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Characterization of the gene encoding Omp85 a novel outer membrane protein of Neisseria gonorrhoeae and introduction of genes into Neisseria gonorrhoeae

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Characterization of the Gene Encoding Omp85, a Novel Outer Membrane Protein of *Neisseria gonorrhoeae* and Introduction of Genes into *Neisseria gonorrhoeae*

Submitted by Dennis Reschke
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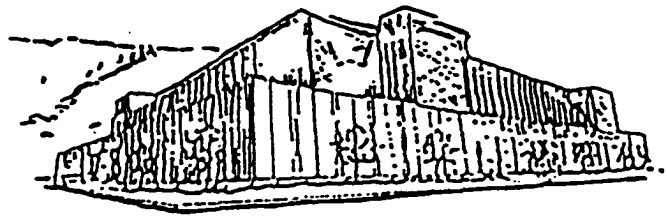

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Characterization of the Gene Encoding Omp85, a Novel Outer Membrane Protein of *Neisseria gonorrhoeae* and Introduction of Genes into *Neisseria gonorrhoeae*

Director: Ralph C. Judd

Abstract

A survey of gonococcal outer membrane proteins using an expression library screened with anti-outer membrane antibody identified sixteen proteins. Among them was an 85,000 Dalton (Omp85) protein that was homologous to *Haemophilus influenzae* protective surface protein D15 and the protective antigen Oma87 of *Pasteurella multocida*. Analogous proteins were present in *Brucella abortus* (Omp1), *Helicobacter pylori*, and some strains of *Escherichia coli*. These data suggested Omp85 was a member of a family of highly conserved proteins which may be important in establishing and/or maintaining mucosal infections. This protein was constitutively and universally expressed in all gonococcal strains tested. The genes encoding the Omp85 protein from *N. gonorrhoeae* and *Neisseria meningitidis* were cloned and sequenced. Both the gonococcal and meningococcal proteins were found to possess a typical leader sequence and terminal Phe residue, characteristic of outer membrane proteins. The gonococcal protein was 792 amino acids in length while the meningococcal protein was 797 amino acids in length. Insertional inactivation of the gene in *N. gonorrhoeae* resulted in a merodiploid state, suggesting the essential nature of the gene.

Since inactivation of *omp85* appeared not to be possible, an alternative approach to study function would be to express Omp85 in a closely related species which naturally lacks Omp85, such as *Branhamella (Moraxella) catarrhalis*. Since very little is known about the transformation parameters or the genetics of this species, a pilot procedure using *N. gonorrhoeae* was developed. The 16S rDNA locus was used as the target sequence for recombintation with a plasmid construct containing the gonococcal promoter *aniA* and the bacterial luciferase reporter gene *luxAB*, inserted within a cloned portion of gonococcal 16S sequence. This construct successfully recombined into the gonococcal 16S rDNA locus. The transformant, 1400.1, emited light in the presence of the substrate n-decyl aldehyde. An integration vector for further manipulations of this kind in *N. gonorrhoeae* was constructed.

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Acknowledgments

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I. Introduction

Neisseria gonorrhoeae is the causative agent of gonorrhea. Manifestations of this sexually transmitted disease include urethritis in both males and females, cervicitis, and pelvic inflammatory disease (PID) in females. Sequelae of these conditions are sterility in both male and female patients and ectopic pregnancies. In 1% or less of infected individuals a disseminated gonococcal infection (DGI) occurs, leading to a purulent arthritis and, infrequently, to endocarditis. An estimated one million new cases occur every year. Treatment in the United States for gonococcal infection of the fallopian tubes alone is currently at a cost of five billion dollars per year (27). Medical concerns of this organism include the rise of antibiotic resistance, the high cost of treatment, and the lack of a vaccine to prevent infection. These problems are exacerbated by the lack of a suitable animal model, making it difficult to identify gonococcal virulence factors.

The gonococcus is a Gram-negative diplococcus. The cell envelope of Gram-negative bacteria is a 3-layered structure consisting of a bilayered membrane, a periplasm containing soluble proteins, a peptidoglycan mesh, membrane-linked oligosaccharides, and an outer membrane with proteins and lipids of unique structure. Outer membrane proteins of most abundance are peptidoglycan-linked lipoproteins with covalent lipid modifications and porins, which are oligomeric B-barrel structures with large aqueous channels. The lipid bilayer of the outer membrane is asymmetric, with an inner leaflet of the same three glycophospholipids as the inner membrane and an outer leaflet of made of lipopolysaccharide (116). Pili and flagella, if present, protrude from the cell surface.

The outer membrane, which is exposed to the environment, carries the

antigenic determinants of the bacterial surface (109). Certain of the proteins and the lipopolysaccharides of the outer membrane function as receptors for bacteriophage and colicins (116). The outer membrane provides the cell with a passive barrier to substances with molecular weights greater than several hundred Daltons. This generally renders Gram-negative bacteria more resistant to antibiotics than are Gram-positive organisms, which lack the structure (102).

Both outer and inner membranes consist of approximately equal proportions of proteins and lipids, but the protein composition of each membrane in unique (109). Gel electrophoresis as well as direct purification reveal that there are a few major outer membrane proteins and nearly 50 additional minor species (109, 126, 133, 135, 136). Genetic studies have elucidated the functions of many of these polypeptides. In general, they confer distinct selective advantages on the cell, but few are actually required for growth (140, 64, 66, 93, 131). The protein composition of the inner membrane is more complex than that of the outer membrane (116), and many of the inner membrane polypeptides are enzymes which catalyze essential biosynthetic reactions (109). Individually, they constitute less than 0.1% of the total cellular protein (109). The inner membrane also contains various transport proteins (109).

Lipopolysaccharide is a substance unique to gram-negative bacteria and is exclusively located in the outer membrane. When injected into animals, it causes endotoxic shock (2). It consists of three components: the outer antigenic sugars, the core sugars, and lipid A (109). The polysaccharide chain projects outward into the environment. The sugar residues differ from various bacteria and even between strains of bacteria. These are generally

referred to as O antigens.

The phospholipid composition of the two membranes is relatively similar (109). 60% of the total phospholipid of *E. coli* is within the outer membrane, while the rest is in the inner membrane (109). Phosphatidyl-ethanolamine is the predominant component, the remainder consists of phosphotidylglycerol and cardiolipin. In general, *E. coli* and most related Gram-negative bacteria do not contain phosphatidyl choline, phosphatidyl inositol, sphingolipids, glycolipids, or steroids which are characteristic of eukaryotic systems (109). All components of the Gram-negative envelope are synthesized on the inner membrane or in the cytoplasm and subsequently translocated across the inner membrane and peptidoglycan layer to the outer membrane. (109).

One purpose of investigating outer membrane proteins is for the potential of vaccine development. Outer membrane proteins are of interest in disease control because of their antigenicity. Vaccination with protein antigens will usually result in both a cellular (T cell) and humoral (B cell) immune response. For protection against bacterial infections, like *N. gonorrhoeae,* bactericidal and opsonic antibodies are of crucial importance, while T cells play a more indirect role by regulating the antibody response in terms of immunoglobin class switch , affinity maturation, and magnitude of response (2). T cells are also necessary for the induction of memory and can indirectly induce killing of bacteria by activating phagocytes (2).

To better understand the infectious process, we must study the bacterial physiology, interaction of the pathogen with host cells, and the immune response. Since the immune system responds predominantly to the outer surface of a pathogen, a good deal of research has focused on components of

the outer membrane, particularly proteins. The recent sequencing of the *Mycoplasma genetalium* genome showed that, of the 482 known genes present, one third were devoted to the membrane (53). Many of these gene products are undoubtedly involved in synthesis rather than being actual components of the cell membrane; nevertheless, a significant portion of a bacterial genome appears to be devoted to the construction of the cell envelope and membrane proteins. Several sources suggest as many as 50 outer membrane proteins exist in the Gram-negative cell envelope (110, 126, 133, 135, 136). If a third of the genes in mycoplasma are devoted to the membrane, then presumably at least that many must be devoted to the membrane of gonorrhea, having approximately twice the genomic complement and having both an inner and outer membrane. To date, only a handful of gonococcal outer membrane proteins have been identified (Table 1).

A portion of the proposed research was to identify and characterize novel outer membrane proteins of *N. gonorrhoeae.* Outer membrane proteins probably play a significant role in adherence and invasion, and in the hosts' humoral and cell mediated immune response to infection. Antigenic and phase variation of many gonococcal outer membrane proteins appear to allow the organism to escape immune clearing (72, 58, 100, 57, 148). Therefore, identification of outer membrane proteins that do not vary may be useful in aiding our understanding of the pathogenesis of this disease and for the development of a subunit vaccine.

Known Outer Membrane Proteins of *N. gonorrhoeae*

Several gonococcal outer membrane proteins have been characterized. The following descriptions will briefly summarize what is known about these proteins. A complete list is shown in Table 1.

Table 1: Known Outer Membrane Proteins of *Neisseria gonorrhoeae*

outer membrane gonococcal protein	letter designation	approximate molecular weight	reference
pilin	Pil	20 kDa	77
porin	Por	36 kDa	73
reduction modifiable protein	Rmp	30 kDa	167
opacity proteins	Opa	30 - 36 kDa	158
transferrin binding proteins	Tbp	95 & 75 kDa	35
haemin-binding proteins	HmB	97 & 44 kDa	90
lactoferrin binding protein	LbpA	103 kDa	149
iron-regulated protein	Frp-B	76 kDa	12
lipid modified protein	Lip	23.5 - 28 kDa	67
multiple transferable resistance CDE	Mtr CDE	44, 110, 50 kDa	60
anaerobically induced proteins	Pan 1,2, 3	54, 45, 32 kDa	30
gonococcal parietal lectin	GPL	65 kDa	11
glycolipid-binding adhesin protein	-	36 kDa	111
sulfhydryl-containing protein	-	41 kDa	105
penicillin binding proteins	PBP-1,2, 3	90, 63, 48 kDa	40

The major gonococcal outer membrane protein is porin (Por), constituting 60% of the outer membrane protein complement (73). The monomeric molecular weight of Por ranges from 32 to 36 kDa. Trimers of Por form a hydrophobic channel creating an anion-specific pore (15). Por is expressed constitutively and does not undergo high-frequency antigenic variation *in vitro* or *in vivo* (73). A given strain expresses either PorA or PorB but never both; these are variants from a single *por* gene locus (56, 24). Antigenic variation exists between strains but Por is invariant within a strain (73). Human convalescent sera had high titers to Por (22) and Por-specific partial immunity was observed in prostitutes recovered from gonococcal infection (114).

Intimately associated with Por in the outer membrane is reduction modifiable protein (Rmp)(96). Rmp was shown to be associated with Por in a ratio of 3:1 and co-precipitated with Por using monoclonal antibodies (mAbs) specific to either Por or Rmp (92, 73, 148). Judd demonstrated the conserved nature of Rmp between strains and the surface exposure of Rmp (71). Antibodies to Rmp, although complement fixing, are not bactericidal and block anti-Por antibodies from their otherwise bactericidal effects (119, 120). By making antibodies to Rmp, the host immune system circumvents its ability to quickly clear the organism. Unlike Por, whose presence in the outer membrane is necessary for survival, null mutants of Rmp are viable (167).

Another component of the outer membrane is the pilus protein (Pil). Pili are filamentous processes composed of multiple subunits which function as adherence ligands. They appear to be necessary for infection, as Pil^- variants are non-infectious in male urethral challenge (77). Generally, only one antigenic type is expressed at a time (65). Expression is controlled at a single

locus, *pilE* (58, 145). Non-reciprocal recombination with a number of silent genes, *pilS*, located throughout the genome, create a wide variety of antigenic recombinations (83, 59, 57, 141). The resultant antigenic variation is so diverse and recombination is so rapid that an immune response to Pil is unlikely to clear the infection. A pilus-based vaccine was tested in Korea but offered only partial immunity to homologous strains (18). Pili share a conserved structure between strains (58), but unfortunately these regions appear not to be surface exposed (125). Work is ongoing in search of conserved surface exposed regions of Pil which may generate broad cross-strain immune activity. Presumably, immune protection would result from the inhibition of attachment to target cells as a result of antibody binding to Pil protein (70).

A third group of outer membrane proteins are designated opacity proteins, (Opas), based on their expression in opaque appearing colonies on solid media (156). There are at least 11 Opa proteins, a single cell expressing from zero to five at any one time (148). Opa expression is regulated by translational frameshifting via the presence of a repeated CTCTT oligo-nucleotide within the signal sequence (152). Conserved regions among Opa proteins appear to be membrane associated whereas hypervariable regions are localized in hydrophilic, surface-exposed regions. There appears to be a correlation between expression and establishment of infection (31, 156), although cells not expressing Opa proteins have been recovered from infected patients during menses and DGI (20, 156). There is also a correlation between the particular Opas expressed and the cell type infected (85).

Another group of characterized outer membrane proteins are the iron regulated protein receptors for transferrin, lactoferrin, and haem. *N. gonorrhoeae* does not secrete siderophores and, since free iron is limited on

mucosal surfaces, various specific iron gathering proteins have evolved. Their expression is regulated by the Fur transcription modulator protein, which in turn is regulated by the intracellular iron concentration.

Transferrin binding proteins, Tbp1 and Tbp2, acquire iron from human transferrin via TonB activation (16, 35). Antibodies against analogous meningococcal proteins block transferrin binding in gonococcus and are bactericidal (153). Lactoferrin is thought to have antibacterial properties by sequestering iron from pathogens. It is found in PMN granules and on mucosal surfaces. Gonococcal lactoferrin binding protein A, LbpA, is also regulated by Fur. Clinical isolates may or may not express LbpA, thus it is assumed that survival is contingent upon the expression of other iron gathering proteins when LbpA is not detectable (149). The haem binding proteins are capable of sustaining gonococcal growth when haem is the only iron source. It is not known at this time whether this gene is regulated by Fur. The molecular weights of these proteins are conserved between all strains tested (90). Fe-regulated protein B, FrpB, is capable of taking iron from haem, though its specificity and function have not yet been established (12).

Lipid modified protein, Lip, is a broadly conserved yet variable molecular weight outer membrane protein. It has been postulated that antibodies bound to Lip may interfere sterically with the interaction of gonococcal surface constituents and host-cell receptors (67). The recently identified multiple transferable resistance efflux pump, MtrRCDE, is homologous to the MexABO proteins of *Pseudomonas aeruginosa* and the AcrAE and EnvCD proteins of *Escherichia coli*. Mutants of these proteins confer a hypersensitivity to hydrophobic agents such as detergent-like fatty acids and bile salts (60). Expression of the Pan proteins are induced by

anaerobic growth using nitrite as the terminal electron acceptor in the respiratory chain. It is thought that within the human host similar anaerobic conditions may exist and that these proteins may play a part in survival under those conditions (30). The 65-kDa gonococcal parietal lectin (GPL) has a lectin-like activity and shares common structural and immunological similarities with GroEL in various Gram-negative and Gram-positive bacteria (11). Glycolipid-binding adhesin protein was found to adhere to various ceramide derivatives and may be involved in epithelial cell receptor binding (111). A sulfhydryl-containing protein was localized to the outer membrane by its ability to fluoresce under ultraviolet light (105). Its function is unknown. Finally, the penicillin binding proteins, PBP-1,2,3, are thought to be involved with membrane synthesis in Gram-negative bacteria and confer penicillin resistance (40).

Basic understanding of bacterial physiology, as it relates to the outer membrane proteins and their function and role in host cell interaction, would increase our understanding of gonococcal pathology in humans. Yet to be fully understood are the roles of known and as yet unidentified outer membrane proteins involved in gonococcal adherence, invasion, and immune evasion. The knowledge gained through understanding of these processes may afford insights into the mechanisms of pathogenesis in other human pathogens.

Mutagenesis and Gene Introduction

One method used to characterize a protein of interest is by insertion of a disrupting genetic element into the open reading frame of the encoding gene, thereby abrogating expression of the gene product. This technique has been used extensively in gonococcal research (97, 167, 142, 61, 23, 82). To

accomplish this, the gene of interest is cloned and an insertional element is introduced, either by site directed or transposon mutagenesis. The resultant construct is then transformed into gonococcus and recombination with the wild type gene yields a mutant form. One draw back of this approach occurs when the gene of interest is essential for growth in the bacteria. In these cases either no mutants are obtained or a state of merodiploidy occurs (117). Merodiploidy is a state describing the existence of both the wild type and mutant forms of the gene in the same cytoplasmic compartment.

Once a gene is disrupted in this manner, complementation with the intact gene introduced on a plasmid vector is often used to demonstrate restoration of gene function in the organism. This corroborates that the original mutant truly contains the disrupted gene of interest and was not an artifact unrelated to the intended purpose. In addition, novel genes can be effectively incorporated into the gene complement of an organism by transformation with a plasmid bearing the gene. Unfortunately in *N. gonorrhoeae,* these latter techniques are either problematic or unavailable.

The latitude of genetic manipulation of Neisserial sp. is limited in comparison with *Escherichia coli.* The isolation of chemically induced mutants is difficult (23) and only certain rather large transposons are compatible in pathogenic Neisseria (76, 103). Transformation in *N. gonorrhoeae,* however, is easily accomplished due to a natural competence, limited only by selection of the proper pilus$^+$ phenotype (147). It has been observed that a 10 bp uptake sequence is necessary for competent cells to take up exogenous DNA (46). Upon uptake, DNA is linearized and then re-circularized once inside the bacterial cell (14). Once inside, homologous recombination can occur via the RecA protein (83). Transformation allows

allelic exchange of disrupted target genes (82) and the generation of hybrid mutants (24). Transposon mutagenesis is limited to transposition in cloned genes in an *E. coli* host prior to transformation and gene disruption in *N. gonorrhoeae* (75, 142).

Plasmids carrying origins of replication other than what are found in Neisserial species do not replicate in *N. gonorrhoeae*. Stein has constructed a shuttle vector for gonococcal transformation (151), however this plasmid does not seem to function consistently (54, personal communication, Stuart Hill). Although phage transduction has not been observed for *N. gonorrhoeae*, conjugative plasmids have been isolated which facilitate genetic exchange between Neisserial sp. (121, 122, 123). In addition to a small, ubiquitous 4.2 kbp cryptic plasmid (84), antibiotic resistance is usually encoded on additional plasmids (124). Several small plasmids encoding resistance to ampicillin have been described which, although not by themselves mobile, can be transferred with the help of certain large conjugative plasmids (121). One of these large plasmids carrying the *tetM* marker has been described for *N. gonorrhoeae* (80). This plasmid, termed p*tetM25.2*, can be efficiently mobilized into non-competent gonococcal strains (129). Techniques have been refined, using a three step process, to introduce novel genes into a host cell without recourse to homologous recombination in the final recipient. The gene of interest is ligated into an *E. coli* vector containing part of the *tetM* gene. This construct is then transformed into the gonococcal recipient strain N214 where it recombines homologously with the resident pTetM25.2 plasmid carrying the entire *tetM* gene. This same N214 strain also carries a conjugative plasmid such that mating with a non-competent strain results in the transfer of pTetM25.2 which now carries the gene of interest. This plasmid remains in

circular form and does not recombine with the new host genomic DNA. Meyer has constructed a fleet of 'Hermes' *E. coli* vectors to facilitate these manipulations (86).

Another method of introducing novel genes into a genome was engineered by Clark (143). This system involves homologous recombination with an intergenic region of the proline biosynthesis operon. The Clark proAB vector has been successfully used as a reporter system to assay Pan (68) and ompS (48) promoter behavior by measuring B-galactosidase activity under various growth conditions. One advantage of this system is that it does not inactivate any of the proline operon genes.

Several problems exist with the aforementioned systems of gene reintroduction. The pTetM conjugation system is cumbersome and technically difficult. The Clark system uses the proline A and B intergenic region as a target site which, unfortunately, exists only at a single locus in the genome. This makes homologous recombination an inefficient proposition.

An alternative method of novel gene introduction into *N. gonorrhoeae* involving recombination with either one or more of the four 16S rDNA loci was exploited. The luciferase reporter gene, *luxAB*, was used together with the gonococcal *aniA* promoter. Due to the light-emitting phenotype of gonococcal transformants, it may be possible to monitor bacteria which contained the gene *in situ* in cell culture systems (33). This same technique may be effective in organ explant models. The luciferase reporter system has been successfully used in following *Salmonella* infection in mice (33) and in assaying promoter activity of virulence factor genes in *Yersinia* (112).

2. Hypotheses:

The **first** hypothesis to be tested is that there are several outer membrane proteins of *N. gonorrhoeae* which have not yet been identified.

Specific goals to this hypothesis:

1. Identify a unique outer membrane protein of *N. gonorrhoeae* strain FA19.
2. Clone and sequence the encoding gene.
3. Generate null mutants by recombination with insertionally inactivated genes.
4. Assay adherence characteristics of *N. gonorrhoeae* MS11 with Omp85-specific antibody in cell culture.

The **second** hypothesis to be tested is that a proposed 16S rDNA-*aniA-luxAB* construct can be successfully recombined into *N. gonorrhoeae* and that this system can be used to assay promoter activity *in vivo*.

Specific goals of second hypothesis:

1. Engineer a reporter gene construct containing the promoterless *luxAB* gene with a *cat* selection marker ligated into the gonococcal 16S rDNA gene.
2. Ligate the *aniA* promoter upstream of the promoterless *luxAB* gene cassette.
3. Transform this construct into *N. gonorrhoeae* MS11 and select recombinants that are chloramphenicol resistant and luciferase$^+$.
4. Assay promoter for activity under various growth conditions by measuring luciferase activity.

3. Materials and Reagents

Bacteria and Transformation

N. gonorrhoeae MS11 (generous gift of Dr. John Swanson) pilus+ cells were selected for transformation by visual inspection as described (147). Transformation was done by established procedure (147). Selection and growth on solid media was done on clear typing media (157) using 10 μg/ml chloramphenicol at 37°C, 100% humidity, 5% CO_2. DH5α *E. coli* (Gibco, BRL - Gaithersburg, MD) cells were used for all genetic manipulations using standard recombinant techniques (8). *E. coli* XL1-Blue and SOLR (Stratagene, LaJolla, CA) cells were used in the generation of genomic libraries as provided by manufacturer. Luria-Bertani medium (LB)(8) was used for all *E. coli* and Enterobacteriaceae broth and agar plate culturing. *Branhamella (Moraxella) catarrhalis* (ATCC# 8193) and all meningococcal and gonococcal strains were grown on clear gonococcal typing media (157).

Genetic Manipulations and Reagents

Restriction endonucleases, Klenow fragment, and T4 DNA ligase were obtained from New England BioLabs (NEB, Beverly, MA) and used according to manufacture protocols. Cloning vectors used were pBluescript+ (Stratagene), and pUP1 (46). Plasmids were isolated using the QIAprep quick spin kit (Qiagen, Chatsworth, CA) and DNA fragments were purified from agarose gels using Gene Clean II glass milk (Bio101, La Jolla, CA). ^{125}I was purchased from New England Nuclear (Boston, MA). Sulfo-NHS-LC-biotin was purchased from Pierce (Rockford, IL). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham (Arlington Heights, IL) and used according to manufacture directions. Immunoblotting was done with Millipore (Bedford, MA) Immobilon polyvinylidene difluoride (PVDF). Goat

anti-rabbit horse radish peroxidase conjugate antibody was purchased from Sigma (St. Louis, MO), streptavidin-peroxidase conjugate was purchased from Amersham, and protein A horse radish peroxidase conjugate antibody was purchased from Boehringer Mannheim (Indianapolis, IN). All other chemicals and reagents were obtained from Sigma.

Southern Blot Analysis

Chromosomal DNA, obtained by phenol extraction (8), and plasmid DNA, obtained by QIAprep columns (Qiagen), were digested with various endonucleases according to procedures of the manufacturer (NEB). Digested DNAs were electrophoretically separated on a 1% agarose gel and the DNAs transferred to nitrocellulose with the Bio-Rad Model 785 Vacuum Blotter (Bio-Rad Laboratories, Hercules, CA) according to instructions and Southern (146). Probe DNA was extracted from agarose gels using glassmilk (Vista, CA) and labeled with ECL universal primer system (Amersham). Blots were visualized using Luminol and autoradiographed on Hyperfilm-MP (Amersham).

SDS-PAGE and Western Blotting

Cells were harvested from confluently grown plate cultures after 16 hr of growth. These were solubilized in solubilizing buffer, 100°C for 10 min, and separated in the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system described by Laemmli (87). Protocols for whole cell lysates have been previously described by Judd (71). Separated proteins were either stained with Coomassie Brilliant Blue R or electrophoretically transferred onto PVDF as previously described (163). Blotting was performed in degassed 20mM sodium phosphate buffer, pH 8.0 for 2 hr at 600 mA. PVDF blots were blocked in 0.05% Tween-20 in Dulbecco's phosphate

buffered saline (dPBS) with 3% skim milk for 1 hr at room temperature. Primary polyclonal rabbit antibody was added at 1:1000 dilution and allowed to adsorb overnight at 4°C with gentle rocking. Rabbit antibody was pre-absorbed with *E. coli* lysate for 2 hr at 4°C with gentle agitation. Primary human antibody from patients with DGI or meningococcemia were diluted 1:100. Secondary antibody was either protein A-horse radish peroxidase (Sigma), goat anti-rabbit horse radish peroxidase (Sigma), or anti-human IgG horse radish peroxidase conjugates (Zymed, South San Francisco, CA). Blots were developed with 4-chloro-1-naphthol and H_2O_2.

Generation of Polyclonal Antisera

New Zealand white rabbits were injected with a series of four intra-venous aqueous preparations of outer membrane proteins of approximately 100 μg total protein per injection. Serum was collected from rabbits before initial injection as a negative control. Initial injection used 1 ml Freund's complete adjuvant, followed by injections using 1 ml Freund's incomplete adjuvant. 0.5 ml inoculums were injected subcutaneously at the axillary and inguinal sites. Injections were performed every two weeks for at total of four injections. Hyperimmune serum was collected intravenously and pooled.

For the generation of Omp85 fusion protein antisera, purified pMal/Omp85 was used with 1 ml Freund's complete adjuvant for the initial injection. Three subsequent injections with 1 ml Freund's incomplete adjuvant was done with cleaved pMal/Omp85 only and Freund's incomplete adjuvant.

Fusion Protein Production and Isolation

A 10 ml overnight culture of *E. coli* DH5α containing the pMal/Omp85

fusion construct was used to inoculate a 1 l volume of LB broth with 50 μg/ml ampicillin. This culture was grown to an OD_{600} = 0.4. Isopropyl-B-D-thio-galacto-pyranoside (IPTG) was then added to a final concentration of 0.3 mM and the culture was incubated for an additional 2 hr at 37°C. The cells were then centrifuged at 4,000xg for 30 min. Cells were resuspended in 50 ml lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 0.25% Tween 20, 10 mM B-ME, 10 mM EDTA, 10 mM EGTA) and sonicated for 5 min at maximum strength for 1 min bursts on ice. Sodium chloride was then added to the lysate to a final concentration of 0.5 M and the suspension centrifuged at 12,000xg for 30 min to clarify the extract.

The clarified extract was batch purified at 4°C with amylose resin (NEB) according to manufactures instruction. Briefly, 15 ml of hydrated resin was added to the extract and gently rocked at room temperature for 1 hr. The crude extract supernatant was removed by centrifugation and the amylose was washed with three volumes of 0.25% Tween-20 column buffer (10 mM Na_2HPO_4, 0.5 M NaCl, 1 mM azide, 10 mM B-ME, 1 mM EGTA, pH 7.0) and five volumes of column buffer without Tween-20. All washings were performed by gentle resuspension followed by centrifugation at 50xg for 5 min. The pellet was resuspended in 25 ml of column buffer with 10 mM maltose added to elute the fusion protein, centrifuged 50xg for 5 min, and the supernatant collected. This process was repeated. The eluate was dialyzed overnight at 4°C in distilled water to remove maltose and then lyophilized. The protein concentration was estimated by absorption at 280nm.

The fusion protein was cleaved from maltose binding protein as follows. A 1:100 ratio of factor Xa was added to dialyzed fusion protein in Xa buffer (20 mM Tris HCl, 100 mM NaCl, 2 mM $CaCl_2$, 1 mM azide) and

incubated at room temperature overnight. Removal of cleaved maltose binding protein was achieved by exposure to amylose resin. The supernatant contained the cleaved fusion protein. This was verified by SDS-PAGE.

Iodination

The method used has been previously described by Judd (71). Briefly, 16 hr cells were harvested and suspended in 4°C dPBS (OD_{600} = 1.36). 1.5 ml were centrifuged (14,000 x G) for 1 min and resuspended in 100 µl of cold dPBS. To this was added ^{125}I (4 µl, 25 µci/µl). The entire mixture was then transferred to a previously iodogenated (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) microfuge tube and incubated at 4°C for 30 min. The reaction was terminated by transferring the cell suspension to a clean tube and washed 3Xs in cold dPBS. Radioactivity was assayed using a Beckman 5500B Gamma counter (Beckman Instruments, Inc, Palo Alto, CA).

Two Dimensional Gel Electrophoresis

As previously described (106), 16 hr plate cultured cells were suspended in cold dPBS (OD_{600} = 0.68); 1.5 ml were centrifuged (14,000 x G) for 1 min and resuspended in 100 µl of first dimension sample buffer, incubated for 2 hr at 37°C with periodic mixing, and centrifuged for 2 hr at 100,000 x G in a Beckman airfuge. 10 µl samples were electrophoresed in the first dimension using a Bio-Rad Mini-PROTEAN II 2-D Cell and Bio-Rad Bio-Lyte 5/7 and 3/10 ampholytes at a 4:1 ratio for 3.5 hr at a constant 750V. Tube gels were then run in the second dimension SDS-PAGE and stained or autoradiographed. Proteins to be N-terminally sequenced were excised from the gels and eluted into buffer using a Bio-Rad Model 422 Electro-Eluter as per manufacture instructions. Proteins were concentrated using Centricon spin columns (Amicon, Beverly, MA).

