrane/envelope biogenesis; 86 proteins), and category E (amino acid transport and metabolism; 79 proteins). Furthermore, 52 proteins were assigned only a putative function (category R), whereas 41 proteins remained without an allocated biological role (category S; Fig. 2B).

**Assessment of Protein Expression**—The relative levels (iTRAQ ratios) of identified proteins were obtained by comparing *N. gonorrhoeae* proteome profiles under individual test conditions to the corresponding protein expression at SGC, as follows: +NHS/SGC (115/114), -Iron/SGC (116/114), and -O<sub>2</sub>/SGC (117/114). Similarly, to our previous studies (21, 37), a >2.0-fold cutoff threshold in the iTRAQ ratios was chosen as a criterion for differential protein abundance. Overall, our experiments revealed that *N. gonorrhoeae* responds to the environmental cues tested, particularly anaerobiosis, with dramatic changes in the cell envelope proteome (Fig. 2C). Expression of 17, 32, and 367 proteins in the presence of NHS, upon iron deprivation, and during anaerobic growth, respectively, was characterized as significantly different compared with SGC (Fig. 2D, Tables I-II and supplemental Tables

S4 and S6). Further, 259 proteins were ubiquitously expressed under all conditions tested (Fig. 2D, supplemental Table S5 and S7).

**Elimination of Cytoplasmic Proteins**—Combining bioinformatic prediction of protein localization with profiling of bacterial subproteomes (cell envelopes, naturally released MVs, surface-exposed proteins, and secreted proteins) is an important part of data analysis because enrichments are never completely free from cytosolic proteins despite thoroughly executed experimental procedures (21, 37, 51–53). In addition, even minute amounts (low femtomoles) of proteins present in a sample can be detected by current MS instruments (54). Some of the cytoplasmic proteins arise from cell lysis during culture or copurify with large protein complexes tethered to the cell envelope; others may be translocated to the cell surface by noncanonical secretory pathways (51, 52).

To eliminate cytoplasmic proteins, a step that follows proteomic mining in our decision tree of vaccine candidates evaluation (Fig. 2D), the subcellular localization of all identified proteins was analyzed using predictive web-based software including PSORTb 3.0.2 (55), SOSUIGramN (56), and CELLO

-O<sub>2</sub>) was compared with the levels of corresponding protein species found in SGC. The color scale covers from fivefold down-regulation (blue), through no change (white), to fivefold up-regulation (red). **D**, Decision tree to select gonorrhea vaccine candidates from the group of 751 common *N. gonorrhoeae* cell envelope-associated proteins identified with 1% FDR in three independent experiments via high-throughput proteomic mining. This group contains 259 uniformly expressed and 416 specifically induced proteins. Cytoplasmic proteins were eliminated using bioinformatics predictions and literature searches. Vaccine candidates were ultimately selected from 92 ubiquitously expressed proteins identified by comparing the 168 uniformly expressed cell envelope proteins of this study with the 305 previously identified cell envelope proteins isolated from FA1090, MS11, F62, and 1291 (21). Subsequently, five of these proteins that were localized to both cell envelopes and MVs fractions (21), and either had pivotal role(s) in other bacterial species (BamA, LptD, TamA) or unknown function (NGO2054 and NGO2139) were subjected to initial assessments as potential gonorrhea vaccine candidates. **E**, Analysis of anaerobic, iron, and NHS-responsive stimulons of *N. gonorrhoeae*. Differentially expressed proteins in response to anoxia, iron deprivation, and the presence of NHS were compared with identify the common anaerobic, iron-depleted, and NHS-responsive protein stimulon.

TABLE II  
Differentially expressed *Neisseria gonorrhoeae* cell envelope proteins in response to iron deprivation

Accession	Name	Gene	Average ratio $\pm$ S.D. <sup>a</sup>	SP <sup>b</sup>	Microarray (-/+ Fe) <sup>c</sup>	Microarray (-/+ Fe) <sup>d</sup>
<b>Outer membrane</b>						
Q5F526 <sup>e</sup>	Putative uncharacterized protein	NGO2111	2.3 $\pm$ 0.37	N	N/A	N/A
Q5F674 <sup>e</sup>	Putative outer membrane protein OmpU	NGO1688	2.6 $\pm$ 0.57	Y	N/A	3.67; 2.96; 3.27; 2.13
Q5F6Q4	Transferrin-binding protein A	<i>tbpA</i> , NGO1495	3.8 $\pm$ 2.56	Y	1.87	4.43; 4.45; 5.20; 4.88
Q5F950	Putative TonB-dependent receptor	<i>tdfG</i> , NGO0553	4.5 $\pm$ 3.9	Y	1.58	2.74; 1.88; 2.16
Q5F543	Ferric enterobactin receptor	<i>fetA</i> , NGO2093	7.6 $\pm$ 6.91	Y	1.73	50.9; 107.5; 39.9; 15.5 qRT-PCR
Q5F6Q3 <sup>f</sup>	Transferrin-binding protein B	<i>tbpB</i> , NGO1496	11.4 $\pm$ 4.27	N	1.51	7.45; 7.06; 12.44; 11.48
<b>Periplasmic</b>						
Q5F5F7 <sup>e</sup>	Putative matB-like adhesion	NGO1971	2.1 $\pm$ 1.25	Y	N/A	2.3
Q5F544 <sup>e</sup>	Ferric enterobactin periplasmic binding protein	<i>fetB</i> , NGO2092	2.8 $\pm$ 1.4	N	1.59	2.92; 3.40; 3.86; 4.00
Q5F7X1 <sup>e</sup>	Putative uncharacterized protein	NGO1044	2.9 $\pm$ 2.88	N	N/A	N/A
Q5FA17 <sup>e</sup>	ABC transporter, periplasmic binding protein, iron related	<i>fbpA</i> , NGO0217	5.2 $\pm$ 4.15	Y	1.58	2.01; 8.72
<b>Inner membrane</b>						
Q5F6S5 <sup>e</sup>	NAD(P) transhydrogenase subunit beta	<i>pntB</i> , NGO1472	2.1 $\pm$ 0.87	N	N/A	1.97
Q5F4Z5 <sup>e</sup>	Putative ATP synthase C chain	<i>atpE</i> , NGO2145	2.1 $\pm$ 2.35	N	N/A	1.88
Q5F6K9 <sup>e</sup>	Penicillin-binding protein 2	<i>penA</i> , NGO1542	2.2 $\pm$ 1.22	N	N/A	N/A
Q5F9N2 <sup>e</sup>	Putative HemY protein	NGO0361	2.5 $\pm$ 1.88	N	N/A	N/A
Q5F6Y2 <sup>e</sup>	Putative uncharacterized protein	NGO1411	2.9 $\pm$ 4.27	N	N/A	N/A
Q5F6V9 <sup>e</sup>	Putative cadmium resistance protein	<i>cadD</i> , NGO1435	2.9 $\pm$ 3.98	N	N/A	N/A
Q5F9V4 <sup>e</sup>	Putative uncharacterized protein	NGO0284	3.2 $\pm$ 3.68	N	N/A	N/A
Q5F767 <sup>e</sup>	Putative paraquat-inducible protein A	<i>pqiA</i> , NGO1319	3.3 $\pm$ 2.34	N	N/A	N/A
Q5FA49 <sup>e</sup>	Sec-independent protein translocase protein TatA	<i>tatA</i> , NGO0183	6 $\pm$ 7.33	N	N/A	N/A
Q5F711	Transport protein	<i>exbB</i> , NGO1378	7.7 $\pm$ 4.57	N	1.53	3.94; 3.27; 6.50; 6.31
<b>Unknown localization</b>						
Q5F9C2 <sup>e</sup>	Putative uncharacterized protein	NGO0477	2.3 $\pm$ 1.8	N	N/A	N/A
Q5FAD1 <sup>e</sup>	Pilin assembly protein	<i>pilP</i> , NGO0095	5 $\pm$ 6.85	Y	N/A	3.53

