

**Pilus Antigenic Variation and Protein II Composition
in the Outer Membrane of Neisseria gonorrhoeae ,
a Case History of Patient 016**

**Submitted in Partial Fulfillment of the Requirements for
Graduation with Honors to the Department of Biology at
Carroll College, Helena, Montana**

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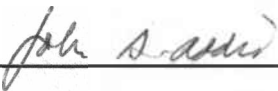
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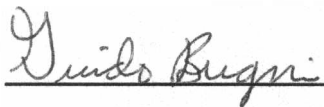
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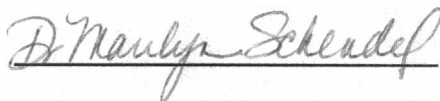
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"Nothing in education is so astonishing as the amount of
ignorance it accumulates in the form of inert facts"

Henry Brooks Adams

I would like to dedicate this thesis to Spuds MacKenzie " The original party animal "

Table of Contents

List of Tables.....	ii
List of Illustrations.....	iii
Acknowledgements.....	iv
Abstract.....	v
Introduction.....	1
Literature Review.....	2
Gonorrhea, the Disease.....	2
<u>Neisseria gonorrhoeae</u>	3
Pili and the Pilus Gene.....	4
PII's and the PII Genes.....	7
Materials and Methods.....	12
Results.....	18
Discussion.....	4
Appendix.....	30
Literature Cited.....	35

List of Tables

<u>Table</u>	<u>Page</u>
1. Intraurethral injection results.....	18
2. pII composition of GC reisolates.....	19
3. Pilus epitope mapping results.....	21

List of Illustrations

<u>Figure</u>	<u>Page</u>
1. Protein II amino acid Sequence.....	9
2. Preparing GC for mRNA extraction.....	15
3. Sequencing results of pilus mRNA with corresponding amino acid sequences.....	22

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Abstract

In this study several male volunteers were infected with piliated gonococci expressing none of the pII proteins. Urine samples were collected from the patients twice daily until the patient developed clinical symptoms of gonorrhea. The samples from each volunteer were then analyzed in order to characterize how pII expression varied while being present in a human host. Pili were also examined for differences in molecular weight. My role was to study the reisolated gonococci from patient 016. Pili were examined by epitope mapping, and the sequences of four pilin genes taken from the last urine sample were determined.

Introduction

Neisseria gonorrhoeae, referred to as gonococci, is the causative agent of gonorrhea. Pathogenicity appears to be related to the presence of thin hairlike appendages known as pili which mediate attachment to human epithelial cells, especially those cells of the urethra.

These pili show much structural diversity among different strains (and even within a single strain). The reason for this diversity is that the gene which codes for the pilin subunit is subject to nonreciprocal, partially homologous recombination. There are several partial pilin genes which can be inserted into the complete pilin gene at different areas within the 3' portion of this gene. The entire partial pilin gene can be used for this recombination event, or only part of it may be used. The result is the pilin subunit is subject to an almost infinite number of amino acid sequences although portions of the gene, and hence the pilin subunit, are conserved, including the 5' end of the gene which encodes for the first 54 amino acids. This diversity prevents the host's antibodies against pili from protecting the body.

Protein II (pII), located on the outer membrane, is also subject to diversity, but not in the same way as pili. In strain MS11_{mk} there are 5 different pII's, designated a through e, which can be expressed independently of one another. The 5 genes coding for these proteins can be in the "on" or "off" position, and a single cell may express one or several of these proteins simultaneously, or not any at all.

In vivo gonococci vary these components quite frequently avoiding the immune response while at the same time allowing this bacterium to survive in a changing environment and colonizing different niches within the host.

In this study gonococci recovered from urine samples of male subjects experimentally infected with this organism were analyzed in order to assess the changes that had occurred in the protein composition of the outer membrane in vivo.

Literature Review

Gonorrhea is one of the most frequently reported diseases in many parts of the world, including the United States (5). The annual incidence of gonorrhea in the United States is around 1 million cases; however, there may be as many as 3 million (3). This disease is caused by the bacterium Neisseria gonorrhoeae (gonococci), and its only natural host is man, although experimental animals can be infected artificially (7,5). This bacterium is susceptible to cold and drying, and thus the disease is transmitted primarily by direct contact, contact which is almost always venereal (7).

Gonococci infect human mucous membranes, and gonorrhea is characterized by destruction of the urethral mucosa (4). The primary site of infection in males is the urethra, and the cervix in females (11).

In males the infection usually consists of acute urethritis and 4 to 5% are asymptomatic (3). In contrast, from 20 to 80% of all females are asymptomatic because no vaginal infections occur as the adult vagina is lined with cornified stratified squamous cells which are resistant to attack (11). The high number of asymptomatic females, coupled with a high degree of transmissibility and a short incubation period, accounts for the difficulty in controlling gonorrhea (3,11).

The importance of this disease lies in the potential of this bacterium to spread to other parts of the body. In Disseminated Gonococcal Infections, gonococci leave the primary site of infection and spread by way of the bloodstream, causing extragenital infections such as arthritis, dermatitis, pharyngitis, sometimes meningitis and endocarditis, and even hepatitis (3).

In 10% of the infected females, fallopian tubes become infected leading to Pelvic Inflammatory Disease (PID) (7). The fallopian tubes can become occluded with scar tissue,

which is a major cause of sterility (3). Gonococci can be transmitted to the newborn during passage through the birth canal, leading to conjunctivitis (3). This is believed to account for 10% of all cases of blindness (11). For this reason, newborns are treated with 1% silver nitrate eyedrops (11). Most gonococcal infections can be treated successfully with penicillin except those bacteria producing penicillinase. In these cases spectinomycin is used (11).

Neisseria gonorrhoeae is a non-motile, gram-negative bacterium existing in the diplococcus state (3). Gonococci are facultatively anaerobic, growing best in an atmosphere of 5 to 10% CO₂ (3). Optimal temperatures for growth are between 35° and 37° with an optimal pH between 7.1 and 7.6 (3). This bacterium has an array of virulence factors including pili, the pII proteins, an antiphagocytic capsule, a potent endotoxin, and an IgA protease (3).

