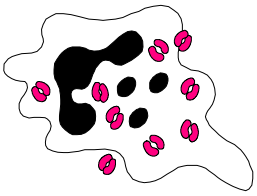

Genetics

Genetics, Plenary Review

The Neisserial genome: Importance and where do we stand?

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In 1995, Fleischman et al. (1) demonstrated that the complete nucleotide sequence of a procaryotic genome (*Haemophilus influenzae* Rd) could be assembled from randomly sheared and cloned genomic DNA, without the benefit of prior restriction site mapping and cosmid cloning. This was a landmark event, as it signaled that automated methods for DNA sequence acquisition and analysis have matured into robust, highly efficient technologies that promise to revolutionize the biological sciences. Indeed, the genomic sequence of several other procaryotes (including *Mycoplasma genitalium* (2), *Escherichia coli*, and others) have or will be completed in the near future. These studies will enhance our understanding of procaryotic molecular biological processes, and provide new avenues for gene discovery and comparative genetics. From a practical standpoint, procaryotic genome sequencing will enhance our ability to understand processes occurring during the pathogenesis of infectious disease. It is highly probable that these studies will provide new approaches for drug discovery, a necessity of increasing importance as microbial antibiotic resistance threatens the ability of the biomedical community to treat bacterial infections. Our own procaryotic genome sequencing efforts are currently centered on the obligate human pathogen Neisseria gonorrhoeae, with other projects either planned or beginning shortly. The purpose of this review is to describe the organization of the gonococcal genome sequencing project, placing this project in the context of the current understanding of Neisserial genome organization and population genetics.

Correlated physical and genetic maps of the *Neisseriae*. Studies were first presented in 1991 by Dempsey et al. (3) and Bihlmaier et al. (4) that determined the physical/genetic maps of the genomes of gonococcal strains FA1090 and MS11, respectively. The organization of the 2.2 Mb genomes of FA1090 and MS11 are remarkably similar, with the locations of most mapped genetic markers being the same, within the limits of resolution of the gel electrophoresis techniques available. A further study by Dempsey et al. placed an additional 28 markers on the FA1090 map (5). By analogy with other gram-negative bacteria, Dempsey et al. suggested that the origin of replication of the gonococcal chromosome may be located near *gyr*B, at 12 o'clock on the FA1090 map. Loci affecting the synthesis and assembly of pili (*pil*C, *pil*D, *pil*F, *pil*T and *reg*G) were not found to be adjacent to the gene encoding the major pilin subunit (*pil*E), as in some other bacterial species. Similarly, genes of the iron regulon (including *tbp*AB, *fur*, *frp*B, *fbp*ABC and *lbp*A) did not appear to be clustered, but rather scattered about the gonococcal chromosome. Recently, Dempsey et al. (6) determined the physical /genetic organization of *N. meningitidis* Z2491, and compared this to strains FA1090 and MS11. A similar study was earlier performed by Bautsch (7) on strain B1940, although no genetic markers were placed on the physical map of that genome. The meningococcal genome size is, within experimental limits, essentially identical to the 2.2 Mb gonococcal genome, and genetic markers were found in the

meningococcus to be in the same approximate location. This may not be surprising, considering the high degree of similarity between the two organisms (8, 9). However, in an approximately 500 kb region of DNA of the meningococcal chromosome, Dempsey et al. found considerable differences between the Z2491 genome and that of gonococcal strain FA1090, suggesting complex translocations and/or inversions between the two strains. These differences occurred in a region thought to be near the terminus of replication, as in the inversion-susceptible region of the enteric terminus.

Neisserial population genetics. Population genetic studies indicate that many bacterial populations are clonal (10). For certain pathogens, specific clonal lineages are uniquely capable of producing disease in a specific host, while other clonal lineages appear relatively avirulent. Thus, strains of the *Bordetella bronchiseptica* ET1 lineage appear uniquely capable of causing respiratory tract infections in pigs, but do not routinely infect other mammalian hosts (11, 12). *Bordetella pertussis* is an exclusively human pathogen, despite a genetic relationship with *B. bronchiseptica* that is so close that Musser et al. concluded that these organisms are biovars of a single species. Similarly, *Salmonella* biovars are differentiated into clones with particular disease characteristics, including host specificity (13-17). Thus, one might expect that certain clonal gonococcal lineages would be specialized for causing specific disease syndromes, suchas disseminated gonococcal infection (DGI) or pelvic inflammatory disease (PID). If this were true, selecting a single gonococcal strain for genome sequencing could miss important information. However, the population structure of *N. gonorrhoeae* is panmictic (18, 19). That is, genetic recombination occurs at such a high frequency that the population is essentially randomized; no single determinant or combination of determinants has been specially selected for virulence fitness. For our purposes, this is extremely important: any strain of *N. gonorrhoeae* will be appropriate for genome sequence analysis (provided that other practical criteria are met; see below); analysis of a single strain should identify all important virulence determinants.

Sequencing the gonococcal genome. Strain selection**.** From the viewpoint of a student of microbial pathogenesis, the *H. influenzae* genome sequence database presents several concerns, despite the singular importance of that sequencing project. As noted by Fleischmann et al. (1): "The nonpathogenic *H. influenzae* strain Rd varies significantly from the pathogenic serotype b strains. Many of the differences between these two strains appear in factors affecting infectivity" (emphasis added). Strain Rd is a derivative of a wild-type *H. influenzae* strain first isolated by Alexander and Leidy (20) in the early 1950s from the throat of a healthy child. Initially, then, the organism was not a disease isolate. Further, the wild-type strain was a serotype d organism, which is only rarely associated with invasive human disease (21). The organism was then passed in vitro to isolate a nonencapsulated variant, yielding strain Rd. Although strain Rd has been extremely useful for in vitro studies (22-24), nonselective in vitro passage may have resulted in loss of information important for *in vivo* growth and survival (i.e., pathogenesis). Indeed, Fleischmann et al. noted sixty potential frameshift mutations in their sequence, by comparison to entries in peptide databases. Many of these probably resulted from nonselective in vitro passage for almost 50 years. An important difference between the strain Rd sequence and virulent serotype b organisms is the complete deletion of the capsular biosynthesis cluster from strain Rd. Importantly, if this region had not already been sequenced from a pathogenic serotype b strain, Fleischmann et al. would not have known that it was missing from their own sequence! How many other silent, extremely important gaps lurk within the Rd database? This is obviously

unknowable; such mutations may diminish this database's usefulness for studies in microbial pathogenesis. (Not to belabor the point, as we recognize the pioneering efforts of Venter and colleagues. It is not so much that we know what is wrong with the *H. influenzae* database. Rather, our concern is that we have no way of knowing what may be missing.) Thus, a primary concern for sequencing the *N. gonorrhoeae* genome is the strain that selected for the sequencing protocol.

With the exception of a few model procaryotes (such as *E. coli*), one of the primary reasons to sequence the genome of a pathogenic procaryote is that these organisms cause human disease; this fact dictates many of our prejudices. Thus, for a strain to be selected for sequencing, we require that at least two conditions be met: 1) The strain must be representative of those organisms isolated from human disease, relying on population genetic and epidemiological data. As noted above, the gonococcus has a panmictic population structure, suggesting that all members of this species have essentially the same disease potential. This greatly simplifies the strain selection problem. 2) The genome of the strain in question must, insofar as it can be determined, be representative of that organism as it existed in the disease state. Thus, the organism must be a low-passage isolate obtained directly from an infection, or at least must retain infectivity in humans and/or relevant animal models. There are currently two gonococcal strains that, to our knowledge, meet this second criterion: strains FA1090 and MS11. Despite long-term in vitro cultivation, each strain still causes disease in human volunteers (25-27). It is important to note two limitations of these in vivo experiments. i) These assays are all performed in male volunteers, as experiments with female subjects is not considered ethical. ii) Experiments in male volunteers are terminated at the onset of inflammation, generally within 48-96 hours. Strictly speaking, we only know that strains FA1090 and MS11 will participate in the early steps of inflammatory disease in males. Later stages of disease, or asymptomatic infection, are not examined in these experiments. These two caveats aside, strains FA1090 and MS11 are well suited for genomic sequencing; we have arbitrarily chosen strain FA1090.

Methods of procedure. The sequencing project is divided into two distinct phases.
1. Primary sequence. Here, the genome is randomly sheared, pUC18 plasmid clones produced, and sequence obtained from the end of each insert using M13 forward and reverse primers and standard dideoxy sequencing technology. In this phase, we will randomly collect sequence, seeking to reach 5-fold coverage of the genome. According to the Smith-Waterman algorithm, assembly of genome sequence representing five-fold coverage should theoretically yields > 99% of the genome (1). Briefly, gonococcal DNA will be randomly fragmented by shearing in a nebulizer (28), which is more efficient than other fragmentation methods. DNA fragments of 800-1000 bp are gel purified, end-repaired, and re-purified using Sephacryl S-500 and phenol extraction. The DNA fragments are ligated into the SmaI site of pUC18, and transformed into *E. coli* XL1-Blue MR. This host strain contains the *mcr*A, *mcr*CB and *mrr* mutations (29), avoiding problems due to the extreme methylation state of gonococcal DNA (30). White colonies are selected, plasmid purified, and cycle sequencing reactions performed using fluorescent-labeled M13 forward and reverse primers. Template isolation employs an automated procedure using the Beckman Biomek 2000 workstation. The four reaction mixtures for each clone are pooled, ethanol precipitated, suspended in loading buffer, boiled, and loaded on a 6% polyacrylamide gel mounted in an ABI 377 sequencer. At present, assembling the sequencing reactions is done manually, but the Roe laboratory is working to automate this process, to

remove this labor-intensive and error-prone manual step. After electrophoresis, the raw information is transferred to a Power Macintosh and reduced to usable sequence data. This analyzed data is then transferred to a Sun SPARCstation 5 where the UNIX script OU-OTTO automatically removes vector sequence and enters the data into one of three sequence assembly databases. (OU-OTTO is a derivative of the original OTTO script obtained from the C. elegans group at Washington University, modified for optimal handling of genomic data by the Roe laboratory). The three sequence assembly databases are generated by: 1) CAP2, from Dr. Huang at the Michigan Technical Institute, 2) FAK2 from Dr. Gene Myers at Arizona, and 3) the Phred/Phrap programs obtained from Dr. Phil Green at the University of Washington. Both the CAP2 and FAK2 programs have been modified by the Roe laboratory to create a final sequence assembly database read by either the Staden XGAP or GAP4 programs. Alternatively, the output from Phred and Phrap is assembled into a database read by the CONSED program (also from Dr. Green's laboratory). In any case, the assembled sequence is manually proofread by reviewing the on-line fluorescent tracings from within XGAP, GAP4 or CONSED, prior to proceeding to the final closure and finishing stages.

2. Sequence closure. Continuing to obtain sequence by random shotgun methods beyond about 5-fold coverage is not cost- or labor-effective, and here we will switch to the second "finishing" or closure phase. Closure strategies will be indicated after manual proofreading of the primary contigs obtained by shotgun sequencing. Problem regions will be identified and sequencing protocols altered (synthesizing new primers close to the problem region, altering polyacrylamide concentration in the resolving gel, or employing dye-terminators). Gaps in the sequence due to "unclonable" regions will be bridged by synthesizing PCR primers that flank gap regions, PCR amplification and direct sequencing of the amplified gap segment. Sequencing the amplicon will be done directly, using this fragment as template, by fragmentation into very short pieces (ca.100 bp), or by walking along the fragment. Using PCR conditions described by Cheng et al . (31), up to 23-42 kb can be amplified for closure purposes. We anticipate that, as found by Fleischmann et al. (1), closure will require the use of a l library. This library will be used for single-pass end sequencing, to order the lambda clones on the genomic sequence, and to resolve gaps in the primary sequence. Finally, the lambda library will be deposited with the ATCC, for availability to the *Neisseria* community. The final contiguous sequence then must be annotated prior to database submission, aided by the FA1090 physical/genetic map. The assembled contigs will be imported into an ACEDB database for storage of sequence data and results of subsequent analyses. Briefly, a contig will first be analyzed by three programs (Blast X, XGrail and GeneFinder) that together will identify ORFs that have similarities in the databases. Potential ORFs without known homologues in the databases will be examined for typical gonococcal codon preference to determine whether these regions represent potential genes. Putative regulatory elements will be searched for by inspecting DNA sequence for consensus sequence elements (promoters, etc.). A rigorous analysis of the gonococcal genomic sequence will require more than simply determining the nucleotide sequence of the genome, and reporting the results of a computer analysis of this sequence. It is important to interpret the sequence data in the larger context of the pathobiological and clinical significance of the organism. We have therefore assembled the Gonococcal Genome Consultants Group (GGCG), bringing together extensive expertise in clinical medicine, microbial pathogenesis and genetics, epidemiology and public health. The GGCG includes MA Apicella, M.D., JG Cannon, Ph.D, SA Morse, MSPH, Ph.D., RF Rest, Ph.D., and PF Sparling, M.D.

