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# Analysis of the bactericidal activity of antiserum raised against the N-terminal half of an 85-kilodalton outer membrane protein of Neisseria meningitidis

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# Analysis of the Bactericidal Activity of Antiserum Raised Against the N-terminal Half of an 85-kilodalton Outer Membrane Protein of *Neisseria meningitidis*

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Analysis of the Bactericidal Activity of Antiserum Raised Against the N-Terminal Half of an 85-kilodalton Outer Membrane Protein of *Neisseria meningitidis* (54pp.)

Director: Ralph C. Judd

The closely related pathogenic *Neisseria* species, *N. meningitidis* and *N. gonorrhoeae*, infect an estimated 500,000 and 250 million people annually worldwide, respectively. Development of effective vaccines to prevent infection has proven difficult. A quadrivalent polysaccharide vaccine is currently available against *N. meningitidis*; however, this vaccine does not confer protection against all major pathogenic serogroups. Furthermore, because the current meningococcal vaccine is not based on a protein antigen, it is poor at inducing immunological memory. There is currently no vaccine available against gonorrhea. Efforts to develop such a vaccine have been hampered by the lack of an appropriate animal model and tremendous antigenic- and phase variation exhibited by most major gonococcal outer membrane proteins. Therefore, attractive vaccine candidates would include less major protein molecules that are universally and constitutively expressed by pathogenic *Neisseria*.

A recently discovered 85-kilodalton outer membrane protein (Omp85) of *Neisseria* appears to be such a vaccine candidate. Omp85 is universally expressed by *Neisseria*, and its expression is highly conserved within species. Furthermore, Omp85 homologues in other pathogenic bacteria were shown to be capable of inducing protective immunity. The vaccine potential of Omp85 was evaluated in this project by analyzing the bactericidal activity of rabbit antiserum raised against the N-terminal 445 amino acids, approximately half entire length, of meningococcal Omp85 ($_{N445}$mcOmp85). The N-terminal half of Omp85 was the focus of this study because it was previously shown that the entire Omp85 molecule is difficult to express in *E. coli*, and systematically evaluating different fragments of Omp85 will allow for pinpointing of a putative bactericidal-eliciting epitope.

Bactericidal assay results indicated that anti-$_{N445}$mcOmp85 rabbit serum was unable to promote strong, specific complement-mediated killing of *Neisseria*. This may be due to the paucity of Omp85 molecules on the bacterial surface, which would decrease the likelihood of complement cascade initiation by antigen cross-linking. However, since the function of Omp85 is still unknown, it is possible that antibodies against Omp85 may disrupt an essential function or prevent cell adhesion. Thus, further studies to examine the vaccine potential of Omp85 are warranted.

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# Abbreviations

| | |
|---|---|
| **Anti-2(.5) mcOmp85:** | Rabbit serum raised against amino acids 445-798 of meningococcal Omp85 |
| **Anti-$_{N445}$mcOmp85:** | Rabbit serum raised against $_{N445}$mcOmp85 |
| **Anti-PMO4:** | Rabbit serum raised against the first 178 amino acids of gonococcal Omp85 |
| **C4bp:** | C4b-binding protein |
| **CBB:** | Coommassie brilliant blue stain |
| **CMP-NANA:** | Cytidiinemonophospho-N-acetylneuramic acid |
| **dPBS:** | Dulbeco's phosphate buffered saline |
| **ELISA:** | Enzyme-linked immunosorbent assay |
| **FMS:** | Fulminant meningococcal sepsis |
| **LOS:** | Lipooligosaccharide |
| **MAC:** | Membrane attack complex |
| **MWM:** | Molecular weight marker |
| **$_{N445}$mcOmp85:** | N-terminal 445 amino acids of meningococcal Omp85 |
| **NHS:** | Normal human serum |
| **NRS:** | Normal rabbit serum |
| **Omp85:** | 85-kilodalton *Neisseria* outer membrane protein |
| **PID:** | Pelvic inflammatory disease |
| **Por:** | *Neisseria* porin protein |
| **Por A:** | Porin A isoform |
| **Por B:** | Porin B isoform |
| **pLG1:** | pTrcHisB vector containing $_{N445}$mcOmp85 insert |
| **pTrcHis:** | Cloning vector containing trc promotor and poly-histidine tag |

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# Introduction

Prevention and control of diseases caused by pathogenic *Neisseria* species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, have been hampered by lack of an effective vaccine. Development of a vaccine against these species has been difficult since a conserved, non-variable, sufficiently immunogenic protein molecule expressed by all strains within a species has yet to be identified. The goal of this project was to evaluate the vaccine potential of a recently discovered 85-kilodalton (kDa) *Neisseria* outer membrane protein (Omp85). Omp85 is invariantly expressed by all *N. gonorrhoeae* and *N. meningitidis* strains studied thus far. In addition, Omp85 is similar at the amino acid level to bacterial outer membrane proteins known to elicit protective immunity. Therefore, it is possible that an Omp85-based vaccine would confer protective immunity against all strains of *N. gonorrhoeae* and *N. meningitidis*.

This project primarily focused on the vaccine potential of meningococcal Omp85. Experiments involving viable meningococci were performed using a non-pathogenic, non-encapsulated strain; however, since the purpose of this work was to evaluate the vaccine potential of Omp85 against pathogenic *Neisseria*, encapsulated *N. meningitidis* would be used in assays if warranted by results from the present experiments. Since meningococcal Omp85 is nearly identical to gonococcal Omp85 at the amino acid level, several strains of *N. gonorrhoeae* were also used to evaluate the vaccine potential of meningococcal Omp85. The high identity between meningococcal- and gonococcal Omp85 would be advantageous to any prospective Omp85-based vaccine, since such a vaccine could potentially confer protection against both meningococcemia and gonorrhea.

### *1-1. Neisseria meningitidis* infection

*N. meningitidis* is a human pathogen that causes fulminant meningococcal sepsis and meningococcal meningitis. Fulminant meningococcal sepsis results from proliferation of *N. meningitidis* in the blood and release of endotoxins; symptoms include fever, chills, muscle aches, hemorrhagic skin rash, diffuse intravascular coagulation, and shock. Death may occur within the first few hours of disease, resulting in mortality rates of up to 80% (28). Meningococcal meningitis results when *N. meningitidis* invades the meninges; symptoms include skin rash, fever, headache, photophobia, irritability, vomiting, and neck stiffness. Death occurs in up to 5% of patients due to intracranial pressure (28). Those that recover may suffer from deafness, mental retardation, or seizures (28).

*N. meningitidis* occurs naturally in the nose of approximately 10% of the population (28). However, 90% of these commensal strains are nonpathogenic. Disease occurs when the blood is accessed by the oropharynx by encapsulated pathogenic strains of *N. meningitidis*, most of which belong to one of the following serogroups: A, B, C, W-135, or Y. In industrialized nations, disease occurrence is 1 to 3 per 100,000; this number increases to 10 to 25 per 100,000 in third world countries (28). Those most susceptible to disease include young children, immune-compromised individuals, and those subject to abject social conditions. Crowding facilitates outbreaks of disease, since meningococci are transmitted via direct contact and respiratory droplets up to a distance of one meter. Consequently, outbreaks are prone to occur in populations of military personnel, school children, and jail inmates.

Early administration of penicillin or cephalosporins is effective at treating meningocccal infection. However, diagnosis during early stages of infection is difficult since early symptoms are ambiguous; fever, chills, muscles aches, and vomiting are symptoms common to non-meningococcal bacterial and viral infections. As a result, treatment may not be initiated until

later in infection, where the likelihood of a complete recovery is decreased. Emphasis must, therefore, be placed on disease prevention.

### *1-2. Neisseria gonorrhoeae* infection

*Neisseria gonorrhoeae* is the causative agent of gonorrhea, a leading cause of urethritis, cervicitis, and pelvic inflammatory disease (PID) in the United States. Occurrence of this sexually transmitted disease is known to date back to 2637 BC, when the Chinese emperor Huang Ti described venereal urethral discharges among his people (23). Symptoms of the disease were also described by Hippocrates in 400 BC (23). Gonorrhea continues to persist as a prominent sexually transmitted disease despite effective antibiotic treatments, including penicillin and tetracycline. This may be partially attributed to the fact that up to 40% of infected men and 80% of infected women are asymptomatic for disease (23). Consequently, although effective treatments are available, many infected individuals do not receive treatment. These asymptomatic individuals may serve as carriers who spread disease.

*N. gonorrhoeae* is specifically adapted for survival in the human urogenital tract. Infection is initiated when the bacterium colonizes the apical side of nonciliated epithelial cells (19). The organism then travels transcellularly to exit through the basolateral side of the host cell, resulting in infection of the basement membrane (19). Symptoms of genital gonococcal infection are a result of damage to the epithelial cells caused by transcellular passage of the bacterium and inflammation at the infected site. In men, symptoms of uncomplicated genital gonococcal infection include urethritis, dysuria, and discharge of purulent exudates (23). Local spread of infection leads to prostatitis, seminal vesiculitis, epididymitis, and inguinal lymphadenitis (23). Chronic inflammation can lead to blockage of the sperm ducts, resulting in sterility (11). In women, uncomplicated gonococcal infection primarily affects the columnar epithelium of the endocervix, resulting in cervicitis. (23). Symptoms include abnormal or increased vaginal

discharge, abnormal uterine bleeding, dysuria, and frequent urinations (23). Ascending gonococcal infection causes acute salpingitis and pelvic inflammatory disease (PID) (23). Chronic scarring of the fallopian tubes may also result, leading to sterility (11). Furthermore, destruction of ciliated cells lining the fallopian tube can lead to ectopic pregnancy (11).

Disseminated gonococcal infection (DGI) results from exit of the organism through the basolateral side of epithelial cells and subsequent entry into the bloodstream. DGI is characterized by several symptoms, including gonococcal meningitis, gonococcal endocarditis, infectious arthritis, and dermatitis. The latter is manifested by petechiae, pustules, hemorrhagic lesions, or ulcerative lesions (23). Non-specific symptoms of DGI include fever, chills, malaise, liver function abnormalities, and leukocytosis (23). *N. gonorrhoeae* also infects the oropharyngeal and ophthalmic regions (11). The latter is most commonly acquired by newborns through infected birth canals (ophthalmia neonatorum) (11).

### 1-3. Vaccines currently available against *N. meningitidis* and *N. gonorrhoeae*

Development of an effective vaccine against pathogenic *Neisseria* has proven difficult. A quadrivalent polysaccharide vaccine is currently available against *N. meningitidis* serogroups A, C, W-135, and Y. However, the efficacy of this vaccine is limited by several factors. First, because the vaccine is not based on a protein antigen, it is poor at inducing immunological memory. Second, it is not very effective in infants. Last, the vaccine is ineffective against serogroup B, which causes one-third of all meningococcal infections in the United States and Europe (28). An effective polysaccharide vaccine against serogroup B will not likely be developed since its capsular polysaccharide is very similar to neuronal cell adhesion molecule, thus posing a risk of autoimmunity (28). Furthermore, serogroup B polysaccharide capsule induces a weak antibody response during natural infection.

There is currently no vaccine available against *N. gonorrhoeae*. Efforts to develop such a vaccine have been hampered by the fact that there is no animal model for gonococcal infection, and most major outer membrane proteins of *N. gonorrhoeae* exhibit tremendous antigenic- and phase variation. Therefore, development of a vaccine against both *N. gonorrhoeae* and *N. meningitidis* would be benefited by less prominent outer membrane protein antigens that are universally and conservatively expressed by *Neisseria*, and are capable of inducing protective immunity. Preliminary evidence suggests that Omp85 may be such an antigen.

**1-4. Omp85**

Manning *et al.* discovered Omp85 in the mid-1990's by screening a genomic library with serum raised against isolated gonoccocal outer membrane proteins (17). Omp85 was found to contain a putative signal peptide and a carboxyl-terminal phenylalanine residue, both of which are characteristic of outer membrane proteins (17). Western blot analysis showed that rabbit serum raised against gonococcal Omp85 cross-reacts with meningococcal whole cell lysates, indicating that Omp85 is also expressed by *N. meningitidis* (17). Presence of the *Omp85* gene in *N. meningitidis* was later confirmed by genome comparison and DNA sequencing (13). The following preliminary evidence suggests that Omp85 may be used to develop an effective vaccine against *N. meningitidis*: 1) Omp85 is highly similar to outer membrane proteins that have been shown to induce protective immunity, 2) Omp85 is universally and constitutively expressed in an invariant manner by all *N. meningitidis* strains studied to date, and 3) Omp85 is a protein antigen.

*Omp85 is highly similar to outer membrane proteins that induce protective immunity.*

A GenBank database search revealed that Omp85 is 61% similar to D-15-Ag of *Haemophilus influenzae* and Oma87 of *Pasteurella multocida* (17). It has been previously shown that D-15-Ag and Oma87 are capable of inducing protective immunity (8,16,18,26). Loosmore *et al.*, demonstrated protective immunity elicited by D-15-Ag when 10 out of 13 rat pups passively

immunized with anti-D-15 rabbit serum were protected against *H. influenzae* challenge (16). Similar findings were reported by Thomas *et al.*, who demonstrated that preabsorbing a suspension of *H. influenzae* with anti-D-15 antibodies prevented the organism from causing disease in rats (26). Protective immunity elicited by Oma87 was demonstrated by Ruffolo and Adler, who showed that anti-Oma87 serum protected mice from *P. multocida* challenge (24).

*Omp85 is universally and constitutively expressed in an invariant manner by N. meningitidis and N. gonorrhoeae.*

Western Blot analysis revealed that Omp85 is expressed by all strains of *N. meningitidis* and *N. gonorrhoeae* studied to date (13). DNA sequence analysis showed that Omp85 is identical in all intraspecies strains (13). Interspecies homology is also high; at the amino acid level, meningococcal- and gonococcal Omp85 are 95% identical and 98% similar (17). Meningococcal- and gonococcal Omp85 are also nearly identical at the nucleic acid level, with only one region of variation occurring between base pairs 720 and 752.

*Omp85 is a protein antigen.*

Shortcomings of the current capsular polysaccharide meningococcal vaccine are outlined above. Since Omp85 is a protein antigen, it is not subject to the limitations of a polysaccharide antigen. Thus, an Omp85-based vaccine may potentially induce immunological memory, confer protection upon infants, and have effectiveness against *N. meningitidis* serogroup B.

The function of Omp85 is currently unknown. However, the following functions have been hypothesized:

1) *Omp85 may be involved in mucosal colonization by Neisseria.* All species of *Neisseria* colonize mucosal surfaces (17). Since Omp85 is expressed by all pathogenic and non-pathogenic

*Neisseria* species studied thus far, it is possible that Omp85 may play a role in mucosal colonization.

2) *Omp85 may be involved in protein transport.* GenBank database search revealed 35% similarity between Omp85 and a chloroplast outer membrane protein (IAP75) (17). IAP75 belongs to a complex of outer membrane proteins that function in polypeptide transport (17). Thus, Omp85 may play a similar role in *Neisseria.*

3) *Omp85 may be involved in signal transduction.* GenBank database search revealed 33% identity between Omp85 and *Photorhabdus luminescens* outer membrane antigen (Oma) (GenBank accession No AJ236920, Chatonnet-Marton, *et al.*, unpublished data). *P. luminescens Oma* is located in a genomic region that promotes pleiotropy in response to various environmental conditions (9). Therefore, it is possible that Omp85 may be involved in signal transduction.

## 1-5. Research Goal, Hypothesis, and Specific Aims

The long-term goal of this work is to evaluate the vaccine potential of meningococcal Omp85. In this project, the ability of Omp85 to induce the generation of bactericidal antibodies was investigated.

### Hypothesis

The hypothesis tested was that rabbit antiserum generated against the first 445 amino acids of meningococcal Omp85 ($_{N445}$mcOmp85) specifically triggers complement-mediated killing of *N. meningitidis* and *N. gonorrhoeae.* The N-terminal half of Omp85 was the focus of this s tudy because it was previously shown that the entire Omp85 protein is difficult to express in *E. coli,* and systematically evaluating different fragments of Omp85 will allow for pinpointing of a putative bactericidal-eliciting epitope.

To test the hypothesis, the following **Specific Aims** were investigated:

1) DNA encoding for $_{N445}$Omp85 was cloned into a hyperexpression vector.

