THE IDENTIFICATION, PURIFICATION, CHARACTERIZATION, AND CONSERVATION OF THE OF THE MAJOR IRON-REGULATED PROTEIN OF *NEISSERIA GONORRHOEAE*

by

Timothy Allen Mietzner

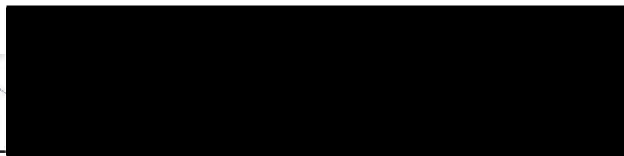
A DISSERTATION

Presented to the Department of Microbiology and Immunology
and the Graduate School
of the
Oregon Health Sciences University
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

September 1985

APPROVED:



(Professor in Charge of Thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

	Page
Introduction	00
Literature Review	1
I. The Gonococcus	1
A. The disease	1
1. Clinical manifestations	1
2. Epidemiology	4
B. Biology of the gonococcus	6
1. Colonial morphology	6
a) Piliation variants	6
b) Opacity variants	7
2. The cell envelope	8
a) The cytoplasmic membrane	9
b) Peptidoglycan	10
c) Outer membrane	12
i) Lipopolysaccharide	14
ii) Outer membrane proteins	19
d) Capsule	30
3. Envelope permeability	31
C. Genetics	35
1. Transformation	36
2. Gonococcal plasmids and conjugation	40

D. Immunopathology of gonococcal infection 44

1. Adherence 44

a) Pili and their role in attachment 44

b) Non-piliated mechanisms of attachment 50

2. Invasion 52

3. Host response to gonococcal infection 54

a) Interaction with polymorphonuclear leukocytes 54

b) Local and humoral antibody response 58

c) Serum resistance 62

4. Other factors 64

II. Microbial growth environments 65

A. Nutrient-limitation and its role in natural immunity 69

B. Iron-metabolism in the human host 70

III. Microbial iron-acquisition-- an overview 75

A. Microbial iron and iron-binding compounds 75

B. Iron-acquisition by *Escherichia coli* 78

1. Enterobactin-mediated iron-acquisition 78

2. Ferrichrome-mediated iron-acquisition 80

3. Aerobactin-mediated iron-acquisition 82

4. Citrate-mediated iron-acquisition 85

C. Iron-acquisition by other enteric pathogens 87

D. Iron-acquisition by pathogenic *Vibrio* spp. 89

E. Iron-acquisition by other pathogenic microorganisms 92

F. Iron-acquisition by *Neisseria* spp. 95

References 103

Manuscript 1. Identification of an iron-regulated 37,000 dalton protein in the cell envelope of Neisseria gonorrhoeae 152

A. Abstract 153

B. Introduction 154

C. Materials and methods 157

1. Organisms 157

2. Growth conditions 157

3. Preparation of outer membranes 158

4. SDS-polyacrylamide gel electrophoresis 159

5. Visualization of OM proteins 159

6. Peptide mapping 160

D. Results 161

1. Identification of an iron-regulated 37,000 dalton protein 161

2. Occurrence of the 37K protein 164

3. Peptide mapping 164

E. Discussion 165

F. Literature cited 169

Manuscript 2. Purification and characterization of the major iron-regulated protein (MIRP) of Neisseria gonorrhoeae 181

A. Introduction 182

B. Materials and methods 184

1. Media and reagents 184

2. Bacteria and growth conditions 185

3. SDS-polyacrylamide gel electrophoresis 185
4. Cell fractionation 186
5. Selective solubilization of the MIRP from gonococcal membranes 186
6. Isolation of crude MIRP preparations 187
7. Ion-exchange chromatography 187
8. Molecular seive chromatography 188
9. Isoelectric focusing 189
10. Amino acid composition and N-terminal sequence analysis 189
C. Results 190
1. Cellular disruption and fractionation 190
2. Preparation of crude detergent fractions 190
3. Isolation of the MIRP from CTB-extracts of the pellet and supernatant whole cell sonicate fractions 191
4. Yield 192
5. Isoelectric focusing 192
6. Amino acid composition 193
7. N-terminal amino acid sequence 193
D. Discussion 194
E. Summary 201
F. References 203
Manuscript 3. The distribution of an antigenically related iron-regulated protein among the *Neisseria* spp. 216
A. Abstract 217

B. Introduction 218

C. Materials and methods 220

1. Bacterial strains 220

2. Growth conditions 220

3. Preparation of crude bacterial membranes 221

4. SDS-polyacrylamide gel electrophoresis 222

5. Preparation of rabbit antiserum specific for the 37K protein 222

6. Preparation of monoclonal antibodies specific for the 37K protein 223

7. Enzyme-linked immunosorbent assay 223

8. Western blot analysis 225

C. Results 226

1. Expression of the gonococcal 37K protein on low-iron agar medium 226

2. Identification of *Neisseria* spp. expressing a protein antigenically related to the 37K protein (AgR-37K) 226

3. Survey of *Neisseria* spp. using an ELISA specific for AgR-37K proteins 227

4. Effect of iron on expression of the AgR-37K proteins 228

5. Reactivity of monoclonal antibodies with the AgR-37K proteins 229

D. Discussion 231

E. References 237

Summary of dissertation work 250

Appendix 255

A. Appendix 1. Effect of desferal on the growth of *N. gonorrhoeae* 256

B. Appendix 2. Effect of decreasing the growth rate on the expression of the 37K protein 258

C. Utilization of lactoferrin-bound iron by gonococci: Requirements for a specific receptor 260

D. Appendix 4. Comparison of peptide maps from the gonococcal 37K protein and an antigenically related iron-regulated 37,000 dalton protein of *N. meningitidis* 276

E. Appendix 5. Pink-to-red color exhibited by a purified, concentrated preparation of the gonococcal MIRP 278

F. Appendix 6. Studies analyzing the iron-binding potential of the MIRP of *N. gonorrhoeae* 280

LIST OF FIGURES

Manuscript 1

Figure | Page
1. OM protein profile (9.5 to 12.5% acrylamide gel) of *N. gonorrhoeae* F62 grown in low-iron medium and in regular medium. 174
2. Effect of acrylamide concentration on the separation of PI and the 37K protein. 175
3. OM protein profile of N. gonorrhoeae F62 grown at pH 6.3 in low-iron medium and regular medium. 176
4. Coomassie blue-stained gradient (9.5 to 12.5% acrylamide) gel of OM prepared from *N. gonorrhoeae* F62 grown as described in the text. 177
5. Coomassie blue-stained gel (9.5 to 12.5%) of OM preparations from *N. gonorrhoeae* F62 grown in regular defined medium, low-iron defined medium supplemented with 100 uM ferric nitrate, and low-iron defined medium without additional iron. 178
6. Autoradiographs of tryptic peptide maps of ^{125}I-labeled 37K and PI molecules. 179

PAGE

Manuscript 2

Figure

1. A silver-stained SDS-PAGE analysis of the purification steps used for the isolation of the gonococcal MIRP. 206

2. SDS-PAGE analysis of the selective solubilization of the gonococcal MIRP by CTB. 207

3. Elution profile of a CM-Sepharose column used to purify the MIRP from crude preparations. 208

4. Gel filtration of the pooled MIRP fractions obtained by ion-exchange chromatography. 209

5. A silver-stained SDS-polyacrylamide gel of the purified MIRP-containing fraction after gel filtration. 210

6. Isoelectric focusing of the purified MIRP isolated from the soluble and the particulate fractions of sonicated whole cells. 211

7. Amino terminal 63 residues of the gonococcal MIRP. 212

8. Elution profile of the non-sedimentable fraction of gonococcal whole cell lysates. 213

9. Local average hydrophobicity of the N-terminal 63 amino acids of the gonococcal MIRP. 214

PAGE

Manuscript 3

Figure

1. SDS-PAGE analysis of crude membrane preparations from *N. gonorrhoeae* strain F62 grown on GC base medium agar supplemented with increasing concentrations of Desferal. 242

2. Analysis of 9 different strains of *Neisseria* spp. and *B. catarrhalis* for the production of AgR-37K proteins. 243

3. SDS-PAGE comparison of membrane preparations from cells grown on GC base medium agar containing 25 uM Desferal and on medium which was not supplemented with this chelator. 244

4. Western blot reactivity of MAbs to strains of *Neisseria* spp. which express AgR-37K proteins. 245

Appendix 1

Figure

1. Growth curves for *N. gonorrhoeae* strain F62 grown in medium containing increasing concentrations of Desferal. 257

Appendix 2

Figure

1. Growth curves for *N. gonorrhoeae* strain F62 grown at different pH values and in the presence and absence of Desferal. 259

PAGE

Appendix 3

Figure

1. Bioassay used to differentiate between those gonococci which utilize the iron from lactoferrin and those which cannot. 269

2. Binding of lactoferrin to strains FA171 and F62. 270

3. Binding of ^{125}I-lactoferrin to strains FA171 and F62 in the presence of unlabeled lactoferrin, unlabeled transferrin, and unlabeled bovine serum albumin. 271

4. Bioassay used to differentiate those strains of gonococci which utilize iron from lactoferrin and those which cannot. 272

5. Time-dependent binding of lactoferrin to strain 171/62L 273

6. Concentration-dependent binding of lactoferrin to strains 171/62L and F62. 274

Appendix 4

Figure

1. Autoradiograph of tryptic peptide maps of ^{125}I-labeled 37K protein from *N. gonorrhoeae* strain F62 and a related protein from *N. meningitidis* strain C-11. 277

Appendix 5

Figure

1. Color photograph demonstrating the pink-to-red color of the gonococcal MIRP. 279

LIST OF TABLES

PAGE

Literature review

Table

1. Summary of ferrated compounds which pathogenic *Neisseria* species can utilize as an iron source *in vitro*. 102

Manuscript 1

Table

1. *N. gonorrhoeae* iron-regulated OM proteins. 180

Manuscript 2

Table

1. Comparison of amino acid compositions of purified gonococcal proteins. 215

Manuscript 3

Table

1. Designations and sources of strains used in this study. 246
2. Comparison of ELISA values with western blot reactivity of different strains of *Neisseria* spp. and *B. catarrhalis*. 247
3. Survey of strains of *Neisseria* spp. and *B. catarrhalis* for the expression of an AgR-37K protein. 248
4. Reactivity of MAbs to the different strains of *Neisseria* spp. producing AgR-37K proteins. 249

PAGE

Appendix 3

Table

1. Characteristics of strains FA171, F62, and 171/62L. 275

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Stephen A. Morse for his patience, encouragement, and advice during by graduate career. Special thanks are extended to the members of the laboratory who were present during my tenure as a student, especially C.Y. Chen, C.S. Parsons, and Drs. Luginbuhl, Sarafian, and Mintz. To these and others I am grateful for their help, consideration, and friendship.

Appreciation is expressed to the faculty, staff, and my fellow students in the Department of Microbiology and Immunology, School of Medicine, Oregon Health Sciences University and at the Sexually Transmitted Diseases Laboratory Program, Center for Infectious Disease, Centers for Disease Control for their friendship and many contributions during my graduate career.

A special thanks is extended to the many individuals who contributed to this thesis project, either through direct efforts or through fruitful scientific discussion. Notably, Dr. R.C. Barnes, Dr. E.W. Hook, III, Dr. J. Knapp, Dr. E.C. Sandstrom, Dr. G.K. Schoolnik, Dr. W.M. Schafer, Dr. G. Luginbuhl, Dr. G. Carlone, and W.O. Schalla. Without their interest, the success of these studies would have been limited considerably.

I acknowledge and express my gratitude for the financial support provided by grants from the National Institute of Allergy and Infectious Diseases (AI 13571 and AI 22148) and from the Medical Research Foundation of Oregon.

I would like to especially thank my friends who could never tell their

parents what I did due to the socially embarassing nature of my work. Without their presence and consideration after many a disappointing experiment it would have made it difficult to try again the next day. So to S.S., M.D., R.&B.H., M.S.M., P.C., H.T.,C.J.V., A.C., and others, I hope I have (or can) given to you what you have given to me.

To the people back in my home town who have enthusiastically encouraged and cultivated my studies and to my family who has always been there for me, I thank you from the bottom of my heart. Most importantly, I wish to thank my mother and my father who have encouraged and supported my education, both through financial sacrifice and by their faith in me. It is to them that I dedicate this work.

ABSTRACT

The limitation of free iron by the human host is considered a non-specific mechanism of defense against microbial infection. *In vitro* growth of the human pathogen *Neisseria gonorrhoeae* under conditions of iron-limitation results in the expression of several unique iron-regulated. The major iron-regulated protein (MIRP) has been shown to have an apparent molecular weight of 37,000 by SDS-polyacrylamide gel electrophoresis. This protein has been purified by selective solubilization with low concentrations of the cationic detergent, hexadecyltrimethylammonium bromide. This extract was further purified by cation-exchange chromatography and gel filtration. Fractions obtained from this purification scheme were pure by the criterion of a single band on SDS-polyacrylamide gels. The purified MIRP has been characterized with respect to its chemical properties including its isoelectric point, amino acid composition, and N-terminal amino acid sequence. MIRP-specific rabbit antiserum and murine monoclonal antibodies have been prepared. These reagents have been used to probe the distribution of proteins which are antigenically related to the gonococcal MIRP among the *Neisseria* spp. All strains of *N. meningitidis*, *N. lactamica*, and *N. cinerea* examined expressed an iron-regulated antigenically related protein. Reactivity patterns of these proteins with epitope-specific monoclonal antibodies indicated that there was species-specific structural heterogeneity. The conservation of this protein, particularly among the pathogenic members of the genus *Neisseria* and its regulation by iron suggest that the MIRP may be an important component of a gonococcal iron-uptake system which is expressed during a natural infection.

INTRODUCTION

Iron is an essential nutrient for the biological processes of most, if not all, forms of life. As a natural mechanism against infection, vertebrates sequester free iron and render it available to themselves but inaccessible to invading microorganisms. In order for pathogens to successfully cause infection, they must be able to compete with host iron-withholding systems. Pathogenic bacteria accomplish this using high affinity iron-uptake systems which are expressed during growth *in vivo*. The ability to obtain iron from the host environment is a trait expressed by all disease-producing microorganisms and represents a necessary determinant of microbial pathogenesis.

Neisseria gonorrhoeae exists in an iron-restricted environment during natural infection. Since the chemical composition of gonococci is influenced by its growth environment, growth under iron-limiting conditions *in vitro* should more closely approximate the *in vivo* chemical composition of this organism. Moreover, the mechanism(s) by which gonococci obtain iron from the host environment is not well-understood. Components of gonococci involved in the acquisition of iron from the environment should be expressed during growth in an iron-limited envrionment. These components may be identified by chemical analysis of gonococci cultivated under these conditions.

Work toward this thesis was initiated by examining proteins expressed by *N. gonorrhoeae* in response to an iron-restricted environment. The following represents the course that these studies have since taken and which make up

the work reported in this thesis:

1. Identification of a major iron-regulated protein expressed by *N. gonorrhoeae* during growth in an iron-deficient environment.

2. Purification of the major iron-regulated protein and molecular analysis of its biochemical properties.

3. Survey of the *Neisseria* spp. for a conserved protein related to the major iron-regulated protein of *N. gonorrhoeae*.

The following sections review literature pertinant to the gonococcus, nutritional immunity, and microbial iron acquisition; all three of which are concepts related to the work presented in this thesis.

I. THE GONOCOCCUS

A. THE DISEASE

Neisseria gonorrhoeae is the causative agent of gonorrhea. Upon microscopic examination, the gonococcus appears as a Gram-negative diplococcus whose opposing edges appear to be flattened. This bacterium was first described by Albert Niesser in 1879 who found the organism in urethral, vaginal, and conjunctival exudates. Interestingly, Neisser's observation made the gonococcus the second bacterial pathogen described (and the first among Gram-negative genera), following only Bacillus anthracis (194). Historically, the major clinical manifestations of gonorrhea in men were recognized in ancient Chinese, Japanese, Egyptian, Roman, and Greek literature, as well as in the Old Testament. Galen (130 A.D.) gave the name gonorrhea (which is Greek for "flow of seed") to the syndrome in the mistaken belief that the urethral exudate characteristic of this syndrome was semen (138).

1. Clinical manifestations. Gonorrhea is a sexually transmitted disease infecting columnar and transitional epithelium. The primary site of infection in heterosexual males is typically confined to the urethra, although pharyngeal infection may occur in rare cases given appropriate exposure. In homosexual men, gonococcal infection commonly involves the urethra, anal canal, and pharnyx. Symptomatic uncomplicated gonorrhea in males typically manifests itself clinically as a purulent urethritis within two weeks post-infection. Asymptomatic infections in males are rare and are thought to

account for only 5-10% of infections. Because of the overt symptomology, most males seek treatment and the disease is usually halted without further complications. Without treatment, symptoms of urethritis last for an average of eight weeks (data taken from the pre-antibiotic era). Those males who are asymptomatic or ingore symptoms not only serve as a primary resevior for the spread of gonorrhea, but also are at risk of developing local or systemic complications (168).

In women, the most common site of infection is the cervix. Additionally, other primary sites of infection include the urethra, anal canal, and pharnyx. The clinical manifestations of gonococcal infection in females are poorly defined (17). Most women present at emergency rooms or gynecologic clinics with one or a combination of symptoms which include: abnormal vaginal discharge; dysuria and frequency of urination; abnormal menstrual bleeding; anorectal discomfort; abnormal labial pain and swelling; or lower abdominal pain. The incubation period in females is probably more variable than in men but most develop symptoms within 10 days (138). Pelvic inflammatory disease (PID) probably occurs in 10 to 20% of women infected with gonorrhea. The proportion of PID cases in the United States in which _N. gonorrhoeae_ has been isolated from the cervix have been reported to be 50% (112). However, a high percentage of PID cases in which _N. gonorrhoeae_ is associated, have other pathogens present in the fallopian tubes. Thus, the overall importance of the gonococcus as a causative agent of PID is unclear (17). The infertility rate caused by PID has been calculated to be 13% after one attack, 36% after two, and 75% after three or more. Furthermore, the risk of ectopic pregnancy increases seven-fold after one attack of PID (425). The total annual cost for gonococcal-associated PID has been estimated to be $300 million (17).

Neonatal gonorrhea infections may involve the conjunctivae, pharnyx, respiratory tract, or anal canal. Conjunctival infection (opthalmia neonatorum) is usually bilateral, occuring two to three days post-partum. In rare cases, a similar disease (conjunctivitis) may occur in adults, generally as the result of autoinoculation. These infections may eventually lead to blindness. Asymptomatic or minimally symptomatic conjunctival infections have also been reported. The most common manifestations of gonorrhea in newborns is gonococcal amniotic infection syndrome. This disease presents itself in the form of chorioamnionitis and nonspecific signs of sepsis in the neonate, together with the presence of N. gonorrhoeae in the orogastric aspirates of the infants affected (168).

A complication of primary and secondary gonococcal infection is bacteremia (disseminated gonococcal infection, DGI) which occurs in ca. 1 to 2% of infected individuals. DGI is marked by fever, malaise, asymmetrical tenosynovitis or oligo arthritis, and by papulopustular or necrotic skin lesions on the extremities. Disseminated infections may occasionally result in endocarditis or meningitis (168). The risk for development of DGI has been correlated with the phenotypic properties of the gonococcal isolates. In areas where a high proportion of isolates require arginine, hypoxanthine, and uracil for growth on chemically defined medium (AHU^-), these strains have been significantly associated with DGI. Similarly, the serogroup of the principle outer membrane protein, Protein I is also correlated with AHU^- strains and consequently with DGI (203). Furthermore, AHU^- isolates are also associated with asymptomatic infections which may contribute to dissemination by allowing prolonged exposure of the host to the organism (86). Most strains causing DGI (and lacking a plasmid-encoded penicillinase)

are highly susceptible to penicillin G (216, 423). Perhaps the most important phenotypic property of strains associated with DGI is their ability to resist the bactericidal action of normal human sera (342). Host factors also predispose individuals to DGI. Patients deficient in the terminal components of complement are especially susceptible to both gonococcemia and meningococcemia (53, 296). Hormonal factors in women may play a role. Most cases of DGI in females occur during pregnancy or close to menses (56).

2. Epidemiology. In the United States, gonorrhea has been the most frequently reported communicable disease since 1965, and in 1979 was reported twice as frequently as the combined number of reported cases of all other notifiable diseases (17). In 1983, some 900,435 cases of gonorrhea were reported to the Centers for Disease Control. In terms of incidence, this represents 387.6 cases per 100,000 population (65). This figure seems even more impressive when one considers that it represents an underestimate (perhaps accounting for only 50% of the total cases) due to underreporting of cases by the private sector. The increase in the incidence of reported cases of gonorrhea increased sharply during and shortly after World War II (1941-1946). The introduction of penicillin therapy correlated with a dramatic reduction in incidence of this disease to a level comparable to the pre-war era. This decrease was a statement for the control of infectious disease by effective drug therapy. Since 1957, the incidence of gonorrhea has steadily increased, reaching a peak in 1975 when this figure exceeded 450 cases per 100,000 population. However, for the past 10 years the incidence has either remained the same or decreased (17).

