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Russell James Shoberg

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Martha H. Mulks
Major professor

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NEISSERIA GONORRHOEAE IgA1 PROTEASE: CONSTRUCTION AND CHARACTERIZATION OF MUTANTS AND THE RESPONSE TO IRON LIMITATION

By

Russell James Shoberg

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ABSTRACT

NEISSERIA GONORRHOEAE IgA1 PROTEASE:
CONSTRUCTION AND CHARACTERIZATION OF
MUTANTS AND THE RESPONSE TO IRON LIMITATION

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The IgA1 proteases produced by *Neisseria gonorrhoeae* are theorized to be important virulence factors for the microbe. One of the objectives of this research was to address the question as to what other functions these enzymes might be involved in. The gene encoding IgA1 protease (*iga*2) was cloned from *N. gonorrhoeae* GCM 740 in *Escherichia coli*. Following characterization, the *iga*2 locus was mutated at two sites by site-specific deletion and disruption and *Iga*$^-$ variants of GCM 740 were constructed by transformation. These *Iga*$^-$ variants, GCM 740Δ4 and GCM 740Δ2, were characterized with regard to potential *Iga*-related phenotypes. Altered membrane protein profiles were consistently observed on stained SDS-PAGE gels. Through analysis of variants with restored *iga*2 alleles, an outer membrane protein of 56 kDa was observed to be expressed in an *Iga*$^+$-dependent fashion as well as a 34.5 kDa cytoplasmic membrane protein which behaved similarly in GCM 740Δ4. A second objective was examination of the IgA1 protease susceptibility of outer membrane proteins in the *Iga*$^-$ variants. Proteins susceptible to IgA1 protease hydrolysis were observed in the outer and cytoplasmic membranes of both of the *Iga*$^-$ variants and the *Iga*$^+$ parental strain. IgA1 protease-susceptible outer membrane proteins were demonstrated in *E. coli* and *Actinobacillus pleuropneumoniae* as well. The identities and

functions of the susceptible proteins are undefined. A third objective was the examination of the effects of ferric iron limitation on IgA1 protease activity. Growth curve experiments with GCM 740 in iron-limited and iron-replete media demonstrated an iron-associated effect as a two to six-fold increase in extracellular IgA1 protease activity was observed under iron-limited conditions. The mature enzyme was shown to be unaffected by the concentration of ferric iron in the media. Further, the increased protease activity was not an effect of the media on the mature enzyme. A potential, well-conserved Fur repressor-binding site was identified in the promoters of two *iga*2 genes. However, this potential regulatory region was not regulated by Fur in a known *E. coli Fur*- strain. Also, DNA hybridization analysis demonstrated the lack of a *fur* homologue in the *Neisseria gonorrhoeae* genome.

This work is dedicated to:

Judi- my life partner and best friend
None of this could (or would) have happened
without all of your efforts

my parents-
for making learning fun

and
Dr. A. E. Duwe
(1920-1991)
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Lastly, to my brothers Uracil and other Adenine- I guess it's time to shut out the lights and head on home, the party's over.

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INTRODUCTION

The nonmotile, gram negative bacterium, *Neisseria gonorrhoeae* (aka the gonococcus) was first described in 1879 by Neisser and was later demonstrated to be the causative agent of the sexually transmitted disease gonorrhea. The microbe shows very little diversity in the sources of energy that it can utilize. Glucose, pyruvate, and lactate are the only carbohydrates that the organism can use as its sole source of carbon, respiring primarily via the Entner-Doudoroff and pentose phosphate pathways (181).

The organism is an obligate human pathogen and has never been isolated from any other species. However, the chimpanzee has been used infrequently as an experimental model system and has been shown to manifest the clinical signs of an uncomplicated gonococcal infection (145) and the ability to transmit the disease venereally (30).

Gonorrhea. *N. gonorrhoeae* infection results in an average of 600-800,000 reported cases of gonorrhea in the United States every year (264). The disease is most often thought of in the context of a sexually transmitted disease affecting the genitourinary tracts of infected persons. This type of infection, known as an uncomplicated gonococcal infection (UGI), is the most frequent outcome of infection (89). The UGI, if diagnosed and treated with an appropriate course of antibiotic therapy is normally resolved without incident. However, there are more serious ramifications of the disease which although

rarely fatal, are certainly dangerous.

These complications are a result of bacterial dissemination via ascension of the reproductive tract or the vascular system. Ascension of the reproductive tract can result in pelvic inflammatory disease (PID) in females and a small array of secondary site infections in the male (89,162). Salpingitis, an inflammation of the fallopian tubes, is a serious complication which is currently thought to be a leading cause of infection-produced infertility in the United States (162). A disseminated gonococcal infection (DGI) can occur in both males and females after the gonococcus gains access to the circulatory system and spreads to remote regions of the body, considering the portal of entry (178).

A UGI in males is characterized by a viscous exudate containing numerous neutrophils which show intracellular gonococci (180). This exudate is stimulated by the colonization, damage, and invasion of the epithelial mucosa in the urethra (267). The invasion and resulting damage to the mucosa triggers infiltration of neutrophils and plasma leakage leading to the clinical signs of inflammation, pain, and swelling (180). Approximately 75% of infected males exhibit these clinical signs with the remainder considered asymptomatic carriers (89). Microscopic examination of the exudate and demonstration of gram negative, intracellular diplococci is considered to be the hallmark of gonococcal infection of the male urethra (89).

Untreated urethritis in males may also result in infection of the periurethral glands, prostate, seminal vesicles, and epididymis (89,267). Although not as potentially dangerous as a DGI, these sequelae have the potential to cause a urethral stricture and result in

sterility in the male (89).

In the female, a UGI typically results in endocervicitis, a slightly different outcome from that in the male. The vaginal mucosal epithelium, unlike the epithelia of the cervix and uterus, is not permissive for attachment and invasion by the gonococcus (267). While there is an inflammatory reaction to the bacteria, the clinical signs may be very slight (89). This is manifested by an alarmingly high percentage of females who carry an asymptomatic infection (30-70%; 180) and are only detected by diagnosis of sexual contacts, or through secondary complications of the disease.

A UGI may also occur at body sites other than the urogenital tract. Conjunctivitis, a rare occurrence in adults, can be acquired either by neonates during passage of the birth canal (one reason for the administration of topical antibiotics in the form of eye drops after birth) or as a common type of laboratory acquired infection due to formation of aerosols. Either (or both) rectal and pharyngeal infections may be commonly encountered in persons who are simultaneously diagnosed with a urogenital infection, with these secondary sites commonly being asymptomatic (>90%; 89).

Pelvic inflammatory disease may arise from about 10-15% of UGI cases and is considered to be due to bacterial ascent through the endometrial cavity by either 1) migration of bacteria in phagocytes; 2) by adherence to spermatazoa (25); or 3) by diffusion in the lumenal fluid (162). As the name indicates, there is a strong inflammatory reaction to the bacteria, resulting in a febrile state, intense pain, and nausea. This sequel to the undiagnosed or untreated UGI requires hospitalization and aggressive antibiotic therapy to prevent the spread of the organism

throughout the body in the form of a DGI.

Salpingitis is a more precisely defined syndrome of PID which results in a severe inflammatory response in the fallopian tubes. The swelling and damage that occurs frequently causes permanent disruption of the fallopian tube, with a final result of infertility or an increased risk of ectopic pregnancy (162). As with PID, salpingitis necessitates hospitalization and aggressive antibiotic therapy for successful resolution and prevention of a DGI. However, even if the course of the infection is halted, there may still have been severe and irreparable harm done to the reproductive tract.

Probably the most severe and certainly, the most difficult syndrome to resolve, is the systemic DGI, occurring in ~1% of males and up to ~3% of females (267). Once the gonococcus has gained entry to the vascular system, it is rapidly transported to other sites of the body showing a tropism for the axial limbs (178). This tropism is postulated to be due to its optimal growth temperature of ~36°C or slightly below human core temperature (183). The presence of these bacteria in circulation results in a strong febrile response which can progress to shock if not treated.

Most commonly, DGI shows a clinical sign of erythematous skin lesions having hemorrhagic and/or necrotic centers (178). These skin lesions often contain culturable gonococci (~50-70% of cases; 180). Also commonly observed is tenosynovitis, an inflammation of the tendon sheaths, particularly of the hands and feet (178). Other clinical signs include polyarthralgias or purulent arthritis, especially of the knees, elbows, ankles, and hands. This interesting pattern of affected joints also indicates the tropism of the gonococcus for slightly cooler regions

of the body. The affected joints frequently may contain viable bacteria in the synovial fluid, as well as an increased number of neutrophils and immune complexes (178). The clinical signs of a DGI can be mitigated by parenteral antibiotic therapy with the infection being eradicated, and the risk of permanent damage to the arthritic joints being minimal (178). There is a hypothesis that some of the arthritic process is due to the cell wall component, peptidoglycan, and this form of arthritis is not directly resolvable by antibiotic therapy (68).

Other unusual, but observed, manifestations of a DGI include endocarditis, hepatitis and perihepatitis, gonococcal meningitis, pneumonia, and osteomyelitis (178).

Persons having a complement deficiency of C6, C7, or C8 have been shown to be at a greater risk of acquiring a DGI (207). Characteristics which have been correlated with the *N. gonorrhoeae* strains which cause DGIs are expression of the PorA serotype (38), resistance to normal human serum killing (243), high susceptibility to penicillin (179), the arginine-hypoxanthine-uracil requiring auxotype (127), and production of a type 1 IgA1 protease (190).

Experimental model systems. A majority of information regarding the pathogenesis of *N. gonorrhoeae* has come from descriptions of the courses of disease in patients. The only whole animal models for the disease which simulate the course of gonorrhea and allow for sexual transmission of the disease are human volunteers and chimpanzees (145). Because of the ethical and/or logistical restrictions in the ability to do informative studies on virulence, a number of experimental systems (mainly heterologous) have been developed for studying gonococcal pathogenesis.

There are two main types of homologous systems, those which use either isolated human cells or tissues. A number of laboratories utilize freshly prepared human cells to study the interactions of the gonococcus with professional phagocytes *in vitro* (226). Similarly, a number of laboratories have utilized transformed human cell culture lines to study the separable events of gonococcal adherence and invasion of human cells (260). Experimental systems have been developed using HEC-1-B, HeLa, Chang conjunctival, and HEp-2 transformed cell lines (251,260). These approaches have provided important information with regard to the host-pathogen interactions at a cellular level, but both types of approach suffer the lack of an immune system and the lack of other microbial flora as would occur in a whole animal model.

An approach which takes the cellular system one step further, is the human fallopian tube organ culture system (FTOC) using explants of normal human tissue as a target for gonococcal adherence and invasion (260). It was the use of this model which provided data implicating: 1) the nonciliated columnar epithelial cells of the mucosa as the target for gonococcal adherence (100), and 2) the cytotoxic effects of lipopolysaccharide and peptidoglycan for mucosal tissues (48). This model does allow for some host immune function as the tissue presumably contains plasma cells, T cells, and macrophages (46). However, the system lacks important portions of the immune response such as the ability to have infiltration of plasma components and the inflammatory response which play a role in the progress of the disease. Experiments performed using chimpanzee FTOC have demonstrated that this tissue is also susceptible to adherence, cytotoxic damage, and invasion by *N. gonorrhoeae* while those tissues of the baboon are not (163).

Animal model systems have been developed and used by various laboratories to address particular phenomena of interest (4). Examples include the mouse intraperitoneal and rabbit intraocular models (176,177); the subcutaneous chamber system applied in guinea pigs, hamsters, and mice to assess microbial ability to scavenge iron or the roles of lipopolysaccharide in the pathogenic process (3); inoculation of uterine strictures or transcervical inoculation in mice in an attempt to simulate urogenital infection (282); the rat model of gonococcal arthrogenesis (68); two different rabbit models used to test the effects of gonococcemia and the somnogenic properties of gonococcal peptidoglycan fragments (238); and the chicken embryo system used to assess relative virulence of colony type variants or strains (32). While these models have all been used to test particular facets of virulence, all fall short of being a *bona fide* model system because *N. gonorrhoeae*: 1) does not establish itself and grow for any length of time, and 2) does not simulate the course of a gonococcal infection (*i. e.* differs in the mode and site of bacterial entry and in the subsequent events to cause the particular phenomenon being tested).

Genetics. *N. gonorrhoeae* possesses a circular chromosome, typical of most bacteria. Estimates have placed its complexity at about 2.1-2.2 Mbp (55). The 51% G+C content is similar to that of *E. coli* (22). No bacteriophages have been described that can infect *N. gonorrhoeae* (39). Progress has been made in attempts to generate both physical and genetic maps of the genome which will allow for evolutionary comparisons to be made (288).

The gonococcus has been shown to possess up to three different types of plasmids in a strain variable manner. Most strains contain either of

the two ~36 kbp conjugative plasmids (24.5 or 25.2 MDa varieties), which have both been implicated in interstrain and interspecies transfer of plasmid DNAs (73,233). The 24.5 MDa plasmid has been shown to mobilize other plasmids to *E. coli* and other species of *Neisseria*, while not mobilizing itself (231). In contrast, the 25.2 MDa plasmid can mobilize both heterologous plasmids as well as itself to other strains and species (233). The two plasmids show extensive DNA homology (>60%) and it has been hypothesized that the 25.2 MDa plasmid arose from the 24.5 MDa plamsid via an insertion event (described below; 231). Prevalence of conjugative plasmid carriage shows both geographic and temporal variation (234).

About 96% of clinical strains also contain an ~4.2 kbp (2.6 MDa) cryptic plasmid (234). Although the complete nucleotide sequence of the cryptic plasmid has been identified (and therefore its open reading frames), no defined functions have been ascribed to any of the ten proposed gene products (133). Interestingly, this plasmid has never been identified in proline-arginine-uracil requiring strains of the gonococcus (57). However, neither the presence or absence of the cryptic plasmid has been correlated with virulence in *N. gonorrhoeae* (133).

The third type of plasmid demonstrated in the gonococcus is the R-factor, with two examples. Gonococci can possess one of five different β-lactamase encoding plasmids, named for the geographic regions from which they were first isolated. Examples are the 4.4 MDa Asian, the 4.0 MDa Nîmes, the 3.2 MDa African, the 3.05 MDa Toronto, and the 2.9 MDa Rio plasmids (58). The Rio and Toronto plasmids are thought to have arisen from the Asian plasmid by deletion, and are not as common as

either of the parental Asian or African plasmids (58). Similarly, the Nîmes plasmid is thought to be an insertion derivative of the African plasmid (82). These β-lactamase plasmids can be mobilized in an interstrain and even in an interspecies fashion, by both of the conjugative plasmids (73).

The second type of R-factor plasmid isolated from *N. gonorrhoeae* specifies tetracycline resistance and is carried on the 25.2 MDa conjugative plasmid (128). The *tetM* gene is considered to be of streptococcal origin and it is presumed to have inserted into the 24.5 MDa plasmid by transposition (231). This marker has been shown to be carried both independently of and in conjunction with a β-lactamase encoding plasmid (128).

Genetic exchange. With regard to gene transfer in *N. gonorrhoeae*, both transformation and conjugation have been shown to occur (39). The gonococcus is naturally competent for DNA uptake throughout its growth cycle (12). Seemingly, the only criteria for this competence are the production of pili, or certain pilin subunits (14); divalent cations; and an energy source (12). Currently, it is not understood what the role of pili are in the transformation process. A ten base pair oligonucleotide uptake sequence (5'-GCCGTCTGAA-3') has been reported to be necessary for high efficiency uptake of DNA by the gonococcus (79). This sequence has been demonstrated to bind specifically to several DNA binding proteins found in the outer membrane and on blebs (59) and is frequently observed near the ends of some *N. gonorrhoeae* genes (79,88,159). These data suggest that the uptake sequence may play an important role in the high frequency and specific uptake of gonococcal DNAs via transformation.

After plasmid DNA is taken up by a transforming gonococcus, it is randomly cleaved into small fragments by a nonspecific endonuclease, and 1) religated to form deleted plasmids (11) or 2) recombined with homologous DNA (13). The latter situation is observed more frequently and seems to require the RecA function (130). These random cleavage events are thought not to be the result of a specific restriction endonuclease (14). However, this mechanism of gene transfer has also been shown to be dependent upon the donor's and the host's restriction-modification systems (259).

Conjugation requires the presence of either the 24.5 or 25.2 MDa conjugative plasmids, but does not require pili (65). Cell to cell contact is required for conjugation and certain of the Opas have been correlated with decreased conjugative efficiencies (10). Aside from pilus independence, conjugation in the gonococcus also differs from that in *E. coli* in that there is no F'-like system. In *N. gonorrhoeae*, a conjugative event results in only the transfer of plasmids and not chromosomal DNA (14). Conjugation allows for the transfer and faithful maintenance of plasmids, which differs from transformation in the gonococcus, apparently in conjugation's independence of the recipient's restriction-modification system (259).

This relatively low degree of genomic variability suggests that *N. gonorrhoeae* possesses a very efficient, or selective host restriction system. In fact, gonococci have been shown to produce at least seven different restriction-modification systems (50). In four of these systems, both the restriction endonuclease and its corresponding methylase have been cloned (50). So far, all of these are isoschizomers of enzymes characterized in other species.

Many gonococcal strains methylate the following sequence 5'-GATC-3' (199), but a corresponding methylase-dependent restriction endonuclease has not been described, suggestive of the *E. coli dam* methylase system. However, *Dam*$^-$ gonococci are not hypermutable (as *Dam*$^-$ *E. coli* are; 199) and there is some evidence of a gonococcal restriction enzyme that cleaves the 5'-G^{Me}ATC-3' sequence in a strain which does not produce the methylase (63). Therefore, the functional role of this putative *N. gonorrhoeae*-Dam methylase is unclear at present.

Genetic regulation. *N. gonorrhoeae* is known for undergoing extensive genetic regulation, exemplified by phase and antigenic variation of some of its surface antigens. Initially, these were described as colonial morphotype and color changes that occurred as the bacteria were serially passaged *in vitro*, leading to the T1-4 colony type/relative virulence classification scheme described by Kellogg and coworkers (113).

Further characterization of these gross differences in colonial morphotype defined the changes as correlating with variable expression of pili (99) and/or outer membrane proteins referred to as opacity proteins (Opas; 266). In summary, T1 colonial types are lightly piliated, intermediate to opaque colonies (*eg.* expressing Opas) and have been shown to be highly virulent in trial infection studies (21,113). The T2 colony type is more heavily piliated and opaque, and is also virulent (113). The T3 and T4 colonies are considered to be nonvirulent and are both nonpiliated, with expression and nonexpression of Opas, respectively (111,267). The regulation of each of these colonial morphotype determinants will be discussed in greater detail in the particular determinant's section to follow.

Other evidence of genetic regulation in *N. gonorrhoeae* comes from

the environmental stimulus-dependent expression or repression of described outer membrane proteins. There are examples of outer membrane proteins or structures which are induced by deprivation of iron, oxygen, and cysteine (42) and heat shock (125). There are also examples of outer membrane proteins which are repressed during oxygen limitation (44). More detail on each of these regulatory phenomena will be provided in the specific sections to follow.

Much of the genetic knowledge that exists regarding *N. gonorrhoeae* is a result of studies done on particular virulence functions. Prime examples include the phase variable phenomena of pilus and opacity protein expression. The study of gonococcal virulence functions has unveiled different phenomena and has resulted in a wealth of information relevant not only to the pathogenic processes involved in gonorrhea, but to pathogenic processes and gene regulation in general.

Virulence functions. The following discussion of the virulence functions of *N. gonorrhoeae*, will address 1) molecules or structures involved in adherence to the host cell, examples being pili and Opas; 2) important structures and antigens implicated in virulence other than as adhesins, examples being Por, Rmp, Lip, lipopolysaccharide (LPS), and peptidoglycan; and 3) functions or attributes involved in pathogenesis, examples being serum resistance, antibiotic resistances, invasion of host cells, and proteases.

Pili. Probably the single most studied component of the gonococcal cell surface has been the pilus. Pili have been shown to be essential for virulence, probably functioning as the primary bacterial adhesin for the host cell (90). Nonpiliated gonococci have been shown to be less virulent than piliated variants of the same strain (21,111), and have

been shown to be important mediators of bacterium-host cell interactions *in vitro* as well (90,283). Additionally, pili are one of the entities responsible for differentiation of gonococcal colonial morphotypes (99; described in the preceding text). The pilus, or the expression of pilin, is also correlated with the ability of *N. gonorrhoeae* to be transformed, but the role of pili in the transformation process is currently undefined (14). Due principally to its role in adherence to the host epithelium, the pilus is considered to be an essential virulence function.

The pilus is composed of polymerized 17-22 kDa pilin molecules and some minor pilus associated accessory proteins (186,236). Pilin production (and hence expression of pili) is a phase variable phenomenon. Phase variable expression has been shown to be due to 1) insertion of a promoterless pilin gene cassette from one of several of the *pilS* (silent or nonexpressing) loci to the *pilE* (expression) locus in an out-of-frame orientation (132) or 2) due to deletions in *pilE*, the expression locus (8,247).

Pili are highly immunogenic *in vivo* (33) and this would suggest that they might be an important vaccine candidate. However, the antigenic type of pilin produced is variable, and this variation has been observed to occur *in vivo* as well (271). A pilus preparation subunit vaccine was tested in a trial and it was found that the vaccinees were not protected against challenge with heterologous pilin producing gonococci, and were only marginally protected against challenge with the homologous pilin producing bacteria (278).

The pilin molecule is comprised of three domains based on comparisons of interstrain antigenic homology: 1) the amino terminal C

region (conserved), the first ~53 amino acids which are highly conserved; 2) the SV region (semivariable), from amino acid 54-114; and 3) the HV region (hypervariable), from amino acid 115-160 (90). Antigenic variability results from the insertion of one of several different pilin gene minicassettes from one of the many *pilS* loci, in a faithful (*i. e.* in frame) orientation behind the *pilE* promoter (171). Recombination is proposed to occur via both intragenomic reciprocal recombination, and during stationary phase by transformation with genomic DNA from lysed cells (171).

The *pilE* locus has also been reported to be regulated in *trans* by the gene products of the *pilA* and *pilB* loci (274). These putative regulatory loci are located ~3-5 kilobase pairs downstream of *pilE* and have amino acid sequence homology with some members of the bacterial two-component regulatory systems (273). PilA reportedly functions as a transcriptional activator and *pilB* serves to down-regulate *pilE* expression by an undefined mechanism (273). It is interesting that *N. gonorrhoeae* has evolved such elegant regulatory mechanisms for the expression of pili. This would suggest that the ability to modulate pilus expression is a vital function for the bacterium in the pathogenic process.

There has been a report describing a 23 kDa gonococcal surface protein which participated in pilus-independent, *in vitro* binding to glycolipids extracted from human cervical epithelial cells (51). Pilus-negative gonococci bound to the glycolipids, but antibodies raised against a pilus preparation immunoprecipitated the 23 kDa putative adhesin, suggesting that it may be a part of the pilus structure (51). This type of pilus-associated adhesin has been described in *E. coli* as

well as being proposed for *N. gonorrhoeae* (146).

Opas. A major proteinaceous component of the gonococcal outer membrane which has also been the subject of intense study is the set of 25-30 kDa proteins referred to as Opas (for opacity proteins; 94) and previously called PIIs (268). Opas exhibit heat-modifiable behavior on SDS-PAGE analysis, manifested as an increase in apparent molecular weight after denaturation at 100°C relative to that of Opas denatured at lower temperatures (161). The term opacity proteins refers to the correlation between the color or opacity of an agar colony and the expression of these surface exposed, phase variable proteins (266). Antigenic variation of these highly immunogenic proteins has been shown to occur, both *in vivo* and *in vitro* (246,262).

Some Opas have been shown to be involved in adherence to eukaryotic epithelial cells (136) and to cohort gonococci (266). Some Opas have also been reported to stimulate association of gonococci with human neutrophils and therefore were once given the name "leukocyte association proteins" (272). Opas can also be the target of serum bactericidal antibody (16). For several or all of these reasons, Opas are considered to be important virulence factors of the gonococcus.

The field of study on Opas grew rapidly due to the problems of phase and antigenic variability. The gene family (thought to be twelve; 171) coding for Opas are all complete genes which are constitutively transcribed (261). Therefore, phase and antigenic variable expression occurs at the level of translation by a mechanism apparently novel to the gonococcus. There is a five nucleotide pair coding repeat (5'-CTCTT-3'), present in the early translated region of the gene which, depending upon the number of repeats present, gives rise to either an

in-frame or out-of-frame gene (261). Therefore, it is the permissive translation (*i. e.* production of a full-length Opa polypeptide) of *opa* genes that is responsible for the "ON" phase and depending upon which *opa* gene(s) is/are "ON", dictates the antigenic type(s) of Opa expressed on the surface of the cell. A gonococcus can express zero to several different Opas simultaneously (246). Variation in the number of coding repeats has been 1) suggested to occur by DNA slippage during replication, 2) shown to be independent of *recA*, and 3) shown to occur in *E. coli* suggesting that specialized gonococcal functions are not necessary for the event to occur (171).

Por. The predominant gonococcal outer membrane protein is the porin protein (Por, formerly called PI; 94,268). Por has been implicated in attachment to host cells *in vitro* (260) and is also the basis of a serotyping scheme used in characterizing strains for epidemiological research (275). A given Por has an apparent molecular weight of ~31-35 kDa by SDS-PAGE, which is not modified by either heat or reduction (268).

There are two mutually exclusive serotypes, PorA and PorB, which branch out further including 24 serovars of PorA and 32 serovars of PorB (240). A given strain can express a Por of only one serovar, unlike the case of Opas, and Por proteins are neither phase nor antigenically variable within an isolate (41). Por serovar expression has been associated with serum resistance, antibiotic resistances, nutritional auxotype, IgA1 protease type, and disseminated infections (106).

The Por protein is the primary constituent of the nonspecific, hydrophilic pore in the gonococcal outer membrane, occurring in a trimeric arrangement (160). There are no described porin proteins in

the gonococcus other than PorA and PorB, and a constitutively *Por*$^-$ gonococcal variant has never been described (40,41). Pors have been shown to interact with Rmp (198) and LPS (93), two other components of the gonococcal cell surface, and peptidoglycan (106), a component of the cell wall. PorA and PorB are oriented differently in the outer membrane as demonstrated by the resistance of PorA to *in situ* cleavage by trypsin and α-chymotrypsin while PorB is susceptible (7).

With regard to its function(s) in virulence, Por can be the target for both serum bactericidal and opsonic antibodies (103,240). However, it is less immunogenic *in vivo* than either pilin or Opas (28). Purified Por has been demonstrated to insert into liposomes, and to fuse liposomes with the plasma membranes of eukaryotic cells and hence may play a role in attachment to and/or invasion of host cells (147,260). This proposed function in invasion is also supported by data demonstrating that anti-PorA and anti-PorB monoclonal antibodies block gonococcal invasion in a monolayer cell culture model system (91).

Rmp. Another major component of the gonococcal outer membrane and the hydrophilic pore is known as Rmp (aka PIII; 20,94). The acronym Rmp, refers to its reduction modifiable behavior on SDS-PAGE (161). When analyzed by SDS-PAGE, reduced-Rmp exhibits an increased apparent molecular weight relative to its non-reduced form. Rmp is considered to be structurally and antigenically conserved among gonococcal strains (105,270), has been cloned in *E. coli* in a faithful manner (*i. e.* it is expressed on the bacterial surface and is immunoreactive; 80), and has also been purified to homogeneity (148).

In the hydrophilic pore structure, Rmp is closely, but noncovalently, associated with Por (20). Crosslinking experiments have

indicated that the pore exists with a 3Por:1Rmp stoichiometry (198). However, this arrangement is not a requisite for *in vitro* growth as *Rmp*$^-$ variants have been constructed (20). At this time the function(s) of Rmp is/are still undescribed.

The antigenic stability and surface exposure of Rmp once suggested that it would make an attractive candidate for a gonococcal vaccine. However, Rmp is only weakly immunogenic *in vivo*, relative to pilin and Opas (137). Also, Rmp is antigenically related to the *E. coli* OmpA protein (a bacteriophage receptor; 18), and anti-OmpA antibodies have been demonstrated to inhibit complement activation in bactericidal assays using *N. gonorrhoeae* (228). By virtue of this property, it is considered to be an important virulence factor for the gonococcus.

Laz and Lip. A minor constituent of the gonococcal outer membrane is now referred to as Lip (for lipoprotein; 94). Lip was originally defined as the H.8 antigen due to its reactivity with a particular monoclonal antibody (37). However, it has since been shown that there are at least two other conserved outer membrane proteins which react with the H.8 antibody (*eg.* Laz; 80).

Both Laz and Lip are lipoproteins (263,292). Lip has been suggested to be associated with both the outer membrane and peptidoglycan (36). Laz has been proposed to function in electron transport but this has yet to be conclusively shown (36,81). Laz is also observed in many of the commensal *Neisseria* species and therefore is not considered to be important in virulence (36). Its mention here is due to the former confusion with Lip, which has been suggested to be involved in virulence (36).

Both of these proteins have been cloned in *E. coli* (15,81). Their

corresponding *N. gonorrhoeae* mutants have been constructed and it appears that the functions of neither Laz nor Lip are required for viability, at least *in vitro* (36). The primary structures of both proteins show a repetitive sequence of AAEAP, found frequently in the N-terminus of the 17 kDa Laz (81) and comprising the entire 6-7 kDa Lip protein (108). One cloned Lip has a predicted molecular mass of 6.3 kDa, yet it migrates with an apparent SDS-PAGE molecular weight of ~20 kDa, the difference potentially being: 1) a function of the post-translationally added lipid moiety, 2) the unusual amino acid composition, or 3) its repetetive structure (36).

Lip is known to vary in molecular mass from strain to strain (from 18-30 kDa) but is invariant within a strain. Expression of Lip has been demonstrated in all isolates of *N. gonorrhoeae* and *N. meningitidis* examined thus far (37). While Lip is immunogenic in patients with gonorrhea and meningococcal infections (28), there is conflicting evidence as to whether Lip is exposed on the surface of viable gonococci (36). In humans, an anti-Lip response is considered to be nonprotective upon challenge as indicated by patients having sequential gonococcal infections and detectable anti-Lip titers (28), but there is also some evidence that a monoclonal antibody directed against the H.8 epitope of Lip can be bactericidal and opsonic *in vitro* (36). Therefore, the importance of Lip as a virulence function is still unresolved. The conservation of Lip among the pathogenic, and its exclusion from the commensal Neisseriae suggests a role in virulence.

Lipopolysaccharide. Also known as endotoxin, and by some authors as lipooligosaccharide (LOS; 94), LPS is an important component of the gonococcal cell surface. Gonococcal LPS is chemically similar to the

rough LPS described in *Salmonella*, in that it is composed of a lipid A moiety covalently bound to a core oligosaccharide, but lacking the repetitive polysaccharide structures known as O-antigen (242,258). The LPS of *N. gonorrhoeae* exhibits a low apparent molecular weight of 3.2-7.1 kDa by SDS-PAGE (84,242).

In *N. gonorrhoeae*, LPS shows intrastrain antigenic variability, both *in vitro* and *in vivo* (155) as well as showing some antigenic similarity to human erythrocyte surface antigens (153). LPS is highly immunogenic *in vivo*, and patient sera from both DGI and UGI cases show reactivity (152). Some types of LPS expressed by *N. gonorrhoeae* have been associated with stable serum resistance (227) and LPS is the target molecule for modification in unstable serum resistance (204). LPS has also been demonstrated as being a major cause of the cytotoxic effects observed in the FTOC model system (83). Due to the cytotoxicity of gonococcal LPS and its roles in serum resistance, LPS is considered to be a very important virulence factor for *N. gonorrhoeae*. Serum resistance will be discussed in further detail in a separate section.

Peptidoglycan. The major component of the gonococcal cell wall, peptidoglycan, has been reported to possess a variety of biological properties. Structurally, it has been reported to be associated with the Por protein, possibly to stabilize the outer membrane (106). It is immunogenic *in vivo* and reportedly activates the classical complement pathway (208). It is also a potent cytotoxin in FTOC analyses (167). Degradative fragments of peptidoglycan have demonstrated arthrogenic potential in an *in vivo* rat model system (68) and induction of slow-wave sleep in a rabbit model system (238). These degradation fragments may be liberated *in vivo* and may be partially responsible for the strong

inflammatory response associated with gonorrhea (237).

Iron acquisition. The ability of the host to withhold iron from invading microbes is an important nonspecific bacteriostatic mechanism. Therefore, the ability of a microbe to acquire iron from its host is an important virulence factor as nearly all of the Fe^{3+} in the human body is rendered unavailable for microbial assimilation due to host produced iron chelators and/or intracellular location (287). In the vascular system, the majority of extracellular iron is bound by transferrin, a glycoprotein functioning in iron transport, while in the external secretions, the glycoprotein lactoferrin is the major host iron binding factor (165,287). Smaller amounts of extracellular iron in the circulatory system are bound by hemopexin and haptoglobin, which bind hemin and hemoglobin, respectively. Intracellular iron in the host is stored as hemoglobin and ferritin, in erythrocytes and all other human cells, respectively (287). Some of the functions of iron in both the host and the invading microbe include serving: 1)as a cofactor in metalloenzymes involved in redox potential, 2) the electron transport chain, 3) DNA synthesis, and 4) tRNA modification (138).

Microbes have evolved with two basic mechanisms of acquiring chelated iron. The first mechanism to be described in detail was the microbe-produced, siderophore-mediated iron binding and uptake system (197). The second mechanism, proposed and described more recently, involves the direct binding of specific host iron binding molecules to a microbial receptor and assimilation of the iron without a soluble microbial iron chelating intermediary (205,206). Typically, the first mechanism is utilized by microbes which spend at least a portion of their existences outside of a host and must compete for iron with other

free-living microbes, while the second mechanism seems to be utilized by more specialized microbes which live dependent upon a suitable host species. There are examples of bacteria which utilize both of these mechanisms (*eg. Shigella spp.*; 205).

N. gonorrhoeae has been a controversial species in the debate over siderophore production. Initially, it was reported that the gonococcus produced its own siderophore, termed gonobactin, which stimulated gonococcal growth in iron-poor media (293). More careful studies indicated that the source of "gonobactin" was the medium itself, and that while it was capable of stimulating growth, the siderophore was not produced by *N. gonorrhoeae* (202,289).

Most strains of *N. gonorrhoeae* are able to assimilate host iron bound by human transferrin (173), human lactoferrin (172), hemoglobin and hemin (173), as well as the siderophore, aerobactin (289). Some gonococci are also able to utilize the iron from a hemoglobin-haptoglobin complex but are unable to acquire iron from a hemin-hemopexin or a heme-albumin complex (64). The ability of *N. gonorrhoeae* to use these iron sources makes sense when it is considered that the gonococcus is strictly a human pathogen and not found free-living at any time. The mechanism by which the gonococcus acquires siderophore-bound iron is undefined at present. Probably this ability is exploited by the microbe as an evolutionary vestige which gives it a competitive edge in the mixed cultures that exist *in vivo*. What is known about the mechanisms involved in iron acquisition from host iron binding proteins will be discussed in the following section.

Iron regulated proteins. There are a number of environmental stress-induced outer membrane proteins that have been suggested to be involved

in the virulence of *N. gonorrhoeae*. With regard to iron-stress, at least 13 different outer membrane Frps (Ferric Regulated Proteins; 94) are inducible under iron-limited conditions (110,174,200,289). West and Sparling have reported that aside from the 37 kDa protein, the number of Frps produced by a given strain may vary depending upon the method used to render the media iron-limited (289).

Of this set of 13 proteins, the only one with a described function is the 37 kDa (Fbp, formerly Mirp; 94,174) which has been demonstrated to bind Fe^{3+} in a specific, reversible manner (9). There are also reported to be outer membrane receptors specific for both lactoferrin and transferrin (140,164). One laboratory has proposed transferrin and lactoferrin binding proteins of 100 and 102 kDa, respectively (49), while another laboratory has proposed molecular weights of 37 kDa and 77 kDa for the gonococcal transferrin and lactoferrin receptors, respectively (281). In *N. meningitidis*, a closely related species, different apparent molecular weights have been reported for the transferrin and lactoferrin receptors, 71 kDa and 105 kDa, respectively (244,245). At present, the true identities of these proteins or receptors remains to be resolved. At least some of these iron-repressible outer membrane proteins are expressed *in vivo*, as convalescent patient serum shows immunoreactivity with them, including Fbp (69).

Oxygen stress. The abilities to respond to both high oxygen tension and/or anaerobic stress are also considered to be virulence functions for bacteria. While *N. gonorrhoeae* was initially considered to be an obligate aerobe, able to survive but not grow under anaerobic conditions (112), it was later demonstrated that the gonococcus is capable of

anaerobic growth, if sufficient nitrite is available for its use as a terminal electron acceptor (126). Further, in continuous culture experiments, it has been reported that if *N. gonorrhoeae* is grown under a lowered oxygen tension, the culture is enriched for the more virulent, piliated organisms (109,151).

It has also been shown that *N. gonorrhoeae* is able to stimulate increased consumption of molecular oxygen in the presence of lactate (26). This ability is presumed to be of significant importance to *N. gonorrhoeae* during its existence in the oxygen radical-rich environment of pus since it has been reported that some gonococcal strains do not produce superoxide dismutase activity (201). Gonococci constit-utively produce both a catalase and a peroxidase which would also assist in survival in pus (31).

Oxis and Anis. When *N. gonorrhoeae* is propagated under anaerobic conditions, a set of oxygen-repressible outer membrane proteins is expressed (Anis), and a set of oxygen-inducible outer membrane proteins is repressed (Oxis; 44). The names Anis and Oxis supercede the previous designations of Pans and Poxs, respectively (94). At least some of the Anis are expressed *in vivo* as patient sera from both uncomplicated infections and from pelvic inflammatory disease show reactivity with Anil (45). Although there are no defined functions for any of the Ani or Oxi proteins they are proposed to be involved in virulence due to the observations that 1) there is an antibody response raised against at least one of them, and 2) they are regulated by relevant host encountered conditions. The expression of other well defined outer membrane antigens is not affected by growth under anaerobic conditions (44).

Heat shock proteins. A third class of environmental stress inducible proteins are the heat shock proteins produced by *N. gonorrhoeae* (125). Little is known about the identities or function(s) of these proteins in *N. gonorrhoeae*. However, since the heat shock response has been suggested to play a role in recovery of microbes from stresses other than heat, the induction of heat shock proteins may play an important role in keeping the gonococcus viable under the rapidly changing, hostile conditions in the host.

Blebs. *N. gonorrhoeae* has been shown to shed membraneous vesicles *in vitro* when grown on agar media or in liquid media (61,269). Blebs of two different densities have been isolated and shown to originate from either the outer or the cyoplasmic membrane (60). The denser bleb fraction, BII, has been shown to contain the following outer membrane antigens by silver stain and immunoanalyses: Por, Rmp, LPS, and Lip while the less dense, BI fraction lacked all of these but Por (60,269).

Chromosomal and plasmid DNAs, as well as RNA, were observed to be associated with both fractions BI and BII and further, the BII fraction was demonstrated to contain plasmid DNA in a DNAase resistant state (60). The BII blebs have been implicated in transfer of plasmids between a donor and a recipient strain and are proposed to play a role in intergonoccal genetic exchange (60).

Serum resistance. The ability to resist complement-mediated killing in normal human sera is an important virulence characteristic of some strains of *N. gonorrhoeae*. There are several means by which this can occur: 1) the absence of bactericidal antibodies against surface exposed antigenic determinants; 2) antigenic variation of surface exposed molecules; 3) the presence of blocking antibodies directed against

surface exposed antigens which inhibit complement fixation at nearby antibody-reactive, surface exposed determinants; and 4) modification of surface exposed determinants rendering them non-immunogenic. *N. gonorrhoeae* is able to escape complement mediated killing in normal human serum by all of these mechanisms (227). However, serum resistant strains are not entirely resistant to complement-mediated killing as they are efficiently killed by immune sera from convalescing DGI patients (102).

Most research on serum resistance/sensitivity has centered around killing by the classical pathway as it has been demonstrated that this pathway is more effective in killing the gonococcus *in vitro* (95). The degree of serum sensitivity of a given gonococcal isolate has been shown to be related to the contribution of the alternate pathway in formation of the C3 convertase (98) and was related to the molecular form of LPS produced (227). The alternate pathway has been shown to kill *N. gonorrhoeae* with the same rate of efficiency as the classical pathway, but the alternate pathway has a slower initiation period (56).

Serum resistance in *N. gonorrhoeae* occurs in both unstable and stable forms. Stability is determined by the effects of *in vitro* subculture and resultant serum resistant phenotype of these passaged bacteria. Unstable serum resistance is exemplified by recovery of a serum resistant isolate from a host, which upon subculture, becomes serum sensitive. This phenotype was first described by Ward, *et al.* who demonstrated that a freshly isolated, serum resistant strain was not killed by serum from other infected patients or immune rabbit sera, but soon became sensitive to normal human serum upon subculture (284).

Unstable serum resistance can also be acquired *in vitro* by

propagation in a medium which contains ultrafiltrates from guinea pig sera (230), human sera (157), or human secretions (158). This shift to serum resistance involves modification of the LPS structure, evidenced by: 1) a shift in the LPS migration on SDS-PAGE (276), 2) altered sensitivity to pyocins (290), and 3) a lack of immunoreactivity with previously reactive antisera (52,276). The molecular alteration was later shown to be due to sialidation of the LPS by a low molecular weight component in the serum in secretions (204) and has also been demonstrated to occur *in vivo*, catalyzed by a gonococcal sialyl transferase (154).

A recent paper described the binding of vitronectin (aka S protein, a host serum glycoprotein) to the surface of *N. gonorrhoeae* (5). A serum function of vitronectin is the inhibition of fluid phase, membrane attack complex formation by preventing C9 polymerization (217). This may be an important mechanism conferring unstable serum resistance to gonococci, as well as a potential aid in invasion of host cells because vitronectin has specific receptors on the surfaces of endothelial cells (239). This report may lead to a better understanding of some of the microbial processes that occur in establishment of a DGI.