2) Recombinant $_{N445}$Omp85 was hyperexpressed in *E. coli*, then purified by affinity chromatography.

3) Rabbit antiserum was generated against purified recombinant $_{N445}$Omp85.

4) Anti-$_{N445}$Omp85 rabbit serum was used in bactericidal assays against *N. meningitidis* and *N. gonorrhoeae*.

# Materials and Methods

## 2-1. Bacterial Strains

| Strain | Source |
|---|---|
| *N. gonorrhoeae* FA19 | Dr. P. Fred Sparling, University of North Carolina, Chapel Hill, NC |
| *N. gonorrhoeae* FA638 | Dr. Keith Joiner, NIH, Bathesda, MD |
| *N. gonorrhoeae* JS1 | Dr. John Swanson, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT |
| *N. gonorrhoeae* MS11 LOS A | Dr. John Swanson, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT |
| *N. meningitidis* 8543 | Dr. Richard Rest, Hahnemann School of Medicine, Philadelphia, PA |
| *N. meningitidis* HH | Dr. Zell McGee, University of Utah, Salt Lake City, UT |

## 2-2. DNA Isolation

*N. meningitidis* HH was grown in 3 ml gonococcal clear typing broth (GC broth, 37°C with 5% $CO_2$) to an $OD_{600nm} \approx 1.0$ . This culture, 0.25 ml, was then used to inoculate 5 ml GC broth. This was grown to an $OD_{600nm} = 0.4 - 0.6$, then centrifuged at 3000 rpm for 10 min. The resulting pellet was resuspended in 567 µl $T_{10}E_1$ (10mM Tris, 1mM EDTA), then treated with 30 µl of 10% SDS, 6 µl proteinase K (20 mg/ml), and 1 µl Rnase A (10 mg/ml). This was mixed and incubated at 37 °C for 1 hr. Phenol extraction was then performed (4), and DNA was resuspended in 100 µl $T_{10}E_1$.

## 2-3. SDS-PAGE and Western blotting

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (15). The protocol for *Neisseria* whole cell lysates was described by Judd (12). Separated proteins were transferred to polyvinylidine diflouride membranes using a Bio-Rad Trans Blot Cell. The transfer was performed in degassed 20 mM sodium phosphate buffer, pH 8.0, for 16 hr at 450 mA. The membrane was blocked for 1

hr in Dulbeco's phosphate buffered saline (dPBS) containing 2% skim milk prior to an overnight incubation in diluted primary antibody (in dPBS). The membrane was then incubated for 1 hr in protein A-horseradish peroxidase (1:2500 dilution dPBS). Blots were developed in 4-chloro-1-naphthol and hydrogen peroxide buffered in methanol.

**2-4. Genetic fusion of $_{n445}$mcOmp85 to poly-histidine tag**

***A. Polymerase Chain Reaction (PCR)***

Primers were designed to amplify bases 76-1396 of meningococcal *Omp85*, 25 nucleotides downstream of the signal peptide cleavage site (Figure 1). Primer designations and sequences were as follows:

5' mOmp85 pTrcHis TOPO: 5' CAAGACATCCGTGTCGAA 3'

3' GC-1(.5) Omp85: 5' GTTAGCTTCGCGAGGC 3'

Plasmid DNA containing *Omp85* from *N. meningitidis* HH (17) was used as the template. Prior to PCR amplification, the template was linearized by treatment with the *PstI* restriction enzyme. Reactions were performed using AccuTaq™ LA DNA Polymerase (Sigma-Aldrich, D8045) and Perkin-Elmer Cetus Thermocycler Model 480. The following parameters were used:

| | | | |
|---|---|---|---|
| **1.** | 95° C | 1 minute | |
| **2.** | 95° C | 1 minute | 25 cycles |
| | 62° C | 30 seconds | |
| | 72° C | 1 minute | |
| **3.** | 72° C | 7 minutes | |

Restriction enzyme analysis using *AccI* and *HincII* was performed on the amplified DNA to verify its identity (see Figure 1 for map of restriction sites).

***B. Plasmid Construction (see Figure 1 for schematic diagram)***

The pTrcHisTOPO TA Cloning Kit (Invitrogen, K4410-01) was used to insert PCR-amplified *$_{N445}$mcOmp85* into the pTrcHisTOPO plasmid. All reactions were performed according to manufacturer's specifications. Transformation of the recombinant plasmid into TOP10 One

Shot® *E. coli* cells was performed according to manufacturer's specifications then plated onto LB agar containing 50 μg/ml ampicillin and 0.2% glucose. Plasmid DNA was isolated from transformants using a QIAprep Spin Plasmid Kit (Qiagen, 27104), then analyzed by restriction digest to check for presence and orientation of the insert. Two sets of double digests using *BamHI/EcoRI* and *AvaI/EcoRI* were used for the analysis (see Figure 1).

***C. Subcloning*** $_{N445}$**mcOmp85** ***into pTrcHisB***

To obtain optimal expression of the fusion protein, $_{N445}$*mcOmp85* was subcloned out of pTrcHisTOPO and into the pTrcHisB plasmid using the Xpress™ System (Invitrogen, V36020). Plasmid DNA consisting of pTrcHisTOPO plus $_{N445}$*mcOmp85* was isolated from transformed TOP10 One Shot® *E. coli* cells using a Midi Plasmid Purification Kit (Qiagen, 12143). *BamHI* and *EcoRI* were then used to excise $_{N445}$*mcOmp85* from pTrcHisTOPO. The resulting fragments were electrophoresed on a 1% agarose gel, and the excised insert was gel purified using a QIAquick Gel Extraction Kit (Qiagen, 28704). The excised $_{N445}$*mcOmp85* was then ligated into the *BamHI* and *EcoRI* sites of pTrcHisB using T4 DNA Ligase (New England Biolabs, Inc.). The resulting plasmid was designated pLG1 (see Figure 1 for schematic diagram). Prior to ligation, pTrcHisB was treated with calf intestine alkaline phosphatase (Boehringer Mannheim) to prevent self-ligation. Plasmid pLG1 was transformed into *E. coli* DH5α cells (Gibco BRL) according to manufacturer's specifications. Transformants were screened for presence and orientation of the insert using PCR- and restriction enzyme analysis. The latter involved a single digest using *AccI* and a double digest using *HindIII/NheI*.

**2-5. Expression of fusion protein**

The fusion protein was expressed according to the protocol provided by Invitrogen. Briefly, Top10 cells containing pLG1 were grown overnight in 1 ml LB broth containing 50 μg/ml ampicillin and 0.2% glucose. From the overnight culture, 0.2 ml was used to inoculate 10 ml LB broth containing 50 μg/ml ampicillin. This was grown to an $OD_{600nm}$ of approximately 0.6, at

which point expression was induced by adding IPTG to a final concentration of 1 mM. Induction was allowed to proceed for 4 hours. Aliquots of cells, 1 ml, were then collected and centrifuged. The resulting pellets were solubalized in 100 µl SDS-PAGE sample buffer. Western blot was then performed as described by Judd (12) to verify that the pelleted cells expressed the fusion protein. Rabbit antiserum, previously generated against the first 178 amino acids of gonococcal Omp85 (17), was used as the primary antibody (1:500 dilution in dPBS). Protein A-horseradish peroxidase was used to detect the primary antibody (1:2500 dilution in dPBS).

**2-6. Purification of fusion protein**

Recombinant $_{N445}$mcOmp85 was purified using pTrcHis Expression and Purification Kit (Invitrogen, K860-01). A 50 ml culture of induced cells was harvested by centrifuging at 3000 rpm for 10 min. Cells were resuspended in 1/10 the culture volume of ice-cold 10 mM Tris-acetate containing 0.75 M sucrose, pH 7.8. Egg white lysozyme was added to an approximate final concentration of 0.1 mg/ml, then the cells were incubated on ice for 2 min. Cells were handled very gently at this point to prevent premature release of the fusion protein. Two volumes of ice-cold 1.5 mM EDTA were slowly added over a period of 10 min, while applying gentle agitation. The cells were then centrifuged at 2500 rpm for 10 min, then resuspended in 1/5 the culture volume of guanidinium lysis buffer. Cells were sonicated on ice (20 second bursts, 10 second rests) for a total of 5 min. The sonicated material was then centrifuged at 13,000 rpm for 15 min to separate out insoluble debris. The supernatant, which contained the fusion protein, was applied in batch mode to a ProBond™ nickel column (Invitrogen). Subsequent binding and wash steps were performed according to manufacturer's specifications using low speed centrifugation. The fusion protein was then eluted off the column in 1 ml fractions. All fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining as described previously (27). Fractions containing purest $_{N445}$mcOmp85 were pooled and dialyzed against water. Protein concentration was approximated using bovine serum albumin standards.

**2-7. Generation of anti-$_{N445}$mcOmp85 rabbit serum**

Polyclonal rabbit serum against recombinant $_{N445}$mcOmp85 was generated as described by Porcella (21). Prior to immunization, serum was collected from the rabbit for use as a control. Primary immunization, consisting of 0.5 mg purified $_{N445}$mcOmp85 (in dPBS) and 0.5 ml Freund's complete adjuvant, was administered subcutaneously at four sites into a New Zealand White rabbit (Western Oregon Rabbit Company). Two weeks later, secondary immunization, consisting of 0.5 mg purified $_{N445}$mcOmp85 (in dPBS) and 0.5 ml Freund's incomplete adjuvant, was administered subcutaneously at four sites. Intravenous boosts, consisting of 1 mg purified $_{N445}$mcOmp85 in dPBS, were then administered weekly over a period of two months. Antibody titer was determined using Western blot strips containing solubalized *N. gonorrheae* MS11 LOSA.

**2-8. Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA using *N. gonorrhoeae* MS11 LOS A as plating antigen was performed to determine antibody titer and to evaluate surface reactivity of anti-$_{N445}$mcOmp85 antibody. *N. gonorrhoeae* MS11 LOS A was grown in GC broth to an $OD_{600nm}$ = 0.68, which corresponds to approximately $5x10^9$ cfu/ml. Cells were pelleted, then resuspended in an equal volulme of PBS. To fix the cells, gluteraldehyde was added to a final concentration of 0.5%, then incubated overnight at 4°C. Cells were then washed three times in PBS. Plating was performed by dispensing 100 µl of $5x10^8$ cfu/ml fixed cells into an Immulon 96-well plate. The plate was centrifuged at 3000 rpm for 15 min, then the supernatant was aspirated off. Cells were air dried onto the plate by incubating at 4°C overnight. Wells were blocked by adding 200 µl of 1% BSA in PBS (BSA-PBS), then incubating at room temperature for 2 hr with gentle agitation. Wells were washed three times in BSA-PBS. Primary antibody was diluted in PBS-BSA, then 100 µl was added to each well. This was incubated overnight at 4°C, followed by three washes with BSA-PBS. Anti-rabbit IgG alkaline phosphatase conjugate (Sigma, A-3687) was diluted 1:5000 in BSA-PBS, then

100 µl was added per well. This was incubated for 1 hr at 4°C, followed by three washes with BSA-PBS. The substrate was prepared by adding 1 tablet of p-nitrophenyl phosphate (NPP) in 10 ml of 1M diethanolamine buffer. The prepared substrate, 100 µl, was then added to each well. Reaction was allowed to proceed for 20 min before measuring optical density of the wells at wavelength 405 nm using a Vmax ELISA plate reader.

**2-9. Bactericidal Assay**

The bactericidal assay performed was a modified version of the assay described by Cadieux (6). Serum was incubated at 56 °C for 45 min to eliminate intrinsic complement proteins, then filter sterilized by passage through a Millex-GP 0.22 µm filter (Fisher, SLGP R25 LS) that was previously blocked with 1% BSA in PBS. Two-fold serial dilutions of the serum were then prepared (1:4 to 1:1024) in Hanks' balanced salt solution containing 1% (w/v) casein hydrolysate (HBSS-cas). Next, 70 µl of each serum dilution was dispensed into separate wells of a 96-well microtiter plate. Fresh cultures of transparent non-piliated *N. gonorrhoeae*, adjusted to a density of $8x10^3$ cells/ml in 31 µl HBSS-cas, were then added to each well. This was incubated for 15 min at 37 °C with 5% $CO_2$. Each well then received 12.5 µl of human complement (Sigma, S-1764) that was preabsorbed with gluteraldehyde-fixed *N. gonorrhoeae*. This was incubated for 45 min at 37 °C with 5% $CO_2$, after which 100 µl from each well was plated onto a GC plate. Plates were incubated overnight, then bactericidal endpoint was calculated.

**2-10. Biotinylation and Immunoprecipitation Combination Assay**

A biotinylation assay was performed using ECL protein biotinylation module (Amersham Pharmacia, RPN 2202). *N. gonorhoeae* MS11 LOS A was grown in GC broth to a density of ~$5x10^9$ cfu/ml ($OD_{600nm}$=0.68). Cells were then washed three times in ice-cold dPBS. After the final wash, cells were resuspended in two-thirds the culture volume of ice-cold 40mM bicarbonate buffer. Biotinylation reagent was added (40 µl per ml of cells), at which point the cells were incubated for precisely 30 min at 4°C with gentle agitation. Cells were then washed

three times in ice-cold dPBS. After the final wash, the pellet was resuspended in an equal volume of lysis buffer (250 mM NaCl; 25 mM Tris-HCl, pH 7.5; 5 mM EDTA, pH 8.0; 1% Triton-X 100), then incubated at 4°C for 45 min with gentle agitation. The lysate was then centrifuged at 13,000 rpm for 10 min. To 100 µl of the supernatant containing soluble biotinylated outer membrane proteins was added 50 mg prewashed protein A-sepharose beads (Sigma, P-3391) and 100 µl anti-$_{N445}$mcOmp85 rabbit serum that was preabsorbed with DH5α/pTrcHisB lysate. This mixture was incubated for 1 hr at 4 °C with gentle agitation. The beads were then washed three times in 1 ml dPBS. After the final wash, beads were solubalized in 100 µl of 1x solubilizing solution. The beads were subsequently collected by centrifugation. To visualize proteins bound to the beads, Western blot was performed on the supernatant as described previously (12). The membrane was blocked in 5% ECL blocking agent in PBS-T for 1 hr, followed by three washes in PBS-T. Streptavidin-horseradish peroxidase conjugate (1:1500 dilution in PBS-T) was then added to the membrane, and incubated at 4°C for 1 hr with gentle agitation. The membrane was then washed three times in PBS-T. To detect biotinylated proteins, equal volumes of ECL detections solutions 1 & 2 were mixed, then applied to the protein side of the membrane. This was incubated for precisely 1 min at room temperature without agitation. Excess detection reagent was then decanted, and the membrane was wrapped in Saran wrap. In a darkroom, the wrapped membrane was placed in an X-ray film cassette together with Hyperfilm MP Autoradiography Film (Amersham Pharmacia, RPN 1677K), with the protein side of the membrane facing the film. The film was exposed for 90 sec, then developed immediately using GBX Developer (Sigma, P-7042).

**2-11. Immunoprecipitation Assay**

*N. gonorrhoeae* MS11 LOS A was grown in GC broth to a density of ~$2x10^9$ cfu/ml ($OD_{600nm}$=0.34). Cells were then washed three times in ice-cold dPBS. To prevent cell lysis, cells were resuspended each time using gentle pipetting. After the final wash, cells were resuspended in one-fifth the culture volume of dPBS. Washed cells, 2 ml, were mixed with 1 ml anti-$_{N445}$mcOmp85 rabbit serum that was preabsorbed with DH5α/pTrcHisB lysate. This was incubated for 1 hr at 4°C with gentle agitation. Cells were then gently washed twice with ice-cold dPBS to eliminate unbound antibodies. After the final wash, cells were resuspended in 3 ml of ice-cold dPBS. Prewashed protein A-sepharose beads (Sigma, P-3391), 150 mg, were then added to the cells and incubated for 1 hr at 4°C. The beads were then centrifuged at 1000 rpm for 2 min, and the supernatant removed. To release antigens, the beads were vigorously resuspended in 3 ml of a mild lysis buffer [250 mM NaCl; 25 mM Tris-HCl, pH 7.5; 5 mM EDTA, pH 8.0; 1% (v/v) Triton-X 100], and incubated at 4°C for 30 min with gentle agitation. The beads were then centrifuged at 1000 rpm for 2 min, and washed in 3 ml dPBS. This was repeated for a total of three washes. The beads were then solubalized in 150 µl of 2x solubilizing solution, after which the beads were spun down and the supernatant was saved. The supernatant was loaded onto an SDS-PAGE gel, then analyzed via Western blot.

