# ABSTRACT

Title of Thesis: THE EFFECT OF THE DELETION OF OPA 5 ON THE ABILITY OF *N. GONORRHOEAE* STRAIN MS11 TO TRANSCYTOSE A POLARIZED T84 EPITHELIAL CELL MONOLAYER

Meredith Ann Davis, Master of Science, 2004

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*Neisseria gonorrhoeae* can express 11 different opacity proteins (Opa). Plasmids were constructed that allowed for the generation of strains containing deletions in each Opa-encoding-gene (amplified by PCR and cloned into pUC19). The Opa-encoding region was replaced with a spectinomycin resistance cassette and transformed into *N. gonorrhoeae*. The biological properties of one of these transformants (MS11 Opa5 delta s) was tested in a transcytosis assay. Wildtype MS11, was able to efficiently cross a polarized epithelial monolayer of T84 cells within 6 hours and occurred in both the apical to

basolateral and the basolateral to apical directions. The number of cells that crossed the monolayer depended upon the dose of the inoculum. Transcytosis of MS11 Opa5 delta s occurred in the apical to basolateral direction, but was delayed. The data indicate that gonococci use multiple mechanisms of transcytosis, and the Opa 5 protein is important for rapid apical to basolateral transcytosis.

# THE EFFECT OF THE DELETION OF OPA 5 ON THE ABILITY OF *N. GONORRHOEAE* STRAIN MS11 TO TRANSCYTOSE A POLARIZED T84 EPITHELIAL CELL MONOLAYER

by

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# DEDICATION

To everyone who has helped me along the way

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LIST OF ABREVIATIONS

β = beta
C = Celsius
$^{0}$ = degrees
Δ = deletion
kb = kilobase
λ = lambda
μg = microgram
μl = microliter
ml = milliliter
PCR = polymerase chain reaction
Xgal = 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
% = percent
PID = pelvic inflammatory disease
DGI = disseminated gonococcal infection
HIV = human immunodeficiency virus
Opa = colony associated opacity total
LOS = lipo-oligosaccharide
HSPG = heparin sulfate proteoglycan receptor
CEACAM = carcino-embryonic cellular adhesion molecule
IgA = immunoglobulin A
IgG = immunoglobulin G
TEM = transmission electron microscopy
EDTA = Ethylenediaminetetraacetic acid
DNA = deoxyribo nucleic acid
ABTS = 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
Ω = Ohms
PBS = phosphate buffered saline
V = volts
mAb = monoclonal antibody
TBE = Tris Borate EDTA
HRP = horseradish peroxidase
MOI = multiplicity of infection

# Introduction

## Epidemiology of *N. gonorrhoeae*

*Neisseria gonorrhoeae*, a member of the family *Neisseriaceae*, is a Gram negative, aerobic, diplococci that is catalase and oxidase positive and can metabolize carbohydrates oxidatively. Many species of *Neisseria* are non-pathogenic, but both *N. meningitidis* and *N. gonorrhoeae* are able to cause disease. While *N. meningitidis* is an opportunistic pathogen that can colonize the nasal passages of healthy individuals and not cause disease, *N. gonorrhoeae* is an obligate human pathogen responsible for causing the disease gonorrhea. Colonization by the gonococcus occurs along mucosal surfaces primarily in the urethra, cervix and other areas of the urogenital tract. Ocular infections also occur in infants during childbirth through an infected birth canal [1].

Despite effective antibiotic therapy, gonorrhea is one of the most common sexually transmitted diseases. Current data from the Center for Disease Control and Prevention shows that there were over 650,000 reported cases of gonorrhea in the United States alone, and there were an estimated 60 million new gonococcal infections worldwide in 1999 [2]. Transmission of *N. gonorrhoeae* occurs primarily by sexual contact.

## Clinical Syndromes of *N. gonorrhoeae*

*N. gonorrhoeae* infection results in a wide variety of diseases, and symptoms vary in men and women. In approximately 95% cases, men experience purulent discharge and dysuria within days of infection and receive treatment [1]. Rarely do complications occur, but some complications, such as prostatisis, epididymitis, and seminal vesculitis, can develop [3, 4].

In women the primary site of colonization is the cervix. Unlike men, women are frequently asymptomatic, and because of this women can serve as a reservoir for infection. Studies have estimated the percentage of asymptomatic female carriers to be between 25% and 80%, and the persistence of gonorrhea in the general population can be largely attributed to the large number of asymptomatic carriers [5]. When symptoms, such as vaginal discharge, dysuria, and abdominal pain do occur, they often pass quickly and are ignored. Because the symptoms are ignored, women with gonococcal infections have a disproportionate number of complicated gonococcal infections such as pelvic inflammatory disease (PID). Inflammation caused by PID can cause fallopian tube scarring which can result in infertility [3-6]. Approximately 1-3% of female cases lead to disseminated gonococcal infections (DGI). The percentage of DGI is much lower in men. Recent work indicates that HIV infections are facilitated by urethritis, particularly those caused by *N. gonorrhoeae* [7-9].

**Pathogenesis of *N. gonorrhoeae***

For a bacterial pathogen to be able to survive in its host and cause disease it must be able to interact with host cells. The disease gonorrhea develops when *N. gonorrhoeae* is inoculated into the urogenital tract of humans. In males initial attachment is essential. In women attachment is not always necessary and it can be advantageous for gonococci to remain unattached during certain times of a woman's menstrual cycle. Because of the varied environments that *N. gonorrhoeae* encounters, it is important that the organism adapts to its surroundings. One mechanism that makes this possible is the variation of antigens present on the surface of gonococci. Upon introduction into the host, *N. gonorrhoeae* can form an initial adhesion to the columnar mucosa via molecules known

as pili. This transient attachment can be replaced by a stronger, more intimate, attachment. While it is unclear what molecules are ultimately needed for this stronger attachment both colony-associated opacity proteins (Opas) and lipooligosaccharide (LOS) have been implicated. From this point the gonococcus can then invade the human cell, transcytose through it, passage between the cells via a loosening of the tight junctions, or simply remain attached to the apical surface of the cell and illicit an immune response which causes the inflammation and tissue damage associated with gonorrhea. It remains unclear what factors, bacterial and environmental, dictate the fate of the gonococcus once it is attached to the human cell. Gonococcal surface molecules undergo both phase and antigenic variation, which allows them to stay viable and infectious in the constantly changing environment of the urogenital tract. Possibly this antigenic variation is responsible for the variation in disease progression and presentation that is seen with gonorrhea.

**Surface Molecules of *N. gonorrhoeae***

The *N. gonorrhoeae* surface contains many molecules important in pathogenesis, many of which are variably expressed. One important group of surface molecules that allows for the initial attachment to human non-ciliated epithelial cells are type IV pili, particularly the PilC protein located at the tip of the pili [10-15]. Non-piliated gonococci are believed to be avirulent in men, and in male human challenge studies pili were found to be necessary for the primary colonization of the male urethra [16, 17]. Once the pili has adhered to the surface of the epithelial cells, the pili retract bringing gonococci closer to the surface of the cell and allowing for a more intimate attachment caused by the binding of other molecules [15, 18]. Between eight and sixteen hours post infection, the

pili appear to be shed, allowing for a tighter attachment with the host cell plasma membrane [18]. Pili are phase-variable, a fact that the gonococcus uses to its advantage. The human urogenital tract is a varied environment and gonococci need to be able to adapt along with the changing environment. Binding to cells is sometimes advantageous, such as in the male urethra where the constant flow of liquid would otherwise wash the gonococci away before colonization occured. Conversely, the surface of the female urogenital tract is in constant flux, and particularly during menstruation, gonococci would not be advantageous to be adhered to the uterine lining because it would be shed along with the uterine epithelial cells that are shed during this time.

Another surface structure important for the pathogenesis of *N. gonorrhoeae* is LOS. LOS consists of three oligosaccharide chains attached to a lipid A core [19]. While the core structure is highly conserved, the terminal oligosaccharides undergo phase variation that is controlled by changes in the number of guanines in the middle of the coding sequences of the glycosyl transferases genes required to build them [20-23]. While conflicting evidence exists as to the importance of LOS in invasion into epithelial cells, human challenge studies have shown a role for LOS in invasion [24-26]. Further research has shown that in the absence of Opa proteins, lacto-*N*-neotetraose LOS promotes invasion into cultured epithelial cells, although in lower numbers than when Opa is present [27].

**Opa Structure**

Opa proteins are an integral surface molecule in gonococcal pathogenesis. These proteins are predicted to consist of an eight-stranded β-barrel in the outer membrane with four extracellular loops [28, 29] Three of those loops contain variable sequence domains

called semivariable (SV) and hypervariable (HV-1 and HV-2), while the fourth loop is highly conserved [29]. They have an approximate molecular mass of 28 kDa.

**Opa Genetics**

The DNA sequence of the chromosome of one strain of *N. gonorrhoeae* has been determined, FA1090. The genome of FA1090 contains 11 distinct opa loci [30]. The 11 Opa proteins are designated A through K based on their descending size in Southern blot of *N. gonorrhoeae* strain MS11 chromosomal DNA [31]. Although the gonococcal chromosome contains 11 Opa alleles, 11 Opa proteins are not always found expressed on the surface of gonococci due to phase and antigenic variation that occurs during infection [32-34]. The genes are all constitutively transcribed but their translation is controlled by changes in the 5' region of each gene known as leader peptide region. The high frequency phase variation has been attributed to changes in the number of the repeated pentameric sequence CTCTT in this region. Addition or deletion of one or several repeats effects on/off switching by positioning the ATG initiation codon and the mature Opa encoding portion of the gene either in or out of frame [35]. This phase variation allows gonococci to express any number of Opa proteins at any point during infection.

**Opa Pathogenesis**

While the precise role of each Opa protein remains to be characterized, human challenge studies point to the overall importance of Opa during infection. Human challenge studies found that gonococci recovered from urogenital, cervical, or rectal infections are typically Opa positive, even when the inoculums were Opa minus [33, 36]. It is widely believed that Opa proteins mediate an intimate attachment to epithelial cells after the initial attachment formed by pili, and in some cases induce invasion into the

eukaryotic cell [37-39]. It has been speculated that the panel of Opa proteins expressed may play a role in whether or not gonococci invade or remain adhered to the outside surface of the epithelial cell.

Opa proteins are broken down into two categories based on their binding specificities. The two major host receptors that have been shown to interact with Opa proteins are the heparan sulphate proteoglycan receptor (HSPG) and the carcino-embryonic antigen cellular adhesion molecule (CEACAM), previously called the CD66 receptor. Only two Opas, Opa A ($Opa_{50}$) and Opa C ($Opa_{52}$) bind to the HSPG receptor, while many of the Opa proteins bind to CEACAMs. The HSPG receptor is found localized at the basolateral surface of polarized epithelial cells [40]. Upon Opa binding to the HSPG receptor, more HSPG receptors are recruited to the cell surface, along with F-actin and tyrosine-phosphorylated proteins [41, 42]. Gonococci have been observed to be internalized with the HSPG receptor by some human cell lines without clathrin-coated pit formation. This internalization involves signaling via a phosphatidylocholine-specific phospholipase and acidic sphingomyelinase [41, 43]. In certain cell lines, adhesion and internalization can also be facilitated by extracellular matrix proteins, including fibronectin or vitronectin [44-47]. This process has been suggested to require phospholipase C [46].

Opas B, C, D, E, F,G, H, I, J, and K all bind to CEACAMs found on the surface of polymorphonuclear, epithelial, and endothelial cells [48, 49]. Table 1 lists the cell types that express each one of the CEACAMs that Opa proteins are known to bind. For a review of the CEACAM nomenclature see Popp et al. [50]. In contrast to the HSPG receptor, these receptors are found predominantly on the apical surface of polarized

epithelial cells. When gonococci are exposed to epithelial cells, their Opa proteins are able to bind residues exposed on the N-terminal domain of CEACAM receptors [50]. The presence of different Opa proteins on the bacterial surface, along with different CEACAM receptors on the eukaryotic cell surface, influence the fate of gonococci, as differential specificities do exist [51-53]. For instance, when OpaE is present along with CEACAM1 or CEACAM5, gonococcal transcytosis across a tight-junction-forming monolayer of epithelial cells has been observed [54]. When OpaD is present along with CEACAM1 or CEACAM5, an opsonin-independent pathway of uptake by professional phagocytes is induced [55].

Once bound to the surface of the eukaryotic cell, *N. gonorrhoeae* can be internalized. When Opa proteins bound to HeLa cells transfected with the CEACAM5 receptor, adherence and invasion were noted [52, 53, 56, 57]. The binding of CEACAMs by gonococci then elicited a Src-like tyrosine kinase and Rac1-dependent signaling pathway [58]. Upon contact with the host cell plasma membrane, gonococci initially form microcolonies that loosely associate with elongated microvilli [37]. Griffiss et al. showed that Opa are vital for microvilli elongation to occur [37]. As has been shown in both epithelial cell lines and primary human urethral epithelial cells, this adherence induces membrane ruffling, rearrangement of the host cell actin cytoskeleton, and eventually the engulfment of gonococci [27, 59]. Controversy exists as to the fate of gonococci once inside the cell [60, 61]. Some studies indicate the presence of a

**Table 1. Summary of Opa-CEACAM Interaction**

| CEACAM | CD66 | Name | Cell Type Present On | Opa Recognized | Reference |
|---|---|---|---|---|---|
| 1 | a | BGP | Epithelial, Endothelial, and Neutrophils | B,C,D,E,G,H,I,J,K | [49, 53, 62, 63] |
| 3 | d | CGM1 | Neutrophils | B,C,G,I | [53, 62, 64] |
| 4 | | CGM7 | Epithelial | none | |
| 5 | e | CEA | Epithelial and Neutrophils | B,C,D,E,F,G,H,I,J,K | [53, 62, 65] |
| 6 | c | NCA | Epithelial and Neutrophils | B,C,G,I | [62, 66] |
| 7 | | CGM2 | Leukocytes | none | |
| 8 | | CGM6 | Neutrophils | | [39] |

*T84 cells express CEACAMs 1,5,6 [54]

phagosomal membrane surrounding intracellular gonococci [67]. Normally cellular material taken up by phagosomes would be degraded in the phagolysosome, but the gonococci's IgA I protease has been implicated in the gonococcal ability to survive in the host cell because of its capacity to cause alteration in lysosomes [68, 69]. Other studies suggest that gonococci do not enter vacuoles, but instead remain in the host cell cytoplasm [60, 70]. Intracellular growth studies and TEM observations suggest that gonococci are able to grow inside cells [60, 70].

**Transcytosis**

Upon binding to epithelial cells the majority of gonococci remain bound to the surface of the cell. Evidence exits to suggest that a small number of bacteria are able to traverse the epithelial cell monolayer [54, 71, 72]. Once internalized into the epithelial cell, some gonococci are able to survive and traverse the host cells basolateral membrane to reach the sub-epithelial mucosa, and Opa binding to CEACAMs has been shown to be sufficient to mediate this traversal [54]. Infection of organ cultures derived from fallopian tubes and nasopharynx have demonstrated that gonococcal traversal to the basolateral region and movement to the stromal matrix [73-75]. Surface molecules such as pili and Opa have been seen to be important for transcytosis, although the amount of time that gonococci took to become detectable at the basolateral surface varied from 12 to 48 hours depending on the cell line used for the study [54, 76]. Most work studying the rate of transcytosis has been done with phenotypically Opa minus strains. Opa minus *N. gonorrhoeae* typically first appear to cross human colonic epithelial cell line T84 monolayers in 36 to 40 hours, although fast trafficking mutants have been isolated [71]. These fast trafficking mutants were linked to an increase in intracellular growth [71].

Recent evidence suggests that passage through the monolayer may occur even before this, within 30 minutes in HEC1-B cells [72]. It is important to note that despite their morphological similarities to primary epithelial cells, HEC1-B cells exhibit inconsistent trans-epithelial resistances and are leaky to [$^{14}$C]-mannitol [72]. The loose tight junctions of HEC1-B cells probably allow gonococci to traverse the monolayer more easily.

Most findings to date imply an intracellular route rather than a paracellular route for gonococcal transcytosis, as evidenced by the sustained trans-epithelial resistance and the presence of intact tight junctions at the apical border of adjacent cells in electron micrographs [54]. Other Gram negative pathogens such as *E. coli* and *Salmonella* have been shown to disrupt organization of tight junctions and inhibit barrier function to allow for paracellular migration to occur. The possibility that gonococci traverse through a paracellular pathway does exist [77-80]. In addition, Mosleh et al. found using electron microscopy of human ureteral cells, that *N. gonorrhoeae* strain MS11, the strain used in this study, was able to disrupt intercellular connections and infiltrate deeper cell layers by a paracellular route [11].

**Polarized Epithelial Cell Model System T84**

The first host cell type that *N. gonorrhoeae* come into contact with are epithelial cells. Epithelial cells cover the internal surfaces of the human body's surface and organs. They form sheets of tightly connected cells and create a barrier between body compartments via the formation of tight junctions. These junctions bring neighboring cells together, preventing passage between cells. Epithelium are one of the important components the innate immune system and provide a first line of protection of from

invading pathogens. Epithelial cells also polarize to form distinct functional domains known as the apical (external) and basolateral (internal) domains [81].

When gonococci are introduced into humans they first come into contact with the apical surface of the host's epithelial cell. To establish infection, gonococcis colonize, invade, and transmigrate across the host epithelium. Because gonococci interact with polarized cells *in vivo*, it is important to replicate this interaction when studying gonococcal infection *in vitro*. Most cell lines used to study gonorrhea, such as HEC1-B, ME180, and HeLa, originated from physiologically relevant tissues such as the cervix or endometrium. These cell lines, however, are not polarizable, meaning they do not form distinct apical and basolateral surfaces that are separated by tight junctions.

The cell line used in this study was a human colon carcinoma cell line named T84. While not from the urogenital tract, T84 cells have been extensively studied and have been used for the investigation of the adhesion, invasion, and transcytosis of other pathogens including *Salmonella*, *Shigella*, *Helicobacter*, and *E.coli*, as well as for polymorphonuclear leukocyte migration [80, 82-88]. These cells form a robust barrier function due to the formation of tight junctions when grown on filter supports that allow them to feed from the basolateral surface [89, 90]. In addition, they generate numerous microvilli on their apical surface, and show structural similarities to epithelial cells *in vivo* [90]. While the colon is not the primary site of infection for *N. gonorrhoeae*, anorectal colonization is seen in approximately 40% of infected women and homosexual men [91]. This cell line has also been used extensively in *Neisserial* studies, particularly those studying the traversal of *Neisseria* through the epithelium [18, 54, 71, 76]. These

characteristics make the T84 cell line one of the best cell lines, among those available, to study gonococcal interaction with a polarized host epithelial cell membrane.

**Thesis Focus:**

*N. gonorrhoeae* possesses many surface molecules important for pathogenesis, Opa proteins being one of them. The presence of these Opa proteins allows gonococci to tightly adhere to human epithelial cells and transmigrate across the cell monolayer. Phase and antigenic variation of these molecules potentially allows them to adapt to the ever changing environment of the urogenital tract. This fact, combined with the idea that the expression of different Opa proteins on the surface of the gonococcus may influence the fate of the bacteria supports the notion of the importance of Opa in gonococcal pathogenesis. Studying individual Opa proteins may shed light as to which Opas or which combinations of Opas are integral for gonococcal traversal of the human epithelium. Construction of individual Opa deletions would allow for the initial characterization of the importance of each individual Opa protein in gonococcal infectivity.

To determine the importance of one Opa protein in traversal of the epithelium, this study seeks to create plasmids with each Opa individually deleted and then to test one of deletions in a transcytosis assay. FA1090 was chosen as the parent strain because it is the only gonococcal strain with a sequenced genome. The putative loci for each of the 11 Opa genes were located in FA1090 chromosome and PCR primers were designed to amplify the genes. These genes were cloned into pUC19 and transformed into *E.coli* DH5α mcr. Upon extraction of the plasmids a deletion PCR was done such that the coding portion of the gene was deleted. Areas upstream and downstream of the coding

region were amplified by PCR, ligated on themselves, and transformed again into *E.coli* DH5α mcr. Upon extraction of the plasmids, they were cut with restriction enzymes and a spectinomycin resistance cassette was inserted to allow for selection when transformed into *N. gonorrhoeae*. These spectinomycin resistant plasmids were again transformed into *E.coli* DH5α mcr and extracted. One plasmid, the Opa 5 deletion plasmid, was then transformed into gonococcal strain MS11. The gonococcal strain MS11 was chosen because this strain has been used in many invasion studies, including male human challenge studies. This strain was used in transcytosis experiments of T84 cells to determine if the deletion of the Opa gene inhibited or enhanced the trancytotic ability of the deletion strain. The data indicated that while the parent strain MS11 was able to transcytose in both the apical to basolateral and the basolateral to apical direction in less than 6 hours, the transcytosis of the Opa deletion strain was delayed. From this it can be concluded that *N. gonorrhoeae* strain MS11 can rapidly transcytose from either side of polarized T84 cells and that the deletion of Opa 5 inhibits this process.

**Specific Goals:**

1) To engineer 11 individual Opa deletion plasmids that can be transformed into from the chromosome of *N. gonorrhoeae*.
2) To determine the effect of the Opa5 deletion on the transcytotic ability of *N. gonorrhoeae* strain MS11.

## Materials and Methods

### Culture Conditions

*N. gonorrhoeae* strains FA1090, F62, and MS11 were grown on GCK agar or GCP broth supplemented with Kellogg's solution and sodium bicarbonate (0.042%) at 37$^{o}$C with 5% $CO_2$. *E. coli* were grown in LB broth or on LB plates. When needed Ampicillin (60µg/ml), Spectinomycin (50µg/ml), or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) (20µg/ml) were added to the growth media [92]. Broth cultures were grown in a rotary shaker at 37$^{o}$C. All bacterial strains are listed in Table 2 and media components are given in Table 3.

### Chemicals, Reagents, and Enzymes

Chemicals were of analytical grade or better and were purchased from Sigma (St. Louis, MO) unless otherwise specified. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) and were used with the supplied buffers according to the manufacturer's instruction.

### Sequence Analysis

Opa encoding-gene sequences were located by searching the CTCTT repeat characteristic of Opa encoding-genes in the *N. gonorrhoeae* sequenced genome found at the University of Oklahoma www.genome.ou.edu/gono.html. Verification of these sequences as true Opa encoding-genes was done by a BLAST search, found at www.ncbi.nlm.nih.gov. Once the sequences were verified as Opa encoding sequences, they were aligned using the ClustalW program from the European Bioinformatics Institute (EMBL-EBI). ClustalW can be found at www.ebi.ac.uk/clustalw.

**Table 2. Bacterial Strains**

| Strain | Genotype/Phenotype | Source |
|---|---|---|
| ***E. coli*** | | |
| DH5αMCR | F-mcrAΔ(mrr-hsdRMS-mcrBC) endA1supE44thi-1recA1gyrA relA1 Δ(lacIZYA-argF) U169 deoR(Φ89dlacD(lacZ)M15) | BRL, Life Technologies |
| ***N. sicca*** | | |
| 342 | | H. Schneider |
| ***N. gonorrhoeae*** | | |
| MS11 | Poly-lactosyl LOS | H. Schneider |
| FA1090 | Full α and β chain LOS | J. Cannon |
| F62 | Full LOS α chain, no β chain | P.F. Sparling |
| F62Δ8-1 | 239-bp *ApoI* deletion in lgtA; truncated lactosyl α chain | D. Stein |
| MS11opa5Δs | pOpa5Δs transformed into MS11 | This work |
| ***C. jejuni*** | | |
| 587 | Non-transcytosing clinical isolate from poultry | Jianghong Meng |

**Table 3. Media composition ***

| | |
|---|---|
| **GCK** | |
| Gonococcal Base Medium (Difco) | 37.0 g |
| Bacto-Agar (US Biologicals) | 5.0 g |
| Kellogg's | 10.0 ml |
| **GCP** | |
| Protease Peptone #3 (Difco) | 15.0 g |
| Soluble Starch (Difco) | 1.0g |
| $KH_2PO_4$ (Sigma) | 1.0g |
| $K_2HPO_4$ (Sigma) | 4.0g |
| NaCl (US Biological) | 5.0g |
| HPLC $H_20$ to 1 L | |
| **Kellogg's** | |
| Glucose (Sigma) | 400 g |
| Glutamine (Sigma) | 5.0 g |
| Ferric nitrate (Sigma) | 0.5 g |
| Thiamine pyrophosphate (?) | 0.02 g |
| HPLC $H_2O$ to 1 L | |
| **LB Broth** | |
| LB Broth Base (US Biological) | 25.0 g |
| HPLC $H_2O$ to 1L | |
| **LB Agar** | |
| LB Agar Base | 40.0 g |
| HPLC $H_2O$ to 1 L | |

* = All media was sterilized by autoclaving except Kellogg's which was filter sterilized.

**TSA with 5% Sheep's Blood** Remel

**Polymerase Chain Reaction (PCR)**

PCR reactions were done using the Expand Long Template PCR System (Roche). PCR reactions were cleaned using the QIAquick PCR Purification Kit (Quiagen). All primers used are listed in Table 4.

**Plasmid and DNA isolations**

All plasmid names are listed in Table 5. Plasmids were isolated by alkaline lysis method [92]. Chromosomal DNA was isolated using the Wizard Genomic DNA purification Kit (Promega).

***E. coli* Transformations**

Competent *E. coli* cells were prepared by the Inoue Method [92]. For transformation, competent cells were thawed on ice for 15 minutes, DNA was added, and the mixture was incubated for 10 minutes. Cells were heat shocked at $37^0$C for 2 minutes. To this 950 µl of LB broth was added, and cells were incubated at $37^0$C in a rotary shaker for 30-45 minutes. Various dilutions were plated onto LB and LB-antibiotic plates [92].

***N. gonorrhoeae* Transformations**

Piliated *N. gonorrhoeae* were incubated with plasmid DNA for 4 hours in the presence of 1mM $MgCl_2$, 0.042% $NaHCO_3$, and 1% Kelloggs solution, in a rotary shaker at $37^0$C. Various dilutions were plated onto GCK and GCK-spectinomycin (50µg/ml) plates and incubated at $37^0$C for 48 hours.