Pili, projecting from the outer membrane of gonococci, are the most important in terms of virulence. There are 3 other major classes of proteins in this outer membrane. These include the major outer membrane protein, designated as the pI protein, the pII or "opacity" proteins, and a third class designated pIII.

When grown on a solid medium, gonococcal colonies are extremely small and have a characteristic appearance when viewed under the binocular microscope. Colonies possessing pili have a well defined border, and those without appear more diffuse with ill defined edges (23). Colonies consisting of cells lacking any form of pII protein are transparent and those expressing a pII have an opaque appearance when viewed with reflected light, the degree of opacity dependent upon the particular pII, or combination of pIIs, expressed (18).

The major outer membrane protein, pI, has a molecular mass between 34,000 and 38,000 daltons (30). This varies between strains but is constant for any one strain (30).

This protein has been implicated in playing a role in resistance to complement killing by normal human serum along with pII (5). Protein III is a surface exposed protein common to most all, if not all, strains of gonococci. Again, as with pI, molecular weight varies but is constant for a single strain. It is believed that these pI and pIII form heteropolymers on the outer membrane of gonococcus (23). This is suggested by the fact that they exist as a complex in zwittergent detergent and are dissociated by SDS. It is believed they form a "hydrophobically associated trimeric unit in situ" (23) stabilized by cross-links.

PILI

Pili represent a major surface antigen projecting from the outer membrane of gonococci which are necessary in order for this bacterium to cause infection. Pili allow for adhesion to the surface of host cells during the initial stages of infection so penetration and multiplication within the cell can occur (14,29). They prevent the bacterium from being washed away by the flow of mucous and other fluid secretions (4).

Pili extend to a length varying from 1000 to 4000 nm and are composed of identical repeating protein subunits, known as pilin (15). Pilin, approximately 18,000 daltons in molecular mass, consist of a hydrophobic amino-terminal and a hydrophilic carboxy-terminal (6). The number of amino acid residues in a pilin molecule is near 165, although this will vary within a few residues.

The pilus gene can be divided into a constant region corresponding to the first 53 amino acids at the amino-terminal of the protein, a semivariable region encoding for amino acid residues 54 to 114 and a hypervariable region corresponding to amino acid 115 to the carboxy-terminus of this subunit (14). However, within this semivariable and hypervariable regions of the protein there are short sequences that are very highly conserved. These occur in amino acid regions 71-75, 93-97 and around cysteine residues located at positions 128 and

158 (25).

Pili are subject to great antigenic variability which is advantageous to this organism in two respects. First, this variability allows gonococci to colonize distinctly different cell types within its human host with greater selectivity (14,16). Gonococci colonize many different sites such as the urethra, cervix, pharynx, rectum and conjunctiva (30). Secondly it allows the bacteria to avoid the host's immune system (14,13). Such variability can in part explain why humans are subject to repeated attacks of gonorrhea (14,26).

The ability to produce pili can also be lost (pilus ⁻ state) at a high frequency of approximately 10^{-3} to 10^{-2} per cell per generation (4). Reversion to the pilus ⁺state also occurs but with a much lower frequency (5). This inability to produce pili may be advantageous to the bacterium by allowing cells to leave the initial site of infection to colonize other sites, and it could also enhance transmission from one host to another (20,23). However, in order for an effective move within the host or to another one, the cells must revert to the piliated state before being washed out of the body (20).

Non-piliated gonococci can be divided into 3 different phenotypes. Those of the pilus ⁻ n phenotype are incapable of reversion to the piliated state as a result of gross DNA rearrangements in the 5' portion of the pilus gene involving deletion of this portion of the gene along with its promotor sequence (23). Cells capable of reversion are designated pilus ⁻ r and can be divided into two classes. Cells with the pilus ⁻ rp⁻ phenotype produce pilin specific mRNA but do not produce any detectable protein as a result of a nonsense mutation (23). Pilus ⁻ rp⁺ cells also produce pilin specific mRNA but its sequence encodes for an "unorthodox" pilin polypeptide, and no pili are formed (23). This is probably the result of an assembly missense mutation in the pilin gene (22).

The transition from the piliated state to the non-piliated state and vice versa, in those capable of reverting, as well as antigenic variation, is a result of nonreciprocal, partially

homologous recombination (gene conversion) (21,22). At many different sites in the gonococcal genome are silent partial pilin gene sequences (lacking the conserved 5' portion of the gene as well as the promotor sequence) which exhibit a great deal of homology to the 3' two-thirds of the complete pilin gene; however, each having its own unique sequence (25).

During a gene conversion event the partial pilin gene is duplicated, and then either all or part of that sequence is translocated into the complete pilin gene (21,22). The analogous portion of the complete pilin gene is then discarded from the genome (21). Whether this results in the reversible transition to the non-piliated state or in the production of a new structurally distinct pilin subunit depends on whether or not a missense or nonsense mutation is generated. In strain MS11_{mk} 6 such silent gene sequences can be found at a locus designated pilS1 (22). Five of these encode for a normal yet antigenically distinct pilin whereas one codes for an assembly missense yielding the pilus⁻ rp⁺ phenotype (22).

New complete pilin gene sequences can result from gene conversion events utilizing different partial pilin genes and also from use of differing lengths of the same partial pilin gene (25). Independent gene conversion events using the same partial and complete pilin gene sequences will thus most likely not yield the same sequence (21). This also means that a previously inserted sequence may not be completely replaced in the next gene conversion event, adding further to the possible antigenic states (25).

These changes in antigenicity are not believed to result from antibody production by the host's immune system (16,21). These changes have been noted long before the body has had time to produce antibodies. Rather, these pilin variants arise in a spontaneous fashion and then undergo selection for the ones that offer the greatest adhesion to the particular niches being colonized (21). Pili isolated from different sites in a patient differ in subunit size (19). During culturing in vitro pilus⁻ forms arise quite frequently; however, seldom, if ever, do

they appear in samples taken from a patient, indicating a very strong selective pressure for piliation (13). Factors other than tissue tropisms may also influence this variation, such as a hormonal influence (30).