<sup>a</sup> Average ratios and standard deviations were calculated for proteins identified in three independent experiments.

<sup>b</sup> Predictions of signal peptide cleavage site using SignalP (42).

<sup>c</sup> Data derived from Ducey et al. (2005), (61).

<sup>d</sup> Data derived from Jackson et al. (2010), (57).

<sup>e</sup> iTRAQ ratios were 0.5 < proteins < 2 in one of the biological triplicate experiments.

<sup>f</sup> The outer membrane localization of TbpB was verified experimentally (104).

TABLE III  
Analysis of polymorphic sites in vaccine candidate proteins among 36 *Neisseria gonorrhoeae* isolates

Name/Gene	Length		Polymorphic sites	
	bp	AA	Nucleotides (%)	AA (%)
LptD	2403	801	18 (0.75)	8 (1)
BamA	2376	792	21 (0.88)	7 (0.88)
TamA	1905	635	14 (0.73)	5 (0.79)
NGO2054	270–273	90–91	8 (2.9)	4 (4.4)
NGO2139	864	288	2 (0.23)	0 (0)

2.5 (41). The primary reason for including these three systems and the majority-votes strategy for proteins assignment was that each of the programs employs different methods for prediction of protein localization.

In our analysis, proteins were allocated to a particular subcellular compartment when at least two of the software systems predicted the same localization. In cases where PSORTb, SOSUIGramN, and CELLO 2.5 assigned different subcellular locations for a particular protein, the protein was allocated to a group of proteins with “unknown subcellular localization”. This approach yielded assignment of 4 extracellular-, 26 outer membrane-, 53 periplasmic-, 116 inner membrane-, 481 cytoplasmic-, and 71 proteins with unknown localization (Fig. 2C, Tables I–II and supplemental Tables S4–S7).

**Differentially Expressed Proteins**—After excluding cytosolic proteins, a total of 13, 22, and 89 proteins showed differential expression during exposure of *N. gonorrhoeae* to NHS, iron deprivation, and anaerobic conditions, respectively (Fig. 2E).

**Analysis of the Common NHS, Iron-depleted, and Anaerobic Stimulon**—Comparison of data sets revealed 10 significantly stimulated proteins in all tested conditions compared with standard aerobic growth (Fig. 2E). The NHS, iron-responsive, and anaerobic stimulon includes F0F1-type ATP synthase C subunit (AtpE, NGO2145), paraquat inducible protein A (PqiA, NGO1319), a putative cadmium resistance protein (CadD, NGO1435), penicillin-binding protein 2 (PBP2, NGO1542), pilin assembly protein PilP (NGO0095), Sec-independent protein translocase protein TatA (NGO0183), a putative HemY protein (NGO0361), and putative uncharacterized proteins NGO0284, NGO0477, and NGO1411 (Tables I–III). None of these proteins were included in the previously described common iron, hydrogen peroxide, and anaerobic stimulon, which consisted of 14 genes and was constructed based on separate global transcriptome analyses (57–59). The poor correspondence between direct comparisons of different data sets has been noted before (59) and is related to bacterial culture methods (solid versus liquid media, different growth phases), sample preparation, sensitivity, detection limit, and cut-off settings of differential expression, as well as the occurrence of post-transcriptional regulatory mechanisms. An example of this latter challenge is exemplified by studies elucidating the manganese-dependent oxidative

stress resistance of *N. gonorrhoeae* where proteomic approaches identified a set of 96 differentially regulated proteins, whereas no changes in gene expression were detected by microarray analysis and qPCR (60).

**NHS Responsive Proteins**—In response to NHS, expression of a total of 13 cell envelope-associated proteins was significantly elevated within a range from 2.1- to 5.8-fold (Fig. 2E, Table I). In addition to the 10 members of the proteome-based stimulon (discussed above), expression of cytochrome c4 (NGO0101), cell division protein FtsQ (NGO1530), and the uncharacterized protein NGO1044 was considerably induced. The increased levels of cytochrome c4 and NGO1044 were also observed during anaerobic growth and upon iron limitation, respectively, whereas FtsQ was solely up-regulated by the presence of NHS (Fig. 2E, Tables I–II, supplemental Table S4).

