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Heptose I Glycan Substitutions on *Neisseria gonorrhoeae* Lipooligosaccharide Influence C4b-Binding Protein Binding and Serum Resistance▽

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Lipooligosaccharide (LOS) heptose (Hep) glycan substitutions influence gonococcal serum resistance. Several gonococcal strains bind the classical complement pathway inhibitor, C4b-binding protein (C4BP), via their porin (Por) molecule to escape complement-dependent killing by normal human serum (NHS). We show that the proximal glucose (Glc) on HepI is required for C4BP binding to Por1B-bearing gonococcal strains MS11 and 1291 but not to FA19 (Por1A). The presence of only the proximal Glc on HepI (*lgtE* mutant) permitted maximal C4BP binding to MS11 but not to 1291. Replacing 1291 lgtE Por with MS11 Por increased C4BP binding to levels that paralleled MS11 lgtE, suggesting that replacement of the Por1B molecule dictated the effects of HepI glycans on C4BP binding. The remainder of the strain background did not affect C4BP binding; replacing the Por of strain F62 with MS11 Por (F62 PorMS11) and truncating HepI mirrored the findings in the MS11 background. C4BP binding correlated with resistance to killing by NHS in most instances. F62 PorMS11 and its *lgtE* mutant were sensitive to NHS despite binding C4BP, secondary to kinetically overwhelming classical pathway activation and possibly increased alternative pathway activation (measured by factor Bb binding) by the F62 background. FA19 lgtF (HepI unsubstituted) resisted killing by only 10% NHS, not 50% NHS, despite binding levels of C4BP similar to those of FA19 and FA19 lgtE (both resistant to 50% serum), suggesting a role for the proximal Glc in serum resistance independently of C4BP binding. This study provides mechanistic insights into how HepI LOS substitutions affect the serum resistance of *N. gonorrhoeae*.

Gonococcal lipooligosaccharide (LOS) plays an important role in several aspects of disease pathophysiology. The lipid A component has proinflammatory properties (22) and may contribute to fallopian tube damage (47) caused by *Neisseria gonorrhoeae* in salpingitis. Oligosaccharide chain extensions from the inner core also play an important role in pathogenesis. One such example is facilitation of adhesion to cells expressing the asialoglycoprotein receptor by bacteria that express the lacto-*N*-neotetraose (LNT) structure (20, 21, 35). Another important aspect of gonococcal pathogenesis is the ability of the bacterium to evade killing by complement. Heptose I (HepI) substitutions of LOS play a key role in complement evasion. The best-characterized example of an LOS structure directly impacting serum resistance is sialylation of LNT LOS (33, 34). LNT LOS sialylation results in enhanced binding of the alternative complement pathway inhibitory protein, factor H (40), which results in complement regulation on the bacterial surface. Another LOS structure that renders gonococci serum resistant upon sialylation but by a mechanism that does not involve factor H binding (16) is the P^K-like structure (Gal→Gal→Glc→HepI, analogous to the L1 LOS immunotype in *Neisseria meningitidis*) (49). Expression of the lactosyl (Gal→Glc→HepI) HepI LOS structure is associated with the serum-resistant phenotype probably because of the lack of naturally occurring antibody against this structure (43).

Clinical isolates of *N. gonorrhoeae* express a minimum of two hexose substitutions on HepI (Gal→Glc→HepI), because the LOS biosynthetic genes responsible for adding the proximal Glc (*lgtF*) and the subsequent Gal (*lgtE*) residues are not phase variable (14). Further glycan modifications of the HepI chain are dictated by the status (either in or out of frame) of the phase-variable *lgtA*, *lgtC*, and *lgtD* genes (14). While the ability to bind to complement-inhibitory proteins such as factor H and/or C4b-binding protein (C4BP) is an important factor in determining resistance to human complement, LOS glycan substitutions may also play a key role in modulating serum resistance independently of complement inhibitor binding and function (46). We undertook this study to determine the molecular basis of HepI glycan substitutions on serum resistance of *N. gonorrhoeae*. The findings presented here shed further light on the critical role of LOS in gonococcal pathogenesis.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and mutants used in this study and their relevant characteristics are listed in Table 1. We insertionally inactivated the *lgtE* and *lgtF* genes in strains MS11, FA19, and 1291 to yield gonococci with LOS HepI substitutions of Glc→HepI and HepI unsubstituted,

TABLE 1. Strains used in this study

Strain	Relevant features	Source or reference
MS11	Por1B; C4BP binder	48
MS11 lgtE	MS11 *lgtE*::*ermC*	This study
MS11 lgtF	MS11 *lgtF*::*spc*	This study
1291	Por1B; C4BP binder	7
1291 lgtE	1291 *lgtE*::*ermC*	This study
1291 lgtF	1291 *lgtF*::*spc*	This study
1291 PorMS11 lgtE	Native Por of 1291 lgtE replaced with MS11 Por	This study
FA19	Por1A; C4BP binder	42
FA19 lgtE	FA19 *lgtE*::*ermC*	This study
FA19 lgtF	FA19 *lgtF*::*spc*	This study
F62	Por1B; non-C4BP binder	44
F62 PorMS11	Native F62 Por replaced with MS11 Por	38
F62 PorMS11 lgtE	F62 Por MS11 *lgtE*::*ermC*	This study
F62 PorMS11 lgtF	F62 Por MS11 *lgtF*::*spc*	This study

respectively, as described previously (37). Plasmid pUNCH61 (a gift from P. Frederick Sparling and Christopher Elkins, University of North Carolina, Chapel Hill [4]) was used for allelic replacement of *porB* of 1291 lgtE, F62, F62 lgtE, and F62 lgtF with MS11 *porB*.

Sera and complement reagents. Fresh sera obtained from 10 healthy adult volunteers (normal human serum [NHS]) were pooled and stored at −80°C until used. C4BP was purified from human plasma as described previously (6). Factor B-depleted serum and purified human factor B were from Complement Technology, Inc. (Tyler, TX). In some experiments, factor B-depleted serum was reconstituted with factor B to a final concentration of 200 μg/ml.

Antibodies. Anti-C4BP monoclonal antibody (MAb) 67 (19) and MAbs against C4c and C4d (both from Quidel Corporation, San Diego, CA) were all used at a concentration of 10 μg/ml in Hanks balanced salt solution ($HBSS^{++}$) to detect C4BP, C4c, and C4d bound to the bacterial surface, respectively. Rabbit polyclonal anti-human C4BP (36, 50), at a dilution of 1:1,000 in phosphate-buffered saline (PBS) containing 0.05% Tween 20, was used to detect C4BP in Western blotting experiments. MAb 5.51, which is specific for MS11 Por loop 5 (8), was used in Western blots. Factor Bb binding to the bacterial surface was measured by whole-cell enzyme-linked immunosorbent assay (ELISA) using MAb against Bb (Quidel) at a dilution of 1:1,000 in PBS, as described previously (29). To ensure equal capture of bacteria to microtiter wells, we used MAb 2-8C-4-1, which is directed against H.8, a common lipoprotein antigen found on all pathogenic neisseriae (3). MAb 2-8C-4-1 was raised by previously described methods (17) and had analytical sensitivity similar to that of another anti-H.8 MAb, called 2C3 (1), that we have used previously to evaluate capture of *N. gonorrhoeae* to microtiter wells (29). Anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC), anti-mouse IgG conjugated to alkaline phosphatase, and anti-rabbit IgG-alkaline phosphatase (all from Sigma) were used as secondary disclosing reagents.