The hypervariable region of the pilin subunit is the immunodominant region and most antibodies raised against intact pili present on the surface of the cell are directed at this region (9). These antibodies will inhibit attachment of gonococci with homologous pili to tissue cultures, but are not very effective in preventing adhesion of heterologous pili (24). The conserved region at the amino-terminus of the protein is immunorecessive, constituting a weak epitope (14). Thus antigenic changes can serve to effectively avoid the immune system of the host.

The presence of conserved regions suggests a critical role in the function of pili and the amino-terminal is thought to bind pili to erythrocytes (29). At the protein level these regions may play some necessary structural or functional role. At the level of DNA itself the conserved nucleotide sequence may be needed to initiate gene conversion (16).

A hydrophobic cleft has been implicated in the binding of pili to host cells (15). The host cell receptor for pili is thought to be a glycoconjugate, possibly a ganglioside, but the situation could be more complex than this (4,15). The receptor is also thought to be tissue specific, dependent upon receptor density and distribution (15). A possible role of pili could be in overcoming the electrostatic repulsion force present between 2 negatively charged cell surfaces (4). This initial attachment would then bring the bacterial cell into close contact with the host cell (4). At this point, adhesion may be further mediated by the pII proteins (4).

Once initial attachment has occurred a second stage of adhesion can then take place, involving direct interaction between the 2 cell surfaces (4). pII's are thought to be involved here, and again a carbohydrate being the receptor (4). pII⁺ cells exhibit a five fold greater ability to attach to buccal cells, although in vivo pII⁻ cells may attach more readily to certain

cell types (4).

Once adhesion is complete, the bacterium will then be able to penetrate the host cell (4). Penetration occurs by way of phagocytosis. Multiplication will subsequently occur, and host cells filled with gonococci will later rupture releasing the bacteria (4).

Pil Proteins

In gonococci virulence is associated with the ability to attach to host cells (27). Pili are clearly the most important as pilus⁻ cells are avirulent unless they can revert to the piliated state before being washed out of the host. The pil proteins are also important virulence factors in many regards.

A single strain of gonococcus can produce as many as 6 to 8 antigenically distinct forms of pil's, differing also in molecular weight (2). A single cell can express zero, one, or up to three different pil proteins on its surface at the same time, in contrast to pili which can only be produced in one form by any one cell (16). Moreover, each different pil is coded for by a separate gene so this antigenic distinction, at least in a direct sense, is not caused by gene conversion. If a cell expresses more than one pil at a time each will be produced in the same quantity as though that cell produced only that particular pil (18).

The pil proteins provide a mechanism for intercellular adhesion between gonococcal cells (16). These adhesions are absent in colonies consisting of pil⁻ cells, and it can be disrupted by treating pil⁺ cells with trypsin, which readily cleaves pil's (2). The result of this intercellular adhesion is an opaque appearance to colonies when viewed with reflected light (2). When expressed, different pil's will cause different degrees of opacity (18). Some pil's confer only a slight opaque appearance to the colony and at one time these colonies were considered to be transparent. However, it is now apparent that some degree of opacity does exist.

pil's function in many regards other than adherence to eukaryotic cells and between

gonococcal cells. A role in resistance to serum killing has been reported as well as a resistance to antibiotics (27). But the main role of pII's may be in the initial interaction of these bacterial cells with leucocytes (28).

Although antigenically distinct, there are certain regions shared in common with other pII's from the same strain and from other strains (18). Antisera raised against purified denatured pII's are reactive against heterologous pII's, but antisera raised against intact gonococcal cells expressing a certain pII are reactive only against homologous pII's, with little cross reaction, although slight ones might exist in certain cases (18). This suggests that the surface exposed regions of the pII molecule are distinct from one another and they may play a role in the serological reactivity of the outer membrane (18).

Strain	Amino Acid Position									
	1	2	3	4	5	6	7	8	9	10
MS11 vo	Ala	Ser	Glu	Asp	Gly	Gly	Arg	Gly	Pro	Tyr
MS11 v28	Ala	Gly	Glu	Asp	His	Gly	Arg	Gly	Pro	Tyr
FA1090 PIIa	Ala	Ser	Glu	Asp	Gly	Gly	Arg	Gly	Pro	Tyr
FA1090 PIIb	Ala	Thr	Glu	Gly	Asn	Gly	Arg	Arg	Pro	Tyr
FA1090 PIIe	Ala	Gly	Glu	Asp	Asn	Gly	Arg	-----		
MS11 ⁺	Ala	Ser	Glu	Glu	Gly	Gly	Arg	Gly	Pro	Tyr
R10 ⁺	Ala	Gly	Glu	Asp	Glu	Gly	-----			

Fig. 1. Protein II amino acid sequences. The sequences of several pII's presented here shows that certain amino acids are conserved, namely those at positions 1,3,6,7,9 and 10 at the amino-terminal of the pII molecule (17).

The common portions of these molecules are embedded in the outer membrane (18). As Fig. 1 shows, there are several conserved amino acids in the amino-terminal region of the pII molecule. This common region is hydrophobic, and it may play a role in anchoring the protein to the outer membrane (17). The variable portions of these proteins are hydrophilic (17).

Different pII's also show several other similarities, such as a high isoelectric point of about 9.0 and most notably heat modifiability (8,18). In immunoblotting reactions, heat modified forms appear to be better antigens (18). This is because lipopolysaccharide (LPS) binds tightly to pII's and stringent conditions are needed to break this association. Variants also differ from one another in certain respects other than the degree to which they cause intercellular adhesion (18). They also have different affinities for binding to different eukaryotic cell types, and they differ in resistance to serum killing, cytotoxicity, and in protease and steroid sensitivities (1).

Given these differences, variation allows for colonization of many different microenvironments within the host (1). Several factors may influence this variation. Studies have shown differences in opacity with respect to the female menstrual cycle (2). Gonococci isolated from the cervix during menses tended to be transparent and during ovulation opaque (2). Variants lacking pII's seem to have a greater resistance to proteolytic enzymes, thus accounting for increased isolation of transparent colonies during the luteal stage of the cycle (30). Other unknown factors may also be involved.