**Effect of Iron Deprivation on the Cell Envelope Protein Profile**—With the application of a >2.0-fold cutoff for differential protein expression, six outer membrane proteins, four periplasmic proteins, 10 inner membrane proteins, and two proteins with unknown localization were designated as up-regulated during growth of *N. gonorrhoeae* under iron limiting conditions (Table II). Among these proteins, 12 were identified as part of the Fur regulon by an examination of the steady-state levels of mRNA in response to iron availability and by microarray (57, 61). Some of these proteins have been well-characterized, including iron acquisition transporters such as the transferrin receptor system composed of TbpA and TbpB, ferric enterobactin receptor FetA and periplasmic iron-binding transporter FetB, ferric binding protein A (FbpA), TonB-dependent transporter TdfG, and ExbB (62–66). The TbpA and TbpB proteins are being pursued as gonorrhea vaccine candidates because of their conservation within specific regions among different isolates, importance in *N. gonorrhoeae* pathophysiology, and abilities to elicit cross-reactive and bactericidal antibodies (48, 67).

Importantly, however, our proteomic approach revealed an additional 10 proteins with expression profiles that were positively regulated by low-iron conditions, including predicted outer membrane proteins, NGO2111 and NGO1688, both containing DUF560 domain of unknown function (Table II). These proteins were identified using iTRAQ-2D-LC-MS/MS as being ubiquitously present in the cell envelopes of *N. gonorrhoeae* strains FA1090, MS11, F62, and 1291 maintained under routine aerobic cultivation in GCBL (21).

**Differentially Expressed Proteins During Anaerobic Growth**—In our proteomic profiling, a total of 367 proteins (about 19% of the *N. gonorrhoeae* FA1090 genome) were differentially regulated during anaerobic growth (supplemental Table S4 and supplemental Table S6). Deep sequencing analysis performed on similarly cultured gonococci revealed differential regulation of 198 genes (59). This discrepancy suggests that expression of many of these proteins is regulated at the post-transcriptional level. After excluding cytoplasmic

proteins, 89 proteins (four outer membrane, 11 periplasmic, 47 inner membrane, and 27 with unknown localization) showed significantly altered quantities and eight of them were also identified using a RNA-seq approach (supplemental Table S4, (59)). Within this group only NGO0372 and NGO2012 were downregulated, with average ratios ( $\pm$  S.D.) of  $0.4 \pm 0.06$  and  $0.2 \pm 0.11$ , respectively. Not surprisingly, proteins that participate in the anaerobic denitrification pathway, including outer membrane proteins AniA (68) and lipid-modified azurin, Laz (69); inner membrane protein NorB (70); and different types of cytochromes, as well as proteins involved in cytochrome biogenesis (71–73), were the most highly up-regulated, with induction reaching 15-fold in comparison to SGC (supplemental Table S4). It is worth noting that AniA is currently being investigated as a gonorrhea vaccine candidate because of its pivotal function in bacterial survival under oxygen-limiting conditions in the presence of nitrite, expression *in vivo*, surface localization, conservation, and ability to induce functional antibodies that inactivate the AniA nitrite reductase activity (45, 74, 75). In addition to AniA, our proteomic profiling revealed induced expression of three outer membrane proteins including vaccine antigens NspA (NGO0203) and porin PilB [NGO1812, (16)], as well as TdfJ [NGO1205, (76)], which is a homolog of the *N. meningitidis* zinc uptake protein ZnuD (77). The presence of TdfJ was not required for survival of *N. gonorrhoeae* within cervical epithelial cells (76). Its expression, however, was significantly elevated in biofilms, where gonococci utilize the anaerobic respiration pathway (78), thus the function of TdfJ in *N. gonorrhoeae* pathophysiology remains to be further elucidated.

**Ubiquitously Expressed Proteins**—In comparison to SGC, the expression of 168 proteins (four extracellular, 18 outer membrane, 37 periplasmic, 67 inner membrane, and 42 with unknown localization) remained unaltered (iTRAQ ratios of  $0.5 < \text{proteins} < 2$ ) upon exposure of *N. gonorrhoeae* to NHS, iron depletion and oxygen deprivation (supplemental Table S5). In our previous proteomic analysis, 305 proteins were designated as ubiquitously present in the cell envelopes isolated from FA1090, MS11, F62, and 1291 (21). A comparison of these two data sets revealed 92 ubiquitously expressed proteins in common (Fig. 2D). This group contains known outer membrane residents such as Phospholipase A (PldA, NGO1492); two potential gonorrhea vaccine antigens: pilus-associated adhesin PilC and an outer membrane channel for pilus extrusion PilQ (16); Omp3; and MafA (NGO1067). Ubiquitous proteins also included homologs of LPS assembly protein LptD (OstA, ImpA, NGO1715); components of the  $\beta$ -barrel assembly machinery (BAM) including BamA and BamD (ComL, NGO0277); the Translocation and Assembly Module [TAM, (79)] consisting of TamA (NGO1956) and TamB (NGO1955); hypothetical proteins NGO1344 and NGO2121; and predicted lipoproteins NGO0834, NGO1985, NGO2054, and NGO2139 [supplemental Table S5 and (21)].

Among the newly identified cell envelope-associated proteins was a predicted outer membrane protein, NGO0952 [TdfH, (76)], comprised of conserved domains TIGR01785 and cl21487, which are characteristic of TonB-dependent heme/hemoglobin receptors/transporters and outer membrane channels, respectively. The function of TdfH remains unknown (76), but global transcriptome analysis revealed that its expression might be subjected to repression by the AraC transcriptional regulator MpeR (76, 80). A homolog of TdfH from *N. meningitidis*, designated as CbpA, has been identified as a receptor for calprotectin and thus plays a role in zinc acquisition (81). Moreover, the levels of efflux pumps MtrCDE and FarAB remained unaltered despite different growth conditions (supplemental Table S5). The MtrCDE efflux pump transports structurally diverse hydrophobic compounds outside the bacterial cell, whereas FarAB is required for resistance against antimicrobial long chained fatty acids (82–84). The surface exposed MtrE serves as an outer membrane channel for both efflux systems (84), and is being developed as a gonorrhea vaccine candidate (16).

