Outer membrane protein P4 is not required for virulence in the human challenge model of Haemophilus ducreyi infection

Diane M Janowicz1*, Beth W Zwickl1, Kate R Fortney2, Barry P Katz1,3 and Margaret E Bauer2

Abstract

Background: Bacterial lipoproteins often play important roles in pathogenesis and can stimulate protective immune responses. Such lipoproteins are viable vaccine candidates. Haemophilus ducreyi, which causes the sexually transmitted disease chancroid, expresses a number of lipoproteins during human infection. One such lipoprotein, OmpP4, is homologous to the outer membrane lipoprotein e(P4) of H. influenzae. In H. influenzae, e(P4) stimulates production of bactericidal and protective antibodies and contributes to pathogenesis by facilitating acquisition of the essential nutrients heme and nicotinamide adenine dinucleotide (NAD). Here, we tested the hypothesis that, like its homolog, H. ducreyi OmpP4 contributes to virulence and stimulates production of bactericidal antibodies.

Results: We determined that OmpP4 is broadly conserved among clinical isolates of H. ducreyi. We next constructed and characterized an isogenic ompP4 mutant, designated 35000HPompP4, in H. ducreyi strain 35000HP. To test whether OmpP4 was necessary for virulence in humans, eight healthy adults were experimentally infected. Each subject was inoculated with a fixed dose of 35000HP on one arm and three doses of 35000HPompP4 on the other arm. The overall parent and mutant pustule formation rates were 52.4% and 47.6%, respectively (P = 0.74). These results indicate that expression of OmpP4 is not necessary for H. ducreyi to initiate disease or progress to pustule formation in humans. Hyperimmune mouse serum raised against purified, recombinant OmpP4 did not promote bactericidal killing of 35000HP or phagocytosis by J774A.1 mouse macrophages in serum bactericidal and phagocytosis assays, respectively.

Conclusions: Our data suggest that, unlike e(P4), H. ducreyi OmpP4 is not a suitable vaccine candidate. OmpP4 may be dispensable for virulence because of redundant mechanisms in H. ducreyi for heme acquisition and NAD utilization.

Keywords: H. ducreyi, GUD, Chancroid, Lipoprotein, Human

Background

Bacterial genomes usually contain a significant portion of open reading frames (ORFs) that encode lipoproteins. For example, the genome of Neisseria meningitidis group B strain MC58 has 70 ORFs that encode surface-exposed or exported putative lipoproteins [1]. Approximately 8% of the ORFs of Borrelia burgdorferi encode putative lipoproteins [2]. The presence of numerous lipoproteins in bacterial genomes suggests their importance for bacterial survival and pathogenesis. Lipoproteins have been demonstrated to have roles in preserving membrane structure, functioning as enzymes, and serving as transporters or toxins. Lipoproteins also serve as immunogens; for example, the lipoprotein outer surface protein A (OspA), which plays important roles in B. burgdorferi’s biology, was used to develop an OspA-based vaccine [3,4].

Haemophilus ducreyi, the etiologic agent of the sexually transmitted genital ulcer disease chancroid, has the capacity to express 67 putative lipoproteins (GenBank accession number AE017143), only four of which have been well characterized: the peptidoglycan associated lipoprotein (PAL), the fibrinogen binding protein (FgbA), the ducreyi lectin A (DltA), and H. ducreyi lipoprotein

* Correspondence: dmjanowi@iu.edu
1Department of Medicine, Indiana University School of Medicine, 545 Barnhill Drive Room EH-435, Indianapolis, IN 46202, USA
Full list of author information is available at the end of the article

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Results

Identification of the ompP4 gene

Analysis of the 35000HP genome identified an 831 bp open reading frame (ORF) that encoded an OmpP4 homologue. Sequence analysis of ompP4 demonstrated an N-terminal signal II peptide and a consensus lipidation sequence, N-VLSGC-C (Figure 1). Based on sorting signals described for Escherichia coli, the presence of a tyrosine at position 2 suggests that OmpP4 sorts to the outer membrane [22,23]. The ompP4 ORF lies within a putative operon (Figure 1). PCR amplification of the ORF of ompP4 demonstrated that the gene was conserved in size and location among 10 different strains of H. ducreyi (Figure 1). Amplicons from two class I and two class II strains were sequenced and the deduced OmpP4 sequences compared. The deduced amino acid sequences and lengths of the ORFs were conserved within each class but differed by 9 amino acids between class I and class II strains (Figure 1).