**Table 4. Primers used in this study**

| Name | Primer Sequence | Restriction Site | Ref |
|---|---|---|---|
| Opa 1F | GCGGAATTCTACATCATCTTCTCCCATAT | EcoRI | A |
| Opa 1R | CGCAAGCTTCATCGCATTACCTTTGGTTG | HindIII | B |
| Opa 2F | GCGGGATCCAGGGCGGTGTCGAAGGCAAA | BamHI | C |
| Opa 2R | CGCAAGCTTTCTCTAGATTCCGCATCC | HindIII | D |
| Opa 3 F | GCGGAATTCGGGGCGACGACTCGTCCAA | EcoRI | E |
| Opa 3 R | GCAAGCTTCCCATTGTTGCGGGAGGCTT | HindIII | F |
| Opa 4F | GCGGAATTCAAGAAGGAATGCCCGAACCG | EcoRI | G |
| Opa 4R | CGTAAGCTTCCGCCTTGAAACACCGGGTT | HindIII | H |
| Opa 5F | GCGGAATTCCCGCCCTGTCGCCTTTAGAC | EcoRI | I |
| Opa 5R | GCTAAGCTTCGCGGATGGTGGGTTTAGGA | HindIII | J |
| Opa 6F | CCATGCAGGCGGGAATTCAAACT | EcoRI | K |
| Opa 6R-Redo | TTTTAAGCTTGGTGTCGTCCACGGCTTTGATGGCTTTG | HindIII | L |
| Opa 7F | GGGAAGCTTAATGCGAACGCTGCTGGCAT | HindIII | M |
| Opa 7R | CGCGAATTCATAGAAATGACGAAATTTTAG | EcoRI | N |
| Opa 8F | GGGAAGCTTGCGTACCGAAGCTTTGTTCG | HindIII | O |
| Opa 8R | GCAGAATTCGTTTGTTATCCCAATAATGCA | EcoRI | P |
| Opa 9F-new | CGTGGATCCGGGGAGAGGGCTCCCCGAATT | BamHI | Q |
| Opa 9R-Redo | AAAAGCATGCCCAAGCCGGTTCAACCAAAGCTGGATTAAAG | | R |
| Opa 10F-new | ATCGAATTCAAAACCGTTTTTCCCG | EcoRI | S |
| Opa 10R-Redo | AAAAGCATGCCTACGCCAGCATTATTTCTACGCTCAAAGAC | SphI | T |
| Opa 11F | GCGAAGCTTGAGGATTTGTACGAAGAGCT | HindIII | U |
| Opa 11R | GGTGAATTCAAAAAACCGATGGTTAAATA | EcoRI | V |
| Omega-Xho-F | GACCTCGAGTTGCAAACCCTCACTGATCC | XhoI | W |
| Omega-Xho-R | CAGTCTCGAGGAGTTAAGCCGCGCCGCGAA | XhoI | X |
| Omega-Pst-F | GACCTGCAGTTGCAAACCCTCACTGATCC | PstI | Y |
| Omega-Pst-R | CAGTCTGCAGGAGTTAAGCCGCGCCGCGAA | PstI | Z |
| Opa3'Del-PstI | ATATCTGCAGACCCACGAAGCCTCATTGGGC | PstI | AA |

| | | | |
|---|---|---|---|
| -MD | | | |
| Opa5' INV PstI | CCCCTGCAGGGTTCCGGGCGGTGTTTCAA | PstI | BB |
| Opa3'del-XhoI -MD | AAACTCGAGACCCACGAAGCCTCGTTGGGC | XhoI | CC |
| Opa5'inv-Pst-Frag5 | CCCCTGCAGAGGTTTGGAGCGATGTGCCAA | PstI | DD |
| Opa5'inv-XhoI -Frag6 | AAACTCGAGGGGTTCCGGGCGGTGTTTCAA | XhoI | EE |
| Opa5'-inv-Pst-Frag9+10 | AAACTGCAGCAGATTTGTTTTGCCGGGATAT | PstI | FF |

**Table 5. Plasmids used in this study**

| Plasmid | Size(kb) | Vector Base | Reference |
|---|---|---|---|
| pUC19 | 2.7 | | New England Biolabs |
| pOpa1 | 2.2 | pUC19 | A,B |
| pOpa2 | 2.4 | pUC19 | C,D |
| pOpa3 | 1.8 | pUC19 | E,F |
| pOpa4 | 2.5 | pUC19 | G,H |
| pOpa5 | 2.3 | pUC19 | I,J |
| pOpa6 | 3.6 | pUC19 | K,L |
| pOpa7 | 2.2 | pUC19 | M,N |
| pOpa8 | 2.3 | pUC19 | O,P |
| pOpa9 | 3.8 | pUC19 | Q,R |
| pOpa10 | 3.3 | pUC19 | S,T |
| pOpa11 | 2.8 | pUC19 | U,V |
| | | | |
| pOpa1Δ | 4.0 | pOpa1 | AA,BB |
| pOpa2Δ | 4.2 | pOpa2 | AA,BB |
| pOpa3Δ | 3.5 | pOpa3 | AA,BB |
| pOpa4Δ | 4.5 | pOpa4 | AA,BB |
| pOpa5Δ | 4.1 | pOpa5 | AA,DD |
| pOpa6Δ | 5.1 | pOpa6 | CC,EE |
| pOpa7Δ | 4.1 | pOpa7 | AA,BB |
| pOpa8Δ | 3.4 | pOpa8 | CC,EE |
| pOpa9Δ | 5.6 | pOpa9 | AA,FF |
| pOpa10Δ | 5.1 | pOpa10 | AA,FF |
| pOpa11Δ | 4.6 | pOpa11 | AA,BB |
| | | | |
| pOpa1Δs | 5.4 | pOpa1Δ | Y,Z |
| pOpa2Δs | 5.6 | pOpa2Δ | Y,Z |
| pOpa3Δs | 4.9 | pOpa3Δ | Y,Z |
| pOpa4Δs | 5.9 | pOpa4Δ | Y,Z |
| pOpa5Δs | 5.5 | pOpa5Δ | Y,Z |
| pOpa6Δs | 6.5 | pOpa6Δ | W,X |
| pOpa7Δs | 5.5 | pOpa7Δ | Y,Z |
| pOpa8Δs | 4.8 | pOpa8Δ | W,X |
| pOpa9Δs | 7.0 | pOpa9Δ | Y,Z |
| pOpa10Δs | 6.5 | pOpa10Δ | Y,Z |
| pOpa11Δs | 6.0 | pOpa11Δ | Y,Z |

**Table 6. Solution compositions**

**DNA Isolation and Analysis**

Solution I:
25 mM Tris (pH 8.0), 50 mM glucose, 10 mM EDTA

Solution II:
0.1% SDS, 0.2 N NaOH

Solution III:
3 M potassium, 5 M Acetic Acid (pH 4.8)

TBE Buffer:
89 mM Tris (pH 8.0), 89 mM Boric Acid, 2 mM EDTA

**Cell Culture Conditions**

Colonic carcinoma cell line T84 (ATCC) were propagated in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 containing 1.2g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate (ATCC), supplemented with 7% fetal bovine serum (HyClone) and 1 % Pen/Strep. Cells were subcultured as necessary by adding 3 ml of 0.25% trypsin, 0.03% EDTA (Mediatech, Inc.) and incubating at $37^0$C until detachment from the cell culture dish occurred. Cells were then split 1:4 with media. Medium was renewed 2 times per week.

**Horseradish Peroxidase Procedure**

A solution of 1 μg/ml horseradish peroxidase in PBS was added to the apical chamber of transwells containing a polarized monolayer of T84 cells. $10^6$ gonococci were added and incubated at $37^0$C with 7% $CO_2$ for 6 hours. At 6 hours, the apical and basolateral media was collected, serially diluted. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate was prepared by dissolving 3 tablets in 20 ml of Sodium Citrate and 2 μl of 30% $H_2O_2$. 100 μl of substrate was added to each well and allowed to develop. The absorbance at 595 nm was measured. As a background control 100 μl of invasion media was mixed with 100 μl of substrate, and the absorbance reading of this was subtracted out from all other readings. The absorbance at 595 nm in the basolateral media was divided by the absorbance in the basolateral media, and that number was multiplied by 100 to give the percentage of HRP in that had passed through the monolayer.

**Invasion Protocol**

Approximately 2 weeks before invasion, 3 x $10^4$ T84 cells were seeded onto polycarbonate Transwell filters with a pore size of 3µm (Costar). Cells were propagated in culture media with fluid renewal every 2-3 days. Trans-epithelial resistance was assessed with an electrode (Millipore), and monolayers with electrical resistances of >1500 $\Omega \cdot cm^2$ were used to transcytosis assays.

Approximately 18 hours prior to the experiment bacterial cultures were struck onto fresh media to ensure that live cells would be used for the invasion experiment.

On the day of experiment bacteria were suspended to a Klett of 100 and diluted to a concentration of 1 x $10^6$ cells/ml for apical to basolateral invasions and 1 x $10^5$ cells/ml for basolateral to apical invasions in media consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5mM sodium pyruvate, supplemented with 5% fetal bovine serum and 0.5% Kellogg's. T84 cells were washed 4 times with phosphate buffered saline (PBS) and 1 time with the invasion media described above. Trans-epithelial resistance was measured to ensure polarization. Approximately 1 x $10^5$ bacteria were added to either the apical or basolateral domains of the transwell depending on the directionality of the invasion experiment. For apical to basolateral experiments 1 ml of invasion media was added to the basolateral domain. For basolateral to apical invasions 200 µl of invasion media was added to the apical domain. Cells were then incubated at $37^0$C with 7% $CO_2$ for indicated times. Apical and basolateral fractions were then collected, diluted, and plated to determine the number of bacteria present in each domain at the indicated time points. In addition, cell associated and internalized bacteria were also quantified. Cell associated bacteria were determined by incubating the

cells with 1% saponin for 15 minutes to lyse the cells and then diluting and plating the lysates onto GCK media. Internalized bacteria were determined by incubating membranes in invasion media supplemented with 100 µg/ml gentamicin for 2 hours to kill all extracellular bacteria, and then lysing the cells with 1% saponin. Lysates were collected, diluted, and plated onto GCK media. After 2 days bacterial colonies were counted.

**Immunofluorescence**

T84 cells (2 $X10^5$) were prepared for confocal microscopy using the protocol described by Bacallao and Stelzer [93]. Solutions used are listed in Table 7. Primary antibodies used were a rat anti-ZO1 (Transduction Laboratories) (2.5µg/ml) to stain the tight junctions, and a mouse anti-gonococcal outer membrane protein (US Biological) (1µg/ml) to stain the gonococci. Secondary antibodies included Alexa Fluor 633 goat-anti rat IgG (Molecular Probes) (2µg/ml) for the ZO-1, Alexa Fluor 488 goat anti-mouse $IgG_1$ (Molecular Probes) (2µg/ml) for the gonococci, and Alexa Fluor 546 phalloidin to stain the actin cytoskeleton of the T84 cells. Stained cells were visualized with a Xeiss 510 Laser Scanning Confocal Microscope.

## Table 7. Immunofluorescence Reagents

| Solution | Reagent | Concentration |
|---|---|---|
| **Fixative One** | Pipes, pH 6.8 | 80 mM |
| | EGTA | 5 mM |
| | $MgCl_2$ | 2mM |
| | paraformaldehyde | 4% |
| | Add PBS to 100 ml | |
| **Fixative Two** | NaBorate, pH11.0 | 100 mM |
| | paraformaldehyde | 4% |
| | Add PBS to 100 ml | |
| **Phosphate Buffered Saline (1x)** | DPBS without $Ca^{2+}$ and $Mg^{+}$ | Media tech Inc. |
| **Quenching Reagent** | $NH_4Cl$ | 75 mM |
| | Glycine | 20 mM |
| **PBS-FSG-Saponin** | Fish Skin Gelatin | 0.66% |
| | Saponin | 0.025% |
| | Add PBS to 100 ml | |
| **0.1% Triton X** | Triton X | 0.10% |
| **Post-Fixative Solution** | NaCacadylate, pH 7.4 | 100 mM |
| | paraformaldehyde | 4% |
| | Add PBS to 100 ml | |

## Results

Colony opacity-associated proteins (Opa) have been demonstrated to be important for the ability of *Neisseria gonorrhoeae* to interact with host cells, and particularly in the ability of the bacteria to transcytose monolayers of human cells [54]. The genome of *N. gonorrhoeae* contains 11 Opa loci, and translation of each of these genes is controlled by a CTCTT repeat downstream of the start codon of each gene. The number of repeats determines whether or not these will be translated into functional proteins, and consequently, there is no way to know how many Opa genes are being expressed in a given gonococci. In order to circumvent this problem, this thesis has undertaken the task to individually delete the coding region of each of the Opa genes, so that the proteins will never be made. This would then allow for experimentation of the effects that each Opa protein has on the ability of gonococci to transcytose a monolayer of polarized epithelial cells.

The experiments necessary to answer this question were undertaken in two parts. First deletions were engineered in each of the Opa strains by cloning the genes into *E. coli*, using PCR amplification to delete the coding region of each gene, and replacing each gene with a spectinomycin resistance cassette. One of these deletions was then transformed into *N. gonorrhoeae* strain MS11 and used to invade a polarized monolayer of human epithelial cells, in order to determine what affect the deletion of that particular Opa would have on the ability of *N. gonorrhoeae* to traverse the monolayer. Below are the results of these experiments.

### Section One: Generation of Opa deletion mutants of *Neisseria gonorrhoeae*

## DNA Sequence Analysis

In order to delete each individual Opa encoding gene, a database search was done to identify the DNA sequences of the Opa encoding-genes found in FA1090 (University of Oklahoma). Opa sequences were identified by searching for the CTCTT repeat region that is characteristic of Opa encoding-genes. Eleven putative genes were identified, and the areas both upstream and downstream of the identified sequences were searched using the BLAST (NCBI) to verify that the sequences were infact Opa encoding-genes. All identified sequences corresponded to Opa encoding-genes. These sequences were then aligned using the multi-sequence alignment program ClustalW (EMBL-EMI) (Fig 1). Significant homology occurred between the start and stop codons of each Opa encoding-genes. The location of all the primers, both those used to amplify the individual genes and those used for the deletion PCR reactions, are labeled in Figure 1. The sequence information was useful in designing the primers that would be used in the construction of the deletions. Regions far upstream and downstream of the beginning and end of the coding region of the genes were identified and used as primer binding sequences for the amplification of each gene. Regions immediately upstream and downstream of the start and stop codons were chosen as primer binding sites for the deletion PCR primers. Although there is a high degree of homologous sequence between the Opa genes, Figure 1 clearly shows that there is unique sequences in each gene, and this distinctness allowed for the amplification of 11 distinct Opa encoding-genes.

## Part One: Construction of Opa deletions by cloning Opa encoding-genes into pUC19

The first step in deleting the Opa encoding-genes was to clone the genes into a plasmid. The general strategy used for this cloning can be found in Figure 2A. To

**Figure 1**. *Neisseria gonorrhoeae* strain FA1090 Opa encoding-gene alignment. Individual Opa encoding-genes were identified and aligned using ClustalW Multiple Sequence Alignment (EMBL-EBI). Each line represents a distinct Opa. An "-" indicates a lack of corresponding nucleic acid. The binding sites for the primers used to amplify the individual Opa fragments can be found at the beginning and end of each sequence. The sequences are indicated by italics and "_", and are labeled to the right of the sequence. Restriction enzyme sites used for cloning that are part of the gene, are highlighted in bold. The binding sites for the primers used to delete the coding region of each gene can be found in the middle of each sequence. They are indicated by italics and "_", and are labeled to the right of the sequence. Start and stop codons are also underlined, bolded and in italics. The codons are also labeled to the right of the sequence. Numbers at the ends of each line signify the nucleotide number in the sequence. An * below each alignment section indicate that the nucleotide found in that position is conserved among all the Opas.

```
CLUSTAL W (1.82) multiple sequence alignment

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------GCGCCTAGGGGCCGTGGCAGTCCC 24
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             CCCGAATTCCTACGCCAGCATTATTTCTACGCTCAAAGACCGCGAATACGTTACCCTTGA 60
Five            ------------------------------------------------------------
Nine            ------------------------------------------------------------

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             AACCCGGTACATAAGGCGCGTCAAAACCGGCTTGGGTTTTGCTGCGGATAATAATGTCTT 84
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             GCAAAAACGCTTCATGCCCACCGACACAGGCGACATCGTCAATAAATTCCTGACCGAACA 120
Five            ------------------------------------------------------------
Nine            ------------------------------------------------------------

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             TAAGAATTTTATTGACGGCATGACCGATATGGATGTCGCCGTTGGCATACGGCGGGCCGT 144
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             CTTCGCCCAATACGTCGATTACCACTTCACCGCCAAACTCGAAGACCAGCTTGACGAAAT 180
Five            ------------------------------------------------------------
Nine            -------------------------------------------------------GCGGA 5

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             CGTGCAGAATGAATTTCGGACGGCCTTTGGCGATTTCGCGCAGTTTTTGGTAACGTTTTT 204
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             CGCCGACGGCAAACGCCGCTGGATTCCCGTGATGGACAAATTCTGGAAACCGTTCATCAA 240
Five            ------------------------------------------------------------
Nine            TCCTGAAATCGGCGAATTCCCAAGCCGGTTCAACCAAAGCTGGATTAAAGTGCAAGACAT 65

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             GCTCGTACCAGCTTTTCAGCCACGCAGGCTCGCGCTTGGCAAGATTGCCGCGCATCGGAA 264
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             ACAAGTGGAAGAAAAAGAGGGCATCGAACGCGCCAAATTCACTACGCAGGAACTTGACGA 300
Five            ------------------------------------------------------------
Nine            GCGCTACGGCGAAAACCCGCATCAGCGCGCCGCGTTTTACCGCGATATTGACCCCGCCGC 125

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ACGGGCTTTCGAGCAGGTTGACGGTTTTACTGTAATCGGTCATTTTTTAATCTCTATTGT 324
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             AACCTGCCCGAAATGCGGCGAACACAAACTGCAAATCAAGTTCGGCAAAATGGGCCGTTT 360
Five            ------------------------------------------------------------
Nine            AGGCAGCCTTTCCGCCTACAATCAACTGCAAGGCAAAGAATTGTCTTACAACAACATCGC 185

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             TACAATATTTCGGTTTCAGACGGCATTGCCCCTCAAACAGTATTTCACAACGGGAAACCC 384
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             CGTTGCGTGTGCCGGTTATCCCGAGTGGAGTTACACCCGCAACGTCAACGAAACCGCCGA 420
Five            ------------------------------------------------------------
Nine            CGATGCCGATGCCGCTTGGGAAGCCGTCAAATCCTTTGACGCGCCCGCCTGCGTGATCGT 245

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             CTATGCCGTCTGAACAGTTAAAAGGCTGATTGTAGCCCAATCGGATGGTTTGTATAAGGT 444
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           ------------------------------------------------------------
Ten             AGAAGCTGCCGAACGCATCGCCAAAGCGGAAGCCGAACAGGTCGAACTCGACGGTCGCGA 480
Five            ------------------------------------------------------------
Nine            GAAACACGCCAATCCGTGCGGCGTAGCCGTTGCAGCCGATACCTTGACCGCCTACAAACT 305
```

Opas 6 and 10 Forward Primers

Opa 9 forward primer

```
Four        ------------------------------------------------------------
Eleven      ------------------------------------------------------------
One         ------------------------------------------------------------
Six         TTTTCTACCAACACTTTGCGGCTTCCATATCGGCTTCAATCCGCCTTTTCAGTTCTTCCA 504
Seven       ------------------------------------------------------------
Two         ------------------------------------------------------------
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         ATGCCCAAATGCGGCGGACGGCTGGTGTACAAATACAGCCGCACCGGCAGTAAATTCATC 540
Five        ------------------------------------------------------------
Nine        CGCCTACGCCACCGACACCACCAGCGCGTTCGGCGGCATCATCGCCTTCAACCGCGAAGT 365

Four        ------------------------------------------------------------
Eleven      ------------------------------------------------------------
One         ------------------------------------------------------------
Six         TACCGTCAAACTTTTCCTCGTCGCGCAGTTTGTGCAGGAAGCGGACGTTCAACCGTTGTC 564
Seven       ------------------------------------------------------------
Two         -------------------------------------------------------GCGGG 5
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         GGTTGCGCCAACTACCCCAAATGCAAACACGTCGAGCCGTTGGAAAAACCCAAAGATACC 600
Five        ------------------------------------------------------------
Nine        CGACGGCGAAACCGTCAAACAGATTACCGACAACCAATTTATGGGAAGTCCTCATGGCGC 425
```

Opa 2 forward primer

```
Four        ------------------------------------------------------------
Eleven      ------------------------------------------------------------
One         ------------------------------------------------------------
Six         CGTACAAATCGCCTTGAAAGTCGAACAAATGG--ACTTCCAGCTTTTGAGAACAGCCGCC 622
Seven       ------------------------------------------------------------
Two         ATCCAGGGCGGTGTCGAAGGCAAAATGCGCGGCAGTACCCAAACTATCGGCATTGCCGCG 65
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         GGCGTCCAATGCCCGCAATGCAAAAAAGGCAACCTCGTCGAGCGCAAATCCGCTACGGCA 660
Five        ------------------------------------------------------------
Nine        CGAAGTTTACCGCCGAAGCCCTTGAAATCGCCGCCGCCAAGAAAAACGTGCGCGTATTGG 485

Four        ------------------------------------------------------------
Eleven      ------------------------------------------------------------
One         ------------------------------------------------------------
Six         ATCAACGGTGGGATTGAAGCCGAAGCTCGCCACGCCGCGCCGCGTGCCGAATGCGCCGTC 682
Seven       ------------------------------------------------------------
Two         AAAACCGGCGAAAATACGACAGCAGC------CGCCACACTGGGCATAGGACGCAGCACA 119
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         AACTGTTTTACAGTTGCAGCACCTATCCCGACTGCAACTACGCCACTTGGAACCCGCCCG 720
Five        ------------------------------------------------------------
Nine        AAGTGCCGCTCAAAGCAGGTGCCAACCGCTTTGAACTCAAACGCGTCGGCGGCGGACTGT 545

Four        ------------------------------------------------------------
Eleven      ------------------------------------------------------------
One         ------------------------------------------------------------
Six         CGCCTCGACGACGAACACGCCGCCGAGTGCATAACGGTGGCCGGGCAGTCGGATGTTGGC 742
Seven       ------------------------------------------------------------
Two         TGG-----AGCGAAAACAGTGCAAATGCAAAAACCGACAGCATTAGTCTGTTTGCAGGC 173
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         TTGCCGAAGAATGCCCGAACTGCCATTGGCCGGTACTGGCCATCAAAACTACCAAACGCC 780
Five        ------------------------------------------------------------
Nine        TGGTACAAACGCCCGACATCAACCGCATCAACCGCGCCGATTTGAAAGTCGTCTCCAAAC 605

Four        ---------------GCGGAATTCAAGAAGGAATGCCCGAACCGTCATTCCCGCCACTTT 45
Eleven      ------------------GGGCTTAAGAAAAAA----CCGATGGTTAAATACATTGCATG 38
One         ------------------------------------------------------------
Six         GGTCGGGGCGTTTAAGGTGCGCCCGAGTTTTCTGCCGTGCACCACCCTGCCCCCCAAAAC 802
Seven       ------------------------------------------------------------
Two         ATACGGCACGATGTGGGCGATATCGGCTATCT-------CAAAGGCCTGTTCTCC----- 221
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         GGGGCGTGGAAAAAGTCTGCCCGCAAAAAGAATG----CGGCTGGAAAGAACAAATCGAA 836
Five        ------------------------------------------------------------
Nine        GCCAACCGACCGAGCAGGAATGGAACGATTTGCTGTTTGTCTGGAACGTCGCAAAATACG 665
```

