The Role of the Exopolyphosphatase PPX in Avoidance by Neisseria meningitidis of Complement-mediated Killing

Qian Zhang, Yanwen Li, and Christoph M. Tang

From the Centre for Molecular Microbiology and Infection, Department of Microbiology, Flowers Building, Imperial College London, London SW7 2AZ, United Kingdom

The complement system is critical for immunity against the important human pathogen Neisseria meningitidis. We describe the isolation of a meningococcal mutant lacking PPX, an exopolyphosphatase responsible for cleaving cellular polyphosphate, a polymer of tens to hundreds of orthophosphate residues found in virtually all living cells. Bacteria lacking PPX exhibit increased resistance to complement-mediated killing. By site directed mutagenesis, we define amino acids necessary for the biochemical activity of PPX. ppx mutants do not result from changes in structures (such as capsule, lipopolysaccharide, and factor H-binding protein), which are known to be required for evasion of this key aspect of host immunity. Instead, expression of PPX modifies the interaction of N. meningitidis with the alternative pathway of complement activation.

Neisseria meningitidis remains a leading cause of septicemia and bacterial meningitis worldwide (1). Although the bacterium is a common commensal of the human upper respiratory tract in between 5 and 40% of healthy adults (2), N. meningitidis occasionally disseminates from this site, entering the bloodstream and the cerebrospinal fluid to cause bacteremic disease and meningitis. Meningococcal disease can progress rapidly, and septicemia still carries a significant case fatality rate of ~10% (3). Furthermore, survivors can be left with disabilities, including hearing and other neurological deficits and loss of digits or limbs. The pathological consequences of meningococcal infection are dependent on the ability of the bacterium to survive and replicate within the systemic circulation, and the level of bacteremia, which can reach as high as $10^9$–$10^9$ cfu/ml (4), is directly correlated with the outcome. To reach such high levels, N. meningitidis must obtain nutrients to sustain its replication and avoid killing by the immune system (5, 6).

Complement is critical to immunity against N. meningitidis. This is demonstrated by the exquisite susceptibility of individuals with complement deficiencies to meningococcal disease (7); people with inherited absence of components of the membrane attack complex (MAC; C5–9-inclusive), which is necessary for the bacteriolytic activity of complement, have over a 1,000-fold increased lifetime risk of developing systemic meningococcal disease and are prone to recurrent infection (7). Additionally, polymorphisms associated with other genes encoding complement factors, including mannose binding lectin and factor H (8, 9), can increase susceptibility to meningococcal disease, and acquired C2 and C3 deficiency is a further risk factor for meningococcal sepsis (7).

The complement system can be activated through any of three pathways: the classical (CP), lectin (LP), and alternative (AP) pathways. All pathways converge at the cleavage of C3 to C3b by a C3 convertase. C3b is covalently linked to the surface of pathogens, which are then eliminated through phagocytosis or lysis following assembly of the MAC (10). The CP is initiated mainly by antibody binding to a foreign antigen, whereas the LP is activated through recognition of carbohydrates on the pathogen surface. In contrast, the AP largely serves to amplify complement activation initiated by the other pathways (10) and is precisely regulated during health by a series of membrane-bound and soluble factors such as factor H (11).

Several factors enable the meningococcus to overcome complement-mediated killing. Virtually all isolates of N. meningitidis from the systemic circulation express a polysaccharide capsule, whereas only around 40% of isolates from the nasopharynx are encapsulated (2). Strains lacking a capsule or expressing truncated forms of lipopolysaccharide (LPS) are highly sensitive to complement-mediated lysis in the presence of human serum (12). LPS can be modified by addition of a sialic acid residue, and this can promote resistance against complement (13). The importance of both capsule and lipopolysaccharide was highlighted in a study of over 4,000 N. meningitidis mutants in which all 18 mutants with enhanced sensitivity to complement were affected in the biogenesis of either capsule or LPS (14). More recently it has also been shown that the bacterium recruits factor H (15, 16), the main negative regulator of the AP, via a lipoprotein called factor H-binding protein (fHbp) to enhance resistance against complement (17).

Here we sought to further understand the interaction of N. meningitidis with the human complement system by screening a library of transposon mutants for those with enhanced resistance against complement-mediated killing. We describe the identification of a mutant lacking an enzyme encoded by NMB1467 that we show is a polyphosphatase (PPX) and is pathway; fHbp, factor H-binding protein; BHI, brain heart infusion; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NHS, normal human serum; PPX, polyphosphate kinase; PPX, exopolyphosphatase.