Construction and characterization of an ompP4 mutant

We constructed and characterized an isogenic ompP4 mutant of H. ducreyi 35000HP, which was designated 35000HPompP4. PCR amplification of the ompP4 ORF in 35000HPompP4 demonstrated the size shift from 859 bp to 1.7 kb expected by addition of the 840 bp kan cassette (Figure 2A). In Southern blotting, the kan probe did not bind to the 35000HP genome but did bind to an 8.6-kb DNA fragment of the mutant genome, as expected. The ompP4 probe bound to a 7.8-kb DNA fragment of the 35000HP genome and to an 8.6-kb fragment of the 35000HPompP4 genome (Figure 2B). Thus, the results from the PCR and Southern blot analyses were consistent with the insertion of a single antibiotic resistance cassette in the appropriate locus for the 35000HPompP4 mutant.

Sarkosyl insoluble membrane fractions were prepared from 35000HPompP4 and 35000HP. The fractions obtained from 35000HPompP4 were similar to those of 35000HP, except for lack of expression of a 30 kDa band (Figure 2C), the predicted size of OmpP4. These data suggest that OmpP4 does sort to the outer membrane [24]. 35000HPompP4 and 35000HP demonstrated similar lipooligosaccharide (LOS) profiles as analyzed by SDS-PAGE (data not shown). 35000HPompP4 and 35000HP demonstrated identical growth rates in broth (data not shown).

Role of OmpP4 in experimental human infection

Eight healthy adults (three males, five females; 5 Caucasian, 3 black; age range 21 to 56; mean age ± standard deviation, 31 ± 11 years) volunteered for the study. One subject withdrew prior to inoculation. Two subjects (volunteers 313 and 314) were inoculated in the first iteration, two subjects (volunteers 316 and 317) in the second iteration, and three
subjects (volunteers 324, 325, and 326) in the third iteration. An escalating dose–response study was used to compare the virulence of the mutant and the parent. In the first iteration, each subject was inoculated with a fixed estimated delivered dose (EDD) (143 CFU) of 35000HP at three sites on one arm and varying EDDs (51, 101 and 202 CFU) of 35000HP ompP4 on the other arm. Pustules formed at 2 of 6 parent sites and 5 of 6 mutant sites. Because the mutant was able to form pustules at doses similar to the parent, a second iteration using similar doses of parent and mutant was performed per protocol: 2 volunteers were inoculated with fixed EDD (128 CFU) of 35000HP on one arm and varying EDD (60, 119 and 238 CFU) of 35000HP ompP4 on the other arm. Pustules formed at 5 of 6 parent sites and 5 of 6 mutant sites (Table 1). After two iterations, pustules formed at 7 of 12 parent sites and 10 of 12 mutant sites, suggesting that the mutant could be more virulent than the parent. As per protocol, an interim analysis was performed in order to determine the number of sites that needed to be inoculated with the mutant and the parent to have sufficient power to detect a difference in the pustule formation rate should 35000HPompP4 be more virulent than 35000HP. In the third iteration, 3 volunteers were inoculated with a parent dose (75 CFU) comparable to that of the mutant (116 CFU); pustules formed at 3 of 9 parent sites and at 1 of 9 mutant sites.