Variants arise at a fairly high frequency of about one per 10^3 to 10^4 cells per generation (18). The ability to switch from expressing one pII by itself to expressing another by itself involves single independent steps (1). The first step involves loss of the ability to produce the first pII, and the second involves gaining the ability to produce the second pII (1).

The mechanism of control, determining whether expression is "on" or "off", is very

interesting. The opacity genes are transcribed constitutively, and control is exerted at the translational level based upon nucleotide sequence changes in the individual genes (17).

These carry the ATG initiation codon but it is out-of-frame, in either of the two possible out-of-frame nonsense sequences (17). Following this codon, a few nucleotides downstream, is a repetitive section of cytosines, followed by a repeating adenine sequence (17). The most interesting portion of these genes is a few nucleotides down from the adenine repeat. This 5' portion of the opacity gene "is composed of identical pentameric pyrimidine units" (17) of the repeating sequence CTCTT. The number of repeating units is variable ranging from 7 to 28 units (17).

The pII proteins are synthesized with a hydrophobic amino-terminal leader peptide. Part of this leader sequence is coded for by the CTCTT repeat region (17). It could be questioned whether or not the number of repeating units could affect the function of the leader peptide. Its length may have an effect on transport of the pII protein to the outer membrane, but no differences were found in this regard with respect to the number of repeats (17).

This repeat region will determine expression of the pII proteins. The number of repeats will determine whether or not the codon specifying the first amino acid in the amino-terminal portion of the processed pII will be in or out of frame (17). If a certain sequence contains, say, 8 repeats, and is in frame, then it will remain in frame if the number of repeats is changed by a multiple of 3. If not then the gene is in the out-of-frame mode, and translation results in a small truncated polypeptide. The number of repeats needed for expression will vary with different genes as the sequence upstream from this region will also vary (17).

Gonococcus alters expression from the "on" to "off" mode or vice versa by changing the number of repeats, and this happens quite frequently (17). Exactly how this is

accomplished is not known. Gene conversion is a possibility but this process may not involve recombinational events of this type (17). At the level of DNA replication, this sequence of pyrimidines could be "slippery" for DNA polymerase resulting in insertions or deletions of these units (17). In any event, this sequence affords gonococci a mechanism of control that can be altered quickly allowing this organism to adapt to new environments efficiently.

In short, variation in both pII expression and pilus antigenicity contribute to evasion of the immune system, survival in a changing host environment and colonization of several distinct niches as both show different binding affinities for different cell types. Since these two vary independently of one another, an extremely large number of variants can arise (28). It is also possible that differences in LPS might contribute to virulence (27). This could only serve to further complicate the quest of controlling gonorrhoea.

Materials and Methods

Gonococci (GC): Strain MS11_{mk}, which were piliated but did not express any form of pII protein, were used to infect the urethra of patient 016, a male army volunteer with no previous cases of gonorrhea. This took place at 10:00 A. M. A urine sample was collected 2 hours later and another at 6:00 P. M. Subsequent samples were taken at 8:00 A. M. and 6:00 P. M. each day until the patient developed clinical symptoms of gonorrhea. Eleven urine samples were concentrated, frozen at -70°C , and labeled u 1 through u 11. This procedure was performed by Dr. John Boslego at the Walter Reed Army Institute of Research.

GC Growth: Fifty microliters of each urine sample was plated out on a clear typing medium, and the same amount on the same medium but containing vancomycin, colistin, and nystatin antibiotics (VCN), used to prevent growth of normal flora present in these samples. The medium consists of each of the following per liter: 3.75 g trypticase peptone, 7.5 g meat peptone, 4 g K_2HPO_4 , 1 g KH_2PO_4 , 5 g NaCl, 1 g soluble starch, and noble agar. GC were grown at 37°C in an atmosphere of 5% CO_2 for 22 - 24h. GC colonies were distinguished by their small size and circular, convex appearance. Several individual colonies, each derived from a single cell, from these plates were passaged once and streaked onto individual plates of clear typing media. After 22 - 24 h of growth these plates were phenotypically characterized, 2 or 3 colonies were suspended in skim milk and frozen at -70°C to serve as stock, and suspensions were made for gel electrophoresis.

Standard suspensions : Suspensions of GC samples were prepared in dulbecco's - phosphate buffer saline (d - PBS) . The bacterial concentration in the suspensions was such that the optical density reading at a wavelenth of 540 nm was 0.6 . Suspensions were vortexed vigorously, and 1.5 ml was then centrifuged at 12,000 RPM for 1.5 min. The supernatant was removed, and the pellet was suspended in 165 µl of 2X solubilizing solution , and then boiled for 10 minutes . These samples were used in protein separation on a sodium dodecyl sulfate polyacrylamide gel .

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS - PAGE) : pII proteins were separated on a 12.5% SDS separating gel, and pili (diluted 10 fold with 2X solubilizing solution) on a 15 % gel . An SDS stacking gel was added (same concentration for both pili and pII's) and 5 µl samples were used for separation . These samples were run against molecular weight markers containing radioactive iodine , and gels were run at 100 volts until the tracking dye ran off the gel. The proteins were then transferred to nitrocellulose paper by blotting for 3 - 4 h at 65 watts in SDS - PAGE / NCP blot buffer .

Monoclonal Antibodies : After blotting the nitrocellulose paper was washed in tris - saline - gelatin - nonidet (TSGN) (see Appendix) for 15 minutes and subsequently sealed in a bag with 2% bovine serum albumin (BSA) TSGN along with the desired monoclonal antibody at an appropriate dilution. These were incubated for 6 to 16 h and then washed in TSGN for 15 min. The papers then were incubated with radioiodinated (^{125}I) protein A, at a 1 : 100 dilution in 2% BSA TSGN, in a sealed plastic bag for 1 h . In some cases the antibodies require a 1 h incubation with rabbit - mouse IgG , at a 1: 500 dilution , prior to

17

The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry should be supported by a valid receipt or invoice. This ensures transparency and allows for easy verification of the data.

