Neisseria gonorrhoeae Virulence Factor NG1686 Is a Bifunctional M23B Family Metallopeptidase That Influences Resistance to Hydrogen Peroxide and Colony Morphology*§

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Background: Deletion of N. gonorrhoeae virulence factor ng1686 results in increased sensitivity to H2O2 and PMN-mediated killing.

Results: NG1686 has endopeptidase and carboxypeptidase activities.

Conclusion: NG1686 is a M23B family zinc metallopeptidase with bifunctional activity.

Significance: This is the first demonstration of a metallopeptidase affecting both resistance to H2O2 and PMN-mediated killing in any bacterium.

Symptomatic gonococcal infection, caused exclusively by the human-specific pathogen Neisseria gonorrhoeae (the gonococcus), is characterized by the influx of polymorphonuclear leukocytes (PMNs) to the site of infection. Although PMNs possess a potent antimicrobial arsenal comprising both oxidative and non-oxidative killing mechanisms, gonococci survive this interaction, suggesting that the gonococcus has evolved many defenses against PMN killing. We previously identified the NG1686 protein as a gonococcal virulence factor that protects against both non-oxidative PMN-mediated killing and oxidative killing by hydrogen peroxide. In this work, we show that deletion of ng1686 affects gonococcal colony morphology but not cell morphology and that overexpression of ng1686 does not confer enhanced survival to hydrogen peroxide on gonococci. NG1686 contains M23B endopeptidase active sites found in proteins that cleave bacterial cell wall peptidoglycan. Strains of N. gonorrhoeae expressing mutant NG1686 proteins with substitutions in many, but not all, conserved metallopeptidase active sites recapitulated the hydrogen peroxide sensitivity and altered colony morphology of the Δng1686 mutant strain. We showed that purified NG1686 protein degrades peptidoglycan in vitro and that mutations in many conserved active site residues abolished its degradative activity. Finally, we demonstrated that NG1686 possesses both Dβ-carboxypeptidase and endopeptidase activities. We conclude that the NG1686 protein is a M23B peptidase with dual activities that targets the cell wall to affect colony morphology and resistance to hydrogen peroxide and PMN-mediated killing.

The obligate human pathogen Neisseria gonorrhoeae (the gonococcus) is the sole causative agent of the sexually transmitted infection gonorrhea, which affects more than 700,000 individuals yearly in the United States and over 88 million worldwide (1). Gonococci infect healthy individuals, causing urethritis in men and cervicitis in women. If left untreated, infection can result in extensive reproductive tract scarring and potentially lead to sterility, pelvic inflammatory disease, and ectopic pregnancy in women (2, 3). Gonococci produce no exotoxins, and this cellular damage is effected by the release of peptidoglycan (PG)6 fragments and lipooligosaccharide, which elicit an inflammatory immune response in the host (4), resulting in damage to host cells (5, 6).

Symptomatic gonococcal infection is characterized by the influx of polymorphonuclear leukocytes (PMNs) to the site of infection. The resulting purulent exudate, consisting almost exclusively of PMNs with attached or internalized gonococci, is the clinical hallmark of a gonococcal infection. PMNs typically kill microorganisms through the combined action of reactive oxygen species (ROS) (e.g. hydrogen peroxide (H2O2), superoxide, and hypochlorous acid) and antimicrobial proteins (e.g. lysozyme, cathepsins, and cationic antimicrobial peptides) (7). Despite this potent two-pronged attack, many gonococci found in the purulent exudate remain viable and cultivable (2), suggesting that the gonococcus has evolved mechanisms to circumvent PMN-mediated killing. Accordingly, the gonococcal genome encodes many antioxidant gene products, some of which may be involved in the resistance to hydrogen peroxide and oxidative stress.
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which have been experimentally shown to detoxify various ROS directly (e.g. catalase, cytochrome c peroxidase, and a manganese-dependent ROS quenching system) (8–11). Other gonococcal gene products repair specific types of cellular damage caused by ROS (e.g. peptide methionine sulfoxide reductase and DNA recombinational repair enzymes) (12, 13). Finally, two efflux pump systems confer resistance to cationic antimicrobial peptides (14, 15) and aid in infection of the murine genital tract (16). A growing body of literature suggests that PMNs kill gonococci primarily by ROS-independent means (16–20). It has additionally been shown that gonococci actively abrogate the oxidative burst of PMNs when growing bacteria predominate in a culture but not when bacteria are non-growing (21). Thus, although gonococci are likely to encounter ROS during human infection and may use ROS production as a signal for late stages of infection, gonococci are relatively unaffected by ROS.

We had previously performed a microarray analysis to detect *N. gonorrhoeae* genes altered in expression in response to the oxidative damaging agent H$_2$O$_2$ for the purpose of identifying novel virulence factors (22). Of the 75 up-regulated genes, several had been previously shown to be important for protection against oxidative damage in gonococci; however, over one-quarter of these genes were predicted to encode proteins with unknown function. Insertional inactivation of a subset of the up-regulated genes (*recN, ng1686*, and *ng554*) revealed that mutant strain Δ*ng554* exhibited increased sensitivity to high levels of H$_2$O$_2$. In contrast, strains *recN* and Δ*ng1686* showed increased sensitivity to both H$_2$O$_2$ and PMN-mediated killing, with strain Δ*ng1686* exhibiting extreme sensitivity to H$_2$O$_2$. Although BLAST searches revealed no characterized homologs of the NG1686 protein, NG1686 contains the active sites of the M23B family of zinc metallopeptidases, and members of this family act to cleave cell wall PG, suggesting that PG could be a substrate of NG1686.