Figure 1 Identification of the ompP4 gene within H. ducreyi 35000HP. A, Map of the ompP4–containing locus. B, PCR amplification of the ompP4 locus from genomic DNA of ten clinical isolates. Lanes 1–6, class I strains 35000HP, HD183, HD188, 82–029362, 6644, and 85–023233, respectively; lanes 7–10, class II strains CIP542 TCC, DMC64, 33921 and HMC112, respectively; lane 11, negative control (no template added). C, Alignment of four deduced OmpP4 sequences among 2 class I strains (35000HP and 82–029362) and 2 class II strains (DMC64 and CIP542). Grey-highlighted residues are conserved within each class but differ between class I and class II strains. Shaded arrows denote the consensus signal peptide cleavage and lipidation site.
The overall papule formation rate for both the parent and the mutant was 100% at 21 sites each. Papules were similar in size at mutant sites (mean, 20.4 mm²) as at parent sites (mean, 27.6 mm²) 24 h after inoculation ($P = 0.23$). 

The overall pustule formation rate was 52.4% (95% CI, 23.3%–81.5%) at 21 mutant sites and 47.6% (95% CI, 21.7%–73.5%) at 21 parent sites ($P = 0.74$). Thus, the $ompP4$ mutant was as virulent as the parent.

For the parent and mutant broth cultures used to prepare the inocula, surface cultures and biopsy specimens had the expected phenotype.

**Biological activity of anti-OmpP4 antiserum**

The abilities of $H. ducreyi$ to resist phagocytosis and complement-mediated bactericidal activity are key features of the organism's pathogenesis [10,25,26]. Although the $H. ducreyi$ $ompP4$ mutant was not attenuated for pustule formation in the human challenge model, immunization with OmpP4 could elicit protective antibodies that enhance bactericidal or phagocytic activity, as has been observed with NTHI e (P4). Therefore, we recombinantly expressed OmpP4 and tested its ability to generate biologically active antibodies in mice. Using Western blot analysis, the polyclonal mouse antiserum uniquely bound to purified recombinant OmpP4 and to a 29.2 kDa membrane protein, the predicted molecular weight of OmpP4, from whole cell lysates prepared from 35000HP (Figure 3).

We used this hyperimmune mouse serum (HMS) raised against recombinant OmpP4 (HMS-P4) and compared the percent survival of 35000HP in 10% HMS-P4. As a positive control for bactericidal antibody activity against $H. ducreyi$, we used hyperimmune pig serum previously shown to enhance bactericidal activity (gift of Thomas Kawula) [27]. As expected, the mean percent survival of 35000HP decreased from 119.9% ± 41.4% in normal pig serum to 53.1% ± 12.4% in hyperimmune pig serum. In contrast, the mean percent survival of 35000HP was 63.0% ± 6.9% in normal mouse serum (NMS) compared with 93.4% ± 16.8% in HMS-P4. Thus, HMS-P4 did not promote bactericidal killing of 35000HP.

We next investigated the ability of HMS-P4 to promote phagocytosis of 35000HP by mouse monocyte-macrophage J774A.1 cells using quantitative phagocytosis assays. After opsonization with NMS, the mean percent phagocytosed 35000HP was 74.6% ± 11.5% compared to 86.3% ± 9.4% of bacteria phagocytosed after opsonization with HMS-P4 ($P = 0.13$); thus, anti-OmpP4 antibodies did not enhance phagocytosis of $H. ducreyi$.

**Discussion**

$H. ducreyi$ expresses at least 11 genes encoding lipoproteins in vivo [13], only three of which have been characterized for their roles in pathogenesis in humans [5,9,10]. For this study, we examined a previously uncharacterized lipoprotein, OmpP4, which has homology to the $H. influenzae$ vaccine candidate e (P4). There are two phenotypic classes of $H. ducreyi$ strains, which express different immunotypes and proteomes [28,29]. $ompP4$ transcripts are expressed both in vivo and during human infection [13], and $ompP4$ was conserved among all class I and class II clinical isolates of $H. ducreyi$ that were tested, although there were minor differences in the deduced amino acid
sequences between the class I and class II ompP4 alleles sequenced. These data, coupled with the protein’s homology to e (P4), led us to hypothesize that OmpP4 may play an essential role in the formation of pustules in the human challenge model. However, 35000HPompP4 caused pustules to form at the same rate as the parent strain, indicating that ompP4 is not necessary for virulence in humans. Whether ompP4 contributes to virulence for class II strains, which are not genetically tractable, is unknown.