The dramatic increase in the number of reported cases of gonorrhea during the last three decades can be attributed to a combination of factors. These

factors can be divided into those which have lead to a "real" increase in the total number of cases and those which have lead to a "perceived" increase but have not contributed to the true morbidity of the disease. The "real" increases in the incidence of gonorrhea have resulted from (i) an increased number of individuals at risk, i.e., sexually active young adults; (ii) changing behavioral and cultural values throughout the United States and developing countries which are reflected in more liberal attitudes; (iii) the advent and popularity of oral contraception or contraceptive intrauterine devices; and (iv) selection of antibiotic resistant strains of _N. gonorrhoeae_ (resulting in increased numbers of treatment failures and exposure of multiple partners to these resistant strains). The "perceived" increases in the incidence of gonorrhea are artifacts of (i) increased culture detection of gonorrhea in women facilitated by the introduction of selective bacteriologic medium; (ii) intensified efforts to trace sexual contacts; (iii) development of an inexpensive transport medium for clinicians without laboratory facilities; and (iv) legal requirements for reporting positive cultures. Recent trends in which the incidence of gonorrhea has remained the same or declined may reflect successes of current public health programs. These trends may also reflect cutbacks in federal funding of public clinics (which will contribute to underreporting and increased number of untreated cases) and/or the decreasing sexual adventurism by an increasingly educated public, especially regarding "new" and "incurable" diseases such as genital Herpes and acquired immune deficiency syndrome (AIDS) (17).

B. BIOLOGY OF THE GONOCOCCUS

1. Colonial morphology. Cultiviation of N. gonorrhoeae on artificial medium was first reported by Lestikow and Leoffler in 1882 (197) and by Bumm in 1885 (51). Since this time, the organism has generally been considered fastidious with respect to its growth requirements. This inaccurate assumption stemmed from the inability to propagate gonococci on simple nutrient agar. Rather than this medium being deficient in a gonococcal growth-essential component, it is now known that growth is inhibited by fatty acids, toxic metals, etc., contributed by the peptone and agar. The toxicity of this medium can be absorbed by the addition of starch, blood, or charcoal (386). The requirements for gonococcal growth on solid medium have been defined and appear to be quite simple (63).

a) Piliation variants. Kellogg et al. (197) described the occurrence of morphologically distinct gonococcal colony types (designated as T_1, T_2, T_3, and T_4) after growth on agar medium. Colony variants T_1 and T_2 were relatively small in size and virulent in male volunteers, while T_3 and T_4 types were larger and avirulent (196). Ultrastructural analysis of organisms from T_1 and T_2 colonial variants by electron microscopy detected the presence of pili. No piliation was observed when organisms from T_3 and T_4 colonies were analyzed by electron microscopy (181, 385). T_1 and T_2 colony variants could be maintained by daily selective subculture on solid medium lacking antibiotics (174, 196). Without selective passage, a rapid shift to the large colony variants occurred. In recent literature, the piliated colony types T_1 and T_2 and nonpiliated colony types T_3 and T_4

have been designated as P^+ and P^-, respectively (386).

The predominant colony type recovered from different gonococcal infections have been analyzed (197, 208, 365). Colony types obtained from primary isolates of males with acute gonorrhea were 90% P^+ (T_1) (197). In another study, Sparling and Yobs (208) found that piliated variants were the predominant colony type in 69% of the primary cultures from males and 67% from females (i.e., P^+ colonies comprised greater than 75% of the total colonies isolated from the infection). Rectal isolates have also been analyzed (365) and piliated colony types also appear to dominate.

b) Opacity variants. A second morphologic distinction between gonococcal colonies was observed by Swanson (377) who described dark and light variants. Dark colony types were associated with decreased leukocyte association, were sensitive to trypsin, and had a unique outer membrane protein associated with them which had an apparent molecular weight of *ca.* 26,000. Swanson (379) later used the terms "transparent" and "opaque" for the light and dark colonial variants, respectively. Opaque colonies displayed extensive intercellular adhesion when analyzed by electron microscopy and were associated with autoagglutinability. Furthermore, there appeared to be varying degrees of opacity, some colonies appearing darker than others. The revised, current nomenclature designates opaque colonies of intermediate darkness as O^+ and very dark as O^{++}, and transparent colonies as O^- (386).

Mayer (233) determined that the average change in colony opacity phenotype for 12 gonococcal strains was 2 X 10^3 per colony forming unit per generation. Furthermore, the state of piliation, the number of passages, alterations in temperature, oxygen tension, or composition of the growth medium did not significantly alter the transition rates.

Similar to piliated colony types, opacity variants have been analyzed with respect to their predominance in several forms of gonococcal infections. Observations by James and Swanson (178, 179) and others (104) suggested that certain opacity phenotypes were found at different anatomical sites. In men, gonococci cultured from the urethra and pharnyx formed opaque colonies. In women, gonococci cultured from the urethra, pharnyx, and endocervix (at mid-cycle) also appeared to be opaque. At menses, cultures tended to consist primarily of transparent colony forms. Fallopian tube isolates were more transparent in appearance than matched endocervical isolates.

2. The cell envelope. Gram-positive and Gram-negative bacteria have distinctly different cell envelopes. Gram-positive organisms contain a cytoplasmic membrane and a thick peptidoglycan layer which is interspersed with carbohydrate and teichoic acid. In contrast, Gram-negative organisms contain a cytoplasmic membrane, a thin peptidoglycan layer, and a lipopolysaccharide-containing outer membrane. Thus, the envelope of Gram-negative bacteria is more highly differentiated than that of Gram-positive bacteria, having an extra membrane system. The presence of this outer membrane and the thin peptidoglycan layer suggest that Gram-negative organisms employ a fundementally different strategy in coping with their environment. The outer membrane has been suggested to perform functions such as holding secreted enzymes or sequestering nutrients near the cytoplasmic membrane by trapping them in the void between the inner and outer membrane (called the periplasmic space). In addition, the outer membrane also provides a selective barrier for the cell, protecting it from toxic molecules in the environment.

The cell envelope of the gonococcus is similar in its ultrastructure to

other Gram-negative organisms. However, it differs from the enterics by its increased permeability to hydrophobic molecules (222). The cell envelopes of wild-type *E. coli* or *S. typhimurium* are relatively impermeable to hydrophobic molecules. This property is critical to their survival in the intestinal tract (270). However, there is no obvious reason for all Gram-negative organisms to possess an identical cell envelope. Considering the different ecological niches that enteric and mucosal pathogens inhabit, it would seem likely that they have evolved different strategies for ensuring their survival (258). Many of these strategies are reflected in the composition of the cell envelope.

a) The cytoplasmic membrane. The cytoplasmic membrane of *N. gonorrhoeae* has not been extensively studied. However, since the cytoplasmic membranes of other Gram-negative (and Gram-positive) organisms are integral components which function in well-conserved systems of transport and metabolism, it is generally accepted that the gonococcal inner membrane is similar to other bacterial cytoplasmic membranes. Furthermore, the lack of antigenic pressure and the protection against toxic compounds conferred by the outer membrane make bacterial cytoplasmic membranes somewhat "immune" to selective pressures and probably also contribute to their conservation. In general, the cytoplasmic membranes of Gram-negative bacteria are characteristic of biologic membranes in that they are composed of a phospholipid bilayer throughout which intrinsic and extrinsic proteins are distributed. Besides its obvious function to confine the cytosol, the cytoplasmic membrane is physiologically active, containing enzymes of the electron transport chain and other biologic activities. It also is responsible for transport of chemical entities in and out of the cytosol.

Johnston and Gotschlich (187) separated the cytoplasmic and outer membranes of N. gonorrhoeae by sucrose density centrifugation of spheroplasts. They reported the cytoplasmic membrane to have a buoyant density of 1.141 g/cm^3, a value which was similar to those reported for cytoplasmic membrane preparations of E. coli and S. typhimurium.

Miller et al. (250) analyzed the proteins of the cytoplasmic membrane and determined that a major protein with an apparent molecular weight of 24,000 was present in spectinomycin-sensitive clinical isolates of N. gonorrhoeae, but was absent in spectinomycin-resistant isolates. This protein comprised ca. 7 % of the total cytoplasmic membrane protein in the spectinomycin-sensitive strains. However, consistant loss of this protein was not observed in laboratory-derived spectinomycin-resistant gonococci. This report demonstrated a wide range of strain-specific cytoplasmic proteins. These investigators reported 21 to 25 bands visible on SDS-polyacrylamide gels after staining with Coomassie blue. Previous reports had observed only 13 protein bands localized in gonococcal cytoplasmic membranes (187, 319). The predominant peptides associated with the cytoplasmic membranes of both spectinomycin-resistant and -sensitive strains were reported to have apparent molecular weights of 59,000, 34,000, and 29,000.

b) Peptidoglycan. The peptidoglycan layer (also referred to as the cell wall) of Gram-negative bacteria is responsible for cellular shape and osmotic rigidity. Peptidoglycan of N. gonorrhoeae has been isolated and reported to comprise 1 to 2% of the cellular dry weight (147). Hebeler and Young (147) analyzed the composition of purified peptidoglycan and reported that it consisted of muramic acid, glutamic acid, alanine, meso-diaminopimelic acid, and glucosamine, in approximate molar ratios of 1:1:2:1:1, respectively. A

lipoprotein covalently attached to the peptidoglycan (analogous to that found in *E. coli*) was not observed (429). However, evidence for association of protein with peptidoglycan has been presented by Hebeler *et al.* (144, 145). The protein-peptidoglycan interactions increased when cells were grown at lower pH values. The absence of gonococcal proteins which covalently attach the peptidoglycan to the outer membrane may be responsible for the apparent loose association of peptidoglycan with the outer membrane as seen in electron micrographs of *N. gonorrhoeae* (429).

Gonococci appear to be unique among Gram-negative bacteria in that they turnover their peptidoglycan layer during exponential growth (323). Peptidoglycan turnover has been reported to occur at rates beween 10 and 50% per generation depending on the strain and growth conditions (148, 256, 419). This high turnover rate might be partly responsible for the highly autolytic nature displayed by *N. gonorrhoeae* during all stages of growth (146).

Rosenthal (320) determined that soluble peptidoglycan fragments were released into liquid medium during exponential-phase growth. Subsequent analysis determined that four forms of peptidoglycan fragments were released. These were (i) cross-linked, *bis*-disaccharide peptide dimer, (ii) uncross-linked, disaccharide peptide monomer, (iii) free peptide, and (iv) free monomer (351). Extracellular peptidoglycan fragments have been recognized as potent biologic effectors that affect host inflammatory and immune responses. Gonococcal peptidoglycan fragments have been shown to possess diverse biologic activities and to invoke host-responses. These include toxicity for human fallopian tubes in organ culture (244), consumption of human complement (297), and arthritogenicity in a rat arthritis model (324).

Vertebrate hosts contain peptidoglycan hydrolases (e.g., lysozyme) as a form of natural immunity against bacterial infection. _O_-Acetylation of gonococcal peptidoglycan renders it resistant to human peptidoglycan-hydrolases resulting in persistant high-molecular weight fragments which are efficient at mediating adverse pathologic effects (321). Extensive _O_-acetylation of peptidoglycan appears to be common among gonococci. However, a strain (RD5) known to be highly autolytic and to turnover its peptidoglycan at high rates had a greatly reduced level of peptidoglycan _O_-acetylation. Therefore, the degree of _O_-acetylation may also play a role in autolysis. Strain-dependent differences in _O_-acetylated peptidoglycan may influence the severity of gonococcal infection.

Recently, Rosenthal _et al_. (322) determined that exposure of gonococci to protein synthesis inhibitors such as chloramphenicol, tetracycline, and streptomycin, rapidly increased the level of _O_-acetylation. The authors speculated that similar conditions encountered by gonococci _in vivo_ might potentiate the pathological consequences of peptidoglycan-host interactions.

c) Outer membrane. The outer membrane of Gram-negative organisms represents the initial permeability barier. It is composed of a lipid bilayer typical of biologic membranes. However, it is unique in that it is asymmetric with respect to its lipid composition. Phospholipid predominates in the inner leaflet of this membrane while lipopolysaccharide (LPS) predominates and is exposed on the outer surface. Proteins, both extrinsic and intrinsic, also make-up an important component of the outer membrane. This structure is especially important in host-parasite interactions because of its direct exposure to the host. The outer membrane acts as the first line of bacterial defense against the specific and non-specific host immune mechanisms.

Therefore, the outer membrane is of particular importance in the infectious process of Gram-negative pathogens.

Johnston and Gotschlich (187) used isopycnic centrifugation of osmotically ruptured gonococcal spheroplasts to separate the outer membrane from the cytoplasmic membrane. The outer membrane fraction banded in sucrose at a buoyant density of 1.219 g/cm^3. This fraction contained nearly all the LPS and greater than half of the total envelope protein. These investigators concluded that the membrane components of _N. gonorrhoeae_ were similar to those of other Gram-negative bacteria. However, other investigators have reported this technique to be unsuccessful in their laboratories (357, 430). More recently, Collins and Salton (67) modified this method by the inclusion of the detergent Brij 58 to lyse the spheroplasts. These investigators reported the successful identification of antigens originating from both the inner and outer membranes of gonococci.

Other procedures have been applied for the isolation of the gonococcal outer membrane. Wolf-Watz _et al._ (429) isolated outer membranes of _N. gonorrhoeae_ by a method originally developed for _E. coli_ (430). In this procedure, spheroplasts prepared by EDTA-lysozyme treatment were passed through a 22-guage needle to shear off the outer membrane. After low-speed centrifugation, the pH of the supernatant was lowered to 5.0 to aggregate the outer membrane. Upon isopycnic sucrose density centrifugation, a single band with a buoyant density of 1.25 g/cm^3 was obtained. Johnston _et al._ (186) extracted whole gonococci with lithium acetate buffer (pH 6.0) containing 10 mM EDTA. This extract was applied to a gel filtration column and the material which eluted in the void volume was collected by isoelectric precipitation and centrifugation. The material isolated by this method was representative of

outer membrane preparations prepared by other procedures. The use of the detergent n-lauryl sarcosinate (Sarkosyl) has been shown to selectively solubilize the cytoplasmic membrane of E. coli under stringent detergent to protein ratios (115). This procedure has been applied to the gonococcus in a number of reports (135, 249, 274). However, a careful analysis of the the material isolated by this procedure has not been carried out.

(i) Lipopolysaccharide (LPS). LPS is ubiquitous among Gram-negative bacteria. The best characterized LPS molecules are those from the Enterobacteriaceae. These molecules are heat-stable long-chain phosphate-containing heteropolymers consisting of a moiety termed lipid A. Lipid A is covalently linked through 2-keto-3-deoxyoctulosonic acid (KDO) to a core polysaccharide (R-core). To this, a high-molecular weight polysaccharide made up of identical oligosaccharide repeating units (O-antigen) is attached. LPS of this basic strucure is referred to as S-type because salmonellae with this LPS composition form smooth colonies on agar. If the LPS of organisms lack the O-antigenic side chains, they give rise to rough colonies with serated edges; these LPS molecules are referred to as R-type. The structure of lipid A is well-conserved among the LPS of different genera, while the O-antigens are structurally heterogeneic (432). LPS molecules are important mediators of toxicity in Gram-negative diseases and are referred to as endotoxin. These molecules are known to stimulate a pyrogenic response as well as other toxic effects in the host (108).

LPS is located on the outer-most aspect of Gram-negative cell envelopes. It can be easily released upon mild chemical treatments of whole organisms (293). Spontaneous release of endotoxin during growth of Gram-negative bacteria is known to occur (293). The production of free endotoxin seems to

be a general phenomenon in all Gram-negative bacteria. The release of free endotoxin by N. meningitidis in a process described as "blebbing" was reported by DeVoe and Gilchrist (97). These blebs were analyzed with respect to their composition. It was determined that they were enriched for LPS (97) and contained little contamination with cytoplasmic membrane components (98). It was of interest that tetramethylphenylenediamine(TMPD)-oxidase activity was associated with these cell wall blebs. This activity is responsible for the oxidase reaction exhibited by Neisseria spp. and is a clinically important property for their identification. Free LPS has been found in culture supernatants of other species of Neisseria (184, 185).

Early studies on the endotoxin of N. gonorrhoeae used a number of extraction methods (223, 224, 390). Tauber and Garison (390) prepared endotoxin by phenol-water extraction and showed that it contained the sugars D-glucosamine, glucose, galactose, heptose, and KDO. Later, Maeland (225) compared gonococcal endotoxin prepared by both phenol-water and aqueous-ether extraction methods. This study indicated that the former consisted mainly of carbohydrate and lipid and contained much less protein than the latter. Both preparations retained an antigenic determinant which appeared to be carbohydrate in nature. The aqueous-ether extracted material contained relatively large quantities of protein and only minor amounts of lipid and carbohydrate.

Stead et al. (266) analyzed five different strains of N. gonorrhoeae for the chemical composition of their phenol-water extracted endotoxin. All LPS preparations contained glucose, galactose, glucosamine, heptose, KDO, and phosphate. The beta-hydroxy fatty acids 10:0, 12:0, 14:0, and the fatty acids 12:0, 14:0, 16:0, and 18:0, and 18:1 were present in the lipid A moiety. This

study detected no differences in the LPS composition of virulent and avirulent gonococcal colony types or penicillin-sensitive and -resistant organisms. These LPS preparations all appeared to lack repeated carbohydrate components analogous to the O-antigenic side chains of enteric species. In a similar study, Perry et al. (292) compared the composition of LPS isolated from gonococcal colony types T_1 and T_4. The LPS from T_4 colonial variants, on mild acid hydrolysis, yeilded a lipid A molecule and a core oligosaccharide that appeared to be common to all strains examined; the latter was composed of 2-amino-2-deoxy-D-glucose, D-glucose, D-galactose, L-glycero-D-manno-heptose, 3-deoxy-D-manno-octulosonic acid. LPS from T_1 colonial variants, upon mild acid hydrolysis, yeilded a lipid A molecule and a high-molecular weight core oligosaccharide which exhibited considerable differences in glycose composition between strains. These investigators suggested that T_4 organisms produce a common R-type LPS whereas T_1 colonies produce S-type LPS with strucurally heterologous O-antigenic side chains. As pointed out by Morse (256), these data must be interpreted with caution since isogenic strains differing in colonial morphology were not compared in this study.

Wiseman and Caird (428) examined the LPS composition of 38 different strains and their isogenic variants. The concentrations of glucose, galactose, and mannose varied from strain to strain, but cells from colony types T_1 and T_2 contained a greater glycose concentration than the avirulent colony types T_3 and T_4. There were higher mannose:KDO, galactose:KDO, and glucose:KDO ratios in the virulent colony types in comparison to the avirulent colony types. This finding suggested that these additional sugars comprise a larger S-type LPS. In contrast to the study of Perry et al. (292), these investigators were unable to confirm the variability

of the putative O-antigen from the virulent colonial variants.

Dienna *et al.* (100) demonstrated that immunization with R-type LPS from one strain of *N. gonorrhoeae* protected against infection by homologous and heterologous gonococcal strains in both the chicken embryo and the mouse animal models. Wallace *et al.* (405) reported that antiserum prepared against R-type LPS recognized 249 of 251 primary gonococcal isolates in a slide agglutination test. This antiserum was specific for *N. gonorrhoeae* and did not recognize other *Neisseria* spp. or organisms of different genera, with the exception of certain streptococci. Streptococcal capsular polysaccharide rich in the disaccharide lactose was responsible for this cross-reactivity. Bundle (52) coupled lactose to bovine serum albumin and used this conjugate to prepare anti-lactose antibody. This antisera was able to agglutinate *Streptococcus faecalis* and was active against LPS from *N. gonorrhoeae* in a passive hemagglutination assay. It has been suggested that antibody against lactose may be a useful component of a gonococcal vaccine (99).

Antigenic analysis of LPS has defined four immunologically distinct acidic polysaccharides from LPS extracts of *N. gonorrhoeae* (7, 8). These polysaccharides have been proposed to be analogous to the R-core of enteric LPS. Specific reactivity to each of these polysaccharides were designated as Gc_1 through Gc_4. Analysis of 163 gonococcal strains indicated that 71.2% contained one of the 4 acidic polysaccharides; 12.3% contained more than one of these polysaccharides; 16.5% were not recognized by these immunological reagents. These studies indicated that the serotypic composition of LPS from different gonococcal strains were variable and complex.

