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Identification of the subcellular location of the a-2,3-sialyltransferase of the bacterial pathogen Neisseria gonorrhoeae

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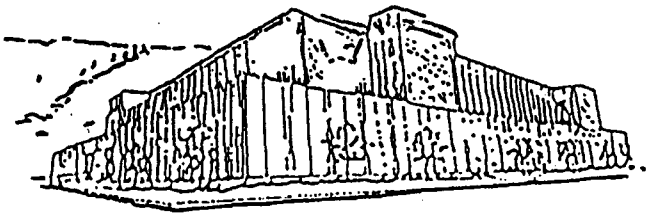

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Identification of the Subcellular Location of the α-2,3-Sialyltransferase of the Bacterial Pathogen *Neisseria gonorrhoeae*

by

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Identification of the Subcellular Location of the α-2,3-sialyltransferase of the Bacterial Pathogen *Neisseria gonorrhoeae* (53pp.)

Director: Ralph C. Judd

Neisseria gonorrhoeae is a sexually transmitted human mucosal pathogen. It causes a variety of diseases ranging from uncomplicated infections of the genitalia to pelvic inflammatory disease and disseminated gonococcal infection. One of the mechanisms used by the bacteria to evade the immune system of the host is the transfer of sialic acid from the host substrate CMP-NANA to lipooligosaccharide molecules found on the surface of the bacteria. This transfer is accomplished by the action of an α-2,3-sialyltransferase (Stase).

The hypothesis that Stase was located in the outer membrane of *N. gonorrhoeae* was tested by enzymatic assay for the presence of Stase and by Western blot detection. Blots were probed with antisera generated to a multiple antigenic peptide system antigen containing a segment of Stase and to a maltose binding protein/Stase fusion protein. The subcellular fractions isolated included the inner and outer membrane, periplasm and cytoplasm. The inner and outer membranes were isolated by sucrose density gradient centrifugation, the periplasm by chloroform extraction and the cytoplasm by sonication and ultracentrifugaton. Culture supernatant was also tested for the presence of Stase.

The enzymatic assay detected the vast majority of Stase activity in the outer membrane. Western blot analysis confirmed that Stase was located only in the outer membrane of the bacteria. The presence of Stase in the outer membrane has implications for vaccine development. If antibodies against Stase can prevent sialylation of the bacteria, the immune system of the host may be able to fight off infection, decreasing the prevalence of gonococcal disease.

TABLE OF CONTENTS

LIST OF FIGURES

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1. INTRODUCTION

1-1. Gonococcal Morphology, Pathology, and Treatment

Neisseria gonorrhoeae (gonococcus) is a gram-negative, kidney shaped bacterium that exists in pairs (Swanson, *et al.*, 1987). It is a facultative anaerobe (Cohen and Sparling, 1992) that infects a variety of human mucosal surfaces including the urethra, endocervix, rectum, pharynx, and conjunctiva (Britigan, *et al.*, 1985). Occasionally it is able to infect the blood and disseminate, causing systemic infection (Cohen and Sparling, 1992). *N. gonorrhoeae* is the causative agent of gonorrhea, a sexually transmitted disease that is reported to affect well over a quarter of a million people every year in the United States (MMWR, 1997). The actual figure is greater because of incomplete reporting and the existence of asymptomatic infections (Britigan, *et al.*, 1985). In most males, gonococcal infection causes urethritis, which results in purulent discharge and painful urination (Britigan, *et al.*, 1985; Tortora, *et al.*, 1992). Many women, however, remain asymptomatic and the disease may progress beyond an infection of the urethra or endocervix (Britigan, *et al.*, 1985). Gonococci are not motile; they must be transported past the endocervix into the fallopian tubes by a carrier, probably refluxed menstrual blood and/or sperm (Cohen and Sparling, 1992). Transport of *N. gonorrhoeae* further into the body may lead to more serious infections.

Serious manifestations of gonococcal infection include disseminating gonococcal infection (DGI), pelvic inflammatory disease (PID), salpingitis, ectopic pregnancy, infertility, and in infants, ophthalmia neonatorum. DGI occurs when *N. gonorrhoeae* causes bacteremia leading to skin lesions, infections of the joints – which can cause arthritis, and infections of the meninges and heart (Cohen and Sparling, 1992; Tortora, *et*

al., 1992). PID is a general term referring to infections in the pelvic area and can include salpingitis (Tortora, *et al.*, 1992). Salpingitis is an inflammatory infection of the fallopian tubes. It may result in the loss of ciliated epithelial cells and in scarring, leading to ectopic pregnancy and infertility (Cohen and Sparling, 1992; McGee, *et al.*, 1992). PID, ectopic pregnancy and infertility are the most costly sequelae of gonococcal infection to treat and ectopic pregnancy can be fatal (McGee, *et al.*, 1992). Gonococcal ophthalmia neonatorum, which may result in blindness, occurs when gonococci enter the eyes of an infant as it moves through an infected birth canal (Brooks, *et al.*, 1995).

Treatment of gonococcal infections is complicated by the increasing emergence of antibiotic-resistant strains of *N. gonorrhoeae* (Britigan, *et al.*, 1985). Gonococcal resistance to penicillin was first described in 1976 (Ison, 1996). Penicillinase-producing *N. gonorrhoeae* (PPNG) carrying a penicillin resistance plasmid have now spread worldwide. Plasmid-mediated tetracycline resistance was described in 1985 (Ison, 1996). Gonococcal resistance to spectinomycin and ceftriaxone has also been reported (Tortora, *et al.*, 1992). Vaccination against *N. gonorrhoeae*, which is not yet possible, would decrease the use of antibiotics and slow the development of resistant strains. Unfortunately, vaccine and diagnostic development are hampered by the fact that humans are the only reservoir for the bacteria (Britigan, *et al.*, 1985). There are no animal models of the disease (Cohen and Sparling, 1992).

In vitro research provides one important level of knowledge about the bacterium. Other information is gleaned from research studies performed on male volunteers. Human trials are limited, however, because of the ethics of allowing an infection to proceed unchecked. Further insights into the mechanisms of pathogenesis come from

human fallopian tubes in organ culture (McGee, *et al.*, 1992) and from tissue culture models.

1-2. Gonococcal Survival Strategies and Major Outer Membrane Proteins

N. gonorrhoeae use a variety of strategies to enhance survival in the host, including the secretion of IgA_1 protease (Kilian, *et al.*, 1996), and the variation of many surface molecules. Pathogenic *Neisseria* secrete an immunoglobulin class A, subclass 1 (IgA_1) protease that may enhance gonococcal colonization of mucosal surfaces by cleaving the hinge region of dimeric IgA_1 antibodies (Cohen and Sparling, 1992). Secretory IgA is the main antibody isotype found in mucosal tissues and secretions (Kilian, *et al.*, 1996). It inhibits microbial colonization in a variety of ways, including agglutination of the bacteria, blockage of adhesins, and increase of bacterial surface hydrophilicity (Britigan, *et al.*, 1985; Kilian, *et al.*, 1996). A decrease in the effectiveness of secretory IgA_1 molecules is likely to enhance the survival of *N. gonorrhoeae* in the host.

Phase and antigenic variation of outer membrane molecules, including glycolipids and proteins, enhance the ability of gonococci to evade the immune defenses of the host. Phase variation is the on/off switching of surface molecule expression. Antigenic variation occurs when the bacteria change the epitopes displayed on their surface molecules. These variations may also allow the bacteria to adapt to the host microenvironment and respond to changes in that environment (van Putten and Robertson, 1995).

Major variable gonococcal outer membrane molecules include pili (Pil), now known as fimbriae, opacity (Opa) proteins, porin (Por), reduction modifiable proteins

(Rmps) and lipooligosaccharide (LOS). Fimbriae are filaments consisting of homopolymers of the polypeptide pilin. They range in molecular weight from 17.5 to 26 kDa and are extremely variable (Swanson and Koomey, 1989). Fimbriae mediate adherence of the bacteria to each other as well as to the mucosa of the host (Swanson, 1990). Either no fimbriae or fimbriae of a single pilin type are expressed at any one time in any given cell. Pil^+ gonococci are able to infect a host while Pil^- organisms are not (Swanson and Koomey, 1989). The phase and antigenic variation of fimbriae are controlled at the level of transcription. Variation is due to recombination of one of at least 12 partial, "silent", pilin coding regions, found throughout the genome, into the complete pilin expression gene (Cohen and Sparling, 1992; Swanson, 1990).

There are at least eleven related Opa proteins encoded throughout the genome of *N. gonorrhoeae*, and either no Opa proteins or up to several may be expressed in a particular gonococcal cell (Cohen and Sparling, 1992; Swanson, 1990). They have molecular weights ranging from 24 to 32 kDa (Brooks, *et al.*, 1995), and give gonococcal colonies various degrees of an opaque phenotype, perhaps due to increased adherence between cells (Cohen and Sparling, 1992). Like fimbriae, Opa proteins mediate adherence of gonococci both to each other and to host cells (Swanson, 1990). Kupsch, *et al.* (1993) demonstrated that different Opa proteins mediated different activities depending on which *opa* allele was expressed. One strain of *N. gonorrhoeae* studied showed increased adherence to and invasion of epithelial cells while other strains, expressing different *opa* alleles were shown to interact with leukocytes. Another study showed that in volunteers initially infected with Opa^- gonococci, only Opa^+ organisms were recovered (Cohen and Sparling, 1992). Thus it appears likely that Opa proteins play

a role in gonococcal infection. Opa proteins vary at a rate of 10^{-3}/cell/generation. In the portions of DNA encoding the signal peptides of the *opa* genes there are $(CTCTT)_n$ repeats which vary in length, controlling the expression of Opa proteins by shifting the translational reading frame. (Cohen and Sparling, 1992; Swanson, 1990) Although relatively stable, the nucleotide sequences encoding Opa proteins can apparently be changed by recombination events with other Opa-encoding genes (Swanson, 1990).

Por is the most abundant outer membrane protein. It has a molecular weight ranging from 32 to 39 kDa. Por proteins associate noncovalently in groups of three (Elkins and Sparling, 1990). Por functions as a pore through the outer membrane of *N. gonorrhoeae* allowing for the transport of hydrophilic molecules (Britigan, *et al.*, 1985). There are two subclasses of Por, PorA and PorB. PorA is associated with stable serum resistance. Gonococci expressing this type of serum resistance are associated with disseminating infections (Cohen and Sparling, 1992; Elkins and Sparling, 1990; Judd and Shafer, 1989). Unlike pilin and Opa proteins, Por type does not vary within a strain of *N. gonorrhoeae*, and only one antigenic type of Por is produced by a particular strain (Judd and Shafer, 1989). The immunogenic, surface-exposed regions of the molecule probably vary as a result of immunological pressure from the host (Elkins and Sparling, 1990).

Reduction modifiable protein has a molecular weight of about 33 kDa, which increases when the protein is reduced (Brooks, *et al.*, 1995; Judd, 1982). It is an antigenically conserved protein constitutively expressed in gonococcal strains and is closely associated with Por (Wetzler, *et al.*, 1989; Judd, 1982). Rmps appear to assist the bacterium in evading the immune system of the host by inducing blocking antibodies that interfere with the action of anti-Por and anti-LOS antibodies. Blocking antibodies may

increase the susceptibility of a host to gonococcal reinfection (Cohen and Sparling, 1992).

1-3. Lipooligosaccharide

Lipooligosaccharide (LOS), a molecule with a molecular weight of 3 to 7 kDa, is found on the surface of *N. gonorrhoeae* (Schneider, *et al.*, 1984). It is composed of three regions: lipid A, the inner core, and the outer core (Brooks, *et al.*, 1995). Lipid A anchors LOS in the outer membrane (van Putten and Robertson, 1995). Variable, non-repeating outer core oligosaccharide units are attached to the lipid A by an inner core of hydrophilic sugar molecules (Brooks, *et al.*, 1995). LOS lacks the repeating O antigens found in the lipopolysaccharide molecules of enteric gram-negative bacteria (Mandrell, *et al.*, 1986; Schneider, *et al.*, 1984).