Future studies. As of this writing, we are in the primary sequence phase; we have approximately 2 Mb of raw sequence, tentatively assembled into over 1000 contigs of about 600 bp to over 10 kb. At our current rate, we should be well into the closure phase by the end of calendar year 1996. Once the gonococcal sequence is complete, our plans include the following:

1. Comparisons with other human mucosal pathogens. The genomes of several other obligate human mucosal pathogens have been or are being sequenced, including *H. influenzae, M. genitalium, Helicobacter pylori, Staphylococcus aureus,* and *Streptococcus pyogenes*. The ecological niches that these organisms occupy have significant similarity which will be most certainly reflected in similarities at the genomic level of these organisms. We anticipate that careful comparison of the gonococcal genomic sequence with that of these other organisms will yield useful insight into the similarities and differences between these organisms, and point towards important comparative studies.

2. Whole-genome transcript mapping. Transcriptional control of gene expression is a common mechanism for controlling how bacteria respond to their environment. The FA1090 genomic sequence will provide the information to globally examine transcriptional regulation of the gonococcal genome. This will be done by preparing hybridization filters carrying all of the identified ORFs from the FA1090 genome, similar to the strategy described by Chuang et al (32) for *E. coli*. This collection of filters will then be hybridized with gonococcal cDNAs prepared by reverse transcription (perhaps with associated PCR amplification) of total RNAs isolated from organisms grown in vitro under environmental conditions that are expected to be important for gonococcal pathogenesis in vivo. Similar experiments can be done using cDNAs prepared from organisms recovered from in vitro infection assays, such as the fallopian tube organ culture model. Ultimately, hybridization probes can be prepared from RNAs obtained from gonococci isolated from experimental or natural infection, to directly examine gonococcal gene expression in vivo. Although some animal models have been proposed for examining gonococcal pathogenesis, none exactly replicates the process of gonococcal infection. Thus, the ability to directly assess gonococcal gene expression in vivo will be extremely important for understanding the intricacies of pathogenesis of this obligate human pathogen.

Acknowledgments: These studies have been supported by USPHS grants AI38399 (to DWD) and HG00313 (to BAR).

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Chromosome organization in *Neisseria gonorrhoeae* and *N. meningitidis*

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Recent construction of chromosome maps of several strains of *N. gonorrhoeae* and *N. meningitidis* provides a means to assess the effects of frequent recombination and horizontal exchange on genome organization, as well as helping to understand the genetic differences contributing to the type of infection caused by each species. Gonococcal strains FA1090 and MS11 are nearly identical in location of mapped genes (1-3), as are meningococcal strains Z2491 (Group A) and B1940 (Group B) (4, 5). Gene order is similar over much of the chromosome for all 4 strains. However, comparing the maps of FA1090 and Z2491 revealed a region of 400 kb showing complex rearrangements (4). The current maps do not reveal the extent of differences in genome organization between and within the two species. We have characterized these differences, digesting neisserial DNA with restriction enzymes recognizing rare sites and resolving the fragments by CHEF electrophoresis. The most extensive restriction site polymorphisms in 7 gonococcal strains were in the region of the chromosome that is rearranged between strains FA1090 and Z2491, although a detailed comparison of gonococcal strains FA1090 and F62 showed polymorphisms in *Spe*I and *Nhe*I fragments mapping in other regions of the chromosome as well. There were also differences between variants of one strain (*N. gonorrhoeae* MS11mk and MS11ms). Gonococcal strains fell into two groups in terms of location of rRNA genes on the chromosome. For meningococci, rRNA genes were located in the same places for all Group A strains analyzed, whereas Group C strains showed extensive variation. However, Group A strains did show polymorphisms in other restriction sites, even among strains of a single clone (isolates of subgroup IV-1 obtained during an epidemic in the Gambia). Just as there is no single type strain of the gonococcus or the meningococcus, we believe that there is not a single chromosome map that is representative of all strains of the two species.

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Genetics

Characterization of a peptidoglycan transglycosylase from *Neisseria gonorrhoeae*

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Peptidoglycan hydrolysis is involved in three important processes in *N. gonorrhoeae*: autolysis (1), genetic transformation (2), and release of toxic peptidoglycan fragments into the surrounding medium (3). We have identified a gene whose deduced amino acid sequence is homologous to peptidoglycan transglycosylases from bacteriophages. We have constructed mutations in the gene and characterized the mutants with regard to autolysis, transformation, and peptidoglycan turnover.

Our results suggest that the gene encodes an autolysin. Although the mutants are capable of lysis in buffer, they do not suffer the rapid death in stationary phase as is seen in the wild type. The mutants undergo a slow reduction in CFUs in late stationary phase culture, surviving 30 hours longer than the wild type strain.

Since the mutants appear deficient in autolysis, they would be expected to be poor donors in transformation. We are investigating the possibility that DNA release is reduced in the mutants and that transformation in mixed culture is reduced. Other peptidoglycan associated proteins have been shown to be necessary for gonococci to serve as recipients in transformation (2,4). However, experiments have shown that our mutants are as competent as the wild type.

The wild type and mutant strains have identical high rates of peptidoglycan turnover during exponential growth, but upon entry into stationary phase, the mutants' rate of hydrolysis slows. The predicted product of the transglycosylase reaction is the 1,6-anhydro disaccharide tetrapeptide monomer, a molecule identical to the tracheal cytolytic toxin released from *Bordetella pertussis* (5) and the toxic substance released by gonococci which kills cultured fallopian tube cells (6). We are investigating the possibility that the enzyme may be required for the formation or release of the cytotoxin and thus important for the organism's pathogenesis.

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Molecular mechanisms of capsule phase variation in group B *Neisseria meningitidis*

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The meningococcal polysaccharide capsules are pivotal virulence factors, which enable the meningococcus to survive during dissemination. However, recent data clearly indicated that the first steps of the infection, i.e. entry and transcytosis of the nasopharyngeal epithelial cells are hindered in the presence of the capsule (1,2). Furthermore, we could demonstrate, that only spontaneous capsule negative variants of an encapsulated meningococcal wildtype strain were able to enter epithelial cells (2). The analysis of these capsule-negative variants of a serogroup B strain (B1940) demonstrated two independent mechanisms of capsule phase variation. In class 1 variants we observed a molecular weight shift from 2 kb to 2.9 kb in one *Eco*RI fragment of the capsule gene locus (*cps*), whereas the class 2 variants exhibited no alterations in the resticition pattern of *cps*. The altered *Eco*RI gene fragment in class 1 variants harbours the *siaA* and *siaB* genes and the 5´ end of the *siaC* gene (3), which encode proteins of the biosynthetic machinery required for α-2,8 linked polysialic acid synthesis. Using PCR and primers which flank each of these genes, we could detect an insertion within the *siaA* gene, which encodes an epimerase catalysing the first step in α-2,8 linked polysialic acid synthesis by converting GlcNAc-6-phosphate to ManNAc, a precursor for NeuNAc synthesis (Frosch, unpublished results). Sequence determination of the enlarged *siaA* gene revealed an insertion at position 587 of the open reading frame, which exhibited characteristics of a transposable genetic element. The size of the insertion sequence element, termed IS*1301*, was 844 bp. The characteristics of this element are described in the accompanying abstract by Hilse *et. al.* Since inactivation of *siaA* causes substrate depriviation for the CMP-NeuNAc synthetase, this defect also resulted in the inability to endogenously sialylate the LOS. Thus, insertion of IS*1301* within *siaA* regulates both, capsule expression and endogenous LOS sialylation.

The biological significance of inactivation of capsular polysaccharide synthesis and of LOS sialylation by IS*1301* depends on its potential to reverse, since expression of the capsular polysaccharide and sialylated LOS are required for survival during dissemination and uncapsulated bacteria have never been observed as disease isolates. To confirm that the capsular polysaccharide can be re-expressed in *siaA*::IS*1301* inactivated meningococci, single colonies were plated and monitored by colony-blotting using mab 735 for capsule expression. This experiment revealed a frequency of 4×10^{-4} for reversion to the encapsulated phenotype.

The defect in the class 2 variants was due to the inactivation of the polysialyltransferase gene, *siaD*. Sequence data obtained by chromosomal sequencing of class 2 capsule negative variants demonstrated a deletion or an insertion of a single cytidine residue within a stretch of seven d(C)

residues at position 89 of the *siaD* sequence. The additional insertion of one dC residue resulted in a frame shift and a translational stop behind the $(dC)_7$ box (4). In a subsequent analysis we determined the number of dC residues in several capsule positive phase revertants of capsule negative clones, which spontaneously appear in a frequency of 10^{-3}. In these clones the original $(dC)_7$ box was reconstituted. These observations suggested a translational regulation of *siaD* by a slipped-strand mispairing mechanism.

There is evidence in two respects that capsule phase variation by the latter mechanism is of major biological significance in the pathogenesis of meningococcal disease. (i) *In vitro* invasion experiments using human epithelial cell lines indicated that the vast majority of all intracellular capsule-negative meningococci (85%) are characterized by the frame-shift within the *siaD* gene, whereas the *siaA*::IS*1301* genotype was observed only in 15% of the unencapsulated variants. (ii) We analyzed several meningococcal isolates collected during the outbreak of endemic and epidemic meningococcal disease. In contrast to the analyzed disease isolates meningococci, which were collected from the nasopharynx of carriers or diseased individuals and which were of clonal identity compared to the disease isolates, were unencapsulated due to the *siaD* mutation. These observations suggest that capsule phase variation due to the slipped-strand mispairing within the *siaD* gene is a prerequisite for meningococcal invasion and the outbreak of disease.

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Genetics

Transcriptional control of antibiotic resistance in *Neisseria gonorrhoeae* due to the *mtr* efflux system

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Energy-dependent efflux systems are possessed by a variety of bacteria and these export systems can enhance bacterial resistance to multiple, often structurally diverse antibiotics (1). Efflux pumps have the capacity to capture antibiotics in the periplasm or cytosol and, through a poorly defined mechanism, export these agents through the inner and outer membranes of Gram-negative bacteria or the peptidoglycan-rich cell wall of Gram-positive bacteria (2). Several classes of efflux pumps have been described in recent years but they all contain a cell membrane-associated translocator protein. Some efflux pumps also contain a periplasmic protein that fuses the inner and outer membranes as well as an outer membrane protein that channels the export of antibiotics to the outside environment. The genes encoding efflux pumps can be constitutively expressed or in certain instances, they can be up-regulated by stresses including antibiotics (2).

We recently described (3) the *mtr* (*m*ultiple *t*ransferable *r*esistance) efflux system possessed by *N. gonorrhoeae* and showed that it is remarkably similar to efflux pumps possessed by *E. coli* and *P. aeruginosa* (2). The *mtr* system was first recognized by Maness and Sparling in 1973 (4) in their studies on multiple antibiotic resistance in gonococci that resulted from a single mutation. The gonococcal efflux pump consists of three membrane proteins (MtrC, MtrD and MtrE) and has the ability to export structurally diverse hydrophobic antimicrobial agents (5), including antibacterial fatty acids and bile salts that bathe certain mucosal surfaces. Levels of gonococcal resistance to these hydrophobic compounds correlate with levels of expression of the *mtrCDE* genes (6), which form a single transcriptional unit.

Recently, we found that expression of the *mtrCDE* efflux system in gonococci is regulated at the level of transcription by both *cis*- and *trans*-acting factors associated with the *mtrR* gene, which is positioned upstream and transcriptionally divergent from the *mtrCDE* gene complex. The MtrR protein is a transcriptional repressor (7) that decreases expression of *mtrCDE*. However, expression of *the mtrR* gene requires the presence of a 13 base pair inverted repeat that is between the -10 and -35 regions of its promoter (3). We report herein that the MtrR repressor recognizes the nucleotide sequence within the -10 and -35 region of the promoter utilized by the *mtrCDE* operon. We also determined that mutations that cause single, radical amino acid substitutions within the helix-turn-helix (HTH) motif or in a downstream region of MtrR result in loss of or reduced MtrR-binding. Although the 13 bp inverted repeat sequence lies just upstream of the MtrR-binding site, we found that a single bp deletion in the inverted repeat resulted in altered MtrR-recognition of its binding site. Thus, the 13 bp inverted repeat can directly modulate transcription of the *mtrRCDE* genes as well as serving a role in MtrR-DNA interactions. We conclude that although tight control of *mtrCDE* gene expression in wild-type

strains is present, the action of the controlling elements described herein can be circumvented through mutations that alter repressor activity or promoter utilization. While these studies have a direct impact on how the *mtr* system is regulated, they also help to serve as a model for how expression of gonococcal virulence genes can be regulated at the level of transcription.

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Genetics

Do sexual bacterial have species?