The experimental model of human infection closely mimics natural infection, but it is limited to the papular and pustular stages of disease. In natural disease, pustules do not evolve into ulcers until several weeks after initial infection. Thus, we cannot rule out a role for OmpP4 during the ulcerative stage of disease. However, during experimental infection, _H. ducreyi_ remains extracellular, where it associates with collagen, fibrin, polymorphonuclear leukocytes and macrophages. These relationships are maintained in natural ulcers [5] and thus it is unlikely that OmpP4 contributes to the ulcerative stage.

One of the attractive characteristics of e (P4) as a vaccine candidate is its ability to generate bactericidal and/or protective antibodies. We therefore examined whether antibodies against OmpP4 could block the organism’s ability to resist either serum bactericidal activity or phagocytosis. OmpP4-specific mouse antiserum had no effect on _H. ducreyi’s_ survival in serum bactericidal assays or on _H. ducreyi’s_ uptake by murine macrophages. It is possible that important conformational epitopes of native OmpP4 lipoprotein were not retained by the recombinant, non-lipidated OmpP4 antigen used. However, similar manipulations did not abrogate the ability of e (P4) to elicit bactericidal antibodies. Overall, our data suggest that, unlike NTHI e (P4), _H. ducreyi_ OmpP4 is not a strong vaccine candidate.

_e (P4)_ is essential for heme uptake by NTHI under aerobic conditions [15,16]. Like _H. influenzae_, _H. ducreyi_ is dependent upon uptake of iron in the context of a porphyrin ring such as heme or hemoglobin for its survival. 35000HPompP4 and 35000HP had similar growth rates under the heme-replete conditions used for the human challenge model, suggesting that ompP4 is not essential for heme uptake. _H. ducreyi_ also expresses several redundant mechanisms for acquiring this essential nutrient, and any contribution of OmpP4 to heme/iron uptake, like those of TdhA or TdX, is likely secondary to the activity of HgbA.

_H. influenzae e (P4)_ is necessary for utilization of the essential coenzyme NAD + (V factor). Members of the _Pasteurellaceae_ cannot synthesize NAD + de novo and must salvage either NAD + or a suitable nicotinamide-based precursor from their environment [32]. So-called V-factor dependent _Pasteurellaceae_ can only utilize NAD + or the precursors nicotinamide mononucleotide

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**Table 1 Response to inoculation of live _H. ducreyi_ strains**

| Volunteer no. | Gendera | Days of observation | Isolateb | No. of initial Papules | No. of Pustules | Final outcome of sites |
|---------------|---------|---------------------|----------|-----------------------|----------------|----------------------|
| 313           | F       | 7                   | P        | 3                     | 2              | 1 2                  |
|               |         |                     |          | M                     | 3              | 3 3                  |
| 314           | M       | 7                   | P        | 3                     | 0              | 3                    |
|               |         |                     |          | M                     | 3              | 2 2 1                |
| 316           | F       | 7                   | P        | 3                     | 3              | 3                    |
|               |         |                     |          | M                     | 3              | 3 3                  |
| 317           | F       | 8                   | P        | 3                     | 2              | 2 1                  |
|               |         |                     |          | M                     | 3              | 2 2 1                |
| 324           | M       | 8                   | P        | 3                     | 1              | 1 2                  |
|               |         |                     |          | M                     | 3              | 1 2                  |
| 325           | M       | 8                   | P        | 3                     | 2              | 2 1                  |
|               |         |                     |          | M                     | 3              | 0 3                  |
| 326           | F       | 6                   | P        | 3                     | 0              | 3                    |
|               |         |                     |          | M                     | 3              | 0 3                  |

Volunteers 313 and 314 were inoculated in the first iteration. Volunteers 316 and 317 were inoculated in the second iteration. Volunteers 324, 325, and 326 were inoculated in the third iteration. aF = female, M = male. bP = 35000HP, M = 35000HompP4.
NMN or nicotinamide riboside (NR) [33,34]. This NAD+ salvage pathway is well characterized in H. influenzae [32,34]: NAD+, NMN, and NR pass through porins into the periplasm, where NAD+ is converted to NMN by the enzyme NadN, and NMN is converted to NR primarily through the catalytic activity of $e^\text{(P4)}$ [17,21,35]. The inner membrane transporter PnuC then transports NR into the cytoplasm, where the enzyme NadR converts NR to NAD+ [36,37].