# Results

## 3-1. Genetic Fusion of $_{N445}$mcOmp85 to Poly-Histidine Tag

### *A. Cloning* $_{N445}$mcOmp85 *into pTrcHis-TOPO*

*$_{N445}$mcOmp85* was inserted into a hyperexpression vector containing a poly-histidine tag to allow for purification and characterization of the expressed protein. The pTrcHis-TOPO vector used allowed for efficient insertion of PCR products. Nucleotides 76-1396 of meningococcal *Omp85* were PCR-amplified and inserted into pTrcHis-TOPO. The vector sequence immediately flanking the 5' end of the insert was similar to nucleotides 64-75 of meningococcal Omp85 at the amino acid level (Figure 2). Thus, in total, nucleotides 64-1396 of meningococcal Omp85 were essentially cloned into pTrcHis-TOPO. The signal sequence of meningococcal Omp85 was omitted in this construct to prevent toxic accumulations of the expressed protein at the surface of transformed cells. Expression of the vector's ampicillin resistance gene occurs only when an insert is present. Thus, all colonies that appeared on an ampicillin plate following cloning and transformation reactions were considered to have taken up a vector containing an insert. To confirm correct orientation of the insert, plasmid DNA from several colonies was analyzed using restriction enzymes *AccI*, *BamHI*, and *EcoRI* (Figure 1).

### *B. Subcloning* $_{N445}$mcOmp85 *into pTrcHisB*

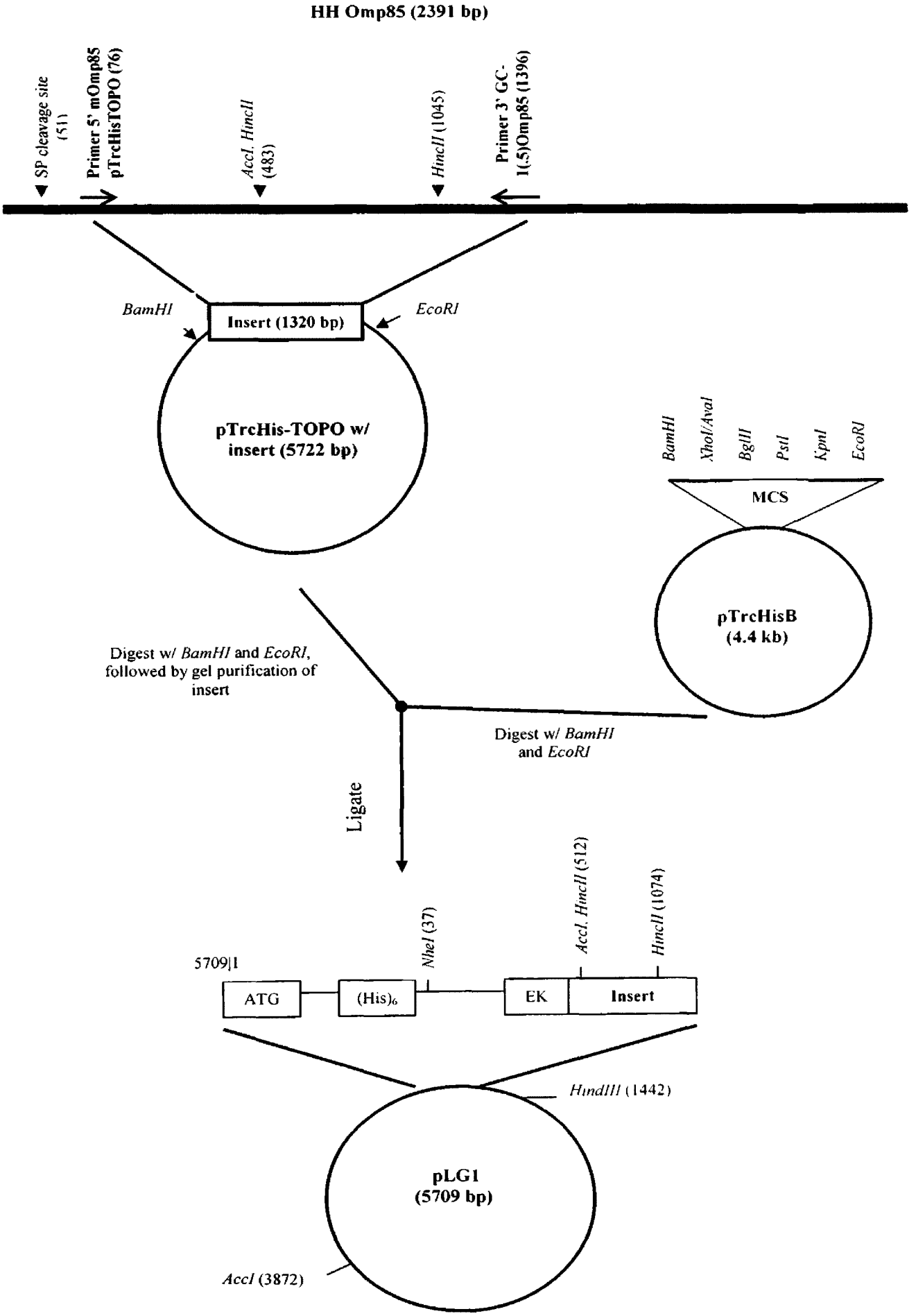
Expression levels of *$_{N445}$Omp85* in the pTrcHis-TOPO vector were not as high as expected . Therefore, *$_{N445}$Omp85* was subcloned out of pTrcHis-TOPO and into pTrcHisB, a similar vector that also contains the poly-histidine tag. The insert was excised from pTrcHis-TOPO using *EcoRI* and *BamHI*, then ligated into pTrcHisB using corresponding restriction sites (Figure 1). The resulting plasmid was designated pLG1 (Figure 3). Following cloning and transformation reactions, several colonies were screened using PCR analysis to confirm presence of the insert.

Plasmids from colonies that contained the insert were subsequently analyzed using restriction enzymes *AccI*, *HindIII*, and *NheI* to confirm correct orientation (Figure 1).

### 3-2. Expression and Purification of Recombinant $_{N445}$mcOmp85

Expression of recombinant $_{N445}$mcOmp85 in transformed TOP10 One Shot® *E. coli* cells was induced with IPTG. To determine optimal induction time, 1 ml samples of transformed cells were collected at 2 hr, 4 hr, and 16 hr post induction. Each sample was separated on a 12.5% SDS-PAGE gel, then analyzed by CBB staining. An induced protein of approximately 55 kDa, which is the expected molecular mass of recombinant $_{N445}$mcOmp85, was present in all samples. Optimal expression of the fusion protein occurred after a 16 hr induction (Figure 4). To confirm identity of the induced protein, each sample of transformed cells was analyzed via Western blot. Antibody previously generated against the first 178 amino acids of gonococcal Omp85 (anti-PMO4) (17) was used as a probe. Anti-PMO4 reacted with a ~55 kDa protein in all samples containing pLG1 (Figure 5), indicating that the induced protein was indeed recombinant $_{N445}$mcOmp85. A ~55 kDa band was visible in the lane containing uninduced $_{N445}$mcOmp85 clone; however, this band was not observed in the CBB-stained SDS-PAGE gel of the uninduced clone. Since Western blot is much more sensitive than CBB staining at detecting proteins, these results may indicate expression of small amounts of the recombinant protein prior to IPTG induction, which is not uncommon. Purification of recombinant $_{N445}$mcOmp85 was performed using affinity chromatography; induced transformants were disrupted by sonication, then applied to a nickel column. The identity of the eluted protein was again confirmed by CBB staining of a 12.5% SDS-PAGE gel, which revealed the presence of a ~55 kDa protein (Figure 6). Identity of the eluted protein was also confirmed by Western blotting. Antibody against the Xpress™ epitope of the recombinant protein (Identified in Figure 3) was used to probe the blot. In agreement with the CBB stain, the probe also detected a protein of approximately 55 kDa (Figure 7).

**Figure 1**. Construction of pLG1. Nucleotides 76-1396 (indicated in parentheses) of *N. meningitidis* HH Omp85 ($_{N445}$mcOmp85) were PCR-amplified using the following primer pair, which was designed to omit the Omp85 signal peptide: 5' mOmp85 pTrcHis TOPO and 3' GC-1(.5) Omp85. The PCR product (designated as a 1320 bp insert) was then ligated into pTrcHis-TOPO. $_{N445}$mcOmp85 was subsequently excised out of pTrcHis-TOPO, gel-purified, and then inserted into the pTrcHisB multiple cloning site (MCS) using *BamHI* and *EcoRI* restriction sites. The resulting plasmid, termed pLG1, was constructed so that $_{N445}$mcOmp85 was directly adjacent to an enterokinase cleavage site (EK) and a poly-histidine tag [$(His)_6$], which allowed for affinity purification of the recombinant protein product. Plasmid LG1 contained several restriction sites (position, relative to recombinant protein start codon, is indicated in parentheses) that were used to confirm presence and orientation of the insert.



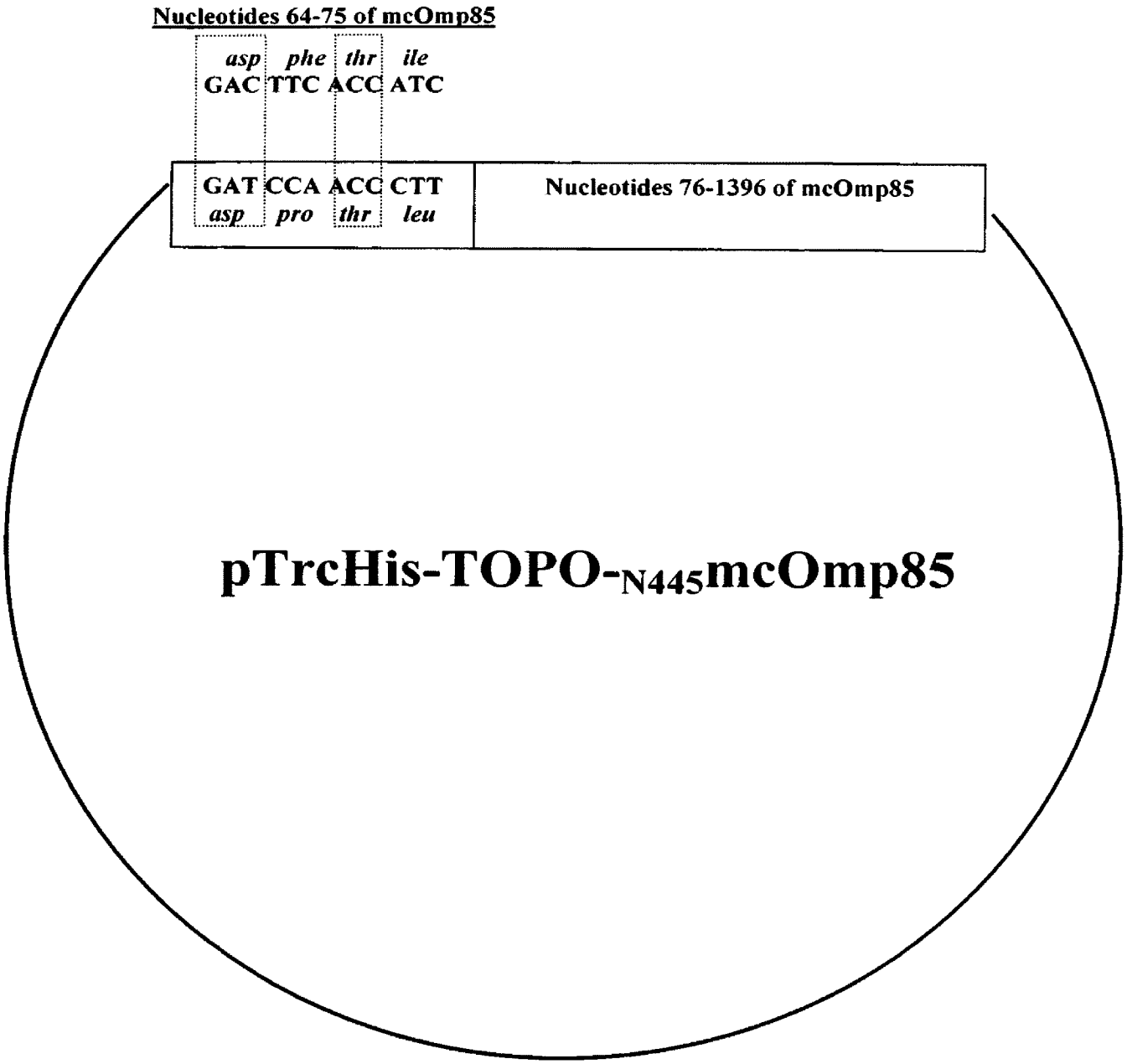



**Figure 2.** Cloning of nucleotides 64-1396 of *N. meningitidis* HH Omp85 into pTrcHis-TOPO. The inserted PCR producted (indicated by gray shading) was comprised of nucleotides 76-1396 of meningococcal Omp85. Vector sequence immediately flanking the N-terminal end of the inserted PCR product was similar, at the amino acid level, to nucleotides 64-75 of meningococcal Omp85; two of the four amino acid residues were conserved (indicated by dashed boxes) between the two sequences. Thus, nucleotides 64-1396 of meningococcal Omp85 were essentially cloned into pTrcHisB.

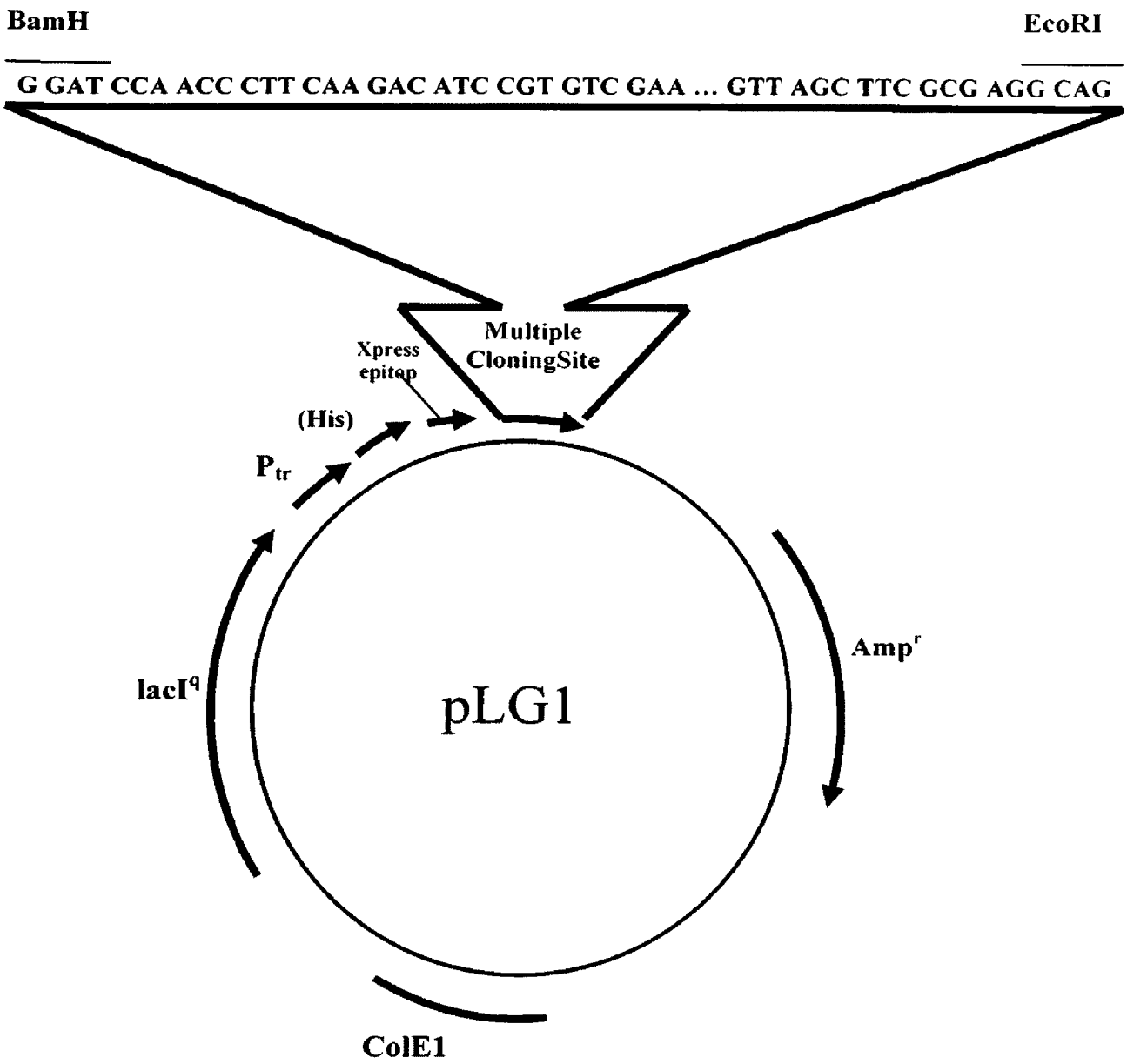



**Figure 3**. Map of pLG1. Nucleotides 64-1396 of meningococcal Omp85 were inserted into the multiple cloning site of pTrcHisB using *BamHI* and *EcoRI* restriction sites. Adjacent to the insertion site were a poly-histidine tag (to allow for affinity purification of the expressed recombinant protein), and an Xpress™ epitope (to allow for detection of the recombinant protein using anti-Xpress antibody). Other plasmid features include an ampicillin resistance gene to allow for screening of transformants; ColE1-derived origin; *lac*$^{q}$ gene encoding for a lac repressor, which regulates the promoter; and *trc* promoter region for high-level inducible expression in *E. coli*.