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We have shown that the exchange of alleles of housekeeping genes between strains of *Neisseria gonorrhoeae* is frequent enough to generate a panmictic population structure (1). High levels of chromosomal exchange were also determined for strains of the meningococcus, although the population structure of this pathogen, which we described as 'epidemic', was obscured by the over-representation of certain strains that had recently increased to high frequency. Although high levels of gene exchange can obscure the identification of organisms we have demonstrated that the rate of gene exchange between *Neisseria meningitidis* and *N. gonorrhoeae* is not high enough to blur the species boundary between them (2); in general, sequences of housekeeping genes derived from *N. gonorrhoeae* can be distinguished from *N. meningitidis* because of differences that are apparently fixed in the gonococcal population. We propose that *N. meningitidis* and *N. gonorrhoeae* represent extremely closely related 'sexual' bacterial populations that appear to be genetically isolated in nature and thus conform to the biological species concept (2). We suggest that ecological isolation - of populations that can colonise the genital tract from those that can colonises the nasopharynx - may be an important component of the barrier to gene exchange between these two pathogens. However, the meningococcus is not ecologically separated from other, closely related, commensal *Neisseria* that occupy the naso-pharynx and the exchange of both highly selected genes (i.e. penicillin resistance determinants) and housekeeping genes between commensal *Neisseria* and strains of meningococci has been reported (3, 4). It is not clear, therefore, if strains of *N. meningitidis* form a valid species according to the biological species concept, or even if such a concept can be applied to organisms that are capable of high levels of chromosomal gene exchange and occupy the same niche.

Recently, a numerical taxonomic study of 315 strains of *Neisseria* for 155 phenotypic tests was published by Barrett and Sneath (the Sneath collection, [5]). This study shows that several of the traditional neisserial species are not readily identified by phenotypic traits; strains of *Neisseria elongata*, for example, were distributed in two phenons, one phenon associated with *N. meningitidis* and the other more distantly related. Furthermore, the relationship between some species, such as the placing of strains of *Neisseria cinerea* within the population of *N. meningitidis*, was unusual. To clarify the phylogenetic relationship of the commensal *Neisseria* and to determine if identifiable groups of organisms (species) exist among bacteria that have the potential for the frequent exchange of housekeeping genes we have undertaken a large scale sequencing project of well characterised strains from the Sneath collection. We have determined over 500 bp of the sequence of the *argF* and *recA* genes of a collection of over twenty five commensal strains of *Neisseria*. The strains sequenced include type strains of *Neisseria sicca*, *Neisseria subflava*, *Neisseria pharyngis* and *Neisseria perflava*, as well as strains identified as *Neisseria lactamica* (7 strains), *Neisseria polysaccharea* (4 strains), *Neisseria cinerea* (4 strains), *Neisseria elongata* (4 strains) and *Neisseria mucosa* (3 strains). These sequences complement

similar data previously derived for 8 strains of *N. meningitidis*, 9 strains of *N. gonorrhoeae* and single isolates of selected commensal strains (4). The total data set contains sequences from over fifty strains. We have concentrated on strains that are considered to be closely related to *N. meningitidis* and *N. gonorrhoeae*.

The sequences of both *recA* and *argF* produced similar groupings of strains although these groups do not necessarily conform to the traditional neisserial species. This concordance between *recA* and *argF* suggests that recombination at these loci is not common between the groups. However, in some cases the relationships between the groups were not necessarily the same for *recA* and *argF*, suggesting that recombination has, in the past, played a role in the phylogenetic history of these genes. We conclude that it may be possible to identify groups of *Neisseria* that conform to the biological species concept but that the phylogenetic history of these groups may depend on the gene chosen for analysis.

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Genetics

Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The closely related pathogenic *Neisseria* species, *Neisseria meningitidis* and *N. gonorrhoeae*, though very similar at the level of DNA sequence (1, 2) produce markedly different disease. The presently-known virulence factors which distinguish the meningococcus from the gonococcus (capsule, Opc, Frp proteins, and type II pilin) have not been shown to account fully for the differential pathogenesis of meningococcal meningitis, in particular the crossing of the blood-brain barrier. We therefore used a genomic subtraction technique to search for new genes specific to the meningococcus, some of which might code for virulence attributes which could help to elucidate the molecular mechanisms of disease caused by this organism.

We adapted the subtractive technique of representational difference analysis (3) to the isolation of probes for genes present in meningococcus strain Z2491, an epidemic group A strain (4) but absent from gonococcus MS11 (5). The libraries achieved were comprehensive and specific in that they contained sequences corresponding to the presently identified meningococcus-specific genes (capsule, *frp*, rotamase, *opc*) but lacked genes more or less homologous between the two species, for example *ppk, pilC1*.

Sequence analysis has shown that a few have some homology with structural genes involved in the virulence of other pathogenic species (haemolysins of *Serratia marcescens* and a ferric-chelate receptor of *Pseudomonas*). However, the large majority have no significant homology to known neisserial or indeed any other sequences, and therefore constitute a bank of previously undiscovered Nm-specific loci. Localization of the Nm-specific genes with respect to the recently-published macro-restriction map of meningococcus Z2491 (6) has revealed that the majority (63%) of the genetic differences between Z2491 and MS11 are clustered in three distinct regions of the chromosome. One of these corresponds to the capsule-related genes, another is restricted to meningococci of serogroup A, whilst one (region 2) of the regions is common to all meningococci tested.

we believe that this technique has wider application as a powerful tool for a rapid and directed analysis of the genetic basis of inter- or intra-specific phenotypic variations between related bacteria. Furthermore among the genes corresponding to the discovered sequences may be some which will help to elucidate the differential pathogenesis of meningococcal meningitis, or provide candidates for new anti-meningococcal vaccines.

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A two-component regulatory system in *Neisseria gonorrhoeae* involved in Opa (P.II) expression.

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In the course of infection pathogenic bacteria encounter numerous different environments within the host. An ability to sense changes in environment, and adapt to them, is of fundamental importance for the bacterium to survive and thrive.

Two-component regulatory systems (2CR's) are recognized as crucial mediators of adaptive responses to environment (1). 2CR's typically consist of an inner-membrane sensor protein which responds to a specific signal to activate a cytoplasmic response protein which mediates response through alteration of gene expression of specific target genes. Sensors, when triggered by signal, autophosphorylate a cytoplasmic residue, usually histidine. The phosphorylated sensor then acts as a kinase, transferring the phosphate to a residue of its cognate response protein (2). The phosphorylated response protein is then able to bind to specific DNA sequences within the promoter region of target genes, causing a change in the level of transcription of these genes that constitutes the response. The phosphorylated response protein may bind to multiple target genes, effecting a global response to a specific signal. (3)

Response regulation in *N. gonorrhoeae* is largely unknown. Many structural genes encoding surface components important for infection have been identified. However, whether these genes are regulated and what possible mechanisms of regulation may exist is unknown. To address this issue, we attempted to identify 2CR's of N. gonorrhoeae as candidates for mediators of N. gonorrhoeae responses to environmental (i.e. human host) signals.

Degenerate oligonucleotide primers, designed to a conserved amino-acid motif in the response proteins of previously characterized 2CR's, were used in a PCR reaction using N. gonorrhoeae strain 1291 as template. A single DNA species of the expected size (322bp) was amplified, cloned and sequenced. The predicted amino-acid sequence of this fragment showed high levels of homology to the corresponding region of other 2CR's. Southern hybridization confirmed that this fragment was amplified from the gonococcal genome. The PCR fragment was used as a probe to identify two contiguous Sau3A fragments of the gonococcal genome that together encode the entire response regulator, and immediately downstream of this, the N-terminal 322 amino-acids of the sensor protein of a 2CR. Flanking regions of this region are probably lethal when cloned at high copy number in *E. coli* as suggested by deletion of the gonococcal DNA from constructs carrying this flanking region.

Comparison of the predicted amino-acid sequence of the putative 2CR to database sequences revealed the highest homology to a 2CR of *S. typhimurium* and *E. coli* (pmrAB and basR/S respectively) involved in LPS phosphorylation.

To investigate the involvement of the gonococcal 2CR in LOS modification, an insertion mutation in the sensor protein encoding gene was constructed. An erythromycin resistance cassette was inserted after the seventh codon of the sensor gene.

The mutant LOS migrated more slowly on SDS-PAGE than wildtype LOS, although this difference was slight. This is perhaps consistent with an alteration in phosphorylation of LOS in which molecular weight differences between mutant and wild-type LOS would be small. Fine detail structural analysis of the mutant LOS is being performed.

Outer membrane protein profiles of the mutant were also analyzed on SDS-PAGE. The mutant does not express a 31Kd protein which is a major component of the wild-type profile. The major outer membrane protein of N. gonorrhoeae in this size range is Opa (P.II). Western blot analysis using MAb 4B12 which recognizes a conserved epitope on all known Opa proteins confirmed that multiple mutants selected after transformation of a 1291 Opa+ express none to markedly reduced amounts of Opa.

The mutant strain is slower to invade confluent monolayers of HepG2 cells when compared to wild-type, as indicated by a ten-fold reduction in cfu's recovered after 30 minutes infection and a four-fold reduction after 4 hours infection. This is consistent with the loss of Opa expression in the mutant. Opa is an important for adhesion of gonococci to host cells and thus loss of Opa expression may be expected to slow the adherence/invasion process (4,5).

In conclusion, we have identified a 2CR in N. gonorrhoeae that is putatively involved in modification of LOS, although the nature of this involvement is at present unknown. The 2CR is also involved in the expression of Opa. Opa expression is subject to phase variation through variation in a repetitive DNA element within the signal sequence of Opa genes (6). Other mechanisms of Opa expression regulation have not been described. 2CR's ultimately mediate effects through alteration of transcription of specific genes. Thus it is likely that the observed effect on Opa expression is at the level of transcription in response to environmental signals.

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A sixteen bp palindrome sequence encompassing the putative ribosomal binding site is conserved in pathogenic *Neisseria rfaC* genes and is involved in the regulation of expression of meningococcal *rfaC*.

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The lipooligosaccharide (LOS) of pathogenic *Neisseria* is an important factor in disease pathogenesis. *Neisseria gonorrhoeae* 1291 and *N. meningitidis* NMB wild type strains express a single LOS that has an Mr of 4.5 kDa and binds monoclonal antibody (MAb) 3F11. We have previously identified the *rfaC* homologues in *N. gonorrhoeae* and *N. meningitidis* that are able to complement the α1,5 LOS heptosyltransferase defect in *Salmonella typhimurium rfaC*630 (SA1377) (1). Sequence analysis of the *rfaC* genes in both *N. gonorrhoeae* and *N. meningitidis* revealed a thirty-two bp A+T-rich region between the putative -35 and -10 elements and the RfaC coding sequences. In addition, a sixteen bp palindrome sequence encompassing the putative ribosomal binding site of *rfaC* was also observed in both *N. gonorrhoeae* and *N. meningitidis*. To investigate whether this palindrome sequence is involved in the regulation of expression of *rfaC*, transcriptional *lacZ* fusions were constructed in *rfaC* and introduced into the chromosome of *N. meningitidis* NMB. The expression of *lacZ* was monitored under a range of environmental conditions to determine the effect, if any on the expression of *rfaC*. These conditions included the growth phase, decreased O2, carbon source, pH and addition of human cell extracts. None of these affected the *lacZ* expression.

To further investigate whether the palindrome sequence is involved in the expression of *rfaC*, site directed mutagenesis was performed to disrupt the palindrome sequence. The length of the palindrome region was unchanged in the mutated sequence. Transcriptional *lacZ* fusions were constructed in *rfaC* with the wild type or the mutated palindrome sequence. These constructs were then introduced into the chromosome of *N. meningitidis*. The expression of *lacZ* was found unchanged in fusions with the intact or mutated palindrome. Consideration was given to the fact that an intact *rfaC* might be necessary for modification of expression through the palindrome. To accomplish this, a single copy of *rfaC* was cloned into a non-essential gene we had previously identified and transformed into the chromosome of *N. meningitidis* containing the reporter fusions with intact or mutated palindromes. Analysis of the new constructs by SDS-PAGE and western blot indicated that the *rfaC* which was incorporated intact into the chromosome was functional and that a full length LOS was produced which bind MAb 3F11. A set of reporter controls was also constructed in which the the non-essential gene was disrupted by a aminoglycoside phosphotransferase gene cassette. SDS-PAGE analysis of these constructs showed the truncated LOS of the original *rfaC* mutant and this LOS did not react with MAb 3F11. The expression of *rfaC* was then analyzed in the presence and absence of wild type *rfaC* in constructs with the palindrome intact and the palindrome altered. Expression of *rfaC* was identical in the constructs containing an intact *rfaC* with the palindrome intact, and in the two construct without *rfaC* with the palindrome either intact or mutated. The level of *lacZ* expression in the construct with *rfaC* intact but with the palindrome altered was four to seven

times higher than that with the wild type palindrome throughout all phases of its growth. Analysis of glucokinase activity indicated that there was no significant change in the activity of this enzyme in any of the constructs. This result suggests that the 16 bp palindrome sequencec between the promoter and the ATG start codon is involved in suppression of expression of *rfaC* and that RfaC may be involved in some way in the regulation of its own expression.