In contrast to H. influenzae, V-factor independent Pasteurellaceae, such as H. ducreyi, can utilize the precursor nicotinamide (NAm) to synthesize NAD+ [34]. In this alternative salvage pathway, NAm diffuses across the cell wall into the cytoplasm, where the nicotinamide phosphoribosyltransferase NadV converts NAm to NMN, which is then converted to NAD+ by an unidentified NMN adenylyltransferase [32,38]. Critical to this alternative salvage pathway is the enzyme NadV; in H. ducreyi strains, the nadV gene is carried on extrachromosomal or integrated copies of plasmid pNAD1, suggesting horizontal transfer of nadV [38,39]. Strain 35000HP, used to generate the ompP4 mutant, contains two tandem, chromosomal copies of pNAD1 [39]. A previous study reported that H. ducreyi 35000HP encodes a complete H. influenzae-like NAD+ salvage pathway [37]. However, at that time the H. ducreyi genome and its annotation were only available in preliminary form. Our analysis of the finalized H. ducreyi 35000HP genome showed that, while 35000HP includes full-length ORFs predicted to encode intact homologs of $e^\text{(P4)}$ (ompP4) and the NR transporter PnuC (HD1041), the homologs of nadN and nadR are pseudogenes. H. influenzae NadR is a bifunctional enzyme whose C-terminus contains NMN adenylyltransferase activity [37]. Possibly, the 3’ end of the H. ducreyi nadR pseudogene may express a truncated NadR with this activity. Alternatively, an as-yet-unidentified enzyme is required to convert NMN to NAD+ in H. ducreyi. Overall, the absence of intact nadN and nadR genes suggests that the H. influenzae-like NAD+ salvage pathway is dispensible in H. ducreyi because of NadV-driven utilization of NAm. NadV activity most likely accounts for our finding that 35000HPompP4 harbored no discernible growth defects. 35000HP is the only H. ducreyi strain whose genome is available to date; thus, whether OmpP4 activity is more critical for NAD+ utilization in other H. ducreyi strains, and whether other strains harbor a complete H. influenzae-like NAD+ salvage pathway, is unknown.

Conclusions

The outer membrane protein OmpP4 is not required for virulence of H. ducreyi in human disease. Antibodies raised against the recombinant OmpP4 protein were not able to enhance phagocytic uptake or serum bactericidal activity, suggesting that OmpP4 would not be a suitable candidate for an H. ducreyi vaccine. The known functions of $e^\text{(P4)}$ in H. influenzae, including heme uptake and NMN conversion to NR in the NAD utilization pathway, are accomplished by different mechanisms in H. ducreyi. A common theme in bacterial pathogenesis is the redundancy of mechanisms used to accomplish tasks critical for a pathogen’s survival. Thus, although $e^\text{(P4)}$ plays an important role in H. influenzae pathogenesis, the activity of its homolog in H. ducreyi appears to be redundant with the virulence factor HgbA and the NadV-dependent NAD+ salvage pathway.

Methods

Bacteria and culture conditions
35000HP is a human-passaged variant of strain 35000 and has been reported previously [40]. H. ducreyi strains were grown on chocolate agar plates supplemented with 1% IsoVitaleX at 33°C in 5% CO2 or in GC base broth.
culture supplemented with bovine hemin (50 mg/ml), 1% IsoVitaleX, and 5% fetal bovine serum.