In the second section, the author outlines the various methods used to collect and analyze the data. This includes both primary and secondary data collection techniques. The primary data was gathered through direct observation and interviews with key stakeholders. Secondary data was obtained from existing reports and databases.

The analysis phase involved using statistical software to identify trends and correlations within the data. The results show a clear upward trend in the number of transactions over the period studied. This is attributed to several factors, including increased market activity and improved operational efficiency.

Finally, the document concludes with a series of recommendations for future research and practice. It suggests that further investigation into the underlying causes of the observed trends would be beneficial. Additionally, it recommends implementing more robust data management systems to enhance the accuracy and reliability of the information.

18

37° C. This was followed by a 5 min. incubation at 65° C to degrade the T4 kinase. The T4 kinase will transfer the gamma phosphate (labeled with radioactive ^{32}P) to the oligopeptide. The radioactive oligopeptide was purified in a pre - spun G - 25 (DNA) Sephadex column by centrifugation at 3,000 RPM for 4 min.

mRNA Sequencing : For each sample to be sequenced the following procedures were performed. Seven and one - half μl RNA, 2.5 μl annealing buffer, and 2.5 μl of the oligopeptide probe were added to a microfuge tube and heated at 90° C for 3 min. and allowed to cool to 45° C. Five presequence mixes (PSM's) were added to 5 microfuge tubes (3 μl each), and reverse transcriptase was added to attain a concentration of 0.3 to 0.6 $\mu\text{g}/\mu\text{l}$. Each of 4 PSM's contains the dideoxy form of one of the 4 nucleotide bases, and the fifth, a control, has no dideoxy form. With the dideoxy form, transcription will stop after this base is inserted on the oligopeptide across from its complimentary base on the message. Along with the dideoxy form of the base, each PSM also contains the normal form of that base so transcription will stop at several places along the message in each tube.

Next, 2 μl of the RNA mixture was added to each PSM tube. These were incubated at 45° C for 35 minutes and were dried in the speed vacuum. The pellets were resuspended in 3 μl of formamide dye solution.

These were then run on an 8% acrylamide gel at 56 watts until the dye ran out. The gel was then fixed in an aqueous solution consisting of 10% methanol and 10% acetic acid. The gel was then placed on a piece of Whatman 3 MM paper and dried. This was then exposed to Kodak film for 3 to 4 days.

Results

Dr. John Boslego infected certain male army volunteer's urethras with pilus ⁺ GC, while others received an inoculum of pilus ⁻ GC. Different numbers of organisms were used for both types of GC. The non-piliated organisms were of the non-reverting type (p⁻ n) so they will not produce pili once inside the patient. The number of volunteers used in the different trials, as well as the number contracting gonorrhea, are shown in Table 1. Penicillin was administered as soon as any volunteer developed the clinical symptoms of gonorrhea.

<u>Number of Cells in the Inoculum</u>	<u>Pilus +</u>	<u>Pilus -</u>
10 ⁴	1/3 (1/1)	0/2
10 ⁵	3/5	0/4
10 ⁸	3/3	0/3

Table 1. Intraurethral Injection Results. The ratios indicate the number of volunteers contracting gonorrhea as a fraction of the number of volunteers infected. For reasons to be discussed in the text the 1/3 ratio could be interpreted as a 1/1 ratio.

The work I performed was done on the urine samples taken from patient 016, a volunteer who did contract gonorrhea. Individual cells from each urine sample were grown and then the resulting colonies were phenotypically characterized. Polyacrylamide gel electrophoresis was then performed on each culture, obtained from cloning single cells, in order to assess the pII composition of each of each sample. We could then see which pII, or combination of pII's, was selected in vivo; that is, which ones were more virulent.

The identity of each pII was determined by running them in a gel against known pII's from patient 020. pIIa gave rise to colonies that ranged from o⁺ to o⁺⁺, pIIb gave mostly o⁺ colonies and the same was true with pIIe. pIIc and pII d were not expressed in this patient. The results are shown in Table 2.

pIIb was the dominant pII expressed among the samples taken from this patient. It was expressed, either alone or in combination with another pII(s), in 85% of the samples from u6 (the sixth urine sample) and in 95.8% of the u11 samples. Cells expressing this pII alone seemed to be favored in patient 016 as 87.5% of the u11 samples, the last urine sample taken from this patient before antibiotic treatment, expressed only pIIb (urine sample 8 contradicts this trend, but reasons for this will be discussed later).

<u>Urine Sample</u>	<u>pIIa⁺</u>	<u>pIIb⁺</u>	<u>pIIe⁺</u>	<u>pIIbe⁺</u>	<u>pIIab⁺</u>	<u>pIIae⁺</u>	<u>pIIabe⁺</u>
U2	0	0	0	0	0	0	0
U4	(no samples present in urine)						
U6	0	65%	5%	10%	5%	10%	5%
U8	33.4%	16.7%	16.7%	0	16.7%	16.7%	0
U11	0	87.5%	0	4.2%	4.2%	4.2%	0

Table 2. pII Composition of GC Reisolates. This table presents the percentage of each pII expressed, or combination of pII's, in each urine sample taken from patient 016; that is, the number of organisms that expressed a certain pII, or certain combination of them.

Monoclonal antibodies reactive against the lipopolysaccharide (LPS) of the input GC were used in immunoblotting reactions to determine the extent of the change in this entity.

The 24 organisms recovered from the eleventh urine sample were used for this purpose and 91.7% of these samples tested positive with the LPS monoclonal antibody, so little change seems to have occurred here.

Polyacrylamide gels of 15% were used to separate the pilin subunits from each sample and to assess differences in molecular weight. Slight differences were found in many samples, but a quantitative assessment was not performed. Pilin from each molecular weight group, as well as each sample from the eleventh urine sample, were then subjected to epitope mapping. The monoclonal antibodies used for this purpose were IH5, 9B9 and 7B4, as well as MC04. However, monoclonal MC04 seems to recognize an epitope which is not very clearly defined but is present on all the pili in our study, so this antibody will not be discussed further.