Preparation of O-deacylated LPS. Bacterial cells were killed with 2% phenol, washed four times with water, and then lyophilized. Cell pellets were resuspended in 200 μl of H_2O containing 5 μg of proteinase K and incubated at 37°C for 5 h. Samples were heated to 70°C for 10 min and then lyophilized. Samples were dissolved in 200 μl of ammonium acetate buffer (20 mM, pH 7.4) containing 1 μg of RNase and 2 μg of DNase, incubated at 37°C for 5 h, and then lyophilized. Crude lipopolysaccharide (LPS)-containing samples were O deacylated by dissolution in 200 μl of anhydrous hydrazine and incubation with stirring at 37°C for 1.5 h. Excess hydrazine was destroyed by the addition of 5 volumes of ice-cold acetone to the chilled samples, which were then washed repeatedly with acetone. O-deacylated LPS (LPS-OH) pellets were redissolved in H_2O and lyophilized.

MS. Capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS) was performed with a crystal model 310 CE instrument (AYI Unicam) coupled to an API 3000 MS (Perkin-Elmer/Sciex) via a MicroIonSpray interface (5). A sheath solution (isopropanol-methanol, 2:1) was delivered at a flow rate of 1 μl/min to a low-dead-volume tee (inner diameter, 250 μm; Chromatographic Specialties). All aqueous solutions were filtered through a 0.45-μm filter (Millipore) before use.

Flow cytometry. C4BP, C4c, and C4d binding to bacteria was performed by flow cytometry, as described previously (38).

Gel electrophoresis and Western blotting. Western blotting was performed to correlate the amount of C4BP binding with Por expression levels. Briefly, 10^8 bacteria were incubated with NHS to a final concentration of 2% in a final reaction volume of 200 μl for 15 min at 37°C. Bacteria were washed three times to remove unbound serum components and lysed with 4× LDS sample buffer (Invitrogen) containing 10% 2-mercaptoethanol, and proteins were separated on a 4 to 12% bis-Tris gel (Invitrogen). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) by Western blotting, and nonspecific binding sites were blocked with PBS containing 1% nonfat dry milk for 30 min. With the 50-kDa marker used as a reference, the blot was cut and proteins above 50 kDa (the α chain of C4BP migrates at ~70 kDa under reducing conditions) were probed with anti-C4BP polyclonal antibody, while the section containing proteins of <50 kDa (Por1B migrates at ~37 kDa) was either stained with Coomassie blue (Imperial Stain; Pierce) or probed with anti-Por1B MAb 5.51. Protein bands on the Coomassie blue-stained PVDF membranes were visualized after destaining with a solution containing 40% methanol and 10% glacial acetic acid. After incubation with the appropriate alkaline phosphatase-conjugated secondary antibody, anti-C4BP- and MAb 5.51-reactive bands were disclosed with alkaline phosphatase substrate (Sigma).

Whole-cell ELISA for factor Bb binding. We measured factor Bb bound to bacteria as a measure of alternative pathway activation, as described previously (29). Briefly, 2×10^8 organisms in $HBSS^{++}$ minus 0.1% gelatin were incubated with 10 μl NHS in a final reaction volume of 100 μl for 10 min at 37°C. Reactions were stopped after 10 min by washing three times with ice-cold HBSS containing 5 mM phenylmethylsulfonyl fluoride in a refrigerated microcentrifuge. Organisms were resuspended in 200 μl of the same buffer, and 50 μl of each sample was applied per well of a 96-well U-bottomed polystyrene microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) for 3 h at 37°C. The plates were washed with PBS containing 0.05% Tween 20. Primary antibodies were diluted in PBS, and secondary antibodies were diluted in PBS–0.05% Tween 20. Factor Bb bound to organisms was measured by anti-factor Bb MAb followed by anti-mouse IgG-alkaline phosphatase conjugate (Sigma). To normalize the measurement of complement components per unit of organism, we determined the H.8 gonococcal antigen concentration coated to wells as a measure of gonococcal density using MAb 2-8C-4-1, followed by anti-mouse IgG-alkaline phosphatase conjugate.

Serum bactericidal assays. Serum bactericidal testing was performed as described previously (30). Briefly, ~2,000 CFU of bacteria grown to the mid-log phase were incubated with NHS (concentration of NHS specified for each experiment) in a final reaction mixture volume of 150 μl. Duplicate aliquots of 25 μl were inoculated onto chocolate agar plates at 0 and 30 min. Survival was calculated as the percentage of the number of colonies that survived at 30 min relative to the baseline colony counts at 0 min. Growth of bacteria over the course of the assay (CFU at 30 min greater than CFU at 0 min) was assigned a survival of 100%.

Statistical analysis. The *t* test (two-tailed) was used to assess differences between strains in resisting complement in serum bactericidal assays and to evaluate differences in factor Bb binding between strains in whole-cell ELISA.

RESULTS

Characterization of the LOSs of strains. A schematic of *N. gonorrhoeae* HepI extensions and the LOS biosynthesis genes responsible for the addition of glycans is shown in Fig. 1. The LOSs of all strains used in this study were verified by mass spectrometry, which confirmed that all *lgtE* and *lgtF* mutants expressed the expected LOS structures (Glc→HepI and HepI unsubstituted, respectively), without alterations in other LOS substitutions (Table 2), thereby excluding polar effects that can result from insertional inactivation of these genes.

C4BP binding to Por1B, but not Por1A, is affected by alterations in HepI LOS chain length. We examined binding of purified C4BP and C4BP in NHS to strains MS11 and 1291 (both Por1B), FA19 (Por1A), and their *lgtA* (Gal→Glc→HepI), *lgtE* (Glc→HepI), and *lgtF* (HepI unsubstituted) mutants. In addition, we studied C4BP binding to a variant of

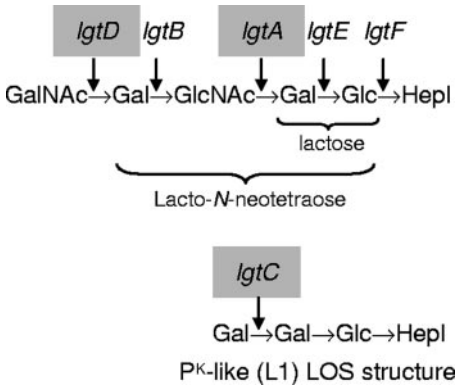

FIG. 1. Schematic diagram of *N. gonorrhoeae* LOS HepI glycan extensions. Genes subject to phase-variable expression are shown in shaded boxes.

strain 1291, selected under pyocin pressure and expressing the P^K-like LOS structure (Gal→Gal→Glc→HepI [28]), called 1291b (23). All of the *lgtA* mutants and 1291b bound similar amounts of C4BP and were equally or more serum resistant than their wild-type parent strains (data not shown); these strains were not studied further.

Truncation of HepI extension by inactivation of *lgtE* to yield a single Glc off of HepI resulted in decreased C4BP binding to Por1B strain 1291 (Fig. 2, middle); however, there was no effect on C4BP binding to the *lgtE* mutant of another Por1B strain, MS11 (Fig. 2, left), or to the *lgtE* mutant of Por1A strain FA19 (Fig. 2, right). Eliminating the proximal Glc on HepI (*lgtF* mutant) resulted in a marked decrease in C4BP binding to both Por1B strains (1291 and MS11) but did not affect binding of C4BP to Por1A strain FA19 (Fig. 2). These data suggest that optimal binding of C4BP to Por1B-bearing strains may require the presence of at least one hexose on HepI and in some instances at least two hexoses (a lactosyl residue [Gal→Glc]), as illustrated by strain 1291. In contrast, elimination of all HepI substitutions (*lgtF* mutation) did not result in a decrease in C4BP binding to the Por1A strain (FA19).