### 3-3. Generation of Anti-$_{N445}$mcOmp85 Rabbit Serum

Three months following primary immunization, anti-$_{N445}$mcOmp85 serum was at a titer of at least 1:32,000, as determined by Western blot strips containing whole cell lysates of *N. gonorrhoeae* MS11 LOS A. To ensure that the rabbit was not previously exposed to an environmental Omp85-expressing bacterial strain, serum was collected from the animal prior to the first immunization (normal rabbit serum). Western blot and ELISA analysis showed that normal rabbit serum did not react with the immunogen (Figures 8 & 9), suggesting no previous exposure to any Omp85-like protein. Reactivity of anti-$_{N445}$mcOmp85 serum against Omp85 was confirmed by Western blot analysis, in which the serum was used to probe the immunogen and various strains of *Neisseria* (Figure 10). When reacted against the immunogen and the *E. coli* clone expressing $_{N445}$Omp85, anti-$_{N445}$mcOmp85 serum detected a protein of approximately 55 kDa; when reacted against a panel of *Neisseria* strains, anti-$_{N445}$mcOmp85 serum detected a protein of approximately 85 kDa. Since the expected molecular weights of $_{N445}$mcOmp85 and Omp85 are 55 kDa and 85 kDa, respectively, these results suggest that antibody response against $_{N445}$mcOmp85 was due to exposure to the immunogen.

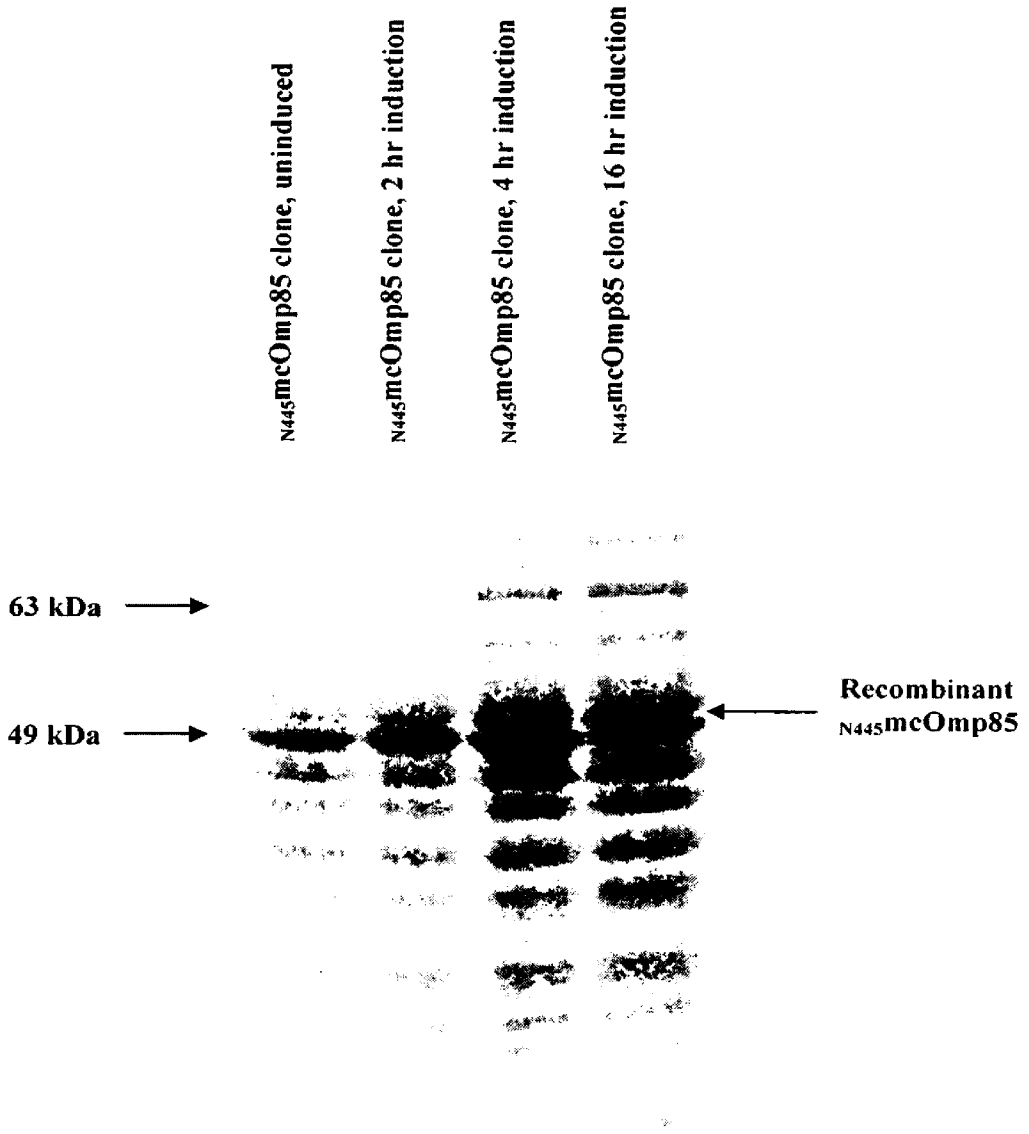



**Figure 4**. CBB-stained polyacrylamide gel illustrating induced expression of recombinant $_{N445}$mcOmp85 in DH5α. Lane 1 contains uninduced clone, lane 2 contains clone following a 2 hr induction, lane 3 contains clone following a 4 hr induction, and lane 4 contains clone following a 16 hr induction. Recombinant $_{N445}$mcOmp85, which has an expected molecular mass of 55 kDa, is indicated with an arrow.

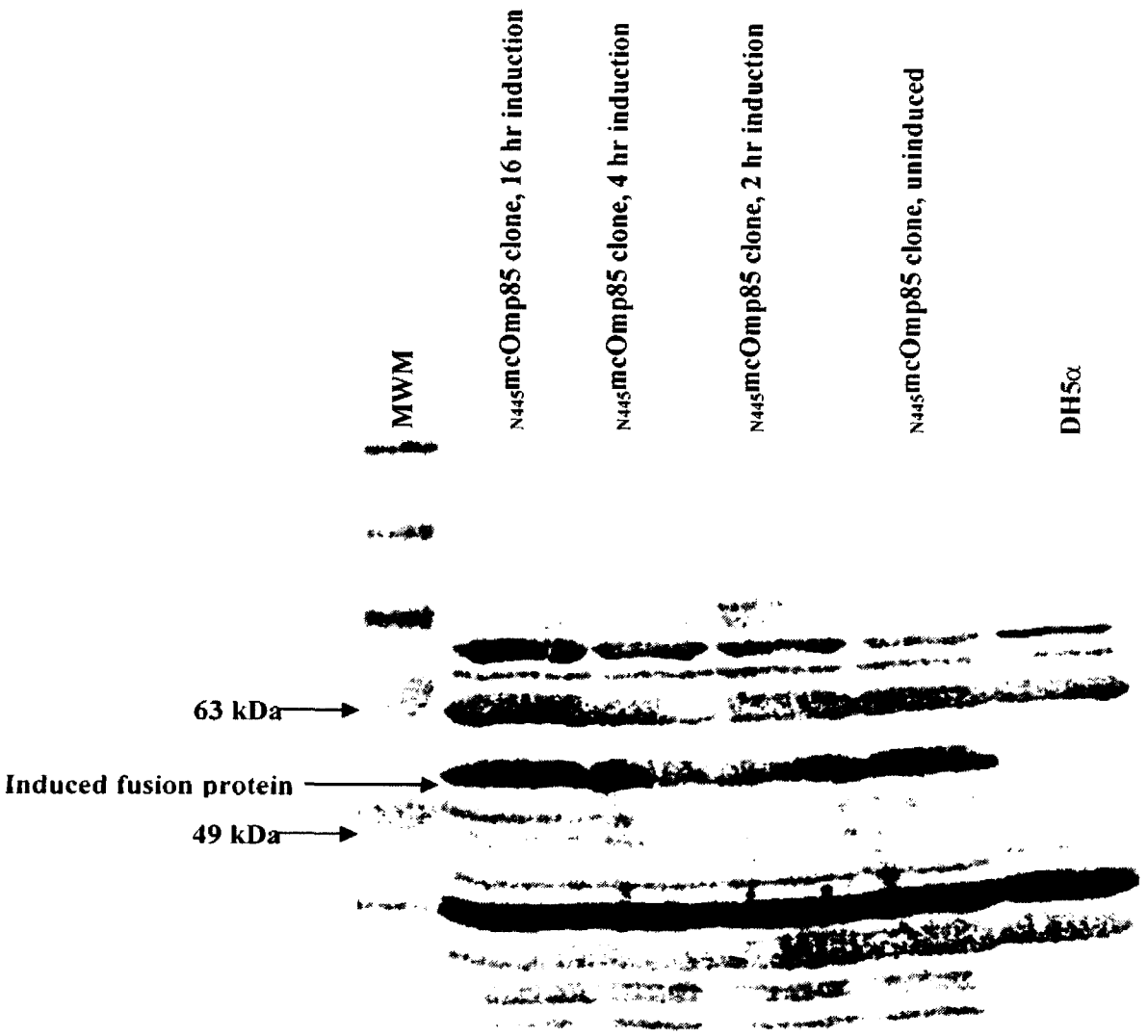



**Figure 5**. Western blot illustrating expression of recombinant $_{N445}$mcOmp85 in *E. coli* DH5α. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, $_{N445}$mcOmp85 clone induced for 16 hr; lane 3, $_{N445}$mcOmp85 clone induced for 4 hr; lane 4, $_{N445}$mcOmp85 clone induced for 2 hr; lane 5, uninduced $_{N445}$mcOmp85 clone; lane 6, DH5α. Blot was probed with antibody against the first 173 amino acids of gonococcal Omp85. Bound antibody was detected using horseradish peroxidase-protein A conjugate. Recombinant $_{N445}$mcOmp85, which has an expected molecular weight of 55 kDa, is indicated with an arrow.

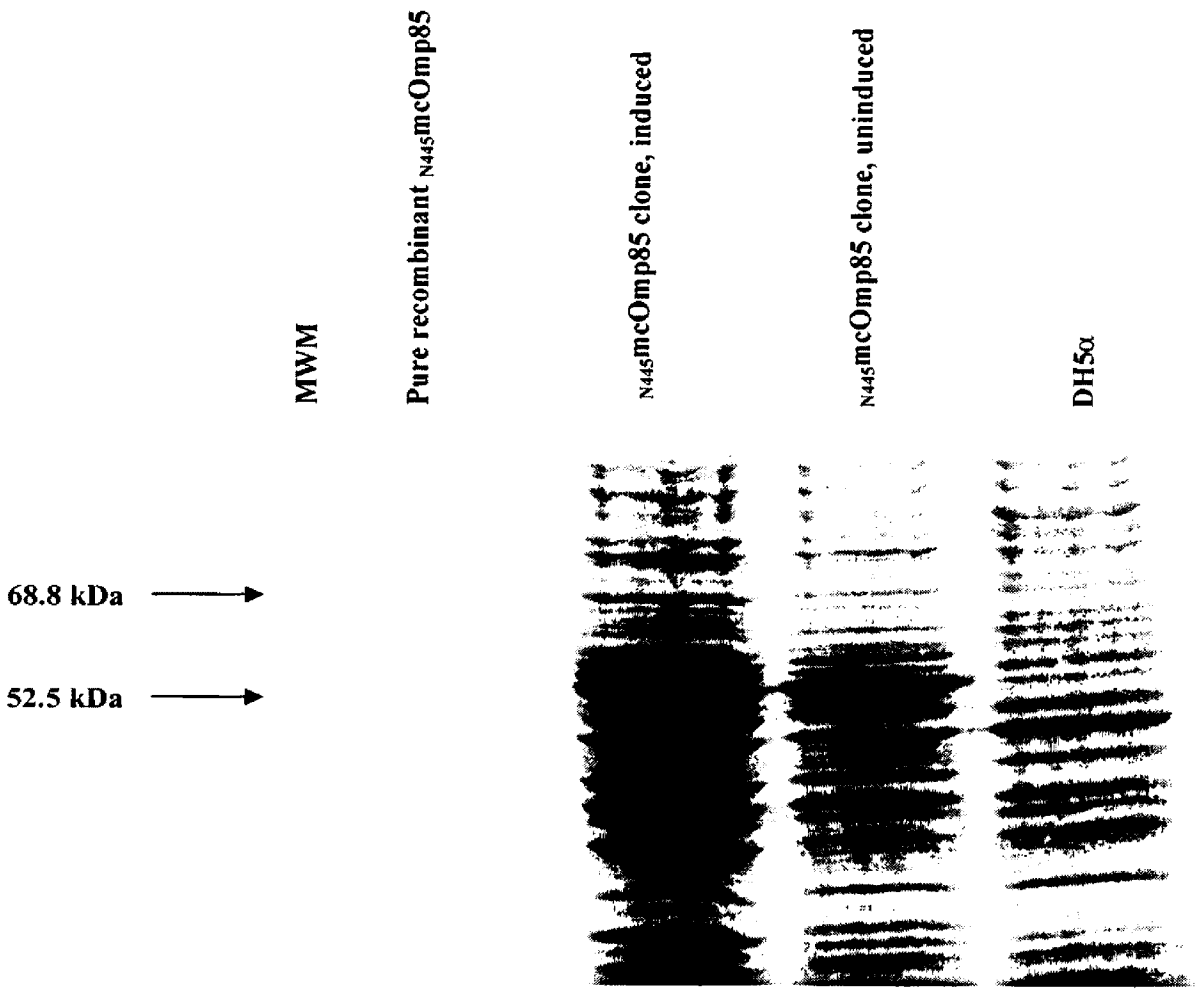



**Figure 6**. Coommassie brilliant blue-stained polyacrylamide gel illustrating purified recombinant $_{N445}$mcOmp85, which has an expected molecular weight of 55 kDa. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, pure recombinant $_{N445}$mcOmp85; lane 3, induced $_{N445}$mcOmp85 clone; lane 4, uninduced $_{N445}$mcOmp85 clone; lane *E. coli* DH5α

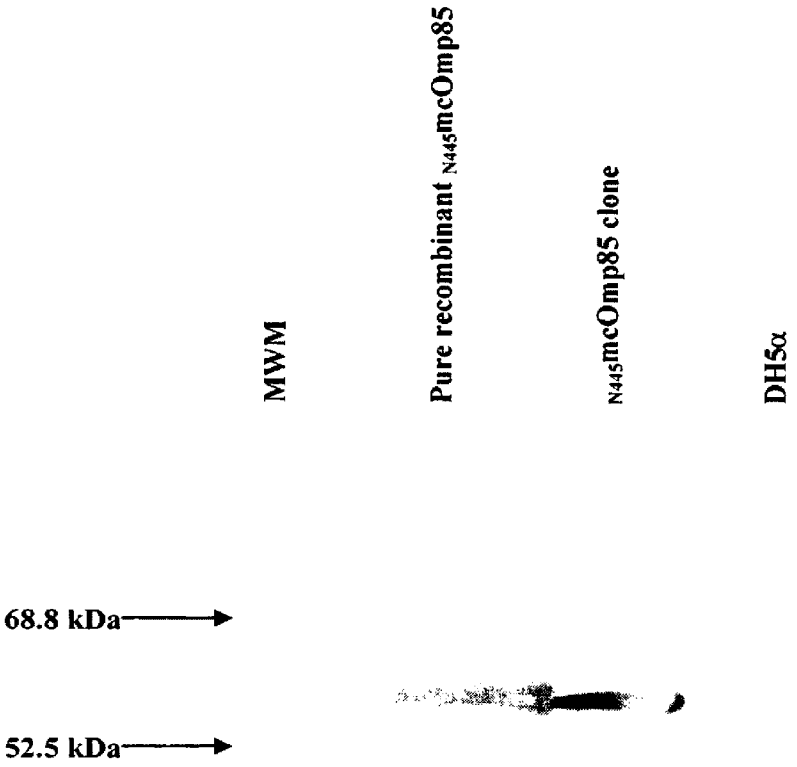



**Figure 7**. Western blot confirming identity of purified recombinant $_{N445}$mcOmp85, which has an expected molecular weight of 55 kDa. Blot was probed with antibody against the Xpress™ epitope of the recombinant protein (see figure 3 for map). Bound antibody was detected using anti-mouse IgG conjugated to horseradish peroxidase. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, purified recombinant $_{N445}$mcOmp85; lane 3, recombinant $_{N445}$mcOmp85-expressing clone; lane 4, *E. coli* DH5α.