Stable serum resistance is often the result of expression of particular forms of LPS and Lip by the gonococcus (209). Expression of the PorA serotype (92,97) and lack of Opa expression (97) have previously been reported to be related to serum resistance. However, a subsequent report indicated that there was no association between the Por subclass expressed and a given strain's serum resistance (209; this study did not address the contribution of Opas).

In particular, the expression of the 2-1-L8 epitope (as defined by a

monoclonal antibody) on the LPS molecule, seems to correlate with stable serum resistance (209,241). This epitope is rarely immunogenic in humans, possibly due to inaccessibility *in situ*, helping to impart serum resistance to its bearer (227). However, if a host does respond to the 2-1-L8 epitope, the antiserum is capable of killing serum resistant strains (227). Exposure of the 2-1-L8 epitope has been shown to increase as the length of the oligosaccharide chains decrease (62).

Another form of stable serum resistance, with regard to a given specimen of normal serum, is the presence of blocking antibodies which prevent complement fixation at other nearby determinants (227). An example of this has been discussed in the previous section on Rmp. Although the mechanism by which blocking antibodies protect the bacterium is unknown, they may prevent deposition of complement at adjacent sites, or result in complement deposition at a site where the membrane attack complex is non-functional (101).

Invasion of host cells. The ability of a pathogen to enter a host cell and isolate itself from the humoral immune system is often critical to the survival of that pathogen and establishment of an infection. *N. gonorrhoeae* has frequently been observed within the nonciliated columnar cells of the urethral, cervical and fallopian tube epithelial mucosae (260), but it was not until more recently that the events of invasion have been studied and described.

The FTOC system demonstrated that gonococci were able to penetrate the epithelial barrier by some undescribed mechanism (100). It was shown that the bacteria bound to the nonciliated host cell's apical surface and entered the cytoplasm in a membrane bound-vacuole (100). These envacuolated gonococci were transported through the host cell's

cytoplasm and released in an unbound state via an orderly parting of the host cell's basal membrane (260). Also, blebs are internalized by these tissues and may be one mechanism for the cytoxicity of LPS and peptidoglycan (48).

More recently, a human epithelium cell culture model (HEC-1-B monolayers) has been described which allows the penetration of gonococci into the host cell's cytoplasm (251). Cytosolic bacteria were observed to be in an unbound state and multiplying until they lysed the host cell (251).

Bacterial entry of these monolayers was suggested to occur in a manner similar to phagocytosis by professional phagocytes, excepting for the lack of bacterial destruction (260). Invasion requires viable gonococci and involves microtubule function (229), suggesting that this is an example of parasite-directed endocytosis. Por is involved in entry (91), the role(s) of Opas is still unclear (53), and pili are not required for invasion (260).

Antibiotic resistance. The ability to survive antibiotic therapy and persist in a host is considered to be an important virulence factor. It should be noted that wild type *N. gonorrhoeae* is resistant to low levels of vancomycin, and this resistance has been exploited in selective media developed for isolation of the gonococcus from clinical specimens (180). Thus far, *N. gonorrhoeae* with several different antibiotic resistances have been isolated either from patients or in the laboratory, and these resistances are summarized in Table 1.

Antibiotic resistance phenotypes have been observed to be encoded on the chromosome, and in some cases on plasmids (also noted in Table 1). For the most part, resistances provided by chromosomal-borne mutations

Table 1. Antibiotic resistances observed in *N. gonorrhoeae* isolates.

Antibiotic	Locus	Location[a]	Reference
ampicillin	*ampA*,*ampB*[b]	C	104
"	*ampC*,*ampD*[b]	C	104
chloramphenicol	*cap*[c]	C	257
erythromycin	*ery*	C	149
fusidic acid	*fus*	C	256
nalidixic acid	*nal*[d]	C	288
penicillin	*pem*	C	285
"	*penA*	C	257
"	*penB*	C	256,285
rifampin	*rif*	C	256
spectinomycin	*spc*	C	149
streptomycin	*str*	C	255
tetracycline	*tet*	C	256
"	*tem*[e]	C	256,285
several	*mtr*[f]	C	149,257
"	*nsr*[g]	C	35
β-lactams	*bla*	P	232
tetracycline	*tetM*	P	182

a - C, chromosomal and P, plasmid-borne.
b - Stepwise additional resistance to *ampA* and *ampB*.
c - Previously called *chl*.
d - Additional resistance in combination with *penA* and *mtr*.
e - Additional resistance in combination with *tet* and *mtr*.
f - Results in penicillin, tetracycline, erythromycin, chloramphenicol, rifampin, and fusidic acid resistances.
g - Results in similar resistances to *mtr* but has been mapped to a different locus.

are low level resistance to a specific drug, with those provided by plasmids being relatively higher. Nonspecific mutations have also been characterized which give a broad range of low level antibiotic resistances (*eg. mtr* and *nsr*), exerting their effects by decreasing the permeability of the gonococcal cell wall to various antibiotics, dyes, and detergents (149,257). Some of these mutations are additive, conferring increased resistance to a drug as compared to a single mutation (*eg. penA*, *penB*, *mtr*, and *tem* for penicillin; 256). Other mutations must occur simultaneously to express the resistant phenotype (*eg. ampA* and *ampB*; 104). All of the chromosomal antibiotic resistances are heritable and can be transformed from a resistant donor strain into a sensitive recipient strain, conferring resistance. Similarly, the plasmid borne resistances can be mobilized by either the 24.5 MDa or 25.2 MDa conjugative plasmids to a sensitive recipient strain where they confer antibiotic resistance, as discussed in the previous sections on genetics and genetic exchange.

Proteases. Production of a few proteolytic enzymes has been described for *N. gonorrhoeae* and a review article proposed some roles in virulence for these enzymes (203). Most of the proteases are nonspecific, and their relevant substrates in pathogenesis have not been proposed or defined. Examples of nonspecific endopeptidases include: 1) a trypsin-like enzyme that hydyrolyzes gelatin, azoalbumin, and *p*-tosyl-L-arginine methyl ester HCl (TAME; 265), and 2) gonocosin, characterized as being a neutral metalloprotease (pH optimum of 7.5-8.0, inhibited by EDTA, and resistant to inhibitors of serine proteases and sulfhydryl inhibitors; 43). Gonocosin is reported to cleave succinylated elastin but was not tested against gelatin, azoalbumin, or TAME (43) so

comparisons to the trypsin-like enzyme can not be made. The ability to cleave elastin may be an indication of gonocosin's potential role in virulence, as elastin is a host protein component of connective tissues.

N. gonorrhoeae has also been described as producing two exopeptidases, an aminopeptidase and a proline iminopeptidase (43). The aminopeptidase was inhibited by EDTA; had an optimum pH of 7.5-8.0; and was described as removing aminoterminal residues adjacent to proline, an activity similar to the aminopeptidase-P of *E. coli* (43). Aminopeptidase activities have been described in some of the nonpathogenic neisseriae as well (286), so the contribution of these enzymes to virulence is questionable.

The proline iminopeptidase is sensitive to both heavy metal and iodoacetamide inactivation, and is activated by dithiothreitol, suggesting that it is a thiol-activated protease (43). Its functional activity involves the removal of aminoterminal proline residues. This enzyme is considered to be similar to a proline iminopeptidase produced by *E. coli* (43). It is more likely involved in the acquisition of amino acids than in pathogenic processes *per se*.

IgA1 proteases. The secreted fluids on mucosal surfaces have secretory IgA (sIgA) as their primary immunoglobulin with a lesser amount of IgG and IgM (165,277). This pool of sIgA is approximately equally comprised of both IgA1 and IgA2 (54). This is in contrast with serum, where the predominant form of IgA is monomeric, and ~80-90 percent of serum IgA is of the IgA1 subclass (54).

The sIgA antibodies are secreted by plasma cells in the lamina propria beneath the mucosal epithelium, in the form of dimeric IgA, two idiotypically identical IgA monomers (M_r of ~160,000) linked non-

covalently via their CH_2 domains through the association of J chain (M_r of 15,000; 2). This IgA dimer diffuses through the interstitial fluids, and is bound by secretory component (SC; a glycoprotein of ~80,000), the IgA receptor on the basolateral side of the epithelial cells (2,169). The IgA dimer-SC complex is internalized by the epithelial cell and extruded through the apical face of the cell into the lumen as an sIgA molecule (2). This receptor-ligand interaction is unique in that the receptor-ligand complex is not dissociated intracellularly, and the receptor is not recycled for further use.

The IgA1 proteases are the best studied of the proteolytic enzymes produced by *N. gonorrhoeae* and are considered to be a virulence factor. Nonpathogenic *Neisseria* spp. do not produce the enzyme (193). These enzymes are classified as extracellular, metal-requiring endopeptidases, which cleave human immunoglobulins of the A1 subclass yielding monovalent Fab_α and intact Fc_α fragments, while not cleaving those of the A2 subclass (187,213). One of the characteristics held in common among the set of bacteria which produce IgA1 proteases is that they are all considered to be human pathogens which reside on the mucosal surfaces.

It has been demonstrated that a given species of bacteria can produce one of a few different types of IgA1 protease differentiated by the site of IgA1 cleavage. These different activities can be identified by the migration of the IgA1 cleavage products (Fd_α and Fc_α) visible after SDS-PAGE (194). *N. gonorrhoeae* has been shown to produce two types of cleavage site specificity, with a pure isolate producing only one specificity (188). The type 1 protease cleaves the PS peptide bond between residues 237-238 and the type 2 protease cleaves the PT peptide

bond between residues 235-236 (190,213). Figure 1 diagrams a portion of the IgA1 hinge region and shows the cleavage sites defined for the two *N. gonorrhoeae* IgA1 proteases. There has been no description of a gonococcal isolate either switching specificity types or ceasing to produce these enzymes upon repetitive subculture, and production has been demonstrated by all four of the Kellogg colony types (213).

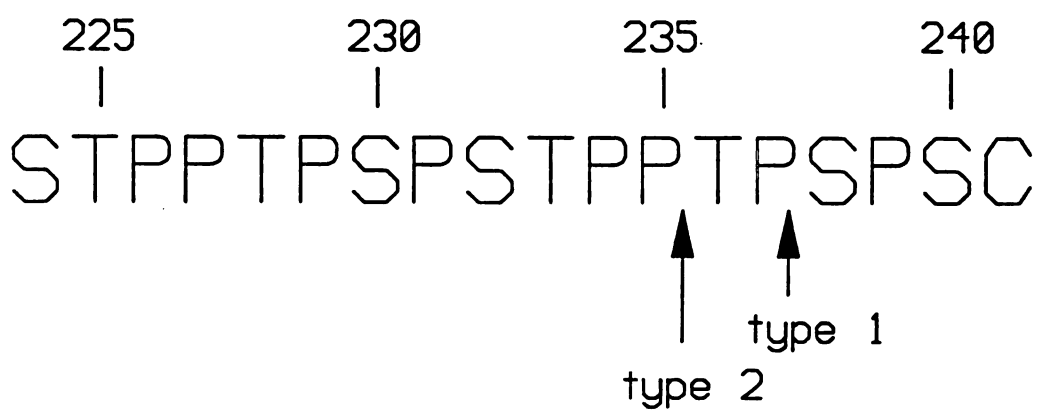


Figure 1. The IgA1 hinge region duplicated amino acid octamer showing the *N. gonorrhoeae* types 1 and 2 IgA1 protease cleavage sites (190,213).

It is intriguing that *N. gonorrhoeae* produces two different IgA1 proteases which cleave IgA1 in the sequence ^{234}PPTPSPS^{240}. In either situation, the products contain an amino-terminal T or S residue which could be removed by the aminopeptidase to expose the adjacent proline, which could be removed in turn by the proline iminopeptidase. In the case of the type 2 IgA1 protease, a second pair of amino acids could be

removed sequentially. A study of *N. gonorrhoeae* defined only 15.5% of type 1 IgA1 protease producers as proline-requiring auxotypes while 46.2% of the type 2 IgA1 protease producers required proline (190; both of these percentage groups include strains which require nutrients in addition to proline). These data suggest a selective advantage for proline-requiring gonococcal strains which also produce a type 2 IgA1 protease.

The type 1 IgA1 protease has been shown to be associated with the expression of only a few Por serovars, namely PorA-1 and PorA-2, with very few examples of PorB-1 and PorB-2 included (190). Additionally, these type 1 IgA1 protease producers were clustered into the arginine-hypoxanthine-uracil- and proline-arginine-hypoxanthine-uracil-requiring auxotypes with a few examples of proline auxotrophs (190). In contrast, type 2 IgA1 protease producers are found among all of the other 45 serovars tested except the PorA-2 group, and are of auxotypes other than the arginine-hypoxanthine-uracil- and proline-arginine-hypoxanthine-uracil-requiring groups (190).

As stated above, the IgA1 subclass is susceptible to cleavage while the IgA2 subclass is not. The molecular basis for this differentiation is a 13 amino acid deletion in the hinge region of IgA2, between the CH1 and CH2 domains (144,170,279). The hinge region of IgA1 contains a duplicated amino acid octamer between residues 225 and 240 (^{N}TPPTPSPS/TPPTPSPS^{C}), where all but two of the defined cleavage sites for IgA1 proteases occur (71,184,187). Ironically, it is the unusual repetitive structure of the hinge region that makes IgA1 more resistant to other commonly used proteinases (168). Also, the presence of secretory component has been reported to provide sIgA with increased

resistance to proteolysis (165), but this is not the case with regard to IgA1 protease hydrolysis as both monomeric IgA1 and sIgA1 are cleaved efficiently (76,150,226).

There are very few examples of other substrates cleaved by the IgA1 proteases. The type 2 enzyme is the better studied of the two gonococcal IgA1 protease types with regard to alternative substrate cleavage. While a large number of potentially relevant serum proteins are not cleaved by these enzymes, it has been shown that there are a small number of other peptides or proteins which are permissive substrates. These include a pair of synthetic IgA1 hinge peptide analogues (291), the type 2 IgA1 protease proenzyme (218), and a set of bacterial membrane proteins with undefined identities (252, and chapter 2 here). These permissive and nonpermissive substrates are listed in Tables 2 and 3, respectively.

The type 2 IgA1 protease of *N. gonorrhoeae* is considered to be a serine protease, on the basis of boronyl peptide inhibitor studies and by comparison of the predicted amino acid sequence with other known serine proteases (6). A comparative study of the two purified enzymes indicated that they have different sizes, the type 2 enzyme being 114 Kda which converts to 109 kDa, and the type 1 enzyme being a 112 kDa form that could not be separated from two smaller proteins (34 and 31 kDa) without a complete loss of activity (253). The same study also suggested that the two enzymes were very different on the basis of stability and response to chemical inhibitors (253). The type 1 protease of *N. gonorrhoeae* was extremely sensitive to the metal chelators tested, phenylmethylsulfonyl fluoride (a serine proteinase inhibitor), and *p*-chloromercuriphenylsulfonic acid (a thiol-specific

Table 2. Permissive substrates for the *N. gonorrhoeae* type 2 IgA1 protease.

Substrate	Cleavage site[a]	Reference
IgA1	PP-TP	213
IgA1 protease proenzyme	a) AP-SP b) PP-SP c) PP-AP	336
Cholera toxin:IgA1 protease fusion proteins	a) PP-AP b) PP-TP	124
Synthetic peptides	A) PP-SP B) PP-AP	291
Bacterial membrane proteins	ND[b]	252

a - Adjoining amino acids given in the one letter code.

b - ND, not determined.

Table 3. Molecules that are nonpermissive substrates for IgA1 proteases.

Molecule	Source[a]	Reference
Albumin	cow,egg,human	119,191
Azocasein		235
Azocoll		187
β-microglobulin	human	187
blood group substances A,B	human	187
casein		213
collagen		213
gelatin		213
glycophorin		187
hemoglobin	cow,human	213
IgA	cow,dog,monkeys,mouse orangutang,pig,rat,rabbit	107,187
IgA2	human	213
IgD		187
IgE	human	213
IgG	human,mouse,rabbit	187
IgM	human	213
Insulin (β chain)	cow	213
J chain	human	117
Light chain (κ,λ)	human	117
Mucin	human	187
Ovomucoid	egg	187
Secretory component	human	117
Synthetic IgA1 hinge peptides		6,34,291
Transferrin		187

a = sources not listed in the table were not defined in the reference.

inhibitor), while the type 2 protease was a minimum of ten times as resistant to these same compounds (253). Kilian, *et al.* also reported that the gonococcal IgA1 proteases were not inhibited by three different serine protease inhibitors (123). These latter sets of chemical data pertaining to serine protease inhibitors are at odds with that presented by Bachovchin, *et al.* (6), but the specific inhibitors used were different so the results can not be compared critically.

Data indicating that the enzymes are produced *in vivo* came from the work of Blake, *et al.*, who demonstrated that active enzyme could be recovered in vaginal wash fluids of patients infected with gonorrhea (17). Another study indicated that some patients infected with gonorrhea also respond with a serum antibody titer against IgA1 protease, detectable by immunoblot analysis (27,28). However, additional data presented by the same group indicated that there was no mucosal immune response to IgA1 protease detectable in genital secretions (27,28). This is in contrast to data involving persons colonized or infected with *Neisseria meningitidis*, who responded to the meningococcal IgA1 protease with serum titers that inhibit the activity of both meningococcal and gonococcal IgA1 proteases (28).

This antibody inhibition data is in agreement with other data comparing the antigenic relatedness of IgA1 proteases from several species. These results showed that a gonococcal IgA1 protease is immunologically similar (*i. e.* is inhibited by heterologous antisera) to an IgA1 protease produced by one of two strains of *N. meningitidis* (123).

These proteases can be inhibited, seemingly paradoxically, by both human secretory IgA and human sera. Two reports by the same group have

determined that serum from four of six patients acutely infected with gonorrhea, inhibited the activity of a type 2 IgA1 protease preparation (76,77). The inhibitor was suggested to be IgG on the basis of chromatographic separations of the inhibitory sera (76,77). In the case of inhibition by sIgA, it was determined that the inhibitory action was via the Fab portion of sIgA, presumably functioning as an antibody with idiotypic specificity for the IgA1 protease molecule (77). These data, while disparate with that of Brooks and Lammel (27,28), may be explained by either: 1) these four patients had truly responded to the gonococcal enzyme, or 2) these sera contained antibodies that were directed against the meningococcal IgA1 protease and happened to inhibit the gonococcal enzyme.

The genes encoding both types of IgA1 protease have been cloned in *Escherichia coli* and characterized by several independent laboratories (67,87,88,131,189,196), and are discussed below in chronological order. The first gene encoding the type 2 enzyme (*iga*2) from strain F62 was cloned by Koomey, *et al.*, who demonstrated that it produced an ~140 kDa extracellular enzyme in *E. coli*, and that the enzyme activity produced in *E. coli* was only ~0.5-2.0 percent of that produced by the parent gonococcal strain (131). The cloned *iga*2 gene was mutated *in vitro* and transformed back into the *iga*2 locus of F62, with no apparent phenotypic effects other than the inability to hydrolyze IgA1 (131). Phenotypes that were reported to be unaffected included: 1) the stained, total protein profiles as analyzed by SDS-PAGE, 2) growth rate and viability on complex solid media, and 3) colonial morphotype conversions (*i. e.* from piliated to nonpiliated, and from transparent to opaque, and vice versa; 131).

A subsequent report by Halter, *et al.* characterized the *iga*2 gene from strain MS11 as having a single copy in the genome, and being expressed at comparable levels extracellularly, in both *E. coli* and the gonococcus (87). They also reported that there were differences in the restriction site maps of the F62 and MS11 clones, the first published observation of restriction site polymorphism among the IgA1 protease genes. This was also the first reported purification of the type 2 enzyme from both *N. gonorrhoeae* and *E. coli*, indicating that the gonococcal form of the protease was a 106/105 kDa doublet while that produced in *E. coli* was a single band of 105 kDa (87).

Mulks and Knapp reported the cloning of an *iga*2 gene from the strain GCM 740, and confirmed that there was a single copy of the gene in the genome (189). They also compared genomic restriction site maps of the three strains from which the *iga*2 gene had been cloned with those of known type 1 enzyme producers (*iga*1). Their data indicated that there is extensive restriction site polymorphism among the *iga*2 loci, and that all three *iga*2 loci are different from the *iga*1 loci (189). The *iga*1 loci however, showed no restriction site heterogeneity among the set analyzed (189). This hypothesis was later expanded by Mulks, *et al.* to include eight different genotypic restriction patterns for strains other than type 1 producers, and a single genotypic pattern for all of the type 1 producers (196).

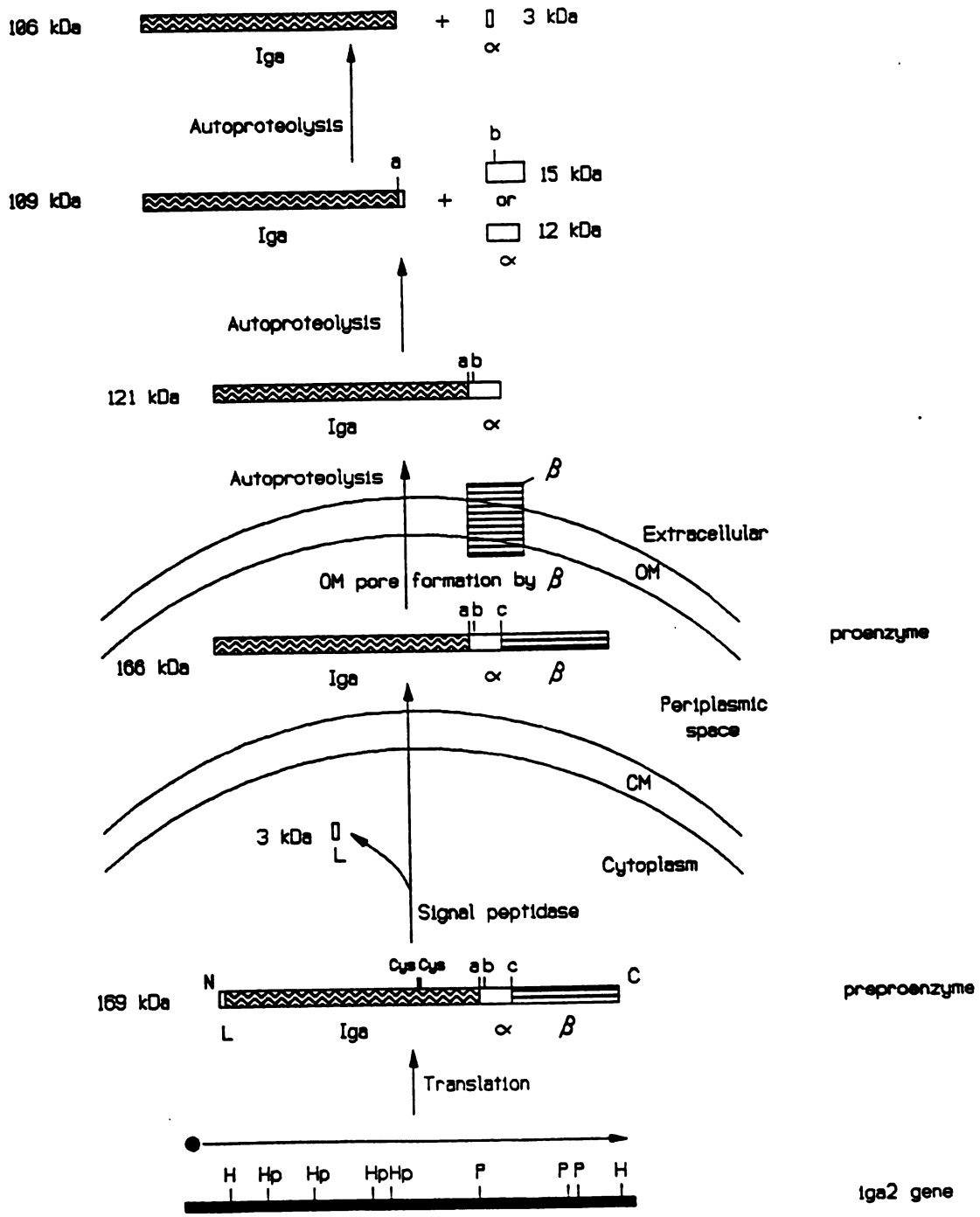
The first report of a cloned *iga*1 gene from *N. gonorrhoeae* (strain 32819) came in 1985 when Fishman, *et al.* reported a recombinant phage and plasmids that encoded the production of an extracellular, type 1 IgA1 protease in *E. coli* (67). They also determined that this *iga*1 clone from *N. gonorrhoeae* was not able to complement in *trans*, a lesion

in a weakly expressed *iga* clone from *Haemophilus influenzae* (24) by cloning the two genes into a single plasmid vector. However, their *iga*1 plasmid subclones were toxic and unstable in *E. coli* and this is the sole report from the group regarding the cloning of the type 1 IgA1 protease.

In 1987, Pohlner, *et al.* determined the complete nucleotide sequence of the *iga*2 gene from MS11 and presented a model for the secretion of the type 2 IgA1 protease in *N. gonorrhoeae* and *E. coli* (218). The gene was characterized as 4,596 base pairs in length, encoding a 1,532 amino acid preproenzyme (169 kDa) with four domains: 1) a 3 kDa leader peptide, 2) the 106 kDa structural IgA1 protease, 3) a 12-15 kDa α-domain with no defined function, and 4) a 45 kDa β-domain necessary for secretion of the protease (218). The leader domain is presumably removed by the cell's leader endopeptidase during the cytoplasmic membrane translocation of the proenzyme. In the periplasmic space the molecule folds into a conformation which allows the β-domain to interact with the outer membrane and form a pore structure through which the proenzyme is extruded. On the extracellular surface, an autoproteolytic event occurs to release the Iga-α intermediate, processed via further autoproteolytic events to yield the mature IgA1 protease and the α-peptide(s) (218). There are two potential cleavage sites at the Iga-α junction (a and b) and one at the α-β junction (c) which are clipped during these processing events, none of which are identical to the sequence cleaved in IgA1 (Figures 1 and 2; 213,218). Selection of the a or b site is apparently nonpreferential as both sites are used. Figure 2 diagrams the events involved in secretion of the IgA1 protease as described by Pohlner, *et al.* (218).

Figure 2. Diagramatic representation of the *N. gonorrhoeae* type 2 IgA1 protease secretion process. Restriction endonuclease sites are designated by the following abbreviations: H, *Hin*dIII; Hp, *Hpa*I; and P, *Pst*I. Designated domains and features of the preproenzyme and proenzyme are in the convention of Pohlner, *et al.* (218; L, leader peptide; Iga, structural IgA1 protease domain; α and β domains, respectively; a, b, c, the three potential internal proteolytic processing sites; and Cys, the two cysteine residues proposed to be in the active site).

Figure 2


106 kDa
Iga
+
3 kDa
α
Autoproteolysis
b
a
15 kDa
109 kDa
+
or
Iga
12 kDa
α
Autoproteolysis
a b
121 kDa
Iga
α
Autoproteolysis
β
Extracellular
OM pore formation by β
OM
a b c
166 kDa
Iga
α
β
proenzyme
Periplasmic space
CM
3 kDa
L
Cytoplasm
Signal peptidase
Cys Cys
a b c
N
C
169 kDa
L
Iga
α
β
preproenzyme
Translation
H Hp Hp HpHp P P P H
Iga2 gene

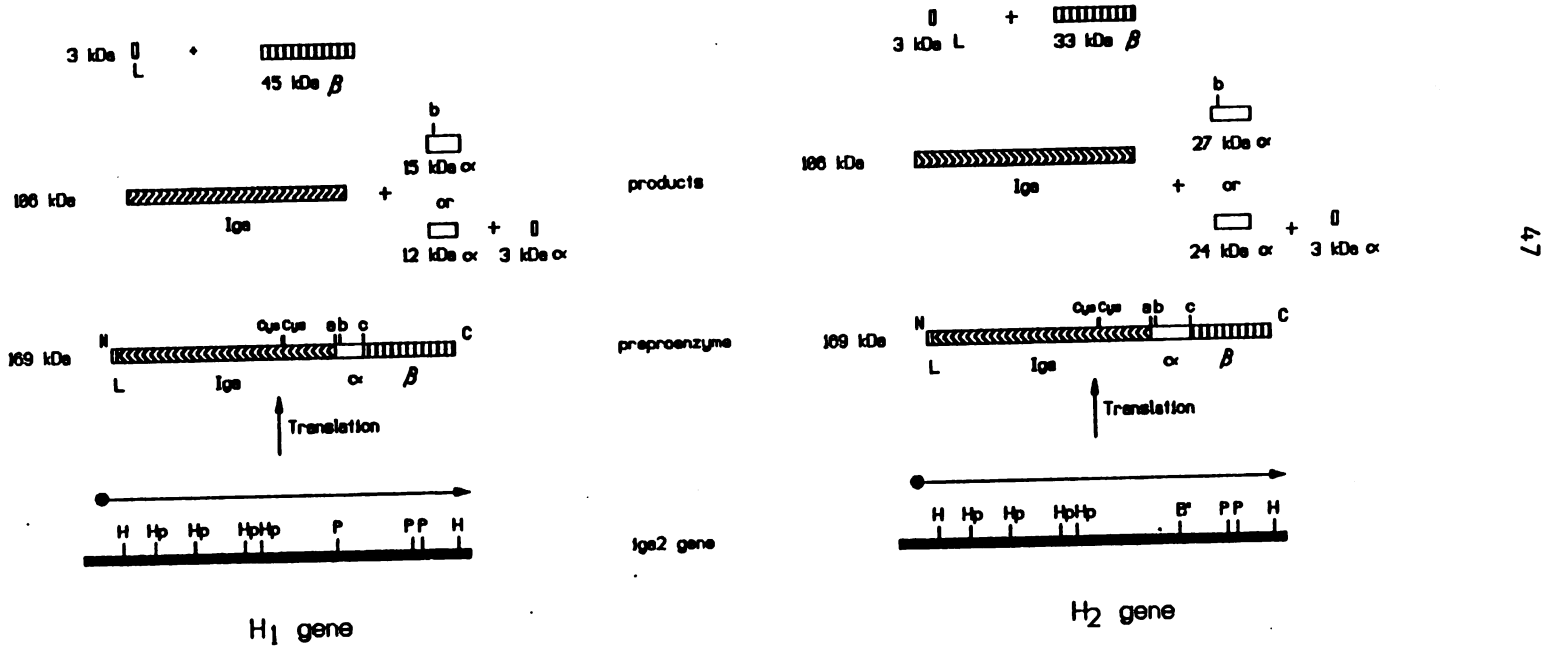
The β-domain contains all of the requisite functions for export as demonstrated by the surface expression of a translational fusion gene (cholera toxin B subunit-β domain) in *Salmonella typhimurium* (124). The exposed fusion protein was susceptible to trypsin and could be released from the cell surface by treatment with exogenous IgA1 protease (124).

The restriction site polymorphism question was later addressed by Halter, *et al.*, who reported that there were several clusters of nucleotide substitutions throughout the gene, in the structural protease domain and in the α-β junction region (88). They also reported the first stable cloning of a gonococcal *iga*1 gene (88). They proposed a scheme for classifying *iga* genes based on the presence or absence of a *Bgl*II or *Pst*I restriction site near the α-β junction, which influences the c autoproteolysis site hydrolyzed during secretion (88). Strains exhibiting a *Bgl*II site process the Iga proenzyme to yield a 33 kDa β-peptide (H_2 strains) while strains with a *Pst*I site process the Iga proenzyme with a 45 kDa β-peptide (H_1 strains; 88). This differential processing is diagrammed in Figure 3 as described by Halter, *et al.* (88). They also reported the presence of the gonococcal uptake sequence (79) at the downstream end of four sequenced *iga* genes, and proposed that the nucleotide sequences of *iga* genes had diverged over time due to interstrain exchanges via transformation (88).

The IgA1 protease genes of *N. gonorrhoeae* show significant DNA homology with those of *N. meningitidis*, as reported by Koomey and Falkow (>80%; 129). When these *iga*2 genes were compared to the IgA1 protease gene of *H. influenzae*, lower homology was observed (67-80%), and no homology was detected between the cloned *iga*2 gene and the genome of seven species of *Neisseria* and *Branhamella catarrhalis*, all considered

Figure 3. Diagramatic representation of the proteins produced during the processing of *N. gonorrhoeae* IgA1 proteases as proposed by Halter, *et al.* (88). Restriction endonuclease sites are designated by the following abbreviations: H, *Hin*dIII; Hp, *Hpa*I; and P, *Pst*I. The domains and features of the preproenzyme and the proenzyme are given in the convention of Pohlner, *et al.* (218; L, leader peptide; Iga, structural IgA1 protease domain; α and β domains, respectively; a, b, c, the three potential internal proteolytic processing sites; and Cys, the two cysteine residues proposed to be in the active site).

Figure 3


3 kDa
L
45 kDa β
b
15 kDa α
166 kDa
Igs
+
or
12 kDa α
+
3 kDa α
products
169 kDa
N
C
Cys Cys
a b
c
L
Igs
α
β
Translation
preproenzyme
H Hp Hp HpHp P PP H
Igs2 gene
H1 gene
3 kDa L
33 kDa β
b
27 kDa α
166 kDa
Igs
+
or
24 kDa α
+
3 kDa α
169 kDa
N
C
Cys Cys
a b
c
L
Igs
α
β
Translation
H Hp Hp HpHp B' PP H
H2 gene

to be commensals (129) and previously shown not to produce IgA1 proteases (193).

The effects of IgA1 protease on the immune system have been studied and data from a variety of investigations suggests that IgA1 protease may play an important role in the virulence of the gonococcus. For example, gonococcal IgA1 protease activity causes deagglutination of particles held in an IgA1 bound-immune complex and lowers the agglutinating titer of antibody preparations that include IgA1 as an agglutinin (215). These data indicate that IgA1 is susceptible to IgA1 protease cleavage when free in solution or when bound to antigen, and implies that the antibody activity of the IgA1 molecules is destroyed.

Further data indicates that treatment of an sIgA preparation with exogenous gonococcal IgA1 protease reduces the ability of that sIgA preparation to inhibit the adherence of gonococci to human cells (195). These experiments were performed by incubating the sIgA preparation with exogenous IgA1 protease prior to exposing the sIgA preparation to the target gonococcal cells, leaving the possibility open that if the sIgA preparation were reacted with the target gonococci first, the results may have been different. If the data presented by Plaut, *et al.* (215) can be extrapolated, then the IgA1 antibodies bound to the gonococci would still have been susceptible to hydrolysis, and a decrease in the inhibition of gonococcal adherence would have been observed.

One of the functions that has been proposed for IgA is a dampening role in the inflammatory response (118). If degradation of IgA1 abrogates this dampening effect, there would be an increased inflammatory response (118), similar to what is observed in cases of gonococcal infection. Many of the clinical signs of gonorrhea are a

result of inflammation and perhaps efficient transmission of the bacteria themselves is dependent upon a strong inflammatory response.

Also, there is some evidence that IgA1 protease treatment of a monomeric IgA1 paraprotein causes a reduction in the ability of IgA1 to inhibit the movement of polymorphonuclear leukocytes, presumably an Fc_{α}-mediated phenomenon (280). Other $Fc_{\alpha 1}$-mediated functions proposed to be inhibited by cleavage of IgA1 include: 1) opsonization and phagocytosis by macrophages and polymorphonuclear leukocytes, 2) monocyte-dependent bactericidal activity, 3) antibody-dependent cellular cytotoxicity, and 4) sIgA enhancement of nonspecific bactericidal mechanisms (*eg.* lactoferrin bactericidal activity) (118).

The monovalent Fab_{α} released after IgA1 protease hydrolysis of IgA1 are reported to retain the ability to bind antigens (156). These Fab_{α} fragments may obscure antigenic determinants susceptible to Fc-mediated events involving other immunoglobulin classes (*eg.* complement activation by IgG or IgM, and opsonophagocytosis by professional phagocytes), or by coating the bacterium with host proteins, render the gonococcus less immunogenic, also an advantage in establishing and maintaining an infection.

Pohlner, *et al.* have proposed that the CD.8 marker antigen on the surface of cytotoxic T cells and a cytokine, granulocyte/macrophage growth stimulating factor (GM-GSF) are potential substrates for the type 2 gonococcal IgA1 protease (219). Cleavage of these two immunologically relevant molecules could potentially alter the normal immune response and change the outcome of an infection.

As a final point regarding the impact of the gonococcal type 2 IgA1 protease on virulence, Cooper, *et al.* compared a set of isogenically

constructed *Iga*$^+$ and *Iga*$^-$ gonococcal strains (131) on the basis of their abilities to adhere, cause cellular damage, and invade the human fallopian tube organ culture model system. They observed that the *Iga*$^-$ mutant strain is as virulent as the wild type parental strain for all three parameters measured (47). The data suggested that IgA1 protease has no effect on the events that lead to initial mucosal colonization. However, it must be kept in mind that the amount of sIgA secreted by the tissue explant may not be equivalent to *in situ* production. These *in vitro* experiments also lack the protective effects of mucus secretion and flow, and can not stimulate inflammation which plays an important role in the establishment of gonococcal infection.

In summary, the role(s) of IgA1 protease in the virulence of *N. gonorrhoeae* is unclear. There is some indirect evidence that it may play a part in neutralizing the IgA1 antibody-mediated effects of sIgA and stimulation of the immune response. However, until the relative virulence of isogenic *Iga*$^+$ and *Iga*$^-$ strains can be examined in either a human or chimpanzee experimental infection, the real contribution(s) of these enzymes in virulence will remain speculative.

IgA1 proteases of other species. A broader discussion of the IgA1 proteases will provide background information and serve to explain why this set of enzymes has been of interest to so many researchers over the years. There have been intense efforts from a variety of laboratories and the information that has been generated merits some attention.

The first description of an enzyme which cleaved human IgA came in 1973 with the isolation of intact Fc_α fragments from the clarified feces of six normal individuals (166). The isolation of the fragment was noteworthy because up to that time, the relative proteinase resistance

of IgA had made it a frustrating molecule for structural studies. Mehta, *et al.* demonstrated that the activity which generated the Fc_{α} fragment was produced by an unidentified microorganism and observed in the clarified supernatant from aerobic broth cultures of fecal material. This activity was proposed to be metal-dependent based on its sensitivity to 5 mM EDTA and was resistant to the effects of serine protease inhibitors. Attempts to identify the specific microbe(s) that produced the enzyme were unsuccessful. However, the authors noted that an organism identified as an *E. coli* did make the enzyme transiently but failed to produce it upon repeated subculture (166).

A subsequent report from the same group provided further information about this enzyme activity but still no producing species was able to be identified, which is the case today. Plaut, *et al.* reported that the enzyme had a molecular weight of 40-60,000 based on its sedimentation in sucrose density gradient ultracentrifugation and that it was inactivated by heating at 55°C for thirty minutes (212). The enzyme was proposed to be a metalloprotease due to its sensitivity to inhibition by EDTA and the fact that activity could be restored by divalent cations (Zn^{++}, Co^{++}, and Mn^{++} were better than Ca^{++} and Mg^{++}; 212). Due to the inability to identify the source of this protease, efforts to find other bacteria which produced the proteolytic activity intensified and many IgA1 protease producing bacteria have been identified.

IgA1 protease methodologies. Many different methods of analyzing a sample for the presence of IgA1 protease have been developed through the years. Some of these are in widespread use by the laboratories in the field and others have predictably been passing fads. Table 4 lists some of the methodologies developed and/or used in this research and gives a

Table 4. Techniques used to analyze the IgA1 proteases.

Assay principles or description	Quantitative[a]	Ref.
I. Those requiring complex formation.		
A. Type 4 group A streptococci Fc_α receptors Uses radiolabeled IgA1 as a substrate Protease activity -> lower number of cpm bound	+	141
B. Beckman Immunochemistry System nephelometry Assays degree of light scatter due to α-IgA:IgA1 complexes Protease activity -> lower amount of scatter	+	142
C. Immunoelectrophoresis Uses α-Hc and α-Lc to detect cleavage products after separation IgA1 protease activity -> altered precipitation pattern	-	166
D. Rocket immunoelectrophoresis Uses α-Hc in the agar solid phase for detection during separation IgA1 protease activity -> altered precipitation with larger area in "cone of rocket"	±	139
E. ELISA Solid phase contains α-Lc:IgA complex Detection with α-Hc after protease treatment IgA1 protease activity -> decreased binding of α-Hc	+	223
II. Those which physically separate IgA1 cleavage products.		
A. Cellulose acetate electrophoresis Uses either unlabeled or radiolabeled IgA1 Detect by staining or autoradiography, respectively IgA1 protease activity -> staining or radioactivity in Fc_α and Fab_α bands	+	216
B. SDS-PAGE		
1. Under reductive conditions Uses either unlabeled or radiolabeled IgA1 Detect by staining or autoradiography, respectively IgA1 protease activity -> staining or radioactivity in Fc_α and Fd_α bands	+	19
2. Under native or nonreductive conditions Detect by immunoblotting with α-Hc IgA1 protease activity -> reactivity in Fc_α and Fab_α bands	-	1

Table 4 (cont'd).