Subsequent serological analysis of gonococcal LPS has suggested that it is

composed of three distinct epitopes; (i) a serotype-specific region; (ii) a variable determinant (this was found to exist on three of the six serogroups described); and (iii) a common region (10). Monoclonal antibodies have been prepared against gonococcal LPS. One of these, designated as 3F11, recognizes the common determinant from all gonococci and cross-reacts, although to a lesser degree, with LPS from meningococci (9). Using pyocin selection, Morse and Apicella (257) determined that pyocin resistant variants lacked reactivity with 3F11. Guymon et al. (134) also reported differences in LPS structure of pyocin-resistant mutants.

The composition of gonococcal LPS may be influenced by its growth environment. Morse et al. (260) determined that growth of strain FA171 in continuous culture under glucose limitation resulted in a growth-rate-dependent change in the LPS. The LPS from cells grown at a low-dilution rate exhibited an eight-fold decrease in serotype antigen when compared to cells grown at a high-dilution rate. The decrease in LPS serotype antigen was associated with an increase in cell surface hydrophobicity. Furthermore, the increased amount of serotype antigen was associated with decreased reactivity of monoclonal 3F11 to formaldehyde-treated gonococci, suggesting that the presence of serotype antigen interferes with accessibility to the common determinate.

Use of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in conjunction with a sensitive silver stain has greatly facilitated the qualitative analysis of gonococcal LPS. Use of this technique in the analysis of neisserial LPS was first reported by Russell and Johnson (327). These investigators detected differences in electrophoretic mobilities (M_r) of LPS preparations isolated from various non-pathogenic Neisseria spp. A number of subsequent studies

have indicated that the M_r and the electrophoretic banding pattern of meningococcal and gonococcal LPS was similar to R-type _Salmonella_ mutants (68, 134, 180, 253, 341). No ladder-like repeating O-antigenic side chains (similar to those observed for enteric S-type LPS) were observed. Recently, Schneider _et al_. (341) reported that individual strains of gonococci and meningococci produce LPS which are heterogeneic with respect to their M_r and antigenic expression. These organisms may express more than one structurally-distinct type of LPS on their surface. Because there is no evidence for long, repeated side chains on gonococcal and meningococcal LPS, it has been suggested that the use of "poly" as applied to the enteric-concept of LPS is a misnomer. Rather, the term lipooligosaccharide (LOS) is thought to more accurately reflect the apparent structure of gonococcal and meningococcal LPS since "oligo" infers a short, unrepeated saccharide polymer.

(ii) Outer membrane proteins. Johnston and Gotschilch (187) analyzed the proteins associated with isolated outer membranes from _N. gonorrhoeae_ strain 2686 (T_4). Protein profiles from SDS-polyacrylamide gels stained with Coomassie blue suggested that three proteins, with apparent molecular weights of 34,500, 22,000, and 11,500, accounted for the majority of the protein. The major outer membrane protein had a molecular weight of 34,500 and made up 66% of the total outer membrane protein. Johnston _et al_. (186) used outer membrane complexes (serotype antigen) isolated by lithium-acetate extraction to assign clinical isolates into 16 distinct serotypes. SDS-PAGE analysis determined that each antigenically distinct serotype contained a major outer membrane protein with a unique subunit molecular weight. Heckels (149) used a slight modification of the lithium-acetate extraction method described by Johnston _et al_ (186). In this procedure, EDTA was omitted from the extraction

buffer because preliminary experiments showed that substantial amounts of LPS were also removed from crude cell envelopes in the presence of EDTA. This preparation was similar in protein composition to that reported by Johnston et al. (186). Treatment with SDS at 37° C revealed five membrane proteins including major bands with corresponding apparent molecular weights of 36,500 (protein I) and 24,000 (protein II). Solubilization of the identical preparations with SDS at 100° C produced similar bands except that the protein II band migrated at a higher molecular weight which corresponded to 29,500 (designated as protein II^{*}). Treatment of whole gonococci with dansyl chloride-cycloheptaamylose to label surface-exposed proteins determined that both proteins I and II (II^{*}) were labeled. Treatment of the lithium-acetate extracted outer membranes with sodium cholate readily solubilized protein II. Fractionation by gel filtration separated protein II from LPS. The insoluble fraction from sodium cholate-extracted membranes contained protein I. This protein could be solubilized with guanidine thiocyanate and purified by gel filtration in the presence of urea to give a homogeneous and antigenically reactive protein I.

A wealth of information regarding the structure, function, and architecture of the gonococcal outer membrane proteins has accumulated since the reports of Johnston and Gotschlich (187), Johnston et al. (186), and Heckels (149). The major gonococcal outer membrane proteins can be divided into three classes based upon their quantitative abundance, molecular weight, and heat-modifiable characteristics when analyzed by SDS-PAGE. The first class of proteins are the protein(s) I; previously referred to by Johnston and Gotschlich (187) as the major outer membrane protein (MOMP), by Johnston et al. (186) as serotype antigen, by Buchanan and Hildebrandt (44) as the

principle outer membrane protein (POMP), and by Heckels (149) as protein I. These proteins are usually the most abundant of the outer membrane proteins and they have a strain-dependent apparent molecular weight of between 32,000 and 36,000 and a pI of between 5.2 and 8.0. All gonococci express only one species of protein I (32).

Localization of protein(s) I to the gonococcal cell surface has been the subject of several reports. Protein(s) I are readily iodinated when whole organisms are exposed to ^{125}I in the presence of a surface-active catalyst (374, 382). Heckels (150) determined that protein(s) I could be cross-linked to peptidoglycan when whole cells were exposed to a bifunctional reagent, suggesting that protein(s) I extended all the way through the membrane. Swanson (380) determined that protein(s) I could be grouped into three different types based on their migration in SDS-polyacrylamide gels. The corresponding molecular weights of these three groups were 34,000, 33,000, and 32,000. Tryptic and chymotryptic peptide mapping determined that the 34,000 and 33,000 dalton protein(s) I were related on the basis of shared peptides. These related molecules were referred to as protein(s) IB. Protein(s) I with a 32,000 dalton subunit molecular weight appeared to have a unique peptide map and were termed protein(s) IA. Blake _et al._ (34) treated intact gonococci with proteolytic enzymes and found that protein(s) IB with subunit molecular weights of 34,000 were susceptible to proteolysis, whereas protein(s) IB with subunit molecular weights of 33,000 were less-susceptible. Protein(s) IA with subunit molecular weights of 32,000 were resistant to proteolysis. The susceptible protein(s) IB retained two membrane-associated peptides after proteolytic digestion. From this analysis a model for the orientation of protein IB molecules in the gonococcal outer membrane was deduced. This model

has protein IB inserted in the membrane as a loop-like structure. The C-terminus extends to the exterior of the organism and back into the membrane. More recently, a similar analysis of the protein(s) IA found a proteinase K-sensitive site on a surface-exposed portion of the molecule. Only one fragment remained membrane-associated after digestion, whereas two membrane-associated fragments were found when whole gonococci with protein(s) IB were exposed to proteinase K (19). Thus, it appears that proteins IA and IB have different surface orientations in the outer membrane with protein(s) IA not exhibiting the loop structure associated with protein(s) IB.

Blake and Gotschlich (31) developed a simple method for the purification of protein(s) I in reasonable quantities. This procedure involved precipitation of protein I by hexadecyltrimethylammonium bromide (CTB) at low ionic strength. Increasing the ionic strength of the buffer allowed solubilization of protein I and nucleic acids. The latter could be removed by precipitation with 20% ethanol. The protein I-containing supernatant was precipitated in 80% ethanol and solubilized in the detergent Zwittergent-3,14 (Z-3,14). This preparation was applied to an ion-exchange column, eluted with a NaCl gradient, and the protein I containing fraction further purified by gel filtration. Protein(s) I purified by this procedure have been analyzed with respect to their amino acid composition and N-terminal amino acid sequences. Protein(s) I appeared to be similar to the major outer membrane proteins from other Gram-negative organisms with respect to the latter two properties. Of interest was that purified protein(s) I eluted from gel filtration columns as trimers in association with a detergent micelle. This is in good agreement with cross-linking analysis of gonococcal outer membranes in which protein(s) I have been reported to exist as trimers (268)

Artificial planar lipid bilayers have been used to examine the functional properties of purified protein(s) I. From these data it has been determined that protein(s) I behave as a voltage-dependent porin, similar to the major outer membrane proteins of other Gram-negative bacteria (103, 126, 436). In contrast to other organisms, the gonococcal porin is anion-selective. There is evidence that each protein I molecule of the trimeric complex may have the capacity to form a pore (436).

A provocative preliminary report has been presented by Blake and Gotschlich (32) regarding the role of protein I in the pathogenesis of gonococcal infections. When whole gonococci were added to the artificial planar bilayer system, the formation of conductive pores resulted (220). This activity was the result of protein(s) I inserting into the lipid bilayer. Transfer of protein(s) I was not the result of protein I releasing from the gonococcal membrane, partitioning into the aqueous phase, and then inserting into the artificial lipid bilayer. Nor did it appear to be the result of fusion with protein I-containing membrane fragments with the bilayer. Rather, convincing evidence was presented for a vectoral transfer of the protein I molecule directly from the gonococcal membrane into the artificial lipid bilayer. This observation suggested that protein(s) I may play an active role in the *in vivo* phagocytosis of gonococci by altering the membrane potential of eukaryotic cells. These preliminary observations await further investigation.

Serologically, protein(s) I were responsible for the antigenic diversity of outer membrane complexes described by Johnston *et al*. (186). More recently, several investigators hane attempted to develop serogrouping systems based on gonococcal protein(s) I (44, 329, 330, 409). The production of monoclonal antibodies to serologically distinct protein(s) I has provided

standardized reagents for the rapid and precise serological characterization of *N. gonorrhoeae* (489). The reactivity of a bank of monoclonal antibodies in a rapid slide-coagglutination assay has led to the grouping of gonococci into several sero-variants referred to as serovars (202). This methodology has greatly facilitated our epidemiologic understanding of gonorrhea and holds great promise for future studies.

A second class of outer membrane proteins have been designated as protein(s) II. These proteins are defined as having heat-modifiable apparent molecular weights of between 24,000 and 30,000 when analyzed by SDS-PAGE. Protein(s) II are easily solubilized by deoxycholate, are readily cleaved when intact gonococci are exposed to proteases, and are surface-labeled by iodination in the presence of surface-active catalysts (379). Protein(s) II were first described by Heckels (149) and Swanson (377). Studies by the latter investigator correlated the presence of a 26,000 dalton protein with the opaque colony phenotype. Subsequent work determined that a number of proteins with apparent molecular weights varying from 24,000 to 33,000 were associated with opaque phenotypes and the property of intragonococcal aggregation (clumping). The presence of these proteins were independent of the state of piliation of the organism.

Lambden *et al*. (210) characterized five different protein II molecules from different variants derived from a single strain (P9) of *N. gonorrhoeae*. Each variant contained none, one, or more protein(s) II. Swanson (383) also reported the occurrence of at least five different protein(s) II from variants of a strain (JS3) of *N. gonorrhoeae*. The heat-modified molecular weights of this class of proteins ranged between 29,000 and 33,000. Furthermore, the migration of some of the protein(s) II in SDS-polyacrylamide gels could be

influenced by 2-mercaptoethanol. Four of the five protein(s) II were consistently associated with colonial opacity.

Blake _et al_. (34) used trypsin and chymotrypisn to cleave the protein II molecules on intact gonococci. This treatment revealed that each enzyme cut at a unique but different site on the protein II molecule. After proteolysis, only one peptide remained membrane-associated, suggesting that protein(s) II were arranged on the gonococcal cell surface with one end buried within the membrane and the other end exposed on the surface.

Swanson (381) and Swanson and Barrera (384) analyzed the relatedness of different protein II molecules. Protein(s) II were purified by cutting them from SDS-polyacrylamide gels after electrophoresis. These preparations were labeled using ^{125}I, treated with trypsin or chymotrypsin, and the peptides analyzed by a two-dimensional separation system. Analysis of the peptide maps indicated that there was a great deal of relatedness among protein(s) II. Antiserum was prepared to different protein II molecules. Whole gonococci were exposed to this antisera prior to solubilization of the membranes by a mild detergent. Immunoprecipitation indicated that reactivity with homologous antisera was specific for the protein(s) II to which the antisera had been prepared. However, when denatured protein(s) II were probed with the same antisera by immunoblotting there was extensive cross-reactivity with all protein II molecules. The authors suggested that all gonococcal protein(s) II share common antigenic determinants, but these common epitopes are not generally accessible to antibody. Furthermore, the surface-exposed determinants of different protein(s) II are generally different from one another. Judd (193) confirmed many of these observations using a similiar system for peptide mapping. This study found that _ca_. two-thirds of

chymotryptic peptides from all protein(s) II were conserved while the remaining one-third varied. In order to examine the variability of surface-exposed peptides, intact gonococci were surface labeled (instead of labeling the denatured band isolated by SDS-PAGE). Peptide maps from this analysis indicated that while some variable peptides could be localized to the surface, others did not appear to be surface-exposed. Judd (193) hypothesized that structural differences in protein(s) II occur at a discrete site(s) within the conserved protein II molecule. These alterations result in different exposures of non-variant portions of the protein II molecule on the surface of the organism. The different orientation of the conserved primary sequence could account for the observed antigenic variability associated with protein(s) II.

Blake and Gotschlich (33) developed a method for purification of protein(s) II. This method involves extraction of cells with Zwittergent-3,14 at pH 4.0 in the presence of high concentrations of $CaCl_2$. Under these conditions, protein(s) II are readily solubilized. Further purification was achieved by cation-exchange and molecular seive chromatography. The isolated protein(s) II exhibited apparent molecular weights corresponding to their heat-modifiable relative mobility, independent of the temperature of solubilization. Gel filtration analysis found that purified protein(s) II eluted as monomers in association with detergent micelles. The isoelectric points of protein(s) II isolated by this procedure varied between 9.0 and 10.0. The amino acid composition of protein II molecules supported this data since they contained a high proportion of basic amino acid residues. The N-terminal amino acid sequences of protein(s) II isolated from two different gonococcal strains (R10 and MS11) shared extensive but not perfect homology.

Stern et al. (372) studied the expression of opacity-associated proteins on the genomic level. A gene encoding for the gonococcal opacity-associated protein was cloned. This sequence was used to probe chromosomal digests from different gonococcal opacity variants. Several restriction fragments related to the cloned opacity-associated protein were determined to be dispersed throughout the genome. No intragenic chromosomal rearrangements were observed between the opacity variants, suggesting that genetic rearrangement may not play a role in the expression of protein(s) II. However, the chromosomal rearrangements may have eluded detection by these methods. The expression of two variant opacity-associated proteins appeared to eminate from the same genomic segment and were probably encoded for, in part, by identical nucleotide sequences. The investigators speculated that opacity-associated protein expression results from recombination and/or gene conversion at the site(s) of protein II expression.

Two reports have examined protein(s) II variation in vivo (346, 437). Zak et al. (437) characterized protein(s) II profiles of gonococcal strains isolated from seven groups of sexual partners. The strains isolated from individuals within each group were identical except that they expressed different protein(s) II. Antibody from each individual was specific for a particular protein(s) II repertoire which often differed from the antibody response mounted by the partner(s). Schwalbe et al. (346) analyzed 54 primary isolates from gonococcal infections which were caused by a single gonococcal strain. At least seven distinct protein(s) II were observed in these isolates. No single protein or combination of protein(s) II predominated among the isolates or were associated with site of infection. Identical gonococcal strains recovered from the same patient at different times

during the infection had different protein(s) II profiles, confirming that protein(s) II variation occurred *in vivo*. A recent description of monoclonal antibodies produced against two different protein(s) II molecules (30) should facilitate the study of this variable class of outer membrane proteins.

Protein(s) III is the third class of gonococcal outer membrane proteins. This class is defined as having an apparent molecular weight of 30,000 in the absence of a reducing agent and 31,000 in the presence of 2-mercaptoethanol. McDade and Johnston (237) first described this protein. They and others (217, 268) found that protein III could be cross-linked to protein I within membranes using bifunctional reagents. In another report, a protein III-specific monoclonal antibody was reacted with whole gonococci, followed by detergent solubilization and immunoprecipitation of antigen-antibody complexes. Analysis of the immunoprecipitate indicated that in addition to protein III, protein I was also precipitated. This observation further substantiated the hypothesis that protein III somehow associates with protein I in the gonococcal outer membrane (387). Data from surface iodination experiments and the ability of the protein III monoclonal antibody to recognize this protein on whole gonococci indicate that protein III is surface exposed. Peptide mapping of surface-labeled chymotryptic peptides of protein(s) III from 4 strains of gonococci with different protein I molecules indicated that protein(s) III were identical (191, 192). All gonococci examined contain this protein, and its apparent molecular weight on SDS-polyacrylamide gels does not vary from strain to strain. Therefore, protein III appears to be highly conserved. To date, no function has been determined for this protein.

There have been other reports of proteins associated with the gonococcal

cell envelope. For the most part, these proteins have not been studied as extensively as proteins I, II, and III. Heckels and Everson (161) described a major outer membrane protein with an apparent molecular weight of 60,000 from a fresh clinical isolate of N. gonorrhoeae. This protein was lost after repeated subculture of this isolate. The 60,000-dalton protein was purified and and found to have a pI of 5.0. However, there have been no subsequent reports confirming or further characterizing this protein. Zak et al. (437) described a surface-exposed protein with an apparent molecular weight of 43,000 which was antigenic during natural infection. This protein was present in all strains examined. Newhall et al. (269) described a high-molecular weight antigenic protein complex in the outer membrane of N. gonorrhoeae. This complex migrated with an apparent molecular weight corresponding to ca. 800,000. It could be purified by solubilization in SDS followed by gel filtration. After reduction and alkylation, this complex migrated with a subunit molecular weight of 76,000. Each macromolecular complex was composed of 10-12 subunits and each subunit had an isoelectric point of ca. 7.6. This protein complex was structurally conserved and common to all gonococci examined (140).

Cannon et al. (56) described an outer membrane antigen from N. gonorrhoeae which was recognized by a monoclonal antibody derived from mice immunized with gonococcal outer membranes. This antigen, designated as H.8, was common to all strains of gonococci (48 of 48 tested) and meningococci (25 of 25 tested) examined. The monoclonal antibody also recognized most strains of N. lactamica (4 of 5 tested) and 1 strain of N. cinerea tested. It did not react with any other of 34 commensal strains of Neisseria and Branhamella tested, with the exception of 1 strain (of 9 examined) of N. sicca. This antigen was

protease-sensitive and heat-modifiable, having an apparent molecular weight of 20,000 when solubilized at 37° C and producing bands at 20,000 and 30,000 daltons when solubilized at 100° C. The location of the H.8 reactive epitope appeared to be on the gonococcal cell surface. More recently, the H.8 antigen from N. gonorrhoeae was cloned in E. coli and the DNA sequence encoding this protein was used to probe Neisseria spp. (29). No reactivity of this DNA probe was observed with organisms that did not express an H.8 antigen, suggesting that those non-reactive Neisseria spp. did not contain a "silent" (unexpressed) gene copy encoding for this antigen.

The presence of iron-regulated proteins in the cell envelope of N. gonorrhoeae has been reported . These are described elsewhere in this review.

d) Capsule. The finding that meningococci have a polysaccharide capsule suggested that gonococci may also be encapsulated. However, the presence or absence of a gonococcal capsule has not been clearly established and capsular polysaccharide has never been isolated from N. gonorrhoeae. Freshly isolated strains of N. gonorrhoeae were reported to possess a capsule demonstrable by light microscopy using India ink and also by electron microscopy of cells exposed to hyperimmune sera (163). Both P^+ and P^- colony types produced capsules (177, 314). Capsules were observed most consistently on recent clinical isolates but were also evident on laboratory-grown organisms. Mild shearing easily removed the capsular material (177). Passage of gonococci in guinea pig subcutaneous chambers enhanced capsular production (93). Synthesis of capsular material in vivo is apparently dependent upon the presence of undefined medium components and logarithmic-phase growth (163). Viridans streptococci stimulated capsule production by gonococci grown on glucose-containing agar medium (177). However, it is not known whether the

streptococci produce a factor which stimulates capsular production, removes chemicals, or otherwise alters the growth medium in order to favor capsular synthesis. Surface polyphosphates have been found in all gonococci and it has been suggested that these may function as a gonococcal capsule (271).

Evidence against the presence of a capsule in N. gonorrhoeae has also been obtained. A method of India ink staining of baterial capsules was employed by Melly et al. (243) followed by visualization using electron microscopy. Capsules were demonstrated for Streptococcus pneumonia and Staphylococcus aureus using this method. However, no true capsules were observed on N. gonorrhoeae grown in vitro or obtained from human urethral exudates. Wheat germ agglutinin, which has a specificty for N-acetyl glucosamine, agglutinates non-capsulate Neisseria spp. by recognizing a sugar residue present on the LPS. This lectin agglutinated strains of N. meningitidis known to be unencapsulated, but failed to agglutinate encapsulated strains of meningococci (338). In contrast, all gonococcal strains were strongly agglutinated by this lectin. Since there was no apparent resistance to agglutination this observation was taken as further evidence against a gonococcal capsule.