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Identification and characterization of *gly1*, a PilA-regulated locus in *Neisseria gonorrhoeae*

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PilA and PilB comprise a two-component regulatory system that controls pilin expression in *N. gonorrhoeae*. We have identified a second locus, *gly1,* that is also controlled by this system.

The *gly1* locus was initially isolated from a *N. gonorrhoeae* gene bank as a clone with hemolytic activity when expressed in *E. coli*. DNA sequence analysis of this locus revealed the presence of two open reading frames (ORFs), which are likely co-transcribed. ORF1 encodes a polypeptide of 18.5 kDa that contains a signal sequence. N-terminal sequencing of this peptide expressed in *E. coli* showed that the 21 amino acid signal sequence is cleaved yielding a mature protein of 15.7 kDa. This protein was observed in outer membrane fractions as well as in culture supernatants of overnight cultures of these strains. ORF2 encodes a protein of 31.8 kDa with homology to no known genes. Both ORFs are required for hemolytic activity.

To demonstrate regulation by PilAPilB, pNG4-26 (*pilA*$^+$*pilB*$^+$) was introduced into an *E. coli* strain also containing *gly1*. Strains containing pNG4-26 had a significantly larger zones of hemolysis compared to otherwise isogenic strains lacking *pilA* and *pilB*. Analysis of a P*gly1-lacZ* transcriptional fusion in *E. coli* showed that *pilA*$^+$*pilB*$^+$ expressing strains had 1.5-fold more b-galactosidase activity than strains lacking *pilA* and *pilB*. This demonstrates that PilAPilB control of *gly1* is at the level of transcription.

DNA-binding by PilA *in vitro* was demonstrated by gel retardation analysis using purified PilA. Sequences 5Õ to ORF1 were bound by PilA in this assay, whereas sequences within or 3Õ of either ORF were not. Deletion analysis to localize the PilA-binding site within this fragment indicated that two regions are required for PilA binding to *gly1* DNA. This region spans from -60 to -383 with respect to the start of transcription (as determined by primer extension analysis). That two regions of the DNA are required for PilA binding is similar to our previous observations with PilA and the *pilE* promoter, which we suspect may involve DNA looping.

Deletion of the *gly1* locus in *N. gonorrhoeae* strain MS11 resulted in viable gonococci, indicating that these genes are not essential. These deletion mutants were analyzed for their interactions with various epithelial cell lines in tissue culture. The results of these experiments showed that adhesion to and invasion of three different cell lines was unaffected by deletion of the *gly1* locus. Interestingly, non-piliated *gly1* mutants were shown to cross polarized T$_{84}$ monolayers more slowly than the otherwise isogenic non-piliated parent. These results could suggest a role for one or both of the *gly1* polypeptides in trafficking of *N. gonorrhoeae* through epithelial cells.

A phase-variable type III restriction-modification system in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae possesses a number of Type II restriction/ modification systems (R/M) but characteristically expresses fewer restriction enzyme activities than corresponding methyltransferases (1). Methyltransferase specificities have been identified for S.NgoI (PuGCGCPy), S.NgoII (GGCC), S.NgoIII (CCGCGG), S.NgoIV (GCCGGC), S.NgoV (GGNNCC), S.NgoVI (GATC), S.NgoVII (GC[C/G]GC), S.NgoVIII (TCACC) (1, 2 and references therein) and characterization by Piekarowicz et. al. (3) of purified methyltransferase which recognizes the sequence $GTAN_5CTC$ (S.NgoIX). Restriction enzymes have been detected which correspond to the sequences for NgoI, II, III, IV, and IX in various strains.

We have identified the genes encoding a Type III restriction modification system (reviewed in 4) present in all strains of *N. gonorrhoeae* tested. Sequence analysis of the *ngo*X locus from strain MS11 indicate the predicted protein for the restriction enzyme (NgoX.R) is 59% identical to the restriction enzyme of the P1 system. The modification enzyme (NgoX.M) is predicted to be 38% identical to the modification enzyme of the P1 system.

The postulated 5' region of the *ngo*X.M gene is unusual in that the predicted start codon is followed by a series of direct pentameric repeats reminiscent of the signal-peptide encoding region of neisserial *opa* genes, responsible for regulating expression of *opa* genes by "phase variation". The *ngo*X.M gene of strain MS11 contains eight repeat elements (an out-of-frame configuration) while strain FA228 contains twelve repeats elements (an in-frame configuration). Mutations constructed in *Escherichia coli* and returned to the gonococcal chromosome by allelic replacement indicate that the *ngo*X.M gene is expressed in strain FA228 but not in strain MS11 but the gene is phase-variable from both the on and off configurations. Phase variation can not be detected in the viable cell population in wild-type strains. Purified heterodimeric NgoX enzyme is active against DNA purified from strains MS11mk (no expression of NgoX.M) and FA228 *ngo*X.M::*lac*Z but not strain FA228 (expresses NgoX.M).

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Detection of single-strand DNA during transformation of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae is naturally competent for DNA transformation; with several factors being required. Pili are essential for DNA uptake into a DNaseI-resistant state, although the mechanism of uptake is not understood. Following uptake, ComA, ComL, and Tpc are thought to be involved in the transfer of DNA to the cytoplasm (1). During uptake, transforming plasmid DNA (pDNA) is converted to linear, double-stranded (ds) molecules (2). RecA-mediated recombination with either a homologous chromosomal locus or linear plasmid completes the transformation process (3). Although RecA is required for transformation, single-strand (ss) DNaseI-resistant DNA, presumed to be the substrate for RecA, has not been previously detected (1,2). In an effort to detect ss pDNA following uptake by *N. gonorrhoeae*, we have re-examined the nature of DNaseI-resistant DNA during transformation.

N. gonorrhoeae MS11 (P^+) or *N. gonorrhoeae* MS11*dud* (P^-) were suspended in broth medium containing one of two isogenic plasmids isolated from *Escherichia coli* DH10. The isogenic plasmids pRML115 and pRML110 differed with respect to the presence or absence, respectively, of the gonococcal DNA uptake sequence (DUS). Following addition of DNaseI (0.5U/ml) to degrade extracellular pDNA, the cells were washed twice and total nucleic acid isolated. The DNaseI-resistant DNA was then analyzed by native blotting to detect ssDNA and by Southern blotting using a radiolabeled probe specific to the pDNA. In control experiments, DNase-resistant pDNA was not detected when either MS11 wild-type (P^+) was incubated with pRML110 (DUS^-) or when MS11*dud* (P^-) was incubated with pRML115 (DUS^+). In contrast, *N. gonorrhoeae* MS11 (P^+) sequestered pRML115 (DUS^+) in a DNaseI-resistant state and both ds linear and ds circular forms were detected by Southern blotting. In addition, native blotting revealed the presence of ss pDNA that corresponded to the linear form of the plasmid based on electrophoretic migration in agarose. Treatment of DNaseI-resistant DNA with S1 nuclease diminished the amount of ssDNA detected by native blotting compared to untreated DNA. Experiments designed to assess the stability of DNaseI-resistant DNA over time indicated that both the ds and ss forms of the pDNA were stable up to 4 hours after uptake.

These preliminary results suggest that ss pDNA is formed during the transformation of *N. gonorrhoeae*, although much ds pDNA is also present. Similar experiments using pDNA isolated from *N. gonorrhoeae* MS11 are being conducted to determine if the ss pDNA is formed specifically by the activity of restriction endonucleases. The ss pDNA detected by native blotting may represent recombinogenic molecules involved in the productive transformation of *N. gonorrhoeae.*

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PilA is unlikely to be an activator of *pilE* transcription in *Neisseria gonorrhoeae*.

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Upstream of the gonococcal pilin subunit gene (*pilE*), is a potentially functional σ^{54} promoter, with an overlapping σ^{70} promoter. The predicted transcription start points (tsp) for these two promoters are within 3 nucleotides, thus making resolution on the basis of primer extension extremely difficult. However, following site-directed mutagenesis of the promoter sequences, we have clearly shown that in *N. gonorrhoeae*, grown under normal *in vitro* conditions, *pilE* is transcribed from the σ^{70} promoter (1). When a P*pilE::cat* fusion is cloned and expressed in *Escherichia coli*, the host σ^{54} -RNA polymerase holoenzyme (σ^{54} -RNAP) competes with the σ^{70}-RNAP for binding to the respective promoter sequences, resulting in relatively low levels of CAT (2). Site-directed mutagenesis of the σ^{54} promoter, or expression of the P*pilE::cat* in an *rpoN* mutant, results in a 30-fold increase in CAT levels (1).

Early studies on the regulation of the gonococcal *pilE* gene (3) were performed with the aim of identifying genes encoding putative transcriptional regulators, using a P*pilE::cat* transcriptional fusion, cloned into an *E. coli* vector as a reporter. A gonococcal library was screened for clones capable of boosting CAT levels. However, this reporter construct contained both the overlapping promoters, thus favouring detection of indirect activation through the inhibition of RpoN binding, via a variety of possible mechanisms. Consequently, two divergently transcribed genes, designated *pilA* and *pilB*, the products of which were believed to activate and/or repress the transcription of *pilE*, were identified (3). Subsequently, despite the demonstration of significant similarity between PilA and the *E. coli* proteins FtsY and Ffh (4,5,6), components of a system involved in the targeting of nascent secretory proteins to the membrane (7), it was reported that *pilA* and *pilB* encoded a two component regulatory system, with PilA functioning as the activator of the *pilE* σ^{54} promoter (4,5). On the basis that gonococci do not use the σ^{54} promoter for transcription of *pilE* during growth *in vitro*, this conclusion seems unlikely.

Purified PilA protein has been shown to bind to a DNA fragment containing the *pilE* promoter (8). In particular, the region from -125 to -161, with respect to the tsp, was shown to be essential for PilA binding *in vitro*. However, we have shown that transcription of *pilE* in the gonococcus is independent of this region. Therefore the potential role of PilA as an essential regulator of the σ^{70} promoter is also unlikely.

We believe that *pilA* does not in fact encode a transcriptional activator of the gonococcal *pilE* gene, but is the gonococcal *ftsY* homologue and has a role in protein translocation.

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Regulation of DNA repair in *Neisseria gonorrhoeae*

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Recombination is indirectly related to virulence in *Neisseria gonorrhoeae* since this process allows phase and antigenic variation of cell surface components (1). Antigenic variation in pilin involves recombination between variant pilin gene sequences and depends on the presence of a functional *recA* gene (2,3). The *Escherichia coli* RecA protein is a multifunctional enzyme having roles in homologous recombination, DNA repair and control over expression of a set of co-regulated genes termed the SOS regulon (4). Many of the genes that comprise this regulon encode enzymes involved in DNA repair or the restoration of DNA replication. The DNA repair capacities of *N. gonorrhoeae* have not been well characterised, however, it is known that the gonococcus possesses an excision repair system (5). The fact that genes in this system are part of the SOS regulon in *E. coli* prompted this investigation into the regulation of genes involved in DNA repair in *N. gonorrhoeae*. Northern (RNA-DNA) dot blot hybridisation was used to investigate potential DNA damage-mediated induction of the gonococcal *recA, uvrA* and *uvrB* genes. In contrast to the situation in *E. coli*, transcription of these genes in *N. gonorrhoeae* was not induced in response to treatment with methyl methanesulphonate (MMS) or UV light. These data indicated that the gonococcus does not possess an SOS-like system that is induced in response to DNA damage. Furthermore, the lack of induction of the gonococcal *recA* gene during the heat shock response suggested that the *recA* gene is not regulated by thermal stress in *N. gonorrhoeae*.

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The gonococcal *rsp* gene appears to have evolved from a two component regulatory system that controls type IV piliation in another species.

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Upstream of the type IV pilin gene *pilE* of *Neisseria gonorrhoeae* is a consensus sigma-54 promoter and an associated upstream activator sequence (UAS) (1). Sigma-54 promoters require the alternative sigma factor, RpoN and an activator protein to function. The activator protein binds to the UAS, which is characteristically located approximately 100 bp upstream of the promoter (2). The type IV pilin gene, *pilA* of *Pseudomonas aeruginosa* is also preceded by a sigma-54 promoter (3). The activator of this gene has been identified as PilR which is part of a two component regulatory system with the sensor protein PilS (4,5). The potential UAS of the *pilE* upstream region was noted, based on its similarity to the sequence required for PilR binding (6), upstream of the *P. aeruginosa pilA* gene. The identified sigma-54 promoter is not involved in *pilE* transcription when gonococci are grown *in vitro* (7). However, it has been shown to be fully functional in *P. aeruginosa*. This transcription is dependent on the presence of the *P. aeruginosa* activator PilR, and the potential UAS of the *pilE* upstream region, implying PilR can bind to gonococcal sequences. On the basis of these results and the conservation of sequences similar to sigma-54 promoters and corresponding upstream sites, it was decided to search for *pilR* like sequences in *N. gonorrhoeae* MS11, with the intention of identifying a regulator of piliation in this bacteria. A fragment that hybridized to a probe derived from the *pilR* gene was cloned and sequenced, and a 1.7 kb open reading frame denoted *rsp* was identified. The derived amino acid sequence of Rsp is similar to regions of both PilS and pillar of *P. aeruginosa*. Homologues of *rsp* appear to be present in *N. meningitidis*, and non-pathogenic *Neisseria*. In each of these, this gene is located downstream of the previously characterised *parC* gene, encoding a topoisomerase subunit (8). Transcriptional analysis indicates *rsp* and *parC* are co-transcribed genes. Expression of *rsp* impairs the growth of piliated *P. aeruginosa* strains. Rsp expression does not result in activation, but rather partial repression of transcription from the gonococcal *pilE* and the *P. aeruginosa pilA* promoters.

