Quantitative Proteomics of the 2016 WHO *Neisseria gonorrhoeae* Reference Strains Surveys Vaccine Candidates and Antimicrobial Resistance Determinants

Authors

Fadi E. El-Rami, Ryszard A. Zielke, Teodora Wi, Aleksandra E. Sikora, and Magnus Unemo

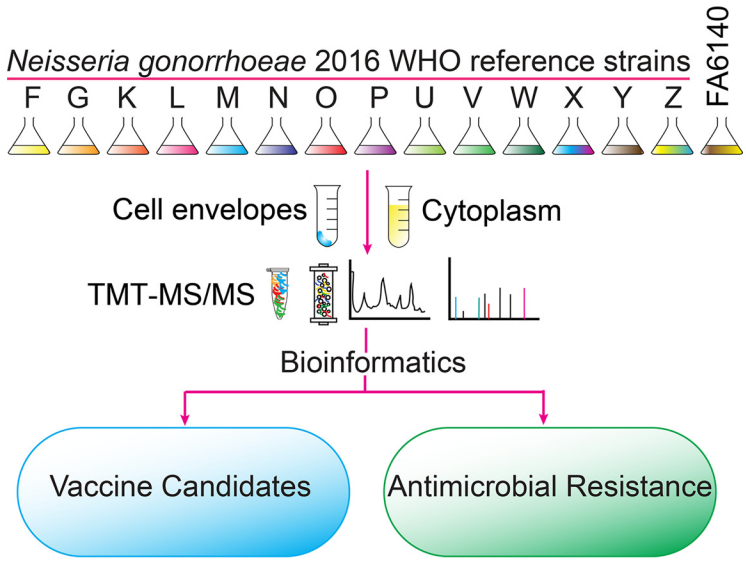
Correspondence

Aleksandra.Sikora@oregonstate.edu; magnus.unemo@regionorebrolan.se

In Brief

We present the first large-scale quantitative MS-based profiling of cell envelopes and cytoplasmic proteins derived from a diverse panel of WHO gonococcal reference strains possessing all existing AMR profiles and intended for quality assurance in laboratory investigations. We describe nine novel gonorrhea vaccine candidates and six potential global proteomic AMR markers. A reference proteomics databank for gonococcal vaccine and AMR research endeavors is provided, which enables microbiological, clinical, or epidemiological projects and enhances the utility of the WHO reference strains.

Graphical Abstract



Highlights

- Quantitative proteomes of the 2016 WHO *Neisseria gonorrhoeae* reference strains.
- Novel gonorrhea vaccine candidates and potential global proteomic AMR markers.
- First large-scale proteomic profiling of gonorrhea vaccine candidates and AMR.
- A reference proteomics databank for gonococcal vaccine and AMR research endeavors.

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Quantitative Proteomics of the 2016 WHO *Neisseria gonorrhoeae* Reference Strains Surveys Vaccine Candidates and Antimicrobial Resistance Determinants*[S]

Fadi E. El-Rami‡, Ryszard A. Zielke‡, Teodora Wi§, Aleksandra E. Sikora‡¶**‡‡, and Magnus Unemo‖§§‡‡

The sexually transmitted disease gonorrhea (causative agent: *Neisseria gonorrhoeae*) remains an urgent public health threat globally because of its reproductive health repercussions, high incidence, widespread antimicrobial resistance (AMR), and absence of a vaccine. To mine gonorrhea antigens and enhance our understanding of gonococcal AMR at the proteome level, we performed the first large-scale proteomic profiling of a diverse panel (n = 15) of gonococcal strains, including the 2016 World Health Organization (WHO) reference strains. These strains show all existing AMR profiles - established through phenotypic characterization and reference genome publication - and are intended for quality assurance in laboratory investigations. Herein, these isolates were subjected to subcellular fractionation and labeling with tandem mass tags coupled to mass spectrometry and multi-combinatorial bioinformatics. Our analyses detected 904 and 723 common proteins in cell envelope and cytoplasmic subproteomes, respectively. We identified nine novel gonorrhea vaccine candidates. Expression and conservation of new and previously selected antigens were investigated. In addition, established gonococcal AMR determinants were evaluated for the first time using quantitative proteomics. Six new proteins, WHO_F_00238, WHO_F_00635c, WHO_F_00745, WHO_F_01139, WHO_F_01144c, and WHO_F_01126, were differentially expressed in all strains, suggesting that they represent global proteomic AMR markers, indicate a predisposition toward developing or compensating gonococcal AMR, and/or act as new antimicrobial targets. Finally, phenotypic clustering based on the isolates' defined antibiograms and common differentially expressed proteins yielded seven matching clusters between established and proteome-derived AMR signatures. Together, our investigations provide a reference proteomics data bank for gonococcal vaccine and AMR research endeavors, which enables microbiological, clinical, or epidemiological projects and enhances the utility of the WHO reference strains. ***Molecular & Cellular Proteomics 18: 127–150, 2019. DOI: 10.1074/mcp.RA118.001125.***

Neisseria gonorrhoeae is an obligate human pathogen and the causative agent of the sexually transmitted disease gonorrhea. Gonorrhea is a global public health concern. In 2012 the World Health Organization (WHO)[1] estimated over 78 million new urogenital cases per year in adults (15–49 years of age) worldwide (1, 2). The spread of gonorrhea is facilitated by the high prevalence of asymptomatic infections. Urogenital gonorrhea is asymptomatic in up to 10–15% of infected men and up to 50% of infected women. Pharyngeal and rectal infections, which have increased in prevalence in both sexes and are predominant among men who have sex with men, are primarily asymptomatic (3, 4). Untreated or inappropriately treated gonorrhea can result in serious consequences on reproductive and neonatal health. Women are disproportionately affected, as gonococcal infection can ascend from the cervix to the uterus, Fallopian tubes, ovaries, and surrounding tissue, causing pelvic inflammatory disease. Long-term sequelae include ectopic pregnancy, chronic pelvic pain, and infertility. Furthermore, gonorrhea is strongly associated with an increased risk of both the acquisition and transmission of HIV (5).

Antimicrobial therapy is the only mainstay in the effective management and control of gonorrhea. However, *N. gonorrhoeae* exhibits an extraordinary capacity to develop antimicrobial resistance (AMR) through mutations and acquisition of AMR genes. The evolution of AMR in *N. gonorrhoeae* has overcome every therapeutic option since the "miracle drug" penicillin was introduced for gonorrhea treatment. Currently, a

From the ‡Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon; §Department of Reproductive Health and Research, World Health Organization, Geneva, Switzerland; ¶Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, Oregon; ‖World Health Organization Collaborating Centre for Gonorrhoea and other Sexually Transmitted Infections, Department of Laboratory Medicine, Clinical Microbiology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

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dual antimicrobial therapy (mainly ceftriaxone and azithromycin) is recommended for treatment of uncomplicated infections (6). Of grave concern over the past decade is the proliferation of resistance or decreased susceptibility to ceftriaxone worldwide. Azithromycin resistance has also emerged in most settings (7). The first failure of one of the recommended dual antimicrobial therapies against pharyngeal gonorrhea was reported in 2016 (8), and the first *N. gonorrhoeae* isolates with resistance to ceftriaxone combined with high-level resistance to azithromycin were identified in the United Kingdom (9, 10) and Australia (11, 12) in early 2018. In consideration of dwindling treatment options, scarce therapeutic alternatives, disease prevalence and morbidity, and lack of a vaccine(s), *N. gonorrhoeae* has been categorized by the WHO as a high priority pathogen globally and by the Centers for Disease Control and Prevention (CDC) as an urgent level threat in the United States (13).

Developing an effective gonococcal vaccine is essential because this is the only sustainable solution to quell the spread of gonococcal AMR and gonorrhea in general. The battle against penicillin-nonsusceptible *Streptococcus pneumoniae* exemplifies a successful vaccination strategy. Introduction of a pneumococcal conjugate vaccine in 2010 reduced the number of infections over 45% (14). Unfortunately, despite its public health importance, gonorrhea vaccine development remains in its infancy. Since 1970, only three small-scale vaccine trials using whole cell (15), pilin (16), and porin proteins (17) have been launched. All were unsuccessful in developing immunity against reinfection with gonorrhea. However, recent breakthroughs, including the development of small animal models for evaluating gonorrhea vaccines (18, 19), increased knowledge about *N. gonorrhoeae* immune evasion mechanisms (20–26), and the development of an effective vaccine for the closely related *N. meningitidis* serogroup B, which provided a low level of cross-protection against gonococcal infection (27), have reinvigorated the interest in gonococcal vaccine development (28).

Proteomic technology offers a powerful toolbox to enable vaccine antigen mining (28–32) and AMR proteome analysis (33–35), and to provide insights into host-pathogen interactions (36–39). Proteomic approaches have an advantage over genomics in drug and vaccine discovery endeavors by delivering information pertaining to protein abundance, post-translational modification(s), structure-function relationships, and protein-protein interactions (40–42). In addition, subcellular fractionation steps preceding proteomic applications reduce sample complexity, increase the likelihood of discovering low-abundance proteins, and aid in defining protein localization, all of which provide further insights into the proteins' functions and interactomes (43, 44). For *N. gonorrhoeae*, proteomic approaches have begun to deliver proteinaceous vaccine candidates (29, 30, 39, 45) and to support elucidation of AMR patterns (46, 47). Current off-gel proteomics, such as isobaric tag labeling (isobaric tagging for absolute quantification, iTRAQ; and tandem mass tags, TMT) coupled with high-pressure liquid chromatography and mass spectrometry techniques (LC-MS/MS), demonstrate superb protein separation and identification and enable detection of proteins in the low femtomole to high attomole range with precision and reliability (29, 48, 49).

To address the need for discovery of additional gonorrhea vaccine and drug candidates and to enhance our understanding of AMR at the proteome level, herein we examined the 2016 WHO *N. gonorrhoeae* reference strains (50) and the FA6140 strain (51) using a global quantitative proteomic approach. The WHO panel consists of 14 *N. gonorrhoeae* reference strains strictly selected and validated internationally to represent the *N. gonorrhoeae* species. All known gonococcal phenotypic and genetic AMR determinants are included for use as quality control strains during phenotypic and genetic laboratory testing. Eight of the strains were initially included in the 2008 WHO reference strains [WHO F, G, K, L, M, N, O, and P; (52)] to which 6 novel strains (U, V, W, X, Y, and Z) were added to constitute the 2016 WHO reference strains (50). All WHO panel strains have been subjected to extensive phenotypic, genomic, and genetic analyses to establish diagnostic markers, molecular epidemiological characteristics, reference genomes, and AMR profiles (phenotypic and genetic) for all antimicrobials currently and previously used for gonorrhea treatment, in addition to novel antimicrobials considered for future interventions. This panel includes WHO X, the first extensively drug-resistant gonococcal strain identified with high-level resistance to ceftriaxone, as well as additional strains with different levels of resistance to ceftriaxone, azithromycin and any additional therapeutic antimicrobials. Complete genomes with detailed annotations are available for all panel strains, providing a fundamental resource for future molecular studies. Accordingly, the well-characterized 2016 WHO reference strains (50) are ideally suited to provide detailed descriptions of the global *N. gonorrhoeae* proteome, a greater understanding of gonococcal AMR at the proteome level, and a source for the identification of broadly conserved novel vaccine candidates. In addition to the WHO panel strains, we have included in our investigations *N. gonorrhoeae* FA6140, which is a penicillin-resistant, β-lactamase-negative isolate that was originally described after a local epidemic outbreak of 199 gonococcal cases in Durham, North Carolina, USA in 1983 (51). It serves as a model for gonococcal AMR studies and has facilitated the characterization of mutations in

[1] The abbreviations used are: WHO, World Health Organization; ACN, acetonitrile; AGC, automatic gain control; AMR, antimicrobial resistance; C, cytoplasmic; CDC, centers for disease control and prevention; CE, cell envelope; COG, cluster of orthologous genes; cRAP, common repository of adventitious proteins; FDR, false discovery rate; GCB, gonococcal base agar; GCBL, gonococcal base liquid medium; KEGG, Kyoto encyclopedia of genes and genomes; LPS, lipopolysaccharide; OMV, outer membrane vesicle; ORF, open reading frame.

genes encoding the "multiple transferable resistance" repressor MtrR (53), ribosomal protein S10 (54), and penicillin-binding protein 2 (55) and their impact on AMR.

Our study is the first to investigate the global proteomic profiles of 15 *N. gonorrhoeae* reference strains using subcellular fractionation to separate cytoplasmic (C) and cell envelope (CE) associated proteomes, which were measured with tandem mass tags coupled to liquid chromatography and tandem mass spectrometry [TMT-LC-MS/MS; (56)], a highly reproducible and sensitive technique. These proteomic studies achieved our three major objectives. First, to enhance progress on gonorrhea vaccine development, novel vaccine candidates were identified, and the expression profiles of currently proposed antigens were established in diverse clinical isolates. Second, to broaden our understanding of AMR, proteomic signatures associated with AMR were defined by conducting a pairwise analysis of differentially expressed proteins to compare FA6140 and the 2016 WHO panel to WHO F, which possesses the largest genome and is susceptible to all relevant antimicrobials (50). Third, to facilitate the use of the 2016 WHO panel in various types of basic research and quality assurance, the complete reference proteomes of all tested strains were defined.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The 2016 WHO *N. gonorrhoeae* reference strains [$n = 14$; (50, 52)] and the *N. gonorrhoeae* FA6140 strain (51) were used in this study. The AMR profiles of all isolates were described previously (50). Gonococcal strains were cultured from frozen stocks (−80 °C) onto gonococcal base agar (GCB) medium (Difco, Sparks, MD) with Kellogg's supplements I and II, diluted 1:100 and 1:1,000, respectively (57). After incubation at 37 °C in a 5% CO_2 enriched atmosphere for 18–20 h, nonpiliated and transparent colonies were subcultured onto GCB and incubated as described above. To initiate growth in liquid medium, nonpiliated colonies were collected from GCB and suspended to the Optical Density (OD_{600}) of 0.1 in prewarmed GCB liquid (GCBL) medium supplemented as described above with the addition of 0.042% sodium bicarbonate. Suspensions were incubated at 37 °C with shaking at 220 rpm.

Subcellular Fractionation and TMT Labeling—All 15 *N. gonorrhoeae* strains were simultaneously cultured in GCBL as described above. Cells were collected by centrifugation (20 min, 6000 × *g*) when the OD_{600} of each culture reached 0.6–0.8, resuspended in PBS and lysed by passage through a French Press. The cell debris was removed by centrifugation and the crude CE fraction was separated from the C proteins using a sodium carbonate extraction procedure and subsequent ultracentrifugation steps. The fraction enriched with CE proteins was reconstituted in PBS supplemented with 0.1% SDS (29, 30). Experiments were conducted in two biological replicates. Sample quality and the overall subproteome profiles were examined by SDS-PAGE coupled with Colloidal Coomassie staining (58, 59). The total protein amount in each fraction was assessed using a Protein Assay Kit (Bio Rad, Hercules, CA). Each CE and C fraction containing 100 μg of protein in 25 μl volume of triethylammonium bicarbonate buffer was reduced with 11.3 mM tris(2-carboxyethyl) phosphine hydrochloride and the cysteines were alkylated using 20.1 mM iodoacetamide. Proteins were digested using trypsin (Promega, Madison, WI) at a 1:40 ratio. TMT reagents (ThermoFisher Scientific, Waltham, MA) were dissolved in acetonitrile (ACN) and used to label proteins in CE and C fractions as follows for the 10-plex experiment (ref 90111, Thermo Fisher Scientific): WHO F strain: TMT^{10}-126, WHO K strain: TMT^{10}-127C, WHO G strain: TMT^{10}-127N, WHO M strain: TMT^{10}-128C, WHO L strain: TMT^{10}-128N, WHO O strain: TMT^{10}-129C, WHO N strain: TMT^{10}-129N, WHO U strain: TMT^{10}-130C, WHO P strain: TMT^{10}-130N, WHO V strain: TMT^{10}-131; for the 6-plex experiment (ref 90402, Thermo Fisher Scientific): WHO F strain: TMT^{6}-126, WHO W strain: TMT^{6}-127, WHO X strain: TMT^{6}-128, WHO Y strain: TMT^{6}-129, WHO Z strain: TMT^{6}-130, FA6140 strain: TMT^{6}-131. Mixtures were incubated for 1 h at room temperature. The reaction was quenched by addition of 8 μl of 5% hydroxylamine. Samples were pooled, dried in a vacuum concentrator and stored at −80 °C before separation by high pressure liquid chromatography (HPLC) and MS analysis.