**Selected Vaccine Candidates**—Our constructed antigen decision tree contains both uniformly expressed and specifically induced gonorrhea vaccine candidates (Fig. 2D). In this report, we subjected to initial assessments as potential vaccine targets five proteins: BamA, LptD, TamA, NGO2054, and NGO2139 (Fig. 2D). These candidates were uniformly expressed during different growth conditions (supplemental Table S5), displayed similar abundance in four *N. gonorrhoeae* strains, were localized to both cell envelopes and MVs fractions (21), and had either pivotal role(s) in other bacterial species (BamA, LptD, TamA) or unknown functions (NGO2054 and NGO2139).

The Bam complex, BamA–E in *E. coli*, folds and inserts beta-barrel proteins into the outer membrane [reviewed in (85)]. The Omp85 protein family member BamA is found in all Gram-negative bacteria (85, 86). Despite the overall functional conservation, BamA homologs recognize their protein substrates in a species-specific manner and display highly divergent sequences located within the surface-exposed loops (87–89). The finding that BamA of *Burkholderia pseudomallei* induced protective immunity in mice (90) highlights the potential of this protein as a vaccine target. We reasoned that BamA of *N. gonorrhoeae*, similar to other bacterial species, is an essential protein. To test this hypothesis, we engineered a strain of FA1090 in which a copy of *bamA* was placed under the control of the IPTG-inducible promoter and inserted within an intergenic chromosomal region between the *lctP* and *aspC* genes (91). Subsequently, the native *bamA* was replaced in-frame with the nonpolar kanamycin resistance cassette using homologous recombination. As expected, the resultant strain FA1090  $\Delta$ *bamA*/*P*<sub>lac</sub>::*bamA* grew robustly in the presence of IPTG, whereas without the inducer, the bacteria failed to grow on solid media and halted proliferation in liquid media within about 6 h from back dilution (data not shown and

supplemental Fig. S1, respectively), concomitant to depletion of BamA levels (Fig. 3).

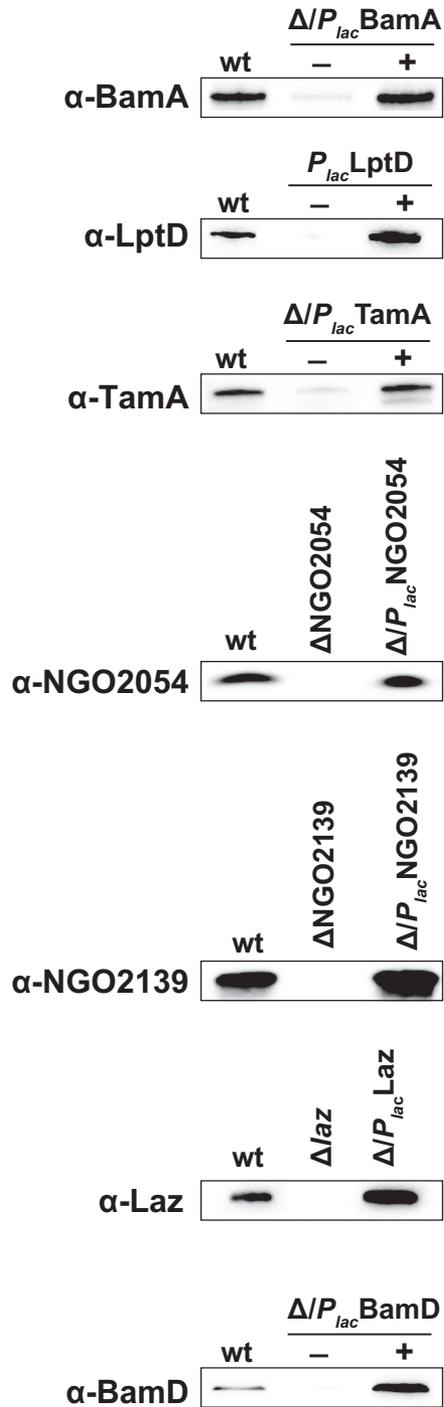
Our second vaccine candidate, LptD, is a low-abundance outer membrane  $\beta$ -barrel protein involved in transport of lipopolysaccharide/lipooligosaccharide into the outer leaflet of the outer membrane (92–94). We have demonstrated that, in contrast to *N. meningitidis* (93) and similar to *E. coli*, LptD is an essential protein for *N. gonorrhoeae* viability (21).

TAM is a newly discovered archetypal protein transport system comprised of TamA (an Omp85 family protein) and TamB. TAM promotes efficient secretion of autotransporters and is required for virulence in proteobacteria (79, 95). Functional characterization of the complex in *E. coli*, *Citrobacter rodentium*, and *Salmonella enterica* demonstrated that TamA is an integral outer membrane protein whereas TamB is a large protein that is encoded in an operon along with the gene for TamA and is located in the inner membrane. A similar genetic organization of a putative TAM complex exists in *N. gonorrhoeae* with TamA and TamB in an operon encoded by NGO1956 and NGO1955, respectively. We discovered both these proteins as being uniformly present in the cell envelopes of different gonococcal strains (21). In agreement with other organisms studied so far, the TAM complex is likely not essential for *N. gonorrhoeae* viability as conditional or in-frame deletion mutants of *tamA* or *tamB*, respectively, did not display noticeable growth defects [data not shown and (21)].

Finally, although bioinformatics predictions allocated NGO2054 and NGO2139 to a group of proteins with unknown subcellular location [supplemental Table S5 and (21)], both proteins contain predicted signal peptides with a lipoprotein box containing the indispensable cysteine residue, which suggest their outer membrane localization (96). The NGO2054 protein encompasses a LambdaBor motif (amino acid residues 3 to 36), a bacterial virulence determinant encoded by lysogenic coliphage lambda (97), whereas NGO2139 is annotated in KEGG as a D-methionine transport system substrate binding protein. The crystal structure of its homolog in *N. meningitidis* (GNA1946) was recently solved and surprisingly revealed an L-methionine bound into the protein cleft (98). Both proteins remain functionally uncharacterized but were found dispensable for *N. gonorrhoeae* viability during SGC in liquid and on solid media (21).

**Characterization of Antigen-specific Polyclonal Antisera**—Recombinant variants of each of the five vaccine candidates that contained a C-terminal 6 $\times$ His extension tag to enable purification from *E. coli* cell lysates using Ni-NTA affinity chromatography were generated. To provide additional controls for our experiments, recombinant BamD and Laz were also prepared.