The relationship between C4BP binding and HepI chain length is determined by the Por1B molecule. We next asked whether differences in C4BP binding to the *lgtE* mutants of Por1B strains 1291 and MS11 were related to differences in the Por molecule. To address this question, we replaced the Por1B molecule of 1291 lgtE with MS11 Por by using plasmid pUNCH61 (4). The resulting mutant, called 1291 PorMS11 lgtE, bound amounts of C4BP similar to that of MS11 lgtE (Fig. 3A), suggesting that the amount of C4BP bound to *N. gonorrhoeae* expressing a specific LOS structure was dependent on the Por molecule present.

Further evidence for the specificity of the Por-LOS interaction in determining C4BP binding was provided by replacing F62 (C4BP nonbinder) *porB* with MS11 *porB* to yield F62 PorMS11 (38) and then truncating LOS by insertional inactivation of *lgtE* or *lgtF*. As seen in Fig. 3B, LOS mutations in the background of F62 PorMS11 affected C4BP binding; C4BP binding persisted in the *lgtE* mutant but was lost in the *lgtF* mutant. Taken together, these data indicate that the effect of the HepI chain length of LOS on C4BP binding was dependent on the specific Por molecule expressed by the strain.

TABLE 2. Negative-ion MS data and proposed compositions of O-deacylated LPS from *N. gonorrhoeae* strains MS11, 1291, FA19, and F62[a]

Strain	Observed ions (*m/z*)		Molecular mass (Da)[b]		Relative intensity[c]	Proposed composition
	$(M-2H)^{2-}$	$(M-3H)^{3-}$	Observed	Calculated		
MS11	1496.3	997.7	2,996.1	2,995.8	1.0	3Hex, 3HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
MS11 lgtE	1,132.2	754.7	2,267.1	2,265.1	1.0	Hex, HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
MS11 lgtF	1,051.1		2,104.2	2,102.9	1.0	HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
1291	1,679.1	1,119.2	3,360.4	3,361.1	1.0	4Hex, 4HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
	1,578.3	1,051.7	3,158.1	3,157.9	0.8	4Hex, 3HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
	1,497.0	997.6	2,995.9	2,995.8	0.4	3Hex, 3HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
	1,396.3	930.5	2,794.5	2,792.6	0.4	3Hex, 2HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
		876.1	2,631.3	2,630.4	0.2	2Hex, 2HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
1291 lgtE	1,132.1	754.7	2,266.6	2,265.1	1.0	Hex, HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
1291 lgtF	1,051.1		2,104.2	2,102.9	1.0	HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
1291 PorMS11 lgtE	1,132.2	754.7	2,266.7	2,265.1	1.0	Hex, HexNAc, 2Hep, 2Kdo, PEtn, lipid A-OH
FA19	1,457.8	971.5	2,917.5	2,995.8	1.0	3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
FA19 lgtE	1,194.2	796.0	2,390.7	2,388.1	1.0	Hex, HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
FA19 lgtF	1,112.6	741.6	2,227.5	2,226.0	1.0	HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
F62	1,457.7	971.6	2,917.6	2,915.6	1.0	3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	1,559.2	1,039.3	3,120.6	3,118.8	0.6	3Hex, 3HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
F62 PorMS11	1,457.3	971.6	2,917.2	2,915.6	0.8	3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
	1,559.8	1,039.2	3,121.5	3,118.8	1.0	3Hex, 3HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
F62 PorMS11 lgtE	1,194.1	795.7	2,390.1	2,388.1	1.0	Hex, HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH
F62 PorMS11 lgtF	1,113.1	741.6	2,228.0	2,226.0	1.0	HexNAc, 2Hep, 2Kdo, 2PEtn, lipid A-OH

[a] The wild type and *lgtE* and *lgtF* mutants were examined for each strain, as indicated; in some cases, the porin from MS11 had replaced the host porin, as indicated. Additionally, for each strain/mutant, there is a consistent variation in the phosphorylation pattern of the lipid A-OH molecule, resulting in lipid A-OH molecules of 952, 1,075, 1,155, and 1,278, as is commonly observed in the pathogenic neisseriae. For simplicity, these "phosphoforms" are not detailed here.

[b] Average mass units were used for calculation of molecular masses, based on proposed compositions, as follows: Sial, 291.00; Hex, 162.15; Hep, 192.17; HexNAc, 203.19; PEtn, 123.05; Kdo, 220.18. The average molecular mass of the O-deacylated lipid A (lipid A-OH) is as indicated.

[c] Intensities are expressed as relative to the largest ions of the indicated charge states.

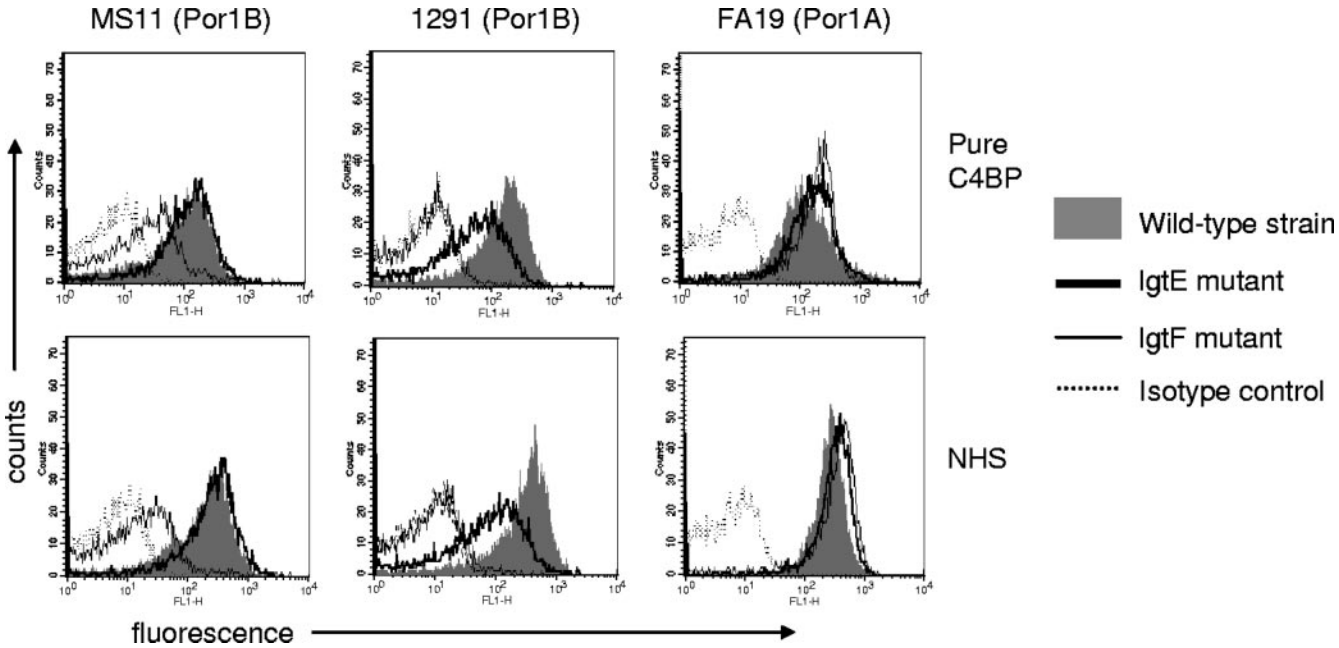

FIG. 2. C4BP binding to *N. gonorrhoeae* strains MS11 (Por1B), 1291 (Por1B), and FA19 (Por1A) and their *lgtE* and *lgtF* mutants. Binding of pure C4BP (1 μg/ml) is shown in the upper panel, and binding of C4BP when bacteria are incubated with NHS at a final concentration of 1% (vol/vol) is shown in the lower panel. Binding to wild-type strains is represented by shaded histograms, *lgtE* mutants by the thick solid lines, and *lgtF* mutants by the thin solid lines. In each instance, a representative isotype control with the wild-type strain (where C4BP or NHS is excluded from the reaction mixture) is shown by the broken line. One representative experiment of at least three reproducibly repeated experiments is shown.