Generation of FA19 Library and Screening

Gonococcal genomic DNA was partially digested with endonuclease *Tsp* 5091. Fragments ranging from 2 to 10 kbp were excised, purified, and cloned into Lambda arms predigested with *Eco*R1 provided by the manufacturer (Stratagene, Lambda Zap II). The resulting recombinants were packaged and transfected onto log phase XL1-Blue host cells. Plaques were transferred to PVDF presoaked in 10mM IPTG. Residual *E. coli* host on PVDF was lysed by chloroform and lysozyme before initial incubation with antisera. Pre-absorbed primary rabbit antibody was applied to PVDF overnight with gentle agitation at room temperature. Circles were washed several times with dPBS before secondary antibody conjugate was applied for one hour. Blots were again washed several times in dPBS to remove unbound secondary antibodies. Positive plaques were visualized by reaction with the chromogen 4-chloro-1-naphthol. Positive plaques were identified back to the master plate and excised from the top agar. Resident plasmids were rescued using helper phage as described by the manufacturer (Stratagene). Potential clones were screened by western blot analysis (163).

Outer Membrane Preparation

The procedure of Osborn (108) was used and modified by Chuck Tilly (34). Briefly, 500 ml of mid-log phase cells were centrifuged at 7,000 rpm for 10 min. (8,000 x G) and resuspended in 6.25 ml of 200 mM Tris (pH 8.0) then diluted with an equal volume of cold (4°C) 1 M sucrose, 200 mM Tris (pH 8.0) solution. To this was sequentially added: 25 µl ice cold 250 mM EDTA, 100 µl of mutanolysin (5 mg/ml in dH_2O)(ICN Biochemical, Inc.), and 12.5 ml dH_2O added forcefully by pipetting. The solution was vortexed after each addition. This suspension was agitated gently at 4°C overnight. Cells were then

sonicated using four 20 second bursts at maximum energy output (550 Sonic Dismembrator, Fisher Scientific). Unbroken cells were removed by centrifugation at 10,000 rpm for 10 minutes (12,000 x G). The supernatant was centrifuged again for 30 minutes at 10,000 rpm. The supernatant to this last spin was again centrifuged at 55,000 rpm (240,000 x G) for 2 hrs. The pellet was resuspended in 1 ml 18% (w/w) sucrose solution.

Sucrose gradients were made using 6 ml of 60% (w/w), then 5 ml of 55, 50, 45, 35, 25, and 20%, respectively, just prior to use and the 1 ml outer membrane suspension applied to the top. After 48 hr of centrifugation at 24,000 rpm using a Beckman SW-41 rotor, fractions were removed using a peristaltic pump and fraction collector. Fractions were dialyzed overnight in dH_2O to remove sucrose. Samples were assayed for total protein content and inner and outer membrane fractions pooled and lyophilized. These fractions were stored at -20°C until used.

Biotinylation and Avidin (monomeric)-agarose Capture

As previously described (55), 1 µl of a 1:20 dilution of sulfo-NHS-LC-biotin dissolved in dimethyl sulfoxide (50 mg/ml) was added to 1.5 ml cell suspension (OD_{600} = 0.68) and incubated on ice for 1 min. Labeled cells were washed 3X in 50 mM Tris-HCl (pH 7.5) to remove unbound biotin.

Once labeled, whole cells were either solubilized in standard SDS-PAGE solubilizing buffer and boiled for 10 min or processed for biotin capture. Solubilized samples were separated by two dimensional gel electrophoresis and blotted onto a PVDF membrane as previously described (163). The membrane was blocked for 1 hr at room temperature in TTBS buffer (0.01% Tween 20, 20 mM Tris, 137 mM NaCl, pH 7.6) containing 3% skim milk, washed three times with TTBS, and incubated with 10 µl streptavidin-

peroxidase conjugate diluted in 50 ml TTBS for 1 hr at room temperature. Unbound streptavidin was removed by washing in TTBS at 60°C for 15 min, followed by two 15 min washes in TTBS at 37°C. All washes were done with gentle agitation. Blots were developed by incubation for 1 min in an equal volume mixture of Luminol reagents one and two supplied by the manufacture (Amersham). The blot was then autoradiographed with hyper-film MP (Amersham) for approximately 1 min.

For biotin capture, labeled cells were lysed by the addition of 200 μl of 2% SDS and 800 μl of PBS. After 1 hr incubation at 37°C, the solution was centrifuged to sediment membrane debris and the supernatant was applied to an avidin (monomeric)-agarose column (Sigma A-2036) at RT to capture biotin labeled proteins. The column was washed with ten column volumes of PBS and captured proteins eluded with ten column volumes of 0.1M glycine (pH 2.0) as per manufacture instruction. The eluate was quickly neutralized with 2M Tris (pH 9.0) to approximately pH 7 and then de-salted and concentrated in a Centricon (Amicon, Beverly, MA) spin column and lyophilized. Proteins were solubilized in 2D solubilizing solution and electrophoresed.

Protein and DNA Sequencing

All N-terminal protein sequencing was done at The University of Montana Molecular Biology Facility (UMMBF) by Joan Strange using an Applied Biosystems 473A gas phase protein sequencer. DNA sequencing was done using the Sanger dideoxy chain termination method (132) by UMMBF using an Applied Biosystems 373A DNA sequencer. DNA sequencing primers were either universal or synthesized. Table 2 shows a list of synthesized primers. Sequencing strategies are shown in appropriate figures and described in the Results section.

Polymerase Chain Reaction (PCR)

Polymerase chain reactions were performed using a Perkin-Elmer Cetus Thermocycler Model 480 and Expand™ High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN). The following conditions were used:

cycle 1: 2 min 94°C
cycle 2: [10 sec 94°C, 30 sec 50°C, 2 min 68°C] for ten repetitions
cycle 3: [10 sec 94°C, 30 sec 50°C, 2 min 68°C - 20 seconds added after each successive cycle] for twenty repetitions
cycle 4: 7 min 72°C

PCR products were purified by either Gene Clean II or by using QIAquick PCR purification kit (Qiagen).

Construction of Plasmids

A. pHP45CΩ

A PCR primer to the upstream portion of the transcription/translation terminator elements of pHP45Ω (115) was synthesized with an *SphI* site included at its 5′ end and ligated to itself (see Figure 25). The resulting plasmid, now without the spectinomycin resistance cassette, was termed pHP45. The chloramphenicol resistance gene cassette (*cam*) from TnMaxI (61) was PCR amplified using primers cat-5′ and cat-3′, both of which contain an *Sph* I site (see Table 2 for primer sequences). This *cam* cassette was inserted into pHP45 in the *Sph* I site to create pHP45CΩ. Figure 25 shows a schematic of these manipulations. *SmaI* was used to remove the Omega fragment for use in the various constructs described below.

B. pDR4 subclones

pDR4 was subcloned using *Hin*cII restriction endonuclease into pBluescript. The resulting clones were designated pDR4L, pDR4.2.1, and

Table 2. List of PCR primers:

1. GC-D15-5'1	5' - CCGAATTCATGAAACTGAAACAGATTG - 3'
2. GC-D15-5'2	5' - CCGAATTCCAAGACATCCGTGTC - 3'
3. 4L T7B.1	5' - CCGGAATTCGACTTTGACGGGCAGGTAGTTG - 3'
4. D15-4L-5'	5' - TCACGCCCAAAGTAACC - 3'
5. D15-P9	5' - AGTGTCGATTGAAGGCG - 3'
6. D15-4.2.1-5'	5' - TTGCCGGTGATGTGGAT - 3'
7. Nru/33-5'	5' - CGTATCGCAGGACAACCTGTTC - 3'
8. PYFT	5' - CGTGAAGTACGG - 3'
9. D15-4.2.1-3'	5' - AGGCATCGACCAGCGTC - 3'
10. D15 -CT-5'	5' - CCGAATTCGAACCCAGGCCGCC - 3'
11. D15-C-T-3'	5' - CGCTGCAGGCAGCGTGTGG - 3'
12. Omp85 CT	5' - A AGC TTA GGA CGT CGT GCC GA - 3'
13. 5'hypoERVQ	5' - GGC GAA CGt/c GTt/c CAA - 3'
14. OmpHELQ	5'- TTG CAG TTT TTG CAA TTC - 3'
15. DR4.H.1-3'	5'- CCGGAATTCAAACCTTCACGCTGATGCTCGGCG - 3'
16. aniA3'	5' - GGGGATCCTTTGTAAGAAAAGTAGGGGGGA - 3'
17. aniAX5'	5' - GGGGATCCGGAATTCCGCCCGCAATGGGACAAC - 3'
18. aniAX5'.1	5' - GAACTTGGTGTGATTATGCGGTTGTCCCATTGCGG - 3'
19. aniA3'.1	5' - AATCACACCAAGTTCTTAACTAATCCCCCCTACTT - 3'
20. 16SP3'	5' - AACTGCAGAGAAAGGAGGTGATCCAGCCGC - 3'
21. 16SK5'	5' - GGGGTACCTGAACATAAGAGTTTGATCCTG - 3'
22. rDNAStu5'	5' - CCATCGATGAACGGGTGAGTAACATATC - 3'
23. rDNAStu3'	5' - GGATCGATTGAACGTCAGTGTTATCCCA - 3'
24. HLuxAB5'	5' - CGGGATCCTACCAACAAATAAGGAAATGTTAT - 3'
25. HLuxAB3'	5' - GGGGTACCCAGCTGTTTTTGTTCCTGCAATGAC - 3'
26. Omega	5' - GCATGCGATCCGGTGGATG - 3'
27. 16SBcat5'	5' - TCATGGCTCAGATTGAACGCTG - 3'
28. 16SBcat3'	5' - TTCCCCTACGGCTACCTTGTTAC - 3'

pDR4.H.1. A map of these is shown in Figure 10. Several constructs of these three subclones were made for the purpose of site-directed mutagenesis of *omp85* by homologous recombination. The *Sma*I Omega fragment from pHP45CΩ was inserted into a unique *Bsi*W1 site in pDR4L which had been blunt-ended with Klenow fragment to create pDR4LCΩ. The aphA-3 fragment from pSL33 (98) was obtained by *Sma*I digestion and inserted into a unique *Nru* I site of pDR4.2.1 to create pDR4.2.1/33 (see Figure 26).

To create constructs with larger flanking regions for more efficient homologous recombination, pOmp85 was created. Primers GC-D15-5'1 and OmpH ELQ (Table 2) were used to amplify the entire *omp85* ORF plus an additional 266 downstream base pairs (see Figure 10). This PCR product was inserted into the blunt-ended *Hin*dIII site of pUP1 (46) and termed pOmp85. Into the unique blunt-ended *Bsi* WI site of pOmp85 was inserted the Omega cassette, and into the unique *Nru* I site, the aphA-3 fragment. The resulting constructs were termed pOmp85CΩ and pOmp85/33, respectively.

C. pDRlux1400.1/pUP1

The *luxAB* genes (47) from pRL1062 (170) were PCR-amplified using primers HLux5' and HLux3' with *Bam*HI and *Kpn*I sites engineered at each end, respectively. This product was ligated into pBluescript (Stratagene) digested with the same restriction enzymes (see Figure 30). The resulting plasmid was called pHlux. The PanI (68) promoter, *aniA*, was constructed using the following primers: aniA3', aniA3'.1, aniAX5'.1, and aniAX5'. Figure 28 shows the arrangement of these primers to one another. Primers aniA3'.1 and aniA5'.1 were allowed to hybridize and *Taq* polymerase (Boehringer Mannheim) was added to fill in the complimentary strands. Primers aniAX5' and aniA3' were then added and PCR-amplified to generate the entire *aniA*

promoter sequence (68). Primers aniA3′ and aniAX5′ were engineered with *BamHI* and *XbaI* sites, respectively. pHlux was digested with the same two endonucleases and the *aniA* promoter inserted. This plasmid was termed pX.1Hlux. The CΩ fragment was then ligated into the filled in *EagI* site of pX.1Hlux. The resulting plasmid was termed pCX.1Hlux.

The C-terminal 1400 bp of the 16S gonococcal rDNA gene (128, 169), traversing base pairs 108 to 1544, was PCR-amplified with primers rDNAStu5′ and 16SP3′ engineered with *ClaI* and *PstI* sites, respectively. This product was ligated into the corresponding sites of pBluescript (Stratagene) and called 16S1400. The 16S1400 insert was digested out with *ClaI* and *PstI*, blunt-ended with Klenow fragment, and inserted into the *SmaI* site of pUP1 (46), resulting in the plasmid called 1400 pUP1. A unique *AflII* site at nucleotide 494 within the 16S fragment of 1400 pUP1 was used to insert the construct of plasmid pCX.1Hlux. This was done by PCR-amplifying out CX.1Hlux using the cat5′ and HLux3′ primers and inserting this into the blunt-ended *AflII* site of 1400pUP1. The resultant plasmid was termed pDRlux1400.1/pUP1 (see Figure 28).

D. pMal/Omp85.

pDR4 was submitted to partial digestion with *Tsp*5091 and the resulting fragments were ligated randomly into pMAL-c2 fusion protein vector (New England Biolabs) digested with *Eco*R1. Colonies were screened by western blotting with anti-outer membrane antiserum and all positive clones were analyzed by restriction analysis and sequenced. The chosen clone included approximately 100 bp upstream of *omp85* ATG codon and 250 bp of *omp85* 5′ end.

***In Vivo* Luciferase Assay**

An *in vivo* luciferase assay was accomplished as previously described (107). Briefly, cells were transferred to ice cold lux buffer (50 mM sodium phosphate, 50 mM 2-mercaptoethanol, 0.5% bovine serum albumin, fraction V, pH 7.0) to an optical density of 1.0 at 600 nm and kept on ice. Samples of 100 μl were added to the same volume of substrate and light emission immediately measured on a Turner Designs model 20 photometer (Turner Designs, Mountain View, CA) for a span of ten seconds. The substrate was prepared by a 1:1000 dilution of n-decyl aldehyde (Sigma, D-7384) in ice cold lux buffer, briefly sonicated for 2 min before use. The high concentration sensitivity setting (1000X) was used in conjunction with a synchronized remote start switch. The average of three separate assays were used for each data point.

Cell Adherence Assay

The technique of van Putten (163a) was used to assess adherence of *N. gonorrhoeae* strain MS11LosA (Opa+, Pil-) to Chang epithelial cells in the absence or presence of Fab fragments (2a) of Omp85-specific antiserum.

Chang human conjunctiva cells (ATCC#CCL 20.2) were grown for three days (to confluence) in Dulbecco's Modified Essential Medium (DMEM) in 24 well microtiter plates which had 12 mm coverslips in the bottom. *N. gonorrhoeae* MS11LosA Opa+/Pil- were harvested after 15 hr growth to an OD_{600} = 0.34 in HEPES-buffered saline, pH 7.4. This preparation was then diluted 1:50 in HEPES-buffered saline and used immediately in the following assay. The medium was removed from the Chang cells and replaced with 1 ml of DMEM-0.1% IsoVitalex (Becton Dickenson, Cockesvilled, MD). Sterile Fab fragments prepared from anti-Omp85 antiserum, anti-bovine serum

albumin and normal rabbit serum were added to a final concentration of 1 μg/ml, 10 μg/ml and 100 μg/ml to individual wells as was sterile DPBS. Ten microliters of gonococci (≈2.5 X 10^5 bacteria), harvested as above, were added to each well and the plate gently shaken. The plate was then incubated at 37°C for 3 hr in 5% CO_2 to allow bacteria to adhere to the Chang cells. Unbound bacteria and unbound Fab fragments were then removed by washing each well 4X with DPBS and the cells and bacteria fixed by adding 1 ml DPBS-2% paraformaldehyde to each well.

Following 16 hr incubation at RT, the paraformaldehyde was removed and 1 ml 50 mM Tris-HCl, pH 7.4 was added to each well. The coverslips were then placed upside down on 100 μl drops of DPBS-0.5% BSA. This was repeated 4X. The coverslips were then placed in 75 μl of a 1:100 dilution of anti-gonococcal outer membrane antiserum, covered and incubated at RT for 1 hr. The coverslips were then washed 3X in 100 μl drops of DPBS-0.5% BSA and then placed in 75 μl of DPBS-Protein-A-gold (10Å gold)(1:100 dilution of commercial preparation - Ted Pella, Redding, CA), covered and incubated at RT for 1 hr.

The coverslips were then washed 3X in 100 μl drops of DPBS and twice in 100 μl drops of dH_2O. The coverslips were returned face-up to the wells of a fresh 24 well plate and 1 ml dH_2O added. Silver stain was prepared: 2.55 g citrate monohydrate and 2.35 g sodium citrate dihydrate was added to 70 ml dH_2O; 800 mg hydroquinone was dissolved in 15 ml dH_2O; 110 mg silver acetate was dissolved in 15 ml dH_2O in subdued light. The dH_2O was removed from the wells of the plate and immediately before use, the silver stain components were mixed and 1 ml added to each well. The reaction proceeded at RT for 10 min, at which point the silver stain was aspirated and

replaced with 1 ml dH_2O. The dH_2O was then removed, replaced with 0.004% crystal violet in dH_2O and the plates incubated at RT for 16 hr.

Adherent bacteria were counted by placing coverslips upside down on a microscope slide and observed under oil immersion (1,000X). Adherent bacteria, which stain black, were counted on 22 cells. The high and low counts were discarded and the remaining 20 counts averaged.

4. Results

Omp85

Figure 1 shows the total protein complement of *N. gonorrhoeae* strain FA19 separated by two dimensional gel electrophoresis. The left panel is stained with Coomassie Brilliant Blue (CBB). The right panel is an autoradiograph of the same strain whose outer membrane proteins were labeled with ^{125}I. There are approximately 12 protein dots visible, ranging in molecular weight from approximately 100 kDa to 14 kDa, each corresponding to a putative outer membrane protein. Upon overexposure, this same gel reveals several additional protein dots, as shown in the left panel of Figure 2. A schematic map of this gel is given in Figure 3. Forty one protein dots could be detected on the autoradiograph, with many more faint protein dots observed but not recorded. An arbitrary numbering system was used for catalog purposes. Several precautions were taken to minimize the possibility of labeling internal proteins via iodine leakage through the membrane including decreased temperature (4°C), limited reaction time (30 min), immobilization of iodagen on wall of reaction vessel, and stabilization of the gonococcal cell wall with cations (31). Por protein, which is approximately 35 kDa in molecular mass, often smears in the first dimension. A horizontal line was used to depict this on the various schematic maps where there was

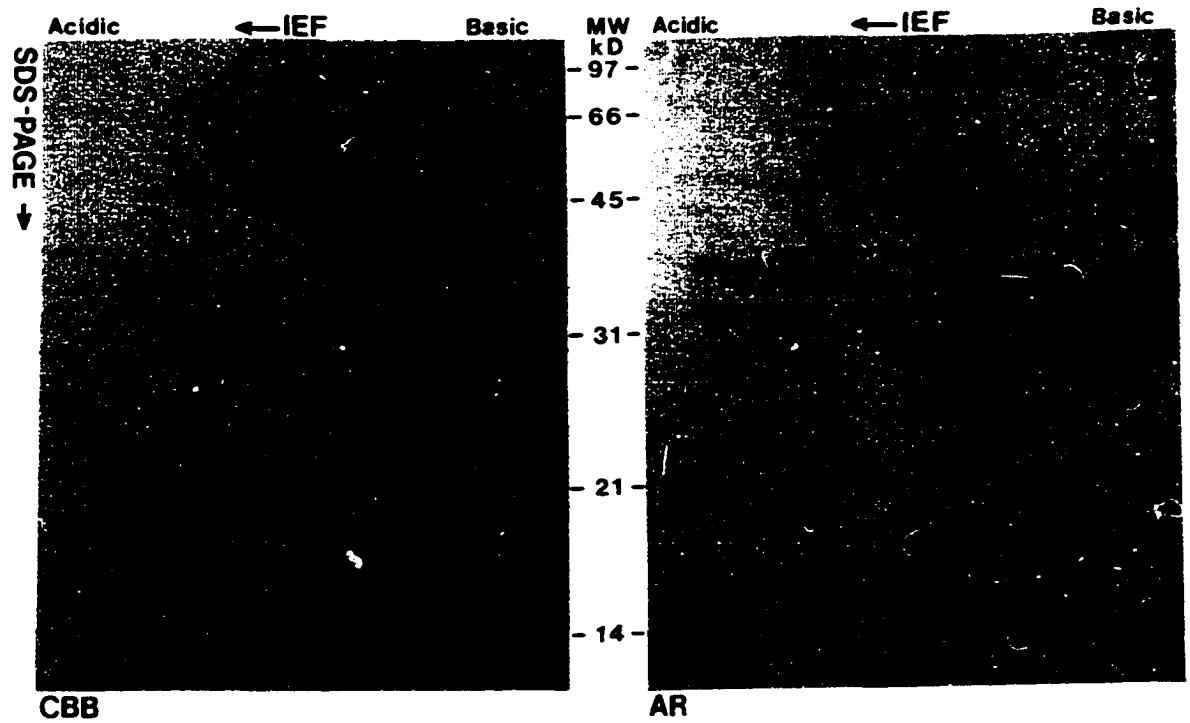


Figure 1. Total protein and surface-iodinated outer membrane proteins of *N. gonorrhoeae* FA19 separated by two dimensional gel electrophoresis. Isoelectric focusing was used to separate solubilized proteins by charge. The horizontal pH gradient ranged between pH 3 to 10 from left to right. Proteins were separated in the second vertical dimension by molecular weight using standard SDS-PAGE techniques. Molecular weight standards are depicted in thousands of Daltons (kD) between the two panels. The left panel is a total protein separation stained with Coomassie Brilliant Blue (CBB). The right panel is an autoradiograph (AR) of a similar gel with the outer membrane proteins labeled with ^{125}I.

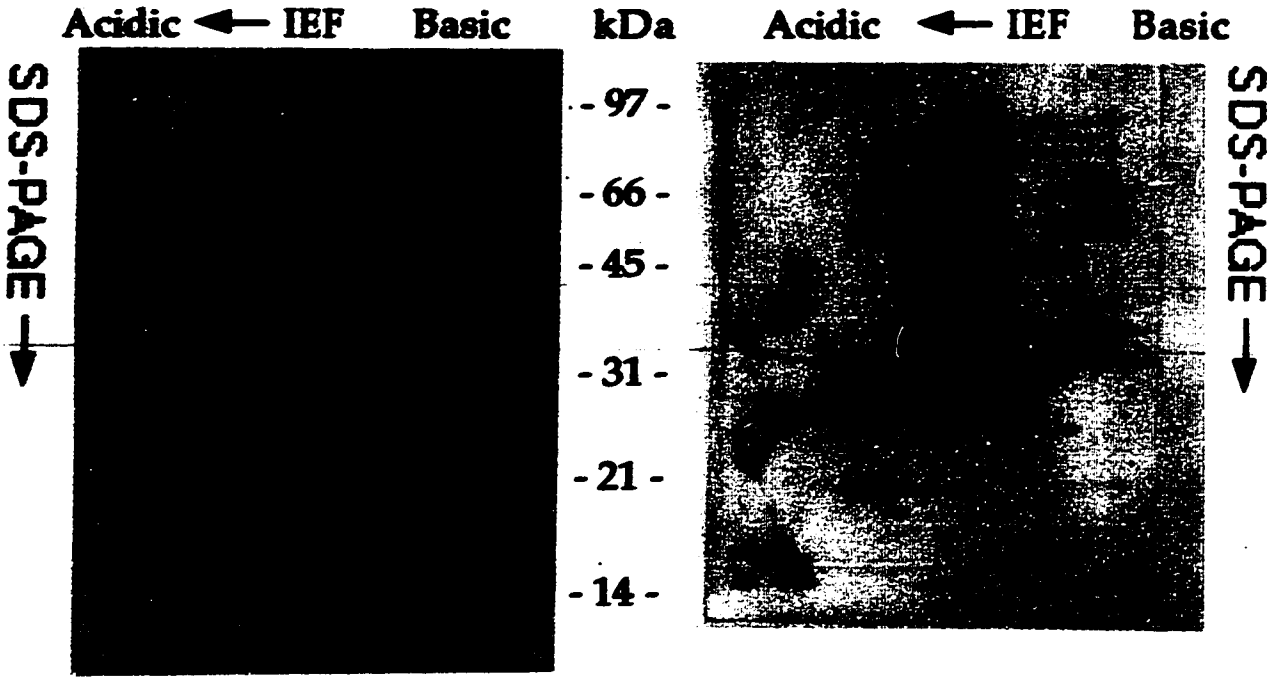


Figure 2. Outer membrane proteins identified by iodination and western blotting. The left panels shows an autoradiograph of total protein from *N. gonorrhoeae* FA19 labeled with ^{125}I and separated by two dimensional gel electrophoresis. The blot was purposely overexposed to reveal proteins with weaker signals. The right panel shows the same proteins labeled with antibodies generated against an outer membrane fraction from gonococcal strain FA19 and visualized by standard western blot techniques (163). The horizontal pH gradient runs from left to right with a pH range of 3 to 10, and the second vertical dimension using SDS-PAGE techniques runs from top to bottom. Molecular weight markers are indicated in the center in thousands of Daltons (kDa).

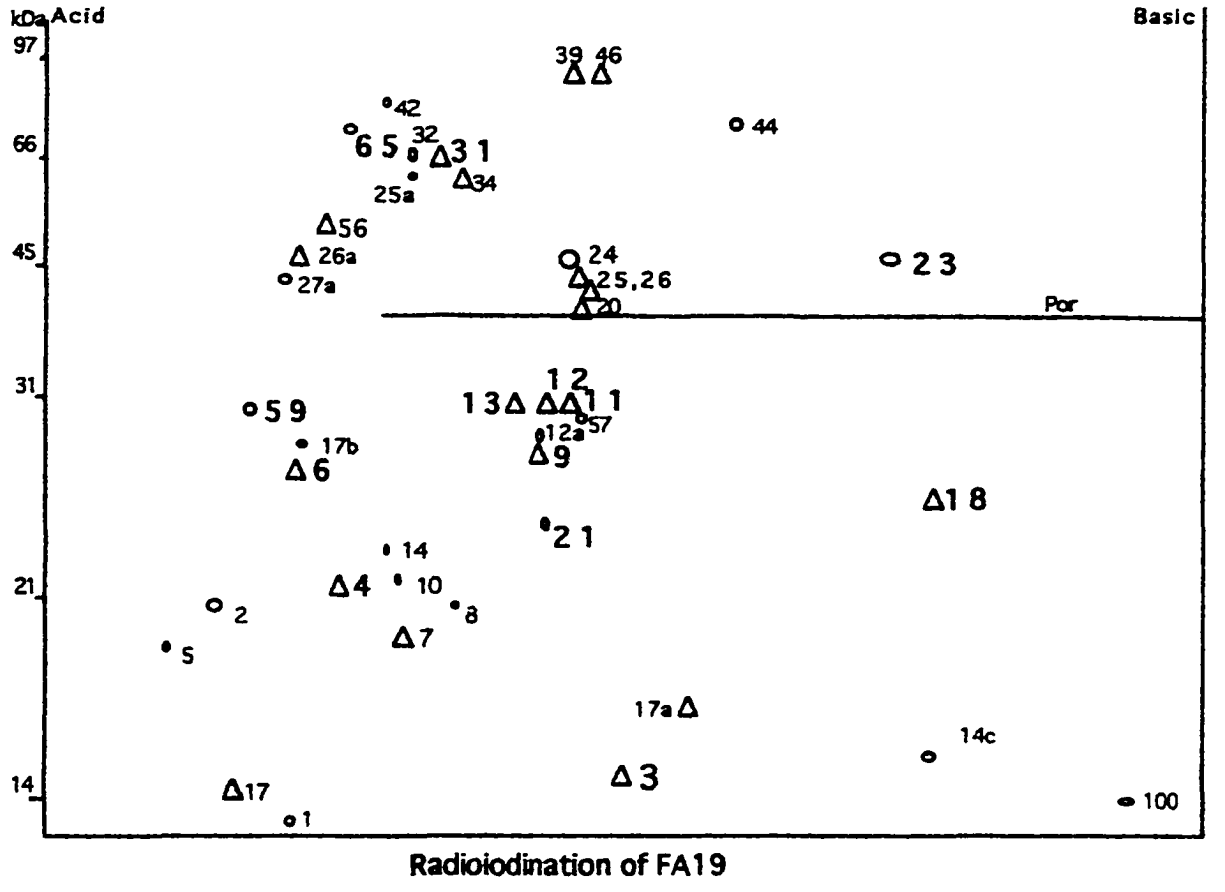


Figure 3. Schematic map of radioiodinated outer membrane proteins of *N. gonorrhoeae* FA19 taken from Figures 1 and 2. Shown here is a depiction of these labeled proteins (o). Numbers are arbitrary given for catalog purposes and have no intrinsic meaning. Molecular weight markers are indicated on left axis in thousands of Daltons (kDa). Horizontal pH gradient extends from left to right, ranging from pH 3 to 10. Δ indicates those proteins also identified on western blot and biotinylation.

smearing. Por was arbitrarily assigned the number '20'.

A second method used to label outer membrane proteins was by western blotting using antibodies generated against the FA19 outer membrane fraction (108, 34). A two dimensional western blot (163) of FA19 is shown in Figure 2, juxtaposed to an overexposed ^{125}I labeled gel, also of FA19. Figure 4 is a schematic map of this western blot to locate the various protein dots relative to each type of labeling system. The numbers used were consistent between the labeling systems. Approximately 47 dots were observed in this two dimensional western blot. Twenty of the 47 protein dots corresponded to those seen using ^{125}I labeling. These are indicated by Δ.

Biotin labeling was a third method used to identify outer membrane proteins using the procedure by Gharbia (55). Biotin is a 244-dalton vitamin that can be conjugated to many proteins via primary amine groups. Biotin also binds with high affinity to avidin and streptavidin. The product used was Sulfo-NHS-LC-Biotin (Pierce), a water-soluble derivative of biotin. A streptavidin-peroxidase conjugate was used as a secondary molecule to illuminate biotin labeled proteins in the presence of Luminol (Amersham) and recorded by autoradiography. The left panel of Figure 5 shows a two dimensional separation of proteins labeled with biotin and the right panel shows those same proteins which were captured on an avidin-agarose bead column, also separated in two dimensions. Sixty one protein dots can be seen in the left panel and eight proteins are seen in the capture blot. All those proteins captured with avidin-agarose were also seen in the biotin labeling blot. These were schematically drawn in Figures 6 & 7, respectively. A composite of proteins common to all three forms of labeling is shown in Figure 8.

