Constitutively Opa-Expressing and Opa-Deficient *Neisseria gonorrhoeae* Strains Differentially Stimulate and Survive Exposure to Human Neutrophils

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The *Neisseria gonorrhoeae* (the gonococcus [Gc]) opacity-associated (Opa) proteins mediate bacterial binding and internalization by human epithelial cells and neutrophils (polymorphonuclear leukocytes [PMNs]). Investigating the contribution of Opa proteins to gonococcal pathogenesis is complicated by high-frequency phase variation of the *opa* genes. We therefore engineered a derivative of Gc strain FA1090 in which all *opa* genes were deleted in frame, termed Opaless. Opaless Gc remained uniformly Opa negative (Opa[−]), whereas cultures of predominantly Opa[−] parental Gc and an intermediate lacking the “translucent” subset of *opa* genes (Δ *opaBEGK*) stochastically gave rise to Opa-positive (Opa⁺) bacterial colonies. Loss of Opa expression did not affect Gc growth. Opaless Gc survived exposure to primary human PMNs and suppressed the PMN oxidative burst akin to parental, Opa[−] bacteria. Notably, unopsonized Opaless Gc was internalized by adherent, chemokine-primed, primary human PMNs, by an actin-dependent process. When a non-phase-variable, in-frame allele of FA1090 *opaD* was reintroduced into Opaless Gc, the bacteria induced the PMN oxidative burst, and OpaD⁺ Gc survived less well after exposure to PMNs compared to Opa[−] bacteria. These derivatives provide a robust system for assessing the role of Opa proteins in Gc biology.

The Gram-negative bacterium *Neisseria gonorrhoeae* (the gonococcus [Gc]) causes the sexually transmitted infection gonorrhea, which is notable for its prevalence—approximately 106 million cases of gonorrhea occur worldwide each year—and its increasing resistance to antibiotics (1–3). The presence of Gc at human mucosal surfaces initiates the recruitment of abundant polymorphonuclear leukocytes (neutrophils; PMNs) to the site of infection (4). Interactions between Gc and human cells are modulated by a variety of bacterial surface structures, including type IV pili, lipooligosaccharide (LOS), porin, and opacity-associated (Opa) proteins (5).

Opa proteins are 25- to 30-kDa outer membrane proteins that were named for their ability to confer an opaque morphology on Gc colonies (6). Gc genomes contain approximately 11 *opa* loci, encoding 7 to 9 unique Opa proteins (7–9). Opa proteins share a conserved β -barrel structure but differ in the sequence of extracellular loops, which dictate Opa receptor specificity (10–12). Most Opa proteins interact with one or more human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (Opa_{CEA}) (reviewed in reference 13). A subset of Opa proteins bind heparan sulfate proteoglycans on epithelial cells or indirectly engage integrins by binding vitronectin (14–17). Opa proteins can also recognize lacto-*N*-neotetraose moieties on LOS (18, 19). Opa expression is phase variable due to slipped-strand mispairing at pentameric repeats in the 5′ end of *opa* genes (20, 21). Thus, Gc can express anywhere from zero to theoretically all Opa proteins. Opa variation occurs at a rate of $\sim 10^{-3}$ to 10^{-4} variants/cell/generation *in vitro* (22, 23), and changes in Opa expression occur during experimental human male urethral infection and in the female mouse genital tract (24–26).

Expression of Opa proteins, particularly Opa_{CEA}, affects PMN antibacterial activities and Gc survival after exposure to PMNs (4). Activated PMNs assemble the NADPH oxidase enzyme to generate reactive oxygen species (ROS), e.g., the oxidative burst (27). Most Opa-positive (Opa⁺) Gc stimulate primary human PMNs

and promyelocytic HL-60 cells to produce ROS (28–32). In contrast, predominantly Opa-negative (Opa[−]) Gc not only fails to induce PMN ROS production but also suppresses the ability of PMNs to produce ROS after exposure to other stimuli (28–30, 33). Opa proteins also affect Gc phagocytosis and killing by PMNs. Human PMNs in suspension can bind and phagocytose unopsonized Gc only if the bacteria express Opa_{CEA} proteins (28–32). Gc phagocytosis by adherent PMNs is more efficient when the bacteria are Opa⁺, but Opa[−] Gc is also internalized (34–36). Regardless of the system used, Opa⁺ Gc is more sensitive than Opa[−] Gc to killing by human PMNs (28, 30, 35, 37).

When investigating the contribution of Opa proteins to Gc interactions with PMNs and other cells, the high rate of Opa phase variation means that any Gc culture in which one Opa phenotype predominates will always include bacteria of a different Opa expression state. Moreover, it is not always possible to identify Opa expression by colony photoopacity. The translucent Opa proteins confer a poorly opaque phenotype on Gc colonies grown on commonly used media, and Opa expression can be reliably detected only by immunoblot (38). Therefore, phenotypically translucent Gc may in fact express one or more Opa proteins. Moreover, during extended growth in liquid culture, bacteria of a different Opa expression state stochastically emerge (our unpublished observations).

Given that *opa* phase variation can confound studies aimed at

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dissecting the role of Opa proteins in Gc pathogenesis, we reasoned that this issue could be circumvented by generating Gc in which Opa expression was constitutively absent or present. We therefore created a derivative of strain FA1090 Gc carrying in-frame deletions in all *opa* genes, which is termed Opaless. Opaless Gc and an intermediate isolate in which the genes encoding translucent Opa proteins were deleted ($\Delta opaBEGK$) behaved similarly to Opa phase-OFF bacteria in *in vitro* growth and survival after exposure to primary human PMNs. Gc constitutively expressing the Opa_{CEA} OpaD recapitulated phenotypes associated with OpaD phase-ON Gc in induction of the PMN oxidative burst and bacterial sensitivity to PMNs. These derivatives provide insight into Opa-mediated interactions of Gc with human PMNs and make a system available for uniformly investigating the contribution of Opa proteins to Gc biology and pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. This study used pilated Gc of strain FA1090 expressing the 1-81-S2 pilin sequence (39) and strains 1291 and F62. The parent of Opaless and OpaD⁺ nonvariable (OpaD⁺_{nv}) Gc has a point mutation in the G-quadruplex region upstream of *pilE* that abrogates pilin antigenic variation (40) (FA1090 1-81-S2 P⁺_{nv}), to ensure all bacteria express the same pilin. FA1090 1-81-S2 Gc predominantly expressing OpaB was previously described (35). OpaB expression was verified by Western blotting (see below). Gc was maintained on Gc medium base (BD Difco) plus Kellogg's supplements I and II (41) (GCB) and routinely grown for 16 to 20 h at 37°C in 5% CO₂. Gc was grown in rich liquid medium (GCB) to obtain viable, exponential-phase cultures as described previously (33).