Assay principles or description	Quantitative[a]	Ref.
C. Solid phase plate assays		
1. Overlay bacterial colonies with immobilized radiolabeled IgA1 in top agar After incubation, overlay with a nitrocellulose filter and allow for diffusion of Fab_α and binding to the filter Detect by autoradiography IgA1 protease activity -> dark spot on X-ray film	-	74
2. Overlay colonies with radiolabeled sIgA1 bound to nitrocellulose filter by α-secretory component After incubation, detect by autoradiography IgA1 protease activity -> light spot on X-ray film	-	87
3. Overlay bacterial colonies with immobilized radiolabeled IgA1 in top agar After incubation, overlay with a nitrocellulose filter having α-Lc to bind free Fab_α Detect by autoradiography IgA1 protease activity -> dark spot on X-ray film	-	29
D. Analytical ultracentrifugation Uses unlabeled IgA1 or sIgA1 Detect by photography of separated bands IgA1 protease activity -> lower density cleavage products (Fc_α and Fab_α)	-	119
E. High Pressure Liquid Chromatography Uses either unlabeled or radiolabeled IgA1 Separate on a gel filtration column Detection by absorbance or determination of radioactivity IgA1 protease activity -> lower molecular weight cleavage products (Fc_α and Fab_α)	+	185

a = +, quantitative; ±, semi-quantitative; -, qualitative only.

brief summary of the relevant points of each.

All have the following in common, the researcher must react a sample containing human IgA1 with the enzyme and analyze the results of that reaction to identify the presence or absence of IgA1 cleavage products. The prototypic reaction was presented by Plaut, *et al.* and involves mixing a culture supernatant, bacterial extract, whole bacteria, or some processed version of these with a buffered reaction mix (pH range 7.0-8.5) containing serum IgA, sIgA, purified IgA1, or purified sIgA1 (214). The reaction mix is incubated at 31-37°C until termination by addition of EDTA, SDS and reducing agents, or heating.

The analysis procedure must entail some means of identifying the cleavage fragments and the original IgA1 molecule. Examples of identification include immunoreactivity of Hc_{α} and Fab_{α} or Lc and physical separation based on size or charge of the cleaved fragments and intact IgA1. For quantitative purposes, the IgA1 protease unit has been defined as that amount of activity cleaving 1 μg of human serum IgA1 in one minute at pH 7.0 and 37°C (214).

These techniques will be discussed in the order listed in Table 4. With the exception of the first technique, the assays in set I are based on the formation of an an immune complex using the IgA1 molecule and its cleavage fragments as antigens, and a specific antibody or anti-serum preparation (*eg.* anti-Hc_{α}, α-Lc, or α-SC) as the antibody component. A common disadvantage to most of these techniques is that their accuracy is dependent upon the quality of the reagents used in detection.

The first assay exploits the class specific, Fc_{α} receptors expressed on the surface of a particular subset of group A streptococci (type 4; incidentally, an IgA1 protease negative group; 141). This particular

assay technique is not widely used. The disadvantages of this procedure include 1) the necessity of a laboratory to culture and work with the virulent streptococci and 2) the necessity of a radioactive probe. The advantage is that the assay is relatively rapid as compared to some of the others.

The second assay described is not used by very many laboratories due to the required instrumentation (142). The Beckman Immunochemistry System is not a commonly held piece of laboratory equipment, except for possibly in a hospital or other clinical laboratory. However, the assay does have the advantages of not requiring a radioactive substrate and being relatively rapid.

The third assay listed, immunoelectrophoresis or IEP, was the method of choice in the early days of IgA1 protease research (166), but its use has declined due to the length of time required to obtain results (~24 hours) and the inability to quantitate enzyme activity. This technique has also been replaced by methods which allow for more samples to be processed in the same length of time (*eg.* ELISA and SDS-PAGE).

Rocket immunoelectrophoresis, a variation of the IEP, offers the advantages of processing more samples in the same length of time and the ability to quantitate a protease preparation (139). The chosen definition of semiquantitative refers to the ability to compare the activity of an experimental sample to the activity of a standard sample but not to determine the activity of a sample *de novo*. This technique is not widely used due to the lack of ability to quantitate unknown samples and the inherently low degree of cleavage specificity that can be assessed.

The final example of a technique based on formation of antigen-

antibody complexes is the ELISA, used by some laboratories in the IgA1 protease field (223). The principle is based on solid phase immune-capture of the the IgA1 molecule with α-Lc. Following treatment with IgA1 protease and rinsing, the secondary antibody (α-Hc_α) is added to identify remaining uncleaved IgA1. The assay is quantitative in that by using a known quantity of IgA1, the amount of Fc_α remaining and the degree of hydrolysis can be determined. The ELISA technique offers the ability to perform many several assays simultaneously, safely, and relatively inexpensively.

The second set of techniques listed is based upon physical separation of the cleavage products from the intact IgA1 molecule. Many of these techniques are commonly used to assay IgA1 proteases today, with the exception of analytical ultracentrifugation.

Cellulose acetate electrophoretic separation is still used by some laboratories, but generally has been replaced by techniques allowing for more samples to be processed in a given time frame (216). The technique is sensitive and specific, can use either unlabeled or radiolabeled substrate, and is a rapid assay to perform and analyze. The chief drawback is that the apparatus limits the number of samples that can be separated in a given run.

The SDS-PAGE-based assays are probably the method of choice in use today by most laboratories studying IgA1 proteases. The technique allows for multiple samples to be processed simultaneously, offers consistency, is amenable to quantitative studies, and is relatively rapid. While the technique is frequently performed with reducing conditions and radiolabeled substrate to maximize sensitivity while conserving substrate, it may also be performed with unlabeled substrate

for detection by staining (19).

A variation of the SDS-PAGE separation technique can be performed with nonreducing conditions to preserve the tertiary structure of the cleavage products for detection with immunoblot analysis (1). This alternate technique is used less frequently but has the advantage of demonstrating that the cleavage fragments were intact rather than dissociated. The disadvantages of the immunoblot procedure are the longer time necessary to obtain results, the inability to quantitate enzyme activity, and the quality limitations of the detection reagents.

The solid-phase plate type assays are used for screening large numbers of isolates simultaneously and are most often used to select an IgA1 protease-producing isolate from a population of non-producers. All of these methods are strictly qualitative. The first described method utilizes radiolabelled IgA1 immobilized via an α-Fc_{α} antibody (74). The basis of the technique involves overlaying a bacteria covered surface with a layer of immobilized IgA1. Secreted IgA1 protease from producing colonies will cleave the immobilized IgA1 allowing the freed Fab_{α} to diffuse so that it can be recovered, usually employing a nitrocellulose membrane (74). IgA1 protease producing isolates are identified by a location-corresponding radioactive signal.

Two variations have arisen from this method. One involves prior immobilization of the labeled IgA1 on the nitrocellulose membrane and incubation on a bacteria covered surface. After allowing for diffusion of the liberated Fab_{α}, IgA1 protease producers can be detected by a decrease in the amount of radioactive signal (87). Another variation incorporates α-Lc antibodies bound to the nitrocellulose membrane to improve the efficiency of Fab_{α} recovery and differentiates IgA1

protease-positive isolates by increased radioactive signal (29).

Analytical ultracentrifugation has been used to verify the composition of IgA1 protease cleavage products but is rarely used today due to the equipment, time, and expense involved in the procedure (119).

Finally, the chromatographic separation of intact and cleaved IgA1 has been used to identify and quantitate proteolytic activity (185). The advantages of the technique are its speed and its consistency in separation of the fragments. The disadvantage is that the HPLC equipment required to perform the assay is not a common piece of laboratory equipment due to the cost.

IgA1 protease comparisons. A fairly extensive listing of bacterial species have now been demon-strated to produce an IgA1 protease and these are listed in Table 5. There are many more examples of gram negative than gram positive species, and a single mycoplasma. Although the number of producing species has grown to include many previously considered to be negative (187,210), all of the bacteria listed in the table still reside on or in the mucosal epithelia. To have been included in this listing, an organism must have met the following criterion: IgA1 is degraded and an intact Fc_{α} must have been demonstrated. An organism may have also shown the capacity to degrade IgG, as in the case of some of the *Bacteroides* spp. (70,114). However, the ability to degrade multiple immunoglobulins was not shown to be a function of a single enzyme, therefore including the possibility that a second type of protease is being produced. Likewise, the unstable nature of enzyme production shown for some of the enteric organisms isolated from patients with urinary tract infections did not disqualify those species from being included due to the original papers'

Table 5. Bacterial species demonstrated to produce IgA1 protease.

Species	% of strains	Site[a]	Refs.
Acinetobacter calcoaceticus var. *anitratus*	14	UT	175
A. calcoaceticus var. *lwoffi*	25	UT	175
Bacteroides asaccharolyticus	38	OC	114
B. buccae	NR[b]	PO	70
B. buccalis	NR	DP	70
B. denticola	NR	DP,PO	70
B. loeschii	NR	PO	70
B. melaninogenicus	47-100	SP	70,114
B. oralis	NR	PO	70
B. oris	NR	PO	70
B. veroralis	NR	OC	70
Bifidobacterium sp.	NR	IBD	72,248
Capnocytophaga gingivalis	NR	NR	70
C. ochracea	100	OC,PO	70
C. sputigena	NR	NR	70
Clostridium ramosum	3	IBD	72,248
Escherichia coli	14	UT	175
Gemella haemolysans	NR	DP	123
Haemophilus aegyptius	NR	Conjunctiva	119
H. influenzae	97	NP,CSF,B,Ear	96,119 150,192
H. parahaemolyticus	21	NP,OC	123,150,192
H. parainfluenzae	0-25[c]	NP	123,150
Klebsiella pneumoniae	17	UT	175
Moraxella spp.	17	UT	175
Neisseria gonorrhoeae	>99	UT	213, *d*
N. meningitidis	99	NP,CSF,B	213
Proteus mirablis	20-100[c]	F,UT,SP,Ear	175,249,250
P. penneri	100	F	250
P. vulgaris	17-50	F,UT,AB,Ear	175,250
Pseudomonas aeruginosa	18	UT	175
Serratia marcescens	20	UT	175
Streptococcus mitis biovar 1	20	DP	225
S. oralis	100	DP	225
S. pneumoniae	100	NP,CSF,B,SP	119,150,192
S. sanguis	100	DP	225
Ureaplasma urealyticum	100	UT	107,115,235

a = Abbreviations are as follow: UT, urinary tract infection; OC, oral cavity; PO, periodontal infection; DP, dental plaque; SP, sputum; IBD, inflammatory bowel disease; NR; not recorded; B, blood; NP, nasopharynx; CSF, cerebrospinal fluid; F, feces; and AB, abcess.

b = NR; not recorded.

c = Discrepancy between the references.

d = M. H. Mulks, unpublished observation.

descriptions of these enzymes as being transiently produced (166,175).

Some of the sites of cleavage have been identified by terminal amino acid sequencing of the cleavage products (117,122,190,194,213,225). Figure 4 shows an alignment of the hinge regions of IgA1 and the allotypes of IgA2 (170). The cleavage specific sites are also indicated as they have been defined by peptide sequencing with the exceptions of the *Ureaplasma urealyticum* (115) and *Capnocytophaga ochracea* (70) proteases which have been suggested, but have not been proven, to cleave the ^{235}PT^{236} and ^{223}PS^{224} peptide bonds, respectivley. With four exceptions, all characterized IgA1 proteases cleave the IgA1 molecule in the duplicated octamer. The proteases that cleave IgA1 beyond amino acids 225 or 240 are produced by *Clostridium ramosum* (71), *Bacteroides melaninogenicus* (184), and *C. ochracea* (all shown in Figure 4) with *Proteus mirablis* as the fourth. Based on SDS-PAGE analyses, the *P. mirablis* IgA1 cleavage site is suggested to be outside of the hinge region but this has not been conclusively demonstrated (249). The enzyme produced by *C. ramosum* is noteworthy in that it also is able to cleave the IgA2m(1) allotype molecule as well as the IgA1 isotype (72).

Some of the species which are able to produce more than one specificity of IgA1 protease show relationships between the enzyme type produced and other phenotypes. Examples are the previously stated relationships between protease type and Por serovar and auxotype in *N. gonorrhoeae* (189), and the relationship between protease type and capsular serotypes in both *N. meningitidis* and *H. influenzae* (96,192,191,194). In *H. influenzae*, similar clustering arrangements were proposed on the basis of antigenic relatedness and serotype (122,123) and between the restriction site polymorphism pattern of *iga*

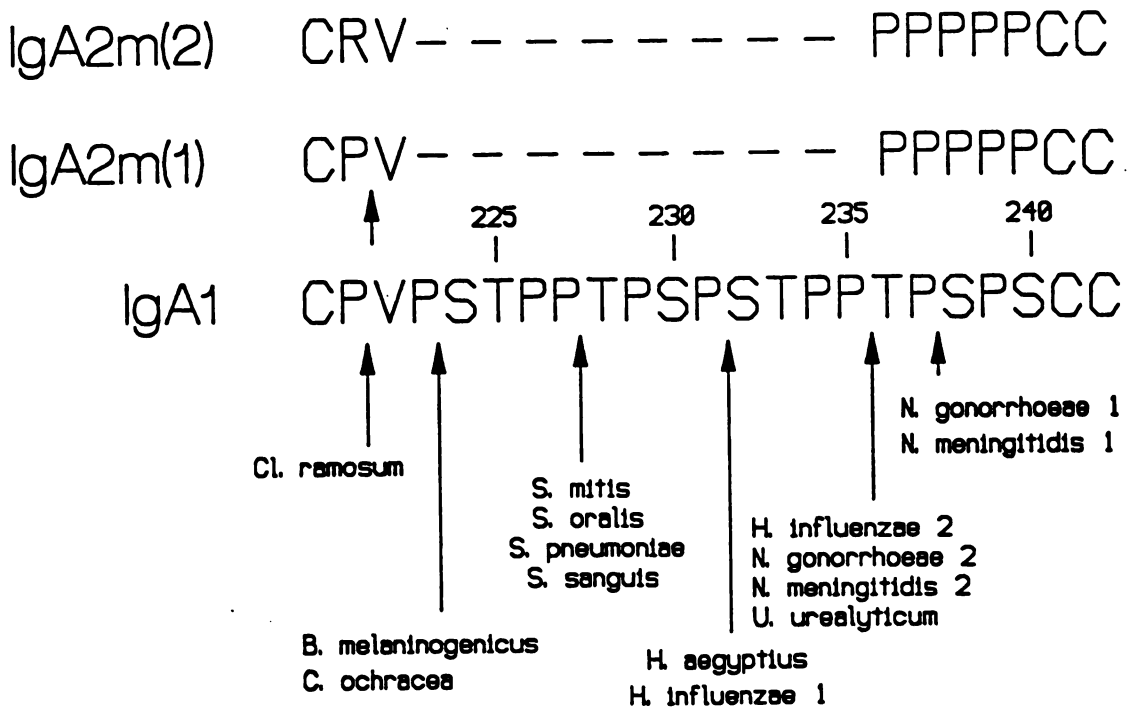


Figure 4. Alignment of the IgA1, IgA2m(1) and IgA2m(2) hinge regions showing the defined and proposed sites of cleavage of several IgA1 protease producing bacteria. The alignment of the molecular hinge regions is as presented by Mestecky and Russell (170) and the IgA1 protease cleavage sites are as reported previously (70,71,115,117, 122,190,194,213,225).

loci and biotype (221). Interestingly, a previous study of *H. influenzae* suggested that there was no relationship between the enzyme specificity and the biotype (191).

Table 6 summarizes some of the information known about the chemistry of these enzymes, including the class of protease that a given IgA1 protease belongs to and the molecular weight, where reported. Many of the species listed in Table 5 are not included in Table 6 due to the lack of information regarding the chemistry of the enzyme. Some of the highlights of the data are the facts that: 1) three of the four classes of proteases are represented, with aspartic proteases being the exception, and 2) these enzymes show a wide range of molecular weights. It appears that this set of proteases with similarly narrow ranges of substrate specificity is actually a grouping of very diverse enzymes which were clustered into a group based solely on that one characteristic.

In addition to *N. gonorrhoeae*, genes encoding IgA1 protease activity have been cloned from *N. meningitidis* (129,222), and *H. influenzae* (23,24,85,86,129,220). One comparative DNA hybridization study of the relationships between these *iga* genes indicates that the two neisserial genes and the *H. influenzae* gene share significant homology, and indicates that the commensal *Neisseria* and *Haemophilus* species lack any significant homology to these cloned *iga* genes (129). A second study confirmed the latter observation within the genus *Haemophilus* (24). Heteroduplex analysis of a *N. gonorrhoeae-N. meningitidis iga* clones-hybrid demonstrated a large region of nonhomology in the 5′ end of the gene with very good homology over the 3′-most one-third (222). A comparison of the *N. gonorrhoeae* and *H. influenzae* gene sequences showed

Table 6. Comparison of the better described IgA1 proteases.

Species	Enzyme class[a]	Mr[b]	References
Bacteroides melaninogenicus	T	62	70,184
Capnocytophaga spp.	M	NR[c]	70
"Fecal"[d]	M	40-60	166,212
Haemophilus influenzae (type 1)	S	108	6,220
Neisseria gonorrhoeae (type 1)	NR	112	253
N. gonorrhoeae (type 2)	S	105-109	6,218,253
N. meningitidis	M	NR	213
Proteus spp.	M	NR	249,250
Streptococcus oralis	NR	100	225
S. pneumoniae	M	NR	117
S. sanguis	M	100-186	78,135,225
Ureaplasma urealyticum	M	NR	235

a = M, metalloprotease; S, serine protease; T, thiol protease.

b = Molecular weight (10^3).

c = Not reported.

d = The first described example of an IgA1 protease.

good homology in the 3′ end of the gene (*i. e.* the β-domain) with clusters of divergence scattered over the upstream sequences (220). This observation coupled with conservation of some of the important amino acid positions suggested to the authors that the *H. influenzae* IgA1 protease may be secreted in a fashion similar to the *N. gonorrhoeae* type 2 enzyme (220).

The *iga* gene from *Streptococcus sanguis* has also recently been cloned (75). Comparative analyses have shown that there was no detectable sequence homology between the *S. sanguis* gene and the genome of non-IgA1 protease producing *S. sanguis* strains, *S. pneumoniae* (all Iga^+), *S. mutans* (all Iga^-), *Clostridium ramosum*, *Bacteroides melaninogenicus*, *H. influenzae*, and *N. gonorrhoeae* (75,78).

When the enzymes are compared on the basis of their abilities to be inhibited by polyclonal anti-IgA1 protease sera, extensive heterogeneity is indicated. Studies by the Kilian laboratory have suggested that there are more than 15 different antigenic types of IgA1 protease produced by *H. influenzae* alone (117,123). These studies also indicate that: 1) the enzyme produced by *Streptococcus pneumoniae* while cleaving the same peptide bond in IgA1 as the enzymes from *S. sanguis* and *S. oralis* (formerly *S. mitior*; 225), is antigenically distinct from that of the latter two; 2) two different strains of *N. meningitidis* each produce two antigenically different IgA1 proteases, one of which is similar to a gonococcal enzyme; and 3) other than the two neisserial enzymes, there are no close immunologic relationships between the IgA1 proteases produced by *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis*, and *S. pneumoniae* (123). Comparison of the IgA1 proteases produced by the *Bacteroides* and *Capnocyophaga* species indicated that each *Bacteroides*

enzyme is serologically distinct and that the *C. ochracea* and *C. sputigena* proteases are identical but unrelated to the enzyme produced by *C. gingivalis* (70). The point of this discussion is to emphasize the heterogeneity that exists among the set of enzymes that we have arbitrarily clustered as "the IgA1 proteases".

Specific IgA1 proteases. Some of the IgA1 proteases have been studied extensively and will be discussed briefly in order to highlight some of the important or interesting data which has been generated. The best studied examples other than *N. gonorrhoeae* come from the bacteria that are most frequently responsible for causing septic meningitis (*N. meningitidis*, *H. influenzae*, and *S. pneumoniae*) and *S. sanguis*, one of the bacteria responsible for the initiation of dental plaque.

The two types of *N. meningitidis* IgA1 protease cleave IgA1 at the same peptide bonds as the gonococcal enzymes (see Figure 4) and are produced in a mutually exclusive fashion with the distribution of type 1- versus type 2-producing strains, approximately equal (194). Enzyme type specificity is correlated with some capsular serotypes as serotype A strains produce only type 1 IgA1 protease and serotypes X and Y only produce a type 2 protease (194). Serotypes B, C, D, W-135, 29-E, Z, and the non-serotypable strains were shown to produce either type of IgA1 protease (194). While *iga* genes have been cloned from *N. meningitidis*, little information is available on these genes (129,222).

H. influenzae has been studied intensively, if not aggressively, with regard to the production of IgA1 proteases. In fact, three different, independent laboratories published the identification of enzyme production by the species (119,150,192). The species has also been controversial with regard to specificity types. *H. influenzae* has

been proposed to produce enzymes with two to four different cleavage site specificities, and enzymes of two different specificities have been proposed to be produced simultaneously by a clonal isolate of *H. influenzae* (23,96,119,122,123,191). This "double cutting" is associated with nonserotypable strains (96,191). Currently, the consensus opinion from the literature is that there are two different specificities of *H. influenzae* enzyme, and that some strains produce both at the same time. Evidence for these unusual strains having more than one copy of the gene has been presented (221).

The type of enzyme specificity produced has been correlated with serotype in *H. influenzae* as serotypes c and e make only the type 2 protease, serotypes b and f only produce the type 1 protease, and serotypes a, d, and non-serotypables make either or both types of protease (96,191,192). Similar correlations between antiserum inhibition profile and serotype (122), biotype and restriction site pattern (221), and serotype and restriction site pattern (23) have been presented.

The *H. influenzae iga* genes encoding for both types 1 and 2 have been cloned by three different laboratories (23,24,85,86,129,220). Comparison of the types 1 and 2 genes showed them to be very homologous to each other with two major regions of divergence; one, near the 5' end of the gene, contained a 370 bp section that was shown to be responsible for the IgA1 cleavage site specificity (86).

Like *H. influenzae*, three separate laboratories published the identification of an IgA1 protease in *Streptococcus pneumoniae* (119,150,191). However, since those reports, the amount of progress has been less than that observed in *H. influenzae* and *N. gonorrhoeae*. Some interesting results have been presented regarding the coproduction of

glycosidases by *S. pneumoniae* and their ability to alter the migration of IgA1 cleavage fragments on IEP and SDS-PAGE analysis (117).

Streptococcus sanguis was the first microbe identified as producing an IgA1 protease (211) and much of the early IgA1 enzymology centered around this enzyme (117,135,143,214). Originally, it was thought that only some strains of the species produced IgA1 protease (~70%; 116,210) but recently, streptococcal taxonomy has been revised and it appears that 100% of strains now identified as *S. sanguis* produce IgA1 protease while those strains which were *Iga*$^-$ are now classified as *S. gordonii* (225).

The gene encoding IgA1 protease activity in *S. sanguis* has been cloned (75). Analysis suggests that it: 1) is a metallo-protease based on conservation of a gram positive bacterial zinc-binding-site sequence, 2) lacks an apparent signal sequence, yet is secreted to the periplasmic space in *E. coli*, and 3) has an unusual P-, G-, and E-rich stretch of 20 amino acids, repeated ten times (78). The classification as a metalloprotease is substantiated by inhibitor studies and by site specific mutagenesis of the proposed zinc binding site (78,214).

Two of the other oral streptococci also produce IgA1 protease, *S. oralis* (previously *S. mitior*) and *S. mitis* biovar 1 (225). Due to the reorganization of this taxonomic group, the organisms formerly classed as *S. mitior* have been redefined as *S. oralis*, all strains of which produce IgA1 protease, and the division of *S. mitis* into two biovars places the IgA1 protease producing strains into biovar 1, 20% of which produce the enzyme while none of the *S. mitis* biovar 2 strains do (225). Of interest is the observation that all of the *S. oralis* strains tested also degrade the carbohydrate moieties on IgA1 as well as proteolyzing

it into Fab_{α} and Fc_{α} (225). A similar phenomenon was observed for the two IgA1 protease producing biovar 1 strains of *S. mitis*. The authors reported that if the IgA1 preparations are pre-treated with either neuraminidase or an *S. mitis* glycosidase-enriched preparation, that IgA1 has increased susceptibility to these streptococcal IgA1 proteases while pretreatment with other commercially prepared endoglycosidases had no effect on the susceptibility of the IgA1 (225). It was reported that the *S. oralis* IgA1 protease is consistently nonimmunogenic in experimental animals (225).

Other oral pathogens involved in periodontal disease, *Bacteroides* spp. and *Capnocytophaga* spp., produce IgA1 proteases (70,114). Some strains of these species also cleave or degrade IgG and/or IgA2 (114), but other of the *Bacteroides* species (*eg. B. gingivalis*) have been demonstrated to produce nonspecific proteases, so it is not out of the question that other species of the genus may too. By definition, the IgA1 proteases cleave human IgA1 and produce intact Fab_{α} and Fc_{α} fragments, and when analyzed, some strains of the species of the *Bacteroides* and *Capnocytophaga* included in Table 5 were observed to have at least an intact $Fc_{\alpha 1}$ remaining (114). Perhaps examination of the taxonomy of these important oral pathogens will clear the picture as it did for the oral streptococci. Also, similar to the observation of low immunogenicity with the *S. oralis* protease, the IgA1 proteases of the *Bacteroides* and *Capnocytophaga* are reported to be difficult to get protease activity inhibiting titers against in experimental animals (70).

Relatively little is known about the IgA1 proteases produced by *Clostridium ramosum* and an unidentified species of *Bifidobacterium*. The

report of enzyme production in *Bifidobacterium* (72) is the only one of its kind and another report from some members of the same research group indicated that they have not been able to find other strains of *Bifidobacterium* which produce the protease (248). That same report also suggested that the ability of *C. ramosum* to produce IgA1 protease is considered to be a rare event. What is interesting about the clostridial enzyme is the fact that it not only cleaves IgA1 but it also is able to specifically hydrolyze IgA2 of the A2m(1) allotype at the same peptide bond that it hydrolyzes IgA1 (71; Figure 4).

Similarly, the IgA1 protease expressed by *Ureaplasma urealyticum* is not very well characterized other than the suggested site of IgA1 cleavage (115) and the suggestion that it may be a metalloprotease (235).

Of interest are the reports of IgA1 protease production by members of the Enterobacteriaceae, recalling that the original description of this enzyme grouping was from a fecal specimen (166,175,249,250). One research group described the unstable production of an IgA1 protease by eight different species of eight different genera isolated from patients with urinary tract infections (175). Also, two reports by another group indicates that several clinical isolates of *Proteus* species produce a specific IgA1 protease (249,250). Every *P. mirablis* and *P. penneri* strain tested cleave IgA1 outside of the hinge region as do one-half of the *P. vulgaris* strains tested (250). These enzymes are sensitive to inhibition by 5 mM EDTA which is also reminiscent of the fecal sample enzyme described by Mehta, *et al.* (166,249,250).

Roles in virulence. In addition to the studies with the gonococcal IgA1 protease and its effect(s) on virulence or immune system functions,

several similar studies have been reported for the IgA1 proteases of the other enzyme producing species, and some of these have been reviewed previously (118, 120,134). Some of these data will be summarized for the purpose of describing the proposed important role for the IgA1 proteases in the virulence of these organisms.

Several studies have described the production of the IgA1 proteases *in vivo* by either isolating the enzyme activity or IgA1 cleavage products from patient specimens including saliva (134), dental plaque (1), intestinal contents (166), cerebrospinal fluid of *H. influenzae* meningitis patients (120), and nasopharyngeal secretions (120). Bacteria with $Fab_{\alpha 1}$ bound to their surfaces have been isolated from fresh dental plaque samples (1). Bacteria (both *Iga*$^{+}$ and *Iga*$^{-}$) which were incubated *in vitro* with whole saliva also bound $Fab_{\alpha 1}$, suggesting that even species that do not produce the IgA1 protease may still encounter benefits from their production *in vivo* (1).

One of the proposed benefits that may occur via hydrolysis of IgA1 is the abrogation of the anti-adherence functions ascribed to IgA1, tested by examining the adherence of oral streptococci to hydroxyapatite, a component of dental enamel (121). One of the conclusions presented was that sIgA has no inhibitory effect on the adherence of IgA1 protease producing bacteria while that the same sIgA preparations are inhibitory for the adherence of non-protease producing strains. That study also suggested that if the sIgA is directed specifically against some undefined antigen(s), potentially an adhesin molecule, production of IgA1 protease is of no advantage for the bacteria, and their adherence is inhibited (121).

A subsequent study suggested that IgA1 protease pretreatment of the

sIgA, or of bacteria that have been incubated with sIgA, destroys the adherence inhibition function of sIgA for these oral streptococci (224). Examination of the bacteria exposed to treated sIgA indicated that they had $Fab_{\alpha 1}$ bound to their surfaces and that it may have actually been of benefit in attaching to the surface (224). A conclusion presented suggested that the production of IgA1 protease promoted bacterial adherence to these surfaces.

It is interesting that the oral streptococci which are among the first bacteria to colonize the surface of the tooth, produce one or more enzymes which inactivate one of the primary antibacterial systems in the secretions. It would also be of interest to examine this suggested cooperative effect with the streptococcal glycosidases and some of the other well characterized IgA1 proteases to see if the effect is general or specific to the streptococcal proteases.

Observations have been presented indicating that the IgA1 protease isolated from *S. oralis* is consistently nonimmunogenic in experimental animals (123,225). These observations prompt the question as to whether the proteins are truly nonimmunogenic or are the vaccinees tolerized to them prior to the trial immunization? Presumably, the test animals have been exposed to these oral streptococci their entire lives and due to continual exposure, may have become tolerized to the antigen. There are no reports of humans raising either a secretory or serum antibody titer to the oral streptococcal IgA1 proteases. Gilbert, *et al.* reported that both serum and secretory IgA which were inhibitory for the type 2 gonococcal protease had no effect on the *S. sanguis* enzyme (77). Perhaps in humans these enzymes are not reacted to by either the serum or mucosal immune systems which predisposes us to dental caries?

Experimental data from an *in vitro* virulence study of *Iga*$^+$ and *Iga*$^-$ *H. influenzae* mirrors that presented from similar experiments with *Iga*$^+$ and *Iga*$^-$ strains of *N. gonorrhoeae* (66). In the nasopharyngeal organ culture system, there is no difference in the abilities of encapsulated *Iga*$^+$ and *Iga*$^-$ *H. influenzae* to attach, damage, and invade the mucosal epithelium. Likewise, the lack of production of a capsule has no detectable influence in the tested virulence of these *Iga*$^+$ and *Iga*$^-$ strains in this *in vitro* system.

Paradoxically, it appears that the ability to produce IgA1 proteases may be of value to the oral streptococci while affording no obvious benefit to pathogens of the nasopharynx or genitourinary tract. One problem with comparing the two types of experimental systems is that in the streptococcal adherence system, the time frames for experiments are on the order of a few hours and the use of whole saliva which had been clarified by centrifugation but had not been sterilized; and in the organ culture systems, the experiments last for up to 72 hours and incorporate conditions which are essentially sterile with regard to normal microbial flora. Perhaps either the long time frames or the near-xenobiotic conditions of the organ culture systems are influencing the measured response(s), or perhaps in the streptococcal adherence system, the suspected presence of other flora is influencing the response(s).

A model has been presented for the invasion of some of the IgA1 protease producing bacteria, specifically for *H. influenzae*, but is applicable to the other invasive species (120). It has been suggested that some children who present with *H. influenzae* type b meningitis have preexisting higher serum and nasopharyngeal titers against these

organisms than children who are carriers of these strains (120). According to the model, IgA1 protease-producing bacteria are able to cleave IgA1 and inactivate its proposed Fc-mediated functions, enabling the bacteria to adhere to the mucosa and colonize a host. The host responds to the bacteria with a secretory immune response (assumed to be sIgA with some of these antibodies directed against, and inhibitory for the IgA1 protease), effectively neutralizing the pathogen and keeping its virulence in check, resulting in carrier status.

However, if an immunized host is subsequently challenged with a larger dose of the same strain, or with antigenically different strain, the bacteria are able to cleave the sIgA1, coating themselves in $Fab_{\alpha 1}$ and shielding themselves from a further immune response, facilitating the adherence and invasion which results in systemic infection.

The key difference between the two outcomes is the presence of IgA1 protease-inhibitory sIgA in the carrier host and the absence of same in the second host. This also lends additional significance to the data from Kilian's group which pointed out the great antigenic diversity observed among the *H. influenzae* IgA1 proteases (117,122,123).

One final observation on the roles of IgA1 protease in immune (dys)function concerns an observation that suggested that children with allergies might be colonized more heavily by IgA1 protease producing bacteria than those children which do not present with allergic symptoms. Kilian, *et al.* presented data from 97 children which shows that greater than sixty percent of these children with allergies have IgA1 cleavage products in their nasopharyngeal secretions while only twelve percent of the non-allergic children do (254). Both groups were reported to produce similar levels of IgA in their secretions, so the

difference is not due to altered production or lack of IgA production by either group (254). Some proposed reasons for this correlation include the inability of the cleaved sIgA1 in allergic children to exclude allergens, or perhaps the proposed down-regulatory effect of sIgA is abrogated due to the inactivated sIgA1, and that predisposes the children to an unregulated immune response allowing for reactions to innocuous allergens.

As evidence for functions of the IgA1 proteases continues to accumulate and is reviewed, it becomes apparent that these enzymes may play important roles in the pathogenic processes of several species of bacteria as well as the regulation of the "normal" immune response. It also points out that the roles of these enzymes are far from well understood and that there likely are functions for these enzymes which still need to be proposed and tested.

The amount of information that exists on the *Neisseria gonorrhoeae* IgA1 proteases is as large as that which exists for other protease-producing species, but needs further study. The observation that the enzymes are stably produced under the relatively "comfortable", nutrient-rich, conditions used *in vitro* prompted examination into the potential for previously undescribed IgA1 protease "housekeeping" functions and the potential for environmental regulation, which may relate to either normal cellular function or to virulence. The previous descriptions of an isogenic set of *N. gonorrhoeae* IgA1 protease mutants did not discuss some phenotypes such as the ability to respond to environmental stresses and the protein profiles of isolated membranes (47,131). With that in mind, the experiments and data presented in this thesis are important steps in addressing these problems.

To address these problems, a pair of *N. gonorrhoeae* deletion-disruption *iga* mutants were constructed by recombinant DNA technology. No obvious phenotypic differences in viability were correlated with the mutant allele. However, when isolated membrane protein profiles of the wild type and mutant variants were compared, differences were consistently observed. Experiments to correlate the production of the type 2 IgA1 protease with membrane protein profiles suggested that isogenic strains which fail to produce the protease also failed to insert a 56 kDa protein into their outer membranes and that one of two Δ*iga* deletion-disruption mutant strains also failed to insert a 34.5 kDa protein into the cyoplasmic membrane. The identities of these IgA1-protease associated proteins are unknown but several potential identities have been ruled out.

Data are also presented which describe new substrates for the type 2 gonococcal IgA1 protease which may later be shown to be important in either normal cellular functions or in pathogenesis. The identities and/or functions of these substrate membrane proteins are not presently known. IgA1 protease-susceptible outer membrane proteins were also demonstrated in an unrelated strain of *N. gonorrhoeae*, *Escherichia coli* and *Actinobacillus pleuropneumoniae* suggesting that the protease may play a broader role in pathogenesis than previously suggested.

Also presented are data which indicate Fe^{3+} concentration-regulation of the type 2 IgA1 protease gene. These data indicated that the observed increase in enzyme activity under low iron conditions was not due to allosteric modulation of the IgA1 protease. These data are suggestive additional evidence for the hypothesis that the IgA1 proteases are a virulence factor in *N. gonorrhoeae*. While a putative

Escherichia coli Fur-binding regulatory sequence was identified in the promoter of the *iga*2 gene, experiments to test its function in a defined *E. coli* genetic background suggested that the sequence was non-functional. Further experiments to identify a proposed *fur* homologue in *N. gonorrhoeae* were unsuccessful.

In conclusion, the data presented here give further evidence that the IgA1 protease is a virulence factor and suggest that it may play other roles in the maintenance of the gonococcal cell during growth *in vitro*.

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Chapter 1

CONSTRUCTION AND CHARACTERIZATION OF ISOGENIC IgA1 PROTEASE-DEFICIENT MUTANTS OF *Neisseria gonorrhoeae*

Russell J. Shoberg and Martha H. Mulks

Department of Microbiology and Public Health

Michigan State University

East Lansing, MI 48824 USA

ABSTRACT

This study was undertaken to determine any role(s) the type 2 IgA1 protease plays in the normal physiology of *Neisseria gonorrhoeae*. A set of site-specific deletion-disruption mutants was constructed in the cloned *iga*2 gene by standard recombinant DNA methodology and these mutated alleles were introduced into the chromosome of strain GCM 740 by transformation. The growth and membrane protein profiles of the Δ*iga* variants, GCM 740Δ4 and GCM 740Δ2, were compared to the parental strain under a number of different standard *in vitro* growth conditions. The wild type and the mutants grew at a similar rate in our standard defined broth medium. However, the outer and cytoplasmic membrane protein profiles of the mutants showed a number of differences detectable by silver-staining of SDS-PAGE gels. Two of these differences were demonstrated to be related to the Iga^{+} phenotype, as they "cured" when the mutant allele was restored to a wild type allele; the p56 outer membrane protein in both Δ-*iga* mutant variants, and a 34.5 kDa cytoplasmic membrane protein in GCM 740Δ4. Some of these unique and/or missing outer membrane proteins were surface exposed. The mutants grew and responded similarly to the wild type under anaerobic conditions and under iron limited conditions, both of which have been shown to induce specific alterations in the outer membrane protein profile. Also, the mutants and wild type were equally capable of posttranslationally processing proteins and being transformed.

INTRODUCTION

The extracellular enzymes known as IgA1 proteases are produced by a set of bacteria which also share the characteristic of being human pathogens capable of initiating infection on the mucosal epithelium (20,34,43). The enzymes are produced by both gram negative and gram positive bacteria in a variety of genera including *Neisseria*, *Haemophilus*, *Bacteroides*, *Capnocytophaga*, *Streptococcus*, *Clostridium*, and *Ureaplasma*. Thus far, only the pathogenic species of the genera *Neisseria* and *Haemophilus* have been demonstrated to produce an IgA1 protease (34,37,43). Both *N. gonorrhoeae* and *N. meningitidis* have been shown to be capable of expressing one of two described IgA1 proteases and in the case of *H. influenzae*, up to four different enzymatic activities have been demonstrated (36,38). The type of enzyme produced (*e. g.* gonococcal type 1 or type 2) is defined by the specific peptide bond cleaved in the hinge region of IgA1 and is determined by apparent molecular weights of the resultant Fc_{α} and Fd_{α} observed on SDS-PAGE (34).

The enzymes were initially defined as having a single substrate, the human immunoglobulin A1, which was later shown to also include the IgA1s produced by the African gorilla and chimpanzee (34). The theorized reason for this substrate exclusivity was proposed to be the presence of a duplicated proline-rich amino acid octamer in the hinge region of IgA1 and absent in IgA2 as well as other sequenced immunoglobulins (34,43).

Since then, the substrate list for the *Neisseria gonorrhoeae* type 2 IgA1 protease has been expanded to include the IgA1 protease proenzyme (45), some synthetic peptides with primary sequence homology to the IgA1 hinge region (62), and a set of gram negative bacterial outer and

cytoplasmic membrane proteins with undefined identities or function (chapter 2 here and 50). For those first two mentioned substrates, the specificity of the protease is at sites with reasonable sequence homology to the IgA1 hinge region. With regard to the final set of new substrates, since the proteins are not defined any further than their cellular compartment and apparent SDS-PAGE molecular weights, the amino acid sequence of each cleavage site is not known.

As stated above, the enzyme has been demonstrated to be extracellular in all species examined. A model for the secretion of the type 2 enzyme from *N. gonorrhoeae* has been proposed (45). According to this model, the primary translational product (preproenzyme) is a 169 kilodalton (kDa) molecule which via the action of a leader endopeptidase (in both *E. coli* and *N. gonorrhoeae*) loses an N-terminal leader peptide in processing and is translocated to the periplasmic space as a 166 kDa proenzyme. The C-terminus of this intermediate is referred to as the β-peptide or helper region (45 kDa) and is required for extracellular secretion. Once the C-terminus of the preproenzyme is in the periplasm, presumably the molecule exists in some conformation which favors insertion of the β-peptide into the outer membrane where it forms a pore through which the rest of the enzyme is extruded. After the preproenzyme is translocated through the outer membrane, an autoproteolytic event releases the 121 kDa proenzyme from the surface. Once the 121 kDa proenzyme is in its cell free form, it undergoes a final autoproteolytic event to release either a 12 or a 15 kDa peptide (both known as α) of unknown function. Both the 109 and 106 kDa forms are presumed to be active against IgA1. It is not known whether the autoproteolytic cleavages are *cis* or *trans*-mediated events. Conversion

from the 109 to 106 kDa forms has also been observed *in vitro* (51).

The gene coding for the *N. gonorrhoeae* type 2 IgA1 protease has been cloned by at least three different laboratories (15,21,35). A set of isogenic *Iga*$^+$ and *Iga*$^-$ variants of the gonococcal strain F62 has been constructed previously and these variants were reported to differ from the parental strain in only the ability to cleave IgA1 (21). This report also stated that there were no differences in total protein profile, in growth on a complex agar medium, or in the ability to alter piliation phenotype and colony color morphotype. A subsequent report failed to demonstrate any differences in the mutant's ability to infect and invade a human fallopian tube organ culture model system (10). However, our laboratory subsequently reported that these two variants possess different outer and cytoplasmic membrane protein profiles when propagated in a defined *in vitro* broth medium (40).

The ability to produce an IgA1 protease has been proposed to be a virulence factor for several years (20,34,43). There is a wealth of indirect evidence to support this hypothesis but there has yet to be a direct comparison of a set of isogenic *iga* variants in an appropriate model system (*i. e.* human or chimpanzee) to answer the question of its contribution to virulence. Some of the indirect evidence includes: 1) only the pathogenic species of a genus produce the enzyme; 2) only pathogens of the human mucosal epithelia produce the enzyme; 3) the enzyme has been demonstrated to be active *in vivo* by isolation of $Fc_{\alpha 1}$ (28) and active enzyme from patient specimens (34,43); 4) infected patients exhibit an antibody response to the enzyme, and these antibodies are inhibitory for enzyme activity (13); and 5) recently, fresh samples taken from dental plaque have demonstrated the presence of

$Fab_{\alpha 1}$ on the surface of oral streptococci, and after incubation of the non-IgA1 protease producing *S. gordonii*, in whole saliva, observed cleaved IgA1 $Fab_{\alpha 1}$ on its surface (1).