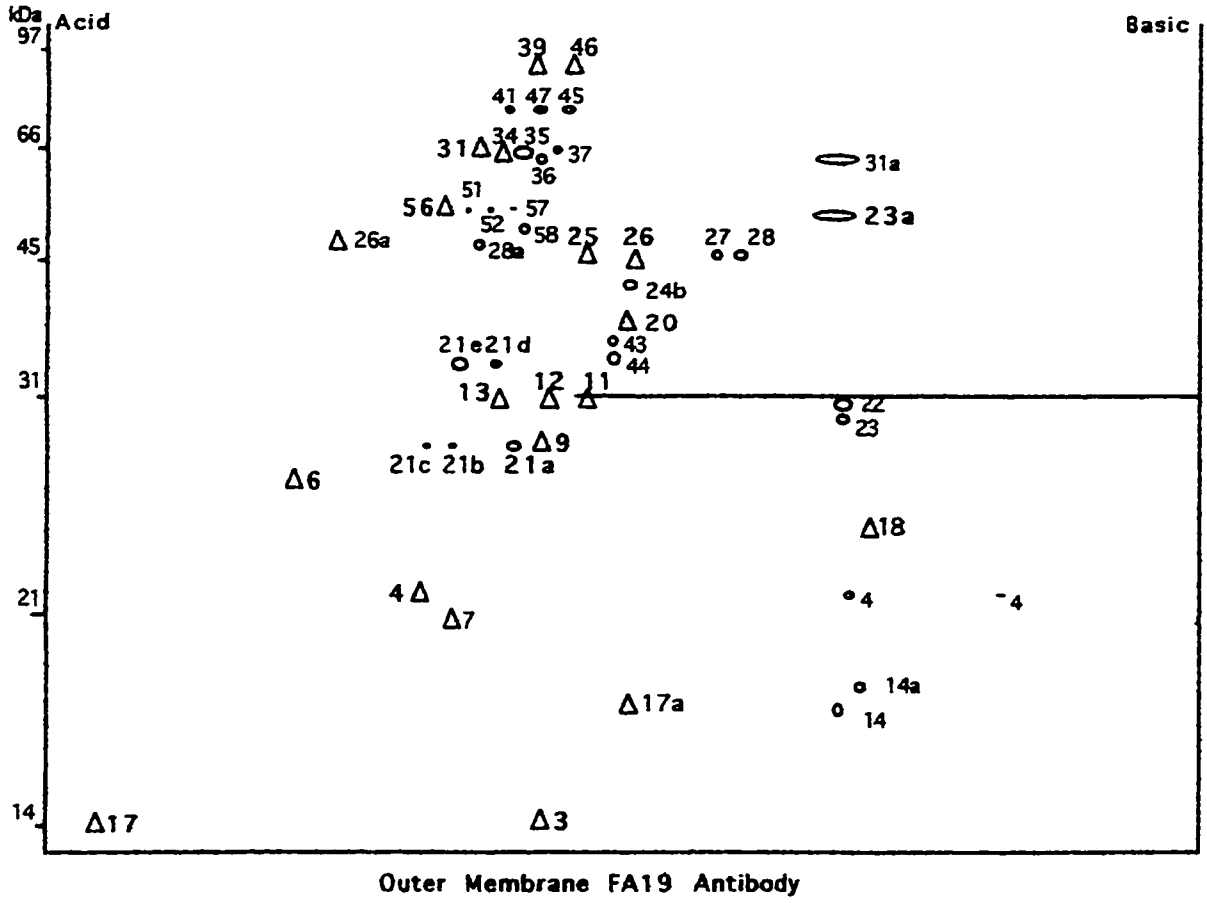


Figure 4. Schematic map of two dimensional western blot of *N. gonorrhoeae* strain FA19 using antibody generated against FA19 outer membrane fraction. Shown are locations of antibody-labeled proteins from the right panel of Figure 3 (o). Δ indicates those proteins also identified by iodination and biotinylation, Figures 1 and 5, respectively. Molecular weight markers indicated on the left axis are in thousands of Daltons (kDa). Horizontal pH gradient extends from left to right ranging from pH 3 to 10.

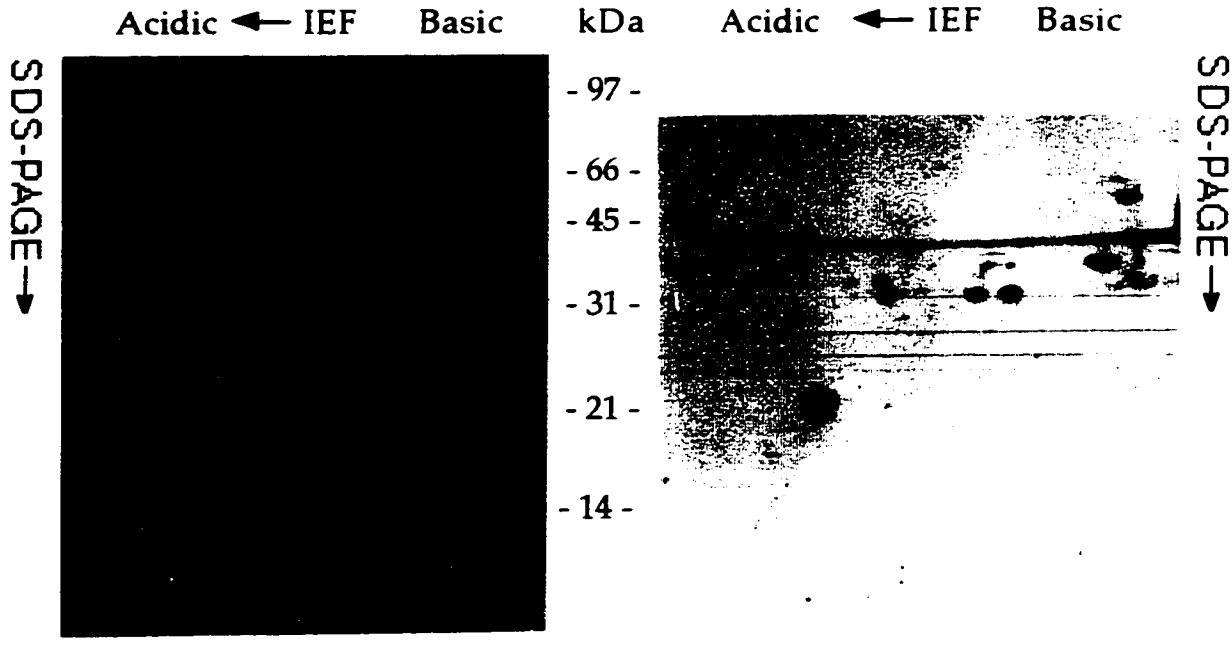


Figure 5. Biotin-labeled proteins from *N. gonorrhoeae* FA19 separated by two dimensional gel electrophoresis. Left panel shows an autoradiograph of approximately 60 proteins labeled with sulfo-NHS-LC-biotin. The horizontal pH gradient extends on both panels from left to right with a pH range of 3 to 10. The second dimension runs from top to bottom with molecular weight markers indicated in the center in thousands of Daltons (kDa). The right panel shows biotin- labeled proteins captured by streptavidin-agarose and separated in two dimensions.

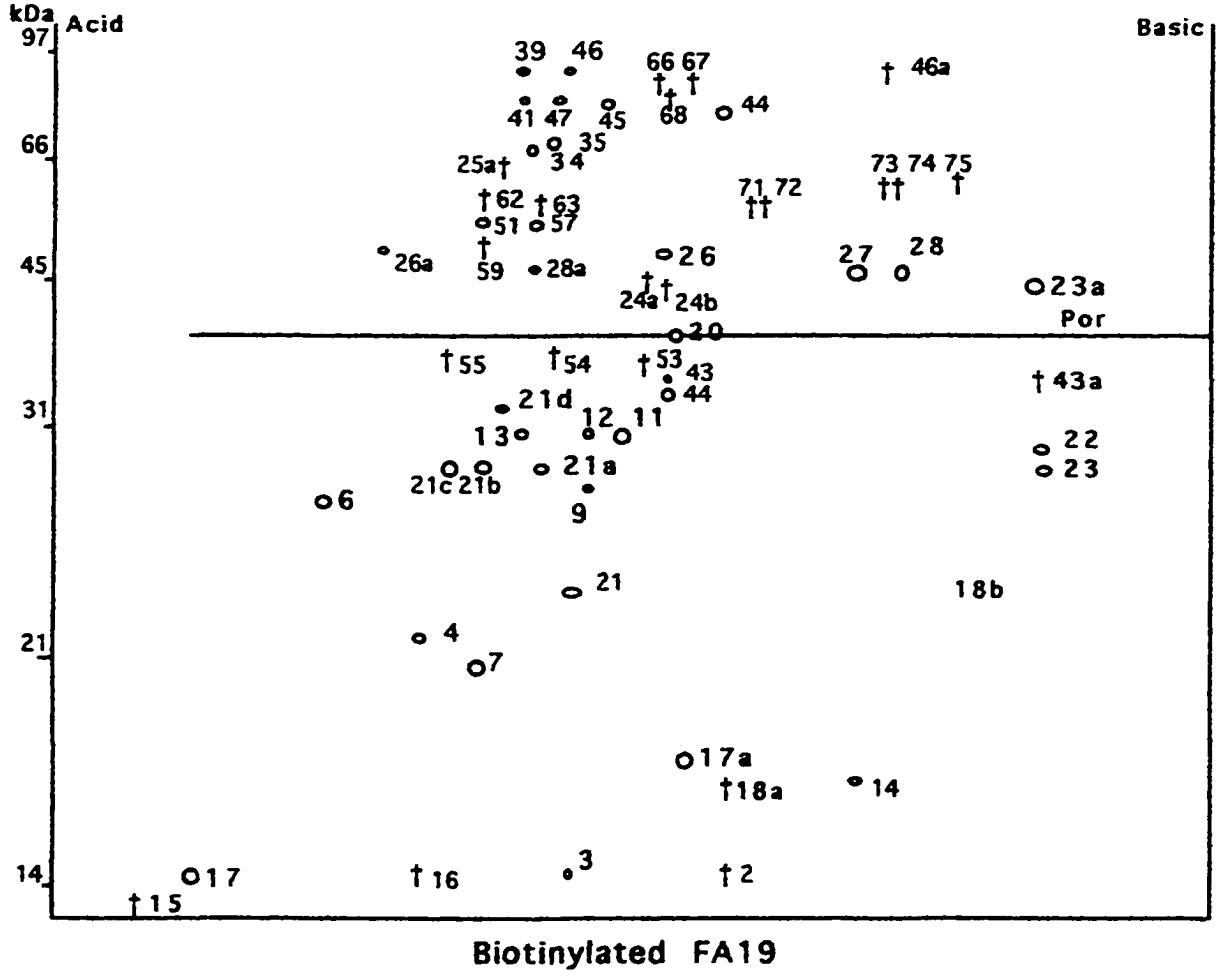


Figure 6. Schematic map of biotin-labeled outer membrane proteins from *N. gonorrhoeae* strain FA19 separated by two dimensional gel electrophoresis. Shown are the locations of biotin-labeled proteins from the left panel of Figure 5 (o). Δ indicates those proteins also identified on western blots and iodination, Figures 1 and 3, respectively. † indicates proteins unique to biotinylation and not found in either western or iodination gels. Molecular weight markers indicated on left axis are in thousands of Daltons (kDa). Horizontal pH gradient extends from left to right ranging from pH 3 to 10.

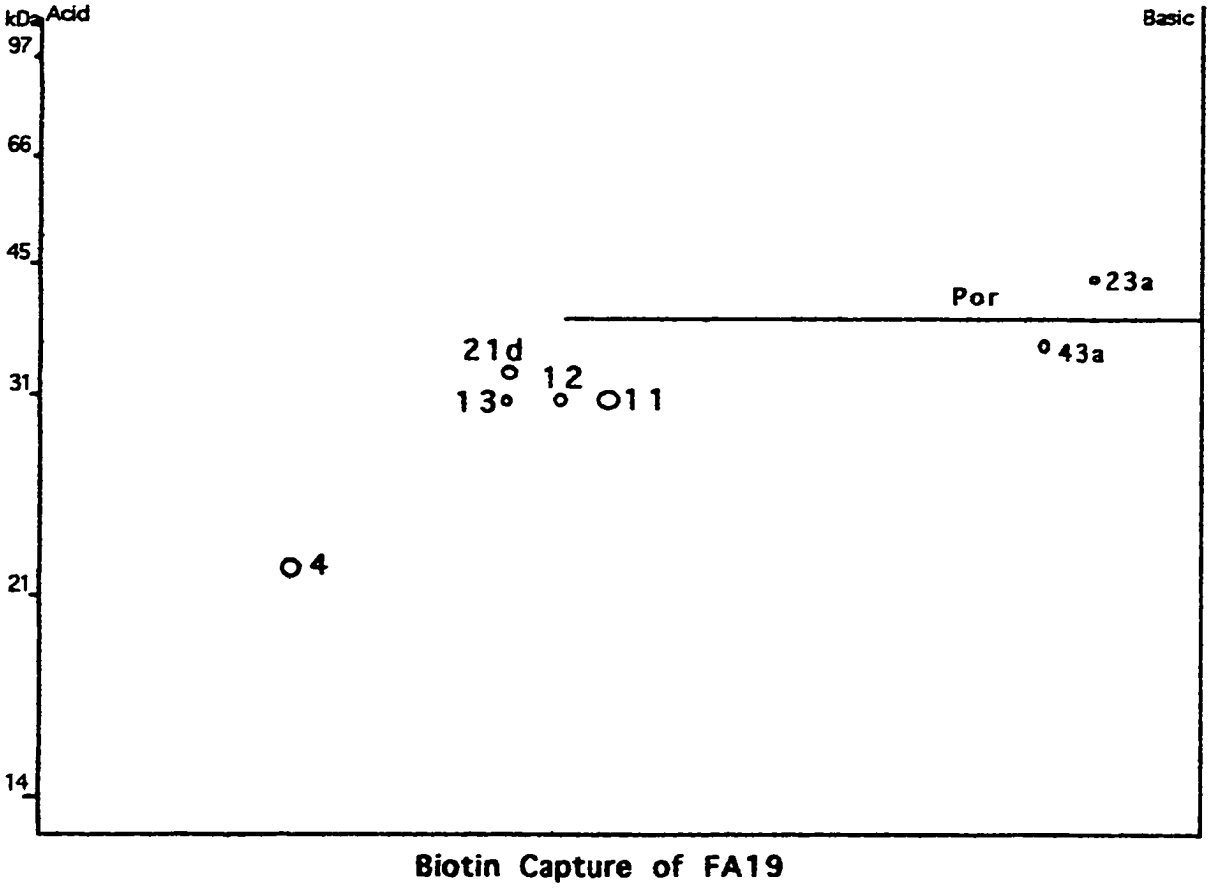


Figure 7. Schematic map of biotin-labeled outer membrane proteins from *N. gonorrhoeae* FA19 captured with streptavidin-agarose. Shown are the locations of labeled proteins from the right panel of Figure 5. Molecular weight markers indicated on left axis are in thousands of Daltons (kDa). Horizontal pH gradient extends from left to right ranging from pH 3 to 10.

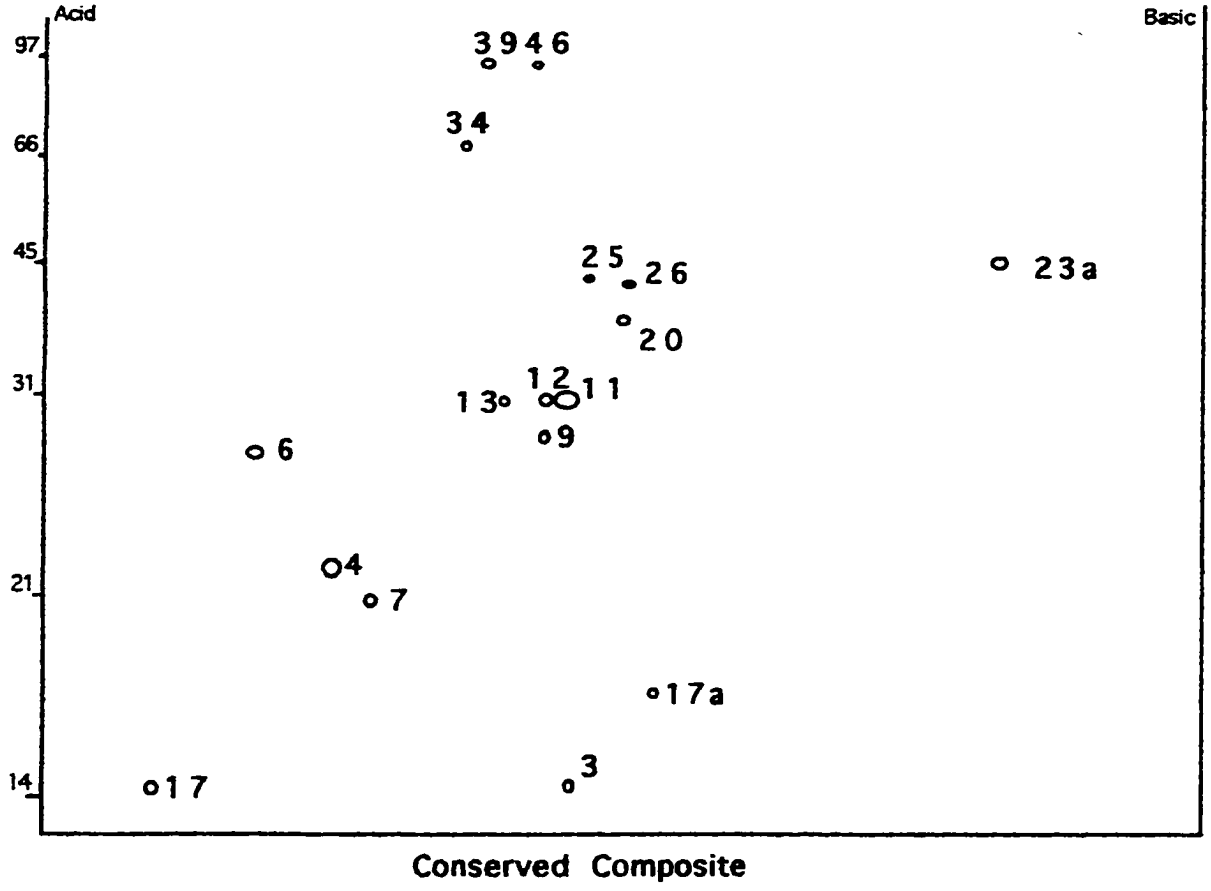


Figure 8. Schematic composite map of outer membrane proteins from *N. gonorrhoeae* FA19 labeled by all three methods of surface protein identification. Shown are the locations of outer membrane proteins found in all three labeling systems separated by two dimensional gel electrophoresis culled from Figures 1, 2, and 5. Molecular weight markers indicated on the left axis are in thousands of Daltons (kDa). Horizontal pH gradient extends from left to right ranging from pH 3 to 10.

Several attempts were made to N-terminally sequence gel purified proteins identified as outer membrane proteins by these methods. Either whole proteins or protein fragments were sequenced. Fragments were generated either by chemical (32) or enzymatic (43) means and separated either by SDS-PAGE (87) or high performance liquid chromatography (43). None of these approaches yielded significant sequence data, presumably for lack of sufficient material and/or N-terminal blockage from oxidation.

A Lambda Zap II (Stratagene) library of *N. gonorrhoeae* FA19 was then prepared and screened with rabbit anti-outer membrane antibody. Several immunoreactive clones ranging in insert product size from approximately 10 kDa to 100 kDa were isolated. Among these, sixteen clones were partially sequenced and a putative identity established by BLAST search (3). Table 3 is a list of these clones together with their identifications.

Of these proteins, the D15 surface protective antigen protein of *H. influenzae* (50), from clone pDR4, was of great interest because of its similarity to outer membrane proteins of four other known bacterial pathogens and because antisera to homologs were protective. These were *E. coli* (17), *Brucella abortus* (10), *Pasteurella multocida* (130), and *Helicobacter pylori* (162). Flack and Thomas discovered that antibodies against the D15 protein in *Haemophilus influenzae* were protective in a rat pup model (159, 49). Further, human convalescent sera to *H. influenzae* was cross reactive to D15 in a wide range of strains tested, making it an attractive candidate for a vaccine component (91). Similarly, specific antibodies to Oma87 of *P. multocida* were shown to be protective in homologous challenge in a mouse model (130). None of the other clones listed in Table 3 were analyzed further and will not be discussed.

Table 3. FA19 Library Clones Identified By Outer Membrane Antiserum

Clones	Significant Homology	Putative MW
pDR4:	Haemophilus surface protective antigen D15 analog	87.5kDa
pDR1.18:	phosphomannomutase	47.3kDa
pDR5L:	translocase for multidrug resistance	51.4kDa
pDR17:	GroEL/chaperonin	57.2kDa
pDR1.2:	RNA polymerase	149.7kDa
pDR3.13/ : pDR3.14	gonococcal cryptic plasmid protein	23.9kDa
pDR3.24:	carbon starvation protein	60.0kDa
pDR22.E.1:	Listeria invasin protein	14.2kDa
pDR27:	lipoprotein carrier	23.1kDa
pDR3.17/: pDR6.2, pDR11, pDR7	inosine-5'-monophosphate dehydrogenase	51.5kDa
pDR1.15	secA translocase	96.3kDa
pDR8:	Salmonella "virulence factor"	57.3kDa
pDR1.19/: pDR2.12, pDR1.5, pDR3.23	catalase	60.0kDa
pDR2.5:	dihydrolipoamide dehydrogenase	51.0kDa
pDR3.7:	leukotoxin	52.8kDa
pDR3.3:	unknown protein	

The full length D15 homolog gonococcal gene was not present in the original pDR4 clone, lacking the final 100 base pairs. Using a downstream primer generated from a contig recently sequenced in the ongoing gonococcal genome sequencing project (41), the full length open reading frame was obtained and cloned into the *Sma*I site of pUP1 (46). The resulting plasmid was termed pOmp85. All PCR primers used and their respective sequences are listed in Table 2. Although pUP1 is a high copy number plasmid, it does not contain a high efficiency *E. coli* promoter contiguous with the multiple cloning site. This allowed suitably moderate expression of the gene.

The entire gene was sequenced (GenBank accession number U81959) and found to contain a typical signal sequence and a consensus terminal phenylalanine residue, indicative of bacterial outer membrane proteins (164, 165, 154). The entire gene encodes a polypeptide having a predicted molecular weight of 87.8 kDa. Removal of this signal peptide yields a mature polypeptide molecular weight of 85.8 kDa. The gene was named *omp85* because of its outer membrane location and its apparent molecular weight of approximately 85 kDa. The gonococcal protein expressed by pOmp85 was designated Omp85. The entire gene sequence is given in Figure 9. A ribosome-binding site sequence preceded the initiation codon of the ORF by seven nucleotides. Potential promoter sequences were not readily apparent. A putative signal sequence cleavage site was identified by the (-3,-1)-rule between the 20 and 21st amino acid (165). The sequencing strategy involved both the subcloning of *Hin*cII fragments into pBluescript and use of the T3 and T7 promoter sequences on the vector, and synthesized primers for those sequences not covered by the available subclones. This strategy is outlined in Figure 10. The entire ORF was sequenced at least twice and most of the DNA was sequenced

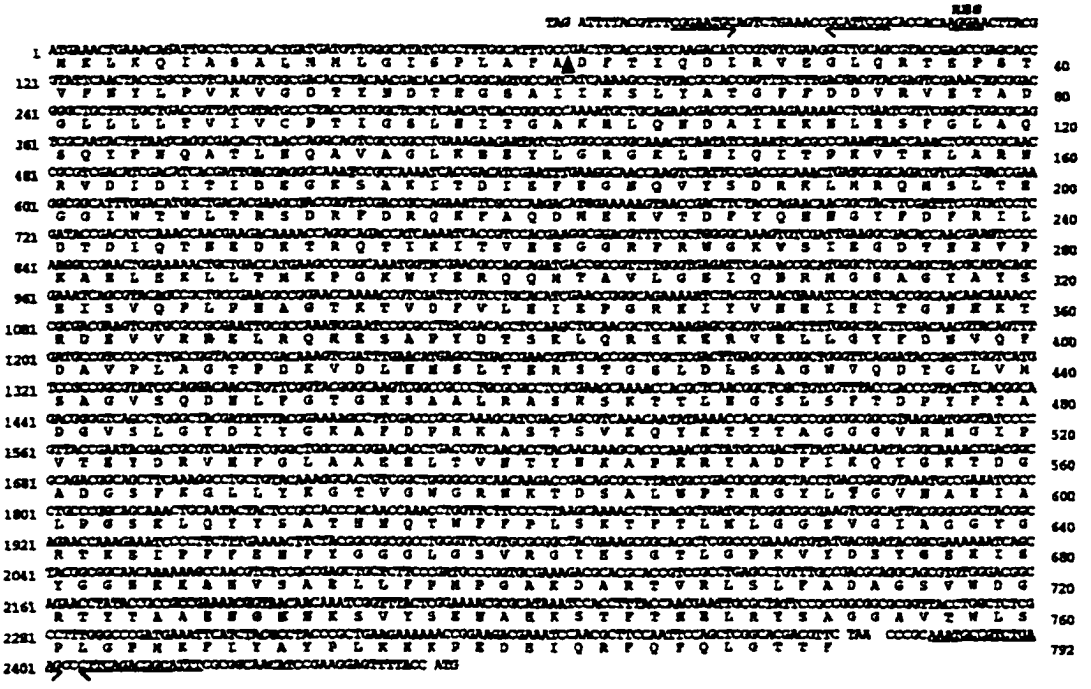


Figure 9. DNA and amino acid sequence of Omp85. There are 2376 base pairs in the open reading frame of *omp85*. The protein consists of 792 amino acids, ending in a phenylalanine residue, similar to D15 and consistent with bacterial outer membrane protein C-termini (154). There is one stop codon (TAA; bp 2379) followed by an underlined rho-independent termination loop. The putative ribsome binding site (RBS) is shown 7 bp upstream of the *omp85* ATG start codon. A putative second stop codon (TAG) and rho-independent termination loop (underlined) is indicated upstream of *omp85*. These are downstream elements of an upstream *yeaL E. coli* analog. Arrowhead indicates putative signal peptide cleavage site (165).

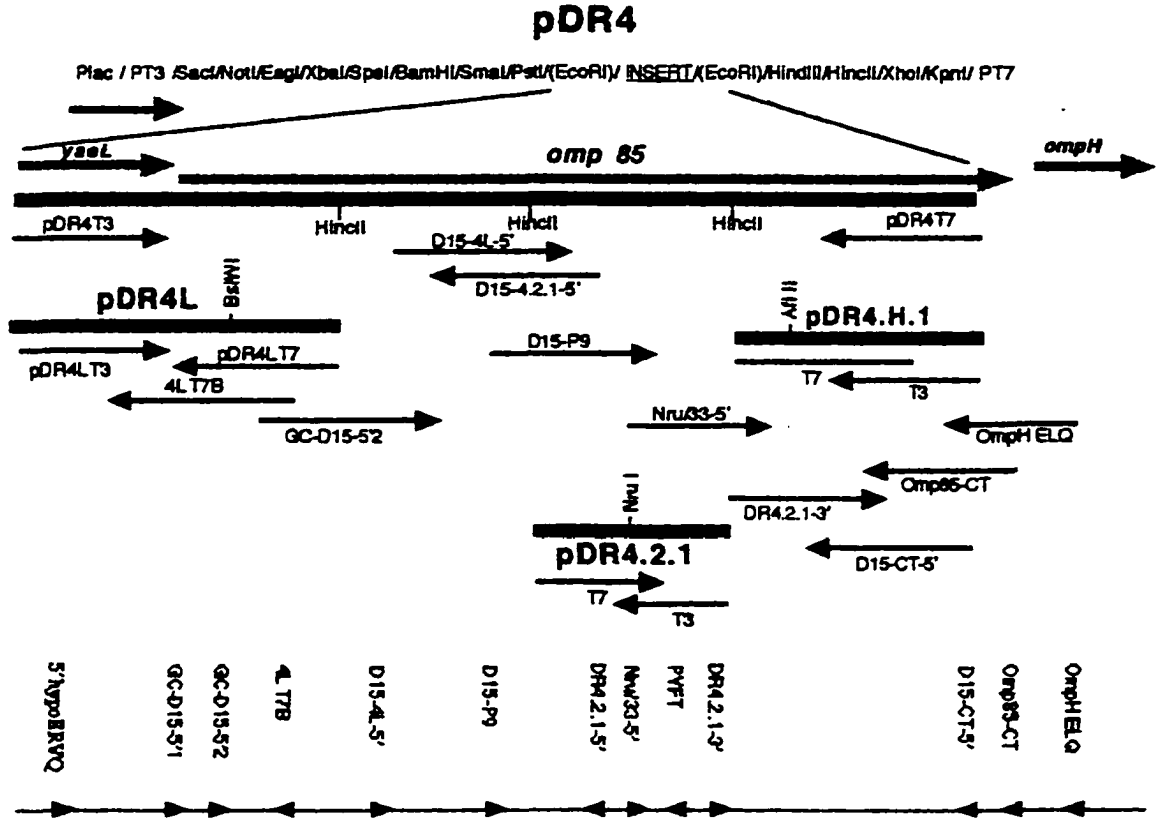


Figure 10. Sequencing strategy for *omp85*. pDR4 contained a 2933 base pair insert in the pBluescript vector rescued from the Lambda Zap II library phage clone. The first 291 base pairs included the 3' end of the *yaeL* gene (17) and the ribosome binding site for *omp85*. The *omp85* open reading frame extends from base pair 292 - 2667. pDR4L, pDR4.2.1, and pDR4.H.1 were subclones of pDR4 generated from *Hin*cII digestions ligated into the *Hin*cII site of pBluescript. Sequencing of these subclones was done from the T7 and T3 promoter sequences found on pBluescript. Arrows indicate direction and approximate extent of sequence per run. Bottom line shows names and directions of synthetic primers used to sequence those sections of the insert not covered by cloned subfragments.

from both strands.