LOS is responsible for many of the pathogenic effects of *N. gonorrhoeae*. For example, McGee, *et al.* (1992) demonstrated that tumor necrosis factor α (TNF- α) was induced by gonococcal LOS in the fallopian tube organ culture model. TNF- α was shown to elicit sloughing of ciliated epithelial cells in a dose dependent manner. When dexamethasone, a chemical that blocked production of TNF, was added, it prevented damage to the mucosa in spite of the presence of the LOS. This suggested that the production of gonococcal products like LOS may damage host cells indirectly by inducing the expression of TNF- α and possibly other cytokines. Destruction of ciliated cells by induced cytokines may inhibit the movement of a fertilized ovum from the fallopian tube to the uterus leading to ectopic pregnancy.

The outer core oligosaccharides of gonococcal LOS play a different role in gonococcal pathogenesis. Mutants deficient in the synthesis of outer core

oligosaccharides exhibited increased sensitivity to complement-mediated serum killing by the alternative complement pathway, i.e. in the absence of antibodies (van Putten and Robertson, 1995). Longer LOS may serve to hide lytic epitopes on other surface components (van Putten and Robertson, 1995), as well as to inhibit the activation of zymogen C1 of the classical complement cascade (Schweinle, *et al.*, 1989). In addition, gonococcal LOS mimics human paraglobosides, precursors of the ABH antigens on human erythrocytes. This may serve to decrease its immunogenicity (Mandrell and Apicella, 1993). The variability of LOS also makes it useful to the bacteria as a method of evading the humoral immune system of the host (van Putten and Robertson, 1995; Schneider, *et al.*, 1985).

LOS is highly variable (van Putten, 1993). A single cell can display antigenically different LOS molecules simultaneously (Apicella, *et al.* 1987) and a single strain may produce up to six structurally different LOS molecules (Schneider, *et al.*, 1988). The most abundant molecule(s) determine the LOS phenotype and vary spontaneously at a rate of 10^{-2} to 10^{-3} (van Putten and Robertson, 1995). The phenotype of LOS may be influenced by the conditions in which the bacterium grows (Schneider, *et al.*, 1988). The regulation of LOS expression is not yet fully understood. It is probable that genes involved in the synthesis of various outer core sugar units can be placed in frame or out of frame by frameshift mutations. For example, three of the genes responsible for the synthesis of a Galβ1-4GluNAc moiety contain poly-G tracts that may prevent the expression of the genes by addition or deletion of a single nucleotide. The resulting frameshift mutation would disrupt the open reading frame and change the LOS phenotype (van Putten and Robertson, 1995).

1-4. Sialyltransferase and Sialylation of Lipooligosaccharide

Further variation of LOS occurs through the action of an α-2,3-sialyltransferase (Stase) (Gilbert, *et al.*, 1996) found in all pathogenic strains of *N. gonorrhoeae* and *Neisseria meningitidis* (Mandrell, *et al.*, 1993). Stase is a 43 kDa enzyme (Gilbert, *et al.*, 1996) that catalyzes the transfer of sialic acid, in a dose-dependent manner (Frangipane and Rest, 1993), from the substrate, cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NANA) to the Galβ1-4GluNAc moiety found on some LOS molecules (Apicella, *et al.*, 1990; Gilbert, *et al.*, 1996; van Putten, 1993). Apicella, *et al.* (1990) demonstrated by immunoelectron microscopy that this transfer occurred *in vivo*. Since CMP-NANA was not produced by *N. gonorrhoeae* (Apicella, *et al.*, 1990; Frosch, *et al.*, 1989), sialic acid was provided by the CMP-NANA found in host serum, secretions and cells (van Putten and Robertson, 1995; Smith, *et al.*, 1995). In an experiment by Parsons, *et al.* (1989) there was no observable *de novo* protein expression induced by the presence of CMP-NANA, indicating that Stase was constitutively expressed. The interconversion between sialylatable LOS (LOS that contains the Galβ1-4GluNAc moiety) and non-sialylatable forms of LOS allows *N. gonorrhoeae* to adapt to different host environments as infection proceeds.

Sialylation appears to play a role in the progression of gonococcal infection. Schneider, *et al.* (1991) showed that, of 36 naturally infected males examined, gonococci recovered from all 36 patients expressed sialylatable LOS. It was further demonstrated that gonococci can change LOS phenotype *in vivo*. Two volunteers were inoculated with gonococci expressing non-sialylatable (3.6 kDa) LOS. Gonococci isolated from the urine sediments of the subjects were examined at different stages of the infection. The

gonococci displayed the initial LOS type until the onset of inflammation and dysuria when sialylatable LOS began to be expressed. No gonococci expressing the original unsialylatable 3.6 kDa LOS were recovered after the onset of urethritis and urethral discharge.

There is evidence that the sialylation of LOS influences the passage of *N. gonorrhoeae* across the "mucosal barrier" and the resistance of the bacteria to serum killing. Sialylated gonococci were inhibited from entering epithelial cells, and the invasiveness of the bacteria appeared to be dependent on the number of sialic acid residues displayed on the bacterial surface (van Putten, 1993). Once the bacteria overcome mucosal defenses and enter the host, sialylation becomes beneficial. Many strains of *N. gonorrhoeae* from fresh isolates exhibited a serum resistance that was lost upon subculture (Ward, *et al.*, 1970). This serum resistance was shown to be a result of the sialylation of LOS (Parsons, *et al.*, 1989).

The bactericidal effect of normal human sera on serum-sensitive *N. gonorrhoeae* was mainly due to the presence of IgM directed against lytic epitopes on LOS (Apicella, *et al.*, 1986; Rice, *et al.*, 1980). It was shown that when LOS was sialylated, these sites were blocked (Parsons, *et al.*, 1989; Smith, *et al.*, 1992). When gonococcal strains wild type F62 and JB1, a mutant deficient in Stase activity (Bramely, *et al*, 1995), were incubated with normal human sera in the presence of CMP-NANA, it was observed that binding of complement component C3 was significantly reduced in the parent strain but not in the mutant strain (Gill, *et al.*, 1996). This indicated that the presence of sialylated LOS decreased the amount of complement that bound to the bacteria. Inhibition of the

classical complement pathway, which mediates serum killing, contributed to serum resistance (Frangipane and Rest, 1993).

Sialylated LOS also protected *N. gonorrhoeae* from complement-mediated killing by anti-PorA and anti-PorB sera as well as from killing by antisera raised against the whole gonococcal cell. The protection appeared to be due to an inhibition of the alternative complement cascade rather than interference with the binding of anti-Por antibodies (Wetzler, *et al.*, 1992). This is significant because *in vitro* experiments showed that the effectiveness of membrane attack complexes was increased 40% by the presence of the alternative complement pathway compared to the classical pathway alone (Rice, 1989).

Sialylation also enhanced gonococcal survival by a delay in complement-mediated opsonophagocytosis (Kim, *et al.*, 1992) and by an increase in gonococcal resistance to phagocytic killing by polymorphonuclear phagocytes (PMNs) (Gill, *et al.*, 1996). In the presence of anti-Por antibodies, phagocytic cells were unable to kill sialylated gonococci by opsonophagocytosis (Wetzler, *et al.*, 1992). The decrease in phagocytic killing by PMNs may be due to decreased binding and cleavage of C3 (Gill, *et al.*, 1996) and/or by impaired attachment of gonococci to PMNs (van Putten, 1993).

Several pieces of evidence indicated, either directly or by process of elimination, that the gonococcal sialyltransferase was located in the outer membrane. First, Stase appears to have a signal peptide (Gilbert, *et al.*, 1996) indicating that it is not a cytoplasmic protein. Second, since gonococci do not produce the CMP-NANA substrate (Frosch, *et al.*, 1989), CMP-NANA would have to be imported if Stase was located in the periplasm, inner membrane or cytoplasm. Unsialylated LOS already on the surface of the

bacterium could be sialylated (Mandrell, *et al.*, 1993), thus the LOS to be sialylated would have to be imported from the surface. Then, after sialylation, it would have to be exported to the surface to be displayed. These import and export processes would probably require special transport proteins. Third, Stase activity can be extracted with Triton X-100, a mild detergent, implying that it is located near the surface of the gonococci (Mandrell and Apicella, 1993). Fourth, it is unlikely that the enzyme is secreted into the surroundings because this would decrease the efficiency of the interaction between the enzyme and substrates with regard to timing and orientation. It would also be costly to the bacteria in terms of protein production, and as mentioned above, no *de novo* protein synthesis was seen in the presence of the substrate CMP-NANA (Parsons, *et al.*, 1989).

If it is shown to be in the outer membrane, Stase may be a vaccine candidate, especially if its immunogenic epitopes are conserved among different strains. If antibodies directed against Stase prevented LOS sialylation, then although adherence and cell invasion may occur (Gill, *et al.*, 1996), host immune defenses would be better able to fight off the infection and possibly prevent the dissemination of the bacteria.

1-5. Research Goal, Hypothesis, and Specific Aims

The goal of this project was to determine which subcellular fraction of the gonococcal cell contained Stase. Rabbit anti-Stase antibodies were made against a Multiple Antigenic Peptide System (MAPS) peptide containing a portion of the Stase amino acid sequence predicted to be antigenic and surface exposed. These antibodies were then used on a Western blot to probe the cytoplasm, inner membrane, periplasm, outer membrane, and culture supernatant fractions to determine the subcellular location

of Stase. As a confirmation, a maltose binding protein (MBP)/Stase fusion protein was generated and used to immunize a rabbit. The rabbit Stase/MBP antiserum generated was also used for Western blot analysis. A gonococcal Stase deficient mutant, strain F62 STO1, with a kanamycin resistance cassette used for an insertional deletion of the gene encoding Stase, served as a negative control (unpublished).

Hypothesis: Sialyltransferase is located in the outer membrane of *Neisseria gonorrhoeae*.

The following **Specific Aims** were investigated to test the hypothesis:

1. The culture supernatant and subcellular fractions, i.e. outer membrane, periplasm, inner membrane and cytoplasm, of *N. gonorrhoeae* strains wild type F62 and Stase deletion mutant F62 STO1 were isolated.

2. In collaboration with Dr. Rick Rest (Allegheny University of the Health Sciences (AUHS), Philadelphia, PA), the subcellular fractions and supernatant of F62 and F62 STO1 were assayed for Stase activity.

3. A peptide sequence from Stase was selected, based on hydrophilicity, surface exposure and antigenicity as predicted by computer model, and used to make a MAPS immunogen.

4. Rabbit Stase antiserum was generated by immunization with the MAPS immunogen.

5. Western blots of the isolated fractions were probed with Stase-MAPS antiserum to identify the fraction containing Stase.

6. The gonococcal gene encoding Stase was cloned into the pMAL-c2 vector system and used to transform *Escherichia coli* cells. Transformed cells expressed a MBP/Stase fusion protein that was purified by batch incubation on amylose resin.

7. Rabbit Stase/MBP antiserum was generated by immunization of a rabbit with the fusion protein and used to confirm results obtained in step 5 above.

2. MATERIALS AND METHODS

2-1. Bacterial strains, Growth, and Harvesting

Neisseria gonorrhoeae strains F62 and F62 STO1, gifts from Dr. Rick Rest (AUHS), were selected for transparency (Opa^-) and lack of pili, and grown for 16 hours in 5% CO_2 at 37°C on clear typing media (Swanson, 1978). Cells were harvested by swabbing into 3ml of GC broth to an OD_{600nm}=1. One milliliter of culture was then inoculated into 50ml of GC broth and grown, with shaking, at 37°C until it had reached mid-log phase as determined by optical density (about 4 hours). Two and one half milliliters of mid-log phase culture were inoculated into 250ml of GC broth and incubated as described above. Once the cells in the 250ml culture were in mid-log phase, they were harvested by centrifugation at 12,000x*g* for 10 minutes.