PG is a complex macromolecule that is an essential component of most bacterial cell walls. PG protects cells from osmotic lysis and helps to regulate cell size and shape (reviewed in Ref. 23). Gram-negative PG is composed of glycan chains formed by 1,4-glycosidic bonds. The muramic acid moiety has an amide-linked peptide side chain repeating subunits of N-acetylglucosamine and N-acetylmuramic acid, which are linked by β-1,4-glycosidic bonds. The muramic acid moiety has an amide-linked peptide side chain usually composed of the four amino acids L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP), and D-alanine or the first three of those without the terminal alanine. Interpeptide cross-bridges formed between adjacent side chains cross-link the glycan chains, reinforcing the PG structure. Although the stability of PG is essential for cell viability, PG is also a dynamic structure that undergoes constant remodeling. Accordingly, bacteria contain peptidoglycanases with a variety of substrate specificities that cleave the bacterial cell wall at prescribed locations and times (reviewed in Ref. 23). In addition to roles in cell wall growth, turnover, and cell separation, peptidoglycanases have also been suggested to contribute to bacterial pathogenesis (24, 25) by generating inflammatory PG fragments (5), releasing virulence factors (26), or altering cellular morphology (27, 28).

In this work, we investigated the pleiotropic effects of the Δ*ng1686* mutation on *N. gonorrhoeae*. We show that NG1686 influences the colony morphology but not the cellular morphology of gonococci and that NG1686 is located within the gonococcal periplasm. Gonococcal strains with mutations in some, but not all, conserved M23B active site residues of NG1686 recapitulate the peroxide-sensitive and altered colony morphology phenotypes of the Δ*ng1686* null mutant. Finally, we demonstrate that NG1686 degrades peptidoglycan in vitro and that it possesses both DD-carboxypeptidase and endopeptidase activities. Importantly, NG1686 differs from the classical M23B family of metallopeptidases in certain active site residues, suggesting that it may represent a new family of M23B metallopeptidases. The unique qualities may be key features in the ability of NG1686 to mediate hydrogen peroxide resistance and survival of killing by PMNs.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis of ng1686**

Site-directed mutants of *ng1686* were created using the QuikChange multisite-directed mutagenesis kit (Stratagene) as described. Primers employed to introduce mutations are listed in supplemental Table S1. A plasmid construct containing the *ng1686* gene under control of its endogenous promoter in the vector pCR-Blunt (pBlunt/1686) was used as a substrate to mutate each putative M23B active site residue individually to an alanine, yielding constructs pBlunt/H295A, pBlunt/D299A, pBlunt/H373A, and pBlunt/H375A. Double mutants for each group of putative active sites were also created, yielding constructs pBlunt/H295A/D299A and pBlunt/H373A/H375A. Mutant genes were sequenced to ensure that only the desired mutations were introduced. Mutant *ng1686* gene sequences were released from the vector with a BamHI digest followed by fill-in with Klenow, subsequent digestion with PciI, gel purification with the Qiaquick kit, and cloning into PacI/PmeI-digested vector pGCC5 (29). These resulting mutant *ng1686* genes, under control of their endogenous promoter, were subsequently recombined into an irrelevant ectopic chromosomal locus of strain Δ*ng1686*. All mutant constructs were verified by both Southern blot and PCR amplification with subsequent sequencing of the mutant *ng1686* alleles from the gonococcal chromosome.

**Protein Isolation from N. gonorrhoeae and Western Blot Analysis**

Protein was isolated from gonococci grown on solid medium as described previously (30) or from gonococci grown in liquid as detailed in the supplemental material. Detection by Western blot analysis is detailed in the supplemental material.

**Construction of Regulatable ng1686 Construct in N. gonorrhoeae**

For construction of an IPTG-regulatable *ng1686* construct, DNA beginning 26 bp upstream of the ATG start codon, including the putative RBS of *ng1686*, was PCR-amplified with primers 1686-6 and 1686-5-Pac (supplemental Table S1) using Pfu polymerase. The resulting PCR product was cloned into pCR-Blunt, yielding the construct pCR-Blunt/1686ORF. The *ng1686*-associated DNA was released with a PacI/SalI double slice.
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digest and cloned into PcaI/SpeI-digested plasmid pKH35 (31), yielding construct pKH35/1686ORF. This construct was then recombined into strains FA1090 nv and Δ1686nv, yielding strains FA1090nv/P_{lac}1686 and Δ1686nv/P_{lac}1686. Proper strain construction was confirmed by PCR and Southern blot analysis.

**H₂O₂ Sensitivity Assays**

H₂O₂ resistance assays were performed as described previously (13) but with the modification of growth of *N. gonorrhoeae* detailed in the supplemental material.