Southern blot analysis of genomic DNA from strain FA19 indicated the presence of the gene as a single copy. Figure 11 showed a single reactive band corresponding to each genomic digest using four different restriction enzymes. In addition, *Cla*I digests of four different strains of *N. meningitidis* also showed a single reactive band to the probe, indicating a single copy of the gene in this closely related species. The meningococcal homolog to *omp85* was then cloned from DNA of strain HH (generously donated by Dr. Zell McGee) into pUP1 (46) using the same PCR primers as with pOmp85. The resulting clone was termed pOmp85MC. The sequence is shown in Figure 12. It was termed *omp85MC* and given the GenBank accession number AF021245. The sequencing strategy is depicted in Figure 13. The meningococcal *omp85MC* gene was comprised of 2391 base pairs and encodes a calculated 797 amino acid polypeptide with a predicted molecular weight of 88.5 kDa. The meningococcal Omp85 was 95% identical and 98% similar (identical plus conserved residues) to gonococcal Omp85 using the MacVector computer algorithm (New Haven, CT). The amino acid region between residues 710 and 730 varied substantially from gonococcal Omp85, including the insertion of five additional amino acids.

omp85 was also found to be present by Southern blot analysis in all other gonococcal strains tested. Figure 14 shows single reactive bands against genomic DNA digests with *Cla*I, indicating a single copy of the gene in eight other gonococcal strains. Figure 15 is another Southern blot of six Neisserial commensal strains, four pathogenic and four non-pathogenic *Escherichia coli* strains showing the presence of a single copy of the homolog to the *omp85* gene. The gene was not found, however, in *E. coli* strain DH5α or in *B.*

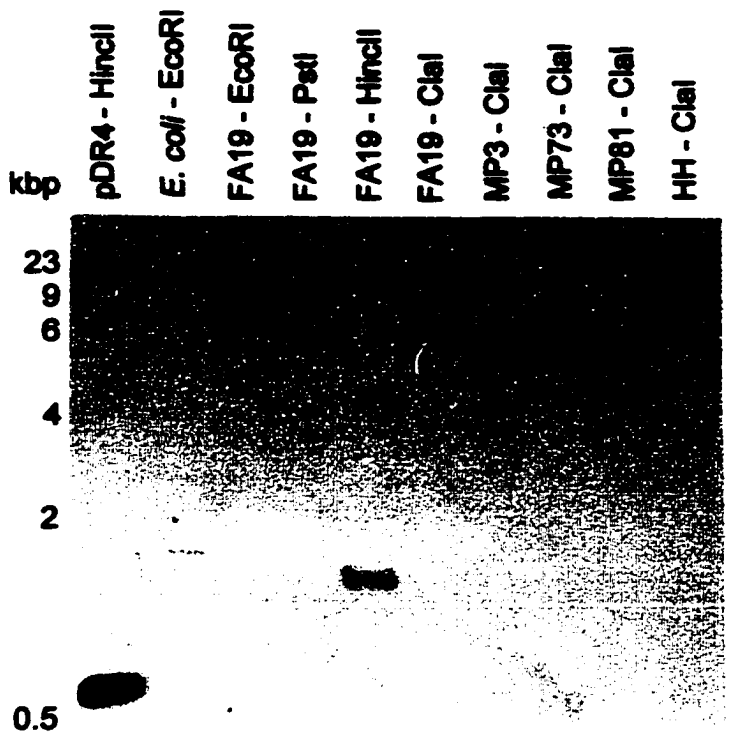


Figure 11. Southern blot analysis of *omp85* in *N. gonorrhoeae* and *N. meningitidis*. Plasmid pDR4 containing *omp85* and chromosomal DNAs from *E. coli* DH5α, *N. gonorrhoeae* strain FA19, and *N. meningitidis* strains MP3, 73, 81, and HH were digested with endonucleases as indicated. pDR4.H.1 was labeled and hybridized under normal stringency conditions. A single hybridizing band is seen in each strain except *E. coli* DH5α, thus a single copy of the *omp85* gene is surmised for each strain. Molecular weight markers are indicated on the left in kilo base pairs (kbp).

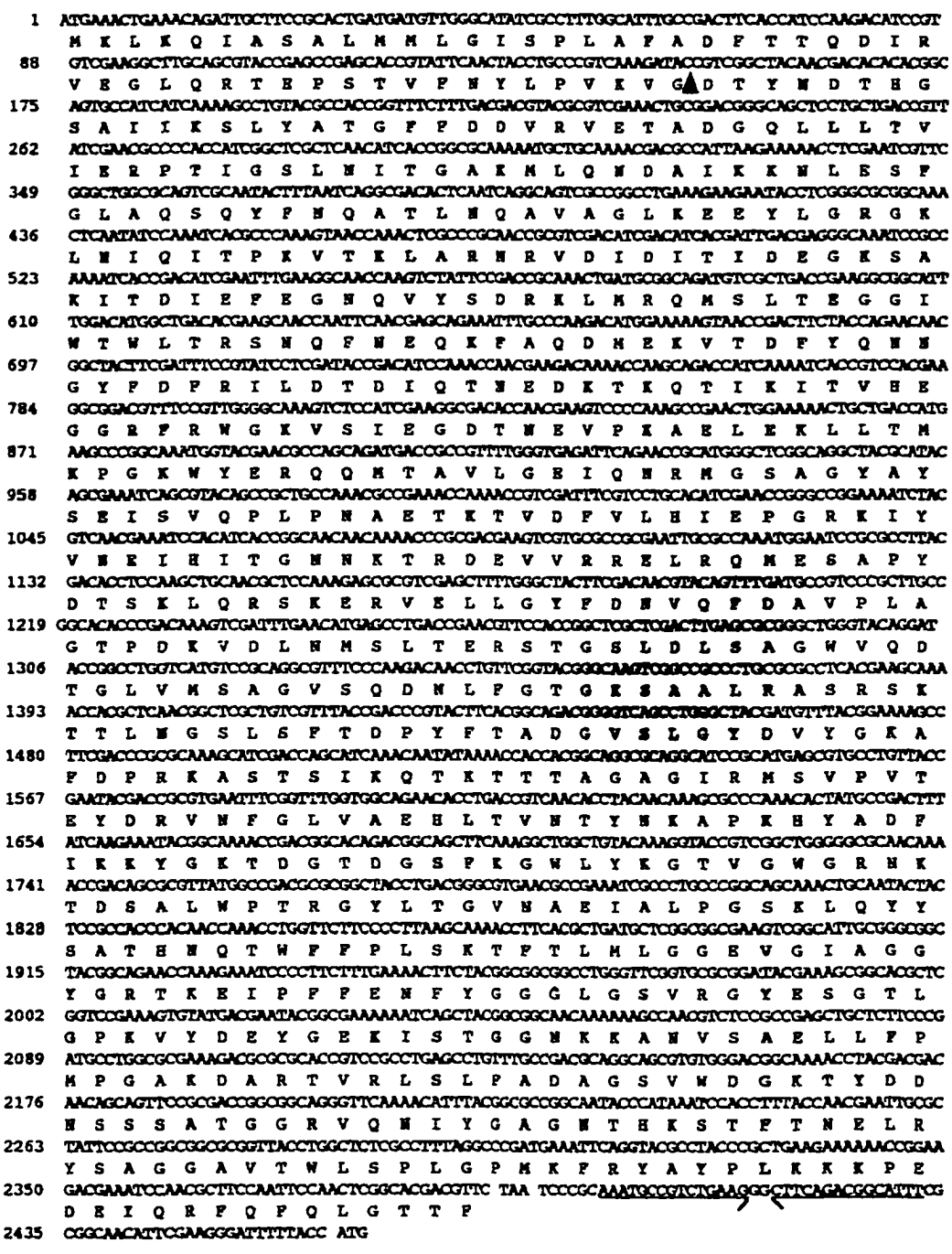


Figure 12. DNA and amino acid sequence of Omp85 MC. There are 2391 base pairs in the open reading frame of *omp85 MC*. The protein consists of 797 amino acids, ending in a phenylalanine residue, similar to Omp85 and consistent with bacterial outer membrane protein C-termini (154). There is one stop codon (TAA) followed by an underlined rho-independent termination loop. The termination loop is identical to Omp85 except for the two intervening base pairs: here is seen two guanosines, while Omp85 contains two cytosine residues. Arrowhead indicates putative signal peptide cleavage site (165).

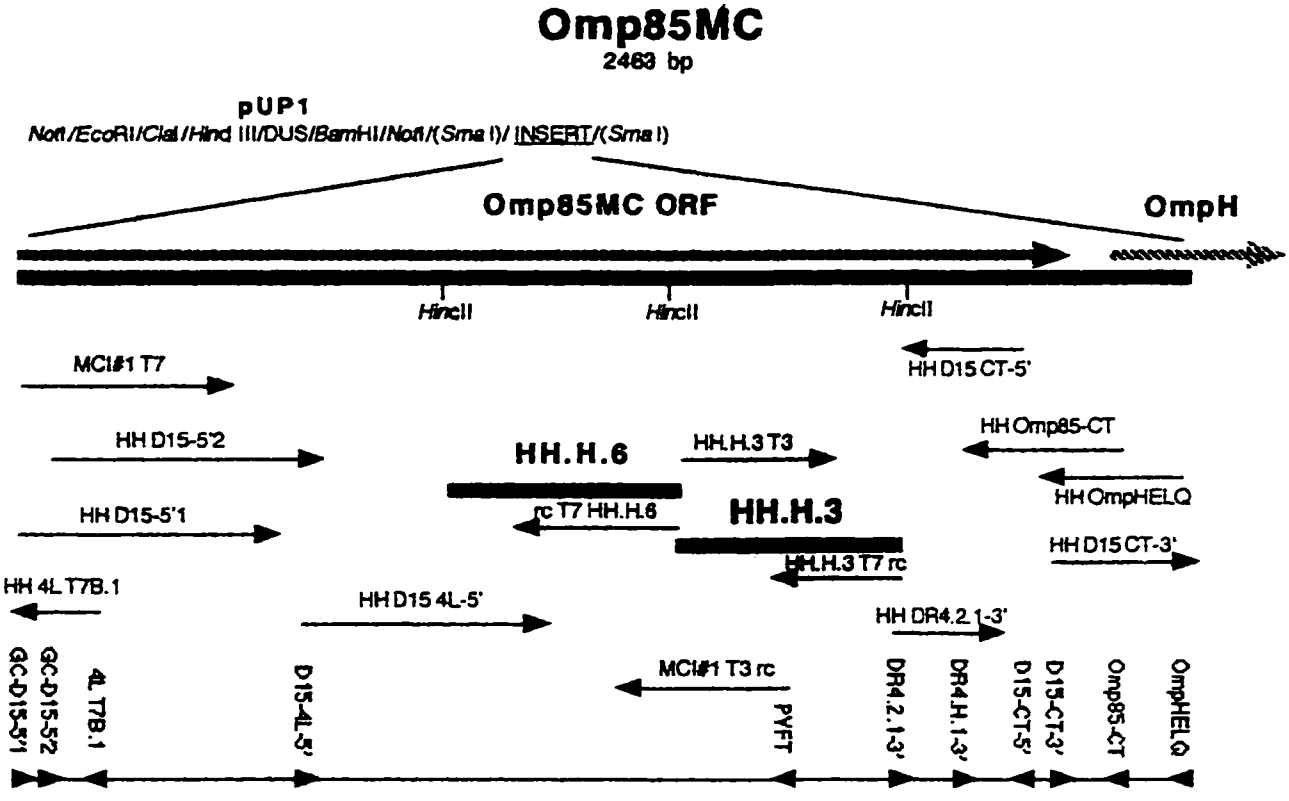


Figure 13. Sequencing strategy of *omp85MC* . pOmp85MC constituted a 2463 bp insert in the pUP1 vector (75) PCR amplified from *N. meningitidis* strain HH (Zell McGee) using primers GC-D15-5'1 and OmpH ELQ (see Table 2). HH.H.6 and HH.H.3 were *Hin*cII digestion fragments of pOmp85MC ligated into pBluescript vector (Stratagene) and sequenced from the universal primers T3 and T7 located on the vector. Arrows indicate direction and approximate extent of sequence obtained per sequence reaction. Bottom line show names and directions of synthetic primers used to sequence those sections of the insert not covered by cloned subfragments.

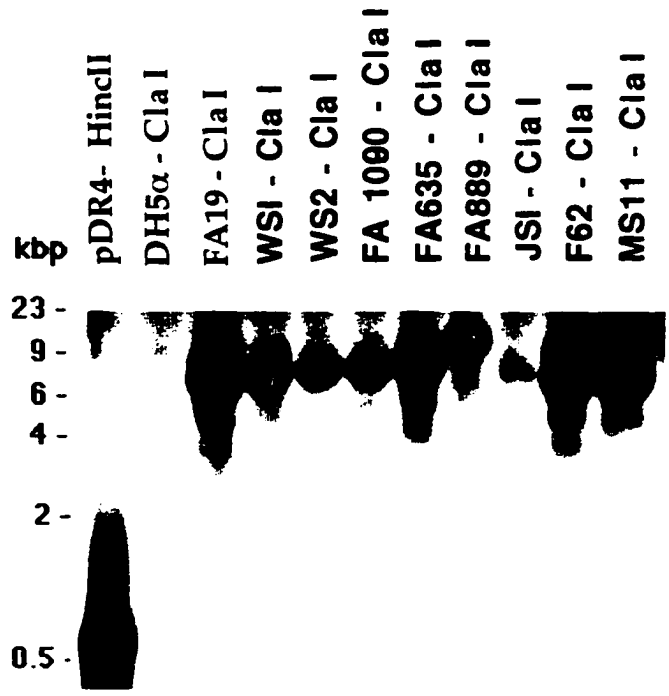


Figure 14. Southern blot analysis of *omp85* in eight other gonococcal strains. Plasmid pDR4 containing *omp85* and chromosomal DNAs from *E. coli* DH5α, gonococcal FA19, and eight other gonococcal strains were digested with endonucleases as indicated. pDR4.H.1 was labeled and hybridized at a single site under normal stringency conditions in each of the strains shown except *E. coli* DH5α. Molecular weight markers are indicated on the left on kilo base pairs (kbp).

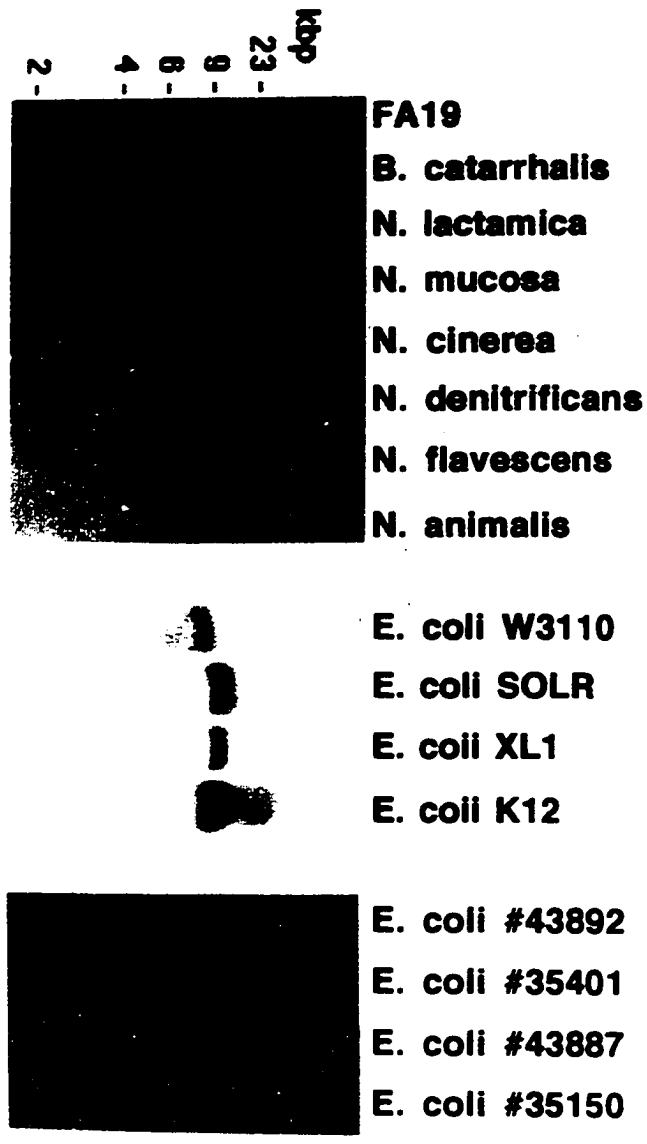


Figure 15. Southern blot analysis of *omp85* in other Neisserial species and *E. coli* strains. *Cla*I digestion of chromosomal DNAs of strains listed were separated in a 1% agarose gel, transferred to nitro cellulose, and probed with labeled pDR4.H.1 under normal stringency conditions. A single hybridizing band appears in all strains shown except *B. (Moraxella) catarrhalis*. A single copy of an *omp85*-type gene is inferred to be present in all these strains except *B. (Moraxella) catarrhalis*. Molecular weight markers are indicated on the left in kilo base pairs (kbp).

(Moraxella) catarrhalis, thus suggesting it may be non-essential.

The GenBank CDS database was then searched (3) for proteins similar to gonococcal Omp85. Identified were the *H. influenzae* D15 protective surface antigen (49), the *P. multocida* Oma87 protein (130), the *Brucella abortus* Omp1 hypothetical protein (GenBank accession #U51683), the *Helicobacter pylori* D15 homolog (162, GenBank accession #AE000579), and an *E. coli* hypothetical protein (17; GenBank accession # U70214). (And most recently *Borrelia burgdorferi* [53]).

The *H. influenzae* D-15-Ag was 31.5% identical and 61.4% similar to gonococcal Omp85. Thomas, et al. (159) demonstrated that The D15 protein was conserved in both typeable and non-typeable strains of *H. influenzae* and reacted with human convalescent sera. They also found that antibodies generated against D15 were protective in the rat pup infection model (161). In addition, Loosmore et al (91) showed that *H. influenzae* serotypes a-f, nontypeable *H. influenzae* and*Haemophilus parainfluenzae* all expressed proteins similar to D15. He also demonstrated that antibodies to recombinant D15 were protective against *H. influenzae* type a and b bacteremia in the infant rat model. The authors claimed that D15 was a universal antigen in *H. influenzae* (91).

The *P. multocida* Oma87 was 31.6% identical and 61.3% similar to gonococcal Omp85. Oma87 was also found to be highly conserved among strains and was recognized by protective antibody. Ruffolo et al (130) demonstrated that it was present in all 16 serotypes of *P. multocida* and was recognized by convalescent animal sera. Antibodies raised to recombinant Oma87 were also protective against homologous challenge in a mouse model.

The *B. abortus* Omp1 hypothetical protein (10) was 24.3% identical and

54.2% similar to gonococcal Omp85. A hypothetical *E. coli* protein was 33% identical and 62% similar to Omp85. And an *H. pylori* putative D15 analog (162) was 21.5% identical and 66.5% similar. Characterization of these last three proteins has not yet been published and the sequences of the last two are from recently completed genome sequencing projects.

A comparison of the seven proteins shown in Figure 16 using the MacVector alignment algorithm shows conserved regions in the C-terminal region and the final Phe residue. The family Neisseriacae is in the beta subdivision of Protobacteria, *H. influenzae, P. multocida,* and *E. coli* are all in the gamma subdivision of Protobacteria, while *B. abortus* and *H. pylori* are in the alpha and epsilon subdivisions, respectively (21). The similarities between the proteins seem to correspond to their respective taxonomic similarities as organisms. A similar variable region between amino acids 710 and 730 was found between Omp85 and Omp85MC. Figure 17 shows the alignment of the conserved 10 amino acid C-terminal outer membrane consensus sequence terminating with a Phe residue. All but *E. coli* have a terminal Phe residue, *E. coli* having a very similar tryptophan.

A known antigenic region in the C-terminal region of Oma87 (Manoha, F., Behar, G., Houlgatte, R., Hellman, U., Wroblewski, H., Delamarche, C. Direct submission; PIR: S47340) shows considerable homology with the various Omp85 analogs as shown in Figure 17A. Identical residues are in capital letters and similar residues are indicated by a "+" sign. It is possible that the C-termini of Omp85 in gonococci and these other organisms are also exposed. This portion of the molecules is also quite homologous by MacVector alignment (Figure 17B).

Expression of Omp85 was observed in western blot analysis in both one

Figure 16. Alignment of Omp85 to Omp85 analogs. MacVector alignment algorithm was used to determine identities (*) and similarities (.) between gonococcal Omp85 amino acid sequences and the amino acid sequences of *Neisseria meningitidis* Omp85MC, *Haemophilus influenzae* D15 (160), *Pasteurella multocida* Oma87 (130), *Escherichia coli* (17), *Brucella abortus* Omp1 (10), and *Helicobacter pylori* (162).

```
                         10          20          30          40          50
N. gonorrhoeae     MKLKQIASAL  MMLGISPLAF  ADFTIQDIRV  EGLQRTEPST  VFNYLPVKVG
    Omp85

1. N. meningitidis MKLKQIASAL  MMLGISPLAF  ADFTIQDIRV  EGLQRTEPST  VFNYLPVKVG
    Omp85 MC       **********  **********  **********  **********  **********
                                             p
                                             |
2. H. influenzae   --mKklliAs  llfGttttvF  AaFvakDIRV  dGvQgdleqq  irasLPVraG
    D15              ****  *   ****.*. .*  *.*. *****  **** .      * . ****.*

3. P. multocida    MK-KlliasL  lfgsttafA-  ApFvvkDIRV  dGvQagtegs  VlatLPVrVG
    Oma87          ** * * ***  *. *.****   * *.******  **** .. **  **. ******

4. E. coli         MK-KlliasL  lfssatvyga  egFvvkDIhf  EGLQRvavga  allsmPVrtG
                   ** * * ***  *. * *  *    .**.*****  *****.. **  .*  ****.*
                                                v
                                                |
5. B. abortus      MsvalvASgt  agfslasvsv  AeaavsrIeV  rGntRvdaqT  irdnidirpG
    Omp1           *.*  ****     *******   ** **  * *   *  *.** *  * * * ** *

6. H. pylori       ----------  ----------  -emkvksIsy  vGLsymsdml  aneivkirVG
                                            *..**.*.    **    .    . * * ****


                         60          70          80          90         100
N. gonorrhoeae     DTYNDTHGSA  FDDVRVETAD  GLLLLTVIVC  PTIGSLNITG  AKMLQNDAIK
    Omp85

1. N. meningitidis DTYNDTHGSA  FDDVRVETAD  GqLLLTVIer  PTIGSLNITG  AKMLQNDAIK
    Omp85 MC       **********  **********  * ******    **********  **********

2. H. influenzae   qrvtDndvan  FDDVkahqeg  dvLvvsVvak  siIsdvkIkG  nsiipteAlK
    D15            *  .*.* *.  *****.* .*  ********.   *.**.***.*  ..**..****

3. P. multocida    qratDndian  yDDVkasreg  ntLvvTVmpk  PvIsnvvIvG  nKsipdeAIK
    Oma87          *  .*.* *.  *****.. .*  . ******    *.**** *.*  .* *.*****

4. E. coli         DTvNDediSn  FeDVRVlrdg  dtLLvqVker  PTIaSitfsG  nKsvkdDmlK
                   ** **.* *.  ******  .*  * *** *     ******.***  .* **** **

5. B. abortus      kaftsadidA  FsDVRihqsg  stLivqVker  svvnnvlfqG  nKkikdpdla
    Omp1           .**..** .*  *.***** **  * *** *     *.*.** * *  .*.*** .*
                                                                y
                                                                |
6. H. pylori       Divdakkidt  FkDVyatfeg  GiLefhfdek  arIagveIkG  gtekekDglK
                   *. *... .*  *.** .. .*  *** *       * ******.*  *.  ******


                        110         120         130         140         150
N. gonorrhoeae     KNLESFGLAQ  SQYFNQATLN  QAVAGLKEEY  LGRGKLNIQI  TPKVTKLARN
    Omp85

1. N. meningitidis KNLESFGLAQ  SQYFNQATLN  QAVAGLKEEY  LGRGKLNIQI  TPKVTKLARN
    Omp85 MC       **********  **********  **********  **********  **********
```