1 To whom correspondence should be addressed. Fax: 44-207-594-3076; E-mail: c.tang@imperial.ac.uk.
2 The abbreviations used are: MAC, membrane attack complex; polyP, polyphosphate; CP, classical pathway; LP, lectin pathway; AP, alternative
TABLE 1

Bacterial strains and plasmids used in this study

| Strains/plasmids | Genotype/description | Ref. |
|------------------|----------------------|------|
| N. meningitidis  |                      |      |
| MC58             | Wild-type serogroup B| 19   |
| MC58::Tn5 or MC58ppx::Tn5 | Insertional inactivation of nmb1467 | This study |
| MC58ΔsiaD        | Insertional inactivation of siaD | 5    |
| MC58::Tn5ΔsiaD  | Insertional inactivation of siaD and ppx | This study |
| MC58Δlst        | Insertional inactivation of lst and ppx | This study |
| MC58::Tn5Δlst   | Insertional inactivation of lst and ppx | This study |
| MC58::Tn5Δppx   | Insertional inactivation of lst and ppx | This study |
| MC58::Tn5Δppx   | Insertional inactivation of lst and ppx | This study |
| MC58ppx::Tn5    | Insertional inactivation of lst and ppx | This study |
| MC58ppx::Tn5    | Insertional inactivation of lst and ppx | This study |
| MC58Δppx        | Insertional inactivation of ppx | This study |
| MC58ΔHpβ        | Insertional inactivation of Hpβ | This study |
| MC58::Tn5ΔHpβ  | Insertional inactivation of ppx | This study |
| E. coli         |                      |      |
| TOP10           | F− mcrA Δ(mrr-ksdRMS-mcrBC) q80lacZΔM15 ΔlacX74 recA1 araD139 Δ araE19 K12 | Invitrogen |
| BL21(DE3)pLysE | F− ompT hsdS(g m c) gal dcm (DE3) pLysE (Cam8) | Novagen |
| TT24554         | E. coli Top10/pHis-ppk | Invitrogen |
| Plasmids        |                      |      |
| pCR2.1-TOPO     | Cloning vector | Invitrogen |
| pET-15b         | Vector for protein expression | Novagen |
| pYH525          | Vector for complementation | This study |
| pmbi1467-pET-15b | pET-15b containing nmb1467 | This study |
| pmbi1467-E147A-pET-15b | pET-15b containing nmb1467 | This study |
| pmbi1467-pYH525 | pYH525 containing nmb1467 | This study |
| PCRs were performed in a 50-µl volume with a 0.2 µM concentration of each primer, 2–50 ng of template DNA, and 2 units of Taq DNA polymerase. Details of the oligonucleotides are given in Table 2. Polymerase with proof reading activity (Expand High Fidelity, Roche Applied Science) was used to amplify DNA fragments for complementation and protein expression. For Southern analysis, genomic DNA was digested, and the fragments were separated by electrophoresis and then transferred to nitrocellulose membranes (Hybond N+, GE Healthcare) by standard methods. Probes were labeled using the random primer method (New England Biolabs), and hybridizations were performed as described previously (19).

The library of mutants was constructed by in vitro mutagenesis with Tn5 (Epicontr). Chromosomal DNA from N. meningitidis strain MC58 was incubated with Tn5 containing a Neisseria DNA uptake sequence and an E. coli origin of replication along with purified transposase. Reaction products were repaired with T4 DNA polymerase and T4 DNA ligase (New England Biolabs) and used to transform N. meningitidis to kanamycin resistance.

For complementation, nmb1467 was amplified from genomic DNA with primers NG551 and NG552, the product was digested with the appropriate restriction enzymes and ligated into pYH525 (20), and the construct was used to transform MC58::Tn5, resulting in MC58ppx·E147A. The same approach was used to generate MC58ppx·E147A. To construct the N. meningitidis pppc mutant (21, 22), the entire nmb1900 ORF was amplified from MC58 genomic DNA with primers NG1153 and NG1157 and cloned into pTrcHisB (Invitrogen). The resulting plasmid was linearized by digestion with EcoRI (at nucleotide 1704 in the ORF) and ligated to a gene encoding tetracycline resistance (tetM) (23). The construct was amplified by PCR and introduced into N. meningitidis by transformation. The identity and genomic location of the transposon in the mutants were confirmed by PCR and Southern analysis.