TOP10 *E. coli* (Invitrogen) was grown in Luria-Bertani (LB) (BD Difco) broth or agar supplemented with 50 µg/ml kanamycin (Sigma-Aldrich) at 37°C for plasmid propagation. The plate medium contained 15 g agar/liter.

Staphylococcus aureus ATCC 25923 was cultured on LB agar and overnight in LB broth at 37°C with rotation. For opsonization, *S. aureus* was incubated with 20% freshly isolated normal human serum for 20 min at 37°C.

FA1090 *opa* gene deletion construction. The locations and characteristics of all *opa* genes in FA1090 Gc are listed in Table S1 in the supplemental material. In-frame deletions of each *opa* gene were created using overlap extension PCR (42). Approximately 0.5 to 1 kb of sequence upstream (fragment A) and downstream (fragment B) of each *opa* locus was amplified by PCR from FA1090 1-81-S2 P⁺_{nv} genomic DNA (see Table S2 in the supplemental material for primer sequences and Table S3 in the supplemental material for PCR conditions). The deletions were designed to replace most of the *opa* open reading frame (ORF) with an HpaI restriction site (underlined in the primer sequence in Table S2 in the supplemental material). The product was cloned into pCR-Blunt TOPO (Invitrogen) or pSmart (Lucigen) and transformed into chemically competent TOP10 *E. coli*, following the manufacturers' instructions, except for *opaC*, where the overlap extension product was confirmed by DNA sequencing and then directly transformed into Gc (see below). Insert-containing plasmids were isolated from *E. coli* using the QiaPrep miniprep kit (Qiagen), and the correct *opa* deletion sequence was verified by DNA sequencing (Genewiz). As overlap extension PCR products from *opaF*, *opaG*, and *opaJ* could not be generated, fragments A and B were separately cloned into pSmart or pCR-Blunt TOPO. The plasmids were digested with HpaI along with a plasmid-specific restriction enzyme and ligated together to create the deletion product. All deletion products were verified by DNA sequencing prior to transformation into Gc.

Screening and validation of *opa* gene deletions in FA1090 Gc. Deletion constructs were sequentially introduced into FA1090 Gc by natural transformation on a solid medium (43). *opa* genes were deleted in the following order: E, B, K, G, C, I, A, D, F, H, and J. From each transforma-

tion, 400 to 600 colonies were screened for the *opa* deletion by PCR, using the conditions listed in Table S3 in the supplemental material. Potential *opa*-deleted clones were verified by PCR, using genomic DNA as the template and the primer combinations listed in Table S3 and/or by Southern blotting. For Southern blotting, genomic DNA was restriction digested (AvaI for *opaA*, C, D, G, and J; ClaI for *opaB*; SalI for *opaE*; XbaI for *opaI*; XbaI and XhoI for *opaK*; all restriction enzymes were from New England BioLabs) and probed with an *opa*-specific gene probe labeled with digoxigenin (Roche). In each subsequent round of *opa* deletion, Southern blotting and/or PCR was used to verify that all *opa* deletions that were previously introduced were retained.

Opa-expressing Gc. (i) Phase-ON Opa⁺ Gc in the $\Delta opaBEGK$ background (OpaD⁺_{var}). FA1090 $\Delta opaBEGK$ Gc was passaged on GCB. Opaque colonies arising in the predominantly translucent culture were streak purified and stored at -80°C. The Opa protein each expressed was determined by Western blotting with a panel of FA1090 Opa-specific antibodies (see below). Among these isolates was variable OpaD⁺ Gc (OpaD⁺_{var}).

(ii) Constitutively expressed OpaD⁺ Gc (OpaD⁺_{nv}). The CTCTT repeat-rich region of FA1090 *opaD* was replaced with a sequence encoding the same amino acids but unable to undergo phase variation (see Fig. 5B). The mutated sequence's codons appear at the same frequency in the Gc genome as the parental, phase-variable codons (http://exon.gatech.edu/metagenome/CodonUsageDatabase/display.cgi?accession=NC_002946). The *opaD*_{nv} amplicon was generated by overlap extension PCR using the primers in Table S2 and conditions in Table S3 in the supplemental material and then transformed into Opaless Gc. Putative transformants were selected by colony photopacity, and successful reintegration of *opaD*_{nv} into the *opaD* locus of Opaless Gc was confirmed by PCR. Opa expression was validated by Western blotting with the 4B12 pan-Opa antibody (see below).

SDS-PAGE and Western blotting for Opa proteins. SDS-PAGE and Western blotting for Opa expression was conducted as previously described (44). The 4B12 pan-Opa antibody hybridoma was obtained from Christof Hauck (University of Konstanz). Chemiluminescent blots were imaged with a ChemiDoc XRS⁺ system (Bio-Rad).

LOS preparation and Western blotting. LOS was prepared from Gc as described by Davis and Goldberg (45), except Gc was collected after overnight growth on GCB, and the protocol was stopped after the proteinase K treatment. Lysates were separated on 10 to 20% Tris-Tricine gradient gels (Bio-Rad) with a broad-range prestained protein marker (New England BioLabs). Gels were silver stained using the Pierce silver stain kit (Fisher) according to the manufacturer's protocols or transferred to polyvinylidene difluoride (PVDF) and immunoblotted with the 3F11 monoclonal antibody, obtained from Michael Apicella (University of Iowa), followed by horseradish peroxidase-coupled goat anti-mouse IgM antibody (Southern Biotech). 3F11 recognizes the terminal *N*-acetylglucosamine of the Gc oligosaccharide α chain of LOS (18).

Gc growth measurements. (i) CFU/colony. The CFU arising per colony of FA1090 Gc with and without *opa* deletions was enumerated as previously described (46). Experiments were performed 3 times.

(ii) Liquid medium. Gc was grown in GCB modified with Kellogg's supplements I and II and 0.042% sodium bicarbonate as previously described (33). After dilution to an optical density at 550 nm (OD₅₅₀) of 0.07, optical densities were measured, and CFU/ml were enumerated from each culture every hour for 3 h. Growth curves were not extended past 3 h since Gc begins to undergo autolysis as it approaches the stationary phase. Experiments were performed 3 times.

Serum survival. Gc grown in supplemented GCB was pelleted at 10,000 × g for 3 min. Bacteria were resuspended in 100% freshly isolated normal human serum for 30 min. CFU/ml were enumerated from the culture before and after serum exposure, and bacterial survival was expressed as (CFU/ml)_{t = 30 min} / (CFU/ml)_{t = 0 min} × 100%.

Gc-PMN coculture. All human subject research was conducted in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Sciences Research. PMNs were isolated

from the venous blood of consenting healthy human donors as previously described (47). PMN preparations routinely contained >95% granulocytes, as assessed by phase-contrast microscopy, and were >99% viable, as assessed by trypan blue exclusion. Care was taken to minimize time between PMN isolation and use in experiments.