3. Envelope permeability. Wolf-Watz et al. (429) suggested that the gonococcal outer membrane was more permeable to hydrophobic compounds than outer membrane of wild-type enteric organisms. Crystal violet, a hydrophobic dye, diffused as rapidly into gonococci as deep rough mutants of E. coli. The low-permeability of wild-type E. coli and S. typhimurium to hydrophobic compounds confers a selective advantage to these organisms by rendering them resistant to the bile salts and the long-chain fatty acids that are abundant in the intestinal tract (270). Relative to the enterics, mucosal pathogens like N. gonorrhoeae, N. meningitidis, Haemophilus influenzae, and Bordetella

pertussis are all sensitive to low-concentrations of the hydrophobic antibiotic erythromycin. Therefore, permeability to hydrophobic molecules may bestow some type of selective advantage for the survival of these organisms on mucosal surfaces.

Gonococci are also extremely sensitive to other hydrophobic agents such as rifampin, acridine orange, ethidium bromide (229), gonadal steroids (118, 221, 252), and fatty acids (251). Since gonococcal LPS appears to resemble the R-type LPS of enteric mutants, it seems likely that susceptibility to hydrophobic compounds results, in part, from the absence of hydrophilic O-antigenic side chains. In addition, accesibility of hydrophobic molecules to phospholipid-rich regions exposed on the surface of gononococci may also contribute to their hypersensitivity to hydrophobic compounds (222).

Genetic studies have proved useful in studying the permeability of the gonococcal cell envelope. A number of genetic loci have been identified which confer low-level antibiotic resistance or susceptibility of gonococci to various antibacterial compounds (363). Mutations in many of these loci exert their effect by altering the target site of the antimicrobial. Others, however, are nonspecific and are thought to act by limiting the permeability of antimicrobial compounds. Several studies (228, 332, 334, 364) have reported genetic loci associated with this apparent permeability phenomenon. Three phenotypes designated Mtr, Env, and Pen have been identified. Strains possessing the Mtr phenotype are characterized by increased resistance to hydrophobic dyes, detergents, and antibiotics. The genetic loci designated as *pen* contribute to increased resistance to the hydrophilic molecule penicillin G (these encode for both altered target site and permeability functions). The *env* loci are responsible for hypersensitivity to hydrophobic molecules.

Presence of the env loci in strains supressess the resistance conferred by the mtr-1, mtr-2, and pen-B loci. The isogenic gonococcal variants BR87 (Env) and FA171 (Mtr) have been prepared by mutagenesis and transformation and presumably differ from their parental strain, FA19 (Wild-type), only in their sensitivity to hydrophobic molecules. These strains have been used to investigate mechanisms of membrane permeability. Lysko and Morse (222) analyzed the exposure of phospholipid head groups on the surface of these three variants. Phospholipids of strain BR87 (Env) were susceptible to digestion by phospholipase-C, whereas those from FA19 (wild-type) and FA171 (Mtr) were not. However, when FA19 and FA171 were pretreated with mixed exoglycosidases, their phospholipid head groups became susceptible to phospholipase-C. This suggested that a glycosylated component was present on strains that were less-permeable to hydrophobic molecules.

Guymon et al. (135) used lysozyme digests of purified peptidoglycan to determine the relative degree of peptidoglycan cross-linking present in the phenotypes described above. A 32% increase in the extent of peptidoglycan cross-linking was associated with variants containing the mtr locus compared to the wild-type phenotype. No differences in rates of peptidoglycan turnover or in the LPS and phospholipid composition of variants exhibiting the Mtr, Env, or Wild-type phenotypes were detected. Acquisition of the mtr locus was accompanied by a seven-fold increase in the amount of a minor 52,000 dalton protein in the cell envelope. This analysis also examined the effect of the pen B locus which confered increased resistance to hydrophilic antibiotics like penicillin. An isogenic variant containing this locus possessed a protein I with a higher molecular weight than the parental strain.

Hydrophilic molecules diffuse through the aqueous channels (porins) of the outer membrane. Therefore, the resistance to penicillin conferred by this genetic locus may be mediated by a new protein I molecule which is more restrictive with respect to the permeability of penicillin.

Gonococcal strains exhibiting the Mtr and Env phenotypes have been isolated from natural infections, suggesting that these phenotypes occur in nature (107, 259). A significant proportion (ca. 15%) of clinical isolates were found to contain env loci. Most of these Env strains also contained a phenotypically suppressed mtr locus (107). Morse et al. (259) showed that a greater proportion of strains isolated from the rectum and urethra of homosexual males contained the Mtr phenotype when compared to matched isolates from the rectum and urethra of heterosexual females or the urethra of heterosexual males. Strains expressing the Mtr phenotype were more resistant to growth inhibition by fecal lipids than were non-Mtr strains. McFarland et al. (238) determined that physiologic levels of long-chain free fatty acids in the feces were inhibitory for gonococcal isolates; strains expressing the Mtr phenotype were inhibited the least. Sensitivity to fecal lipids was greatest in strains displaying an Env phenotype. However, envelope phenotype was not the only important factor in determining sensitivity to fecal lipids. AHU^- strains uniformly exhibited a wild-type resistance to hydrophobic compounds but were as sensitive to fecal lipids as Env strains. Gonococcal strains containing the Mtr phenotype would be more resistant to the adverse environment encountered in the rectum and thus the predominance of this phenotype among homosexual males is logical. However, the selective advantage conferred by the env locus in the host environment is not clear.

C. GENETICS

The chromosome of the gononococcus is composed of *ca.* 1.5×10^3 kilobases with a corresponding molecular weight of *ca.* 9.8×10^2 megadaltons (200). This represents a coding capacity of some 1000 to 5000 genes. Relative to the genomic size of *Salmonella typhimurium*, the size of the gonococcal genome is small; containing only 35% of the chromosomal content compared to *S. typhimurium*. The small relative size of the gonococcal genome suggests that it may be limited in its genetic potential. The lower DNA content may be typical of pathogenic organisms which are highly adapted to growth in a specific environment (256).

Studies employing thermal renaturation techniques to analyze DNA hybridization have indicated that all members of the genus *Neisseria* are closely related; each member hybridizing with others at levels which represent greater than 62% homology. *N. meningitidis* and *N. gonorrhoeae* were determined to be very closely related, hybridizing with each other at a value of 93% (165). More restrictive analyses have reported lower but similar values (109). The high-degree of relatedness between meningococci and gonococci is interesting in light of the fact that these are the only two members of the genus *Neisseria* which are considered pathogenic for man. Part of the related DNA sequences may reflect conserved mechanisms of pathogenesis and virulence.

Gonococci have two mechanisms for the exchange of genetic information, transformation and conjugation. Genetic exchange by transduction does not appear to occur since no viruses which infect gonococci have been identified. It has been suggested that gonococcal immunity to transduction may be due to

an efficient restriction system which prevents viral replication (386). The presence of 3 site-specific restriction endonucleases NgoI, NgoII, and NgoIII, have been identified and characterized. From the recognition sites of these enzymes it can be predicted that cytosine-methylation probably occurs (386). Adenosine-methylation has also been reported for some but not all gonococcal strains (386).

Experiments have analyzed the DNA repair systems utilized by N. gonorrhoeae. Gonococci were readily mutagenized by ethylmethanesulfonate and nitrosoguanidine, both direct mutagens which cause mutations by mispairing mechanisms. However, mutagenesis with UV-irradiation was not an effective means for inducing gonococcal mutations. Gonococci appear to have an active excision repair system which acts on pyrimidine dimers (55). UV-irradiation may act by inducing error-prone post-replication repair systems. Since gonococci were not mutagenized by UV-irradiation (but can repair photoactivated pyrimidine dimers), it has been suggested that these organisms lack an error-prone repair system (54).

1. Transformation. Transformation of Neisseria spp. was first reported by Alexander and Redman in 1953 (4) but was not observed for gonococci until 1966 (361). The ease by which gonococci can be genetically transformed with naked DNA is unusual among microoganisms. Freshly isolated gonococcal strains are nearly always transformable (57, 361). Competence for genetic transformation is significantly higher (10^3-fold) for P^+ cells than for isogenic P^- variants (361). P^+ variants have been reported to take up transforming DNA into a DNase-resistant state, whereas P^- cells could not (27, 102). Non-piliated cells treated with EDTA and lysozyme to increase cell envelope permeability showed only a moderate increase in transforming

competence, however this treatment resulted in a marked decrease in viability (27). It has been estimated that 35 to 100% of piliated cells are competent for transformation. Competence of piliated organisms is expressed constitutively throughout all phases of growth. Phenotypic expression of competence *in vitro* requires only a utilizable energy source and cations, preferably divalent cations such as Mg^{2+} and Ca^{2+} (27).

Since competence of gonococci is lost coincident with pilation, the possibility that pili may play a direct role in transformation has been suggested. Sparling *et al*. (362) investigated whether pili served as receptors of DNA during transformation. Neutral sucrose gradients were used to isolate bound DNA-pili complexes. No binding between ^{3}H-DNA and purified unlabeled pili, or between unlabeled DNA and ^{125}I-pili could be demonstrated. However, the disruption of pili by high concentrations of sucrose (39) make these experiments difficult to interpret. Mathis and Scocca (231) also have presented evidence against the direct role of pili in gonococcal transformation. Addition of anti-pilus antibodies did not inhibit the frequency of gonococcal transformation. Binding of labeled DNA to specific gonococcal proteins which were separated by SDS-PAGE and transferred to nitrocellulose paper could be detected, but none of these proteins corresponded to the gonococcal pilin subunit. To date no evidence is available to support a role for pili in gonococcal transformation. It is equally possible that the expression of pili merely reflects a complicated phenotype that includes competence for transformation (58).

Dougherty *et al*. (102) hypothesized that two types of receptors which bound transforming DNA were present on the surface of competent gonococci. Homologous (gonococcal) and heterologous (non-gonococcal) DNA absorbed to

receptor 1 and was accessible to external deoxyribonuclease (DNase). Receptor 2 bound homologous DNA or DNA isolated from taxonomically related species, only a portion of which became resistant to DNase treatment. Homologous, but not heterologous DNA, could compete with gonococcal DNA for uptake. DNA uptake by competent gonococci was followed by a period of 20 min of relative inability to be transformed by another molecule of transforming DNA. The presence of the DNA binding sites on gonococci remain speculative and await identification and characterization.

There appear to be obvious similarities between the gonococcal system of transformation and the well-characterized mechanism of transformation by *Haemophillus*. Transforming DNA is taken up and remains as double stranded molecules until shortly before recombination with the recipient chromosome in both organisms (27). Competent gonococci, like *Haemophillus*, preferentially take-up homologous DNA (125). In *Haemophillus*, uptake of homologous DNA is facilitated by an 11 base pair sequence distributed throughout the genome. This sequence is recognized by a cellular receptor and accounts for the specificity of DNA uptake (355). If gonococci possess a similar sequence for transforming DNA, it is apparently different from the *Haemophillus* sequence as DNA from *Haemophillus* does not inhibit uptake of gonococcal DNA by competent gonococci(125).

Uptake of plasmid DNA by gonococci is inefficient (360). However, transformation efficiencies are increased about 10-fold in hybrid plasmids containing gonococcal DNA, probably because they contain the necessary DNA sequences for efficient DNA uptake (125). Plasmids recovered from transformants usually suffer deletions unless the recipient strain contains a homologous plasmid; the presence of a homologous plasmid markedly increases

plasmid transformation efficiencies (26).

The *in vivo* potential for gonococcal transformation has been investigated. Sarubbi and Sparling (333) demonstrated that genetic exchange took place when two strains of *N. gonorrhoeae* carrying different antibiotic resistance markers were co-cultivated *in vitro*. The direction of gene transfer was dependent on the colony types of the two strains. It is likely that transformation may represent the principle means of exchange of chromosomal DNA between gonococci in nature (125). Transformation might also represent a means of genetic exchange between gonococci and other *Neisseria*. Sparling *et al*. (362) observed that intraspecific transformation of gonococci and other taxonomically related organisms could occur *in vitro*. Intraspecific transformation frequencies were quite high for certain genetic loci between the meningococcus and the gonococcus. Ison *et al*. (172) recently demonstrated that meningococcal DNA could transform gonococci for certain characteristics commonly used to differentiate these organisms, including the ability to utilize maltose. Thus, transformation *in vivo* may theoretically produce intraspecies hybrids that would pose problems for diagnostic laboratories, as well as provide a means of selection for more virulent or antibiotic-resistant strains.

Genetic transformation has been used to map a number of chromosomal antibiotic resistance genes in *N. gonorrhoeae* (362). Resistance to a variety of antibacterial agents was found to be controlled by several genetic loci which fell into three linkage groups (227, 228). Group I comprised linked ribosomal markers, as well as loci affecting the cell surface of gonococci. Resistance to streptomycin, tetracycline, chloramphenicol, erythromycin, rifampin, and spectinomycin have genetic loci within this group. In addition,

loci responsible for resistance to killing by normal human serum (serum antibody complement, sac) and loci which encode for a new membrane protein (nmp; refers to protein I) also cluster in this region (58). The genotypically identical envelope permeability mutations (mtr) studied by Maier et al. (228) and Sparing et al. (364) were not linked to any other known genetic loci and comprise group II. Group III contain a locus (pen) which specifies resistance to relatively high levels of penicillin. Genetic transformation has also been used, but less extensively, for the mapping of nutritional markers.

2. Gonococcal plasmids and conjugation. The presence of covalently closed circular DNA in N. gonorrhoeae was first described by Manness and Sparling (229) and by Engelkirk and Schoenhard (111). While these studies confirmed the presence of plasmid DNA in the gonococcus, the first biochemical characterization of these plasmids were not reported until later by Mayer et al. (234). In this report a 2.6 megadalton plasmid was found in five of six gonococcal isolates. This plasmid was present in the gonococcus as a multicopy pool of 24 to 32 copies per chromosomal equivalent. Other studies reported the occurence of similar plasmids in gonococcal strains (109). However, no phenotypic properties were identified for this plasmid (373). Therefore, it has been referred to as the phenotypically "cryptic" 2.6 megadalton plasmid.

Stiffler et al. (373) reported the presence of a large 24.5 megadalton plasmid in addition to the phenotypically cryptic 2.6 megadalton plasmid in a penicillin-resistant and a penicillin-sensitive sensitive isolate. There was no apparent correlation between the presence of this plasmid and resistance to chloramphenicol and tetracycline, or to the amino acid requirements of the

particular strain examined. Elwell and Falkow (109) found a similar plasmid in only 1 of 12 isolates examined These investigators noted that the large mass of this plasmid was similar to plasmids of the enteric species which encoded for genes mediating conjugation.

Elwell and Falkow (109) speculated that the presence of a plasmid gene pool in N. gonorrhoeae coupled with the emergence of R-plasmids which mediated penicillin-resistance in H. influenzae would make gonococci a prime candidate to acquire plasmid-mediated resistance. Shortly thereafter, simultaneous reports documented the isolation of beta-lactamase producing gonococci throughout the world (15, 291, 298, 299). Eisenstein et al. (106) demonstrated that beta-lactamase producing gonococcal strains contained a small plasmid, in addition to the cryptic 2.6 megadalton plasmid. Penicillinase-producing gonococcal strains originating in the Far East carried a 4.4 megadalton plasmid while strains originating in England carried a 3.2 megadalton plasmid. In addition, 50% of the strains carrying the 4.4 megadalton plasmid also found to harbored the 24.5 megadalton plasmid, while none of the strains carrying the 3.2 megadalton plasmid contained the larger plasmid.

Elwell et al. (110) determined that the structural gene for beta-lactamase production resided on the 4.4 megadalton plasmid. The substrate profile, molecular weight, and isoelectric point of the gonococcal penicillinase produced by both the 4.4 and 3.2 megadalton plasmids were similar to the TEM-1 beta-lactamase produced by many Gram-negative bacilli (21). In E. coli the gene specifying TEM beta-lactamase production resides upon a common 3.6 megadalton sequence of plasmid DNA (TnA, later called Tn2). This sequence has the ability to translocate from one replicon to another, similar to insertion

sequences in *E. coli*. This property has probably been responsible for the wide distribution of the TnA sequence among different R-plasmids (162). Roberts *et al*. (315) determined that both gonococcal R-plasmids (3.2 and 4.4 megadaltons) hybridized with *ca*. 40% of the TnA sequence. Furthermore, both gonococcal plasmids shared *ca*. 70% sequence homology with a 4.1 megadalton beta-lactamase producing plasmid (RSF0880) found in *H. influenzae*. These investigators suggested that the gonococcal R-plasmids were an extension of the enteric R-plasmid pool. However, the means by which the R-plasmids were acquired by *N. gonorrhoeae* remains speculative.

Conjugal gene transfer was described by several groups shortly after the discovery of the gonococcal R-plasmids (18, 106, 201, 315, 316, 317). Gonococcal conjugation requires the presence of the large 24.5 megadalton plasmid. This plasmid can mediate transfer of itself or the R-plasmids. Roberts *et al*. (315) observed that in the absence of selective pressure, the R-plasmid of clinical gonococcal isolates containing the conjugative 24.5 megadalton plasmid appeared to be maintained *in vitro* at a higher level than clinical isolates which did not carry the conjugative plasmid. Thus, the 24.5 megadalton conjugative plasmid may play an important role in maintaining the R-plasmid in nature.

A report that the 24.5 megadalton plasmid could mediate transfer of chromosomal genes (317) was met with enthusiasm, but could not be confirmed in subsequent studies (25, 272, 371). The recombinants observed in this report which were thought to be the result of conjugal chromosomal transfer probably resulted from low-frequency transformation between gonococci (58). If the chromosome of *N. gonorrhoeae* could be mobilized, a genomic mapping system might be developed which could facilitate the understanding of the

pathobiology of gonococci.

Recently, Stein *et al.* (370) characterized a chimeric beta-lactamase plasmid which could function both in *E. coli* and *N. gonorrhoeae*. This plasmid was a fusion product of the gonococcal 4.4 megadalton penicillinase plasmid and the 2.6 megadalton cryptic plasmid. This hybrid contained several unique restriction sites, a selectable penicillinase-producing phenotype, and it readily transformed *E. coli*. However, the frequency of transformation of gonococci was very low unless this vector contained a gonococcal DNA insertion (58). In lieu of an apparent system of chromosomal conjugation, use of this or similar shuttle vectors could give information about the gonococcus analogous to that obtained from conjugation in other organisms. Towards this end, Stein *et al.* (369) have cloned gonococcal DNA into this shuttle vector and transformed a strain of *E. coli* deficient in proline biosynthesis (*pro* AB). Transformants were selected by their ability to complement the *E. coli* proline biosynthetic lesion. Characterization of two transformant plasmids indicated that they shared a common 3.2 kilobase piece of DNA. Further restriction and subcloning of this fragment identified clones which corrected the *pro* A lesion of *E. coli*; similar analysis obtained clones which corrected the *pro* B loci. Subsequent transformation of *N. gonorrhoeae* strain F62 which is a proline requiring strain, indicated that it could be corrected to auxotrophy by the plasmid which contained the *pro* A sequence. Similar approaches in the future may help to unravel the genetic basis for gonococcal pathogenicity.

D. IMMUNOPATHOLOGY OF GONOCOCCAL INFECTIONS

1. Adherence. The transmission of gonorrhea typically relies on the deposition of organisms on genital, rectal, or pharyngeal mucosal surfaces. These surfaces represent a hostile environment employing both immune and non-immune mechanisms in order to antagonize the initiation of a microbial infection. A critical factor for the adaptation of life in such an environment is a mechanism of attachment to a surface which is continuously being washed by flows of mucous and other secretions (416). Mucosal cells taken from the male urethra of patients suffering with early symptoms of gonorrhea have been analyzed by electron microscopy. These studies indicated that gonococci attached to the surface of epithelial cells (410). Similarly, Evans (113) observed that gonococci firmly attached to the stratified squamous epithelium (but not to mucous secreting columnar epithelium) when preparations of cervical biopsies from patients presenting with gonorrhea were analyzed by electron microscopy. Thus, gonococci appear to employ attachment as an initial step in the establishment of an infection.

a) Pili and their role in attachment. The proteinaceous appendages which extend from the gonococcal cell surface were first reported by Jephcott *et al.* (181) and Swanson *et al.* (385). Pili are associated with virulent colonial variants of gonococci. *In vitro* studies have determined that piliated gonococci readily attach to human erythrocytes (45) and spermatazoa (173), tissue culture cell lines derived from epithelial cells (376), and to human fallopian tubes in organ culture (411). Purified pili attach to human buccal and vaginal mucosal cells (290). The eukaryotic pilus receptor has not been

fully characterized. Pretreatment of buccal epithelial cells with a mixture of neuraminidase and exoglycosidase inhibits binding of both piliated gonococci (46) and labeled purified pili (397), suggesting that the buccal cell receptor may be a carbohydrate. Antibody directed against pili in urogenital secretions inhibits attachment of piliated gonococci to epithelial cells (395). Thus, gonococcal pili are considered the primary mediators of adherence to mucosal cells.