The chimeric BamA, LptD, and TamA formed insoluble aggregates and were purified under denaturing conditions, whereas NGO2054, NGO2139, BamD and Laz were obtained under native conditions. Subsequently, all proteins were used to immunize rabbits and obtain polyclonal antisera. The reac-



**FIG. 3. Generation of polyclonal antibodies and immunoblotting analysis.** Recombinant versions of vaccine candidates BamA, LptD, TamA, NGO2054, and NGO2139, as well as proteins employed as controls in the experiments (Laz and BamD) were purified by Ni<sup>2+</sup> affinity chromatography and subsequently used to obtain polyclonal rabbit antisera. Wild type, isogenic conditional mutants, or knockouts, as well as complemented variants of FA1090 (as indicated above each immunoblot) were harvested from GCB. Whole-cell lysates matched by equivalent OD<sub>600</sub> units were separated in 4–20% Mini-PROTEAN TGX precast gels, transferred onto nitrocellulose membranes and probed with different polyclonal rabbit antibodies.

tivity of these newly generated antibodies was examined using wild type *N. gonorrhoeae* FA1090 and isogenic conditional or in-frame deletion mutants, as well as corresponding complemented strains. As expected, the individual antisera cross-reacted with whole-cell lysates originating from the wild type and complemented gonococci but not from the mutant strains (Fig. 3).

**Secondary Validation of iTRAQ Results**—The reliability of protein quantitation using iTRAQ on MALDI- and ESI-based MS instrumentation has been demonstrated for alterations of up to two orders of magnitude in a wide range of biological samples assessed in technical and biological replicates. However, it has been considered that low-signal species may have higher relative variability. In addition, underestimation of large changes when using an iTRAQ-based protein quantitation approach has also been reported [reviewed in (99)]. Therefore, to provide additional validation of protein expression profiles, the cell envelopes extracted from FA1090 cultured concurrently under SGC, in the presence of NHS, during iron deprivation, and anaerobically were subjected to SDS-PAGE and immunoblotting analyses (supplemental Fig. S2). SDS-PAGE coupled with colloidal Coomassie G-250 staining showed that, as expected (Fig. 2C, supplemental Table S4), the most altered was the overall cell envelope protein profile of *N. gonorrhoeae* maintained under anaerobic conditions (supplemental Fig. S2A). In agreement with the iTRAQ analyses (supplemental Table S5), similar levels of MtrE, BamA, LptD, TamA, NGO2054, NGO2139, BamD, and Ng-MIP were detected by immunoblotting during all tested conditions (supplemental Fig. S2B). As controls for differentially expressed proteins, we utilized antisera against well-recognized protein markers for anaerobic [AniA (21) and Laz (68)] and iron-limited [TbpB (48)] conditions. The iTRAQ quantitation indicated that in comparison to SGC, the relative levels of AniA and Laz increased on average  $9.5 \pm 2.81$  and  $2.9 \pm 0.59$  fold, respectively (supplemental Table S4), whereas there was  $11.4 \pm 4.27$  fold increase in TbpB abundance during iron limitation (Table II). Corroborating these findings, as shown in supplemental Fig. S2B, increased amounts of AniA and Laz, as well as TbpB were detected in the cell envelope fractions of gonococci grown anaerobically and during iron deprivation, correspondingly.

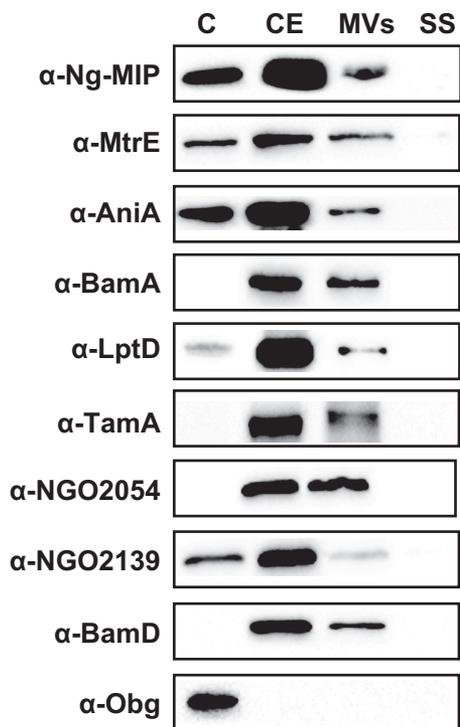
Additionally, to examine the expression of our vaccine candidates throughout *N. gonorrhoeae* growth, wild type FA1090 was maintained under routine aerobic cultivation in GCBL. Bacterial proliferation was monitored by measurements of cell density at OD<sub>600</sub> within 6 h of the experiment (supplemental Fig. S3A). Every hour samples were withdrawn and the whole cell lysates were probed with anti-BamA, anti-LptD, anti-TamA, anti-NGO2054, and anti-NGO2139 antisera. The representative immunoblots (supplemental Fig. S3B) showed that the vaccine candidates were constitutively expressed throughout the duration of the experiment.

Together, these analyses further supported iTRAQ-based proteome relative quantitation and demonstrated that the selected candidates are ubiquitously expressed during exposure of *N. gonorrhoeae* to different environmental cues as well as throughout different growth phases.

**Assessment of Vaccine Candidates' Localization and Surface Exposure**—Bioinformatic predictions of proteins' subcellular locations are invaluable tools for creating a “decision tree” to evaluate vaccine candidates. Experimental verification, however, is essential for final subcellular protein assignment. The subcellular localization of AniA and Ng-MIP are two great examples of discrepancies between *in silico* predictions and experimental evidence. According to the predictive bioinformatic tools (SOSUIGramN, PSORTb, and CELLO) and the majority-votes strategy, both proteins were assigned to the periplasmic compartment (supplemental Tables S3 and S4, respectively). In contrast, Shewell, L.K. *et al.* (45) demonstrated the outer membrane localization and surface-exposure of AniA by immune-S.E.M. and trypsin-accessibility studies. Likewise, subcellular fractionation of *N. gonorrhoeae* cells coupled with immunoblotting and flow-cytometry experiments revealed the surface-localization of Ng-MIP (100).