Sample Fractionation and MS Analysis—Samples were fractionated by strong cation exchange (SCX) with a Paradigm (Michrom Biosciences, San Diego, CA) HPLC with mobile phases of 5 mM potassium phosphate monobasic in 30% ACN/70% water (v/v) pH 2.7 (buffer A) and 5 mM potassium phosphate monobasic in 30% ACN/70% water (v/v) pH 2.7 with 500 mM potassium chloride (buffer B). The sample was brought up in buffer A (200 μl). The peptides were separated using a 2.1 mm × 100 mm Polysulfoethyl A column (PolyLC) over 60 min at a flow rate of 200 μl/min. The separation profile was as follows: hold 2% B for 5 min, 2% to 8% B in 0.1 min, 8% to 18% B in 14.9 min, 18% to 34% B in 12 min, 34% to 60% B in 18 min, 60% to 98% B in 0.1 min and hold for 10 min. Fractions were collected in 96-well microtiter plates at 1 min/fraction. Sixty fractions were pooled into 12 and dried using a speed vac. The samples were desalted using Oasis HLB 1cc cartridges. The cartridges were washed with 70% ACN/0.1% trifluoroacetic acid (TFA) and equilibrated with 0.1% TFA. Samples were loaded onto the cartridge in 0.1% TFA, washed with 0.1% TFA, and eluted in 1 ml 70% ACN/0.1% TFA. The samples were dried by vacuum centrifugation.

Desalted SCX fractions were analyzed by liquid chromatography electrospray ionization mass spectrometry (LC/ESI MS/MS) with a Thermo Scientific Easy-nLC II (Thermo Scientific) nano HPLC system coupled to a hybrid Orbitrap Elite ETD (Thermo Scientific) mass spectrometer. In-line de-salting was accomplished using a reversed-phase trap column (100 μm × 20 mm) packed with Magic C_{18}AQ (5-μm 200Å resin; Michrom Bioresources) followed by peptide separations on a reversed-phase column (75 μm × 250 mm) packed with Magic C_{18}AQ (5-μm 100Å resin; Michrom Bioresources) directly mounted on the electrospray ion source. A 90-min gradient from 7% to 35% ACN 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 2750 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 [automatic gain control (AGC) target value 1,000,000; resolution 120,000; and injection time 250 msec] with MS/MS spectra acquisition in the Orbitrap (AGC target value of 50,000; 15,000 resolution; and injection time 250 msec). The 15 most intense ions from the Fourier-transform full scan were selected for fragmentation in the higher-energy C-trap dissociation (HCD) cell by higher-energy collisional dissociation with a normalized collision energy of 40%. Selected ions were dynamically excluded for 30 s with a list size of 500 and exclusion mass by mass width ± 10ppm. HPLC and MS/MS analyses were performed in the Proteomic Facility at the Fred Hutchinson Cancer Center, Seattle.

Proteomic Data Analysis—Data analysis was performed using Proteome Discoverer 1.4 (Thermo Scientific). The data were searched against WHO_F_CDS (supplemental Material S1; 2449 entries) with the common Repository of Adventitious Proteins (cRAP, http://

www.thegpm.org/crap/) fasta file. Trypsin was set as the enzyme with maximum missed cleavages set to 2. The precursor ion tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. Variable modifications included TMT 6Plex (+229.163 Da) on any N-Terminus, oxidation on methionine (+15.995 Da), carbamidomethyl on cysteine (+57.021 Da), and TMT 6Plex on lysine (+229.163 Da).

Experimental Design and Statistical Rationale—All experiments described above were performed using the 15 bacterial strains in biological duplicates. Data were searched using Sequest HT. All search results were run through Percolator for scoring. Quantification was performed using the canned TMT 6plex or TMT 10plex methods through Proteome Discoverer with stringent criteria for protein identification including 1% False Discovery Rate (FDR), at least one unique peptide per protein, each identified peptide restricted to a single protein, and the score for every detected peptide of ≥1. Differential protein expression between CE and C fractions was determined by comparing the normalized total reporter ion intensities of groups using the WHO F protein expression profile as a reference.

Bioinformatic Analysis—To detect potential homologous proteins, amino acid sequences of each identified *N. gonorrhoeae* vaccine candidate were downloaded and compared against the GenBank proteome database (https://www.ncbi.nlm.nih.gov/genbank/) using our in-house designed program based on the Reciprocal Best Blast Hit approach (60) using BLASTP with the following parameters: percentage identity ≥50%, and E-value ≤1.0 e-5. The NCBI codes for the bacterial species included in this study are listed in supplemental Table S7.

Differential protein expression in four proteomics data sets (CE and C fractions in two biological replicates) was designated by fold changes ≥1.5 or ≤0.667 in reference to strain WHO F. Because of the variable nature of protein expression in *N. gonorrhoeae*, we took a conservative approach to designate protein expression and a protein was categorized as "upregulated" or "downregulated" solely when the fold change abundance was higher than 1.5 or lower than 0.667, respectively, to that of WHO F consistently in two biological experiments. A protein was designated as "ubiquitous" when its abundance was between 0.667–1.5-fold compared with WHO F in both experiments, or "variable" when its levels were not consistent between experiments.

A comprehensive assessment of predicted subcellular protein localization was accomplished by using the CELLO (61), PsortB 3.0.2 (62), SOSUI-GramN (63), SignalP 4.1 (64), LipoP 1.0 (65), and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) prediction algorithms and a majority voting strategy. Furthermore, for proteins whose subcellular localization was not predicted using the aforementioned algorithms, we relied on the difference between their unique peptide counts in the CE and C fractions as follows:

$$\textit{Unique Peptide Count Difference (UPCD)} = \sum_{i=1}^{2} xCE - \sum_{i=1}^{2} xC$$

where "*i*" is the sequential number assigned for samples and "*x*" is the total number of peptides detected in each fraction. Cytoplasmic proteins had more of their unique peptides detected in the C fraction (UPCD < 0), whereas membrane proteins had unique peptides enriched in the CE fraction (UPCD > 0). Proteins with UPCD $= 0$ were excluded from analysis using this UPCD formula. Proteins were categorized as follows: outer membrane, periplasmic, inner membrane, C proteins, and proteins with unknown localization.

The phenotypic and proteotypic clusters of all strains were constructed using as variables both their AMR (50) and proteomic profiles obtained in this study. These clusters were designed based on the Hamming distance between tested strains, which counts how many elements differ between two vectors, and is equivalent to Manhattan distance on binary data. Average linkage was used to determine distances between clusters.

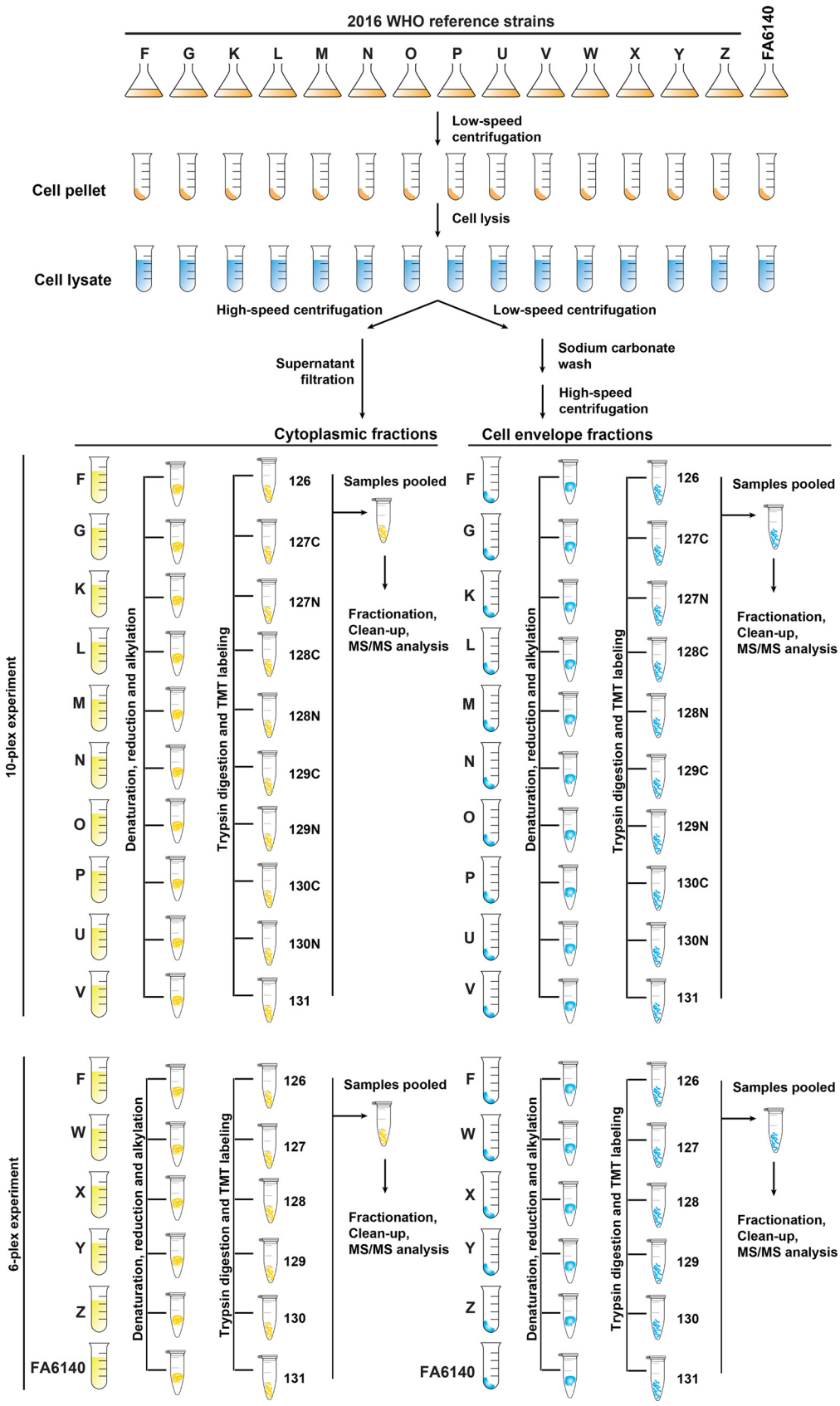
Graphs were generated with GraphPad Prism version 7 for Mac (GraphPad Software). The proteotypes of strains that belong to the same phenotypic cluster were compared, highlighting proteins that are significantly up- or downregulated with respect to those proteins of WHO F.

RESULTS AND DISCUSSION

Study Rationale—In our study design (Fig. 1), all 15 strains were cultured concurrently to mid-logarithmic growth, harvested, and subjected to subcellular fractionation to separate CE (outer membrane, periplasmic, inner membrane) and C proteins. We utilized TMT reagent technology for protein identification and quantitation as it provides a highly sensitive method for peptide labeling (56) and allows up to 10 biological samples to be analyzed in a single experiment (67). TMT-labeling, two-dimensional liquid chromatography fractionation, and subsequent MS/MS analyses were conducted on every 6-plex and 10-plex experiment pertaining to the CE and C fractions derived from each strain (Fig. 1). We selected WHO F as the reference strain for protein identification and quantitation because it has the largest genome (2,292,467 bp) and proteome (2,449 ORFs) among the 2016 WHO reference strains (50) and FA6140 (68), and it is susceptible to most antimicrobials currently or historically used for gonorrhea treatment.

Subcellular fractionation experiments coupled with proteomics repeatedly show cytoplasmic proteins associated with the membranes, which are commonly regarded as "contaminating" or "moonlighting" proteins (29, 30, 69, 70). Therefore, to focus solely on the enriched proteins in individual subproteomes, we first eliminated C and CE proteins that were detected in the CE and C protein fractions, respectively, from further analyses. Complete lists of all identified proteins are in supplemental Tables S1–S2. Subsequently, we performed two-armed proteomic data analyses: 1) for vaccine antigen mining, we focused on common proteins identified in all strains in the CE fraction with the overarching goal to discover omnipresent *N. gonorrhoeae* proteins; 2) to profile AMR signatures, we performed a pairwise comparison of individual strains to WHO F to enhance the discovery of strain-specific feature(s).

Overview of Cell Envelope and Cytoplasmic Proteomes—The 10-plex biological replicate experiments identified a total of 1150 proteins in the CE fraction of all ten strains, of which 1010 were common in both sets (Fig. 2*A*). In the two 6-plex experiments, 1194 proteins were identified; 975 were shared in all six isolates (Fig. 2*A*). Taken together, the 10-plex and the 6-plex experiments resulted in identification of 1100 proteins in the CE fractions, of which 904 were common among all examined *N. gonorrhoeae* strains (Fig. 2*A*). The proteome coverage per strain ranged from 41.22% (981 proteins) for WHO Y to 45.32% (1042 proteins) for WHO G (supplemental Table S3).


2016 WHO reference strains
F
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O
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W
X
Y
Z
FA6140
Low-speed centrifugation
Cell pellet
Cell lysis
Cell lysate
High-speed centrifugation
Low-speed centrifugation
Supernatant filtration
Sodium carbonate wash
High-speed centrifugation
Cytoplasmic fractions
Cell envelope fractions
10-plex experiment
Denaturation, reduction and alkylation
Trypsin digestion and TMT labeling
126
127C
127N
128C
128N
129C
129N
130C
130N
131
Samples pooled
Fractionation, Clean-up, MS/MS analysis
6-plex experiment
127
128
129
130

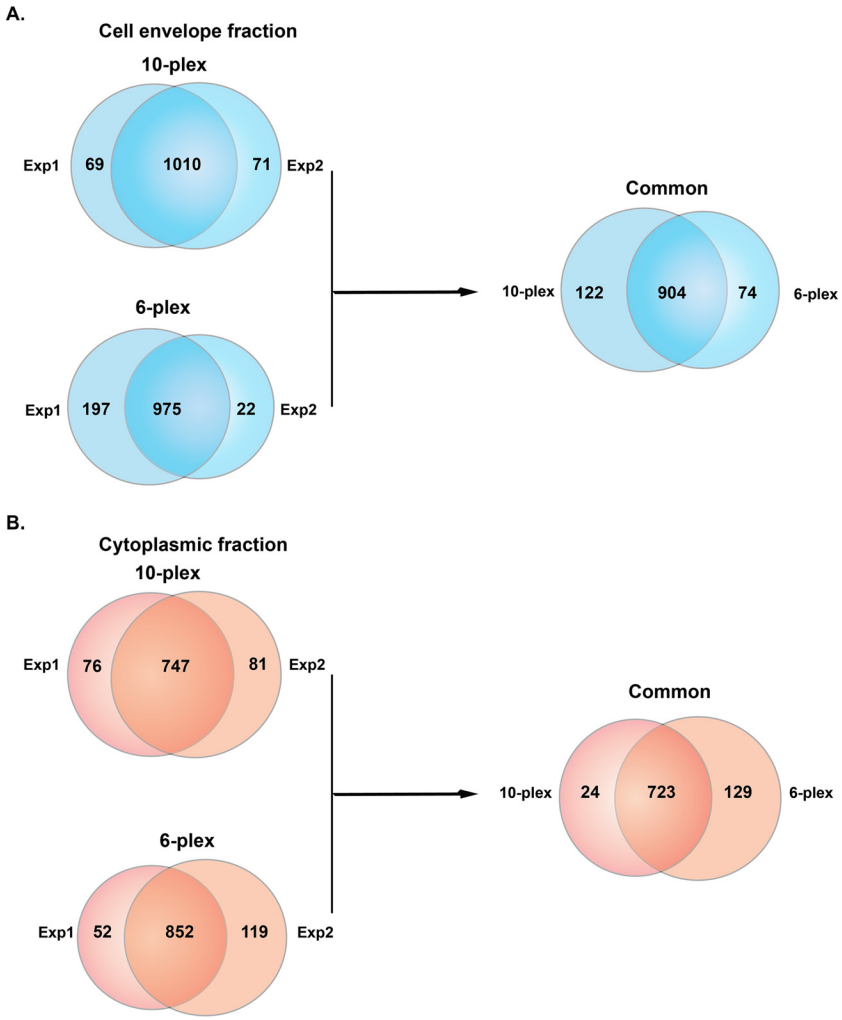


FIG. 2. **Venn diagrams illustrating the distribution of proteins identified in cell envelope and cytoplasmic fractions in two independent proteomic experiments.** *A*, Cell envelope proteomes derived from the 2016 WHO reference strains and FA6140 were analyzed in 10-plex and 6-plex experiments performed in biological duplicates. A total of 1079 and 1081 proteins was identified in Experiments 1 and 2, respectively, and 1010 common proteins were found in both 10-plex experiments. The 6-plex TMT labeling revealed 975 common proteins as well as 197 and 22 unique proteins in Experiments 1 and 2, respectively. Further analyses were applied to 904 proteins mutually identified in both 10-plex and 6-plex experiments. *B*, The proteomic profiling of cytoplasmic fractions yielded 904 proteins shared among all 10 strains, of which 747 were common in both experiments. The 6-plex TMT identified 904 and 971 proteins in Experiments 1 and 2, respectively; of which 852 were common between replicates. In further analyses solely the 723 proteins shared between both experiments were included. Exp 1 - experiment 1; Exp 2 - experiment 2.