**Construction of NG1686 and Related Site-directed Mutant Proteins for Overexpression**

The protein coding sequences of ng1686 and the six site-directed mutants, not including the signal sequences, were amplified by PCR with primers 1686-For-Nhe and 1686-Rev-eco (supplemental Table S1), cloned into pCR-Blunt (Invitrogen), and sequenced to verify that no unwanted mutations had been introduced. The genes were released from these plasmids with a Nhel-EcoRI double digest and ligated to the Nhel-EcoRI sites in pET28a, yielding constructs pET/HIS-1686, pET/HIS-H295A, pET/HIS-D299A, pET/HIS-H295A/D299A, pET/HIS-H373A, pET/HIS-H375A, and pET/HIS-H373A/H375A.

**Anti-NG1686 Antibody Production**

HIS-1686 (NG1686) protein was purified from *Escherichia coli* BL21 (DE3) cells overexpressing pET/HIS-1686 by Creative Dynamics, Inc. (Port Jefferson Station, NY). Anti-1686 polyclonal antibodies were generated in a rabbit and purified using a protein G column by the same company.

**PG Isolation**

From *E. coli*—PG was isolated from *E. coli* strain TOP10 (Invitrogen) using the technique described by Zahrl et al. (32). 1 liter of cells in stationary phase was harvested, washed with 40 ml of 10 mM Tris-HCl (pH 6), and resuspended in 30 ml of the same buffer. The resuspended cells were added dropwise to 300 ml of boiling 4% SDS, followed by an additional 45 min of boiling. PG sacculi were collected by ultracentrifugation at 200,000 × g for 20 min at 20 °C. The resulting pellet was resuspended in 150 ml of 2 M NaCl and incubated overnight at room temperature. After ultracentrifugation, sacculi were washed with water and resuspended in 20 ml of 0.1 mM MgCl₂. The suspension was treated with 50 μg/ml DNase, 50 μg/ml RNase A, and 200 μg/ml α-amylase (Roche Applied Science) for 90 min at 37 °C. Pronase was added to a final concentration of 200 μg/ml and further incubated at 60 °C for 60 min. Enzymes were inactivated by the addition of SDS to 8% final concentration and 15 min of boiling. PG was collected by ultracentrifugation followed by two washes with water. Pellet was resuspended in 5 ml of water, and the yield was quantified by lyophilizing 1 ml of the sample and weighing. Purified PG was stored at −20 °C.

From *N. gonorrhoeae* for Zymogram Analysis, HPLC-based Assays, and LC/MS-based Assays—PG was isolated from *N. gonorrhoeae* strain FA1090 or KH530 (pacA mutant) as described earlier (33) but with the following modifications. The final ultracentrifugation step was eliminated, and the PG preparation was suspended in 500 μl of 25 mM sodium phosphate buffer (pH 6). To inactivate any PG-associated enzymes that may degrade PG, the PG preparation was incubated with either trypsin (5–7.5 μg) or Pronase (60–100 μg) at 37 °C for 2–16 h. To inactivate trypsin and Pronase, the PG was boiled in 25 mM sodium phosphate buffer (pH 6) containing 4% SDS for 1–1.5 h. The PG preparation was centrifuged for 30 min at 30,000 × g at 15 °C, and the pellet was washed five times by suspension in 10 ml of phosphate buffer prior to suspension in 500 μl of 50 mM sodium phosphate buffer (pH 7.5).

**Zymogram Analysis of PG Degradation**

Zymogram analysis was performed adapting protocols described by others (34, 35). *E. coli* BL21 (DE3) cells expressing pET/HIS-1686, the site-directed mutant NG1686 proteins, or purified NG1686 protein were subjected to electrophoresis on a 12% SDS gel containing 0.05% (w/v) *E. coli* murein sacculi, 0.1% (w/v) *N. gonorrhoeae* murein sacculi, or 0.2% (w/v) lyophilized *Micrococcus lyodeikticus* cells (Sigma). Gels were run at 4 °C at 75 V. Following electrophoresis, gels were rinsed with water twice for 30 min each at room temperature with gentle shaking to remove SDS. Gels were transferred to renaturing buffer (0.5% Triton X-100, 25 mM Tris-HCl (pH 7.5)) for 2 × 30 min, and renaturing was continued overnight (~16 h) at room temperature. Fresh renaturing buffer was added, and gels were shifted to 37 °C for 2.5 h. Gels were stained with 0.1% methylene blue dissolved in 0.01% KOH for 60 min and destained with water to visualize zones of PG clearing. Gels were finally stained with Coomassie Brilliant Blue to allow visualization of protein in the gel.

**Solubilization of PG Sacculi**

*N. gonorrhoeae* PG (2 μl, ~140,000 cpm) labeled with [³H]glucosamine was added to 1.5 μg of purified enzyme in 1.2 ml of a 25 mM Tris-HCl (pH 7.5), 0.5% Triton X-100 buffer in the presence or absence of 1 mM phenanthroline. The reaction was incubated at 37 °C. Samples of 200 μl were taken at various time points and added to 500 μl of 20% TCA and 20 μl of unlabeled PG to serve as carrier. Samples were placed on ice for 30 min and then centrifuged for 30 min at 48,000 × g at 4 °C. Peptidoglycan in 500 μl of each soluble fraction was determined by scintillation counting.