Therefore, the subcellular localization and surface exposure of BamA, LptD, TamA, NGO2054, and NGO2139 were investigated. In these experiments, controls included protein markers for surface-exposed (Ng-MIP, MtrE, and AniA), periplasmic side of the outer membrane [BamD, (85)] and cytoplasmic [Obg<sub>GC</sub> (43)] localization, respectively. The *N. gonorrhoeae* FA1090 cells were harvested at mid-logarithmic phase of growth in GCBL, lysed and the whole cell lysate was subjected to a carbonate extraction method to enrich for outer membrane proteins in the cell envelope fraction (21). Separation of MVs from soluble proteins was achieved by high-speed ultracentrifugation of culture supernatant (21). The same total amounts of purified subproteomes were loaded onto SDS-PAGE followed by immunoblotting analyses with antisera against individual proteins. As expected, the Obg<sub>GC</sub> was found exclusively in the cytosolic protein fraction (Fig. 4). None of the proteins were detected in the soluble fraction of the supernatant. Ng-MIP, MtrE, AniA, BamA, LptD, TamA, NGO2054, NGO2139, and BamD were found predominantly in the cell envelopes. In addition, these proteins were also identified in MVs and some of them were detected in the cytosol, which suggests that during the exponential phase they were still tightly associated with the ribosomes.

Subsequently, to investigate the surface accessibility of selected vaccine candidates, dot blotting experiments and protease accessibility studies were conducted (Fig. 5A). In the first approach, intact or lysed *N. gonorrhoeae* FA1090 cells, cultured as described above, were spotted on the nitrocellulose membrane and probed with primary antisera against individual vaccine candidates as well as proteins utilized as controls. The antibodies against BamA, LptD, TamA, NGO2054, and NGO2139 cross-reacted with intact gono-

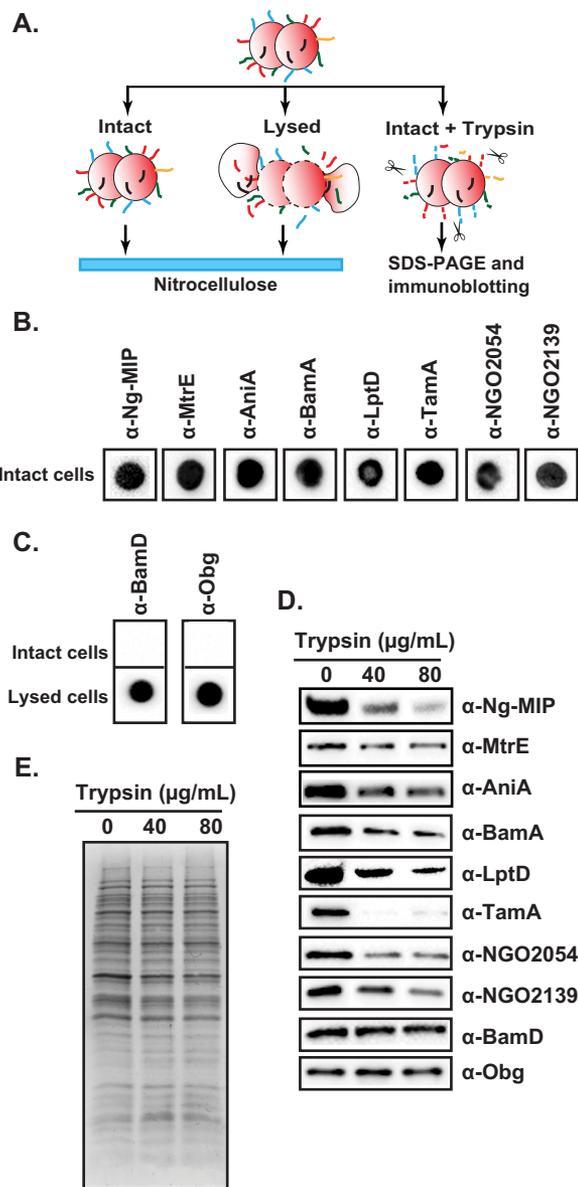


**FIG. 4. Subcellular localization of selected vaccine candidates.** Equal amounts of subproteome fractions (C-cytoplasmic, CE-cell envelopes, MVs-naturally released membrane vesicles, SS-soluble fractions of supernatants) derived from FA1090 cultured under standard growth conditions in GCBL were separated in 4–20% gradient gels. The proteins were transferred to nitrocellulose and probed with individual polyclonal rabbit antibodies against tested proteins, as indicated on the left. Controls included protein markers for the periplasmic face of the outer membrane (BamD) and cytoplasm ( $Obg_{GC}$ ) protein.

cocci similarly to anti-Ng-MIP, anti-MtrE, and anti-AniA antisera (Fig. 5B). In contrast, BamD and  $Obg_{GC}$  were not detected unless cell lysates were applied to the nitrocellulose and probed with the respective antibodies (Fig. 5C).

In the second approach, intact *N. gonorrhoeae* cells were treated with increasing amounts of trypsin. Reactions were stopped with PMSF, cells were lysed by addition of SDS loading buffer and probed with appropriate antibodies. As anticipated, these experiments revealed that the surface exposed proteins Ng-MIP, MtrE, and AniA were susceptible to digestion by trypsin as their amounts significantly decreased upon incubation with increasing concentrations of protease (Fig. 5D). Similar results were obtained by probing samples with antisera against vaccine candidates BamA, LptD, TamA, NGO2054, and NGO2139. The vast majority of other proteins remained unaltered as evidenced by comparison of protein profiles from trypsin-treated and untreated samples by either immunoblotting analyses of BamD and  $Obg_{GC}$  or by staining using colloidal Coomassie G-250 (Fig. 5D and 5E, respectively), which demonstrated the intactness of the cells.