A possible explanation for altered C4BP binding as a result of allelic *por* replacements could be different levels of Por expression among the mutants. For example, decreased C4BP binding of 1291 lgtE relative to1291 PorMS11 lgtE could result from less expression of Por by the former strain. We examined C4BP binding and Por expression simultaneously in 1291, MS11, their *lgtE* and *lgtF* mutants, and 1291 PorMS11 lgtE by Western blotting after the strains were incubated with NHS. As seen in Fig. 4A, the lower part (proteins with molecular masses of <50 kDa) of the blot, stained with Coomassie blue, revealed no differences in Por expression among the strains. C4BP binding to strains in the same blot was examined by probing proteins of >50 kDa (Fig. 4A, top) with anti-C4BP and showed results consistent with fluorescence-activated cell sorting analysis (Fig. 1, middle and left tracings). These data strongly suggested that the differences in C4BP binding among strains were not explained by differences in levels of Por molecule expression. The availability of a MAb against MS11 Por allowed us to assess Por expression across all MS11 Por-expressing mutants while simultaneously examining C4BP binding. As seen in Fig. 4B, MS11 Por expression (lower panel) by the MS11 LOS mutants and the corresponding LOS mutants in the background of F62 PorMS11 was similar. Simultaneous detection of C4BP bound to bacteria on the same blot (Fig. 4B, top) confirmed the previously demonstrated differences in C4BP binding among strains determined by fluorescence-activated cell sorting analysis (Fig. 1, left tracing, and Fig. 3, right tracing). Taken together, these data provide firm evidence that Por expression levels were similar across strains, are not influenced by the process of allelic replacement, and therefore are not directly responsible for the differences in C4BP binding levels among the strains studied.

Correlation between C4BP binding and resistance to killing by NHS (serum resistance). We examined the ability of the strains used in this study to resist killing by NHS as a functional correlate of C4BP bound to the different strains (Table 3). The effect of HepI truncations on serum resistance of the Por1B-bearing strains MS11 and 1291 were first examined. As expected, both wild-type strains survived 100% in 10% NHS. Increasing the NHS concentration to 50% resulted in partial survival of both wild-type strains (59.6% and 51.6% for MS11 and 1291, respectively). Mutants that were low or nonbinders of C4BP (MS11 lgtF and 1291 lgtF) were fully sensitive (100% killing) when incubated with even 10% NHS. 1291 lgtE, which bound "intermediate" levels of C4BP, showed only ~9% survival in 10% NHS ($P < 0.001$ [compared to wild-type 1291]). MS11 lgtE was more serum resistant than MS11 (100% survival in 50% NHS; $P < 0.001$ [compared to MS11]).

Evidence that the lower level of C4BP binding of 1291 lgtE was most likely responsible for its serum-sensitive phenotype was provided by allelic replacement of 1291 lgtE with MS11 Por. The resulting mutant, 1291 PorMS11 lgtE, which bound more C4BP than did 1291 lgtE (Fig. 3A), also showed enhanced serum resistance, thus providing strong evidence for a correlation between increased C4BP binding and enhanced serum resistance in an isogenic background.

However, replacement of Por of F62 and F62 lgtE with MS11 Por did not restore serum resistance (100% killing in even 10% NHS), despite the ability of both of these mutants (F62 PorMS11 and F62 PorMS11 lgtE) to bind C4BP (Fig.