2-2. Isolation of Inner and Outer Membrane Fractions

A modification of the procedure of Osborn and Munson (1974) was used. Briefly, the pellet generated above (representing 250ml of original culture) was resuspended in 6.25ml of cold 200mM Tris-HCl, pH 8.0 (measured at room temperature) and diluted with an equal volume of cold 1M sucrose, 200mM Tris-HCl, pH 8.0 (measured at room temperature). Two resuspended pellets were combined and frozen at –70°C. The suspension was thawed on ice and the following pre-chilled reagents were added sequentially, with vortex mixing after the addition of each reagent: 25µl of 250mM EDTA, 100µl of N-acetylmuramidase (13,000 units/ml in triple distilled water [3xdH_2O]) (Sigma, St. Louis, MO; mutanolysin M9901) and 12.5ml of ice cold 3xdH_2O. The 3xdH_2O was added forcefully by pipet. The cells were gently rocked overnight at 4°C, then sonicated on ice by four, 20 second bursts at 50% power (setting 3) using the

microtip on a Fisher 550 Sonic Dismembranator (Fisher Scientific, Santa Clara, CA). The preparation was then centrifuged at 12,000x*g* for 10 min. at 4°C. The supernatant was decanted and centrifuged for 30 min. more at 12,000x*g*. Supernatant was removed and ultracentrifuged at 4°C for 2 hours at 225,000x*g* in a Ti80 rotor (Beckman, Fullerton, CA). The resulting membrane pellets were resuspended in 1ml of 18% (w/w) sucrose containing 1mM EDTA and 0.2mM dithiothreitol (DTT). An isopycnic sucrose gradient was prepared in a 37ml polypropylene tube by layering the following sucrose (w/w) solutions containing 1mM EDTA and 0.2mM DTT: 6ml of 60% sucrose solution followed by 5ml each of 55%, 50%, 45%, 35%, 25% and 20% sucrose. One milliliter of the membrane suspension was then layered on top and the gradient was centrifuged at 80,000x*g* for 48 hours at 4°C in an SW28 rotor (Beckman). Using a peristaltic pump, fractions were collected off the top of the gradient in 1ml aliquots. Fractions were analyzed by refractometry and absorbance at 280nm. Fractions corresponding to the central portion of the outer membrane peak were combined as were the fractions corresponding to the main portion of the inner membrane peak.

2-3. Isolation of Periplasm, Cytoplasm, and Culture Supernatant

Periplasm was isolated using a modification of the procedure described by Judd and Porcella (1993). Briefly, cells were grown on plates as described above and harvested to OD_{600nm}= 0.68 in ice cold Dulbecco's phosphate-buffered saline, pH 7.4 (DPBS) containing magnesium and calcium ions. The cells were washed three times in cold DPBS. The final wash was removed and the cells were resuspended, with vortexing, in 20µl of chloroform (Baker, Phillipsburg, NJ)/1.5ml of original suspension. After a 15 min. incubation at 22°C (standard) or 4°C (to preserve enzymatic activity), 100µl of ice

cold 0.01M Tris-HCl, pH 8.0 (measured at room temperature) was added with vortexing, and cells were pelleted by centrifugation at full speed for 5 min. in a microfuge. The supernatant contained the periplasmic contents.

Cytoplasm was isolated by growing, harvesting and washing cells as described in the periplasm extraction procedure above. After the final wash, cells were resuspended in 100µl DPBS/1.5ml original suspension, and sonicated with four, 20 sec. bursts at 50% power (setting 3) on a Fisher 550 Sonic Dismembranator using a microtip. The suspension was then centrifuged 10 min. at full speed in a microfuge. The supernatant was removed and ultracentrifuged at 130,000x*g* for one hour. The clarified supernatant contained the cytoplasmic contents.

Supernatant was isolated from cells grown and harvested as described above. The supernatant was removed from the cell pellet and ultracentrifuged at 225,000x*g* for 4 hours at 4°C with a Ti80 rotor (Beckman). The resulting supernatant was the culture supernatant used in the following experiments.

2-4. Protein Assay

To determine the protein concentrations of the subcellular fractions and supernatant, a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL; kit # 23225) was used. Protein concentration was determined as described in the microtiter plate protocol with the absorbance readings made at 570nm (within the acceptable range of 540-590nm) and the incubation was performed as described for the enhanced protocol (60°C for 30 min.). BSA standards were used to generate a standard curve.

2-5. SDS-PAGE and Western Blotting

Contents of the subcellular fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed using a modification of the tris-glycine discontinuous system of Laemmli (1970) as described by Judd (1987). Western blotting was performed essentially as previously described (Batteiger, *et al.*, 1982; Judd, 1988). Briefly, proteins were transferred to a Millipor (Bedford, MA) Immobilon polyvinylidene difluoride (PVDF) membranes by immunoblotting in 20 mM sodium phosphate buffer, pH 8.0 for approximately 4 hours at 0.6 Amps, using a Bio-Rad (Richmond, CA) Trans-Blot Cell. The blots were blocked with 0.05% Tween-20 in DPBS and probed with either rabbit anti-Stase-MAPS-AP1 (1:50 dilution in DPBS), rabbit anti-Stase/MBP (preabsorbed 1:2 with *E. coli* DH5α cell lysate, at a 1:134 dilution in DPBS), or anti-Stase sera, a gift from Dr. Rick Rest (AUHS) generated against a purified Stase recombinant protein. Other antisera were generated as described below. Secondary antibody, Protein A-horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) was added in a 1:2500 dilution. A buffered 4-chloro-1-naphthol, hydrogen peroxide solution was used to develop the blots.

2-6. Sialyltransferase Enzymatic Assay

An enzymatic assay for the presence of sialyltransferase was performed on the subcellular fractions, isolated as described above, by Dr. Rick Rest's laboratory (AUHS). Incorporation of the ^{14}C labeled N-Acetyl Neuraminic Acid (NANA) portion of CMP-NANA into purified LOS molecules was used to detect Stase activity essentially as described by Mandrell, *et al.* (1993). The assay mixtures contained sodium phosphate (0.5M, pH 6.8), 2% bovine serum albumin, purified LOS from *N. gonorrhoeae* F62 (1µl,

10mg/ml), CMP-NANA (Sigma, 0.86ng) and CMP-[sialic-4,5,6,7,8,9-^{14}C]NANA (Dupont-NEN, Boston, MA; 0.03μCi/ml, 1.5ul). Each assay had a final volume of 50μl with 0.5% Triton X-100 as the diluent. Two or 4μl aliquots of one of the subcellular fractions prepared as described above were added to each assay mixture to start the reaction. Reaction mixtures were incubated at 37°C for 15 min. The reaction was then stopped by addition of 0.5ml of a 5% phosphotungstic acid-15% trichloroacetic acid solution (PTA-TCA), which precipitates BSA-LOS complexes. After centrifugation, the precipitate was washed twice with PTA-TCA, solubilized in 0.5ml of Solvable (Packard, Meriden, CT), scintillation fluid was added and the sample was counted in a liquid scintillation counter. Background counts of approximately 150 cpm were subtracted from the experimental value of each sample. Stase specific activity was expressed as cpm/μg of protein in sample.

2-7. Selection of MAPS Peptide

The MAPS molecule used to generate rabbit polyclonal antiserum consisted of eight identical peptides (^{83}ENRNEKYDYYF^{93}) attached to a polylysine backbone at F^{93} by alanine residues. The peptide sequence was selected from hydrophilicity, antigenicity, flexibility and surface probability plots of the amino acid sequence of sialyltransferase generated by MacVector software (Kyte-Doolittle algorithm). Stase-MAPS-antigenic peptide 1 (Stase-MAPS-AP1) was synthesized by the University of Montana Molecular Biology Facility (UMMBF-Missoula, MT).

2-8. Generation of Peptide-Specific Polyclonal Antiserum

A New Zealand White rabbit was used to generate polyclonal antisera against Stase-MAPS-AP1. Primary immunization was 1mg/ml Stase-MAPS-AP1 in 500μl of

3xdH_2O and 500µl complete Freund's adjuvant (CFA) injected subcutaneously in four sites. Secondary immunization was the same as the primary except incomplete Freund's adjuvant (IFA) replaced complete Freund's adjuvant. Boosters were given every two to three weeks and ranged from 0.2mg to 1mg of Stase-MAPS-AP1. The first booster was intramuscular in IFA and subsequent boosters were intravenous in 3xdH_2O.

2-9. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed in 96 well Immulon II plates (Dynatech Laboratories, Chantilly, VA) coated with the Stase-MAPS-AP1 (10µg/ml in 3xdH_2O and/or DPBS) for 18 hours at 4°C, (100µl/well). Wells were then blocked with DPBS, 0.05% Tween-20 for 18 hours at 4°C to prevent binding of non-specific antibodies. Antiserum was added at a starting dilution of 1:100 in blocking buffer (100µl/well) and two-fold serial dilutions were made to a final dilution of 1:12,800. Negative controls consisted of normal rabbit sera as the primary antisera and wells coated with an irrelevant MAPS antigen containing a peptide from the gonococcal multiple transferable resistance protein C (MtrC). After an 18 hour incubation at 4°C, wells were washed 5x with 0.05% Tween-20 in DPBS (100µl/well/wash) and incubated with goat anti-rabbit IgG whole molecule, alkaline phosphatase conjugate (1:1000 in blocking buffer, 100µl/well) for two hours at 37°C. Wells were washed 5x with 0.05% Tween-20 in DPBS (100µl/well/wash). To detect the presence of anti-Stase-MAPS-AP1 antibodies, 150µl/well of p-nitro phenyl phosphate (PNPP) in diethanolamine buffer, pH 9.8 (one tablet/15ml at room temperature) were added and incubated at room temperature for 30 min. Absorbance was read by V_{max}

ELISA plate reader (Molecular Devices, Sunnyvale, CA) running the Softmax program at a wavelength of 405nm. ELISA was used to determine antibody titer.

2-10. Generation of Plasmid Vector pMAL-Stase

The pMAL-c2 vector (New England BioLabs (NEB), Beverly, MA; kit #800-645) was engineered to contain a Stase-encoding insert and was used to generate a Stase/MBP fusion protein. The vector contained an ampicillin resistance marker, the inducible P_{tac} promoter, the gene for MBP (*malE*), followed by a polylinker containing restriction endonuclease cleavage sites and *lacZα*. It also contained the gene *lacI*, encoding the Lac repressor that prevented transcription from P_{tac} until IPTG was added. Interference with plasmid function by transcription from P_{tac} was prevented by an *rrnB* terminator sequence. A unique set of oligonucleotide primers containing engineered endonuclease cleavage sites and complementary to the ends of the Stase gene (GenBank accession # NGU60664) were synthesized by the UMMBF. The forward primer (Stase 5'): 5'-**GGATTC**ATGGGGTTGAAAAAAGTCTGTTTGACC (*Eco*R I site in bold) and the reverse primer (Stase 3'): 5'**GCTCTAGA**TTAATTTTTATCGTCAAATGTCAAAAT (*Xba* I site in bold) were used to amplify the gonococcal Stase gene from strain F62 by PCR (Boehringer Mannheim Expand High Fidelity PCR System), using genomic DNA as the template. The product was purified using the QIAquick method for DNA purification from PCR reactions (QIAGEN, Los Angeles, CA) and double-digested with *Xba* I and *Eco*R I restriction endonucleases (NEB). The pMAL-c2 vector (NEB) was double-digested with *Xba* I and *Eco*R I, calf intestine alkaline phosphatase (CIP) (NEB) treated and purified by the QIAquick method. The ligation reaction was performed using the Ready-To-Go T4

DNA Ligase kit (Pharmacia Biotech, Piscataway, NJ; kit #27-0361-01) and the resulting insert-containing plasmids (pMAL-Stase) were transformed into Library Efficiency DH5αMCR Competent Cells (Life Technologies, Grand Island, NY). Transformed cells were plated onto LB-$Amp_{120ug/ml}$, X-$gal_{40ug/ml}$, $IPTG_{0.1mM}$ media. IPTG induced transcription from the P_{tac} promoter allowing the expression of the MBP/Stase fusion protein. The termination codon on the end of the fusion protein prevents the expression of β-galactosidase α resulting in white colonies on X-gal plates. QIAGEN plasmid preparations were made from selected white colonies, double digests (*Xba* I and *Eco*R I) were performed and the products run on a 1% agarose DNA gel stained with ethidium bromide to confirm the presence of the pMAL-Stase plasmid. 1 Kb DNA Ladder (GibcoBRL, Grand Island, NY) was used as the size standard. Plasmid DNA was sequenced using the pMAL primer (UMMBF) to confirm the correct insertion of the clone.