Conservation of ompP4 in H. ducreyi clinical isolates

H. ducreyi strains have been categorized into one of two different classes, based on their OMP profiles and LOS migration patterns [5,28]. To examine whether ompP4 was conserved among strains of both classes, we isolated genomic DNA from the following six class I strains: 35000HP (Winnipeg), HD183 (Singapore), HD188 (Kenya), 82–029362 (California), 6644 (Boston), and 85–023233 (New York). Genomic DNA was also isolated from the following four class II strains: CIP542 ATCC (Hanoi), HMC112 (CDC), 33921 (Kenya), DMC64 (Bangladesh). The ompP4 ORF was PCR amplified, using primers 5′- GCGATATTAAGTGGCAACTAGCGG-3′ and 5′-GCA AATTCACTCTCCACAGCGCTG-3′ that were external to the ORF, from genomic DNAs isolated from the above strains. Amplicons from two class I and two class II strains were sequenced and compared.

Construction and characterization of an ompP4 mutant of strain 35000HP

An 840 bp kan cassette that consists almost entirely of aphA-3 coding sequence from pUC18K3 [41] was ligated into a 3.9 kb ompP4-encoding region of the 35000HP genome that had been cloned into the pBluescript plasmid. Because ompP4 lies within a putative operon (Figure 1), a non-polar kan cassette was used, in which the 840 bp selectable kanamycin resistance gene (aphA-3) is immediately followed by a consensus ribosomal-binding site and a start codon [41].

Sequence analysis of one resulting plasmid confirmed that the kan cassette was inserted 187 bp downstream from the transcriptional start of the 831-bp ompP4 ORF. A 4.7-kb EcoRI/XhoI fragment of this plasmid was subcloned into pRSM2072, which utilizes lacZ as a counter-selectable marker to facilitate allelic exchange [42]. The resulting plasmid was electroporated into strain 35000HP. Selection was performed on plates containing kanamycin (30 μg/mL). Colonies were then picked and grown on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and kanamycin. Coincident appeared as small blue colonies because the growth of 35000HP –lacZ- deficient colonies in which a second cross-over event had occurred appeared as white, larger colonies. An ompP4 mutant was recovered and designated 35000H0mpP4.

Construction of the mutant was confirmed using PCR amplification and Southern blotting. PCR amplification of the ompP4 ORFs of 35000H0mpP4 and 35000HP was performed using primers (5′-TGTACTTTATCATCTAATACGGAATCAT-3′ and 5′-TTTGTAGATTACGATTACG-3′) specific to the intergenic regions flanking ompP4, followed by agarose gel electrophoresis. For Southern blot analysis, H. ducreyi DNA was digested to completion with PstI, electrophoresed on 0.8% agarose gels and probed with either the cloned ompP4 insert or the kan cassette.

LOS and OMPs were purified from 35000HP and 35000H0mpP4 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described [9]. The growth rates of parent and mutant in broth used to prepare the challenge inocula were also compared.

Human inoculation protocol

Stocks of 35000HP and 35000H0mpP4 were prepared according to the US Food and Drug Administration guidelines (BB-IND 13046). For the human inoculation protocol, healthy adult male and female volunteers over 21 years of age were recruited for the study. Subjects gave informed consent for participation and for human immunodeficiency virus (HIV) serology, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and the institutional ethics committee of Indiana University-Purdue University of Indianapolis. The experimental protocol, preparation and inoculation of the bacteria, calculation of the EDD, and clinical observations were all done exactly as described previously [12,43]. Subjects were observed until they reached clinical endpoint, which was defined as resolution of all sites, development of a pustule that was either painful or > 4 mm in diameter, or 14 days after inoculation. Subjects were then treated with one dose of oral ciprofloxacin as described [44].

Comparison of papule and pustule formation rates for the two strains was performed using a logistic regression model with generalized estimating equations (GEE) to account for the correlation among sites within the same individual, as previously described [43]. The GEE sandwich estimate for the standard errors was used to calculate 95% confidence intervals (95% CI) for these rates.