Opas 4 and 11 forward primers

```
Four        TCGTCATTCCCGCGAAAGCG---GGA-ATCTAGGACGCAGGGTTAAGAAAACCTACATCC 101
Eleven      ATGCCGATGGCGTAAGCCTG---AGGCATTTCCCCCTTTCAATTAGGAGTAATTTTAT-- 93
One         ------------------------------------------------------------
Six         GTAGTCGTGCCCCAAAAGTTTTTTCGCATAGGCAAGGTTGCCGTCTGAAAGGGCTTGGCG 862
Seven       ------------------------------------------------------------
Two         -TACGGACGCTACAAAAA-----CAGCATCAGCCGCAGCACCGGTGCGGATGAATATGCG 275
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         CCGCCTGCACCGCAAGAGTGAAGCGGTTGGGATTAAGATGAAAGAAAATGCCGTCTGAAA 896
Five        ------------------------------------------------------------
Nine        TCAAATCCAACGCCATCGTCTTCGGCAAAGGCGGCCAAACCTACGGTATCGGCGCAGGCC 725

Four        CGTCATTCCCTCAAAAACAGAAAACCAAAATCAGAAACCTAAAATCCCGTCATTCCCGCG 161
Eleven      -GAATACCCTTCAAAAAGGCTTTACCCTTATCG--AGCTGATGATT--GTGAT---CGCT 145
One         ------------------------------------------------------------
Six         GACGGCGGTACTGCTGGTGCGGATGTCTTCGACAATGACAGAAGGCGTGCGCTCGGTCTG 922
Seven       ------------------------------------------------------------
Two         GAAGGCAGCGTCAACGGCACGCTGATGCAGCTGGGCGCACTGGGTGGTGTCAACGTTCCG 335
Eight       ------------------------------------------------------------
Three       ------------------------------------------------------------
Ten         GGGGTTTCAGACGGCATTTGTAAATTAGAAGGGGCTGTCCCAGATGGCTGGGGAAATTTA 956
Five        ------------------------------------------------------------
Nine        AAATGAGCCGCGTGGACAGCACCCGCATCGCCGCCCGCAAAGCGCAAGATGCCGGTCTCG 785

Four        AAAGCGGGAATCTAGAATCTCGGACTTTCAGATAATCT--TTGAATATTGCCGTTGTTCC 219
Eleven      ATCGTCGGCATTTTGG----CGG-----CAGTCGCCCT--TC-----CCGCC----TACC 185
```

```
One         ------------------------------------------------------------
Six         CATATCG-GGCTGTTGTGCCAAAAGTTCAAAACAGCCT--TC------CCGCCCCGCCCC 973
Seven       ------------------------------------------------------------
Two         TTTGCCGCAACGGGAGATTTGACGGTTGAAGGCGGTCT--GCG-----CCACGACCTGCT 388
Eight       ------------------------------------------------------------
Three       ----------------------------------------------------GCGGAATT 8      Opa 3 forward
Ten         AATTAAGTCAGAATTATCCCTATGAGAAAAGCCGTCCAAGCCGGTACAAACAAAAGAAAC 1016   primer
Five        ------------------------------------------------------------
Nine        ACCTCAACGGCGCATGTGCCGCT--TCCGATGCCTTCTTCCCATTCCGCGACGGCGTGGA 843

Four        AAGGTCCGGATTCCCGCCCGCGCGCAAGTTTC-CGAAGCCATCCTTTTGGCCGAAGGTCA 278
Eleven      AAGACTA----CACCGCCCGCGCGCAAGTTTC-CGAAGCCATCCTTTTGGCCGAAGGTCA 240
One         ------------------------------------------------------------
Six         GAAACGGAAATCATCGCCGACGAGCAAATAGCGCGTATTCAAGGTTTGACGCAGCAGGCG 1033
Seven       ------------------------------------------------------------
Two         CAAACAGGATGCATT--CGCCGAA-AAAGGCAGTGCTTTGGGCTGGAGCGGCAACAGCCT 445
Eight       ------------------------------------------------------------
Three       CGGGGCGAGCGACTCGTCCAACTATGCCGCCTTCCTCCGCTACGGCATCCGCACCAGCCT 68
Ten         CCATCGGACTGTTTGCCGCAGGGGTAACTGCAAGAACGGCAGCAGGGTTGGCAGCAGTTA 1076
Five        ------------------------------------------------------------
Nine        CGTGATTGCCGAACAGGGCATCAAAGCCATCATCCATCCGGCAGGCTCGATGCGCGATCA 903

Four        AAAATCAGCCGTCACCGAGTATTACCTGAATCACGGCACATGGCCGGAAAACAACACTTC 338
Eleven      AAAATCAGCCGTTACCGGGTATTACCTGAATCACGGCATATGGCCGGAAGACAACACTTC 300
One         ------------------------------------------------------------
Six         GTCGATAAATGCTTGCGCGGATATTTCGGAAAAATTCCGATCGAAACGCAAAACCCAGGC 1093
Seven       ------------------------------------------------------------
Two         CACTGAAGGCACACTGGTCGGACTCGCGGGTCTGAAACTGTCGCAACCCTTGAGCGATAA 505
Eight       ------------------------------------------------------------
Three       GCAATCCAAATACGTCCTGCACAACTTGGGCTTCCGC-TGCGCAAGCCGATAACCCCTTC 127
Ten         ATCAAAACACGGCAGCCTGTTATTTTCTATGGCGAAATAGTGTTTGGCACATCATCGGGA 1136
Five        ------------------------------------------------------------
Nine        GGAAGTTTTCGACGCGGCGGACGAACACGGCATCGCCATGGCGGTAACCGGCATCCGCCA 963

Four        TGCCGGCGTGGCATCCTCCGCCACCGAC-ATCAAAGGCAAATATGTTCAAAGCG-TTACG 396
Eleven      TGCCGGCGTGGCATCCCCC-CCTCCGAC-ATCAAAGGCAAATATGTTCAAAGCG-TTACG 357
One         ------------------------------------------------------------
Six         GGCATCGACGCAACCCGTCCCTTCCAGCAATTCCAGTTTGGTACGCAGGGGGCTGATACG 1153   Opa 7 forward
Seven       ----------------------------TATTTTA--TAGAAATGACGAAATTTTAGATT 30     primer
Two         AGCCGTCCTGTCTGCGACGGCGGGCGTGGAACGCGACCTGA-ACGGACGCGACT-ACGCG 563
Eight       ------------------------------------------------------------
Three       AATTATAGTGGATTAACAAAAACCAGTACGGCGTTGCCTCGCCTTGCCGTACTGGTTTTT 187
Ten         AAACTTCCTTTACACGCATTTGGACGAAATCTGCGAAATTATGGAGGCATACGACATATC 1196
Five        ------------------------------------------------------------
Nine        CTTCCGCCATTAAGGCAGACGAACAAGGCAATGCCGTCTGAAGGGTTTTCAGACGGCATT 1023

Four        GTCGCAAACGGCGTCGTTACCG---CCGA----AATGAAATCAGACGGCGTAAACAAAGA 449
Eleven      GTCGCAAACGGCGTCGTTACCG---CCGA----AATGAAATCAGACGGCGTAAACAAAGA 410
One         ------------------------------------------------------------
Six         GCAGGGCGGGGTTTTGCCGGTA---CGGAGTGCGAAAAATTCTTTGGGTTGGGGTTCGAA 1210
Seven       GCAAGAACTTATCCCCTCCGCC---GTCATTCCCACGGA-------AGTGGGAATCTAGA 80
Two         GTAACGGGCGGCTTTACCGGCG---CGG-CTGCAGCAAC--CGGCAAGACGGGTGCACGC 617
Eight       ------------------------------------------------------------
Three       GTTAATCCGCTATATTCCGCCA---TCTCTAAGATTTACAGCGATACACGGGTAATTTAA 244
Ten         GTTTACGATTACTTATCCGTCAAAACAGTCCGCATTTGGAAATGTATGACGGCGAAGCAG 1256
Five        ------------------------------------------------------------
Nine        TTTGCGCTATTTACCGTAATCC--TGAAATCCCGTCATTCCCACGCAGGCAGGAATCTAG 1081

Four        AATCCAAGGCA--AAAGACTCTCCCTGTGGGCCAGGCGTGAAGCCGGTTCGGTAAAATGG 507
Eleven      AATCAAAGGCA--AAAAACTCTCCCTGTGGGGCAGGCGTCAAGACGGTTCGGTAAAATGG 468
One         ------------------------------------------------------------
Six         AACGACGGCCACGACGGGCAATCCGCGTGCGTCGGCTTCGAGGCGGAGTTTTTGGAGGAT 1270
Seven       AATAAAAAGCA--GCAGG-AATTTATCGGAAATAACT--GAAACCGAACGGACCGGATTC 135   Opa 5 forward
Two         AATATGCCGCACACCCGCCGGGTTGCCGGTCTGGGGGTGGATGTCGAATTCGGCAACGGC 677   primer
Eight       ------------------------------------------------------------
Three       GGAATGCCCGAACCGTCATTCCCGCAACTTTTCGTCATTCCCGCGAAAGCGGGAATCTAG 304
Ten         GGGCGGGTGAAAGTTATTTCCGTAAAGGCAGACGCGGCCGCAGTGCCGCCGGCAAAGCCG 1316
Five        ---------------------------------------------------GCGGAATTCCC 11
Nine        ATGAGAATCCATCCTGTCAGAAATACCCGAATCGTCAGGAATACCCGTGTCATCATTCCC 1141

Four        TTCT--GCGGA-CAGCCGGTTACGC--GCGCCAAAGCCAAAGACGCCGACGACGTTACCG 562
Eleven      TTCT--GCGGA-CAGCCGGTTACGC--GCAACGACGCCAAAG---CCGACGACGTCA--- 517   Opas 1 and 8
One         -----------------------------------GCGGAAT---TCTACATCATCTTCT 22    forward primers
Six         GTGT--TTGTGTCCGAGGTGTACGCCGTCGAAATTGCCTATGGTTACGGCGGCCCCGTGC 1328
Seven       CCGC--CTGCG--CGGGAATGACGATTCATAAGTTTCCCGAAATTCCAACATAACCG--- 188
Two         TGGA--ACGGCTTGGCACGTTACAGCTACACCGGTTCCAAACAGTACGGCAACCACA-GC 734
Eight       ---------------------------------------------TTTGTTATCCCAATAA 16
Three       AATC--CCGGACTTTCAGATAATCT--TTGAATATTGCTGTTGTTCCAAGGTCCGGA--T 358
Ten         CCGTATTCGGGCTTTTGAAGCGCAACGGCAAGGTTTGTACGGTTACTGTCGGACACTCGA 1376
Five        G---CCCTGTCGCCTTTAGACAG------CGCACGCAAACAGGAACTTGCCGCCCATGAC 62
Nine        GATACACCGTAATCCTGAAACCCGTCATTCCCGCGCAGACAGAAATCCGGATTTGTCCGC 1201

Four        ACGACGCCGGCACCG----ACAACGGCGGCAAAGGCAAAATCGACACCAA------GCAC 612
Eleven      ---AAGCCGACGCCG----CCAACGCC-----------ATCGAAACCAA------GCAC 552
One         CCCATATTGCTTCTG----ACAATCCGAACATCCCGACACGCGACATCCC------GCAC 72
Six         GGAAAGTCGGCGCGTTGTGCCGCCCCGGCCTGATTTTCATTGTATTTCC------TTTT 1382
Seven       --AAACCTGACAGTA----ACCGTAGCAACCGAACCGTCATT-----CCC------GCGA 231
Two         GGACAAATCGGCGTAGGCTACCGGTTCTGACGGACAGAAAACAGACAGCC------GCAA 788
Eight       TGCACGCCGCGGCTG-----ATGCCGCCGTAGAGGAAATGATGCCCGCCC------GCAT 65
Three       TCCCGCCTGCGCGGGAATGACGAATCCATCCGTACGGAAACCTGCACCAC------GTCA 412
Ten         ACCGCTATTTTATTGCCTATTATCCGCGGACGGGTGAAACCCGGCAGCATT-----GTTT 1431
Five        G-GCGCACTCATCCGC--GTCCTCCCTTCGGAAAACGGACTCGACATC------CTGCAA 113
Nine        GCGGAAACTTATGCGCC-GCCATTCCCGCGAAGGCGGGAATCCGGACCGTTCGGTTTCGG 1260

Four        CTGCCGTCAACCTG---CCGCGATAAATCAACTGCCAAATAAGGCAAATTAGGCCTTAAA 669
Eleven      CTGCCGTCAACCTG---CCGCGATGAACCAACTGCCAAATAAGGCAAATTAGGCCTTAAA 609
One         GAAACA-CAAGCCG---CCCCGAAAGGCAAATGCCGAAAAATCGCGATAAAAACCATCAA 128
Six         CGGTTGAAACCCCGCC-ACTCGGACATCCGTCCTTCGGGGCGGCAGGATCAGACTTTATT 1441
Seven       AAGCGGGAATCTAGAA-TCTCGGACTTTCAG-----ATAATCTTTGAATATTGCTGTTGT 285
Two         AGATCACGGTCT-----TTGCGGCTGTTTCT---------TATGAAAAGAAAACCCTATT 834
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Eight           TGATTTCGCGCGACAC-GCCCAAGCCGTAGCGCAAACCGTGTGCCTTTTGCGGCAGGCTG 124
Three           TTCCTACGAACCTACA-TCCCGTCATTCCCAC---GAAAGTGGGAATCTAGAAATAAAAA 468
Ten             ATACGGATTGTTCGTAGTTACGATGTATCAGATTGTAAGCGGATTTGGCCGTTTCCGTAT 1491
Five            GTGTTTGAAGAAATTTCTTGCAGCAT--CAACACCACAGCCGC----CGGCGGCAACGCC 167
Nine            TTATTTCCGATAAATTCCTGCTGCTTTTCATTCCTAGATTCCCACTTTCGCGGGAATGAC 1320
                                    *

Four            TTTTAAATAAA------TCAAACGGTAAGTGATTTCCCACGGCCGCCCGGATCAACCCGG 723
Eleven          TTTTAAATAAA------TCAAACGGTAAGTGATTTCCCACGGCCGCCCGGATCAACCCGG 663
One             TCGGAAAATAT------TGCATAAACAGGCGAAAATATATCATTGAAAAGAA-AACCCAT 181
Six             CGGGAGGGGTGCA-ACCCCTTCCAAATCAGGACGACACATAGGGCGGTGC--TTGATGTG 1498
Seven           TCTAAGGTCCGGA-CTCCCGCCCGCGCGGGAATGACG-GCGGAAAGATGC--CCGACGGT 341
Two             CCAATTGCCTG----CTTCTATTGTTTCAAGACTTCTTCCAAAGATTCGGCATCAATCAG 890
Eight           TCGGCGGTTTTCG-TCCAGCTTCTGCCCGCAAATTCAATCGTTTTTTCGGACGAAGCGTT 183
Three           GCAGCAGGAAT----TTATCGGAAATAACTGAAATTCAATACCGCAAAAATCTACCCGAA 524
Ten             CAATCGCGGCACA-CATTTTGCCGAACGGCAAAACCATATCAACGCAATTGGGAACTTTT 1550
Five            GGCCTGATGG-----CGGTTG-------CCGACTGATTTTGCCAAACCGCCCGGGCGCGG 215
Nine            GAAGGAGTGGGAATCCGGTTTTTGAGTTCCGGCCATTTCCGACAAATTGCTTTGGCATTG 1380

Four            GCG-GCTTGTCTTTTAAGGGTTTGCAAGGCGGGCGGGGTCGTCCGTTCCGGTGGAAATAA 782
Eleven          GCG-GCTTGTCTTTTAAGGGTTTGCAAGGCGGGCGGGGTCGTCCGTTCCGGTGGAAATAA 722
One             ACA-A--TGTCTGACAGAA----ACAAGCC--GCTTTGCCATTGTTTCC--TAACAGTTA 230
Six             CCG-TCCCGTGTGTTGAAACATTGTTGCGTTCGGGAGTGTGGTGAAACAGGCGGTCATTG 1557
Seven           CTT-TATAGCGGATTAACAAAAATCAG-----GACAAGGCGGCGGGCC--GCAGACAGTA 393
Two             ACG-TATAGCGGATTAACAAAAATCAG-----GACAAGGCGGCGGGCC--GCAGACGGTA 942
Eight           GTT-TATAGCGGATTAACAAAAATCAG-----GACAAGGCGGCGGGCC--GCAGGCAGTA 235
Three           ATGATATAGCGGATTAACAAAAATCAG-----GACAAGGCGGCGGGCC--GCAGGCAGTA 577
Ten             GGA-----ATCGGGCAAACGTCATTTGCGCA-AGTTTGACGGCATTCCCAAAGAGCATTT 1604
Five            -------CCCGT---GAACAAATGCCGT-------CTGAAAACCTTTCAGGCGGCATTTT 258
Nine            GATATTTCCTATTTTAATCCACTATAGTGGATTAACTTTAAACCAGTACGGCGTTGCCTC 1440

Four            TATATCGATTGC--GCTTCAAGGCCCTGCAT---GTGCCTCATTGCCACCCGTTTAAAC- 836
Eleven          TATATCGATTGC--GCTTCAAGGCCCTGCAT---GTGCCTCATTGCCACCCGTTTAAAC- 776
One             AACCCCG----C--CCTTCAAGGCGGCGGGGC-AGGGCTTGATTCAAAATGGCGCAAGC- 282
Six             TAAACGGTATTGCGGTTTATAGACAGTGTGCCGCCCGTTCCGCCCGCCCGACGCGGGAA- 1616
Seven           CAAATGGTACGG--------AACCGATCCGCCCGGTGCTTCATCACCTTA----GGGAA- 440
Two             CAAATGGTACGG--------AACCGATCCGCCCGGTGCTTGGGCGTCTTA----GGGAA- 989
Eight           CGGATGGTACGG--------AACCGGTTCGCCCGGTGCTTGGACGCCTTA----GGGAA- 282
Three           CGGATGGTACGG--------AACCGGTTCGCCCGGTGCTTGGGCGCCTTA----GGGAA- 624
Ten             CGGGCTGTATTT-------GAAGGAGTGCGGACGGCGTTTTGACAACAGTGGGGTAAAAG 1657
Five            ATAGTGGATTAA----CAAAAATCAGGAC--AAGGCGGCGGGCCGCAGA----------- 301
Nine            GCCTTAGCTCAA----AGAGAACGATTCTCTAAGGTGCTGAAGCACCAAGTGAATCGGT- 1495
                      *             *               *

Four            -ACGGTTTT----T---ATCTGACAGGCGCG-CAAT-----------CCGCCCCCTCATT 876
Eleven          -ACGGTTTT----T---ATCTGACAGGCGCG-CAAT-----------CCGCCCCCTCATT 816
One             -CCCTGCCC----TCAAATCCAACACGCA-G-GATT-----------AAACCATAATAGC 324
Six             -AAGTAGGCAAATTTCCCGCCGCCGAACGCGCCAAA-----------C-GCACAAAAACG 1663
Seven           -CCGTTCCC----TTTGAGCCG--GGGCGGGGCAAC-----------CCGTACCGGTTTT 482
Two             -CCGTTCCC----TTTGAGCCG--GGGCGGGGCAAC-----------CCGTACCGGTTTT 1031
Eight           -CCGTTCCC----TTTGAGCCG--GGGCGGGGCAAC-----------CCGTACCGGTTTT 324
Three           -CCGTTCCC----TTTGAGCCG--GGGCGGGGCAACG----------CCGTACCGGTTTT 667
Ten             TTCGAATTTCCATTTTAAAACAATTGGTAAAGCAGGATTTATCCCGGCTGGCCGGGATTT 1717
Five            -CAGTACAA----ATAGTACGGCAAGGCGGGCCAACGC----------TGTACTGGTTTA 346
Nine            TCCGTACTA----TTTGTACTGTCTGCGGCTTCGCCGCC--------TTGTCCTGATTTT 1543

Four            TGT----T-AATCCGCCATAT-----------TGTATTGAAACACCGCCCGG--AACCC- 917   Opa 5' deletion
Eleven          TGT----T-AATCCGCCATAT-----------TGTATTGAAACACCGCCCGG--AACCC- 857   primer
One             GGC----T-TTCTTATTATTT-----------CTTATTGAAACACCGCCCGG--AACCC- 365
Six             CGC----AGCAGGCGCGGTGCTA---------TGTGTTGAAACACCGCCCGG--AACCC- 1707
Seven           TGT----T-CATCCGCCATAT-----------TGTGTTGAAACACCGCCCGG--AACCC- 523
Two             TGT----T-CATCCGCCATAT-----------TGTGTTGAAACACCGCCCGG--AACCC- 1072
Eight           TGT----T-CATCCGCCATAT-----------TGTGTTGAAACACCGCCCGG--AACCC- 365
Three           TGT----T-AATCCGCCATAT-----------TGTGTTGAAACACCGCCCGG--AACCC- 708
Ten             TAAAAAATAAATACATTCTTTAA---------CAAAACAAATCATATCCCGGCAAAACA- 1767
Five            AAT----TCAATTCACTATAT------------GTGTTGGCACATCGCTCCA--AACCT- 387
Nine            TGT----T-AATCCGCCATATCGCCGCACCGGAATTTTAAAAAATAAATACA--TTCTTT 1596
                                  *                        *

Four            ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 954
Eleven          ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 894
One             ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 402
Six             ---GATATAATCCGCCTTTGAAGCA------TCAGTGAAAAT------CTTTT------- 1745
Seven           ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 560
Two             ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 1109
Eight           ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 402
Three           ---GATATAATCCGCCCTTCAA-CA------TCAGTGAAAAT------CTTTT------- 745   Opa 5' deletion
Ten             ---AATCTGATAAAATACTTGGCGT------TTATTAACAAT------CTTTT------- 1805   primer
Five            ---GATATAATCCGCCTTTCAACA-------TCAGTGAAAAT------CGTTC------- 424
Nine            AACAAAAAAAATCATATCCCGGCAAAACAAATCTGATAAAATGCTTGGCGTTTATTAACA 1656
                    *    *                     *     * ***      * **

Four            ---TTT-AACCGGTTAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 1006
Eleven          ---TTT-AACCGGTTAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 946   Start codon
One             ---TTT-AACCGGTCAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 454
Six             ---TTT-AACCGGTCAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 1797
Seven           ---TTT-AACCGGTTAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 612
Two             ---TTTTAACCGGTTAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 1162
Eight           ---TTTTAACCGGTTAAACC--GA-ATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 455
Three           ---TTTTAACCGGTCAAACC--GATATAAGGAGCCGAAAA-TGAATCCAGCCCGCAAAAA 799
Ten             ---T---AATAATTTAATCTTAGATATTCGGAGTTGATA--TGCATACGGCCGACAAAAA 1857
Five            --TTTTTAGTCAGTTAACATTAAAT-TTCGGAGTCGAAAAATGAATCCAGCCCGCAAAAA 481
Nine            ATCTTTTAATAATTTAATCTTAAATATTCGGAGTTGATA--TGCATACGGTCGACAAAAA 1714
                   *   *     * **      *  *  ****  ** *  ** ** * * *  ******

Four            ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTC-- 1064
Eleven          ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTC------------ 994
One             ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTT 514
Six             ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTT 1857
Seven           ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCT--------------------------CTT 652
Two             ACCTTCTCTTCTCTTCC------------------------------------------- 1179
Eight           ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTT 515
Three           ACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTC------- 852
Ten             AC-TTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTT 1916
Five            AC-----------------------------CTTCTCTTCTCTTCTC------------ 499
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Nine            AC-TTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTC------------ 1761
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Four            ----------------------------------------TTCTCTTCGGCAGCGCAGGCGG 1086
Eleven          ----------------------------------------TTCTCTTCGGCAGCGCAGGCGG 1016
One             CTCTTCTCTTCTCTTCTC----------------------TTCTCTTCCGCAGCGCAGGCGG 554
Six             CTC-------------------------------------TTCTCTTCGGCAGCGCAGGCGG 1882
Seven           CTC-------------------------------------TTCTCTTCGGCAGCGCAGGCGG 677
Two             -----------------------------------------------GCAGCGCAGGCGG 1192
Eight           CTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGG 575
Three           ----------------------------------------TTCTCTTCCGCAGCGCAGGCGG 874
Ten             CTC-------------------------------------TTCTCTTCCGCAGCGCGGGCGG 1941
Five            ----------------------------------------TTCTCTTCCGCAGCGCAGGCGG 521
Nine            ----------------------------------------TTCTCTTCCGCAGCGCGGGCGG 1783
                                                               ******* *****

Four            CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 1146
Eleven          CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 1076
One             CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 614
Six             CAAGTGAAGACGGCGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 1942
Seven           CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 737
Two             CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 1252
Eight           CAAGTGAAGACAATGGCCGCGGCCCGTATGTGCAGGCGGATCTGGCTTACGCCTACGAGC 635
Three           CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 934
Ten             CGGGTGAAGACCATGGGCGCGGCCCGTATGTGCAGGCGGATCTGGCTTACGCCTACGAGC 2001
Five            CAAGTGAAGGCAATGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCCTACGCCGCCGAAC 581
Nine            CGGGTGAAGACCATGGGCGCGGCCCGTATGTGCAGGCGGATCTGGCTTACGCCTACGAGC 1843
                *  ****** *   ** ************************ * ** ******  *** *

Four            GCATTACCCACGATTATCCGGAACCAACCGCTCCAGGCAAAAACAAAA---TAAGCACGG 1203
Eleven          GCATTACCCACGATTATCCGGAACCAACCGCTCCAGGCAAAAACAAAA---TAAGCACGG 1133
One             GCATTACCCACGATTATCCGGAACCAACCGGTGCAAAAAAAGACAAAAAAATAAGCACGG 674
Six             GCATTACCCACGATTATCCGGAACCAACCGGTGCAAAAAAAGGCAAAA---TAAGCACGG 1999
Seven           GCATTACCCACGATTATCCGGAACCAACCGCTCCAGGCAAAAACAAAA---TAAGCACGG 794
Two             GCATTACCCACGATTATCCGGAACCAACCGGTACAAAAAAAGACAAAA---TAAGCACGG 1309
Eight           ACATCACCCGCGATTATCCCGATGCAGCCGGTGCAAACCAAGGCAAAAAAATAAGCACGG 695
Three           GCATTACCCACGATTATCCGGAACCAACCGGTGCAAAAAAAGGCAAAA---TAAGCACGG 991
Ten             ACATCACCCGCGATTATCCCGATGCAGCCGGTGCAAACCAAGGCAAAAAAATAAGCACGG 2061
Five            GCATTACCCACGATTATCCGGAACCAACCGGTGCAAAAAAAGACAAAAAAATAAGCACGG 641
Nine            ACATTACCCGCGATTATCCCGATGCAGCCGGTGCAAACAAAGGCAA---AATAAGCACGG 1900
                 *** **** ********* **  ** *** * **    **  ***     *********

Four            TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 1263
Eleven          TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 1193
One             TAAGCGATTATTTCAGAAACATCCGTACGCATTCCGTCCACCCCAGGGTGTCGGTCGGCT 734
Six             TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 2059
Seven           TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 854
Two             TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 1369
Eight           TAAGCGATTATTTCAAAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 755
Three           TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 1051
Ten             TAAGCGATTATTTCAAAAACATCCGCACCCGCTCCGTCCACCCCCGGCTTGCCTTCGGCT 2121
Five            TAAGCGATTATTTCAGAAACATCCGTACGCATTCCGTCCACCCCAGGGTGTCGGTCGGCT 701
Nine            TAAGCGATTATTTCAGAAACATCCGTACGCATTCCATCCACCCCAGGGTGTCGGTCGGCT 1960
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Four            ACGACTTCGGCGGCTGGCGCATCGCCGCGGATTATGCCCGTTACAGGAAATGGCACAACA 1323
Eleven          ACGACTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACGACA 1253
One             ACGATTTCGGCAGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACAACA 794
Six             ACGACTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACAACA 2119
Seven           ACGACTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACGACA 914
Two             ACGACTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACAACA 1429
Eight           ACGACTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACGACA 815
Three           ACGACTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACGACA 1111
Ten             ACGATTTCGGCGGCTGGCGCATCGCCGCGGATTATGCCCGTTACAGGAAATGGCACAACA 2181
Five            ACGATTTCGGCAGCTGGAGGATAGCGGCAGATTATGCCCGTTACAGAAAGTGGAACAACA 761
Nine            ACGACTTCGGCGGCTGGCGCATCGCCGCGGATTATGCCCGTTACAGGAAATGGCACAACA 2020
                **** ****** ***** * ** ** ** ***************** ** *** ** ***

Four            ATAAATATTCCGTGAACATAAAAGAGTTGGAAAGAAAGAATAATAAAACTTTTGGCGGCA 1383
Eleven          ATAAATATTCCGTCGACATAAAAGAGTTGGAAAACAAGAAT------------------- 1294
One             GTAAATATTCCGTCAACATAAAAAGGGTGAAAGAAAACAAT------------GGCAGCG 842
Six             GTAAATATTCCGTCAACACAAAAAAGGTGAACGAAAACAAG------------GGCGAAA 2167
Seven           ATAAATATTCCGTCGACATAAAAGAGTTG---GAAAACAAG------------AATCAGA 959
Two             GTAAATATTCCGTCAACACAAAAAAGGTGAACGAAAACAAG------------GGCGAAA 1477
Eight           ATAAATATTCCGTCGACATAAAAGAGTTGGAAAACAAGAAT-----------------CA 858
Three           ATAAATATTCCGTGAACATAAAAGAGTTGGGAAGAAAGGATGGTACCTCTTCTAGCGGCC 1171
Ten             ATAAATATTCCGTGAACATAAAAGAGTTGGGAAGAAACGATAATAGCGCTTCTGACAGCA 2241
Five            GTAAATATTCCGTCAACATAAAAAGGGTGAAAGAAAACAATGGCA---------GCGGGA 812
Nine            ATAAATATTCCGTGAACATAAAAGAGTTGGAAAGAAAGAATAATAAAACTTTTGGCGGCA 2080
                 ************  *** ****  * **      **  *

Four            ACCAGCTTAACATAAAATACCAAAAGACGGAACATCAGGAAAACGGCACATTCCACGCCG 1443
Eleven          --CAG---AATAAGAGAGACCTGAAGACGGAAAATCAGGAAAACGGCAGCTTCCACGCCG 1349
One             GGAAAAAACTGACGCAAGACCTGAAGACGGAAAATCAGGAAAACGGTACGTTCCACGCCG 902
Six             AGATAAACGTGACGCAATATCTGAAGGCGGAAAATCAGGAAAACGGTACGTTCCACGCCG 2227
Seven           --ATAAGAGAGAC-------CTGAAGACGGAAAATCAGGAAAACGGCAGCTTCCACGCCG 1010
Two             AGATAAACGTGACGCAATATCTGAAGGCGGAAAATCAGGAAAACGGTACGTTCCACGCCG 1537
Eight           GAATAAGAGAGAC-------CTGAAGACGGAAAATCAGGAAAACGGCAGCTTCCACGCCG 911
Three           GCTATCTTAACATACAAACCCGAAAGACGGAAAATCAGGAAAACGGTACGTTCCACGCCG 1231
Ten             AGCATCTTAACATAAAAACCCAAAAGACGGAACATCAAGAAAACGGCACATTCCACGCCG 2301
Five            AAAAACT-GACGCAAGA--CCTGAAGACGGAAAATCAGGAAAACGGTACGTTCCACGCCG 869
Nine            ACCAGCTTAACATAAAATACCAAAAGACGGAACATCAGGAAAACGGCACATTCCACGCCG 2140
                                    *  *** ***** **** ******** *  **********

Four            TTTCTTCTCTCGGCTTGTCCGCCGTTTACGATTTCAAACTCAACGACAAATTCAAACCCT 1503
Eleven          TTTCTTCTCTCGGCTTATCAGCCGTTTACGATTTCAAACTCAACGACAAATTCAAACCCT 1409
One             TTTCTTCTCTCGGCTTGTCCGCCGTTTACGATTTCGATACCGGTTCCCGCTTCAAACCCT 962