Immunofluorescence. Binding and phagocytosis of Gc after exposure to interleukin-8 (IL-8)-primed PMNs, attached to serum-coated glass coverslips, were measured as previously described (48). The heat-inactivated normal human serum (Sigma) used to coat the coverslips was unable to opsonize Gc (K. Daily and A. Criss, unpublished observations). To block actin-dependent phagocytosis, cytochalasin D (10 μ g/ml) (Sigma) was added to PMNs 30 min prior to infection and kept in the infection medium for the duration of the experiment. Images were acquired from a Nikon Eclipse E800 upright fluorescence microscope with a Hamamatsu digital camera using Openlab software (PerkinElmer). Over 2,000 bacteria were examined for their localization. Experiments were performed 3 times.

Bacterial survival. Gc survival after exposure to adherent, IL-8-primed PMNs was measured as previously described (47). Experiments were performed 3 to 4 times.

PMN oxidative burst. Reactive oxygen species generation from PMNs exposed to Gc was measured by luminol-dependent chemiluminescence, using a Victor 3 multilabel plate reader (Perkin-Elmer), as described in reference 33. Luminescence was measured in 1-s intervals every 5 min for 30 to 60 min.

Polymyxin B survival. Mid-logarithmic phase Gc was exposed to increasing concentrations of polymyxin B (Alexis Biochemicals) as described in reference 49, except that incubations occurred in 0.5 $\times$ GCBL. Bacterial survival at each concentration was calculated as the ratio of the number of CFU recovered after 45 min divided by the number of CFU present at the start of the experiment and is presented relative to survival in the absence of polymyxin B (100%). Results are the averages $\pm$ standard errors from 11 to 14 replicate samples per strain.

Statistics. Differences between Gc strains were compared using two-tailed Student's *t* test. Differences were considered statistically significant at a *P* value of <0.05.

RESULTS

Generating Opaless Gc in strain FA1090. To delete the *opa* genes from piliated Gc of strain FA1090, the majority of each open reading frame was replaced in frame with a restriction enzyme recognition sequence (Fig. 1A). CFU arising after nonselective transformation were screened by PCR for replacement of the full-length *opa* with the deletion construct. One mutant clone served as the parent for the next round of transformation, until all 11 *opa* genes were sequentially deleted. The first four *opa* genes deleted encoded the translucent Opa proteins OpaB, E, G, and K to yield FA1090 Δ *opaBEGK* Gc. The remaining seven were then deleted to yield the complete *opa* deletion, here called Opaless. PCR analysis showed the four intended *opa* genes and no others were deleted in Δ *opaBEGK* Gc, while all 11 *opa* genes were deleted in Opaless Gc (Fig. 1B).

Opacity-associated phenotypes and growth characteristics of Opa-deficient Gc. Parental Gc and *opa*-deleted progeny were examined for Opa expression by Western blotting and colony photoopacity. Although most colonies of parental Gc were phenotypically translucent, the population contained some Opa-expressing bacteria. The parental bacterial lysate contained one or more heat-modifiable proteins that reacted with a pan-Opa antibody by Western blotting (Fig. 2A), and opaque Gc colonies were detected in cultures of parental Gc (Fig. 2B and E). For subsequent experiments, parental Gc was subcloned to yield an isolate that was predominantly Opa negative (Opa⁻) by Western blotting and

FIG 1 Creation of Opaless FA1090 Gc. (A) The internal portion of each *opa* gene (top) was replaced in frame with a HpaI restriction enzyme recognition site (H) by overlap extension PCR, using primers with homology to the 5' (solid line) and 3' (dotted line) ends of *opa*. The deletion construct (middle) was transformed into the chromosome of FA1090 Gc to produce *opa*-deleted progeny. One clone with the *opa* deletion was transformed with the next *opa* deletion construct, until all 11 *opa* genes were replaced in the genome. (B) Genomic DNA from parental, Δ *opaBEGK*, and Opaless Gc was used as the template for PCR, using primer sets homologous to the sequences flanking each of the 11 *opa* genes. Loss of *opa* is indicated by the faster-migrating PCR product for each primer set.

colony morphology (data not shown). Phenotypically translucent Δ *opaBEGK* Gc did not express any Opa proteins by Western blotting (Fig. 2A). When nonselectively passaged, Δ *opaBEGK* Gc sporadically yielded opaque colonies (Fig. 2C and F), and the opaque subpopulation expressed Opa proteins by Western blotting

FIG 2 Absence of Opa protein expression in Opaless Gc. (A) Lysates from equal CFU of parental Gc, phenotypically translucent $\Delta opaBEGK$ ($\Delta opaBEGK$ translucent) Gc, phenotypically opaque $\Delta opaBEGK$ ($\Delta opaBEGK$ opaque) Gc, and Opaless Gc were incubated at 37°C or 100°C and then separated by SDS-PAGE and immunoblotted with a pan-Opa antibody. Expression of one or more Opa proteins in parental and $\Delta opaBEGK$ opaque Gc is indicated by a heat-modifiable shift in reactivity with the Opa antibody. (B to G) Colonies of parental (B, E), $\Delta opaBEGK$ (C, F), and Opaless (D, G) Gc were imaged using white light transilluminated with a mirrored (B to D) or frosted (E to G) substage for opacity-associated phenotypes. Opa⁺ colonies (arrows) are optically reflective when illuminated with the mirrored substage and dark in color when illuminated with the frosted substage. Scale bar = 1 mm.

(Fig. 2A). These observations suggest that deleting the translucent *opaBEGK* genes from Gc yielded an isolate in which Opa protein expression could be monitored by colony phenotype. In contrast to parental and $\Delta opaBEGK$ Gc, Opaless Gc was negative for Opa protein expression by Western blotting (Fig. 2A). Opaless Gc colonies appeared uniformly translucent (Fig. 2D, G) and remained translucent for 25 nonselective passages (data not shown). Thus, deletion of *opa* genes yielded a derivative of Gc that is constitutively Opa[−].

Given the small-colony phenotype of a previously constructed FA1090 *opa* deletion strain (50), we measured the *in vitro* growth of FA1090 parental, $\Delta opaBEGK$, and Opaless Gc. The three strains produced similarly sized colonies (Fig. 2B to G) and yielded equivalent numbers of CFU per colony after 24 h of growth on solid medium (see Fig. S2A in the supplemental material). When cultured in rich liquid medium, parental, $\Delta opaBEGK$, and Opaless Gc grew with kinetics identical to those of midexponential phase, as measured by optical density (see Fig. S2B) and CFU/ml of culture (see Fig. S2C). No differences were observed in protein or LOS composition among parental, $\Delta opaBEGK$, and Opaless Gc (see Fig. S1A, C, and D), and all derivatives retained the piliated colony morphology of the parent (Fig. 2E to G). Opaless Gc also remained resistant to freshly isolated normal human serum, which is characteristic of strain FA1090 (51) (146% $\pm$ 27% survival of parental Gc versus 119% $\pm$ 31% survival of Opaless Gc after 30 min of incubation in 100% serum).