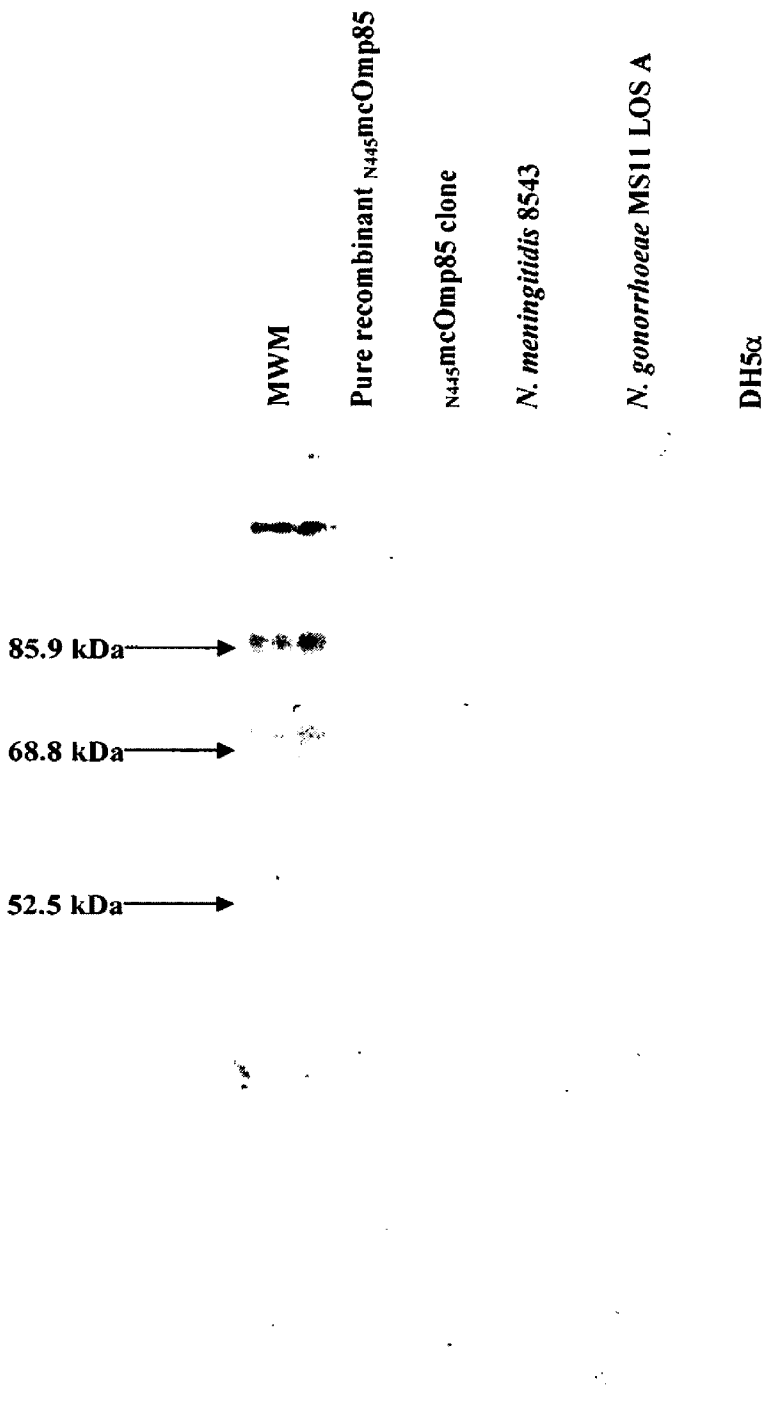



**Figure 8**. Western blot depicting lack of reactivity of normal rabbit serum against various Omp85-expressing isolates. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, pure recombinant $_{N445}$mcOmp85; lane 3, $_{N445}$mcOmp85-expressing clone; lane 4, *N. meningitidis* 8543 whole cell lysate; lane 5, *N. gonorrhoeae* MS11 LOS A whole cell lysate; *E. coli* DH5α.

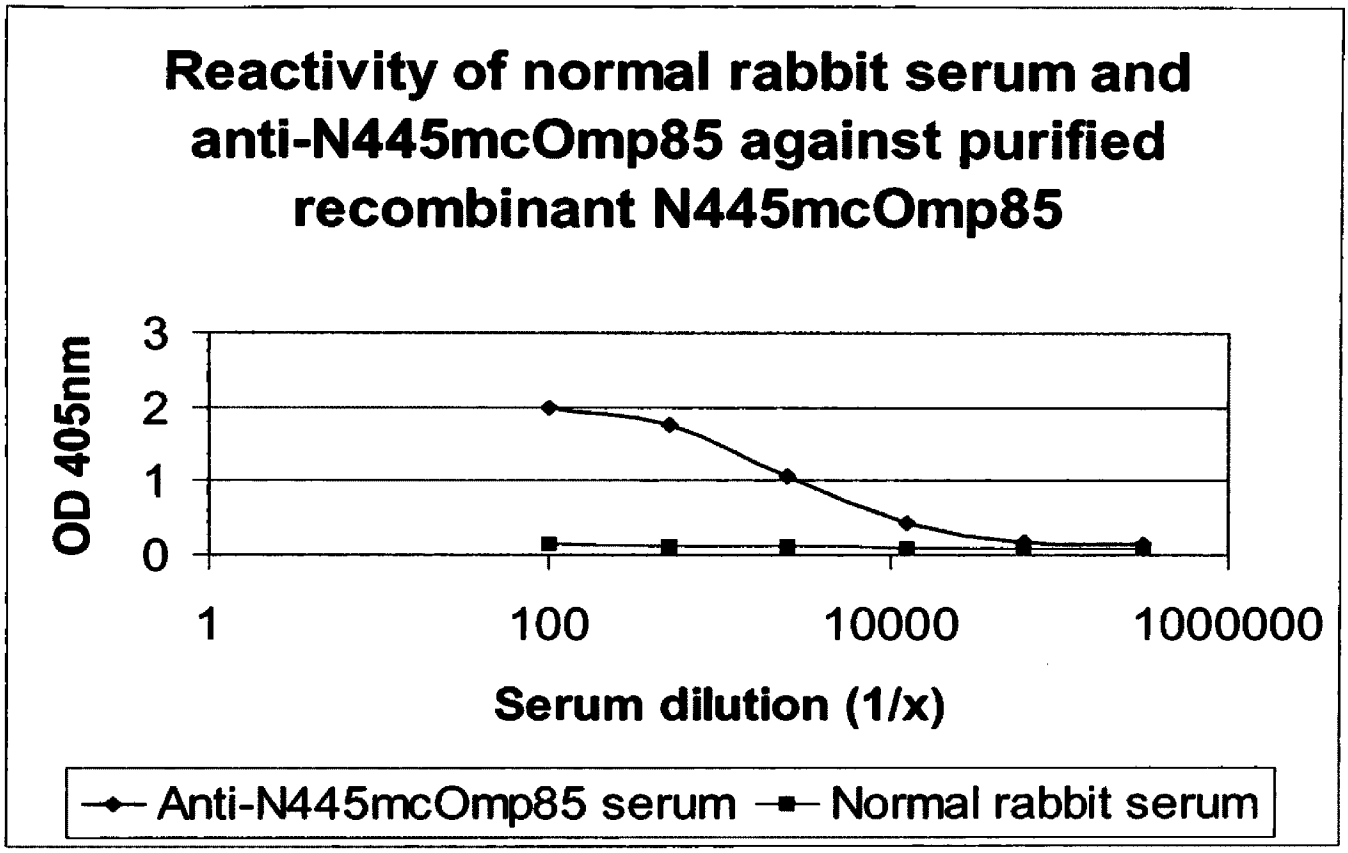



**Figure 9**. ELISA depicting reactivity of normal rabbit serum and anti-$_{N445}$mcOmp85 serum against Omp85. Purified recombinant $_{N445}$mcOmp85 was used as the plating antigen. Optical density of the wells was measured 25 min after addition of substrate.

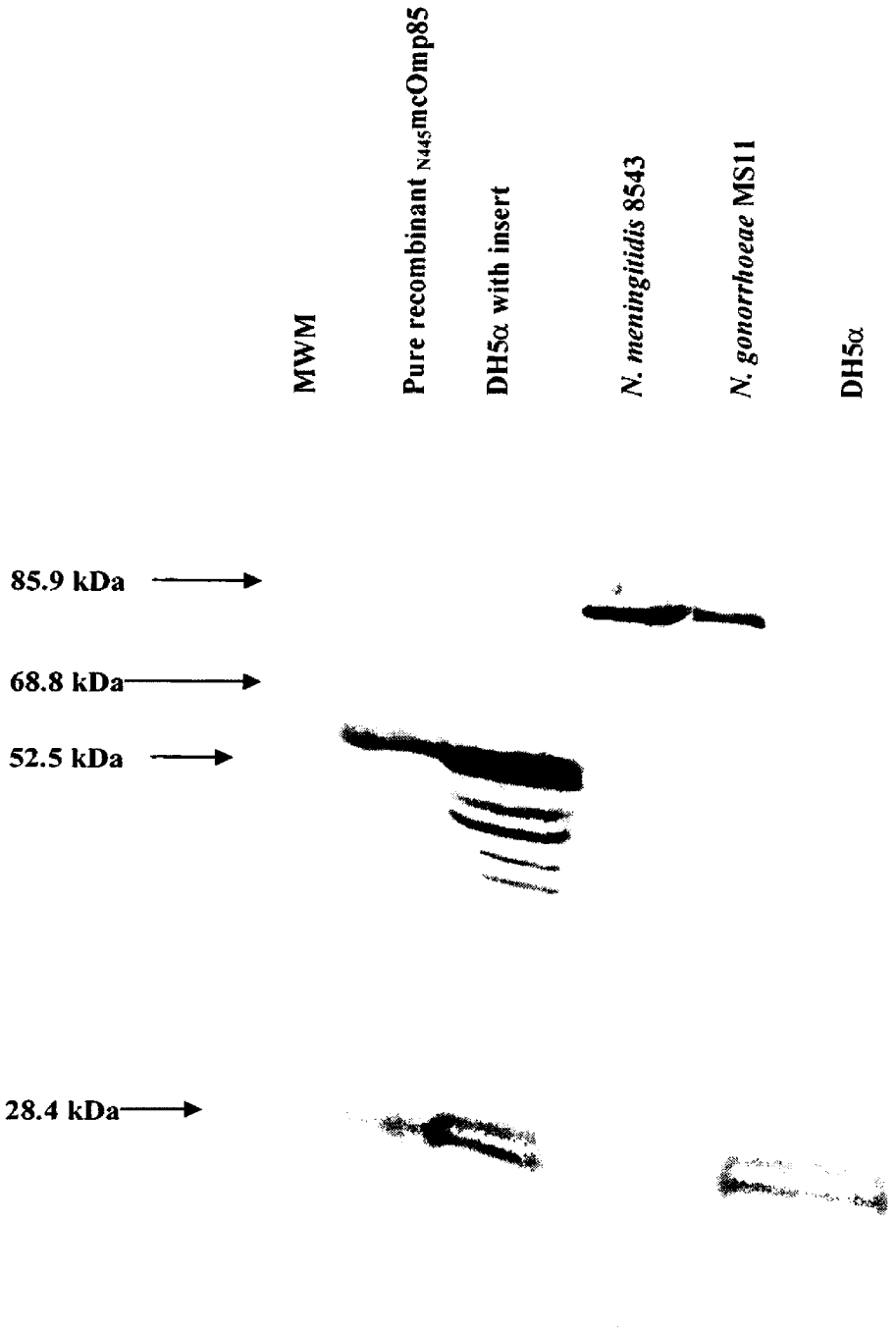



**Figure 10**. Western blot demonstrating reactivity of anti-$_{N445}$mcOmp85 serum against various Omp85-expressing isolates. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, purified recombinant $_{N445}$mcOmp85; lane 3, lysate of $_{N445}$mcOmp85-expressing clone; lane 4, *N. meningitidis* 8543 whole cell lysate; lane 5, *N. gonorrhoeae* MS11 LOS A whole cell lysate; lane 6, *E. coli* DH5α lysate. Recombinant $_{N445}$mcOmp85 has an expected molecular mass of 55 kDa.

### 3-4. Bactericidal Assay

The bactericidal assay was performed as described by Cadieux (6), with modifications. Human complement was preabsorbed with gluteraldehyde-fixed *N. gonorrhoeae* prior to inclusion into killing mixtures. This was done to remove proteins that may mediate non-specific, antibody-independent killing. One nonpathogenic, unencapsulated strain of *N. meningitidis* and four strains of *N. gonorrhoeae* were assayed for their susceptibility to $\alpha$-$_{N445}$mcOmp85-mediated complement killing. Two-fold serial dilutions of $\alpha$-$_{N445}$mcOmp85 serum were prepared to allow for determination of killing titer, which was defined as the reciprocal of the last serum dilution that produced no bacterial growth. Results are shown in Tables 1-5. Table 1 shows bactericidal assay results for *N. meningitidis* 8542. This strain was comparatively sensitive to complement killing, as 10% complement prevented growth in all dilutions of both $\alpha$-$_{N445}$mcOmp85 and normal rabbit serum; killing mixtures in which complement was not added back resulted in growth of large numbers of colonies in all serum dilutions. Therefore, to allow for calculation of antibody-mediated killing titer, complement concentration was reduced to 5%. Anti-$_{N445}$mcOmp85 serum produced a killing titer of approximately 8; however, no significant difference in killing titer was observed between anti-$_{N445}$mcOmp85 and normal rabbit serum. Table 2 shows bactericidal assay results for *N. gonorrhoeae* MS11 LOS A. Large numbers of colonies grew in all serum dilutions, indicating that this strain was resistant to serum killing. Bactericidal assay results for *N. gonorrhoeae* FA19 are shown in Table 3. None of the serum dilutions hindered growth, indicating that this strain was also resistant to serum killing. Table 4 shows bactericidal assay results for *N. gonorrhoeae* JS1. Anti-$_{N445}$mcOmp85 serum produced a killing titer of approximately 2; however, no significant difference in killing titer was observed between anti-$_{N445}$mcOmp85 and normal rabbit serum. Table 5 shows bactericidal assay results for *N. gonorrhoae* FA638. Compared to other test strains, *N. gonorrhoeae* FA638 was especially sensitive to rabbit serum-mediated complement killing, as indicated by the relatively high killing

titer for both anti-$_{N445}$mcOmp85 and normal rabbit serum; serum dilutions in which complement was not added back did not seem to inhibit bacterial growth. An approximately two-fold difference in killing titer was observed between anti-$_{N445}$mcOmp85 and normal rabbit serum. This suggests that *N. gonorrhoeae* FA638 is slightly susceptible to complement killing that is mediated by antibody against the first 445 amino acids of Omp85.