Gonococcal pili exhibit a tremendous range of serologic diversity. Pili are immunogenic during natural infection (39, 52, 348, 394). Host humoral and secretory antibody develops to both common and type-specific antigenic determinants (43, 277). Brinton _et al_. (39) prepared antisera to pili from 37 different gonococcal isolates. Cross-reactivity of each antisera to the different pili preparations was analyzed by an ELISA. Cross-reactivity between different pili preparations ranged from 5 to 15%. This observation exemplifies the serologic diversity exhibited by gonococcal pili. This is certainly an important strategy used by the gonococcus to evade the host immune response.

Each pilus filament is composed of up to 10,000 pilin subunits which polymerize to form a linear structure _ca_. 6 nm in diameter and 1,000 to 4,000 nm in length (385). Pili purified from different gonococcal strains possess different subunit molecular weights (ranging between 17,500 and 21,000) as well as different isoelectric points and buoyant densities (328). Similar differences have been observed for pilin isolated from isogenic variants of the same strain (211). The amino acid composition of pilin isolated from several different strains has been determined and shown to be similar but not identical (164, 318). The N-terminal amino acid sequence has been reported to

be identical through the 59th residue of pilin isolated from two isogenic opacity variants (345); and in another study, through the 29th residue of pilin isolated from four different strains (164). Of the first 20 N-terminal residues, 18 are hydrophobic. The N-terminal amino acid present for all pilin is an unusual residue, N-methylphenylalanine. It is interesting that pilin from *Moraxalla nonliquefaciens* and *Pseudomonas aeruginosa* exhibits almost identical sequence homology with the gonococcal pilin N-terminal 20 amino acids, including N-methylphenylalanine. No such homology is apparent with the N-terminal amino acid sequence from the common *E. coli* pili (345). Tryptic peptide maps from two gonococcal pilus preparations with 5% shared antigenicity and different subunit molecular weights have been analyzed in order to determine structural relatedness. Of the 22 peptides resolved by this system only 14 were identical (345). The similarities and differences in pilin primary structure substantiates the serologic evidence suggesting that pili contain both common and unique determinants.

In an interesting preliminary report, Schoolnik *et al.* (345) used a hemagglutination assay to assess the adherence function of pili purified from different gonococcal strains and different opacity variants from the same strain. In this assay, the capacity of pili to agglutinate human erythrocytes was used as an index of their ability to adhere to these cells. Pili isolated from different strains and different opacity variants of the same strain agglutinated erythrocytes with markedly different efficiencies. Thus, functional heterogeneity, in addition to structural and serologic heterogeneity, is also a property of gonococcal pili.

Pili from gonococci were cleaved at their methionine residues with cyanogen bromide to produce 3 fragments (345). These fragments were

designated CNBr-1 through 3, and respectively comprised residues 1-7 (N-terminus), 8- ca. 84, and ca. 84-160 (C-terminus). Competition of these fragments with whole pili in the hemagglutination assay elucidated several significant properties of gonococcal pili. These included the following: (1) the CNBr-2 fragment encompassed the receptor-binding domain which determined adherence function; (2) the CNBr-2 fragments of pili differing in their efficiencies for agglutinating erythrocytes, recognized the same receptor; (3) each pilin subunit of a fibril contained a receptor-binding domain which, if accessible in quarternary structure, contributed to the pili macromolecule and formed a polyvalent ligand with a linear array of binding regions along its longitudinal axis; (4) N-terminal analysis, tryptic maps, and amino acid compositions of the CNBr-2 fragments suggested that this domain was highly conserved among preparations of heterologous pili; and (5) CNBr-3 was not involved in the pili adherence function. Therefore, the functional properties attributable to gonococcal pili were proposed to reside within the CNBr-2 fragment.

Antibody against serologically diverse pilus preparations and against their respective CNBr-2 and CNBr-3 fragments were prepared. Anti-CNBr-2 antisera reacted with the CNBr-2 fragment to which it had been prepared and showed extensive cross-reactivity with the CNBr-2 fragments generated from serologically diverse pili as well as with the heterologous uncleaved and undenatured pili. In contrast, anti-CNBr-3 antibody bound only the pilus antigen from which the CNBr-3 fragment was prepared. Peptide maps of different CNBr-3 fragments suggested that this fragment is structurally unconserved. Furthermore, when the intrachain disulfide bond residing in the

CNBr-3 fragment was reduced and alkylated, the type-specific antigenic determinant was destroyed, suggesting that the tertiary structure contributed by this disulfide loop was important for type-specific epitope formation.

From this work, Schoolnik et al. (345) have proposed that gonococcal pili are composed of a functional and antigenic domains. The functional domain resides within the N-terminal half of the the molecule, is conserved among serologically diverse pili, and represents an immunorecessive region when entire pili are used as an immunogen. This region is antigenic but not immunogenic. The C-terminal end of the pilin molecule accounts for the type-specific diversity of different pili preparations and is immunodominant with respect to host response. This domain is structurally diverse between different pili preparations.

More recent reports by Schoolnik et al. (343) and Rothbard et al. (325) have presented a more detailed analysis of the work described above. Using human endometrial carcinoma cell monolayers, Rothbard et al. (326) have shown that antisera prepared against a common peptide from CNBr-2 blocked adherence, suggesting that the receptor binding domain for this cell type is also encompassed in this conserved pilin sequence. These studies have supported the preliminary observations reported by Schoolnik et al. (345). The implications of the studies described above are potentially exciting in terms of prevention of gonorrhea by immunoprophylaxis. Despite biologic evidence suggesting that gonococcal pili might be protective, the serologic diversity of pili has hindered the reality of using it as a component of a gonococcal vaccine. The use of an immunogenic common peptide analogous to CNBr-2 may be useful in overcomming the problem of antigenic diversity. However, it should

be noted that not all studies support these conclusions. Using buccal mucosal cells and Chang conjunctival epithelial cells, Virji and Heckels (403) have demonstrated that binding is blocked by type-specific but not cross-reacting monoclonal pilus antibody. This report may indicate that the interaction of pili and eukaryotic cells may be much more complex than suggested by Schoolnik and coworkers.

A genetic system must exist in the gonococcus to account for the intragenic and intergenic structural heterogeneity of pili and for the regulation of expression. Meyer *et al*. (246) have cloned a pilin structural gene. Using this sequence as a probe, a chromosomal rearrangement was correlated with a conversion of gonococci from the P^+ to the P^- state. Several copies of pilin gene sequences were found to be dispersed throughout the gonococcal genome, most of which were "silent" (i.e., non-expressing) loci. Two regions of the chromosome were identified as expression loci for pilin synthesis such that pilin sequences inserted within this site were expressed. More recently, Hagblom *et al*. (136) have determined the DNA sequence of pili expressed from different derivatives of a single strain. These derivatives were derived by picking independent P^- variants from a parental P^+ strain. Each isolate represented the start of a line in which alternating P^+ and P^- variants were then selected. Within each line of derivatives, switching from the P^- to the P^+ state resulted in either the expression of a new variant or the re-expression of a former variant. Since re-expression of a former variant was observed, pilin information is not lost during phase variation or antigenic switching. The C-terminal portion of the pili DNA sequence (encoding amino acid residues from *ca*. 50 to the end of the

pilus gene) is composed of a semivariable and a hypervariable region. The semivariable region (residues 54-114) exhibited many amino acid substitutions between variant pilin sequences. The carboxy-terminal portion of the molecule was highly variable; in addition to amino acid substitutions, in-frame insertions of one to four codons occurred. Two regions, 10 and 13 amino acids in length respectively, bracketed a hypervariable segment in the C-terminal half of the pilin sequence and were always conserved; each centered around one of the two cysteine residues of the pilin molecule. The switch from P^+ to P^- involved the deletion of pilin sequences from one of the two expression loci (245). However, pilin expression may also involve a switch-induced regulator as non-piliated gonococci can have a functional pilin in the expression loci. These data strongly suggest that antigenic variation results from recombinational mixing between the hypervariable and semivariable regions of the many silent pilin gene copies. The exact relationship between the genomic rearrangements and expression of pilin genes awaits further analysis.

b) Non-piliated mechanisms of attachment. The observation by Novotny *et al.* (277) that pili are rarely seen when gonococci in urethral exudate are examined by electron microscopy suggest that pili may not be an absolute virulence determinant *in vivo*. Non-piliated mechanisms of gonococcal attachment have not been pursued to the same degree as pili-mediated attachment; however, some studies have approached this issue. Swanson *et al.* (388) and King and Swanson (199) have demonstrated that P^- gonococci can exhibit either higher or lower levels of association with human PMNs than isogenic P^+ variants. The factor responsible for this association was termed leukocyte-association factor. The molecular entity responsible for

this property was determined to be a protein exhibiting a strain-dependent molecular weight (28,000 and 29,000). This protein was localized to the gonococcal cell surface and was termed leukocyte association protein (LAP). Other investigators reported that a decreased association with human PMNs was associated with the presence of one or more variable proteins with molecular weights similar to the LAP (416). The proteins described in both of these reports can be classified as protein(s) II.

James et al. (176) have determined that opaque gonococcal colony variants attach better to human cells in tissue culture than do isogenic transparent variants. Protein(s) II (other than LAP) were associated with the ability to promote attachment to various types of eukaryotic cells (210, 388). Lambden et al. (210) observed differences in adherence to eukaryotic cells depending upon the protein(s) II composition of isogenic gonococcal variants. Some variants attached better to buccal cells, whereas others adhered more efficiently to erythrocytes. Similar differences were noted with respect to attachment to polymorphonuclear leukocytes.. Recently, a protein II-specific monoclonal antibody was shown to inhibit attachment of a gonococcal strain expressing a cross-reactive protein II (375). Thus, protein(s) II may also be important mediators of adherence.

The surface of mucosal cells have an overall net negative charge. N. gonorrhoeae, like other Gram-negative bacteria, also has a net-negative charge. Thus, there is an apparent overall repulsive force between gonococci and the mucosal surface which must be overcome before attachment can occur. Heckels et al. (160) chemically modified the charge of the gonococcal cell surface and found that pili only facilitated attachment when gonococci bore a

net-negative charge. It has been suggested that pili facilitate attachment by initially overcoming the repulsive electrostatic barrier which exists between gonococci and the host cell. The role of protein(s) II as well as other gonococcal ligands may be particularly important after the "long-range" repulsive forces have been overcome by pili.

2. Invasion. Healthy mucosal surfaces of the body are covered with adherent commensal bacteria which comprise the hosts normal flora. Thus, after attachment the critical determinant of pathogenicity must be the ability to penetrate the epithelial cell lining (415). Harkness (142) summarized data showing that by the third day of acute infections, gonococci appeared to penetrate mucosal surfaces and multiply within the subepithelial connective tissues. Other studies have observed that urethral epithelial cells (including mucous-secreting cells) and endocervical cells from individuals with gonorrhea appear to be "studded" with gonococci on the periphery of the mucosal cell (386). A more thorough understanding of how gonococci invade has been obtained by observing infected human fallopian tubes in organ culture. Using this model, the initial steps in the course of gonococcal infection have been delineated (239). These steps are identified as (i) attachment, (ii) phagocytosis, (iii) transport of gonococci to the base of the cell, and (iv) exocytosis into the subepithelial tissues.

Shortly after infection, gonococci attach to the tips of microvilli of non-ciliated cells. Attachment of gonococci is dependent on surface components such as pili, outer membrane proteins, and other undefined exposed molecular entities (241). Gonococci exhibit a tropism for human fallopian tube mucosa in comparison to oviduct mucosa of guinea pigs, rabbits, cows, and

pigs (182). In the human fallopian tube organ culture model, attachment increased for _ca_. 24 h. During this period there appeared to be a 'sloughing' of large numbers of predominantly ciliated cells. P^+ colonial variants attached and damaged the mucosa more rapidly than P^- organisms (240). Supernatants of infected organ cultures have been shown to contain toxic activity (183) and evidence suggested that LPS accounted for most of the toxicity (127). Gonococcal peptidoglycan fragments also appeared to be toxic in this model (244).

After infection has been allowed to proceed for _ca_. 20 h, microvilli to which gonococci were attached retract, pulling the organisms to the host mucosal cell surface. The organisms are phagocytized and gonococci-containing vesicles are transported rapidly to the base of the mucosal cell. While pili facilitated attachment, they were not a critical determinant of invasiveness (415). Once gonococci are internalized within these vesicles they may multiply, the vesicles may enlarge, and some can be observed fusing with each other. At _ca_. 40 h post-infection, gonococci were exocytosed into subepithelial tissues where abscesses eventually formed. Electron microscopic evidence and experiments which employ intracellular protection to killing by antibiotics suggested that gonococci may remain viable during phagocytosis and exocytosis by non-professional phagocytic mucosal cells. Non-pathogenic _Neisseria_ species did not invade mucosal cells in this model. The validity of the human fallopian tube model as an accurate reflection of the disease process _in vivo_ must be considered when evaluating whether a similar situation exists during a human gonococcal infection. For instance, it is unclear whether or not a similar process operates in acute infections which take place

on urethral and endocervical mucosae (as opposed to fallopian tube mucosa which represents the tissue type infected in pelvic inflammatory disease). Exposure of gonococci to the rich tissue culture medium used in this procedure certainly does not reflect the in vivo composition of the natural growth environment. Finally, it is unclear whether all host-defense mechanisms are operable in this model. However, this model does give a detailed analysis of the invasive capabilities of gonococci interacting with human mucosal cells.

3. Host response to gonococcal infection. Progressive mucosal and submucosal gonococcal invasion are often accompanied by a vigorous polymorphonuclear leukocyte (PMN) response. This response results in a copious urethral discharge containing numerous PMNs which is the hallmark of patients presenting with symptomatic gonococcal urethritis. The host also responds by evoking a specific local and humoral antibody response, as well as utilizing natural mechanisms of resistance such as the bactericidal action of complement.

a) Interaction with host polymorphonuclear leukocytes. It has been suggested that gonococci stimulate the infiltration of PMNs into the colonized area by a process of chemotactic attraction. James and Williams (175) and Schiller (340) showed that gonococci produced a factor in liquid culture filtrates which was chemotactic for human PMNs. Sandstrom et al. (331) found that sonicated whole cells in one of three gonococcal strains tested stimulated in vitro neutrophil migration. This activity was also present in the culture supernatant and could be pelleted by centrifugation at 100,000 x g, suggesting that this activity was associated with a high molecular weight complex. Rank and Holmes (307) found chemotactic activity to be produced by a

viable gonococcal strain. This activity could also be found in lysates prepared from this strain. This chemotactic activity was heat-stable and was not sedimented by centrifugation at low g-forces. This factor did not appear to be the same as that described by Sandstrom et al (331) as gel filtration of the lysate indicated that it had a low-molecular weight. The chemotactic activity was sensitive to digestion by trypsin, pronase, and amyloglucosidase, suggesting that it may be a small glycopeptide. Whether or not chemotactic activity produced by gonococci plays a significant role during infection is speculative.

It is likely that complement consumption by gonococci stimulates PMN-attractive factors. Watt and Medlin (414), Schiller (340), and Densen et al. (95, 96) have observed that the activation of complement was required for chemotactic activity. These studies did not find any evidence for chemotactin production by gonococci in the absence of human serum. The chemotactic activity was shown to result from the generation of C5a (a known chemotactic effector) during complement consumption by N. gonorrhoeae in normal human serum. Densen et al. (96) compared the kinetics of chemotactic factor generation between strains isolated from uncomplicated and disseminated gonococcal infections. In the presence of normal human serum, the former stimulated a rapid chemotactic response (about 60% maximal in 5 min), whereas the response was delayed for the latter (about 50% maximal in 20 min). Both isolates consumed the same amount of complement after 60 min, although apparently by different pathways; isolates from uncomplicated infections preferentially used the classical pathway while the disseminated isolates utilized the alternative pathway. These observations prompted speculation

that delayed stimulation of chemotactic factors by disseminated gonococcal isolates may, in part, account for their association with asymptomatic infection and their eventual dissemination.

Piliated gonococci readily attach to PMNs but apparently resist phagocytosis to a greater extent than non-piliated gonococci (101, 122, 282, 306, 393). However, piliated gonococci stimulate neutrophil respiratory metabolism despite the fact that they are not ingested (94). Protein(s) II may also play a role in leukocyte association. Swanson et al. (388) and King and Swanson (199) demonstrated that non-piliated gonococci may exhibit higner or lower levels of association with PMNs than piliated gonococci of the same strain. Increased leukocyte attachment was correlated with the presence of a leukocyte association protein which falls into the catgory of gonococcal protein(s) II.

Serum factors may play a role in neutrophil interactions with gonococci. Densen and Mandell (94) found that, in the absence of serum, P^{+} gonococci attached to PMNs to a greater extent than their P^{-} isogenic variants. However, in the presence of non-immune serum, both piliated and non-piliated gonococci associated equally well with PMNs. Rest et al. (309) determined that isogenic gonococcal variants which were piliated and/or opaque associated with human neutrophils in the absence of serum, whereas a non-piliated transparent variant required the addition of non-immune serum in order to be efficiently phagocytized. The presence of immune antiserum increased the susceptibility of gonococci to phagocytic killing (402). Jones et al. (190) determined that the opsonins of immune antiserum could be removed by absorption with purified gonococcal antigens. Incubation with purified pili

decreased the opsonic potential of immune antiserum to the greatest extent, with purified LPS and purified protein I having lower, but detectable effects. The opsonic activity appeared to be mediated by a specific immunoglobulin which was recognized by the Fc receptor of the phagocyte.

The fate of PMN-ingested gonococci is unclear. Most studies have indicated that few, if any, gonococci survive the intracellular environment of the PMN (59). However, other studies have found that gonococci can survive and even replicate within human phagocytic cells (60, 61, 401). Evidence for the cytotoxicity of gonococci for PMNs has also been presented. This cytotoxic effect was due to factor(s) produced by viable gonococci and was not related to the endotoxic effect of LPS (62). The mechanisms by which intracellular gonococci might survive the toxic environment of the phagocyte are unknown.

After ingestion, phagolysosome-mediated killing of gonococci is accomplished by both oxygen-dependent and -independent mechanisms (308). *In vitro* killing of gonococci by neutrophil granule extracts has been examined in a number of studies. Buck and Rest (48) determined that macromolecular synthesis was stopped without cellular lysis by an oxygen-independent mechanism 45 min after treatment of gonococci with crude granule extract. Further analysis suggested that a bactericidal component(s) of neutrophil granules adhered to gonococci, altering their outer membrane permeability and their ability to divide. Daly *et al.* (92) found that isogenic gonococcal variants which contained the *mtr*-2 or *pen*A2 loci were much more sensitive to killing by an oxygen-independent activity of neutrophil granule extracts than the wild-type phenotype. These observations might explain the low frequency

of mtr-2 and penA2 in strains isolated from patients with disseminated gonococcal infections. Proteases from granule extracts were found to be active against protein(s) I and II of whole gonococci. However, these proteases were only bacteristatic and not directly responsible for the killing of gonococci by the unfractionated granule extract (310). Casey *et al*. (59) determined that acid-extracts of PMN granules and a purified granule protein (57,000 daltons, 57K) were, at low concentrations, bactericidal under aerobic conditions for actively growing gonococci. Resistance to the cidal activity could be induced by bacteriostasis imposed by anaerobiasis. Since neutrophils must encounter gonococci under conditions of low-oxygen tension *in vivo*, the expression of oxygen-independent mechanisms of killing may be important for an effective defense against gonorrhea. The results by Casey *et al*. (59) suggest that the efficacy of these oxygen-independent mechanisms of PMN killing may be partly inhibited by the bacteriostatic conditions imposed by hypoxia within the host.

b) Local and humoral antibody response to infection. Local immunoglobulins develop in both men and women in response to gonococcal infection (195, 284, 394, 396). Kearns *et al*. (195) detected IgA antibodies to the gonococcus in urethral exudates of 29 of 35 men diagnosed as having gonococcal urethritis and further demonstrated that these antibodies were chiefly secretory IgA. O'Reilly *et al*. (284) analyzed the cervicovaginal secretions of women suffering from gonorrhea and found specific secretory IgA. Local antibody produced during infection in both urethral exudates and vaginal washings were confirmed by Tramont (394). This report also described the presence of specific IgG antibody as well. The presence of substantial

levels of IgG in secretions maybe the result of non-specific transudation through inflamed mucous membranes (242). Both specific IgA and IgG can block the binding of the homologous (infecting) strain of gonococci to human epithelial cells (394). The ability to block the attachment to human epithelial cells could be absorbed from the genital secretions by purified pili (396).