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Identification and characterization of PilP, a lipoprotein essential for type IV pilus biogenesis in *Neisseria gonorrhoeae*

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Expression of type IV pili appears to play a critical role in the colonization of the human host by *Neisseria gonorrhoeae* (Gc) (1) and contributes to a number of distinct phenotypes, including competence for natural transformation (2), autoagglutination (3), and adherence to epithelial tissue (4). Previous studies have shown that PilQ, a member of the GspD/PulD/pIV protein family, forms a multimeric complex in the outer membrane, and is essential for pilus biogenesis (7). The function of the PilQ multimer is as yet undetermined, however it is speculated to form a channel through which pili are extruded, or perhaps act as a platform for extracellular assembly of pili.

DNA sequencing of the region upstream of *pilQ* revealed the presence of four open reading frames (ORFs) which display significant sequence homology and similar organization to the pilM-P gene cluster of *Pseudomonas aeruginosa* (8) and *P. syringae* (9). Furthermore, an ORF upstream of *pilM* was identified which showed homology to the penicillin binding protein, PonA. This finding was also consistent with the organization of Pseudomonas spp.

Gonococcal mutants bearing transposon insertions in *pilO* and *pilP* were non-piliated and failed to express pilus-associated phenotypes. As predicted by the presence of a consensus lipoprotein signal sequence, [3H]-palmitic acid labeled PilP was detected in both *E. coli* and Gc. The piliation defects in the mutants could not be ascribed to polarity on distal *pilQ* expression as shown by direct measurement of PilQ antigen in those backgrounds and the use of a novel technique to create tandem duplications in the Gc genome. Both the PilP- and PilQ- mutants shed PilC, a protein which facilitates pilus assembly and is implicated in epithelial cell adherence (10). Combined with the finding that levels of multimeric PilQ were greatly reduced in PilP- mutants, the results suggest that PilP is required for PilQ function and that PilQ and PilC may interact during the terminal stages of pilus biogenesis.

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Expression of capsular polysaccharide in *Neisseria meningitidis*: Comparison of biosynthetic and transport genetic loci responsible for serogroup A, B, C, Y and W-135 capsules

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Meningococci of serogroups A, B, C, Y, and W-135 are leading worldwide causes of meningitis and septicemia in otherwise healthy individuals. Dramatic increases in serogroup B and C meningococcal disease have occurred in parts of the United States between 1991-1995 (1,2), emphasizing the need for improved prevention strategies. One of the most important virulence determinants elaborated by the meningococcus is extracellular capsule. Capsules of serogroups B, C, Y and W-135 *N. meningitidis* are composed of polysialic acid or sialic acid linked to glucose or galactose, while serogroup A capsule contains N-acetyl-D-mannosamine. We have studied the biosynthetic and membrane transport gene clusters of Regions A and C of the meningococcal *cps* capsule gene complex (3). Region A encodes four biosynthetic genes (*synX,B,C,D*) and Region C encodes four genes (*ctrA,B,C,D*) necessary for membrane transport of capsule. Transcription of *synX-D* and *ctrA-D* appears to be divergently initiated from promoters present within a 134 bp intergenic region that separates Regions A and C. PCR and Southern DNA hybridization studies demonstrated DNA homology between the sialic acid capsule producing serogroups B, C, Y, and W-135 in *ctrA*, in the intergenic region and in *synX, B,* and *C*. However, nucleotide sequencing outward from the 3¢-end of *synC* demonstrated that the sequence downstream of *synC* was unique between sialic acid producing serogroups B, C and Y/W-135. Sequence diverged at the last codon of *synC* or directly following the *synC* ORF. In contrast, serogroup A *N. meningitidis* did not show homology with *synX-D* or the intergenic region, but did contain a *ctrA* homologue as defined by PCR and Southern analysis. These results show the close genetic relationship of biosynthesis and transport genes of the sialic acid capsule-producing meningococcal serogroups B, C, Y, and W-135, which appear to differ only at the capsular sialyltransferase. However, the capsule biosynthesis region of serogroup A meningococcal strains is clearly different.

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Integration host factor is required for efficient transcription of *pilE* in *Neisseria gonorrhoeae*

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Integration host factor (IHF) is a heterodimeric DNA binding protein found in gram- negative bacteria. IHF binds DNA in a sequence specific manner that usually results in bending of the target DNA molecule. IHF is pleiotropic with respect to function. Biological phenomena influenced by IHF include transcriptional regulation, plasmid partitioning, DNA replication, and genetic recombination (reviewed 1).

We purified IHF to homogeneity from *N. gonorrhoeae* using Fast Performance Liquid Chromatography (FPLC). Gel retardation assays demonstrated specific binding of gonococcal IHF to the *pilE* promoter region. The IHF binding site was defined by DNaseI footprint analysis and mapped proximal to the three previously identified *pilE* promoters; deletion of the putative IHF binding site negated retardation of *pilE* promoter DNA fragments. Binding of IHF to *pilE* promoter DNA was confirmed by Kleinschmidt Electron Microscopy, where binding of the protein induced bending of the promoter DNA fragment.

Isogenic *N. gonorrhoeae* strains were constructed that contained either a wild type *pilE* locus or a deleted *pilE* locus where the promoter IHF binding site was removed. Primer extension analysis of *pilE* and Northern blotting of total gonococcal RNA indicated that in the absence of the IHF binding site transcription was reduced approximately tenfold. A recombinant assay was developed whereby the *N. gonorrhoeae ihf* genes were expressed in *E. coli* in conjunction with *pilE* on a compatible plasmid. Again, efficient transcription required the presence of gonococcal IHF. However, a surprising finding was a translational defect in pilin expression in the absence of IHF.

Overall, these data indicate that efficient transcription of *pilE* requires binding of IHF to a site within the *pilE* promoter region. The mode of action of IHF appears to be the induction of a static bend in the promoter DNA that possibly allows the correct alignment of a transcriptional regulator with its cognate promoter.

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IS*1301*, a novel IS element of *Neisseria meningitidis*: Site-specific insertion and variable distribution in genetically related strains

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Reversible insertional inactivation of the *siaA* gene by transposition of IS*1301* leads to phase variation of capsular polysaccharide in serogroup B *Neisseria meningitidis* (1).
We determined the target site specificity of this element by cloning and sequencing the insertion sites of 12 identical IS*1301* copies found in *N. meningitidis* strain B1940 (2). A target consensus core of 5'-AYTAG-3' was identified, but the presence of additional features around the target sites may indicate that other factors like DNA secondary structure are involved in target recognition; these features include extended palindromic symmetry, stem-loop formation, and the high incidence of A/T-tracts. The left inverted repeat of an IS*1016*-like element common in *N. meningitidis* acts as a hot-spot for insertion and one IS combination was located upstream of the *frpC* gene. By sequence analysis we were able to place IS*1301* into the IS*5* subgroup within the IS*4* family of elements.

IS*1301* did not occur in *N. gonorrhoeae* or in apathogenic Neisseriae. 28.4% of 341 *N. meningitidis* strains from different global sources contained IS*1301*. It was found in serogroups A, B, C, E, W135, X, Y, and among nongroupable isolates. MEE (multilocus enzyme electrophoresis) data were available for 223 of the meningococcal strains. The collection included serogroup A strains from 5 subgroups and serogroup B and C isolates from clusters A4, the ET-5 complex, and the ET-37 complex, as well as other bacteria representative of the genetic diversity of these serogroups (3, 4).

Among serogroup A strains, only subgroup VI strains isolated in the German Democratic Republic and two new ETs contained IS*1301*. In cluster A4, all IS*1301*-containing strains were serogroup B. 27% of strains of the ET-37 complex, 14% of the A4 cluster and 7% of strains of the ET-5 complex possessed IS*1301*. IS*1301* was found in 22 of 32 strains of cluster A3 (69%), which contains many serogroup Y and X strains, and other bacteria from the whole spectrum of genetic diversity also contained IS*1301*. These results indicate, that IS*1301* was probably imported by horizontal genetic exchange after speciation of *N. meningitidis* and has been spreading by horizontal genetic exchange.

Southern blot hybridizations with 23 meningococcal strains revealed an average of 10 (range 2-18) copies of IS*1301* per strain. The band patterns differed among strains belonging to identical or related ETs indicating that transposition events are more frequent than changes in housekeeping enzymes. Four isolates from one healthy carrier of the same ET differed in both capsular polysaccharide and presence of IS*1301*. Three consecutive isolates were nongroupable and did not carry IS*1301*, while the fourth isolate carried the IS element and expressed a serogroup W capsular polysaccharide. However, in a few cases, closely related strains presented

a similar restriction pattern. These results suggest that testing for presence of IS*1301* and its restriction pattern might be useful for fine epidemiology of related carrier strains that are indistinguishable by other criteria.

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Promoter strength influences phase variation of Neisserial *opa* genes

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The *opa* multigene family of *Neisseria gonorrhoeae* MS11mk encodes eleven related outer-membrane proteins which phase vary *in vitro* and *in vivo*. Illegitimate recombination within direct pentameric DNA repeats, encoding the signal-peptide region of pre-Opas, leads to switches in expression states (1). Despite the conserved nature of the variation mechanism, expression of specific genes is favored in switching from an Opa- to an Opa$^+$ population in vitro and in vivo (2). The genes which are highly expressed differ from the rest of the family with respect to promoter structure, based on sequence comparisons between the *opa* genes of strain MS11mk (3).

We have analyzed transcription of the *opa* gene family of *N. gonorrhoeae* MS11mk, focussing on the different promoters found among the eleven genes to determine whether increased levels of expression are associated with increased phase variation rates. Primer extension and Northern blotting were used to assess the levels of transcription of three representative *opa* genes (*opa*A, B and C) in "on" and "off" states. Full length *opa* mRNA was detected primarily in strains expressing the homologous gene. Truncated *opa* mRNA was constitutively expressed from all *opa* genes regardless of their expression state. Quantitative comparisons of *opa* mRNA in *N. gonorrhoeae* were complicated by the simultaneous expression of all eleven genes and the cross-reactivity of mRNA probes.

Expression levels from the individual promoters were therefore assessed by creating transcriptional and translational *lac*Z fusions to each of the representative *opa* promoters, lacking the DNA repeats responsible for variation. The expression levels were compared to the phase variation rates of translational *opa::pho*A fusions containing the same promoters in addition to the corresponding coding repeat regions. A strong correlation was found between expression levels from the different promoters and the variation rates at which "on" variants appeared from an "off" population (i.e. *opa*A > *opa*B > *opa*C). These results provide an explanation for the favored expression of specific Opa proteins and indicate that expression of *opa* genes may be regulated at the level of transcription.

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Analysis of the recombination producing pilin antigenic variation using insertions in silent and expressed pilin loci

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Gonococcal pilus phase and antigenic variation support immune evasion (1,2) and alter receptor binding specificities (3,4). Antigenic and some phase variants are generated when variant DNA from one of several silent pilin genes replaces sequences in an expressed pilin gene (5,6). These RecA-dependent recombination events (7) are usually non-reciprocal. The original purpose of the study was to devise an assay to quantitate antigenic variation. To this end, we constructed a Gc strain derived from MS11 containing a promoterless *cat* gene ('*cat*) in the conserved *cys2* region of silent pilin copy3 of the *pilS*1 locus. Presence of an inducible *recA* allele, *recA6* (Seifert, in preparation) in the chloramphenicol sensitive (Cm^s) assay strain, BHA*cat1*, allowed control of when antigenic variation could and could not occur. The plan was to measure recombination of *'cat* into the expressed pilin gene (*pilE*), by selecting chloramphenicol resistant (Cm^r) recombinants after RecA induction.