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RNA Isolation and RT-PCR—For RNA isolation, bacteria were grown in liquid BHI with gentle agitation to midlog phase and harvested by centrifugation at 4,000 × g, and RNA was extracted with the RNeasy method (Qiagen). Genomic DNA was removed by DNase treatment (Qiagen), and RNA was reverse transcribed to cDNA using SuperScript III First Strand Synthesis (Invitrogen). Transcript levels were measured by quantitative real time RT-PCR with SYBR Green detection strategy. The upstream region of nmb1467 was amplified using primers NG552 and NG559, and the product was digested with NdeI and ligated into pET-15b (Novagen) to generate pET-15b/nmb1467. pET-15b was constructed using the QuickChange site-directed mutagenesis kit (Stratagene) with pET-15b as DNA template and primers NG607 and NG608. Alanine substitution of residues Glu117, Asp140, and Ser145 was performed using a two-step PCR strategy. The upstream region of nmb1467 was amplified using primers NG552 with NG1984, NG1985, NG1986, or NG1987 (for ppk) and NG559 with NG1985, NG1986, NG1987, or NG1988 (for gdh) for qRT-PCR. The downstream region was amplified with NG552 with NG1984, NG1986, or NG1988 (for nmb1467) or with NG552 with NG1985, NG1986, or NG1987 (for gdh or the tet complement, mutants from the library (105 cfu) were incubated with proteinase K (10 mM dithiothreitol) and incubated with protease K (100 μg/ml final concentration) at 37 °C overnight. Samples were separated by Tricine SDS-PAGE as described previously (5, 6). LPS was visualized by silver staining (GE Healthcare).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Western Blot, and LPS Analysis—Whole cell lysates were prepared by mixing 1010 cfu/ml bacteria with an equal volume of 2 × SDS-PAGE loading buffer (100 mM Tris-Cl, pH 6.8, 20 μg/μl β-mercaptoethanol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and then boiling for 10 min. Proteins were separated by SDS-PAGE as described previously (5) and stained with Coomassie Blue or transferred to Immobilon P polyvinylidene fluoride membranes (Millipore). Membranes were incubated with primary antibodies (concentration and incubation times were optimized for each antibody), washed with PBS and 0.1% Tween 20 (PBS-T), and then incubated with an HRP-conjugated secondary antibody. Binding was detected with the ECL reagent (Amersham Biosciences).

For LPS analysis, bacterial suspensions were mixed with an equal volume of 2 × LPS loading buffer (100 mM Tris-Cl, pH 8.0, 6% β-mercaptoethanol, 6% SDS, 0.2% bromphenol blue, 46% glycerol, 10 mM dithiothreitol) and incubated with proteinase K (100 μg/ml final concentration) at 37 °C overnight. Samples were separated by Tricine SDS-PAGE as described previously (5, 6). LPS was visualized by silver staining (GE Healthcare).

Purification and Modification of NMB1467 and Generation of Immune Sera—nmb1467 was amplified with primers NG552 and NG559, and the product was digested with Ndel and BamHI and ligated into pET-15b (Novagen) to generate pnmbl467-pET-15b. pnmbl467-pET-15b was constructed using the QuickChange site-directed mutagenesis kit (Stratagene) with pET-15b as DNA template and primers NG607 and NG608. Alanine substitution of residues Glu117, Asp140, and Ser145 was performed using a two-step PCR strategy. The upstream region of nmb1467 was amplified using primers NG552 with NG1984, NG1985, or NG1987, and NG1988 (for ppk or gdh) or with NG552 with NG1985, NG1986, or NG1987 (for nmb1467) or with NG552 with NG1985, NG1986, or NG1987 (for gdh or the tet complement, mutants from the library (105 cfu) were incubated with proteinase K (10 mM dithiothreitol) and incubated with protease K (100 μg/ml final concentration) at 37 °C overnight. Samples were separated by Tricine SDS-PAGE as described previously (5, 6). LPS was visualized by silver staining (GE Healthcare).

Screen for Resistant Mutants and Complement Binding Studies—To screen for genes involved in resistance against complement, mutants from the library (105 cfu) were incubated with dilutions of heat-inactivated convalescent sera with baby rabbit complement (Pelfreeze; 1:4 final dilution) in a 2-ml volume for 1 h (24). Bacteria were recovered by plating to solid medium. For serum assays, bacteria were diluted to a final concentration of 1 × 105 cfu/ml in Dulbecco's modified Eagle's

### TABLE 2

#### Oligonucleotides used in this study

| Primer   | Sequence                                  | For         |
|----------|-------------------------------------------|-------------|
| NG551    | GGCTTAACTTCGTCCGCGACATTCGGTTGGGG        | ppp complementation |
| NG552    | GGCGATCCATGCCGCTGACATTCGGTTGGGG        | ppp complementation |
| NG559    | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | ppp purification |
| NG552    | GCGGATCCATGCCGCTGACATTCGGTTGGGG        | Amplification of pppE147A |
| NG607    | GGCGATCCATGCCGCTGACATTCGGTTGGGG        | Amplification of pppE147A |
| NG608    | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG1984   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG1985   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG1986   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG1987   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG1988   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG1989   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Amplification of pppE147A |
| NG886    | AGTGGTGGATTTCGCATTACCCGGG            | gdh for RT-PCR |
| NG887    | TOAAGTAGCCATTCTGACGCT                  | gdh for RT-PCR |
| NG955    | CGACGAGTGTTTTGGG                      | ppp for qRT-PCR |
| NG956    | GAATTCATTTGTACAG                     | ppp for qRT-PCR |
| NG957    | GGCGATCCATGCCGCTGACATTCGGTTGGGG      | Construction of ppp::tet mutant |
| NG559    | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Construction of ppp::tet mutant |
| NG1985   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Upstream of Hbp |
| NG1986   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Downstream of Hbp |
| NG1987   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Downstream of Hbp |
| NG1988   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Downstream of Hbp |
| NG1989   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Downstream of Hbp |
| NG1986   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Downstream of Hbp |
| NG1987   | GCACTAGGACATCCGGCTGACATTCGGTTGGGG      | Downstream of Hbp |

Relevant restriction sites and codon changes are underlined. Modified nucleotides are shown in bold. qRT-PCR, quantitative RT-PCR.
medium without CMP-N-acetyl neuraminic acid and incubated with different concentrations of normal human serum (NHS) at 37 °C for 1 h. Human serum was heated at 56 °C for 30 min to inactivate complement as required.