**3-5. Biotinylation and Immunoprecipitation Combination Assay**

To test for surface exposure of Omp85, an immunoprecipitation assay was performed on gonococcal whole cells using anti-$_{N445}$mcOmp85 rabbit serum. Omp85 comprises only a small percentage of total *Neisseria* outer membrane proteins, as indicated by SDS-PAGE and CBB staining of whole cell lysates. Therefore, to amplify any potential signal produced by antibody binding to Omp85, gonococcal whole cells were biotin-labeled prior to immunoprecipitation. ECL detection reagents failed to detect any biotinylated proteins immunoprecipitated by anti-$_{N445}$mcOmp85 rabbit serum. Lysate of biotinylated whole cells was detected, indicating that the biotinylation and detection procedure was not flawed. To test for the possibility that biotin labeling blocked binding sites for anti-$_{N445}$mcOmp85, the antiserum was used to probe biotinylated and unlabeled whole cells via Western blot and ELISA. The Western blot revealed that anti-$_{N445}$mcOmp85 detected an ~85 kDa protein from unlabeled cells; anti-$_{N445}$mcOmp85 failed to detect Omp85 from biotinylated cells (Figure 11). Likewise, the ELISA showed greater reactivity of anti-$_{N445}$mcOmp85 against unlabeled whole cells, as compared to reactivity against biotinylated cells. Also tested were antibody against the first 178 amino acids of gonococcal Omp85 (anti-PMO4) and antibody against amino acids 445-798 of meningococcal Omp85 (anti-2(.5)mcOmp85). Both Anti-PMO4 and anti-2(.5)mcOmp85 exhibited decreased reactivity against biotinylated gonococcal whole cells, as determined by Western blot and ELISA

| | $\alpha_{N445}$mcOmp85 +10% C' | Normal rabbit serum +10% C' | $\alpha_{N445}$mcOmp85 +5% C' | Normal rabbit serum + 5% C' | $\alpha_{N445}$mcOmp85 without C' |
|---|---|---|---|---|---|
| **1:2** | 0 | 0 | 0 | 1 | 250 |
| **1:4** | 0 | 0 | 0 | 2 | 250 |
| **1:8** | 0 | 1 | 6 | 11 | 250 |
| **1:16** | 1 | 0 | 40 | 100 | 250 |
| **1:32** | 0 | 0 | 100 | 150 | 200 |
| **1:64** | 0 | 0 | 100 | 150 | 200 |
| **1:128** | 17 | 0 | 100 | 200 | 200 |
| **1:256** | 1 | 0 | 75 | 150 | 150 |
| **1:512** | 0 | 0 | 75 | 150 | 150 |
| **1:1024** | 0 | 1 | 250 | 150 | 200 |

**Table 1**: Bactericidal assay to evaluate complement-mediated killing of *N. meningitidis* 8542 induced by anti-$_{N445}$mcOmp85. Number of colonies counted on each plate is indicated.

| | $\alpha_{N445}$mcOmp85 +10% C' | Normal rabbit serum +10% C' | $\alpha_{N445}$mcOmp85 without C' |
|---|---|---|---|
| **1:2** | 200 | 225 | 150 |
| **1:4** | 225 | 200 | 150 |
| **1:8** | 225 | 150 | 175 |
| **1:16** | 225 | 125 | 175 |
| **1:32** | 200 | 150 | 125 |
| **1:64** | 225 | 150 | 125 |
| **1:128** | 225 | 150 | 125 |
| **1:256** | 200 | 175 | 50 |
| **1:512** | 225 | 175 | 35 |
| **1:1024** | 225 | 200 | 22 |

**Table 2**: Bactericidal assay to evaluate complement-mediated killing of *N. gonorrhoeae* MS11 LOS A induced by $\alpha_{N445}$mcOmp85. Number of colonies counted on each plate is indicated.

| | $\alpha_{N445}$mcOmp85 +10% C' | Normal rabbit serum +10% C' | $\alpha_{N445}$mcOmp85 without C' |
|---|---|---|---|
| **1:2** | 200 | 200 | 200 |
| **1:4** | 200 | 200 | 200 |
| **1:8** | 200 | 200 | 200 |
| **1:16** | 200 | 200 | 200 |
| **1:32** | 200 | 200 | 200 |
| **1:64** | 200 | 200 | 200 |
| **1:128** | 200 | 200 | 200 |
| **1:256** | 200 | 200 | 200 |
| **1:512** | 200 | 200 | 200 |
| **1:1024** | 200 | 200 | 200 |

**Table 3**: Bactericidal assay to evaluate complement-mediated killing of *N. gonorrhoeae* FA19 induced by $\alpha_{N445}$mcOmp85. Number of colonies counted on each plate is indicated.

| | $\alpha_{N445}$mcOmp85 +10% C' | Normal rabbit serum +10% C' | $\alpha_{N445}$mcOmp85 without C' |
|---|---|---|---|
| **1:2** | 2 | 1 | 75 |
| **1:4** | 50 | 75 | 75 |
| **1:8** | 50 | 100 | 75 |
| **1:16** | 150 | 100 | 50 |
| **1:32** | 200 | 150 | 75 |
| **1:64** | 200 | 200 | 75 |
| **1:128** | 200 | 200 | 75 |
| **1:256** | 200 | 150 | 50 |
| **1:512** | 270 | 200 | 50 |
| **1:1024** | 270 | 270 | 50 |

**Table 4**: Bactericidal assay to evaluate complement-mediated killing of *N. gonorrhoeae* JS1 induced by $\alpha_{N445}$mcOmp85. Number of colonies counted on each plate is indicated.

| | $\alpha_{N445}$mcOmp85 +10% C' | Normal rabbit serum +10% C' | $\alpha_{N445}$mcOmp85 without C' |
|---|---|---|---|
| **1:2** | 0 | 0 | 50 |
| **1:4** | 0 | 0 | 100 |
| **1:8** | 0 | 0 | 100 |
| **1:16** | 0 | 0 | 80 |
| **1:32** | 0 | 0 | 100 |
| **1:64** | 0 | 2 | 100 |
| **1:128** | 0 | 4 | 80 |
| **1:256** | 0 | 8 | 40 |
| **1:512** | 4 | 17 | 22 |
| **1:1024** | 14 | 16 | 75 |

**Table 5**: Bactericidal assay to evaluate complement-mediated killing of *N. gonorrhoeae* FA638 induced by $\alpha_{N445}$mcOmp85. Number of colonies counted on each plate is indicated.

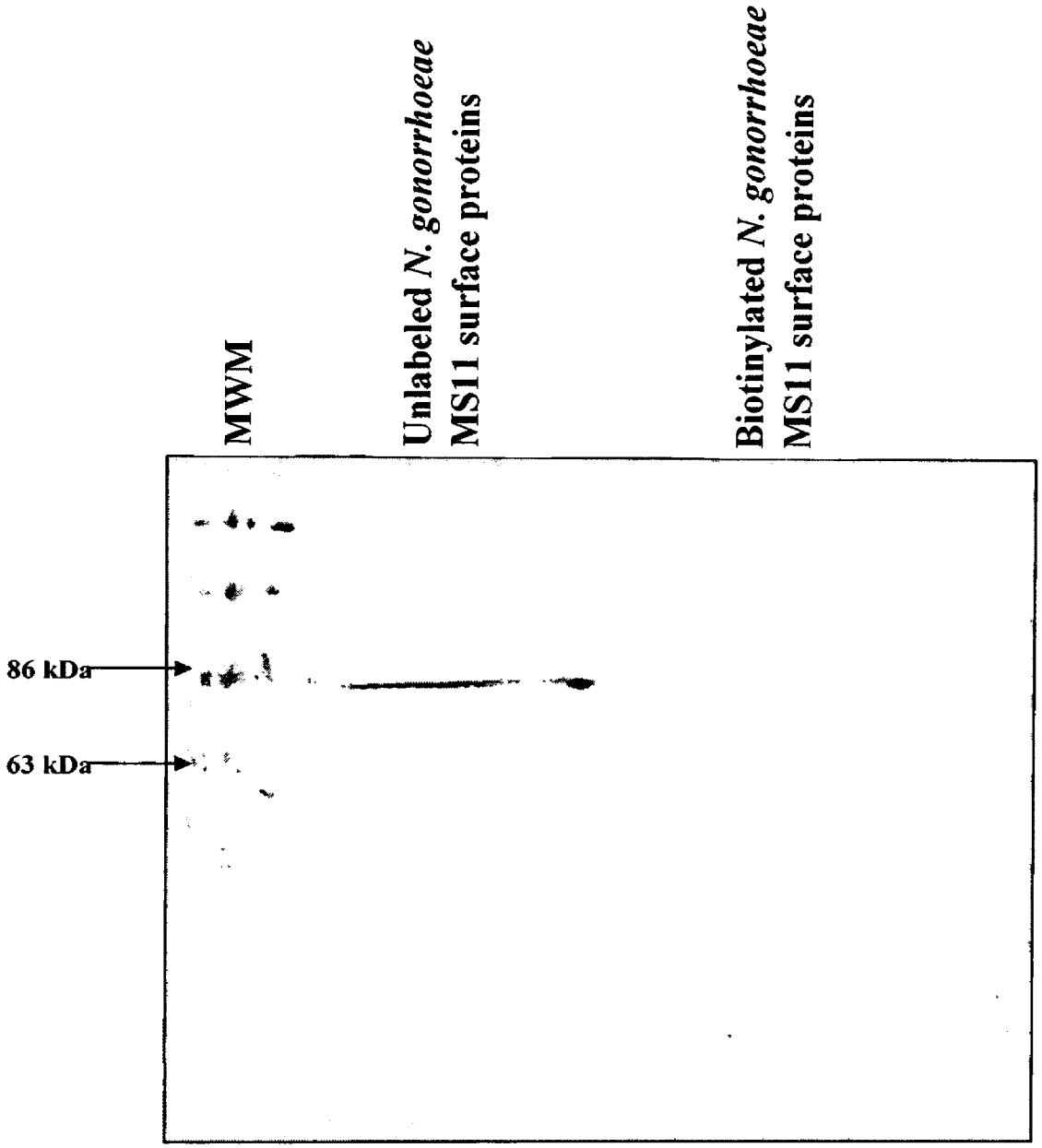



**Figure 11**. Western blot comparing reactivity of biotinylated gonococcal cells to that of unlabeled gonococcal cells. Anti-$_{N445}$mcOmp85 serum was used as the probe. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, unlabeled *N. gonorrhoeae* MS11 surface proteins; lane 2, biotinylated *N. gonorrhoeae* MS11 surface proteins.

(Figure 12). These results suggest that biotin labeling whole cells severely hinders binding of anti-$_{N445}$mcOmp85.

**3-6. Immunoprecipitation**

Since biotin labeling was found to prevent binding of anti-$_{N445}$mcOmp85 to whole cells, an immunoprecipitation experiment was performed on unlabeled cells to assay surface exposure of Omp85. Whole cells were incubated with anti-$_{N445}$mcOmp85 serum for 1 hr, at which point the cells were washed to eliminate unbound antibodies. The cells were then incubated with protein A-sepharose beads for 1 hr, followed by addition of a mild lysis buffer to release antigen-antibody complexes from the cell membrane. The beads were subsequently washed. Proteins bound to the beads were visualized by solubilizing the beads in SDS-PAGE buffer, then analyzing the solute via Western blot. Probing the blot with anti-$_{N445}$mcOmp85 revealed a band of ~85 kDa (Figure 13). The intensity of the ~85 kDa band increased when the same number of cells were sonicated prior to immunoprecipitation. This was expected, since disrupting the membrane would increase the number of epitopes exposed. An ~85 kDa protein was not detected from the sepharose beads when whole cells were immunoprecipitated with either normal rabbit serum or dPBS, indicating that the ~85 kDa protein was immunoprecipitated specifically by anti-$_{N445}$mcOmp85 serum. The multiple bands that appeared below the 52.5 kDa molecular weight marker resulted from protein A being solubalized off the sepharose beads, since protein A-sepharose beads, on their own, produced the same banding pattern.

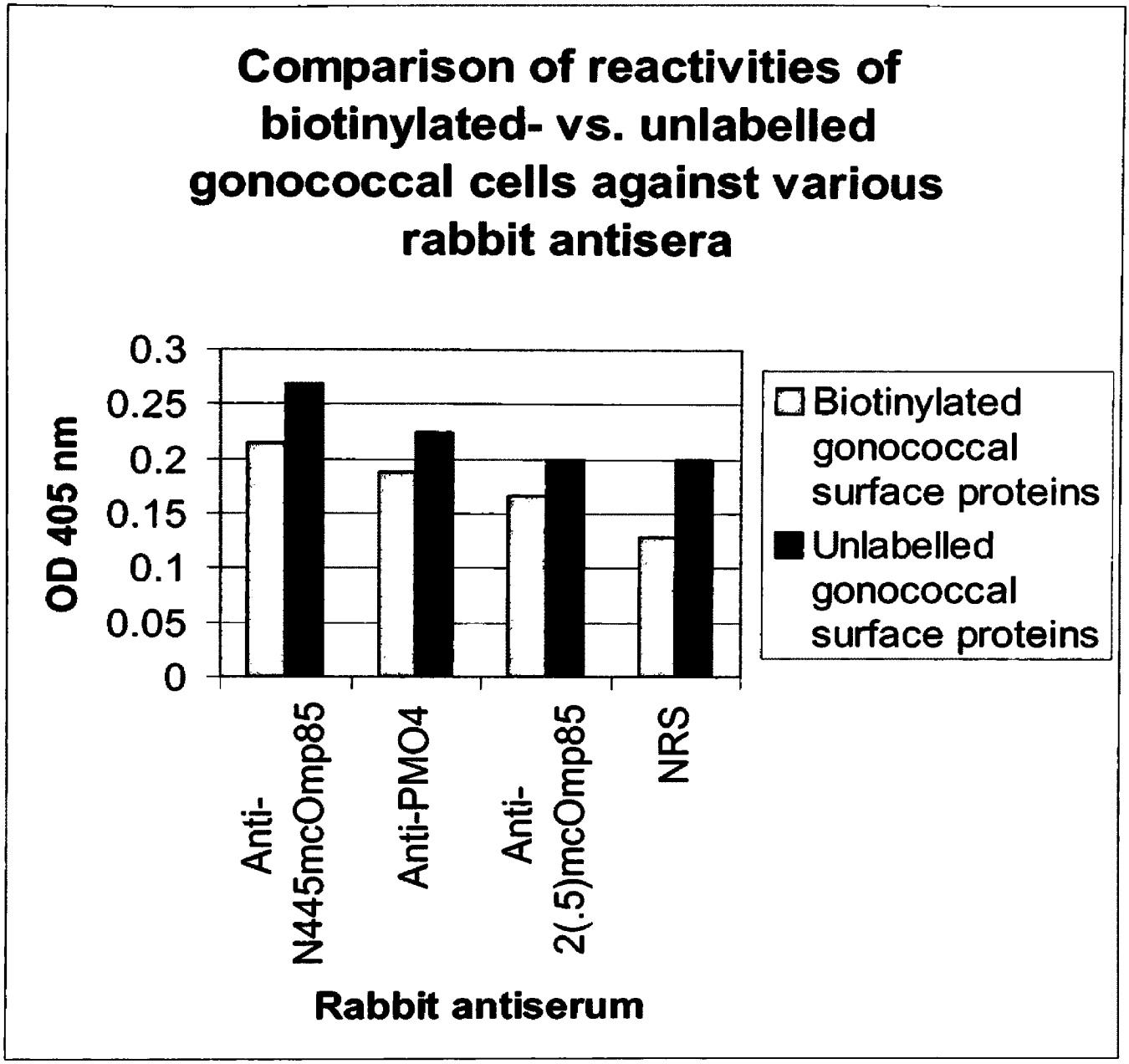



**Figure 12.** ELISA demonstrating reactivity of various rabbit antisera against biotinylated- and unlabeled *N. gonorrhoeae* MS11 LOS A. Biotinylated cells are depicted with blue bars; unlabeled cells are depicted with maroon bars. The following rabbit antisera were used to assay reactivity: anti-$_{N445}$mcOmp85; serum raised against the first 173 amino acids of gonococcal Omp85 (anti-PMO4); serum raised against amino acids 445-798 of meningococcal Omp85 (2(.5)mcOmp85); and normal rabbit serum (NRS).