Proteomics of the C fraction in the 10-plex set conducted in biological replicates yielded 904 proteins that were shared among all 10 strains, of which 747 were common in both experiments (Fig. 2*B*). The two 6-plex experiments identified 1023 shared proteins, with 852 common among the two replicates (Fig. 2*B*). Cumulatively, C fraction profiling resulted in identification of 876 proteins with 723 common in all 15 *N. gonorrhoeae* strains (Fig. 2*B*). Proteome coverage ranged from 31.37% (746 proteins) in WHO U to 38.43% (852 proteins) in FA6140 (supplemental Table S3).

Subsequently, we allocated common proteins that were identified in all 15 *N. gonorrhoeae* strains to outer membrane,

FIG. 1. **Experimental paradigm of quantitative proteomic profiling of the *N. gonorrhoeae* 2016 WHO reference strains and the FA6140 strain.** All gonococci were cultured concurrently in liquid medium until reaching mid-logarithmic growth. Bacterial cells were harvested, lysed, and subjected to subcellular fractionation to separate the crude cell envelope (CE) and cytoplasmic (C) proteomes. CE proteins were enriched using a sodium carbonate wash and ultracentrifugation. The obtained CE and C protein samples (100 μg) were denatured, reduced, alkylated, trypsinized, and the peptides from each strain were labeled using 10-plex and 6-plex Tandem mass tag (TMT) reagents, as indicated. Finally, samples were pooled, fractionated by strong cation exchange, and analyzed by liquid chromatography electrospray ionization mass spectrometry. Experiments were performed in biological duplicates.

inner membrane, periplasm, cytoplasm, or unknown localization categories based on PSORTb 3.0.2 (62), SOSUIGramN (63), and CELLO (61) predictions and the majority-voting strategy. We used these software packages to take advantage of their different algorithms and statistical approaches for the prediction of protein subcellular localization. As expected from our subcellular fractionation approach (30, 49, 69), the CE fraction was enriched in membrane proteins compared with the C sample, with outer membrane (26 *versus* 8), periplasmic (51 *versus* 38), and inner membrane proteins (145 *versus* 6) that were also identified with considerably higher peptide counts (Fig. 3*A*–3*C*, and supplemental Tables S1–S2 and S4–S5). The C preparations yielded 592 cytoplasmic proteins that were identified with greater peptide counts compared with the cytoplasmic proteins associated with the CE fraction (Fig. 3*D*, supplemental Tables S5–S6). Furthermore, to increase the discovery of potential vaccine candidates, we searched the 149 proteins of unknown localization identified in the CE fraction (Fig. 3*E*) for the presence of signal peptides and transmembrane motifs using SignalP 4.1 (64), LipoP 1.0 (65), and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The results of all software programs and the majority votes strategy revealed six additional CE proteins, four of which were found in the majority of examined strains (supplemental Table S6). In addition, literature searches for experimental evidence of protein surface exposure allowed assignment of BamE [NGO1780; (71)], SliC [NGO1063; (72)], MetQ [NGO2139; (30, 73)], Ng-MIP [NGO1225; (30, 74)], and BamG [NGO1985; (75)] to the cell surface. Subcellular location predictions for all proteins are listed in supplemental Table S8.

Expression Patterns of Common Identified Proteins Compared with WHO F—Proteins were categorized as ubiquitous, up- or downregulated, or variable based on their abundance in relation to the corresponding protein in WHO F in biological duplicate experiments. We investigated expression patterns of detected proteins in both subproteome fractions that were shared among all strains (Figs. 4–5, supplemental Tables S4–S5). Annotated cell envelope proteins were predominantly ubiquitous in the CE fraction. The proportion of ubiquitous CE outer membrane proteins (n = 26) ranged from 73% (n = 19 in WHO N) to 46.15% (n = 12 in WHO L; Fig. 4*A*). Ubiquitous periplasmic proteins ranged from 76.47% (n = 39 in WHO N) to 35.2% (n = 18 in WHO U) of the total number of proteins annotated to localize to the periplasm (n = 51; Fig. 4*B*). Finally, between 83.4% (n = 121 in WHO M) and 44.1% (n = 64 in WHO U) of inner membrane proteins (n = 145) were ubiquitously expressed (Fig. 4*C*). Upregulated outer membrane (0–19.23%), periplasmic (0–7.8%) and inner membrane (0–4.8%) proteins made up the smallest proportion of proteins in the CE fraction (supplemental Table S4), whereas downregulated outer membrane (3.85–30.77%), periplasmic (0–23.53%) and inner membrane (1.38–24.8%) proteins were moderately prevalent (supplemental Table S4). Further analysis of the CE fraction detected 121 common proteins with

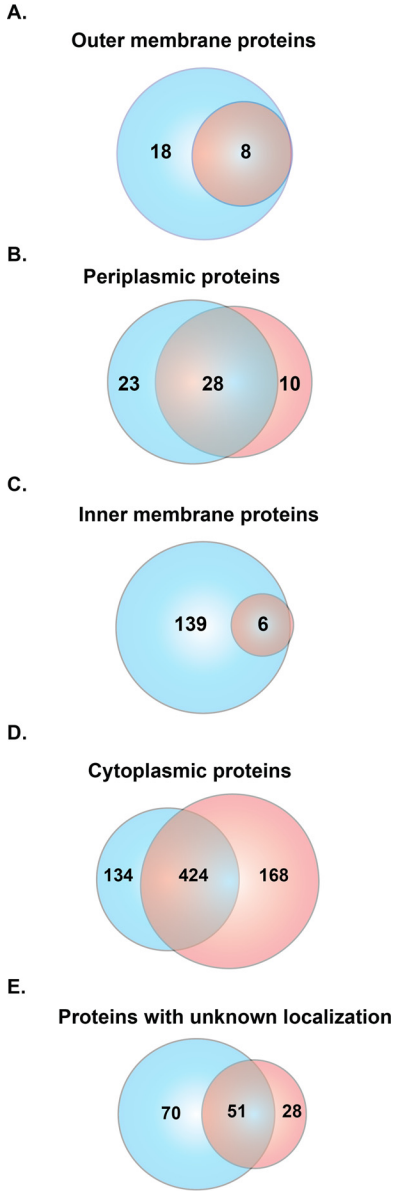
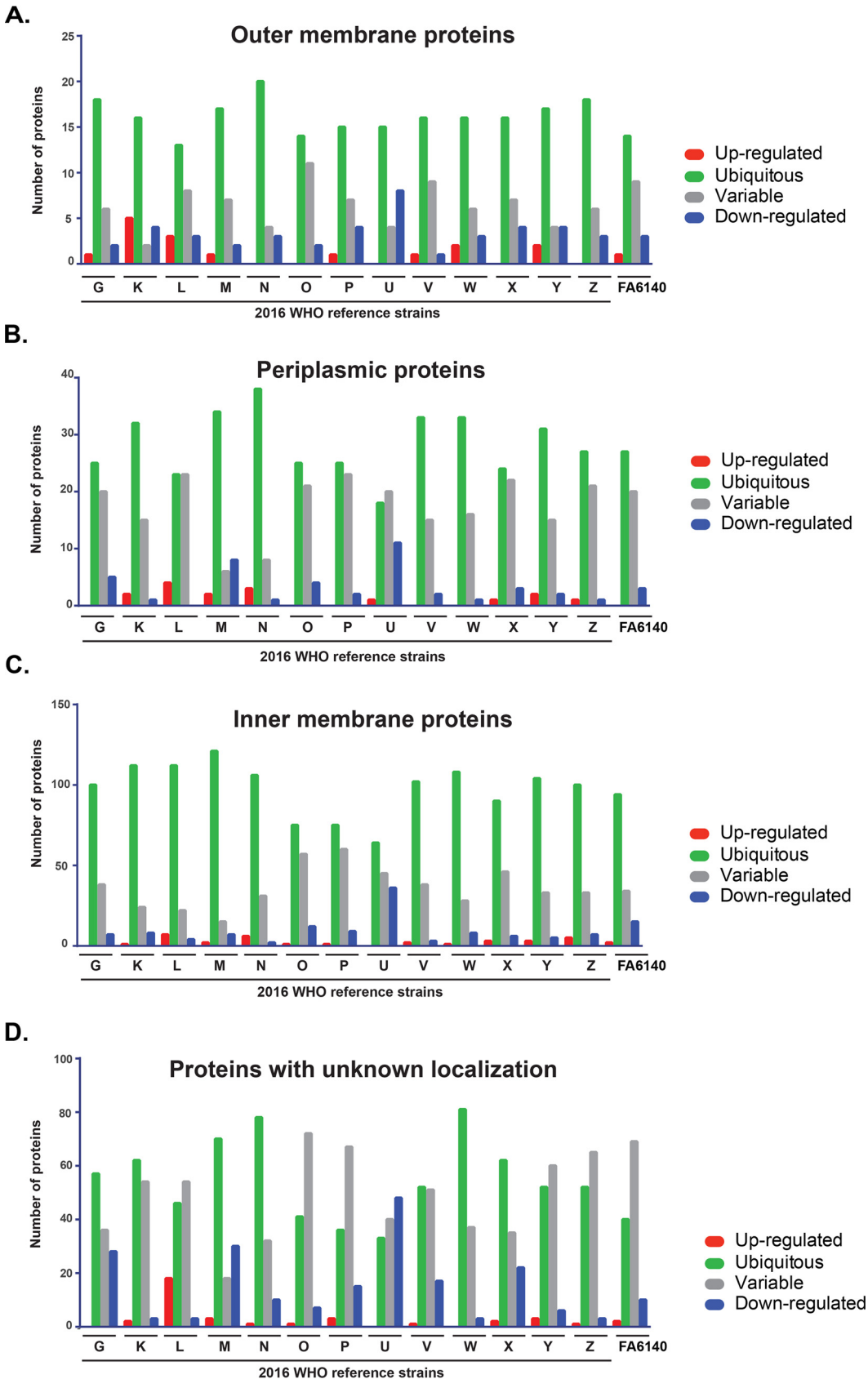


FIG. 3. **Subcellular localization of proteins identified in cell envelope and cytoplasmic subproteomes.** Proteins identified in the cell envelope (blue circle) and cytoplasmic (red circle) fractions were subjected to comprehensive assessments of subcellular localization using different prediction algorithms and were allocated into the outer membrane (*A*), periplasm (*B*), inner membrane (*C*), cytoplasm (*D*), or unknown localization (*E*).

unknown localization (Fig. 3*E*, supplemental Table S4). Ubiquitous expression was the dominant pattern for these proteins in WHO G, K, M, N, V, W, and X and ranged from 42.97% (n = 52, WHO V) to 66.94% (n = 81, WHO W). Variable expression of proteins with unknown localization dominated in WHO L, O, P, Y, Z, and FA6140. WHO U had the highest number of downregulated proteins (39.67%, n = 48) with respect to those in WHO F (Fig. 4*D*).

In contrast to the CE expression pattern, we observed a striking increase in the number of variably expressed cyto-


A.
Outer membrane proteins
Number of proteins
25
20
15
10
5
0
G K L M N O P U V W X Y Z FA6140
2016 WHO reference strains
Up-regulated
Ubiquitous
Variable
Down-regulated
B.
Periplasmic proteins
Number of proteins
40
30
20
10
0
G K L M N O P U V W X Y Z FA6140
2016 WHO reference strains
Up-regulated
Ubiquitous
Variable
Down-regulated
C.
Inner membrane proteins
Number of proteins
150
100
50
0
G K L M N O P U V W X Y Z FA6140
2016 WHO reference strains
Up-regulated
Ubiquitous
Variable
Down-regulated
D.
Proteins with unknown localization
Number of proteins
100
80
60
40
20
0
G K L M N O P U V W X Y Z FA6140
2016 WHO reference strains
Up-regulated
Ubiquitous
Variable
Down-regulated