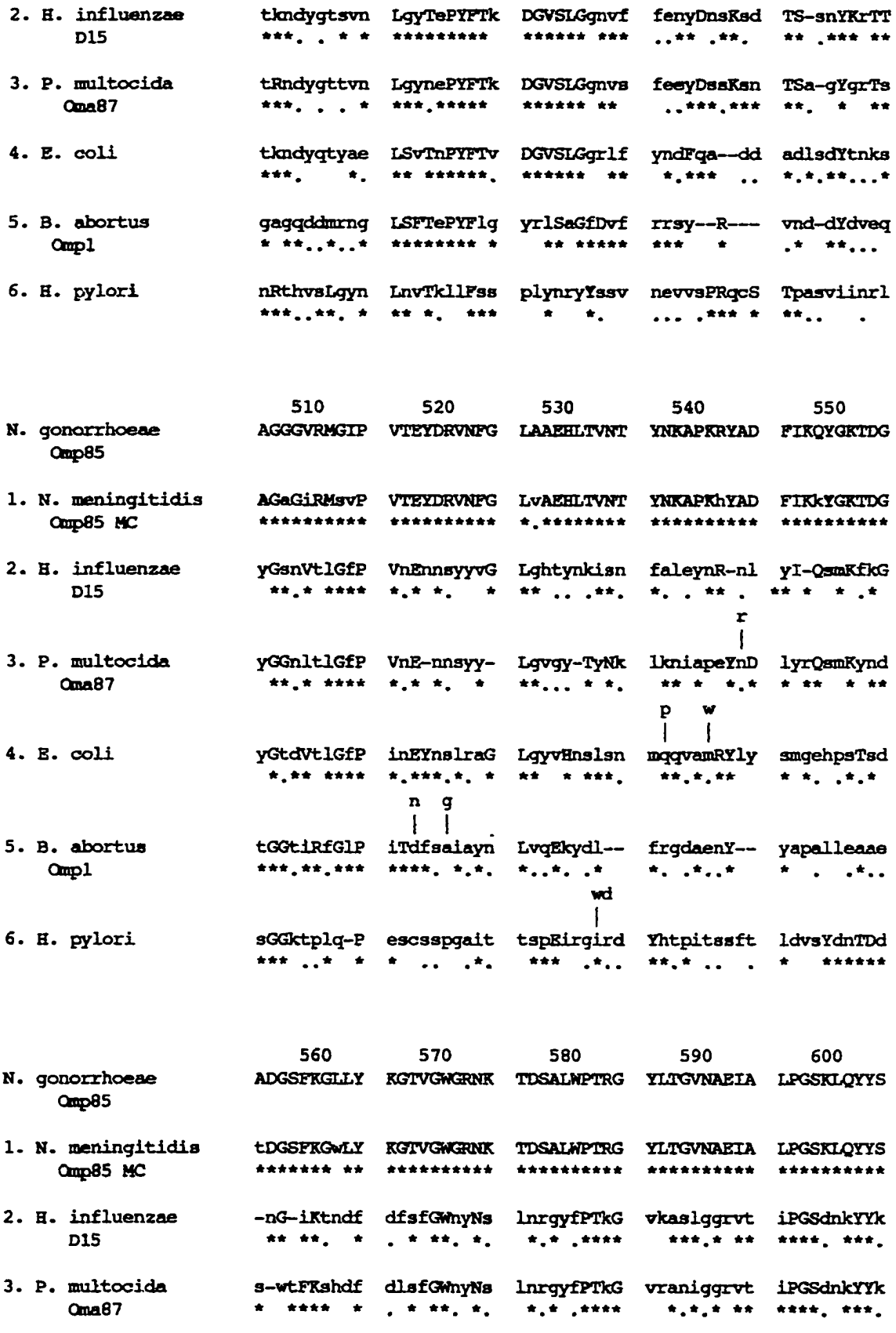
```
2. H. influenzae      qNLdanGfkv  gdvlirekLN  efaksvKEhY  asvGryNatv  ePiVntLpnN
     D15              ***** **    ** * *..**  * . ******  * ** * *  .* *..**.*

3. P. multodicda      qNLdanGfkv  gdvlNrAkLe  efrkGivEhY  nsvGryNakv  daiVntLpnN
     Oma87            ***** **    ** ****.**  *   ** ***  * ** * **  .* *..**.*

4. E. coli            qNLEasGvrv  gesldrtTia  diekGLedfY  ysvGKysasv  kavVTpLpRN
                      ***** **    ** ******.  *   **.* *  * ** * *  .* ** ****

5. B. abortus         pcrq-lk-pr  ap-FdmATme  adkeaiKaaY  shiGrtdatv  nartvdLgqg
     Omp1                * *  **  *. ** ****  .. .***..*    ** * *  .**...***.

6. H. pylori          sqm---Gikk  gdtFdeqkLe  hAktaLKtal  eGqGyygsvv  evrteKvseg
                      .**   ** *  ** ***..**  ** ****..   ***  . *  . *..*** .

                          160         170         180         190         200
N. gonorrhoeae        RVDIDITIDE  GKSAKITDIE  FEGNQVYSDR  KLMRQMSLTE  GGIWTWLTRS
     Omp85

1. N. meningitidis    RVDIDITIDE  GKSAKITDIE  FEGNQVYSDR  KLMRQMSLTE  GGIWTWLTRS
     Omp85 MC         **********  **********  **********  **********  **********

2. H. influenzae      RaeIlIqInE  ddkAKlaslt  FkGNesvSss  tLqeQMeL-q  pdsW-Wklwg
     D15              *.** * ***  *..****.*.  *.***  *..  .*  **.* *  * * * **

3. P. multocida       saeIkIqInE  ddvAlfkeIt  FEGNeafSsg  KLadQMeL-q  tdsW-Wklfg
     Oma87            ..**.* ***  *. * *.**.  *****.**.  **  **.* *  .* * *  *

4. E. coli            RVDlklvfqE  GvSAeIqqIn  ivGNhafttd  eLishfqLrd  evpW-Wnvvg
                      ****.*.***  * **.* ***  * ***.**.  .**.*. * *  .  * * . *

5. B. abortus         RVnvvyeInE  GsrtKIanIE  FvGNQafSqR  rLrdviStkr  snplsWLTRn
     Omp1             ****  .***  *..*******  * ***.****  **.  ** .  *.  ******
                                                          es
                                                          |
6. H. pylori          allIvfdvnr  GdSiyIkqsi  yEGsaklkrR  milsankqrd  fmgWmWglnd
                       * * *.**   *.*  *.*    ****.  . *  .**.. . *    * *  ..

                          210         220         230         240         250
N. gonorrhoeae        DRFDRQKFAQ  DMEKVTDFYQ  NNGYFDFRIL  DTDIQTNEDK  TRQTIKITVH
     Omp85

1. N. meningitidis    nqFneQKFAQ  DMEKVTDFYQ  NNGYFDFRIL  DTDIQTNEDK  TRQTIKITVH
     Omp85 MC         **********  **********  **********  **********  **********

2. H. influenzae      nkFegaqFek  DlqsirDyYl  NNGYakaqIt  kTDvQlNdeK  TkvnvtIdVn
     D15              **** .**.*  ***.* ***  **** . **  .**** ****  ** .*.*.**

3. P. multocida       nkFDqtqFnk  DlEtlrsyYl  drGYaqFqIL  DTDvklsdDK  -kepclIs-e
     Oma87            ***** **.*  ***.* .**  *.** *****  ***** ****  **.  ** *
                       k
                       |
4. E. coli            DRyqkQKlAg  DlEtlrsyYl  drGYarFnId  sTqvsltpDK  kgiyvtvnit
                      *********   ***.* .**  *.**  *.*  .***  . **  .  *.*.*
```

```
                                                                        avld         w
                                                                         |           |
5. B. abortus          DvyDegrlqa  DeEtlrrFYy  NrGYaDFRvL  ssnpsTNEyr  sRspmKvrVi
   Ompl                * **  **..  * *.*  **   *.** *****  .**  *** *  ** .*** *

6. H. pylori           gklrldqley  DsmriqDvYm  rrGYlDahIs  spflkTdfst  hdaklhykVk
                       ***  ***.   *  ** * *  ..**** **   .. **** ..    ..*. .*.


                          260         270         280         290         300
N. gonorrhoeae         EGGRFRWGKV  SIEGDTNEVP  KAELEKLLTM  KPGKWYERQQ  MTAVLGEIQN
   Omp85

1. N. meningitidis     EGGRFRWGKV  SIEGDTNEVP  KAELEKLLTM  KPGKWYERQQ  MTAVLGEIQN
   Omp85 MC           **********  **********  **********  **********  **********
                                                   s
                                                   |
2. H. influenzae       EGlqydlrsa  rIiGnlggms  -AELEpLLal  hlndtfrRsd  iadVenaIka
   D15                 ** **   ..  .* ** ..**   **** ****  . .. * * *  **.* ..**.
                                                   e
                                                   |
3. P. multocida        EGdlyt-vKt  rvsGgmwggm  sAELapiLTi  qlnglfrRts  vleVeqrnks
   Oma87               *** *   *.  .*.**    .  .***. ****  * .   * *   * .*    **

4. E. coli             EGdqyklsgV  evsGnlag-h  sAEiEqLtki  ePGelYngtk  vTkmeddIkk
                       ****** * *  .*.** .. .  .****** .*  .**. **  *  ** * *****

5. B. abortus          pfGRckrGKq  plm--vs-ir  rrlivwsrpv  rasltsakei  edsVLs-vte
   Ompl                ** ****   **   .* *.  *  *    .*  ***    .**   .**** * *

6. H. pylori           EGiqyRisdi  lIEiDnpvVP  lktLEKaLkv  Krkdvfnieh  lrAdaqilkt
                       ** *** *.*   ** *.  **    .*** *.*  *.  . ** **  * *    **.


                          310         320         330         340         350
N. gonorrhoeae         RMGSAGYAYS  EISVQPLPNA  GTKTVDFVLH  IEPGRKIYVN  EIHITGNNKT
   Omp85

1. N. meningiditis     RMGSAGYAYS  EISVQPLPNA  GTKTVDFVLH  IEPGRKIYVN  EIHITGNNKT
   Omp85 MC           **********  **********  **********  **********  **********

2. H. influenzae       klGerGYgsa  tvnsvPdfdd  anKTlaitLv  vdaGRrltVr  qlrfeGNtvs
   D15                 ***. *** *  .**  *  *.  *.***.*.*   ******* *  ****.**. *

3. P. multocida        klGerGYAta  qvnVhPtfde  qdKTisldfi  vEaGksytVr  qIrfeGNtss
   Oma87               ***. *** *  ****** *.    .***.* *   *****.  *.  ****.**..*

4. E. coli             llGryGYAYp  rvqsmPeiNd  adKTVklrvn  vdaGnrfYVr  kIrfeGNdts
                       **. *****   *   *  *.   *.***.* **  ****.****.  .***.***.*

5. B. abortus          svagsGYAfa  kveprgdrNf  enhTisvVys  vdqGpraYiq  rIeIrGNdKT
   Ompl                .*********  .*. *  .*   ...**. *    **.*.* ***   *** *****
                                                  l
                                                  |
6. H. pylori           eiadkGYAfa  vvkpd-Ldkd  ekngVkviyr  IEvGdmvYiN  dviIsGNqrT
                        **. *****   *. * * *.  ..*.*. * *  ** * .****  ** *******
```

```
                          360        370        380        390        400
N. gonorrhoeae       RDEVVRRELR QMESAPYDTS KLQRSKERVE LLGYFDNVQF DAVPLAGTPD
     Omp85

1. N. meningitidis   RDEVVRRELR QMESAPYDTS KLQRSKERVE LLGYFDNVQF DAVPLAGTPD
     Omp85 MC       ********** ********** ********** ********** **********

2. H. influenzae     aDstlRqEmR QqEgtwYnsq lvelgKiRld rtGfFetVen ridPinGsnD
     D15             *..****** * *** *** ** ** *** ****.** **.** *

3. P. multocida      aDstlRqEmR QqEgAwlsse lvelgKlRld rtGYFesVet kteaipG-sD
     Oma87           *..****** * *** .*. ** ** *** ******* .* **** **

4. E. coli           kDaVlRREmR QMEgAwlgsd lvdqgKERln rLGfFetVdt DtqrvpGsPD
                     **.******* ***** **. ********* *****.** ** .******

5. B. abortus        RDyViRREfd lnEgdafnqv mvQRaKrRlE aLdfFqtVni staP-gsdPD
     Omp1            ** ****** **.*** .***** *** *****.*** .*.* **.**

6. H. pylori         sDriiRRELl lgpkdkYnlt KLrnSenslr rLGfFskVki eekrvnssl-
                     .* ****** .. ** * ***.*.*.* ****.**** *. .*.**
```

```
                          410        420        430        440        450
N. gonorrhoeae       KVDLNMSLTE RSTGSLDLSA GWVQDTGLVM SAGVSQDNLF GTGKSAALRA
     Omp85

1. N. meningitidis   KVDLNMSLTE RSTGSLDLSA GWVQDTGLVM SAGVSQDNLF GTGKSAALRA
     Omp85 MC       ********** ********** ********** ********** **********

2. H. influenzae     eVDvvykvkE RnTGSinfgi GygtesGisy qAsVkQDNfl GTGaavsiag
     D15             .*** .*.* ********* *. **** ***.***** *** *.** *

3. P. multocida      qVDviykvkE RnTGSinfgi GygtesGLsy qAsikQDNfl GmGsSisLgg
     Oma87           **** .*.* ********* *. **** ***.***** * *.* ** *

4. E. coli           qVDvvykvkE RnTGSfnfgi GygtesGvsf qAGVqQDNwl GTGyavging
                     **** .*.* ********* *. **** . *** *** * *** *.**.*
                                            ttg
                                            |
5. B. abortus        qViLvvdvvE kSTGefsigg GygespGaqv eAaiterNfl GrGqyirisA
     Omp1            ** * *.*.* ****.*.*** *. *..* * .***** *** * **   *.*

  (yaniatg ggrsypgmpk gagrmfagnl sltnprifds wysstinlya dyrisyqyiq qgggfgvnvg rmlg)
                                                                        |
6. H. pylori         -mDLlvSveE grTGqLqfgl GygsygGLml ngsVSerNLF GTGqSmsL--
                      *** ***.* .** **** *. .**** ****** *** ***** **
```

```
                          460        470        480        490        500
N. gonorrhoeae       SRSKTTLNGS LSFTDPYFTA DGVSLGYDIY GKAFDPRKAS TSVKQYKTTT
     Omp85

1. N. meningitidis   SRSKTTLNGS LSFTDPYFTA DGVSLGYDvY GKAFDPRKAS TSiKQYKTTT
     Omp85 MC       ********** ********** ********** ********** **********
```



```
4. E. coli          qDnSFKtddf  tfnyGWtyNK  lDrgyfPTdG  srvnltgkvt  iPGSdneYYk
                    .*.***.  *  . . **. **  *.* .** *    ..*.*.**  ****. ***.

5. B. abortus       nspwlrssv-  sysltys-si  dDikn-PhdG  lygkfiqEfA  glGgdakYvk
   Omp1             ..  *** *  . **..* *  .*    * *    .   .***   **. ** .

6. H. pylori        yyfprnGvif  ssyatmsglp  ssgtL-nswn  gLgGnvrntk  vyG-KfaaY-
                      * *****  .* .. *     *.*** **.  *.*   *.   * * **. *


                        610         620         630         640         650
N. gonorrhoeae      ATHNQIWFFP  LSKTFTLMLG  GEVGIAGGYG  RTKEIPFFEN  FYGGGLGSVR
   Omp85

1. N. meningitidis  ATHNQIWFFP  LSKTFTLMLG  GEVGIAGGYG  RTKEIPFFEN  FYGGGLGSVR
   Omp85 MC        **********  **********  **********  **********  **********
                                           ka
                                           |
2. H. influenzae    lsadvqgFyP  Ldrdhlwvvs  asaGyAnGfG  -nKrlPFyqt  ytaGGiGSlR
      D15           * *   ***  *.*.   ***  *..* *.***  .* *****.  * ********
                                            sa
                                            |
3. P. multocida     lnaeaqgFyP  LdrehgwvLs  srisfAdGfs  -gKrlPFyqy  ysaGGiGSlR
   Oma87             . *.  ***  *.*. . ***  * ********  .* *****   * ********
                                 w           w
                                 |           |
4. E. coli          vTldtatyvP  idddhkvvLG  rtrGygdGlG  -gKEmPFyEN  FYaGGsstVR
                    .* * * * *  *... .****   . * *** *  .********  ***** ****
                                 ea
                                 |
5. B. abortus       tTfkgnyyqt  LSqdivglLG  vgaGyihefG  -ddgvriFd-  lfknssdiiR
   Omp1             ** * ..* .  ***.*. ***  ..*   .**  ...*.***   ** .* * **

6. H. pylori        -hHlQk-yl-  Li-dliarfk  tqgGyifrYn  tddylPlnst  FYmGGvttVR
                      * *. **   *  .*. .*   .* *   *.   .. *** ..  ** ***.***


                        660         670         680         690         700
N. gonorrhoeae      GYESGTLGPK  VYDEYGEKIS  YGGNKKANVS  AELLFPMPGA  KDARTVRLSL
   Omp85

1. N. meningitidis  GYESGTLGPK  VYDEYGEKIS  YGGNKKANVS  AELLFPMPGA  KDARTVRLSL
   Omp85 MC        **********  **********  **********  **********  **********
                                    fk
                                    |
                                a   gngn    dvi                 fv
                                |   | |     |                   |
2. H. influenzae    GfayGsiGPn  iYaEhGtKIS  sGGNaittaS  AELivPtPsd  KsqnTVRtSL
      D15           **. ******  **.*.*.***   ***  *..*  **** * **.  *...*** **
                                   cl
                                   |
                                a  rtrqcp                       fv
                                |  ||                          |
3. P. multocida     GfayGaiGPn  iYDsYvssdv  iGGNamvtaS  tELivPtPad  KnqnsVRtSL
   Oma87            **. ******  ***.* ..     *** ....*  **** * **.  **..*** **
                                (fph qasnydpdyd yecatqdgak)
```

```
                                        a  |
                                        |  |
4. E. coli              GfqSnTiGPK   VYDlcksdda  vGGNamAvaS  lEfitPtPsd  KyAnsVRtSf
                        ****.*****   *** . .. *   *** .* .*   *** * **.  * *.*** **

5. B. abortus           GfkfngiGPy   qdaknGkryw  mGGttyfsgt  AEvqFPMPvl  peslgVRgaf
     Omp1               **. ..***      .. *.*    **..  * *  *** ****     ** .** **

6. H. pylori            GfrnGsvtPK   --DEfG--lw  lGGdgiftaS  tELsygvlkA  akmRlawfgf
                        ** ****.**     ****  *    ***   ..*  *** * *  *   . * .****
```

```
                          710          720          730          740          750
N. gonorrhoeae          FADAGSVWDG   RTYTAAENGN   NKSVYSENAH   KSTFTNELRY   SAGGAVTWLS
     Omp85                           ddns         g
                                     |            |
1. N. meningitidis      FADAGSVWDG   kTYssAtgGr   vqniYagNtH   KSTFTNELRY   SAGGAVTWLS
     Omp85 MC           **********   ******..*.   *****.***   **********   **********

2. H. influenzae        FvDAaSVWnt   k-wksdksGl   dnnVlkslpd   ygk-ssriRa   StGvgfqWqS
       D15               *.*******.   * ..*..**    **** .. **    *. ** **   *** *  * *

3. P. multocida         FvDAaSVWnt   R-wkAedkak   fakl-nvpdy   -Sd-psrvRa   SAGvAlqWqS
       Oma87             *.*******.   * ..*.****    .* *  ..    *. .* **   *** ** * *

4. E. coli              FwDmGtVWDt   -nw---dssq   y-SqYpd--y   -Sd-psniRm   SAGiAlqWmS
                        * * *****.     ..   ****     * ***  .    *. .****   *** ** ***

5. B. abortus           FADAatly-G   nd-TpdisGd   ----------   ----dKklRa   SvGvslmWaS
     Omp1               *******. *   .. **. ***                    .*.**   *.* ** * *

6. H. pylori            ltfktptrgs   ffYnApvtta   NfkdYgviga   gfe-ratwRa   StGlqieWiS
                        **  .*.***     *.** ...   * . **  *     .  .. *   *** .*.***
```

```
                          760          770          780
N. gonorrhoeae          PLGPMKFIYP   YPLKKKPEDE   IQRFQFQLGT   TF
     Omp85

1. N. meningitidis      PLGPMKFrYa   YPLKKKPEDE   IQRFQFQLGT   TF
     Omp85 MC           ******* **   **********   **********   **

2. H. influenzae        PiGPlvFsYa   kPiKKyenDd   veqFQFsiGg   sF
       D15               ***** * **    ****  ***   ****** **.   **

3. P. multocida         PiGPlvFsYa   kPLKKyqgDE   IeqFQFsiGg   TF
       Oma87             ***** * **    **** ..**   ****** **.   **

4. E. coli              PLGPlvFsYa   qPfKKydgDk   aeqFQFniGk   Tw
                        ***** * **    ****  .*.    ********.   **

5. B. abortus           PfGPlrFdYa   fPvaKadtDk   vQnFnFgvst   kF
     Omp1               ******* **   *** *  .*.   **.*** ***   .*

6. H. pylori            PmGPlvlIfP   iaf
                        ***** ****    **
```

```
P. mul.   GVALQWQSPIGPLVFSYAKPLKKYQGDEIEQFQFSIGGTF
H. inf.   GV  QWQSPIGPLVFSYAKP+KKY+ D++EQFQFSIGG+F
E. coli.  G+ALQW SP+GP+ F YA PLKK   DEI++FQF +G TF
N. gon.   G A+ W SP+GP+ F YA PLKK   DEI++FQF +G TF
N. men.   G A+ W SP+GP+ F YA PLKK   DEI++FQF +G TF
B. abo.   GV+L W SP GPL F YA P+ K   D+++ F F +   F
H. pyl.   G+ ++W SP+GPLV  +         GD  + F+FS+G  F
```

Figure 17A. Homology to *P. multocida* Oma87 antigenic C-terminus with Omp85 and Omp85 homologs. Identical amino acids are lettered and similar residues are marked with +. Organisms listed in order of similarity to Oma87 C-terminus of *P. multocida* using MacVector alignment algorithm.

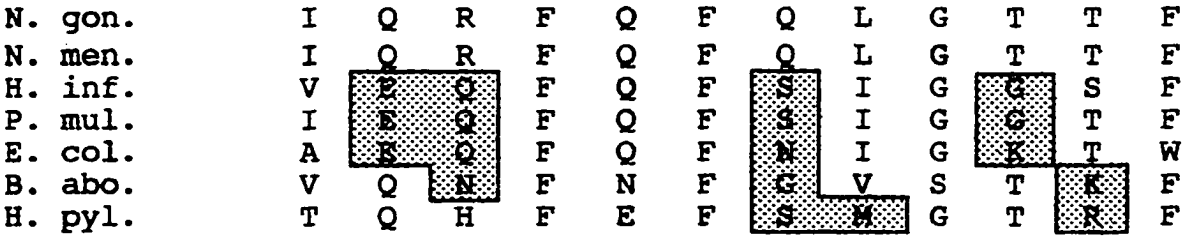


Figure 17B. Alignment of last twelve amino acid residues of Omp85 using MacVector alignment algorithm to Omp85 homologs. Dark stippled area shows dissimilarities, all other areas are similar or identical.

and two dimensions. Figure 18 shows Omp85 expression in *E. coli* DH5α via the pOmp85 plasmid. No expression of the protein was observed in *E. coli* DH5α alone. Adjacent is shown the same cell lysates on SDS-PAGE. In addition, Omp85 expression was observed in all other gonococcal strains tested as well as in several meningococcal strains (Figure 19). Figure 20 shows a two dimensional blot of gonococcal strain MS11 using antisera from Omp85 fusion protein, locating the specific position of Omp85. Figure 21 shows a western blot of several gonococcal and meningococcal strains using the same fusion antibody to show its specificity. The numbering system of Figures 2, 4, and 6, identify Omp85 as protein number 46.

If Omp85 were required for mucosal colonization, it was hypothesized that commensal mucosal Neisserial species would harbor the gene. Thus several Neisserial commensal strains were tested for the expression of Omp85. Figure 22 shows the results of this survey. All species tested showed evidence of a reactive protein in the 85 kDa range. The molecular weights of these Omp85 analogs varied slightly in molecular weight. Interestingly, *B. (Moraxella) catarrhalis,* a species demonstrated to be closely related to the Neisseria genus by 16S rDNA analysis (127), showed no expression of Omp85. To assay the extent of Omp85 expression in the Gram-negative bacteria, a western blot was performed using whole cell lysates of various pathogenic *E. coli* strains as well as *Salmonella typhimurium* and *Shigella flexneri.* As shown in Figure 23, all *E. coli* and Enterobacteriaceae strains failed to react with the Omp85 antiserum.

This is an interesting result when compared with the Southern data previously mentioned which showed the presence of the homolog in all *E. coli* strains tested, with the exception of DH5α. These data either suggest that

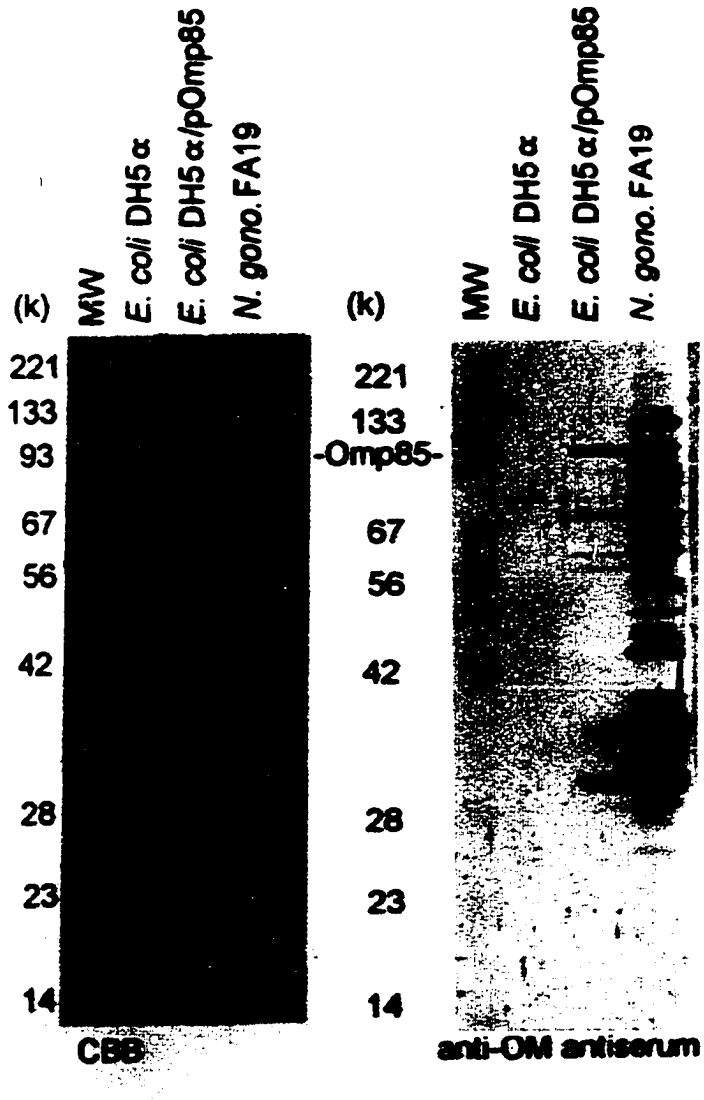


Figure 18. Western blot analysis of Omp85. Cell lysates of *E. coli* DH5α, *E. coli* DH5α with pOmp85 plasmid, and *N. gonorrhoeae* strain FA19 were separated by SDS-PAGE (12.5%)(left panel) blotted to PVDF and probed with antibody to outer membrane fraction of *N. gonorrhoeae* FA19 (right panel). Omp85 position is indicated in the center column. Molecular weight markers are in thousands of Daltons (k).

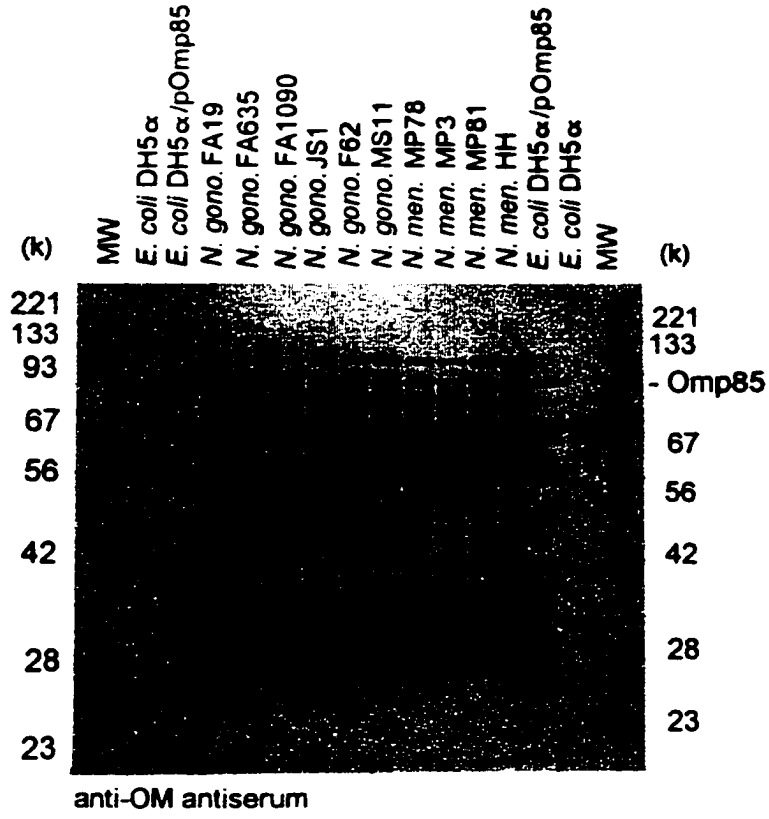


Figure 19. Western blot of Omp85 in other gonococcal sp. and in clinical isolates of *N. meningitidis*. Cell lysates of *E. coli*, *E. coli* expressing Omp85, and several strains of both *N. gonorrhoeae* and *N. meningitidis* were separated by SDS-PAGE (12.5%) and blotted onto PVDF. Rabbit antibody to outer membrane fractions of *N. gonorrhoeae* FA19 was used to indicate the presence of Omp85 in all strains tested except *E. coli* DH5α. The position of Omp85 is indicated on the right. Molecular weight markers are indicated in thousands of Daltons (k).

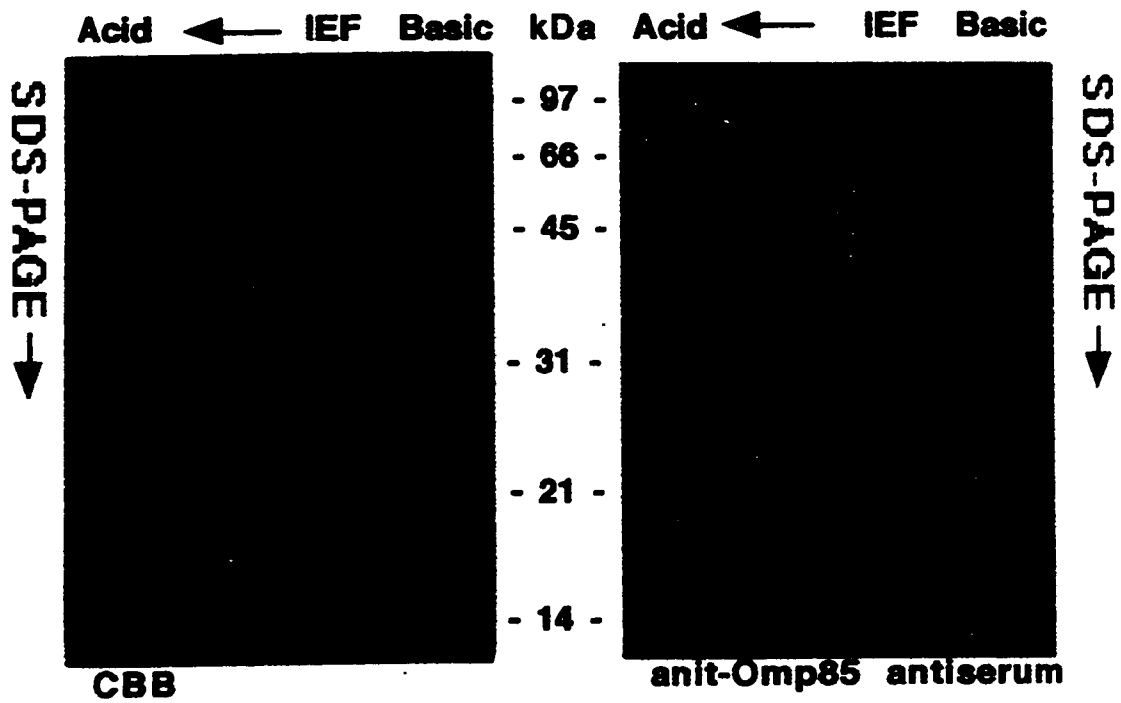


Figure 20. Two dimensional western blot of Omp85 in *N. gonorrhoeae* strain MS11. Cell lysates of MS11 were prepared as described in Materials and Methods and the proteins separated in two dimensions. The left panel is stained with Coomassie Brilliant Blue and the right panel is a western blot of the same gel probed with antibody generated against Omp85 fusion protein. Small arrow indicates the location of Omp85. Molecular weight markers are in thousands of Daltons (kDa). Horizontal pH gradient extends from left to right in each gel, ranging from pH 3 to 10.

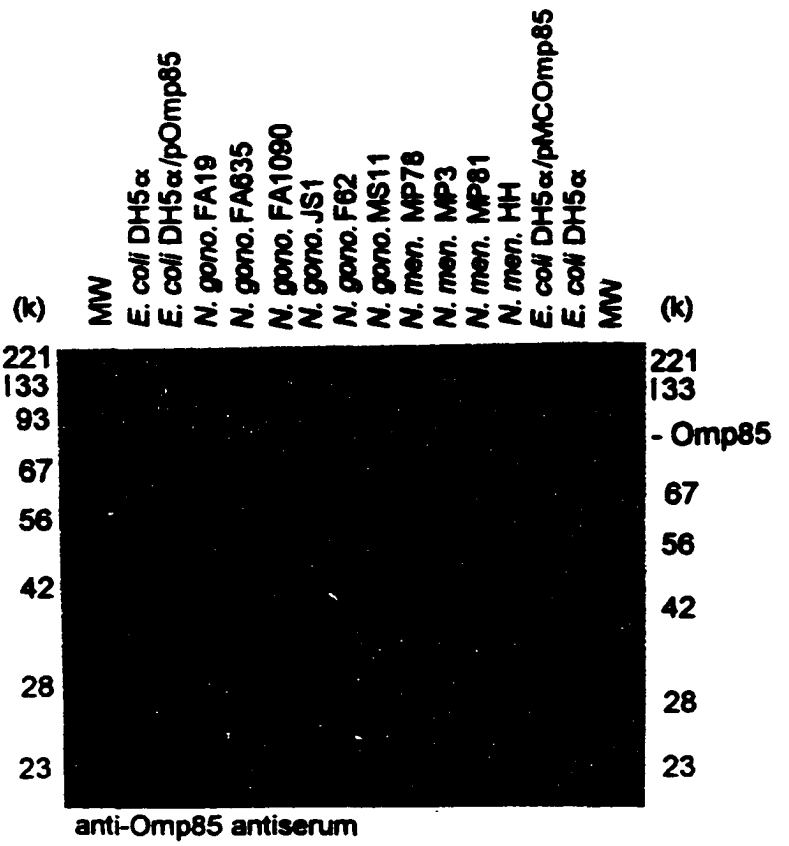


Figure 21. Western blot analysis using fusion protein-generated antibody. Cell lysates of *E. coli* DH5α, *E. coli* DH5α expressing Omp85 and Omp85 MC, and various gonococcal and meningococcal strains were separated by SDS-PAGE (12.5%) and transferred to PVDF. Rabbit antibody to Omp85 fusion protein reacted with Omp85 proteins as indicated on right. Molecular weight markers are indicated on either side in thousands of Daltons (k).