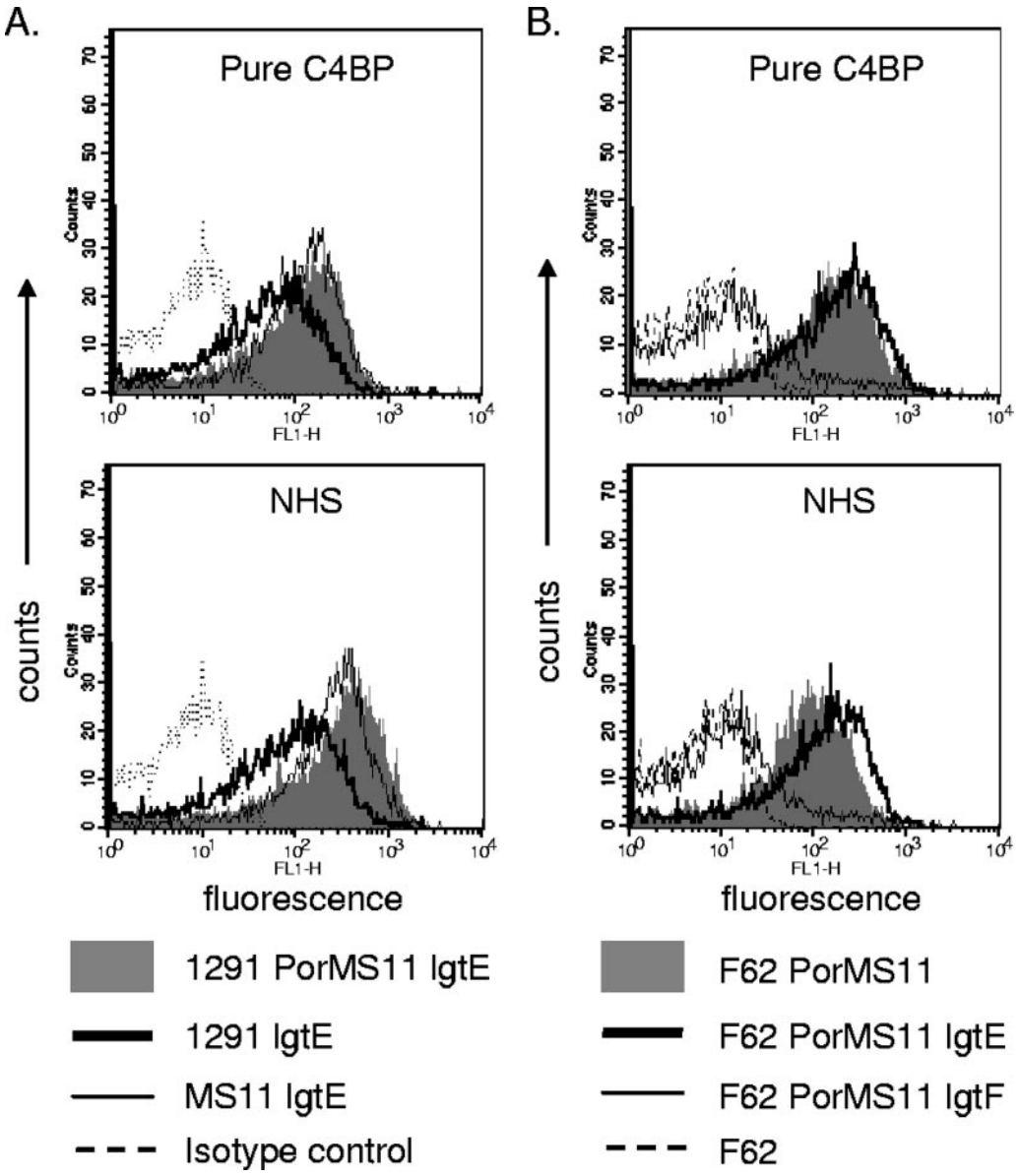

FIG. 3. The effect of LOS HepI substitutions on C4BP binding is Por specific and independent of strain background. (A) Replacing Por of 1291 lgtE with MS11 increases C4BP binding to levels seen with MS11 lgtE. The upper graph shows binding of pure C4BP (1 μg/ml), and the lower graph shows binding of C4BP in 1% (vol/vol) NHS. Binding to 1291 lgtE and 1291 PorMS11 lgtE is shown by the thick solid line and shaded histogram, respectively. Binding to control strain MS11 lgtE is shown by the thin line. A representative isotype control (where either pure C4BP or NHS is excluded from the reaction mixture) is shown by the broken line. (B) The effects of HepI LOS substitutions on C4BP binding to MS11 Por1B in the background of F62 simulate binding in the MS11 background. Binding of purified C4BP (1 μg/ml) (upper graph) or C4BP in 1% (vol/vol) NHS (lower graph) to F62 PorMS11 (shaded histogram), F62 PorMS11 lgtE (thick solid line), and F62 PorMS11 lgtF (thin solid line) is shown. C4BP binding to negative control strain F62 is shown by the broken line. One representative experiment of three reproducibly repeated experiments is shown.

3B). We have previously analyzed complement binding to F62 PorMS11 and have ascribed its serum sensitivity (despite C4BP binding) to kinetically overwhelming classical pathway activation (38). Another possible explanation for the serum-sensitive phenotype of F62 PorMS11 and F62 PorMS11 lgtE could be the increased ability of F62 to activate the alternative pathway of complement; this is addressed below. As expected, F62 PorMS11 lgtF, which did not bind C4BP, was serum sensitive.

Por1A strain FA19 showed 100% survival in both 10% and 50% NHS. Unlike the Por1B strains, truncation of the LOS of Por1A strain FA19 did not affect C4BP binding (Fig. 2). As expected, FA19 lgtE was fully resistant to killing by 50% NHS (100% survival). Consistent with its ability to bind to C4BP, the *lgtF* mutant of Por1A strain FA19 was resistant to 10% NHS but was fully killed (0% survival) by 50% NHS. These data suggested a role for the proximal Glc on HepI in enhancing resistance to complement, which was independent of C4BP binding.

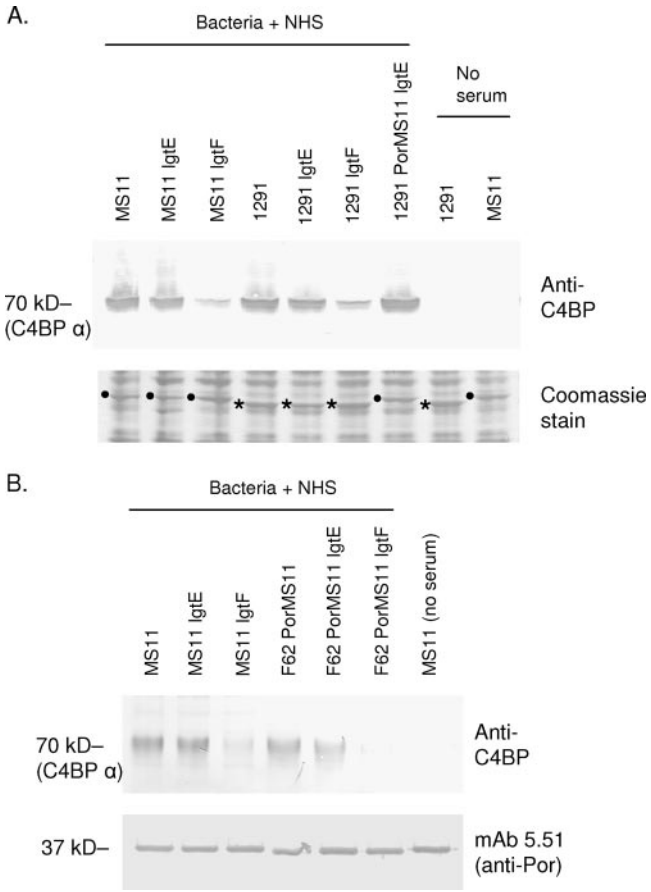

FIG. 4. Changes in C4BP binding seen with HepI LOS mutants are not related to differences in Por expression levels. (A) Strains MS11, 1291, their *lgtE* and *lgtF* mutants, and 1291 PorMS11 lgtE were incubated with 1% NHS. Following extensive washing, bacterial lysates were subjected to electrophoresis and Western blotting. Control lanes included bacteria alone (no NHS in reaction mixture). With the 50-kDa marker used as a guide, the blot was cut horizontally; the upper portion (proteins of >50 kDa) was probed with polyclonal anti-human C4BP, and the lower portion (proteins of <50 kDa) was stained with Coomassie blue. The location of the 70-kDa C4BP α chain is shown. The positions of the ~37-kDa MS11 and 1291 Por1B molecules are indicated on the Coomassie-stained blots by black dots and asterisks, respectively. (B) C4BP binding and Por expression on MS11, F62 PorMS11, and their *lgtE* and *lgtF* mutants was examined. As described above, bacteria were incubated with NHS and Western blotting was performed. Proteins of >50 kDa were probed with anti-C4BP, and proteins of <50 kDa were probed with anti-MS11 Por1B MAb 5.51.

Alternative pathway activation on mutants in the F62 background. We have shown previously that kinetically overwhelming classical pathway activation on F62 PorMS11 is probably the reason for its serum-sensitive phenotype, despite its ability to bind to C4BP (38). However, F62 PorMS11 lgtE was also serum sensitive despite its binding to C4BP and possessing the same LOS structure as the serum-resistant strain MS11 lgtE. In addition to increased classical pathway activation, we addressed the possibility of enhanced alternative pathway activation, as has been previously reported for serum-sensitive strains such as F62 (15). We used whole-cell ELISA to measure factor Bb binding to the bacterial surface as a measure of alternative pathway activation. As seen in Fig. 5A, F62 bound significantly more factor Bb than did MS11. In addition, we also noted that a decrease in HepI chain length resulted in increasing alternative pathway activation (measured by Bb binding) on the F62 PorMS11 LOS mutants (F62 PorMS11 lgtF > F62 PorMS11 lgtE > F62 PorMS11). In contrast, MS11 lgtE and MS11 lgtF did not show the marked increments in factor Bb binding compared to the parent strain MS11. Equal capture of bacteria to microtiter wells was confirmed with an anti-H.8 lipoprotein MAb, where the readings of the optical density at 410 nm (OD_{410}) ranged between 0.783 and 0.851 (mean ± standard deviation [SD], 0.813 ± 0.02). Collectively, these data point to a role for higher levels of alternative pathway activation in the background of F62, which could contribute to increased killing of F62 PorMS11 lgtE and F62 PorMS11 lgtF, despite the ability of the former to bind to C4BP.