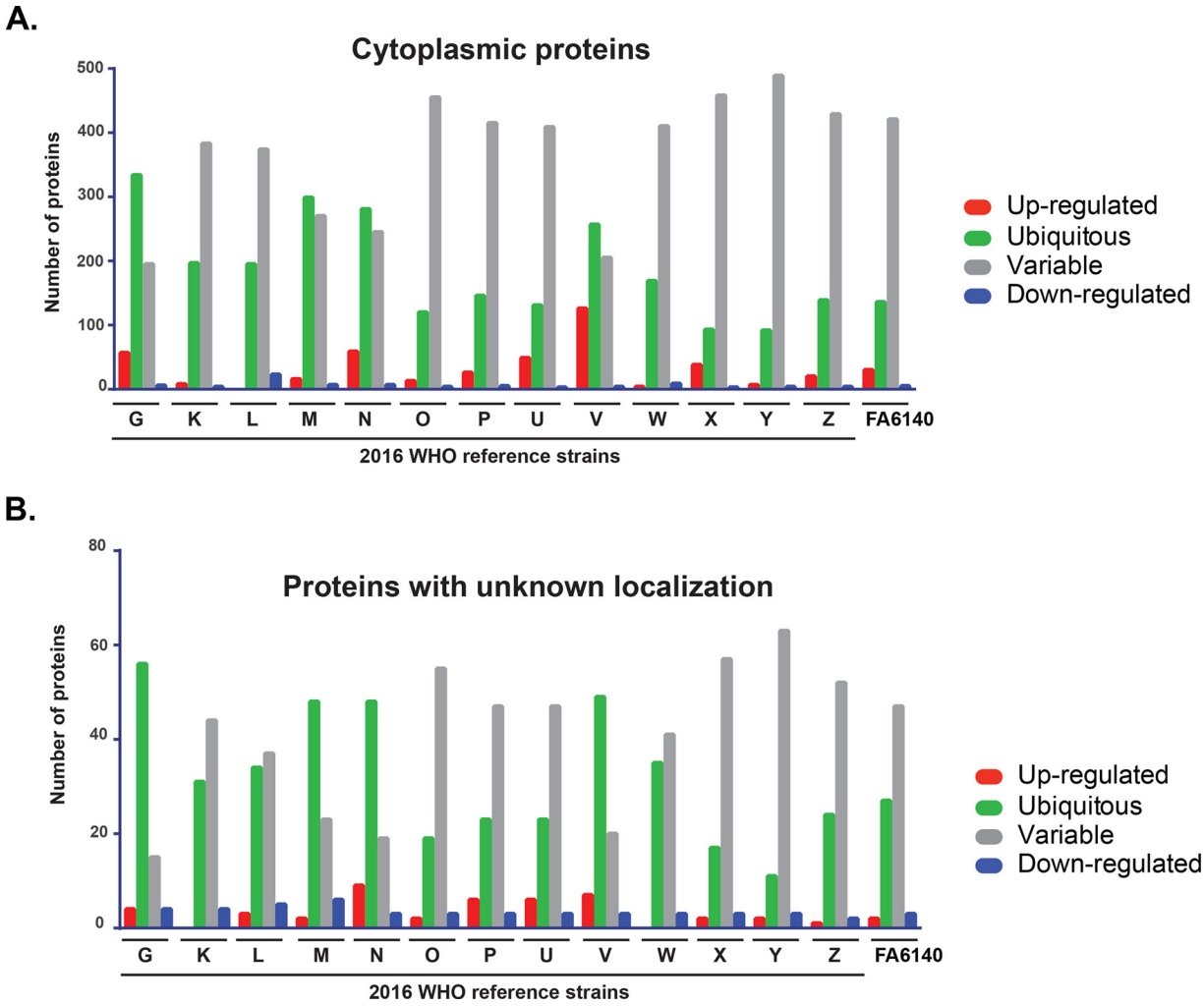


FIG. 5. **Expression patterns of common proteins identified in the cytoplasmic proteome.** Cytoplasmic (*A*) and proteins with unknown localization (*B*) are shown. Protein levels in individual gonococcal strains were compared with the protein level in the reference WHO F isolate. Protein expression is categorized as ubiquitous (green bars); upregulated (red bar); downregulated (blue bar); and variable (gray bar).

plasmic proteins in the C fraction of analyzed strains compared with WHO F (Fig. 5*A*, supplemental Table S5). The percentage of variable proteins ranged from 32.9% to 82.6% for WHO G and WHO Y, respectively (supplemental Table S5). Ubiquitous proteins were the next most common category and oscillated from 15.5% in WHO Y to 56.4% in WHO G. The third group contained upregulated proteins (0–21.28%), and downregulated proteins ranged from 0.5–3.88% (supplemental Table S5). For proteins with no assigned localization, variable expression was the most prevalent pattern in WHO K, L, O, P, U, W, X, Y, Z, and FA6140 (Fig. 5*B*), ranging from 79.75% (n = 63, WHO Y) to 46.83% (n = 37, WHO L). Ubiquitous proteins were the dominating group in WHO G, M, N, and V (Fig. 5*B*). Finally, up- and downregulated proteins constituted up to 11.39% and 7.59%, respectively, of the total proteins with unknown localization in the C fraction.

Together, the first quantitative proteomic profiling of the 15 *N. gonorrhoeae* strains demonstrated distinct differences in their proteomes and showed that a pattern of ubiquitous protein expression was prevalent in the CE fraction, whereas variably expressed proteins were the dominant group in the C subproteome.

Antigen Mining Decision Tree—To identify novel gonorrhea antigens and to gain information about expression of previously identified vaccine candidates (26, 30, 49, 76), we designed an antigen mining decision tree (Fig. 6). We included in this process 25 outer membrane proteins (all outer membrane proteins identified except RmpM) and 121 proteins of un-

FIG. 4. **Expression patterns of common proteins identified in the cell envelope fraction.** Outer membrane (*A*), periplasmic (*B*), inner membrane (*C*), or proteins with unknown localization (*D*) are shown. Expression of each protein in each gonococcal strain was compared with the protein level in the reference WHO F isolate. Protein expression is categorized as ubiquitous (green bars); upregulated (red bar); downregulated (blue bar); and variable (gray bar).

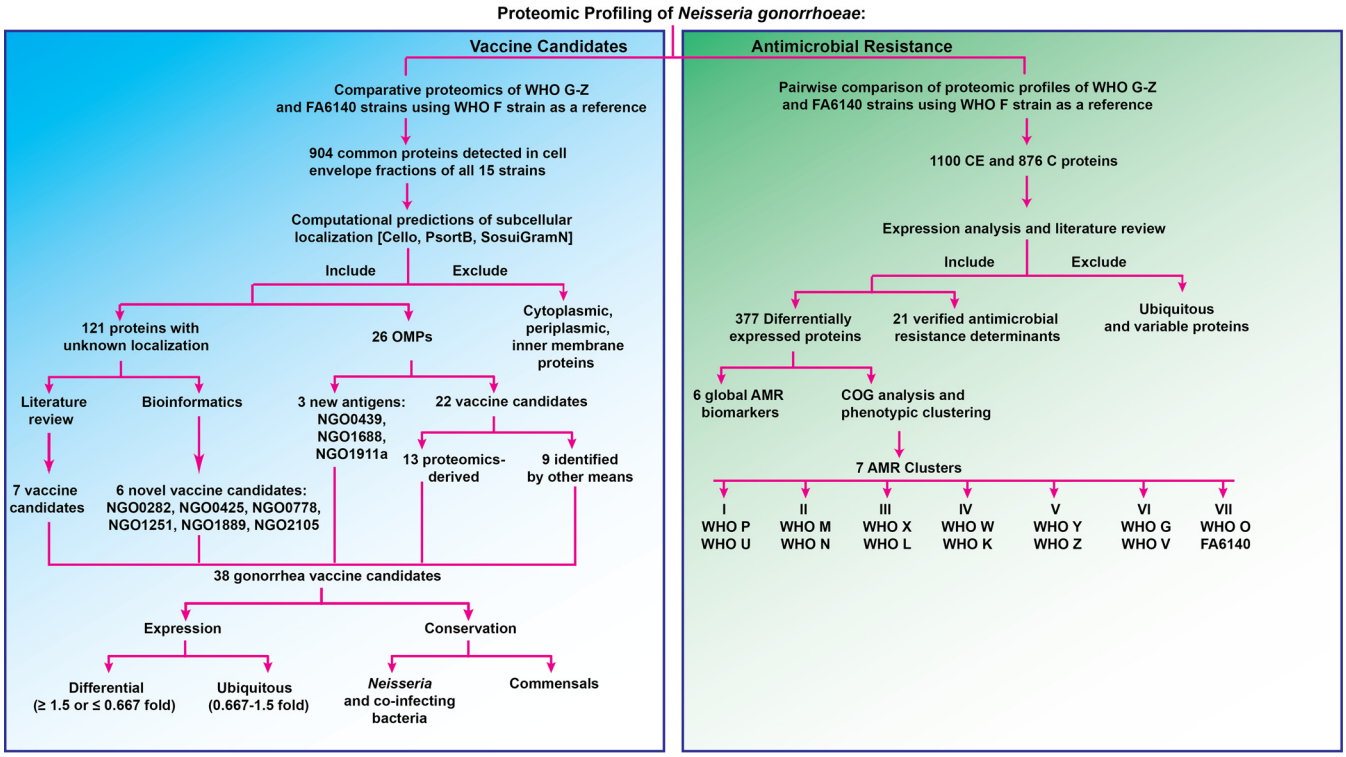


FIG. 6. **Decision tree designed for proteomic mining of *Neisseria gonorrhoeae* vaccine candidates and antimicrobial resistance (AMR) determinants.** For vaccine antigen mining, we focused on 904 common proteins identified in all strains in the CE fraction with the overarching goal to discover omnipresent *N. gonorrhoeae* proteins. Computational predictions were employed to predict protein subcellular localization. Solely 26 OMPs and 121 proteins with unknown localization were included in further analyses. Bioinformatics and literature review yielded six novel vaccine candidates in the latter group of proteins. The OMPs constituted three new antigens and 22 previously proposed gonorrhea vaccine candidates including 13 proteomics-derived. Finally, the 38 identified gonorrhea vaccine candidates were subjected to expression and conservation analyses. To profile AMR signatures, we performed a pairwise comparison of individual strains to the antimicrobial susceptible WHO F strain to enhance the discovery of strain-specific feature(s). The maximum number of identified proteins reached 1100 and 876 for CE and C fractions, respectively. Expression profiling and literature review yielded 398 differentially expressed proteins including 21 verified AMR determinants. Ubiquitous and variably expressed proteins were excluded from the analysis. To link AMR phenotypes with proteomic signatures, COG analysis and phenotypic clustering of gonococcal strains based on their defined antibiograms and the 377 differentially expressed proteins were performed generating seven phenotypic clusters that matched between established and proteome-derived AMR signatures. OMPs - outer membrane proteins; CE - cell envelope; C - cytoplasm; COG - Cluster of Orthologous Genes.

known localization identified in CE proteomic profiling (Fig. 6 and supplemental Table S1). The latter group of proteins was subjected to signal sequence and transmembrane motif analyses to increase the coverage of potential vaccine candidates. Together, these investigations yielded nine novel antigens including NGO0282, NGO0425, NGO0439, NGO0778, NGO1251, NGO1688, NGO1889, NGO1911a, and NGO2105 in addition to previously discovered proteomics-derived antigens [(29, 30); Table I] and vaccine candidates identified by other means (Table II).

Further bioinformatics and literature searches were performed to gain insights into the new proteomics-derived vaccine candidates. The putative lipoprotein NGO0282 is a homolog of the outer membrane localized LptE, which is a component of the trans-envelope LptA-G machinery involved in the transport of lipopolysaccharide/lipooligosaccharide (LPS/LOS) molecules to the *E. coli* and *N. meningitidis* outer membrane, respectively. LptE's chaperone-like role in LptD biogenesis is conserved in both bacteria but LptE works in concert with LptD to translocate LPS to the cell surface only in *E. coli* (77, 78). LptD is essential for *E. coli* and *N. gonorrhoeae* viability but is dispensable for *N. meningitidis* (79–81). Therefore, the function of *N. gonorrhoeae* LptE in both LOS transport and LptD biogenesis needs to be elucidated. NGO0425 contains a tetratricopeptide repeat-like domain and a transmembrane helix. Together with its *E. coli* homolog, YfgM, NGO0425 belongs to the UPF0070 family. In *E. coli*, YfgM was proposed to act within the β-barrel trafficking chaperone network and its depletion in Δ*skp* and Δ*surA* knockout backgrounds contributed to further alterations in outer membrane integrity (82). The vaccine candidate protein NGO0439 is homologous to the *E. coli* outer membrane protein LolB, which is involved in lipoprotein trafficking to the outer membrane (83, 84). We consider *N. gonorrhoeae* LolB to be a vaccine candidate antigen because its surface localization should be experimentally verified. Differences in the lo-

TABLE I

Expression and homologs of proteomics-derived Neisseria gonorrhoeae vaccine candidates[#]

Accession	Protein homolog in *Neisseria gonorrhoeae* FA1090	Localization	Protein description	Protein name	Protein expression: WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z	FA6140	Protein conservation: *Gardnerella vaginalis* ATCC14019	*Chlamydia trachomatis* E/11023	*Chlamydia trachomatis* J/6276tet1	*Prevotella ruminicola*	*Mycoplasma hominis* ATCC27545 LBD-4	*Neisseria meningitidis* MC58	*Neisseria meningitidis* serogroup A Z2491	*Neisseria meningitidis* serogroup C 053442	*Neisseria gonorrhoeae* F62	*Neisseria gonorrhoeae* FA1090	*Neisseria gonorrhoeae* NCCP11945	*Neisseria gonorrhoeae* MS11	*Neisseria lactamica* 020-06	*Neisseria weaveri* NCTC13585	*Lactobacillus crispatus* ST1	*Lactobacillus gasseri* ATCC 33323 JCM 1131	*Lactobacillus jensenii* strain SNUV360	*Lactobacillus iners* DSM 13335
WHO_F_00053c	NGO0055	OM	Pilus assembly protein	PilC																					+	+	+	+	+	+	+				
WHO_F_00104c	NGO0097	CE	Pilus assembly protein	PilN																					+	+	+	+	+	+	+				
WHO_F_00296c**	NGO0277	CE	Outer membrane biogenesis protein	BamD																					+	+	+	+	+	+	+				
WHO_F_00301***	NGO0282	CE	LPS-assembly lipoprotein	LptE																					+	+	+	+	+	+	+				
WHO_F_00453c**	NGO0425	CE	Hypothetical protein																						+	+	+	+	+		+				
WHO_F_00467*	NGO0439	OM	Outer membrane lipoprotein LolB	LolB																					+	+	+	+	+	+	+				
WHO_F_00839c	NGO0778	CE	Membrane protein																						+	+	+	+	+	+	+				
WHO_F_00962	NGO0834	OM	Membrane protein	CsgG																					+	+	+	+	+	+	+				
WHO_F_01229c***	NGO1063	OM	Surface-exposed lysozyme inhibitor	SliC																						+	+	+	+	+	+				
WHO_F_01388	NGO1205	OM	Zinc uptake component D protein	ZnuD																		+			+	+	+	+	+	+	+				
WHO_F_01407c*	NGO1225	OM	Ng-MIP	Ng-MIP															+	+	+	+			+	+	+	+	+	+	+				
WHO_F_01438c*	NGO1251	CE	Lipoprotein																						+	+		+	+	+	+				
WHO_F_01539	NGO1344	CE	AsmA-like protein																						+	+	+	+	+	+	+				
WHO_F_01599c	NGO1393	CE	Adhesin	MafA																					+	+	+	+	+	+					
WHO_F_01745c***	NGO1492	OM	Phospholipase A1	PldA																					+	+	+	+	+	+	+				
WHO_F_01817*	NGO1559	CE	OmpA/MotB domain-containing protein																	+	+	+						+	+	+	+		+			
WHO_F_01959c	NGO1688	OM	Outer membrane protein	OmpU																					+	+	+	+	+	+	+				
WHO_F_01995c**	NGO1715	OM	LPS-assembly protein	LptD																					+	+	+	+	+	+	+				
WHO_F_02071***	NGO1780	OM	Outer membrane biogenesis protein	BamE																					+	+	+	+	+	+	+				
WHO_F_02094***	NGO1801	OM	Outer membrane biogenesis protein	BamA																+	+	+			+	+	+	+	+	+	+				
WHO_F_02195c*	NGO1889	CE	Lipoprotein	Lpr																					+	+	+	+	+	+					
WHO_F_02224c	NGO1911a	OM	Pilus assembly protein																							+	+	+	+	+					
WHO_F_02269c**	NGO1956	OM	Translocation and assembly module A	TamA																					+	+	+	+	+	+	+				
WHO_F_02304c***	NGO1985	OM	Lipoprotein	BamG																					+	+	+	+	+	+	+				
WHO_F_02385c*	NGO2054	OM	Hypothetical protein																						+		+	+	+		+				
WHO_F_02440c	NGO2092	CE	Iron ABC transporter protein																						+	+	+	+	+	+					
WHO_F_02455c*	NGO2105	CE	Adhesion and penetration protein	AidA																					+	+	+	+	+	+					
WHO_F_02462	NGO2111	OM	Hypothetical protein																						+	+	+	+	+	+	+				
WHO_F_02473	NGO2121	CE	Lipoprotein component	MlaA																					+	+	+	+	+	+	+				
WHO_F_02490**	NGO2139	OM	Methionine ABC transporter protein	MetQ															+						+	+	+	+	+	+	+	+	+	+	+

[#]Color legends for protein expression: Ubiquitous (green), up-regulated (red), down-regulated (blue), variable (grey). Ubiquitous expression among all 15 strains is marked with (***), 14 strains with (**), 12–13 strains with (*). Abbreviations: OM: Outer membrane, CE: cell envelope, +: protein homolog detected.