Some of the better characterized gonococcal virulence factors are major proteinaceous components of the outer membrane. Most often, these have been described from bacteria grown in either complex or defined *in vitro* laboratory media under standard conditions of increased CO_2 tension (5-10%) at 35-37°C. The outer membrane proteins relevant to these studies are discussed below. There are also some outer membrane components which have been described in detail which are induced or repressed by relevant environmental stresses. These will be discussed later in this section.

The major gonococcal outer membrane protein is the porin protein (Por, formerly called PI; 16) which is the basis of a serotyping scheme used in characterizing strains for epidemiological research (58). There are two major Por serotypes, PorA and PorB. These proteins are neither phase nor antigenically variable within an isolate. Por has an apparent molecular weight of ~34-38 kDa by SDS-PAGE and is neither heat nor reduction modifiable (57). There are no other described porin proteins in the gonococcus. A role in virulence has been proposed as the Por proteins are known to be targets of serum bactericidal and opsonic antibodies (17,47).

Another component of the gonococcal pore is a 34 kDa protein known as Rmp (formerly PIII, 16). The acronym Rmp refers to its reduction modifiable behavior on SDS-PAGE (26,57). Rmp is invariant with regard to apparent molecular weight or antigenic type among all gonococcal strains tested. Rmp is antigenically related to the *E. coli* porin

protein, OmpA (3). This relationship gives it a passive role in virulence as anti-OmpA antibodies have been demonstrated to function as blocking antibody and prevent complement activation via the classical pathway (46).

A third major protein component of the outer membrane which has been the subject of intense study is the set of 24-30 kDa proteins referred to as Opas (opacity, 16) and previously called PIIs. Opas exhibit heat-modifiable behavior on SDS-PAGE, increasing in apparent molecular weight after denaturation at 100°C relative to that of Opas denatured at lower temperatures (26,57). The term opacity proteins refers to the correlation between the color or opacity of a colony grown on agar and the expression of these surface exposed, phase variable proteins (55). Opas are also antigenically variable (48). Their functional role in virulence has been suggested as an adhesin (23).

There are a number of environmental stress-induced outer membrane proteins that are also implicated in virulence of the organism. With regard to iron-stress, outer membrane proteins of 104 or 103; 97; 88 or 86; 80 or 79; 76, 74, or 73; 70; 45 or 41; 37 or 36; 29; 25; 23; 20.5; and 19.5 or 19.0 kDa are inducible under iron-limited conditions (19,29,30,41,60). Numbers with "or" between them refer to the same protein described in different laboratories. Of this set of proteins, the only one with a described function is that of 36 or 37 kDa (aka Fbp and Mirp; 16) which has been demonstrated to bind Fe^{3+} in a specific, reversible manner (32). There are reported to be specific outer membrane receptors for lactoferrin and transferrin, two human iron binding proteins, but no particular molecular weight has been reported for either receptor in the gonococcus (4,24). These receptor proteins

would function in the essential role of iron acquisition from the host and therefore would be vital for virulence. Presumably, at least some of these iron-repressible outer membrane proteins are expressed *in vivo*, as convalescent human serum shows immunoreactivity with them, including Fbp (11).

When *N. gonorrhoeae* is propagated under anaerobic conditions, a set of oxygen-repressible outer membrane proteins is expressed (Ani, 16; previously Pan) and a set of oxygen-inducible outer membrane proteins is repressed (Oxi, 16; previously Pox, 7). Anis 1-3 have been defined as having molecular weights of 54, 46, and 31 kDa while Oxis 1-5 have defined molecular weights of 62,27, 27, 22, and 16.5 kDa. At least one of the Anis is expressed *in vivo* as patient sera from uncomplicated infections and cases of pelvic inflammatory disease show reactivity with Ani1 (8). Although there are no known functions for any of the Ani or Oxi proteins, they are implicated in virulence due to the fact that there is an antibody response raised against at least one of them.

Compared to the outer membrane, very little is known about the identities of any of the proteins which are part of the cytoplasmic membrane. In fact, other than a report on the specific density, only one other paper describes the protein components (18,31).

Although a set of variants had been previously constructed and described, we felt that there were more questions to be addressed than those referred to in the previous report (21).

We decided to address the question as to what other functions the IgA1 protease might perform for the gonococcus *in vitro* by constructing a set of isogenic *Iga*$^+$ and *Iga*$^-$ variants with a defined background that we could consider an IgA1 protease-deficient phenotype. The gonococcus

expresses IgA1 protease activity constitutively *in vitro* (and presumably *in vivo*) and has never been demonstrated to lose the ability to produce the enzyme *in vitro*, even after repeated subculture. Therefore, we hypothesized that the IgA1 protease had some required function *in vitro*. We compared the relative abilities of the wild type (WT) and mutant variants to grow in a standard defined medium for the gonococcus, to posttranslationally process proteins, to be transformed, to shift colony color and morphotype, and also compared the membrane protein profiles.

Due to our previous observation that IgA1 protease-susceptible proteins were present in the outer membrane of the gonococcus, and the fact that the enzyme has been shown to function in the extracellular milieu, we chose to concentrate on the role of the enzyme in outer membrane protein profile responses *in situ*. Some of the other questions that we attempted to answer involved the potential role of the IgA1 protease in maturation of the membrane protein profiles and in modification of those profiles in response to relevant environmental stimuli such as iron limitation and growth under anaerobic conditions. These two environmental stress stimuli are particularly relevant given the first niche that the gonococcus occupies in the human host. The male urethra and the female vagina would have very low levels of available iron due to the secretion of lactoferrin by the mucosal epithelium (27). Due to the presence of normal anaerobic microbial flora in the genitourinary tract and cervix, these microenvironments are also considered to be anaerobic.

A second set of questions that we attempted to address was, would the gonococcal protein secretion apparatus be confused or disrupted by translation of truncated Δ-*iga*2 gene products? We attempted to

determine whether these truncated polypeptides would be detectable in the membranes of the organism and if their synthesis interfered with the normal secretion of other membrane proteins.

[Portions of this work have been presented at the 6th. International Pathogenic Neisseria Conference (40) and at the 90th. Annual meeting of the American Society for Microbiology (49).]

MATERIALS AND METHODS

Bacterial strains. The *Neisseria gonorrhoeae* strain GCM 740 is a prototrophic, type 2 IgA1 protease producing (genotype *iga*2b), serotype PorIB2 clinical isolate which has been described previously (39). The Δ-*iga*2 isogenic variants GCM 740Δ2 and GCM 740Δ4 were constructed in this laboratory via deletion mutagenesis and insertion of a β-lactamase gene cassette. Construction of these isogenic variants has been presented previously (40). The revertant variants, GCM 740Δ2R-2, GCM 740Δ2R-40 and GCM 740Δ4R-83, were constructed in this laboratory by transformation of the Δ*iga*2 variants with the cloned *iga*2b gene. Construction of the Δ-*iga*2 isogenic variants will be described in a separate section of the Materials and Methods.

The *Escherichia coli* strains DH1 and HB101 were used in the manipulation of recombinant plasmids and have been described (25). The *E. coli* strain Q358 has also been described previously and was used as a host for the recombinant phage, λ710 EMBL3. This recombinant bacteriophage contained gonococcal insert DNA which encoded IgA1 protease activity in a lysate and has also been described (35). A listing of plasmid vectors and recombinant *iga*2b plasmid constructs is given in Table 1 and restriction maps of the various constructs are

Table 1. Properties of plasmids constructed and used.

Plasmid	Insert size[a]	*Iga2*[b]	*Bla*[c]	Source
pRJ1	4.3	-	R	This study
pRJ2	9.4	+	R	This study
pRJ21	9.4	+	S	This study
pRJ3	6.6	+	R	This study
pRJ21Δ1	8.6	-	S	This study
pRJ21Δ2	8.3	-	S	This study
pRJ21Δ2A^r	10.7	-	R	This study
pRJ21Δ3	7.1	-	S	This study
pRJ21Δ4A^r	7.5	-	R	This study
pBR322	NA[d]	NA	R	This laboratory
pBR322'	NA	NA	S	This study
pLES2	NA	NA	R	V. L. Clark (54)
pNal6	10	NA	S	D. C. Stein (52)

a = Insert size in kilobase pairs

b = IgA1 protease phenotype denoted as follows: +, *Iga2*$^+$; -, *Iga2*$^-$.

c = Beta-lactamase phenotype denoted as follows: R, resistant; S, sensitive.

[d] NA, not applicable.

presented in Figure 1.

Media. *N. gonorrhoeae* was usually propagated on GC Base medium (Difco Laboratories, Detroit, MI) containing Kellogg's supplement (61) at 37°C in a 5-10% CO_2 atmosphere. If it was necessary to propagate the bacteria in broth, the bacteria were grown in the defined medium of Morse and Bartenstein (33). Hereafter, these media will be referred to as GCB and NEDA, respectively.

For selection and antibiotic sensitivity characterization of Δ-*iga*2 variants, gonococci were propagated on GCB containing either nalidixic acid at 1.0 μg per ml or penicillin G at 0.025 μg per ml. All antibiotics used in these studies were from Sigma Chemical Co. (St. Louis, MO).

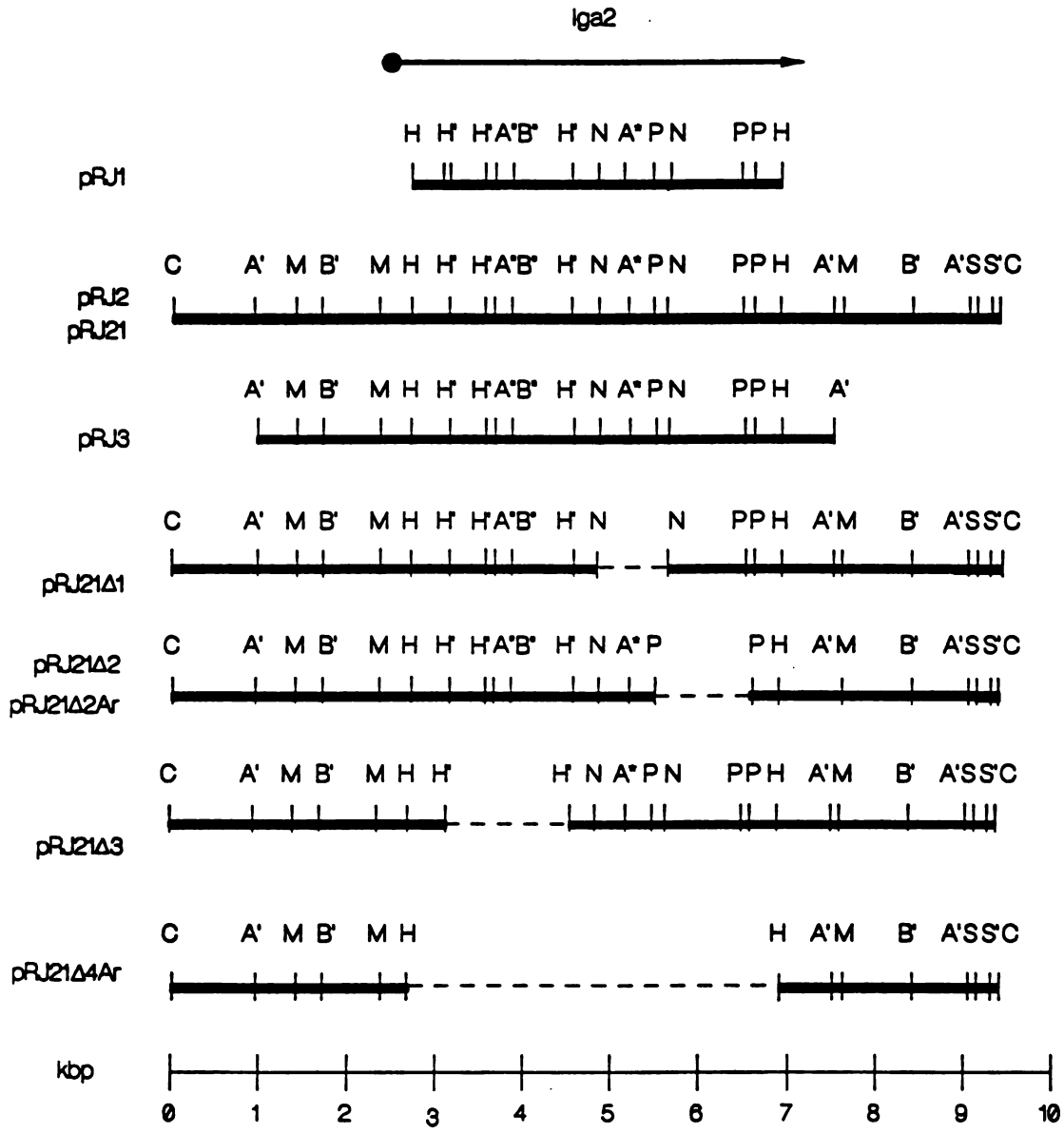
E. coli variants were propagated in LB broth or on LB agar plates (25). When required for selective purposes, ampicillin or tetracyline was added to the medium at 50-100 μg per ml and 12.5 μg per ml, respectively. Propagation of λ710 EMBL3 in *E. coli* Q358 was in LB broth containing 0.2% maltose.

Subcloning of the *iga*2b gene. The recombinant bacteriophage λ710 EMBL3 was identified by plaque hybridization (25) using a cloned *iga* gene from *Haemophilus influenzae* (6) as a probe. A restriction site map was determined and presented by Mulks and Knapp (35). DNA modification enzymes and restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD), Boehringer Mannheim Biochemicals (Indianapolis, IN), or New England BioLabs, Inc. (Beverly, MA) and were used as recommended by the suppliers.

Using this restriction site data, portions of the insert DNA from λ 710 EMBL3 were subcloned into pBR322 as a series of plasmids (pRJ1,

Figure 1. Restriction site maps of recombinant plasmids constructed in this study. Restriction sites are indicated as follows: A*, *Acc*I; A', *Ava*I; A", *Ava*II; B', *Bgl*I; B", *Bgl*II; C, *Cla*I; H, *Hin*dIII; M, *Mlu*I; N, *Nsi*I; P, *Pst*I; S, *Sma*I; and S', *Sal*I. The direction of transcription is as reported previously (45) and confirmed in our laboratory.

Figure 1


lga2
H H' H'A*B* H' N A* P N PP H
pRJ1
C A' M B' M H H' H'A*B* H' N A* P N PP H A'M B' A'S S'C
pRJ2
pRJ21
A' M B' M H H' H'A*B* H' N A* P N PP H A'
pRJ3
C A' M B' M H H' H'A*B* H' N N PP H A'M B' A'S S'C
pRJ21Δ1
C A' M B' M H H' H'A*B* H' N A* P P H A'M B' A'S S'C
pRJ21Δ2
pRJ21Δ2Ar
C A' M B' M H H' H' N A* P N PP H A'M B' A'S S'C
pRJ21Δ3
C A' M B' M H H A'M B' A'S S'C
pRJ21Δ4Ar
kbp
0 1 2 3 4 5 6 7 8 9 10

pRJ2, and pRJ3), in order to allow for the study of the IgA1 protease gene in *E. coli* on a multicopy vector. Transformation of *E. coli* HB101 was by the $CaCl_2$ method (9). Restriction site maps are presented in Figure 1 and pertinent information is summarized in Table 1.

Qualitative IgA1 protease assays were performed as described by Plaut, *et al.* (44). Briefly, experimental samples were incubated from one to twelve hours at 37°C with four volumes of a reaction mix comprised of ^{125}I-labeled human IgA1 in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.5% bovine serum albumin (w/v). Reactions were terminated by addition of four volumes of SDS-PAGE sample buffer (22). Samples were boiled for five minutes and then resolved over 9.0% SDS-PAGE (22). Reaction products (Hc_α, Fc_α, and Fd_α) were identified by autoradiography at -70°C with an intensifying screen and X-Omat AR film (Eastman Kodak Co., Rochester, NY).

Construction of Δ-*iga* mutants in *E. coli*. The schema used to construct the Δ-*iga*2b mutants are summarized in Figure 2. This first involved the conversion of pBR322 (Amp^r) to pBR322′ (Amp^s) by frameshift mutagenesis; this was accomplished by linearization of pBR322 with *Pst*I, blunt ending with the Klenow fragment of DNA polymerase plus dNTPs, and religation with T4 DNA ligase. *E. coli* HB101 was transformed with the ligation mix and propagated on LB containing tetracycline. Mutated plasmids were identified by growth of the transformed strain on LB containing tetracycline and failure to grow on LB containing ampicillin. Site specificity of the mutation was confirmed by failure to be cleaved by *Pst*I, assayed visually by gel electrophoresis.

To allow for further selection based on ampicillin resistance/sensitivity, pRJ21 was constructed by subcloning the 9.4 kbp

Figure 2. Diagram depicting the steps involved in construction of the various recombinant deletion-disruption plasmids used in this study.

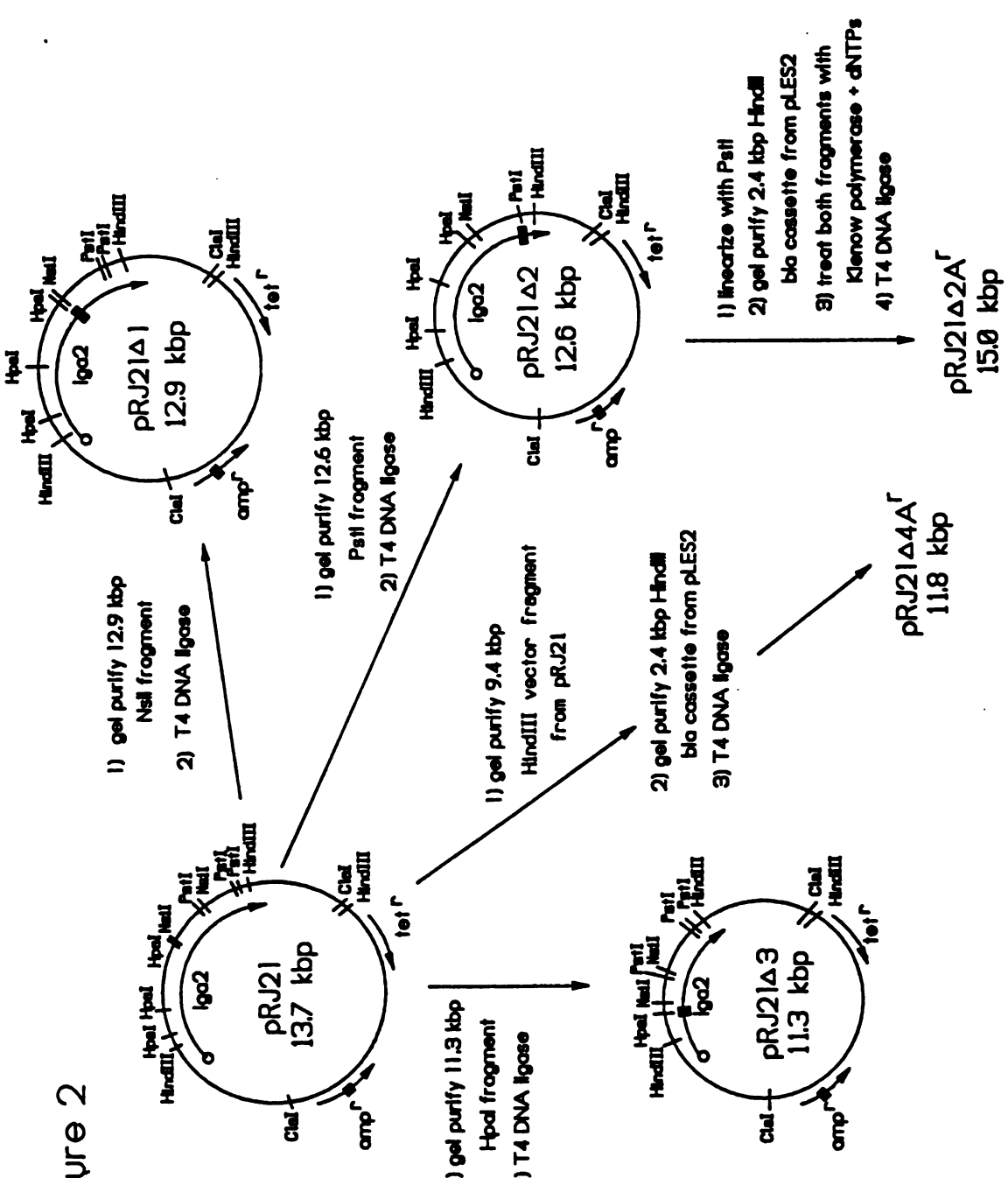

pRJ21
13.7 kbp
pRJ21Δ1
12.9 kbp
pRJ21Δ2
12.6 kbp
pRJ21Δ3
11.3 kbp
pRJ21Δ4A^r
11.8 kbp
pRJ21Δ2A^r
15.0 kbp
1) gel purify 12.9 kbp NsiI fragment
2) T4 DNA ligase
1) gel purify 12.6 kbp PstI fragment
2) T4 DNA ligase
1) gel purify 9.4 kbp HindIII vector fragment from pRJ21
1) gel purify 11.3 kbp HpaI fragment
2) T4 DNA ligase
2) gel purify 2.4 kbp HindIII blo cassette from pLES2
3) T4 DNA ligase
1) linearize with PstI
2) gel purify 2.4 kbp HindIII blo cassette from pLES2
3) treat both fragments with Klenow polymerase + dNTPs
4) T4 DNA ligase
lac2
tet^r
amp^r

Figure 2

*Cla*I-bounded insert from pRJ2 (*Iga2*$^{+}$) into the unique *Cla*I site of pBR322'. Ligation mixes were used to transform *E. coli* DH1 and recombinant constructs were identified by colony hybridization using the 4.3 kbp *Hin*dIII fragment of pRJ2 as a probe (25). This construct served as the basis for all subsequent deletion mutants.

Deletion mutants were constructed by digestion of pRJ21 with either *Hpa*I, *Nsi*I, or *Pst*I followed by elution of the appropriately sized band from agarose gels, religation, and transformation of *E. coli* DH1 with selection on LB containing tetracycline. DNA was prepared from the transformants by an alkaline lysis miniprep procedure and analyzed for loss of the appropriate restriction sites by endonuclease digestion and agarose gel electrophoresis. Mutants constructed in this manner were pRJ21Δ1 (deleted 0.8 kbp *Nsi*I fragment), pRJ21Δ2 (deleted 1.1 kbp *Pst*I fragment), and pRJ21Δ3 (deleted 2.3 kbp *Hpa*I fragment). Figure 1 gives a graphic representation of the restriction sites used to generate these mutants.

In order to mark these deleted constructs with a phenotype which could be used to select for eventual gonococcal transformants, a scheme described by Koomey, *et al.* was utilized (21). This involved insertion of a β-lactamase gene (*bla*) cassette at the original sites of deletion and the schema are summarized in Figure 2. The *bla* cassette was prepared initially from pLES2, an *E. coli-N. gonorrhoeae* shuttle vector (54), by gel purification (25) of the 2.4 kbp *Hin*dIII fragment. The *Hin*dIII termini were blunted by treatment with Klenow DNA polymerase plus dNTPs, and this blunt ended fragment was ligated into the similarily blunt ended site of the recipient Δ-*iga*2 construct.

A notable exception to this scheme was the construction of

pRJ21Δ4A^r. This mutant construct was generated by performing a partial *Hin*dIII restriction digest on pRJ21, elution of the ~10 kbp band from an agarose gel (25), and direct ligation of the 2.4 kbp *Hin*dIII *bla* gene cassette, utilizing the cohesive ends of each fragment. *E. coli* DH1 was transformed with recombinant constructs selected on LB containing ampicillin. Due to the presence of the complicating *Hin*dIII site in the vector, and the presence of cohesive termini, this strategy was used rather than the blunt end ligation scheme described above.

Transformation of *N. gonorrhoeae* GCM 740 to *Iga2*$^-$. Initially, several attempts were made to transform the gonococcal WT strain GCM 740 with the band purified, 10.7 kbp insert of pRJ21Δ2A^r by a standard procedure (2). Prior to all transformations, a T1 colony morphotype was selected and expanded on GCB to ensure competence. Competent gonococci were resuspended in NEDA and one microgram of purified DNA was added. After an incubation period at 37°C, the bacteria were plated onto sterile membrane filters (Millipore Corp., Bedford, MA) preplaced on warm GCB plates and the bacteria were incubated at 37°C under 5-10% CO_2 for a 6 hour expression period. Following the expression period, the membranes were aseptically transferred to a warm GCB plate containing 0.025 μg per ml penicillin G and reincubated at 37°C for 48-72 hours. At this time, penicillin resistant colonies were identified and selected for further testing, including IgA1 protease production (12,44), penicillin resistance, and restriction mapping by Southern analysis (25).

Due to difficulties with the timing of the expression period, the consistency of penicillin G concentrations in the lots of media, and the inability to recover transformants with the desired constellation of

phenotypes, an alternate selection procedure was employed to identify putative transformants. As described above, competent gonococci were selected and expanded on GCB, and were incubated at 37°C with the transforming DNA (the 7.9 kbp *Cla*I insert of pRJ21Δ4A^r or the 10.7 kbp *Cla*I insert of pRJ21Δ2A^r). The expression period however, involved spreading of the transformation mix directly onto the surface of a GCB plate and a 6 hour incubation at 37°C under 5-10% CO_2. Following this expression period, 0.1 ml of a 10 μg per ml penicillin G solution was spread over the central one-half of the plate. The plate was reincubated as above, and the pencillin G was allowed to form a diffusion gradient. At 48-96 hours after application of the antibiotic, penicillin resistant colonies were observed growing in the zone between the site of application and the periphery. These putative transformants were selected and tested further for IgA1 protease production (12,44) and penicillin resistance, and were restriction mapped by Southern analysis (25).

Preparation of bacterial membranes. Membranes were prepared from bacteria grown to late exponential phase in NEDA by the sucrose-lysozyme-EDTA spheroplasting method (42). Spheroplasts were disrupted by ultrasonication and membranes were separated by isopycnic sucrose density gradient ultracentrifugation (18). Membrane protein concentrations were determined by the Bradford dye binding assay (5) using a commercial kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, CA).

Membrane protein profile analysis. Protein profiles were analyzed by separation of 3 μg of membrane proteins per lane over 7.5-12.5% linear polyacrylamide gradient SDS-PAGE (22) and a combination Coomassie blue-

silver staining procedure (14). Specific, described gonococcal outer membrane proteins were identified by their relative abundance and mobility, and in some cases by their heat- or reduction-modifiable behavior (26,57). Apparent molecular weights were determined by comparison to mobilities of a set of molecular weight standards (Bio-Rad).

Assessment of surface exposed outer membrane proteins. To determine whether any of the proteins which differed between the variants were exposed on the surface of the bacterium, we radiolabeled the surface exposed proteins using $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) and lactoperoxidase (Sigma) by the procedure of Swanson (56). Briefly, a 24 hour culture of gonococci were swabbed off a GCB plate and washed once with phosphate buffered saline (PBS). Cells were resuspended in PBS, 100 μg of lactoperoxidase in PBS and 100 μCi of $Na^{125}I$ were added, followed by 40 μl of 0.6% H_2O_2 (v/v). Additional H_2O_2 was added at three minute intervals until fifteen minutes, when an excess volume of PBS containing 5 mM cysteine was added to terminate the reaction. Cells were pelleted by centrifugation and washed once in PBS plus cysteine prior to final resuspension in PBS. Specific activity as cpm per ml was determined by direct counting of aliquots in a Gamma5500B gamma counter (Beckman Instruments, Inc., Fullerton, CA). Analysis was performed by separation on 7.5-12.5% SDS-PAGE gels, Coomassie blue staining, and autoradiography of dried gels. Identification of described gonococcal outer membrane proteins was accomplished by relative abundance and mobilities, and/or heat- or reduction-modifiable behavior as described above.

Growth of Δ-*iga*2 mutant gonococci under anaerobic conditions. The methodology described by Clark, *et al.* was used to propagate this set of variants on GCB in an anaerobic environment (7). Gonococci were inoculated on GCB with a 1.5 cm filter paper disc (Whatman, Inc., Clifton, NJ) placed at the center of the plate. The disk was inoculated with 120 μl of 1 M $NaNO_2$ and the plate was placed in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD). The jar was sealed and filled with an anaerobic gas mixture containing 5% CO_2 (BBL). Incubation was at 37°C with growth assessed at 48 hours.

Outer membranes were prepared from 24 hour cultures of anaerobically grown bacteria as well as from bacteria grown under standard aerobic conditions by the sucrose-lysozyme-osmotic shock procedure of Clark, *et al.* followed by ultrasonication and extraction of outer membrane proteins by Na n-lauroylsarcosine (7). Membrane protein concentrations were determined and analysis of outer membrane proteins was performed as described above.

Growth of Δ-*iga*2 mutant gonococci in iron-limited media. Two methods of rendering media iron-limited were utilized. One involved the extraction and removal of ferric iron from NEDA by the chelator 8-hydroxyquinoline (8-HQ; Sigma; 59) and the second involved chelation of ferric iron in NEDA by the siderophore, deferrioxamine B mesylate (Desferal; CIBA-GEIGY Corp., Summit, NJ). Gonococci have previously been shown to be unable to assimilate iron chelated by Desferal (41).

Ability to grow and growth rate under conditions of iron limitation were assessed by growth in 8-HQ extracted NEDA. The ability to express previously described iron-repressible outer membrane proteins was determined by preparation of outer membranes as described above and

separation of the cytoplasmic and outer membranes by isopycnic sucrose density gradient ultracentrifugation. Outer membrane protein concentrations were determined, and analyses of membrane protein profiles were performed as described above.

Pulse-chase analysis of wild type and Δ-*iga*2 mutant gonococcal proteins. Gonococci were passaged twice in methionine-deficient NEDA and radiolabeled in a 25 ml culture during either mid-exponential phase or in stationary phase with ^{35}S-Met (20 μCi per ml). Labelling was accomplished with a one minute pulse followed by a chase period from 0-120 minutes with excess unlabeled methionine (0.1 mM or 5000-fold excess). At intervals, 1 ml aliquots of bacteria were removed and disrupted in an equal volume of cold 10% trichloroacetic acid (w/v). The precipitate was recovered by 15 minutes of centrifugation at 10,000 xg in a microfuge (Brinkmann Instruments, Inc., Westbury, CT). Insoluble pellets were washed twice with cold 5% trichloroacetic acid and twice more with a cold ethanol:ether (1:1) solution to remove the trichloroacetic acid with excess solvent drained after each centrifugation step. After the final centrifugation, excess solvent was drained and evaporated overnight at 37°C. Dried pellets were resolubilized in SDS-PAGE sample buffer (22) and if necessary, neutralized with 1 N NaOH, boiled for five minutes and centrifuged for two minutes to remove any insoluble debris. Incorporated radioactivity was quantitated by liquid scintillation spectrophotometry in a Beckmann LS counter using EcoLume aqueous scintillation cocktail (ICN). Equivalent amounts of radiolabeled protein were loaded in each lane of a 7.5-12.5% SDS-PAGE gel as described above. Resolved proteins were identified by Coomassie blue staining and autoradiography of dried gels

at room temperature.

Restoration of the Δ-*iga*2 loci to wild type. As *N. gonorrhoeae* is transformable with a frequency of 0.2-1.0% (2,52), two different procedures were employed in an attempt to identify the nonselectable, WT transformants from the population of gonococci in a transformation mix.

Initially, competent *Iga*$^-$ gonococci (GCM 740Δ4 and GCM 740Δ2) were transformed as per Atkinson and Mulks (2) with the gel purified 9.4 kbp *Cla*I insert from pRJ2. Appropriate dilutions were plated directly onto GCB to yield 100-200 colonies per plate. Isolated colonies were transferred in duplicate to GCB and to GCB containing 0.025 μg per ml penicillin G in an attempt to identify the desired Pen^r to Pen^s transformants.

A screening variation utilizing the IgA1 protease plate assay described by Gilbert and Plaut was also attempted (12). Following transformation as described above, dilutions of cells were plated on GCB and allowed to grow for 24 hours. At that time, a top agar overlay containing ^{125}I-labeled IgA1 (immobilized on polyacrylamide beads by anti-human α chain; Bio-Rad) was applied. After a digestion period of 2 hours at 37°C, a nitrocellulose membrane (BA85; Schleicher & Schuell, Inc., Keene, NH) was applied. Following a diffusion period of 20-30 minutes at 37°C, the membrane was removed, washed twice in PBS, and air dried. The membrane was exposed to X-ray film overnight at -70°C with an intensifying screen. By aligning the membrane, the autoradiograph, and the original plate, putative IgA1 protease producing colonies were identified, picked through the overlay, and transferred to GCB plates for expansion.

A second method of recovering the desired transformants was

attempted in order to increase the probability of screening gonococci that had been transformed. Gonococci can be simultaneously transformed with multiple pieces of DNA by a process termed congression (52). This trait was exploited in order to reduce the number of colonies that needed to be screened to isolate the desired restored phenotype. A congression experiment using a limited amount of the non-linked nalidixic acid resistance gene and an excess amount of the non-selectable pRJ2 DNA, was performed, similar to that described by Stein (52). Briefly, competent gonococci were resuspended in GC broth containing 10 mM $MgCl_2$ and 0.042% HCO_3^- (53) and a predetermined, limiting amount of pNal6 DNA (the cloned Nal^r gene) plus microgram quantities of pRJ2 were added. Following a 30 minute transformation period at 37°C, DNAaseI (Sigma) was added to 50 μg per ml, and the mix was reincubated at 37°C for a 6 hour expression period, after which dilutions were spread directly onto GCB plates containing 1.0 μg per ml nalidixic acid. After 24 hours of incubation at 37°C under 5-10% CO_2, Nal^r colonies were identified and transferred in duplicate to GCB plates for direct IgA1 protease plate assays.

IgA1 protease producing, Nal^r isolates were confirmed by single cloning of isolated colonies and subsequent testing of antibiotic resistance/susceptibility as well as IgA1 protease phenotype. Isolates which were identified as being the desired revertants were restriction mapped by Southern blot analysis with the 4.3 kbp *Hin*dIII *iga*2b gene fragment and the 2.4 kbp *Hin*dIII *bla* gene cassette to verify that the locus had been restored faithfully.

Transformants of the desired genotype were analyzed by comparison of prepared outer and cytoplasmic membranes with those of the initial

parental type (GCM 740) and the intermediate Δ-*iga*2 mutants (GCM 740Δ2 and GCM 740Δ4). Membranes were prepared by procedures described above involving separation of outer and cytoplasmic membranes by ultracentrifugation over isopycnic sucrose density gradients. Comparison of resolved membrane protein profiles was by analysis of Coomassie blue-silver stained 7.5-12.5% SDS-PAGE.

RESULTS

Cloning of the *iga*2b gene and construction of Δ-*iga* mutants. Three plasmid constructs were generated which contained insert DNA corresponding to portions of the *iga*2b gene. Two of these produced active IgA1 protease (pRJ2 and pRJ3; inserts of 9.4 kbp and 6.6 kbp, respectively; Figure 1) while the third, pRJ1, contained ~90% of the structural gene (insert of 4.3 kbp), presumably lacking the promoter (45).

Four different deletion mutant constructs were generated. One construct, pRJ21Δ3, deleted 2.4 kbp in the 5′ half of the gene, presumably covering the active site (Figure 1; 45). Two of the constructs, pRJ21Δ1 and pRJ21Δ2, each deleted ~17-25% (0.8 kbp and 1.1 kbp, respectively) of the gene covering the approximated junctions of the structural protease domain, the α domain, and the β domain (Figure 1; 45). One further deletion was generated (pRJ21Δ4, 4.3 kbp deleted), which removed ~90% of the structural protease domain, the entire α domain and most of the β domain (Figure 1; 45). As predicted, all of these constructs failed to produce active IgA1 protease as assayed using cell free supernatants and extracts of sonicated whole cells (data not shown).

Two of these deletion constructs were successfully interrupted with the *bla* gene cassette and introduced via transformation into GCM 740. These were pRJ21Δ2A^r and pRJ21Δ4A^r, with their recipient variants being designated GCM 740Δ2 and GCM 740Δ4, repectively (Figure 3). Disruption of pRJ21Δ1 and pRJ21Δ3 with the *bla* cassette was not successful and therefore, these constructs were not transformed into the gonococcus.

Characterization of the Δ-*iga* mutants. When the growth rates of these two mutant gonococcal variants were compared with that of the parental WT, GCM 740, in a defined medium (Figure 4), no differences were observed. During propagation on GCB, colony color and morphotype changes were observed indicating that the Δ-*iga* mutants were capable of undergoing both pilin and Opa phase variations (data not shown).

Numerous profile differences were observed when the isolated outer and cytoplasmic membranes of the mutant variants were compared with those of GCM 740. Figures 5 and 6 depict representative stained SDS-PAGE gels of these comparisons for outer and cytoplasmic membranes, respectively.

GCM 740 outer membranes contained unique bands of 130, 88, 48, and 25 kDa (Figure 5., lane 1) which were not present in either GCM 740Δ4 or GCM 740Δ2 (Figure 5, lanes 2 and 3). GCM 740Δ4 was also observed to lack a band at 94 kDa, present in both the WT and in the other mutant, GCM 740Δ2. GCM 740Δ2 was observed to lack bands of 34, and 26 kDa that were present in both GCM 740Δ4 and the WT, and to contain a unique band of 28 kDa which was missing in the WT and in GCM 740Δ4. When the set of isogenic *Iga* variants constructed by Koomey *et al.* was compared, there were no differences observed in protein profile between the WT and the mutant (data not shown;40).

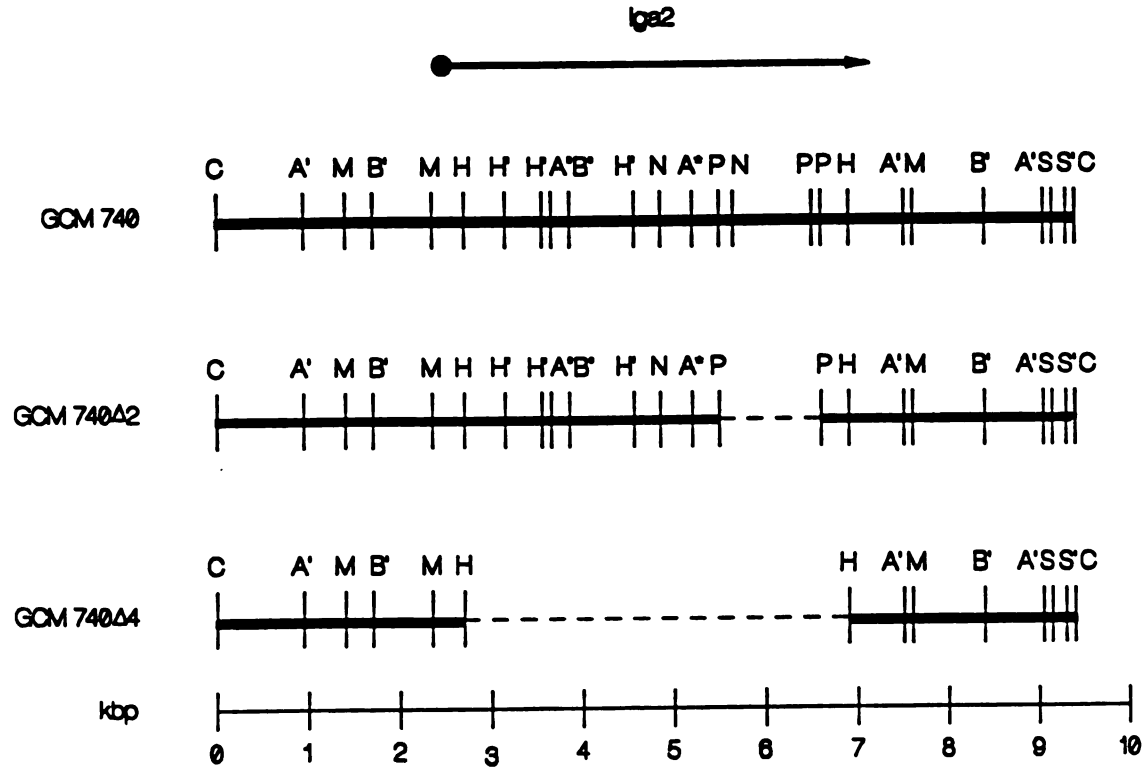


Figure 3. Restriction site maps of the region around the *iga*2b locus in strain GCM 740 and the Δ-*iga*2b loci of its isogenic strains GCM 740Δ2 and GCM 740Δ4. Sites are designated as follows: A*, *Acc*I; A′, *Ava*I; A", *Ava*II; B′, *Bgl*I; B", *Bgl*II; C, *Cla*I; H, *Hind*III; M, *Mlu*I; N, *Nsi*I; P, *Pst*I; S, *Sma*I; and S′, *Sal*I.