To further define the role of factor B in complement activation and killing of these mutants, we tested the strains using factor B-depleted serum in serum bactericidal assays. Parent strain F62 and all of the F62 PorMS11 mutants were completely killed by even 5% factor B-depleted serum, supporting increased classical pathway activation on these strains. Decreasing the factor B-depleted serum concentration to 2.5% in the bactericidal assays revealed differences among the strains (Fig. 5B). As expected, the C4BP-binding mutants (F62 PorMS11 and F62 PorMS11 lgtE) showed significantly increased survival compared to the C4BP-nonbinding strains F62 and F62 PorMS11 lgtF. Factor B-depleted serum reconstituted with pure factor B served as a control in these experiments but did not enhance killing at this concentration of serum.

C4BP cofactor activity on and complement binding to FA19 lgtF. We analyzed complement binding to FA19 lgtF to better understand the reason for its relatively serum-sensitive phenotype (resistant to 10% NHS but killed by 50% NHS) despite C4BP binding, which would shed light on the role of the proximal Glc on HepI in complement evasion. We first focused on an analysis of C4b degradation (a measure of the cofactor activity of C4BP) on FA19 and its *lgtF* mutant, to assess

TABLE 3. Serum bactericidal assays

Strain	Mean % survival (SD) in[a]:	
	10% NHS	50% NHS
MS11	100	59.6 (8.9)*
MS11 lgtE	100	100
MS11 lgtF	0	0
1291	100	51.6 (3.6)
1291 lgtE	9.1 (3.6)	0
1291 lgtF	0	0
1291PorMS11 lgtE	100†	54.6 (13.1)†
FA19	100	100
FA19 lgtE	100	100
FA19 lgtF	100	0
F62	0	0
F62 PorMS11	0	0
F62 PorMS11 lgtE	0	0
F62 PorMS11 lgtF	0	0

[a] Symbols: *, $P < 0.001$ (compared to MS11 lgtE); †, $P < 0.001$ (compared to 1291 lgtE at the corresponding serum concentration).

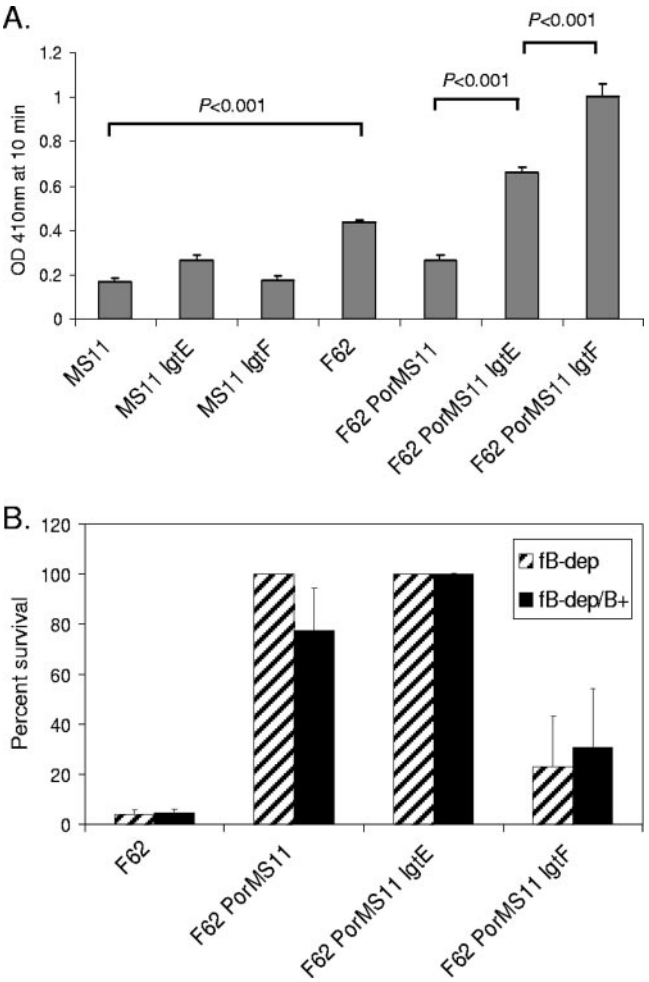

FIG. 5. (A) Increased alternative pathway activation on F62 and mutants in an F62 background. Factor Bb binding to F62 PorMS11 and its LOS mutants was measured by whole-cell ELISA using anti-factor Bb MAb. As controls in this experiment, we used the corresponding LOS mutants in the background of MS11 that contained its native (MS11) Por and wild-type strain F62. Bacteria were incubated with NHS at a final concentration of 10% (vol/vol). (B) Resistance of strains to killing by factor B-depleted serum. F62, F62 PorMS11, F62 PorMS11 lgtE, and F62 PorMS11 lgtF were incubated with factor B-depleted serum (2.5% [vol/vol]), and bactericidal assays were performed (hatched bars). Factor B-depleted serum reconstituted with physiological amounts of factor B was used as a control (solid black bars). The survival of F62 PorMS11 lgtF was significantly lower than those of F62 PorMS11 and F62 PorMS11 lgtE ($P \leq 0.05$).

whether C4BP bound to FA19 lgtF was functionally less effective than C4BP bound to the wild-type FA19 strain.

C4BP regulates classical complement pathway activation by serving as a cofactor in the inactivation of C4b by factor I and yields the C4 fragments C4d (which remains bound to the bacterial surface) and C4c (released into solution). Cofactor function of C4BP was assessed with MAbs directed against C4c and C4d. Anti-C4d is specific for the parent molecule C4b as well as for the fragment C4d; MAb against C4c recognizes C4b and C4c but not C4d. Therefore, cofactor activity will not alter the amount of C4 measured on the bacterial surface by the MAb against C4d but will decrease the amount of C4 bound to the organism measured by MAb against C4c, resulting in a higher C4d/C4c ratio (27).

As a negative control for the lack of C4BP cofactor activity in these experiments (a C4d/C4c ratio approaching unity), we used strain MS11 lgtF. Strains MS11 and FA19 have been shown previously to have high C4d/C4c ratios (38) and served as positive controls for cofactor activity on their surfaces. As seen in Fig. 6, no difference in the measurement of C4d that bound to FA19 and FA19 lgtF was detected, suggesting that the numbers of C4b molecules deposited (a function of Ig binding) were similar for both strains. The C4d/C4c ratios were also similar for both strains, suggesting that C4BP cofactor function measured at 30 min was equivalent. In contrast, the negative control strain MS11 showed a C4d/C4c ratio of 1.1, while the parent strain MS11 showed C4b processing by C4BP (C4d/C4c ratio of 3.7).

We also compared factor H and total C3 binding to FA19 and FA19 lgtF and did not observe differences in levels of binding of either of these complement components (data not shown). Factor Bb binding experiments were also performed, and the OD_{410} (mean ± SD) of Bb bound to FA19 lgtF was 0.257 ± 0.05, compared to 0.069 ± 0.001 for Bb bound to FA19 ($P < 0.001$). Although the absolute amount of Bb binding to FA19 lgtF was less than that seen with serum-sensitive strains such as F62 (Fig. 5), it may have contributed to the diminished survival of this mutant at higher serum concentrations.