Cumulatively, these data showed that the five vaccine candidates are surface-localized proteins and supported our ear-



**FIG. 5. Assessment of surface exposure of candidate antigens BamA, LptD, TamA, NGO2054, and NGO2139.** A, Experimental outline of dot blotting of either intact or lysed *N. gonorrhoeae* FA1090 cells and protease accessibility studies using intact cells. B, Intact cells of FA1090 were spotted on nitrocellulose and probed with different polyclonal antibodies, as shown above individual dot blots. C, Intact and lysed cells were used to detect BamD and  $Obg$ , which were utilized as periplasmic and cytoplasmic protein markers, respectively. D, Intact FA1090 cells were incubated with increasing concentrations of trypsin (as indicated), lysed, separated in 4–20% Tris-glycine precast gels and probed with individual antisera. E, Intactness of the cells during trypsin treatment was verified by separation of total-cell lysates by SDS-PAGE and visualization of protein profiles with colloidal Coomassie G-250 staining.

lier observation (21) that they are also constituents of naturally released MVs.

*Complement-mediated Antibody-dependent Serum Bactericidal Activity*—The ability of recombinant antigens to elicit

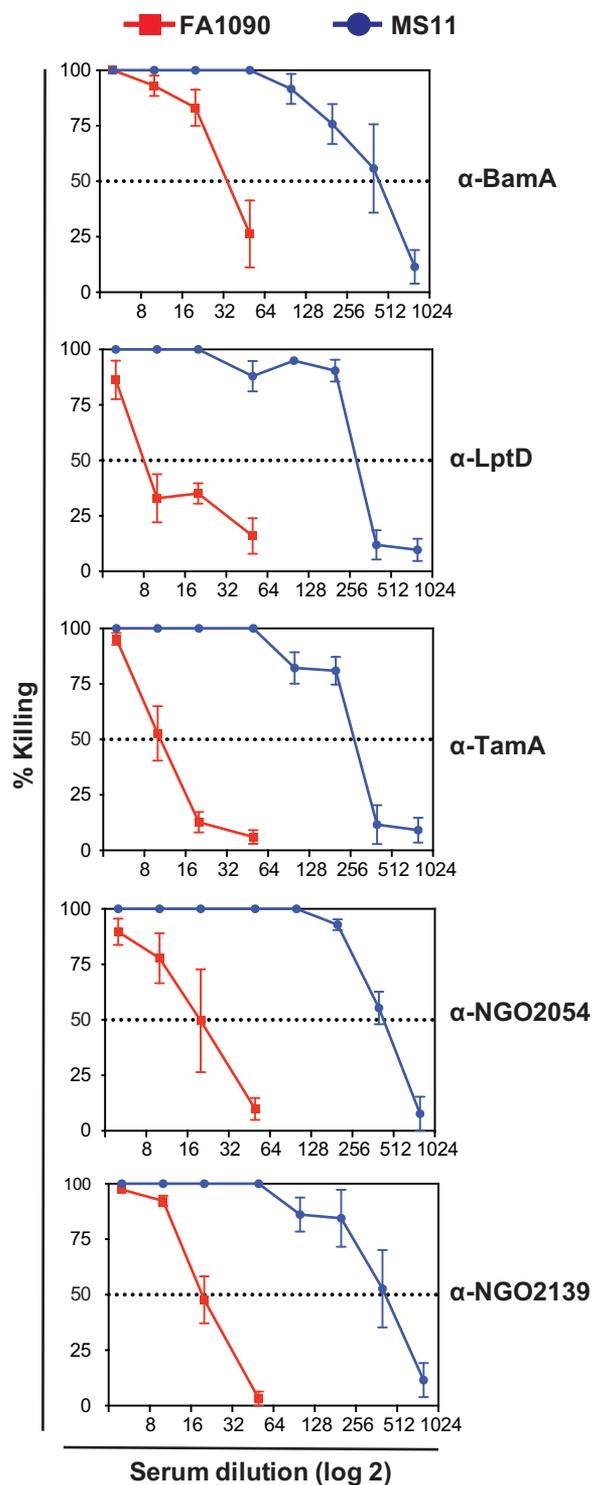


FIG. 6. The purified recombinant variants of BamA, LptD, TamA, NGO2054, and NGO2139 elicit bactericidal antibodies. Serum bactericidal assays were conducted against  $5 \times 10^3$  CFUs/ml of FA1090 (serum resistant) and MS11 (serum sensitive) *N. gonorrhoeae* strains using the rabbit anti-BamA, anti-LptD, anti-TamA, anti-NGO2054, and anti-NGO2139 post-immune sera and NHS as the complement source. The rabbit sera were heat-inactivated at 56 °C for 30 min. The bacterial cells were pre-sensitized with dilutions of the antigen-specific heat-inactivated sera for 15 min, followed by the addition of NHS

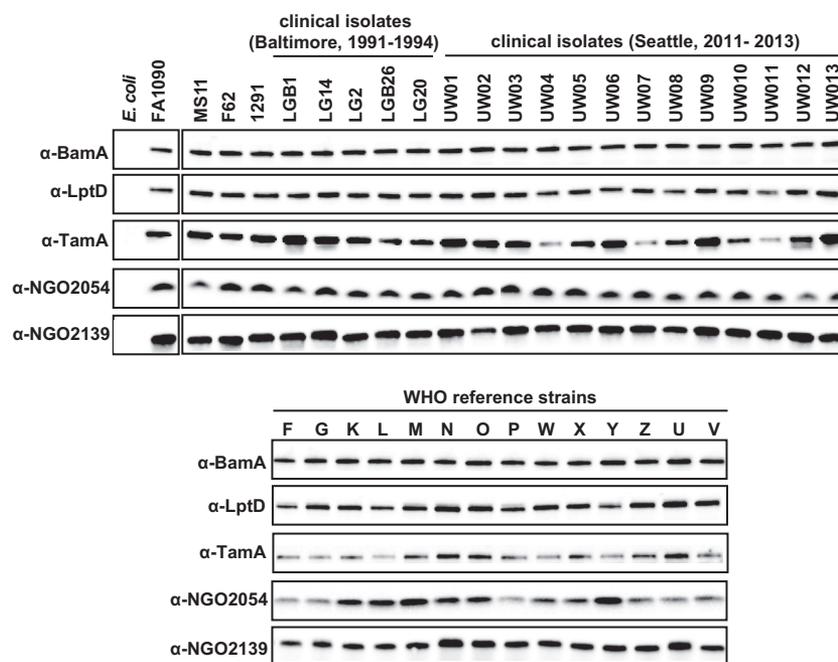
protective antibodies against homologous and heterologous *N. gonorrhoeae* strains FA1090 (serum resistant) and MS11 (serum-sensitive) was tested using the rabbit anti-BamA, anti-LptD, anti-TamA, anti-NGO2054, and anti-NGO2139 post-immune sera and NHS as the complement source (101). As expected (67), FA1090 was more resistant than MS11 to bactericidal killing by all the sera examined (Fig. 6). All antisera displayed bactericidal activity, and overall, the most potent against both FA1090 and MS11 were anti-BamA, anti-NGO2054, and anti-NGO2139. In particular, the bactericidal<sub>50</sub> titers against FA1090 and MS11 were 32 and 512 for anti-BamA, respectively, and 20 and 400 for anti-NGO2054 and anti-NGO2139.