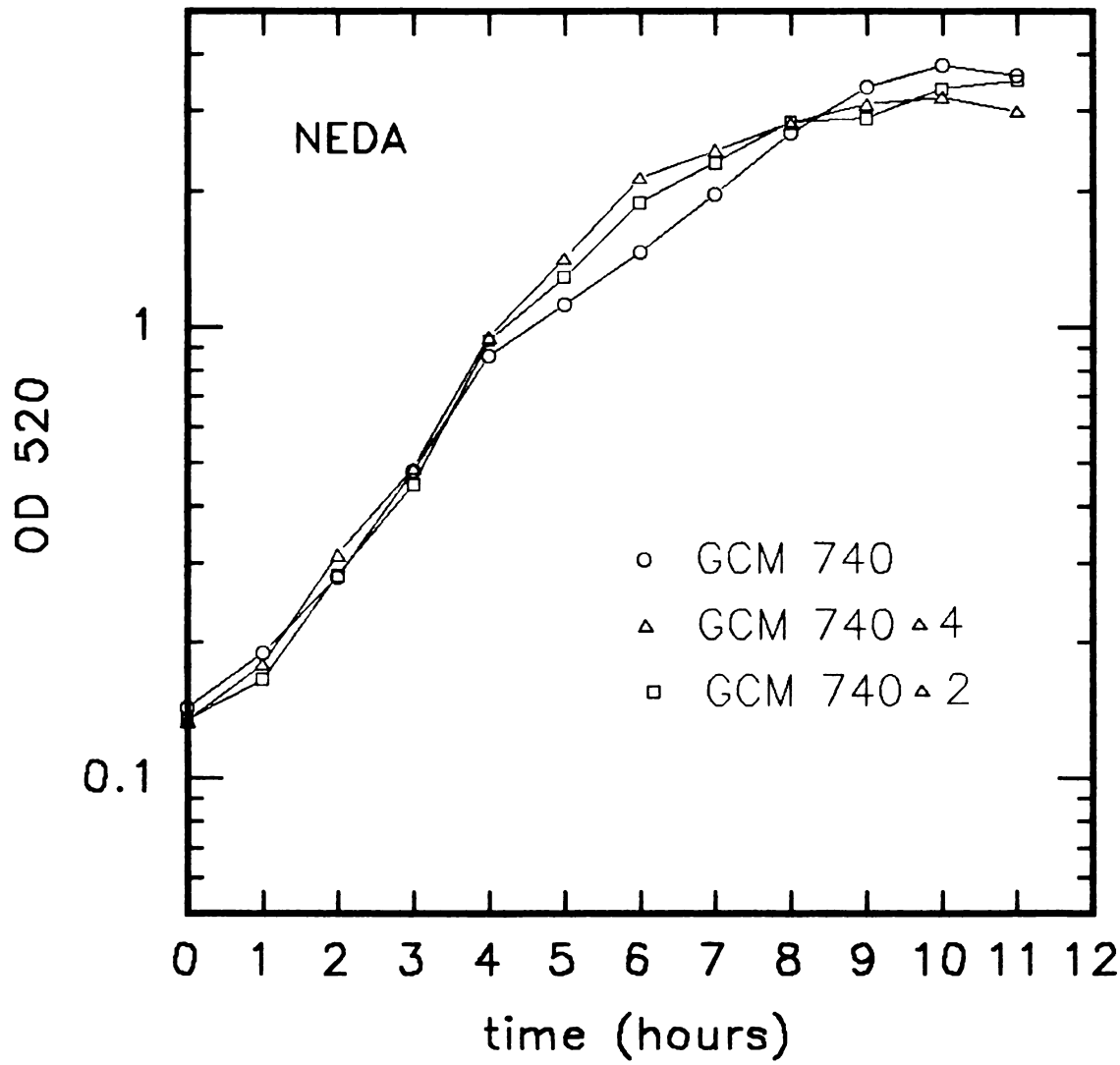
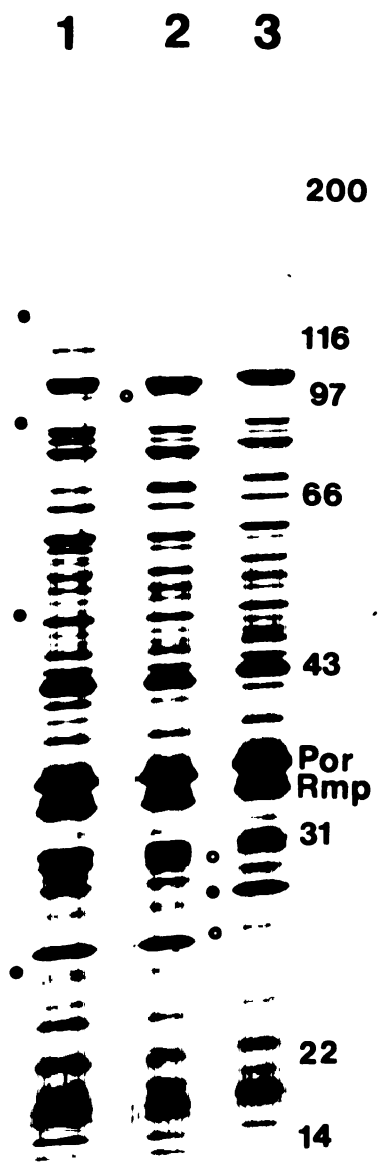


Figure 4. Growth rates of *N. gonorrhoeae* GCM 740 and two isogenic Δ*iga*2 mutants in a defined broth medium (33) at 37°C.

Figure 5. Coomassie blue-silver stained SDS-PAGE gel of isolated outer membranes of GCM 740 and its two isogenic Δ-*iga*2 mutants, GCM 740Δ4 and GCM 740Δ2. The gel contains three micrograms of outer membrane proteins per lane and is a 7.5-12.5% polyacrylamide linear gradient, stained as per Gorg, *et al*. (14). Lane 1, GCM 740; lane 2, GCM 740Δ4; and lane 3, GCM 740Δ2. Missing protein bands are represented by o and unique bands are represented by ●. Positions of major gonococcal outer membrane proteins and molecular weight standards are shown along the right margin (kDa; Bio-Rad).

Figure 5



The only protein which has been possible to define by standard methods is the 26 kDa protein present in GCM 740 and GCM 740Δ4 which showed heat modifiability and therefore belonged to the Opa class of proteins (data not shown). All other differing proteins were demonstrated not to be Por, Rmp, or Opas by standard procedures (data not shown).

GCM 740 cytoplasmic membranes were observed to contain a unique doublet band at 24 kDa and to be missing a band at 19 kDa (Figure 6, lane 1). GCM 740Δ4 cytoplasmic membranes contained a unique band of 39 kDa which was missing in both the WT and GCM 740Δ2 (Figure 6, lane 2). GCM 740Δ2 was missing bands of 49 and 27 kDa which the other variants exhibited, and had unique bands of 110, 62, and 44 kDa. Similarly, when the isogenic set of variants constructed by Koomey, *et al.* was compared, the *Iga*$^-$ mutant was observed to lack a band of 65 kDa and to express a novel band of 56 kDa (data not shown;40).

Surface exposure of the variant outer membrane proteins. As demonstrated by the ability to be labeled with ^{125}I by the lactoperoxidase procedure of Swanson, some of the variant proteins described above for GCM 740Δ2 were exposed on the cell surface (56). In particular, the major protein of 28 kDa, unique to GCM 740Δ2 was labeled (Figure 7, lane 3) while the missing band at 26 kDa (present and labeled weakly in both GCM 740 and in GCM 740Δ4) was observed in lanes 1 and 2. Also, a second protein band of 12.5 kDa was absent in GCM 740Δ2 that was present and labeled in the other variants. By virtue of the apparent SDS-PAGE molecular weight and heat-modifiable behavior, the 26 kDa band present in GCM 740 and in GCM 740Δ4 was considered to be an Opa while the unique 28 kDa protein observed in GCM 740Δ2 was not heat-modifiable

Figure 6. Coomassie blue-silver stained SDS-PAGE gel of isolated cytoplasmic membranes of GCM 740 and its two isogenic Δ-*iga*2 mutants, GCM 740Δ4 and GCM 740Δ2. The gel contains three micrograms of cytoplasmic membrane proteins per lane and is a 7.5-12.5% polyacrylamide linear gradient, stained as per Gorg, *et al.* (14). Lane 1, GCM 740; lane 2, GCM 740Δ4; and lane 3, GCM 740Δ2. Missing protein bands are represented by o and unique bands are represented by ●. Positions of the molecular weight standards are shown along the left margin in kDa (Bio-Rad).

Figure 6

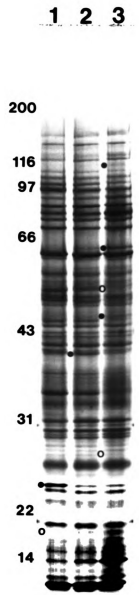
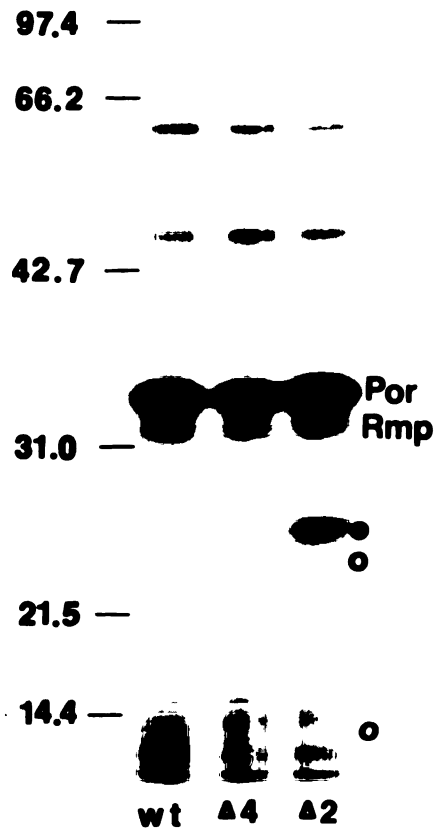


Figure 7. Autoradiograph of ^{125}I surface labeled *N. gonorrhoeae* cells separated over a 7.5-12.5% SDS-PAGE. Cells were labeled with lactoperoxidase as described previously (56). Lane 1, GCM 740; 2, GCM 740Δ4; and 3, GCM 740Δ2. Unique proteins are represented by ● and missing proteins are represented by o. Positions of major gonococcal outer membrane proteins are given for reference and molecular weight standards are given along the left margin in kDa (Bio-Rad).

Figure 7



and therefore was not an Opa (data not shown; 26,57). None of the other unique bands described in Figure 5 were observed to be surface exposed by their ability to be labeled.

Growth and analysis of outer membrane protein profiles under anaerobic conditions. All three variants of *N. gonorrhoeae* were capable of growth on GCB solid medium supplemented with $NaNO_2$ (data not shown).

The outer membrane protein profiles of the three variants showed some intervariant differences when grown under anaerobic conditions. These interstrain novel proteins expressed under anaerobiosis differed from those observed when the variants were grown under aerobic (*i. e.* normal) conditions. GCM 740 expressed a unique protein band of 31.5 kDa, GCM 740Δ4 expressed a unique band of 66 kDa, and GCM 740Δ2 expressed a unique band of 20.5 kDa (Figure 8; lanes 7-9).

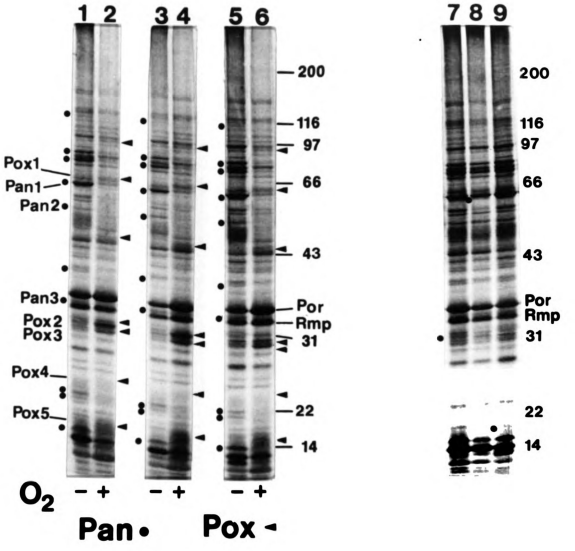
The Δ*iga*2 variants were demonstrated to be capable of expressing the appropriate anaerobic stress proteins in their outer membranes as demonstrated in Figure 8. All three variants expressed Anis 1-3 (lanes 1, 3, and 5) and repressed the synthesis of Oxis 1-5 (lanes 2, 4, and 6), as defined by Clark, *et al.* (7).

A number of other oxygen-repressible outer membrane proteins were observed for the set of three variants including proteins of: 125, 94, 64, 38, 25, 22.5, and 16 kDa which were not defined by Clark, *et al.* (7). Similarly, two previously undescribed oxygen-inducible outer membrane proteins of 92 and 45 kDa were expressed by all three variants.

Growth and analysis of outer membrane protein profiles under iron-limited conditions. All three variants were capable of growth under iron-limited conditions (Figure 9). All three variants grew to a higher final optical density when propagated in iron-replete media as compared

Figure 8. Extracted outer membrane proteins from *N. gonorrhoeae* GCM 740, GCM 740Δ4, and GCM 740Δ2 grown under aerobic and anaerobic conditions on GCB solid medium. Each lane contains 3 μg of n-lauroylsarcosine-insoluble proteins (as per Clark, *et al.*, 7) separated by 7.5-12.5% linear polyacrylamide gradient SDS-PAGE and stained with Coomassie blue-silver. For lanes 1 through 6, odd numbered lanes contain membranes from cells grown under anaerobic conditions and even numbered lanes contain membranes from cells grown under aerobic conditions. Lanes 1 and 2, GCM 740; 3 and 4, GCM 740Δ4; 5 and 6, GCM 740Δ2. Positions of oxygen-repressible outer membrane proteins (Pans) are given to the left of each pair and are represented by ● while oxygen-induced outer membrane proteins (Poxs) are represented by ◄ on the right edge of each pair. Positions of previously described Pan and Pox (7) and major gonococcal outer membrane proteins are given for reference points. Lanes 7 through 9 contain n-lauroylsarcosine-insoluble proteins extracted from cells grown under anaerobic conditions (7, GCM 740; 8, GCM 740Δ4; and 9, GCM 740Δ2). Positions of unique outer membrane proteins are shown by ● to the left of each lane. The positions of molecular weight standard proteins are given in kDa along the right edges of the figure (Bio-Rad).

Figure 8


1 2
3 4
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7 8 9
200
116
97
66
43
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Rmp
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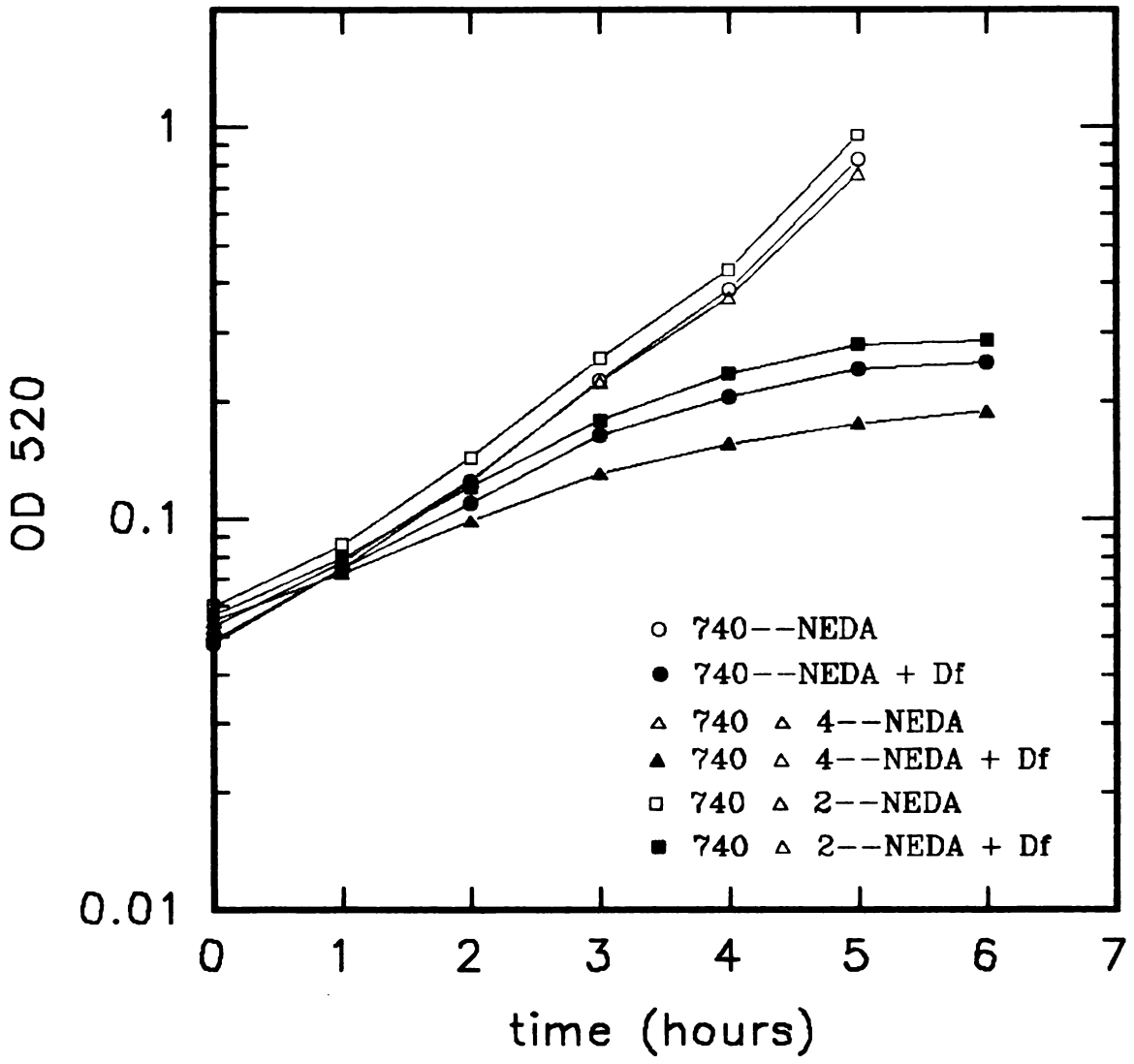
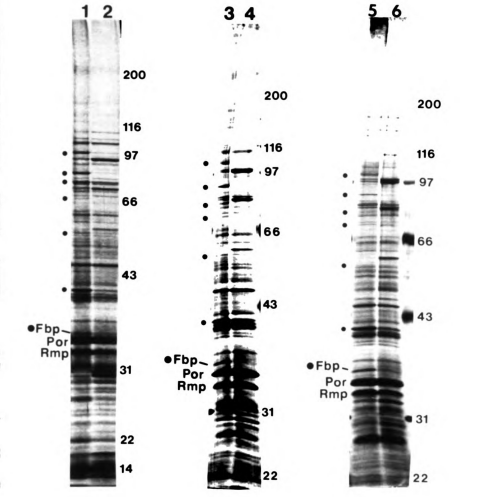


Figure 9. Growth curves of *N. gonorrhoeae* GCM 740, GCM 740Δ4, and GCM 740Δ2 grown under iron-replete and iron-limited conditions. Bacteria were grown in either NEDA plus 10 μM Fe^{3+} (added as $Fe(NO_3)_3$-$9H_2O$; iron-replete; NEDA) or in the same medium plus 50 μM Desferal (iron-limited; NEDA + Df). Both media were supplemented with 0.252 mM $CaCl_2$ and 0.042% $NaHCO_3$ (w/v) and incubated at 37°C with shaking.

Figure 10. Coomassie blue-silver stained SDS-PAGE gels of isolated outer membranes from *N. gonorrhoeae* GCM 740, GCM 740Δ4, and GCM 740Δ2 separated over a 7.5-12.5% linear polyacrylamide gradient. Lanes 1, GCM 740 in iron-limited medium; 2, GCM 740 in iron-replete medium; 3, GCM 740Δ4 in iron-limited medium; 4, GCM 740Δ4 in iron-limited medium; 5, GCM 740Δ2 in iron-limited medium; and 6, GCM 740Δ2 in iron-limited medium. Iron-replete medium was NEDA plus 10 μM Fe^{3+}, 0.252 mM $CaCl_2$, and 0.042% $NaHCO_3$ (w/v). Iron-limited medium was prepared by addition of 50 μM Desferal to the same medium. Iron-repressible outer membrane proteins are represented by ● and positions of major gonococcal outer membrane proteins are given for reference. Positions of molecular weight standards are given along the right margins in kDa (Bio-Rad).

Figure 10



to iron-limited media.

All three of the variants were also able to express some of the appropriate described iron-regulated outer membrane proteins as demonstrated in Figure 10. In particular, the conserved Fbp protein (35 kDa here; 29) was expressed. Other previously described iron-repressible outer membrane proteins that were observed included: 102, 85, 78, 73, 57, and 41 kDa.

Pulse-chase labeling analysis of WT and Δ-*iga*2 variants. To determine whether differences existed in the ability to process proteins between the WT and the mutant gonococci, we performed a series of pulse-chase labeling experiments using Tran^{35}S-label (ICN Pharmaceuticals Inc., Irvine, CA) in methionine-deficient NEDA. When *N. gonorrhoeae* cells were pulsed during exponential phase, proteins were observed to chase "out" (*i. e.* decrease in density over time) after addition of an excess of cold carrier methionine while others chased "in". This indicated that both of the mutant variants are capable of post-translational processing with similar efficiency as the WT. These data are summarized in Table 2 and shown in Figure 11.

In general, the set of variants were observed to process relatively few proteins during exponential growth, as only 2 proteins were observed to chase "out" for GCM 740 (Figure 11A), and one unique protein was observed for each of the two mutant variants (Figure 11B and C). The proteins observed in the WT were of 35.5 and 27 kDa. A unique protein of 48 kDa was observed in GCM 740Δ4 and a unique protein of 113 kDa was observed for GCM 740Δ2 (Figure 11B and C, respectively).

When these autoradiographs were analyzed with regard to the protein bands which chased "in" (*i. e.* increased in density over time), we

Table 2. Summary of pulse-chase labelling data.

Bands processed during exponential phase[a]

Bands chasing "out"			Bands chasing "in"		
GCM 740	GCM 740Δ4	GCM 740Δ2	GCM 740	GCM 740Δ4	GCM 740Δ2
		113		116	116
	48				112
35.5			97	97	97
27					89
			67		
				55	
					39
			28		

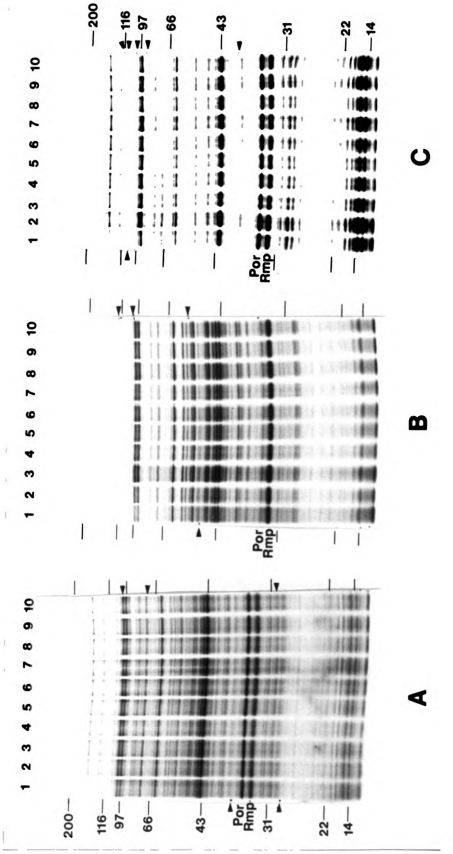
Bands processed during stationary phase[a]

Bands chasing "out"			Bands chasing "in"		
GCM 740	GCM 740Δ4	GCM 740Δ2	GCM 740	GCM 740Δ4	GCM 740Δ2
		35.5			215
				162	
			143	143	
				140	
			120	120	120
			116		
				109	
				107	107
			104		
				95	95
			89		
			78	78	78
				69	69
				66	
			61.5		
			47.5		
			46		
				33	

[a] Apparent molecular weights given in kDa.

Figure 11. Autoradiographs of *N. gonorrhoeae* cells pulse-chase labeled during exponential phase with ^{35}S-methionine. Macromolecules were precipitated with trichloroacetic acid, resolubilized by boiling in SDS-PAGE sample buffer, and separated by 7.5-12.5% SDS-PAGE. Panels are as follow: A) GCM 740, B) GCM 740Δ4, and C) GCM 740Δ2. Lanes are samples taken at intervals post-chase as follow: lane 1, 0 minutes; 2, 30 seconds; 3,1 minute; 4, 2 minutes; 5, 4 minutes; 6, 8 minutes; 7, 16 minutes; 8, 32 minutes; 9, 64 minutes, and 10, 128 minutes. Positions of protein bands which chased "out" following the pulse of label are given along the left margin as are some major gonococcal outer membrane proteins. Positions of protein bands which were observed to chase "in" after the labeling period as well as the molecular weight standards (in kDa, Bio-Rad) are shown along the right margin.

Figure 11



observed a similar situation in that a low number of proteins were being processed (Figure 11). Three different proteins were observed to chase "in" for the WT with each of the mutants having similarly few bands, 3 for GCM 740Δ4 and 5 for GCM 740Δ2. A band of 97 kDa was observed to be processed by all three variants. A band of 116 kDa was processed by both of the mutants, but not by the WT. GCM 740 also processed unique proteins of 67 and 28 kDa. GCM 740Δ4 uniquely processed an additional protein of 55 kDa while GCM 740Δ2 uniquely processed proteins of 112, 89, and 39 kDa.

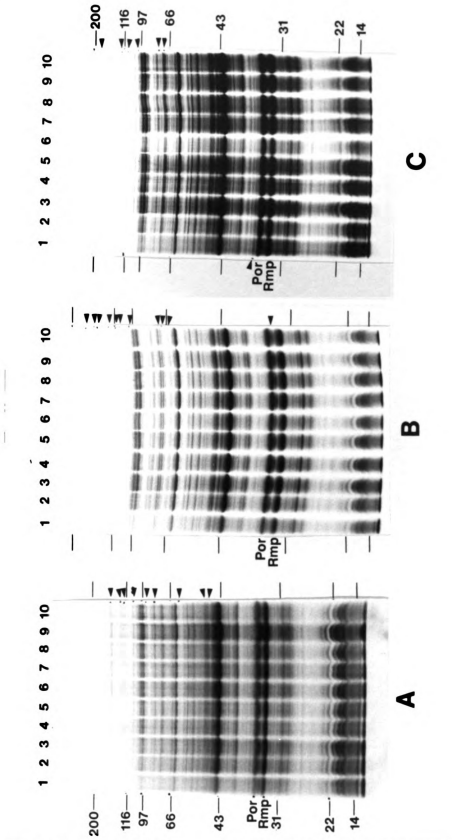
When the cells were labeled during stationary phase, virtually no protein bands were observed to be chased "out" while a relatively larger number chased "in". These data from stationary phase cells are also summarized in Table 2 and are shown in Figure 12. With regard to the number of proteins chased "out", only a single protein was observed to be processed in GCM 740Δ2 (Figure 12C; 35.5 kDa)

This low amount of processing was not observed however when the variants were analyzed for proteins which chased "in", as the WT processed 9, GCM 740Δ4 processed 11, and GCM 740Δ2 processsed 6 proteins. Two proteins of 120 and 78 kDa were processed by all three variants. Three proteins of 107, 95, and 69 kDa were processed by both of the mutants but not by the WT. A protein of 143 kDa was processed by both the WT and GCM 740Δ4, but not by GCM 740Δ2. GCM 740 processed unique proteins of 116, 104, 89, 61.5, 47.5, and 46 kDa. GCM 740Δ4 processed unique proteins of 162, 140, 109, 66, and 33 kDa while GCM 740Δ2 processed a single unique protein of 215 kDa.

The 89 kDa protein uniquely processed by GCM 740 during stationary phase (Figure 12A) and the protein of 28 kDa uniquely processed during

Figure 12. Autoradiographs of *N. gonorrhoeae* cells pulse-chase labeled during stationary phase with ^{35}S-methionine. Macromolecules were precipitated with trichloroacetic acid, resolubilized by boiling in SDS-PAGE sample buffer, and separated over 7.5-12.5% SDS-PAGE. Panels are as follow: A) GCM 740, B) GCM 740Δ4, and C) GCM 740Δ2. Lanes are samples taken at intervals post-chase as follow: lane 1, 0 minutes; 2, 30 seconds; 3,1 minute; 4, 2 minutes; 5, 4 minutes; 6, 8 minutes; 7, 16 minutes; 8, 32 minutes; 9, 64 minutes, and 10, 128 minutes. Positions of protein bands which chased "out" following the pulse of label are given along the left margin as are some major gonococcal outer membrane proteins. Positions of protein bands which were observed to chase "in" after the labeling period as well as the molecular weight standards (in kDa, Bio-Rad) are shown along the right margin.

Figure 12



exponential phase (Figure 11A), may correspond to the unique GCM 740 outer membrane protein bands of 88 and 25 kDa shown in Figure 5 with the differences in apparent molecular weight due to experimental variation. However, since the pulse-chase analysis utilized disrupted whole cells, this is not considered proof of identity.

Restoration of the *iga*2b allele in the Δ*iga* variants. In general, the proposed schema for identification of *Iga*$^-$ to *Iga*$^+$ revertants constructed by transformation with the 9.4 kbp *Cla*I fragment was found to be unreliable. Attempts to identify the reverted *iga* allele by a shift from Pen^r to Pen^s were difficult due to the degree of subjectivity in differentiating Pen^r from Pen^s colonies. The use of a direct IgA1 protease plate assay (12) to screen the plated transformation mix for *Iga*$^+$ colonies was also considered unreliable in that even though putative *Iga*$^+$ transformants could be identified, recovery of viable organisms from beneath the top agar overlay was not consistently successful. Using these two screening methods, over 18,000 GCM 740Δ4 and 23,000 GCM 740Δ2 potential transformants were tested without success. In general, the low transformation frequency expected also made these attempts to identify a nonselectable transformant extremely difficult.

Use of the congression experimental approach (52) was successful in identifying *Iga*$^+$ revertants for both GCM 740Δ4 and GCM 740Δ2. By first selecting for transformants using Nal^r as a selectable phenotype, two GCM 740Δ2 revertants, GCM 740Δ2R-2 and GCM 740Δ2R-40, were isolated from 117 screened (1.71% congression) and one GCM 740Δ4 revertant, GCM 740Δ4R-83, was isolated from 708 screened (0.14% congression). Secondary screening for the desired *Iga*$^+$ revertants involved duplicate

plating on GCB and a direct IgA1 protease plate assay to identify any *Iga*$^+$ colonies from the pool of Nal^r transformants selected.

Characterization of the Δ-*iga*2b revertant variants. Use of a standard qualitative IgA1 protease assay (44) confirmed that these three revertants were producing the specific IgA1 protease (Figure 13).

Southern analysis of chromosomal DNA from the set of variants demonstrated that the restoration event had taken place faithfully. *Hin*dIII digested DNA from the revertants showed an *iga*2b homologous band of the appropriate size (4.3 kbp; Figure 14A). The revertants had lost any DNA homology with the *bla* gene cassette (Figure 14B). Finally, the appropriate variants lacked homology with the 1.1 kbp *Pst*I fragment of an *iga*2b gene while the revertants regained homology (Figure 14C). Southern analysis using the ~10 kbp *Pst*I insert fragment of pNal6, demonstrated that this probe hybridized to a different band (~6 kbp) than that of the 4.3 kbp *Hin*dIII *iga*2b probe indicating that the two loci were not physically linked (data not shown).

Isolated outer membranes were analyzed by protein profile (Figures 15 and 16). Some profile differences were observed consistently, such as the novel bands of 102, 86, and 80 kDa and the missing band of 82 kDa which correlated with Nal^r. One protein band of 56 kDa was observed to correlate with the *Iga*$^+$ phenotype as it was not observed in either GCM 740Δ4 or GCM 740Δ2 (Figure 16). However, this protein was not noted as being unique to the WT in Figure 5.

Isolated cytoplasmic membrane proteins were also analyzed by protein profile also (Figure 17). One of the proteins which varied between mutant and WT was observed to be "cured" by reversion of the Δ-*iga*2b lesions. The 34.5 kDa band which is missing in GCM 740Δ4 and present in

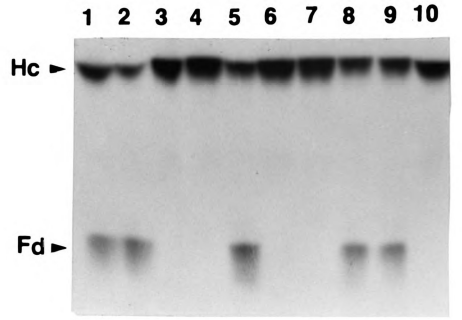


Figure 13. Autoradiograph of IgA1 protease assay cleavage products. Assays were performed as described in Materials and methods. Lanes are as follow: 1, GCM 740; 2, GCM 740Nal^r; 3, GCM 740Δ4; 4, GCM 740Δ4Nal^r; 5, GCM 740Δ4R-83; 6, GCM 740Δ2; 7, GCM 740Δ2Nal^r; 8, GCM 740Δ2R-2; 9, GCM 740Δ2R-40; and 10, negative control. Positions of intact heavy chain (Hc_α) and one of the cleavage products (Fd_α) are shown along the left margin.

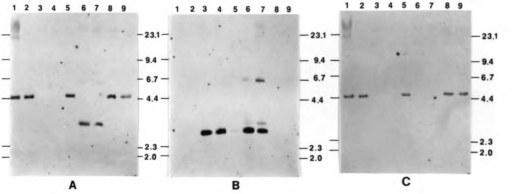


Figure 14. Southern blot analysis of WT, Δ-*iga*2 mutant, and revertant strains of *N. gonorrhoeae* GCM 740. All DNAs were digested with *Hin*dIII and separated over 0.65% agarose gels in TBE (25). Panels were probed as follow: A) 4.3 kbp *Hin*dIII *iga*2b fragment, B) 2.4 kbp *Hin*dIII *bla* cassette, and C) 1.1 kbp *Pst*I fragment of *iga*2b gene. For each panel, lanes are as follow: 1, GCM 740; 2, GCM 740Nal^r; 3, GCM 740Δ4; 4, GCM 740Δ4Nal^r; 5, GCM 740Δ4R-83; 6, GCM 740Δ2; 7, GCM 740Δ2Nal^r; 8, GCM 740Δ2R-2; and 9, GCM 740Δ2R-40. Molecular weight standards are shown along the right margin of each panel (λ DNA digested with *Hin*dIII; Bethesda Research Laboratories, Inc., Gaithersburg, MD).

Figure 15. Coomassie blue-silver stained 7.5-12.5% SDS-PAGE gel of isolated outer membranes from WT, Δ-*iga*2 mutant, and revertant strains of *N. gonorrhoeae* GCM 740. Three micrograms of outer membrane proteins were loaded per lane as follow: 1, GCM 740; 2, GCM 740Nal^r; 3, GCM 740; 4, GCM 740Δ4; 5, GCM 740Δ4Nal^r; 6, GCM 740Δ4R-83; 7, GCM 740Nal^r; 8, GCM 740; 9, GCM 740Δ2; 10, GCM 740Δ2Nal^r; 11, GCM 740Δ2R-2; 12, GCM 740Δ2R-40; and 13, GCM 740Δ4Nal^r. Unique protein bands are represented by ● and missing proteins are represented by o. Both are shown to the left of the indicated band. The 56 kDa protein band which correlated with the Δ*iga*2 lesion in strain GCM 740-Δ4 is represented by + . Positions of major gonococcal outer membrane proteins are given along the right margin as are the molecular weight standards in kDa (Bio-Rad). The position of p56 is noted and the positions of the set of three bands which correlated with Nal^r is represented by N_3.

Figure 15

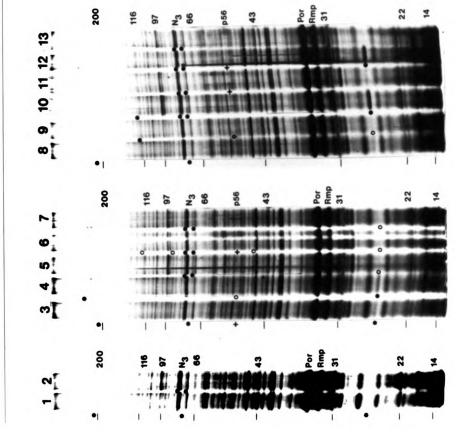


Figure 16. Coomassie blue-silver stained 7.5-12.5% SDS-PAGE gel of isolated outer membranes from WT and Δ-*iga*2 mutant variants of *N. gonorrhoeae* GCM 740. Three micrograms of outer membrane proteins were loaded per lane as follow: 1, GCM 740; 2, GCM 740Δ4; and 3, GCM 740Δ2. Unique protein bands are represented by ● and missing proteins are represented by o. Both are shown to the left of the indicated band. Positions of major gonococcal outer membrane proteins are given along the right margin as are the molecular weight standards in kDa (Bio-Rad). The position of p56 is noted.

Figure 16

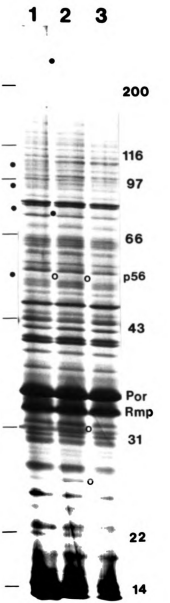
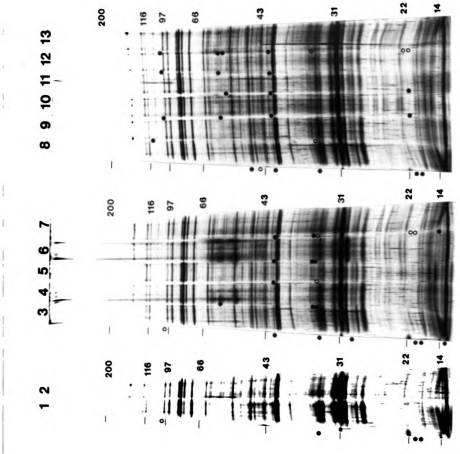


Figure 17. Coomassie blue-silver stained 7.5-12.5% SDS-PAGE gel of isolated cytoplasmic membranes from WT, Δ-*iga*2 mutant, and revertant strains of *N. gonorrhoeae* GCM 740. Three micrograms of cytoplasmic membrane proteins were loaded per lane as follow: 1, GCM 740; 2, GCM 740Nal^r; 3, GCM 740; 4, GCM 740Δ4; 5, GCM 740Δ4Nal^r; 6, GCM 740Δ4R-83; 7, GCM 740Nal^r; 8, GCM 740; 9, GCM 740Δ2; 10, GCM 740Δ2Nal^r; 11, GCM 740Δ2R-2; 12, GCM 740Δ2R-40; and 13, GCM 740Δ4Nal^r. Unique protein bands are represented by ● and missing proteins are represented by o. Both are shown to the left of the indicated band. Positions of molecular weight standards are shown along the right margin in kDa (Bio-Rad).

Figure 17



the WT, does show up in the revertant GCM 740Δ4R-83 (Figure 17; lanes 3-6). Again, this band was not observed to be a variant band in Figure 6.

DISCUSSION

We have been able to successfully construct a pair of *Neisseria gonorrhoeae* Δ-*iga*2 variants isogenic with GCM 740, a WT clinical isolate. These two deletion-disruption mutants were capable of growing at a similar rate in a standard defined medium for *N. gonorrhoeae* (Figure 4) and were capable of posttranslationally processing proteins during both exponential growth and stationary phases. Both of the mutants were competent for transformation as demonstrated by recovery of transformed variants with a faithfully restored *iga*2 locus (Figure 14). They were also capable of phase variable pilus expression, evidenced by the recovery of both P^+ and P^- colony variants (data not shown). Likewise, the mutants were able to express Opas in a phase variable manner as determined both grossly, by changes in colonial color on GCB and molecularly, by assessment of heat modifiable behavior of outer membrane proteins (data not shown).

The mutants exhibited altered membrane protein profiles (Figures 5,6,15,16, and 17) as compared to their WT parent. HOwever, except in the cases of one outer membrane protein (p56, present only in the WT) and a cytoplasmic membrane protein of 34.5 kDa missing in GCM 740Δ4 and GCM 740Δ2 and present in GCM 740Δ4R-83), none of these other membrane protein differences appeared to "cure" with restoration of the WT *iga*2 locus (Figures 15-17).

With regard to the differences observed in the outer membrane protein profiles between WT and Δ-*iga*2 mutants, it was not possible to

identify the majority of these differing minor component proteins as previously described outer membrane proteins or environmental stress response outer membrane proteins. The sole exception to this was the 26 kDa protein missing in GCM 740Δ2 but present in both the WT and in GCM 740Δ4. In the two variants which exhibited this protein band, it was identified as an Opa based on heat-modifiable behavior (data not shown).

We had initially hypothesized that the mutants would not be capable of inserting the relevant environmental stress proteins into their outer membranes based on the separate observations that 1) the membrane protein profiles were different when propagated in a "normal" defined medium and 2) that there are proteins present in the outer membrane that were permissive substrates for IgA1 protease *in vitro* (39,46). We hypothesized that these differences were the result of some IgA1 protease *trans*-mediated effect. When this hypothesis was tested we observed that the mutants were capable of growth under both iron-limited (Figure 9) and anaerobic conditions. Furthermore, both of the mutants were completely capable of expressing a full range of previously described oxygen-repressible and iron-repressible outer membrane proteins (Figures 8 and 10).

We tested the abilities of the mutants to process proteins posttranslationally in an attempt to correlate these differences in membrane protein profiles with the *iga*2 locus. Our results indicated that the mutants had the ability to process proteins with similar efficiency as their WT parent (Figures 11 and 12). Therefore, the hypothesized pleiotrophic phenotype of the *iga* locus was not supported by these data.

The 28 kDa protein which was observed to be unique to GCM 740Δ2

(Figure 5), exhibited phase variable expression as it was not observed in all membrane preparations from this variant (Figures 5 and 16). This, considered with its apparent SDS-PAGE molecular weight and surface exposure (Figure 7), was suggestive of an Opa. However, when preparations which contained the 28 kDa protein were analyzed to define heat modifiable proteins, the protein did not exhibit heat modifiable behavior (data not shown). This apparent contradiction suggests that the 28 kDa outer membrane protein is not an Opa but rather some undescribed, phase variable outer membrane protein.