Pathogenic *Neisseria* spp. (*N. gonorrhoeae* and *N. meningitidis*) elaborate an enzyme that cleaves primate IgA_1 molecules into two fragments, Fc_a and FAb_a (300). This IgA_1-protease is capable of cleaving both serum and secretory IgA. Blake and Swanson (36) and Blake *et al*. (35) analyzed vaginal washings from patients infected with *N. gonorrhoeae*. In 76% of patients suffering from gonorrhea, IgA_1-protease activity could be detected. Of individuals which were culture negative for *N. gonorrhoeae*, only 44% contained IgA_1-protease activity in vaginal washings. However, the IgA_1 cleavage products produced from washings of uninfected women resembled those produced by the cleavage of IgA_1 with trypsin or chymotrypsin. On the other hand, specific IgA_1 cleavage products which resembled those produced by the partially purified IgA_1-protease of *N. gonorrhoeae* were found after the exposure of IgA_1 to vaginal washings from individuals with culture positive gonorrhea. This finding suggested that the gonococcal IgA_1-protease was functional *in vivo*. Since the role which IgA_1 plays in the host defense against gonorrhea is not understood, it is difficult to assess the pathogenic implications of the gonococcal IgA_1-protease.

Koomey *et al*. (207) have cloned the IgA_1-protease gene from *N. gonorrhoeae* into *E. coli*. Clones expressing protease activity were identified

by an enzymatic assay. Expression of IgA_1-protease activity in *E. coli* was associated with the presence of a peptide with a molecular weight of 140,000. In order to create a mutant deficient in IgA_1-protease activity, a segment from the plasmid containing the IgA_1-protease-associated sequences was excised and a fragment of a similiar size which encoded for the synthesis of beta-lactamase was inserted in its place. *E. coli* containing a plasmid of this construct did not express IgA_1-protease activity. The entire insert including both the IgA_1-protease associated sequences and the beta-lactamase gene was excised from this plasmid and used to transform *N. gonorrhoeae*. Transformants were selected by screening for penicillin resistance. The presence of this sequence in the gonococcal genome was confirmed by Southern blot hybridization. Transformed gonococci were IgA_1-protease negative by enzymatic analysis and no other phenotypic differences were detected. This report demonstrates the applicability of *in vitro* mutagenesis in the construction of isogenic gonococcal mutants defective in a potential virulence factor.

IgA_1-protease deficient gonococcal strains constructed as described above were examined for decreased virulence as assessed by infection of human fallopian tubes in organ culture (69). No differences were detected in the ability of the IgA_1-protease deficient mutants to attach, damage, and invade human fallopian tube mucosa when compared to the parental strains. These investigators suggested that, in the initial encounter with previously uninfected human mucosa, the production of IgA_1 protease is not a critical determinant for the ability of the gonococcus to act as a mucosal pathogen.

The humoral antibody response to *N. gonorrhoeae* has been investigated.

Cohen (66) noted that sera contained both "natural" and induced antibodies which were reactive with *N. gonorrhoeae*; these antibodies were of the IgG, IgM, and IgA classes. Antibodies directed against pili, LPS, and other cell-associated gonococcal proteins have been demonstrated in sera of patients with complicated and uncomplicated gonococcal infections (47, 170, 311, 396, 437). Zak *et al.* (437) detected antibodies which were produced to pili, protein I, protein(s) II, and a common surface protein with an apparent molecular weight of 43,000.

Antibody directed to LPS is bactericidal in the presence of complement for gonococci *in vitro* (311). Hook *et al.* (170) analyzed sera from 13 patients with complicated infections. Of these, IgG antibodies directed toward protein I were found in 12 sera. Antibodies specific for LPS were detected in 9 of these sera. Complement-mediated bactericidal activity was present after absorption of one sera (which reacted with both protein I and LPS) with purified LPS, suggesting that antibody directed against protein I was also bactericidal. Joiner *et al.* (189) used monoclonal antibodies specific for protein I to determine their capacity to kill gonococci in the presence of complement. Monoclonals directed against closely-associated surface-exposed epitopes of protein I differed markedly in bactericidal activity, despite consuming nearly equal amounts of complement.

These analysis suggest that gonococci induce a humoral antibody response in the host. Other studies have detected a cell-mediated immune response, although the significance of this response has yet to be determined (212). In light of the host-immune response to gonococcal infections, it is interesting that repeated episodes of acute gonorrhea are common for untreated

infections. This phenomena may be a consequence of gonococcal heterogeneity, production of an antibody-inactivating IgA_1-protease, and/or other gonococcal determinants which aid in the evasion of the host immune system.

c) Serum-resistance. The role of complement in mucosal resistance to gonococcal infections is uncertain. The concentration of complement found at mucosal surfaces is less than that found in serum (304) and these levels may be too low to mediate many complement-associated activities (40). In addition, seminal plasma contains an inhibitor of complement activation which may protect mucosal gonococci from bactericidal activity (41). Most freshly isolated strains of gonococci are resistant to complement-mediated killing by normal human serum (242, 412). Most isolates from uncomplicated infections lose phenotypic serum resistance upon subculture. Serum-resistance among these isolates can be induced by a heat-labile low-molecular weight factor in human serum (230). Morse et al. (260) have determined that serum-resistance can be modulated by the nature of the growth environment. In this study, the dilution-rate (with glucose as a limiting nutrient) of chemostat grown gonococci was correlated with changes in LPS composition and changes in the ability of gonococci to evade the bactericidal activity of normal human serum. These studies indicate that some gonococcal isolates express a complex phenotype in which an unstable form of serum resistance is regulated by host factors, the nature of the growth environment, and other undefined components.

Certain gonococcal isolates exhibit stable phenotypic serum-resistance which is not lost upon subculture. Schoolnik et al. (342) determined that this property was correlated with strains causing disseminated gonococcal infection. Stable serum-resistance appeared to be a necessary but not

sufficient property of organisms causing disseminated gonococcal infections. The importance of serum resistance to dissemination can be inferred by the observation that individuals deficient in the terminal components of complement are at increased risk to disseminated gonococcal infection (296).

The mechanism of *in vitro* complement-mediated killing of *N. gonorrhoeae* by normal human serum has been examined. It is known that both the classical and alternative pathways may be involved. Natural antibody against gonococcal components have been found in individuals with no history of gonococcal infection (124, 335, 344). The susceptibility of serum-sensitive strains appeared to be mediated by natural IgM antibodies via the classical complement pathway (344). These antibodies appeared to be directed against LPS (124). Stable serum-resistance may also result from a gonococcal antigenic component which is recognized by natural IgG antibody and which is able to block serum-mediated killing of gonococci (236, 312). Although serum-sensitive and serum-resistant strains activate equivalent amounts of complement, the ability of the membrane attack complex does not insert in membranes of serum-resistant organisms (143, 188). Thus, killing of gonococci by normal human sera is a complex reaction relying on components from both gonococci and host fluids and secretions. Moreover, the relevence of these observations to *in vivo* immunity are not well-understood.

Antibody developed during disseminated gonococcal infections are bactericidal for the homologous strain in *ca.* 50% of cases examined (312). Rice and Kaspar (311) have reported that convalescent IgG directed against gonococcal LPS enhances the bactericidal action of complement. Hook *et al.* (170) have presented similar evidence for the induction of bactericidal

antibody directed against protein I.

4. Other factors. The ability of gonococci to evade the host immune system plays a major factor in the outcome of gonococcal infections. In addition, the ability to multiply within the host is an important factor. The latter is largely the result of the physiologic capabilities of gonococci (reviewed by Morse, ref. 256). Sequestration of nutrients, in particular iron, is also an important factor for *N. gonorrhoeae* to multiply *in vivo*. This subject is detailed in the following two sections of this review.

From this non-exhaustive description of the pathobiology of *N. gonorrhoeae*, it is apparent that the pathogenesis of gonococcal infection is a multifactoral process. This process involves the ability of the gonococcus to adhere, invade, multiply, produce toxins, and evade the host mechanisms of immunity. Understanding the biologic basis for these processes is necessary in order to impose effective treatment, surveillance, and/or immunoprophylactic measures required for the control of gonorrhea.

II. MICROBIAL GROWTH ENVIRONMENTS

For pathogenic microorganisms, the host represents a natural ecosystem in which they must typically invade, survive, cause disease (in most cases), and disseminate to other individuals in the population. This environment is defined by many parameters; temperature, pH, humidity, and the concentration of essential nutrients are but a few examples. The latter is particularly important in light of the fact that natural environments are frequently limited in one or more essential nutrients due to their depletion by the host and by endogenous microbial populations (391). Hence, the natural environment is nearly always nutrient-limited so that nutrient insufficiency is the most common environmental extreme to which a pathogen is exposed (141).

In the laboratory, microorganisms are generally propagated in batch culture. This system is closed with respect to the input of nutrients. In batch culture, the concentrations of all essential nutrients are initially present in excess, so that growth of microorganisms is limited by their ability to assimilate these nutrients. As the microbial population expands, nutrients are consumed thereby continually decreasing their concentration in the medium. In addition, metabolic end products accumulate in the medium. The result is an environment which is changing constantly with respect to its composition. In response, microorganisms must continually alter their physiological status in order to cope with this changing environment. Presuming that toxic by-products of metabolism do not become inhibitory,

growth will terminate upon the depletion of one or more of the essential nutrients. Such an environment is far removed from that likely to be encountered in the host.

Despite the apparent limitations of analyzing organisms from batch culture, the practice has remained common in the laboratory. Perspectives on the evolution and institution of this cultivation technique in the laboratory have been described in the following excerpt from Tempest et al. (392):

"No doubt all will agree that a landmark in the history of microbiology was the development of the now classical procedures whereby specific microbes could be isolated and cultured free from the 'contaminating' organisms with which they were naturally associated. Isolation of organisms in a 'pure' state was, of course, necessary in order to investigate and rationalize the many biological phenomena, ranging from disease and putrefaction to fermentation, that could be observed in Nature. But, in order to study the physiological properties of pure strains, larger quantities of organisms were required; this, it was found, generally could be accomplished by simply inoculating cells of the isolated strain into batches of nutrient medium contained in a closed vessel, incubating at a suitable temperature (with or without aeration) and allowing events to run their course. The success of this process--the 'batch culture' process--was such that it quickly became the routine method for culturing microbial cells, which it remains today. It is ironic, however, that many microbiologists now seem to look upon this 'batch' process as being essentially natural whereas this it clearly is not. The environment in a batch culture generally is far removed from that likely to be found in Nature and there is good reason for believing that the behaviour of

the organisms, so cultured, also is considerably different from that expressed in natural environments. Indeed so much is this the case that, as the late Professor Kluyver is reported to have argued, one should look upon all pure cultures as 'laboratory artifacts'."

From the preceeding discussion it is apparent that a natural environment such as the human host is not a closed system, rather it is considered an open system. Nutrients are continually being supplied, consumed, or transported so that the balance between these processes yields a relatively constant supply of essential nutrients. However, this environment generally will contain insufficient concentrations of nutrients to permit microorganisms to grow at maximum rates (392). In the laboratory, a similar environment can be simulated by the use of a continuous culture apparatus--the "chemostat" (276). This system utilizes a continuous, but variable, flow of medium, containing a limiting nutrient to control the growth of microorganisms. Continuous culture results in a relatively homogeneous population of cells which have been grown under rigorously controlled environmental conditions. The functional significance of biologic activities and cell ultrastructure can be assessed by studying the changes in cell physiology associated with specific changes in the environment.

While the chemostat provides a mechanism to establish an environment which more closely resembles the natural microbial environment, it also has its limitations. Most of these are practical considerations such as time, equipment, and limited capacity to produce sufficient quantities of material for analysis. Because of this, batch culture remains a necessary and successful technique in which microorganisms are propagated for study. The

contrast between batch and continuous culture points out the importance of considering the environment when attempting to understand structure-function relationships. The field of bacterial pathogenesis attempts to relate pathologic damage to specific bacterial components. Since expression of many microbial virulence components are tightly coupled to growth conditions, it is imperative that one consider the host environment when studying these factors.

A. NUTRIENT-LIMITATION AND ITS ROLE IN NATURAL IMMUNITY.

From a physiologic perspective, nutrient-limitation might be regarded as the consequence of a complex interaction between availability and assimilation of a particular nutrient within a microenvironment. However, with respect to bacterial pathogenesis it can represent a form of natural immunity in which the host is protected from infection due to the lack of an essential nutrient required for the growth of the invading bacterium. Natural immunity has been recognized since the 1880's as an effective system of host protection. However, knowledge concerning specific mechanisms of natural immunity have not evolved to the same extent as our knowledge of acquired immunity over the same period. Examples of natural immunity include the alternative complement pathway, lysozyme, beta-lysins, and interferon. These systems operate nonspecifically in order to prevent the growth of pathogens within the host.

Conditions of nutrient-limitation act to exclude growth of pathogenic microorganisms within the human host. Specific systems which are responsible for withholding growth-essential nutrients from invading pathogens make up the host's system of nutritional immunity. The concept of nutritional immunity was first suggested by Kochan (204) in reference to the ability of the human host to withhold iron. Vertebrates have developed an elaborate yet remarkably efficient strategem to withhold growth-essential iron from microbial and neoplastic invaders while retaining their own access to this metal (422). The withholding of other nutrilites such as inorganic phosphate and zinc may also be involved in nutritional immunity (420). However, the host iron-withholding defense mechanism has been studied most extensively.

B. IRON-METABOLISM IN THE HUMAN HOST AND ITS ROLE IN NATURAL IMMUNITY.

Iron-metabolism in humans is under strict regulation. The normal adult male contains _ca._ 50-70 mg of iron per kilogram of body weight (398). This iron is efficiently recycled through the body as tissue requirements dictate (352). Unlike other elements, the concentration of iron in the body is maintained relatively constant, not by excretion of excess iron, but by the ability of the intestine to regulate absorption (399). Three phases of iron absorption are involved. The first is an intraluminal phase in which incoming iron is processed for absorption. Dietary iron is present mainly in the form of ferric-chelates. The stability of the iron-chelate complex in the gastrointestinal tract will determine the extent to which free iron will be absorbed by the intestinal mucosa. If the iron-chelate complexes are very stable, their uptake will depend upon the efficiency by which the intact complex is absorbed by the intestinal mucosa. Ferric and ferrous iron are equally well absorbed provided they are chelated by suitable ligands. Heme iron is more efficiently absorbed than nonheme iron (28).

The second phase of iron absorption involves the movement of iron across the mucosa. This movement is accomplished by two processes operating simultaneously. The first is the result of simple diffusion of iron and is limited by the impermeability of the intestinal mucosa and the concentration of iron in the lumen. This process operates when iron stores are high. The second process has a limited capacity, displays saturation kinetics and competitive inhibition. This system operates when iron reserves are low (399).

The third phase of iron absorption has been referred to as the corporeal phase. In this phase, iron is bound to plasma transferrin for delivery to the tissues (399). The transferrin-bound iron which finds its way into the blood stream delivers iron predominantly to the bone marrow for erythropoiesis. The majority of body iron (60 to 70%) is transiently bound to hemoglobin in red blood cells. Senescent red blood cells are taken up by the reticuloendothelial cells in the liver and spleen. The iron is then processed and returned to the circulating transferrin pool or tissue storage sites in the forms of ferritin and hemosiderin where it may be released when necessary (64). The latter two compounds serve as the major iron-storage components of nonheme iron and are found in all tissues (although the highest concentrations generally occur in the liver, spleen and bone marrow). Ferritin and hemosiderin are chemically distinct yet intimately related in function. Ferritin is soluble in water and increased serum ferritin levels are often observed in liver disease or conditions which increase red cell turnover. It has a molecular weight of 460,000 and in its crystalline state contains up to 20% iron. Hemosiderin is a water-insoluble protein-free aggregate composed of up to 35% iron in the form of ferric hydroxide (64).

Body fluids of vertebrates contain _ca._ 10^{-5} M of total iron (49, 105). However, due to the presence of iron-binding proteins (transferrins) the concentration of free ionic iron is _ca._ 10^{-18} M (49). The transferrins comprise a group of structurally and functionally related iron-binding glycoproteins (3) which are distributed throughout the body at sites frequently infiltrated by microorganisms. The protein transferrin circulates in blood at concentrations of 2-3 g/l, delivering iron between sites of

absorption, storage, and hemoglobin synthesis (119). Historically, iron has been recognized as a component of plasma since 1898; that this iron was predominantly associated with a protein component has been known since 1927. However, the transferrin molecule was not identified until 1946 (422). Schade and Caroline (336) discovered that a protein component of egg white (conalbumin) retarded bacterial and fungal growth by virtue of its avidity for iron. Insightfully, they predicted that body fluids would contain a similar component for withholding iron. Subsequent to their prediction, they demonstrated an iron-reversible antimicrobial activity in a protein fraction of human plasma (337).

Lactoferrin is the iron-binding glycoprotein found predominantly in mucosal secretions and granules of circulating polymorphonuclear leukocytes. Human breast milk contains up to 20% of the total protein content as lactoferrin (422). An analogous protein found in egg white, conalbumin, also belongs to the transferrin family. All three molecules consist of a single polypeptide chains with apparent molecular weights of 80,000 to which one or two carbohydrate groups are attached. These proteins possess two metal binding sites, each of which can reversibly bind a ferric ion together with a bicarbonate or carbonate anion. All contain a two-fold internal homology with one metal binding site located in each half. The metal is bound by coordinating with tyrosine and histidine residues at each site (235). The estimated association constant of transferrin for iron is 10^{36} (1). Lactoferrin has an equally high affinity for iron but unlike transferrin, it does not relinquish its iron at pH values below 5 (283). The transferrins have very low affinity for ferrous iron, suggesting that a reductive mechanism

would provide a means for the removal of iron (352). Other possible mechanisms for removing iron from transferrin are chelation, protonation, or labilization of the bicarbonate ion (205).

The major biologic activity of the transferrins is to bind iron. As a consequence of this activity, serum transferrin serves as the principle carrier of iron in the blood. Therefore, it plays a central role in iron metabolism although serum ferritin is also believed to serve a transport function (356). Of equal importance, is the role which the transferrins play as part of the host's natural defense against infection. Only 30% of the transferrin binding sites in normal human sera are occupied by iron (50). The iron saturation levels of lactoferrin in colostrum are reported to be less than 10% (120). This enables the transferrins to scavenge free iron which may be present from excess absorption or release during cellular destruction. Furthermore, the transferrins maintain the concentration of free iron in serum and external secretions at levels far below that necessary to sustain microbial life. Consequently, these proteins are also involved in bacteriostasis; and in concert with other host components such as specific antibodies and complement, are bactericidal for a variety of microbes. The contributions of the transferrins to the antibacterial activities of serum and colostrum can, in many cases, be reversed by the addition of iron (352). Certain bacteria may resist the antibacterial properties of serum and cause disseminating infections. However, in order to survive within the host, these microorganisms must possess an effective mechanism for the acquistion of host iron.

Iron in the form of heme is found in serum often complexed to proteins

like hemoglobin. Hemoglobin is a readily useable form of iron for various microbes, and as a result, the potential for infection at sites of hemolysis is great. Evidence suggests that hemoglobin may be withheld as a microbial iron source by haptoglobin, a hemoglobin-binding protein whose concentration in serum increases significantly during infection. Haptoglobin forms stable complexes with hemoglobin ($K_{diss} = 2 \times 10^{-7}$ M), which are rapidly cleared by the reticuloendothelial system (105).

The human host has utilized iron-deprivation as a form of natural defense. Weinberg *et al.* (421) suggested that perhaps all vertebrates make "considerable metabolic adjustments during infection that have the effect of depriving inactive microorganisms of iron". Possible metabolic mechanisms which the human host adjusts in response to infection have been proposed and experimently examined (421). These include decreased intestinal absorption of exogenous iron; shift of plasma iron to iron stores resulting in a hypoferremia; infiltration of iron binding proteins to the site of infection; and increased synthesis of host iron binding proteins (421). All of these mechanisms operate to impose a nutritional barrier designed to breach the infectious process by preventing bacterial multiplication.