**Conservation of Vaccine Candidates**—The conservation of the selected vaccine candidates (BamA, LptD, TamA, NGO2054, and NGO2139) was examined by analyzing the presence of polymorphic sites at both nucleotide and amino acid levels using the completed genome sequence of strain FA1090 (Gen Bank accession number AE004969), the draft genome sequences of 14 different *N. gonorrhoeae* strains (downloaded from the Broad Institute [http://www.broadinstitute.org/annotation/genome/Neisseria\\_gonorrhoeae/MultiHome.html](http://www.broadinstitute.org/annotation/genome/Neisseria_gonorrhoeae/MultiHome.html), and the 14 WHO 2015 reference strains (10, 28, 29). Overall, these analyses demonstrated that our five vaccine candidates were well conserved among 29 examined gonococcal isolates, with the number of nucleotide/amino acid polymorphic sites ranging from 2 (0.23%)/0 (0%) for NGO2139 to 21 (0.88%)/7 (0.88%) for BamA (Table III).

Subsequently, the expression of selected vaccine candidates was assessed using whole-cell lysates derived from a panel of temporally and geographically diverse *N. gonorrhoeae* strains (Fig. 7, supplemental Table S1). Because BamA and LptD have homologs in other Gram-negative bacteria, the specificities of the antisera were also tested using *E. coli*. All of the vaccine candidates were expressed by the 36 *N. gonorrhoeae* isolates; BamA, LptD, and NGO2139 showed similar abundance, whereas TamA and NGO2054 seemed to be expressed at variable levels in some gonococcal strains. In addition, none of the antisera cross-reacted with the *E. coli* whole-cell extracts (Fig. 7).

Together, these studies demonstrated that BamA, LptD, TamA, NGO2054, and NGO2139 are conserved and expressed not only in the commonly used laboratory strains (FA1090, MS11, F62, and 1291) but also clinical isolates from

at 10% final concentration, and incubation continued for 30 min. The CFUs were determined by plating bacteria on solid media. The average percent killing was determined from at least four independent experiments and was calculated as the number of CFUs in samples incubated with rabbit post-immune sera and NHS to the number of CFUs recovered from samples treated with rabbit post-immune sera and HI-NHS. *N. gonorrhoeae* viability was not affected in any of the control samples.



**FIG. 7. Expression of potential vaccine targets was evaluated using a panel of 36 temporally and spatially diversified GC isolates.** Clinical isolates of *N. gonorrhoeae* including WHO reference strains (as indicated) were harvested from GCB, matched by equivalent OD<sub>600</sub> units and resolved in 4–20% Tris-glycine precast gels. Following transfer onto nitrocellulose membrane and blocking, the proteins were probed with polyclonal rabbit antisera against BamA, LptD, TamA, NGO2054, and NGO2139. An *E. coli* strain (ER2566) was used as a negative control.

Baltimore and Seattle, as well as the 2015 WHO reference strains (10, 28, 29).

**Final Remarks**—Gonorrhea occurs at a high incidence and has a major impact on reproductive and neonatal health worldwide (2). Alarming, with each new antibiotic introduced for gonorrhea, resistance has emerged, including resistance to penicillins, tetracycline, macrolides, fluoroquinolones, and recently the third-generation cephalosporins. Current treatment options are seriously limited, and the development of a gonorrhea vaccine is the only long-term solution to this problem. Progress on gonorrhea vaccines has been slow, in part because of the high number of surface-exposed molecules in *N. gonorrhoeae* that undergo phase or antigenic variation and a lack of understanding of protective responses. Gonorrhea vaccine development can therefore benefit from a comprehensive, unbiased approach for antigen discovery. The application of comparative proteomics described in this study, together with previous comprehensive analyses of the *N. gonorrhoeae* cell envelopes and MVs (21), as well as other global proteomic approaches are ideally suited for identifying promising *N. gonorrhoeae* antigens and to guide future gonorrhea vaccine development (102). These newly identified cell envelope-associated proteins can then be subjected to rigorous evaluation that includes their expression as recombinant proteins in *E. coli*, examination of their surface localization and conservation among temporally, geographically, and genetically diverse panels of gonococcal strains, assessment of their ability to induce antibodies that

react against heterologous *N. gonorrhoeae* strains and are bactericidal, functional characterization, identification of protective epitopes, testing different combinations of antigens with various adjuvants and routes of immunization, as well as their protective capabilities in the gonorrhea mouse models. These studies could ideally lead to a selection of rational vaccine candidates that should be further tested alone and in different combination(s) in the human male challenge model of gonococcal infection to examine protection against *N. gonorrhoeae*, as well as the safety and nature of the immune responses generated. Finally, our studies also offer an unbiased overview of the *N. gonorrhoeae* cell envelope at the proteome level. Accordingly, the new research leads generated could provide novel paths for the scientific community to investigate the mechanisms that underpin *N. gonorrhoeae* pathophysiology. This, in turn, may drive the rational design of new preventive, diagnostic, and therapeutic interventions.

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§§ To whom correspondence should be addressed: Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, 433 Weniger Hall, 103 SW Memorial Pl., Corvallis, OR 97331. Tel.: 541.737.5811; E-mail: Aleksandra.Sikora@oregonstate.edu.

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