The fact that not all of the differences were observed on all gels with a single preparation, and that all differing bands could not be observed from preparation to preparation, indicated that the majority of these detected differences were not directly related to the Δ-*iga*2 lesion. Rather, the fact that only one of the bands, p56, correlated with the *iga*2 phenotype suggests that the bands which differed from preparation to preparation were likely due to the relative yields of a particular protein in that particular preparation. Alternatively, there may have been secondary mutations which had arisen either as a compensatory result of the original Δ-*iga*2 lesion or as completely unrelated lesions.

Most of the observed differences in cytoplasmic membrane protein profiles were not observed to be directly related to the *iga*2 phenotype. This was indicated by the failure of most of the profile differing bands to be "cured" by restoration of the WT allele in the deletion mutant variants. The sole exception was a 34.5 kDa protein band present in WT but not in GCM 740Δ4 or GCM 740Δ2 and expressed in the revertant GCM 740Δ4R-83 (Figure 17).

In summary, most of the observed differences in the outer and cytoplasmic membrane protein profiles that were presented in the Results section were apparently not the result of the particular *iga*2 allele present in the bacterium. However, the p56 outer membrane protein and the 34.5 kDa cytoplasmic membrane protein did "cure" when the Δ-*iga*2 allele was restored to WT and therefore must be associated with the *Iga* phenotype in some fashion. It is not known whether the genes might be physically linked or if the production of IgA1 protease influences these other proteins in some posttranslational fashion. Overall, the observed intervariant variability in the membrane protein profiles is not solely accounted for by the *Iga* phenotype.

Finally, the phenotypic ability to produce an *iga*1 protease does not affect the physiologic status of *N. gonorrhoeae* under the sets of conditions tested in the laboratory. Two phenotypic differences were observed to correlate with the Δ-*iga*2 allele: 1) the inability to hydrolyze human IgA1 and 2) the lack of expression of a 56 kDa outer membrane protein and a 34.5 kDa cytoplasmic membrane protein.

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Chapter 2

PROTEOLYSIS OF BACTERIAL MEMBRANE PROTEINS BY *Neisseria gonorrhoeae* TYPE 2 IgA1 PROTEASE

Russell J. Shoberg and Martha H. Mulks

Department of Microbiology and Public Health,

Michigan State University,

East Lansing, MI 48824 USA

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ABSTRACT

The IgA1 proteases of *Neisseria gonorrhoeae* have been defined as having human IgA1 as their single permissive substrate. However, in recent years there have been reports of other proteins which are susceptible to the proteolytic activity of these enzymes. To examine the possibility that gonococcal membrane proteins are potential substrates for these enzymes, isolated outer and cytoplasmic membranes of *N. gonorrhoeae* were treated *in vitro* with exogenous pure IgA1 protease. Analysis of silver stained SDS-PAGE gels of outer membranes indicated that there were two outer membrane proteins of 78 and 68 kDa which were cleaved by IgA1 protease *in vitro*, in GCM 740 (a wild type strain) and in two isogenic IgA1 protease negative variants. Similar results were observed with a second gonococcal strain, F62, and its isogenic IgA1 protease negative derivative. When GCM 740 cytoplasmic membranes were treated with protease, three minor proteins of 24.5, 23.5, and 21.5 kDa were cleaved. In addition, when outer membranes of *E. coli* DH1 were treated with IgA1 protease, several proteins were hydrolyzed. While the identities of all of these proteolyzed proteins are unknown, the data presented indicate that there are several proteins found in the isolated membranes of gram-negative bacteria which are permissive *in vitro* substrates for gonococcal IgA1 protease.

INTRODUCTION

The enzymatic activity now labelled IgA1 protease was first described in 1973 by Mehta, *et al.*, when they demonstrated the presence of a bacterial enzyme in human stool samples which produced intact Fab_{α} and Fc_{α} fragments from human immunoglobulin A (19). Since that time, several bacteria have been reported to produce enzymes with this activity and these enzymes have been the subject of several review articles (13,21,30). Bacteria which produce IgA1 proteases are clustered into a broad grouping consisting of human mucosal surface pathogens. This group includes the gram-negative bacteria *Neisseria gonorrhoeae* and *N. meningitidis* (31), *Haemophilus influenzae* (14,17,25), *Bacteroides melaninogenicus* (11), *B. asaccharolyticus* (11), and *Capnocytophaga sp.* (11). There are also several gram positive IgA1 protease producers such as *Streptococcus pneumoniae* (14,17,25), *S. sanguis* (32), *S. mitis* (35), *S. oralis* (12,35) and *Clostridium ramosum*, (6) as well as one mycoplasma, *Ureaplasma urealyticum* (36). Because many of the organisms that produce IgA1 proteases are mucosal pathogens of man and the IgA1 proteases specifically cleave a major component of the secretory immune system, the production of IgA1 protease has been postulated to be a virulence factor for these organisms (13).

It has been reported repeatedly that this group of enzymes is specific for a single substrate, the hinge region of human IgA1 (21,30). While a number of relevant human and animal proteins have been tested as potential substrates, the only permissive substrates for these enzymes were the IgA1 molecules from man and higher primates. Each IgA1 protease cleaves a single peptide bond within the duplicated octet of

amino acids that is present in the hinge region of human IgA1 and missing in IgA2 due to a deletion (21,30). *N. gonorrhoeae*, as well as *N. meningitidis* and *H. influenzae*, produce at least two types of IgA1 proteases as defined by the specific peptide bond cleaved (22,24,26). Other of the IgA1 protease producing species cleave within this same hinge region but at different peptide bonds (21,30).

The enzymes from *N. gonorrhoeae* have been particularly well studied and the genes encoding them have been cloned by several laboratories (8,15,23). The entire nucleotide sequence of an *iga* gene has been reported as well as a proposed model for the secretion of the gene product from the gonococcus (33). According to the model, an N-terminal leader peptide is responsible for secretion of the entire 169 kDa *iga*2 gene product through the cytoplasmic membrane of the gonococcus. Next, a 45 kDa carboxyl terminal helper region (β protein) mediates attachment to the outer membrane and forms a pore through which the proenzyme is extruded. Once exposed on the extracellular side of the outer membrane, the proenzyme releases itself from the outer membrane via an autoproteolytic event. Finally, in the fluid phase, IgA1 protease makes a second clip in its own sequence to release either a 12 or 15 kDa peptide (α) of unknown function (33). This report was the first description of a substrate other than human IgA1 for the gonococcal enzyme. This same research group has subsequently proposed that the gonococcal IgA1 protease may be capable of cleaving two other human proteins, the granulocyte macrophage growth stimulatory factor (GM-GSF) and the CD.8 surface antigen of cytotoxic T lymphocytes (34). These data raise the question that there might be other unrecognized substrates for the IgA1 proteases.

We have developed procedures for the purification of both the types 1 and 2 gonococcal IgA1 proteases (39). The ability to produce pure gonococcal type 2 IgA1 protease and the construction of an isogenic set of IgA1 protease positive and negative variants of *N. gonorrhoeae* has allowed us to examine the question of whether IgA1 proteases may be involved in the modification of the gonococcal cell surface via proteolysis of gonococcal membrane proteins and whether these enzymes may have more permissive substrates than had previously been described. We have examined the susceptibility of isolated outer and cytoplasmic membranes to IgA1 protease in an *in vitro* assay system using both wild type gonococcal strains and IgA1 protease-deficient variants. We also examined the effect of IgA1 protease on the isolated outer membrane of an *E. coli* laboratory strain. The data presented here demonstrate that the type 2 gonococcal IgA1 protease is capable of hydrolyzing several proteins in the outer and cytoplasmic membranes of the gonococcus and further indicate that this effect is neither strain nor species limited.

(Portions of this work were presented at the 6th International Pathogenic Neisseria Conference in Pine Mountain, GA,in 1988 [28].)

MATERIALS AND METHODS

Bacterial strains and media. *N. gonorrhoeae* GCM 740 is a prototrophic type 2 IgA1 protease-producing strain of PorB2 serovar that has been described previously (23). The pair of Δ*iga*2 deletion-disruption mutants, 740Δ4 and 740Δ2 were constructed in this laboratory for use in other experiments and their construction will be described below (Figure 1). In GCM 740Δ2, the deletion removes 1.1 kbp (24% of

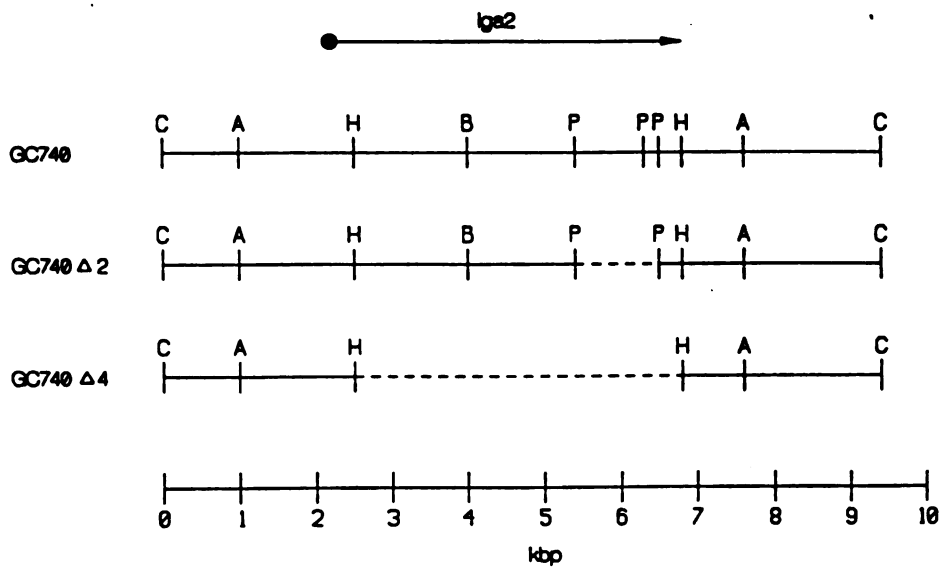


Figure 1. Restriction maps of *Neisseria gonorrhoeae* GCM 740 and two isogenic gene disruption mutants. Deleted regions are represented by dashed lines and both resultant constructs were negative for IgA1 protease activity (restriction site abbreviations: A, *Ava*I; B", *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I).

the *iga* gene), and in GCM 740Δ4, the deletion encompasses 4.3 kbp (93% of the *iga* gene). The gonococcal strain F62 and its *Iga2*$^-$ isogen F62-VD111, (PorB7, proline requiring) have been described previously by Koomey, *et al.* (15). As well as differing in Por serovar and auxotype, these two sets of strains have different *iga*2 genotypes as defined by restriction site polymorphism (27). The *E. coli* strains DH1 and HB101 were used in cloning of the *iga*2 locus from GCM 740 and in construction of the deletion-disruption mutants. *Actinobacillus pleuropneumoniae* ATCC 27088 is a serotype 1 type strain.

Gonococci were propagated on GC base solid medium (Difco Laboratories, Detroit, MI) plus Kellogg's defined supplement (43, GCB) and grown in the defined broth medium of Morse and Bartenstein (20). The Δ*iga* mutants were isolated after transformation on GCB containing a diffusion gradient of penicillin G. Following isolation and confirmation of phenotype, these mutants were propagated on GCB. *A. pleuropneumoniae* was propagated on brain heart infusion agar and in brain heart infusion broth, both containing 10 μg/ml NAD (Difco). *E. coli* strains were propagated in LB broth or on LB agar solid medium (18) containing either ampicillin (100 μg/ml) or tetracycline (12.5 μg/ml) as necessary. Antibiotics and NAD were from Sigma Chemical Co., St. Louis, MO.

Construction of Δ*iga* mutants. Cloning of the *iga*2 gene from GCM 740 has been reported previously (23). Standard recombinant DNA methodology (18) involving endonuclease restriction and fragment elution from agarose gels was performed to construct deletion mutants (Figure 1). A 2.4 kbp β-lactamase gene cassette was removed from pLES2, a *N. gonorrhoeae-E. coli* shuttle vector (40) and ligated into these deleted

constructs in order to provide a marker which could be used to select for gonococcal transformants. Resultant *Iga*$^{-}$*Amp*^{r} plasmids were identified and amplified in *E. coli*. Insert DNA was removed by endonuclease digestion and gel purified prior to transformation of GCM 740.

Transformation of GCM 740 was performed as previously described (1) with the following modifications. Following the transformation period (60 minutes at 37°C), cells were plated on GCB for a 6 hour expression period at 37°C under 5% CO_2. Following expression, 100 μl of a penicillin G solution (20 μg/ml) was spread over the inner two-thirds of the plate and allowed to form a diffusion gradient. Penicillin resistant colonies were isolated at 72 hours and subcultured on GCB plus 0.025 μg/ml penicillin G to verify the phenotypic stability. Penicillin resistant colonies were assayed for IgA1 protease phenotype by SDS-PAGE analysis of ^{125}I-labeled IgA1 cleavage products (24). Southern blots of endonuclease restricted genomic DNA also were probed with the 4.3 kbp *Hin*dIII *iga* fragment and the 2.4 kbp β-lactamase cassette fragment to verify the genotype of the mutants (18,27).

Preparation of cytoplasmic and outer membranes. Bacteria were grown to mid-exponential phase in appropriate broth media and harvested by low speed centrifugation. Spheroplasts were prepared by the sucrose-lysozyme-EDTA method (29) and were disrupted by ultrasonication. Cytoplasmic (CM) and outer (OM) membrane fractions were separated by ultracentrifugation over isopycnic sucrose density gradients (10). Protein concentrations of each fraction were determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard (2, Bio-Rad Laboratories, Richmond, CA).

IgA1 protease purification and quantitation. IgA1 protease was purified from GCM 740 as previously described (39). Briefly, cell-free supernatant from cells grown in defined medium was harvested and concentrated by ultrafiltration in a positive pressure filtration cell (Amicon Corp., Lexington, MA). Bulk protein was removed by batch anion exchange treatment with DE52 at pH 7.5 (Whatman Inc., Clifton, NJ). Two FPLC chromatographic steps were used to prepare the pure IgA1 protease, a Mono-P chromatofocussing column followed by a Superose 12 gel filtration column (both Pharmacia, Inc., Piscataway, NJ). After both column runs, fractions containing IgA1 protease activity and silver stainable IgA1 protease were pooled, concentrated, and dialyzed into Iga2 buffer [50 mM TrisHCl (pH 7.5) + 5 mM EDTA] by ultrafiltration in a Centricon-30 (Amicon Corp.). Determination of protease units, defined as μg IgA1 cleaved $minute^{-1}$ ml^{-1}, was by quantitative SDS-PAGE analysis as described previously (32).

IgA1 protease treatment of membrane preparations. Three protein micrograms of each membrane preparation was mixed with one-tenth volume of 10X IgA1 protease assay buffer [0.5 M TrisHCl (pH 7.5), 0.1 M $MgCl_2$, 0.1 M $CaCl_2$] and from 0.17-4.0 units of IgA1 protease per microgram of membrane protein. In control reactions, either Iga2 buffer or heat-inactivated IgA1 protease was added instead of active enzyme. Incubation was at 37°C overnight and the reaction was terminated by addition of one-half volume of 2X sample buffer (16) with boiling for five minutes. Reaction mixtures were separated by discontinuous system SDS-PAGE (16) over a 7.5-12.5% linear polyacrylamide gradient followed by dual Coomassie blue-silver stain (7). Wet gels were analyzed with transillumination to detect the presence of bands which had changed in

staining intensity. A decrease in staining intensity was assumed to be due to proteolysis and a resultant decrease in the concentration of protein at that position since the buffer control lane had been treated in an identical fashion save the presence of the protease. An increase in the staining intensity of a protein band was assumed to be due to an increase in the concentration of protein at that position.

For control experiments utilizing heat-inactivated IgA1 protease, the enzyme was inactivated by heating at 95°C for ten minutes. A nonheated sample with an equivalent volume of the same preparation was used as the active enzyme control.

Antiserum preparation and immunoblot analysis. A polyclonal antiserum was prepared in New Zealand white rabbits by standard procedures. Injections were subcutaneous using electrophoretically pure IgA1 protease (prepared as described above) in Freund's adjuvant (Sigma) as the antigen. Antisera titers were determined by assaying dilutions of the antiserum for inhibition of IgA1 protease activity in an SDS-PAGE assay (32). This antiserum preparation gives 50%-inhibition of IgA1 protease activity at a dilution of 1:595.

Western blot analysis was used in an attempt to determine whether the low molecular weight bands that increased in staining intensity were due to degradation of the exogenously supplied IgA1 protease by intrinsic membrane proteases. Following SDS-PAGE as described above, proteins were electroblotted onto a nitrocellulose membrane (BA 85; Schleicher & Schuell, Inc., Keene, NH) by the method of Towbin, *et al.* (42) for sixty minutes at 100 V, constant voltage. Filters were blocked with 5% powdered skim milk in PBS-Tween 20 (0.05%) and probed overnight with anti-IgA1 protease antiserum. Washes were in PBS-Tween 20 and

^{125}I-labelled Protein A was used to detect antigen-antibody complexes with exposure to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

Human serum proteins. Human serum albumin (Cohn fraction V) and human gamma globulins (purified from Cohn fractions II and III) were purchased from Sigma. The human IgA1 and IgA2 paraproteins (Mor and Mapp) were purified from patient sera by standard procedures.

Digestion of human serum proteins. Aliquots of purified type 2 IgA1 protease from *Neisseria gonorrhoeae* GCM 740 (0.5 units) were incubated overnight with 0.25 μg of IgA1 (Mor), 0.25 μg of IgA2 (Mapp), 0.50 μg of gamma globulins, or 0.25 μg of albumin in a reaction mix containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Reactions were terminated by addition of one-half volume of double strength SDS-PAGE sample buffer (16) and boiled for five minutes. Reaction products were resolved over either 9.0% or 7.5-12.5% SDS-PAGE gels, stained, and analyzed with transillumination as described above for any degradation which had occurred in the presence of the added IgA1 protease.

RESULTS

Specificity of purified IgA1 protease preparation. The purified IgA1 protease preparation was demonstrated to be specifically active against a human IgA1 (Mor) paraprotein and to lack proteolytic activity against: 1) a human IgA2 (Mapp) paraprotein, 2) a human gamma globulin preparation, and 3) human serum albumin following overnight incubation (data not shown).

Incubation of isolated outer membranes with exogenous IgA1 protease. Outer membranes of GCM 740 and its two isogenic IgA1 protease negative

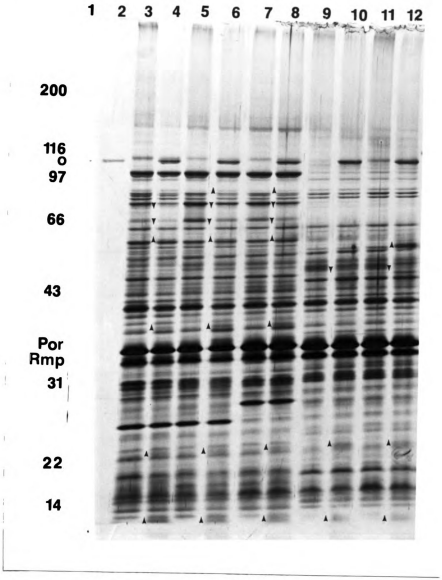
derivatives, 740Δ2 and 740Δ4, were incubated with electrophoretically pure type 2 gonococcal IgA1 protease prepared from GCM 740 to determine whether this enzyme could proteolyze gonococcal OM proteins. Figure 2 shows a typical silver stained SDS-PAGE gel comparing the protein composition of OMs incubated either with or without exogenous pure protease.

Two proteins of 78 and 68 kDa were proteolyzed, *i. e.* decreased in staining intensity, in all three of the variants in the GCM 740 set (Figure 2; lanes 4, 6, and 8). In contrast, an increase in staining intensity was observed for bands of 61, 38, 24.5, and 14 kDa in all three of these variants. In addition, a band of 88 kDa which was present in the wild type (lanes 3 and 4) and missing in both mutants in the absence of exogenous protease, was observed to increase in staining intensity for both of the mutant variants (lanes 6 and 8). Control experiments where OM were incubated with either buffer alone (Figure 2, lanes 3, 5, and 7) or with heat-inactivated IgA1 protease (data not shown) indicated that the proteolytic activity observed was heat-labile and associated with the addition of electrophoretically pure IgA1 protease. Immunoblot analysis with anti-IgA1 protease antiserum demonstrated that the lower molecular weight bands which appeared post-IgA1 protease treatment were not degradation products of the added IgA1 protease (data not shown).

Based on relative staining intensity, all of the proteins which were affected by the addition of protease were minor components of the OM. The identities of these protein bands are not known. However, it was determined by SDS-PAGE analysis and with heat and reduction modification experiments that they did not correspond to some of the previously

Figure 2. Silver stained SDS-PAGE gel of *N. gonorrhoeae* OMs following incubation with and without exogenous pure gonococcal type 2 IgA1 protease. Three micrograms of OM proteins were incubated for 14 hours at 37°C with either Iga2 buffer or 12 protease units of IgA1 protease in Iga2 buffer. Following incubation, mixtures were boiled for five minutes in sample buffer and separated over a linear 7.5-12.5% gradient SDS-PAGE gel. Lanes: 1, 12 units of IgA1 protease; 2, 12 units of IgA1 protease after 14 hours of incubation at 37°C; 3 and 4, GCM 740; 5 and 6, GCM 740Δ4; 7 and 8, GCM 740Δ2; 9 and 10, F62; 11 and 12, F62-VD111. Odd numbered lanes contain OMs incubated with Iga2 buffer and even numbered lanes contain OMs incubated with protease. Down-pointed triangles indicate bands which decreased in intensity following incubation with protease and up-pointed triangles indicate bands which increased in intensity. Positions of molecular weight standards (Bio-Rad) are shown along the left margin in kilodaltons as are the major gonococcal OM proteins (Por and Rmp) and IgA1 protease (o).

Figure 2



described gonococcal OMPs or antigens; *i. e.* they were not Por (PI), Rmp (PIII), or Hmps (PIIs) based on apparent molecular weights (9,41).

To determine whether proteolysis of gonococcal OM proteins was strain specific, outer membranes of *N. gonorrhoeae* F62, a type 2a IgA1 protease producer (27), and its isogenic protease-negative mutant, F62-VD111, were incubated with purified type 2b protease from GCM 740.

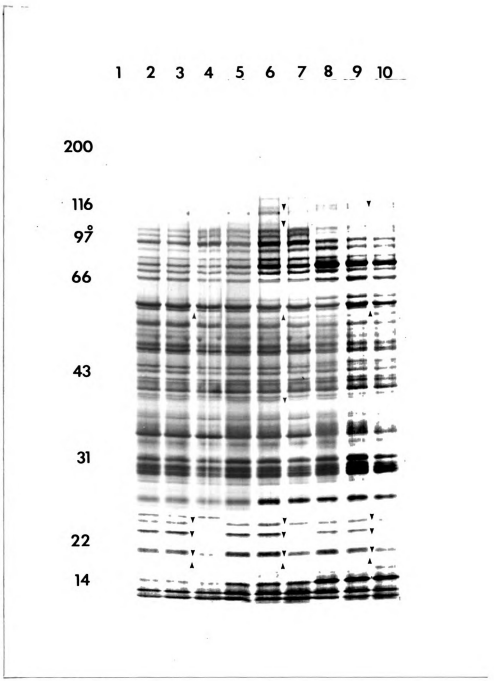
When the F62 set was analyzed, the two variants had a single proteolyzed band of 48 kDa in common (Figure 2, lanes 10 and 12). Protein bands of 24.5 and 14 kDa increased in staining intensity for both of these variants and these corresponded to bands of the same molecular weight also observed to increase in the GCM 740 set. The IgA1 protease deficient mutant, F62-VD111, exhibited a band of 58 kDa which increased in staining intensity following treatment with protease (Figure 2, lane 12). As stated above, the identities of these bands were not determined but some identities were ruled out based on apparent molecular weight and behavior.

Incubation of isolated cytoplasmic membranes with exogenous IgA1 protease. These experiments were performed in a similar fashion to those with the OMs to determine whether the observed proteolysis was limited to OM proteins. We found that there were also CM proteins which altered in staining intensity following incubation with exogenous, pure IgA1 protease (Figure 3; lanes 4, 7, and 10). Control reactions incubated with buffer alone or with heat-inactivated IgA1 protease demonstrated that this proteolysis was heat labile and dependent upon the addition of active IgA1 protease (Figure 3, lanes 3, 6, and 9).

When the GCM 740 isogenic set was analyzed (Figure 3, lanes 2-10), three protein bands of 24.5, 23.5, and 21.5 kDa were proteolyzed and two

Figure 3. Silver stained SDS-PAGE gel of *N. gonorrhoeae* CMs following incubation with Iga2 buffer, heat-inactivated type 2 gonococcal IgA1 protease in Iga2 buffer, and active IgA1 protease in Iga2 buffer. Three micrograms of CM proteins were incubated for 13.5 hours at 37°C as described below. Following incubation, mixtures were boiled for 5 minutes in sample buffer and separated over a linear 7.5-12.5% gradient SDS-PAGE gel. Lanes: 1, 6 units of IgA1 protease after 13.5 hours of incubation at 37°C; 2, 3, and 4, GCM 740; 5, 6, and 7, GCM 740Δ4; 8, 9, and 10, GCM 740Δ2. Lanes 2, 5, and 8 contain CMs plus buffer; 3, 6, and 9 contain CMs plus inactivated protease (6 protease unit equivalents); and lanes 4, 7, and 10 contain CMs plus 6 units of protease (2 units per microgram of CMP). Down-pointed triangles indicate bands which decreased in intensity following incubation with protease and up-pointed triangles indicate bands which increased in intensity. Positions of molecular weight standards (Bio-Rad) are shown along the left margin in kilodaltons as is IgA1 protease (o).

Figure 3



protein bands of 57 and 20.5 kDa were observed to increase in intensity in all three variants. The two mutants, GCM 740Δ4 and 740Δ2, also shared a protein band of 121 kDa which was proteolyzed (lanes 7 and 10). Strain GCM 740Δ4 exhibited two protein bands of 107 and 39 kDa which were proteolyzed (lane 7); these protein bands were not observed for either GCM 740 or GCM 740Δ2.

When analyzed in a similar experiment, the F62 set of variants showed a single CM protein band of 45 kDa in both the wild type and the mutant which increased in staining intensity following protease treatment (data not shown).

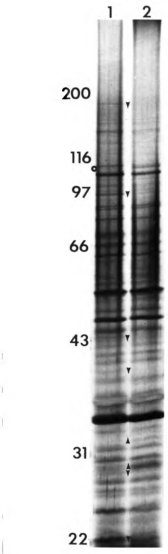
Incubation of *E. coli* outer membranes with exogenous IgA1 protease. To determine whether the observed proteolysis was restricted to gonococcal OM proteins, OMs of two other gram negative bacteria, *E. coli* DH1 and *Actinobacillus pleuropneumoniae*, were incubated with purified type 2 gonococcal IgA1 protease. Several *E. coli* DH1 OM proteins changed in staining intensity following incubation with protease (Figure 4).

E. coli DH1 OM bands which were proteolyzed included protein bands of 177, 90, 43, 38.5, 27.5, 22.5, and 19.7 kDa. Two minor bands of 31 and 28.5 kDa increased in staining intensity. *A. pleuropneumoniae* OMs exhibited a band of 37 kDa which was cleaved and two bands of 33 and 25 kDa that increased in staining intensity (data not shown).

While the identities of these protein bands are unknown, the fact that some protein bands are observed to be degraded while others increase in staining intensity supports the hypothesis that the type 2 gonococcal IgA1 protease has the ability to cleave OM proteins of gram negative species in addition to the gonococcus.

Figure 4. Silver stained SDS-PAGE gel of *E. coli* DH1 OMs following incubation with and without exogenous pure type 2 gonococcal IgA1 protease. Three micrograms of OM proteins were incubated for 14 hours at 37°C with either Iga2 buffer or IgA1 protease (0.17 protease units per microgram of OMs) in Iga2 buffer. Following incubation, mixtures were boiled for 5 minutes in sample buffer and separated over a linear 7.5-12.5% gradient SDS-PAGE gel. Lane 1, OMs plus Iga2 buffer and lane 2, OMs plus protease. Down-pointed triangles indicate bands which decreased in intensity following incubation with protease and up-pointed triangles indicate bands which increased in intensity. Positions of molecular weight standards (Bio-Rad) are shown along the left margin in kilodaltons as is IgA1 protease (o).

Figure 4



DISCUSSION

In this study, we have demonstrated that the type 2 IgA1 protease of *Neisseria gonorrhoeae* is capable of cleaving proteins found in both the outer and cytoplasmic membranes of the gonococcus, and also of cleaving outer membrane proteins of other gram-negative bacteria. This proteolytic activity was associated with a silver stainable, electrophoretically pure, heat labile preparation of IgA1 protease. Proteolysis of bacterial membrane proteins, or of any substrate of bacterial origin other than the protease itself, by any IgA1 protease, has not been reported previously.

Our conclusion that the phenomenon observed, i.e., the disappearance of some proteins and appearance of others in membrane preparations incubated with IgA1 protease, is due to specific proteolysis of membrane proteins by this enzyme, is supported by several control experiments. First, this phenomenon requires addition of active IgA1 protease; incubation of OM or CM preparations with heat-inactivated protease led to no alteration of proteins. Second, appearance of new protein bands was not due to autodegradation of the IgA1 protease, as evidenced by the integrity of the pure protease post-incubation overnight at 37°C (Figure 2, lanes 1 and 2). Third, appearance of new proteins was not due to degradation of the added protease by any hydrolytic activity present in the membrane preparations; immunoblot experiments demonstrated no bands other than the 109 kDa intact protease reactive with monospecific antiserum against IgA1 protease. Fourth, the lack of non-specific, contaminating proteolytic activity in the purified IgA1 protease preparation was demonstrated by the lack of digestion of some relevant human serum proteins, including human IgA2.

We constructed *Iga*$^-$ isogenic variants specifically to allow us to examine membrane proteins produced in the absence of protease as potential substrates. The ability to isolate and propagate these IgA1 protease deficient *N. gonorrhoeae* variants indicates that neither the ability to produce IgA1 protease nor the events of enzyme production and secretion are required for *in vitro* growth of the gonococcus. Our *Iga*$^-$ mutant variants grow equivalently to the WT in standard broth media and are capable of growing anaerobically and under iron limitation, and of expressing the appropriate previously defined stress response OM proteins (4,5,37,38). However, we did observe some protein profile differences between the WT and *Iga*$^-$ isogenic variants (Figure 2, lanes 3, 5, and 7) (28,38). We initially hypothesized that these differences would be "cured" by the addition of protease to the membrane preparations. This did occur in the case of an 88 kDa OM protein, present in the WT GCM 740 but not in the mutants in the absence of protease, and found in both mutants after incubation with active protease. However, in general the proteins which differed between WT and mutant variants were not apparent substrates for the protease.

Most of the altered proteins, whether in the outer or cytoplasmic membrane, were found in all members of an isogenic set. This is strongly supportive of specific proteolysis of unique common substrate proteins rather than generalized degradation. The presence of IgA1 protease susceptible proteins in the OM and CM of WT gonococcal strains (including both GCM 740 and F62) which produced active enzyme, as well as in the *Iga*$^-$ mutants, may be due to the *in vitro* accessibility of both sides of the OM and CM. In contrast, in an *in vivo* situation the active form of IgA1 protease, as measured against human IgA1, is restricted to

the extracellular fluids (33). However, during the process of secretion, the intact IgA1 protease proenzyme (enzyme plus α and β peptides) does exist in the periplasmic space, and therefore has access to the periplasmic sides of both the OM and CM. While it has been reported that this intermediate form of the enzyme has no activity against human IgA1, it is not known whether the proenzyme has proteolytic activity against any other substrate (33). It is therefore possible that proteolysis of membrane proteins not exposed on the cell surface could occur *in vivo*.

We do not know the specific identities of any of these membrane proteins that are degraded by IgA1 protease. However, we do know that none of these are among the previously well described gonococcal major outer membrane proteins; i.e., not Por, Rmp, or Hmp. We also have examined the susceptibility of defined iron limitation and anaerobiosis induced outer membrane proteins, and have found that these stress induced proteins also are not substrates for IgA1 protease hydrolysis (38). The data does suggest that some of the substrate proteins may be conserved gonococcal membrane proteins, since degradation products of the same apparent molecular weight were observed in all five variants examined (Figure 2).

Alteration of protein bands in both the GCM 740 and F62 sets of strains by protease purified from GCM 740 indicated that the proteolysis of membrane proteins is not strain specific (Figure 2). This proteolytic effect is also not restricted by species, as demonstrated in Figure 4, which shows that OM proteins of *E. coli* DH1 are susceptible to cleavage by the exogenously added IgA1 protease. Similar results were observed when isolated OMs of a porcine pathogen, *Actinobacillus*

pleuropneumoniae, were treated with IgA1 protease.

The fact that there are proteins in the OM of the gonococcus that are permissive substrates for the IgA1 protease prompts the question as to whether the enzyme performs this function *in vivo*. If membrane proteins were processed *in vivo* by IgA1 protease, one would expect that either there would be proteins found in the mutants (and not in the WT) which would be degraded by added protease, or that there would be proteins absent in the mutants (and found in the WT) that would appear after incubation with added protease. The 88 kDa OM protein present in WT GCM 740 and absent in the mutants, which appears post-IgA1 protease treatment of the mutants, is suggestive that this type of processing of membrane proteins by IgA1 protease does occur *in vivo*.

In summary, the data presented here indicate that there are proteins present in the outer and cytoplasmic membranes of *N. gonorrhoeae* that are permissive *in vitro* substrates for gonococcal type 2 IgA1 protease. Proteolysis of these membrane proteins was dependent on the addition of active IgA1 protease and was neither strain nor species restricted. This report defines an entire range of new potential substrates for the IgA1 proteases, whose role in the physiology of the organism or the pathogenesis of gonococcal infection remains to be determined.

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Chapter 3

EXPRESSION OF THE *Neisseria gonorrhoeae* TYPE 2 IgA1 PROTEASE GENE UNDER IRON LIMITED CONDITIONS

Russell J. Shoberg and Martha H. Mulks

Department of Microbiology and Public Health

Michigan State University

East Lansing, MI 48824-1101

ABSTRACT

The effect of iron limitation on *Neisseria gonorrhoeae* and its expression of IgA1 protease activity was examined by growing bacteria under conditions of iron limitation and iron excess. When bacteria were grown in an iron limited medium, there was an initial burst of high IgA1 protease activity in the supernatant, which was more than double that observed with cells grown in an iron replete medium and over five-fold more than cells that were grown in a high iron medium. This effect was consistently observed regardless of the chemical means of iron limitation. The activity of the mature enzyme was not affected by either the medium iron concentration or the type of medium used for bacterial propagation. The existence of a well conserved nucleotide sequence homology to an *Escherichia coli* operator *fur* binding site in the promoter of the *iga*2 gene is presented. However, when the cloned *N. gonorrhoeae iga*2b gene was transformed into a set of *Escherichia coli* $Fur^{+/-}$ isogenic strains, *fur*-mediated regulation of the *iga*2b gene in *E. coli* was not observed. Southern analysis failed to detect a chromosomal *fur* homolog in *N. gonorrhoeae* when the cloned *E. coli fur* gene was used as a probe. These apparent discrepancies are discussed further.

INTRODUCTION

The ability to produce the enzymes referred to as IgA1 proteases is a phenotype shared among a relatively small set of bacteria having a number of other characteristics in common, such as being human pathogens which initiate infections of the mucosal surfaces (37,45). Representative IgA1 protease producing bacteria include: *Neisseria gonorrhoeae* (46), *N. meningitidis* (46), *Haemophilus influenzae* (25,29,38), *Streptococcus pneumoniae* (25,29,38), *S. sanguis* (47), *S. oralis* (23,49), *S. mitis* (49), *Clostridium ramosum* (16), *Bacteroides melaninogenicus* (23), *B. asaccharolyticus* (23), *Capnocytophaga spp.* (23), and *Ureaplasma urealyticum* (50).

Production of an IgA1 protease has long been postulated to be a virulence factor for these microbes and there is a body of indirect evidence for this (see 24 for a review of the subject). However, since many of these microbes are obligate human pathogens and lack a *bona fide* animal model system (*e. g.*, *N. gonorrhoeae*) and since the "relevant" substrate for IgA1 protease is human IgA1, convincing experiments have yet to be performed.

The sites at which *N. gonorrhoeae* may invade and inhabit a host include the mucosal surfaces of the urogenital tract, rectum, pharynx, and conjunctiva in an uncomplicated gonococcal infection; as well as the bloodstream, synovial fluid, and cerebrospinal fluid during cases of diseminated gonococcal infection. The glycoprotein lactoferrin is secreted on the mucosal surfaces of the host where it functions as a nonspecific, antimicrobial mechanism by reducing the availability of free Fe^{3+} (31). Evidence that the urogenital tract is a low iron environment for bacterial growth came from the work of Shand, *et al.*,

who demonstrated that bacteria isolated directly from a patient with a urinary tract infection were expressing iron repressible outer membrane proteins (52). In the vascular system and interstitial fluids, transferrin, an iron transport glycoprotein is secreted, where it also functions as a nonspecific, antimicrobial mechanism. Therefore, regardless of the site of colonization, the gonococcus resides in an environment with little available iron, and must respond to that metabolic stress. Most strains of gonococci are able to acquire and utilize iron bound to the following human organic iron sources: transferrin, lactoferrin, hemin, and hemoglobin (59). *N. gonorrhoeae* does not produce siderophores (low molecular weight iron acquisition molecules) but interestingly, has been demonstrated to assimilate iron bound to the siderophore aerobactin (59).

A number of putative gonococcal virulence functions that are regulated by the *in vitro* medium concentration of iron have been reported. These include the Fbp (*F*erric *b*inding *p*rotein), a 37,000 dalton major outer membrane protein which has been demonstrated to bind iron reversibly (4,34), and the separate outer membrane protein receptors specific for human transferrin and lactoferrin (6,28). The expression of these proteins has been demonstrated to be regulated in a coordinate fashion by the limited availability of iron in the medium (34,159). There are also a number of other iron-repressible outer membrane proteins (*i. e.* induced by the limitation of iron in the medium) that have been described for *N. gonorrhoeae* but most of these have no described function as yet (22,43,59). The subset of iron repressible outer membrane proteins expressed by a given strain has been demonstrated to be dependent upon the mode of iron limitation (59).

Most of these have been demonstrated to be expressed *in vivo* as well, evidenced by the host's immune response to the protein (15a).

Several bacterial toxins are regulated by iron concentrations including diphtheria toxin of *Corynebacterium diphtheriae* (57), exotoxin A produced by *Pseudomonas aeruginosa* (5), the *Shigella dysenteriae* Shiga toxin (13), shiga-like toxin produced by strains of *Escherichia coli* (8), and the hemolysins of *Vibrio cholerae* (56) and *Listeria monocytogenes* (11). Also under iron regulation are several bacterial siderophore iron acquisition systems (12) and a variety of bacterial surface proteins (42).

There are two major described mechanisms by which bacteria regulate gene expression with regard to iron-mediated expression. The first mechanism, used by a variety of bacteria, is the Fur transcriptional repression system as first described in *Salmonella typhimurium* (14) and further characterized by Hantke (19,20). According to the model, the repressor exists as a Fur homodimer in the cytosol. In the presence of sufficient cytoplasmic ferrous iron, the Fur dimer complexes with Fe^{2+}, subsequently binding to an operator sequence (the "iron box") in the promoter region of Fur regulated genes, therein preventing the association of RNA polymerase with these genes and resulting in a lack of gene expression (3). To date, Fur regulatory systems have been proposed and described in such varied organisms as *Yersinia pestis* (55), *Vibrio cholerae* (8), and *Corynebacterium diphtheriae* (7). The second described mechanism involves the cooperative transcriptional activation of regulatory genes governing the synthesis and uptake of the *Vibrio anguillarium* siderophore, anguibactin, by the *trans*-acting factors Taf and AngR (12,51).

Since a number of *N. gonorrhoeae* genes are known to be regulated by the availability of ferric iron, and since a number of microbial virulence factors are also regulated by iron limitation, we felt that it would be prudent to study the effects of low iron concentration on the expression of IgA1 protease. Since we had evidence that both wild type and *Iga*$^-$ gonococci could respond to iron limitation and express the appropriate, previously described iron regulable outer membrane proteins (Chapter 1 of this thesis), we concluded that the ability to produce IgA1 protease was not required to respond to a low iron stress. Since the IgA1 proteases have been postulated and presented as virulence factors (24,37,45), we wondered whether they might be induced or regulated in some fashion by the available iron concentration.

To examine the effect(s) of iron on the activity of the *Neisseria gonorrhoeae* IgA1 protease gene we studied it in comparison to the well described *fur* regulatory system. We measured enzyme activity expressed under controlled conditions of iron limitation and iron excess in the gonococcus, and in a characterized *E. coli Fur* background to examine the effect of the *fur* locus on expression of the cloned *iga*2 gene. We also used the cloned *E. coli fur* gene as a molecular probe to search for a gonococcal *fur* homologue.

We present evidence that the type 2 IgA1 protease of *N. gonorrhoeae* is influenced by the medium iron concentration and further, that this effect is not due to modulation of the activity of the mature enzyme. We were unable to demonstrate *fur* regulation of the cloned *iga*2 gene in *E. coli* or the presence of a *fur* homologue on the gonococcal chromosome. Potential pitfalls in the experimental design are discussed concerning the lack of *fur* regulation in *E. coli*.

(Portions of this data were presented at the 90th. Annual Meeting of the American Society for Microbiology in Anaheim, CA, in 1990 [53].)