2-11. Expression and Purification of MBP/Stase Fusion Protein

A modification of the NEB protocol for Protein Fusion and Purification System #800 was used to express and purify the Maltose Binding Protein (MBP)/Stase fusion protein. Briefly, a 10ml overnight culture (grown in LB-$Amp_{120ug/ml}$) of cells containing pMAL-Stase was diluted 1:10 in LB-$Amp_{120ug/ml}$ and grown to an OD_{600} of approximately 0.5. The culture was then induced with IPTG, at a final concentration of 1mM, for 3 hours. Cells were centrifuged at 4,000x*g* for 20 min., resuspended in 40ml of lysis buffer (described in the NEB protocol), and frozen overnight at –20°C. Cells were thawed in ice water and sonicated with ten 15 second bursts at 50% power (setting 3) on a Fisher 550 Sonic Dismembranator using a microtip. NaCl was added to a final concentration of

0.5M and the mixture was centrifuged for 30 min. at 9,000x*g*. Supernatant was removed, placed on amylose resin (5ml bed volume/tube) (NEB) and rocked overnight. Batches of resin were centrifuged at 3000 rpm in a Beckman Model TJ-6 swinging bucket centrifuge and washed with three bed volumes of column buffer+0.25% Tween-20 (described in the NEB protocol) followed by a wash with five bed volumes of column buffer. Each wash was followed by a low speed (3000 rpm) spin in a Beckman TJ-6 centrifuge. Ten milliliters of column buffer+10mM maltose was added to each batch. Tubes were spun down as described for the washes and the column buffer containing the MBP/Stase fusion protein was aspirated off, dialyzed against distilled water and lyophilized.

2-12. Generation of Rabbit Polyclonal Stase Antiserum

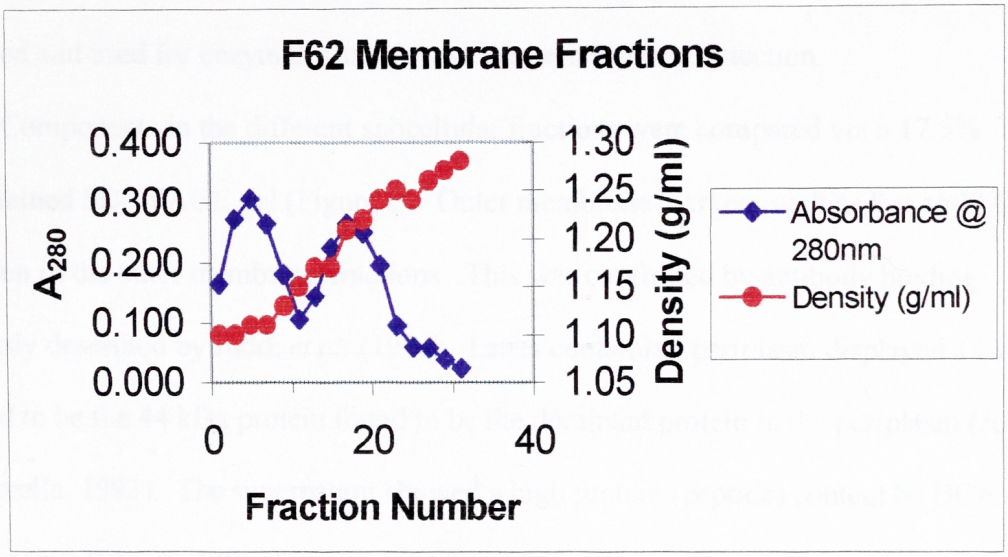
The purified fusion protein obtained above was solubilized in DPBS (0.5 mg/ml as determined by BCA protein assay) and used to immunize a New Zealand White rabbit. The primary immunization was 0.26mg of MBP/Stase fusion protein in CFA given subcutaneously in four sites. The secondary immunization was 0.38mg of fusion protein in IFA given subcutaneously in four sites. IV boosts were given two weeks apart and consisted of 100mg of fusion protein and 140mg of fusion protein respectively.

3. RESULTS

3-1. Isolation of Subcellular Fractions

Outer membrane, periplasmic, inner membrane and cytoplasmic fractions were isolated from both *N. gonorrhoeae* strain F62 (parent) and *N. gonorrhoeae* F62 STO1 (Stase deletent). The outer membrane of each strain was separated from the inner membrane by isopycnic sucrose density gradient centrifugation. The gradients were fractionated into 1ml fractions and the absorbance at 280nm (the wavelength corresponding to the absorbance peak of proteins) of each fraction was measured. When the A_{280} of every other fraction was plotted sequentially from the top of the gradient to the bottom, two protein absorbance peaks were observed (Figures 1A and 1B). In the gradient separation of strain F62, the first peak was found in fractions 1-11 and the main portion of the second peak corresponded to fractions 12-23. In the STO1 fractions, the first peak was found in fractions 1-13 and the second in fractions 14-26. The density of every other fraction (beginning with fraction 1 in each gradient) was measured by refractometry. For strain F62, the density ranges of the first and second peaks (averaged over two gradients) were 1.09g/cm^3 to 1.15g/cm^3, and 1.17g/cm^3 to 1.25g/cm^3, respectively. The peaks of strain STO1 had a density range of 1.08 g/cm^3 to 1.15g/cm^3 for the first peak and 1.17g/cm^3 to 1.25g/cm^3 for the second peak. The densities of the inner and outer membranes of other Gram negative bacteria are approximately 1.1g/cm^3 and 1.22g/cm^3 respectively (Judd, *et al.*, 1996). These values fell within the density ranges corresponding to two protein peaks identified by absorbance, which led to the conclusion that the first peak contained inner membrane proteins and the second peak contained outer membrane proteins.

A.



B.

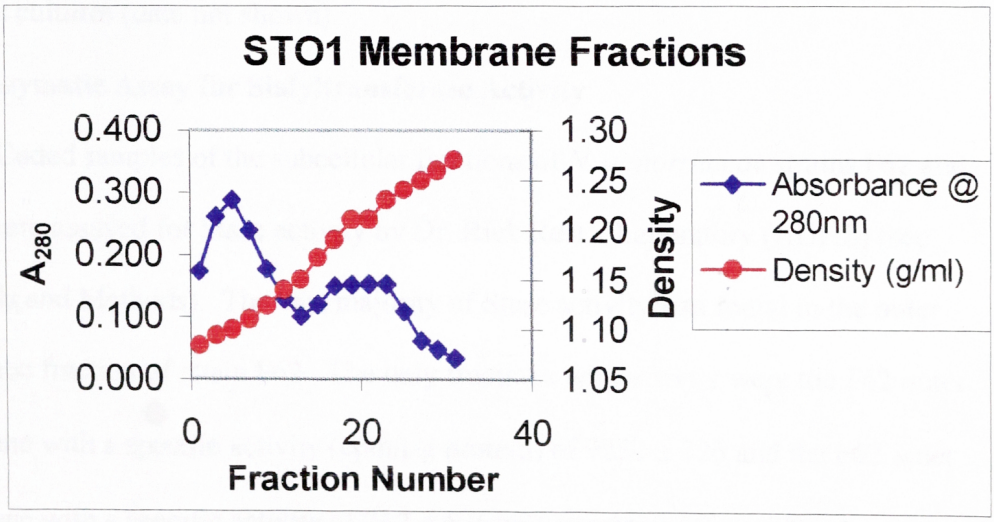


Figure 1. Absorbance$_{280}$ and density (g/ml) of fractions showing sucrose gradient membrane separations of **(A)** *N. gonorrhoeae* F62 and **(B)** *N. gonorrhoeae* F62 STO1 by isopycnic centrifugation. The first peak corresponded to the inner membrane and the second peak corresponded to the outer membrane.

This was confirmed by cytochrome, sugar dehydrogenase and immunological assays (Dr. Ralph Judd, personal communication). Fractions from the middle of each peak were combined and used for enzymatic assays and Western blotting detection.

Components in the different subcellular fractions were compared via a 17.5% silver-stained SDS-PAGE gel (Figure 2). Outer membrane markers such as Por and LOS were seen in the outer membrane fractions. This was confirmed by antibody binding previously described by Judd, *et al.* (1996). Lanes containing periplasm displayed a band assumed to be the 44 kDa protein found to be the dominant protein in the periplasm (Judd and Porcella, 1993). The supernatant showed a high protein (peptide) content by BCA protein assay, but very little if any was attributable to gonococci as the control supernatant (sterile) contained more protein (peptides) than supernatant taken from bacterial cultures (data not shown).

3-2. Enzymatic Assay for Sialyltransferase Activity

Coded samples of the subcellular fractions of *N. gonorrhoeae* strains F62 and STO1 were assayed for Stase activity by Dr. Rick Rest's Laboratory (AUHS) (see Materials and Methods). The vast majority of Stase activity was found in the outer membrane fraction of strain F62. The only fractions with activity were the F62 outer membrane with a specific activity (cpm/μg protein) of 9050 ± 326 and the F62 inner membrane with a specific activity of 267 ± 6.0cpm/μg protein (Figure 3). As expected, the STO1 fractions (negative controls) showed no Stase activity, and no Stase activity was detected in the supernatant fractions.

Subcellular Fractions of *N. gonorrhoeae* Wild Type Strain F62 and Stase-Deficient Mutant Strain F62 STO1 as Detected by Silver Stain

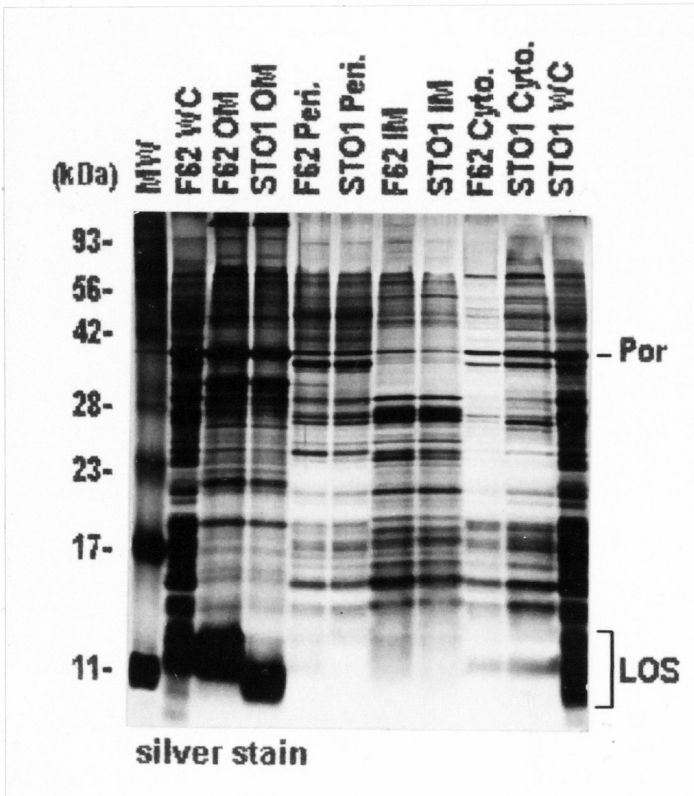


Figure 2. Comparison of the different protein banding patterns of the subcellular fractions of *N. gonorrhoeae* strains F62 and F62 STO1 by silver stain. Proteins were separated on a 17.5% SDS-PAGE gel. Outer membrane markers Por and LOS are indicated. WC- whole cell lysate, OM- outer membrane, Peri.- periplasm, IM- inner membrane, Cyto.- cytoplasm, MW- molecular weight markers, expressed in kilodaltons (kDa).