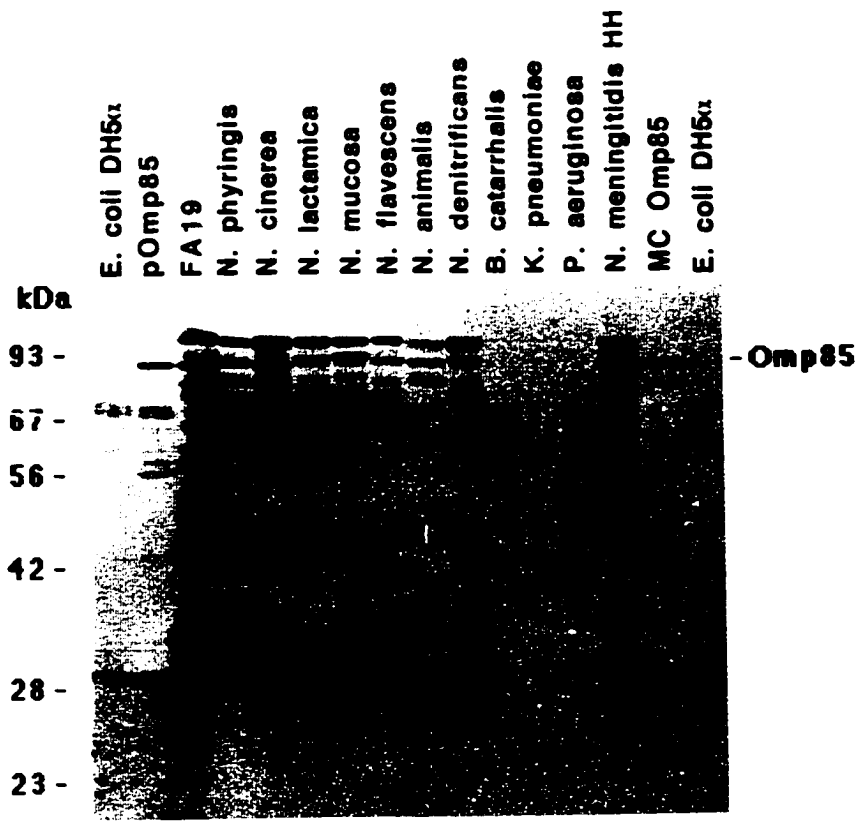


Figure 22. Western blot analysis of Omp85 in Neisserial commensals, *B. (Moraxella) catarrhalis,* and two species of the Enterobacteriaceae. Cell lysates of *E. coli, E. coli* expressing Omp85, *N. gonorrhoeae* FA19, several Neisserial commensal species, *B. (Moraxella) catarrhalis, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* were separated by SDS-PAGE (12.5%) and transferred to PVDF. Omp85 was identified only in Neisserial species by anti-outer membrane fraction antibody. Its position is shown on the right. Molecular weight markers are in thousands of Daltons (kDa).

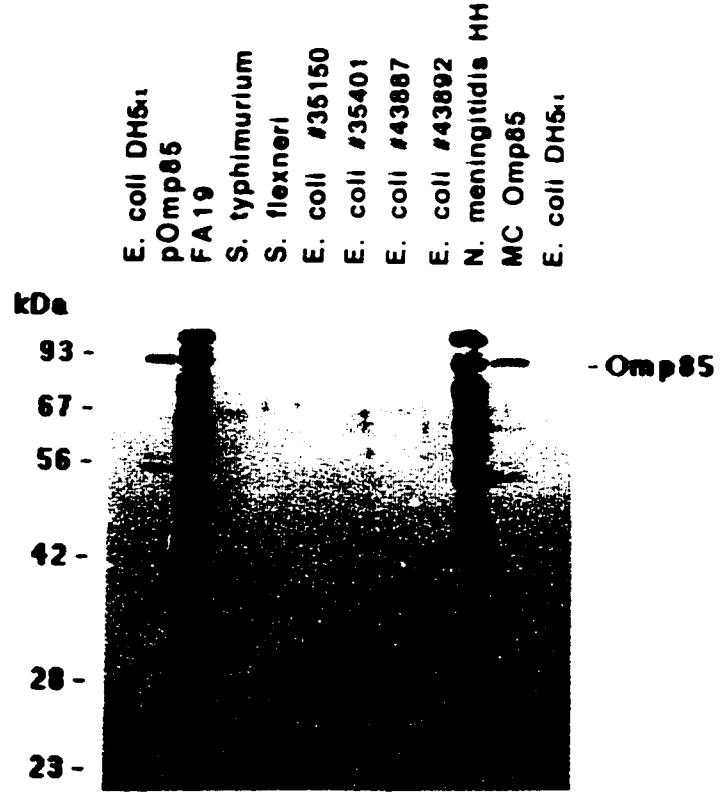


Figure 23. Western blot of Omp85 in the Enterobacteriaceae. Cell lysates of *E. coli* DH5α, *E. coli* DH5α expressing Omp85, *N. gonorrhoeae* FA19, *Salmonella typhimurium*, *Shigella flexneri*, *E. coli* (ATCC# 35150 - cytotoxic strain; ATCC# 35401 - enterotoxic strain; ATCC# 43887 strain - mucosal adherence strain; ATCC# 43892 strain - enteroinvasive strain), *N. meningitidis* strain HH, and *E. coli* expressing Omp85 MC were separated by SDS-PAGE (12.5%). These were blotted onto PVDF and probed with antibody to outer membrane fraction of *N. gonorrhoeae* FA19. The position of Omp85 is indicated on the right. Molecular weight markers are in thousands of Daltons (kDa).

omp85 is not essential in the Enterobacteriaceae and that expression of Omp85 is inhibited, or, more likely, the antibodies used did not recognized the epitopes of *E. coli* Omp85 exposed on the outer surface.

Figure 24 shows Omp85 to be recognized by antisera from human patients with meningococcemia and diseminated gonococcal infection by western blotting. Naive human sera did not react with Omp85. These data indicate that Omp85 is expressed *in vivo* and that it is antigenic.

Antibody generated against a pMal/Omp85 fusion protein was used to identify the location of Omp85 on a two dimensional gel. Figure 20 shows Omp85, indicated by the arrow. The predicted pI of Omp85 is approximately 8.75. Several two dimensional western blots were made using the fusion protein antibody. The position of Omp85 varied somewhat. The pH gradient here is not linear, the majority of the proteins falling within the 5 to 7 pH range.

In order to further characterize the Omp85 protein, attempts were made to insertionally inactivate *omp85* by homologous recombination. This would allow phenotypic changes to be observed with the absent gene product and possible function ascertained. Several constructs of the gene were made in *E. coli* using the Omega cassette and the aphA-3 fragment. The Omega fragment consists of a chloramphenicol resistance gene flanked by transcription and translational terminators at either end (see Figure 25). Insertion of this fragment in an open reading frame causes termination of transcription and translation of that gene as well as that of any downstream genes that might be in an operon. Should Omp85 be in an operon, the entire operon could be disrupted using this form of mutagenesis. Phenotypic changes in the organism as a result of gene inactivation may give clues to the

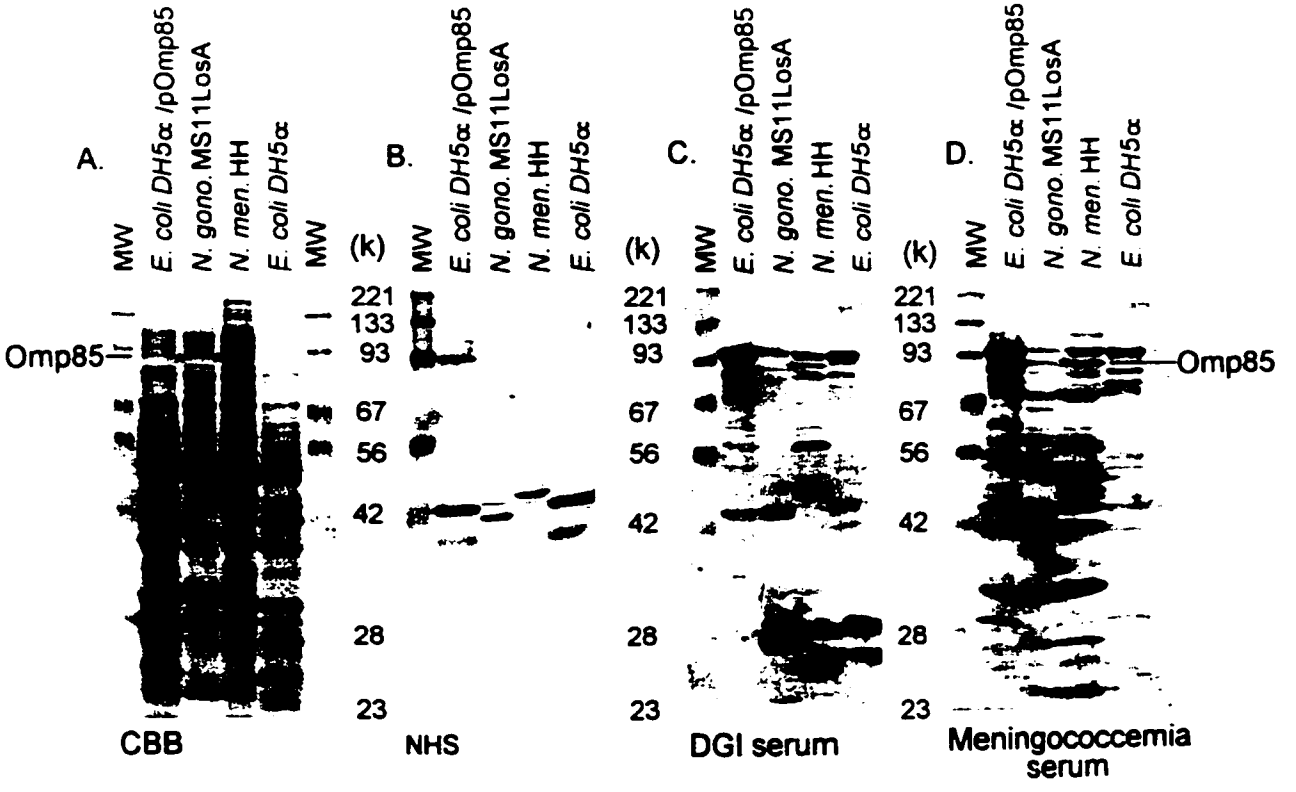


Figure 24. Western blot of pOmp85/*E. coli* DH5α, *N. gonorrhoeae* MS11 LosA, and *N. meningitidis* HH probed with human convalescent serum. Whole cell lysates were separated in a 12.5% SDS-PAGE gel and either stained with Coomassie Brilliant Blue (CBB; panel A) or blotted and probed with normal human serum (pool of 25 patients with no history of gonococcal or meningococcal disease (NHS; panel B), serum from a female patient with disseminated gonococcal infection (DGI; panel C), or serum from a male patient (HH) suffering from meningococcemia with *N. meningitidis* strain HH. The meningococcus strain HH was isolated from patient HH whose serum was used in this blot. All sera was used at 1:25 dilution. Patient sera reacted with Omp85 in *N. gonorrhoeae* and *N. meningitidis* indicating Omp85 was expressed during infection. The sera did not react with cloned Omp85, either because of topological differences when expressed in *E. coli* or due to antigenic variability of Omp85 in the infecting strains of gonococci and meningococci. Molecular weight markers in thousands of Daltons (k).

Figure 25. Omega and aphA-3 fragment construct. The omega primer was used to PCR-amplify the pHP45Ω plasmid without the streptomycin/ spectinomycin resistance gene cassette. The resulting plasmid, termed pHP45, still retains the ampicillin resistance gene marker. In the place of the Spc^r/Sm^r gene was inserted the chloramphenicol resistance gene (*cam*) from TnMax1 which has a gonococcal Opa promoter. The resulting plasmid was termed pHP45CΩ. A *Sma*I digest of pHP45CΩ yields the Omega fragment with the transcription-translation termination elements flanking either end of the *cam* cassette. Shown is only one end; the other end is identical but in the opposite orientation. The bi-directionality of the Omega fragment abrogates the need for screening clones for correct orientation of insertion.

At the core of the aphA-3 fragment lies a promoterless *cat* cassette. Upstream of the *cat* cassette contains three stop codons in all three possible reading frames and downstream of the cassette lies a ribosome binding site and an in frame ATG start codon. A *Sma*I digest of pSL33 yields the blunt-ended 650 bp aphA-3 fragment. This fragment must be ligated in frame with any ORF and can only function in the orientation shown. Downstream genes of a possible operon are allowed to be translated normally with this construct.

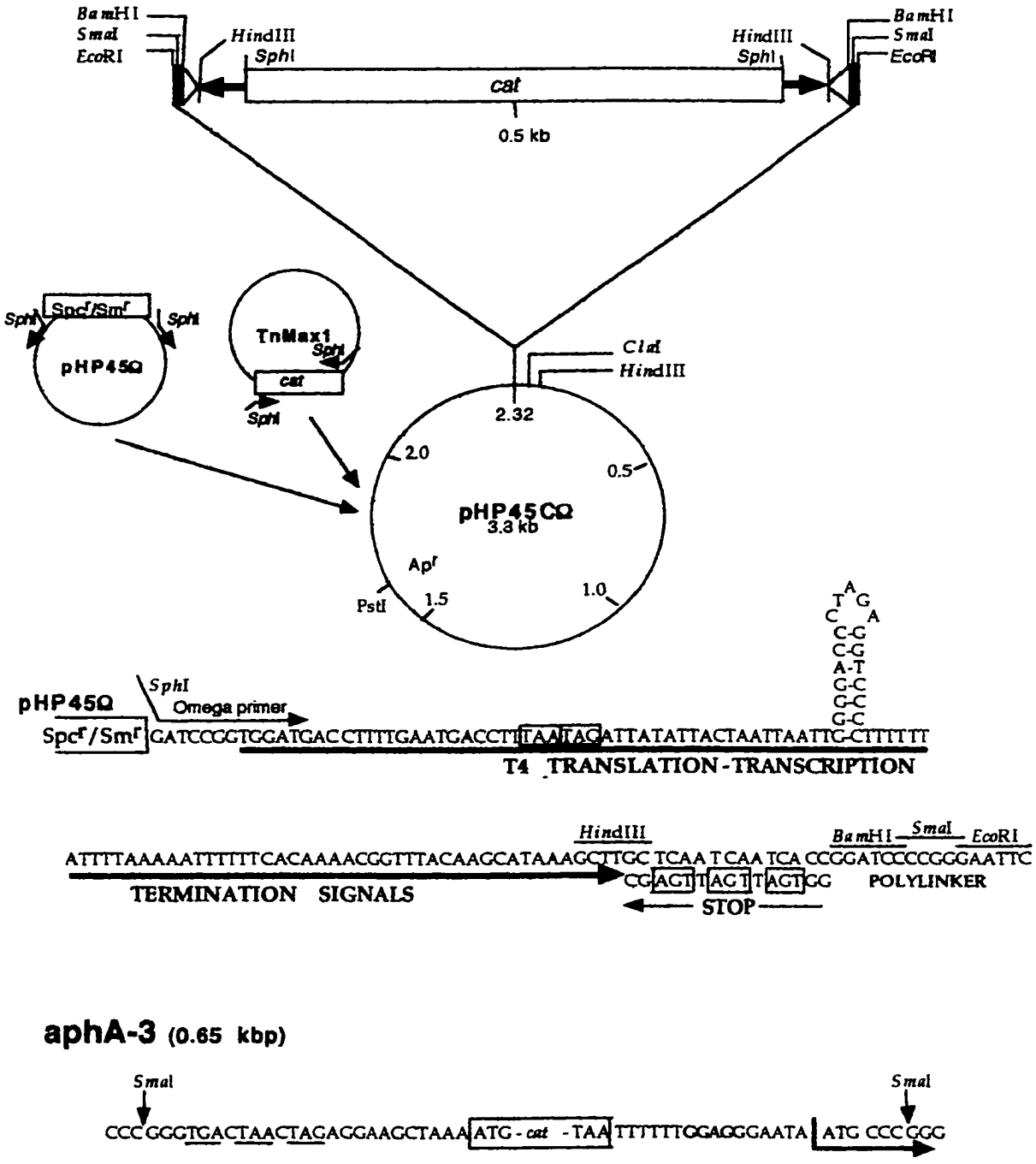


Figure 25. Omega and aphA-3 fragments.

original function of the gene, e.g. lose of adherence ability. The aphA-3 cassette consists of stop codons in all three reading frames at the 5′ end followed by a promoterless chloramphenicol resistance gene, with a down-stream Shine Dalgarno and ATG site. Orientation of this cassette must be in frame and codirectional with the gene of interest that it is to be disrupted. It is designed to terminate translation of a gene in an operon yet allow translation of any downstream sequences on the same messenger RNA. A cartoon of these constructs is shown in Figure 25.

pDR4 was subcloned by *Hinc*II digestion and ligation into pBluescript. The plasmids pDR4L, pDR4.2.1 and pDR4.H.1 were thus generated. A series of constructs were generated from these plasmids in anticipation of insertionally inactivating *omp85* in *N. gonorrhoeae* by homologous recombination. Figure 26 shows the orientation and construction of these plasmids. pDR4L contains a unique *Bsi*WI site into which the Omega fragment was inserted by blunt-end ligation. The Omega fragment used was a modification of the Omega fragment from pHP45Ω (115). PCR amplification was used to retrieve the host plasmid including the transcription/translation termination elements and omit the streptomycin/spectinomycin resistance gene cassette. This was done because of natural resistance to streptomycin in many strains of gonorrhea. In its stead was placed the chloramphenicol resistance gene from TnMax1 (61). The promoter for this *cam* cassette is from one of the *opa* genes of gono-coccus. The resulting plasmid was termed pHP45CΩ. A *Sma*I digest of pHP45CΩ results in an approximately 1000 bp blunt end fragment containing the *cam* cassette and flanking transcription/translation terminating elements. This fragment was inserted into the unique *Bsi*W1 site in pDR4L which had been blunt-ended with Klenow fragment. The resulting plasmid was termed

Figure 26. Omp85 mutagenesis constructs. Four plasmids were created to insertionally inactivate Omp85 by homologous recombination. The relative position of these plasmids are shown in relation to pDR4 and *omp85*. pDR4LCΩ was created by inserting the Omega fragment from pHP45CΩ into the unique *Bsi*WI site, blunt-ended with Klenow fragment. pDR4.2.1/33 was assembled by inserting the aphA-3 fragment from pSL33 into the unique *Nru*I site of pDR4.2.1. pOmp85, containing the entire *omp85* gene and 266 downstream base pairs, had the Omega fragment inserted at the unique *Bsi*WI site, blunt-ended with Klenow fragment. The resultant construct was termed pOmp85CΩ. pOmp85/33 was constructed by inserting the aphA-3 fragment into the unique *Nru*I site of pOmp85.

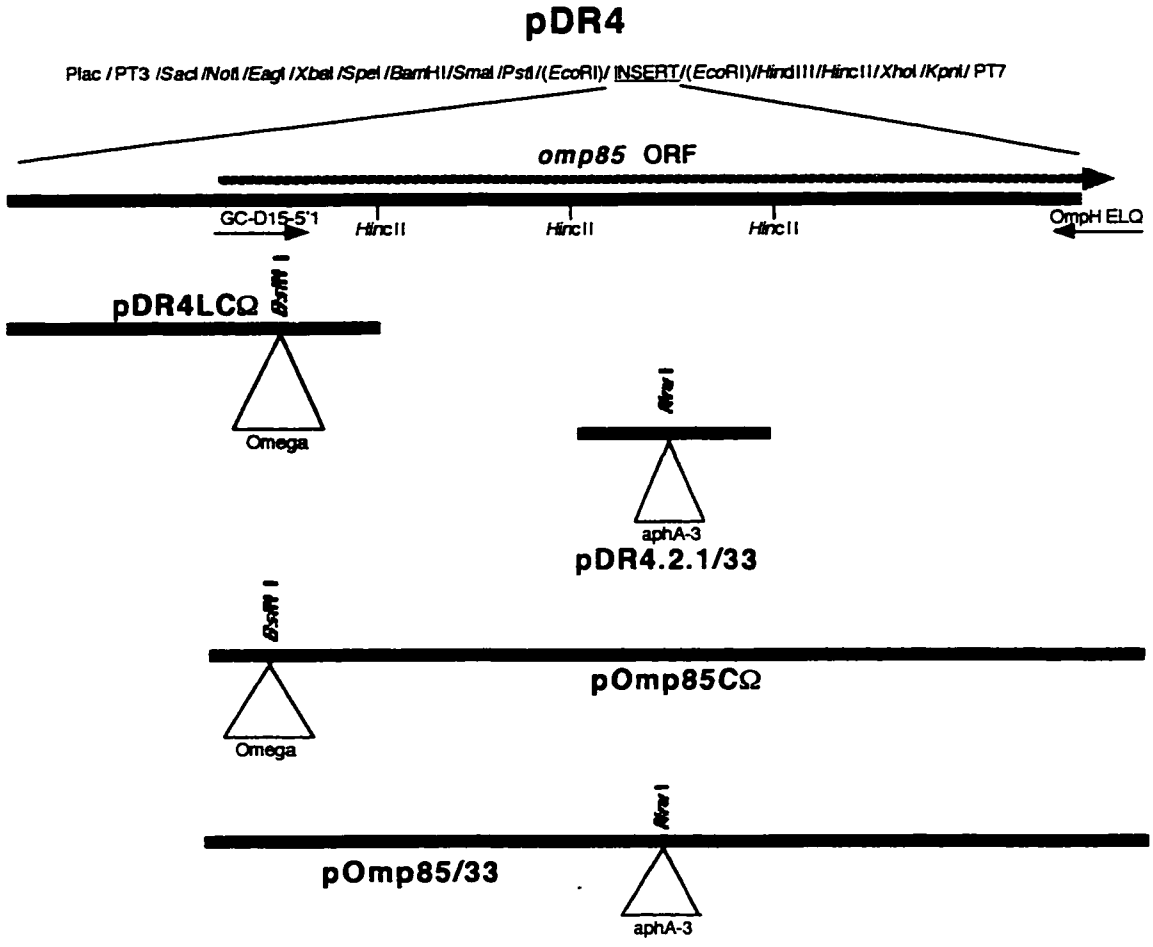


Figure 26. Mutagenesis constructs

pDR4LCΩ.

The aphA-3 fragment from pSL33 (98) contains a chloramphenicol resistance gene but with a ribosome binding site 3′ to the stop codon followed by an ATG codon, instead of a transcription/translation terminator element as in the Omega fragment. It also lacks a promoter, thus in-frame ligation is necessary. The aphA-3 fragment allows continued translation of downstream genes in the operon messenger RNA, should there be any polar effects of disrupting *omp85*. A *Sma*I fragment from pSL33 containing the aphA-3 fragment was inserted into the *Nru*I site resulting in the construct termed pDR4.2.1/33. This *Nru*I site was the only convenient site in the *omp85* ORF which would result in an in frame insertion of the aphA-3 fragment.

Double digests of all these constructs using *Sac* I and *Xho* I followed by blunt-ending with Klenow fragment was used to excise these disrupted *omp85* subfragments from pBluescript and place them into the unique *Sma*I site of pUPI. This was done to include the gonococcal uptake sequence (DUS) in the construct . The DUS element is contiguous to the *Sma*I site and is necessary for gonococcal transformation (46).

Finally, the CΩ and aphA-3 fragments were inserted into the unique *Bsi*WI and *Nru*I sites of pOmp85, respectively, resulting in plasmids pOmp85CΩ and pOmp85/33. This was done to increase the probability of recombination with gonococcal *omp85* by increasing the length of flanking DNA.

Transformations using circular and linearized pDR4LCΩ, pOmp85CΩ, pOmp85/33, and pDR4.2.1/33 were attempted. pDR4.2.1/33 and pDR4LCΩ transformants resulted in merodiploid mutants. These were termed Nru and Bsi, respectively. Efforts to resolve the merodiploid state into a full mutant

were unsuccessful. Transformation using genomic DNA from these merodiploid mutants, continued passage on agar containing increasing amounts of antibiotic, and transformation using the construct digested out from the vector to facilitate double cross over and diminish single cross over events failed to resolve the merodiploid state. The former method was an attempt to coax the mutant gene to recombine with the wild type gene because of the added pressure of antibiotic in the environment, thus creating the need for a second copy of the gene. Because the merodiploid state could not be resolved to a full mutant state, that is, two copies of the disrupted gene and no wild type copies, it was determined that the gene is essential for existence in *N. gonorrhoeae*. Figure 27 is a western blot of the merodiploids showing no significant difference in Omp85 expression. Figure 28 shows PCR products of these merodiploids indicating the presence of both the mutant and wild-type Omp85 gene.

Figure 29 shows the results of gonococcal adherence to Chang congunctiva cells in the presense of Omp85-specific rabbit Fab fragments. Although there appears to be some non-specific adherence inhibition using anti-BSA and normal rabbit serum Fab fragments, clear inhibition was seen using Omp85-specific Fab, increasing in proportion to concentration. These results corroborate the surface location of Omp85 and support the possibility that the protein plays a role in adherence.

Gene Integration into the 16S rDNA Locus

Having failed to create a full Omp85 mutant in gonococci, it was of interest to further elucidate the function of Omp85 by placing the entire gene into the genome of a related bacteria which did not carry the gene. The likely candidate for this was *B. (Moraxella) catarrhalis* because of its relatedness to

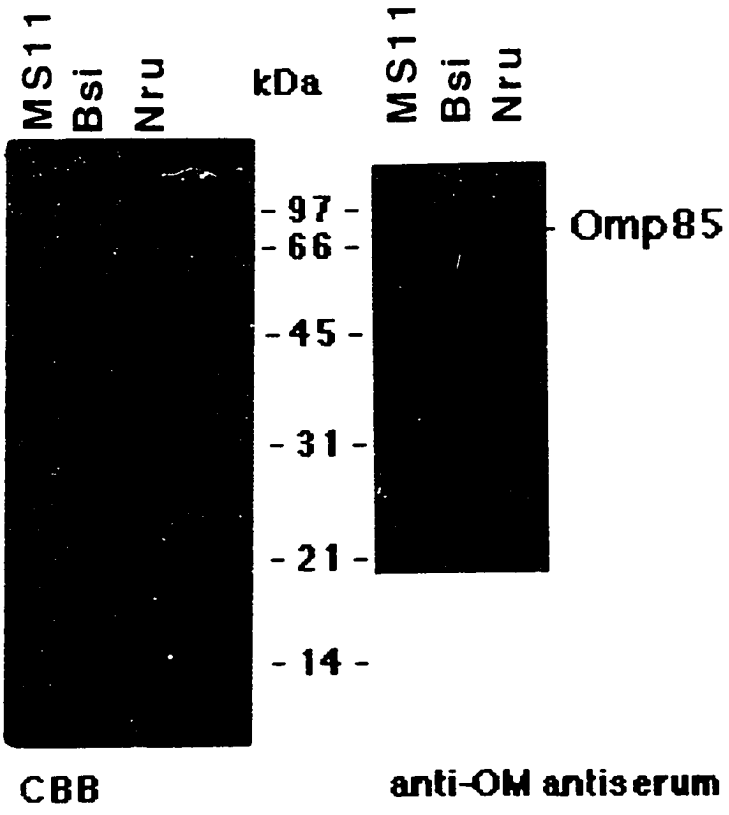


Figure 27. Western blot analysis of Omp85 merodiploids. Cell lysates of MS11, Bsi, and Nru Omp85 transformants were separated by SDS-PAGE (12.5%) as shown in the left panel. Identical gel was transferred to PVDF and probed with anti-outer membrane antibody. Omp85 is indicated on right. Molecular weight markers are in thousands of Daltons (kDa).

Figure 28. PCR analysis of Omp85 merodiploid mutants. 1% agarose gel of PCR products using primers as indicated. Primers 5'hypoERVQ and PYFT produce a product of 1594 bp in MS11 and an additional product of 2594 bp in merodiploid Bsi and an additional product of 2244 bp in Nru. This indicates the presense of a wild type and mutated Omp85 allele in the merodiploid strains. Primers PYFT and cat3' produce a product of 2218 bp in Bsi and 2189 in Nru. This indicates that the construct had recombined with the host *omp85* gene via a double recombination event, not in another site within the genome, and that pUP1 vector DNA was not included in this event. Molecular weight markers in kilobase pairs (kbp).