To assess the binding of complement factors, bacteria were grown overnight and then fixed in 3% paraformaldehyde for at least 1 h, and $2 \times 10^7$ cells were incubated with pooled immune sera from 10 individuals for 30 min at 37 °C, washed twice, and then resuspended in PBS-T with a FITC-conjugated goat anti-human C3 polyclonal antibody (ICN; at 1:300 dilution) or a rabbit anti-human C5b-9 polyclonal antibody (Calbiochem; at 1:200 dilution). Binding was detected with a goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen; at a 1:200 dilution). The activity of the CP and LP was inhibited in sera in the presence of 10 mM MgCl$_2$ and 10 mM EGTA (25).

Analysis of Capsule Expression and LPS Sialylation—Strains were grown to midlogarithmic phase in liquid medium, collected by centrifugation, fixed as above, and then washed three times with PBS. Next, bacteria ($2 \times 10^7$ cells) were incubated with mAb 3F11 (26) or an anti-serogroup B capsule mAb (National Institute for Biological Standards and Control, code 95/750). The cells were washed twice, resuspended in PBS-T containing a FITC-conjugated donkey anti-mouse polyclonal antibody (Jackson ImmunoResearch Laboratories; at a 1:200 dilution), and incubated for 30 min on ice. After washing with PBS-T, fluorescence was measured using a FACS Calibur analyzer (BD Biosciences) recording at least $10^4$ events. The gate was set at ~2% of cells after incubation with PBS without a primary antibody. Results were expressed as the mean fluorescence index calculated as geometric mean fluorescence multiplied by the percentage of positive cells.

Polyphosphatase and PolyP Assays—The activity of NMB1467 was measured by its ability to reduce the concentration of Type 75 polyP (Sigma) with 1 unit of enzyme defined as the amount causing the release of 1 pmol of P$_i$ from polyP/min at 37 °C. Reactions containing 50 mM Tricine/KOH, pH 8, 175 mM KCl, 15 mM polyP, and 1 mM MgCl$_2$ or CaCl$_2$ were incubated at 37 °C for 15 min. To measure the concentration of polyP, 10-μl aliquots of reactions were added to toluidine blue (6 mg/liter), and the ratio of A at 530 and 630 nm was determined. As a polyanion, polyP induces a shift in the absorption spectrum of toluidine blue from 630 to 530 nm, and the concentration of polyP directly corresponds to changes in the ratio of absorbance at these wavelengths (27). Standard curves were generated with known concentrations of polyP.

To measure polyP levels in N. meningitidis, polyP was extracted from cells using glass milk and converted to ATP, which was quantified by measuring bioluminescence in the presence of luciferase as described previously (28). Briefly, 2 $\times 10^9$ cfu of N. meningitidis were harvested after overnight growth on plates and lysed in 4 mM guanidine isothiocyanate and 50 mM Tris-Cl, pH 7. Whole cell lysates were incubated with glass milk, treated with DNase and RNase (New England Biolabs), and washed, and the polyP was eluted with 150 μl of 50 mM Tris-Cl, pH 8 and then measured by adding 50 μl of the sample to an equal volume of 40 mM (NH$_4$)$_2$SO$_4$, 4 mM MgCl$_2$, and 5 mM ADP with 1 μg of purified His-tagged polyphosphate kinase (PPK) (29). The reaction was incubated for 40 min at 37 °C and then at 90 °C for 2 min to generate ATP from polyP.

RESULTS

Loss of nmb1467 Enhances Survival of N. meningitidis in Human Serum.—To further understand interactions between N. meningitidis and the complement system, we isolated mutants with enhanced resistance against complement-mediated killing by screening a library of ~40,000 MC58 mutants constructed by in vitro Tn5 mutagenesis. Mutants in the library or the wild-type strain MC58 were incubated for 2 h in the presence of dilutions of heat-inactivated convalescent sera from two patients with baby rabbit complement. No colonies were recovered after the wild-type strain was incubated in a 1:512 dilution of immune sera from the patients. However, several colonies were recovered when bacteria from the library were incubated under identical conditions. Two independent nmb1467 mutants, with Tn5 inserted at nucleotide 165 or nucleotide 187 of the predicted ORF, were recovered in separate screens with sera from the two patients. nmb1467 is present in the five available N. meningitidis genomes and 21 of 21 other isolates (detected by PCR; not shown).