To confirm that the inocula contained or lacked the kan cassette and that the kan cassette was not lost by the mutant during the course of infection, individual colonies from the inocula, surface cultures and biopsy specimens were picked, suspended in freezing medium and frozen in 96-well plates. If available, thirty colonies from an individual specimen were scored for susceptibility to kanamycin on kanamycin-containing chocolate agar plates as described [31].

Recombinant fusion protein construction and expression

The ompP4 ORF, without the signal peptide sequence, was amplified from 35000HP genomic DNA using synthetic primers (5′-TGTACTTTACATCTAATACGGAATCAT-3′ and 5′-TGAATAAAGAGTTAATCCTACAAA
A-3’) and then cloned into the pCR-XL-TOPO vector using the TOPO XL Cloning Kit (Invitrogen Corp, San Diego, Calif). The fragment was excised using EcoRI and then cloned into pRSETB (Invitrogen). Transformation of recombinant plasmid into BL21(DE3)pLysS cells allowed for fusion protein expression. Recombinant OmpP4 was expressed in inclusion bodies and was purified under conditions using urea following the QIAexpressionist System (Qiagen, Inc, Valencia, Calif). Stepwise dialysis with decreasing urea concentrations was used to remove urea from the recombinant proteins and then concentrated with a Centricon-10 microconcentrator (Amicon Corp., Beverly, Mass). Purified recombinant OmpP4 was used to inoculate BALB/c mice to produce polyclonal antibodies (Harlan Bioproducts for Science) that were used in bactericidal and phagocytosis assays.

Immune serum bactericidal assays

35000HP was grown for 16–18 h from a freezer stock on chocolate agar plates at 33°C with 5% CO2 and harvested in phosphate-buffered saline. After vortexing for 30 sec, cells were suspended in GC medium and diluted to a final concentration of approximately $10^3$ to $10^4$ CFU/ml. Bactericidal assays were performed in 96-well plates. Each well received 50 μl 35000HP and 10 μl (or 10%) of heat-inactivated NMS or HMS-P4 and brought to 65 μl with GC broth. Plates were incubated for 30 min at 33°C with 5% CO2. Then, 25 μl of either active or heat-inactivated normal human serum, which was used as the complement source, was added and the plates were incubated for an additional 60 min at 33°C with 5% CO2. Bacteria were quantified by plating 100 μl from each well onto chocolate agar and incubating for 48 h at 33°C with 5% CO2. Heat-inactivated hyperimmune pig serum collected after multiple inoculations with *H. ducreyi*, which has been shown to promote bactericidal activity against *H. ducreyi*, was used as a positive control (kindly provided by Thomas Kawula, University of North Carolina, Chapel Hill) [27]. Data were reported as percent survival in active NHS compared to that in heat-inactivated-NHS. Each experiment was repeated three times, and arithmetic mean and standard deviation of the percent survival were calculated.

Phagocytosis assays

The mouse monocyte-macrophage cell line J774A.1 (TIB-67; American Type Culture Collection) was cultured in Dulbecco’s modified Eagle’s medium (DMEM; BioWhittaker) supplemented with 4 mM GlutaMAX, 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), and 1 mM sodium pyruvate. The cells, which were kept in culture for less than 1 month, were used only at low passage numbers. Twenty hours before infection, the cells were allowed to adhere to coverslips in 24-well tissue culture plates ($2 \times 10^5$ cells/well). The following day, nonadherent cells were removed by washing twice with RPMI-F. 35000HP containing the green fluorescent protein-expressing plasmid pRB157K (courtesy of R. J. Blick and E. J. Hansen) was grown to mid-logarithmic phase in Columbia broth without FBS and with streptomycin (100 μg/ml) and then centrifuged at 6,500 x g for 10 min. 35000HP(pRB157K) was suspended to an OD$_{660}$ of 0.2, yielding approximately $10^7$ CFU/ml. A 900 μl portion of bacteria was opsonized with 100 μl of either NMS or HMS-P4 and incubated for 30 min at RT. The suspensions were subjected to centrifugation, and the resulting pellets were suspended in 900 μl of RPMI-F. Approximately $2 \times 10^5$ CFU of opsonized bacteria were added to wells containing J774A.1 cells ($2 \times 10^5$ cells) for a multiplicity of infection of 10:1. Samples were centrifuged at 150 x g for 2 minutes, and phagocytosis was allowed to proceed at 37°C for 40 min. Phagocytosis was stopped by placing the tissue culture plate on ice. Cells were then fixed with 3.7% paraformaldehyde in PBS.