Monoclonal IH5 recognizes an epitope in the region between amino acids 128 and 141. To be recognized by this antibody the epitope must have an aspartic acid residue at position 136. Other amino acids composing this epitope are undefined (25).

Monoclonal 9B9 recognizes an epitope in the amino acid 76 to 91 region of the pilin subunit, and data suggests that amino acids 86 and 87 must be lysine and glutamic acid respectively (25).

For specific reactivity with monoclonal 7B4, amino acid 143 must be alanine and amino acid 148 must be glutamic acid (Swanson, unpublished data).

Pili from input GC were recognized by all three monoclonal antibodies, and by MC04 as well.. Recognition by all three seemed to be a common theme throughout the samples. However, a pilin sequence which was recognized by only monoclonal 9B9 was very prominent in urine sample u11, comprising 54.2% of the individual samples. Still though, 29.4% of the u11 samples reacted positively with each of the three monoclonals. Table 3 shows the results of this study.

<u>Urine Sample</u>	<u>IH5</u>	<u>9B9</u>	<u>7B4</u>	<u>IH5.9B9</u>	<u>IH5.7B4</u>	<u>All 3</u>	<u>None</u>
U2						100%	
U6		10%			10%	70%	10%
U8	(to few samples mapped for accurate data ro be obtained)						
U11		54.2%		4.2%		29.4%	12.6%

Table 3. Pilus Epitope Mapping Results. This table shows the percentage of pilin subunits from each sample that were reactive with each monoclonal or some combination of them. Only in urine samples 2 and 11 were all samples tested, but a sufficient number of those from sample u6 that differed in molecular weight was used to give accurate results.

Four samples (u11 E,G,K and O) were chosen for pilin mRNA sequencing based on differences in molecular weight, colony edge morphology, and epitope mapping. Sample u11 E reacted with all 3 monoclonal antibodies, K with only 9B9, and G did not react with any of the monoclonal antibodies. Originally u11 O expressed a pilin subunit not recognizable by any of the three antibodies but as the cells were plated a pilus⁻ variant arose in culture from u11 K, and it was grown and was substituted for u11 O for use in the sequencing. The designation u11 O was kept for reasons of clarity, although this is not the original sample u11 O.

The sequences of the 4 genes were compared to the sequence of the plasmid pVD203, which is the sequence of the pilin gene of input GC, and is the preferred sequence in vitro. The various DNA probes used in the hybridization for the sequencing were about 60 to 90 base pairs apart and were complementary to the sequences in pVD203. The results are depicted in Fig. 2.

1 10 20
 met asn thr leu gln lys gly phe thr leu ile glu leu met ile val ile ala ile val
 Input GC ATG AAT AAC CTT CAA AAA GGC TTT ACC CTT ATC GAG CTG ATG ATT GTG ATC GCT ATC GTC
 u11 E
 u11 G
 u11 K
 u11 O

30 40
 gly ile leu ala ala val ala leu pro ala tyr gln asp tyr thr ala arg ala gln val
 Input GC GCC ATT TTG GCG GCA GTC GCC CTT CCC GCC TAC CAA GAC TAC ACC GCC CGC GCG CAA GTT
 u11 E
 u11 G
 u11 K
 u11 O

50 60
 ser glu ala ile leu leu ala glu gly gln lys ser ala val thr glu tyr tyr leu asn
 Input GC TCC GAA GCC ATC CTT TTG GCC GAA GGT CAA AAA TCA GCC GTC ACC GCG TAT TAC CTG AAT
 gln asp
 u11 EG G.....
 u11 G
 u11 K
 u11 OT.....
 val

70 80
 his gly lys trp pro glu asn asn thr ser ala gly val ala ser pro pro ser asp ile
 Input GC CAC GGC AAA TGG CCG GAA AAC AAC ACT TCT GCC GGC GTG GCA TCC CCC CCC TCC GAC ATC
 ile
 u11 ET.....
 glu asp ala
 u11 GG.....G.....G.C.....(4 bases of 17 missing) ..
 u11 K
 glu lys asp ala trp his
 u11 OG.....A..G.....CG TGG CAT (4 of 15 bases missing) ..

90 100
 lys gly lys tyr val lys glu val glu val lys asn gly val val thr ala thr met leu
 Input GC AAA GGC AAA TAT GTT AAA GAG GTT GAA GTT AAA AAC GGC GTC GTT ACC GCC ACA ATG CTT
 u11 E
 ile lys gln gln ala
 u11 G .T.....G C.A.....CA.....GC.
 u11 K
 lys glu ala
 u11 OG..A.....??? ???.....GC.

Discussion

The results from the experiments performed by Dr. John Boslego showed fairly conclusively that piliation is necessary for the colonization of the human host (needed to initiate infection). No volunteer infected with non-piliated GC contracted gonorrhea.

However, there was one discrepancy in the data that is worth mentioning. Three volunteers were infected with 10^4 piliated organisms, and of those three only one contracted the disease. As it turned out the other two subjects had anti-pilus antibodies present in their blood, either from a prior infection or from a vaccine administered at some time in their lives. In immunoblotting reactions, these antibodies did react with pili from this strain. So even though they were probably formed against a heterologous strain they could be responsible for preventing the infection. Therefore, we look at these results as one volunteer contracting gonorrhea out of one inoculated, rather than one out of three.

The data presented in Table 2 indicated that GC expressing pIIb alone is favored in this patient. In urine sample two, this condition represented 65% of the organisms and only 10% did not express this pII at all. Similarly in u11 87.5% of the organisms expressed pIIb alone and only 4.2% did not express it at all. The data from u8 does not seem to be consistent with this trend; however, there were only 6 organisms recovered from this urine sample and thus the apparent lack of conformity of the data is a result of too few organisms to allow valid results.

In urine sample 2, no pII were expressed by any of the organisms. This is due to the fact that the input GC were devoid of any pII's, and this urine sample simply represents a washout of the inoculate. This sample does not represent infection but represents input GC which were recovered 2 hours after injection into the host allowing insufficient time for changes to occur.