Examination of ORFs around nmb1467 suggests that transposon insertion in this gene is unlikely to cause polar effects (Fig. 1A). However, to exclude this possibility, the MC58nmb1467::Tn5 mutant was complemented by introducing a single wild-type copy of the gene under the control of its own promoter in the intergenic region between nmb102 and nmb103 (20), generating MC58nmb1467::com (Fig. 1A). To examine the contribution of nmb1467 to survival in the presence of human complement, the recovery of MC58, MC58nmb1467::Tn5, and MC58nmb1467::com was compared after 1 h in NHS. In a 1:8 dilution of NHS, approximately twice the number of MC58nmb1467::Tn5 survived compared with the wild-type strain (Fig. 1B; $p < 0.001$). This relative resistance was not observed in the complemented strain MC58nmb1467::com (Fig. 1B), demonstrating that inactivation of nmb1467 is responsible for the enhanced resistance of the nmb1467 mutant. In contrast, there was no significant difference in the recovery of the strains after incubation in heat-treated serum (Fig. 1C; $p = 0.086$ for MC58 versus MC58nmb1467::Tn5, and $p = 0.25$ for MC58nmb1467::Tn5 versus MC58nmb1467::com).

nmb1467 Encodes PPK, an Exopolyphosphatase—nmb1467 is predicted to encode a protein with a molecular mass of 56.6 kDa that shares 36% amino acid identity with a characterized exopolyphosphatase from E. coli, PPK (Fig. 2A) (28). This enzyme releases terminal P$_i$ from polyP, which is synthesized in bacteria by PPK. To determine the biochemical function of NMB1467, the gene was expressed in E. coli as a recombinant protein, which was purified by binding to a HisTrap column and eluted in the presence of 200 mM imidazole (Fig. 2B). The biochemical activity of NMB1467 was examined using polyP as
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the substrate and measuring the fall in polyP concentrations over time. NMB1467 caused a significant reduction in the concentration of polyP (Fig. 2C; p < 0.0001), whereas no change was detected following incubation with buffer alone or with a control protein, bovine serum albumin (not shown). The specific activity of the recombinant protein was ~2 × 10^6 units/mg as determined by performing assays with different concentrations of NMB1467 (Fig. 2C). The activity of NMB1467 was dependent on Mg^{2+} with optimal activity observed with between 1 and 2 mM Mg^{2+}, similar to E. coli PPX (28); in the presence of Ca^{2+}, the activity was ~20% of levels with Mg^{2+} (not shown). As NMB1467 is the first meningococcal protein with demonstrated exopolyphosphatase activity, NMB1467 was designated PPX.

Identification of Residues Critical for PPX Activity and Resistance against Complement-mediated Killing—Like E. coli PPX, the N. meningitidis enzyme is predicted to contain four domains with the catalytic site located in a cleft between domains I and II (30, 31). The subsequent domains are necessary for the formation of homotypic dimers that allow the enzyme to engage longer (>50 units) polyP substrates and allow high processivity. In contrast, a related enzyme from Aquifex aeolicus (30, 31) only possesses the first two domains, which, along with the lack of PPK in this bacterium, indicates that it does not act as an exopolyphosphatase (30, 31). However, the structures of domains I and II of the E. coli and A. aeolicus enzymes are closely related with a root mean square deviation of 1.6 Å for 282 Cα pairs (30, 31), and both contain a predicted variant Walker B motif, (D/E)XGG(G/A)SXE, which harbors a P-loop domain ((D/E)XG) that is critical for phosphate binding. The A. aeolicus enzyme structure contains a Ca^{2+} ion that is engaged via side chain oxygens of residues Ser^{146} and Glu^{148} (corresponding to Ser^{145} and Glu^{147} in the N. meningitidis homologue); the position of Ca^{2+} is thought to be occupied by the cofactor metal Mg^{2+} in the active enzyme. Additionally, there is a conserved Glu^{117} residue in close proximity to this cation that is likely to be involved in the catalytic process (30, 31).

To further define the basis of the contribution of PPX to resistance against complement, MC58ppx::Tn5 was complemented with the non-functional ppx^{E147A} allele to generate MC58ppx^{E147A}. Western blot analysis with anti-PPX serum confirmed that PPX is expressed by MC58ppx^{E147A} at levels similar to those of MC58 and the complemented strain MC58ppx^{com} (Fig. 3A). The non-functional ppx allele failed to reverse the relative resistance of MC58ppx::Tn5 to complement-mediated killing; in a 1:8 dilution of NHS, the survival of MC58ppx^{E147A} was significantly higher than that of the wild-type strain and MC58ppx^{com} (Fig. 3B; p < 0.005 for MC58ppx^{E147A} versus both strains), demonstrating that the biochemical activity of PPX is crucial for interactions with the complement system.