Surprisingly, the simple recombination of '*cat* into *pilE* was not observed among Cm^r variants, thus BHAcat1 could not be used to quantitate the rate of antigenic variation. However, study of the Cm^r variants provided great insight into the mechanism of antigenic variation. Each Cm^r variant contained a new hybrid *pilE/pilS1::cat* locus while both starting loci, *pilS1::cat* and *pilE*, usually remained intact. Among the Cm^r variants, three classes of hybrid loci were defined based on the size and composition of each hybrid locus. Class I hybrid loci were created by recombination between *pilE* and *pilS1* copy3::*cat*. Class II hybrid loci were created by recombination between *pilE* and *pilS1* copy4, and class III hybrid loci by recombination between *pilE* and *pilS1* copy5. The presence of '*cat* in the *cys2* of copy3 during recombination between *pilE* and copy3::*cat* did not appear to affect the frequency or location of recombination producing class I hybrid loci compared to the other two classes. Further analysis of the hybrid loci revealed that they could be created directly through transformation or through intracellular recombination. Each class of hybrid locus was mapped and found to occur in the 10 kb region between *pilS1::cat* and *pilE*. This 10 kb region was duplicated during the formation of each hybrid locus such that each hybrid locus was flanked by 10 kb of directly repeated gonococcal DNA.

It was possible that the inability to detect recombination of '*cat* into *pilE* was due to the interruption of the *cys2* sequence since this sequence, conserved at the nucleotide level, may play a role in pilin copy recombination. To test whether an intact *cys2* sequence was required for recombination of '*cat* into *pilE*, strain BHA*cat2* was constructed by inserting '*cat* into *pilS1* copy3 at the hypervariable region (HV) which lies immediately upstream of *cys2*. Again, simple recombination of '*cat* into *pilE* was not detected. Instead, class II and class III hybrid loci were detected in Cm^r variants, but class I hybrid loci were never observed. This data suggested that at least a portion of *pilS cys2* must be available for interaction with *pilE cys2* in order for recombination between the two copies to occur. These two '*cat* studies demonstrated that the recombination producing hybrid loci was dependent upon pilin sequences and occurred regardless of whether '*cat* was present in, or absent from, the silent copy undergoing recombination with *pilE*. The data strongly suggest that the hybrid loci represented intermediates in the pathway that leads to antigenic variation. The first step, duplication and recombination between *pilE* and *pilS1*, produced a hybrid *pilE/pilS* copy which typically would resolve at an intact *pilE*. Failure of the hybrid intermediate to resolve at *pilE* allowed a low frequency integration of the hybrid intermediate, detected by selection. The duplication of the 10 kb region between *pilS1*::*cat* and *pilE* suggested that a circular intermediate was created during the formation of each *pilE/pilS1* hybrid locus.

Studies using the small 10 bp *Not*I linker in place of *'cat* revealed that both the size of heterologous sequence and its position in a *pilS* copy affect recombination of the heterologous sequence into *pilE*. Preliminary studies of *'cat* and *Not*I linker insertions in *pilE* showed that recombination of *pilS* sequence to replace a heterologous sequence in *pilE* behaves differently than recombination of heterologous sequence from *pilS* into *pilE*. Further studies using insertion in *pilS* and *pilE* will help to define the mechanisms of antigenic variation, the sequences required for antigenic variation, and the limitations imposed during recombination of heterologous sequences.

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Regulation of the prepilin-peptidase *pilD* gene of *Neisseria gonorrhoeae* and *meningitidis*

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The *pilD* gene of *Neisseria gonorrhoeae* encodes a type IV prepilin-peptidase (1, 2). This gene and the surrounding genes show strong homology with a family of genes implicated in pilus biogenesis in*Pseudomonas aeruginosa* and *Klebsiella oxitoca (3).* We analyse the regulation of this locus and investigate the hypothesis of a role of *pilA/pilB*, a pleiotropic regulatory system first characterized in the pilin gene regulation (4, 5). We show that a partially purified extract of PilA is able to retardate the migration of a DNA fragment overlapping the pilD promoter region of *Neisseria gonorrhoeae*. The size of the major transcript of the *pilD* gene corresponds to potential promoter with a consensus -12- 24 sequence precedently proposed (1)
We have constructed a *pilD-lacZ* fusion which was introduced in wild type, *pilA*, and *pilB* mutants strains of *Neisseria meningitidis*. The results obtained indicated *that pilA and pilB* are probably implicated in the regulation of this locus.

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Characterization of an IPTG-inducible *pilE* strain: relationships between *pilE* transcription, piliation and DNA transformation in *Neisseria gonorrhoeae*

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The gonococcal pilus, a member of the type IV family of pili, is important in both adhesion to eukaryotic cells and DNA transformation. Pili have been suggested to play an important role in the initiation of disease by providing the initial attachment of the bacterial cell to human mucosal tissue (1). The piliation state also correlates with efficiency of DNA transformation; piliated gonococci (Gc) transform at a rate several logs higher than non-piliated Gc (2). In addition to *pilE*, other genes involved with pilus assembly, namely *pilC* (3) and *pilT* (4), are required for full transformation competence. Biogenesis of type IV pili is thought to involve transport of pilin protein to the inner or outer membrane prior to assembly of pili (5). The relationship between assembly of gonococcal pili, piliation state, and DNA transformation efficiency is the focus of the present study.

We have constructed a derivative of MS11-C9 (Δ*pilE1*) in which the *lac IOP* regulatory sequences control *pilE* transcription. In this strain, levels of pilin mRNA and protein correlated directly with levels of IPTG in the growth medium, although wild-type levels of expression were never attained. Transmission electron micrographs demonstrated that the number of full-length pili per bacterial cell directly correlated with the induction level, and that at low levels of induction, single long pili were observed. This result suggests that a threshold level of pilin accumulates in localized pools prior to assembly of pilin monomer into pili. The transformation studies showed that transformation efficiency also directly correlated with the level of *pilE* transcription. However, the transformation efficiency plateaued at an intermediate level of pilin protein expression and never reached the wild-type level. These data support the hypothesis that assembly of pilin into pili is critical for efficient DNA transformation competence.

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Molecular characterization of the pyruvate dehydrogenase gene cluster of *Neisseria meningitidis.*

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The pyruvate dehydrogenase (PDH) complex is a multisubunit enzyme which catalyzes the oxidative decarboxylation of pyruvate, yielding acetyl-CoA and CO_2 as end products with the simultaneous reduction of NAD^+ (1). In *Escherichia coli* PDH is formed by the product of three clustered genes, *aceE* (pyruvate dehydrogenase), *aceF* (dihydrolipoyl transacetylase) and *lpd* (lipoamide dehydrogenase), whose sequence and transcriptional organization have been well studied (1).

Our group has previously characterized the neisserial *lpdA* gene, coding for a lipoamide dehydrogenase which has an N-terminal lipoyl-binding domain similar to those present in dihydrolipoyl transacetylases (2,3,4). During the screening and sequence analysis of a λgt11 meningococcal genomic library with a rabbit antiserum to an unrelated antigen of *N. meningitidis*, we identified a clone bearing 2 truncated open reading frames (ORF) equivalent by sequence homology to the C-terminal third and most of the bacterial *aceE* and *aceF* genes, respectively. Using a combination of restriction analysis, Southern blotting and sequencing of the λEMBL3 clone from which the *lpdA* gene was cloned, these ORF were shown to be linked to the *lpdA* gene in the order *aceE-aceF-lpdA*, forming a putative PDH gene cluster. In addition, a fourth ORF was located in the intergenic *aceF-lpdA* region, with no homologous counterparts in the available sequence databases. This ORF is most probably not translated, since it lacks a ribosome binding site and does not match the meningococcal codon usage pattern.

To further characterize this complex, meningococcal *aceE* and *aceF* insertional mutants were prepared and analyzed by Northern blotting concurrently with an *lpdA* deletion mutant, with the goal of dissecting its transcriptional organization. Our data suggest that the transcription of the whole gene cluster is driven from the same promoter upstream *aceE*, terminating either after *aceF* or *lpdA* and thus generating 2 mRNA species 5 and 7 kb long. This sharply contrasts with the situation in *E. coli*, where one promoter coordinately transcribes the 3 genes into one mRNA molecule and *lpd* is further transcribed from a separate promoter (5).

Such differences in the differential regulation of *aceE-aceF* vs. *lpd* probably reflect divergent biochemical requirements for Lpd in these two organisms, and may help shed light on the biological function of LpdA.

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Isolation of mutants deficient in pilin antigenic variation

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The gonococcal (Gc) pilus is subject to antigenic variation by unidirectional, *recA* dependent homologous recombination from silent to expressed pilin loci (1). Located at the 3' end of all Gc pilin loci is the *Sma/Cla* repeat, deletion of which results in decreased antigenic variation (2). Several distinct *Sma/Cla* binding activities have been observed (3). We hypothesize that antigenic variation depends on gene products involved in general recombination processes, such as RecA, as well as those involved in specific recombination processes, such as *Sma/Cla* DNA binding proteins.

We have adapted Shuttle Mutagenesis (4) to insert mini-transposons throughout the Gc genome in a random fashion. A size-restricted plasmid library of strain FA1090 was mutated with the erythromycin resistant mini-transposon mTnEGNS. Pools of randomly mutated plasmid DNA were used to transform FA1090 *recA*6. This strain contains *recA* under the control of an IPTG-inducible promoter, thus controlling the process of antigenic variation in response to IPTG. The resulting erythromycin resistant mutants were screened for the ability to undergo antigenic variation using a colony-based PCR assay. Mutants found to be antigenic variation deficient (AVD) by this PCR analysis have been classified on the ability to transform exogenous DNA and survive UV exposure. We are characterizing the roles of the mutated genes in general recombination, DNA repair, and pilin antigenic variation.

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Genetic analysis of the *ton*B, *exb*B, and *exb*D operon in *Neisseria gonorrhoeae*

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The acquisition of iron from transferrin (TF) and lactoferrin (LF) by *Neisseria gonorrhoeae* and *Neisseria meningitidis* requires the interaction of these glycoproteins with specific outer membrane (OM) receptors. The TF receptor of *N. gonorrhoeae* and *N. meningitidis* is comprised of two proteins, Tbp1 and Tbp2 (1). Sequence analysis of Tbp1 revealed homology with several TonB-dependent OM receptors including FepA, FecA, IutA, FhuA, and FhuE (1); a similar homology has been observed for the neisserial LF-binding protein LbpA (2). These results suggested that a TonB analog must exist in pathogenic *Neisseria* species. The acquisition of TF-bound iron was shown to be an energy-dependent process (3). The energy-coupled transport of iron complexes across the OM of many gram negative bacteria following their interaction with specific OM receptors has been shown to require a complex of proteins consisting of TonB, ExbB, and ExbD.

In order to identify the neisserial TonB, an *Escherichia coli* heme-requiring mutant expressing the meningococcal HmbR protein was screened with a *N. meningitidis* cosmid library (4). Several colonies were isolated that were able to utilize hemoglobin as both a porphyrin and iron source. Nucleotide sequence of one of the clones revealed homology with *exb*D. Oligonucleotides were synthesized and used to amplify the nearly identical gene from *N. gonorrhoeae* strain F62. Sequence analysis of the region upstream of *exb*D revealed the presence of sequences with homology to *exb*B and *ton*B. The putative gonococcal *exb*D sequence was found to encode for a protein of 145 amino acids; the DNA sequence exhibited 98% homology with that of *N. meningitidis* and 57% homology with the *E. coli* homologue. There is a 6bp intervening sequence between the start of *exb*D and the end of *exb*B. The putative gonococcal *exb*B sequence was found to encode for a protein of 221 amino acids; the DNA sequence exhibited 98% and 55% homology with the meningococcal and *E. coli* homologues, respectively. The putative gonococcal and meningococcal *ton*B sequences terminated 65bp upstream of *exb*B and exhibited considerable divergence from other reported *ton*B genes as well as from each other. The two proline-rich regions (Lys-Pro and Glu-Pro) commonly observed in other TonB proteins are reversed in the neisserial homologue. The organization of the gonococcal and meningococcal genes is unique in that they are arranged in an operon in the order of *ton*B, *exb*B, and *exb*D. In many other bacteria, *ton*B exists as a single transcriptional unit located at a distance from *exb*B and *exb*D. Or, as in *Pseudomonas putida* (5) and *Haemophilus influenzae* (6), *ton*B follows *exb*B and *exb*D.

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Primary structure of the *rpoB* gene of *Neisseria meningitidis*, coding for the beta subunit of RNA polymerase, which is organized within an operon and description of a new attenuator-like sequence.

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The *rpoB* gene codes for the second largest (beta) subunit of DNA directed RNA polymerase (EC 2.7.7.6). Rifampin (syn. rifampicin), an antibiotic drug, is known to act on the beta-subunit, resulting in the inhibition of elongation (1). Recently the mechanism conferring resistance against this drug could be demonstrated for *Neisseria meningitidis* (2, 3). A single point mutation in a specific region of the gene is responsible. As meningococci represent a bacterial species which is known to be of panmictic population structure, another mechanism for acquiring resistance against rifampin could be the horizontal transfer of at least parts of the *rpoB* gene, either inter- or intraspecific. To recognize those events the knowledge of the primary structure of the gene is of interest.

Here the nucleotide sequence and the deduced amino acid sequence for the *rpoB* gene from a rifampin sensitive reference strain (BNCV-strain) is presented together with information on the organization of the gene in an operon.