TABLE II

Expression and homologs of Neisseria gonorrhoeae vaccine candidates identified by other means[#]

Accession	Protein homolog in *Neisseria gonorrhoeae* FA1090	Localization	Protein description	Protein name	Protein expression: WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z	FA6140	Protein conservation: *Gardnerella vaginalis* ATCC14019	*Chlamydia trachomatis* E/11023	*Chlamydia trachomatis* J/6276tet1	*Prevotella ruminicola*	*Mycoplasma hominis* ATCC27545 LBD-4	*Neisseria meningitidis* MC58	*Neisseria meningitidis* serogroup A Z2491	*Neisseria meningitidis* serogroup C 053442	*Neisseria gonorrhoeae* F62	*Neisseria gonorrhoeae* FA1090	*Neisseria gonorrhoeae* NCCP11945	*Neisseria gonorrhoeae* MS11	*Neisseria lactamica* 020-06	*Neisseria weaveri* NCTC13585	*Lactobacillus crispatus* ST1	*Lactobacillus gasseri* ATCC 33323 JCM 1131	*Lactobacillus jensenii* strain SNUV360	*Lactobacillus iners* DSM 13335
WHO_F_00101c	NGO0094	OM	Type IV pilus biogenesis and competence protein	PilQ																+	+				+	+	+	+	+	+		+	+		
WHO_F_00249c	NGO0233	OM	NspA protein	NspA																					+	+	+	+	+	+	+				
WHO_F_00279		OM	Lactoferrin binding protein A	LbpA																					+	+	+	+		+	+				
WHO_F_00294c	NGO0275	OM	IgA-specific protease	IgA1																					+	+	+	+	+	+					
WHO_F_00995	NGO0868	OM	OpcA protein	OpcA																					+	+	+			+					
WHO_F_01461	NGO1276	OM	Copper-containing nitrite reductase	AniA																					+	+		+	+	+	+				
WHO_F_01561c	NGO1363	OM	Multidrug transporter	MtrE																			+		+	+	+	+	+	+	+				
WHO_F_01749c	NGO1495	OM	Transferrin-binding protein A	TbpA																					+	+	+	+		+	+				
WHO_F_01750c	NGO1496	OM	Transferrin-binding protein B	TbpB																					+	+	+	+	+	+	+				
WHO_F_02106	NGO1812	OM	Porin	PorB																					+	+	+	+	+	+	+				
WHO_F_02300c	NGO1981	OM	Adhesin complex protein	ACP																					+	+	+	+	+	+	+				
WHO_F_01083	*Multiple	OM	PIIC_3 opacity protein	PiiC_3																					+	+	+	+	+						
WHO_F_01464	*Multiple	OM	PIIC_6 opacity protein	PiiC_6																					+	+	+	+							
WHO_F_01766	*Multiple	OM	PIIC_9 opacity protein	PiiC_9																					+	+	+	+							
WHO_F_02168c	*Multiple	OM	PIIC_11 opacity protein	PiiC_11																					+	+	+	+	+	+					

[#]Color legends for protein expression in cell envelope fraction: Ubiquitous (green), up-regulated (red), down-regulated (blue), and variable (grey). *Multiple protein homologs in *Neisseria gonorrhoeae* FA1090 are detected: NGO0066a, NGO0070, NGO0950a, NGO1040a, NGO1073a, NGO1277a, NGO1463a, NGO1513, NGO1553a, NGO1861a, NGO2060a. Abbreviations: OM: Outer membrane, +: protein homolog detected.

calization of homologous proteins exist. For instance, fHbp is a surface-displayed lipoprotein in *N. meningitidis* but not in *N. gonorrhoeae* (85), whereas BamE is on the surface of gonococci but faces the periplasmic side of the outer membrane in *E. coli* (71). Protein NGO1688, annotated as OmpU, is a putative iron uptake outer membrane protein that is positively regulated by the oxygen-sensing transcription factor, FNR (86). NGO1911a is a predicted pilus assembly protein that is associated with the adhesin PilY (87). Finally, NGO0778, NGO1251 (a putative lipoprotein), and NGO1889 are hypothetical proteins. NGO1889 belongs to the LprI family (PFO7007) that comprises bacterial proteins of ~120 amino acids in length that contain four conserved cysteine residues. LprI from *Mycobacterium tuberculosis* acts as a lysozyme inhibitor (88), providing the exciting possibility that *N. gonorrhoeae* LprI contributes to residual lysozyme resistance observed in gonococci deprived of surface-exposed lysozyme inhibitors SliC and ACP (72, 89). Lastly, NGO2105 contains peptidase S6 (residues 43–310) and autotransporter (residues 1215–1468) domains potentially involved in proteolytic activity and auto-translocation, respectively, suggesting that this is a newly identified autotransporter protein in *N. gonorrhoeae*. In support of this notion, the NGO2105 locus, also known as adhesion and penetration protein or "NEIS1959 (iga2)" in the PubMLST database, encodes IgA2 protease (AidA) and has homologs in other *Neisseria* (Table I) as well as *Haemophilus influenzae* (90).

Together, our investigations yielded nine novel gonorrhea vaccine candidates, including proteins with implications in pathogenesis such as IgA2 (AidA) and LprI, and provided valuable information regarding the expression patterns of previously selected vaccine candidates.

Expression and Homologs of Gonorrhea Vaccine Candidates—We first evaluated expression profiles of extensively studied gonorrhea vaccine candidates including MtrE (91–93), PorB (94, 95), PilQ (96), TbpA (97, 98), Opa (99, 100), and AniA (19, 101–103). MtrE was upregulated in 8 isolates (Table II). Compared with WHO F, PorB was present at similar levels only in WHO G and N and was downregulated in 12 strains. PilQ (96) was ubiquitously expressed in 10 strains, whereas expression of Opa proteins was widely variable, as expected (104, 105). The TbpA level was similar in 8 strains; however, we did not detect TbpB (106). Nor did we detect ACP (107, 108) or OpcA (109, 110) under the standard growth conditions used in our studies, which suggested that they might be specifically regulated. AniA was present at different levels in 7 strains, ubiquitous in five, and upregulated in two isolates. Immunoblotting experiments with anti-AniA antisera corroborated these findings (103). The cellular pool of NspA (111) varied in ten isolates, whereas lactoferrin binding protein LbpA (112) was variable in five strains and was ubiquitous in WHO L and G (Table II).

Strikingly, most of the proteome-derived vaccine candidates showed ubiquitous expression among numerous strains (Table I). SliC, PldA, BamE, BamA, and BamG were ubiquitous in all 15 isolates. Similar results for these proteins were obtained by iTRAQ-MS/MS applied to the proteomic profiling of cell envelopes and outer membrane vesicles (OMVs) isolated from four different strains of *N. gonorrhoeae* (29). Further, LolB, Ng-MIP, NGO1559, and NGO2054 were unvaryingly expressed in at least 12 isolates. Among the novel vaccine candidates identified in our study, LptE, LolB, IgA2, and NGO1251 were found ubiquitous in at least 13 strains. In addition, LprI and NGO0778 were similarly expressed in 12 and 9 isolates, respectively. In support of our proteomics data, immunoblotting analyses demonstrated similar cellular levels of BamA, MetQ, TamA, LptD, NGO2054 (30), BamE-D (71), SliC (72), and BamG (75) in whole cell lysates of the 2016 WHO strains as well as geographically and temporally diverse clinical isolates of *N. gonorrhoeae* from Baltimore ($n = 5$) and Seattle ($n = 13$). Our previous studies showed that PorB, PilQ, BamA-D, SliC, MafA, PldA, MetQ, IgA1 protease, and LptD are cargo proteins present at similar levels in naturally released gonococcal OMVs (29, 71, 72), which further highlights their potential as vaccine antigens considering the success of *N. meningitidis* OMV-based vaccines (26, 113).

Finally, we examined the presence of homologs of the gonorrhea vaccine candidates among non-gonococcal *Neisseria* species, other commensal bacteria (114, 115), and co-infecting microbes (116–119) that inhabit the same ecological niche as *N. gonorrhoeae.* Antigens conserved between these pathogens and preferably not in commensals have the potential to eradicate several sexually transmitted infections, if formulated into a protective vaccine(s). Our comparative analyses showed that all the proteomics-based antigens have homologous proteins in the majority of investigated *N. meningitidis* strains, and none are present in *M. hominis* (Table I). In addition, Ng-MIP-like proteins exist in *C. trachomatis*, *G. vaginalis*, and *P. ruminicola;* BamA and NGO1559 homologs were found in *C. trachomatis* and *P. ruminicola*. MetQ, a methionine transporter (73), was the only proteomics-derived vaccine candidate with homologs across all examined bacteria except for *C. trachomatis*, *M. hominis*, and *P. ruminicola*. Further, we detected protein homologs of PilQ in two *C. trachomatis* strains and MtrE and ZnuD in *P. ruminicola*; these three proteins were absent in commensal species.

Together, our investigations provide pioneering information into newly identified and existing gonorrhea vaccine candidates. We have established each candidate's expression pattern in diverse *N. gonorrhoeae* isolates and identified homologs among other pathogenic and/or commensal bacteria that share the same ecological niche. Stable expression in the WHO gonococcal panel coupled to presence in *N. meningitidis* and co-infecting agents—but rarely in urogenital commensals—further highlights the importance of including these antigens in gonorrhea vaccine(s).

Proteomics Profiling of N. gonorrhoeae Antimicrobial Resistance—Various genome-based AMR determinants have

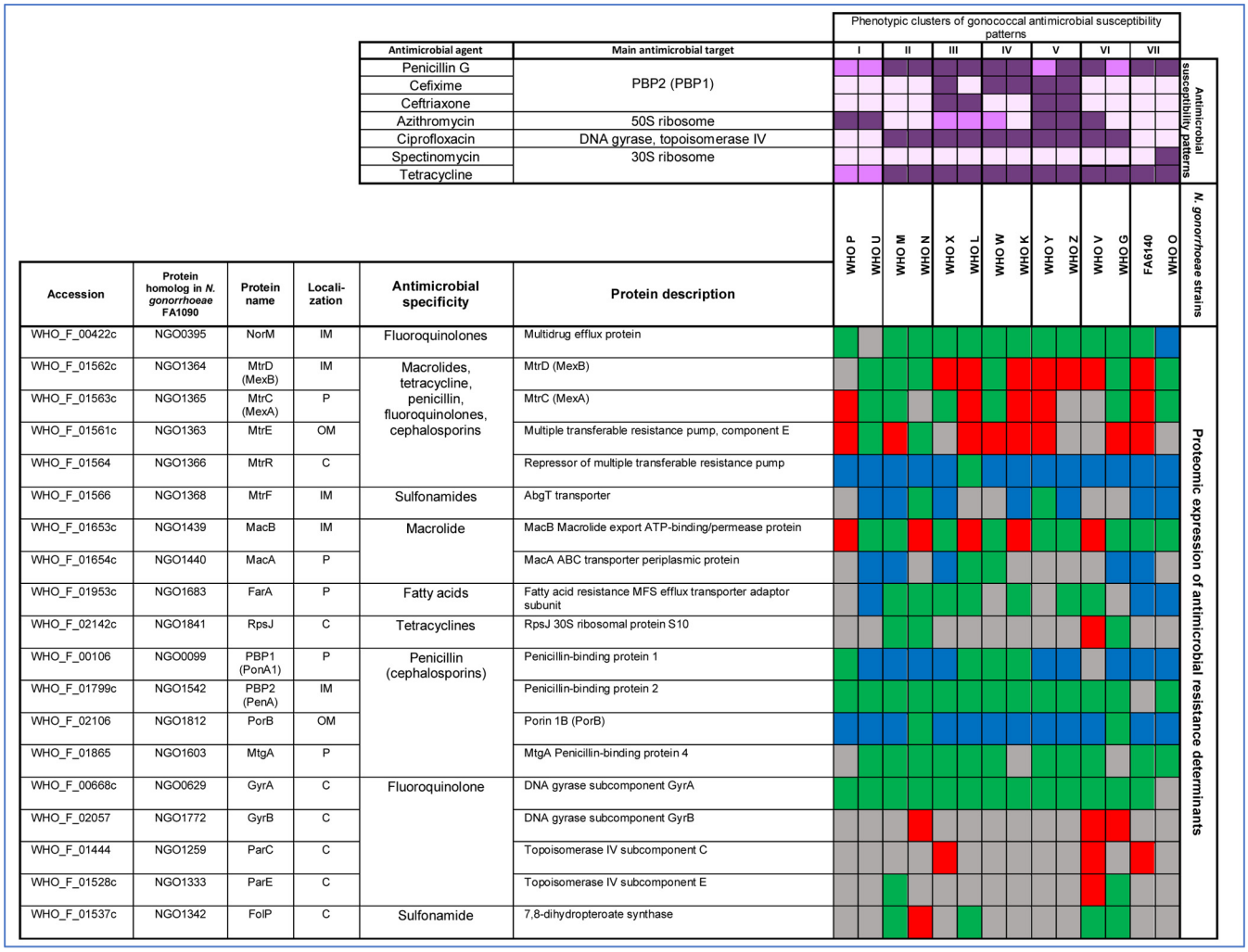
been deciphered in the gonococcus over the past decades (51, 120–125). However, many AMR determinants remain to be identified and characterized, *e.g.* the chromosomally encoded penicillin and cephalosporin resistance determinant "factor X" (126–128) and the AMR mechanisms that contribute to a large proportion of azithromycin resistance (129). The uncertainty behind these AMR determinants illustrates the need for alternative approaches to enhance our understanding of gonococcal AMR complexity. At the proteomic level, only two studies have attempted to address this challenge, both of which used 2D-SDS-PAGE exclusively (47, 130). Therefore, we focused on identifying proteomic AMR signatures that exist in the absence of antimicrobial pressure during standard *in vitro* growth conditions by performing a pairwise comparison of all identified proteins in each individual strain to the WHO F reference strain (Fig. 6, supplemental Tables S1–S2). As expected, we identified different numbers of proteins in the CE and C fractions in each comparison set because of differences in the number of open reading frames (ORFs) between the gonococcal strains (supplemental Table S3). A maximum of 1100 and 876 CE and C proteins were identified, respectively (Fig. 6, supplemental Tables S1–S2). Similarly to our previous analysis, we excluded typical cytoplasmic proteins from the CE subproteome and cell envelope proteins from the C fraction. We solely focused on 398 proteins with significantly different expression in the examined strains compared with the fully antimicrobial-susceptible strain WHO F with the rationale that these proteins may provide clues about the proteomic basis of AMR (Fig. 6). For instance, we identified MtrE as upregulated in many strains with increased resistance to numerous antimicrobials even in the absence of antimicrobial exposure, which represents an upregulation of the multidrug MtrCDE efflux pump and possibly additional efflux pumps for which MtrE acts as the outer membrane channel (29, 92, 131–133). Overall, we identified 162 and 95 proteins with known and unpredicted subcellular locations, respectively (Tables III–V). Peptide counting performed for the latter group of proteins yielded 55 and 36 proteins that are likely localized to the CE and C, respectively, and four proteins with ambiguous localization. Next, we separated 21 proteins that have been previously verified as *N. gonorrhoeae* AMR determinants (Fig. 6, Table III) from new potential proteomic AMR signatures (Tables IV–V).