In Bacillus cereus, loss of PPX is compensated for by a reduction in expression of PPK, the enzyme responsible for polyP synthesis (32). Therefore, we determined whether levels of pppk mRNA are influenced by the presence of PPX in N. meningitidis. The amount of pppk transcript was determined by quantitative real time RT-PCR and normalized to mRNA levels of gdh, a housekeeping control (Fig. 3C). We found that pppk mRNA levels were identical in the ppx mutant and the wild-type strain. We also determined the amount of total cellular polyP present in MC58 and MC58ppx::Tn5; a mutant lacking pppk was constructed as a control. Strains without a functional PPX enzyme (i.e. MC58ppx::Tn5 and MC58ppx^{E147A}) had significantly higher levels of polyP than the wild-type or complemented strains (Fig. 3D; p < 0.005 for MC58ppx::Tn5 and MC58ppx^{E147A} versus MC58). In contrast, the amount of polyP detected in the pppk mutant was ~10% of levels in MC58.
Resistance to Complement-mediated Killing of ppx Mutant Is Independent of Capsule, LPS Sialylation, and fHbp—To determine the basis of the increased complement resistance of the ppx mutant, we first examined whether loss of PPX enhances the growth or survival of N. meningitidis in complete medium. MC58 and MC58ppx::Tn5 grew at equal rates in complete laboratory medium and in heat-inactivated sera (Fig. 4). Therefore, the increased survival of MC58ppx::Tn5 cannot be accounted for by increased growth. Furthermore, loss of PPX did not affect the colony or cell morphology as determined by transmission electron microscopy (not shown).

Next, we analyzed whether PPX affects the amount of the polysaccharide capsule on bacteria, which is necessary for resistance of N. meningitidis against complement-mediated lysis (12). FACS analysis with a mAb against the serogroup B capsule demonstrated that inactivation of ppx does not result in a significant change in the level of capsule (Fig. 5A; \( p = 0.178 \)) with comparable levels of capsule produced by MC58 and MC58ppx::Tn5. Furthermore, the effect of PPX on resistance against complement was examined in a capsule-deficient strain, MC58ppx::Tn5/siaD. More dilute NHS was used in assays with capsule-deficient strains because of their inherent sensitivity to complement; in the presence of a 1:16 dilution of NHS, the strain lacking PPX (MC58ppx::Tn5/siaD) was still relatively resistant compared with the strain expressing this protein, MC58ppx::Tn5/siaD (Fig. 5B; \( p < 0.005 \)). Taken together, these results indicate that the enhanced resistance resulting from loss of ppx is not mediated by alterations in the polysaccharide capsule.

LPS sialylation also contributes to the resistance of some Neisseria spp. against complement-mediated killing (13). To investigate the effect of loss of PPX on LPS, we examined the LPS profile of bacteria by SDS-PAGE and silver staining. There was no apparent difference in the appearance of LPS from MC58 and MC58ppx::Tn5 (not shown). Additionally, the extent of LPS sialylation was analyzed with a mAb that recognizes L3,7,9 immunotype LPS and mAb 3F11, which binds to the terminal Gal1–4GlcNAc epitope on unsialylated LPS (26). FACS analy-
sis demonstrated that the degree of LPS sialylation on MC58ppx::Tn5 was not significantly different from that of the wild-type strain MC58 (Fig. 5C; \( p = 0.066 \)). Furthermore, loss of ppx still leads to a significant increase in survival in NHS of bacteria lacking lst, which encodes the LPS-specific sialyltransferase (Ref. 33 and Fig. 5D; \( p < 0.005 \)), indicating that alteration of LPS sialylation is not the basis for the contribution of PPX to serum resistance.

*N. meningitidis* recruits factor H, the major negative regulator of the complement AP, to its surface by a lipoprotein called fHbp. Therefore, we next investigated whether fHbp expression is influenced by PPX. Western blot (Fig. 6A) and FACS (not shown) analyses showed that loss of PPX does not affect levels of fHbp. We also constructed mutants lacking fHbp alone (MC58\( \Delta \)fHbp) or lacking fHbp and PPX (MC58ppx::Tn5\( \Delta \)fHbp) and tested their survival in the presence of human serum. PPX still affected the sensitivity of the meningococcus to complement even in the absence of fHbp (Fig. 6B; \( p < 0.005 \)).

**Loss of PPX Leads to Decrease in AP Activation on Bacterial Surface**—To further characterize the contribution of PPX to interactions with the complement system, the deposition of complement factors on the surface of bacteria was examined by FACS analysis after incubation in NHS (Fig. 6). As expected for a strain with increased survival, deposition of the MAC was significantly lower on MC58ppx::Tn5 compared with MC58 (Fig. 7A; \( p < 0.001 \)). Of note, this was accompanied by lower levels of C3 on the surface of the strain lacking PPX (Fig. 7B; \( p < 0.005 \)). To address which complement pathway contributes to the lower C3 deposition, we repeated the experiment while inhibiting the LP and CP by addition of EGTA and Mg\(^{2+} \) to NHS. Although the overall amount of C3 on the strains was markedly reduced, there was still significantly less C3 on the strain lacking PPX than on the wild-type isolate (Fig. 7B; \( p < 0.01 \)), indicating that the activity of the AP is lowered on the ppx mutant. The significance of these results to complement-mediated killing was confirmed by performing serum assays with only the AP active. Under these conditions, the ppx mutant still had a significant survival advantage compared with MC58 (Fig. 7C; \( p < 0.02 \)).