From a gene bank (kindly provided by E.C. Gotschlich, New York), constructed using DNA from the so called BNCV strain, four overlapping clones had been captured. A 624 bp PCR generated probe which was characterized extensively (4) was used to screen the library. Analysis of the four clones yielded sequence data about a ribosomal protein gene, *rpoB* and *rpoC*, which codes for the largest subunit of RNA polymerase. The nucleotide sequence of *rpoB* from the BNCV strain is 4170 bp in length (EMBL/GenBank/DDBJ Ac.No. Z54353), being with its 1389 codons somewhat longer than homologous genes from other bacterial species. Amplification of the *rpoB* gene from a set of meningococcal strains and from four further *Neisseria* species indicated that the length of the subunit is conserved throughout the genus. Analysis of the base composition revealed a G/C content of about 49.66 mol%. The percent identity (amino acids) to the homologous gene from *Escherichia coli* (SwissProt Ac.No. P00575) is about 62.37 %. The deduced amino acid sequence was used to construct a phylogenetic tree together with *rpoB* sequences from a set of gram negative and gram positive bacteria as well as common tobacco as outgroup. Clustering of the neisserial gene is within the gram negative bacteria, as expected.

Like in some species of *Enterobacteriaceae* (5) the *rpoB* gene from *N. meningitidis* seems to be located within an operon, consisting of four ribosomal protein genes, *rpoB* and *rpoC*. In *N. meningitidis*, this organization is strengthened by the lack of a promoter sequence or a ribosomal binding site for both the *rpoB* and *rpoC* gene. Further evidence for an operon structure but with differential expression of *rpoB* and *rpoC* in relation to the ribosomal genes gives a hypothetical attenuator like sequence.

Characterized by a large inverted repeat this hypothetical attenuator is able to build a stemloop. It is of completely different sequence than the attenuator described for *E. coli* (6). In *N. meningitidis* the following sequence is found (the inverted repeats are underlined):

TGTTTACATTTATTTGCTTAGTTTTTATCAAATCATTGCAAATAAATGTAAAACA.

This hypothetical attenuator is located in the intergenic region between the *rplL* gene, coding for one of the ribosomal proteins and *rpoB*, thus dividing the operon in two functional subunits. It could lead to a more quantitative expression of the four ribosomal proteins and to a lower expression of both polymerase subunits, *rpoB* and *rpoC*. A differential expression will allow to keep the amount of ap 20000 ribosomes vs ap. 6000 RNA polymerase molecules per cell (Bautz, pers. comm.).

Acknowledgment. This work was done with financial support from G.N.W. (Hygiene Institute, Heidelberg) and by generous help from Prof. E.C. Gotschlich (Rockefeller University, New York).

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Genetic transfer between Neisseriae: simulation in microcosm.

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The horizontal gene transfer between commensal and pathogenic Neisseriae has been proposed to be the mechanism by which these later species have acquired altered portions of the *penA* gene that encodes for the PBP2 (Penicillin-Binding-Protein 2) (1,2). These changes in the protein have resulted in a moderately penicillin resistant phenotype in the meningococci, that have been isolated in Spain during the last few years (3). As these isolates did not show an clonal origin (4), this hypothesis could become actual.

Little is known about the possibility of the gene transfer in the nature and about its simulation in the laboratory. Works on soil and water microcosms have been performed (5) but few ones have been done in simulated human environments. We have design a simple microcosm, formed by an agar layer and liquid medium, that mimics the upper human respiratory tract with a co-cultivation of naturally resistant and commensal strain, *Neisseria polysaccharea* with a MIC of penicillin G of 0.4 µg/ml and a sensitive strain of *N. meningitidis* (MIC=0.025 µg/ml).

In a first step of transformation, we obtained transformants with a MIC of 0.1 µg/ml in all stages of growth and a MIC of 0.3 µg/ml in a second step. There was an increase of the transformation efficiency in the exponential phase of growth respect to the lag and stationary ones. This increase was probably due to the cell autolysis that characterizes Neisseriae (6), and the release of the DNA to the medium, because no competence factors have been described in Gram-negative bacteria (7). Surprisingly, when purified DNA from donor strain was added to the medium in a saturating concentration to transform meningococcus, there was a lesser efficiency than when the two strains were co-cultivated.

When we compared the efficiencies between a static microcosm or one that is shaken, we found greater values in the first way of cultivation than in the second; perhaps the good conditions of a shaking culture inhibited the cell autolysis mentioned above.

The presence of DNase into the medium, simulating those secreted by the variety of microorganisms that colonizes the human throat, decreased the transfer of the DNA between both strains but did not inhibit it in its totality. On the other hand, when purified DNA was used in a normal culture, no transformants were obtained when DNase was added. We proposed that a direct contact cell to cell could explain these results and, although conjugation has been described as the mechanism that could occur more frequently in the environment because the DNA is protected against the action of free nucleases (8), transformation is then possible too. The DNA was transferred when an agar layer was present in the culture, protecting it from the action of the enzyme, simulating the possible interaction between the liquid and epithelial surfaces of the oropharynx, as was described in other natural environments, like soils or waters (8).

From these results, we could conclude that the origin of penicillin resistance in the meningococci possibly was the commensal Neisseriae that colonize the naso and oropharynx and, although in stress conditions and in presence of free nucleases released by other microorganisms, an efficient genetic transfer could be done.

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Identification and characterization of *pilU*, a gene whose product modifies pilus-associated phenotypes in *Neisseria gonorrhoeae*

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Expression of type IV fimbriae is correlated with the ability of *Neisseria gonorrhoeae* to colonize the human host (1). Fimbriae are also associated with other phenotypes, including twitching motility (2), competence for natural transformation (3), and autoagglutination (4). Several *N. gonorrhoeae* genes have been recently found that exhibit close similarity to other members of a family of putative nucleotide-binding proteins which are involved in protein secretion and assembly (5,6). Among those gene products, gonococcal PilF protein was most closely related to the pilus assembly protein PilB of *P. aeruginosa* while the product of the gonococcal *pilT* gene is most similar to the PilT protein of *P. aeruginosa* which is involved in pilus-associated twitching motility and sensitivity to phages (7,8). The nucleotide sequence analysis of the gonococcal *pilT* locus revealed the presence of a large open reading frame located 0.2 kb downstream of the *pilT* gene. This ORF encodes a 408-amino acid protein which shows 33% identity with the gonococcal PilT protein and 45% identity with PilU protein in *P. aeruginosa* (8). A 46 kDa protein corresponding to this ORF, was detected in both *E. coli* and *N. gonorrhoeae*, and the gene was denoted *pilU*. Gonococcal PilU$^-$ mutants did not autoagglutinate in a pilus-specific manner although electron-microscopic examination showed that the defect in autoagglutination was not due to a decrease in a pilus expression. Unlike PilT$^-$ mutants, the PilU$^-$ mutants were fully competent for DNA transformation as compared to the wild type strain. In contrast to what was observed in *P. aeruginosa* (8), gonococcal PilU$^-$ mutants were capable of twitching, indicating that PilU protein is not required for this type of motility in *N. gonorrhoeae*. Furthermore, binding to human epithelial cells was increased eight fold in PilU$^-$ mutants. This finding was in contrast to the studies in meningococci, showing that autoagglutination appeared to promote binding to epithelial cells (9). There appears to be no absolute correlation between autoagglutination and adherence in gonococci. The results show that PilU promotes pilus autoagglutination, but appears to have an inhibitory effect on pilus-mediated adherence to the epithelial cells.

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Genetic analysis of post translational modifications of meningococcal pilin.

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The pilin protein, PilE, is the major structural component of the meningococcal class I pilus. Natural variation in amino acid sequence of PilE may result in functional modifications, notably differential adhesion to human cell lines (1). Phosphate and sugar groups, which have long been supposed to be associated with the neisserial pilin protein (2), have recently been shown to be covalently attached to the pilins of some strains of *Neisseria meningitidis* and *Neisseria gonorrhoeae* (3,4,5). However, the presence and role of such post translational modifications is unclear and variations in these additional moieties may also affect pilus assembly and/or function. The PilE protein of *Neisseria meningitidis* C311 has been well characterised and is known to be a glycoprotein with multiple substitutions which include α-glycerophosphate attached to Ser_{93} (3,4). Mutants have been created in this strain to analyse the role of such modifications in pilus structure and function.

C311 pilin is post-translationally modified with the novel O-linked trisaccharide digalactosyl 2,4-diacetamido-2,4,6-trideoxhexose. Mutants defective in *galE* were constructed that contain an unaltered *pilE* sequence, but which lack the terminal di-gal moiety whilst retaining the unusual diacetamido sugar. Adhesion assays and electron microscopy showed no difference between GalE mutants and their parental strains, indicating that di-gal has no discernible effect on pilus assembly or adhesion *in vitro*. However, the effects of the di-gal modification *in vivo*, or on other unknown pilus functions, or the possible effects of the diacetamido-2,4,6-trideoxhexose alone cannot be ruled out by these studies.

Site-directed mutagenesis was used to remove sites of possible O-linked modifications targeting the highly conserved serine residues at positions 63, 69 and 93 of mature C311 pilin. Pilin genes were constructed that are identical to the parental *pilE* locus except for base substitutions at the relevant codons. These genes were introduced by transformation into the high adhesion variant C311 #16 to replace the parental *pilE* locus. The *pilE* genes of these transformants were amplified by PCR and sequenced to confirm that only the desired mutated *pilE* sequence was present. Stable meningococcal clones containing alterations only at positions 69 or 93 were easily isolated. However, meningococcal transformants containing alterations to codon Ser_{63} of *pilE* frequently reverted back to producing pilin containing the original amino acid at this position, with simultaneous possibly compensating alterations further downstream, particularly to Ser_{79}. This suggests that the Ser_{63} is critical to pilin function and/or assembly. Ser_{63} has been shown to be glycosylated with a disaccharide in gonococcal pilin (5). Our results suggest that this residue may be essential to the structural integrity and for functional properties of class I meningococcal pilin. Phenotypic studies on pilus structure and adhesive function with these mutant *pilE* loci will be described.

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Tetrameric repeats in *Neisseria*

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Short, repetitive DNA motifs are implicated in reversible on/off switching (phase-variation) of several genes in the pathogenic *Neisseria*. Examples include homopolymeric tracts in the promoters of *opc* (1) and *pilC* (2), as well as in the coding region of the glycosyl-transferases *lgtA* and *lgtC* (3, 4). Similarly, phase variation of Opa proteins is influenced by variation in number of the repeat unit 5'-CTCTT-3' within the promoter.

Recently, a locus has been described in Nm serogroup B strain MC58 containing four copies of the tetramer 5'-GCAA-3', (6) now designated *nmrep4* and we have recently demonstrated the presence of three further loci containing the motif 5'-$(GCAA)_n$-3' (7).

One of these loci, *nmrep2*, has been isolated from a λ library and sequence analysis has revealed the presence of the motif 5'-$(GCAA)_8$-3'. There is 59% similarity between the DNA sequences of *nmrep2* and *nmrep4*. In common with *nmrep4*, *nmrep2* has no obvious translational start site, but the predicted amino acid sequence downstream of the tetramers of *nmrep2* shows homology to the virulence determinants Icsa of *Shigella flexneri* and Aida-1 of *Escherichia coli*, with 48% and 46% similarity respectively.

We have examined a number of isolates of *N. meningitidis* for the presence of *nmrep2* and *nmrep4*. PCR amplification and direct sequencing indicated that *nmrep2* is conserved in 29/29 serogroup B strains and 20/20 strains of other serogroups. The majority (29/29 and 16/20 respectively) contain eight copies of the tetramer. Similarly, *nmrep4* appears to be widely conserved in these strains. However there is greater variation between strains of the number of repeat units at this locus (5'-$(GCAA)_1$-3' to 5'-$(GCAA)_{12}$-3'), and we were unable to amplify *nmrep2* from several of the serogroup B strains.