Proteomic Signature Of Previously Verified Gonococcal Antimicrobial Resistance Determinants—Our proteomic analysis detected subcomponents of all five efflux pumps described in *N. gonorrhoeae, i.e.* MtrCDE, MtrF, FarAB, MacAB, and NorM (Table III). The outer membrane-barrel protein MtrE serves as the channel for the tripartite MtrCDE pump and likely fulfills this same function in the MacAB and FarAB efflux pumps (131, 133). The MtrCDE complex is the most studied efflux pump system in *N. gonorrhoeae*. The multiple transferable resistance (*mtr*) locus contains the *mtrCDE* operon (134) that is negatively regulated by the repressor MtrR (135). Mutations that abrogate *mtrR* activity result in an overexpression of the MtrCDE efflux pump and decreased susceptibility to numerous antimicrobials, *e.g.* macrolides, penicillins, cephalosporins, and tetracycline (53, 133). AMR mutations in the *mtrR* promoter were previously identified in WHO G, K, M, O, P, V, W, X, Y, and Z and within the *mtrR* gene (G45D or a frameshift mutation resulting in a truncated peptide) in WHO K, L, M, P, and W (50), suggesting an overexpression of the MtrCDE efflux pump. Our proteomic profiling also verified that the levels of MtrE were significantly increased or variable in all isolates except for two strains lacking any type of *mtrR* AMR determinant (WHO U and N; Table III). Accordingly, MtrE proved to be an effective indicator of expression of the MtrCDE efflux pump. Our findings were further supported by the downregulation of MtrR in all examined strains except WHO L (Table III). WHO L is also the only examined strain that contains an mtr_{120} mutation, which generates a novel promoter for *mtrCDE* transcription that further enhances the expression of the MtrCDE efflux pump (50, 136). The second efflux system that showed differential expression was the MacAB efflux pump, which can decrease macrolide susceptibility (137). MacA expression varied across the isolates. Expression of the inner membrane component, MacB, was enhanced in the azithromycin resistant strains WHO P and V, but also in the azithromycin susceptible strains WHO N and K, as well as WHO L, which is intermediately susceptible to azithromycin. Interestingly, MacB was the most highly expressed in WHO V, the only strain with high-level azithromycin resistance [MIC$>$256 μg/ml; because of the 23S rRNA A2059G mutation in all four alleles (50)], which indicates that overexpression of the MacAB efflux pump may contribute to the high MICs of azithromycin and other macrolides in WHO V. The FarA component of the FarAB efflux pump system, which exports long-chain fatty acids and other hydrophobic agents (138), was not overexpressed in any of the examined strains and was instead ubiquitously expressed in seven WHO strains (M, N, K, L, X, Z, and V) and downregulated in WHO U, O, and FA6140. Our proteomic profiling also revealed that the NorM and MtrF efflux pumps, which can decrease the susceptibility to fluoroquinolones and sulfonamides, respectively (139, 140), were not overexpressed in any of the examined strains.

Among other established AMR determinants that were differentially expressed was the major porin of *N. gonorrhoeae*, PorB (141, 142), which was downregulated in all strains except for WHO G and N (Table III). This downregulation suggests reduced import of antimicrobials such as penicillins, cephalosporins and tetracyclines, which can contribute to a decreased antimicrobial susceptibility. Furthermore, WHO F, G, and N express PorB1a, which is associated with a lack of the AMR determinant *penB* and consequently high-level chromosomally mediated resistance to penicillins and cephalosporins (125). All other strains express PorB1b. All WHO strains with PorB1b ($n = 11$), except WHO U, contained the AMR determinant *penB*. Consequently, our proteomic data

TABLE III

Proteomic signature of previously verified gonococcal antimicrobial resistance determinants[#]



[#]Color legends for protein expression: Ubiquitous (green), up-regulated (red), down-regulated (blue), variable (grey). Color legends for phenotype against antimicrobial agents are as follows: resistant (dark purple), intermediate susceptible/resistant (purple), susceptible (light purple). Abbreviations: OM: Outer membrane, IM: inner membrane, C: cytoplasmic, P: periplasmic.

suggest that *penB* may also be associated with a decreased expression of PorB1b in addition to the previously documented decreased penetration through PorB1b, resulting in a decreased susceptibility to several antimicrobials. The expression of penicillin-binding protein 1 (PBP1) was significantly downregulated in nine out of the twelve WHO strains that possess the *ponA1* resistance determinant, which encodes a L421P amino acid substitution in PBP1 that contributes to high-level chromosomally mediated penicillin resistance (50). Accordingly, our proteomic data indicate that the decreased PBP1 expression, in addition to the PBP1 L421P amino acid alteration might contribute to high-level chromosomally mediated penicillin resistance. In contrast, PBP2 (the main lethal target for penicillins and cephalosporins) was ubiquitously expressed in 13 of the 14 strains. Similarly, GyrA expression was ubiquitous in 13 of the 14 strains. No association between GyrA expression and the main fluoroquinolone resistance mutations [amino acids S91 and D95 (50)] was identified. GyrB and/or the second fluoroquinolone target, ParC, were overexpressed in the ciprofloxacin-resistant WHO strains G, N, V and X, which may suggest that these strains upregulate GyrB and/or ParC to compensate for the mutated main fluoroquinolone target GyrA (Table III).

New Potential Proteomic-derived Antimicrobial Resistance Signatures—In the CE fraction, two hypothetical proteins predicted to localize to the inner membrane, WHO_F_00238c and WHO_F_01126, were downregulated in all examined strains compared with the antimicrobial-susceptible WHO F strain (Table IV). WHO_F_00238c, which corresponds to NGO0222 in the FA1090 genome, is a small protein with a predicted molecular weight of 8.32 kDa that contains two predicted transmembrane domains but no signal peptide. WHO_F_

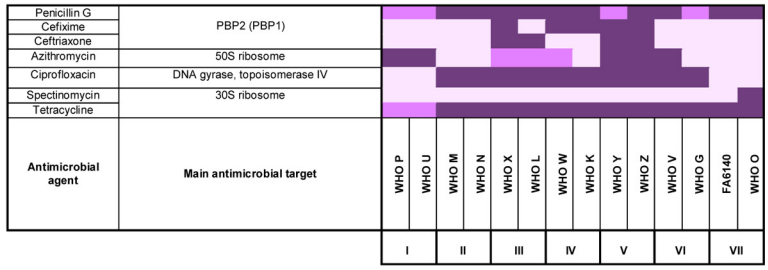
TABLE IV

New potential proteomic-derived antimicrobial resistance signatures with defined subcellular localizations[#]

Accession	Protein homolog in GC FA1090	Localization	Protein description	COG	Function	WHO P	WHO U	WHO M	WHO N	WHO X	WHO L	WHO W	WHO K	WHO Y	WHO Z	WHO V	WHO G	FA6140	WHO O
WHO_F_02461c		E	Putative hemoglobin receptor component HpuA																
WHO_F_00057	NGO0057	IM	Thioredoxin	COG0526OC	Protein turnover														
WHO_F_00073	NGO0071	IM	Lipoprotein signal peptidase II	COG0597MU	Cell wall/membrane/envelope biogenesis														
WHO_F_00089c	NGO0083	IM	Pilin glycosylation protein	COG1086MG	Cell wall/membrane/envelope biogenesis														
WHO_F_00091c	NGO0085	IM	UDP-glucose lipid carrier transferase	COG2148M	Cell wall/membrane/envelope biogenesis														
WHO_F_00139c	NGO0127	IM	Ribonuclease BN-like family	COG1295S	Function unknown														
WHO_F_00155	NGO0143	IM	Sodium/proton antiporter	COG1757C	Energy production and conversion														
WHO_F_00191c	NGO0178	IM	Lipid A core - O-antigen ligase	COG3307M	Cell wall/membrane/envelope biogenesis														
WHO_F_00211	NGO0196	IM	Putative spermidine/putrescine transport system permease,	COG1177E	Amino acid transport and metabolism														
WHO_F_00213c	NGO0198	IM	Ammonia transporter	COG0004P	Inorganic ion transport and metabolism														
WHO_F_00238c	NGO0222	IM	Hypothetical protein																
WHO_F_00246c	NGO0230	IM	Potassium transporter	COG0168P	Inorganic ion transport and metabolism														
WHO_F_00303	NGO0284	IM	Membrane protein																
WHO_F_00311	NGO0291	IM	Potassium/proton antiporter	COG3263P	Inorganic ion transport and metabolism														
WHO_F_00328	NGO0307	IM	Phage T7 F exclusion suppressor FxsA	COG3030R	General function prediction only														
WHO_F_00385	NGO0360	IM	Putative uroporphyrinogen-III C-methyltransferase	COG2959H	Coenzyme transport and metabolism														
WHO_F_00386	NGO0361	IM	Uncharacterized enzyme of heme biosynthesis	COG3071H	Coenzyme transport and metabolism														
WHO_F_00425c	NGO0399	IM	Putative Zn-dependent protease	COG0501O	Protein turnover														
WHO_F_00585	NGO0551	IM	Predicted membrane protein	COG3671S	Function unknown														
WHO_F_00627c	NGO0589	IM	Uracil transporter	COG2233F	Nucleotide transport and metabolism														
WHO_F_00666c	NGO0627	IM	Site-specific recombinase	COG4389L	Replication, recombination and repair														
WHO_F_00696	NGO0656	IM	Oxalate/formate antiporter family transporter	COG2807P	Inorganic ion transport and metabolism														
WHO_F_00816c	NGO0753	IM	Nitrate/nitrite sensor protein	COG3850T	Signal transduction mechanisms														
WHO_F_00996	NGO0869	IM	Hypothetical protein	COG0586S	Function unknown														
WHO_F_01057c	NGO0923	IM	Putative succinate dehydrogenase cytochrome	COG2009C	Energy production and conversion														
WHO_F_01100c	NGO0968	IM	Glutamine transport system permease protein glnP	COG0765E	Amino acid transport and metabolism														
WHO_F_01106c	NGO0974	IM	Lysophospholipid transporter lplT																
WHO_F_01110c	NGO0978	IM	Thiol:disulfide interchange protein DsbD	COG4232OC	Protein turnover														
WHO_F_01126		IM	Hypothetical protein																
WHO_F_01200c	NGO1032	IM	Inner membrane transport protein yajR	COG2814G	Carbohydrate transport and metabolism														
WHO_F_01225c	NGO1059	IM	Membrane protein	COG0861P	Inorganic ion transport and metabolism														
WHO_F_01371	NGO1188	IM	Magnesium transporter	COG2239P	Inorganic ion transport and metabolism														
WHO_F_01381	NGO1198	IM	Phosphoethanolamine transferase eptB	COG2194R	General function prediction only														
WHO_F_01398c	NGO1216	IM	Diacylglycerol kinase	COG0818M	Cell wall/membrane/envelope biogenesis														
WHO_F_01428c	NGO1246	IM	Signal peptide peptidase SppA	COG0616OU	Protein turnover														
WHO_F_01445	NGO1260	IM	Transcriptional regulatory protein ZraR	COG3829KT	Transcription														
WHO_F_01460c	NGO1275	IM	Nitric oxide reductase subunit B	COG3256P	Inorganic ion transport and metabolism														
WHO_F_01535c	NGO1340	IM	DedA-family integral membrane protein	COG0586S	Function unknown														
WHO_F_01568c	NGO1370	IM	Uncharacterized iron-regulated membrane protein	COG3182S	Function unknown														
WHO_F_01572c	NGO1374	IM	Cbb3-type cytochrome oxidase, subunit 1	COG3278O	Protein turnover														
WHO_F_01579	NGO1380	IM	Zn-dependent proteases	COG1994R	General function prediction only														
WHO_F_01621	NGO1410	IM	Inner membrane protein ybaN	COG2832S	Function unknown														
WHO_F_01623	NGO1411	IM	Citrate transporter	COG1055P	Inorganic ion transport and metabolism														
WHO_F_01626	NGO1414	IM	Na( )-translocating NADH-quinone reductase subunit B	COG1805C	Energy production and conversion														
WHO_F_01629	NGO1417	IM	Na( )-translocating NADH-quinone reductase subunit E	COG2209C	Energy production and conversion														
WHO_F_01669c	NGO1455	IM	Manganese transport protein MntH	COG1914P	Inorganic ion transport and metabolism														
WHO_F_01739c	NGO1485	IM	Inner membrane protein ybhI	COG0471P	Inorganic ion transport and metabolism														
WHO_F_01797c	NGO1540	IM	Putative phosphoethanolamine transferase ybiP	COG2194R	General function prediction only														
WHO_F_01811	NGO1552	IM	Proline:sodium symporter PutP	COG0591ER	Amino acid transport and metabolism														
WHO_F_01835c	NGO1574	IM	Phosphatidylglycerophosphatase A	COG1267I	Lipid transport and metabolism														
WHO_F_01979c	NGO1699	IM	Inner membrane protein ypjD	COG4137R	General function prediction only														
WHO_F_01990c	NGO1710	IM	O-acetyltransferase OatA	COG1835I	Lipid transport and metabolism														
WHO_F_01998	NGO1718	IM	Virulence factor MviN	COG0728R	General function prediction only														
WHO_F_02013	NGO1732	IM	Putative multidrug export ATP-binding/permease protein	COG1132V	Defense mechanisms														
WHO_F_02018c	NGO1737	IM	NADH dehydrogenase subunit N	COG1007C	Energy production and conversion														
WHO_F_02019c	NGO1738	IM	NADH dehydrogenase subunit M	COG1008C	Energy production and conversion														
WHO_F_02021c	NGO1740	IM	NADH:ubiquinone dehydrogenase, L subunit	COG1009CP	Energy production and conversion														
WHO_F_02025c	NGO1744	IM	NADH-quinone oxidoreductase subunit H	COG1005C	Energy production and conversion														
WHO_F_02033c	NGO1751	IM	NADH dehydrogenase I subunit A	COG0838C	Energy production and conversion														
WHO_F_02034c	NGO1752	IM	Putative integral membrane protein	COG0421E	Amino acid transport and metabolism														
WHO_F_02035c	NGO1753	IM	Putative integral membrane protein	COG4262R	General function prediction only														
WHO_F_02053	NGO1768	IM	Integral membrane protein	COG1971S	Function unknown														
WHO_F_02078	NGO1787	IM	Na /alanine symporter	COG1115E	Amino acid transport and metabolism														
WHO_F_02091	NGO1798	IM	Phosphatidate cytidylyltransferase	COG0575I	Lipid transport and metabolism														
WHO_F_02174c	NGO1867	IM	Two-component system sensor kinase	COG5000T	Signal transduction mechanisms														
WHO_F_02207c	NGO1900	IM	Membrane protein	COG1297S	Function unknown														
WHO_F_02222	NGO1910	IM	Inner membrane protein yccS	COG1289S	Function unknown														
WHO_F_02262c	NGO1948	IM	Membrane protein	COG2259S	Function unknown														
WHO_F_02267c	NGO1954	IM	Amino acid/peptide transporter (Peptide:H symporter)	COG3104E	Amino acid transport and metabolism														
WHO_F_02308c		IM	Putative cytochrome B561	COG3038C	Energy production and conversion														
WHO_F_02419	NGO2071	IM	Putative integral membrane protein	COG1368M	Cell wall/membrane/envelope biogenesis														
WHO_F_02432	NGO2084	IM	Integral membrane protein	COG0670R	General function prediction only														
WHO_F_02468	NGO2116	IM	ABC-type spermidine/putrescine transport systems	COG1127Q	Secondary metabolites biosynthesis														
WHO_F_02481	NGO2128	IM	Inner membrane protein	COG1807M	Cell wall/membrane/envelope biogenesis														
WHO_F_01084	NGO0952	OM	TonB-dependent heme/hemoglobin receptor family protein	COG1629P	Inorganic ion transport and metabolism														
WHO_F_02460c	NGO2109	OM	Outer membrane cobalamin receptor protein	COG1629P	Inorganic ion transport and metabolism														
WHO_F_00222	NGO0206	P	Spermidine/putrescine ABC transporter	COG0687E	Amino acid transport and metabolism														
WHO_F_00233c	NGO0217	P	ABC-type thiamine transport system	COG1840P	Inorganic ion transport and metabolism														
WHO_F_00242	NGO0225	P	Protein of unknown function																
WHO_F_00268c	NGO0250	P	Cryptic protein cnp1																
WHO_F_00397	NGO0372	P	Lysine-arginine-ornithine-binding periplasmic protein	COG0834ET	Amino acid transport and metabolism														
WHO_F_00650c	NGO0613	P	Hypothetical protein																
WHO_F_00702c	NGO0662	P	Aspartyl/glutamyl-tRNA amidotransferase subunit A	COG0154J	Translation, ribosomal structure and biogenesis														
WHO_F_00717c	NGO0678	P	Lipoprotein																
WHO_F_00728c	NGO0690	P	Lipoprotein																
WHO_F_00809	NGO0747	P	Lipoprotein	COG1729S	Function unknown														
WHO_F_00828	NGO0766	P	Peptidyl-prolyl cis-trans isomerase D																
WHO_F_01000	NGO0873	P	DNA modification methylase	COG0270L	Replication, recombination and repair														
WHO_F_01209c	NGO1044	P	Hypothetical protein																
WHO_F_01214	NGO1049	P	Nickel uptake substrate protein	COG5266P	Inorganic ion transport and metabolism														
WHO_F_01249		P	Putative serotype-1-specific antigen																
WHO_F_01256	NGO1080	P	C-type cytochrome	COG3909C	Energy production and conversion														
WHO_F_01289		P	Predicted transcriptional regulator	COG1396K	Transcription														
WHO_F_01440c	NGO1253	P	Putrescine-binding periplasmic protein	COG0687E	Amino acid transport and metabolism														
WHO_F_01652	NGO1438	P	Probable thiol:disulfide interchange protein DsbC	COG1651O	Protein turnover														
WHO_F_01747	NGO1494	P	Spermidine/putrescine ABC transporter substrate-binding	COG0687E	Amino acid transport and metabolism														
WHO_F_01754	NGO1502	P	N-acetylmuramoyl-l-alanine amidase I	COG0860M	Cell wall/membrane/envelope biogenesis														
WHO_F_01757c	NGO1505	P	Hypothetical protein																
WHO_F_01925c	NGO1655	P	Putative peptidyl-prolyl isomerase																
WHO_F_01926c	NGO1656	P	Cell-binding factor	COG0760O	Protein turnover														