DISCUSSION

Complement is a key component of innate immune defenses against pathogenic neisseriae. *N. gonorrhoeae* has evolved several mechanisms to evade human complement. One such mechanism is binding of the complement-inhibitory proteins factor H and C4BP. Sialylation of gonococcal LNT LOS enhances factor H binding (40). Factor H can also bind to select Por1A-bearing strains, independently of LOS sialylation (39). C4BP is a key inhibitory molecule of the classical pathway of complement because it acts as a cofactor in factor I-mediated cleavage of C4b (10, 11) and also causes irreversible decay of the classical pathway C3 convertase, C4b2a (13). We have detailed previously the interactions between C4BP and gonococcal Por and have shown that preventing C4BP binding to the bacteria with a competitive inhibitor renders otherwise serum-resistant strains susceptible to killing by human complement.

LOS structure has been shown previously to play a key role in the resistance of *N. gonorrhoeae* to complement-dependent killing. Morse and Apicella (31) isolated an LOS variant of serum-resistant strain JW31, called JW31R, under *Pseudomonas aeruginosa* pyocin pressure that was serum sensitive. Subsequent analysis of the JW31R LOS showed that it possessed an unusual GalNAc→Hex→Hex→Hex→HepII extension and either a lactose or Gal→lactose (P^K-like LOS) on HepI (12). A study by Guymon et al., who also used pyocin pressure to select mutants of strain FA19, noted that two mutants that expressed truncated LOS molecules, one called FA5100 (lacking Glc, Gal, and Hep and having greatly reduced GlcNAc content) and another called FA5000 (expressing smaller amounts of Glc, Gal, and GlcNAc), were both serum sensitive compared to parent strain FA19 (18). FA5100 was shown to be killed

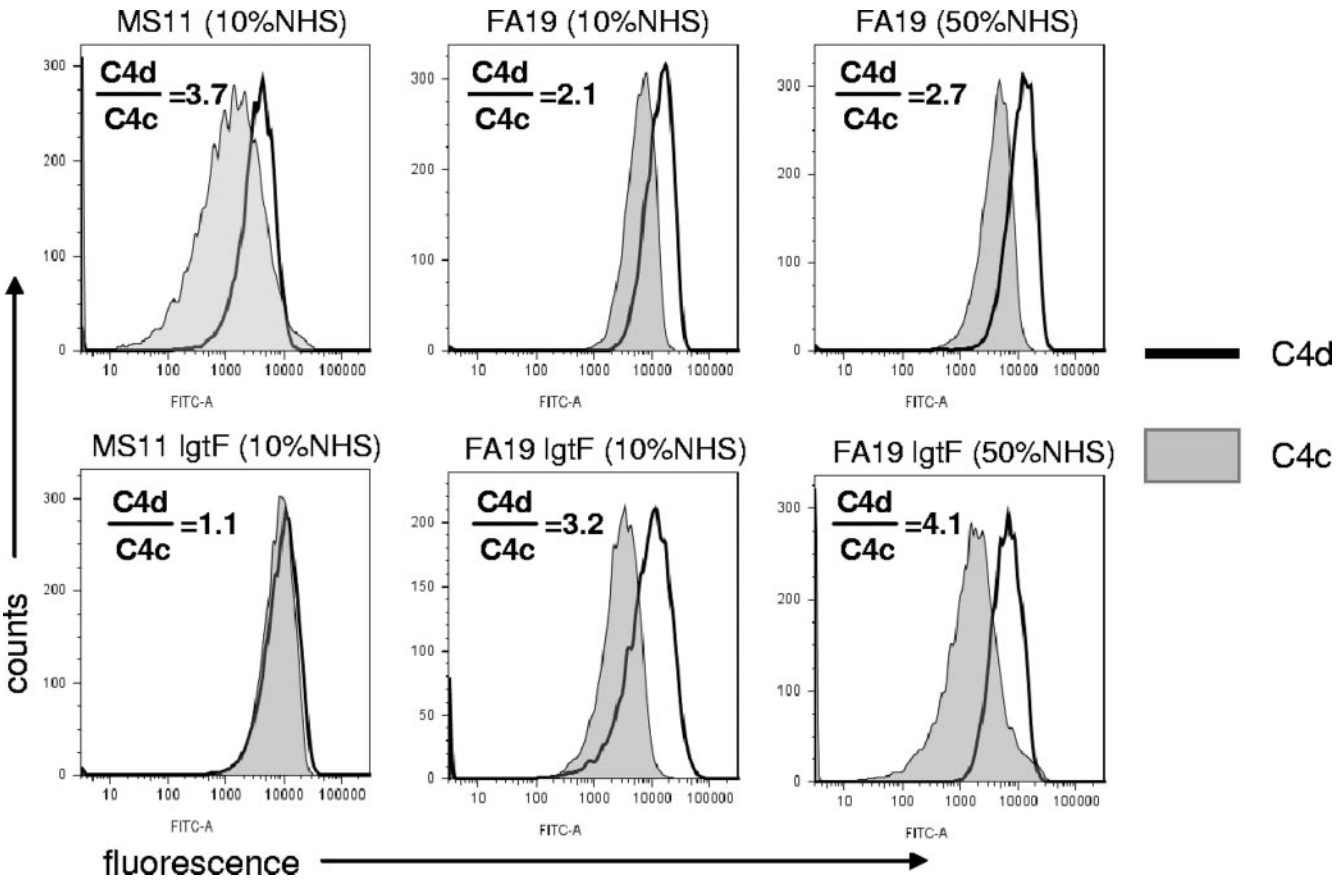

FIG. 6. C4BP cofactor activity on FA19 and FA19 lgtF. C4c and C4d binding to FA19 and FA19 lgtF was compared to assess C4BP cofactor activity on the two strains. MS11 (C4BP binder, serum resistant) and MS11 lgtF (C4BP nonbinder, serum sensitive) were used as controls. A high C4d/C4c ratio indicates C4b cleavage (and C4BP cofactor activity) on the bacterial surface.

even by agammaglobulinemic serum, suggesting increased susceptibility to the alternative pathway of complement.

While the mutants discussed above highlight the importance of LOS extensions beyond 2-keto-3-deoxyoctulosonic acid in serum resistance, they represent unusual structures selected by pyocin pressure and are of uncertain pathophysiological significance. Three phase-variable genes in the *lgtABCDE* operon, namely, *lgtA*, *lgtC*, and *lgtD*, determine gonococcal HepI LOS substitutions. Expression of a terminal GalNAc on HepI LOS of *N. gonorrhoeae* (because of *lgtD* expression with *lgtA* concomitantly "on") enabled binding of naturally occurring IgM, which resulted in killing of strains that also activated the alternative pathway secondarily to direct binding of properdin (15). Shafer et al. (46) examined serum resistance of LOS phase variants of a transformant of strain FA19 in which the native Por1A molecule was replaced by the Por1B molecule. A variant in which *lgtA* was "off" as a result of slipped-strand mispairing (and therefore expressed only a lactose extending from HepI LOS) was fully serum resistant (~100% survival) in 33% NHS. In contrast, a variant that had both *lgtA* and *lgtD* phase varied on (and therefore expressed a GalNAc distal to the LNT on HepI) showed <1% survival under similar conditions. Likewise, *N. gonorrhoeae* strain 15253, whose *lgtB* to *lgtD* genes are naturally deleted and whose *lgtA* gene is defective (9), extends lactose (only) from HepI LOS, binds C4BP, and is serum resistant (32, 38). Collectively, the above data clearly implicate LOS structure in modulating serum resistance.