Taken together, these results show that *opa* genes can be successfully deleted from the FA1090 Gc chromosome to yield a strain incapable of expressing any Opa proteins. We conclude that the complete loss of *opa* genes from FA1090 Gc does not affect bacterial viability, growth rate, or protein and LOS expression.

Opaless Gc behaves similarly to phenotypically Opa[−] bacteria in interactions with primary human PMNs. To compare the functionality of *opa*-deleted Gc to Opa phase-OFF bacteria, we assessed parental, $\Delta opaBEGK$, and Opaless Gc interactions with primary human PMNs. Parental and $\Delta opaBEGK$ Gc were predominantly Opa[−] by Western blotting and colony opacity (data not shown).

We first determined how genetically *opa*-deficient Gc modulated the PMN oxidative burst. Parental and $\Delta opaBEGK$ Gc induced minor amounts of ROS production from PMNs, but the oxidative burst was smaller and delayed compared to PMNs infected with *S. aureus* (Fig. 3A). In contrast, PMNs exposed to Opaless Gc were identical to uninfected PMNs and generated no ROS (Fig. 3A). Parental, $\Delta opaBEGK$, and Opaless Gc all suppressed the oxidative burst of PMNs infected with *S. aureus* (Fig. 3B). These results indicate that *opa*-deficient Gc, like Opa phase-OFF parental Gc, retains the ability to suppress PMN ROS production. Assuming the minimal ROS production in PMNs exposed to parental and $\Delta opaBEGK$ Gc was due to a subpopulation of Opa⁺ bacteria, this finding illustrates that Opaless Gc is a preferable genetic background in situations where Opa variation influences the experimental readout.

To examine the survival of genetically *opa*-deficient Gc after PMN challenge, adherent, IL-8-primed human PMNs were infected with $\Delta opaBEGK$ and Opaless Gc. Bacterial survival was compared to Opa[−] parental Gc and phase-ON Gc for expression of the Opa_{CEA} OpaB. We previously showed that OpaB⁺ Gc survives less well than Opa[−] bacteria after PMN exposure (35). Opaless, $\Delta opaBEGK$, and parental Opa phase-OFF Gc were statistically indistinguishable from PMNs in their survival profile, and all survived significantly better than OpaB⁺ bacteria (Fig. 3C). These results show that Opaless Gc behaves akin to phenotypically Opa[−] bacteria after challenge with human PMNs.

Gc does not require Opa expression for nonopsonic internalization by primary human PMNs. Several groups have shown that PMN phagocytosis of unopsonized Gc requires bacterial expression of Opa_{CEA} proteins (28–32). In contrast, we reported that predominantly Opa[−] Gc can be internalized by adherent, IL-8-treated human PMNs (35). However, the Opa[−] Gc population in our study could have contained a subpopulation of Opa expressors that represented the internalized population. Therefore, we assessed the ability of adherent, primed PMNs to internalize genetically Opa-deficient Gc by immunofluorescence. Approximately 60% of PMN-associated Opaless Gc was intracellular, a percentage statistically indistinguishable from the Opa[−] parental or $\Delta opaBEGK$ Gc (Fig. 4). Thus, Gc does not need to express Opa proteins to be internalized by PMNs in this assay system. Phagocytosis of unopsonized Opaless Gc was dependent on the PMN actin cytoskeleton, since bacterial internalization was inhibited in PMNs treated with cytochalasin D (Fig. 4D). Together, these results conclusively show that adherent, primed human PMNs can phagocytose Gc, in the absence of Opa protein expression or known opsonins, by an actin-dependent process.

Complementation of Opaless Gc with non-phase-variable OpaD restores Opa-dependent phenotypes in Gc. Opaless Gc

FIG 3 Interactions of Opaless Gc with primary human PMNs. (A) PMNs were left untreated or exposed to opsonized *S. aureus*, phenotypically translucent parental Gc (parent), $\Delta opaBEGK$ Gc, or Opaless Gc. ROS production was measured as luminol-dependent chemiluminescence produced over 1 h. (B) PMNs were left untreated or exposed to opsonized *S. aureus*, alone or with an equivalent multiplicity of infection (MOI) of phenotypically translucent parental Gc (parent), $\Delta opaBEGK$ Gc, or Opaless Gc. Luminol-dependent chemiluminescence was measured as described for panel A. (C) Parental Gc that was predominantly Opa⁺ by Western blotting (parent) or expressed the translucent OpaB (OpaB⁺), $\Delta opaBEGK$ Gc, and Opaless Gc were exposed to adherent, IL-8-primed PMNs. Bacterial survival over time was calculated as the CFU enumerated from PMN lysates at each time point, divided by the CFU present at 0 min. *, $P < 0.05$ (Student's two-tailed *t* test) for OpaB⁺ Gc compared to the parent, $\Delta opaBEGK$, or Opaless Gc at matched time points.

was complemented with a constitutively expressed allele of the FA1090 *opaD* gene. OpaD is an Opa_{CEA} protein that confers a strongly opaque phenotype on Gc colonies. OpaD expressors are isolated from the male urethra and mouse genital tract after infection with FA1090 Gc, and OpaD⁺ Gc stimulates a strong oxidative

FIG 4 Opa-independent, actin-dependent phagocytosis of unopsonized Gc by primary human PMNs. Adherent, IL-8-primed PMNs were exposed to phenotypically translucent parental Gc (A), $\Delta opaBEGK$ Gc (B), Opaless Gc (C), or Opaless Gc in the presence of cytochalasin D (CytoD) (D) for 1 h. Intracellular bacteria appear green, while extracellular bacteria appear red/yellow. Percent internalization (E) was calculated as the number of intracellular Gc divided by the total number of cell-associated Gc. Scale bar = 10 μ m. *, $P < 0.025$ (Student's two-tailed *t* test) for Opaless Gc $\pm$ cytochalasin D.

burst in PMNs (25, 30, 50, 52, 53) (Fig. 5A). We took advantage of the redundancy of the genetic code to engineer an OpaD allele in which the CTCCTT repeat tract was replaced with a DNA sequence that encoded the same amino acids but cannot phase vary (Fig. 5B). The construct was transformed into Opaless Gc to yield OpaD⁺_{nv} (nonvariable) Gc.