TABLE IV—*continued*

WHO_F_01981	NGO1701	P	Hypothetical protein		
WHO_F_02051	NGO1767	P	Catalase	COG0753P	Inorganic ion transport and metabolism
WHO_F_02387	NGO2056	P	Thiamine transporter substrate binding subunit	COG4143H	Coenzyme transport and metabolism
WHO_F_00060	NGO0059	C	4-hydroxyphenylacetate 3-monooxygenase reductase	COG1853R	Coenzyme transport and metabolism
WHO_F_00082c	NGO0078	C	DNA polymerase III subunit alpha	COG0587L	Replication, recombination and repair
WHO_F_00198c	NGO0185	C	Phosphoribosyl-ATP pyrophosphatase	COG0140E	Amino acid transport and metabolism
WHO_F_00206	NGO0191	C	30S ribosomal protein S15	COG0184J	Translation, ribosomal structure and biogenesis
WHO_F_00288c	NGO0269	C	Amino acid ABC transporter ATP-binding protein	COG1126E	Amino acid transport and metabolism
WHO_F_00434	NGO0407	C	type I restriction-modification system endonuclease	COG0610V	Defense mechanisms
WHO_F_00452c	NGO0424	C	GTP-binding protein EngA	COG1160R	General function prediction only
WHO_F_00481c		C	SodB_2 superoxide dismutase	COG0605P	Inorganic ion transport and metabolism
WHO_F_00610	NGO0573	C	Excinuclease uvr subunit B	COG0556L	Replication, recombination and repair
WHO_F_00635c	NGO0597	C	Ndk nucleoside diphosphate kinase	COG0105F	Nucleotide transport and metabolism
WHO_F_00663	NGO0624	C	Acyl-CoA dehydrogenase	COG1960I	Lipid transport and metabolism
WHO_F_00690c	NGO0650	C	ATP-dependent RNA helicase	COG0513LK	Replication, recombination and repair
WHO_F_00697c	NGO0657	C	Pseudouridine synthase	COG1187J	Translation, ribosomal structure and biogenesis
WHO_F_00718c	NGO0679	C	Isopropylmalate isomerase large subunit	COG0065E	Amino acid transport and metabolism
WHO_F_00745	NGO0701	C	Hypothetical protein		
WHO_F_00840c	NGO0779	C	Homoserine dehydrogenase	COG0460E	Amino acid transport and metabolism
WHO_F_00930c	NGO0803	C	GTPases - translation elongation factors	COG1217T	Signal transduction mechanisms
WHO_F_01222	NGO1055	C	Acyl-CoA hydrolase	COG1607I	Lipid transport and metabolism
WHO_F_01314c	NGO1141	C	Hypothetical protein	COG3680S	Function unknown
WHO_F_01421	NGO1239	C	Hypothetical protein	COG3680S	Function unknown
WHO_F_01425	NGO1243	C	Hgd 3-hydroxyacid dehydrogenase	COG2084I	Lipid transport and metabolism
WHO_F_01585c	NGO07315	C	Uncharacterized protein		
WHO_F_01684c	NGO1473	C	NADPH quinone reductase	COG2249R	Coenzyme transport and metabolism
WHO_F_01759c	NGO1507	C	Holo-ACP synthase	COG0736I	Lipid transport and metabolism
WHO_F_01996	NGO1716	C	Phosphotransferase	COG3178R	General function prediction only
WHO_F_02076	NGO1785	C	Ribonuclease E	COG1530J	Translation, ribosomal structure and biogenesis
WHO_F_02135c	NGO1833	C	50S ribosomal protein L22	COG0091J	Translation, ribosomal structure and biogenesis
WHO_F_02136c	NGO1834	C	30S ribosomal protein S19	COG0185J	Translation, ribosomal structure and biogenesis
WHO_F_02137c	NGO1835	C	50S ribosomal protein L2	COG0090J	Translation, ribosomal structure and biogenesis
WHO_F_02156c	NGO1854	C	50S ribosomal protein L1	COG0081J	Translation, ribosomal structure and biogenesis
WHO_F_02177c	NGO1870	C	Methionyl-tRNA formyltransferase	COG0223J	Translation, ribosomal structure and biogenesis
WHO_F_02198c		C	Restriction endonuclease		DNA repair
WHO_F_02422	NGO2074	C	Ubiquinone biosynthesis O-methyltransferase	COG2227H	Coenzyme transport and metabolism
WHO_F_02437c	NGO2089	C	Hypothetical protein	COG2191C	Energy production and conversion



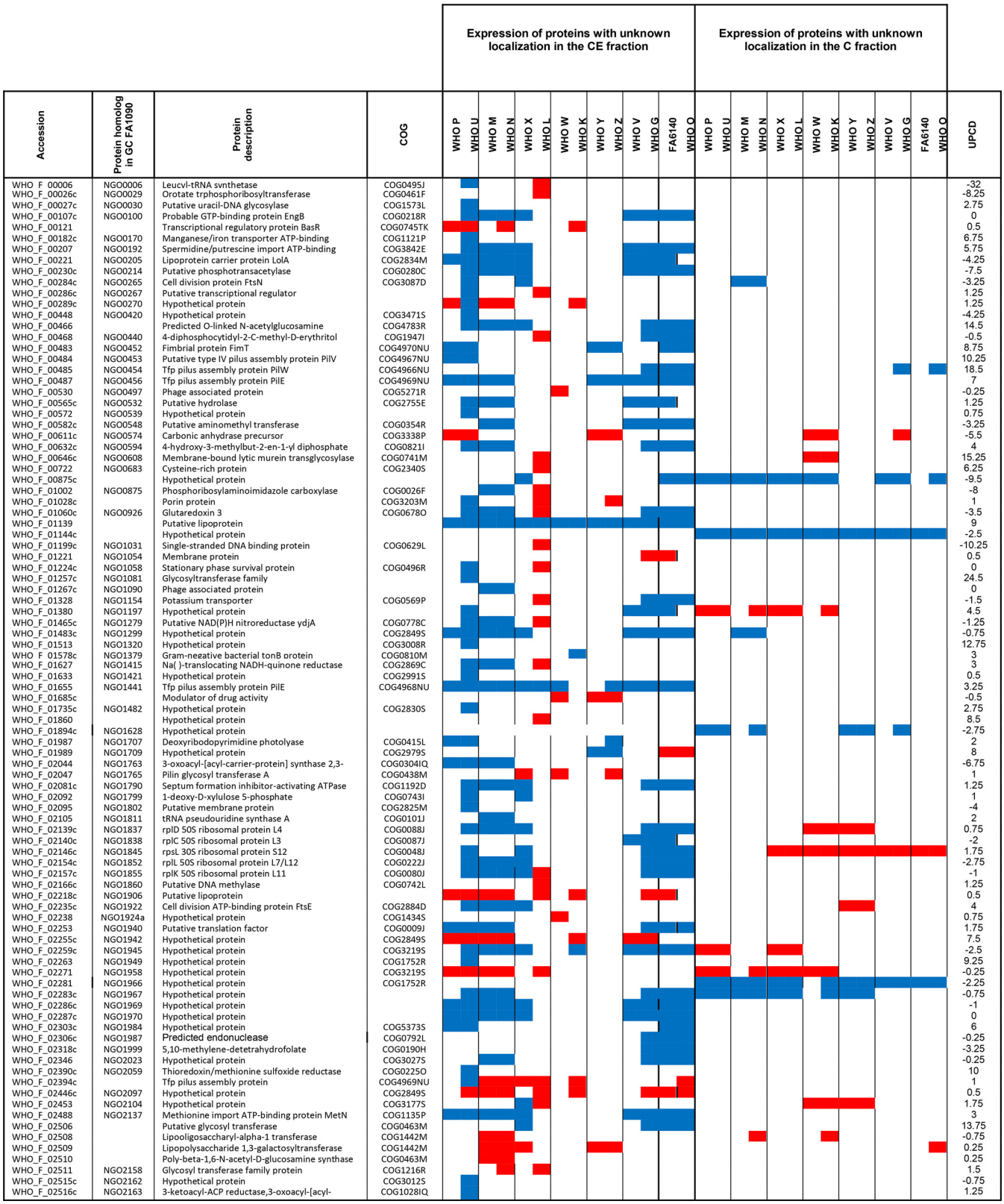
[#]Color legends for protein expression: up-regulated (red), down-regulated (blue), and undetected (white). Abbreviations: OM: outer membrane, IM: inner membrane, C: cytoplasmic, COG: cluster of orthologous genes, PBP: penicillin binding protein. Color legends for phenotype against antimicrobial agents are as follows: resistant (dark purple), intermediate susceptible/resistant (purple), susceptible (light purple).

01126 lacks a homologous protein in FA1090. This is also a small protein (5.39 kDa) with no peptides predicted to be recognized by signal peptidase I or II. In the C fraction, no protein was differentially expressed in all strains, but two cytoplasmic proteins, NGO0597 and NGO0701, were upregulated in all strains except WHO L. NGO0597 is a nucleoside diphosphate kinase (Ndk; 15.4 KDa) involved in DNA and RNA synthesis (143), regulation of gene transcription (144), and peptide chain elongation during translation (145), all processes that are targets for different antimicrobials. NdK is secreted from *Pseudomonas aeruginosa* (144), *M. tuberculosis* (146), *and Leishmania* (147) to modulate interaction with host cells, block phagosome maturation in macrophages (146, 148), and promote host cell apoptosis and necrosis (149). It remains to be investigated whether the gonococcal Ndk is secreted during infection and whether it may serve as an anti-virulence or antimicrobial target. Finally, we detected two proteins with undefined subcellular localization displaying global differential expression. WHO_F_01139 and WHO_F_01144c, which have no homologs in the FA1090 genome, were downregulated in all strains. Our use of UPCD predicted WHO_F_01139 to localize to the cell envelope. WHO_F_01139 is a putative lipoprotein (16.9 KDa) with a predicted signal peptide II domain. Based on UPCD, in addition to the lack of a predicted signal peptide and the absence of transmembrane domains, we predict the hypothetical protein WHO_F_01144c (7.4 kDa) is cytoplasmic. The impact of these six proteins on AMR is yet to be elucidated; however, our data suggest that they may represent general proteomic markers for gonococcal AMR, a predisposition toward developing or compensating for gonococcal AMR, and/or new antimicrobial targets.

Phenotypic Clustering Based on Antibiograms and Common Differentially Expressed Proteins—To link AMR phenotypes with proteomic signatures, we performed phenotypic clustering of gonococcal strains based on their defined antibiograms (50, 53–55, 150) and common differentially expressed proteins (Tables IV–V). We additionally investigated each protein's Cluster of Orthologous Genes (COG) annotations and inferred the functional relevance to the observed phenotypes. These analyses generated seven phenotypic clusters that matched between established and proteome-derived AMR signatures (I-VII; Tables IV–VI).

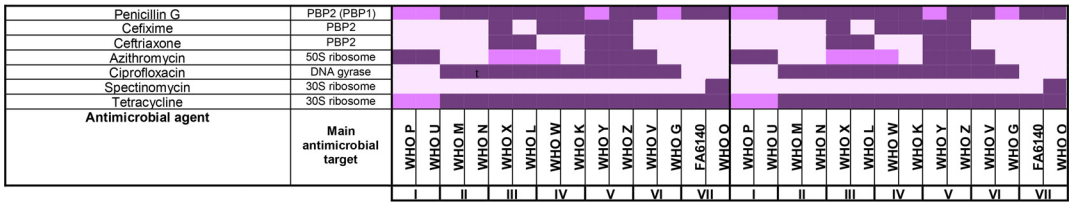
TABLE V

New potential proteomic-derived antimicrobial resistance signatures with undefined localization[#]