In patient 020 (Swanson, unpublished data) pIIb was also dominant during the initial stages of infection. It was expressed alone in 86% of the cells from urine sample 4, and in 66% of urine sample 5. It was also dominant in urine sample 6, however its percentage dropped to 39% of the organisms, and to only 25% in sample 7. In sample 7, 58% of the cells expressed pIIa and pIIc simultaneously. So this combination might have been even more favored in patient 020 than the pIIb⁺ cells, or it may have been favored in the latter part of initial infection.

pIIc was not expressed in any organism taken from patient 016 all the way through urine sample 11. It may have been selected against in this patient, but it could have also been the result of a mutation in the gene encoding for pIIc which prevented its expression. Thus the pIIac combination could not have resulted and hence organisms expressing pIIb were the most favored. In the seventh urine sample taken from patient 020, the last one taken from this subject, organisms expressing pIIb alone did represent 25% of the sample and was second only to the pIIac combination.

One thing our group was interested in was the frequency at which pII variants arose from pII⁻ organisms. To determine this a transparent (pII⁻) colony of input GC was suspended in ddH₂O and plated on three dishes of the clear typing medium and one containing the VCN inhibitor.

Each cell gave rise to a colony. Since there was a fairly uniform distribution of colonies over the plate the number of colonies in one-fourth of the plate was counted to estimate the number of colonies on each plate. The number of opaque (pII⁺) variants was also counted, and each was passaged onto fresh media to be certain of their opacity.

On one regular clear typing media plate, 11 opaque variants were recovered and 10 on the VCN plate. Thus we reason that there is no difference between the two media in favoring opaque variants. Overall 23 opaque variants were recovered out of a total of 7,572 colonies

resulting in a variant frequency of 0.308% which agrees with the 0.5% frequency that this species commonly shows.

All opaque variants were shown, through immunoblotting, to express pIIa alone (in a single step change such as this, one would expect that only one pII gene out of the five would switch to the "on" state). Thus it may have been that the original transparent colony, which was suspended, had a foci of pIIa⁺ organisms originating from one variant within that colony. Since this experiment used three such transparent colonies one would not expect each to do this, but they did. Later on input GC were plated, and all opaque variants were subject to immunoblotting. Forty-two of forty-four variants which arose turned out to be pIIa⁺ and the other two were pIIb⁺.

So our results were consistent. pIIa⁺ organisms may be selected by this medium or this may represent a difference between in vivo and in vitro expression of pII's, pIIa being favored in vitro. GC expressing pIIa by itself were not found at a high frequency in vivo.

This experiment did have a more serious implication concerning our results. In many immunoblots, from both patients we saw a faint trace of pIIa in several of the organisms, and in urine sample 11 from patient 016, virtually all the GC showed this phenomena. However, there was only a faint trace of pIIa in these samples, and thus the cells were not considered to be pIIa⁺. We can now see that this phenomenon may have been due to passage of GC on our clear typing media two or three times before immunoblotting was performed, a procedure needed to obtain enough cells from the urine sample for suspension. If the media did select organisms expressing pIIa, then this could explain the faint trace of pIIa on the immunoblots as some of the cells began to express this pII.

Input GC expressed a pilin subunit that gave rise to a fairly sharp colony edge (p^{++/+}) when assembled into pili. In urine sample 2, this was still the case, but in u6 colony edge morphology ranged from very sharp (p⁺⁺) to not very sharp (p^{+/-}). But most of the

colonies did still have a fairly sharp edge ($p^{++/+}$ or $p^{+/++}$), and this theme seemed to continue in the eleventh urine sample (with the exception that no colonies in u11 exhibited the $p^{+/-}$ morphotype). No relationship between colony edge morphology and pilin subunit molecular weight could be discovered.

There was only slight differences in the molecular weights of the pilin subunits from the various organisms in each urine sample, and most seemed to have the same molecular weight, although this cannot be taken to mean that there were no changes in the amino acid sequences of these pilins.

The mRNA sequences of the four pilin genes provided some interesting results. Sample u11 E showed some single base changes, as compared to pVD203, in the amino acid 50 - 65 region and in the 135 to 140 region, but there did not seem to be any great changes in this gene. The protein encoded by this gene reacted with all three monoclonal antibodies, and the sequence showed this should be the case. There was a single base substitution in the triplet code for amino acid residue 143 which must encode for alanine in order for monoclonal 7B4 to react with this protein, but this change did not result in an amino acid substitution.

The pilin gene from u11 K did not show any changes what-so-ever when compared to pVD203, yet its protein only reacted with monoclonal 9B9. This could have resulted from an error in immunoblotting. Sometimes positive reactions are obscured for one reason or another. For example, a couple of gels had to be rerun because fewer samples reacted positive by would be expected, and the second time through many more did react positive. There is also another explanation which deserves to be noted. When autoradiographs are being read to determine base sequence, one wishes to have only one of the four wells show radiation at each depth (each base position on the gene), but many times 2 or more wells (each well corresponding to a different dideoxy base) show radioactivity and sometimes one cannot distinguish the exact base. When the sequence is homologous to pVD203 upstream

and downstream from these points the base is assumed to be the one in pVD203 at that position. This assumption may have been wrong in certain regions and thus the discrepancy.

That this gene is exactly like pVD203, or so we assume, and u11 E is similar enough to be considered pVD203, is not odd. This seems to be the preferred sequence in strain MS11_{mk}. Three groups of scientists (Schoolnik, 1984; Meyer, 1984; Bergstrom, 1986) have found "virtually identical pDV203 sequences for pilus + organisms of this strain geneologically separated by multiple in vitro passages" (25). PVD203 - like sequences have been found to occupy several of the 12 - 16 silent partial pilin genes of this strain, and such a broad homology may control the preferred use of this sequence in recombinational events (25).

The sequence provided by u11G also shows some interesting results. Sequence changes are found at several places in the variable regions of this gene, and most of its sequence differs from the input GC sequence from amino acid residue 135 to the end of the gene. However, the highly conserved cysteine residue, and surrounding residues, at position 128 is conserved, as well as at position 158, although the base sequence surrounding position 158 is somewhat different it still encodes for the same amino acids. Base changes have also resulted in different amino acids being incorporated into the protein at areas in which certain ones are needed for positive monoclonal antibody reactivity, thus showing why this pilin subunit did not react with any of the three monoclonals.