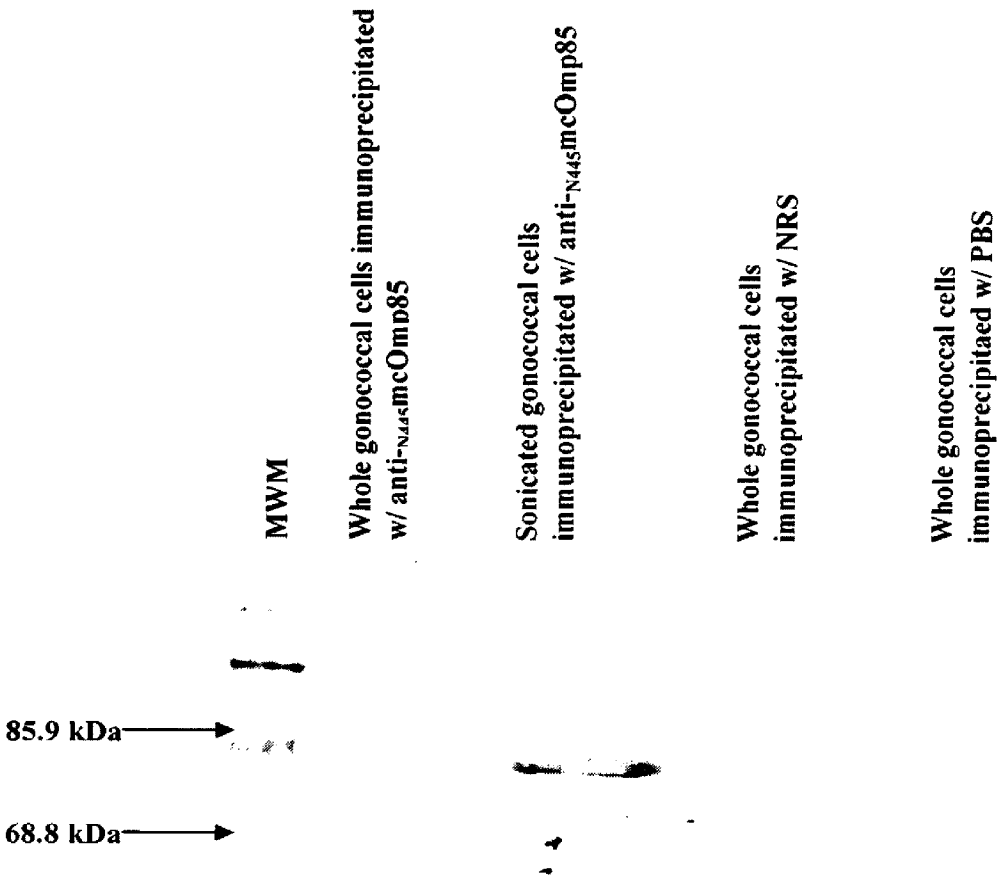



**Figure 13.** Western blot to visualize results of immunoprecipitation assay on gonococcal cells. Immunoprecipitation of gonococcal cells was performed using anti-$_{N445}$mcOmp85 antiserum and protein A-sepharose beads. Proteins subsequently bound to the sepharose beads were visualized by solubilizing the sepharose beads, then analyzed via Western blot, using anti-$_{N445}$mcOmp85 as a probe. Lane 1, GIBCO Benchmark prestained molecular weight marker (MWM), expressed in kilodaltons; lane 2, *N. gonorrhoeae* MS11 LOS A whole cells immunoprecipitated with anti-$_{N445}$mcOmp85; lane 3, *N. gonorrhoeae* MS11 LOS A that was sonicated prior to immunoprecipitation with anti-$_{N445}$mcOmp85; lane 4, *N. gonorrhoeae* MS11 LOS A whole cells immunoprecipitated with normal rabbit serum (NRS); lane 5, N. *gonorrhoeae* MS11 LOS A whole cells immunoprecipitated with PBS.

# Discussion

Pathogenic strains of *Neisseria* infect up to 250 million people worldwide annually (1-3,5,28). *N. meningitidis* causes fulminant meningococcal sepsis and meningococcal meningitis, which can have mortality rates of up to 80% and 5%, respectively (28). *N. gonorrhoeae* is the causative agent of gonorrhea, a leading cause of urethritis, cervicitis, and pelvic inflammatory disease in the United States. Early diagnosis of these diseases is often difficult; early symptoms of meningococcal disease are very similar to those of viral and other bacterial diseases, while detection of gonococcal infection is often hindered by the fact that up to 40% of infected men and 80% of infected women are asymptomatic for disease (23). Consequently, initiation of treatment often does not occur until later during infection, resulting in decreased effectiveness of the treatment and increased opportunity for unidentified carriers to spread the disease. Therefore, disease prevention would most likely be the most effective cure. Unfortunately, the current vaccine against *N. meningitidis* has limited effectiveness, and there is no vaccine available against *N. gonorrhoeae*.

The hypothesis tested here was that rabbit antiserum generated against the first 445 amino acids of a meningococcal 85 kilodalton (kDa) outer membrane protein (Omp85) promotes specific complement-mediated killing of *N. meningitidis* and *N. gonorrhoeae*. Omp85 is a potential vaccine candidate since it is universally expressed by *Neisseria*, and its expression is highly conserved (17). At the nucleic acid level, all strains within a species have identical Omp85 (17). High interspecies homology exists at the amino acid level; meningococcal Omp85 is 95% identical and 98% similar to gonococcal Omp85 (17). In addition, Omp85 homologues in *Haemophilus influenzae* and *Pasteurella multocida* were shown to induce protective immunity in rats (8,16,18,26). Therefore, antibodies against Omp85 could potentially confer protection against all strains of both *N. meningitidis* and *N. gonorrhoeae*. Evaluating the bactericidal

activity of serum against the first 445 amino acids of meningococcal Omp85 (anti-$_{N445}$mcOmp85) was one approach to assay the vaccine potential of Omp85. The N-terminal half of Omp85 was focused upon here since it was previously shown that the entire molecule is difficult to express in *E. coli*. In addition, systematic evaluation of different Omp85 fragments would allow for pinpointing of a putative bactericidal-eliciting epitope.

To purify adequate quantities of $_{N445}$mcOmp85 for rabbit immunization, DNA encoding for $_{N445}$mcOmp85, minus the signal sequence, was cloned into a hyperexpression vector. The pTrcHis-TOPO® vector was used, which was designed to allow for insertion of PCR-amplified products. This vector system included the following features: poly-histidine tag to allow for affinity purification of the recombinant protein via a nickel column; *trc* promoter region for high-level inducible expression in *E. coli*; *lac* operator binding site for the *lac* repressor; *lacI*$^q$ gene encoding for the *lac* repressor, which regulates the promoter; ampicillin resistance gene to allow for selection of transformants; origin derived from pBR322, a commonly used *E. coli* cloning vector; and an Xpress™ epitope for detection of the recombinant protein. Plasmid DNA from the resulting transformants were analyzed using restriction enzymes *AccI*, *BamHI*, and *EcoRI* to confirm correct orientation of the insert. Expression of recombinant $_{N445}$mcOmp85 was induced with 1 mM IPTG, which prevents binding of the *lac* repressor to its corresponding operator. Subsequent SDS-PAGE and CBB stain analysis revealed that expression levels were not as high as expected. This may have been due to toxicity of the expressed recombinant protein to *E. coli*, or a sub-optimal vector system. To address the latter possibility, $_{N445}$mcOmp85 was subcloned into pTrcHisB. The pTrcHisB vector differs from pTrcHis-TOPO® in that it lacks the TOPO cloning site, and it has a ColE1-derived origin. Otherwise, pTrcHisB retains all other features of pTrcHis-TOPO®. $_{N445}$mcOmp85 was inserted into the multiple cloning site of pTrcHisB using *EcoRI* and *BamHI* (Figure 1). Following the transformation reaction, plasmid DNA from resulting colonies was analyzed by PCR to confirm presence of the insert. Those clones that

contained the insert were analyzed using restriction enzymes *AccI*, *HindIII*, and *NheI* to confirm correct orientation (see Figure 1 for map of restriction sites). Analysis of recombinant $_{N445}$mcOmp85 expression levels via SDS-PAGE and CBB staining showed increased protein expression as compared to that achieved in pTrcHisTOPO (Figure 4). Therefore, the pTrcHisTOPO vector was inefficient at expressing recombinant $_{N445}$mcOmp85. Purification of the fusion protein was performed via affinity chromatography using a nickel column. CBB staining and Western blot analysis was used to confirm the identity of the purified protein as $_{N445}$mcOmp85 (Figures 6&7).

To generate anti-$_{N445}$mcOmp85 serum, rabbits were immunized with purified recombinant $_{N445}$mcOmp85. Three months after primary immunization, anti-$_{N445}$mcOmp85 serum was at a titer of at least 1:32,000, as determined by Western blot strips containing whole cell lysates of *N. gonorrhoeae* MS11 LOS A. To ensure that the rabbits were not previously exposed to Omp85 of environmental strains, serum was collected prior to immunization (normal rabbit serum, NRS). Western blot analysis showed that NRS did not react to either purified recombinant $_{N445}$mcOmp85 or *Neisseria* whole cell lysates (Figure 8). Thus, reactivity of anti-$_{N445}$mcOmp85 was a result of exposure to the immunogen, and not by exposure to environmental Omp85-expressing strains. The reactivity of anti-$_{N445}$mcOmp85 serum was further tested via Western blot against the immunogen, $_{N445}$mcOmp85-expressing clone, and various *Neisseria* strains. Recombinant $_{N445}$mcOmp85 has an expected molecular mass of 55 kDa; anti-$_{N445}$mcOmp85 detected a ~55 kDa protein in the lanes containing purified recombinant $_{N445}$mcOmp85 and the $_{N445}$mcOmp85-expressing clone (Figure 10). This confirmed reactivity of anti-$_{N445}$mcOmp85 serum against the immunogen. Anti-$_{N445}$mcOmp85 detected a ~85 kDa protein in all lanes containing whole cell lysates of various *Neisseria* strains (Figure 10), supporting previous observations that Omp85 is universally expressed by *Neisseria*.

To evaluate anti-$_{N445}$mcOmp85 serum for its ability to specifically promote complement-mediated killing of *Neisseria*, bactericidal assays were performed. One unencapsulated strain of

*N. meningitidis* and four strains of *N. gonorrhoeae* were assayed for their susceptibility to complement-mediated killing specifically promoted by anti-$_{N445}$mcOmp85 rabbit serum (Tables 1-5). To quantify bactericidal activity, two-fold serial dilutions of the antiserum were performed to allow for determination of a killing titer, which was defined as the reciprocal of the last serum dilution that produced no bacterial growth. Various degrees of sensitivity to anti-$_{N445}$mcOmp85 serum was observed among the strains tested. However, most strains that exhibited sensitivity to anti-$_{N445}$mcOmp85 serum were equally sensitive to NRS, indicating that killing was a result of serum components other than anti-$_{N445}$mcOmp85 antibodies. *N. meningitidis* 8543 was unable to grow in any serum dilution containing 10% human complement. This agreed with previous work, which showed that unencapsulated meningococcal strains are much more sensitive to normal human serum (NHS) as compared to encapsulated strains; all unencapsulated, non-sialylated strains of *N. meningitidis* showed some degree of serum sensitivity to 10% NHS (22). Therefore, to determine killing titer of anti-$_{N445}$mcOmp85 rabbit serum against *N. meningitidis* 8543, complement concentration was reduced to 5%. This resulted in a killing titer of approximately 4 (Table 1). However, NRS also produced a killing titer of approximately 4, indicating that killing observed with this meningococcal strain was not due to anti-$_{N445}$mcOmp85 antibodies.

*N. gonorrhoeae* MS11 LOS A was resistant to all dilutions of anti-$_{N445}$mcOmp85 serum and NRS.

*N. gonorrhoeae* FA19 was also resistant to all dilutions of anti-$_{N445}$mcOmp85 and NRS. Resistance to NRS killing observed here agrees with previous work by Pettit *et al.* (20), which demonstrated that *N. gonorrhoeae* FA19 exhibited ≥50% survival in 25% pooled NHS. This particular gonococcal strain was isolated from a patient with disseminated gonococcal infection (DGI). Since DGI strains tend to be more serum resistant as compared to strains from uncomplicated gonococcal infections (20), NRS resistance exhibited by *N. gonorrhoeae* FA19 agreed with previous results.

*N. gonorrhoeae* JS1 was sensitive to killing by anti-$_{N445}$mcOmp85, producing a killing titer of approximately 2. However, NRS also produced a killing titer of approximately 2, suggesting that the killing induced by anti-$_{N445}$mcOmp85 was non-specific. Pettit *et al.* (20) found *N. gonorrhoeae* JS1 to be resistant to killing by 25% pooled normal serum. This discrepancy in sensitivities to normal serum may be due to differing serum components between NRS and NHS. *N. gonorrhoeae* JS1 was isolated from a patient with uncomplicated gonococcal infection; since such strains are often sensitive to serum killing (20), the observation that JS1 is sensitive to NRS is likely not anomalous.

*N. gonorrhoeae* FA638 was sensitive to killing by anti-$_{N445}$mcOmp85, producing a killing titer of 512. This was approximately twice the killing titer produced by NRS, suggesting that antibodies against $_{N445}$mcOmp85 promoted some degree of complement-mediated killing against this strain. *N. gonorrhoeae* FA638 was previously shown to have truncated lipooligosaccharide (LOS) (20). Thus, it is possible that this strain's sensitivity to anti-$_{N445}$mcOmp85-specific killing was due to increased surface exposure of Omp85 epitopes. It may also be possible that *N. gonorrhoeae* FA638 expressed more Omp85 than other strains, although such differences in outer membrane protein expression levels between strains have never been addressed.

The strains tested here exhibited varying degrees of sensitivity to NRS killing. Several factors contributing to serum sensitivity in *Neisseria* have been previously described. Cytidinemonophospho-N-acetylneuramic acid (CMP-NANA), the nucleotide sugar of sialic acid, confers serum resistance upon binding to LOS (22). Both meningococci and gonococci are capable of being sialylated; however, only meningococci are capable of endogenous LOS sialylation (22). Human secretions serve as the source of sialic acid for most resistant gonococcal strains; therefore serum resistance is often lost after repeated subcultures (22). Sialylated gonococci resist serum killing by exhibiting increased binding to factor H (22), which regulates the complement cascade by inactivating surface-bound C3b. Binding of factor H to surface-bound C3b was demonstrated using non-sialylated gonococci, which bound factor H only if the

cells were previously incubated in NHS; non-sialylated gonococci that were previously incubated in heat-inactivated serum did not bind factor H (22). Since heating serum causes the inactivation of complement proteins, these observations indicated that factor H binds non-sialylated cells only if they were previously coated with C3b.

Porin (Por) is another factor that contributes to serum resistance. Gonococci express one of two porin isoforms, Por A or Por B (22). Strains that express Por A are often associated with DGI, which in turn are associated with serum resistance (22). Gonococci that express Por B are more closely associated with local urogenital disease (22), and tend to be more serum sensitive. Therefore, Por A expression is often indicative of a serum resistant phenotype. Serum resistance in Por A-expressing strains may be due to the fact that factor H preferentially binds Por A over Por B (22). As a result, Por A strains have less surface-bound C3b, which is inactivated by factor H. The role of Por A-factor H binding in conferring serum resistance was studied using monoclonal antibodies against the fifth loop of Por A ($\alpha$-$_{5L}$Por A), which serves as the binding site for factor H. Serum resistant strains that were incubated with $\alpha$-$_{5L}$Por A antibodies were subsequently rendered sensitive to NHS (22). Thus, binding of factor H to Por A plays a role in conferring serum resistance in gonococci.