Accession	Protein homolog in GC FA1090	Protein description	COG	Expression of proteins with unknown localization in the CE fraction: WHO P	WHO U	WHO M	WHO N	WHO X	WHO L	WHO W	WHO K	WHO Y	WHO Z	WHO V	WHO G	FA6140	WHO O	Expression of proteins with unknown localization in the C fraction: WHO P	WHO U	WHO M	WHO N	WHO X	WHO L	WHO W	WHO K	WHO Y	WHO Z	WHO V	WHO G	FA6140	WHO O	UPCD
WHO_F_00006	NGO0006	Leucyl-tRNA synthetase	COG0495J																												-32
WHO_F_00026c	NGO0029	Orotate trphosphoribosyltransferase	COG0461F																												-8.25
WHO_F_00027c	NGO0030	Putative uracil-DNA glycosylase	COG1573L																												2.75
WHO_F_00107c	NGO0100	Probable GTP-binding protein EngB	COG0218R																												0
WHO_F_00121		Transcriptional regulatory protein BasR	COG0745TK																												0.5
WHO_F_00182c	NGO0170	Manganese/iron transporter ATP-binding	COG1121P																												6.75
WHO_F_00207	NGO0192	Spermidine/putrescine import ATP-binding	COG3842E																												5.75
WHO_F_00221	NGO0205	Lipoprotein carrier protein LolA	COG2834M																												-4.25
WHO_F_00230c	NGO0214	Putative phosphotransacetylase	COG0280C																												-7.5
WHO_F_00284c	NGO0265	Cell division protein FtsN	COG3087D																												-3.25
WHO_F_00286c	NGO0267	Putative transcriptional regulator																													1.25
WHO_F_00289c	NGO0270	Hypothetical protein																													1.25
WHO_F_00448	NGO0420	Hypothetical protein	COG3471S																												-4.25
WHO_F_00466		Predicted O-linked N-acetylglucosamine	COG4783R																												14.5
WHO_F_00468	NGO0440	4-diphosphocytidyl-2-C-methyl-D-erythritol	COG1947I																												-0.5
WHO_F_00483	NGO0452	Fimbrial protein FimT	COG4970NU																												8.75
WHO_F_00484	NGO0453	Putative type IV pilus assembly protein PilV	COG4967NU																												10.25
WHO_F_00485	NGO0454	Tfp pilus assembly protein PilW	COG4966NU																												18.5
WHO_F_00487	NGO0456	Tfp pilus assembly protein PilE	COG4969NU																												7
WHO_F_00530	NGO0497	Phage associated protein	COG5271R																												-0.25
WHO_F_00565c	NGO0532	Putative hydrolase	COG2755E																												1.25
WHO_F_00572	NGO0539	Hypothetical protein																													0.75
WHO_F_00582c	NGO0548	Putative aminomethyl transferase	COG0354R																												-3.25
WHO_F_00611c	NGO0574	Carbonic anhydrase precursor	COG3338P																												-5.5
WHO_F_00632c	NGO0594	4-hydroxy-3-methylbut-2-en-1-yl diphosphate	COG0821I																												4
WHO_F_00646c	NGO0608	Membrane-bound lytic murein transglycosylase	COG0741M																												15.25
WHO_F_00722	NGO0683	Cysteine-rich protein	COG2340S																												6.25
WHO_F_00875c		Hypothetical protein																													-9.5
WHO_F_01002	NGO0875	Phosphoribosylaminoimidazole carboxylase	COG0026F																												-8
WHO_F_01028c		Porin protein	COG3203M																												1
WHO_F_01060c	NGO0926	Glutaredoxin 3	COG0678O																												-3.5
WHO_F_01139		Putative lipoprotein																													9
WHO_F_01144c		Hypothetical protein																													-2.5
WHO_F_01199c	NGO1031	Single-stranded DNA binding protein	COG0629L																												-10.25
WHO_F_01221	NGO1054	Membrane protein																													0.5
WHO_F_01224c	NGO1058	Stationary phase survival protein	COG0496R																												0
WHO_F_01257c	NGO1081	Glycosyltransferase family																													24.5
WHO_F_01267c	NGO1090	Phage associated protein																													0
WHO_F_01328	NGO1154	Potassium transporter	COG0569P																												-1.5
WHO_F_01380	NGO1197	Hypothetical protein																													4.5
WHO_F_01465c	NGO1279	Putative NAD(P)H nitroreductase ydjA	COG0778C																												-1.25
WHO_F_01483c	NGO1299	Hypothetical protein	COG2849S																												-0.75
WHO_F_01513	NGO1320	Hypothetical protein	COG3008R																												12.75
WHO_F_01578c	NGO1379	Gram-negative bacterial tonB protein	COG0810M																												3
WHO_F_01627	NGO1415	Na( )-translocating NADH-quinone reductase	COG2869C																												3
WHO_F_01633	NGO1421	Hypothetical protein	COG2991S																												0.5
WHO_F_01655	NGO1441	Tfp pilus assembly protein PilE	COG4968NU																												3.25
WHO_F_01685c		Modulator of drug activity																													-0.5
WHO_F_01735c	NGO1482	Hypothetical protein	COG2830S																												2.75
WHO_F_01860		Hypothetical protein																													8.5
WHO_F_01894c	NGO1628	Hypothetical protein																													-2.75
WHO_F_01987	NGO1707	Deoxyribodopyrimidine photolyase	COG0415L																												2
WHO_F_01989	NGO1709	Hypothetical protein	COG2979S																												8
WHO_F_02044	NGO1763	3-oxoacyl-[acyl-carrier-protein] synthase 2,3-	COG0304IQ																												-6.75
WHO_F_02047	NGO1765	Pilin glycosyl transferase A	COG0438M																												1
WHO_F_02081c	NGO1790	Septum formation inhibitor-activating ATPase	COG1192D																												1.25
WHO_F_02092	NGO1799	1-deoxy-D-xylulose 5-phosphate	COG0743I																												1
WHO_F_02095	NGO1802	Putative membrane protein	COG2825M																												-4
WHO_F_02105	NGO1811	tRNA pseudouridine synthase A	COG0101J																												2
WHO_F_02139c	NGO1837	rplD 50S ribosomal protein L4	COG0088J																												0.75
WHO_F_02140c	NGO1838	rplC 50S ribosomal protein L3	COG0087J																												-2
WHO_F_02146c	NGO1845	rpsL 30S ribosomal protein S12	COG0048J																												1.75
WHO_F_02154c	NGO1852	rplL 50S ribosomal protein L7/L12	COG0222J																												-2.75
WHO_F_02157c	NGO1855	rplK 50S ribosomal protein L11	COG0080J																												-1
WHO_F_02166c	NGO1860	Putative DNA methylase	COG0742L																												1.25
WHO_F_02218c	NGO1906	Putative lipoprotein																													0.5
WHO_F_02235c	NGO1922	Cell division ATP-binding protein FtsE	COG2884D																												4
WHO_F_02238	NGO1924a	Hypothetical protein	COG1434S																												0.75
WHO_F_02253	NGO1940	Putative translation factor	COG0009J																												1.75
WHO_F_02255c	NGO1942	Hypothetical protein	COG2849S																												7.5
WHO_F_02259c	NGO1945	Hypothetical protein	COG3219S																												-2.5
WHO_F_02263	NGO1949	Hypothetical protein	COG1752R																												9.25
WHO_F_02271	NGO1958	Hypothetical protein	COG3219S																												-0.25
WHO_F_02281	NGO1966	Hypothetical protein	COG1752R																												-2.25
WHO_F_02283c	NGO1967	Hypothetical protein																													-0.75
WHO_F_02286c	NGO1969	Hypothetical protein																													-1
WHO_F_02287c	NGO1970	Hypothetical protein																													0
WHO_F_02303c	NGO1984	Hypothetical protein	COG5373S																												6
WHO_F_02306c	NGO1987	Predicted endonuclease	COG0792L																												-0.25
WHO_F_02318c	NGO1999	5,10-methylene-detetrahydrofolate	COG0190H																												-3.25
WHO_F_02346	NGO2023	Hypothetical protein	COG3027S																												-0.25
WHO_F_02390c	NGO2059	Thioredoxin/methionine sulfoxide reductase	COG0225O																												10
WHO_F_02394c		Tfp pilus assembly protein	COG4969NU																												1
WHO_F_02446c	NGO2097	Hypothetical protein	COG2849S																												0.5
WHO_F_02453	NGO2104	Hypothetical protein	COG3177S																												1.75
WHO_F_02488	NGO2137	Methionine import ATP-binding protein MetN	COG1135P																												3
WHO_F_02506		Putative glycosyl transferase	COG0463M																												13.75
WHO_F_02508		Lipooligosaccharyl-alpha-1 transferase	COG1442M																												-0.75
WHO_F_02509		Lipopolysaccharide 1,3-galactosyltransferase	COG1442M																												0.25
WHO_F_02510		Poly-beta-1,6-N-acetyl-D-glucosamine synthase	COG0463M																												0.25
WHO_F_02511	NGO2158	Glycosyl transferase family protein	COG1216R																												1.5
WHO_F_02515c	NGO2162	Hypothetical protein	COG3012S																												-0.75
WHO_F_02516c	NGO2163	3-ketoacyl-ACP reductase,3-oxoacyl-[acyl-	COG1028IQ																												1.25

TABLE V—*continued*



#Color legends for protein expression: Up-regulated (red), down-regulated (blue). Color legends for phenotype against antimicrobial agents are as follows: resistant (dark purple), intermediate susceptible/resistant (purple), susceptible (light purple). Abbreviations: UPCD: unique peptide count difference; PBP: penicillin binding protein.

Cluster I strains, WHO P and U, exhibit resistance to azithromycin and the majority of upregulated proteins identified were involved in ribosomal biogenesis: 30S ribosomal proteins S15 and S19; 50S ribosomal proteins L1, L2, and L22; the small GTPase EngA; pseudouridine synthase; RNA helicase; and ribonuclease E. In contrast, proteins involved in cell envelope biogenesis - PilE, LolA, and PglB - were downregulated in both strains, which may be associated with the strains' decreased susceptibility to penicillin G.

Cluster II strains, WHO M and N, exhibit resistance to penicillin G, tetracycline, and ciprofloxacin. Upregulated proteins included DNA repair factors (DnaE, a putative type I-site specific deoxyribonuclease NGO0407, and exonuclease UvrB). Proteins involved in amino acid metabolism (NGO0269, NGO0679); and translation (NGO0803, NGO1870) were also upregulated, which suggested strains in Cluster II possess compensatory mechanisms for ciprofloxacin and tetracycline resistance, respectively. Hypothetical proteins represented most downregulated proteins and included WHO_F_00875c, NGO1299, NGO1945, NGO1967, NGO1969, NGO1970, and NGO2089.

Cluster III, IV, and V are comprised of WHO X and L, WHO W and K, and WHO Y and Z, respectively, and exhibit resistance to at least four different antimicrobials, with ciprofloxacin and tetracycline in common (Table IV-V). Three proteins in common between strains in Cluster III and IV were identified. Of these, homoserine dehydrogenase and holo-ACP synthase—involved in amino acid and lipid metabolism, respectively—were downregulated, whereas thioredoxin, which is involved in defense against oxidative stress and protein turnover, was upregulated (151, 152). The NADP quinone reductase (MdaB, modulator of drug activity B) was upregulated in Cluster IV and V strains. In *E. coli*, MdaB protects against polyketide compound toxicity (153), whereas overproduction of this protein defends *P. aeruginosa* from oxidative stress (154).

Strains in Cluster VI (WHO V and G) are resistant to penicillin G (WHO G intermediate susceptible), tetracycline and ciprofloxacin. Differentially regulated proteins in this cluster were strikingly like Cluster II, with 13 proteins in common (Table IV–VI). Seven proteins involved in DNA repair, amino acid metabolism and translation were upregulated, further strengthening a possible compensatory mechanism for the resistance to ciprofloxacin and tetracycline. Six proteins functioning in coenzyme metabolism (NGO2056) and with unknown functions (NGO1299, NGO1945, NGO1969, NGO1970, NGO2089) were downregulated (Table VI).

Finally, the cluster VII strains (WHO O and FA6140), displaying resistance to penicillin G and tetracycline, had eight common differentially expressed proteins (Table VI). Among these proteins, two metabolic coenzymes (NGO0360 and NGO2056) and a putative cytochrome *b*561 involved in energy production were downregulated. This cluster possessed a similar expression profile to strains in Cluster I that are intermediately susceptibility to penicillin G and tetracycline. Finally, NGO2071, a putative integral inner membrane protein; NGO0291, a potassium proton/antiporter; NGO452, annotated as a putative FimT protein; PilW (NGO0454); and PilE were also downregulated in the cluster VII strains (Table VI).

Our proteomic findings elucidate many differentially regulated proteins as potential general proteomic markers for gonococcal AMR, a predisposition toward developing or compensating for gonococcal AMR, and/or new antimicrobial targets, *e.g.* NGO0222, WHO_F_01126, NGO0597, NGO0701, WHO_F_01139, WHO_F_01144c. Deeper analysis of gonococcal proteotypes that relied on AMR-based phenotypic clustering identified additional proteomic markers potentially associated with (or compensating for) AMR in clusters I, II, VI, and VII. Further studies should examine the proteomic profiles of wild type and AMR gonococcal strains during exposure to varying levels of different antimicrobials. In line with this, the expression of eight outer membrane proteins was enhanced in ampicillin resistant *E. coli* strains upon exposure to the minimal inhibitory concentration of ampicillin (33). Additionally, the functional role(s) of the differentially regulated hypothetical proteins potentially involved in gonococcal AMR need to be elucidated, which would help decode the intricate AMR network and promote the design of ways to curb the spread of AMR among *N. gonorrhoeae* strains.

CONCLUSIONS

The present study provides the first global quantitative proteomic characterization of the 2016 WHO *N. gonorrhoeae*

TABLE VI

Common differentially expressed proteins in phenotypically clustered Neisseria gonorrhoeae strains

	Cluster I		Cluster II		Cluster III		Cluster IV		Cluster V		Cluster VI		Cluster VII	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Amino acid metabolism	NGO0185		NGO0269 NGO0679		NGO1752	NGO0779		NGO0779			NGO0185 NGO0269 NGO0679	NGO0192		
Cell envelope biogenesis		NGO0085 NGO0456 LolA												NGO2071
Coenzyme metabolism	NGO2074	NGO2056 NGO0360		NGO2056			MdaB		MdaB	NGO0059	NGO2074	NGO2056		NGO2056 NGO0360
DNA repair		NGO1707	NGO0078 NGO0407 UvrB						WHO_F_02198c		NGO0078 NGO0407 UvrB			
Energy production		WHO_F_02308c								NGO0143				WHO_F_02308
Hypothetical protein	NGO1239	WHO_F_00875c,	NGO1942	WHO_F_00875c		WHO_F_00875c		NGO1141	NGO1239	NGO1628		NGO1299		
	NGO1958	NGO1299	NGO2097	NGO1299						NGO1966		NGO1945		
		NGO1969		NGO1945						NGO7315		NGO1969		
		NGO1970		NGO1967								NGO1970		
		NGO2089		NGO1969 NGO1970 NGO2089								NGO2089		
Inorganic ion metabolism	NGO0952	NGO1059	NGO0952						NGO1411 WHO_F_00481c			NGO2137	WHO_F_00481	NGO0291
		NGO2137	NGO1188											
Intracellular trafficking and secretion				PilE	WHO_F_02394c	PilE						NGO0456		NGO0452 NGO0454 PilE
Lipid metabolism	NGO1243	NGO1763	NGO0624	NGO1507		NGO1507		NGO1507			NGO1243			
	NGO1906		NGO1055 NGO1243					NGO1710						
Protein turnover		NGO0399 NGO1655		NGO0926	NGO0057		NGO0057							
Translation and Ribosomal biogenesis	S15, S19, EngA, RNA helicase, Ribonuclease E L1, L2, L22 , NGO0657		NGO0803 NGO1870								NGO1870			

reference strains (50) and FA6140 (51) to identify new vaccine candidates, gain information about expression of previously identified antigens, and enhance our understanding of AMR in *N. gonorrhoeae*. To our knowledge, this is also the largest quantitative proteomics study performed on bacterial subproteomes to date. Importantly, nine novel vaccine candidates have been identified, significantly broadening the gonorrhea antigen repertoire. Further, expression of 21 previously verified AMR determinants at the proteome level was investigated and six new proteomic signatures were identified that may be associated with AMR or may indicate a strain's likelihood of developing or compensating for the physiological consequences of gonococcal AMR. The proteomic signatures we identified may also represent new antimicrobial targets. Expression patterns of antimicrobial targets and AMR determinants provide proteomic signatures that can complement, verify, and enhance our phenotypic- and genetic-derived understanding of gonococcal AMR complexity. Considering that commensal *Neisseria* species are among the most highly abundant bacteria of the human oropharyngeal microbiota, in addition to the substantial genetic, molecular and phenotypic diversity existing within the *Neisseria* genus, further analyses of the conservation of gonococcal vaccine candidates and AMR determinants in additional commensal *Neisseria* species are crucial.

Cumulatively, our studies provide a wealth of information regarding gonococcal proteomic profiles and will contribute to ongoing efforts in vaccine/drug development as well as elucidation of AMR mechanisms in *N. gonorrhoeae.*

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DATA AVAILABILITY

The raw mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE (66) partner repository with the data set identifier PXD008412.

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** To whom correspondence may be addressed: Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon. E-mail: Aleksandra.Sikora@oregonstate.edu.

§§ To whom correspondence may be addressed: World Health Organization Collaborating Centre for Gonorrhoea and other Sexually Transmitted Infections, Department of Laboratory Medicine, Clinical Microbiology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden. E-mail: magnus.unemo@regionorebrolan.se.

‡‡ Joint senior authors.

Author contributions: A.E.S. designed research with input from M.U.; R.A.Z. performed the experiments; F.E.E. performed computational data analysis; F.E.E., A.E.S., T.W., and M.U. interpreted the data; F.E.E., A.E.S., and M.U. wrote the paper. All authors commented on and approved the final version of the paper.

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