The growth characteristics of OpaD⁺_{nv} Gc were compared to *opa*-deficient bacteria and to OpaD phase-ON, $\Delta opaBEGK$ Gc (OpaD⁺_{var}) (see Materials and Methods). Here, the $\Delta opaBEGK$ Gc background was used instead of the *opa*-intact parent to ensure the OpaD phase-ON bacteria did not coexpress any translucent Opa proteins. OpaD⁺_{nv} and OpaD⁺_{var} Gc expressed similar amounts of Opa protein (Fig. 5C). While translucent colonies were occasionally detected in the OpaD⁺_{var} Gc population, OpaD⁺_{nv} Gc colonies were uniformly opaque (Fig. 5D to G). Total protein expression was similar between the two strains (see Fig. S1B in the supplemental material), and OpaD⁺_{nv} Gc retained the LOS profile of Opaless Gc (see Fig. S1C and D). No differences in growth were noted between OpaD⁺_{nv} and OpaD⁺_{var} Gc, whether cultured on solid or in liquid medium (see Fig. S3 in the supplemental material). Thus, reintroduction of a nonvarying *opa* gene

FIG 5 Complementation of Opalesc Gc with a nonvariable, constitutively expressed allele of FA1090 *opaD*. (A) ROS production was measured in human PMNs exposed to *S. aureus*, $\Delta opaBEGK$ Gc (Opa⁻ Gc), or $\Delta opaBEGK$ Gc phase-ON for OpaA, OpaD, OpaF, or OpaI, as described for Fig. 3A. (B) The 5' end of FA1090 *opaD* was mutated to replace the phase-variable CTCTT repeats (line above the DNA sequences) with nucleotides encoding the same amino acids (written below the DNA sequences) yet unable to phase vary (black boxes). (C) Lysates prepared from equal CFU of $\Delta opaBEGK$ Gc, OpaD⁺_{var} Gc, Opalesc Gc, and OpaD⁺_{nv} Gc were separated by SDS-PAGE and Western blotted with a pan-Opa antibody. (D to G) Colonies of OpaD⁺_{var} Gc (D, F) and OpaD⁺_{nv} Gc (E, G) were imaged as described for Fig. 2B to G for opacity-associated phenotypes. Arrows point to translucent colonies in the OpaD⁺_{var} population. Scale bar = 1 mm.

into Opaless Gc allows for constitutive Opa protein expression, without affecting Gc growth.

OpaD⁺_{nv} and OpaD⁺_{var} Gc induced similar amounts of ROS production in PMNs, whereas Opaless Gc did not induce detectable ROS (Fig. 6A). While the magnitude of the oxidative burst was slightly larger in response to OpaD⁺_{nv} Gc, this difference was

FIG 6 Sensitivity of OpaD⁺ Gc to killing by human PMNs but not polymyxin B. (A) Primary human PMNs were exposed to Opaless Gc, OpaD⁺_{var} Gc, or OpaD⁺_{nv} Gc, and ROS production was measured as described for Fig. 3A. (B) Opaless Gc, OpaD⁺_{var} Gc, and OpaD⁺_{nv} Gc were exposed to adherent, IL-8-primed PMNs, and bacterial survival was calculated as described for Fig. 3C. *, *P* < 0.05 (Student's two-tailed *t* test) for Opaless Gc compared to either OpaD⁺_{var} Gc or OpaD⁺_{nv} Gc at matched time points. (C) Opaless Gc, OpaD⁺_{var} Gc, and OpaD⁺_{nv} Gc were exposed to polymyxin B at the indicated concentrations. Bacterial survival was calculated as the CFU after 45 min of exposure, divided by the input CFU.

not statistically significant. These results confirm that OpaD is a strong stimulator of PMN ROS production. Assuming the reduced ROS in response to OpaD⁺_{var} Gc is due to a minor fraction of Opa⁻ bacteria, these findings highlight the advantage of using Gc expressing a nonvarying *opaD* allele to examine Opa-dependent bacterial interactions with host cells.

To assess how expression of OpaD influenced FA1090 Gc sur-

vival after challenge with PMNs, adherent, primed human PMNs were exposed to Opaless, OpaD⁺_{var}, and OpaD⁺_{nv} Gc. OpaD-expressing Gc survived significantly less well after PMN challenge compared with Opaless bacteria (Fig. 6B). There was no statistical difference in survival between OpaD⁺_{nv} and OpaD⁺_{var} Gc (Fig. 6B). We conclude that expression of the Opa_{CEA} OpaD renders FA1090 Gc more sensitive to PMNs.

PMNs' antionococcal activity is due primarily to nonoxidative species such as cationic antimicrobial peptides (35, 54–56). To begin to understand the mechanism underlying the increased killing of OpaD⁺ Gc by PMNs, we measured bacterial survival after exposure to the model cationic peptide polymyxin B. No significant difference in sensitivity to polymyxin B was measured between Opaless and OpaD⁺ Gc (Fig. 6C). This observation suggests that the PMN survival defect of OpaD⁺ Gc is due to antionococcal properties of PMNs other than cationic antimicrobial peptides.

DISCUSSION

Gc varies its Opa proteins and other surface components during human infection (5). While Opa variation may enable bacterial evasion of host immunity and adaptation to new hosts or sites within a host, it complicates the assessment of how Opa expression influences specific aspects of Gc pathogenesis. We circumvented this issue by creating Opaless Gc in which all *opa* genes were deleted from the chromosome of strain FA1090 bacteria and complementing it with a nonvariable *opaD* allele. Using these derivatives, we made several conclusions about the contribution of Opa protein expression to Gc biology and pathogenesis. First, genetically *opa*-deficient Gc has no growth defect, and Gc growth *in vitro* is not affected by Opa expression. Second, genetically *opa*-deficient Gc survives exposure to primary human PMNs and suppresses the PMN oxidative burst, analogous to predominantly Opa[−] bacteria. Third, OpaD⁺ Gc has a survival disadvantage after PMN challenge and stimulates a strong PMN oxidative burst. Fourth, Opa expression is dispensable for Gc phagocytosis by adherent, chemokine-primed human PMNs. The use of these derivatives provides a robust and flexible system for quantifying the effect of Opa proteins on bacteria-bacteria and bacteria-host interactions.