III. MICROBIAL IRON-ACQUISITION--AN OVERVIEW

A. MICROBIAL IRON AND IRON-BINDING COMPOUNDS

The trace mineral iron is one of 27 elements known to be essential for life (398). This metal plays an irreplaceable role in a variety of critical metabolic reactions. A diverse range of biologic activities are associated with iron-containing proteins. These include enzymes of the electron transport chain, H_2O_2 and O_2 metabolism, tricarboxylic acid cycle, DNA biosynthesis, and nitrogen fixation (266). Because iron is one of the most abundant and ubiquitous elements on earth, it might seem unlikely that an organism would find itself in a situation of iron-insufficiency. However, the chemical nature of iron severely restricts its bio-availability (352). In aqueous solutions at physiologic pH, ferric ions exceeding 10^{-18} M precipitate as polymers of ferric oxyhydroxide. Therefore, in order to satisfy basal requirements for iron which are in the micromolar range, organisms have evolved specific molecules for maintaining iron in a soluble, useable form (262)

Microorganisms obtain iron from an iron-sufficient environment using low-affinity systems (264). There are inherent difficulties in dissecting the low-affinity system from the high affinity iron-uptake system. Therefore, the low-affinity mechanisms of bacteria are not well understood. Much more is known regarding the high affinity iron-uptake systems of bacteria. Many microbes employ siderophores in order to obtain iron from the environment.

Siderophores are low-molecular weight, nonporphyrin, nonprotein, iron-binding compounds (266). Neilands (264) has recently expanded the definition of a siderophore by suggesting that they must also be: (i) repressed in the presence of iron; (ii) have a high formation constant for iron (on the order of 10^{30}); and (iii) act as a source of iron for the producing, and perhaps other, microbial species. Most siderophores examined to date fall into two chemical classes, hydroxamates and phenolates (catechols). Although the chemical structures of different siderophores are diverse, their role in bacterial iron-metabolism remains the same.

The prototypical siderophore of the phenolate group is enterochelin, more commonly referred to as enterobactin. O'Brien and Gibson (279) first isolated this compound from supernatants of E. coli in 1970. At about the same time, Pollack and Neilands (302) found enterochelin in culture supernatants of S. typhimurium. It has since been shown to be produced by Enterobacter aerogenes, E. cloacae, and Klebsiella pneumoniae (264). Enterobactin is a cylic trimer of 2,3-dihydroxy-N-benzoyl-L-serine and has an association constant for ferric ions of 10^{52} (266). At neutral pH this value drops to 10^{37} due to proton competition, but it still exceeds the binding affinity of transferrin. Other bacteria also synthesize catechol-type siderophores of related structure. These include Agrobacter tumefaciens, Paracoccus denitrificans, Bacillus species, and Pseudomonas aeruginosa. The aromatic structure of this group of siderophores enables them to be readily detected by their fluoresence and characteristic UV absorption spectra. Furthermore, they are easily isolated by extraction with organic solvents (264).

The group of hydroxamate siderophores are more diverse in their structures. The first naturally occuring hydroxamate siderophore to be recognized was ferrichrome. This compound is a cyclic hexapeptide produced by various fungi and may serve as an iron source for other microorganisms in addition to the fungus responsible for its synthesis. Bacterial citrate-hydroxamate siderophores include arthrobactin (*Arthrobacter* spp.), schizokinen (*B. megaterium*), and aerobactin (*E. aerogenes*, and several other enteric organisms). There are other examples of hydroxamate siderophores produced by bacteria in addition to those mentioned. All possess binding constants for the ferric ion in the range of 10^{30} (264), a value less than that of their phenolate counterparts.

The strategy used by microorganisms with respect to iron-acquisition is to secrete the siderophore into the envirionment. Taking advantage of it's high-affinity for iron, the siderophore competes with endogenous chelators for ferric ions and renders the iron available to specific uptake components of the bacterium. The best characterized high affinity microbial iron-uptake system is that found in *E. coli*.

B. IRON-ACQUISITION BY *E. COLI*

1. Enterobactin-mediated iron-acquisition. When confronted with an iron-restricted environment, *E. coli* synthesizes and secretes the catechol-type siderophore known as deferri-enterobactin. In addition, they produce several new high molecular weight outer membrane proteins (iron-regulated proteins) (267). The iron-regulated proteins of *E. coli* K12 have apparent molecular weights of 83,000, 81,000, and 74,000 (267). Hancock *et al.* (137) observed that mutants defective in ferric-enterobactin uptake were unable to absorb colicin B and were also defective in the production of the 81,000 dalton protein. This protein is the product of the *fep* A gene (88). A receptor specific for ferri-enterobactin complexes had been predicted by Wayne and Neilands (417) to explain the data of Gutterman (133), who found that hyperexcreting mutants of enterobactin were immune to colicin B. Hollifield and Neilands (167) determined that Triton X-100 extracts of outer membranes from *E. coli* K12 grown in low-iron medium retained ferric-enterobactin binding activity. A similar extract was used by Fiss *et al.* (117) to purify the Fep A protein. This purified preparation and ferric-enterbactin were shown to have a dissociation constant of *ca.* 10 nM.

The Fep A protein recognizes the ferri-enterobactin complex at the cell surface. This complex is transported intact across both the outer and cytoplasmic membranes (264). Transport across the latter is thought to be the result of a permease which has been designated as Fep B (431). Once inside, the intact ferric-enterobactin complex is dissociated by the *fes* gene product

(264). The fes gene product is thought to be an esterase which operates on the enterobactin structure, releasing iron to the intracellular environment (213). Alternatively, the fes gene product may represent a reductase which operates, in some capacity, to reduce the ferric ion off of the ferric-enterobactin complex (264). Also involved in the transport of ferri-enterobactin is the ton B gene. The product of this gene is a protein with an apparent molecular weight of 36,000 (303). This gene appears to play a key role in many surface-related phenomena including colicin sensitivity, high affinity transport systems, and vitamin B_{12} transport (265). It has been suggested, with respect to vitamin B_{12} transport, that the ton B gene product is a cytoplasmic membrane protein which exists as a diffussible component and functions by releasing adsorbed substrate from the outer membrane receptor. This hypothesis has not been tested for the high-affinity iron uptake systems (265).

Evidence that enterobactin-mediated iron transport occurs in vivo was obtained by analyzing E. coli recovered from peritoneal washings of lethally infected guinea pigs. Analysis of membrane protein profiles of the organisms detected the expression of the three iron-regulated proteins (81,000, 78,000,and 74,000 daltons) described previously. The 81,000 dalton ferri-enterobactin receptor and the 74,000 dalton protein were expressed in relative quantities nearly equal to those of the major outer membrane proteins which are constitutively produced (131). In another study, Griffiths and Humphreys (129) detected enterochelin from peritoneal washings of lethally infected guinea pigs. Moore and Earhart (255) found that normal human sera contains enterobactin-specific antibody. This may account for a portion of

the anti-bacterial activity of sera to *E. coli*. These studies provide evidence for expression of iron-sequestering components *in vivo*.

2. Ferrichrome-mediated iron-acquisition. Iron complexed to the hydroxamate siderophore ferrichrome may also be utilized as an iron source by *E. coli*. This represents a case in which *E. coli* effectively utilizes a siderophore produced by a different organism. *E. coli* maintains a receptor on their surface which is specific for ferri-ferrichrome. This receptor was first described by Wayne and Neilands (417) who reported that mutants resistant to bacteriophage T1 and phi80 (Ton A and Ton B) were also resistant to albomycin, an antimicrobial analogue of ferrichrome. Futhermore, they showed that ferrichrome could compete with and inhibit infection of wild-type *E. coli* by bacteriophage phi80. This receptor, designated as the Ton A protein, has an apparent molecular weight of 78,000. It has been partially purified by mild alkaline extraction, followed by diffential centrifugation and ion-exchange chromatography. This preparation was used in competition experiments with ferrichrome and ferrichrome analogues (219). It was determined that ferrichrome could efficiently compete with bacteriophage T1, as could the analogues chromichrome, alumichrome, and di- and trimethyl esters of ferrichrome. Other hydroxamate siderophores such as ferrioxamine B and rhodotorulic acid did not compete. Measurement of the direct binding of radiolabeled ferrichrome by the Ton A protein was unsuccessful, suggesting that another bacterial component might be required for binding (263). Alternatively, the ferri-ferichrome iron binding activity might be chemically labile or the turnover rate too rapid to allow detection.

Fecker and Brown (114) cloned the region involved in the utilization of

ferric-hydroxamates (Fhu) by E. coli. Their analysis indicated that this region consisted of four genes designated in transcriptional order as fhu A (ton A), fhu C, fhu D, and fhu B. The fhu C and fhu D genes encoded for 30,000 and 26,000 dalton proteins, respectively, both of which appeared to be localized in the cytoplasmic membrane. The fhu A gene encoded for the 78,000 dalton outer membrane receptor. In this study, no gene product could be detected for the fhu B region. More recently, evidence has been obtained indicating that the fhu B gene product encodes a 20,000 dalton protein. This protein appeared to be unstable and susceptible to proteolytic degradation (305). The fhu CDB genes are thought to be components of the system used for the general transport of ferri-hydroxamate complexes from the periplasm to the cytosol. Mutants deficient in the outer membrane protein, Protein II*, exhibited altered ferrichrome transport (70). Only the fhu A gene product exhibited specificity for the hydroxamate ferri-ferrichrome complex. The ton B gene product is also involved in ferrichrome transport. The presence of the Ton B protein was not required for transport of ferrichrome-bound iron by spheroplasts, suggesting that its role is required for transport across the outer membrane (418). It is not clear whether the synthesis of the outer membrane Ton A receptor protein (78,000 daltons) is derepressed at low-iron concentrations (264). However, it has been determined that the uptake of ferri-ferrichrome proceeds much faster in iron-starved cells (264). E. coli appears to internalize ferri-ferrichrome complexes and excrete the deferrated siderophore after removal of the iron (218). The mechanism by which iron is removed from ferichrome is presumed to involve reduction.

Other hydroxamate siderophores can be utilized by E. coli. These include

coprogen, rhodotorulic acid, and ferri-oxamine B. Iron complexed to these compounds is transported by the same components used for ferrichrome transport with the exception of the Ton A (Fhu A) receptor. In place of the Ton A receptor, the outer membrane receptor designated Fhu E is used (232).

3. Aerobactin-mediated iron acquisition. Aerobactin is a siderophore which was first identified as a product of E. aerogenes (123). More recently, it has been identified in iron-limited cultures of Shigella flexneri (289) and E. cloacae (88). It has a formation constant for Fe(III) of 10^{23} (265). Production of this siderophore by E. coli was first described by Williams (426) who was investigating the virulence-linked plasmid which encodes for the production of colicin V (Col V). The ColV plasmid was known to be associated with pathogenic strains of E. coli (88). Williams observed that the ColV-K30 plasmid conferred a strong selective advantage to E. coli in lethally infected mice unless iron was administered with the inoculum. Futhermore, defined medium in which transferrin-bound iron was the sole iron source supported limited growth by plasmid free strains but had no effect on the growth of ColV-containing strains. This activity was independent of enterobactin-mediated iron-uptake. The fact that this novel iron uptake system was mediated by aerobactin was described shortly thereafter by Warner et al. (413) and Braun (37).

Konopka et al. (206) compared the ability of aerobactin to remove the iron from transferrin with that of other catechol and hydroxamate siderophores. Their findings indicated that aerobactin removed the iron from transferrin but did so at a rate slower than that of enterobactin. The rate of aerobactin-mediated iron removal was accelerated by the addition of

pyrophosphate, suggesting that the removal of iron(III) from transferrin proceded through a ternary complex. When the analysis was performed in serum with viable E. coli, the rate of aerobactin removal of iron (III) from transferrin was greater than that of enterobactin despite the fact that the formation constant of aerobactin is several orders of magnitude lower than that of enterobactin. In this system, E. coli acted as a "sink" to facilitate the aerobactin-mediated removal of iron from serum transferrin. These attributes of aerobactin-mediated iron assimilation may account for its status as a virulence determinant of E. coli.

The surface receptor in E. coli for the iron-aerobactin complex has been identified as a 74,000 dalton protein. This protein also serves as the receptor for cloacin DF13, a bacteriocin produced by E. cloacae (22, 128). Using a cir^- background (which was necessary due to a 74,000 dalton protein that is the receptor for colicin V and Ia), Grewal et al. (128) showed that strains carrying mutant ColV plasmids in which iron-aerobactin complexes are not transported also lacked the plasmid-specified 74,000 dalton outer membrane protein. The genes responsible for the regulation of aerobactin biosynthesis and the ferri-aerobactin receptor have been cloned from the ColV-K30 plasmid by selecting cloacin-sesitive clones (23, 209) in a cloacin-resistant background. Although the proteins responsible for aerobactin bisynthesis were not identified, it was suggested that they consist of between four and five gene products (23). Krone et al. (209) also cloned the ferri-aerobactin receptor using a rationale similar to that described above. The latter study indicated that there was a 50,000 dalton polypeptide encoded on a sequence adjacent to the 74,000 dalton receptor gene product. The role of this protein

in aerobactin-mediated iron-uptake is presently unclear.

In addition to the plasmid-encoded aerobactin genes, Braun _et al._ (38) found that four chromosomal genes--_ton_ A (_fhu_ A), _fhu_ B (the gene region now known to consist of _fhu_ BCD), _ton_ B, and _exb_ B--were required for utilization of iron from aerobactin. The _fhu_ A gene product is not directly involved in the transport process, but mutations in this gene exert a polar effect on the expression of the linked _fhu_ B, C, and D genes (88). Therefore, with the exception of the surface receptor, uptake of aerobactin by _E. coli_ appears to procede by a mechanism characteristic of hydroxamate siderophores.

The ColV plasmids are known to be associated with virulence. However, not all ColV plasmids contain the genes for aerobactin-mediated iron-transport (e.g., pColV, I-K94) (88). Several investigators have studied the distribution of aerobactin production by _E. coli_ (24, 400). Stuart _et al._ (368) analyzed clinical isolates of _E. coli_ from several different sources for iron-suppressible production of hydroxamates. Since aerobactin is the only known hydroxamate siderophore produced by _E. coli_, it is likely that these investigators were detecting production of this siderophore. Their findings indicated that greater than 50% of the isolates analyzed from human sources (feces and blood) produced hydroxamates. This was in contrast to only 6% of isolates from non-animal sources. These investigators also analyzed isolates from swine, cattle, and poultry. They concluded that the ability to carry out hydroxamate-dependent iron transport was widely distributed among natural isolates of _E. coli_. They further suggested that hydroxamate-mediated iron transport operating independently or in conjunction with enterobactin-mediated iron transport may offer a selective advantage. Montgomerie _et al._ (254)

analyzed strains of *E. coli* isolated from humans for hydroxamate production and for the ability of the hydroxamates to cross-feed an aerobactin-requiring strain of *E. coli*. The incidence of aerobactin-positive strains that were isolated from blood was greater than the incidence of these strains isolated from other sites (ascites, urine, and rectal). Using a murine model of renal infection, it was determined that production of aerobactin was correlated with lethality, but was not correlated with renal infection.

The chromosomal location of the aerobactin-mediated iron uptake genes in a strain of *E. coli* isolated from the cerebral spinal fluid of a neonate with meningitis was recently reported by Valvano and Crosa (400). Bindereif and Neilands (24) analyzed the location of the aerobactin-mediated iron uptake genes in eight aerobactin-positive clinical isolates. In two of the eight isolates, the genes were found on a plasmid; in the remaining six, the genes were most likely chromosomal. Both studies detected IS-1 (or IS1-like) sequences which flanked the genes. Perez-Casal and Crosa (285) determined that a replication region, in addition to the IS-1 sequences, bordered the aerobactin genes of the ColV plasmid. They postulated that these features may have played an important role in the preservation of the aerobactin-related sequences as well as contributed to their spread.

4. Citrate-mediated iron acquisition. A fourth system of iron-acquisition by *E. coli* involves the functional iron-chelator citrate. Frost and Rosenberg (121) found that mutants of *E. coli* K12 which were defective in enterobactin-mediated iron-acquisition took up iron by a citrate-dependent mechanism. They also determined that induction of this system in the presence of citrate required new protein synthesis. Hancock *et*

al. (137) identified a citrate-inducible protein which had an apparent molecular weight of ca. 81,000. It was suggested that this protein was the ferric-citrate receptor. Waggeg and Braun (404) isolated mutants defective in citrate-mediated iron transport and found that 25% of these mutants lacked the citrate-inducible protein; these mutants were designated as fec A. Outer membranes bound iron-citrate complexes in a manner proportional to the amount of the Fec A protein in the membranes. Further analysis indicated that induction of this system was dependent upon citrate (as well as the analogues fluorocitrate and phosphocitrate) but was not induced by other closely related organic acids such as tricarballylate, isocitrate, hydroxycitrate, cis-aconitate, fumarate, oxaloacetate, and succinate (171). In the absence of iron (but in medium containing citrate) the citrate-dependent iron transport system was not induced. Transport of labeled iron was observed in a five to ten-fold excess over labeled citrate in this system, suggesting that citrate was not transported. Mutants defective in citrate-mediated iron acquisition that produce the Fec A protein are designated as fec B. While it is known that the two phenotypes are closely linked, the function or gene product(s) of fec B have not been characterized.

C. IRON ACQUISITION BY OTHER ENTERIC PATHOGENS

Iron transport by S. typhimurium procedes in much the same manner as in E. coli (264). The major difference between E. coli and S.typhimurium with respect to iron-acquisition is that the latter lacks a ferric-citrate transport system (264). It is interesting that S. typhimurium utilizes citrate as a carbon source while E. coli does not (264). Since a citrate-mediated iron transport system would require specificity for ferric-citrate only, free citrate could not be efficiently assimilated. Therefore, S. typhimurium appears to sacrifice a citrate-mediated iron uptake system in favor of using citrate as a carbon source.

S. typhimirium produces and secretes the siderophore enterobactin (302) and can utilize the iron bound to enterochelin (301). It also has a common receptor that binds ferrichrome, albomycin, and bacteriophage ES18; apparently the genetic equivalent of the ferrichrome-receptor (Ton A protein) in E. coli. Loss of the ability to synthesize enterobactin greatly decreases the virulence of S. typhimurium for mice as well as inhibits its ability to grow in human serum (433). Hoiseth and Stocker (169) described mutants defective in the ability to synthesize all aromatic compounds, including enterobactin. These mutants were virtually non-virulent and their use as a live vaccine conferred excellent protection against challenge with a virulent strain.

Bennett et al. (20) described iron-regulated outer membrane proteins with apparent molecular weights of 82,000, 79,000, and 77,000. The function of these proteins has not been determined. However, given the similarities

between the iron-acquisition systems of E. coli and S. typhimurium, it seems likely that they will have functions analogous to those of the iron-regulated outer membrane proteins of E. coli.

Klebsiella spp. produce enterochelin and aerobactin in response to iron-stress (266). They also appear to use exogenously supplied iron chelated by the hydroxymate siderophore deferrichrome B (198). The iron-regulated proteins of K. aerogenes were recently examined; outer membrane proteins were identified with apparent molecular weights of 69,000, 70,000, 75,000, 78,000 and 83,000 (427).

Shigella sonnei and S. boydii synthesize both phenolate and hydroxymate siderophores (295). S. flexerii strains normally synthesize only aerobactin (286). However, rare isolates of this organism have been identified which produce enterobactin (289). Lawler and Payne (215) determined that the genes for the synthesis and transport of aerobactin are linked and are chromosomally located in S. flexreri, S. boydii, and S. sonnei. The genes were not found in the one strain of S. dysenteriae examined. The aerobactin genes of Shigella spp. exhibited considerable homology with the ColV-plasmid aerobactin genes, including the IS-1 sequences. Griffiths et al. (130) found that the synthesis of aerobactin and a 76,000 dalton outer membrane protein could be transferred to E. coli K12 by conjugation. Furthermore, enteroinvasive strains of E. coli, which produce shigella-like infections, synthesize aerobactin and a 76,000 dalton protein during iron-limitation. The 76,000 dalton protein has a greater molecular weight than the protein (74,000 daltons) associated with aerobactin-mediated iron uptake in E. coli strains carrying the ColV (K30) plasmid.

D. IRON-ACQUISITION BY PATHOGENIC *VIBRIO* SPP.

Payne and Finkelstein (288) reported that iron-starved cultures of *Vibrio cholerae* contained a phenolate-type siderophore. This compound was similar, but not identical to enterobactin. This siderophore, called vibriobactin, has recently been isolated and characterized (132). In addition to vibriobactin, other phenolates such as agrobactin and enterobactin can cross-feed *V. cholerae*, the former being favored over the latter. The hydroxymate ferrichrome was also more efficient in its ability to cross-feed this organism than was enterobactin. Iron-regulated proteins with apparent molecular weights of 220,000, 77,000, 76,000, 75,000,73,000, and 62,000 were expressed by this organism (349). Sciortino and Finkelstein (347) determined that *V. cholerae* grown *in vivo* in infant rabbits expressed novel outer membrane proteins which, in part, were similar to those observed in *V. cholerae* grown *in vitro* under conditions of iron-deprivation. Recently, Sigel *et al*. (350) used mutants defective in iron-vibriobactin transport to show that a functional iron-vibriobactin transport system was not required for organisms to multiply and elicit diarrhea in infant mice. These mutants, like the wild-type strains, could assimilate iron using a mechanism similar to the ferric-citrate uptake system in *E. coli*. Both citrate or asparagine could be utilized for growth in low-iron medium. These investigators suggested that compounds of this type may increase the availability of iron to *V. cholerae* in the host.