Serum resistance associated with Por A-expressing strains may also be partially attributed to the fact that C4b-binding protein (C4bp) preferentially binds Por A over Por B (22). C4bp is a fluid-phase regulator of the classical pathway, and is involved in mediating inactivation of C4b. Since C4b is required for the formation of C3 convertase in the classical pathway, C4b inactivation results in serum resistance by preventing accumulation of C3b.

In meningococci, encapsulation and sialylation are the two main factors contributing to serum resistance and virulence. It was previously shown that strains isolated from patients with invasive meningococcal disease possess both a capsule and sialylated LOS (22). In addition, only strains possessing both a capsule and sialylated LOS were capable of causing bacteremia in

infanat rats (22). All encapsulated strains exhibited at least 20% survival in 10% NHS (22), while non-encapsulated, non-sialylated strains showed some degree of sensitivity to the same concentration of NHS (22). Encapsulation and sialylation have an additive effect on conferring serum resistance, since encapsulated, sialylated strains were more serum resistant than encapsulated, non-sialylated strains (22). Furthermore, exogenous addition of CMP-NANA to encapsulated strains increased serum resistance in encapsulated, non-sialylated strains (22). Encapsulation likely confers serum resistance by masking outer membrane binding sites for C4 and C5b (22). C4 participates in the classical pathway by serving as the precursor to C4b, which is one of the components of the C3 convertase. C5b binds cell surfaces, and provides a foundation for the formation of membrane attack complex (MAC). Thus, serum resistance in meningococci results primary from thwarting MAC formation on the cell surface. This is in contrast to gonococci, which resist serum primarily by the prevention of C3b deposition on the cell surface.

Previous studies showed that bactericidal activity of antibodies against major Neisserial surface antigens is affected by sialylation (7). Antibodies against LOS and Por are less bactericidal to sialylated cells, as compared to non-sialylated cells (7). Therefore, sialylation may also prevent bactericidal activity of antibodies against Omp85.

In this project, 4 out of the 5 *Neisseria* strains assayed were not sensitive to complement-mediated killing that was specifically promoted by anti-$_{N445}$mcOmp85 rabbit serum. One strain showed only slight sensitivity to anti-$_{N445}$mcOmp85-mediated complement killing; there was only an approximately two-fold difference in killing titer between anti-$_{N445}$mcOmp85 and NRS (Table). The inability of anti-$_{N445}$mcOmp85 rabbit serum to generate a strong, specific bactericidal response may be attributed to any of the factors, described above, that confer serum resistance upon *Neisseria*. It is also likely that the paucity of Omp85 molecules on the cell surface may be a contributing factor preventing anti-$_{N445}$mcOmp85 serum from exhibiting specific bactericidal activity. Quantifiable CBB staining of *Neisseria* whole cell lysates indicated that

Omp85 comprises only a minute percentage of total outer membrane proteins. This would hinder anti-$_{N445}$mcOmp85-mediated complement killing by the classical pathway, since it drastically decreases the chance that two Omp85 molecules would be in close enough proximity to allow for cross-linking by IgG. The pentameric nature of IgM would allow it to cross-link a single Omp85 molecule, and thus potentially initiate the classical pathway; however, because the high titer serum used in these bactericidal assays was collected after several months of immunization, the vast majority of immunoglobulins had already experienced a class-switch to the IgG isotype. Thus, it might be difficult for Omp85 to elicit bactericidal antibodies. Previous work showed that *Neisseria* are not susceptible to antibody-independent complement killing via the alternative pathway (22). This may explain the complete inability of anti-$_{N445}$mcOmp85 rabbit serum to produce killing in some of the strains tested here.

To discount the possibility that anti-$_{N445}$mcOmp85 serum did not exhibit bactericidal activity due to lack of Omp85 surface exposure, immunoprecipitation assays were performed on biotinylated gonococcal whole cells. Cells were biotinylated prior to immunoprecipitation for the purpose of amplifying any potential signal, by treatment with streptavidin-peroxidase conjugate, that may result from immunoprecipitation by anti-$_{N445}$mcOmp85 serum. Signal amplification was desired since Omp85 molecules appeared to be sparse in the outer membrane; thus, anti-$_{N445}$mcOmp85 antibodies likely would not immunoprecipitate sufficient quantities of Omp85 molecules to allow for detection without signal amplification. Biotinylated whole cells were treated with a mild lysis buffer to release antigens. The lysed cells were then incubated with protein A-sepharose beads and anti-$_{N445}$mcOmp85 serum to precipitate out any labeled Omp85 molecules. The sepharose beads were subsequently washed. To determine if the beads had bound any biotin-labeled Omp85 molecules, the beads were solubalized, separated on SDS-PAGE gel, and then transferred to a PVDF membrane. To detect the presence of any biotin-labeled proteins, the membrane was incubated with streptavidin-peroxidase conjugate, then developed on autoradiography film. This procedure failed to detect any 85 kDa proteins, which

indicated that anti-$_{N445}$mcOmp85 serum was unable to bind epitopes on biotin-labelled whole cells. This suggested that the epitope recognized by anti-$_{N445}$mcOmp85 was not surface exposed; alternatively, the epitope may have been surface exposed, but steric hindrance caused by biotin labeling may have prevented binding of anti-$_{N445}$mcOmp85 antibodies. To test the latter possibility, biotinylated- and unlabeled gonococci were analyzed by Western blotting, using anti-$_{N445}$mcOmp85 serum as a probe. No bands were detected in the lane containing biotinylated cells, while an 85 kDa band was detected in the lane containing unlabeled cells (Figure 11). Similar results were observed via ELISA: biotinylated cells produced a diminished signal, as compared to unlabeled cells (Figure 12). Biotinylation also hindered binding of antibodies against the first 173 amino acids of gonococcal Omp85 (anti-PMO4) and antibodies against amino acids 445-798 of meningococcal Omp85 (anti-2(.5)mcOmp85) (Figure 12). Collectively, these results suggested that biotinylation sterically hindered antibody binding to the majority of Omp85 epitopes. This indirectly pointed to the surface-exposed nature of Omp85. Because biotinylation exclusively labeled surface-exposed proteins of whole cells, and biotinylation hindered antibody binding to Omp85, it may be deduced that Omp85 is a surface-exposed molecule.

To obtain more direct evidence of Omp85 surface exposure, an immunoprecipitation experiment was performed on unlabeled gonococcal whole cells. Whole cells were incubated with anti-$_{N445}$mcOmp85 serum, after which unbound antibodies were washed away. The cells were then incubated with protein A-sepharose beads, followed by treatment with a mild lysis buffer to release antigens. The beads were then washed. To determine if the beads had bound any Omp85 molecules, the beads were solubalized, then analyzed by Western blot, using anti-$_{N445}$mcOmp85 serum as a probe. This resulted in detection of an ~85 kDa protein (Figure 13). A much more intense 85 kDa band was detected when the cells were sonicated prior to immunoprecipitation. This indicated that many Omp85 surface epitopes are inaccessible to antibodies due to a transmembrane or intracellular location, or due to masking by LOS. The

increased signal caused by sonication also suggests that immunoprecipitation of Omp85 from whole cells was due to surface exposure of Omp85, rather than release of Omp85 from autolyzed cells.

Taken together, these results indicated that the Omp85 epitope recognized by anti-$_{N445}$mcOmp85 serum is surface-exposed. This agrees with previous observations suggesting that Omp85 is an outer membrane protein. These observations include the presence of a signal peptide (29) and a C-terminal phenylalanine (25), a 10 amino acid C-terminal motif indicative of outer membrane proteins (25), and reactivity of anti-Omp85 antibodies against isolated gonococcal outer membranes (Quick and Judd, see Figure 14). Therefore, anti-$_{N445}$mcOmp85 serum was not prevented from exhibiting strong bactericidal activity due to lack of Omp85 surface exposure. More likely, other factors contributed to the lack of bactericidal activity of anti-$_{N445}$mcOmp85. These include the scarcity of Omp85 in the outer membrane, which may have prevented antigen cross-linking by antibody, and therefore thwarted initiation of the complement cascade via the classical pathway. It is also possible that the nature of the $_{N445}$mcOmp85 antigen and the method used to immunize the rabbit prevented a predominant IgG3 response, which is the subclass of IgG most effective at activating complement (10). It was previously shown that variations in LOS phenotype altered surface-exposure of other *Neisseria* outer membrane proteins (14). Therefore, it is possible that the lack of anti-$_{N445}$mcOmp85 bactericidal activity was due to an outer membrane protein-masking LOS phenotype expressed by the strains tested.

Despite the lack of bactericidal activity exhibited by anti-$_{N445}$mcOmp85 serum, Omp85 is still a promising vaccine candidate. Since the antiserum studied in this project was raised against the N-terminal half of Omp85, it may be possible that epitopes located in the C-terminal half elicit bactericidal antibodies. Since the function of Omp85 has yet to be determined, it is also possible that antibodies against Omp85 may hinder an essential function or block cell adhesion.

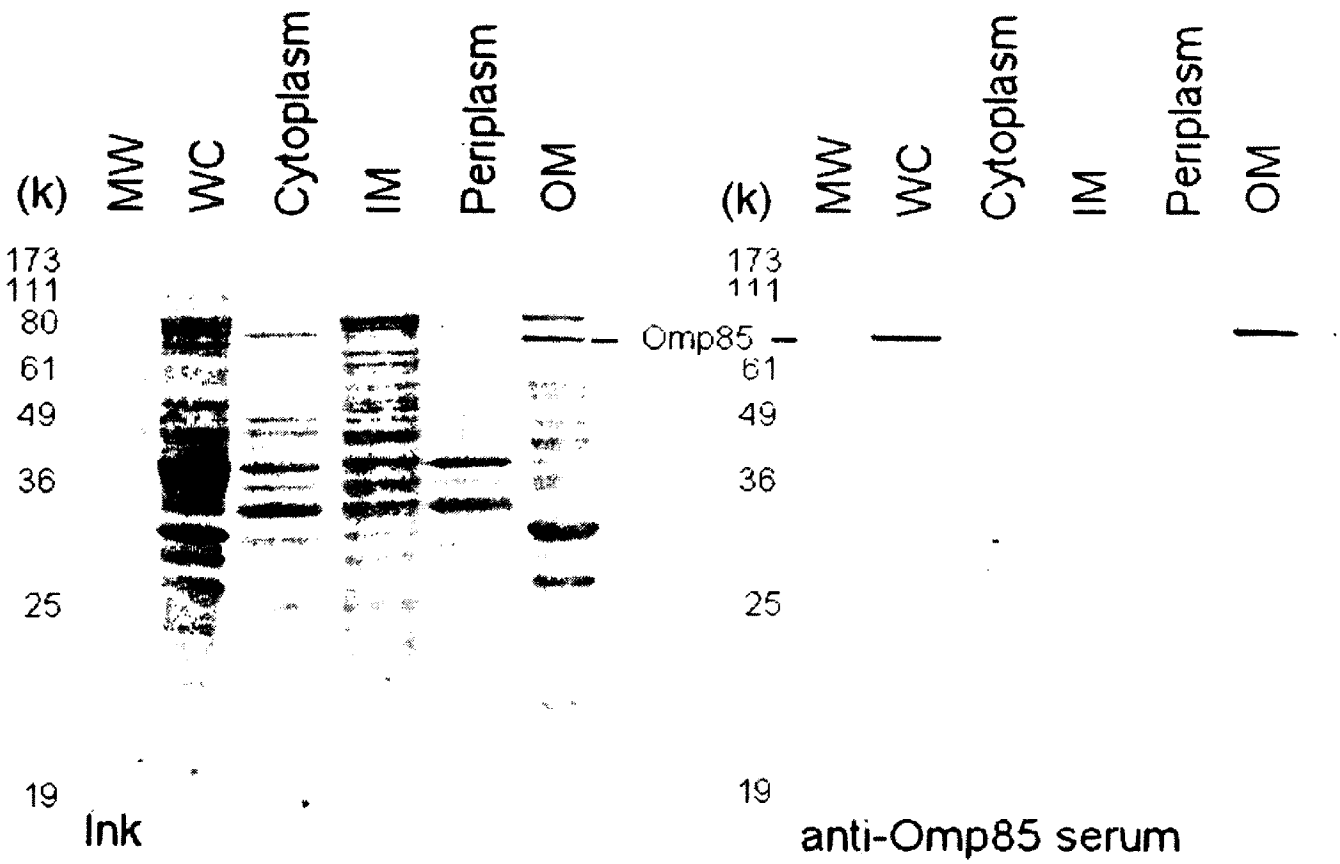



**Figure 14** (Courtesy of Quick and Judd). Cellular location of Omp85 by Western blot analysis. Lysates of whole cels (**WC**), cytoplasm, isolated inner membranes (**IM**), periplasm and isolated outer membranes (**OM**) of *Neisseria gonorrhoeae* MS11 LOSA separated in a 12.5% SDS-PAGE gel, blotted and probed with antiserum raised to the first 178 amino acids of Omp85 (**anti-Omp85 serum**). The Omp85 is identified. Molecular mass markers (**MW**), expressed in kilodaltons (**k**), were GIBCO Benchmark prestained markers. These results indicated that Omp85 was found only in whole cells and isolated outer membranes, confirming that Omp85 is an outer membrane protein.

The vaccine potential of Omp85 is further implicated by its universal and conservative expression in all pathogenic *Neiseria*. Furthermore, Omp85 homologues in *Haemophilus influenzae* and *Pasteurella multocida* were shown to induce protective immunity in rats (8,16,18,26). Thus, further studies examining the vaccine potential of Omp85 are warranted.

In summary, anti-$_{N445}$mcOmp85 rabbit serum did not exhibit specific bactericidal activity against 4 of 5 *Neisseria* strains. *N. gonorrhoeae* FA638 was slightly susceptible to anti-$_{N445}$mcOmp85-mediated complement killing; the killing titer produced by anti-$_{N445}$mcOmp85 against FA638 was approximately twice that produced by NRS. The slight sensitivity of FA638 to anti-$_{N445}$mcOmp85 killing may be attributed to truncated LOS (20), which may allow for greater surface exposure of the Omp85 molecule. Alternatively, this strain may express more Omp85 as compared to other *Neisseria* strains. To determine if bactericidal activity of anti-$_{N445}$mcOmp85 was hampered due to lack of surface exposure of the corresponding epitope, immunoprecipitation assays were performed on gonococcal whole cells using anti-$_{N445}$mcOmp85 serum. Results from these experiments indicated that the epitope recognized by anti-$_{N445}$mcOmp85 was indeed surface-exposed. Therefore, the lack of specific bactericidal activity exhibited by anti-$_{N445}$mcOmp85 was more likely a consequence of other factors. These include the low abundance of Omp85 molecules on the outer membrane, which would decrease the likelihood of antibody cross-linking to two adjacent antigens, thus preventing initiation of the classical complement pathway. It is also possible that the nature of the $_{N445}$mcOmp85 antigen and the method of immunization prevented a sufficient IgG3 response, thus hindering complement activation. LOS phenotype of the test strains may have also played a role, since previous work showed that variations in LOS may mask surface exposure of other *Neisseria* outer membrane proteins (14).

Although anti-$_{N445}$mcOmp85 serum lacked bactericidal activity, Omp85 is still an attractive vaccine candidate. Unlike most previous *Neisseria* outer membrane protein vaccine

candidates, Omp85 is universally and conservatively expressed by *Neisseria*. Moreover, Omp85 homologues in *Haemophilus influenzae* and *Pasteurella multocida* were shown to induce protective immunity in rats (8,16,18,26); therefore, antibody binding to Omp85 may disrupt some essential function, or may prevent cell adhesion. In addition, since the antiserum used in this project was raised against the N-terminal half of Omp85, it may be possible that epitopes in the C-terminal half of the molecule may elicit generation of bactericidal antibodies. Thus, further studies examining the function of Omp85 and the ability of anti-Omp85 antibodies to hinder pathogenicity of *Neisseria* warranted.

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