Six             TTTCTTCTCTCGGCTTGTCCGCCGTTTACGATTTCAAACTCAACGACAAATTCAAACCCT 2287
Seven           TTTCTTCTCTCGGCTTATCAGCCGTTTACGATTTCAAACTCAACGACAAATTCAAACCCT 1070
Two             TTTCTTCTCTCGGCTTGTCCGCCGTTTACGATTTCAAACTCAACGACAAATTCAAACCCT 1597
Eight           TTTCTTCTCTCGGCTTATCAGCCGTTTACGATTTCAAACTCAACGACAAATTCAAACCCT 971
Three           TTTCTTCTCTCGGCTTGTCAACCGTTTACGATTTCAGAGCCAACGATAAATTCAAACCCT 1291
Ten             TTTCTTCTCTCGGCTTGTCAACCGTTTACGATTTCAGAGCCAACGATAAATTCAAACCCT 2361
Five            TTTCTTCTCTCGGCTTGTCCGCCGTTTACGATTTCGATACCGGTTCCCGCTTCAAACCCT 929
Nine            TTTCTTCTCTCGGCTTGTCAACCGTTTACGATTTCAGAGTCAACGATAAATTCAAACCCT 2200
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Four            ATATCGGTGCGCGCGTCGCCTACGGACACGTCAGACACAGCATCGATTCGACTAAAAAAA 1563
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Eleven          ATATCGGTGCGCGCGTCGCCTACGGACACGTCAGACACAGCATCGATTCGACTAAAAAAA 1469
One             ATGCAGGCGTGCGCGTCAGCTACGGACACGTCAGACACAGCATCGATTCGACCAAAAAAA 1022
Six             ATATCGGCATGCGCGTCGGCTACGGGCACGTCAGACATCAGGTTCGTTCGGTTGAACAAG 2347
Seven           ATATCGGTGCGCGCGTCGCCTACGGACACGTCAGACACAGCATCGATTCGACTAAAAAAA 1130
Two             ATATCGGTGCGCGCGTCGCCTACGGACACGTCAGACACAGCATCGATTCGACCAAAAAAA 1657
Eight           ATATCGGTGCGCGCGTCGCCTACGGACACGTCAGACACAGCATCGATTCGACCAAAAAAA 1031
Three           ATATCGGCGTGCGCGTCGCCTACGGACACGTCAGACATCAGGTTCATTCAATGGAAAAAG 1351
Ten             ATATCGGCGTGCGCGTCGCCTACGGACACGTCAGACATCAGGTTCATTCAATGGAAAAAG 2421
Five            ATGCAGGCGTGCGCGTCAGCTACGGACACGTCAGACACAGCATCGATTCGACCAAAAAAA 989
Nine            ATATCGGTGTGCGTGTCGGCTACGGACACGTCAGACACGGTATCGATTCGACTAAAAAAA 2260
                **   **   *** ***  ****** ***********     *   ***     ** **

Four            TAACAGGTACTCTTACCGCCTACCCTAGTGATGCTGACGCAGC-AGTTACGGTTTATCCT 1622
Eleven          TAACAGGTACTCTTACCGCCTACCCTAGTGATGCTGACGCAGC-AGTTACGGTTTATCCT 1528
One             CAACAGATGTTATTACCGCCCCCCCACTACTTCTGACGGAGC-ACCTACAACTTATAAT 1081
Six             AAACCACGACTGTTACCACTTACCTACAGAGTGGTAAGCCA---AGTCCTATCGTACGAG 2404
Seven           TAACAGGTACTCTTACCGCCTACCCTAGTGATGCTGACGCAGC-AGTTACGGTTTATCCT 1189
Two             CAACAGAGTTTCTTACCGCCGCCGGTCAGGATGGCGGAGCG----CCTACGGTTTATAAT 1713
Eight           CAACAGAGTTTCTTACCGCCGCCGGTCAGGATGGCGGAGCG----CCTACGGTTTATAAT 1087
Three           AAACCACGACTGTTACCACTTACCCAAGCGACGGTAGTGCGAAAACTTCTGTTCCATCAG 1411
Ten             AAACCACGACTGTTACCACTTACCCAAGCGACGGTAGTGCGAAAACTTCTGTTCCATCAG 2481
Five            CAACAGATGTTATTACCGCCCCCCCACTACTTCTGACGGAGC-ACCTACAACTTATAAT 1048
Nine            CGAAAAATACTCTTACCGCCTACCATAGTGCT---GGCACAAA-ACCTACGTATTATGAT 2316
                  *       * ***** *   *                                *

Four            GACGGACATCCGC---------AAAAAAACACCTATCAAAAAAGCAACAGCAGCCGCCGC 1673
Eleven          GACGGACATCCGC---------AAAAAAACACCTATCAAAAAAGCAACAGCAGCCGCCGC 1579
One             GCTAATCCACAGA---------CGCAAAACCCTTATCACCAAAGCGACAGCATCCGCCGC 1132
Six             GTTCGACCCTC-------------AAACTTCCCCATCACGAAAGCCGCAGCAGCCGCCGC 2451
Seven           GACGGACATCCGC---------AAAAAAACACCTATCAAAAAAGCAACAGCAGCCGCCGC 1240
Two             AACGGAAGTACGC------------AAGACGCCCATCAAGAAAGCGACAGCATCCGCCGC 1761
Eight           AACGGAAGTACGC------------AAGACGCCCATCAAGAAAGCGACAGCATCCGCCGC 1135
Three           AAATGCCCCCC-------------AAACCTGCCTATCACGAAAACCGCAGCAGCCGCCGC 1458
Ten             AAATGCCCCCC-------------AAACCTGCCTATCACGAAAACCGCAGCAGCCGCCGC 2528
Five            GCTA---ATCC----ACAGAC--GCAAAACCCTTATCACCAAAGCGACAGCATCCGCCGC 1099
Nine            GATATAGATTCGGGAAAAAACCAAAAAAACACTTATCGCCAAAACCGCAGCAGCCGCCGC 2376
                                         **    *  ***   *** *  ***** *******

Four            TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGCCTGACCTTGGAC 1733
Eleven          TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGCCTGACCTTGGAC 1639
One             GTGGGCCTCGGCGTCATCGCCGGCGTCGGTTTCGACATCACGCCCAACCTGACCCTGGAC 1192
Six             TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGTCTGACCTTGGAC 2511
Seven           TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGCCTGACCTTGGAC 1300
Two             GTGGGCCTCGGCGTCATCGCCGGCGTCGGTTTCGACATCACGCCCAACCTGACCTTGGAC 1821
Eight           GTGGGCCTCGGCGTCATCGCCGGTATCGGTTTCGACATCACGCCCAAGCTGACCCTGGAC 1195
Three           TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGTCTGACCTTGGAC 1518
Ten             TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGCCTGACCTTGGAC 2588
Five            GTGGGCCTCGGCGTCATCGCCGGCGTCGGTTTCGACATCACGCCCAACCTGACCCTGGAC 1159
Nine            TTGGGCTTCGGCGCGATGGCGGGCGTGGGCATAGACGTCGCGCCCGGCCTGACCTTGGAC 2436
                 ***** ******  ** ** **  * **  * *** ** *****   ****** *****

Four            GCCGGCTACCGCTACCACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAA 1793
Eleven          GCCGGCTACCGCTACCACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAA 1699
One             ACCGGCTACCGCTACCACAACTGGGGACGCCTGGAAAACACCCGCTTCAAAACCCACGAA 1252
Six             GCCGGCTACCGCTACCACTATTGGGGACGCCTGGAAAACACCCGCTTCAAAACCCACGAA 2571
Seven           GCCGGCTACCGCTACCACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAA 1360
Two             GCCGGGTACCGCTACCACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAA 1881
Eight           ACCGGCTACCGCTACCACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAA 1255
Three           GCCGGCTACCGCTACCACTATTGGGGACGCCTGGAAAACACCCGCTTCAAAACCCACGAA 1578
Ten             GCCGGCTACCGCTACCACTATTGGGGACGCCTGGAAAACACCCGCTTCAAAACCCACGAA 2648
Five            ACCGGCTACCGCTACCACAACTGGGGACGCCTGGAAAACACCCGCTTCAAAACCCACGAA 1219
Nine            GCCGGCTACCGCTACCACTATTGGGGACGCCTGGAAAACACCCGCTTCAAAACCCACGAA 2496
                 **** ************ * ********* *****************************

Four            GCCTCGTTGGGCATGCGCTACCGCTTCTGATTCCCCGATACCGATGCCGTCTGAACCTTC 1853
Eleven          GCCTCATTGGGCATGCGCTACCGCTTCTGATTCCCCGATACCGATGCCGTCTGAACCTTC 1759
One             GCCTCATTGGGCATGCGCTACCGCTTCTGATTCCCCGATACCGATGCCGTCTGAACCTTC 1312
Six             GCCTCGTTGGGCGTGCGCTACCGCTTCTGATTCCCCGATACCGATGCCGTCTGAACCTTC 2631
Seven           GCCTCATTGGGCATGCGCTACCGCTTCTGATTCCCCGATACCGATGCCGTCTGAACCTTC 1420
Two             GCCTCGTTGGGCATGCGCTACCGCTTCTGATTCGCCGATTCCGATGCCGTCTGAACCTTC 1941
Eight           GCCTCATTGGGCGTGCGCTACCGCTTCTGATTCCCCGACACCGATGCCGTCTGAACCTTC 1315
Three           GCCTCATTGGGCATGCGCTACCGCTTCTGATTCCCCGACACCGATGCCGTCTGAACCTTC 1638
Ten             GCCTCATTGGGCGTGCGCTACCGCTTCTGATTCGCCGATTCCGATGCCGTCTGAACCTTC 2708
Five            GCCTCATTGGGCATGCGCTACCGCTTCTGATTCCCCGATACCGATGCCGTCTGAACCTTC 1279
Nine            GCCTCATTGGGCGTGCGCTACCGCTTCTGATTCGCCGATTCCGATGCCGTCTGAACCTTC 2556
                ***** ****** ******************** ****  ********************

Four            AGACGGCATTTTTAAT-CGCCCGCCGTTTACA--GGCGCGGGGCGGGCGCAGTAAAATAC 1910
Eleven          AGACGGCATTTTTAAT-CGCCCGCCGTTTACA--GGCGCGGGGCGGGCGCGGGGAAATAC 1816
One             AGACGGCATTTTTGATGCACCTGCCGTTTACA--GGCGCGGGGCGGGCGCAGTAAAATAC 1370
Six             AGACGGCATTTTTGATGCACCTGCCGTTTACG--GGCGCGGGGCGGGCGCAGTAAAATAC 2689
Seven           AGACGGCATTTTTGATGCACCTGCCGTTTACA--GGCGCGGGGCGGGCGCGGGGAAATAC 1478
Two             AGACGGCATTTTTGATGCACCTGCCGTTTACA--GGCGCGGGGCGGGCGCAGTAAAATAC 1999
Eight           AGACGGCATTTTTAAT-CGCCCGCCGTTTACA--GGCGCGGGGCGGGCTTGG--AAATAC 1370
Three           AGACGGCATTTT-GTTTGAGATTCCGTTTACC--AATGGCTGACAAACGCTTCCAAAT-- 1693
Ten             AGACGGCATTTTTGACGCGCTCGCCGTTTACG--GGCGCGGGGCAGGTGCGGGGAAATAC 2766
Five            AGACGGCATTTT------------------------------------------------ 1292
Nine            AGACGGCATTTTTTCT-GATTCGCCGATTCCGATGCCGTCTGAACCTTCAGACGGCATTT 2615
                ************

Four            CCGAACCGTCATTCCCGACAA----------------------------CACCGCAATCT 1942
Eleven          CCGAACCGTCATTCCCGACAATACCGCAATCTCGAAACCCGTCCGACAACACCGCAATCT 1876
One             CCGAACCGTCATTCCCGACAATACCGTAATCTCGAAACCCGTCCGACAACACCGCAATCT 1430
Six             CCGAACCGTCATTCCCGACAACACCGTAATCTCGAAACCCGTC----------------- 2732
Seven           CCGAACCGTCATTCCCGACAATACCGCAATCTCGAAACCCGTC----------------- 1521
Two             CCGAACCGTCATTCCCGACAATACCGTAATCTCGAAACCCGTCCGACAACACCGCAATCT 2059
Eight           CCGAACCGTCATTCCCGACAATACCGTAATCTCGAAACCCGTCCGACAACACCGCAATCT 1430
Three           CGGTATTCTTGGGCTTATGCACTTCCTCTGTCGGCGTGCCGACCATCATCAGCCCGATGA 1753
Ten             CCGTACCGCCATTGCCGAT-ACACCGTAACCCTAAAACCCGCC----------------- 2808
Five            --AAGGCGCAAGATCGGGCAA-ACGGCATTTTCAGACGGCATA-ACTGACAGTATAATCC 1348
Nine            TTAAGGCGCAAGATCGGGCAA-ACGGCATTTTCAGACGGCATA-ACTGACAGTATAATCC 2673
                                    *

Four            CGAAATTCG-TCATTCCCGCGCAGGCGGGAATCCGG--ACCT--GTCCGC--ACGGAAAC 1995
Eleven          CGAAATTCG-TCATTCCCGCGCAGGCGGAAATCCGG--ACCT--GTCCGC--ACGGAAAC 1929
One             TAAAACCCG-TCATTCCCGCGCAGGCGGGAATCCGG--ACCT--GTCCGC--ACGGAAAC 1483
Six             ------------ATTCCCGCGCAGGCGGAAATCCGG--ACCCCCGACGCG--GCGGGAAT 2776
Seven           ------------ATTCCCGCGCAGGCGGAAATCCGG--ACCT--GTCCGC--ACGGAAAC 1563
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Opa 3' deletion primer

Stop codon

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Two             TAAAACCCG-TCATTCCCGCGCAGGCGGGAATCCGG--ACCT--GTCCGC--ACGGAAAC 2112
Eight           CGAAATTCG-TCATTCCCGCGCAGGCGGAAATCCGG--ACCT--GTCCGC--ACGGAAAC 1483
Three           TTTATCC--TTATCCTCGCAACCGAAAGCCTCCCGCAACAATGGGCTGTTGACCCACAT 1811
Ten             ------------ATTCCCGCGCAGGCGGGAATCCGG--ATTTGTCGGGATTCGCTCAAAT 2854
Five            GAACATCCGGCCGCTTTCGCGCGGCCTTCGGCATTATCCGATTTTTTCCGAAAGCCGAAC 1408
Nine            GAACATCCGGCCGCTTTCGCGCGGCCTTCAGCATTATCCGATTTTTTCCGAAAGCCGAAC 2733
                                 ***                                      *

Four            TTATCGG-ATAAAACGGTTGCCCAAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 2054
Eleven          TTATCGG-ATAAAACGGTTGCCCAAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 1988
One             TTATCGG-ATAAAACGGTTGCCCAAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 1542
Six             CTATCGG-A---AATGACTG---AAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 2829
Seven           TTATCGG-ATAAAACGGTTGCCCAAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 1622
Two             TTATCGG-ATAAAACGGTTGCCCAAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 2171
Eight           TTATCGG-ATAAAACGGTTGCCCAAACCCCGCGTCCTAGATTCCCACTTCCGTGGGAATG 1542
Three           CCCCGTA-ATCCAGACATTGTCGAATCCCTGAGCCGTTGCCGCCAGTTGCAGCGCATACG 1870
Ten             CGGGTGGCGGAGGGATGGTGTTGCAGATTAACGATGTGGATACCAACAGCGGGGAATTTG 2914
Five            CATGCAATACAAACCCCTCCTGCTCGCCCTGATGCTTGGCAGGCGGCGG--GTGAAGACC 1466
Nine            CATGCAATACAAACCCCTCCTGCTCGCCCTGATGCTTGGCAGGCGGCGG--GTGAAGACC 2791
                                                    * *    *       * *

Four            ACGGTTCAGTTGCATAGGGCCGGGTGG-----TCGAA-------AGAGGCGGATTCG--- 2099
Eleven          ACGGTTCGGTCTGCCGTTTTCGGACGGC-ATTTCGACTCAATCCAGCAGTGCGTCCACAA 2047
One             ACGGTTCGGTTGCGTAGGGCCGGGTGG-----TCGA--------AAGGGCGGATTCG--- 1586
Six             ACGGTTCGGTTGCATAGGGTCAGATTG-----TCGAA-------AGGGGCGGATTCG--- 2874
Seven           ACGGTTCGGTTGC-TACGGCCCGCT----------------------------------- 1646
Two             ACGGTTCGGTTGC----------------------------------------------- 2184
Eight           ACGGTTCAGCAAGCGTAGGCCGGATACTTGTATCCGACAAAACCTTTAACATTCCCATCA 1602
Three           CCGCACAACCCGCCGTCAGCATCTGCTCCCATTCCGGTTTCGGCTTAGGCACATCGC--- 1927
Ten             CAATCTATACCGCTCAGGATGCATCGGT----------------AAAGCTGGATTTGTT- 2957
Five            ATGGGCGCGGCCCGTATGTGCAGGCGGATCTGGCTTACGCCTACGAGCACATCACCCGCG 1526
Nine            ATGGGCGCGGCCCGTATGTGCAGGCGGATCTGGCTTACGCCTACGAGCACATCACCCGCG 2851

Four            ATGGATTCGATGAAAACGGTAGAA--------ATG---TTGGATTGATGGGA-ATGGCGG 2147
Eleven          ACGCGCGCGCGTCAAACGGGCGCAGGTCGTCTATGCCTTCGCCCACGCCGAT-GTAGCGG 2106
One             ---------ATGAAAACGGTAGAC--------ATG---TTGGATTGATTGTTTATTGTTG 1626
Six             ATGGATTCGATGAAAACGGTAGAA--------ATG---TTGGATTGATGGGA-ATGGCGG 2922
Seven           ---GATTCCCCGACACCGAT------------------------------------GCCG 1667
Two             ---GTTCCGACAACACCG------------------------------------------ 2199
Eight           TTGCAATCCATTGCAGCAATGTCC--------AAAATGTCGAATTCAAGAAT-CCGGCCT 1653
Three           ---GGTTCGGCACAAACGTTACCCCGATGACCATCGGCGCCATATTGCCCACTTTTTCCG 1984
Ten             AAAAATGGAATTGAAACACTGCAAA---------------GAAATGTTGGAA--CAAAAG 3000
Five            ATTATCCCGAAGCAACCGGTGCAAAAAAAGGCACAACAATAAGCACGGTAAGCGATTATT 1586
Nine            ATTATCCCGAAGCAACCGGTGCAAAAAAAGGCACAACAATAAGCACGGTAAGCGATTATT 2911
                              * *

Four            ACTGAAGCCCACCGATTC--ATCGACTCCAACGTTTACGATGCTTCCAACGGTTTCAGAC 2205
Eleven          ACGGGGACGGGGCGGTCGGAAGCAAGCGCGGCGAGGATGCCGCCTTTTGCCGTGCC-GTC 2165
One             AGGAAAATATGA-GATCTTCTTTCCGGTTGAAGCCGATTTGTTTTTATCTTATGGGTGTT 1685
Six             ACTGAAGCCCACCGATTC--ATCGACTCCAACGTTTACGATGCTTCCAACGGTTTCAGAC 2980
Seven           TCTGAA-CCTTCAGA-------------CGGCATTTTTGATGCGCTCGCCGTTTACAGGC 1713
Two             --TAG----------------------------TCTCGAAAC------------------ 2211
Eight           ACAAAATCATTCCGAGCA----TAATACTATGAAATACCGTCGTTTTTACCGCAATGGCG 1709
Three           CCTTTTTCATCGCATCGTCGCCGAAATTCAGTTCGGCAACCGTTTGCTTCAACACATCGC 2044
Ten             ACAAAGAAATCGAGTTGCTCCGCAAG-CTGACCGAAACCGTTTAAACAGATATGCCGTCT 3059
Five            TCAAAAACATCCGCACCCGCTCCGTCCACCCCCGACTCGCCCTCGGCTACGATTTCGGCG 1646
Nine            TCAAAAACATCCGCACCCGCTCCGTCCACCCCCGACTCGCCCTCGGCTACGATTTCGGCG 2971

Four            GGCATTTTTTAC-ACAATTCCCGCCATTTTCCAT-CATTCCCGACAACACCG--CA-ATC 2260
Eleven          GAGTTTGGTAACGATAAGCCCCGTCAGCCCCAATGCGTCGTCAAAGGCTTTG--AC-TTG 2222
One             ATGCTATATCATCATAGTTATGCCGAAGATGCA---GGGCGCGCGGGCAGCG--AG-GCG 1739
Six             GGCATTTTTTAC-ACAATTCCCGCCATTTTCCAT-CATTCCCGACAACACCG--CA-ATC 3035
Seven           G-CGGGGCGGGC-GCAGTAAAATACCCGAACCGT-CATCCCCGACAACACCG--TA-ATC 1767
Two             ------------------------------CCGT-C-----CGACAACACCG--CA-ATC 2232
Eight           GCACTTACTTTT-----TTACGGTTGTAACCAATAAACGGCAGAAGATTTTGACCG-ATG 1763
Three           GAAAACGTTGCAATCCTACCTCGCCTTGAATCACGGTAAAACGGAAGGGGCGCATG-TTG 2103
Ten             GAAAAAAGTTTT--CAGGCGGCATATTCTTCGACAGGTCTTGTATAATACCGTTTG-AAC 3116
Five            GCTGGCGCGTCTTTTCCGCCCCCGCCGTTGCCGCCGGCCTCCACCAATCCCTTCAATATT 1706
Nine            GCTGGCGCGTCTTTTCCGCCCCCGCCGTTGCCGCCGGCCTCCACCAATCCCTTCAATATT 3031

Four            TCGAAACCCGTCATTTCCGACA-ACACCG-CAATCTTGA----------AATTCGTCA-T 2307
Eleven          GTTGACGGCGTTTTGCCCGATA-TTGGCATCGAGTACGACGA-----TGATTTCGTGC-G 2275
One             CAGATACAGGTTTTGGAAGATG-TGCACGTCAAG-GCGA----------AGCGCGTAC-C 1786
Six             TCGAAACCCGTCATTCCCG-CG-CAGGCGGGAATCTAGATC--TGTCAGTGCAGGAAC-T 3090
Seven           TCGAAACCCGTCATTCCCG-CG-CAGGCGGGAATCTAGATC--TGTCAGTGCAGGAAC-T 1822
Two             TTAAAACCCGTCATTCCCG-CG-CAGGCGGGAATCCGGACCCCTGACGCGGCGGGAAT-C 2289
Eight           ATGCGGTGCGTTTGGCTTTACGGCAGGCGGTAATGGCGGTG--------CGCGAACGG-A 1814
Three           CCGTGATCGGAAACTTGGGTTG-CCGCCTGCAATATTTGTTCCAACTCCGCCGCATCG-G 2161
Ten             TTTCAGGCTTTTGATTATGGCGGCAGGCAAACATACCAAACACAGCAACCGGGTACGCAT 3176
Five            ACCGATCCCGCCATTGCCGCCG-ATTCCGTCAAATCCATCAATTCCGCCGAATCACGCCT 1765
Nine            ACCGATCCCGCCATTGCCGCCG-ATTCCGTCAAATCCATCAATTCCGCCGAATCACGCCT 3090
                                           *    *

Four            TCCCGATA-ACACCGCAATC-TCGAAACCCGTCATTCCCGCGC---AGGCGGGAATCCA- 2361
Eleven          GCGCGCCG-GGAATGGCTTT-TTGCAGCACGCGCTTCACTTTT---TTGATTTCTTCCAT 2330
One             GAAAGACA-AAAAAGTGTTTACCGATGCGCGTGCCGTATCGAC---CCGTCAGGATGTG- 1841
Six             TATCGGGC-AAAACGGTTTCTTGAGATTTTGAGTCCTGGATTC---CCACTTTCGCGGG- 3145
Seven           TATCGGGC-AAAACGGTTTCTTGAGATTTTGAGTCCTGGATTC---CCACTTTCGCGGG- 1877
Two             TATCGGAA-ATGAC-------TGAAACCCCGCGTCCTAGATTC---CCACTTCCGTGGG- 2337
Eight           ATCCGTTT-GAAATTTTGGCATGGGTGTTGATGCCCGATCATC---TGCATACCATATGG 1870
Three           GGGCGGGGTGCTTCAGCTTTTTGGAAGATCGGCGGTTCGTCAA---TAATTTTAAAGCAT 2218
Ten             TATCGGCGGGCAATGCCGGGGCAGGAAATTGAGTTTTGCATCCG--CCGACGGACTGCG- 3233
Five            ATTCCCCA-AAAACCTTGATGCGGCGGGCTGAAGCCCGCCCTGCAACCCTCTCTATGCAC 1824
Nine            ATTCCCCA-AAAACCTTGATGCGGCGGGCTGAAGCCCGCCCTGCAACCCTCTCTATGCAC 3149

Four            -GA-CCCCCGACGCGGCGGGAATCT---ATCGGAAATGACTGAAACCCCG-CGTCCTAGA 2415
Eleven          CAA-ATGAAGCTGCGTGGGCAGGCG---GCCGGCGGTGTCGGCAAGCACGATGTCGATCC 2386
One             -----TTCAAATCCGGCGAAAACCTC--GACAACATCGTACGCAGCATACCCGGTGCGTT 1894
Six             --A-ATGACAATTCATAAGTTTCCC-------GAAATTCCAACATAACCGAAACCTGA-- 3193
Seven           --A-ATGACAATTCATAAGTTTCCC-------GAAATTCCAACATAACCGAAACCTGA-- 1925
Two             --A-ATGACGGTTC---GGTTGC------------GTTCCGACAACACCGTAGTCT---- 2375
Eight           CGG-CTGCCGGACAATGATTCTGCTT--ATTCGGAACGCTGGCGGCAAATCAAGCGGC-- 1925
Three           CCATATCGTTATACTCCGGTCATCGG--TCGGGTGTTTCGGACACATATGCCGTGAAGGC 2276
Ten             ----CCCAACCCCCGACAGCGTGCG------TGAAAAGCTGTTTAACTGGCTGGGACAAG 3283
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Five      CCCCTTGCGAGCCCGACACTACGCAACATCTTGAGAACCCATCCTGTCAAGAATACCCGA 1884
Nine      CCCCTTGCGAGCCCGACACTACGCAACATCTTGAGAACCCATCCTGTCAAGAATACCCGA 3209

Four      TTCCCACTTCCGTGGGAATGACGGTTCGGTT--GC-TACGGCCCGCTGATTCCCCGACAC 2472
Eleven    CGCGCGCTTTCGCGGCTTGGACGGCATCGAA--GCACACGGCGGCGGAATCGCCCGTGGT 2444
One       TACACAGCAAGATAAAAGCTCGGGCATTGTG--TCTTTGAATATTCGCGGCGACAGCGGG 1952
Six       CAGTAACCGTAGCAACTGAACCGTCATTCCC--GCGCAGGCGGGAATCTAGAACGTAGAA 3251
Seven     CAGTAACCGTAGCAACTGAACCGTCATTCCC--GACAACACCGTAATCT------TAAAA 1977
Two       -------CGAAAC-------CCGTCATTCCC--GCGCAGGCGGGAATCTAGGATGCGGAA 2419
Eight     --ACAGCCAATATTTAATCGGCGGCAATCTCAGGCTTTGGCAAAACGCTTTTGGGAACAT 1983
Three     TTCAGACGGCATATCCGGCATCAGCGGACGGCGGCAGGCTGCCAATATATCCATTTCC 2336
Ten       ATTTGACGGGAAAAACGGTTTTGAATTCGAT----------------------------- 3314
Five      ACCGTCCCGATACACCGTAATCCTAAAACCC-GTCATTCCCGCGCTGCAATGGGACATCG 1943
Nine      ACCGTCCCGATACACCGTAATCCTAAAACCC-GCCATTCCCGCGCAG-GCGGGAATCCAG 3267

Four      C-----GATGCC--GTCTGAACCTTCAGACGGCATCCAAAACCCGGTGTTTC--AAG--- 2520
Eleven    TTGTGAAATGAC--GGTTACATTGTTGCGCC-CGCCCCAAGCCTGAAGCTGC--TCGCGC 2499
One       TTCGGGCGGGTC--AA-TACGATGGTGGACGGCATCACGCAGACCTTTTATT--CGACTT 2007
Six       -CTGAAGAAACC--GTTTTGCCCGATAAGTTTCCGTGCGGACAA----GTTT--GAATTC 3302
Seven     -CCCCTCCG-CC--ATTATGAA-GACAAATCGCGGCACAAAAAATGCCGTCT--GAAATA 2030
Two       TCTAGAGAAACC------------------------------------------------ 2431
Eight     ACTATCCGCGGT--GAGGCCGATTTTGCCTGCCATTTTGATTATCTGCATTT--CAATCC 2039
Three     TTCCGATAGGTTTGGCTGTTGGAAATGTCCATCAGCCCCAATACCGTGCTGAAATAGTGG 2396
Ten       ------------------------------------------------------------
Five      GCGGCAGCGGGGCGGT--TTTCCCTTCGCT--CGCACTGTTTCTGCTCTG--TTTCATCA 1997
Nine      TCCGTTCGGTTTCAGTCATTTCCAAAAATTGCCGTAGCGTTAAGTTTCTAGATTCCCACT 3327

Four      GCGG-------------------------------------------------------- 2524
Eleven    GCGGCGGCGCGGAAAGTATCGCCTGCCGCCAGCAATACGGATTTGCCCTGCGCTTGGAAA 2559
One       CTACCGATGCGGGCAGGGCAGGCGGTTCATCTCAATTCGG--------TGCATCTGTCGA 2059
Six       CCGCCTGCATGGAAATGACGACATATCGACAGGCGCAGGGTGTTTTGCATTTGATAAAAA 3362
Seven     CTGTTTGGCGGTTTCAGACGGCATTT------GCTCAAACTTTATCAGGCGTAATGGCG- 2083
Two       ------------------------------------------------------------
Eight     GGTCAAACATGGCTATGTAGGACAAATTTCCGATTGGCGGTTTTCTACGTTTCACCGTTA 2099
Three     TCGTGCGAATATTCGTTTTCCGCCGCTTTTTGTTTGAGGCATTGGAAATCTATGCCCCCG 2456
Ten       ------------------------------------------------------------
Five      TAGGTATGCACAACACGGGGATGACGCTTCTGCCGGGCGGTGCAATCCGTTCGACGCACA 2057
Nine      TTCGGAGGAATGACGCGGTGCAGGTTTCCGTGC-GGACGGATTCGTCATTCCCACACAGA 3386

Four      ------------------------------------------------------------
Eleven    TATTTGGCGAGTTTGCCGATAGACGTGGTTTTGCCCGCGCCGTT--GATACCGGCAAGCA 2617
One       CA----GCAATTTTATTGCCGGACTGGATGTCGTCAAAGGCAGC--TTCAGCGGCTCG-G 2112
Six       TGCCGTCTGAAAGCTTCAGACGGCATTTCTGCGGCAATCGGATT--ATTTCCAAACCAAA 3420
Seven     ---CGTTTCGCCTTCTCCGCCGACATTCTCCGCACAGC--GTTT--GCAGACGGTTTCAT 2136
Two       ------------------------------------------------------------
Eight     TGTCAAACAGGGTATTTATCCG-CATAATTGGGGTGGCGGCAAT--GCGGACTTTTCTAT 2156
Three     TGTTGGCGGAAGGCTTTGGAAAACCACATAACCATCGGGATATGCGTCTGTCCGGAAGGC 2516
Ten       ------------------------------------------------------------
Five      TGG----CCCGGCACGGCAGCC----GACTTGGGCATCGAAAT---CCCGCGCGTGCCGT 2106
Nine      CAGGAATCCGGATTTGTCCGCGCGGAAACTTATGCGCCGTCATT--CCCGCACAGGCGGG 3444

Four      ------------------------------------------------------------
Eleven    TAATCACGAAAGGCTCTTTAGTTTCGGGCAAGACCAGCGGTTTTTCCAACGGCTTAATCA 2677
One       CAGGCATCAACAGCCTTGCCGGTTCGGCGAATCTGCGGACTTTAGGCGTGGATGACGTCG 2172
Six       AGCGCGTGGTTGCGTTTGCCGCGCCGGAGGATGGTGTATTTGCCGAAACGTTTGTATTCG 3480
Seven     AACCTGCAACCGCGCCCACATCGCGGG-------TGTAATGCCAGCAGCGTTCGCATTAA 2189
Two       ------------------------------------------------------------
Eight     TGAATACGATTGAAGTAATGTCGGATTCGAGAATCCGACCTACGGAAAACTGAAAGAGCA 2216
Three     GCGATGGCGTAAGGCGCGGCGTGCAGGTACATCCCGTTTTCGCCCAAACTTTCGCCGTGG 2576
Ten       ------------------------------------------------------------
Five      ACTATAG-TGGATTAACAAAAACCAGTACGGCGTTGGCC---CGCCTTAGCTCAAAGAGA 2162
Nine      AATTCAGGTCTGTCGGCATAGGAACTTATCGGATAAAACGGTTGCCCCAACCCTGCGTTC 3504

Four      ------------------------------------------------------------
Eleven    GGTCGTACAAGGCTTCTTTCAACGCGCCGCGCAATTCGTTG-CCGTCTTTCAGCCCTTTG 2736
One       TTCAGGGCAATAATACCTACGGCCTGCTGCTAAAAGGTCTGACCGGCACCAATTCAACCA 2232
Six       CCTATCAACAGATAGGCATCGTCGGGGCGTTCGGCGGCGTGGTTGGGGTTGTTGGCTTCA 3540
Seven     GCTTCCC----------------------------------------------------- 2196
Two       ------------------------------------------------------------
Eight     TCGGCTGCTGGACGGCATTATGCGCAAAGCCCGCCGCAACCGCCCGCTGACGGAGGCGCA 2276
Three     TCGGAAACATAATACACCGCGCTTTCCAAATCGTCGCGGTTTTCAAGTTTGCGGATAACC 2636
Ten       ------------------------------------------------------------
Five      ACGATTCTCTAAGGTGCTGAAGCACCAAGTGAATCGGTTCCGTACTATCTGTACTGTCTG 2222
Nine      TAGATTCCCACTTTCGTGGGAATGACGATTCAGGTA--TTCCTAATAGCAACCCGCCACA 3562

Four      ------------------------------------------------------------
Eleven    AGGCTGACGCGGCCGCGCACGTCTTTCATCAGGTATTCGGTGGCCTCCATGCCCATATCG 2796
One       AAGGTAATGCGAT----------------------------------------------- 2245
Six       GCCGGTTTGCCGTTGAGCAGAACCGCTTTGGCATTGACAAAGCCGCGCCCATGGCGC--- 3597
Seven     ------------------------------------------------------------
Two       ------------------------------------------------------------
Eight     AACCAAACGCAACCGATATTTGTCGAAGACCCGTTATGTGGTCGAACAAAGCTTCGGTAC 2336
Three     TTGTCGATAAACTGATCCACATACAAAACCGTATTGTCGTAAGTGTTGACCAACGTGGCG 2696
Ten       ------------------------------------------------------------
Five      CGGCTTCGCCGCCTTGTCCTGATTTTTGTTAATCCACTATATTCCTAAACCCACCATCCG 2282
Nine      ACCCACCTACGCCCATCCTACGCATCTGCCATACAGCCCTGTTCCGGAGCGCGCGGATAA 3622

Four      ------------------------------------------------------------
Eleven    CCGGTAATCAGCACGGTTTCCAGCTCTTCGTACAAATCCTCAACTTCGC----------- 2845
One       ------------------------------------------------------------
Six       ------------------------------------------------------------
Seven     ------------------------------------------------------------
Two       ------------------------------------------------------------
Eight     GCAAGCTTCCC------------------------------------------------- 2347
Three     CGGGCGCATTTGTCGATTTCGTTGGTATCGCATGTCGGCGTGAATTTGCGCTCGGCTTCG 2756
Ten       ------------------------------------------------------------
Five      CGTTCGAACGC------------------------------------------------- 2293
Nine      GGTGCGATGCCGCCGGCGGGCATCATAGTCCTCTCTCCCTGTGGGAGAGAGCTAGAAAGC 3682
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Opa 10 reverse primer

Opa 2 reverse primer

Opas 4 and 6 reverse primers

Opa 7 reverse primer

Opa 1 reverse primer

Opas 5, 8, and 11 reverse primers