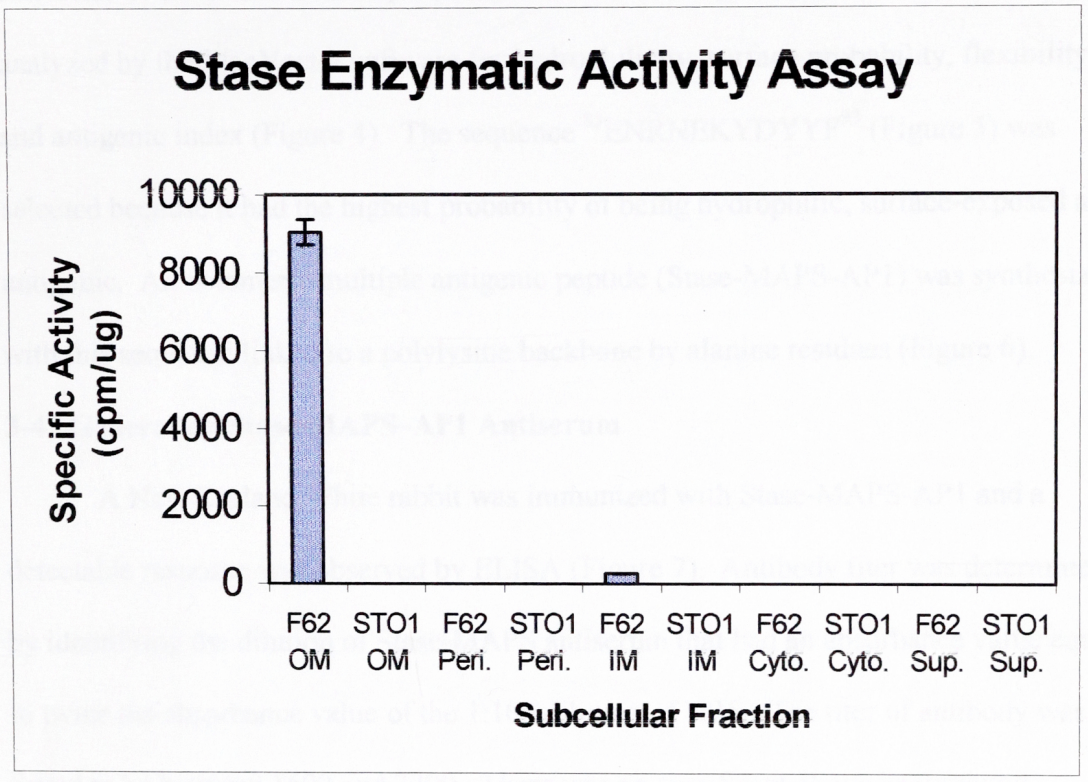


Figure 3. Subcellular fractions and culture supernatants of *N. gonorrhoeae* strains F62 and STO1 were assayed for Stase activity. Only the outer membrane of strain F62 showed significant activity with 9050 ± 326cpm/μg of protein. The inner membrane fraction of strain F62 had a minor amount of Stase based on its activity of 267 ± 6.0 cpm/μg of protein. None of the STO1 fractions (negative controls) showed any Stase activity. OM- outer membrane, Peri.- periplasm, IM- inner membrane, Cyto.- cytoplasm, Sup.- supernatant.

3-3. Selection of Peptide Sequence for MAPS Immunogen

The nucleotide sequence for Stase (GenBank accession number NGU60664) was converted to the amino acid sequence by the DNA Strider computer software and analyzed by the MacVector software for hydrophilicity, surface probability, flexibility and antigenic index (Figure 4). The sequence ^{83}ENRNEKYDYYF^{93} (Figure 5) was selected because it had the highest probability of being hydrophilic, surface-exposed and antigenic. An octomeric multiple antigenic peptide (Stase-MAPS-AP1) was synthesized with this sequence linked to a polylysine backbone by alanine residues (Figure 6).

3-4. Generation Stase-MAPS–AP1 Antiserum

A New Zealand White rabbit was immunized with Stase-MAPS-AP1 and a detectable response was observed by ELISA (Figure 7). Antibody titer was determined by identifying the dilution of Stase-MAPS antiserum that had an absorbance value equal to twice the absorbance value of the 1.100 dilution of NRS. The titer of antibody was found to be between 1600 and 3200. There was no significant above background recognition of an irrelevant MAPS antigen containing a peptide from the gonococcal multiple transferable resistance protein C (MtrC), indicating that the lysine backbone did not act as an epitope for antibody generation. Typically, antibodies are not generated to the lysine core of MAPS antigens (Yen, 1995). Normal rabbit serum (NRS) was used as a negative control and did not display an above background reaction to MtrC-MAPS coated wells.

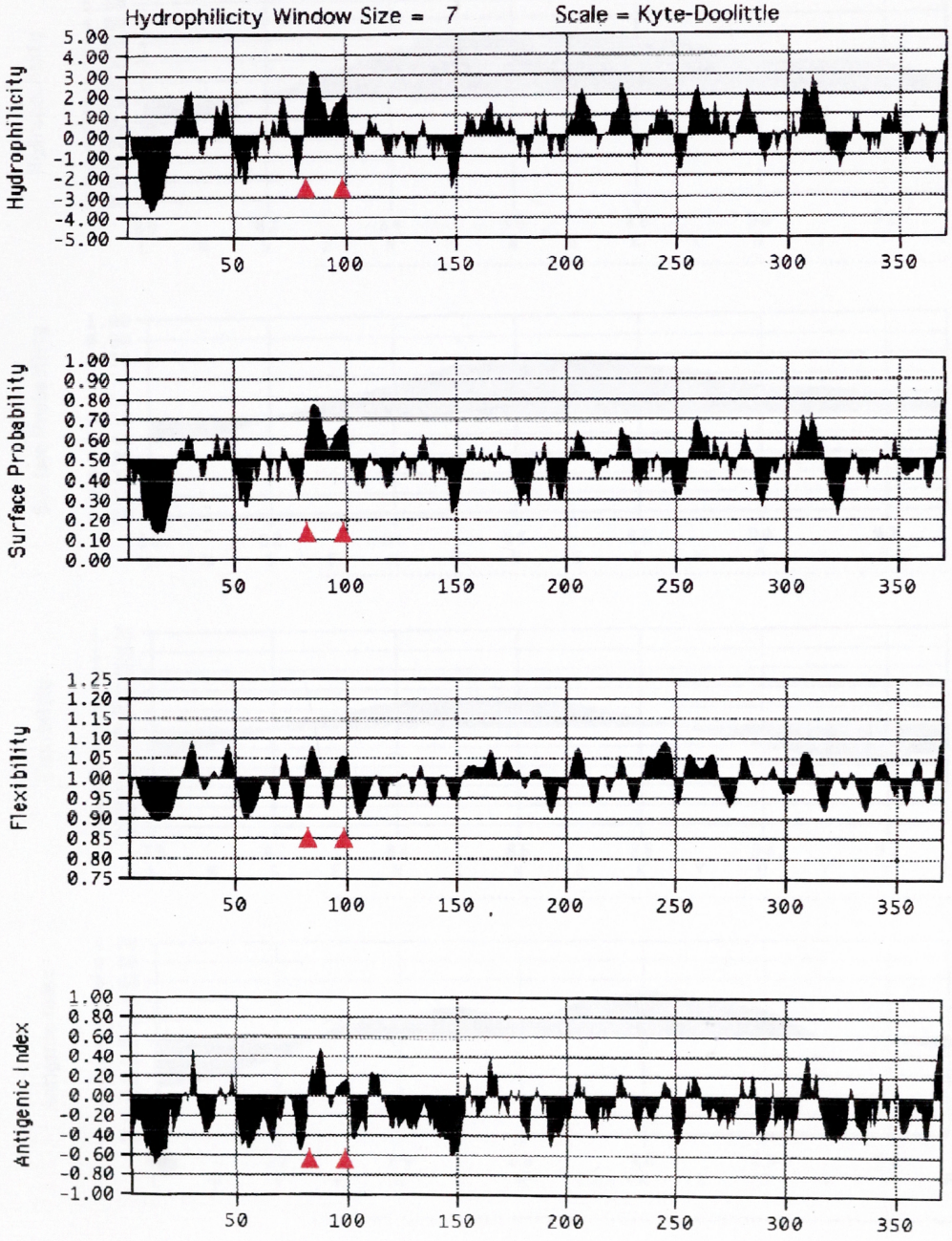


Figure 4. MacVector computer algorithm analysis of the Stase amino acid sequence. Numbers along the horizontal axis indicate the amino acid number beginning with the N-terminus. Arrowheads indicate the sequence selected for Stase-MAPS-AP1 (amino acids 83-93).

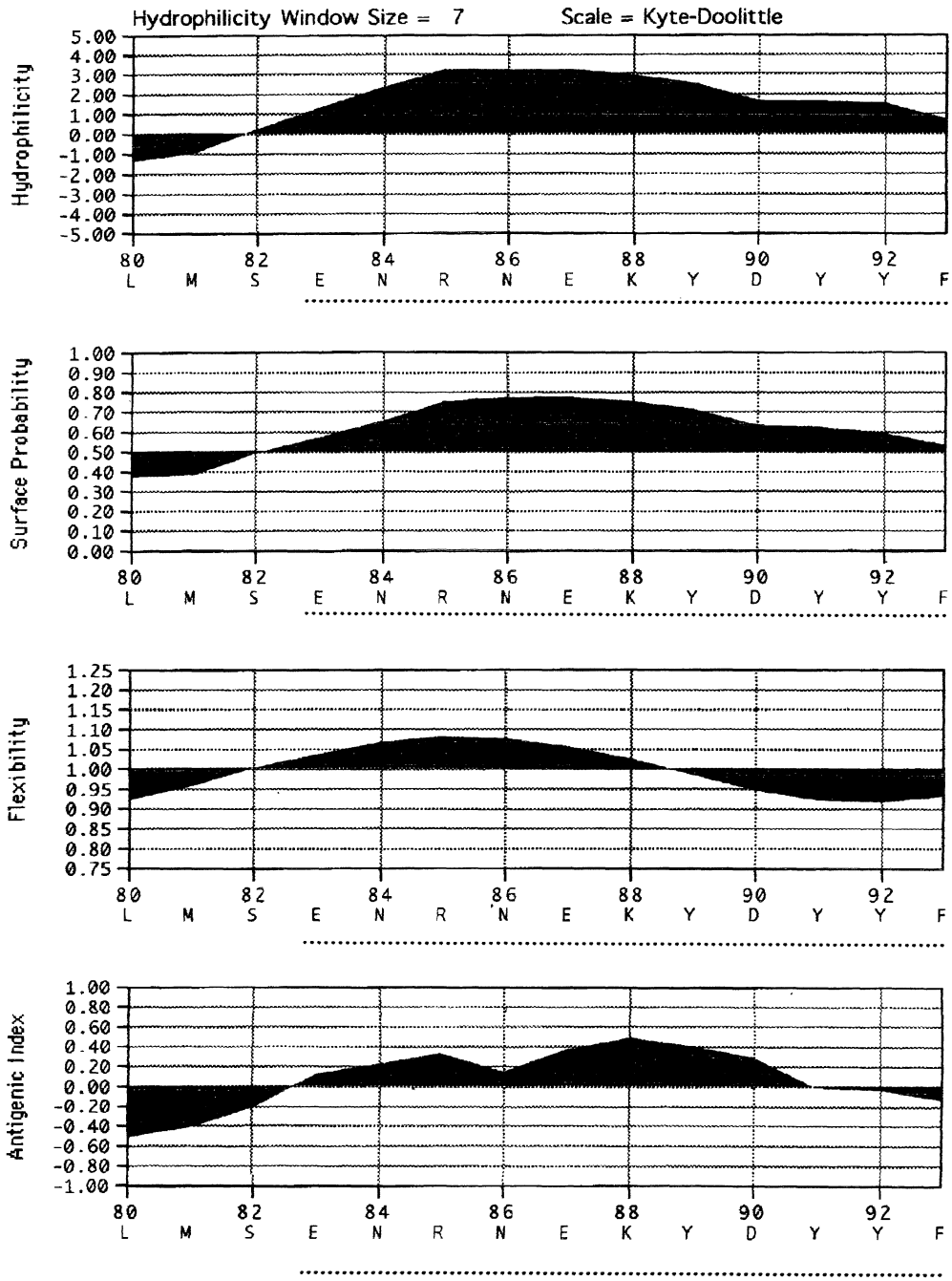


Figure 5. Predicted characteristics of the sequence selected for generation of MAPS peptide. The sequence ^{83}ENRNEKYDYYF^{93} (underscored with dotted line) was selected based on computer algorithm predicted (MacVector software) hydrophilicity, surface probability, flexibility and antigenic index.

Multiple Antigenic Peptide

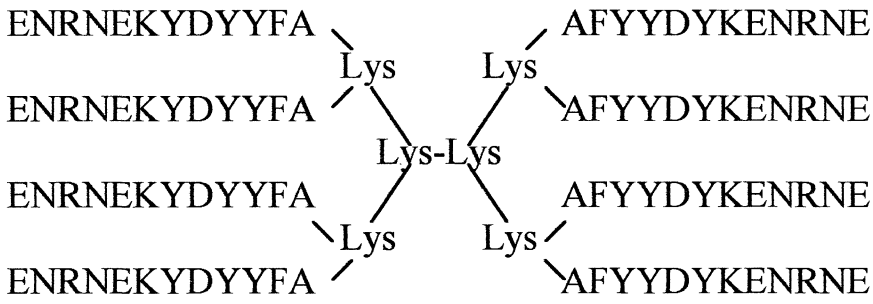


Figure 6. Stase-MAPS-AP1 is an octomeric peptide antigen containing eight copies of the selected Stase peptide sequence ^{83}ENRNEKYDYYF^{93} linked to a lysine backbone. The alanine residue directly linked to the lysine backbone was used as a spacer and was not part of the selected amino acid sequence.