To examine whether exogenous polyP affects complement-mediated lysis, serum assays were performed with wild-type MC58 in the presence of increasing amounts of polyP. A significant, dose-dependent increase in bacterial survival was detected following addition of polyP, indicating that this polyanion can directly impair the function of the complement system.

**DISCUSSION**

PolyP is found in virtually all living cells, including prokaryotes and eukaryotes, and has been shown to have many functions, including acting as an energy source, providing a phosphate store for chelating metal ions, and acting as a molecular scaffold (28). Studies of *ppk* mutants have demonstrated that polyP is required for the virulence of pathogens such as *E. coli* (34), *Shigella flexneri*, *Salmonella enterica* (35), and *Pseudomonas aeruginosa* (36), contributing to growth, motility, responses to stress and starvation, acid and heat intolerance, and cell entry. In the meningococcus, PPK is required for resistance against complement-mediated lysis (21, 22). In contrast, there have been notably few studies on the effect of PPX in prokaryotes as the enzyme is often encoded in a monocistronic operon upstream of *ppk* unlike in the meningococcus. Until now, the contribution of PPX has only been examined in *B. cereus* in which loss of this enzyme affects motility and biofilm formation (18).

We identified the meningococcal PPX through a genetic screen for
PPX and Meningococcal Complement Resistance

FIGURE 5. A, expression of capsule is not influenced by loss of PPX. Capsule expression was analyzed by FACS with anti- \textit{N. meningitidis} serogroup B mAb and is shown as the mean fluorescence index (MFI). The amount of capsule was not significantly altered in the \textit{ppx} mutant compared with MC58. B, resistance of MC58\textit{ppx::Tn5} is independent of expression of the polysaccharide capsule. Strains were incubated in a 1:16 dilution of NHS; MC58\textit{ppx::Tn5asid} was relatively resistant compared with MC58\textit{asid}. C, the extent of LPS sialylation is unaffected by PPX by FACS analysis with the mAb 3F11. D, resistance of MC58\textit{ppx::Tn5} does not require sialylation of LPS. Genomic DNA of MC58\textit{ppx::Tn5} was extracted and transferred into MC58\textit{lst}, which is unable to sialylate LPS. The resulting mutant MC58\textit{ppx::Tn5lst} was incubated with NHS, and there was significantly increased survival compared with MC58\textit{lst}. The resistance of MC58\textit{ppx::Tn5} is independent of expression of LPS sialylation and is still observed in the absence of \textit{lst}. Error bars show the S.E. of experiments performed in triplicate.

FIGURE 6. A, PPX does not affect expression of fHbp by \textit{N. meningitidis}. There was no difference in the level of fHbp production in MC58 compared with MC58\textit{ppx::Tn5} as detected by Western blot analysis of whole cell lysates. The loading of samples was judged by probing replicate blots with polyclonal antibodies against RecA. The sizes of a molecular mass marker in kDa are shown. Survival of strains following incubation in normal human serum for 1 h is shown as a percentage of the number of bacteria in the input. Results are shown as the mean of three independent experiments performed in duplicate, and error bars show the S.E.

FIGURE 7. A, reduced deposition of MAC on MC58\textit{ppx::Tn5} compared with MC58. Deposition of MAC on the bacterial surface was detected by FACS using anti-C5–9 complex mAb after incubating bacteria with NHS, and results are shown as the mean fluorescence index (MFI). The level of MAC on the surface of MC58\textit{ppx::Tn5} is significantly decreased compared with MC58. B, C3 binding to MC58\textit{ppx::Tn5} is reduced compared with MC58. Binding of C3 to bacteria was detected by FACS analysis after incubation with NHS using an anti-C3 mAb. The level of C3 on MC58\textit{ppx::Tn5} surface is significantly less than that on the MC58 either with (NHS) or without CP and LP pathway (–CP/LP). Results are shown as the amount of C3 binding to the \textit{ppx} mutant relative to MC58. C, in the absence of the CP and LP, the relative resistance of MC58\textit{ppx::Tn5} compared with MC58 in human serum assays is still observed. D, addition of millimolar concentrations (indicated) of polyP enhances survival of MC58 in serum assays. Bacteria (10^5 cfu in 100 \mu l) were incubated for 1 h in NHS (at a 1:8 dilution), and then aliquots were plated to solid medium to determine the number of cfu. Assays were performed in triplicate, and error bars show the S.D.

transposon mutants that are relatively resistant to complement-mediated killing in the presence of bactericidal antibodies in sera from convalescent patients. The main advantage of this approach is that mutants of interest are identified by positive selection. The main constraint for the screen was the amount of available human immune serum (which was in limited supply) and the complement source. We chose to use baby rabbit complement in the initial screen as this is routinely included in serum bactericidal assays as recommended by the Centers for Disease Control but subsequently validated our findings in serum assays using human complement. The assay could be modified by adjusting bacterial growth conditions (by propagating them in iron-restricted conditions for example) to more closely reflect environments \textit{in vivo} as this might affect the expression of bacterial structures involved in interactions with complement.