```
Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------------------------------
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           GTATAGCGTTCGTAATACGTCGGCCCGTGGCTGCCGATGGTATGCAGGATTAAAACCGCG 2816
Ten             ------------------------------------------------------------
Five            ------------------------------------------------------------
Nine            TGCAAGGCTTGTATTTTAGAAGACTAAGGGATTTGGGAAAGATTGTCGAAATTCGGGGAG 3742

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------------------------------
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           TCTTTATCGTTTTTGTTGAGGGCTTCGTCGAACTTGGTCAACAGGATATTGTCGAGGCAC 2876
Ten             ------------------------------------------------------------
Five            ------------------------------------------------------------
Nine            CCCTCTCCCCGGATCCACG----------------------------------------- 3761
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Opa 9 reverse primer

```
Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------------------------------
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           TCGCCGTTGCGGCAGTATTCGGGCAGGTTGAGCGAGGTAACGTCGGTATTCGGCACTTTG 2936
Ten             ------------------------------------------------------------
Five            ------------------------------------------------------------
Nine            ------------------------------------------------------------

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------------------------------
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           CCGCACACGCCCTTGCAGCCGGAATCGTTTTCCAACCAAGTAACTTCCACGCCGGCGCGC 2996
Ten             ------------------------------------------------------------
Five            ------------------------------------------------------------
Nine            ------------------------------------------------------------

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------------------------------
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           TGCACGATGTCCAGCAGGTTGTCTTGGTGTTCGGCTTTGATTTCGTCATAATCCGTGCGG 3056
Ten             ------------------------------------------------------------
Five            ------------------------------------------------------------
Nine            ------------------------------------------------------------

Four            ------------------------------------------------------------
Eleven          ------------------------------------------------------------
One             ------------------------------------------------------------
Six             ------------------------------------------------------------
Seven           ------------------------------------------------------------
Two             ------------------------------------------------------------
Eight           ------------------------------------------------------------
Three           TCGAAGGTTGAGAACATACACGGCAGGGAGTGCGCGGTCGATGTGCCGCAGCTTCTGACC 3116
Ten             ------------------------------------------------------------
Five            ------------------------------------------------------------
Nine            ------------------------------------------------------------

Four            ---------------------------------------------------------
Eleven          ---------------------------------------------------------
One             ---------------------------------------------------------
Six             ---------------------------------------------------------
Seven           ---------------------------------------------------------
Two             ---------------------------------------------------------
Eight           ---------------------------------------------------------
Three           TGCGGGAAATTGACAATTTCATCGCCGCGCGCGGCAAGCAGCGGCGTAGTTTGGCGG 3173
Ten             ---------------------------------------------------------
Five            ---------------------------------------------------------
Nine            ---------------------------------------------------------
```

Opa 3 reverse primer

*PLEASE NOTE: Showing colors on large alignments is slow.*

delete each individual Opa, the Opa encoding DNA was cloned into the plasmid pUC19 by restriction enzyme digestion and transformed into *E.coli* DH5αmcr. Each Opa encoding-gene was individually amplified using PCR. The size of each amplicon was determined by analyzing an aliquot of the PCR reaction on an agarose gel (Fig. 2B). The observed sizes agreed with the predicted size of each Opa amplicon as shown in Table 8. Each Opa amplicon is shown in Figure 2B. Each lane contains only one band, indicating that the primers were specific for the individual Opa, despite the high degree of sequence similarity between the Opa encoding-genes. The Opa fragments were digested with specific restriction enzymes, whose recognition sequences had been incorporated into the PCR primers. The same restriction enzymes were used to digest the plasmid pUC19. Restriction enzymes were chosen so that the *Pst*I site in the polylinker region of pUC19 would be removed since this enzyme would be used later in the cloning scheme, and so that they would only digest each Opa fragment once. For most of the Opa cloning, *EcoRI* and *HindIII* were used, but when one of these restriction sites was found in the sequence of the Opa encoding-gene, as in Opas 6, 8, 9, and 10, *SphI* or *BamHI* were substituted. After digesting each Opa fragment and pUC19 with the designated enzymes, the DNA was ligated into pUC19 and transformed into *E.coli* DH5αmcr. White colonies were chosen after plating onto LB media plus the antibiotic ampicillin and the chemical indicator X-gal. The plasmids were extracted and digested with the enzymes used in the cloning to verify that the correct PCR amplicon had been cloned. As can be seen in Figures 2C and 2D, all of the constructs that were extracted from *E.coli* contained a DNA fragment with the same mobility as the PCR amplicon that was used in the cloning. These sizes agree with the predicted sizes in Table 8. This data indicates that each

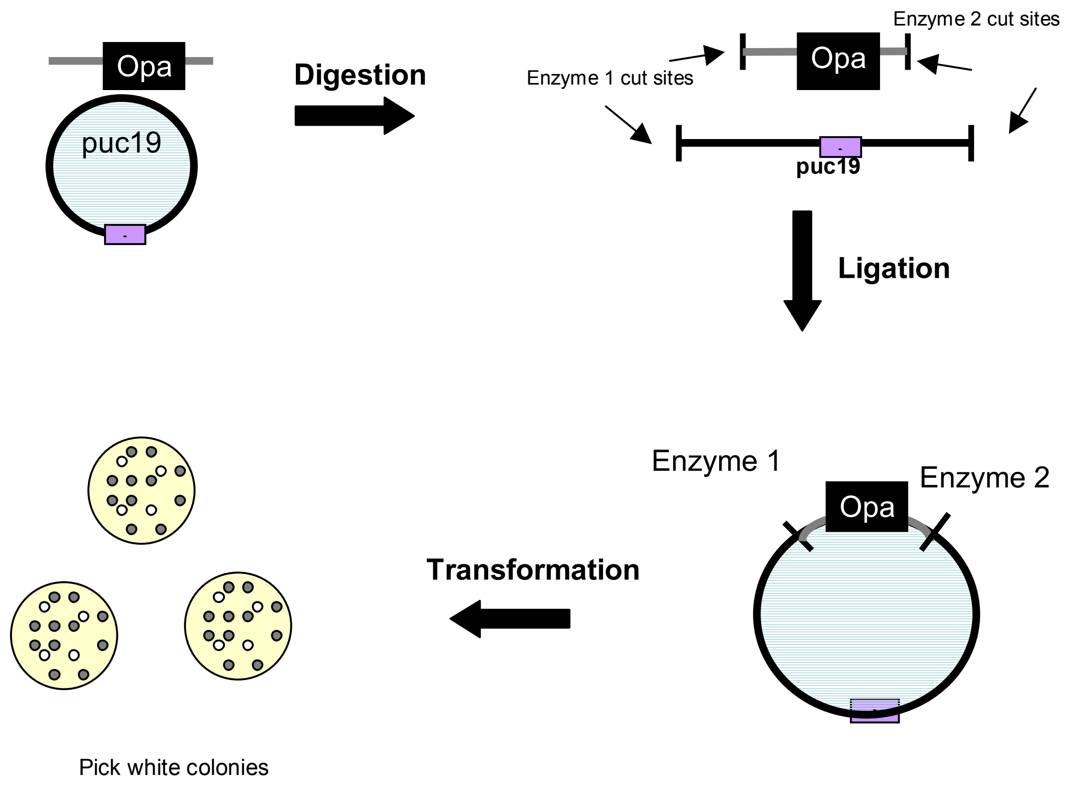



**Figure 2A**. Diagram of the construction of the plasmids pOpaX

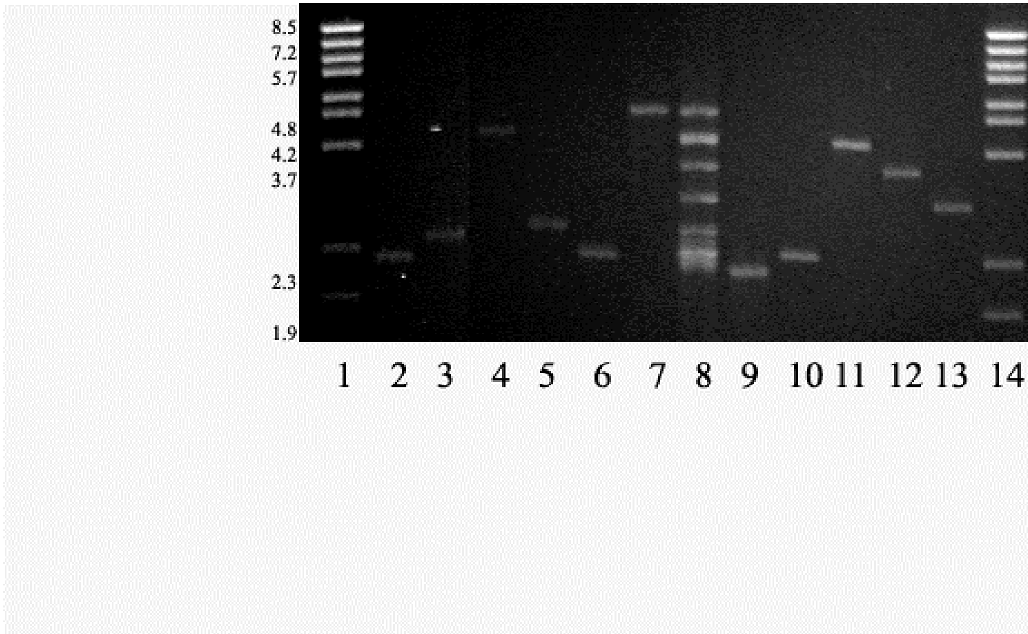



**Figure 2B**. Opa PCR Fragments. Individual Opa fragments were amplified by PCR and run on a 1% agarose gel for 1 hour at 95 V constant voltage to verify the correct sizes of the fragments. The lanes represent:

1. λ DNA digested with *BstEII*
2. Opa 1
3. Opa 2
4. Opa 3
5. Opa 4
6. Opa 5
7. Opa 6
8. All 11 Opas
9. Opa 7
10. Opa 8
11. Opa 9
12. Opa 10
13. Opa 11
14. λ DNA digested with *BstEII*

**Table 8**. Predicted sizes of Opa fragments

| | | Predicted Sizes (kb) | |
|---|---|---|---|
| Opa Number | pOpa | pOpaΔ | pOpaΔs |
| 1 | 2.2 | 4.0 | 5.4 |
| 2 | 2.4 | 4.2 | 5.6 |
| 3 | 1.8 | 3.5 | 5.0 |
| 4 | 2.5 | 4.6 | 6.0 |
| 5 | 2.3 | 4.2 | 5.6 |
| 6 | 3.6 | 5.0 | 6.5 |
| 7 | 2.2 | 3.7 or 4.0 | 5.1 or 5.4 |
| 8 | 2.3 | 3.4 | 4.7 |
| 9 | 3.8 | 5.6 | 7.0 |
| 10 | 3.3 | 5.1 | 6.5 |
| 11 | 2.8 | 4.6 | 6.0 |

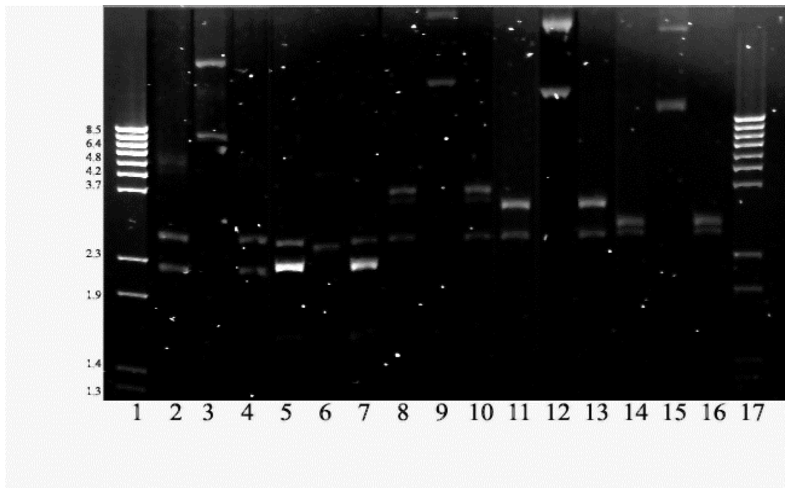
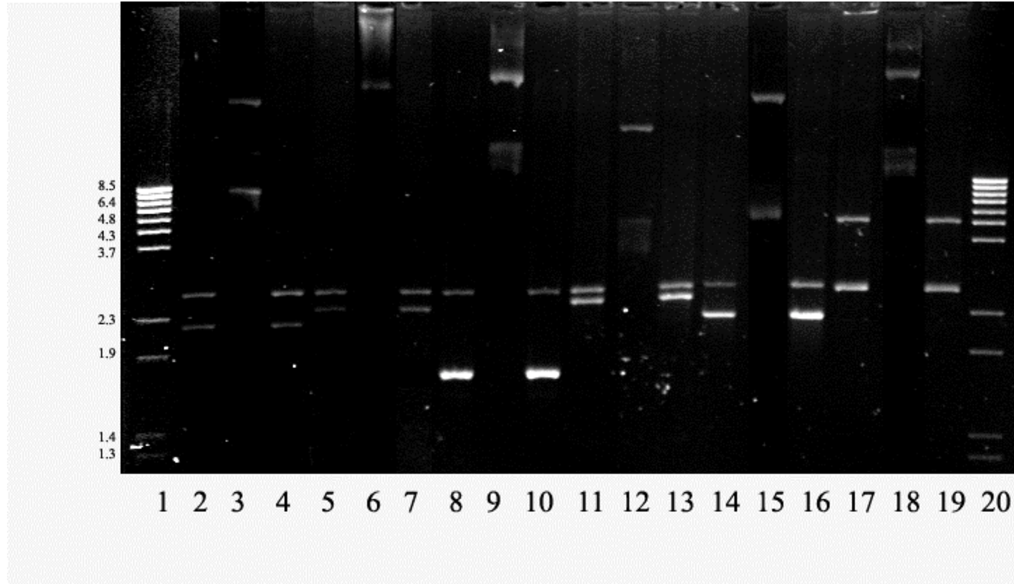



**Figure 2C and 2D**. Opa fragments cloned into pUC19. Opa fragments were cut with the appropriate restriction enzymes as was pUC19 at 37$^0$C for 2 hours. The products of the digestions were cleaned, and ligated over night at 16$^0$C. Ligated products were transformed into *E.coli* DH5αmcr and selected for Lac$^-$ ampicillin resistant phenotype. Clones were verified by restriction digestion with the enzymes used in the original cloning, and restriction fragments were run on a 1% agarose gel in TBE buffer for 1 hour at 95 V constant voltage. The lanes represent

**2C**

1. λ DNA cleaved with *BstE*II
2. Opa 1 and pUC19
3. Miniprep of Opa 1 and pUC 19 undigested
4. *EcoRI* and *HindIII* digestion of Opa 1 and pUC19 miniprep
5. Opa 2 and pUC19
6. Miniprep of Opa 2 and pUC 19 undigested
7. *BamHI* and *HindIII* digestion of Opa 2 and pUC19 miniprep
8. Opa 3 and pUC19
9. Miniprep of Opa 3 and pUC 19 undigested
10. *EcoRI* and *HindIII* digestion of Opa 3 and pUC19 miniprep
11. Opa 4 and pUC19
12. Miniprep of Opa 4 and pUC 19 undigested
13. *EcoRI* and *HindIII* digestion of Opa 4 and pUC19 miniprep
14. Opa 5 and pUC19
15. Miniprep of Opa 5 and pUC 19 undigested
16. *EcoRI* and *HindIII* digestion of Opa 5 and pUC19 miniprep
17. λ DNA cleaved with *BstE*II




**2D**

1. λ DNA cleaved with *BstE*II
2. Opa 6 and pUC19
3. Miniprep of Opa 6 and pUC 19 undigested
4. *EcoRI* and *HindIII* digestion of Opa 6 and pUC19 miniprep
5. Opa 7 and pUC19
6. Miniprep of Opa 7 and pUC 19 undigested
7. *EcoRI* and *HindIII* digestion of Opa 7 and pUC19 miniprep
8. Opa 8 and pUC19
9. Miniprep of Opa 8 and pUC 19 undigested
10. *EcoRI* and *HindIII* digestion of Opa 8 and pUC19 miniprep
11. Opa 9 and pUC19
12. Miniprep of Opa 9and pUC 19 undigested
13. *EcoRI* and *HindIII* digestion of Opa 9 and pUC19 miniprep
14. Opa 10 and pUC19
15. Miniprep of Opa 10 and pUC 19 undigested
16. *EcoRI* and *HindIII* digestion of Opa 10 and pUC19 miniprep
17. Opa 11 and pUC19
18. Miniprep of Opa 11 and pUC 19 undigested
19. *EcoRI* and *HindIII* digestion of Opa 11 and pUC19 miniprep
20. λ DNA cleaved with *BstE*II

Opa containing fragment had been individually cloned into the plasmid pUC19.

**Part Two: Deletion of Opa Coding Sequence**

The second step of the gene deletion process was to use PCR to delete the coding region of each gene. The general scheme for the deletion is outlined in Figure 3A. First the start and stop codons were located (Fig. 1). Primers were designed to amplify a sequence around the coding region. This resulted in a PCR amplicon that included pUC19 and the regions flanking the coding sequence. The predicted size of these fragments was calculated by subtracting the size of the fragment between the deletion primers from the total size of the Opa PCR fragment plus the size of pUC19. The deletion PCR fragments were analyzed on an agarose gel to verify their size (Fig. 3B) and agree with the predicted sizes. The PCR primers used for the PCR products were flanked with either *Pst*I restriction sites for Opas 1, 2, 3, 4, 5, 7, 9, 10 and 11, or *Xho*I restriction sites for Opas 6 and 8. The amplicons were digested with the appropriate enzymes, ligated onto themselves, and transformed into *E.coli* DH5αmcr. Upon extraction of the plasmids from *E.coli*, the plasmids were cut with *Pst*I or *Xho*I as appropriate, and analyzed on an agarose gel to assure that the correct deletion plasmid had been identified (Fig. 3C and 3D). As can be seen in Figures 3C and 3D, the size of the Opa deletion amplicons are the same as the digestion products isolated after the transformation, verifying that the constructs were created correctly.

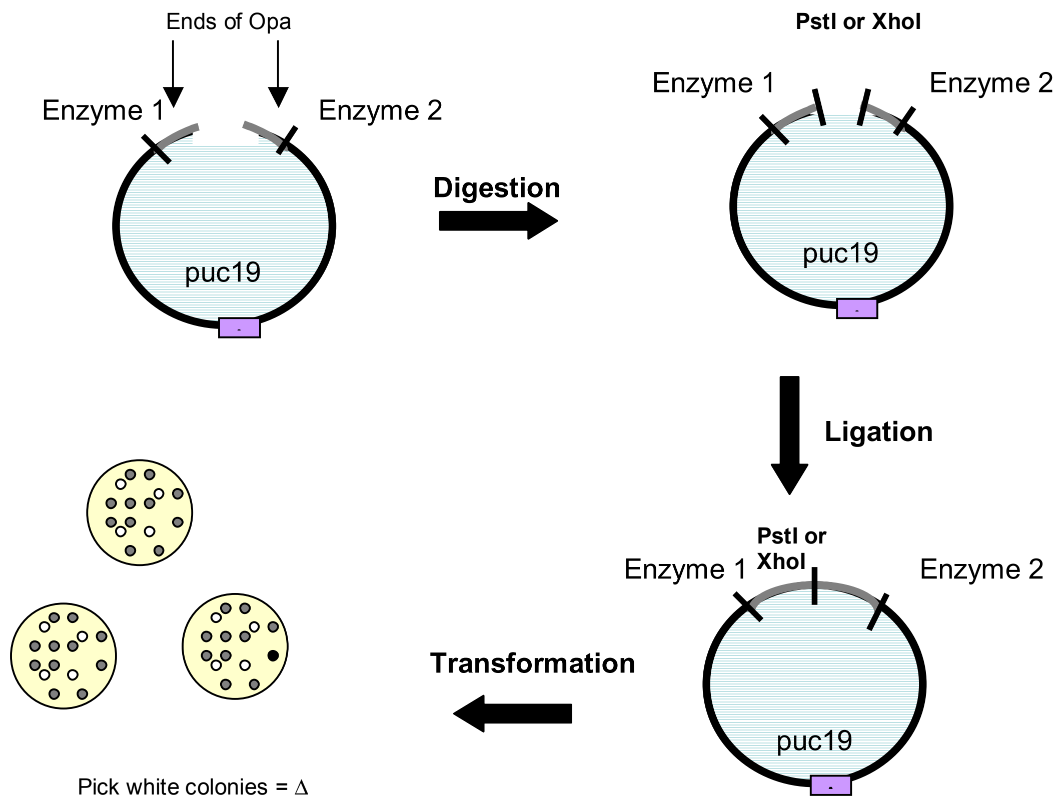



**Figure 3A**. Diagram of the construction of the plasmids pOpaXΔ.

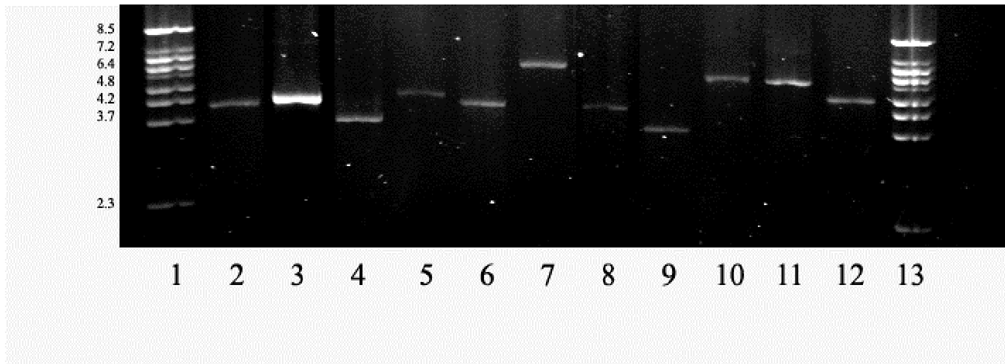



**Figure 3B**. Opa deletion PCR fragments. PCR fragments were verified by running on a 1% agarose gel in TBE buffer for 1 hour at 95 V constant voltage and comparing the sizes of the fragments to the predicted sizes in Table 8. The lanes represent

1. λ DNA cleaved with *BstE*II
2. Opa 1Δ
3. Opa 2Δ
4. Opa 3Δ
5. Opa 4Δ
6. Opa 5Δ
7. Opa 6Δ
8. Opa 7Δ
9. Opa 8Δ
10. Opa 9Δ
11. Opa 10Δ
12. Opa 11Δ
13. λ DNA cleaved with *BstE*II

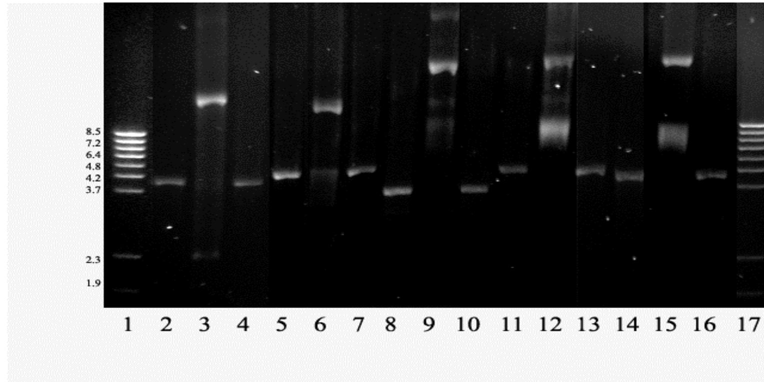
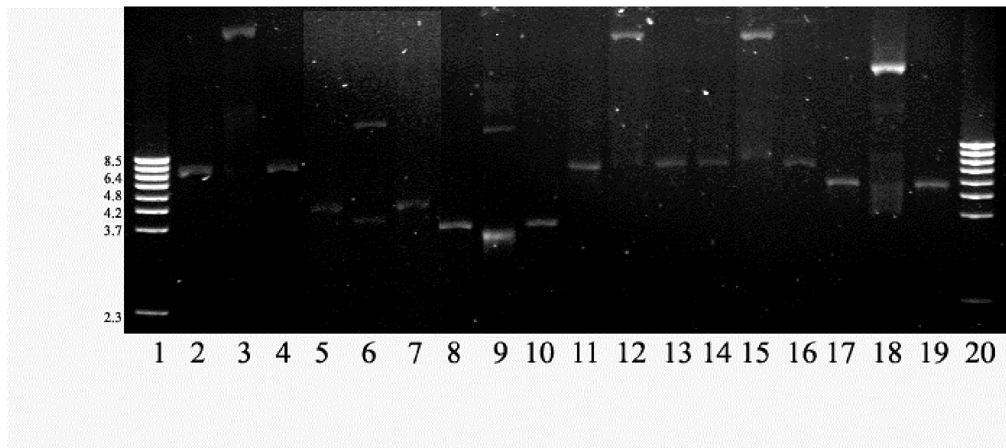



**Figure 3C and 3D**. Opa deletion fragments in pUC 19. Opa minipreps were cut with the appropriate restriction enzymes at 37$^0$C for 2 hours. The products of the digestions were cleaned, and ligated over night at 16$^0$ C. Ligated products were transformed into *E.coli* DH5αmcr and selected for Lac$^-$ ampicillin resistant phenotype. Clones were verified by restriction digestion with the enzymes used in the original cloning, and restriction fragments were run on a 1% agarose gel in TBE buffer for 1 hour at 95 V constant voltage. The lanes represent

**3C**

1. λ DNA cleaved with *BstE*II
2. Opa 1Δ PCR fragment
3. Opa 1Δ miniprep undigested
4. Opa 1Δ miniprep digested with *PstI*
5. Opa 2Δ PCR fragment
6. Opa 2Δ miniprep undigested
7. Opa 2Δ miniprep digested with *PstI*
8. Opa 3Δ PCR fragment
9. Opa 3Δ miniprep undigested
10. Opa 3Δ miniprep digested with *PstI*
11. Opa 4Δ PCR fragment
12. Opa 4Δ miniprep undigested
13. Opa 4Δ miniprep digested with *PstI*
14. Opa 5Δ PCR fragment
15. Opa 5Δ miniprep undigested
16. Opa 5Δ miniprep digested with *PstI*
17. λ DNA cleaved with *BstE*II




**3D**

1. λ DNA cleaved with *BstE*II
2. Opa 6Δ PCR fragment
3. Opa 6Δ miniprep undigested
4. Opa 6Δ miniprep digested with *XhoI*
5. Opa 7Δ PCR fragment
6. Opa 7Δ miniprep undigested
7. Opa 7Δ miniprep digested with *PstI*
8. Opa 8Δ PCR fragment
9. Opa 8Δ miniprep undigested
10. Opa 8Δ miniprep digested with *XhoI*
11. Opa 9Δ PCR fragment
12. Opa 9Δ miniprep undigested
13. Opa 9Δ miniprep digested with *PstI*
14. Opa 10Δ PCR fragment
15. Opa 10Δ miniprep undigested
16. Opa 10Δ miniprep digested with *PstI*
17. Opa 11Δ PCR fragment
18. Opa 11Δ miniprep undigested
19. Opa 11Δ miniprep digested with *PstI*
20. λ DNA cleaved with *BstE*II

**Part Three: Insertion of Spectinomycin Resistance Cassette**

To allow for selection of transformants carrying the Opa deletion, a spectinomycin resistance cassette was inserted into the region where the Opa coding sequence had been (Fig. 4A). The spectinomycin resistance cassette was amplified fromthe plasmid pHP45. PCR primers for the spectinomycin resistance cassette were flanked with either *PstI* or *Xho*I sites. Both the deletion plasmid and the spectinomycin resistance cassette were cut with these enzymes and ligated together. This construct was then transformed into DH5αmcr, and spectinomycin resistant colonies were isolated. Plasmid DNA was purified from the transformants, digested with either *Pst*I or *Xho*I, and analyzed on an agarose gel to verify the construct. As shown in Figures 4B and 4C, the constructs that were recovered from the *E.coli* produced the expected digestion products. Each lane contains two bands, one around 1.4 kb, the size of the spectinomycin resistance cassette, and another the size of each Opa deletion PCR product (Table 8). The size of the fragments, along with the ability of the *E.coli* to grow on spectinomycin, indicates that the spectinomycin resistance cassette had successfully been inserted into the Opa coding region of each Opa encoding-gene.