**Characterization of NG1686 Reaction Products**

For the HPLC-based assays containing NG1686 and H373A/H375A, reactions contained 50 mM sodium phosphate buffer (pH 7.5), 75 μl of purified FA1090 PG, and 1.5 μM enzyme in a total reaction volume of 150 μl. The reactions were incubated overnight at 37 °C. For the assays of HIS-1686 in the presence of zinc or EDTA, reactions contained 50 mM sodium phosphate buffer (pH 7.5), 25 μl of purified FA1090 PG, 1.5 μM NG1686, and either 0.5 μM ZnSO₄ or 1 mM EDTA in a total reaction volume of 150 μl; the reactions were incubated for 5 h at 37 °C. To stop the reactions, samples were boiled for 10–15 min. The insoluble material was removed by centrifugation, and the supernatants were applied to Centricon 10,000 MWCO spin columns, which had been preswashed with 50 mM sodium phosphate buffer (pH 7.5). HPLC analysis was carried out using a Prevail (Alltech) C18 HPLC column (5 μm, 25 × 4.6 mm).
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Reaction products were separated using a 0–15% gradient of 60% acetonitrile, 0.05% TFA over 60 min at a flow rate of 1 ml/min, and the elution of the products was monitored at 210 nm.

LC/MS analysis of soluble reaction products was performed at the University of Wisconsin-Madison Biotechnology Center. Reaction mixtures analyzed by LC/MS were set up as described above except that 75 µl of FA1090 PG was used. The filtered reaction products were analyzed at the University of Wisconsin-Madison Biotechnology Center using a Zorbax SB-C18 column (1.8 µm, 2.1 × 50 mm) run on an Agilent 1200 HPLC with a linear gradient of 99.9% water, 0.1% formic acid to 99.9% acetonitrile, 0.1% formic acid over 60 min at a flow rate of 0.25 ml/min. Peaks were analyzed using an Agilent LC/MSD TOF using electrospray ionization in positive ion mode.

Analysis of NG1686 Reaction Products Digested with Mutanolysin

Reaction mixtures contained 50 mM sodium phosphate buffer (pH 7.5), 75 µl of purified KH530 PG, 1.5 µM NG1686, and 0.5 µM ZnSO4 in a total reaction volume of 200 µl. The reactions were incubated on a rotator overnight at 37 °C. NG1686 was heat-killed by boiling for 5 min, and the insoluble material was removed by centrifugation. The soluble products were pooled and filtered using a Centricon 10,000 MWCO spin column, and the filtrate was divided into two aliquots. To one sample, 10 µl of a stock solution of mutanolysin (1 mg/ml) was added; to the second sample, 10 µl of water was added as a control. The reaction mixtures were incubated on a rotator at 37 °C for 2 h and then filtered using a Centricon 10,000 MWCO spin column. LC/MS analysis was performed following the same protocol described above.

Determination of Peptidoglycan Cleavage Site

Reaction mixtures contained 50 mM sodium phosphate buffer (pH 7.5), 75 µl of purified FA1090 PG, 1.5 µM NG1686, and 0.5 µM ZnSO4 in a total reaction volume of 200 µl. Control reactions containing H373A/H375A or EDTA (1 mM) were also set up in parallel, as was a reaction lacking PG. Following overnight incubation at 37 °C, the samples were boiled for 5 min to stop the reactions, and the soluble products were filtered using a Centricon 10,000 MWCO spin column. The reaction mixtures were incubated on a rotator at 37 °C and then filtered using a Centricon 10,000 MWCO spin column. To each sample, 25 µl of 10% K2B7O4 (pH 9) and 0.5 µl of FDNB were added in a total reaction volume of 250 µl, and the reactions were incubated at 65 °C for 30 min in the dark. DNP products were dried under vacuum, resuspended in 30% acetonitrile, and analyzed by HPLC as described above for the cleavage site experiment. Peaks of interest were collected, dried under vacuum, and analyzed by ESI-MS in negative ion mode at the University of Wisconsin-Madison Biotechnology Center.

RESULTS

NG1686 Is a Periplasmic Protein—NG1686 has a canonical signal sequence (residues 1–31) and a putative signal peptide cleavage site, suggesting that it is secreted across the cytoplasmic membrane. Kye-Doolittle hydropathy analysis predicted that NG1686 is largely a hydrophilic protein, apart from a short hydrophobic region at the N terminus of the protein corresponding to the signal sequence (data not shown) (39). Additionally, NG1686 lacks a canonical lipobox motif (see the LipoServer Web site), indicating that the protein is not likely to be membrane-associated. To experimentally determine the location of NG1686, strains FA1090 and FA1090Δng1686 (referred to as Δ1686 or Δng1686), which contains a deletion of the ng1686 gene, and the complement strain Δ1686/1686+ (22) were grown in liquid medium, the supernatant and total cellular protein fractions were isolated, and a Western blot analysis was performed. A ~46 kDa band of equal intensity was detected from cell extracts of strains FA1090 and Δ1686/1686+ but not from cell extracts of strain Δng1686 (Fig. 1), demonstrating that the complement strain expresses equivalent amounts of NG1686 as the parent strain FA1090. Moreover, no NG1686 protein was observed in the concentrated supernatant fractions from strain FA1090 or Δ1686/1686+ (data not shown). The presence of a signal sequence combined with a lack of detectable protein in the culture supernatant strongly suggests that the NG1686 protein is located in the periplasm.