Phagocytosis was evaluated by confocal microscopy, as described previously [43]. Briefly, after washing in DMEM-FBS, samples were stained with affinity-purified rat anti-mouse CD45 monoclonal antibody (R&D Systems, Minneapolis, MN) followed by DyLight Fluor 649-conjugated goat anti-rat secondary antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.). Nuclei were visualized with Hoechst 33342. Samples were mounted onto slides with Vectashield mounting medium (Vector Laboratories) and examined under an Olympus FV1000-MPE confocal laser-scanning microscope. To assess whether bacteria were phagocytosed or remained extracellular, arbitrary fields in each sample were optically sectioned in 0.2 μm steps. The optical sections were stacked and animated using ImageJ software (Rashband, W.S., Image), U. S. National Institutes of Health, Bethesda, Maryland, USA) to allow for examination of the relative positions of the bacteria and eukaryotic cells in three dimensions. Numbers of intracellular and extracellular bacteria were recorded to determine percent of bacteria phagocytosed, which was calculated as: (total number of intracellular bacteria/total number of bacteria) x 100. Three independent experiments were performed and the mean percent phagocytosed bacteria was calculated and compared between bacteria opsonized with NMS and bacteria opsonized with HMS-P4. Statistical analysis was performed using paired Student’s t tests.

**Abbreviations**

EDD: Estimated delivered dose; HMS-P4: Hyperimmune mouse serum raised against recombinant *H. ducreyi* OmpP4; kan cassette: Nonpolar mutagenic cassette encoding kanamycin resistance gene; LOS: Lipooligosaccharide; NMS: Normal mouse serum; NTHI: Nontypeable *Haemophilus influenzae*; OMP(s): Outer membrane protein(s); ORF: Open reading frame.
Competing interests
The authors declare that they have no competing interests.

Authors' contributions
DMJ conceived and designed the study, carried out the in vitro work, directed the human challenge studies, and drafted the manuscript. BWZ carried out the clinical component of the human challenge studies and participated in the bactericidal assays. BPK provided statistical design and analysis for the human challenge studies and analysis for the in vitro assays. MEB participated in the phagocytosis assays and analysis of data and contributed to drafting the manuscript. All authors read and approved the final draft.

Acknowledgments
This work was supported by National Institutes of Health (NIH) National Institute of Allergy and Infectious Diseases (NIAID) grant AI074657 to D.M.J. The human challenge trials were supported by NIH NIAID Public Health Service grant U19 AI14194 and by the Indiana Clinical and Translational Sciences Institute and the Indiana Clinical Research Center (UL RR052761). We thank Sheila Ellinger for assistance with regulatory documents for the human trials and S. Spinola and B. E. Batteiger for their helpful discussions and critical reviews of the manuscript. We thank the volunteers who enrolled in the human challenge study.

Author details
1Department of Medicine, Indiana University School of Medicine, 545 Barnhill Drive Room EH-435, Indianapolis, IN 46202, USA. 2Department of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Drive Room MS-218, Indianapolis, IN 46202, USA. 3Department of Biostatistics, Indiana University School of Medicine, 635 Barnhill Drive Room MS-218, Indianapolis, IN 46202, USA.

Received: 14 February 2014 Accepted: 4 June 2014 Published: 24 June 2014

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doi:10.1186/1471-2180-14-166

Cite this article as: Janowicz et al.: Outer membrane protein P4 is not required for virulence in the human challenge model of *Haemophilus ducreyi* infection. *BMC Microbiology* 2014, 14:166.