We selected Gc strain FA1090 for this study for several reasons. FA1090 has been used extensively in human urethral challenge studies (57) and the murine genital tract model of gonorrhea (58). The FA1090 genome has been sequenced and annotated (www.genome.ou.edu/gono.html; <http://stdgen.northwestern.edu>), the locations of all 11 *opa* genes in the FA1090 chromosome are known (8) (see Table S1 in the supplemental material), and the colony opacity phenotype for each FA1090 Opa protein has been elucidated (25, 53). In addition, antibodies have been raised against individual FA1090 Opa proteins (23, 24). Prior to this work, a derivative of FA1090 in which all 11 *opa* genes had been deleted, the Δ *opaA-K* mutant, was constructed (50, 53). However, the FA1090 Δ *opaA-K* mutant has a significant growth defect on solid and in liquid media (53) (our unpublished observations). This observation suggested that Opa expression, even residual expression in predominantly Opa[−] bacteria, was required for Gc growth. In contrast, the Opaless FA1090 derivative described in this work grew with similar kinetics to the *opa*-intact parent. While it is possible that we selected for a mutation that allows for Gc growth in the absence of all Opa expression, *opa*-deficient de-

rivatives of strain MS11 Gc and strain H44/76 *Neisseria meningitidis* also show no *in vitro* growth defect (19, 59). Each of the 11 transformation events that yielded Opaless occurred at a frequency of ≥ 0.02 per CFU, which is similar to the frequency we measure for transformation of non-*opa* constructs (our unpublished observations). Additionally, complementation with nonvarying *opaD* did not change the growth characteristics of Opaless Gc. Therefore, we conclude that Opa expression is not required for Gc growth *in vitro*.

One of the main reasons for creating Opaless Gc was to avoid changes in expression of Opa proteins during bacterial culture that adversely affect Gc-host interaction studies. Using Opaless and OpaD⁺_{nv} Gc, we found that constitutively Opa[−] and Opa⁺ Gc behave similarly to their phase-varying counterparts in survival after challenge with primary human PMNs and in modulating the PMN oxidative burst. Interestingly, there was a small increase in ROS production from PMNs exposed to cultures of predominantly translucent parental and Δ *opaBEGK* Gc, whereas Opaless Gc stimulated no detectable ROS from PMNs. This effect was likely due to a subset of bacteria that phase varied ON Opa expression in the parental and Δ *opaBEGK* Gc cultures. Thus, FA1090 Opaless Gc and nonvarying Opa derivatives can be used in place of predominantly Opa⁺ and Opa[−] bacteria to avoid the confounding effects of Opa variation during infection.

In this work, we showed that unopsonized Opaless Gc is readily phagocytosed by PMNs, revealing a new mode of Gc internalization. Since phagocytosis of Opaless Gc requires the PMN actin cytoskeleton, PMNs may be internalizing Opa[−] bacteria by actin-dependent macropinocytosis. As support for this possibility, PMNs that are attached to a substrate and primed with chemokines, like the PMNs in our assay system, show increased motility and membrane ruffling, features that enhance macropinocytosis (60). Alternatively, adherent, primed PMNs would also undergo increased degranulation, to potentially present a receptor on the PMN surface for internalization of Opa[−] Gc.

Introducing a non-phase-varying allele of OpaD from strain FA1090 into Opaless Gc allowed us to examine the effect of constitutive Opa protein expression on Gc interactions with human PMNs. Constitutive OpaD expression did not affect Gc growth characteristics *in vitro*, indicating that the ability to phase vary Opa expression is not essential to Gc viability. However, OpaD expression reduced Gc survival after exposure to primary human PMNs. We are currently testing possibilities to explain the OpaD survival defect in PMNs. Given the potent ROS response of PMNs to OpaD⁺ Gc, one possibility is that Opa⁺ bacteria are killed by PMNs' extensive oxidative arsenal. Another possibility is that PMNs are more adept at binding and engulfing OpaD⁺ bacteria, leading to increased bacterial killing. In support of this notion, we and others have shown that expression of CEACAM-binding Opa proteins (for instance, FA1090 OpaB) enhances the association of Gc with PMNs (30–32, 35). A third possibility is that Opa⁺ bacteria are more sensitive to PMN antimicrobial proteins. However, OpaD⁺ Gc showed no increased sensitivity to polymyxin B, a surrogate for the cationic antimicrobial peptides produced by PMNs. Opa proteins have high isoelectric points (pI of mature OpaD = 9.83 [available via <http://web.expasy.org>]), which may help the negatively charged bacterial surface resist binding of positively charged antimicrobial peptides. Opa⁺ Gc may show more sensitivity to killing by PMN serine proteases, which have antionococcal activity *in vitro* and in PMN phagosomes (48, 61), as

Opa expression makes Gc more sensitive to the serine proteases trypsin and chymotrypsin (50).

Although expression of Opa proteins like OpaD is detrimental to Gc survival after exposure to PMNs, Opa⁺ Gc is recovered from the male urethra and the female cervix during the follicular phase of the menstrual cycle (25, 26, 52, 62). These *in vivo* observations indicate that factors other than PMNs select for bacterial expression of Opa proteins during acute infection. One such pressure may come from the requirement for Gc to attach to the apical surface of the mucosal epithelium and be internalized by epithelial cells (63). Opa expression also facilitates Gc transcytosis of polarized epithelial monolayers, allowing the bacteria to gain access to subepithelial regions (64). Another pressure may stem from the human adaptive immune system. Opa⁺ Gc proteins prevent maturation and activation of CD4⁺ T cells and block Th1- and Th2-mediated immunity (65, 66). Opa⁺ Gc engagement of CEACAM1 also leads to B cell death (67). Additionally, studies in mice have shown that Opa⁺ Gc is more resistant to serum bactericidal activity and to as-yet unidentified factors in genital tract secretions (50). Therefore, the ability to phase vary Opa protein expression allows Gc to fine-tune its interactions with epithelial and immune cells as needed during the course of infection.

Opaless Gc and Opa-expressing derivatives provide a robust system for studies of Opa biology and Gc pathogenesis. For instance, complementation of Opaless with non-phase-varying *opa* alleles, for example, OpaD⁺_{nv}, will address how individual Opa proteins contribute to different models of infection. *opa* genes can also be introduced into Opaless Gc under the control of regulatable promoters to assess how changes in Opa expression affect infection of a given cell type over time. Opaless Gc can serve as a recipient for Opa variants expressed by other strains of *Neisseria* or Opa proteins synthesized *in vitro*, in order to evaluate these proteins' receptor binding profiles and other biochemical properties. Finally, the contribution of putative (non-Opa) Gc virulence factors can be investigated in constitutively Opa⁺ or Opa⁻ genetic backgrounds, to determine if and how the presence of Opa affects their function. These and other approaches will help reveal the contribution of Opa proteins, individually or in combination, to Gc-host interactions and enhance understanding of how these interactions influence the course of human disease.