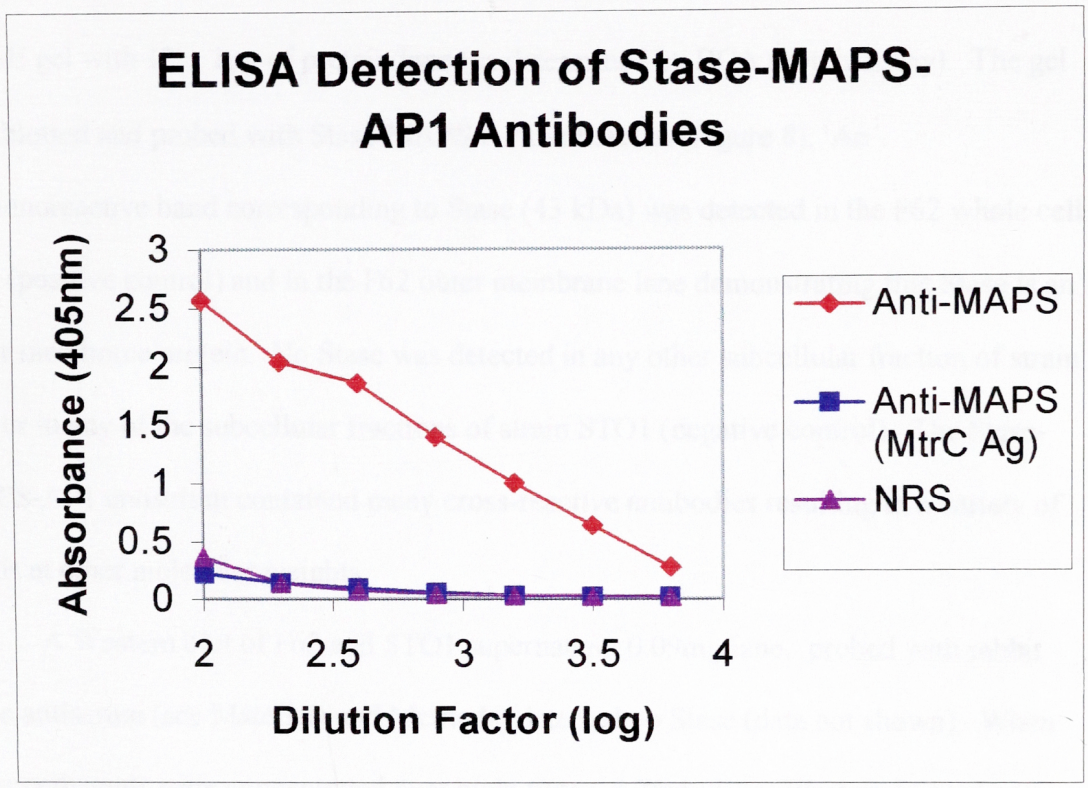


Figure 7. Detection of Stase-MAPS-AP1 antibodies was accomplished by ELISA (see Materials and Methods). Wells were coated with Stase-MAPS-AP1 and incubated with two-fold serial dilutions (100-6400) of Stase-MAPS-AP1 antiserum (Anti-MAPS, diamonds) or normal rabbit sera (NRS, triangles). A third set of wells was coated with an irrelevant MAPS antigen (negative control) containing a peptide from MtrC. These wells were incubated with two-fold serial dilutions of Stase-MAPS-AP1 antiserum, as described above (Anti-MAPS (MtrC Ag), squares).

3-5. Western Blot Detection of Stase

The subcellular fractions of both strains F62 and STO1 were run on a 10% SDS-PAGE gel with 12 ± 1μg of protein/lane (as determined by BCA protein assay). The gel was blotted and probed with Stase-MAPS-AP1 antiserum (Figure 8). An immunoreactive band corresponding to Stase (43 kDa) was detected in the F62 whole cell lane (positive control) and in the F62 outer membrane lane demonstrating that Stase is an outer membrane protein. No Stase was detected in any other subcellular fraction of strain F62 or in any of the subcellular fractions of strain STO1 (negative control). The Stase-MAPS-AP1 antiserum contained many cross-reactive antibodies resulting in a variety of bands at other molecular weights.

A Western blot of F62 and STO1 supernatant, 0.09mg/lane, probed with rabbit Stase antiserum (see Materials and Methods) detected no Stase (data not shown). When the supernatants were concentrated over eight times, 0.75mg/lane, Western blotted and probed with Stase/MBP antiserum, there was still no evidence of Stase (Figure 9).

3-6. Cloning of Stase into pMAL Fusion System

The PCR product containing the gene encoding Stase with the engineered *Xba* I and *Eco*R I endonuclease cleavage sites (see Materials and Methods) was cloned into the pMAL-c2 vector and transformed into DH5α cells. The colonies of transformed cells were white and grew on LB-Amp-X-gal-IPTG plates. When plasmid preparations were made from cultures of these colonies, double digested with *Xba* I and *Eco*R I, and run on an agarose gel, two bands corresponding to the 1.1 kb Stase insert and the 6.6 kb plasmid vector were apparent.

Coomassie Stain and Western Blot of the Subcellular Fractions of *N. gonorrhoeae* Strains F62 and STO1 Probed with Stase-MAPS-AP1 Antiserum

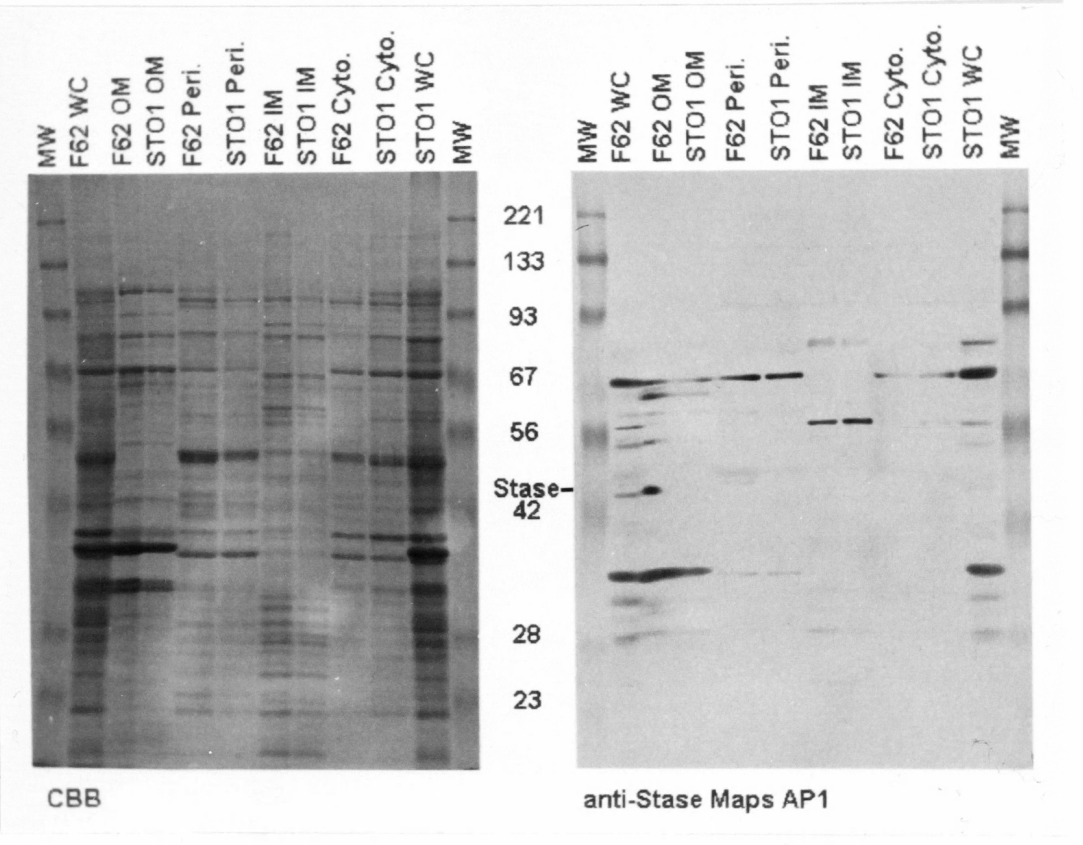


Figure 8. Detection of Stase in strains F62 and F62 STO1 of *N. gonorrhoeae* was performed by Western blot of a 10% SDS-PAGE gel probed with Stase-MAPS-AP1 antiserum. Subcellular fractions were run with 12 ±1μg of protein per lane. The left panel shows a Coomassie Brilliant Blue (CBB) stain of a 10% SDS-PAGE gel corresponding to the accompanying Western blot. Bands corresponding to Stase were detected in the lanes containing F62 whole cell lysate (F62 WC) and F62 outer membrane (F62 OM). WC- whole cell lysate, OM- outer membrane, Peri.- periplasm, IM- inner membrane, Cyto.- cytoplasm, MW- molecular weight markers, expressed in kilodaltons (kDa).

Coomassie Stain and Western Blot of Culture Supernatant of *N. gonorrhoeae* Strains F62 and F62 STO1 Probed with Stase/MBP Antiserum

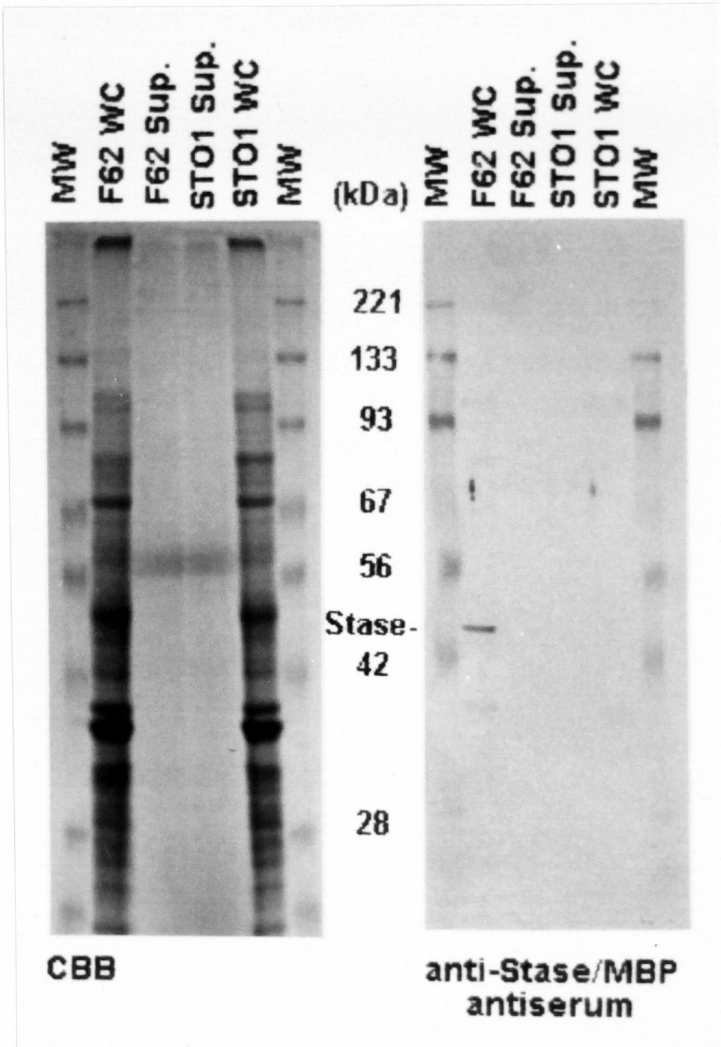


Figure 9. A 10% SDS-PAGE gel stained with Coomassie Brilliant Blue and a Western blot probed with Stase/MBP antiserum failed to detect the presence of Stase in concentrated supernatant fractions of either *N. gonorrhoeae* strain F62 or strain F62 STO1 (negative control) although the antiserum was capable of detecting Stase (F62 WC lane, positive control). WC- whole cell lysate, Sup.- supernatant, MW- molecular weight markers, expressed in kilodaltons (kDa).