Because *lgtE* and *lgtF* are not phase variable, the shortest HepI chain extension that has been described in clinical isolates of *N. gonorrhoeae* is a lactose (Gal→Glc→HepI), which results when *lgtA* and *lgtC* are both phase varied off or, as in the case of strain 15253, where *lgtA* is deficient and *lgtC* absent (2). Expression of this 3.6-kDa LOS molecule has also been associated with serum resistance in other gonococcal strains (43). We observed that C4BP binding was not affected in *lgtA* deletion mutants that express the L8-like LOS (data not shown). Turning *lgtA* off would enable gonococci to bind less naturally occurring antibody (as might otherwise occur if phase-variable genes such as *lgtD* and *lgtA* are both on concomitantly) (43) and yet retain the ability to inhibit complement by binding C4BP.

Expression of LNT LOS confers certain advantages on the organism: sialylation of LNT LOS enables gonococci to decrease nonopsonic opacity-associated protein-mediated uptake by polymorphonuclear neutrophils (PMNs) (41) and, as a result of factor H binding and complement regulation (40), also may reduce the amount of opsonic mediated uptake of serum-sensitive strains by PMNs (26). In the unsialylated state, LNT increases the ability of bacteria to invade epithelial cells via binding to the asialoglycoprotein receptor (20, 35). Host-spe-

cific immune pressures may select for expression of certain LOS immunotypes. Our data suggest that the HepI LOS extensions that are expressed naturally (such as lactose, LNT, and P^K) by clinical isolates as a result of phase variation of *lgtA*, *lgtD*, and *lgtC* allow optimal C4BP binding, which may be important in bacterial survival in vivo.

We noted in the present study that the proximal Glc on HepI was critical for C4BP binding to Por1B strains. Deletion of *lgtF* resulted in almost complete abrogation of C4BP binding to the two C4BP-binding Por1B strains that we studied, MS11 and 1291 (Fig. 2). The amount of C4BP binding correlated well with resistance to serum killing; for example, replacement of the 1291 Por 1B with MS11 Por 1B in 1291 lgtE resulted in higher C4BP binding and restoration of high-level serum resistance (Table 3). These data also suggested that the effects of HepI LOS truncation on C4BP binding were specific for each Por molecule independently of the background of the strain; i.e., Por 1B from MS11 was shown to bind to C4BP when its gene was transformed into either 1291 lgtE or F62 lgtE. In addition, we showed that changes in C4BP binding when LOS was altered or when allelic replacement of *porB* was performed were not due to differences in the levels of Por expression (Fig. 4).

While the loss of C4BP binding was clearly associated with increased sensitivity to killing by NHS, the converse was not always observed; i.e., not all mutants that bound C4BP were resistant to serum. F62 PorMS11 and F62 PorMS11 lgtE are illustrative examples, where 100% killing in 10% NHS ensued despite C4BP binding. We have previously analyzed Ig and complement C3, C4, and C5b-9 binding to F62 PorMS11 and showed that high levels of IgM binding to the F62 background resulted in kinetically overwhelming complement activation, which could overcome regulation by C4BP (38). Griffiss et al. (15) have shown that binding of naturally occurring IgM against the terminal GalNAc of neisserial LOS (as expressed by F62), plus added alternative pathway activation, as evidenced by direct binding of properdin to a 39-kDa molecule (specifically seen on serum-sensitive strains such as F62), accounts for bacterial lysis. In agreement with the findings of Griffiss et al., we demonstrated increased alternative pathway activation (measured by factor Bb binding) on mutants where MS11 Por was introduced into the F62 background. Another noteworthy finding was that truncation of the HepI chain of LOS was associated with increased alternative pathway activation (Fig. 5A). It is possible that decreasing LOS HepI length allows greater access of properdin to its target on F62, resulting in greater stability of the C3bBb convertase. We attempted to determine the functional role that the alternative pathway contributed in killing of F62 PorMS11 and its LOS mutants but noted that even 5% factor B-depleted serum killed these strains, suggesting overwhelming classical pathway activation. We did observe the expected relative differences in serum resistance among the strains when serum concentrations were further decreased to 2.5% but at this lower serum concentration, reconstituting factor B-depleted serum with pure factor B did not enhance bacterial killing because alternative pathway activation is concentration dependent and does not function at serum dilutions below 1:16 (45). The molecular mechanism and the physiological relevance of the high levels of alternative pathway activation on serum-sensitive *N. gonorrhoeae* strains such as F62 is not fully clear and merits further consideration. We have shown previously that replacement of F62 Por with FA19 Por resulted in a serum-resistant phenotype (38). Factor H binds to the Por1A molecule of FA19 and inhibits activation of the alternative pathway (39), which may not occur on F62 PorMS11 (Por1B; serum sensitive). Furthermore, the function of factor H binding to FA19 Por1A may be reflected by the observation that FA19 shows 100% survival in 50% NHS, while both wild-type Por1B strains in the present study that bind only C4BP show intermediate serum resistance at this higher serum concentration (Table 3). Collectively, these results emphasize that killing of neisseriae by complement is a determined by several variables, and the final outcome (i.e., survival versus killing) reflects the balance between complement activation and inhibition that occurs on the bacterial surface.

While the proximal Glc on HepI was required for C4BP binding to Por1B strains, we observed that Por1A strain FA19 bound C4BP even when it lacked any HepI glycan substitution. The lgtF (HepI unsubstituted) mutant was fully resistant to 10% NHS (in contrast to the lgtF mutants of Por1B strains that bound C4BP weakly), further underscoring an important role for C4BP in serum resistance. However, FA19 lgtF was sensitive to 50% NHS, while FA19 and FA19 lgtE were both resistant at this concentration of NHS (Table 3). Similar results were observed by Kahler et al., who showed that a *pgm* mutant of FA19 (which also has no glycan substitutions on HepI and therefore makes an LOS that is phenotypically similar to that of FA19 lgtF) showed 100% survival in 10% NHS but ~90% killing in 50% NHS (25). In an attempt to understand the basis for the relative serum sensitivity of FA19 lgtF, we examined cofactor activity of C4BP on its surface to ensure that surface-bound C4BP was functional. We saw no difference in C4b processing when FA19 was compared with its parent strain (Fig. 6). We also did not observe differences in the amounts of factor H, C3, or C5b-9 (data not shown) bound to FA19 lgtF and FA19. However, we detected more factor Bb on FA19 lgtF, which suggests that more alternative pathway components were recruited to the surface of this strain. We did not, however, detect differences in the total amount of complement activation in a 30-min period as measured by C5b-9 binding. It is possible that subtle differences in the rate of complement activation may contribute to increased susceptibility of FA19 lgtF to complement when exposed to 50% NHS. In addition, killing by complement requires optimal MAC insertion in the bacterial membrane. Joiner et al. (24) have shown that C5b-9 is associated with distinct proteins in serum-resistant and serum-sensitive gonococcal strains. It is possible that the proximal Glc residue on HepI plays a role in protecting bacteria against the effects of the C5b-9 complex; loss of this Glc may render bacteria more susceptible to the lytic effects of the terminal complement cascade.

In conclusion, we have identified a mechanism by which HepI substitutions of gonococcal LOS can affect serum resistance. Binding of complement-inhibitory proteins such as C4BP and factor H constitutes an important mechanism of complement evasion by *Neisseria gonorrhoeae* (38–40). Most naturally occurring gonococcal strains express at least a lactosyl residue on HepI, and we have shown that expression of the proximal Glc (and in some instances expression of the lactosyl substitution) is critical for optimal C4BP binding and comple-

ment evasion. In light of these findings, the effects of other LOS modifications (as may occur on HepII and lipid A) strongly merit consideration.

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