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REFERENCES

- Allen VG, Mitterni L, Seah C, Rebbapragada A, Martin IE, Lee C, Siebert H, Towns L, Melano RG, Low DE. 2013. *Neisseria gonorrhoeae* treatment failure and susceptibility to cefixime in Toronto, Canada. *JAMA* 309:163–170.
- Unemo M, Shafer WM. 2011. Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. *Ann. N. Y. Acad. Sci.* 1230:E19–E28.
- Schmid G. 2009. Global incidence and prevalence of four curable sexually transmitted infections (STIs): new estimates from WHO. The 2nd Global HIV/AIDS Surveillance Meeting, Bangkok, Thailand.
- Criss AK, Seifert HS. 2012. A bacterial siren song: intimate interactions between *Neisseria* and neutrophils. *Nat. Rev. Microbiol.* 10:178–190.
- Virji M. 2009. Pathogenic *Neisseriae*: surface modulation, pathogenesis and infection control. *Nat. Rev. Microbiol.* 7:274–286.
- Swanson J. 1978. Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of *Neisseria gonorrhoeae*. *Infect. Immun.* 21:292–302.
- Bhat KS, Gibbs CP, Barrera O, Morrison SG, Jahnig F, Stern A, Kupsch EM, Meyer TF, Swanson J. 1991. The opacity proteins of *Neisseria gonorrhoeae* strain MS11 are encoded by a family of 11 complete genes. *Mol. Microbiol.* 5:1889–1901.
- Dempsey JA, Litaker W, Madhure A, Snodgrass TL, Cannon JG. 1991. Physical map of the chromosome of *Neisseria gonorrhoeae* FA1090 with locations of genetic markers, including *opa* and *pil* genes. *J. Bacteriol.* 173:5476–5486.
- Connell TD, Shaffer D, Cannon JG. 1990. Characterization of the repertoire of hypervariable regions in the protein II (*opa*) gene family of *Neisseria gonorrhoeae*. *Mol. Microbiol.* 4:439–449.
- Vandeputte-Rutten L, Bos MP, Tommessen J, Gros P. 2003. Crystal structure of neisserial surface protein A (NspA), a conserved outer membrane protein with vaccine potential. *J. Biol. Chem.* 278:24825–24830.
- de Jonge MI, Hamstra HJ, van Alphen L, Dankert J, van der Ley P. 2003. Mapping the binding domains on meningococcal Opa proteins for CEACAM1 and CEA receptors. *Mol. Microbiol.* 50:1005–1015.
- Malorny B, Morelli G, Kusecek B, Kolberg J, Achtman M. 1998. Sequence diversity, predicted two-dimensional protein structure, and epitope mapping of *Neisserial* Opa proteins. *J. Bacteriol.* 180:1323–1330.
- Sadarangani M, Pollard AJ, Gray-Owen SD. 2011. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic *Neisseria*. *FEMS Microbiol. Rev.* 35:498–514.
- Chen T, Belland RJ, Wilson J, Swanson J. 1995. Adherence of pilus⁺ Opa⁺ gonococci to epithelial cells *in vitro* involves heparan sulfate. *J. Exp. Med.* 182:511–517.
- van Putten JP, Paul SM. 1995. Binding of syndecan-like cell surface proteoglycan receptors is required for *Neisseria gonorrhoeae* entry into human mucosal cells. *EMBO J.* 14:2144–2154.
- Duensing TD, van Putten JP. 1997. Vitronectin mediates internalization of *Neisseria gonorrhoeae* by Chinese hamster ovary cells. *Infect. Immun.* 65:964–970.
- Gomez-Duarte OG, Dehio M, Guzman CA, Chhatwal GS, Dehio C, Meyer TF. 1997. Binding of vitronectin to *opa*-expressing *Neisseria gonorrhoeae* mediates invasion of HeLa cells. *Infect. Immun.* 65:3857–3866.
- Blake MS, Blake CM, Apicella MA, Mandrell RE. 1995. Gonococcal opacity: lectin-like interactions between Opa proteins and lipooligosaccharide. *Infect. Immun.* 63:1434–1439.
- Levan A, Zimmerman LI, Mahle AC, Swanson KV, Deshong P, Park J, Edwards VL, Song W, Stein DC. 2012. Construction and characterization of a derivative of *Neisseria gonorrhoeae* strain MS11 devoid of all *opa* genes. *J. Bacteriol.* 194:6468–6478.
- Stern A, Brown M, Nickel P, Meyer TF. 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell* 47:61–71.
- Murphy GL, Connell TD, Barritt DS, Koomey M, Cannon JG. 1989. Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. *Cell* 56:539–547.
- Mayer LW. 1982. Rates *in vitro* changes of gonococcal colony opacity phenotypes. *Infect. Immun.* 37:481–485.
- Black WJ, Schwalbe RS, Nachamkin I, Cannon JG. 1984. Characterization of *Neisseria gonorrhoeae* protein II phase variation by use of monoclonal antibodies. *Infect. Immun.* 45:453–457.
- Simms AN, Jerse AE. 2006. *In vivo* selection for *Neisseria gonorrhoeae* opacity protein expression in the absence of human carcinoembryonic antigen cell adhesion molecules. *Infect. Immun.* 74:2965–2974.
- Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, Seifert HS, Cannon JG. 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *J. Exp. Med.* 179:911–920.
- Swanson J, Barrera O, Sola J, Boslego J. 1988. Expression of outer