MATERIALS AND METHODS

Bacterial strains and media. *Neisseria gonorrhoeae* GCM 740 is a prototrophic, type 2 IgA1 protease-producing (*iga*2b genotype) strain of serovar PorB2 and *N. gonorrhoeae* NRL 30465 is an arginine-, hypoxanthine-, and uracil-requiring (AHU^-), type 1 IgA1 protease-producing (*iga*1 genotype) strain of PorA1 serovar. These strains have been described previously (39). The *Escherichia coli* strains BN402 and BN4020 are *fur* isogens, being Fur^+ and Fur^-, respectively, and have been described previously (2).

Gonococci were grown in NEDA, the chemically defined medium of Morse and Bartenstein (36). The medium was made iron-limiting in one of two manners, either extraction with the chelator 8-hydroxyquinolone (8-HQ; Sigma Chemical Co., St. Louis, MO; 58) or by addition of the hydroxamate siderophore, desferrioxamine B mesylate (Desferal; CIBA-GEIGY Corp., Summit, NJ), to 50 μM (43). To ensure that media induced the iron-limitation response, gonococci were grown to mid-exponential phase and outer membranes were isolated for analysis as described below.

E. coli strains were grown on LB agar medium (30) prior to transformation (10) with the previously described type 2 IgA1 protease-encoding plasmid pRJ2 (Figure 1; 40). Following transformation, the recombinant strains were selected and propagated on LB agar medium containing 12.5 μg tetracycline per ml (Sigma Chemical Co.). For growth curves and IgA1 protease assays, the *E. coli* strains were propagated in fBHI containing 12.5 μg tetracycline per ml (fBHIt). We prepared fBHI

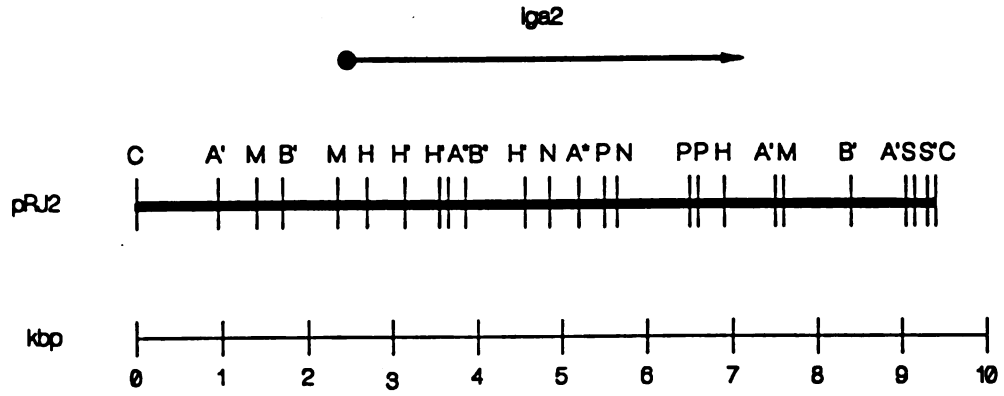


Figure 1. Restriction map of pRJ2, a pBR322-*iga*2b recombinant construct encoding IgA1 protease activity. The direction of transcription is indicated and restriction sites are designated as follows: A*, *Acc*I; A', *Ava*I; A", *Ava*II; B', *Bgl*I; B", *Bgl*II; C, *Cla*I; H, *Hin*dIII; M, *Mlu*I; N, *Nsi*I; P, *Pst*I; S, *Sma*I; and S', *Sal*I.

by ultrafiltration of brain heart infusion broth medium (Difco Laboratories, Detroit, MI) through a 10,000 molecular weight cutoff membrane (type YM10; Amicon Corp., Lexington, MA) under positive nitrogen pressure. Media prepared in this fashion had been shown to be iron-replete by its failure to induce the expression of iron-limitation outer membrane proteins in *N. gonorrhoeae*, analyzed as described below.

Isolation of outer membranes and analysis. Gonococci were grown to mid-exponential phase in the medium being tested. Cells were harvested by low speed centrifugation and spheroplasts were prepared by sucrose-lysozyme-EDTA treatment (44). Spheroplasts were disrupted by ultrasonication and outer and cytoplasmic membranes were separated by ultracentrifugation over isopycnic sucrose gradients (21). Membrane protein concentrations were determined with a Bradford assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (Sigma Chemical Co.) as a standard. Membrane proteins were resolved on 7.5-12.5% linear gradient SDS-PAGE gels (27) and visualized using a combination Coomassie blue-silver stain (18). Wet gels were analyzed with transillumination and iron-repressible proteins were identified by comparison of membranes from cells grown under iron replete conditions with membranes from cells grown under iron-limited conditions. Expression of the ~37,000 dalton Fbp protein was considered evidence of iron-limitation by the medium (34,59).

IgA1 protease assays. Quantitation of IgA1 protease production was determined by digestion of a ^{125}I-labelled human IgA1 paraprotein (Mor) with a clarified supernatant sample prepared by centrifugation of an aliquot of broth culture for two minutes in a microcentrifuge (Brinkmann Instruments Inc., Westbury, NY). IgA1 cleavage products were separated

by 9.0% SDS-PAGE as previously described (47). The amount of radioactivity in the IgA1 cleavage products (Fc_α and Fd_α) was determined using a Beckman Gamma5500B gamma counter (Beckman Instruments Inc., Fullerton, CA). These values were used to calculate the percent cleavage and in turn, the number of units of IgA1 protease per digest and the number of units per ml of culture supernatant. One IgA1 protease unit has been previously defined as one μg of IgA1 cleaved per minute at 37°C (47).

Assays examining the effect(s) of exogenous ferric iron on the activity of preformed IgA1 protease were performed by diluting an enriched IgA1 protease preparation (54) 1:400 in assay buffer [50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5% bovine serum albumin (w/v)] and incubating with exogenous iron in the assay mix. Iron was added in the form of $Fe(NO_3)_3$ $9H_2O$. Assays were performed in triplicate at 37°C for fifteen minutes. The number of IgA1 protease units per ml was calculated for each replicate along with the mean enzymatic activity per group. The mean number of units per ml for each treatment group was compared to that of the control group (no Fe^{3+} added) by a Student's t-test to compare sample means.

To examine the effect(s) of the media on the activity of preformed IgA1 protease, assays were performed by diluting an electrophoretically pure preparation of IgA1 protease (54) 1:100 and 1:200 in the medium being tested. Media tested were 8-HQ extracted NEDA containing 252 μM Ca^{2+} and 0.042% HCO_3^- (w/v) and NEDA containing 50 μM Desferal, 10 μM Fe^{3+}, 252 μM Ca^{2+}, and 0.042% HCO_3^- (w/v). Assay mixes were incubated for fifteen minutes at 37°C in triplicate. The number of IgA1 protease units per ml was calculated and likewise, the mean proteolytic activity

for each treatment group. Mean treatment group protease activities were compared to that of a control group [NEDA containing 10 μM Fe^{3+}, 250 μM Ca^{2+}, and 0.042 % HCO_3^- (w/v)] using a Student's t-test to compare sample means.

Nucleotide sequencing and analysis. The reported promoter region of the type 2b IgA1 protease gene was subcloned into the bacteriophage vector M13mp18 by standard recombinant DNA techniques (30) and single stranded DNA was isolated. Dideoxynucleotide sequencing reactions using [α-^{35}S]dATP (New England Nuclear Corp., Boston, MA) were performed using the Sequenase Version 2.0 kit as recommended by the supplier (United States Biochemical, Cleveland, OH). Sequencing primers included the M13 universal primer supplied with the kit, and a 20-mer oligonucleotide primer (GTTTGTTGACATCTGCAACG), selected and synthesized according to our preliminary sequence data (Research Genetics, Huntsville, AL). Sequencing reaction products were resolved over 8% polyacrylamide denaturing gels and identified by autoradiography.

Comparisons of the nucleotide sequences of the *iga*2b and *iga*2c (48) promoter regions to each other, to an *E. coli* consensus Fur binding site (41), and the *fbp* promoter region (4) were performed using the GenePro version 4.20 software system (Riverside Scientific, Seattle, WA).

E. coli fur **experiments.** The *E. coli* strains BN402 (*Fur*$^+$) and BN4020 (*Fur*$^-$) were transformed to the *Iga*2b$^+$ phenotype (*i. e.* producing IgA1 protease) with pRJ2 by the $CaCl_2$ procedure (10), selecting for transformants on LB agar plus 12.5 μg tetracycline per ml. *Fur* phenotypes were confirmed by the Arnow test for siderophore production (1) and *Iga*2b phenotype was confirmed by IgA1 protease assay (47).

The IgA1 protease-producing *fur* isogens were propagated in iron-rich

fBHIt for two passages and transferred to fBHIt for analysis of growth rate (OD_{600}) to late exponential phase and IgA1 protease production as described above.

Southern blot analysis. To examine the possibility that the gonococcal genome contained a *fur* homologue, we performed Southern analysis by standard procedures (30). Chromosomal DNA was prepared from *N. gonorrhoeae* strains GCM 740 and NRL 30465 and the *E. coli fur* isogens, BN402 and BN4020, digested with the restriction endonuclease *Cla*I as recommended by the supplier (Boehringer Mannheim Biochemicals, Indianapolis, IN), and separated over a 0.7% agarose gel in Tris-boric acid-EDTA buffer (30). Transfer to a nitrocellulose sheet (BA85; Schleicher & Schuell, Inc., Keene, NH) was by capillary action with 10X SSC overnight at room temperature (30). Sheets were blocked and probed in an aqueous hybridization solution at 6X SSC (0.9 M NaCl) overnight at either 68°C (A; high stringency) or 42°C (B; low stringency). Two washes of thirty minutes each were done at room temperature in either: (A) 2X SSC, 0.1% SDS; or in (B) 6X SSC, 0.1% SDS. Two subsequent washes of thirty minutes each at 42°C were done in these respective solutions for both A and B. The sheets were reblocked, probed with the anti-hapten antibody, and developed as per the kit supplier's instructions.

The *E. coli fur* gene probe was prepared by agarose gel electrophoretic band elution of the 2.0 kilobase pair *Hin*dIII fragment of pF3 (3,30). The probe was labeled by random priming using Klenow DNA polymerase and a hapten-dUTP conjugate as per the Genius Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals).

RESULTS

Growth and IgA1 protease production in iron limited media. When GCM 740 was propagated in 8-HQ extracted NEDA without additional Fe^{3+} (Figure 2A; C), it grew at a slower rate compared to growth in 8-HQ extracted NEDA containing either 10 or 50 μM Fe^{3+} (Figure 2A; CF and CF5, respectively). The iron limited gonococci were incapable of growing to a final optical density as high as either of the iron replete cultures. When the levels of IgA1 protease per ml of culture supernatants were normalized with respect to the optical density and compared (Figure 2B), the iron limited culture (C) produced nearly twice the amount of IgA1 protease per ml per OD as that of the culture with 10 μM Fe^{3+} (CF) and over four times that of the culture with 50 μM Fe^{3+} (CF5). This initial trend of increased IgA1 protease activity was observed up to mid-exponential phase (~6 hours), where IgA1 protease activity in the iron replete culture (10 μM Fe^{3+}; CF) surpassed the activity of the iron limited culture, until these cultures reached late-exponential phase (~9 hours). At late-exponential phase, the iron limited culture (C) again exhibited more IgA1 protease in the medium than did either of the iron replete cultures (CF and CF5). Final stationary phase IgA1 protease production was 37 units per ml for the iron limited culture, 24 units per ml for the culture containing 10 μM Fe^{3+}, and 12 units per ml for the culture containing 50 μM Fe^{3+}.

Because these data were generated using a method of iron limitation not previously described for *N. gonorrhoeae*, a similar experiment was performed with GCM 740 in NEDA using Desferal to chelate the ferric iron and render it nonassimilable to the gonococci (43). When optical

Figure 2A

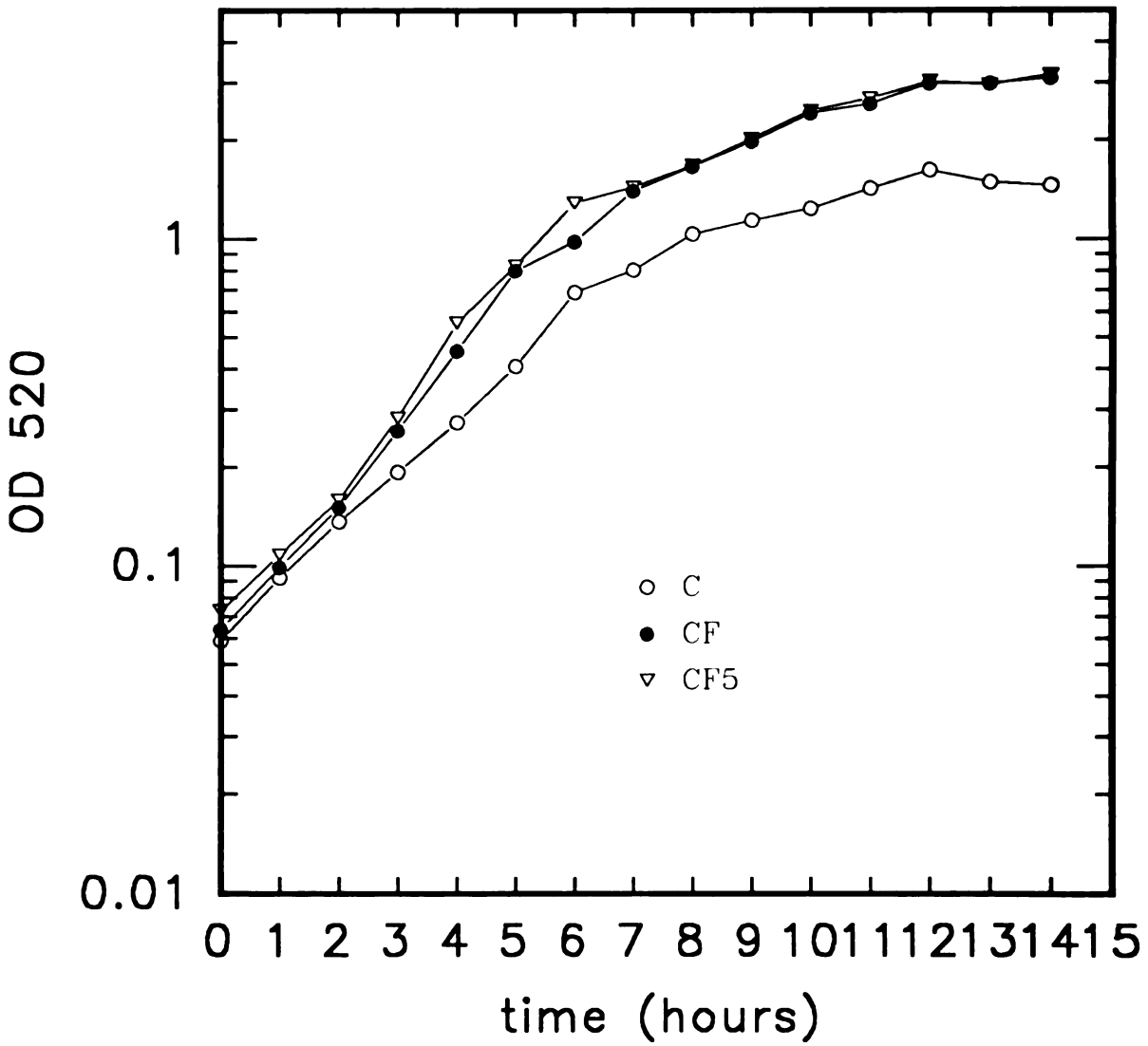
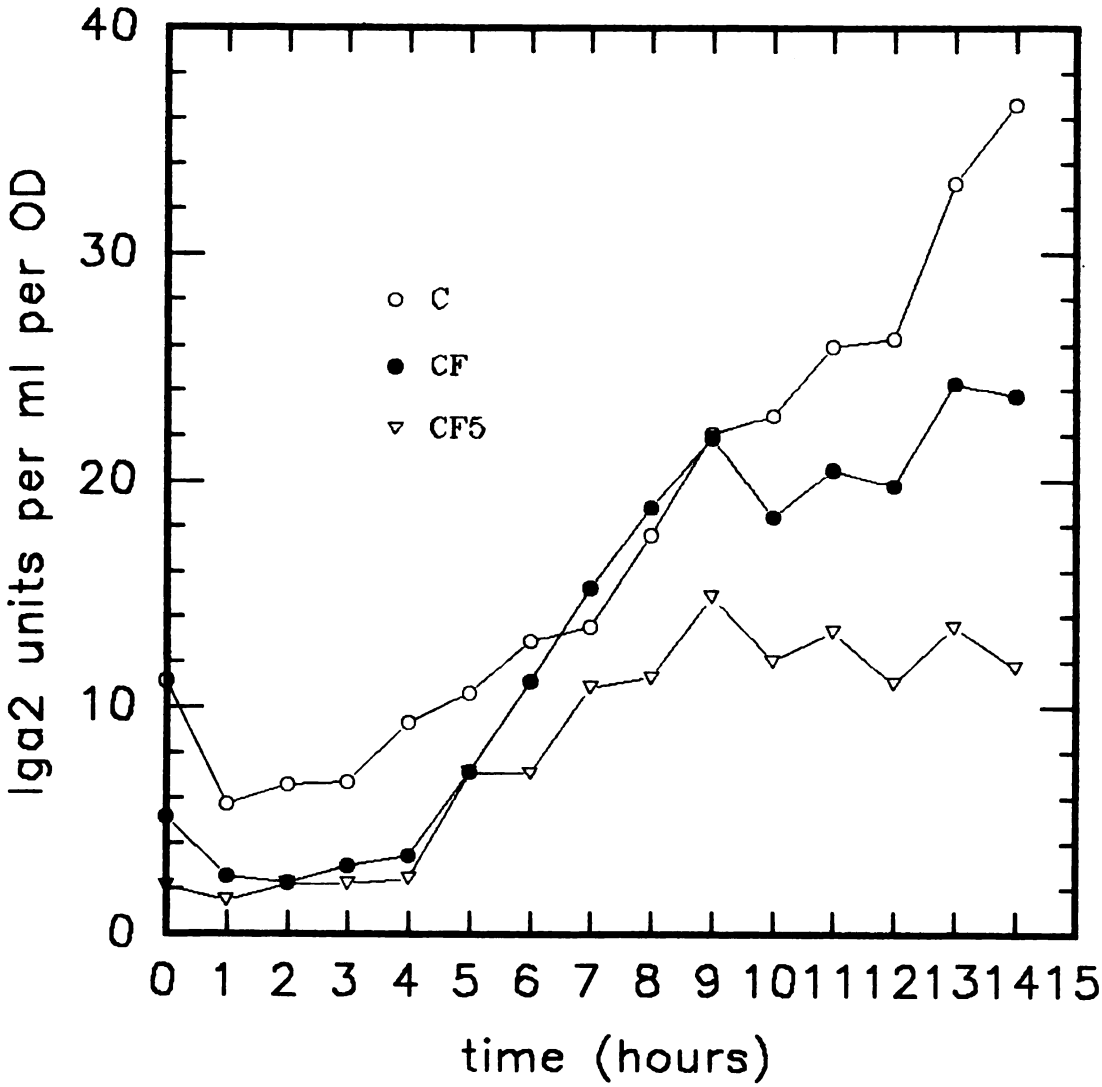


Figure 2. The effects of 8-hydroxyquinolone-extracted defined media on growth rate (A) and extracellular IgA1 protease activity (B) of *Neisseria gonorrhoeae* GCM 740. Media tested were as follows: 8-HQ extracted NEDA (C), 8-HQ extracted NEDA plus 10 μM $Fe(NO)_3$ (CF), and 8-HQ extracted NEDA plus 50 μM $Fe(NO)_3$ (CF5). All cultures were incubated with shaking at 37°C with 0.252 mM Ca^{2+} and 0.042% $NaHCO_3$ (w/v) added. IgA1 protease assays were performed as previously described (47).

Figure 2B


40
30
20
10
0
lga2 units per ml per OD
C
CF
CF5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
time (hours)

density data were analyzed (Figure 3A), the iron limited culture (Df) grew at a greatly reduced rate and was unable to reach a maximal optical density comparable to that of an iron replete culture (N). When the data comparing units of IgA1 protease per ml of culture supernatant were normalized with respect to the optical density (Figure 3B), the iron limited culture (Df) exhibited at least three-fold more enzyme activity initially than the iron replete culture (N). Though the enzyme activity of the iron limited culture declined over time to a level approaching that of the iron replete culture, the iron limited culture still expressed more than twice the amount of IgA1 protease activity per ml of culture as that expressed by the iron replete culture.

These data suggested that some iron-mediated phenomenon was occurring and resulting in increased amounts of IgA1 protease activity being expressed in the culture medium. We hypothesized that this could be due to: 1) activation of preformed IgA1 protease by the lowered Fe^{3+} concentrations, 2) destabilization of preformed IgA1 protease by the increased Fe^{3+} concentrations, or 3) a low iron-inductive effect on IgA1 protease synthesis.

To confirm that the media conditions tested were inducing a low iron response, we prepared outer membranes of gonococci grown under our assumed iron limited and iron replete conditions. A silver-stained SDS-PAGE gel (shown in Figure 4) demonstrated that under the appropriate medium conditions, previously described iron-repressible outer membrane proteins were expressed (22,34,43,59). In particular, the ~37 kilodalton Fbp protein was only expressed in media containing Desferal or in 8-HQ extracted media that was not supplemented with exogenous Fe^{3+} (lanes 2 and 4).

Figure 3A

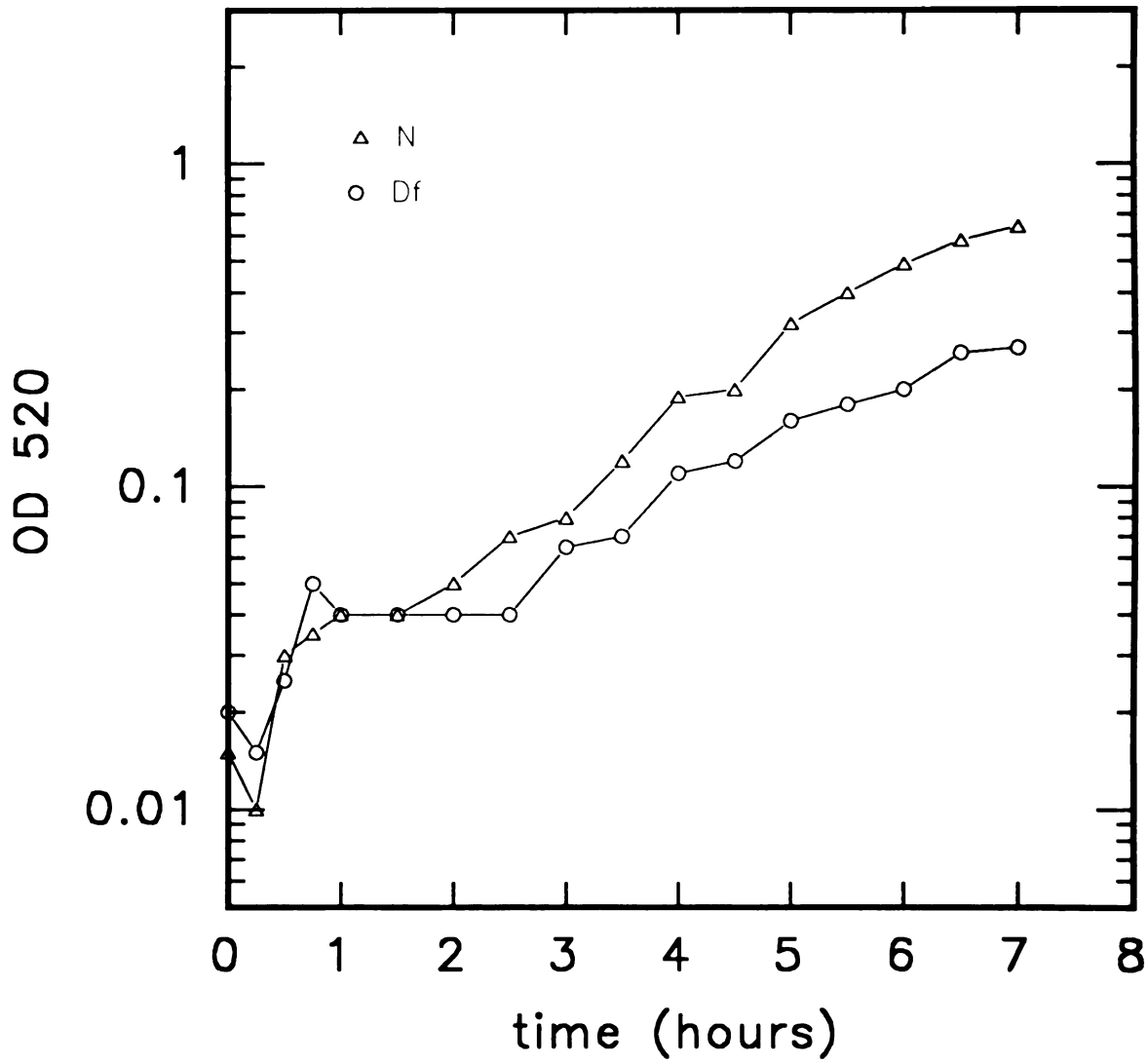


Figure 3. The effects of Desferal-induced iron-limitation on growth rate (A) and IgA1 protease expression (B) of *Neisseria gonorrhoeae* GCM 740. Media tested were NEDA (N), and NEDA plus 50 μM Desferal (Df). All cultures were incubated with shaking at 37°C with 10 μM $Fe(NO)_3$, 0.252 mM Ca^{2+} and 0.042% $NaHCO_3$ (w/v) added. IgA1 protease assays were performed as previously described (47).

Figure 3B

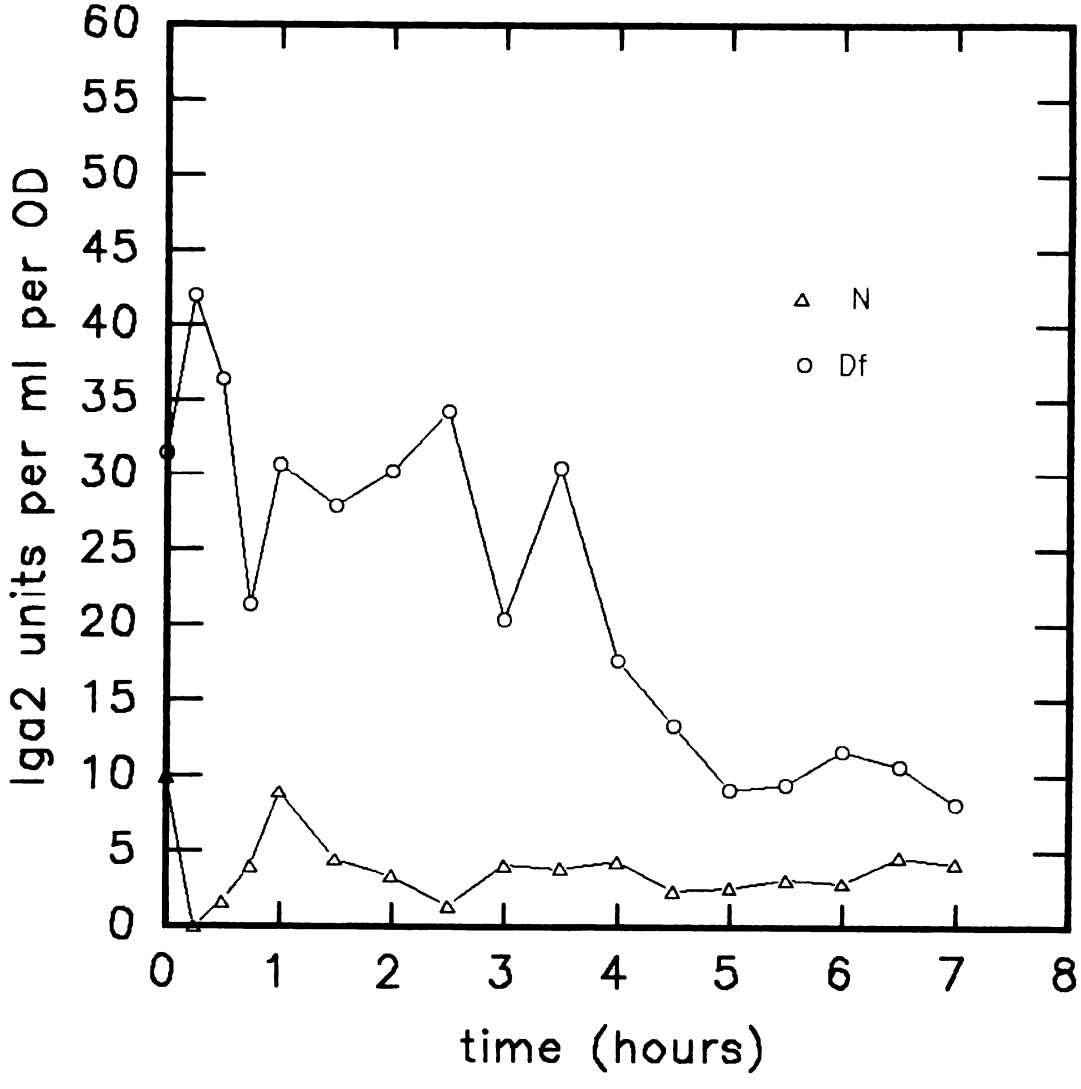
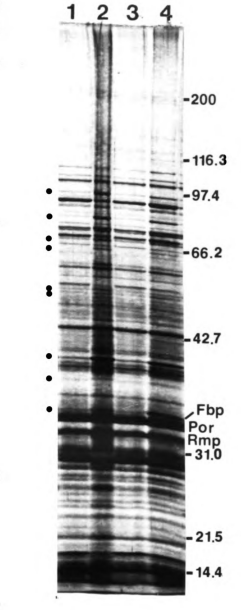

60
55
50
45
40
35
30
25
20
15
10
5
0
lga2 units per ml per OD
0
1
2
3
4
5
6
7
8
time (hours)
N
Df

Figure 4. Silver stained 7.5-12.5% linear gradient SDS-PAGE analysis of outer membranes of *N. gonorrhoeae* GCM 740 grown under iron-limited and iron replete conditions. Bacteria were grown in broth media as follows: lane 1, NEDA + 10 μM $Fe(NO)_3$; lane 2, NEDA + 10 μM $Fe(NO)_3$ + 50 μM Desferal; lane 3, 8-HQ extracted NEDA + 10 μM $Fe(NO)_3$; and lane 4, 8-HQ extracted NEDA. All media contained 0.252 mM Ca^{2+} and 0.042% HCO_3^- (w/v). Iron-repressible proteins are shown along the left margin (●) as are some of the defined major gonococcal outer membrane proteins. Positions of molecular weight standards are shown along the right margin in kDa (Bio-Rad).

Figure 4



Effects of exogenous Fe^{3+} and various media on activity of preformed IgA1 protease. When an enriched preparation of IgA1 protease was incubated with exogenous Fe^{3+}, there were no statistically significant differences between enzyme activities in the presence of exogenous iron (concentration range from 0.1-100 μM Fe^{3+}) as compared to a control reaction lacking exogenous Fe^{3+}. Table 1 is a summary of the data.

When a preparation of purified IgA1 protease was diluted and incubated in the various media tested, there was not a statistically significant increase in the IgA1 protease activity between the treated samples and the control samples (Table 2). However, there was a statistically significant decrease in enzyme activity when IgA1 protease in 8-HQ extracted NEDA (plus Ca^{2+} and HCO_3^-) was compared to the control IgA1 protease samples in NEDA (plus Fe^{3+}, Ca^{2+}, and HCO_3^-).

These data taken together, indicated that the increase in extracellular IgA1 protease activity was not due to an activating or destabilizing effect on preformed enzyme, caused by either treatment of the medium or the medium iron concentration.

Examination of the promoter sequences of two *iga*2 genes and comparison to the *E. coli* Fur binding site. The promoter nucleotide sequence of the *iga*2b gene was identical to that of the previously reported *iga*2c gene over the region of interest (48; Figure 5). These two sequences exhibited high homology (14 of 19 bases or ~74%) to an *E. coli* consensus iron box, the Fur binding site in the operator of genes regulated by the iron-dependent Fur repressor system (41). When the putative *iga*2 iron box was compared to the putative gonococcal *fbp* iron box (4,35), less intraspecies homology was detected. The *fbp* iron box

Table 1. The effect of exogenous iron concentration on activity of an enriched preparation of IgA1 protease.[a]

$\mu M\ Fe^{3+}$	$\mu g\ IgA1\ min^{-1}\ ml^{-1}$ [b]	$p > 0.05$[c]
100	14.13 ± 0.20	-
10	14.56 ± 0.79	-
1	13.92 ± 0.60	-
0.1	14.72 ± 0.57	-
Control	14.72 ± 0.35	ND[d]

a - Enzyme preparation was diluted 1:400 in 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5% bovine serum albumin (w/v) plus the stated concentration of $Fe(NO_3)_3\ 9H_2O$ and quantitative assays using ^{125}I-labeled IgA1 were performed for 15 minutes at 37°C as previously described (47).

b - Mean ± SD of three assays (x 10^3)

c - $t_{\alpha(2)\ 0.05} = \pm 2.776$

d - Not determined

Table 2. The effect of relevant growth media on activity of a purified IgA1 protease preparation.[a]

Medium	$\mu g\ IgA1\ min^{-1} ml^{-1}$ [b]	$p > 0.05$[c]
8-HQ extracted NEDA + Ca^{2+}, HCO_3^-	2.05 ± 0.16	+
NEDA + 50 μM Desferal + Ca^{2+}, Fe^{3+}, HCO_3^-	3.23 ± 0.30	-
NEDA control + Ca^{2+}, Fe^{3+}, HCO_3^-	3.00 ± 0.40	ND[d]

a - Enzyme preparation was diluted 1:100 and 1:200 in the stated medium and quantitative assays using ^{125}I-labeled IgA1 were performed for 15 minutes at 37°C as previously described (47).

b - Mean ± SD of six assays (x 10^3)

c - $t_{\alpha(2)\ 0.05} = \pm 2.228$

d - Not determined.

		HOMOLOGY
E. coli consensus	GATAATGAT$\frac{A}{T}$AT$\frac{T}{C}$ATT$\frac{TT}{AG}$C	
*iga*2b	tAaAATGcaAAaCATTATC	14/19
	⋮ ⋮⋮⋮ ⋮⋮⋮⋮	
fbp	aATAATtATTtgCATTTat	13/19
E. coli consensus	GATAATGAT$\frac{A}{T}$AT$\frac{T}{C}$ATT$\frac{TT}{AG}$C	

Figure 5. Nucleotide sequence alignments of parts of the IgA1 protease promoter region and the promoter region of the gonococcal *fbp* gene (4) with a reported consensus *E. coli* Fur binding site (41). Conserved nucleotides are marked with dots and those shared among either gonococcal sequence and the consensus are in upper case letters.

shared 13 of 19 bases with the *E. coli* consensus (~68% homology), while the *iga*2 and *fbp* iron boxes had only 8 of 19 bases in common (~42% homology). These data suggested that the gonococcal *iga*2 gene may have the potential to be regulated in a manner similar to the Fur repressor system described in *E. coli* (3,20).

Response of a cloned *iga*2b gene in a known *Fur* background. The *E. coli Fur* isogens were transformed with pRJ2, a recombinant pBR322 plasmid encoding the type 2 IgA1 protease of GCM 740 (*iga*2b, Figure 1). Transformants were tested for both the *Fur* and *Iga*2 phenotypes by the Arnow test (1) and qualitative IgA1 protease assays (47) prior to the growth curve experiment with quantitative IgA1 protease assays. The growth curve experiment was performed using bacteria grown in an iron rich medium with the presumption that Fur repressed genes would be derepressed in the *Fur*$^-$ strain (BN4020). Therefore, if the cloned *iga*2 gene was *fur*-regulable in *E. coli*, we predicted that we would see an increase in IgA1 protease activity with the *Fur*$^-$ mutant due to

derepression.

In fBHIt, BN402 containing pRJ2 (*Fur*$^+$) did not produce catechol, an intermediate in siderophore production, and did produce IgA1 protease (data not shown). The mutant BN4020 containing pRJ2 (*Fur*$^-$) produced catechol in the same medium and produced IgA1 protease (data not shown).

The two strains grew with similar growth rates in fBHIt (Figure 6A). The two strains also expressed nearly identical amounts of IgA1 protease at all points along the growth curve as followed (Figure 6B). From these data we concluded that the gonococcal *iga*2b gene is not regulated by the *E. coli fur* system, as shown by the equivalent expression in the *E. coli Fur* isogens grown in an iron rich medium.

Southern analysis of the *Neisseria gonorrhoeae* chromosome with the *E. coli fur* gene. Figure 7 shows Southern analysis of chromosomal DNAs from two strains of *N. gonorrhoeae*, GCM 740 and NRL 30465 and from *E. coli* BN402 and BN4020, the *Fur*$^+$ and *Fur*$^-$ isogens. Figure 7a shows homologies detected under high stringency hybridization and wash conditions. No homologies were detected between the *E. coli fur* probe and the chromosome of either gonococcal strain. Figure 7b shows homologies detected under low stringency hybridization and wash conditions. Note the hybridization of the *E. coli fur* probe to the molecular weight standards, λ DNA digested with *Hin*dIII (lane 1); the lack of hybridization to an empty lane (lane 2); the lack of apparent specific hybridization to any region of the digested gonococcal DNAs (lanes 3 and 4); and the still detectable specific, as well as nonspecific hybridization to the digested *E. coli* DNAs (lanes 5 and 6). These data suggested that there is no appreciable homology between the

Figure 6A

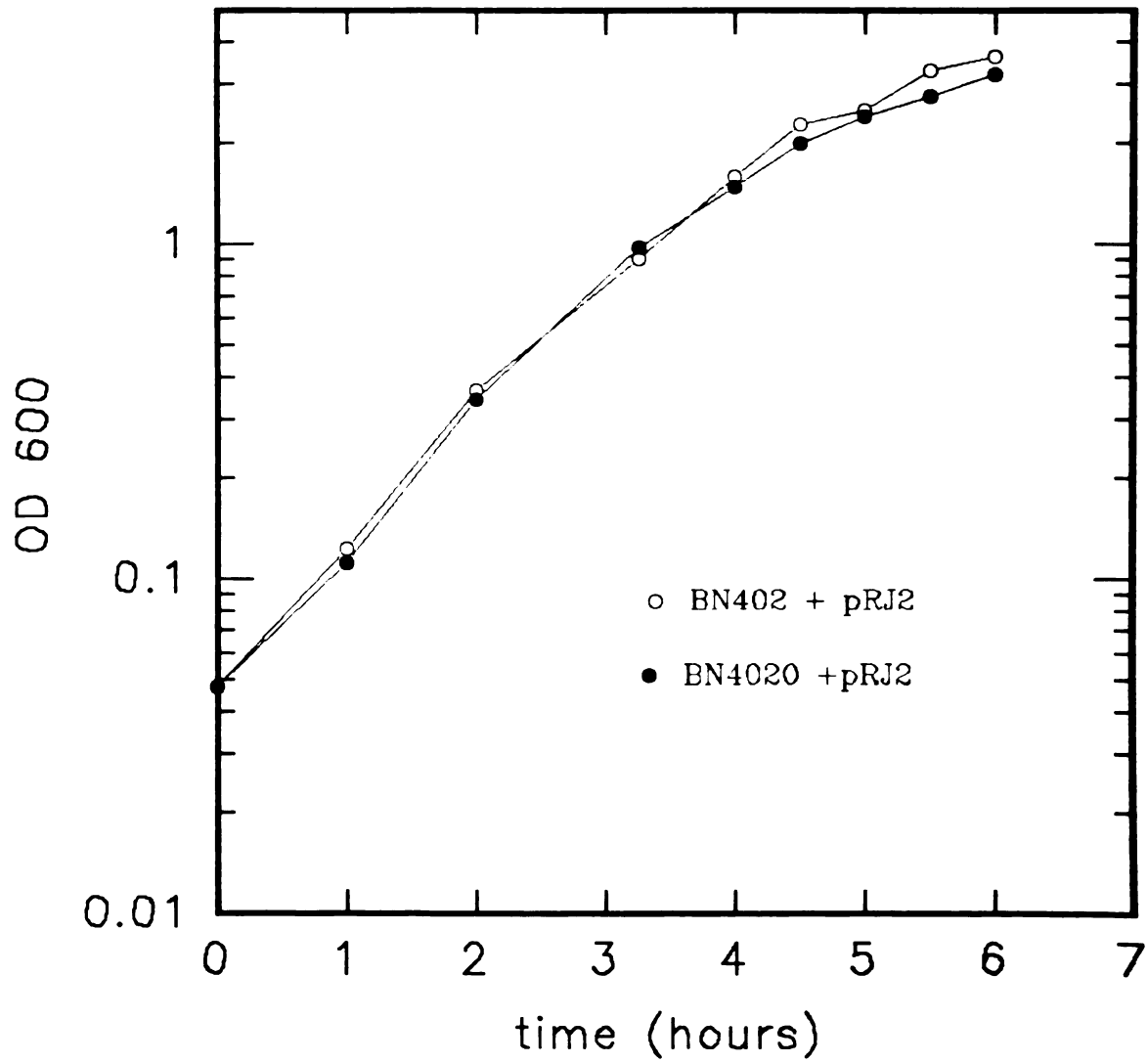


Figure 6. Growth (A) and IgA1 protease expression (B) of *E. coli* BN402 (*Fur*$^+$) plus pRJ2 and *E. coli* BN4020 (*Fur*$^-$) plus pRJ2 in fBHIt, an iron rich medium. Cultures were incubated with shaking at 37°C and contained 12.5 μg tetracycline per ml. IgA1 protease assays were performed as previously described (47).