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Characterization of cell division gene homologues *fts*Z, *fts*E and *fts*X in *Neisseria gonorrhoeae* strain CH811

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We have identified cell division gene homologues *fts*Z, *fts*E and *fts*X in *Neisseria gonorrhoeae* strain CH811. In many bacteria, FtsZ plays a critical role in cell division and aggregates to form a circumferential ring on the inner surface of the cytoplasmic membrane in *Escherichia coli* (1). The gonococcal *fts*Z gene was amplified from the chromosome using a combination of PCR and inverse PCR strategies. The translated gonococcal *fts*Z sequence shares 40% to 50% similarity with FtsZ from various bacterial species. The GTP-binding motif common to all other FtsZ proteins (2) is conserved in the gonococcal FtsZ. In vitro transcription/translation of the gonococcal *fts*Z gene produced a protein of the expected size. The gonococcal FtsZ protein was identified by Western blot analysis of cell extracts of Neisseria species with a polyclonal antiserum to the *E. coli* FtsZ. The gonococcal *fts*Z gene, without its upstream region, was cloned downstream of an inducible lac promoter in pTAg (R&D Systems) and expression of the gonococcal *fts*Z in *E. coli* led to filamentation. In *E. coli*, *fts*E and *fts*X encode cell division proteins which may be constituents of the septalsome (3). The gonococcal *fts*X gene was originally isolated from a genomic library in pBluescript KS+ (Stratagene). A partial *fts*E gene was identified immediately upstream of *fts*X and the remainder of *fts*E was subsequently amplified from the chromosome by inverse PCR. The gonococcal *fts*E and *fts*X genes are linked and overlap by 4 base pairs. The translated *fts*E sequence shares approximately 68% similarity with FtsE from *E. coli* and also *Haemophilus influenzae*. As with its homologues, the gonococcal FtsE contains ATP-binding motifs similar to members of the ATP-Binding Cassette (ABC) family (4). The translated *fts*X sequence shares approximately 50% similarity with FtsX from *E. coli* and also *H. influenzae*. Protein sequence analysis of all known FtsX revealed that it is an integral membrane protein. In vitro transcription/translation experiments produced a protein of the expected size for FtsX. We have also ascertained that as in *E. coli* no promoter lies immediately upstream of *fts*X. The genes flanking *fts*E and *ftsX* are not the same as found in *E. coli* or *H. influenzae* indicating a different organization.

Insertion of cat cassettes into the *fts*Z and *fts*X genes of *N. gonorrhoeae* strain CH811 indicates that ftsZ may be an essential gene.

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Studies on FA1090 S-Pilin Variants

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Neisseria gonorrhoeae commonly undergoes both pilus phase and antigenic variation. Phase variation occurs via several recombination-dependent and recombination-independent mechanisms. Pilus antigenic variation occurs predominantly by non-reciprocal homologous recombination of silent-copy pilin DNA into the pilin expression locus (1,2). One form of pilin variant results from incorporation of sequence information which creates pilin protein monomers that are excreted as soluble protein, S-pilin (3). S-pilin is an altered form of pilin missing 39 amino acids from the amino terminus in addition to the seven amino acid leader peptide.

We have isolated several strain FA1090 variants based on their colony morphology to characterize S-pilin production. All variants contain *recA6(tetM-lacIOP-recA)* and are phenotypically RecA$^-$ until induced with IPTG. Western blot analysis showed both forms of pilin in all variants. Variants with a non-piliated colony morphology were PilC+ as determined by immunoblot analysis. Variants with a piliated colony morphology and visible pili by transmission electron microscopy had more full length pilin than S-pilin as detected by Western Blots. Although many of the piliated colony morphology variants had received semivariable and/or hypervariable sequences from the same silent copy, no direct correlation could be made between primary sequence changes in the semivariable and hypervariable regions of the *pilE* gene and the relative amounts of S-pilin and full length pilin produced. Our studies further substantiate previous studies which have shown that S-pilin variants express a few pili and also produce full length pilin (3). The finding that all variants produce detectable amounts of both S-pilin and full length pilin suggests that S-pilin variation is not a classical form of ON/OFF phase variation. Rather, it represents a spectrum of phenotypes between mostly piliated and mostly S-pilin producing. Rarely is a variant detected that is exclusively piliated or exclusively S-pilin producing.

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Identification of a second homologue of the lysophosphatidic acid acyltransferase in *Neisseria meningitidis* and implications for meningococcal membrane phospholipid biosynthesis

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Lysophosphatidic acid (LPA) acyltransferase is an enzyme intermediate involved in membrane phospholipid biosynthesis in a number of prokaryotes and eukaryotes (1). A meningococcal homologue of LPA acyltransferase (*nlaA* - *n*eisserial *L*PA *a*cyltransferase) has recently been characterized (2). This study reports the identification of a second meningococcal homologue of LPA acyltransferase and notes its potential role in meningococcal phospholipid biosynthesis.

A meningococcal mutant designated 469, derived by Tn*916* insertional mutagenesis, was isolated on the basis of its severely truncated LOS phenotype. Sequencing of chromosomal DNA flanking the transposon insertion in mutant 469 revealed an open reading frame (*orfB*) with predicted homology (27% identity, 45% similarity over a 239 amino acid region) to the PlsC LPA acyltransferase of *E. coli*. The nucleotide and predicted amino acid sequence of *nlaA* was distinct from *orfA*. Enzymatic assays revealed that mutant 469 exhibited 40-fold less LPA acyltransferase activity than the parental strain, in contrast to the *nlaA* mutant, which demonstrated a three-fold increase in LPA acyltransferase activity (2). Although LPA acyltransferase activity was severely reduced in mutant 469, it did not accumulate significant levels of LPA, as was seen in the *nlaA* mutant (2). These observations suggest that, in addition to *nlaA*, *orfB*, and/or other genes interrupted in mutant 469, are involved in meningococcal membrane phospholipid metabolism.

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Regulatory pathways of adhesion in pathogenic *Neisseriae*

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Adherence to eukaryotic cells is essential in the pathogenesis of *Neisseria meningitidis* (Mc) and *N. gonorrrhoeae* (Gc). Pilus-mediated adhesion has been shown to play an essential role in this process. Pilin (PilE) , the pilus major subunit, and two pilus associated proteins, PilC1 and PilC2, are key components in meningococcal adhesiveness. Phase and/or antigenic variation of these molecules are the only identified means by which the pathogenic *Neisseriae* modulates pilus-mediated adhesion.

We are presenting data indicating that a regulatory system coexist with genic variations. Our data suggest that *pilE* promoters are structurally distinct in Gc and Mc, thus being in agreement with different regulatory pathways between the two species.

In Mc, *pilC1* and *pilC2* loci have different roles and distinct regulatory regions (1, 2). These data are in good agreement with the different functions of these proteins in terms of adhesion. Only PilC1 in Mc is ivolved in adhesion (1). *pilC1* promoter has a specific fragment which encompasses a PilA binding domain, thus suggesting that this promoter is controlled by the regulatory protein PilA(2). The acquisition of this region confers to *pilC1* another level of regulation of gene expression compared to *pilC2*. This specific region is analyzed in different meningococcal isolates and its role is studied using site-directed mutagenesis. Moreover, this region seems to be present in other chromosomal loci indicating a common regulatory pathway. A central and pleiotropic regulatory system of genes could allow the pathogenic *Neisseriae* to adapt in a more coordinate and responsive manner to environmental changes.

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A repetitive sequence element cause polymorphism in the PilQ protein of *Neisseria meningitidis*

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Omp-mc was originally characterized as an antigenically conserved, abundant outer membrane protein of *Neisseria gonorrhoeae* (1, 2) and attracted attention as a potential vaccine component (1, 3). Gonococcal mutants expressing defective forms of the protein were found to be non-piliated (4). In line with observations found for related molecules in *Pseudomonas aeruginosa* (5), gonococcal Omp-mc was renamed PilQ (4). The C-terminus of PilQ contains polar residues with a strong probability of assuming an amphipathic b-sheet conformation. This region shares identity with similarly located domains found in members of a large protein family required for translocation of macromolecules across the outer membrane. Accumulating evidence suggests that the molecule functions in pilus biogenesis by serving as a gated channel or as a pore, composed of 10-12 monomers.

Based on its potential to serve as a critical pilus component and protective immunogen in *Neisseria meningitidis*, the gene was cloned and characterized from strain 44/76. Mutants expressing truncated forms of the PilQ protein were constructed and were all devoid of pili and pilus-related phenotypes. Sera from patients recovering from meningococcal disease reacted with the *N. meningitidis* PilQ protein by immunoblotting, whereas acute sera did not. This finding shows that PilQ is expressed *in vivo*.

Sequence analysis of the 5' portion of the *pilQ* gene of strain 44/76 showed the presence of 7 copies of a repetitive element with the motif (CCG)GCAAAACAACAG(GCTGCCGC). The repetitive sequences were located in an area of the gene encoding a structurally and functionally important part of the molecule. This region was studied in more detail in a collection of 52 *N. meningitidis* strains from various geographic origins, including different serogroups and the dominant clone-complexes associated with meningococcal disease. The strains were screened for polymorphism in the size of the PCR fragment of the 5’ region of the gene and 21 of these were selected for further analysis by DNA sequencing. Meningococcal strains were found to harbor from 4 to 7 copies of the repetitive element. No association between the number of copies and the serogroup, geographic origin or multilocus genotype of the strains was evident. Strains belonging to the clone ET-5 (6), for example, presented 5, 6 or 7 copies of the element. In most cases, variation in repeat number was also reflected in altered mobility of the molecule in SDS-PAGE.

Although intrastrain repeat variation in meningococcal PilQ has not been formally demonstrated, the variability within clones of the ET-5 complex strongly suggest that intrastrain variation does occur.

The consequences of changes in PilQ repeat number remain unclear and it is difficult to envision that significant changes in antigenicity ensue from the simple gain or loss of repeats. Given its role in pilus biogenesis, changes in PilQ may represent a means of fine tuning assembly to accommodate variability in pilin and PilC expression. In any event, the findings here expand the repertoire of mechanisms by which *N. meningitidis* generates plasticity in biologically important molecules.

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PCRDOP Amplification and Analysis of a 1.8Kb fragment of a potentially novel two-component regulatory system in *Neisseria meningitidis*

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Neisseria meningitidis normally colonises the nasopharynx but during systemic infection meningococci can colonise other sites such as the blood and CSF. The bacteria must be able to sense and adapt to environmental stimuli such as pH, osmotic tension, nutrient availability etc. which may increase survival potential in specific microenvironments encountered. Many of the identified mechanisms for sensing environmental changes involve two-component regulatory systems which may also be an important factor in virulence regulation. Deletion mutants in one such response regulator, *ompR*, has been shown to severely impair virulence of *S. typhi* and *S. typhimurium*. Comparisons of published amino acid sequence data from several global sensory and regulatory proteins revealed conserved regions between the different families. By constructing degenerate oligonucleotide primers to these regions of patch homology we have been able to amplify a 1.8 kb fragment from *N. meningitidis* chromosomal DNA. The PCR product was cloned into the pGEM vector and subsequently sequenced. Comparisons of known response regulator sequences with the sequence obtained showed an amino acid percentage homology range of 32.2% - 49.1% involving seventeen different response regulators and twenty six different organisms. The sensor region showed a range of 23.5% - 30.3% involving just six different sensor proteins from five organisms. The sequence analysis described implies that a potentially novel two-component operon has been amplified. The operon will be mutated and introduced into the wild type strain to produce defined isogenic mutants. These mutants will be used to define the role(s) of this potentially novel response operon in the survival and pathophysiology of *N. meningitidis.*

Transformation defects in genetically defined pilus mutants of *Neisseria gonorrhoeae*

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In *Neisseria gonorrhoeae*, chromosomal gene transfer appears to occur exclusively by transformation. Analysis of clinical isolates has revealed an exceptionally high rate of horizontal gene exchange at all loci tested (1). These results indicate that natural transformation is not just a laboratory phenomenon, but a significant mechanism for genetic exchange within the host.

Competence for natural transformation is tightly associated with the expression of type IV pili (2). Recently, a number of genes involved in pilus biogenesis have been identified (3,4,5). The products of these genes share identity with gene products involved in transformation in other Gram-negative and Gram-positive species. Conservation of transformation components among species suggests that a common machinery may be involved.

Pilus biogenesis mutants are deficient in natural transformation. The step at which these mutations block transformation has not been determined, but appears to be an early event in transformation, before DNA can be recovered from the periplasm (2). As such, the block appears to be at the level of uptake of DNA into a site inaccessible to DNase I. Other transformation deficient mutants have been described, but the step at which they are affected appears to be subsequent to outer membrane translocation (2,6).

Recovery of transforming DNA from gonococci as well as successful transformation requires a specific 10 base pair sequence to be carried by the transforming DNA (7). We have developed an assay using radiolabeled PCR products to distinguish between the initial pilus-mediated events of transformation, which consist of DNA binding and uptake across the outer membrane. The assay has revealed that the 10 base pair sequence is required for the earliest detectable step in transformation, DNA binding.

Specific non-piliated mutants are defective in DNA binding, although the precise association between pilus expression and binding remains unclear. Either the molecule(s) that engage DNA are physically associated with the pilus filament or alternatively, require pilus biogenesis to achieve their proper localization or functional state. Additionally, PilC and PilT mutants which express pili (2,8), are incapable of binding sequence specific DNA, suggesting that pilus expression is necessary, but not sufficient for DNA binding.

It has been shown that second site suppressor mutants which restore wild type levels of pili in a PilC$^-$ background are defective in DNA uptake (9). Our results show that the PilC mutation alone can account for this defect, and that the defect occurs specifically at the level of DNA binding. However, a defect in PilC expression does not seem to account for the findings in PilT mutants since they bind well to human epithelial cell lines (a correlate of functional PilC expression (10)). In addition to their DNA binding defect, PilT mutants display altered

expression of autoagglutination and twitching motility (also pilus associated phenotypes). This argues that the defect in PilT mutants is the result of a qualitative change in pilus filament structure or conformation.

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