Deletion of ng1686 Affects Colony Morphology but Not Cell Morphology or Sensitivity to Cell Wall-targeting Antibiotics—We previously noted that the Δng1686 mutant exhibits a larger and flatter colony morphology relative to strain FA1090 (22), which is complemented in strain Δ1686/1686+ (Fig. 3A) (data not shown). Because NG1686 encodes a protein with sequence similarity to the M23B family of endopeptidases, many of which act to degrade cell walls, we sought to determine whether the
FIGURE 1. Western blot analysis of NG1686 protein expression in parent, mutant, and complement strains. SDS-polyacrylamide gels containing 10 μg of total cellular protein per lane of strains FA1090, Δng1686, and Δng1686/1686+ were run and transferred to PVDF membrane, with subsequent Western blot analysis using anti-1686 antiserum in the ECL Plus detection kit.

overall cell morphology or cell size of the Δng1686 mutant was altered. Transmission electron microscopy of strains FA1090 and Δng1686 was used to visualize the size of individual cells grown on solid medium. Measurement of >100 individual cells of each strain revealed no difference in cell size between cells of the two strains (data not shown). Moreover, no qualitative difference in the overall morphology of individual cells was noted in the transmission electron microscopy analysis, suggesting that NG1686 does not affect septum formation or cell separation (data not shown).

Mutation of ng1686 does not affect the general cellular permeability of gonococci because resistance to the oxidative damage agents paraquat and diamide and the antibiotics naladixic acid, chloramphenicol, and streptomycin is not altered in the Δng1686 mutant strain (22). To specifically test the resistance of strains FA1090, Δng1686, and Δng1686/1686+ to antibiotics that act on the cell wall, we measured the minimum inhibitory concentration of these three strains to the antibiotics ampicillin, cephalothin, ceftazidime, vancomycin, and polymyxin B (data not shown). All three strains showed identical resistance profiles to these antibiotics. These results suggest that NG1686 does not affect septum formation or cell separation (data not shown).

NG1686 Does Not Act to Detoxify H2O2—We have previously observed that deletion of ng1686 in the gonococcus results in increased sensitivity to H2O2 (22), which could suggest that NG1686 acts to detoxify H2O2. Therefore, we tested whether overexpression of NG1686 was able to decrease the sensitivity of a strain to H2O2. An IPTG-inducible version of ng1686 was created by cloning the ng1686 binding site into plasmid pH35 (31) under control of the lac regulatable promoter regulatory sequences, resulting in NG1686 protein expression that is dependent on the addition of IPTG. This construct was then recombined into an ectopic locus in the chromosome of strain FA1090nv and the corresponding Δng1686 mutant strain, Δng1686nv (the subscript “nv” in the designation indicates that a strain cannot undergo pilin antigenic variation (40)), yielding strains FA1090nv/Δlac1686 and Δng1686nv/Δlac1686, respectively. A 2–3-fold increase in NG1686 protein levels was observed in strain FA1090nv/Δlac1686 (designated Δng1686+, in the absence of IPTG (~IPTG) is statistically the same as strain Δng1686nv at 20 and 50 mM H2O2 doses (*, p < 0.05) and statistically different from strain FA1090nv (†, p < 0.04) at the same doses by Student’s t test. Indicated strains are statistically the same as strain FA1090nv at all doses (‡, p > 0.05).
2–3 times more NG1686 protein than strain FA1090, (Fig. 2). Taken together, these results indicate that low level sensitivity to \( \text{H}_2\text{O}_2 \) is mediated by extremely small amounts of NG1686 and that overexpression of NG1686 does not result in decreased sensitivity to \( \text{H}_2\text{O}_2 \). These results suggest that NG1686 does not directly detoxify \( \text{H}_2\text{O}_2 \) to influence survival.

**Subset of M23B Active Site Residues Is Required for NG1686 Phenotypes in Gonococcus**—The NG1686 protein contains conserved active sites of the M23B family of zinc metallopeptidases, as classified by the MEROPS peptidase database. The active site residues of this family occur in the motifs HXXXD and HXXH, with the histidine shown in boldface type serving as the catalytic residue (41), and these residues correspond to amino acids 295–299 and 373–375 of NG1686. To test the contribution of these M23B active sites to NG1686 functions, the DNAs encoding each of these residues were mutated individually and in combination to encode alanine residues, and the mutant genes were subsequently recombined into strain H373A/H375A. We then tested whether the mutant genes were able to restore the parental colony morphology and \( \text{H}_2\text{O}_2 \) sensitivity to strain \( \Delta \text{ng1686} \). Strains D299A, H295A/D299A, H375A, and H373A/H375A recapitulated the altered colony morphology of strain \( \Delta \text{ng1686} \) whereas the colony morphology of strains H373A and H295A recapitated that of the complement strain \( \Delta \text{ng1686}/1686^+ \) (Fig. 3A). The \( \text{H}_2\text{O}_2 \) sensitivity phenotype of the mutant strains mirrored the observed colony morphologies, with strains D299A, H295A/D299A, H375A, and H373A/H375A showing statistically the same \( \text{H}_2\text{O}_2 \) sensitivity as strain \( \Delta \text{ng1686} \) and strains H373A and H295A showing statistically the same sensitivity as the complement strain \( \Delta \text{ng1686}/1686^+ \) (Fig. 3B). These results indicate that the His-295 and His-373 residues of the NG1686 protein are not essential for activity and therefore suggest that NG1686 may have activities different from those of other M23B family proteins.