Independent \textit{ppx} mutants with distinct transposon insertion sites were isolated from separate screens performed with sera from two individuals, highlighting the role of this gene for survival in the presence of an active complement system. Both insertions were in the initial portion of the ORF and would be expected to result in a null phenotype. Our finding that PPX is involved in resistance against complement is consistent with previous work showing that \textit{N. meningitidis} lacking PPK is highly sensitive to complement-mediated lysis even though the underlying mechanism for this observation was not defined (21, 22).

The dependence of \textit{N. meningitidis} PPX activity on residues Ser^{145} and Glu^{147} (which engage Ca^{2+} in the protein structure...
from *A. aeolicus*) is consistent with the involvement of Mg$^{2+}$ in the catalytic activity of the enzyme. The precise role of Mg$^{2+}$ in PPX function is not clear, although it could allow access of the negatively charged polyP into a region containing acidic amino acids in the Walker B motif or stabilize reaction intermediates. To date, others have not been able to obtain the crystal structure of PPX with bound Mg$^{2+}$, suggesting that its interaction with the protein might be transient and involved in forming reaction intermediates. On the other hand, the highly conserved Glu$^{117}$ residue is likely to be required for direct hydrolytic attack of polyP or attack via a water molecule. Studies with a “non-catalytic” enzyme that can still engage Mg$^{2+}$ (such as PPX$^{E117A}$) may provide further insights into the activity of PPX by enabling solution of the structure of the enzyme with its cation cofactor along with its substrate.

We did not identify any other predicted exopolyphosphatases in the meningococcal genome by searching for homologues of biochemically characterized enzymes from *E. coli* and *Saccharomyces cerevisiae*. Therefore, loss of PPX was expected to lead to an overall increase in levels of cellular polyP unless there was compensatory up-regulation of the expression of PPX as in *B. cereus* (32). However, there was no change in *ppk* mRNA levels following loss of *ppx*, and total cellular polyP levels were elevated compared with the wild type. We were able to exclude the possibility that the enhanced recovery of the *ppx* mutant in the presence of human complement resulted from an increased growth rate or survival compared with the wild-type strain. This was a distinct possibility as polyP has pleiotropic effects in other organisms. However, we examined the survival of the *ppx* mutant in several conditions, including growth in rich medium (BHI) and survival in heat-treated serum (not shown), but did not detect an increase in survival of the mutant under any circumstance. In *E. coli*, polyP affects responses to exogenous stress by modulating transcription of *rpoS* (37), the alternative σ factor that regulates around 50 genes during the transition from logarithmic into stationary phase. The repertoire of transcription factors in *N. meningitidis* is limited compared with *E. coli* (19), and the absence of a functional copy of RpoS might explain the relative lack of pleiotropic effects upon loss of PPX in the meningococcus.

Alternatively, polyP could act as a protective shield around the bacterium as proposed for the gonococcus (38). Despite extensive attempts, we were unable to identify extracellular polyP from the meningococcus even though we could isolate polyP from culture supernatants of *Neisseria gonorrhoeae* (not shown). There are several reasons why we were unable to detect extracellular polyP. First, the chain length of extracellular polyP on *N. meningitidis* might be less than 20 phosphate residues, which is below the length of molecule that can be detected by enzymatic assays based on PPK (39). However, given the length of polyP in other organisms, this is unlikely. Second, the polysaccharide capsule might have affected our ability to extract polyP, although this can be discounted as we could not detect external polyP on a capsule-minus strain (not shown). Finally, polyP might be more tightly attached to the surface of *N. meningitidis* than the gonococcus, preventing its successful extraction.

To gain further insights into the basis of the enhanced complement resistance of the *ppx* mutant, we examined the deposition of complement C3 on bacteria following incubation in human complement. We found that PPX affects interactions mainly through the AP of complement. This pathway acts as an amplification step for complement activation after initiation by the other pathways or through spontaneous hydrolysis of C3 (10). As a key pathway, several microbes have evolved a variety of strategies to down-regulate the AP. Indeed, the polysialic acid residues of the capsule and recruitment of factor H by the meningococcus both reduce AP activity and promote evasion of complement-mediated lysis (40). Precisely how PPX contributes to AP activity on *N. meningitidis* is not clear, although our data indicate that its influence is not due to changes in the expression of capsule, LPS sialylation, or fHbp individually (although we cannot exclude the possibility that its effect is mediated by modification of a combination of factors); requires the biochemical activity of the enzyme; and is related to the amount of polyP produced by the strains. Interestingly, we found that millimolar concentrations polyP itself can inhibit complement-mediated killing, although how this relates to levels found at the surface of bacteria is unclear. Further studies are underway to examine both the exact cellular location of polyP and the transcriptional profiles of bacteria with and without PPX.

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