**Part Four: Transformation into *N. gonorrhoeae* strain MS11**

Once the Opa replacement plasmids had been verified one was chosen to be transformed into *N. gonorrhoeae* strain MS11 for use in a transcytosis experiment. The Opa 5 deletion was chosen to be transformed because of its 97% sequence homology to Opa C, an Opa associated with invasive disease, and the gonococcal strain MS11 was chosen because of its extensive use in invasion studies. The pOpa5Δs was cleaned and transformed into MS11 for 4 hours at $37^0$C with constant shaking. After growth on

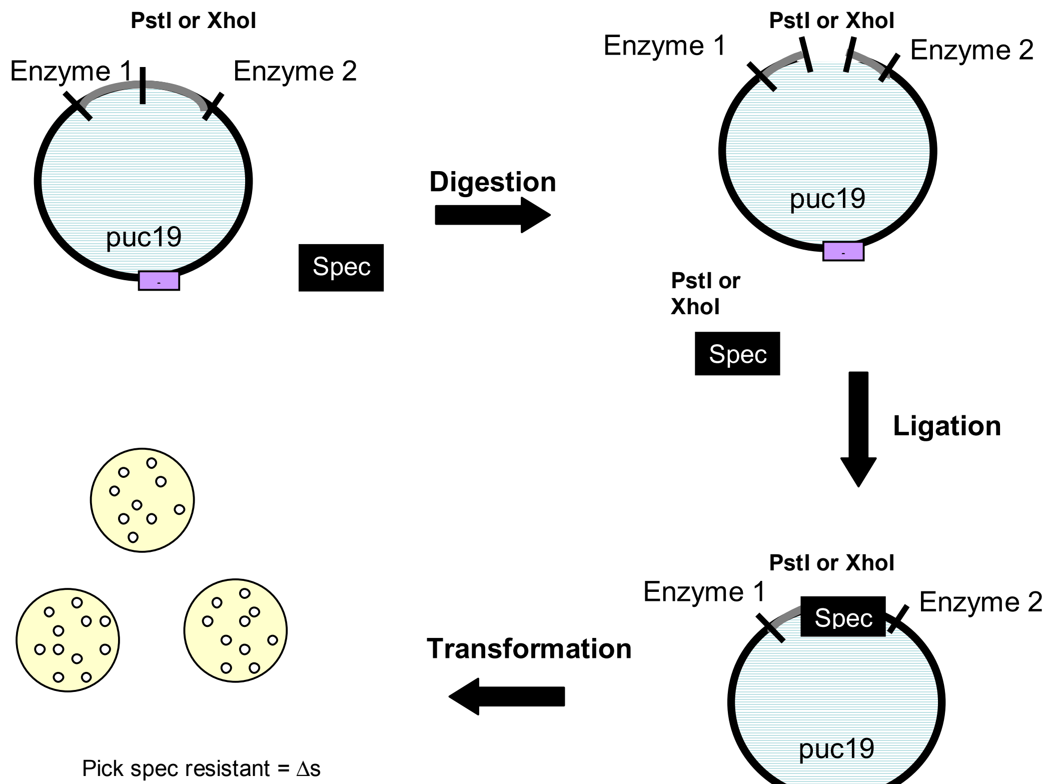



**Figure 4A**. Diagram of the construction of the plasmids pOpaΔs.

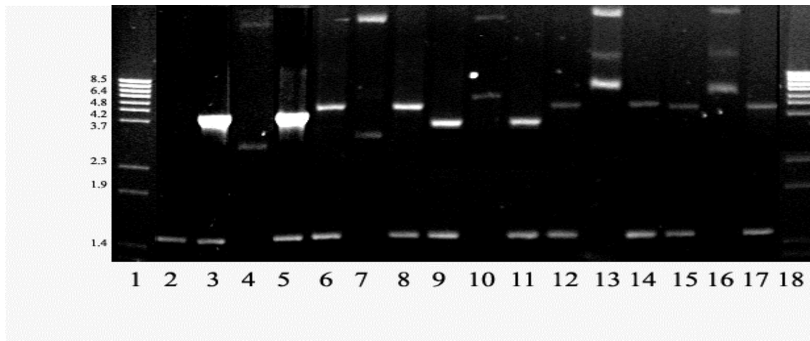
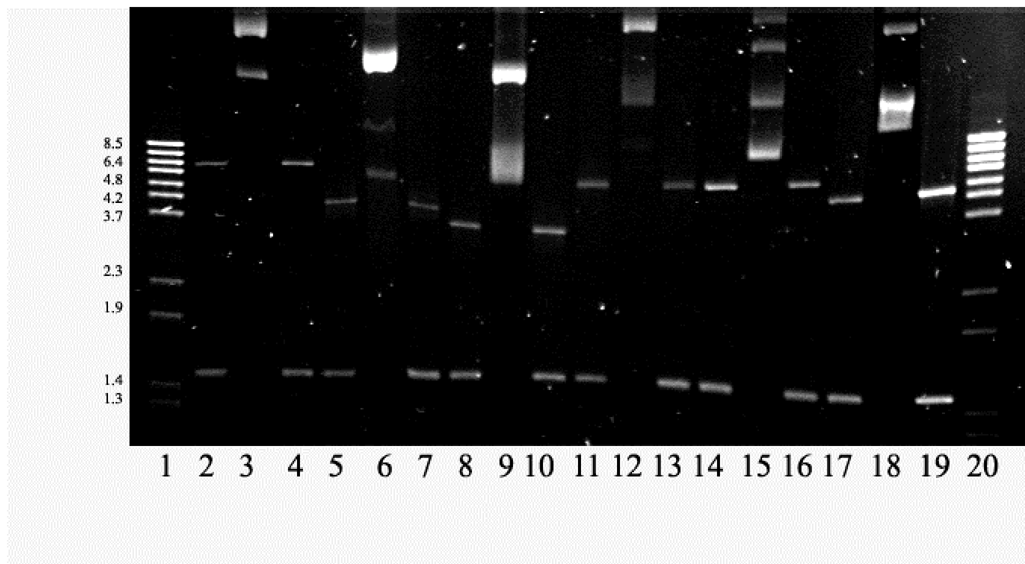



**Figure 4B and 4C**. Opa deletion fragments with spectinomycin cassette inserted cloned into pUC 19. OpaΔ minipreps along with the spectinomycin resistance cassette were cut with the appropriate restriction enzymes at $37^0$C for 2 hours. Digestions were cleaned, and ligated over night at $16^0$C. Ligated products were transformed into *E.coli* DH5αmcr and selected for spectinomycin resistant phenotype. Clones were verified by restriction digestion with the enzymes used in the original cloning, and restriction fragments were run on a 1% agarose gel in TBE buffer for 1 hour at 95 V constant voltage. The lanes represent

**4B**

1. λ DNA cleaved with *BstE*II
2. Spec cassette
3. Opa 1Δ PCR fragment and Spec cassette digested with *PstI*
4. Opa 1Δs miniprep undigested
5. Opa 1Δs miniprep digested with *PstI*
6. Opa 2Δ PCR fragment and Spec cassette digested with *PstI*
7. Opa 2Δs miniprep undigested
8. Opa 2Δs miniprep digested with *PstI*
9. Opa 3Δ PCR fragment and Spec cassette digested with *PstI*
10. Opa 3Δs miniprep undigested
11. Opa 3Δs miniprep digested with *PstI*
12. Opa 4Δ PCR fragment and Spec cassette digested with *PstI*
13. Opa 4Δs miniprep undigested
14. Opa 4Δs miniprep digested with *PstI*
15. Opa 5Δ PCR fragment and Spec cassette digested with *PstI*
16. Opa 5Δs miniprep undigested
17. Opa 5Δs miniprep digested with *PstI*
18. λ DNA cleaved with *BstE*II




**4C**

1. λ DNA cleaved with *BstE*II
2. Opa 6Δ PCR fragment and Spec cassette digested with *XhoI*
3. Opa 6Δs miniprep undigested
4. Opa 6Δs miniprep digested with *XhoI*
5. Opa 7Δ PCR fragment and Spec cassette digested with *PstI*
6. Opa 7Δs miniprep undigested
7. Opa 7Δs miniprep digested with *PstI*
8. Opa 8Δ PCR fragment and Spec cassette digested with *PstI*
9. Opa 8Δs miniprep undigested
10. Opa 8Δs miniprep digested with *PstI*
11. Opa 9Δ PCR fragment and Spec cassette digested with *XhoI*
12. Opa 9Δs miniprep undigested
13. Opa 9Δs miniprep digested with *XhoI*
14. Opa 10Δ PCR fragment and Spec cassette digested with *XhoI*
15. Opa 10Δs miniprep undigested
16. Opa 10Δs miniprep digested with *XhoI*
17. Opa 11Δ PCR fragment and Spec cassette digested with *PstI*
18. Opa 11Δs miniprep undigested
19. Opa 11Δs miniprep digested with *PstI*
20. λ DNA cleaved with *BstE*II

media containing spectinomycin, resistant colonies were selected and their genomic DNA was extracted. This DNA was used as a template for PCR with the Opa5 primers. MS11 chromosomal DNA was also used as a template for a separate control PCR reaction. The projected size for the Opa 5 amplicon was approximately 2.3 kb, and the band in lane one agrees with this prediction (Fig. 5). The Opa5Δs amplicon was predicted to be slightly larger, about 2.8 kb, and the band agrees with the predicted size (Fig. 5). The Opa5Δs amplicon is a combination of three bands, a 0.4 kb band 5' of the spectinomycin resistance cassette, a 1.0 kb band 3' of the spectinomycin resistance cassette, and a 1.4 kb band which is the spectinomycin resistance cassette. These three bands are liberated from one another upon *PstI* digestion. As can be seen in Figure 5, lane 4, all three of these bands are present. This demonstrates that the correct Opa has been replaced by the spectinomycin resistance cassette. This clone was then used to study the affects of the Opa 5 deletion on the ability of MS11 to traverse a monolayer of polarized T84 cells.

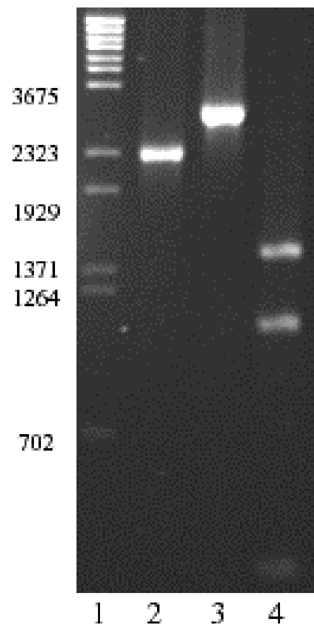



**Figure 5**. Opa 5 deletion in MS11. pOpa5Δs was transformed into *N. gonorrhoeae* strain MS11, and genomic DNA was extracted from spectinomycin resistant clones. DNA was digested with *PstI* at 37$^0$C for 2 hours, and verified by running on a 1% agarose gel in TBE buffer for 1 hour at 95 V constant voltage. The lanes represent

1. λ DNA cleaved with *BstEII*
2. Opa 5 PCR fragment from MS11 chromosomal DNA
3. Opa5Δs PCR fragment from spectinomycin resistant clone
4. Opa5Δs PCR fragment digested with *PstI*

**Section Two: Analysis of the ability of *N. gonorrhoeae* strain MS11 to traverse a polarized epithelial cell monolayer**

Previous research has shown that Opa proteins are important in the pathogenesis of *N. gonorrhoeae*, but the significance of each individual protein has yet to be characterized. The experiments described in this section begin the characterization of the importance of one of the Opa proteins, Opa 5, in the ability of gonococci to transmigrate across a monolayer of polarized human epithelial cells. The ability to transmigrate across the monolayer has implications in the ability of *N. gonorrhoeae* to spread beyond its initial point of colonization and lead to syndromes like pelvic inflammatory disease. Once the OpaΔs mutant was constructed it could be tested in a transcytosis experiment. Before commencing with this, control experiments needed to be done to characterize the polarized T84 cell monolayer, and to see how wildtype gonococci interact with the monolayer. Briefly, the experiments in this section involve an initial characterization of the polarized T84 cells, visualization of the interaction of *N. gonorrhoeae* with the T84 cell monolayer, both by laser scanning confocal microscopy. Finally, an analysis of the ability of gonococci to transmigrate across the polarized T84 cell monolayer both in the basolateral to apical direction and the apical to basolateral direction was done.

**Part One: Characterization of polarized human epithelial cell line T84**

Before commencing the study of the ability of *N. gonorrhoeae* strain MS11 to traverse a polarized T84 monolayer, the ability of MS11 to interact with the monolayer was first assessed using laser scanning confocal microscopy. For these experiments, T84 cells were grown on transwells (3µm pore size) (Fig. 6). T84 cells grown on the filters become polarized with their apical surface contacting the medium and their basolateral

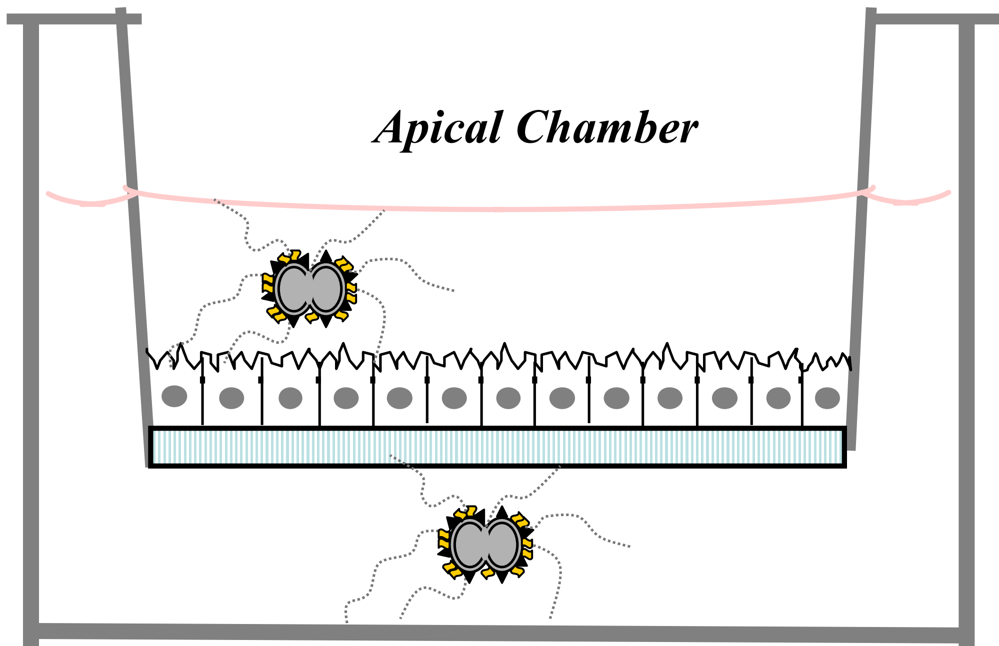



**Figure 6**. Diagram of transwells used for transcytosis experiments

surface contacting the filter. This arrangement mimics epithelial cells *in vivo*, and provides a good model for which to study the passage of gonococci from one side of the cell to the other. Bacteria can be added to either chamber and allowed to passage through the monolayer of cells that grows on the filters. Initially the monolayer was visualized by staining with a fluorescent antibody to the tight junction protein ZO-1. Tight junctions separate the apical and basolateral surfaces of the epithelial cell. The ability to visualize the tight junctions assured that it would be possible to determine the location of the cell-associated gonococci relative to the apical or basolateral poles of the T84 cells in later experiments. Figure 7 shows that staining for tight junctions was possible. The tight junctions looked well formed, as red rings around the cells were seen. Figure 8, in which both tight junctions and F-actin (stained with phalloidin) were visualized, indicated that the F-actin was at the tight junctions and apical surface when no bacteria are present. F-actin was present both at the cell periphery as well as along the tight junctions as can be seen by the colocalization of F-actin and ZO-1, shown as yellow (Fig. 8B and 8C).

**Visualization of the Interaction of *N. gonorrhoeae* With a Polarized T84 Monolayer**

After visualizing the tight junctions, the monolayers were infected with three strains of gonococci, F62, F62Δ8-1, and MS11, from the apical surface, to see where the bacteria would be localized and whether or not there would be any differences between the strains. This was done using fluorescence microscopy. F62 was chosen as a wildtype control, while F62Δ8-1 was chosen as a negative control because previous research indicated that F62Δ8-1 did not invade non-polarized epithelial cells [27]. MS11 however, has been demonstrated to be an invasive strain, and it was hypothesized that the F62 strains would not interact with the monolayers as well as MS11. To determine the

**Figure 7.** Cellular distribution of ZO-1 in polarized T84 cells. T84 cells, grown on transwells, were washed, fixed, and permeablized. The tight junctions were visualized with a Z-01 specific antibody and an Alexa-Fluor 633-conjugated goat anti-rat IgG antibody (shown as red). The cells were analyzed using laser scanning confocal microscopy. The image was taken from the optical sections where the tight junctions were visible. The bar in the upper left corner indicates 10 μm.

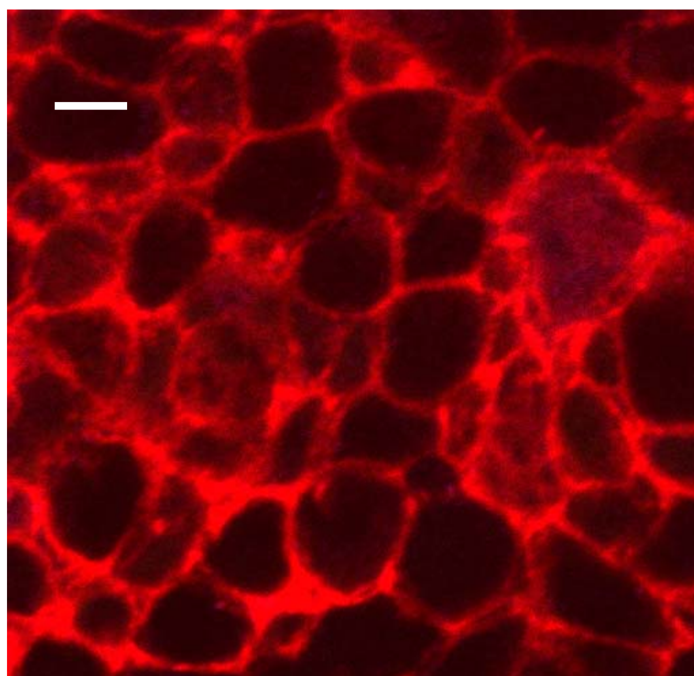
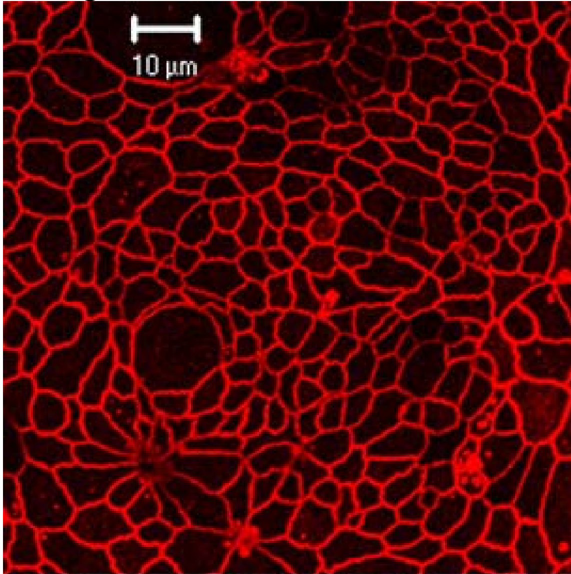


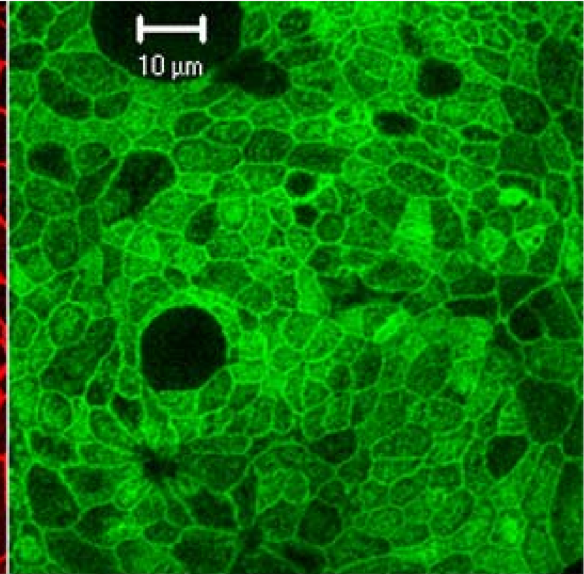
**Figure 8**. Cellular distribution of F-actin and ZO-1 in polarized T84 cell monolayer. T84 cells, grown on filter supports, were washed, fixed, and permeablized. The actin cytoskeleton was visualized with Alexa Fluor 488-phalloidin (green) and tight junctions were visualized with a Z-01 specific antibody and an Alexa-Flour 633-conjugated goat anti-rat IgG antibody (shown as red). Actin and tight junction colocalization can be seen as yellow. The cells were analyzed using laser scanning confocal microscopy. The images were taken from the optical sections where the tight junctions were visible. The bar in the upper left corner indicates 10 µm. Shown are the representative images:

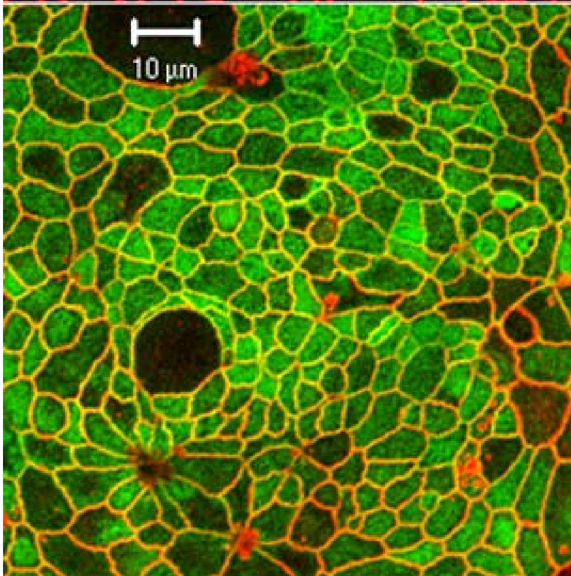
A. Tight junctions
B. Actin cytoskeleton
C. Overlay of tight junctions and actin cytoskeleton

A. Tight Junctions




B. F-Actin




C. Overlay

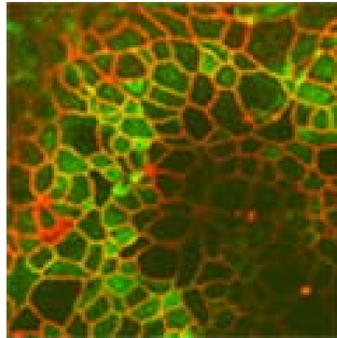
best time to visualize interaction, different time points were chosen. Shorter time points, 0, 6, and 12 hours, were used as well as one longer time point of 24 hours after inoculation. Bacteria were incubated with T84 cells for the given amount of time, removed, the monolayers were stained with fluorescent antibodies, and then viewed with a laser scanning confocal microscope. Figure 9 shows that there was a difference seen in the ability of F62 and F62Δ8-1 versus MS11 to interact with the monolayer. The 0 hour control represents monolayers that had not yet been infected with bacteria, and hence no bacteria were seen. By 6 hours all three strains of bacteria were detected in the apical region of the T84 cells as indicated by the white spots. In particular, the number of MS11 was much higher than the other two strains. This trend continued through the other time points, and was especially evident at the 24 hour time point. At 24 hours, the apical area of the monolayer was almost completely covered with MS11, while the F62 and F62Δ8-1 still appeared scattered. All strains appeared to be in aggregates, or micro-colonies. No overlap of white and red was seen meaning that the bacteria were not located in association with the tight junctions, but rather dispersed throughout the apical surface of the cell. Also, no overlap of white and green was seen, indicating that F-actin was not recruited to the site of bacterial attachment.