- membrane protein II by gonococci in experimental gonorrhea. *J. Exp. Med.* 168:2121–2129.
27. Roos D, van Bruggen R, Meischl C. 2003. Oxidative killing of microbes by neutrophils. *Microbes Infect.* 5:1307–1315.
 28. Rest RF, Fischer SH, Ingham ZZ, Jones JF. 1982. Interactions of *Neisseria gonorrhoeae* with human neutrophils: effects of serum and gonococcal opacity on phagocyte killing and chemiluminescence. *Infect. Immun.* 36:737–744.
 29. Virji M, Heckels JE. 1986. The effect of protein II and pili on the interaction of *Neisseria gonorrhoeae* with human polymorphonuclear leukocytes. *J. Gen. Microbiol.* 132:503–512.
 30. Fischer SH, Rest RF. 1988. Gonococci possessing only certain P.II outer membrane proteins interact with human neutrophils. *Infect. Immun.* 56:1574–1579.
 31. Kupsch EM, Knepper B, Kuroki T, Heuer I, Meyer TF. 1993. Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. *EMBO J.* 12:641–650.
 32. King GJ, Swanson J. 1978. Studies on gonococcus infection. XV. Identification of surface proteins of *Neisseria gonorrhoeae* correlated with leukocyte association. *Infect. Immun.* 21:575–584.
 33. Criss AK, Seifert HS. 2008. *Neisseria gonorrhoeae* suppresses the oxidative burst of human polymorphonuclear leukocytes. *Cell. Microbiol.* 10:2257–2270.
 34. Simons MP, Nauseef WM, Apicella MA. 2005. Interactions of *Neisseria gonorrhoeae* with adherent polymorphonuclear leukocytes. *Infect. Immun.* 73:1971–1977.
 35. Criss AK, Katz BZ, Seifert HS. 2009. Resistance of *Neisseria gonorrhoeae* to non-oxidative killing by adherent human polymorphonuclear leukocytes. *Cell. Microbiol.* 11:1074–1087.
 36. Farrell CF, Rest RF. 1990. Up-regulation of human neutrophil receptors for *Neisseria gonorrhoeae* expressing PII outer membrane proteins. *Infect. Immun.* 58:2777–2784.
 37. Sarantis H, Gray-Owen SD. 2007. The specific innate immune receptor CEACAM3 triggers neutrophil bactericidal activities via a Syk kinase-dependent pathway. *Cell. Microbiol.* 9:2167–2180.
 38. Dekker NP, Lammel CJ, Mandrell RE, Brooks GF. 1990. Opa (protein II) influences gonococcal organization in colonies, surface appearance, size and attachment to human fallopian tube tissues. *Microb. Pathog.* 9:19–31.
 39. Seifert HS, Wright CJ, Jerse AE, Cohen MS, Cannon JG. 1994. Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J. Clin. Invest.* 93:2744–2749.
 40. Cahoon LA, Seifert HS. 2009. An alternative DNA structure is necessary for pilin antigenic variation in *Neisseria gonorrhoeae*. *Science* 325:764–767.
 41. Kellogg DS, Jr, Peacock WL, Jr, Deacon WE, Brown L, Pirkle DI. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* 85:1274–1279.
 42. Heckman KL, Pease LR. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat. Protoc.* 2:924–932.
 43. Stohl EA, Seifert HS. 2001. The *recX* gene potentiates homologous recombination in *Neisseria gonorrhoeae*. *Mol. Microbiol.* 40:1301–1310.
 44. Criss AK, Seifert HS. 2006. Gonococci exit apically and basally from polarized epithelial cells and exhibit dynamic changes in type IV pili. *Cell. Microbiol.* 8:1430–1443.
 45. Davis MR, Jr, Goldberg JB. 2012. Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction. *J. Vis. Exp.* 63:pii=3916. doi:10.3791/3916.
 46. Sechman EV, Kline KA, Seifert HS. 2006. Loss of both Holliday junction processing pathways is synthetically lethal in the presence of gonococcal pilin antigenic variation. *Mol. Microbiol.* 61:185–193.
 47. Stohl EA, Criss AK, Seifert HS. 2005. The transcriptome response of *Neisseria gonorrhoeae* to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Mol. Microbiol.* 58:520–532.
 48. Johnson MB, Criss AK. 4 February 2013, posting date. *Neisseria gonorrhoeae* phagosomes delay fusion with primary granules to enhance bacterial survival inside human neutrophils. *Cell. Microbiol.* [Epub ahead of print.] doi:10.1111/cmi.12117.
 49. Shafer WM, Qu X, Waring AJ, Lehrer RI. 1998. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/modulation/division efflux pump family. *Proc. Natl. Acad. Sci. U. S. A.* 95:1829–1833.
 50. Cole JG, Fulcher NB, Jerse AE. 2010. Opacity proteins increase *Neisseria gonorrhoeae* fitness in the female genital tract due to a factor under ovarian control. *Infect. Immun.* 78:1629–1641.
 51. Cohen MS, Cannon JG, Jerse AE, Charniga LM, Isbey SF, Whicker LG. 1994. Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. *J. Infect. Dis.* 169:532–537.
 52. Jerse AE. 1999. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. *Infect. Immun.* 67:5699–5708.
 53. Fulcher NB. 2004. The role of *Neisseria gonorrhoeae* opacity proteins in host cell interactions and pathogenesis. Ph.D. thesis. University of North Carolina, Chapel Hill, NC.
 54. Frangipane JV, Rest RF. 1992. Anaerobic growth of gonococci does not alter their Opa-mediated interactions with human neutrophils. *Infect. Immun.* 60:1793–1799.
 55. Seib KL, Simons MP, Wu HJ, McEwan AG, Nauseef WM, Apicella MA, Jennings MP. 2005. Investigation of oxidative stress defenses of *Neisseria gonorrhoeae* by using a human polymorphonuclear leukocyte survival assay. *Infect. Immun.* 73:5269–5272.
 56. Wu H, Soler-Garcia AA, Jerse AE. 2009. A strain-specific catalase mutation and mutation of the metal-binding transporter gene *mmtC* attenuate *Neisseria gonorrhoeae* *in vivo* but not by increasing susceptibility to oxidative killing by phagocytes. *Infect. Immun.* 77:1091–1102.
 57. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE. 2011. Experimental gonococcal infection in male volunteers: cumulative experience with *Neisseria gonorrhoeae* strains FA1090 and MS11mkC. *Front. Microbiol.* 2:123.
 58. Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE. 2011. Estradiol-treated female mice as surrogate hosts for *Neisseria gonorrhoeae* genital tract infections. *Front. Microbiol.* 2:107.
 59. Sadarangani M, Hoe JC, Callaghan MJ, Jones C, Chan H, Makepeace K, Daniels-Treffandier H, Deadman ME, Bayliss C, Feavers I, van der Ley P, Pollard AJ. 2012. Construction of Opa-positive and Opa-negative strains of *Neisseria meningitidis* to evaluate a novel meningococcal vaccine. *PLoS One* 7:e51045. doi:10.1371/journal.pone.0051045.
 60. Lim JP, Gleeson PA. 2011. Macropinocytosis: an endocytic pathway for internalising large gulps. *Immunol. Cell Biol.* 89:836–843.
 61. Shafer WM, Onunka VC, Martin LE. 1986. Antigonococcal activity of human neutrophil cathepsin G. *Infect. Immun.* 54:184–188.
 62. James JF, Swanson J. 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. *Infect. Immun.* 19:332–340.
 63. Merz AJ, So M. 2000. Interactions of pathogenic *Neisseriae* with epithelial cell membranes. *Annu. Rev. Cell Dev. Biol.* 16:423–457.
 64. Wang J, Gray-Owen SD, Knorre A, Meyer TF, Dehio C. 1998. Opa binding to cellular CD66 receptors mediates the transcellular traversal of *Neisseria gonorrhoeae* across polarized T84 epithelial cell monolayers. *Mol. Microbiol.* 30:657–671.
 65. Boulton IC, Gray-Owen SD. 2002. Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. *Nat. Immunol.* 3:229–236.
 66. Liu Y, Islam EA, Jarvis GA, Gray-Owen SD, Russell MW. 2012. *Neisseria gonorrhoeae* selectively suppresses the development of Th1 and Th2 cells, and enhances Th17 cell responses, through TGF-beta-dependent mechanisms. *Mucosal Immunol.* 5:320–331.
 67. Pantelic M, Kim YJ, Bolland S, Chen I, Shively J, Chen T. 2005. *Neisseria gonorrhoeae* kills carcinoembryonic antigen-related cellular adhesion molecule 1 (CD66a)-expressing human B cells and inhibits antibody production. *Infect. Immun.* 73:4171–4179.