There were two discrepancies with this gene. One was the appearance of a stop codon at amino acid position 137. This could have been the result of missreading the sequence off of the autoradiograph or just a poor reaction with the presequence mixes. The second problem was in the amino acid 74 - 80 region. In this region 4 bases were missing and 13 others could not be distinguished as to their identity. If 4 bases are missing the entire reading frame will be wrong downstream from this point. Perhaps only three bases were missing, and it

was interpreted as 4. The autorad was very confusing and hard to read in this section. But there is another possible explanation. Looking at the autorad a little further downstream from this point one find another discrepancy. The bases in the sequence are separated on the film by a certain distance but at one point there was a radioactive entity in the dideoxy guanine well half way where 2 bases in sequence should be. At first this was regarded as an error in the gel separation, but this could supply the base we are missing. If this is so then the reading frame would be restored so the cysteines would be conserved but the amino acid sequence in the region between where the bases are missing, as compared to pVD203, and where the extra base showed up would be different. The amino acid sequence difference would be fine since this is the variable region of the pilin subunit.

The sequence in u11 O was used to show a recombinational event and how it can result in the loss of piliation. The same problem of missing 4 bases came into view, but the same possible "extra" base was also evident. If in fact 4 bases were missing then the reading frame would not be read correctly resulting in an abnormal and perhaps truncated protein. Even if not so a stop codon did arise at position 136. Even though a truncated peptide did arise, the cysteines and other surrounding conserved residues were present, although the second cysteine is not at position 158, and one has to extend beyond the stop codon to reach it, it is present.

Characterizing the changes that occur in the outer membrane of Neisseria gonorrhoeae in vivo may someday help lead to the development of an effective vaccine for gonorrhea. Perhaps a certain sequence on the pilin subunit that is necessary in vivo can be found and used for antibody preparations. We can only hope that DNA sequencing will help in the elucidation of this entity. What remains in the future is much more basic research before the dream of a vaccine can be actualized.

Appendix

Buffers and other solutions :

D-Phosphate Buffer Saline (PBS):

Solution B stock :

NaCl	8.0 g
KCl	0.2 g
Na ₂ HPO ₄	1.15 g
KH ₂ PO ₄	0.2 g
ddH ₂ O	100 ml

Solution B stock :

CaCl ₂	1.0 g
MgCl ₂	1.0 g
ddH ₂ O	100 ml

Final Solution :

A stock	10 ml
B stock	1 ml
ddH ₂ O	89 ml

2X Solubilizing Solution :

10% SDS	2 ml
2-mercaptoethanol	0.4 ml
glycerol	1.0 ml
1 M Tris pH 6.8	0.625 ml
dH ₂ O	6.0 ml
bromophenol blue	add till blue color persists

SDS - Page Running Buffer :

Tris (Sigma 7 - 9)	15.125 g
glycine	72.0 g
SDS	5.0 g
dH ₂ O	fill to 5 l

SDS - PAGE / NCP Blot Buffer :

5X concentrate :

glycine	360 g
Tris base (Ultra Pure)	75.75 g
dH ₂ O	fill to 5 l

Final :

5X concentrate	500 ml
methanol	500 ml
dH ₂ O	1500 ml

2% BSA TSGN :

TSGN	200 ml
bovine serum albumin (BSA)	4 g

Guanine Lysis Buffer :

guanine isothiocyanide	47.5 g
0.5 M Sodium Citrate pH 7.0	5.0 ml
2- mercaptoethanol	1.0 ml
dH ₂ O	50 ml
0.1 N NaOH	adjust to pH 7.0
adjust volume to 100 ml with dH ₂ O	

Acidic Phenol for RNA Extraction :

To 100 ml of distilled phenol add 100 ml 0.5 Sodium Acetate pH 5.0. Dissolve phenol and discard Sodium Acetate after it settles to the top. Add 100 ml 0.05 M Sodium Acetate and extract it again. Add 100 ml 0.05 M Sodium Acetate.

Annealing Buffer :

1.0 M Tris HCl pH 8.3, 1.2 M NaCl	200 µl
1.0 M DTT	40 µl
0.5 M EDTA	8 µl
ddH ₂ O	552 µl

Solutions for Acrylamide Gels :

Acrylamide : Bis Acrylamide (30 : 0.8) :

acrylamide	90 g
N, N' - methalene - bis - acrylamide	2.4 g
dH ₂ O	fill to 300 ml

1.875 M Tris pH 8.8 :

Tris (Sigma 7 - 9)	22.7 g
concentrated HCl	adjust to pH 8.8
dH ₂ O	fill to 100 ml

1.0 M Tris pH 6.8 :

Tris (Sigma 7 - 9)	12.1 g
concentrated HCl	adjust to pH 6.8
dH ₂ O	adjust to 100 ml

0.2 M EDTA :

EDTA	6.72 g
dH ₂ O	100 ml

10% Ammonium Persulfate :

ammonium persulfate	0.5 g
dH ₂ O	5.0 ml

Acrylamide Gels :

Separating Gel (for protein separation) :

	<u>12.5%</u>	<u>15%</u>
acrylamide : bis	12.5 ml	15 ml
1.875 M Tris pH 8.8	6 ml	6 ml
0.2 M EDTA	0.3 ml	0.3 ml
dH ₂ O	10.9 ml	8.4 ml
Temid	0.015 ml	0.015 ml
10% ammonium persulfate	0.3 ml	0.3 ml

Stacking Gel (for protein separation) :

acrylamide : bis	2.5 ml
1.0 M Tris pH 6.8	1.88 ml
0.2 M EDTA	0.15 ml
dH ₂ O	10.3 ml
Temid	0.0075 ml
10% ammonium persulfate	0.15 ml

8% Acrylamide Gel (for RNA separation) :

8% acrylamide stock	80 ml
Temid	45 µl
10% ammonium persulfate	1.0 ml

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