Western blot analysis of NG1686 protein levels in the \( \Delta \text{ng1686}/1686^+ \) and six point mutant strains revealed similar levels of NG1686 protein in all strains. The largest difference was in strain H373A/H375A, which showed 3-fold less protein than strain \( \Delta \text{ng1686}/1686^+ \) (Fig. 3C). Because strain \( \Delta \text{ng1686} \) expressed \( \sim 70 \) fold less NG1686 protein than strain FA1090, yet still showed \( \text{H}_2\text{O}_2 \) sensitivity (Fig. 2), the small differences in protein levels are not responsible for the observed differences in \( \text{H}_2\text{O}_2 \) sensitivity and colony morphology. Taken together, these data show that some, but not all, of the defined M23B active site residues are required for NG1686 function in gonococci.

**NG1686 Depgrades *E. coli* and *N. gonorrhoeae* PG in Vitro**—There were no consistent changes in protein profiles recorded between the parent strain FA1090 and the \( \Delta \text{ng1686} \) mutant in a two-dimensional gel analysis (data not shown), and several active site residues required for the \( \Delta \text{ng1686} \) phenotypes corresponded to the M23B clade of metallopeptidase, indicating that the target of NG1686 could be peptidoglycan.

To determine whether PG is a target of NG1686, we assayed the hydrolytic activity of NG1686 using zymogram gels containing PG. *E. coli* bacterial cell lysates overproducing NG1686 and each of the site-directed mutant proteins as well as the purified NG1686 protein were run on zymogram gels containing PG purified from either *E. coli* (Fig. 4, A and D) or *N. gonorrhoeae* (Fig. 4C). We observed zones of clearing that corresponded to the location of the NG1686 protein (Fig. 4), as determined by subsequent staining of the gel with Coomassie Blue (Fig. 4, B and E), as well as to lysozyme, which was added to lyse the bacterial cells for cell extracts (Fig. 4, A, C, and D). In contrast, zymogram gels containing desiccated *Micrococcus lysodeikticus* did not show zones of clearing dependent on NG1686 protein but did for lysozyme, indicating that this...
Gram-positive PG is not cleaved by the NG1686 protein (data not shown). There are several factors that could contribute to the comparatively low activity of NG1686 observed relative to that of lysozyme. First, the site of PG cleavage (e.g., PG backbone or PG cross-links) influences the zone size that is created by peptidoglycanase activity. Because lysozyme degrades the PG backbone, the resulting PG monomers can easily diffuse out of the gel. Second, NG1686 has a molecular mass that is about 3-fold larger than lysozyme, and this may impede its diffusion in the gel; third, the efficiency of protein renaturation within the zymogram gel also influences zone size.

To demonstrate the relative abilities of the mutant proteins to cleave *E. coli* PG, we ran differing amounts of cell lysates on zymogram gels (Fig. 4, D and E). *E. coli* lysates carrying construct pET/HIS-D299A, pET/HIS-H295A/D299A, or pET/HIS-H373A/H375A showed no zone of clearing at the location of NG1686 protein migration (Fig. 4, D and E); lysates carrying construct pET/HIS-H295A or pET/HIS-H373A showed less robust zones of clearing relative to the pET/HIS-NG1686 lysate; and the pET/HIS-H375A lysate showed an extremely faint zone of clearing at the location of the protein (Fig. 4D). We attribute the additional bands with PG degradative activities to proteins present in *E. coli* because they directly correlate with the amount of lysate loaded (Fig. 4, D and E) and are not present in the lanes containing purified protein (Fig. 4A). Identical results were observed for zymogram gels containing gonococcal PG (Fig. 4C).

To further demonstrate the ability of NG1686 to degrade PG, we performed PG sacculi solubilization studies using purified NG1686 protein or the double point mutant H373A/H375A protein. NG1686 converted radiolabeled insoluble gonococcal sacculi to soluble PG fragments, whereas the mutant H373A/H375A protein lacked this ability (Fig. 5). The soluble PG fragments released from gonococcal sacculi by NG1686 were further analyzed by LC/MS. This enabled detection of 22 reaction products with masses consistent with PG fragments, nine of which were the most abundant (Fig. 6 and Table 1). Importantly, these products were absent in NG1686 reactions containing the zinc chelating agent EDTA or phenanthroline (supplemental Figs. S2 and S3) and reactions containing the mutant H373A/H375A enzyme (supplemental Fig. S1). We have shown
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**DISCUSSION**

This study reveals the ability of the *N. gonorrhoeae* virulence factor NG1686 to degrade PG and demonstrates that NG1686 belongs to the M23B family of zinc metallopeptidases. We show that NG1686 is a bifunctional enzyme, possessing both endopeptidase and δδ-carboxypeptidase activities (Fig. 9). We therefore conclude that the lack of NG1686 PG degradative activity in the Δng1686 mutant manifests itself in the varied phenotypes seen in this strain, specifically altered colony morphology, extreme sensitivity to H₂O₂, and sensitivity to PMN-mediated killing. This is the first suggestion that a PG-degrad-

...
ing enzyme has roles in resistance to H$_2$O$_2$ and PMN-mediated killing.