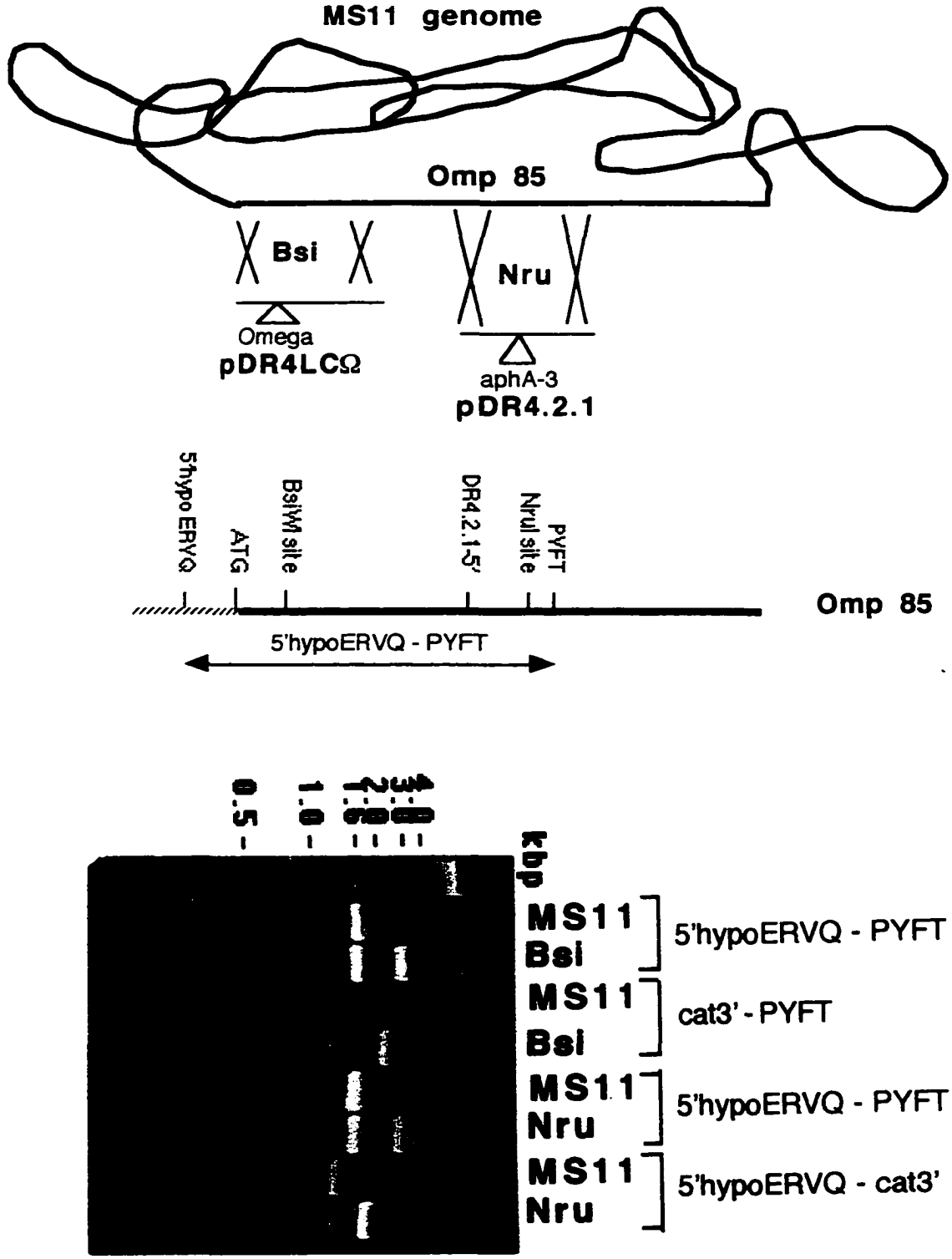
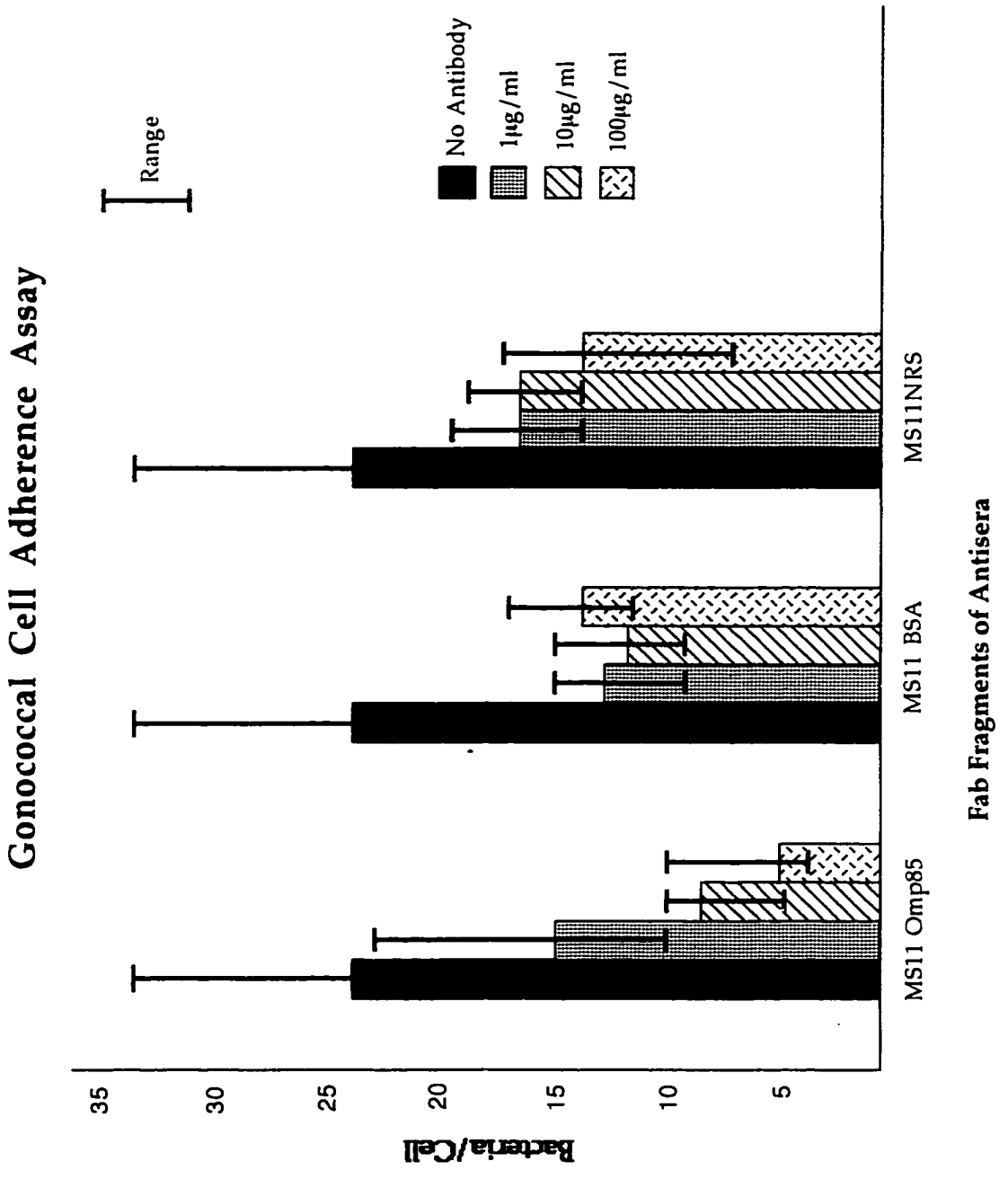


Figure 28. Schematic view of construct recombination and PCR analysis

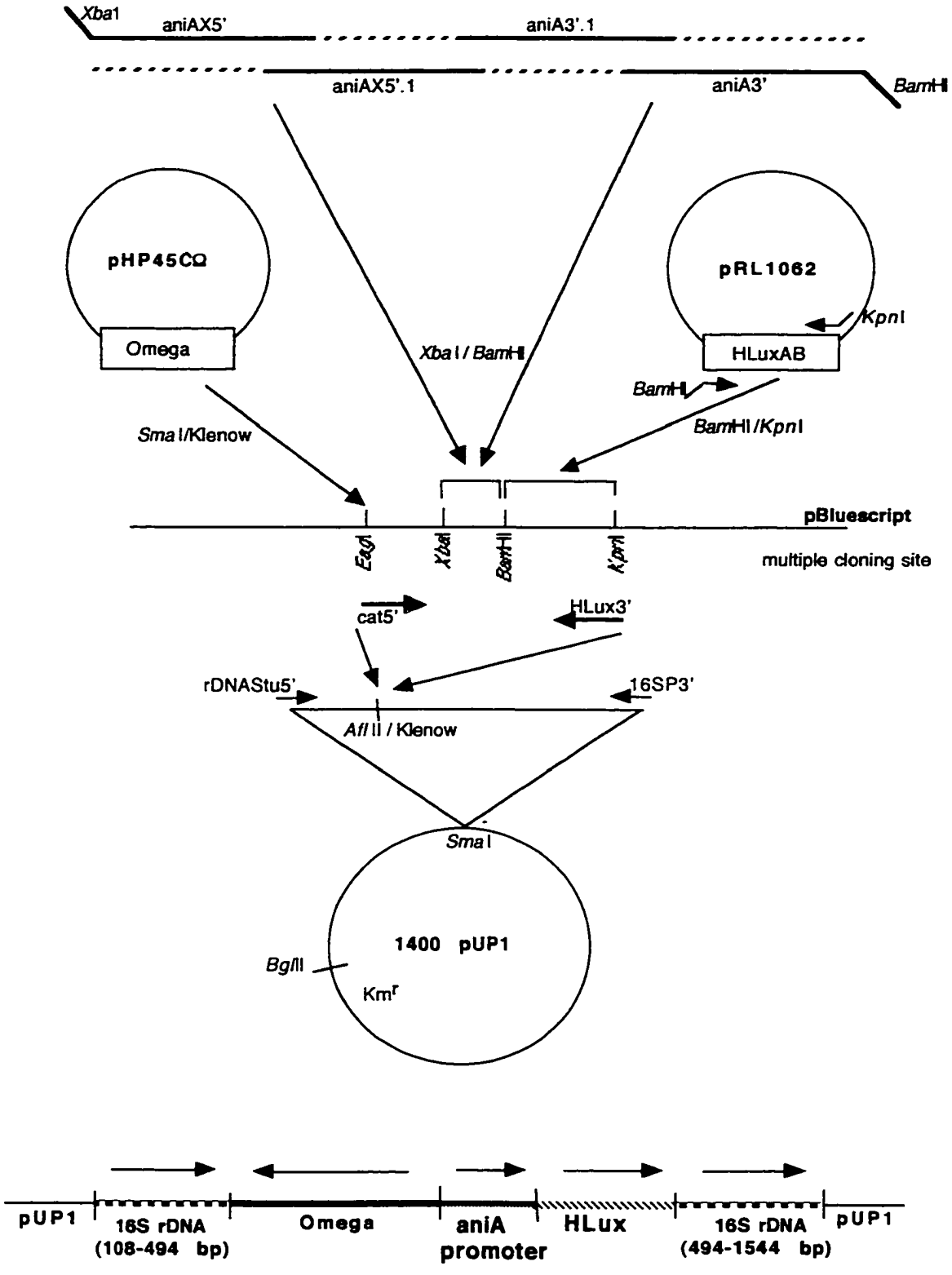
Figure 29. Cell adherence assay. Omp85-specific, BSA-specific, and normal rabbit sera Fab fragments were added to gonococcal MS11LosA bacteria in the various concentrations shown. Bacteria were then allowed to adhere to Chang conjunctival epithelial cells. Non-adherent cells and excess Fab fragments were washed off and the bacteria and host cells visualized by staining. Each data set represents the average number of gonococci left adhering per Chang cell.



the Neisseria and the fact that it lacked *omp85*. Because the parameters of transformation and the genetics of *B. (Moraxella) catarrhalis* are not well understood at this time, a pilot project using gonococcal 16S sequences was attempted. A 300 base pair section of the gonococcal 16S sequence was PCR amplified and cloned into the pBluescript vector using primers rDNAStu5′ and rDNAStu3′. A chloramphenicol resistance gene marker was inserted into the *Stu*I site of this segment, and the 16S construct placed into the *Sma*I site of pUP1 (46). Transformation and subsequent recombination with the genomic 16S region with this construct was successful. Pursuing the feasibility of novel gene integration into the gonococcal genome, a construct was made using the *luxAB* reporter gene (47) with the gonococcal *aniA* (68) promoter. Successful recombination was not achieved. It was thought that the 16S portion of the construct was too small in comparison with the insert size and therefore a larger section of 16S target DNA was cloned. A 1400 bp section of the 16S gonococcal rDNA from strain MS11 was cloned into pUP1 (46), using primers rDNAStu5′ and 16SP3′, resulting in the plasmid 1400 pUP1. This strategy proved successful. Figure 30 shows the construction of pDRLux1400.1/pUP1. The Omega - *aniA* - *luxAB* construct was PCR amplified out of pBluescript and placed into the blunt-ended *Afl*II site of 1400 pUP1. Following transformation, several recombinants were chosen and analyzed by PCR methods. All ten clones tested were identical; one was arbitrarily chosen and termed 1400.1. The PCR analysis, as seen in Figure 30, confirmed the incorporation of the construct into one of the rDNA genes. The *cat* gene was shown to be present and presumably expressing under selection conditions. PCR amplification with the 16SK5′ primer insured that the construct had indeed recombined with the genomic 16S site and not another random site in the

Figure 30. pDRLux1400.1/pUP1 plasmid construction. The *aniA* promoter from Pan1 protein (68) was synthetically constructed by first annealing primers aniAX5'.1 and aniA3'.1 together and filling in the complementary strands with *Taq* (Boehringer Mannheim) polymerase. Primers aniAX5' and aniA3', with engineered *Xba*I and *Bam*HI sites, respectively, were then added in a PCR reaction to amplify the entire *aniA* promoter. This product was inserted into pBluescript at the *Xba*I and *Bam*HI sites. The *luxAB* genes (47) from pRL1062 (170) were PCR-amplified using primers HLux5' and HLux3' with *Bam*HI and *Kpn*I sites at each end, respectively. This product was ligated into pBluescript digested with the same restriction enzymes. The CΩ fragment was then ligated into the *Eag* I site blunt-ended with Klenow fragment. The resulting plasmid was termed pCX.1Hlux.

The end 1436 base pairs of the 1544 bp 16S gonococcal rDNA gene (128, 169), traversing base pairs 108 to 1544, was PCR-amplified with primers rDNAStu5' and 16SP3' engineered with *Cla*I and *Pst*I sites, respectively. This product was ligated into the corresponding sites of pBluescript and called 16S1400. The 16S1400 insert was digested out with *Cla*I and *Pst*I, blunt-ended with Klenow fragment, and inserted into the *Sma*I site of pUP1 (46), resulting in the plasmid called 1400 pUP1. A unique *Afl*II site within the 16S fragment of 1400 pUP1 was used to insert the construct of plasmid pCX.1Hlux. This was done by PCR-amplifying out CX.1Hlux using the cat5' and HLux3' primers and inserting this into the blunt-ended *Afl*II site of 1400 pUP1. The resultant plasmid was termed pDRlux1400.1/pUP1. All orientations were verified by PCR. Selection of *E. coli* was done on LB plates containing 50 μg/ml ampicillin, 30 μg/ml chloramphenicol. Selection of gonococcal recombinants was done on clear typing media containing 10 μg/ml chloramphenicol. Arrows indicate direction of translation.



pDRLux1400.1/pUP1

genome. This is the case because the section of 16S DNA to which this primer anneals is upstream from the integration site and is not in the original pDRLux1400.1/pUP1 construct. A map of the positions of the primers used is shown in Figure 31.

Figure 32 shows an SDS-PAGE analysis of MS11 transformant 1400.1 grown under aerobic, microaerophilic, and anaerobic conditions. The 54 kDa Pan 1 protein can be seen somewhat in the microaerophilic growth condition, and very strongly expressed under anaerobic conditions. Pan 1 was not noticeably expressed under aerobic conditions. Under all three of these growth conditions, luciferase activity was measured using the same amount of cells with no significant difference in activity. This is shown in Figure 33. Thus the *aniA* promoter appears to be constitutively active. A growth curve (Figure 34) of the parent strain MS11 compared with the transformant strain 1400.1 shows a higher growth rate for the wild type MS11, the slop of log phase growth being twice that of the mutant. The burden of an additional two genes and the absence of one or more of the four 16S gene loci seems to affect growth of the transformant. Should more than one of the four 16S loci become inactivated by recombination, significant growth reduction would be expected. Primers upstream and downstream of each of the 16S loci would need to be constructed to determine precisely which and how many of the four 16S loci are occupied.

5. Discussion

Identification of Outer Membrane Proteins

Outer membrane proteins can be identified by several methods. An example of a technique not used in these experiments but one that has been used in gonococcal research by others is transposon mutagenesis. A genomic

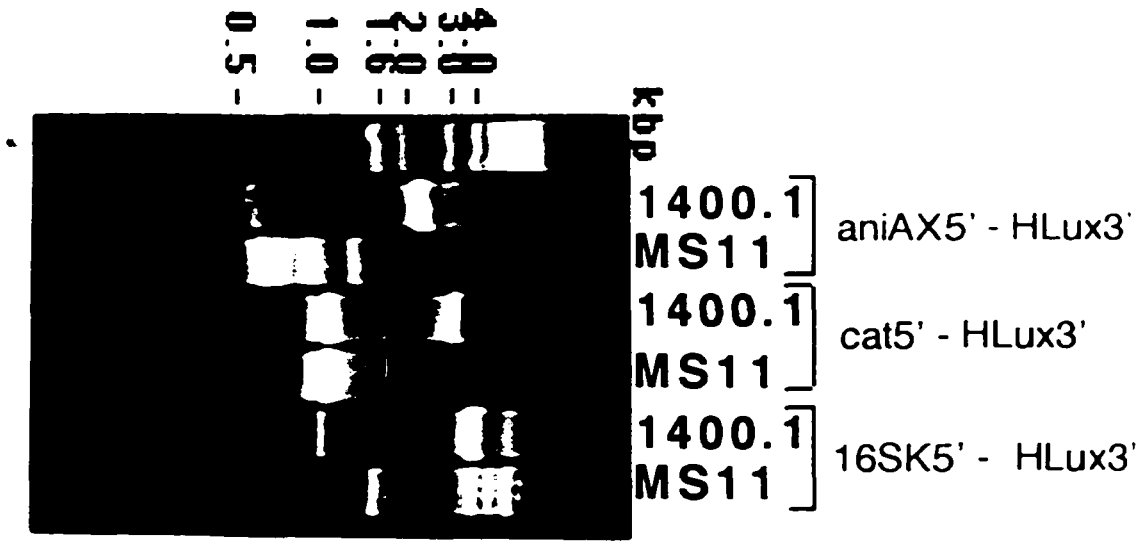
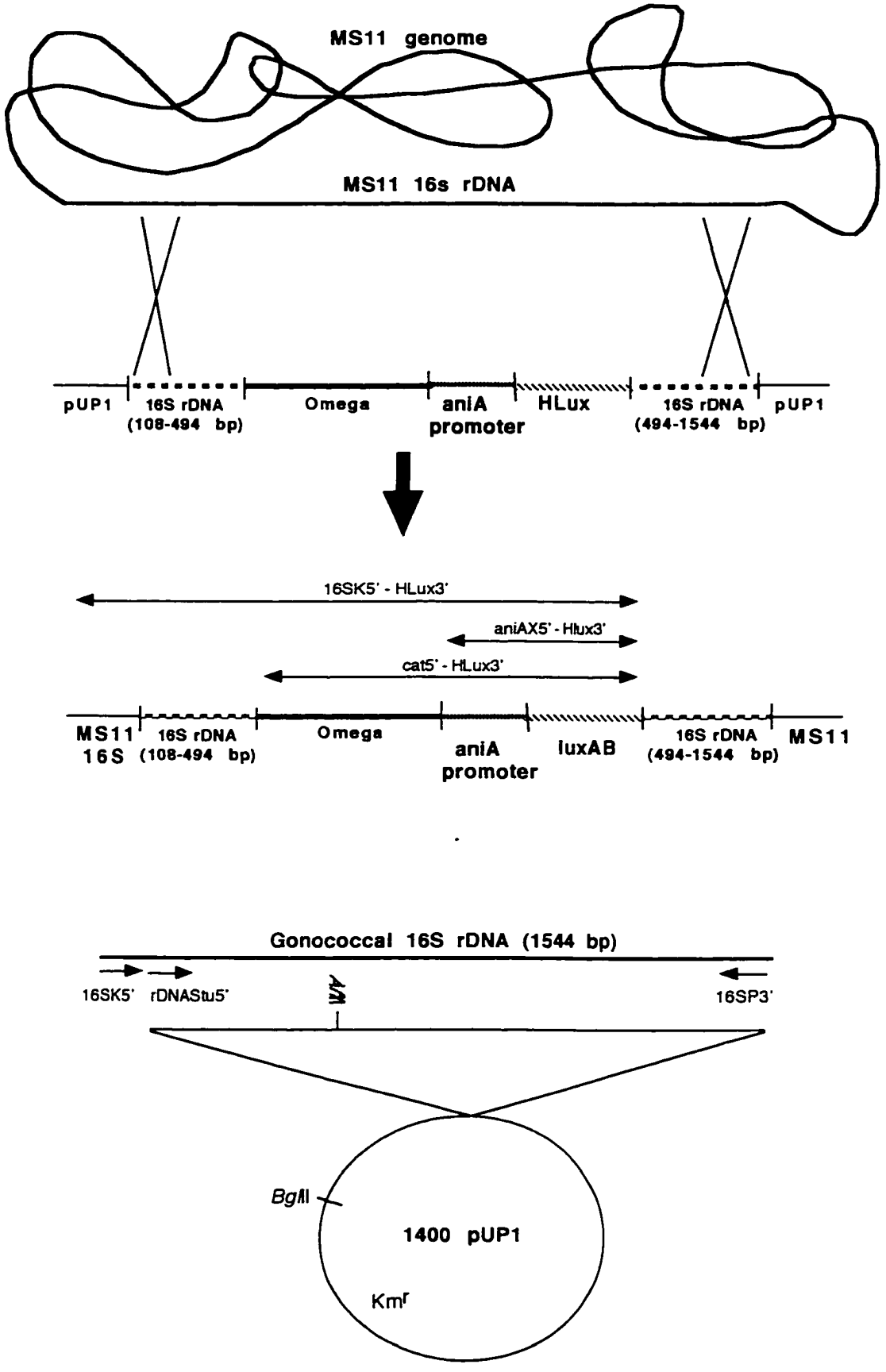


Figure 31. PCR analysis of 1400.1 and construction of 1400 pUP1. Comparisons between pDRLux1400.1/pUP1 transformant 1400.1 and host gonococcal strain MS11 using three sets of primers as indicated. Primers aniAX5′ and HLux3′ constitute the *aniA* promoter and *luxAB* gene yielding a product of approximately 2400 bp. Primers cat5′ and Hlux3′ constitute the *aniA* promoter and *luxAB* gene together with the chloramphenicol resistance gene yielding a product of approximately 3300 bp. Primers 16SK5′ and Hlux3′ constitute the chloramphenicol resistance gene, the *aniA* promoter and *luxAB* genes together with the upstream portion of the 16S rDNA segment yielding a product of approximately 3900 bp. Molecular weight markers on left in kilo base pairs (kbp). Adjacent is a map showing the location of various primers used in this analysis and in the construction of 1400 pUP1 relative to the 16S rDNA gene.



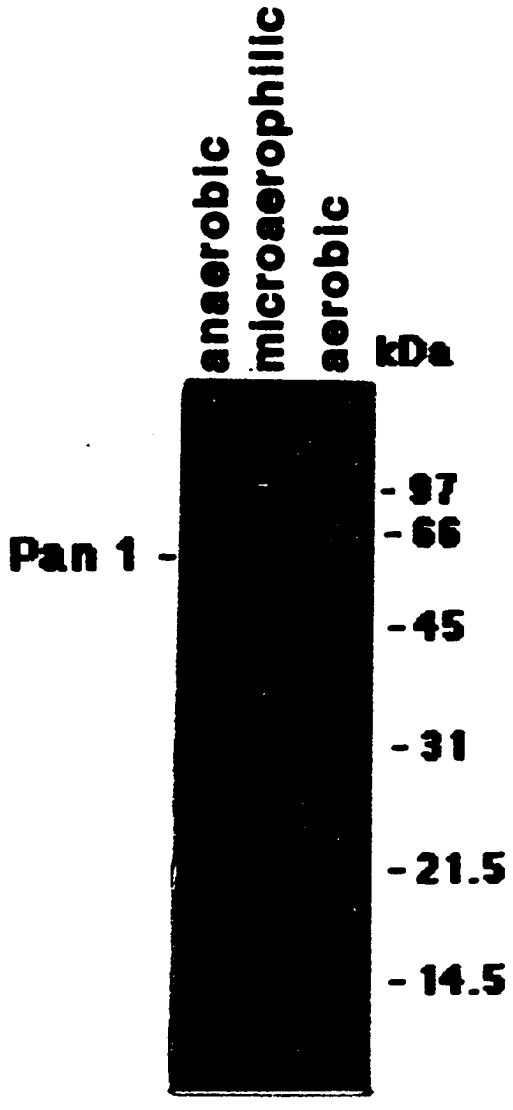
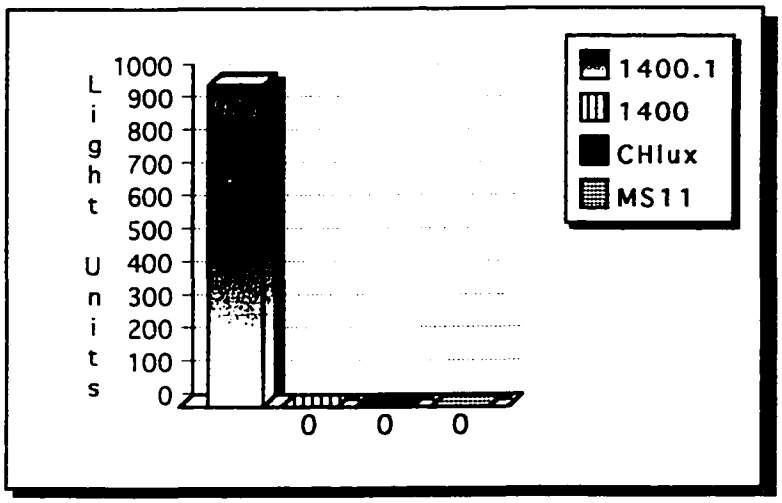
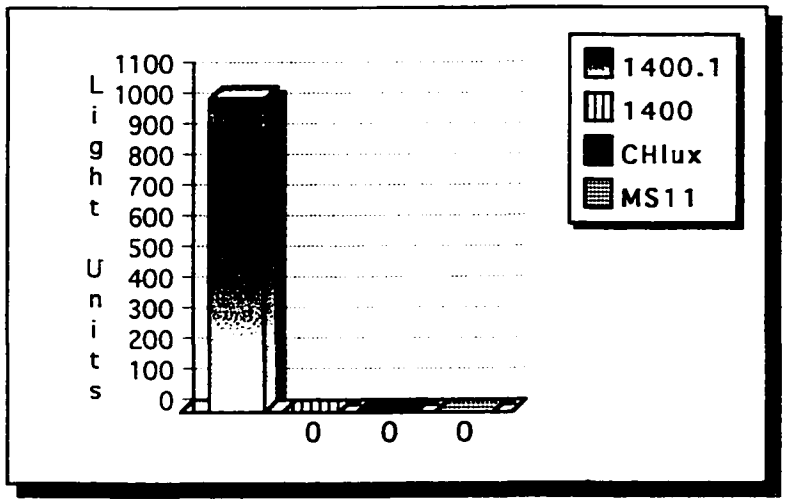


Figure 32. SDS-PAGE of MS11 transformant 1400.1 used in luciferase assay. Cells were grown either aerobically on plates, microaerophilically in broth, or anaerobically on plates in an anaerobe chamber. Pan1 is indicated on left. Molecular weight standards in thousands of Daltons (kDa).

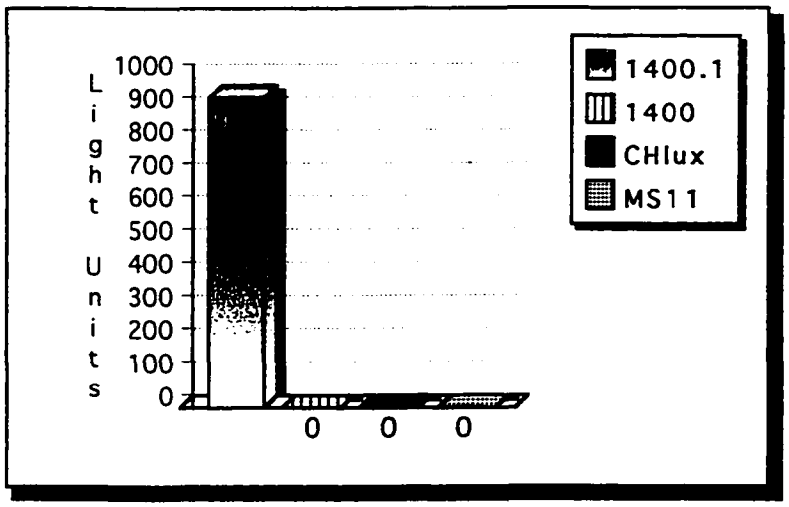
Figure 33. Luciferase assay of gonococcal transformant 1400.1 (*aniA - lux*). Aerobically grown cells were inoculated onto clear typing media (157) for 16 hr and then harvested. Microaerophilically grown cells used clear typing media without agar and 4 ml of $NaHCO_3$ (8.4%) and 2 mM $NaNO_2$ added per liter. Bacteria were added to an OD_{600} of 0.1 and incubated at 37°C for 16 hr with flasks completely full of media and not shaken to prevent aeration. Anaerobically grown cells were inoculated onto clear typing media and a sterile filter disk (1.3 cm) containing 20 µl of a 20% $NaNO_2$ solution was place in the center of each plate (97). Incubation was done in a 2.5 l GasPack Anaerobe chamber (BBL) containing AnaeroPacks (Mitsubishi Gas Chemical Co., New York, NY) at 37°C and the reducing environment monitored using anaerobic indicator strips (MedPak, Montvale, NJ).'CHLux1400' is identical to 1400.1 but without the *aniA* promoter. '1400' is identical to '1400.1' excepting a chimeric *aniA* promoter.