Figure 6B

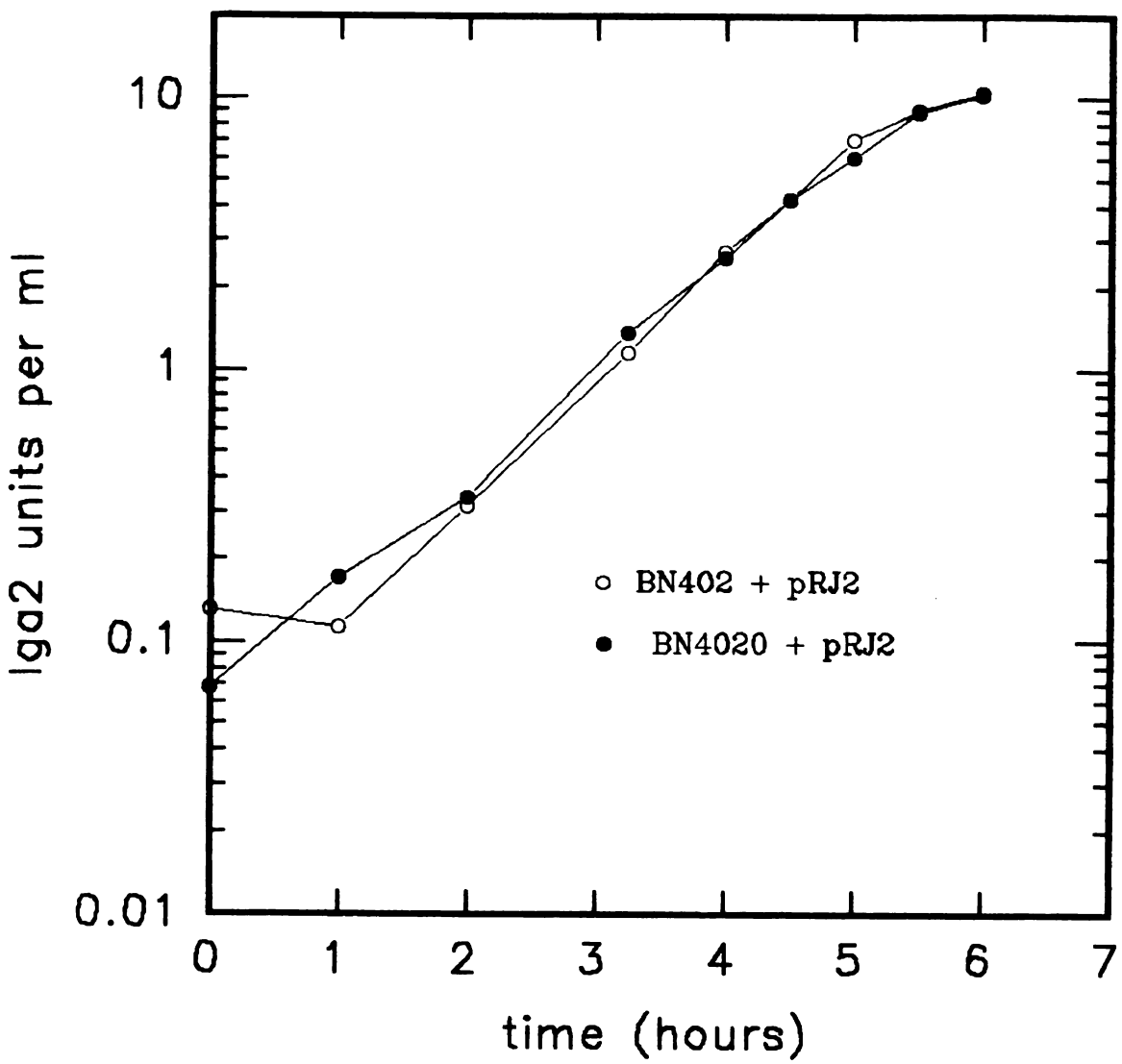
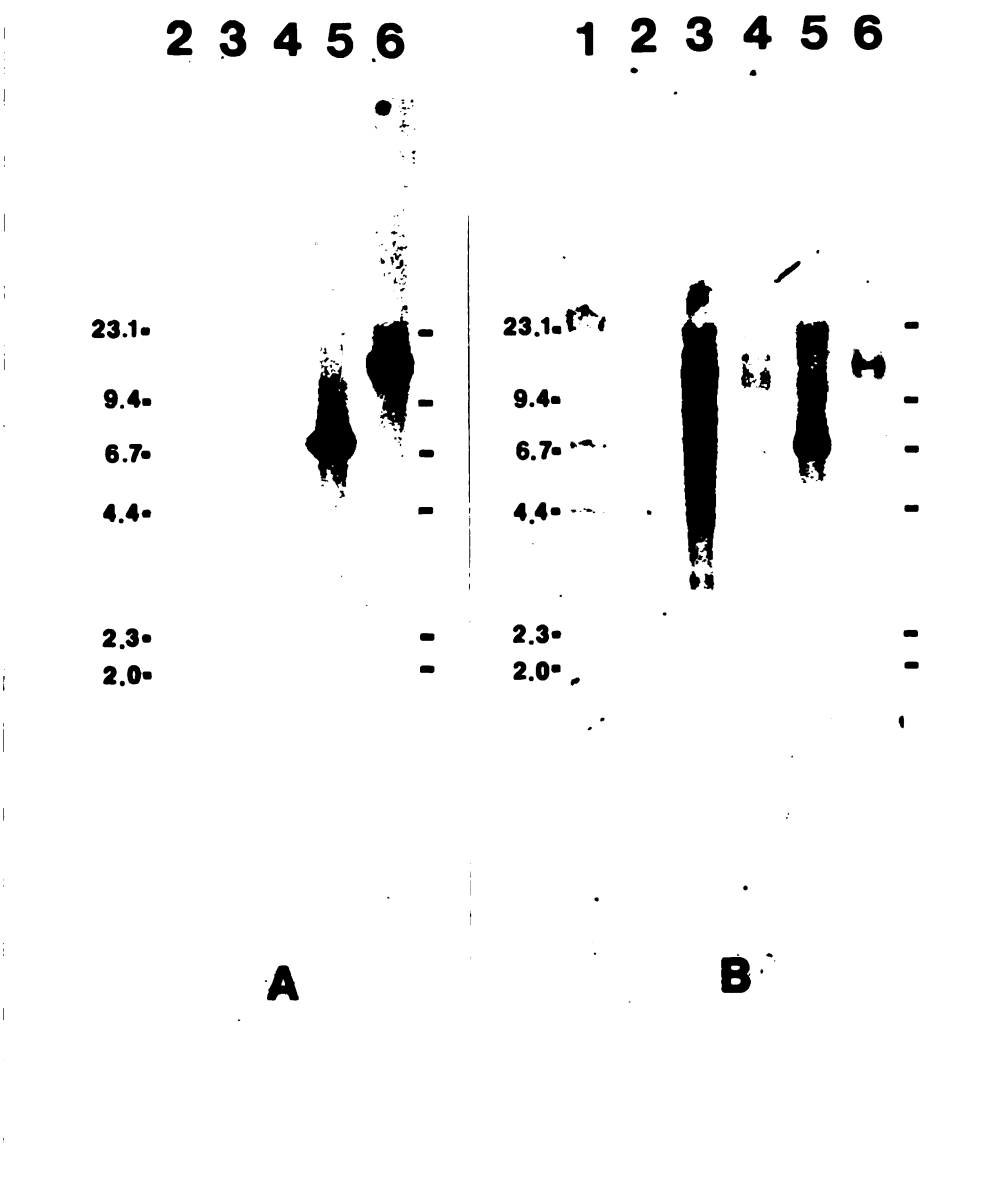

10
1
0.1
0.01
lga2 units per ml
0
1
2
3
4
5
6
7
time (hours)
BN402 + pRJ2
BN4020 + pRJ2

Figure 7. Southern analysis of *Neisseria gonorrhoeae* and *E. coli* genomic DNAs probed with an *E. coli fur* gene probe. Panel A was hybridized and washed at high stringency. Panel B was hybridized and washed at a low stringency (see Materials and Methods for conditions). All DNAs were digested with *Cla*I unless specified otherwise. Lane 1, λ DNA digested with *Hin*dIII; lane 2, no DNA; lane 3, *N. gonorrhoeae* GCM 740; lane 4, *N. gonorrhoeae* NRL 30465; lane 5, *E. coli* BN402; and lane 6, *E. coli* BN4020. Positions of molecular weight standards are given along the margins in kilobase pairs (Bethesda Research Laboratories).

Figure 7



fur gene of *E. coli* K-12 and the genome of either strain of *N. gonorrhoeae* tested.

DISCUSSION

We have demonstrated an iron-mediated effect which resulted in an increase in the extracellular activity of the type 2 IgA1 protease of *Neisseria gonorrhoeae*. This increase in enzymatic activity when gonococci were propagated in iron limited media correlated with the expression of several described iron-repressible outer membrane proteins (22,34,43,59). The increased IgA1 protease activity was not due to an effect of either high or low ferric iron concentrations, altering the activity of preformed enzyme, and was not due to an effect of the medium on the activity of preformed enzyme.

The promoter regions of both the *iga*2b and *iga*2c genes showed good nucleotide sequence homology to a consensus *E. coli* Fur binding site which suggested that the gonococcal IgA1 protease gene might be regulated by Fur in an iron-dependent manner. However, when we examined the effect of iron limitation in a known *Fur* background, it indicated that the *E. coli fur* system did not regulate the cloned *N. gonorrhoeae iga*2b gene. It was also shown that there is no detectable DNA homology between the cloned *fur* gene of *E. coli* K-12 and the genomes of two *N. gonorrhoeae* strains, GCM 740 and NRL 30465.

It is not known at the present time how the medium iron concentration influences or regulates the expression of the gonococcal *iga*2b gene, and whether the putative iron box in the promoter of the *iga*2 genes is functional or anecdotal. Experiments to address these problems are proposed in the following discussion.

We initially were interested in defining the gonococcal response to

iron limitation with regard to the expression of IgA1 protease because of its proposed function as a virulence factor for the organism (24,37,45). We hypothesized that if IgA1 protease were truly a virulence factor, it might be upregulated in response to environmental stimuli. One of the pertinent stimuli that we felt was important to test was the limitation of available iron, as there is very little free iron in or on the human body. Estimates have reported the free Fe^{3+} concentration as low as 10^{-18} M (15).

The first niche that *N. gonorrhoeae* would be expected to encounter in a host is a mucosal epithelial surface, and any free iron which might exist in these sites would be rapidly chelated by the host glycoprotein, lactoferrin (31). Gonococci are able to acquire and utilize the iron chelated by lactoferrin (32), but the events necessary for production of the lactoferrin binding protein and expression on the surface of the gonococcus are themselves triggered by a low iron response (6,28). Therefore, the microbe is required to respond to the stress of iron limitation prior to being able to express virulence functions which may serve to alleviate this stress. There are a number of gonococcal outer membrane proteins which are regulated by the available iron concentration, many of which have no defined function (22,43,59). Some of these outer membrane proteins with described functions include the Fbp (demonstrated to bind Fe^{3+}, 35) and the undefined receptors for lactoferrin and transferrin (6,28).

We observed an increase in the level of extracellular IgA1 protease when the bacteria were grown in iron limited media (Figures 2B and 3B), regardless of the chemical reason for limitation of Fe^{3+}. These same media induced expression of Fbp (Figure 4) and other iron repressible

outer membrane proteins. The gonococci grown under iron limited conditions produced from two to six-fold more enzyme than gonococci grown in the same medium supplemented with 10 μM Fe^{3+} (Figures 2B and 3B). For one set of media tested, gonococci grown to stationary phase in a five-fold excess of Fe^{3+} demonstrated less than one-third the amount of IgA1 protease expressed by bacteria grown in an iron limited culture (Figure 2B; cultures C and CF5). From these data, we concluded that the IgA1 protease response was due to the limitation of iron.

Although the expression of IgA1 protease activity is induced by only two to six-fold when the cells are grown in iron limited media, we feel that this response is of an acceptable magnitude to be considered genuine. There are a number of previously described iron regulated functions which have inductive responses of this same order of magnitude.

The *E. coli* outer membrane protein FhuA (aka TonA) shows an induction of 2-3 fold under iron limited conditions in contrast to other iron regulated functions such as Cir and FepA, 12 and 26 fold, respectively (19). This moderate overexpression of FhuA has been observed by two different laboratories (19,42). A study of the kinetics of the *fhu*A induction response demonstrated a rapid increase in protein synthesis (as measured by ^{3}H-leucine incorporation) which was followed by reaching a maximal rate of synthesis, and then a steady decline over time (26). This short term induction response is unusual in that only one other iron regulated membrane protein with an undefined function showed a similar response, while three different iron regulated proteins (including FepA and Cir) showed a slower induction time but had a continually increasing rate of synthesis (26).

Another example of a low induction ratio in *E. coli* is the manganese containing metalloenzyme, superoxide dismutase (SodA). In qualitative enzyme activity assays, there appeared to be no activity regulation with regard to the tested strain's *Fur* phenotype. However, when the *sodA* promoter was fused to the quantifiable *lacZ* reporter gene, a 3-4 fold induction was observed (41).

Further examples of low inductive ratios have been demonstrated with an *E. coli ompA-lacZ* transcriptional fusion construct, pSC27.1 (containing a synthetic *E. coli* consensus iron box), which shows inductive ratios of 3.3 to 4.4-fold in two different *E. coli* host strains (9), and about 2 fold in *Yersinia pestis* (55). Also, the cloned diptheria toxin gene from *Corynebacterium diphtheriae*, shows a 4 to 5-fold inductive ratio in *E. coli* (57).

We hypothesized that there could be two broad mechanisms that would result in this outcome: 1) the change in enzymatic activity was due to an effect of either increased or decreased Fe^{3+} concentration upon the preformed IgA1 protease enzyme or 2) that the decrease in available Fe^{3+} induced *de novo* synthesis of the *iga2* gene product with a resultant increase in enzyme activity.

We tested the former hypothesis by performing *in vitro* quantitative enzyme assays using preformed or mature IgA1 protease. Incubating the enzyme in the presence of from 0.1-100 μM Fe^{3+}, one order of magnitude greater and two orders lower than the Fe^{3+} concentration in the iron replete media tested, had no significant effect on its proteolytic activity (Table 1). Similarly, when we incubated IgA1 protease in the iron limited media and compared its proteolytic activity to that when incubated in iron-replete media, no significant increase in activity was

detected (Table 2). Rather, in the media rendered iron limited by 8-hydroxyquinolone extraction, we observed a statistically significant decrease in IgA1 protease activity. From these data we concluded that the effect of iron limitation on the activity of IgA1 protease was not due to activation or destabilization of mature enzyme by the media concentration of Fe^{3+}.

The observation that a sequence bearing good homology to a consensus iron box is present in the promoter of the *iga*2 genes prompts the question as to whether it is functional (Figure 5). Our data suggest that this putative iron box is not functional, at least as far as the gonococcal *iga*2 gene being *fur*-regulable in the *E. coli* strains. It was determined by phenotypic assays that the strains were functioning in an appropriate manner (i. e. BN4020, the *Fur*$^-$ strain, was expressing *fur*-regulated functions such as catechol production in an iron rich media) so the experiment yielded valid results with respect to the function of the *fur* repressor system. However, the degree of gene expression is at least four-fold lower in *E. coli* relative to the gonococcus (data not shown) and in order to perform quantitative IgA1 protease assays which are statistically valid, one must incubate for long periods of time during which the breakdown of the IgA1 protease molecule itself may affect the results obtained.

Another potential problem with the system used to address the question of the *fur*-regulability of *iga*2 is the fact that the IgA1 protease molecule is a foreign protein and has no known homologue in *E. coli*. That, coupled with the fact that the IgA1 protease molecule is secreted by a mechanism which also has no described homologue in *E. coli*, may introduce unknown and uncontrolled variables into the system.

Addressing the question of cross functionality of different regulatory systems, we also observed that there is not a well conserved gonococcal *fur* homology at the DNA level, as detected by Southern hybridization analysis with the cloned *E. coli fur* gene (Figure 7). Therefore, if *N. gonorrhoeae* has a *fur*-like repressor system for iron regulation, it may be sufficiently divergent from the broadly conserved enteric *fur* system that the two systems may not cofunction. Even among closely related bacteria, there are reports of known Fur regulable genes being weakly regulated in other systems. An example is the poorly regulated cloned *E. coli* aerobactin operon in *Shigella flexneri* (2).

However, we do not feel that this is the case due to a recent report which indicated that the purified Fur protein binds to the promoter region of the gonococcal *fbp* gene, demonstrated in a gel retardation assay (35). This data suggests that indeed there may be a Fur-like mediated system in *N. gonorrhoeae* and that it may be similar enough to the *E. coli* system to allow for cross-function.

In order to better address the problem, we propose that an *iga*2b-*E. coli* fusion protein be constructed in order to allow for the putative iron regulated expression of an *E. coli* protein which may be permissively regulated in a *fur*-dependent fashion. A suggested fusion protein would be the promoterless *pho*A gene used in Tn*pho*A mutagenesis as described by Goldberg, *et al.* (17). Briefly, the *pho*A coding sequence is randomly inserted downstream from the promoter of the gene under study via Tn*pho*A mutagenesis. Resultant mutants can be identified by plating on a differential, low-iron inductive medium also containing 5-bromo-4-chloro-3-indolylphosphate, a chromogenic substrate for alkaline phosphatase (the *pho*A gene product; 17). Alkaline phosphatase

activity can be quantitated spectrophotometrically, which would allow for assays to be done at points along the growth curve in a manner similar to those done for IgA1 protease. We propose that this sort of assay using the putative regulatory region of the *iga*2b gene fused to an *E. coli* reporter gene may produce more reliable data and allow the question of whether the putative IgA1 protease iron box is really a functional entity or just an interesting looking stretch of DNA.

In summary, we have demonstrated that there was an increase in the amount of extracellular IgA1 protease activity expressed when *Neisseria gonorrhoeae* was grown in an iron limited media. This increase in enzyme activity was accompanied by the expression of previously described iron stress outer membrane proteins and occurred under two different forms of iron limitation. The increase in IgA1 protease activity was not due to an activation of the preformed enzyme caused by a shift to a lower Fe^{3+} concentration nor to a destabilization of preformed enzyme caused by an increased Fe^{3+} concentration.

A potential Fur repressor binding site was identified in the promoter of the *iga*2 gene, having 74% nucleotide sequence homology with the *E. coli* consensus Fur binding site sequence. Functional assays involving the cloned gonococcal type 2 IgA1 protease gene in a known *Fur*$^-$ *E. coli* background indicated that the *iga*2b gene is not under iron regulation as controlled by the *E. coli* Fur repressor system. Also, DNA hybridization analysis failed to detect specific homology between a cloned *E. coli fur* gene and chromosomal DNA from two strains of *N. gonorrhoeae*. The significance of the putative gonococcal iron box located in the promoter of the *iga*2 gene and the apparent lack of *fur* regulation are under further investigation.

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SUMMARY AND CONCLUSIONS

One of the objectives of this research was to identify other potential functions of the *Neisseria gonorrhoeae* type 2 IgA1 protease. Two different deletion-disruption mutant variants of the strain GCM 740 were constructed, GCM 740Δ4 and GCM 740Δ2, isogenic except for their abilities to produce the IgA1 protease.

After comparison of the *Iga*$^-$ variants with GCM 740, the parental strain, only minor differences were observed between the two phenotypes. The *Iga* status of a variant did not influence its ability to: 1) grow in a chemically defined medium, 2) grow under anaerobic conditions on a complex agar medium, 3) grow under iron limited conditions in defined broth media, 4) process polypeptides, either co- or post-translationally, or 5) to be transformed.

Consistent differences in the outer (OM) and cytoplasmic membrane (CM) protein profiles of the WT and mutants were observed. This suggested a relationship between the variant's *iga* phenotype and the membrane protein profiles. Differing proteins were undefined with regard to identity or function, but based on relative abundance, apparent SDS-PAGE molecular weight, and heat or reduction modifiable behavior, several previously described OM proteins were ruled out. Other than an OM protein of 26 kDa, which corresponded to an Opa, these variant-specific proteins were identified not to be any of the other previously well described gonococcal antigens (*i. e.* Por, Rmp, or environmental stress proteins). The Δ-*iga* mutants were also capable of

expressing appropriate, previously described OM stress response proteins under anaerobic or iron limited conditions.

In an attempt to verify that some of these intervariant protein profile differences were linked to the *iga* allele, the Δ-*iga* allele in each mutant was restored by transformation. These *Iga*$^+$ transformants were demonstrated to produce IgA1 protease and to have a restriction site map identical to the parental strain. The *Iga*$^+$ revertants expressed a 56 kDa OM protein of unknown function, which exhibited *Iga*$^+$-dependent expression. One of the variants, GCM 740Δ4R-83 also exhibited a similar *Iga*$^+$-dependent 34.5 kDa cytoplasmic membrane protein, that was not observed in either of two GCM 740Δ2 *Iga*$^+$ revertants.

Another objective was to examine the susceptibility of the OM proteins in the Δ-*iga* mutants to exogenous IgA1 protease. The outer and cytoplasmic membranes of these three variants were shown to contain proteins which can be cleaved by exogenous, pure type 2 IgA1 protease *in vitro*. This was the first evidence of bacterially produced substrates for these enzymes, save the autohydrolysis of the IgA1 protease pro-enzyme. The ability of the type 2 IgA1 protease to alter some of the membrane proteins *in vitro*, suggested that it might also perform this function *in vivo*. The ability of IgA1 protease to cleave bacterial membrane proteins was not limited to the particular enzyme-producing strain or its variants (*eg*. GCM 740), but was also observed with another *N. gonorrhoeae* strain, F62 and an Δ-*iga* variant; *Escherichia coli* DH1; and a reference strain of *Actinobacillus pleuropneumoniae*.

When *N. gonorrhoeae* was propagated in an iron-limited medium, the initially measured IgA1 protease activity was from 2 to 6-fold as great as that of the same strain propagated in similar media to which Fe^{3+} had

been added. This overall trend of increased protease activity was maintained throughout mid-exponential phase and resumed in stationary phase. Increased enzyme activity was not due to modulation of the activity of preformed mature IgA1 protease by either the ambient Fe^{3+} concentration or the chemical means of rendering the medium iron limited, which suggests an increase in enzyme production.

Comparison of the DNA sequence of type 2 IgA1 protease genes with an *E. coli* consensus Fur binding site suggested the presence of an "iron box", as the *iga*2 genes and the consensus sequence shared 74% nucleotide identity. However, functional analyses examining the expression of the cloned *iga*2 gene in characterized *E. coli Fur* host strains indicated that the cloned *iga*2 gene was not Fur-regulated in *E. coli*. We were also unable to detect a *fur* homologue in the *N. gonorrhoeae* genome by DNA hybridization analysis. The mechanism of IgA1 protease-regulation by ferric iron and the presence of a putative Fur-binding site in the promoter of the *N. gonorrhoeae iga*2 gene is presently unexplained.

APPENDIX

RESTRICTION SITE POLYMORPHISM IN GENES ENCODING TYPE 2 BUT NOT TYPE 1 GONOCOCCAL IgA1 PROTEASES

M. H. Mulks, D. A. Simpson, and R. J. Shoberg

Department of Microbiology and Public Health

Michigan State University

East Lansing, Michigan 48824 USA

Antonie van Leeuwenhoek J. Microbiol.

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This paper is appended to the dissertation because of the contributions of R. J. S. including: subcloning of the *iga*2b gene used as a molecular probe, experimental preparation of some of the blot panels shown in Figure 1, interpretation of the data, and presentation of the data in poster format at the 5th International Pathogenic Neisseria Conference in Noordwijkerhout, the Netherlands (1986).

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Restriction site polymorphism in genes encoding type 2 but not type 1 gonococcal IgA1 proteases

M. H. MULKS, D. A. SIMPSON, & R. J. SHOBERG
Dept. of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824, USA

Abstract. *Neisseria gonorrhoeae* produces two phenotypically distinct types of IgA1 proteases, each of which cleaves a specific peptide bond in the hinge region of the human IgA1 heavy chain. The genes encoding IgA1 protease from twenty-eight different strains of *N. gonorrhoeae*, including twelve which produce type 1 enzyme, thirteen which produce type 2 enzyme, and three which are protease negative, were analyzed. Nine restriction site patterns were found in the *iga* genes. All twelve type 1 strains showed identical restriction maps of the *iga* gene, which differed from all the *iga*-2 variants. The three protease negative strains each contained DNA homologous to the probe. While strain to strain variation in restriction maps of specific genes is not unique and has been reported in *N. gonorrhoeae* previously, the existence of such restriction site polymorphism among *iga*-2 genes contrasts strongly with the lack of such variation among *iga*-1 genes. The basis for this lack of diversity among the *iga*-1 genes is under further investigation.

Introduction

The immunoglobulin A1 proteases are extracellular proteolytic enzymes that have been correlated with virulence in several species of bacteria which are pathogenic for humans (Mulks 1985; Plaut 1983). *Neisseria gonorrhoeae* produces two distinct phenotypic types of IgA1 protease, each of which cleaves a different specific bond in the hinge region of the human IgA1 heavy chain. A given isolate produces one but not both of the two phenotypic types of enzyme. The type of IgA1 protease produced correlates with nutritional auxotype, outer membrane Protein I serovar, and *dam* methylation in this organism. Strains which produce type 1 IgA1 protease generally require arginine, hypoxanthine, and uracil for growth, belong to the Protein IA-1 or IA-2 serovars, and are *dam* methylase positive (Mulks & Knapp 1985). Strains which produce type 2 enzyme are extremely diverse in both auxotype and serotype, and may be either *dam*+ or *dam*−. Initial studies in this laboratory (Mulks & Knapp 1985) indicated that there is also polymorphism in the physical structure of the *iga* gene, as determined by restriction site maps. We report here that there is extensive restriction site polymorphism in genes encoding type 2 but not type 1 IgA1 proteases.

Materials and methods

Bacterial strains. Isolates of *Neisseria gonorrhoeae* used in this study are listed in Table 1. All isolates with either NRL or CDC designations were received from Dr Joan S. Knapp, Neisseria Reference Laboratory, Seattle, Washington. Isolates were grown on GC base medium (Difco Laboratories) plus 1% Kellogg's supplement (Knapp & Holmes 1975) or in defined broth medium (Morse & Bartenstein 1980), and were stored at −70C in 2% tryptone + 20% glycerol.

IgA1 protease assays. IgA1 protease type was determined by analysis of IgA1 cleavage products on 9% polyacrylamide SDS gels (Mulks et al. 1980).

Auxotyping. Each isolate was auxotyped on defined media (Knapp & Holmes 1975) and scored for requirements for arginine, hypoxanthine, uracil, proline, and methionine. In some cases, isolates were also tested for requirements for ornithine, serine, leucine, and isoleucine/valine. All isolates required cystine-cysteine for growth.

Serotyping. Serotyping of isolates into Protein I serovars by coagglutination with monoclonal antibody reagents (Knapp et al. 1984) was performed by Dr J. S. Knapp.

DNA purification. Chromosomal DNA from all gonococcal strains was prepared (Bricker et al. 1983) from cultures grown in defined broth medium (Morse & Bartenstein 1980).

Restriction analysis and southern hybridizations. Restriction enzymes (Bethesda Research Laboratories, Inc., and New England BioLabs, Inc.) were used according to the suppliers' recommendations. Electrophoresis of DNA was on agarose submarine gels in SB3 buffer, (Bricker et al. 1983). Molecular weight standards were phage λ-*Hind*III and 1 kilobase ladder fragments (Bethesda Research Laboratories, Inc.). DNA was transferred onto nitrocellulose paper by the Southern procedure and hybridized with P^{32}-labeled probe DNA at 42C in 50% formamide (Maniatis et al. 1982). The probe was the 4.2 Kb *Hind*III fragment from a cloned *iga*-2b gene (see Fig. 2), isolated from an agarose gel after electrophoresis and labelled with P^{32}-dCTP by nick translation, (Mulks & Knapp 1985).

Dam methylase determinations. Purified chromosomal DNA from each gonococcal isolate was digested with the following restriction endonucleases: *Mbo*I, which only recognizes and cleaves the DNA sequence GATC if the adenine re-

sidue is not methylated. *Dpn*I, which only cleaves this sequence if the adenine is methylated, and *Sau*3A1, which cleaves regardless of methylation. If the DNA was cleaved by *Dpn*I and/or *Sau*3A1 but not *Mbo*I, the DNA was considered

Table 1. Characteristics of *N. gonorrhoeae* strains used in this study.

Strain	Auxotype[a]	Serovar	Dam	IgA1 Protease		Source[b]
			Methylase	Activity	Genotype	
1. NRL 30465	AHU	IA1	+	1	iga-1	Japan
2. NRL 32819	AHU	IA1	+	1	iga-1	Wash.
3. NRL 905	AHU	IA1	+	1	iga-1	*
4. NRL 9396	AHU	IA1	+	1	iga-1	Copen.
5. CDC 6	AHU (argC)	IA1	+	1	iga-1	Iowa
6. CDC 22	AHU (argO) IV	IA1	+	1	iga-1	Fla.
7. CDC 209	AHU IV Leu Met	IA1	+	1	iga-1	Ohio
8. CDC 54	AHU (argO) Ser	IB2	+	1	iga-1	Iowa
9. CDC 125	AHU	IB2	+	1	iga-1	Ohio
10. CDC 208	AHU (argO) IV	IB2	+	1	iga-1	Ohio
11. NRL 31774	Pro	IB1	+	1	iga-1	Copen.
12. NRL 33296	Pro	IB1	+	1	iga-1	Copen.
13. F62	Pro	IB7	−	2	iga-2a	*
14. MKB	Pro	IB7	−	2	iga-2a	*
15. CDC 3	WT	IB3	−	2	iga-2a	Colo.
16. CDC 179	ArgO	IA1	−	2	iga-2a	Colo.
17. GCM 740	WT	IB2	−	2	iga-2b	Mass.
18. MS-11	WT	IB9	−	2	iga-2c	*
19. IF-2	WT	IB9	−	2	iga-2c	*
20. CDC 96	Pro	IA3	+	2	iga-2d	N.J.
21. CDC 253	ArgO Met	IB2	−	2	iga-2d	Colo.
22. CDC 34	Pro Arg Met Leu	IB1	−	2	iga-2e	Iowa
23. CDC 50	Pro	IB1	+	2	iga-2e	Fla.
24. CDC 1	ArgO	IB24	−	2	iga-2f	Fla.
25. CDC 9	Pro	IA3	−	2	iga-2g	Mass.
26. NRL 7904	WT	IA5	+	−	iga-2a	Copen.
27. NRL 7908	WT	IA5	+	−	iga-2a	Copen.
28. NRL 31778	Met	IB15	+	−	iga-2h	Copen.

[a] Auxotype: WT = prototrophic; Arg = requires arginine for growth; ArgO = arginine requirement can be satisfied by ornithine, citrulline, or arginine; ArgC = arginine requirement can be satisfied by citrulline or arginine but not ornithine; AHU = requires arginine, hypoxanthine, and uracil; Pro = requires proline; Met = requires methionine; Ser = requires serine; and IV = requires isoleucine and/or valine.

[b] Source: Copen. = Copenhagen, Denmark; others include Colorado, Florida, Iowa, Massachusetts, New Jersey, Ohio, and Washington states.

* NRL 905 was received from J. S. Knapp; F62 was received from P. F. Sparling; MKB is the F62 derivative used by J. M. Koomey and was received from H. D. Cooper; MS-11 and its derivative strain IF-2 were received from M.So.

to be methylated and the strain designated *dam*+; if the DNA was cleaved by *Mbo*I and *Sau*3A1 but not *Dpn*I, the strain was designated *dam*- (Kolodkin et al. 1982).

Results and discussion

IgA1 protease genes from several strains of *N. gonorrhoeae* have been cloned and carefully mapped with restriction enzymes (Mulks & Knapp 1985; Fishman et al. 1985; Rahr et al. 1985; Halter et al. 1984; Koomey et al. 1982). Comparison of these physical maps of the *iga* genes and their flanking sequences (Mulks & Knapp 1985) demonstrated significant strain-to-strain variation in the number and location of restriction sites within the *iga* gene. In order to evaluate the extent and significance of these variations in the genes encoding gonococcal IgA1 protease activity, we analyzed twenty-eight different isolates of *Neisseria gonorrhoeae* (Table 1). Each isolate was assayed for IgA1 protease type, auxotype, serotype, and production of *dam* methylase activity. Specific isolates to be studied were selected as representative of the phenotypic diversity to be found among protease type 1 and type 2 strains. In our collection of over 1100 gonococcal strains, only the three which are included in this study have been found to be protease negative.

Chromosal DNA was purified from each isolate and digested with four restriction enzymes: *Hind*III, *Ava*I, *Bgl*II, and *Pst*I. The restriction fragments were separated by electrophoresis on agarose gels and the fragments homologous to the *iga* gene were identified by Southern hybridization, using as a probe the 4.2 Kb *Hind*III fragment from our cloned *iga*-2b gene (see Fig. 2). Representative Southern blots are shown in Fig. 1. Using the data from these blots plus restriction maps of cloned *iga* genes, nine distinct restriction maps of gonococcal *iga* genes were identified (Fig. 2). Each of these varied slightly in pattern within the region specifying IgA1 protease activity, but all nine were essentially identical in the structure of the flanking sequences. Among 13 isolates producing type 2 protease, 7 variations in the restriction map of the *iga* gene (which we have designated genotypes *iga*-2a to *iga*-2g) were found. No correlation could be made between specific *iga*-2 genotypes and auxotype, Protein 1 serovar, production of *dam* methylase, or source of the isolates. In contrast, 12 isolates producing type 1 protease, regardless of the auxotype, serovar, or source of the isolate (all were *dam*+), had identical restriction maps of the *iga* gene (*iga*-1), which differed from all 7 *iga*-2 genotypes. The three strains which do not produce detectable levels of IgA1 protease activity contained apparently intact *iga* genes with either *iga*-2a restriction patterns or an eight variant (*iga*-2h), suggesting that the *iga* gene is present in these strains but is defective or that other genes are necessary for full expression of protease activity.

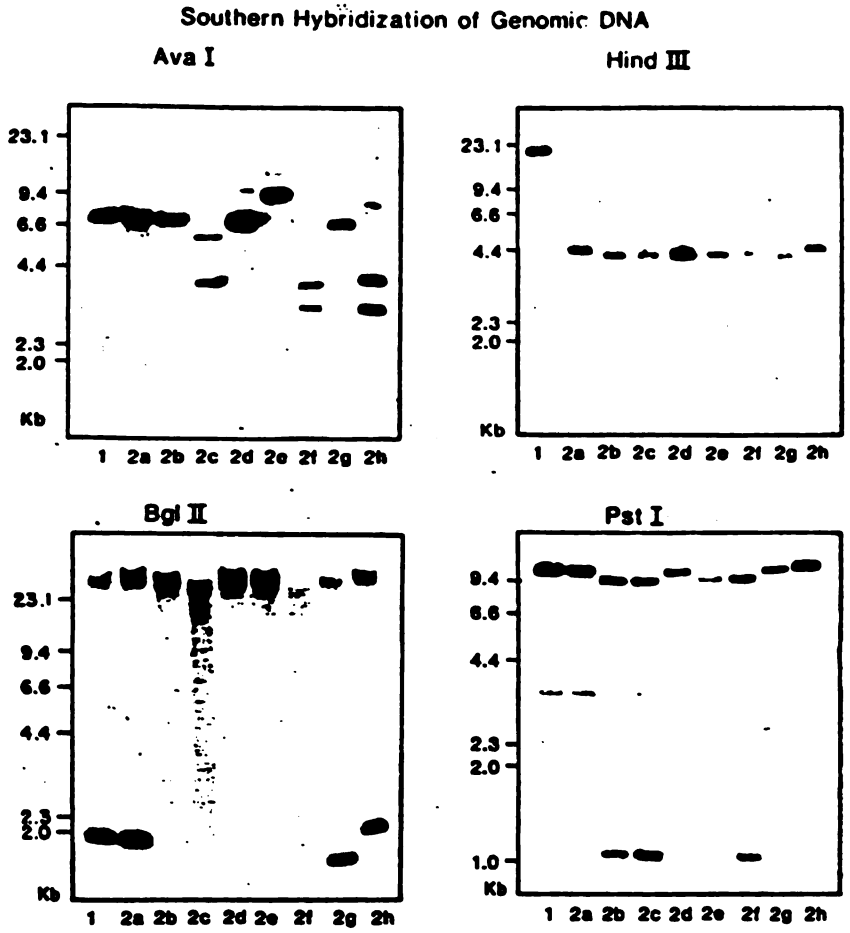

Fig. 1. Restriction and Southern blot analysis of genomic DNA purified from nine different strains of *N. gonorrhoeae*. DNA from each strain was digested with each of four restriction enzymes, including *Ava* I, *Bgl* II, *Hind* III, and *Pst* I. DNA fragements were separated on 0.7% agarose gels, transferred onto nitrocellulose, and hybridized to the P^{32}-labelled 4.2 Kb *Hind* III fragment isolated from a cloned *iga*-2b gene (see Fig. 2). In all four gels, lanes are designated according to the *iga* genotype of the strain, i.e., *iga*-1 and *iga*-2a through 2h. Sizes of DNA molecular weight markers, in Kb, are indicated on the left of each autoradiograph.

The significance of these variations in gonococcal *iga* genes to the physiology of the organism or the pathogenesis of infection is not clear. Differences in the genes encoding type 1 and 2 proteases were expected; despite the fact that these genes are highly homologous at the DNA level, the type 1 and type 2 enzyme proteins are biochemically and antigenically distinct (Simpson, Hausinger & Mulks, J. Bacteriology, in press). However, while the genes encoding type 1 enzymes are unique and distinct from those encoding type 2 enzymes, the varia-

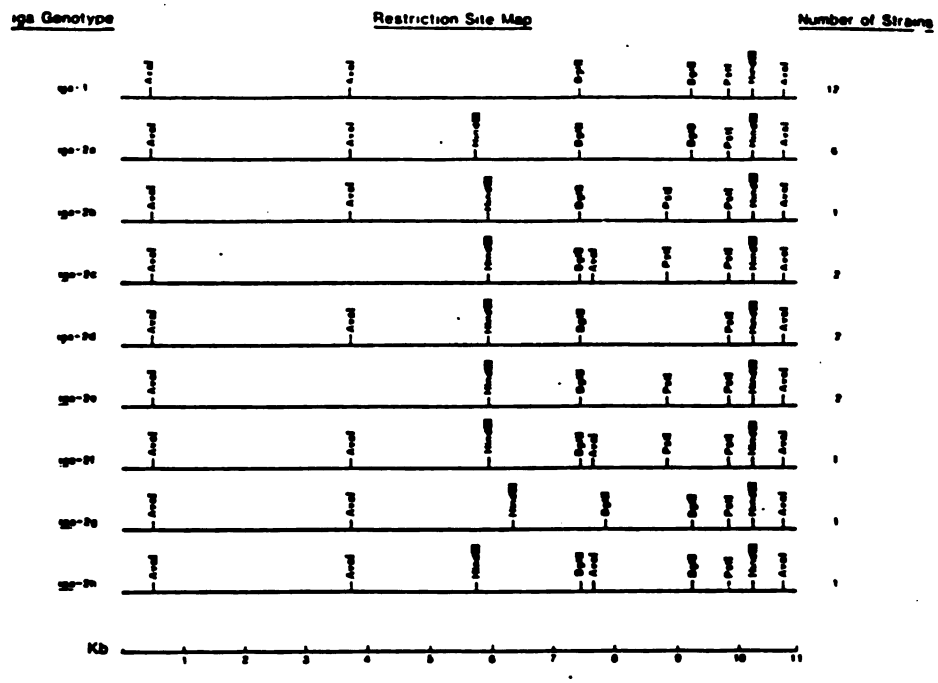

Fig. 2. Physical maps of gonococcal IgA1 protease (*iga*) genes and flanking sequences, as determined by restriction enzyme analysis of cloned genes and by chromosomal Southern blot analysis with the 4.2 Kb *Hind* III fragment from a cloned *iga*-2b gene used as a probe. Nine distinct arrangements of restriction sites were found, each varying slightly in pattern within the region specifying IgA1 protease activity but all being essentially identical in the structure of the flanking sequences. The *iga* structural gene spans the region between the two *Hind* III sites, and is transcribed from left to right from a promoter ~0.3 Kb upstream from the *Hind* III site. The number of strains belonging to each genotype is indicated on the right; further information concerning the phenotypes of each strain is given in Table 1.

tions in *iga*-2 genes are not correspondingly responsible for obvious differences in the proteins they encode. Whether subtle differences in the type 2 enzyme proteins exist has not been determined.

Strain-to-strain variation in DNA sequences of specific genes is not unique to the IgA1 protease genes of *N. gonorrhoeae*. Both genotypic and phenotypic polymorphism also occurs among the IgA1 proteases of *Haemophilus influenzae* (Bricker et al. 1983, 1985; Mulks et al. 1982). In this species, however, the type of protease produced and the genotype of the *iga* gene correlates with the capsular serotype of the organism. No similar correlation of *iga* genotypes to other known phenotypes has been detected in *N. gonorrhoeae*. Other gonococcal genes which demonstrate restriction site polymorphism include *pro*A and *pro*B, which specify two enzymes involved in the biosynthesis of proline (Stein et al. 1984). A variety of other enzymes and proteins produced by the gonococcus also show considerable strain-to-strain antigenic or electrophoretic diversity. The multiple

serovars of outer membrane protein I and the multiple isozymes used to establish "electrophoretic types" are examples which probably reflect significant differences in the DNA sequences of those genes. It is of particular interest that, while extensive diversity in these genes is apparently the norm for this species, AHU strains of *N. gonorrhoeae* are highly conserved for protein I serovars (Knapp et al. 1985; Mulks & Knapp 1985) and isozyme electrophoretic types (J. Musser, pers. comm.), as well as for IgA1 protease genotype. The mechanism which mediates this apparent limitation of genetic variation in AHU strains of *N. gonorrhoeae* remains to be determined.

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