The most well studied members of the M23B family of zinc metallopeptidases are LytM and lysostaphin, both of which cleave the pentaglycine cross-bridges found in staphylococcal PG (41). The NG1686 protein clearly belongs to this family, possessing all of the active site residues of this family (HXXXD, HXH), demonstrating the ability to degrade PG from both *E. coli* and *N. gonorrhoeae* in vitro, and being sensitive to metal chelation by EDTA or phenanthroline (supplemental Figs. S2 and S3). However, NG1686 must differ fundamentally from these well characterized M23B class endopeptidases because neither *E. coli* nor *N. gonorrhoeae* PG contains pentaglycine cross-bridges and because NG1686 does not require the HXH motif for activity. The crystal structure of LytM, an M23B auto-

### TABLE 2

| Peak | Calculated mass | Observed mass (Da) | 1686 + mutanolysin | 1686 + water |
|------|----------------|--------------------|--------------------|--------------|
| Monomers |                  |                    |                    |              |
| Tetra' (anh$^a$) | 850.3 | 850.4 | 850.4 | |
| Tetra (red$^b$) | 921.4 | 921.4 | 921.4 | |
| Tetra (OAc) (red) | 939.4 | 939.4 | ND$^a$ | |
| Penta (anh) | 981.4 | 981.4 | ND | |
| Penta (red) | 992.4 | 992.4 | ND | |
| Penta (OAc) (red) | 1010.4 | 1010.4 | ND | |
| Dimer |                  |                    |                    |              |
| Tetra-tri (anh) or tri-tetra (anh) | 1771.7 | ND | 1771.8 | |
| Tetra (OAc)-tri (anh) or tri (OAc)-tetra (anh) | 1813.7 | ND | 1813.7 | |
| Tetra (anh) | 1842.8 | ND | 1842.8 | |
| Tetra (OAc)-tetra (anh) | 1884.8 | ND | 1884.8 | |
| Penta-tetra (anh) or tetra-penta (anh) | 1913.8 | ND | 1913.8 | |
| Penta (OAc)-tetra (anh) or tetra (OAc)-penta (anh) | 1955.8 | ND | 1955.8 | |
| Trimer |                  |                    |                    |              |
| Tetra (OAc)-tetra or (OAc)-tetra (anh) | 2848.2 | ND | 2848.2 | |

$^a$ Tri, disaccharide tripeptide.
$^b$ anh, 1,6-anhydromuramic acid.
$^c$ Tetra, disaccharide tetrapeptide.
$^d$ red, N-acetylmuramic acid with reducing end.
$^e$ ND, not detected.
$^f$ Penta, disaccharide pentapeptide (disaccharide = N-acetylglucosamine-N-acetylmuramic acid).

**FIGURE 7.** HPLC analysis of FDNB-derivatized reaction mixtures containing NG1686 (A), H373A/H375A (B), or NG1686 + EDTA (C) and NG1686 reaction lacking PG (D). The arrows indicate elution products that were collected, dried, and analyzed by ESI-MS. Based on ESI-MS and the elution of standards, the first arrow corresponds to the mono-DNP-DAP; the second arrow corresponds to DNP-alanine. mAU, milliabsorbance units.

**FIGURE 8.** HPLC analysis of FDNB-derivatized reaction mixtures containing D-alanine (A), MurNAc-pentapeptide + NG1686 (B), or MurNAc-pentapeptide + H373A/H375A (C) and NG1686 reaction lacking substrate (D). The arrows indicate the elution products corresponding to DNP-alanine. mAU, milliabsorbance units.

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lysin from *Staphylococcus aureus*, identifies the His and Asp residues of the HXXXD motif and the second His of the HXH motif as ligands for the Zn$^{2+}$ ion; the first His of the HXH motif is the catalytic residue (41). Mutagenesis of these active sites completely abolishes the activity of the LytM protein (41). In contrast, mutation of the analogous residues in the NG1686 protein revealed that only the Asp-299 residue is absolutely critical for activity in vitro, whereas mutation of the His-295, His-373, and His-375 residues resulted in decreased activity (Fig. 4). These results demonstrate that the His-373 residue is not the catalytic residue and suggest that NG1686 is a structurally distinct member of the M23B family of metallopeptidases.

The recent elucidation of the crystal structure of the NMB0315 protein from *N. meningitidis* (98% sequence iden-
tity; 99% sequence similarity to the NG1686 protein from *N. gonorrhoeae*) allowed us to interpret the site-directed mutant data of NG1686 in the context of the newly solved three-dimensional structure of this meningococcal protein (42). In the crystal structure of NMB0315, the metal ion is coordinated by three spatially adjacent residues (His-295, Asp-299, and His-375) and two water molecules. Residue His-373 interacts with the metal ion through one of the water molecules (42). Consistent with the crystal structure, mutation of residues His-295, Asp-299, His-373, and His-375 in NG1686 resulted in decreased or no observed activity of NG1686 by zymogram (Fig. 4), although the H295A and H373A mutants were able to mediate the colony morphology and H2O2 resistance phenotypes of *N. gonorrhoeae* as well as the parental NG1686 protein (Fig. 3). Interestingly, although the domain structure of NMB0315 suggests that NMB0315 is in an autoinhibited conformational state (42), our *in vitro* data argue either for the lack of autoinhibition or autoinhibition that is overcome upon the addition of appropriate substrate (e.g. PG or a peptide) with no need for further processing of the NG1686 to activate the protein.