Aerobic



Microaerophilic



Anaerobic

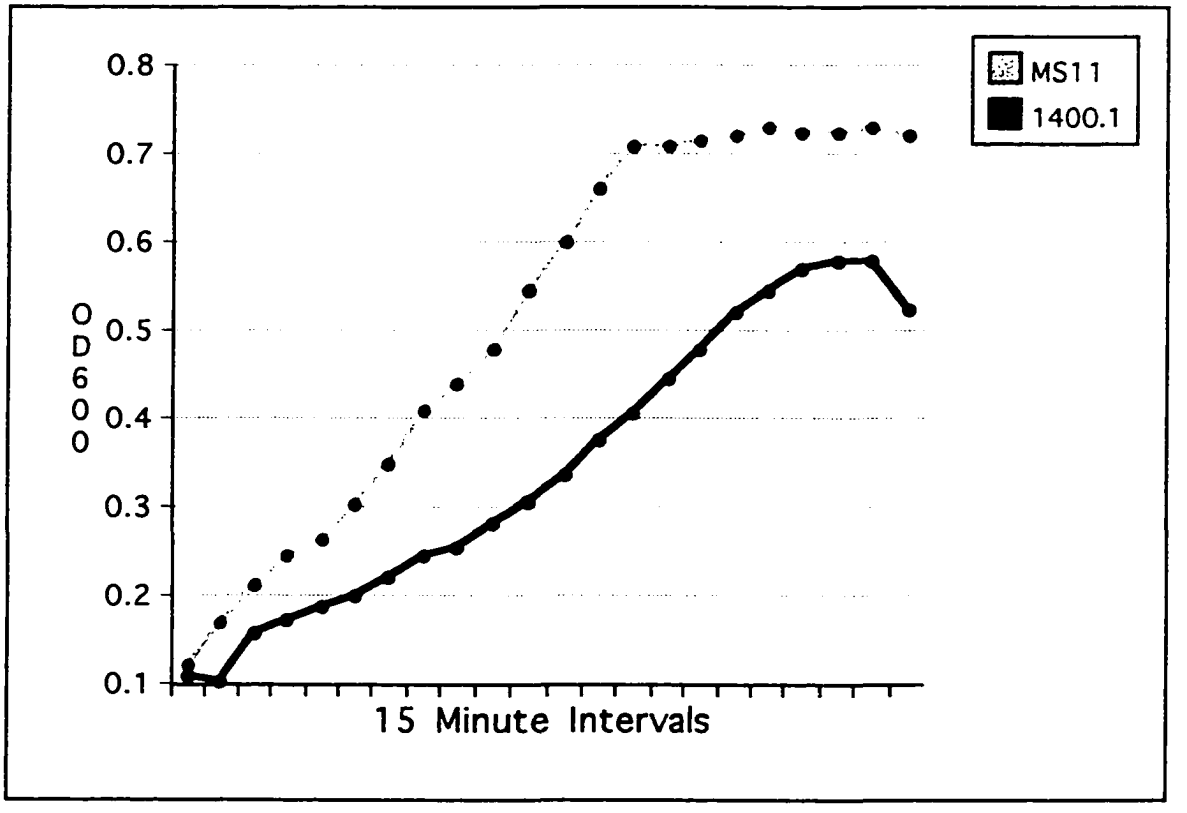


Figure 34. Growth curve of MS11 and 1400.1. Cells were harvested from 16 hr plates and placed in 3 ml of gonococcol broth to an OD_{600} of 1.0. 1 ml was added to 25 ml of gonococcol broth and incubated at 37°C, 225 rpm in a 500 ml side arm flask. Chloramphenicol was added to 10 μg/ml in the flask containing 1400.1.

library is subjected to transposon mutagenesis whose inserting element includes a *pho*A gene which, when associated with a signal peptide, will yield a colored phenotype (75). Outer membrane proteins are usually equipped with a signal peptide to traverse the inner membrane to transport it to the outer membrane (154). Another method would be to directly search the genome sequence data bank against known outer membrane proteins or to search for know functional domains of virulence determinants.

The first approach taken in these experiments was iodination of outer membrane proteins. Iodination of whole cells has been an accepted method for identifying outer membrane proteins for many years (71, 42, 81, 168, 99, 19). Because the iodagen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, is bound to the reaction vessel wall where it cannot diffuse through the membrane, covalent binding of ^{125}I to proteins is confined to outer membrane proteins (1). Fractionation of labeled cells has shown that very little contamination of inner membrane proteins occurs (1). In the two iodination gels, one exposed longer than the other, approximately 41 protein dots were identified. Some of these proteins yielded stronger signals than others, some disproportionate to their quantity. For instance, "protein 3" is barely visible in a Coomassie blue stained gel, yet its iodination signal is quite strong. Conversely, "protein 18", for example, shows significant quantity on SDS-PAGE and yet shows very little uptake of ^{125}I. This indicates chemical and/or topological differences between these two proteins.

Because iodine binds predominantly to tyrosine and somewhat to histidine and phenylalanine (42), some outer membrane proteins could be missed because these residues are not exposed on the outer loops of these proteins. Corroborating methods, therefore, were used to establish the

identity of these outer membrane as surface located.

Biotin labeling of outer membrane proteins is also a common procedure used (19, 88, 1). In this system, biotin covalently binds to primary amines of amino acid residues of proteins; enzyme-conjugated avidin is added which vigorously binds to biotin, and the complex is then identified by adding the substrate of the enzyme. Some properties of the avidin - biotin system that lend themselves for use as technical tools include high affinity ($KD<10^{-15}M$), multi- or monovalent binding of conjugated avidin, great stability, relatively small size, and ready availability. Using this system, several additional proteins were identified as compared with iodine. These were: 2, 15, 16, 18a, 18b, 24a, 24b, 25a, 43a, 46a, 53, 54, 55, 59, 62, 63, 66, 67, 68, 71, 72, 73, 74, 75. The biotin capture method retrieved 8 proteins, most of which were dominant in the iodination method, and all of which were also seen with biotin labeling alone (Figure 6). This capture method could be used as a method to acquire large quantities of specific proteins for use in N-terminal sequencing of amino acids and antibody production.

Variability between iodination and biotinylation results may be in part due to chemical differences in binding: iodine binds mainly to tyrosine and biotin mainly to lysine residues. Because biotin is a small molecule (MW = 556.58) diffusion through the membrane may be more of a problem (1).

Outer Membrane Fractionation and Generation of Antibody

The separation of the outer and inner membranes of the gonococcus has been accomplished in the past by various techniques. These include solubilization with sarkosyl (118, 9), and the use of salt coupled with differential centrifugation (39). The purity of these fractions were less than optimal. This was partially due to the unusual O-acetylation of the gonococcal

peptidoglycan which is refractory to lysozyme action. Recently, Judd, *et al.*, (74) purified the gonococcal outer and inner membranes to homogeneity. Chemical and immunological analysis revealed a clean separation, demonstrating the presence of known components (e.g. cytochromes - inner membrane; LOS - outer membrane) of each membrane restricted to their respective fractions.

The purified gonococcal outer membrane was injected into rabbits to produce polyclonal antibody specific to outer membrane components. Figure 4 shows 47 gonococcal proteins recognized by this antibody. Western blotting of two dimensional gels details many proteins common with iodination of the same proteins. This same result was obtained by others with *Yersinia,* comparing western blots using antibodies generated to whole cells and iodination (95, 144). Specifically, proteins 3, 4, 6, 7, 9, 11, 12, 13, 17, 17a, 18, 20, 25, 26, 26a, 31, 34, 39, 46, 56 were found to be common between the two labeling techniques.

Differences seen between the two techniques may be explained by noting that the antibody was generated to outer membrane fraction of . vigorously growing log phase cells. These proteins may vary quantitatively and qualitatively from gonococci grown on 16 hr plates. It should also be noted that neither of these growth conditions may reflect the protein profile of gonococci resident in a human host. Thus, some outer membrane proteins may not be identified by antibody or iodination (e.g. Pan1 [68]). In addition, the enzymatic and sonication method of extraction used in the fractionation technique used in the preparation of the outer membrane antisera could generate epitopes not included on the reducing conditions of two dimensional gel electrophoresis. For this reason, some proteins may not be labeled

or others labeled that should not be. Also, isoelectric focusing can yield slight variations in protein dot coordinates from gel to gel and thus some proteins may be misidentified, especially those in the 44 kDa region where there is a high density of protein types. These differences may explain some of the variation seen between these two systems of labeling.

Seventeen proteins were found to be common to all three labeling techniques as shown in Figure 8.

Isolation of Specific Proteins

Once proteins were identified as being outer membrane in origin, the first approach taken in these experiments to isolate and characterize the protein was by direct N-terminal sequencing. The amino acid sequence of the protein would then lead to function by homology to other known outer membrane proteins. Several attempts were made using either whole proteins or fragments of proteins without success. Direct N-terminal sequencing has many problems associated with it, including sensitivity to oxidation and terminal blockage, and the necessity for relatively large amounts of material (32). Due to these limitations this approach was dropped.

The anti-outer membrane antibody was used to screen a genomic FA19 library. Sixteen proteins were identified from homology searches (3) using the partial DNA sequence of the inserts. Only one clone, pDR3.3, did not hybridize with any known protein in the DNA data bank. Several of these proteins seemed to be involved in general 'housekeeping' type functions (e.g. inosine-5'-monophosphate dehydrogenase, dihydrolipoamide dehydrogenase) while others piqued interest as possible virulence determinants: pDR22.E.1 - Listeria invasin protein, pDR3.7 - leukotoxin. By no means do these 16 proteins represent the entire complement of outer membrane

proteins. There was no indication that the library was exhausted of further unique clones. Many positive archived clones were never sequenced.

Omp85

The protein of interest to our lab was the analog of the *Haemophilus influenzae* D15 protective surface antigen (71, 161, 166, 89, 166). Antisera to D15 antigen was shown to be protective in an infant rat model study against live *H. influenzae* challenge (49). In addition, human convalescent sera from patients with epiglottitis or meningitis were reactive to D15, and antigenicity in typeable and nontypeable *H. influenzae* strains appears to be universal (91).

There are no good animal models for *N. gonorrhoeae* infection, thus testing the hypothesis that antibodies to Omp85 would be protective against challenge, as was with *H. influenzae* in rat pups, is not possible. Human convalescent sera from patients with DGI and meningococcemia were shown by western blotting to be reactive. These results demonstrate that Omp85 is expressed *in vivo* and that the protein is antigenic in humans. The antigenic universality seen in *H. influenzae* was also observed in *N. gonorrhoeae.* All strains tested contained the Omp85 gene and expressed the protein. Omp85 was also shown to be expressed in all Neisserial commensals tested, suggesting that is necessary for establishing and maintaining mucosal infection. These characteristics, *in vivo* expression, antigenicity, and universal expression, are primary considerations in subunit vaccine development. Since antibodies to Omp85 analogs in *H. influenzae* and *P. multocida* were protective against challenge, it may be true that antibody to gonococcal Omp85 generated in humans during infection may contribute to the elimination of the bacteria by the immune system.

The gonococcal DNA sequence of the region surrounding Omp85 is

known (41). These genes are involved with lipid A biosynthesis. These same genes are found in several of the pathogenic organisms discussed as containing analogs of Omp85. The linkage of these is given here:

Omp85	lpxA - fabZ - lpxD - hlpA - Omp85 - hypothetical protein - cdsA
Omp85MC (150)	lpxA - fabZ - lpxD Omp85
P. multocida (36)	firA - skp - Oma87
H. influenzae D15 (50)	firA - skp - D15 - hypothetical protein - cdsA
E. coli (17)	lpxA - fabZ - lpxD - hlpA - Omp85 - yeaL
B. abortus (10)	lpxA - fabZ - lpxD ..... Omp1 - hypo. - hypo. - cdsA

[Protein hlpA is also known as skp or OmpH; protein lpxD has also been termed firA. Dotted sections indicate that the intervening sequences at the juncture are not known.]

With the exception of *yaeL* (*E. coli*) and *omp85*, all these gene functions are known and have been characterized as being involved in lipid membrane synthesis. Whether Omp85 is also involved in lipid synthesis can only be hypothesized by association with these genes. In gonococci, the intergenic spaces between each of these genes are less than 100 bp, perhaps suggesting that it is part of an operon. The idea of an operon is also suggested by the apparent lack of a *omp85* promoter. Schnaitman (135) has suggested that these genes are in a so-called 'macromolecular II operon". Briefly, the proposed operon consists of: *lpxA* gene, whose product catalyzes the first step in lipid A biosynthesis (4); *polC* (*dnaE*) which encodes the α core subunit of DNA polymerase III; the rnh gene encoding RNaseH; the *lpxB* and *lpxD* genes which also function in the lipid A biosynthetic pathway (136); the cdsA gene which encodes CDP-diglyceride synthetase, an essential step in biosynthesis of

glycerophospholipids (136); and the *ompH* gene (also referred to as *skp* or *hlpA*) gene which encodes a 16 kDa protein active in folding protein in the periplasm (101).

Argument against an operon can be made by noting that the genes involved in membrane phospholipids synthesis in *E. coli* are scattered throughout the genome yet are coordinately transcribed (116). In addition, rho-independent terminators were found immediately upstream and downstream of Omp85, suggesting independent regulation.

A search of the Omp85 amino acid sequence against the GenBank data base also revealed a homology with a cyanobacterium protein (75a). The cyanobacterium protein was named IAP75 because of its similarity to the 75 kD chloroplast import associated protein, IAP75 (139). The chloroplast IAP75 was located in the outer membrane of chloroplasts and was one of four components of a polypeptide transport complex. This suggests that Omp85 might be part of a similar transport complex.

The sequences of other proteins from the chloroplast import associated complex were searched against the gonococcal genome sequence data base (41). A gonococcal protein similar to a purported GTP-binding protein, IAP34 (78a), was identified. The areas of highest homology were in the regions identified as GTP-binding protein motifs. Surface-crosslinkage studies by Dr. Ralph Judd were performed to determine if Omp85 might participate in a system analogous to the IAP complex. Studies using the crosslinker DTBP (104a) showed that Omp85 was within 11.9Å of up to five proteins, one of ≈34 kDa, possibly being a IAP34 homolog. These data support the possibility that Omp85 might participate in a complex analogous with the chloroplast IAP complex.

In summary, Omp85 may be involved in the membrane lipid A biosynthetic machinery, or in a polypeptide transport complex located on the outer membrane. Its surface location is evidenced by iodination, biotinylation, anti-outer membrane antibody adherence, the presence of a signal peptide, and homology to several other known Omp85-like outer membrane proteins.

Omp85 Mutants

Attempts at insertional inactivation of Omp85 by homologous recombination resulted in a state of merodiploidy, a common occurrence in gonococcal recombination (personal communication W. Shaffer). This is probably due to the dual chromosome nature of diplococci: one chromosome containing the mutated version to survive on selective media, the other maintaining the wild type. Attempts at resolving the merodiploid to a complete mutant were not successful. Because *E. coli* DH5α and *B.(Moraxella) catarrhalis* seem to survive without the gene, it is tempting to speculate the non-essential nature of Omp85. The aphA-3 mutant, Nru, theoretically insures transcription of downstream elements within a possible operon; thus if an operon is involved with Omp85 production, the other components of that operon would not be affected. This does not abrogate the necessity of any essential genes, however. It is possible that Omp85 in gonorrhea has additional functions not found in *E. coli* DH5α or *B. (Moraxella) catarrhalis*, thus making its existence essential.

The conclusion of the mutagenesis studies is that Omp85 is essential to gonococcus. It is interesting to note in Figure 26 that wild type Omp85 levels of expression is similar to levels in the merodiploid mutants, suggesting some compensatory transcription or translation action to maintain the same

protein concentrations. Thus there seems to be some variation in the amount of Omp85 able to express in the gonococcal genome.

Cell Adherence

Because there was some inhibition of adherence of gonococcal cells to Chang conjunctiva cells using non-specific Fab fragments, it could be interpreted that the Fab fragments cause a steric hindrance to adherence. The decrease of adherence to gonococci to Chang cells with anti-Omp85 Fab could be simply a dosage effect: since there are presumably numerically more Omp85 molecules, and therefore antibody binding sites, in the outer membrae that there are random sites to which anti-BSA or normal rabbit serum Fab binds, that there was a commensurate decrease in adherence due to steric hindrance. Should Omp85 be involved in peptide transport or membrane synthesis as hypothesized, antibody adherence, under described experimental conditions, may interfere with Omp85 function. It would seem unlikely that aberation of function of this type molecule would directly cause a decrease in adherence.

It is possible that Omp85 is indeed directly involved with adherence via a eukaryotic receptor. Based on known components of the outer membrane of gonococci involved with this reaciton, it appears that several, not just one or two components, are necessary. Therefore, if Omp85 has evolved to play a role in these actions, it would be only one component of many whose additive effect promotes adherence.

Gene Integration

Omp85 expression was observed in all Neisserial species tested. One method of discerning gene function is to knock out the gene and note phenotypic changes in the mutant as compared to the wild type. Since this

was not possible, an alternative is to find a strain that did not carry the gene and observe any phenotypic changes as compared to those strains which did. This also was not possible. *B. (Moraxella) catarrhalis* is closely related to the genus Neisseria genetically (127) and is also a mucosal pathogen. It was hypothesized that *omp85* could be introduced into *B. (Moraxella) catarrhalis* by insertion into one of the 16S rDNA sequences in the genome by homologous recombination with a construct containing the 16S rDNA with *omp85* inserted within it. A pilot project was attempted using 16S sequences from both *N. gonorrhoeae* and *B. (Moraxella) catarrhalis* with a chloramphenicol resistance gene marker inserted within the ORF. Transformation of these constructs into their respective hosts resulted in recombination only in *N. gonorrhoeae*. Reasons for failure in *B. (Moraxella) catarrhalis* could be several: one, *B. (Moraxella) catarrhalis* does not recognize the *cat* promoter used (gonococcal Opa promoter(61)); two, transformation was never achieved: no one has transformed *B. (Moraxella) catarrhalis* with anything but chromosomal DNA from other *B. (Moraxella) catarrhalis* strains using the pilus adherence technique (28). There could also be a methylation problem resulting in total restriction of the *E. coli* -derived plasmid DNA. Transformation was also attempted by electroporation (63) with out success; or thirdly, though not likely, there is only one 16S rDNA loci and interruption of this gene would be lethal. Should *B. (Moraxella) catarrhalis* be used in the future perhaps different strains might work better.

As a pilot experiment for introducing novel genes into the genome of gonococcus or related species, a project was begun to insert a reporter gene regulated with a gonococcal promoter into the genome of gonorrhea by homologous recombination with the 16S rDNA. The promoter chosen was

aniA from Pan 1 (69). Pan 1 is an inducible nitrite reductase (97) that is transcriptionally unregulated under anaerobic conditions. Anaerobiosis alters the expression of at least seven other gonococcal outer membrane proteins (97). Human convalescent serum reacts with Pan 1 suggesting anaerobiosis is a normal mode of metabolism for the gonococcus in humans. The terminal electron acceptor supporting growth in vivo appears to be nitrite (80). Nitrite is present in biological fluids, including urine, blood and saliva (80), and although gonococci cannot reduce nitrate to nitrite, other bacterial species which are normal flora in these sites can reduce nitrate to nitrite. When gonococci are grown anaerobically, *aniA* is transcribed and Pan 1 is produced (Figure 31).

The *aniA* promoter is complex, containing σ^{70} and *E. coli* "Gear box" consensus sites (68). Its expression may involve the presence of a repressor protein (68) similar to those genes in *E. coli* upregulated under anaerobic conditions. The full sequence of the *aniA* upstream region has not been published and the sequences of Pan 2 and Pan 3 proteins, also upregulated under anaerobic conditions in gonococci, are not known. The gonococcal sequencing project is not yet complete and searches using the *aniA* promoter as a probe did not find any homologous regions. Thus it may be that the sequences are not in the data bank or the promoters of Pan 2 and 3 are sufficiently different so as not to hybridize with the *aniA* promoter. What was used was the upstream 159 base pairs which included the gearbox promoter element but not the σ^{70} element, or any elements upstream of that. The original strain used for this sequence was R10 (68). This particular strain was not available but a related strain, F62, which Hoehn and Clark used for other

Pan 1 work, was available. After synthesis of primers based on the published sequence of the *aniA* promoter (68), a PCR amplification product was cloned and sequenced. The approximately 100 bp fragment proved to be chimeric, having the correct 5' and 3'-end sequences but the incorrect internal sequences, presumably found only in strain R10. Figure 28 shows the format of constructed internal primers to obtain the correct sequence for the *aniA* promoter. As a negative control, the incorrect promoter was also used to assemble the 16S reporter construct. It was termed pDRLux1400/pUP1. The MS11 transformant was termed simply 1400. In addition, an identical construct, CHLux, was created without the *aniA* promoter entirely.

The reporter gene chosen was the *Vibrio harveyi* luciferase gene, *lux* (GenBank accession # X58791, 47). The *luxAB* gene is derived from a six member operon. The luciferase enzyme, coded by *luxA* and *luxB*, catalyzes the following reaction: $FMNH_2 + RCHO + O_2 = FMN + RCOOH + H_2O +$ light, in which R is a straight chain aldehyde of seven or more carbon atoms, FMN is flavin mononucleotide, and $FMNH_2$ is reduced flavin mononucleotide. The production of light from bacterial luciferase can be detected with a photometer. A linear relationship exists between the amount of light produced to the amount of enzyme present. Bacteria which contain luciferase but no substrate (aldehyde) are phenotypically dark. Such cells can be made to emit light by the external addition of substrate to the growth media (25).

There are many reasons why bacterial luciferase is an excellent reporter gene. Comparing it with another common reporter gene, B galactosidase, 10^{11} molecules are needed to generate a measurable signal (50 U of enzyme; $A_{420} \approx 0.1$); in contrast, 10^5 molecules of luciferase emits 2.7 X 10^4 quanta of light which yields about 2,500 cpm in a scintillation counter with the coinci-

dence turned off. This is a difference in magnitude of 5 to 6 orders (25). The lower limit of light detection of luciferase is 100 molecules (62). The half life of *V. harveyi* luciferase is approximately 2 hrs. This is convenient for reactivation and regeneration of the protein for real time experiments, as opposed to enzymatic assays which are generally one time use only. Although oxygen is needed for the reaction, vanishingly small quantities are necessary. The threshold of oxygen requirement is 0.1 nM. The normal oxygen tension within eukaryotic cells in culture is 1 nM (33). Luciferase has been expressed in the anaerobe *Clostridium perfringens* (113), thus oxygen presence is of no practical concern. Luciferase has been used for the detection of gene expression (107, 112) as well as following infection in living hosts (33). And finally, the aldehyde substrate is readily absorbed through the cell membrane of both eukaryotic and procaryotic organisms with no apparent consequences (170), whereas the substrate for B-galactosidase is toxic to gonorrhea (143).

The *aniA-lux* transformant, 1400.1, emitted light, upon addition of substrate, under aerobic, microaerophilic, and anaerobic conditions, while the negative control group did not. Since the promoter was active in aerobic and anaerobic conditions, it appeared that the entire *aniA* upstream sequences were not present to yield proper anaerobic/aerobic control. A three hundred base pair upstream sequence was successfully used in a similar experiment using B- galactosidase as the reporter system in gonorrhea (143), but, these sequences were not available. Apparently the 'gearbox' portion of the *aniA* promoter is sufficient for constitutive expression. The construct did successfully integrate into the 16S region, as verified by PCR analysis (Figure 29). There was no background transcription of the promoter-less *lux* gene by virtue of its placement in the 16S rDNA region, as evidenced by no light

emission from the promoterless CHLux construct.

The growth curve comparison between wild type MS11 and the *aniA-lux* transformant, 1400.1, shows a decrease in growth in 1400.1. The inactivation of more than one of the 16S rDNA sequences could be the cause. PCR primers specific to each 16S loci would answer the question of which and how many sites are occupied. A growth rate comparison between 1400.1 and a transformant whose identical 16S rDNA locus was inactivated with a *cam* cassette only would also help answer the question whether the LuxAB proteins are at all toxic to gonorrhea, and therefore causing a decrease in growth rate.

Possible applications for use of 1400.1 could be to follow the course of infection in real time in cell culture and organ explant assays, or following ingestion in phagocytosis. Further manipulation could easily be obtained by replacement of the present promoter with the full functional *aniA* promoter once the sequence is published, or any other functional promoter.

Considering the general use of a recombination system using the 16S rDNA target for integration of novel genes into the genome of *Neisseria gonorrhoeae*, two caveats need to be addressed. Because there are four 16S rDNA loci in the gonococcal genome (169), and presumably only one of the four is involved with a recombination event for any given transformant, each locus may have its own unique genetic characteristics based on its respective genetic environments. It is possible that local DNA-dependent RNA polymerase activity may be different for each loci, making background transcription a problem. Each transformant should be assayed for this possibility. The other consideration is that of titration of promoter activity. Any promoter used whose counterpart is still intact in the genome would

cause a diluting effect on activity with the native promoter. This would not be a problem with novel promoter elements or where the host genomic copy of the promoter is completely inactivated.

The 1400 pUP1 vector is now poised to be a vehicle of transformation and recombination in *N. gonorrhoeae*. Any suitable construct could be inserted into the *Afl*II site within the 16S region. An engineered multiple cloning site could also be constructed into this site, allowing for convenient insertions. A unique *Bgl*II site on the pUP1 vector makes a convenient cut site for linearization, a scheme which often adds great efficiency to transformation in the gonococcus (54). The 1400 base pair 16S construct was chosen over the original 300 base pair 16S fragment because of efficiency of recombination: all transformants had integrated into a 16S region with the 1400 base pair version, whereas with the 300 bp constructs only a small fraction of transformants actually integrated into the 16S site. The remainder apparently integrated elsewhere in the genome.

6. Summary

A gonococcal expression library was screened with antibody against an outer membrane fraction. Sixteen unique proteins were identified. Among them was an 85,000 Dalton protein (Omp85) that was homologous to similar proteins in six other bacterial pathogens. Antibodies to the Omp85 analogs in two of these species, *Haemophilus influenzae* and *Pasteurella multocida,* were protective to live challenge in animal models. Omp85 protein was constitutively and universally expressed in all gonococcal and meningococcal strains tested. The genes encoding the Omp85 proteins from *N. gonorrhoeae* and *N. meningitidis* were cloned and sequenced. Insertional inactivation of the gene in *N. gonorrhoeae* resulted in a merodiploid state, suggesting the

essential nature of the gene.

A method of gene introduction into the genome of *N. gonorrhoeae* was developed using the 16S rDNA locus as an integration site. A construct using the gonococcal promoter, *aniA*, and the bacterial luciferase gene, *luxAB*, was created to assay potential function of the integrated gene. The transformant, 1400.1, was isolated and assayed for luciferase activity. Expression was constitutive under aerobic and anaerobic conditions. An integration vector, 1400 pUP1, has been constructed for further manipulations of this kind in *N. gonorrhoeae.*

7. Literature

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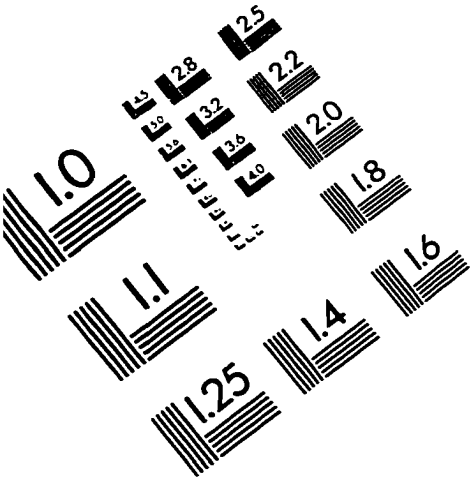
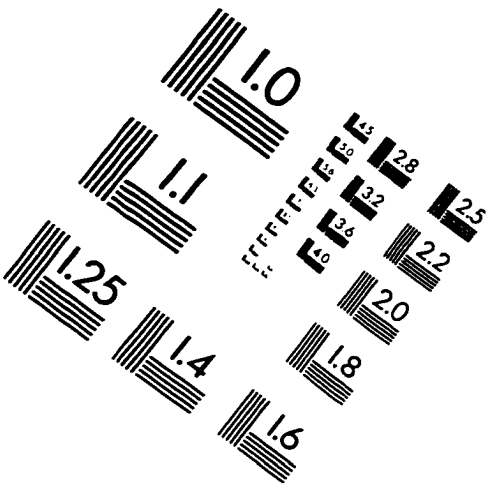
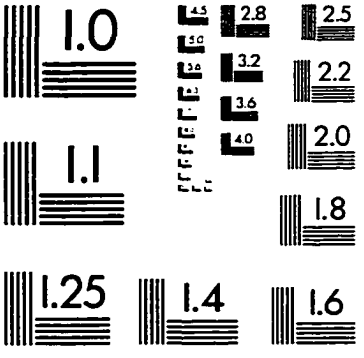
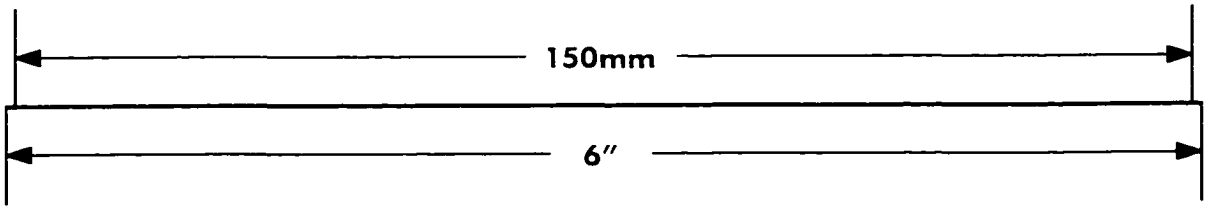
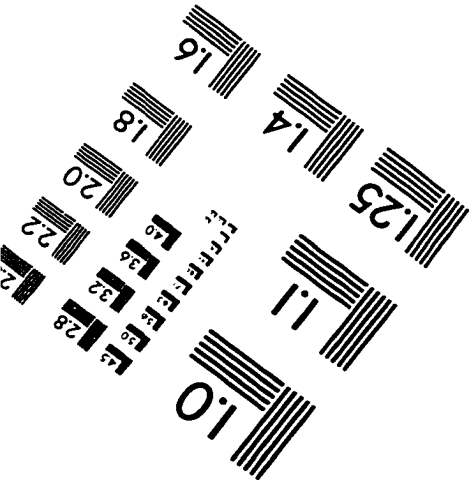
IMAGE EVALUATION TEST TARGET (QA-3)

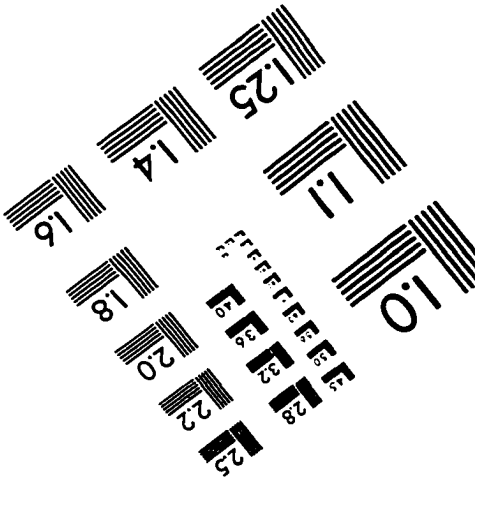












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