Sequence data confirmed the correct orientation and location of the engineered Stase insert. The pMAL-Stase plasmid encodes a MBP/Stase fusion protein (Figure 10). The ochre stop codon of the Stase gene prevents the expression of the *lacZα* gene product, β-galactosidase α as demonstrated by the white color of pMAL-Stase transformed colonies.

3-7. Expression of MBP/Stase Fusion Protein

Cells transformed with the plasmid described above were grown, induced and treated as described in Materials and Methods. Fusion protein eluted from the amylose resin was visualized on a coomassie brilliant blue stained 10% SDS-PAGE gel (Figure 11), which showed a band at slightly less than 93 kDa. The expected molecular weight of the fusion was approximately 85 kDa. The identity of the fusion protein was further confirmed by Western blot using Stase antiserum provided by Dr. Rick Rest (AUHS) (see Materials and Methods), which reacted with the fusion protein, but not with a MBP control (Figure 12).

3-8. Generation of Stase/MBP Antisera and Detection of Stase

The MBP/Stase fusion protein was used to immunize New Zealand white rabbit (see Materials and Methods). When the antisera generated was used to probe a Western blot containing the subcellular fractions of both strains F62 and STO1, Stase was only detected in the lanes containing F62 whole cell lysate and F62 outer membrane (Figure 13). This confirmed the hypothesis that the cellular location of Stase was the outer membrane. Most of the cross-reactive antibodies were preabsorbed by *E. coli* DH5α cell lysate (see Materials and Methods).

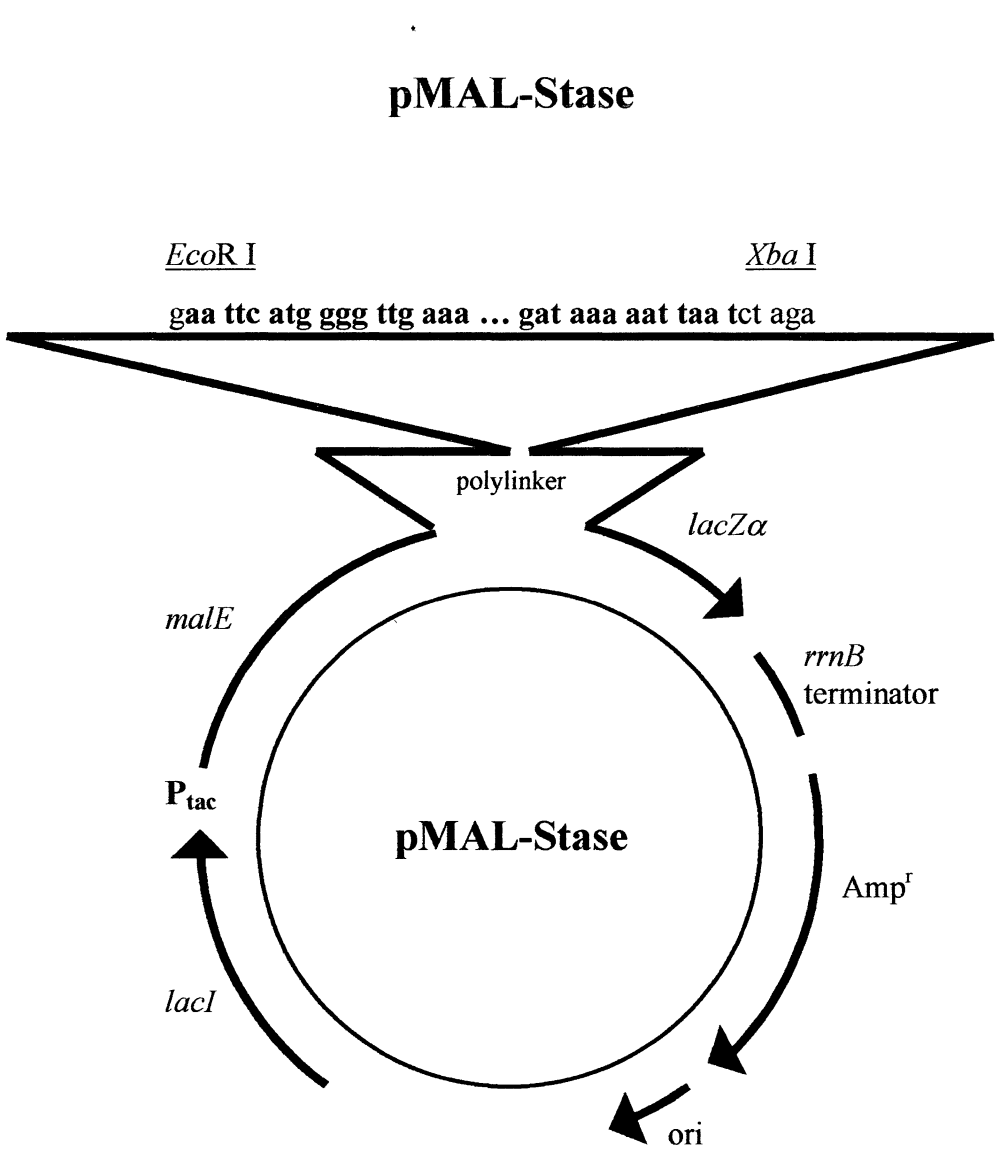


Figure 10. The pMAL-Stase plasmid construct contains an insert (bold) of the Stase gene with engineered restriction endonuclease sites (*Eco*R I and *Xba* I). P_{tac} is an inducible promoter under the repressor control of the *lacI* gene product. Derepression occurred when IPTG was added to the system. The *lacZα* gene product was not expressed because of the stop codon found at the end of the Stase gene. The *malE* gene encodes MBP (without a signal sequence) which was fused to Stase and expressed in the cytoplasm. Ampicillin resistance provided a selectable marker for the plasmid.

Coomassie Stain Detection of Fusion Protein MBP/Stase

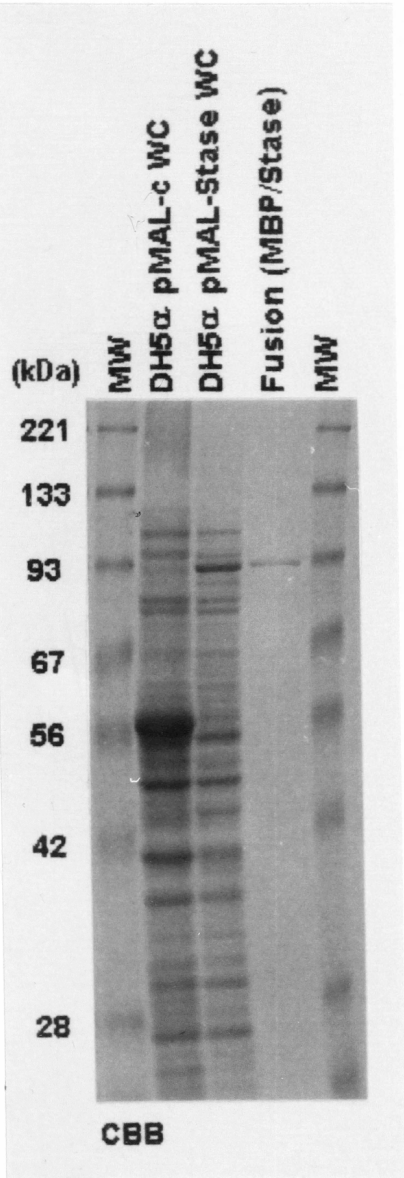


Figure 11. Purified fusion protein MBP/Stase was detected by Coomassie Brilliant Blue stain of a 10% SDS-PAGE gel. The single band in the Fusion lane correspond to the expected M_r of the MBP/Stase fusion protein (85 kDa). Induced *E. coli* DH5α cells containing the pMAL-Stase plasmid show a corresponding band that is essentially absent from the whole cell lysate of DH5α cells containing the pMAL-c plasmid without the Stase gene insert. MW- molecular weight markers, expressed in kilodaltons (kDa).

Western Blot Detection of the Fusion Protein MBP/Stase

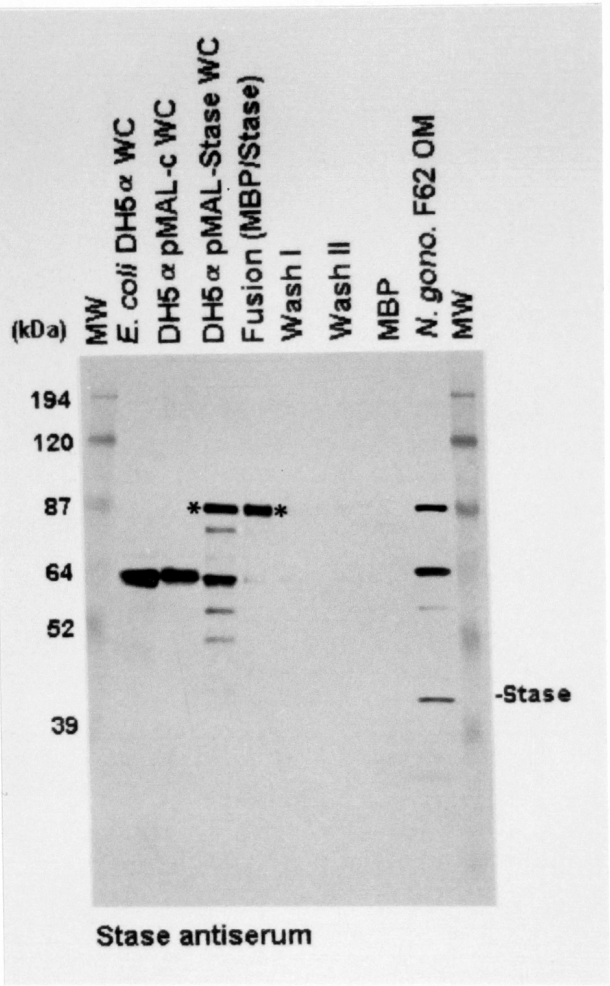


Figure 12. A Western blot probed with Stase antiserum (a gift from Dr. Rick Rest, see Materials and Methods) showed the presence of the fusion protein in *E. coli* DH5α cells harboring the pMAL-Stase plasmid, as well as confirming the identity of the purified plasmid protein (asterisks indicate fusion protein). Stase antiserum did not react with a band of the predicted fusion M_r (85 kDa) in DH5α cells or DH5α cells containing the pMAL-c plasmid without the Stase gene insert. Neither did it react with MBP alone. A band corresponding the Stase (43 kDa) was detected in the *N. gonorrhoeae* F62 outer membrane lane (positive control). MW- molecular weight markers, expressed in kilodaltons (kDa), WC- whole cell lysate, OM- outer membrane.