The HdpA protein from *Helicobacter pylori* was recently shown to also carry dual carboxypeptidase and endopeptidase activities (28). Whereas site-directed mutagenesis of one of the predicted active site residues (H259A) of HdpA disrupted activity of HdpA, the analogous H295A mutation of NG1686 had little effect on NG1686 phenotypes. Therefore, although NG1686 and HdpA may share dual activities, the residues responsible for activity differ between the proteins. The two other known gonococcal PG endopeptidases, PBP3 and PBP4, also have both endopeptidase and DD-carboxypeptidase activity, although they are not members of the M23B family (43, 44). It is unknown what factors determine which activity of NG1686 is performed or what the mechanism is behind the dual action of NG1686. Therefore, it would be interesting to both determine the crystal structure of NG1686 in the context of its different substrates and separately test the effect of the mutations on the endopeptidase and DD-carboxypeptidase activities of NG1686 *in vitro*.

The large colony morphology phenotype of the Δng1686 mutant is the easiest to reconcile with the demonstrated peptidoglycanase activities of NG1686. The loss of a protein that acts on PG has previously been shown to affect both the colony morphology (45, 46) and the cellular morphology of gonococci (45, 47). Much recent work has sought to define the cellular roles of these M23B/LytM family endopeptidase enzymes in a variety of other Gram-negative bacteria (27, 48–51). Several M23B family proteins function in the cleavage of septal PG to allow for efficient cell separation (51–54). In *H. pylori*, four M23B family proteins have been found to affect PG cross-linking, contributing to the helical curvature of this bacterium and its ability to colonize the stomach; however, these proteins do not affect cell separation in *H. pylori* (27, 28). In contrast, the ng1686 mutant does not show an altered cellular morphology or cell separation defect. Moreover, HPLC analysis of the PG from strains FA1090 and Δng1686 revealed no discernable differences in the type or amount of PG components in mutanolysin-digested PG isolated from strain FA1090 or Δng1686 (supplemental Fig. S5), indicating that NG1686 does not affect PG structure overall.

Our current hypothesis is that NG1686 causes subtle, localized, changes in the PG structure. Localized actions of peptidoglycanases have been proposed to explain the requirement of specific peptidoglycanases for the function of various trans-envelope machines, such as flagella or type III or IV secretion systems (55–57). None of these are present in *N. gonorrhoeae* strain FA1090, so if NG1686 is acting in the localized degradation of PG, it is unclear what the localized breaks in the PG would facilitate.

There are several hypotheses that explain how the lack of PG degradative ability results in the susceptibility of the Δng1686 strain to both H2O2 and PMN-mediated killing. The most con-
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cise explanation has both phenotypes resulting from the NG1686 protein acting on a common substrate. Therefore, the simplest hypothesis is that deletion of ng1686 affects the overall permeability of the cell wall. If this were the case, the ∆ng1686 strain should be more susceptible to killing by a variety of chemicals. However, we previously only observed increased susceptibility to killing by H2O2 and the inorganic peroxide cumene hydroperoxide (22) and not to other oxidants or antibiotics, suggesting that small defects in PG structure in the ∆ng1686 mutant result in discernable phenotypes only for peroxide and PMN-mediated killing. An alternate hypothesis is that NG1686 has more than one cellular target and that the phenotypes of altered colony morphology and increased sensitivity to H2O2 and PMN-mediated killing result from the action of NG1686 on different substrates. Because the results of a two-dimensional gel electrophoresis analysis performed on total cell lysates from strains FA1090 and ∆ng1686 (data not shown) revealed no gross differences in the protein profiles of the two strains resulting from the loss of ng1686, we do not favor this hypothesis. Therefore, our preferred hypothesis is that NG1686 acts exclusively on PG in the gonococcus.

Criss et al. (20) has confirmed that N. gonorrhoeae is neither affected by oxidative killing mechanisms of PMN (17, 18) nor protected by its antioxidant gene products against killing by PMNs (19), further suggesting that PMNs kill gonococci exclusively through non-oxidative factors, such as cationic antimicrobial peptides or degradative enzymes (14, 16). The ∆ng1686 mutant is more susceptible to non-oxidative killing by PMNs than the parent strain FA1090 (20, 22). Moreover, this increased sensitivity is only manifest in the ∆ng1686 bacterium located extracellularly, not ∆ng1686 bacteria that are internalized by PMNs (20), suggesting that exocytosed PMN factors, such as antimicrobial peptides or DNA nets, are responsible for the enhanced susceptibility of the ∆ng1686 strain. Our current work also shows that the NG1686 protein is localized inside the gonococcal cell, so the enhanced susceptibility of the ∆ng1686 strain to extracellular killing by PMNs could reflect susceptibility to a PMN factor that is internalized by gonococci. Although two-dimensional gel electrophoresis did not identify any gonococcal proteins that were substrates of NG1686 (data not shown), the NG1686 protein could additionally act on proteins or peptides produced by PMNs. Thus, if the ∆ng1686 mutant were unable to cleave some PMN antimicrobial factor, this would render the mutant more susceptible to killing by PMNs. However, the simplest explanation for the influence of NG1686 activity on these diverse phenotypes is that the localized cleavage of PG is required for expression of one or more bacterial factors that directly increase resistance to ROS and non-oxidative PMN killing.

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