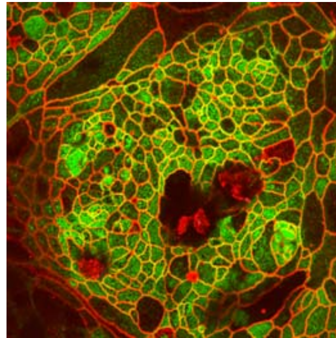
To further determine where host cell-associated gonococci were located, 1μm optical slices were taken, from the apical pole to basolateral pole of the T84 cell, to determine the location of the bacteria relative to the cell surface. As seen in Figure 10, all of the green bacteria were detected in the slices before or in the same slice as the tight junctions. This indicated that most of the bacteria were located in the

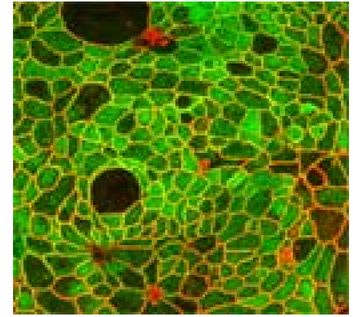
**Figure 9**. Interaction of *N. gonorrhoeae* strains F62, F62Δ8-1, and MS11 with polarized T84 monolayer. T84 cells grown on transwells, were incubated with $10^6$ gonococci at $37^0$C for 6, 12, and 24 hours. After washing, fixation, and permeabilization, gonococci were visualized with a mouse anti-gonococcal outer membrane protein mAb and an Alexa Fluor 488 goat anti-mouse $IgG_1$ (shown as white). Actin filaments were stained with an Alexa Fluor 546 phalloidin (shown as green), only at 0 and 24 hours. Tight junctions were stained with a rat anti-ZO1 antibody and Alexa Fluor 633 goat anti rat IgG (shown as red) (only at 0 and 24 hours). The cells were analyzed using laser scanning confocal fluorescence microscopy. The images were taken from the optical sections where tight junctions were visible. The bar in the upper left corner indicates 10 μm. Shown are the representative images:

0 hour
6 hours
12 hours
24 hours

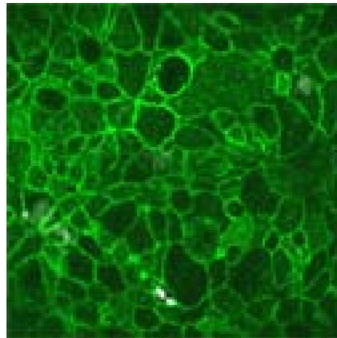
0 hours

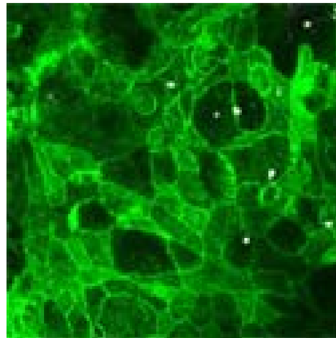


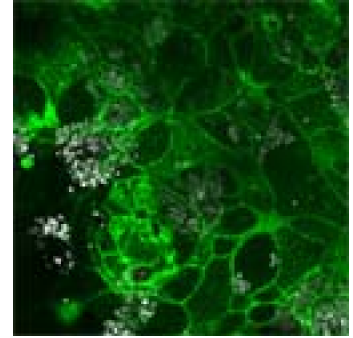




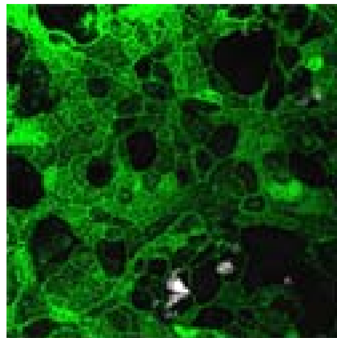
6 hours

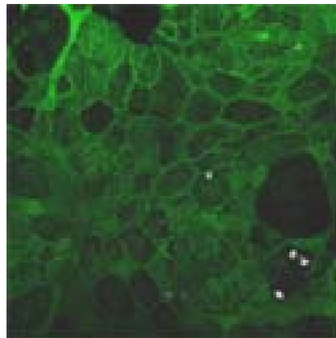


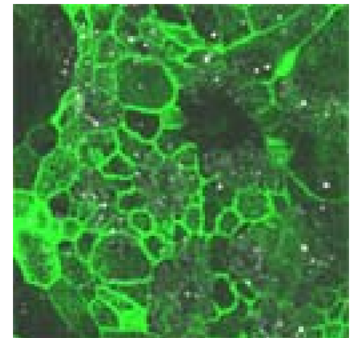




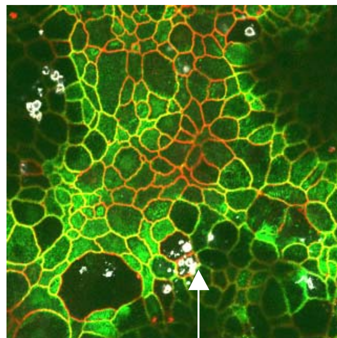
12 hours

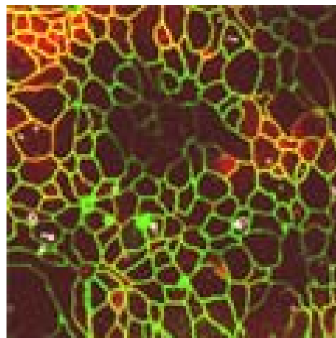


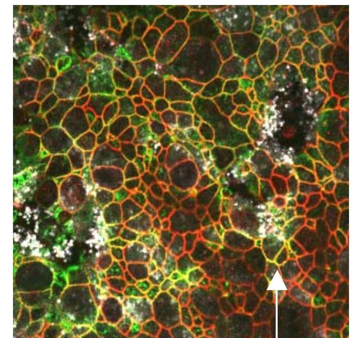




24 hours







few bacteria | | many bacteria
F62 | F62Δ8-1 | MS11

apical portion of the cell, either on the cell surface where actin was visible, or just underneath the cell surface. No bacteria were seen below the level where the tight junctions were visible, indicating that there were few, if any, gonococci in the basolateral area or associated with the basolateral surface. Because no staining was done to highlight the basolateral membrane of the T84 cells it can not be concluded with certainty that the bacteria were not associated here, but even when 20 1μm slices were taken from the apical to the basolateral surface of the cells, no bacteria were ever found in the basolateral portion of the cell, for any of the strains.

All three strains appeared to be either on the apical surface or just below the surface.

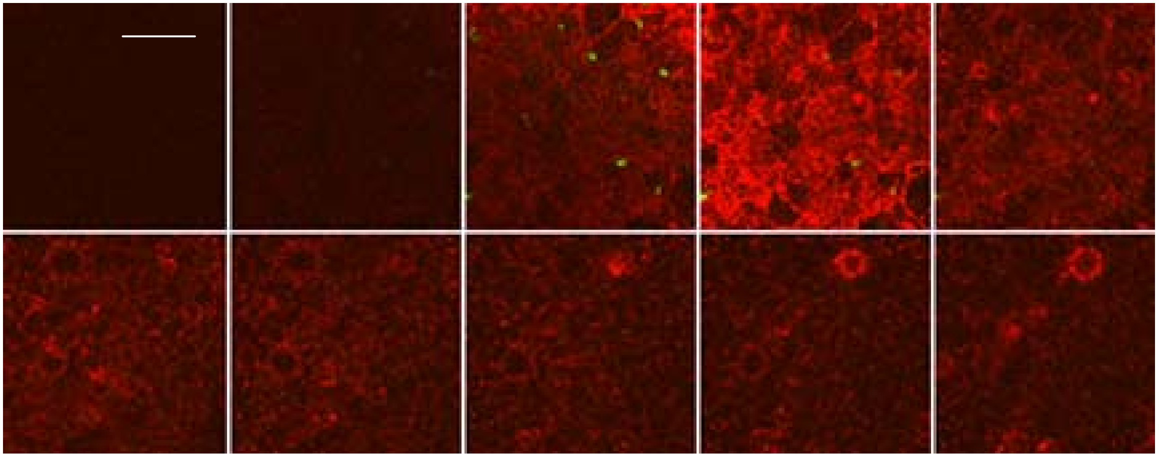
**General Strategy for Transcytosis Experiments**

Once the interaction of gonococci with the T84 cell monolayer was characterized, experiments were conducted to quantitatively determine the ability of *N. gonorrhoeae* to transcytose the monolayer. The general process used for these experiments is diagramed in Figure 11. Briefly, approximately two weeks prior to the experiment T84 cells were seeded onto the transwells and incubated with medium renewal every other day. When the trans-epithelial resistance (TER) reached above 1500 Ω, transcytosis experiments were conducted. Either $10^5$ or $10^6$ gonococci were added to the basolateral or apical chamber and incubated for varying lengths of time at $37^0$C. The TER was assessed at the beginning of the experiment and at all subsequent time points. At the designated time points, apical and basolateral media was collected, diluted, and plated onto GCK or Blood Agar plates. Cell associated and internalized bacteria were assessed by lysing the T84 cells with 1% saponin and 1μg/ml gentamicin, and the lysates were diluted and plated.

**Figure 10**. Cellular distribution of T84 cell-associated *N. gonorrhoeae* strains F62Δ8-1 and MS11. T84 cells grown on transwells, were incubated with $10^6$ gonococci at $37^0$C for 6 hours. After washing, fixation, and permeabilization, gonococci were visualized with a mouse anti-gonococcal outer membrane protein specific mAb and an Alexa Fluor 488 goat anti-mouse $IgG_1$ (green). Actin filaments were stained with Alexa Fluor 546 phalloidin (red). The cells were analyzed using laser scanning confocal fluorescence microscopy. Images of 1µm optical sections were taken from the apical side to the basolateral side of the transwell. The bar in the upper left corner indicates 10 µm. Shown are representative images:

A. F62Δ8-1
B. MS11

A.



B.

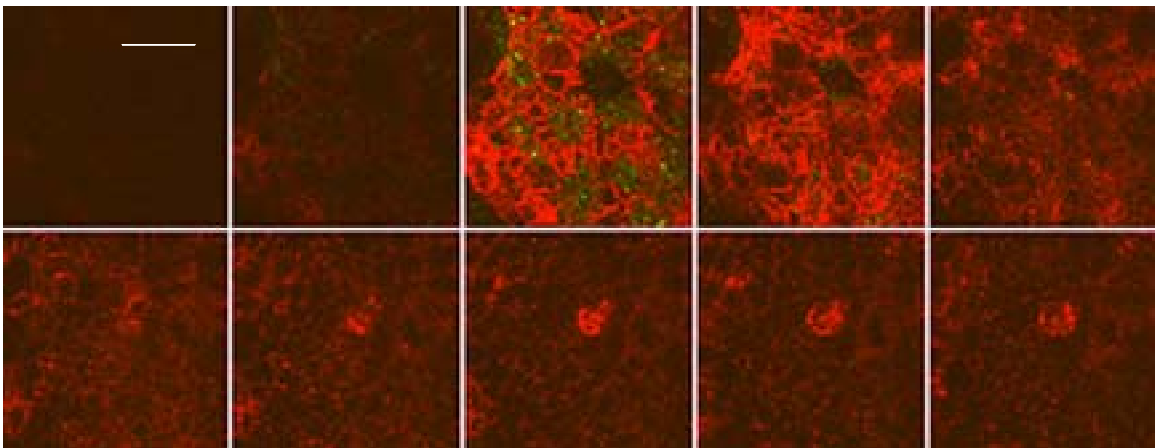
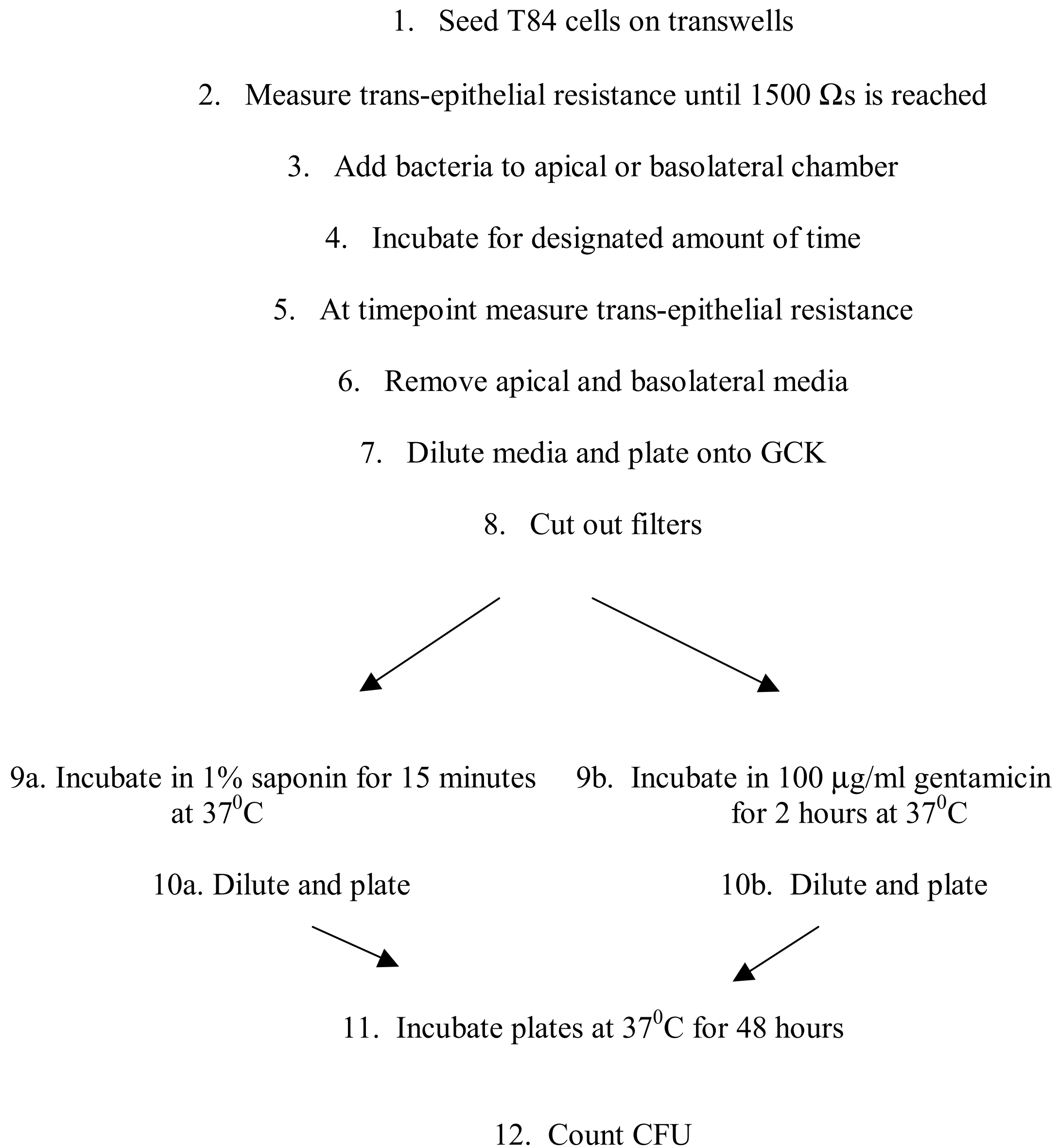


**Figure 11**. Diagram of protocol used for transcytosis experiments



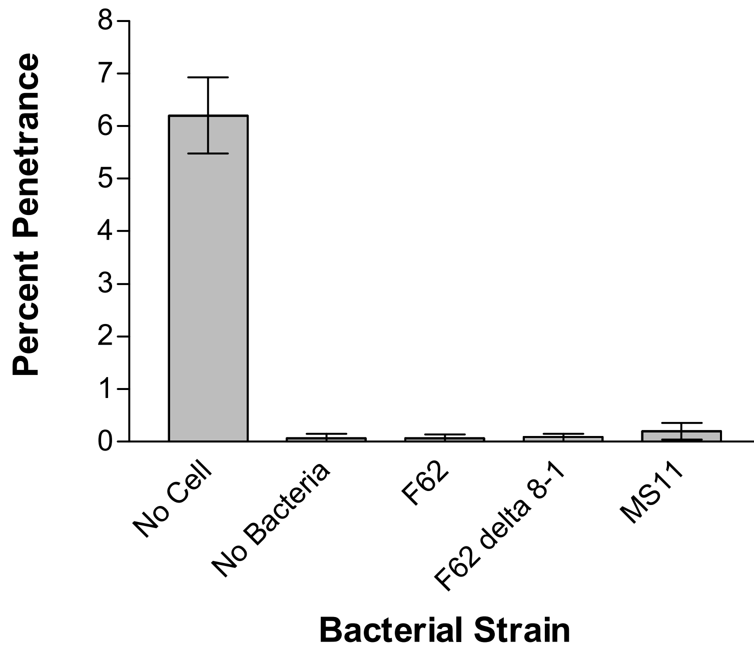
**Assessment of the T84 Cell Monolayer Integrity**

To study the ability of MS11 to transmigrate across a polarized monolayer it was necessary to assess the integrity of the monolayer before starting the transcytosis assays. This was done in two ways. First bacteria were added along with horseradish peroxidase (HRP) to monolayers, incubated for six hours, apical and basolateral supernatants were collected, and the percentage of HRP that crossed the monolayer was determined. As can be seen in Figure 12, when HRP was added in the absence of bacteria virtually none, about 0.1%, of the HRP was able to pass from the apical chamber to the basolateral chamber. This showed that the monolayers were intact and were not permeable to soluble proteins. To ensure that the monolayers would remain intact even during incubation with bacteria, HRP was added along with *N. gonorrhoeae* strains F62, F62Δ8-1, and MS11, to the apical chamber, and the amount of HRP in the basolateral chamber was again compared to the amount left in the apical chamber. All three strains of gonococci F62, F62Δ8-1, and MS11 showed statistically the same amount of HRP was able to cross the monolayer, around 0.1 to 0.2%, which was similar to the amount of HRp able to cross an untreated monolayer. When HRP was added to the apical chamber of the transwell containing no cells a much greater amount, 6%, was able to pass through the monolayers (Fig. 12A). This result showed that the monolayer of cells maintained its polarity and prevented the passage of HRP during the infection. 0.1% is much smaller than 6%, and it can be concluded that the monolayer remained intact even during bacterial infection. The second method used to assess the integrity of the monolayer was through the monitoring of the TER. Once the epithelial cells form a monolayer they are able to block the passage of electricity between the cells. An

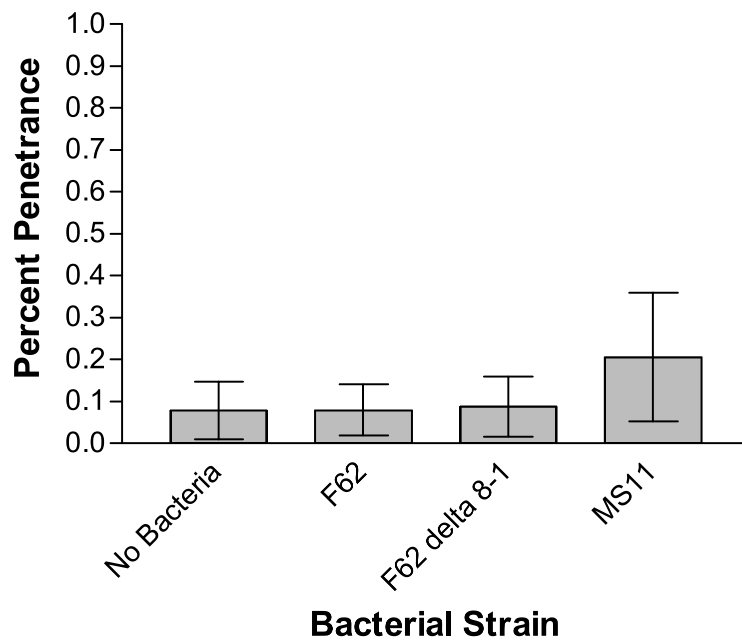
**Figure 12**. Permeability of polarized T84 cell monolayer to horseradish peroxidase (HRP). To assess the non-selective permeability of the monolayers 1μg/ml HRP was added to monolayers either alone (no bacteria control), or with *N. gonorrhoeae* strains F62, F62Δ8-1, and MS11. After six hours of incubation the absorbance at 595 nm was recorded for the apical and basolateral supernatants. To determine the percentage of horseradish peroxidase able to pass through the filter, the absorbance of the basolateral supernatant was divided by that of the apical and multiplied by 100. Data illustrated the means using the two independent experiments.

A. Percentage of HRP in basolateral domain at 6 hours
B. Percentage of HRP in basolateral domain at 6 hours, no cell control omitted to highlight differences between strains

A.)




B.)



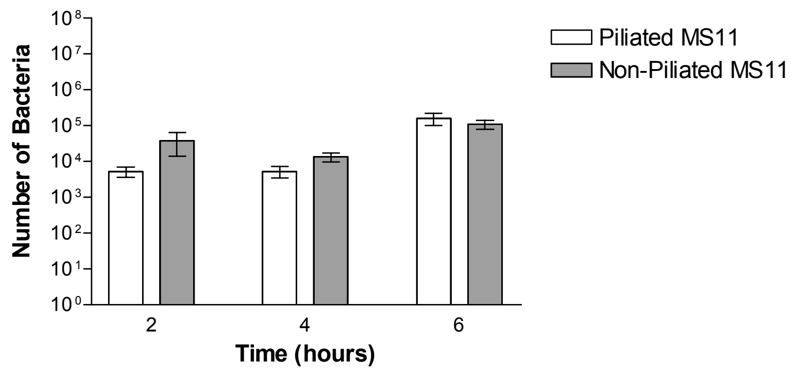
electrical pulse was applied to the monolayer and the amount of electricity the monolayer could withstand was measured. In general, a resistance reading of 700 Ωs is considered polarized, but for the beginning of each of the experiments in this thesis, a resistance of 1500 Ωs was used. During the course of the experiments, if the resistance dropped below 1000 Ωs, that filter was not used in data calculations. This assured that the monolayer remained polarized during the course of the experiment and that any transcytosis that occurred was not due to a breakdown of the monolayer.

**Transcytosis Experiments with $10^6$ Gonococci in the Basolateral to Apical Direction**

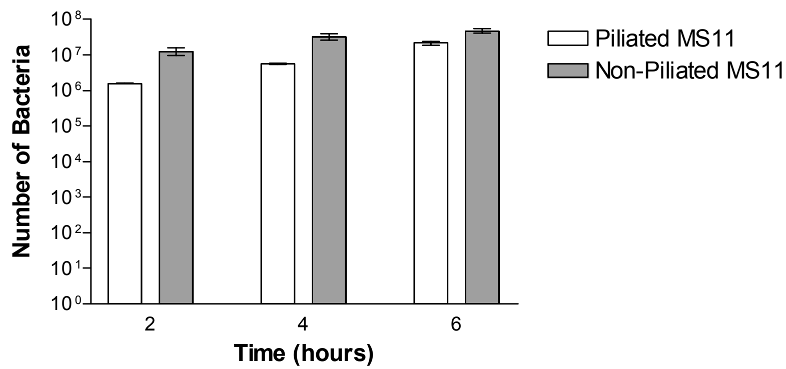
The ability of MS11 to passage through the monolayer was assessed in both the basolateral to apical direction and the apical to basolateral direction, in addition to being tested at two different doses. During a typical gonococcal infection, bacteria first come into contact with the apical surface of epithelial cells. Since gonococci are able to breach the epithelial cell monolayer, either by transcytosis or passage between the cells, then gonococci also come into contact with the basolateral surface of the epithelial cells. Both of these interactions were tested. First basolateral to apical traversal was tested at a multiplicity of infection (MOI) of 100, $10^6$ bacteria per filter, to determine if transcytosis was possible in this direction. Initially piliated MS11 were chosen for this experiment because of their likely interaction with T84 cells, but later non-piliated MS11 were used. It was predicted that the non-piliated MS11 would interact poorly with the T84 cells because of their lack of pili, but as Figure 13A shows, this was not the case. At 2 hours approximately $10^4$ piliated MS11 were able to transmigrate across the monolayer and this number increased slightly over time (Fig. 13A). The number of non-piliated MS11 able to transmigrate across the monolayer in 2 hours was slightly higher than that

**Figure 13**. Transcytosis of piliated and non-piliated *N. gonorrhoeae* strain MS11 wildtype from the basolateral to apical surface of polarized T84 cells. $10^6$ bacteria were used to infect polarized T84 monolayers from the basolateral direction. At the indicated times, the presence of bacteria in the apical (A) and basolateral (B) domains, as well as cell associated (C), were determined as described in Materials and Methods. A total of 4 independent wells were infected with piliated (white) and 8 with non-piliated MS11(gray). Data illustrated represent the means using the two to four independent experiments performed in duplicate.

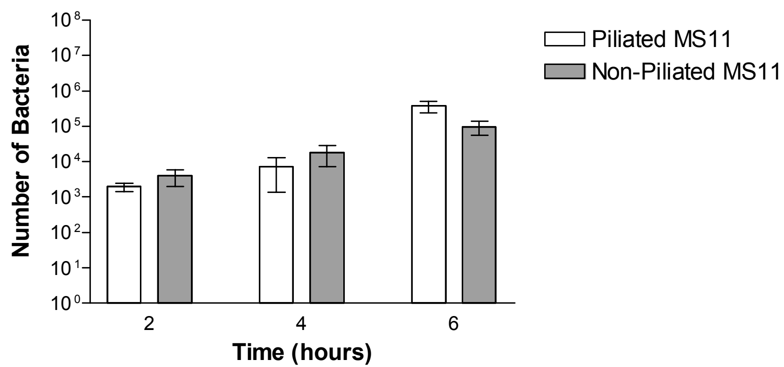
A.) Apical
(Transcytosed)




B.) Basolateral




C.) Cell Associated



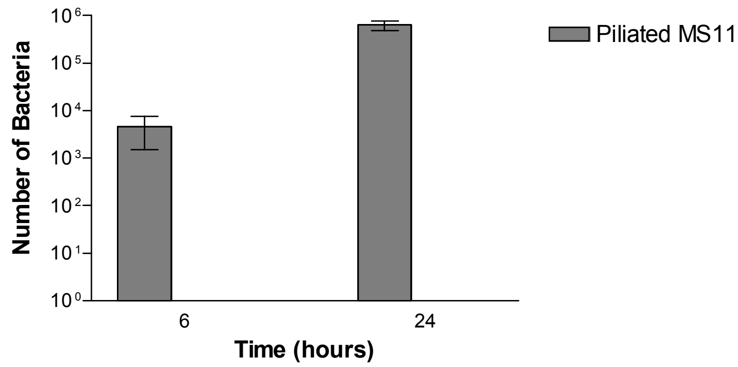
of the piliated MS11, around 5 x $10^4$. The number of transcytosed bacteria at 4 hours was similar to that of transcytosed bacteria at 2 hours, but by 6 hours the number had increased. At 6 hours the number of transcytosed piliated and non-piliated MS11 reached about $10^5$. The number of cell associated non-piliated MS11 exceeded the number of piliated MS11 at each timepoint, which was surprising because pili are necessary to form the initial attachment to epithelial cells. Because non-piliated MS11 were initially to be used as a negative control, but were later found to be able to both interact with the T84 cells and transcytose, they were not used in future experiments.

**Transcytosis Experiments with $10^5$ Gonococci in the Basolateral to Apical Direction**

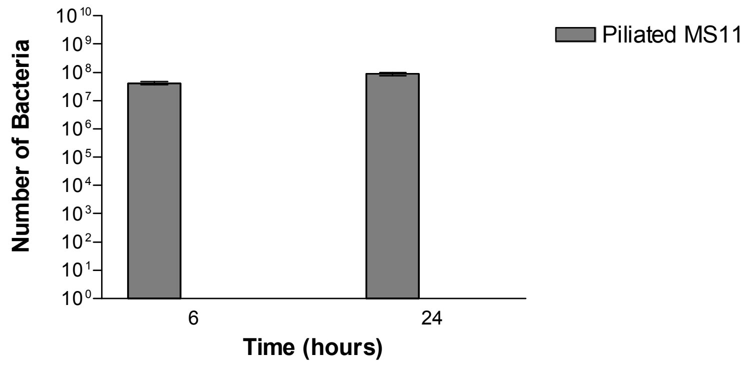
The ability of MS11 to transmigrate across the monolayer from the basolateral to the apical surface of the T84 cells was also tested at a lower MOI of 10 ($10^5$ bacteria per filter). Since previous results showed there to be no significant difference between the number of transcytosed bacteria in 2, 4, and 6 hours, longer timepoints, 6 and 24 hours, were tested, to see if differences occurred later on in the infection. Figure 14A shows that at 6 hours about 5 x $10^4$ MS11 were able to transmigrate. This number was as expected. When $10^6$ gonococci were used, approximately $10^5$ were able to transmigrate in 6 hours. In this experiment $10^5$ gonococci were incubated with the monolayers, and about 5 x $10^3$ were able to transmigrate. At 24 hours, the number of MS11 increased significantly to about $10^6$. The number of MS11 found associated with T84 cells at 6 hours was about $10^4$, and increased to 5 x $10^5$ at 24 hours. The data indicated that at an MOI of 10, wildtype MS11 were able to transmigrate across the monolayer in the basolateral to apical direction in appreciable numbers, and increased in number as time passes.

**Figure 14.** Transcytosis of *N. gonorrhoeae* strain MS11 wildtype versus from the basolateral to the apical domain of the polarized T84 cell monolayer after 6 and 24 hours. $10^5$ bacteria were used to infect polarized T84 monolayers. At the indicated times, the presence of bacteria in the basolateral apical (A) and basolateral (B) domains, as well as cell associated (C), were determined as described in Materials and Methods. A total of 6 independent wells were infected with MS11 (gray). Data illustrated represent the means of three independent experiments performed in duplicate.