Coomassie Stain and Western Blot of the Subcellular Fractions of *N. gonorrhoeae* Strains F62 and F62 STO1 Probed with Stase/MBP Antiserum

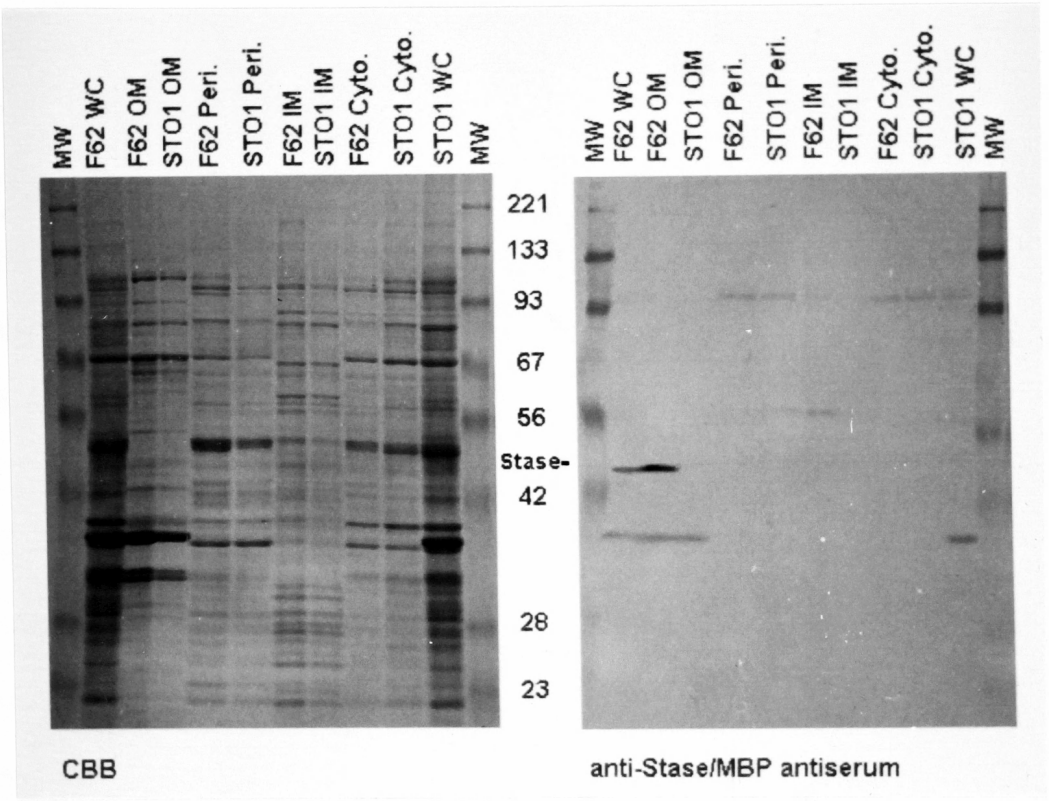


Figure 13. Detection of Stase was performed by Western blot of a 10% SDS-PAGE gel probed with Stase/MBP antiserum. Subcellular fractions were run with 12 ±1μg of protein per lane. The left panel shows a Coomassie Brilliant Blue (CBB) stain of the SDS-PAGE gel corresponding to the accompanying Western blot. Only the lanes with F62 whole cell lysate (F62 WC) or F62 outer membrane (F62 OM) contained Stase. WC- whole cell lysate, OM- outer membrane, Peri.- periplasm, IM- inner membrane, Cyto.- cytoplasm, MW- molecular weight markers, expressed in kilodaltons (kDa).

4. DISCUSSION

Gonococcal diseases are a significant problem among populations in the United States and Europe that exhibit high risk behaviors. These populations include adolescents, certain minority groups and people living in inner cities. Many developing countries also have high rates of gonococcal disease, and gonococci continue to develop antibiotic resistance (Mabey, 1996; Piot and Islam, 1994). Penicillin and tetracycline-resistant gonococci make up more than 50% of gonococcal isolates in many countries in Africa and Asia (Mabey, 1996). Furthermore, evidence indicates that transmission of the HIV virus is increased among partners with gonococcal infection or other bacterial sexually transmitted diseases (Sparling, *et al.*, 1994; Mabey, 1996). To date, there is no effective vaccine available.

This study focused on the identification of the subcellular location of the gonococcal α-2,3-sialyltransferase (Stase), an enzyme that appears to enhance the persistence of gonococcal infection by the transfer of sialic acid from the host donor molecule, CMP-NANA, to the bacterial surface LOS (Mandrell, *et al.*, 1993). The presence of sialic acid on the Galβ1-4NAcGlu moiety of a 4.5 kDa LOS molecule expressed by many strains of *N. gonorrhoeae* enhances the serum resistance of the bacteria (Smith, *et al.*, 1995). Serum resistance is enhanced by decreasing the effectiveness of IgM mediated killing (Smith, *et al.*, 1995), by reducing the binding of complement protein C3, by enhancing the cleavage of C3b to an inactive form, and by increasing the binding of Factor H which binds C3b preventing the formation of the C3 convertase of the alternative complement pathway (Smith, *et al.*, 1995; Kuby, 1997). Sialylation has also been shown to delay complement-mediated opsonophagocytosis

(Kim, *et al.*, 1992) and to increase the resistance of gonococci to phagocytic killing by PMNs (Gill, *et al.*, 1996).

Determining the cellular location of Stase is a relevant step toward the goal of understanding and defeating the mechanisms used by gonococci to persist in the host. If Stase is located on the bacterial surface (i.e. in the outer membrane), further studies of the action of anti-Stase antibodies are warranted. The hypothesis that Stase was located primarily in the outer membrane of *N. gonorrhoeae* was tested by enzymatic assays and Western blots of subcellular fractions of the bacterium. The first step in locating Stase was the isolation of the various subcellular fractions, including the outer membrane, periplasm, inner membrane and cytoplasm. It seemed unlikely that gonococci would secrete Stase into the surroundings as this would be wasteful and inefficient, but the supernatant was also collected to test this possibility.

Separations of the inner and outer membranes were accomplished by a sucrose density gradient centrifugation procedure previously shown to give clearly identifiable inner and outer membrane fractions (Dr. Ralph Judd, personal communication). The membrane separations of the wild type gonococcal strain F62 and the Stase-deficient mutant strain F62 STO1 used as a negative control, showed protein peaks at virtually identical densities (Figures 1A and 1B) further demonstrating the reliability of this membrane isolation procedure.

The vast majority of Stase enzymatic activity was found in the outer membrane of strain F62, with the specific activity of the outer membrane being over 30 times higher than the specific activity in the inner membrane fraction. Outer membrane proteins must pass through the inner membrane on their way to insertion in the outer membrane, thus

the slight amount of activity found in the inner membrane may be due to Stase in transit. It is also possible that a small overlap in the membrane peaks (Figure 1A), may account for the presence of Stase activity in the inner membrane fraction. No Stase activity was detected in any other fraction tested.

The hypothesis that Stase was located in the outer membrane was further confirmed by Western blot using rabbit antiserum generated to an octomeric MAPS peptide bearing the Stase amino acid sequence: ^{83}ENRNEKYDYYF^{93}. This sequence was shown by MacVector software using the Kyte-Doolittle algorithm to have the highest hydrophilicity In addition, it had a high predicted surface probability, flexibility, and a high antigenic index that takes into account the previous information as well as the predicted secondary structure (Jameson and Wolf, 1988). The MAPS peptide was shown by ELISA to be adequately immunogenic (Figure 7). When Stase-MAPS-AP1 antiserum was used to probe a Western blot containing the various subcellular fractions, a band of the appropriate (43 kDa) molecular mass was seen in the whole cell lysate of strain F62 and in the F62 outer membrane fraction, but not in any other subcellular fraction, confirming that Stase was located in the outer membrane. *N. gonorrhoeae* is a typical gram-negative bacteria, so the presence of cross-reactive antibodies in the Stase-MAPS-AP1 antiserum, resulting in the detection of other bands of various molecular weights, was not surprising.

The absence of a 43 kDa protein band corresponding to Stase in lanes containing the mutant strain F62 STO1 whole cell lysate and outer membrane, confirmed the identity of the Stase protein band in the wild type F62 whole cell and outer membrane lanes. Evidence that the Stase gene in strain STO1 was disrupted by a kanamycin resistance

cassette, and therefore not expressed, came from PCR that used STO1 genomic DNA as the template and primers complementary to the 5' and 3' ends of the gene encoding Stase. The resulting PCR product contained a major band roughly 1000 base pairs higher than the PCR product obtained when wild type F62 DNA was used as the template (data not shown). This confirmed the insertion of the kanamycin resistance cassette into the gene.

One of the advantages of MAPS immunogens is that they generate antibody to a very small number of epitopes. It is a rapid and easy way to generate specific antibodies (Yen, 1995). However, this specificity makes them more difficult to use in Western blot detection when the protein of interest is present in small quantities because each molecule of protein may have just a few binding sites. If antibody were generated to a mixture of immunogenic MAPS antigens, this problem might be overcome.

The antibody-generating response against a whole protein tends to be stronger than the response to a MAPS immunogen, perhaps because of the abundance of B and T cell epitopes available. Purified Stase is very difficult to obtain, presumably, because it is a minor outer membrane protein. Generating the recombinant protein in *E. coli* was thus a useful method for obtaining enough protein for antisera production. To this end the gene encoding Stase was cloned into the pMAL-c2 plasmid and used to transform *E. coli*. Cells containing the plasmid were induced with IPTG, and the resulting fusion protein, MBP/Stase, was affinity-purified on amylose resin. Addition of maltose allowed the removal of the fusion protein from the resin. The purified fusion protein was used to generate Stase/MBP antiserum, which was used to probe a Western blot identical to the

one described above, with similar results, further confirming that Stase was located in the outer membrane.

The only data at variance with the hypothesis that Stase was an outer membrane protein was the presence of an N-terminal signal peptide that was predicted by computer algorithms to be uncleavable and to target Stase to the inner membrane (Gilbert, *et al.*, 1996). The protein sequence used for the prediction was based on the Stase gene from *N. meningitidis*, which differs from the gonococcal strain F62 protein by 16 or 17 amino acids depending on the *N. meningitidis* strain used for comparison (Gilbert, *et al.*, 1996). However, only one of the amino acid differences occurs in the region predicted to contain the signal peptide. The algorithms used to predict protein localization have an overall success rate of 83% (Nakai and Kanehisa, 1991). The data in this study clearly showed the computer prediction to be inaccurate by demonstrating that Stase was an outer membrane protein.

Sialylation is known to abrogate the bactericidal effects of normal rabbit sera, rabbit anti-PorA and anti-PorB sera, rabbit anti-gonococcal whole cell sera, and normal human antisera that contains anti-LOS antibodies (Wetzler, *et al.*, 1992). It has been suggested that the structural and antigenic similarity of sialylatable LOS to paragloboside, a precursor for the major blood group antigens (ABH), may mask the bacterium decreasing the immune reaction of the host (Mandrell and Apicella, 1993). Sialylation of these LOS molecules increases their similarity to sialylated glycoproteins and certain glycosphingolipids (Smith, *et al.*, 1995; Mandrell, *et al.*, 1990) and may serve to enhance this effect. Sialylation also interferes with complement-mediated serum killing and phagocytosis as described above, leading to a serum-resistant phenotype.

The serum resistance caused by sialylation can be lost on subculture and is termed unstable serum resistance. It is most common in cases of uncomplicated gonococcal infection and in cases such as acute salpingitis where a significant inflammatory response is induced (Rice, 1989). Gonococci with a stable serum resistant phenotype have been found in most cases of DGI, which occur in 1 to 3% of gonococcal infections (Rice, 1989; Schoolnik, *et al.*, 1976). Stable serum-resistant gonococci did not become serum-sensitive when treated with neuraminidase, an enzyme that cleaves sialic acid from LOS molecules, indicating that this type of serum resistance was not caused by sialylation (Parsons, *et al.*, 1992). A vaccine that prevented unstable serum resistance by decreasing the ability of the gonococci to sialylate themselves would be useful in the majority of cases and could lead to a decrease in the prevalence of gonococcal infection.

Since it was shown by two methods, enzymatic assay and Western blotting, that Stase was an outer membrane protein, experiments determining whether Stase antiserum inhibits sialylation of the bacteria are warranted. If this can be shown to occur *in vitro*, Stase may be a potential vaccine candidate because it may serve to decrease the pathogenicity of the bacteria and allow the immune system to destroy them. Due to the similarity of gonococcal Stase to the α-2,3-sialyltransferase of *N. meningitidis*, and the conserved nature of Stase among the meningococcal strains examined (Gilbert, *et al.*, 1996), it is likely that Stase has a fairly conserved sequence among gonococcal strains. It would be of interest to know if Stase antiserum is bactericidal in serum killing assays. If so, Stase alone may be an excellent vaccine target. If antibodies against Stase are not sufficient to cause serum killing, but are able to block sialylation of the gonococcal LOS, it would be worth considering Stase vaccination in combination with other gonococcal

outer membrane protein epitopes. Since the most immunodominant epitopes on the gonococcal surface tend to be quite variable, antibodies against a conserved immunorecessive epitope might exhibit increased bactericidal activity if the bacteria are unable to sialylate their LOS. The antisera generated in this project will be useful in assessing the potential of Stase as a vaccine candidate.

In summary, the hypothesis that Stase is located in the outer membrane of *N. gonorrhoeae* was supported by enzymatic assay data and Western blot detection. Stase-MAPS-AP1 antiserum was made against a MAPS immunogen that consisted of eight copies of an eleven amino acid peptide from Stase, selected based on its predicted hydrophilicity, surface probability, flexibility and antigenic index. Stase/MBP antiserum was generated against a fusion protein, MBP/Stase, expressed by *E. coli* harboring the pMAL-Stase plasmid vector. Both antisera were used in Western blot detection of Stase and will be helpful in evaluating the potential of Stase as a vaccine candidate either by itself or in conjunction with epitope(s) from other gonococcal outer membrane proteins.

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