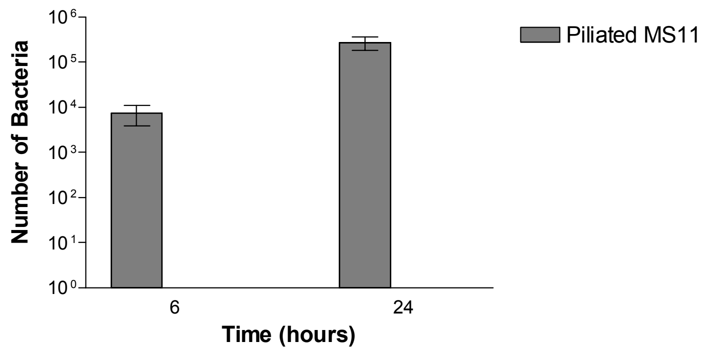
A.) Apical
(Transcytosed)




B.) Basolateral




C.) Cell Associated



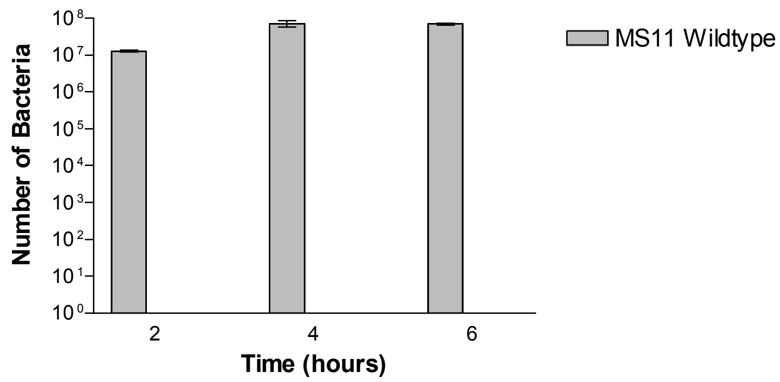
**Transcytosis Experiments with $10^6$ Gonococci in the Apical to Basolateral Direction**

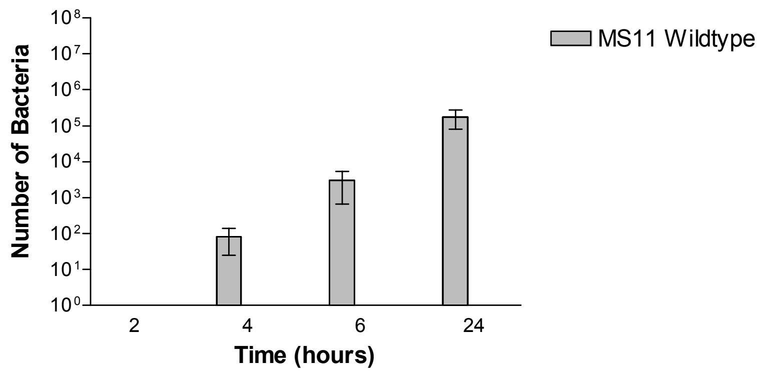
During a typical infection, *N. gonorrhoeae* initially interacts with the apical surface of epithelial cells, and so the ability of MS11 to traverse the monolayer was also tested in this direction. An MOI of 100 ($10^6$ bacteria per filter) piliated MS11 were used to infect the polarized T84 cell monolayer for 2, 4, and 6 hours. As can be seen from Figure 15A, the number of bacteria in the apical domain increased slightly over the course of the 6 hour incubation. At 2 hours approximately $10^4$ bacteria were associated with the T84 cells. This number increased over time, and eventually reached approximately $10^6$ bacteria at 24 hours. The number of MS11 able to transmigrate across the monolayer appeared to increase over time (Fig. 15B). While no bacteria were found at 2 hours, a small number appeared at 4 hours, about $10^2$ bacteria. This number increased to $5 \times 10^3$ in 6 hours and $5 \times 10^5$ in 24 hours. This data shows that MS11 was able to rapidly cross a polarized monolayer of T84 cells from the apical to the basolateral surface in appreciable numbers. However, the transmigration rate of MS11 from the apical to the basolateral surface was slightly slower than in the basolateral to apical direction.

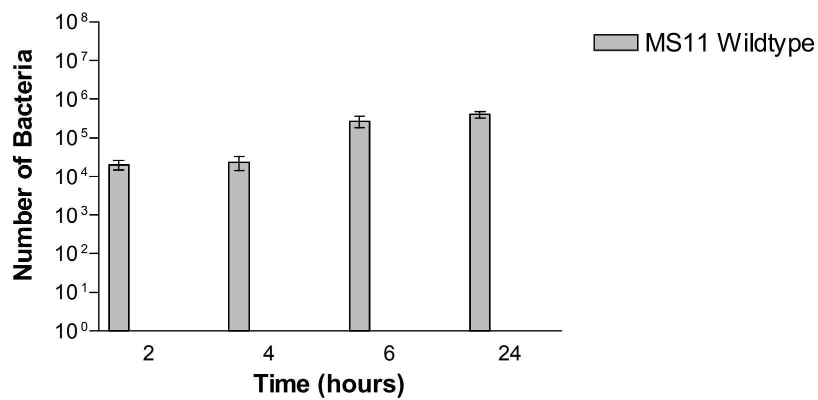
**Transcytosis Experiments with $10^5$ Gonococci in the Apical to Basolateral Direction**

As a final test of the ability of *N. gonorrhoeae* strain MS11 to transmigrate across the monolayer in the apical to basolateral direction an MOI of 10 ($10^5$ bacteria) was also tested. For this experiment one of the Opa deletions created in the first part of this thesis was also tested, the Opa 5 (Opa C) deletion. As a control a strain of *Campylobacter jejuni*, 587, was used, as it had previously been demonstrated to not transmigrate across

**Figure 15.** Transcytosis of *N. gonorrhoeae* strain MS11 wildtype from the apical to the basolateral surface of the polarized T84 cell monolayer after 2, 4, and 6 hours. $10^6$ bacteria were used to infect polarized T84 monolayers from the apical direction. At the indicated times, the presence of bacteria in the apical (A) and basolateral (B) chambers, as well as cell associated (C), were determined as described in Materials and Methods. A total of 6 independent wells were infected with MS11 (gray). Data illustrated represent the means of one to three independent experiments performed in duplicate.


A.) Apical
Number of Bacteria
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
2
4
6
Time (hours)
MS11 Wildtype

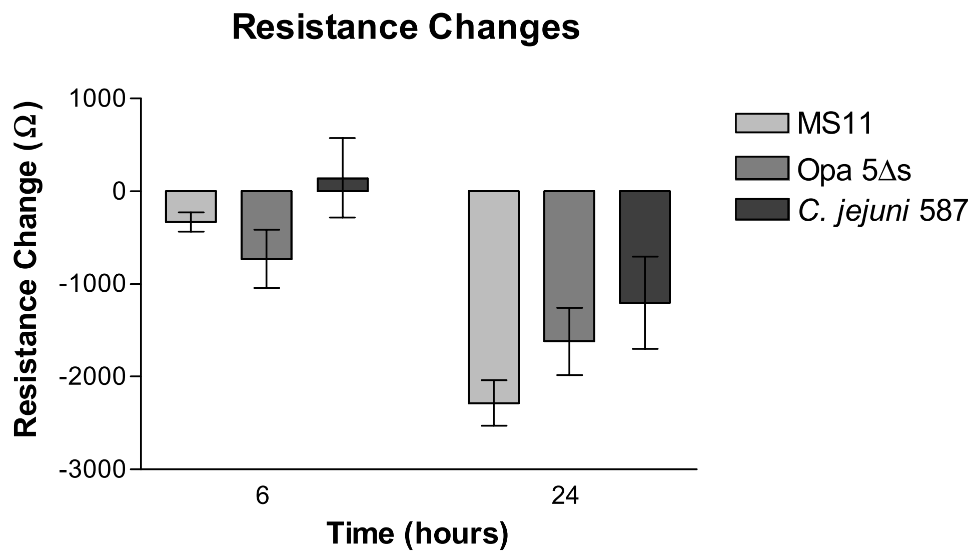

B.) Basolateral
(Transcytosed)
Number of Bacteria
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
2
4
6
24
Time (hours)
MS11 Wildtype


C.) Cell Associated
Number of Bacteria
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
2
4
6
24
Time (hours)
MS11 Wildtype

polarized T84 monolayers (personal communication). In addition, the TER for each well used in this experiment was documented at each time point, and the results of this can be seen in Figure 16. Both strains of MS11 induced a significant reduction in the TER, while the *Campylobacter* strain only caused a decrease after 24 hours. This suggests that the MS11 strains were initially loosening the tight junctions, whereas the *Campylobacter* was not.

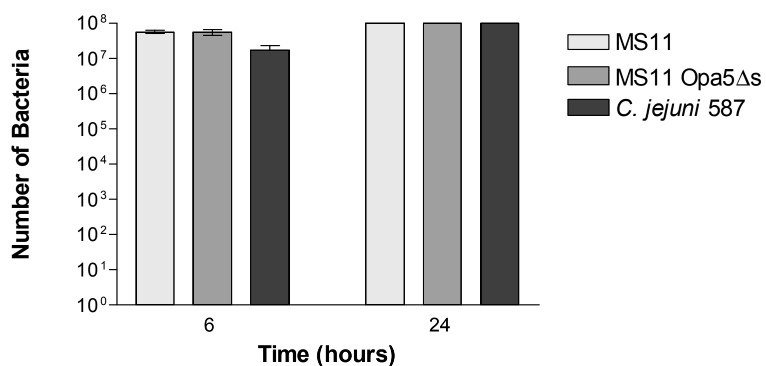
Over the course of the experiment the number of bacteria in the apical domain increased slightly for all three strains, about 10 fold (Fig. 17A). As can be seen in Figure 17B there was a significant difference between the strains in their ability to passage through the monolayer. At 6 hours approximately $5 \times 10^5$ MS11 were found in the basolateral domain, while only $10^3$ MS11 Opa 5Δs, and $10^1$ *C. jejuni* 587 were found there. This is a 100 fold difference between the wildtype MS11 and the Opa 5 deletion, despite the fact that both strains adhered equally as well (Fig. 17C). This data indicates that Opa 5 is important in the ability of MS11 to traverse the monolayer, but is not important for MS11 to attach to the cells. The fact that the two strains were able to adhere equally as well but didn't transmigrate equally, indicated that adherence was not important for traversal of the monolayer. After 24 hours though, the Opa 5 deletions appeared to almost reach the same level as the wildtype, approximately $1 \times 10^5$ as compared to $5 \times 10^5$.

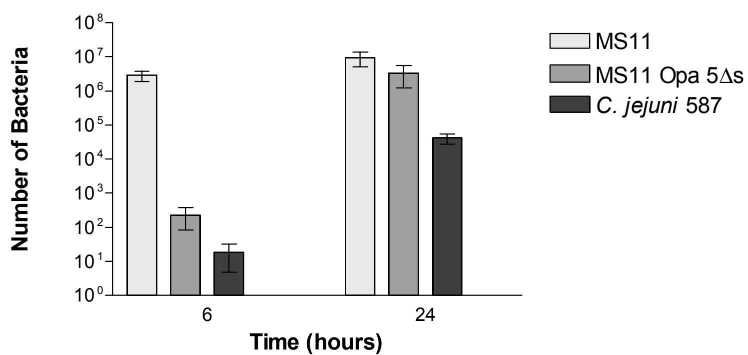
**Figure 16.** Change in resistance of T84 cell monolayer during transcytosis of *N. gonorrhoeae* strain MS11 wildtype, MS11 Opa5Δs, and *C.jejuni* strain 587 from the apical to the basolateral domain of polarized T84 cell monolayer at 6 and 24 hours. $10^5$ bacteria were used to infect polarized T84 monolayers from the apical direction. At the indicated times, resistance readings were measured with an electrode. The difference between the resistance reading at the start of the experiment and the indicated time point was calculated and plotted.

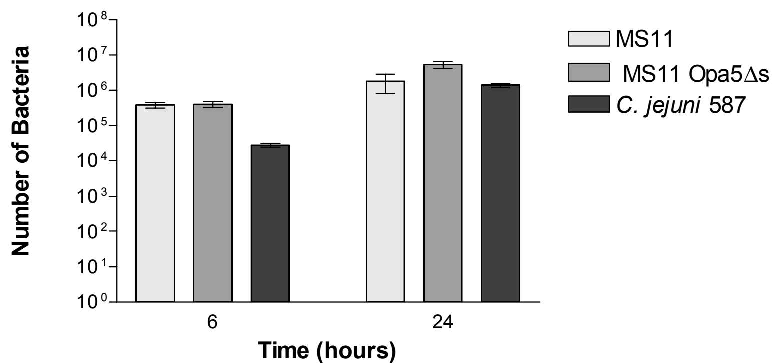


Resistance Changes
Resistance Change (Ω)
1000
0
-1000
-2000
-3000
6
24
Time (hours)
MS11
Opa 5Δs
C. jejuni 587

**Figure 17**. Transcytosis of N. *gonorrhoeae* strain MS11 wildtype, MS11 strain Opa 5Δ, and *C. jejuni* strain 587 from the apical to the basolateral domain of a polarized T84 cell monolayer after 6 and 24 hours. $10^5$ bacteria were used to infect polarized T84 monolayers from the apical direction. At the indicated times, the presence of bacteria in the apical (A) and basolateral (B) domains, as well as cell associated (C), were determined as described in Materials and Methods. A total of 9 independent wells were infected with MS11 (light gray) and MS11 Opa5Δs (dark gray), and *C. jejuni* 587 (black). Data illustrated represent the means using three independent experiments performed in duplicate.


A. Apical
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
Number of Bacteria
6
24
Time (hours)
MS11
MS11 Opa5Δs
*C. jejuni* 587


B. Basolateral
(Transcytosed)
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
Number of Bacteria
6
24
Time (hours)
MS11
MS11 Opa 5Δs
*C. jejuni* 587


C. Cell Associated
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
Number of Bacteria
6
24
Time (hours)
MS11
MS11 Opa5Δs
*C. jejuni* 587

# Discussion

## Overview

Opa proteins are one group of structures found on the surface of the human pathogen *Neisseria gonorrhoeae*. The gonococcal genome contains loci for 11 distinct Opa encoding-genes, but because of genetic variation, 11 Opa proteins are not always expressed on the surface of gonococci. This genetic variation is mediated by a CTCTT repeat in the region immediately downstream of the start codon of each gene. If the number of repeats places the start codon in frame, the gene will be translated. Conversely, if the repeats place the start codon out of frame with the rest of the gene, a missense protein will be made and ultimately degraded. This genetic variation complicates the study of the importance of Opa proteins in the pathogenesis of *N. gonorrhoeae*, because there is no way to know if a particular Opa is being expressed or not at a given time, and therefore, if it plays a role in disease pathogenesis. Even when gonococci used for invasion studies begin phenotypically as Opa minus, they still have the ability to express their Opa genes at any point during the infection. This phenomenon is significant because spontaneous frameshifting occurs at a rate of $10^3$ / cell / generation. For this reason, to be able to study the importance of Opa proteins in disease pathogenesis it was necessary to solve this problem. The purpose of the work in this thesis was to create deletion plasmids with genetically defined Opa deletions that could be transformed into *N. gonorrhoeae*, and then to test the effects of one of these deletions in a transcytosis experiment, to determine its role in this process.

## Successful deletion of Opa encoding-genes

The first goal of this study was to produce genetically defined Opa deletions. In order to complete this task, Opa encoding-genes needed to be identified from the FA1090 genome, and the data in Figure 1 shows that 11 sequences were identified. PCR amplification was able to generate 11 distinct sequences, indicating that the 11 primer sets that were developed could amplify the 11 Opa-encoding-genes (Fig. 2B). To delete the coding region of these genes, the amplicons needed to be cloned into a plasmid, pUC19. To verify that this occurred, the plasmids were extracted and digested. The sizes of the bands after restriction digestion match the predicted sizes found in Table 8, indicating that this was done successfully (Fig. 2C and 2D). A second PCR amplification was used to delete the coding region of the genes. Figure 3B shows that the sizes of the deletion PCR fragments and these sizes agree with the predicted sizes of the Opa fragment plus the plasmid pUC19, minus the coding region. The coding region of the gene was then replaced with a spectinomycin resistance cassette (Fig 4B and 4C).

The Opa 5 deletion was chosen to be transformed into *N. gonorrhoeae* strain MS11 to be used for the transcytosis experiments because of its sequence similarity to Opa C, an Opa associated with invasive disease. The Opa 5 deletion construct was introduced into *N. gonorrhoeae* strain MS11 by transformation. A PCR reaction was done from chromosomal DNA extracted from spectinomycin resistance MS11 clones with the Opa 5 PCR primers, and the PCR product was digested to verify that the construct had correctly replaced the Opa 5 encoding-gene in the gonococcal chromosome. The creation of this strain made the study of its importance in the transcytotic ability of MS11 possible.

**Importance of Opa 5 in the ability of *N. gonorrhoeae* strain MS11 to transcytose across human intestinal epithelial cell line T84**

Upon introduction into the urogenital tract, gonococci contact the epithelial cells. These cells become polarized *in vivo*, hence it was important to choose a cell line that would mimic the orientation that the gonococcus would interact with the cells during a natural infection. A polarizable urogenital tract cell line model has yet to be well established. Despite the fact that the intestine is not a primary site of infection for gonococci, the T84 intestinal epithelial cells have been used many time for *N. gonorrhoeae* studies because these cells share many of the same properties of urogenital tract epithelial cells.

The second goal of this work was to test the effects of the deletion of Opa 5 on the transcytotic ability of MS11. The results from the trancytosis experiments demonstrate that wild type MS11 was able to transcytose the polarized T84 monolayer well (Figures 13, 14, 15, and 17). This is in stark contrast to the Opa5Δs strain. Only about $10^3$ Opa5Δs were able to transcytose from the apical to basolateral surface in 6 hours, as compared to $5 \times 10^6$ wildtype MS11. This represents over a 1000 fold reduction in the ability of MS11 Opa5Δs to transcytose as compared to wildtype MS11. Since the only known difference between these two strains was in their ability to express the Opa 5 gene product, this data indicated that Opa 5 was important for the ability of MS11 to transcytose across polarized T84 cells. Given that the amount of the Opa5Δs strain to transcytose in 24 hours was nearly equal to that of the MS11 wildtype, the data suggests that Opa5Δs was able to overcome any initial impediment the Opa5 deletion caused. This result is significant because during a gonococcal infection, gonococci normally

interact with the apical surface of epithelial cells. The fact that the deletion of the Opa 5 gene had such a strong impact on the ability of the bacteria to transcytose has implications for the disease progression. If gonococci are not able to transcytose then they cannot spread past the point of initial infection and dissemination would not occur.

While there is a difference in the ability of MS11 and MS11 Opa5Δs to transcytose across polarized T84 cells, there did not appear to be any difference in the ability of the two strains to adhere to the monolayer (Figure 17). At both 6 and 24 hours, the same numbers of bacteria, approximately $5 \times 10^5$, were found associated with the monolayers for both strains. The deletion of Opa 5 inhibited the ability of gonococci to transcytose without affecting the ability to bind to the T84 cells. These two results together imply that adhesion and transcytosis are independent of one another. One possible explanation for this phenomenon is that tight adherence of gonococci to host cells may make transcytosis more difficult because the bacteria would be less likely to detach from the apical surface of the cell for transcytosis. Another possible explanation is that the host receptors that are engaged when Opa 5 is present are different from those engaged by gonococci lacking Opa 5. These receptors may influence whether or not the bacteria remains attached to the apical surface or transcytoses. The independence of transcytosis from invasion adhesion, was further supported by the finding that non-piliated MS11 were able to transcytose from the basolateral to apical surface better than piliated MS11 (Fig. 13).

One of the major findings of this work was that MS11 is able to transcytose in both the apical to basolateral direction and the basolateral to apical direction (Fig. 13, 14, 15, 17). The fact that apical to basolateral transcytosis occurred was not surprising, as

this phenomenon had already been demonstrated [54, 71]. The fact that basolateral to apical transcytosis was possible with MS11 was interesting because this phenomenon provides another mechanism for gonococcal survival in the urogential tract and also another way to spread after transmission. In 6 hours, approximately $5 \times 10^5$ piliated and non-piliated gonococci were able to transcytose from the basolateral to apical side of the polarized T84 cell monolayer, when and MOI of 100 was used (Fig. 13). At an MOI of 10, the number of piliated gonococci able to transcytose in this direction was about 100 fold less, $5 \times 10^3$ (Fig 14). Gonococci normally contact with the apical surface of epithelial cells when they are first introduced into the urogenital tract. However, since transcytosis in the apical to basolateral direction is possible, then gonococci would have access to the basolateral surface of the epithelial cell and would need mechanisms to interact with this side of the cell to remain infectious. Basolateral to apical transcytosis would be beneficial to gonococci, especially in women, because the lining of the uterus is shed monthly and if gonococci were to remain tightly attached to the apical domain of the epithelial cell from the uterus, then they would be shed along with the cells. It would be advantageous for gonococci to remain below the epithelial cell lining until the epithelial cell had been replaced, and once this had occurred, gonococci could transcytose from the basolateral to apical pole of the new cell, and cause disease again.

A second important finding involved the time at which transcytosis occurred. Previous work indicated that gonococcal transcytosis did not occur for more than 24 hours from the time of inoculation [71]. The results of the experiments in this work show that rapid gonococcal transcytosis occurred, as early as 4 to 6 hours. This was probably a result of the fact that adhesion did not influence the rapid transcytosis measured here.

Pili have been shown to be necessary for the initial adhesion to epithelial cells, and the time it takes for pili to adhere and then be shed is considerable, between 8 and 16 hours [18]. The transcytosis measured in this thesis, occurred very rapidly, in 4 to 6 hours, which is much shorter than 8 to 16 hours. Since the transcytosis being measured in this work did not appear to be dependent upon ability of gonococci to adhere, this may explain why the process occurred in a short amount of time. It is possible that this type of gonococcal transcytosis occurs before a tight adhesion to the host cell is established. If gonococci were able to form a loose attachment to the surface of the T84 cells using a surface molecule other than pili, then they would be able to transmigrate more quickly, without the need to shed their pili.

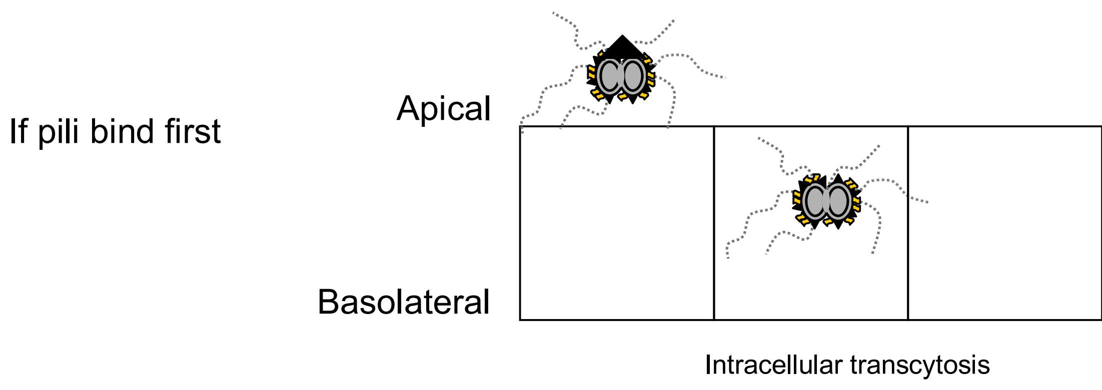
The dosage of inoculated bacteria had an effect on the amount of gonococci that transcytosed. At 6 hours in the basolateral to apical direction, at the high dose, $2 \times 10^5$ gonococci were able to transcytose, as compared to $5 \times 10^3$ for the low dose. This is a 100 fold increase when 10 fold more bacteria were used. In contrast, in the apical to basolateral direction, with the high dose, $3 \times 10^3$ were able to transcytose, whereas with the low dose, $3.5 \times 10^6$ were able to transcytose. This result was surprising because when more bacteria were used, less transcytosed. One possible explanation for this is that the higher numbers of bacteria were clumping together and forming aggregates. This would affect the number of colonies in two ways. First, if gonococci form aggregates this might prohibit their ability to enter the tight junctions. Secondly, when the bacteria were plated, one clump would produce one colony as would one single bacterium. The higher the number of bacteria in the starting inoculum, the more likely it would be that gonococci

would come into contact with one another, thereby increasing the chances that a clump of bacteria would form.

The results from these experiments support the notion that gonococci use a paracellular, rather than an intracellular, route to transmigrate across the polarized epithelial cell monolayer (Fig. 18). Gentamicin treatment of T84 monolayers infected with MS11 never yielded any viable intracellular gonococci, while treatment of *C. jejuni* 587 infected T84 monolayers did (data not shown). It is possible that gonococci were engulfed by the T84 cell and then killed intracellularly. Immunofluorescence experiments supports the hypothesis that gonococci were not inside of the cells, as no gonococci were never detected in the basolateral region of the cells, indicating that gonococci were always located at the apical pole of the cell (Fig. 9). Also, supporting the notion of a paracellular route of transmigration was the effect that gonococci had on the trans-epithelial resistance. The TER readings decreased during the course of the experiments, indicating that the tight junctions were loosened to allow for the passage of gonococci. (Fig. 16).

**Potential Problems**

Although the experiments in this work were well controlled, some problems inherent with the experiments existed. First was the problem of bacterial growth. Over the course of the transcytosis experiments, gonococci were actively growing. This phenomenon is exemplified by the number of bacteria recovered after incubation in the domain to which the bacteria were added. The doubling time for gonococci is no shorter than one hour, and all of the bacterial numbers in these fractions in which the bacteria were added are consistant with growth. If $10^6$ bacteria were added at time zero, then by 6



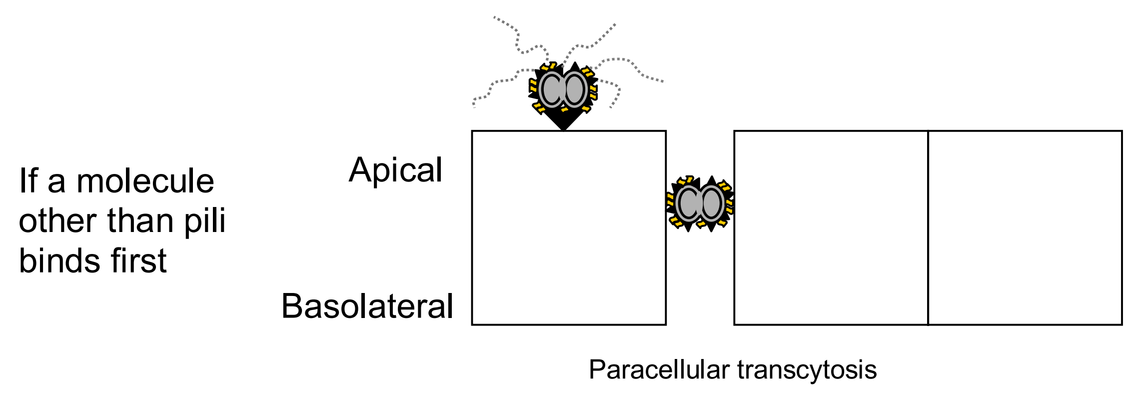




Figure 18. Model of paracellular transcytosis.

hours approximately $6.4x10^7$ bacteria would be expected. For example, in the apical to basolateral high dose experiments, $10^6$ bacteria were added and at 6 hours $6.99 X10^7$ bacteria were recovered, close to the predicted $6.4x10^7$. Results such as this are consistant with active bacterial growth throughout the experiment. The fact that the bacteria were growing during the course of the experiments makes the calculation of the actual number of bacteria that have transcytosed difficult. The number of bacteria that were counted after plating represents not only individual bacteria that transcytosed, but also bacteria that have grown from the bacteria that transcytosed. Future studies could label the bacteria, possibly with radioactivity, to track the individual bacteria that were able to transcytose. Also, since the bacteria were able to transcytose quickly, very short timepoints could be used in the future so that growth would be at a minimum.

Not only was growth a factor in the experiments, but so was viability. Since these experiments all involved plating cellular fractions onto GCK and blood agar plates, only bacteria that were alive were accounted for. The possibility remains that there were some bacteria that were able to transcytose, and for a variety of reasons, were killed, and therefore not included in the plate count. This means that there is the potential that even more bacteria were able to transcytose but were not accounted for. Because viability could affect the results, it would be important in the future to measure transcytosis by a method that did not involve plating the cellular fractions, such as by radioactively labeling a bacterial surface structure, and then measuring radioactivity.

Despite the fact that no gonococci were seen in association with tight junctions, this does not mean that the bacteria were not between the cells. The immunofluorescence images were taken from the apical to basolateral direction, looking down on the cell. The

bacteria could be located between the cells, and if images were taken from the zy plane instead for from the xy plane, then possibly the bacteria would be visible. Also, the basolateral membrane was not stained, and so it is possible that more slices were needed to see the basolateral domain of the polarized T84 cells. In the future, the location of gonococci inside of the cell should be studied, possibly with the use of electron microscopy, to make certain that gonococci are only located in the apical domain.

A problem specific to the experiment involving the Opa5Δs strain, involve the comparisons that are made between the results generated with this strain and the results from the wildtype strain. Because of the variation that occurs in the Opa genes, there is no way to say with certainty that the Opa 5 protein is expressed in the wildtype. While the gene is present in the genome of wildtype MS11 it may be turned off. For these kinds of comparisons to be made with certainty, a strain of MS11 which constitutively produces Opa 5 would need to be created. Only then could definitive experiments be done to conclude the importance of Opa 5 on transcytosis.

**Future Directions**

In summary, deletions constructs for each individual Opa gene were made in *E. coli* and the Opa 5 deletion was transformed into *N. gonorrhoeae* strain MS11 to test in a transcytosis experiment. The deletion yielded a significant decrease in the ability of the Opa5Δs to rapidly transcytose in the apical to basolateral direction, but not in the ability to adhere to the T84 cell monolayer. In the future, it would be interesting to see if the deletion of other Opa genes had a similar or different affect on the ability of MS11 to transcytose. With this in mind, it would also be interesting to create a completely genetically Opa deficient strain. This strain could be compared to different deletion

strains and to wildtype, to determine whether gonococci are able to transcytose in the absence of all Opa proteins. Overall, the work in this thesis represents an important first step in the process of creating the Opa minus strain, but the results generated are also relevant themselves to pathogenesis because the deletion of Opa 5 did have such a large impact on the ability of MS11 to transcytose. Transcytosis is the first major step in the spread of gonococcal disease, and if the bacteria can be stopped from spreading than much of the negative disease outcome could be circumvented.

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