Andrus *et al*. (5) analyzed the siderphores produced by pathogenic *Vibrio*

species. Their results suggested that phenolate compounds were produced by *V. cholerae*, *V. fluvialis*, *V. vulnificus*, and *V. anguillarum*, but not by *V. parahemolyticus* or *V. alginolyticus*. Furthermore, cross-feeding experiments and chemical assays determined that while there were some similarities between the siderophores secreted by these organisms, there appeared to be a number of different iron-transport systems expressed among members of this genus.

V. anguillarum causes a hemorrhagic septicemia in salmonids. Crosa *et al.* (91) correlated a high-virulence phenotype of this organism with the presence of a plasmid class (pJM1) found in strains isolated in the Pacific Northwest. Curing the plasmid from the high-virulence strains resulted in the loss of virulence for juvenile Coho salmon (90). Analysis of the iron-sequestration system of *V. anguillarum* which contained or lacked pJM1, determined that strains which contained the plasmid grew much faster in a minimal medium with transferrin as the sole iron source (87). In a similar experiment, Crosa and Hodges (89) found that highly virulent *V. anguillarum* (containing pJM1) expressed two novel iron-regulated outer membrane proteins with apparent molecular weights of 86,000 (OM2) and 79,000 (OM3). One of these, OM2, was only expressed in the isogenic parent strain containing pJM1. This study also correlated the presence of this plasmid with an energy-dependent uptake of ^{55}Fe. Using transposon-mediated mutagenesis, Walter *et al.* (408) isolated derivatives of *V. anguillarum* strain 775 containg pJM1 which were defective in their ability to grow in medium in which the iron chelator EDDA was added. Analysis of pJM1 insertions provided convincing evidence that genes associated with expression of the high affinity iron transport system were localized on the plasmid. Actis *et al.* (2) were able to clone from pJM1 the sequence

encoding the OM2 structural gene in *E. coli*. It was also shown that OM2 was exposed on the surface of *V. anguillarum* (2). This finding supports the contention that OM2 may be the receptor for a putative ferri-siderophore complex. To date, the siderophore from this organism has not been isolated and characterized (88).

The similarity of the pJM1 plasmid-mediated iron-uptake system of *V. anguillarum* and the aerobactin-mediated pColV (K30) iron-uptake system of *E. coli* prompted Walter *et al*. (408) to look for homology between the two systems. Their findings indicated that mutants deficient in the production of siderophore could not be cross-fed by the heterologous bacterial culture supernatant. Furthermore, DNA hybridization studies indicated that the two plasmids lacked any extensive homologous sequences. The occurence of two unrelated plasmid-encoded iron-acquisition systems which are associated with the ability to confer virulence to organisms which harbor them, underscores the critical link between iron and infection.

E. IRON-ACQUISITION BY OTHER PATHOGENIC MICROORGANISMS

Pseudomonas spp. produce various siderophores of diverse structure (267). *P. aeruginosa* produces the siderophore pyochelin (84, 85). Cox (81) found iron reductase activities in cell free extracts which appeared to operate on Fe(III)-pyochelin and Fe(III)-citrate. These two activities appeared to be mediated by different enzymes based upon differences in thermal sensitivity, locations in fractions of cell-free extracts, and in apparent sizes during gel filtration. Cox (82) also showed that ferri-pyochelin complexes as well as ferric-citrate were taken up by *P. aeruginosa* through an initial energy-independent surface-associated binding step followed by an energy-dependent accumulation of iron. Sokol and Woods (359) identified a protein component in cell envelopes of *P. aeruginosa* which bound [^{59}Fe]-pyochelin but not $^{59}FeCl_3$. This protein had an apparent molecular weight of 14,000 and was produced in large quantities by cells cultivated in iron-straved medium. This protein has been purified; antibodies specific for this protein block the binding of [^{59}Fe]-pyochelin in an *in vitro* iron-binding assay.

A second compound can be used as a siderophore by *P. aeruginosa* is pyoverdin (83). Pyoverdin stimulates growth in medium containing either transferrin or human serum. Ankenbauer *et al.* (6) compared pyochelin and pyoverdin for their ability to stimulate growth of *P. aeruginosa* in the presence of transferrin or human serum. They concluded that pyoverdin was the most effective siderophore for growth in human serum. Further evidence for an

operative iron-sequestration system *in vivo* was obtained by Brown *et al*. (42) who analyzed the outer membrane protein composition of *P. aeruginosa* taken directly from the sputum of a cystic fibrosis patient. Proteins with apparent molecular weights of between 80,000 and 90,000 were observed which corresponded to the proteins expressed when organisms were grown *in vitro* under iron-limiting conditions.

Yersiniae species were analyzed for their ability to accumulate iron in an iron-deficient medium by Perry and Brubaker (294). While the organisms were able to obtain iron by an energy-dependent mechanism, no siderophore production was detected. Hemin could be efficiently utilized as a sole source of iron for *Yersiniae*. The investigators suggested that this organism may possess an efficient cell-bound transport system for Fe(III).

Iron acquisition by the acid fast bacilli has been examined and a class of iron-chelating growth factors, called mycobactins, have been identified (358). These compounds are cell-associated iron-binding compounds which assist in the acquisition of iron from the environment. Like siderophores, mycobactins are produced under conditions of iron-deprivation. Further work has demonstrated that low-molecular weight iron-binding compounds known as exochelins are also produced by certain species of mycobacteria. Two main classes of exochelins have been described in members of this genus--those that are soluble in chloroform and those that are soluble in aqueous solvents. The structures of both types of exochelins have yet to be elucidated (16). However, transport of iron by these exochelins has been analyzed and it has been suggested that iron-acquisition is not necessarily mediated by the cell-associated mycobactin (which would seem a logical intermediate) (367).

Organisms of the Gram-positive genus Bacillus produce siderophores. B. megaterium produce a hydroxamate-type siderophore which has been given the name schizokinen (214). This compound is closely related in structure to aerobactin (264). Transport of ferric-schizokinen in B. megaterium procedes by a shuttle mechanism in which the intact complex is transported into the cell. The iron is then removed, probably by reduction, and the deferri-siderophore then excreted (264). B. subtilis may also produce a hydroxymate-type siderophore; however, the phenolate 2,3-dihydroxy-N-benzolglycine is known to be produced and effectively utilized for iron-acquisition by this organism (264).

F. IRON-ACQUISITION BY *NEISSERIA* SPP.

There have been a number of studies attempting to link virulence of *Neisseria* spp. with it's ability to utilize iron. Payne and Finkelstein (287) found that when avirulent gonococci (non-piliated colonial variants) were inoculated along with iron into chick embryos, their virulence increased. No increase in virulence was observed when the more virulent piliated isogenic variants were used to infect the chick embryo model. They suggested that iron might compensate for the absence of a virulence factor in non-piliated variants by interfering with the chick embryo defense system. The validity of this model as a measure of gonococcal virulence is questionable and consequently the results must be interpreted with appropriate reservations. Using a mouse model, Holbein *et al.* (166) found that LD_{50} values for some strains of *N. meningitidis* could be decreased 10^9-fold (i.e., virulence enhanced) by the addition of iron-dextran to the inoculum. However, the LD_{50} of other strains were not affected by the addition of iron-dextran. This finding substantiated the report by Payne and Finkelstein (287) in terms of iron-enhanced virulence of gonococci.

Odugbemi *et al.* (280) analyzed the iron content of isogenic gonococcal colonial variants and found that there was a lower iron content in virulent, piliated colony types than in avirulent, non-piliated variants. In another study, Odugbemi *et al.* (281) suggested that the rate of reversion of non-piliated gonococcal variants to piliated variants could be influenced by the concentration of iron in the medium as well as by the addition of various

iron-chelators. Intermediate levels of iron and the weak chelators nitrilotriacetic acid, ammonium citrate, and glutamic acid were reported to favor reversion.

Acquisition of iron by N. meningitidis in vitro was studied by Archibald and DeVoe (11, 13), Archibald et al. (14), and Simonson et al. (354). These closely related studies are summarized in the following description. Meningococci could be grown in batch culture using a defined medium (NDM) in which iron was the limiting nutrient. Using this defined medium in conjunction with continuous culture, the minimum requirement of iron was determined to be ca. 2.5×10^{-9} nanograms per cell. This value was ca. 10% of the maximum amount of iron which could be accumulated by each cell (i.e., the amount of iron accumulated per cell when iron was in excess). Total heme iron (e.g., that which is present in compounds such as cytochromes, oxidases, catalases) was decreased by 2-fold under iron-privation. Non-heme iron which comprises the large majority of cellular iron and which is present in compounds that include a wide variety of storage, transport, and catalytic molecules was decreased by ca. 10-fold. The difference between the decrease in the non-heme iron (10-fold reduction) and the decrease in the heme iron (2-fold reduction) suggests that a priority is given to respiration during growth in a low-iron environment.

In examining the iron-uptake system of meningococci grown under iron-privation, it was found that acquisition of iron from $FeCl_3$ was a biphasic process. The initial energy-independent uptake was rapid, occuring within the first minute. A secondary energy-dependent uptake was much slower and was sensitive to cyanide, antimycin, and the proton ionophore

tetrachlorosalicylanilide. Meningococci also internalized iron complexed to the chelators citrate and nitriloacetate in a biphasic manner. Heating of active and cyanide-treated iron-starved cells at 60° C for five minutes completely inactivated the ferric-citrate mediated energy-independent iron uptake system. On the other hand, binding of $FeCl_3$ was not completely inactivated by heating at 60° C for 5 min, suggesting that a heat-labile protein(s) might be involved.

Iron could be removed from citrate by isolated outer membranes obtained from cells propagated in low-iron medium. Removal of iron from the ferric-citrate complex was energy-independent; citrate was not bound by the membranes concominantly with the iron that it had previously been complexed with. Of the total ^{55}ferric-citrate associated with these membranes, ca. 70% was resistant to Sarkosyl extraction, suggesting that the radioactivity was associated with the outer membrane. The majority of labeled-iron (88%) appeared to be bound to protein and not lipid. Separation of membrane components by SDS-polyacrylamide gel electrophoresis after their exposure to ^{55}ferric-citrate determined that nearly all the radioactivity migrated at a position corresponding to the meningococcal (strain SD1C) major outer membrane protein (apparent molecular weight, 36,500). It was suggested that this protein band observed in the gel was not a single polypeptide species, but rather a comigrating complex of different proteins. Cells maintained in an iron-replete environment were unable to remove ^{55}Fe from ferric-citrate. The authors speculated that this was due to saturation of the iron-binding sites by excess unlabeled iron which was present in the medium used. It is equally possible that this could be due to an induction of an iron-uptake

mechanism in a low-iron environment.

Several compounds were tested with respect to their ability to donate iron using a plate bioassay in which meningococci were seeded into low-iron medium. The different iron-binding compounds tested are listed in Table 1. The authors suggested that meningococci possessed a high-affinity iron-acquisition system with a predilection for phosphate ester or carboxylic acid-bound iron.

Simonson _et al_. (353) described a high-affinity mechanism for the acquisition of transferrin-bound iron by meningococci. Previous to this, Archibald and DeVoe (12) had shown that _N. meningitidis_ could utilize the iron from transferrin. Utilization was dependent upon contact between the organism and the transferrin molecule. This system appeared to involve an energy-independent, saturable binding to the meningococcal cell surface. This binding was not discriminate between deferrated or ferrated-transferrin. The unloading of the transferrin-bound iron was both energy- and pH-dependent (i.e., cells grown at pH 6.6 had a greater capacity to acquire ^{55}Fe from transferrin than cells grown at pH 7.2) (353).

Yancey and Finkelstein (116, 434, 435) analyzed the ability of _Neisseria_ spp. to assimilate iron. Using a number of well-characterized microbial siderophores, they found that only ferrated-dihydroxymate derivatives such as schizokinen, aerobactin, and arthrobactin could stimulate growth of gonococci and meningococci in an iron-restricted environment. Furthermore, they went on to partially characterize the putative siderophore activity found in meningococcal and gonococcal culture supernatants. The criterion for assessing this activity was the ability of spent supernatants to crossfeed

Arthrobacter flavescens strain JG-9 (an organism which does not produce a siderophore, yet can utilize ferrated-hydroxymate derivatives or hemin as an iron source). This report initially provided exciting evidence for a neisserial siderophore-mediate iron-uptake system in Neisseria spp. However, other researchers have been unable to demonstrate the existance of a siderophore. Norrod and Williams (275) could were unable to detect siderophore activity produced by gonococci grown in defined medium containing EDDA. Likewise, Archibald and DeVoe (13) found no evidence for siderophore activity in meningococci. The descrepency in these reports may have been explained recently by West et al. (424) who ascribed siderophore activity to a contaminating component of the complex growth medium (probably from proteose peptone). While no assay can prove the absence of a siderophore, the accumulating body of evidence presently does not support the existence of a "conventional" siderophore-mediated iron-uptake system among the pathogenic Neisseria spp.

Mickelsen and Sparling(247) and Mickelsen et al. (248) examined the ability of Neisseria spp. to obtain iron from various compounds. In particular, they examined human iron-binding. A compilation of these results are included in Table 1. In general, all strains of gonococci and meningococci utilize iron bound to transferrin. Two commensal species, N. lactamica and N. flavescens could also utilize the iron from transferrin and human serum. Other commensal Neisseria spp. could not obtain iron from transferrin and were variable in their ability to obtain iron from human serum. None of the Neisseria spp. tested could utilize conalbumin as an iron source. They also found that 100% of meningococcal isolates, 53% of

unselected gonococcal isolates, and 24% of the strains from the commensal Neisseria spp. could utilize lactoferrin as an iron source. The ability to obtain iron from lactoferrin by gonococci did not correlate with the presence or absence of any detectable plasmid. However, it did correlate reasonably well with auxotype; 86% (19 of 22 tested) of nutritionally prototrophic gonococci could utilize lactoferrin-bound iron, whereas only 14% (4 of 28 tested) of auxotrophic strains requiring arginine, hypoxanthine, and uracil (AHU) were able to obtain iron from lactoferrin. The inability of AHU^{-} gonococci to utilize iron bound to lactoferrin has been suggested to result in the high association of these strains with asymptomatic infection (40).

Norqvist et al. (274) examined the outer membrane protein profiles of gonococci grown in low-iron medium and in regular (iron not limiting) medium. The presence of proteins with molecular weights of between 70,000 and 100,000 were observed in Sarkosyl-insoluble cell envelopes from cells grown in low-iron medium when compared to cells grown in regular medium. These proteins were suppressed by the addition of iron to the medium followed by several generations of growth. Production of these proteins was not due to a decreased growth rate in the low-iron medium. These iron-regulated proteins varied between strains with respect to both relative migration and the number produced. Only one protein, migrating with an apparent molecular weight of 97,000 appeared to be conserved among all gonococcal strains that were analyzed. A subsequent report by Magnusson et al. (226) found alterations in the surface charge on gonococci grown in low-iron medium. It was suggested that alterations in surface charge might be due, in part, to the expression of new proteins produced in the low-iron environment.

The observations regarding the presence of iron-regulated proteins gonococci were confirmed by Mietzner et al. (249). In addition, lower molecular weight iron-regulated proteins were also detected in the Sarkosyl-insoluble cell envelopes of N. gonorrhoeae. In particular, an iron-regulated protein was identified which had an apparent molecular weight of 37,000. While most of the other iron-regulated proteins exhibited intrastrain variation, the 37,000 dalton protein was common to all strains examined. Peptide map analysis of this protein from two unrelated gonococcal strains suggested that the iron-regulated 37,000 dalton proteins were identical. West et al. (424) analyzed the iron-regulated proteins in the crude cell envelopes on N. gonorrhoeae. This report confirmed the previous studies regarding the expression of gonococcal iron-regulated proteins. In addition, the expression of these proteins during growth of gonococci in the presence of several iron-sources was examined. It was found that most of the iron-regulated gonococcal proteins were not coordinately regulated. However, the common iron-regulated 36,000 dalton protein (37,000 daltons in previous reports) was expressed under all conditions of iron-limitation. The expression of this protein in the presence of all iron-sources examined and in all strains examined prompted speculation that the 36,000 dalton iron-regulated protein might play a key role in iron-acquisition by gonococci.

Other mechanisms of iron-acquisition can be found in the literature. In addition to pathogenic bacteria, environmental isolates and higher organisms such as fungi and parasities also exhibit high affinity iron-sequestration systems. A unifying theme from the extensive literature on iron-related metabolism is that the dependence of living organisms on the trace mineral iron has resulted in the development of mechanisms which ensure the acquisition of this element.

Table 1. Summary of ferrated-compounds which pathogenic *Neisseria* spp. can utilize as an iron source *in vitro*.

COMPOUND	REPORTING REFERENCE *N. meningitidis*	*N. gonorrhoeae*
Chemical iron chelators		
1. Nitriloacetate	13	
2. Citrate	248, 13	248
3. *cis*-Aconitate	13	
4. Isocitrate	13	
5. Malate	13	
6. Oxalate	13	
7. Pyruvate	13	
8. Pyrophosphate	13	
9. Adenosine triphosphate	13	
10. Adenosine diphosphate	13	
Microbial siderophores		
1. Rhodotorulic acid	13	
2. Enterochelin	434, 13	434
3. Albomycin	13	
4. Schizokinen	434	434
5. Arthrobactin	434	434
6. Aerobactin	434	434
Iron-containing proteins		
1. Hemoglobin	248, 13	248 (v)[a]
2. Hemin	248	248
3. Ferritin	13	
4. Transferrin	13, 248	248
5. Lactoferrin	247	247 (v)

a. The symbol v indicates that there were strain-to-strain variations in the ability of this species to utilize iron from this compound.

REFERENCES

1. Aasa, R., B.G. Malmstrom, P. Saltman, and T. Vanngard. 1963. The specific binding of iron (III) and copper (II) to transferrin and conalbumin. Biochim. Biophys. Acta. 75: 202-223.

2. Actis, L.A., S.A. Potter, and J.H. Crosa. 1985. Iron-regulated outer membrane protein OM2 of Vibrio anguillarum is encoded by virulence plasmid pJM1. J. Bacteriol. 161: 736-742.

3. Aisen, P., and A. Leibman. 1972. Lactoferrin and transferrin: A comparative study. Biochim. Biophys. Acta. 257: 341-323.

4. Alexander, H.E., and W. Redman. 1953. Transformation of type-specificity of meningococci. Change in heritable type induced by type-specific extracts containing desoxyribonucleic acid. J. Exp. Med. 97: 797-806.

5. Andrus, C.R.., M.A. Walter, J.H. Crosa, and S.M. Payne. 1983. Synthesis of siderophores by pathogenic Vibrio species. Curr. Microbiol. 9: 209-214.

6. Ankenbauer, R., S. Sriyosachati, and C.D. Cox. 1985. Effects of siderophores on the growth of Pseudomonas aeruginosa in human serum and transferrin. Infect. Immun. 49: 132-140.

7. Apicella, M.A. 1974. Antigenically distinct populations of Neisseria gonorrhoeae: Isolation and characterization of the responsible determinants. J. Infect. Dis. 130: 619-625.

8. Apicella, M.A. 1976. Serogrouping of Neisseria gonorrhoeae:

Identification of four immunologically distinct acidic polysaccharides. J. Infect. Dis. 134: 377-383.

9. Apicella, M.A., K.M. Bennett, C.A. Hermerath, and D.E. Roberts. 1981. Monoclonal antibody analysis of lipopolysaccharide from Neisseria gonorrhoeae and Neisseria meningitidis. Infect. Immun. 34: 751-756.

10. Apicella, M.A., and N. Gagliardi. 1979. Antigenic heterogeneity of the of the non-serogroup antigen structure of the lipopolysaccharides of Neisseria gonorrhoeae. Infect. Immun. 26: 870-874.

11. Archibald, F.S., and I.W. DeVoe. 1978. Iron in Neisseria meningitidis: Minimum requirements, effects of limitation, and characteristics of uptake. J. Bacteriol. 136: 35-48.

12. Archibald, F.S., and I.W. DeVoe. 1979. Removal of iron from human transferrin by Neisseria meningitidis. FEMS Microbiol. Lett. 6: 159-162.

13. Archibald, F.S., and I.W. Devoe. 1980. Iron acquisition by Neisseria meningitidis in vitro. Infect. Immun. 27: 322-334.

14. Archibald, F.S., C. Simonson, and I.W. DeVoe. 1981. Comparison of iron binding and uptake from $FeCl_3$ and Fe-citrate by Nesseria meningitidis. Can. J. Microbiol. 27: 1066-1070.

15. Ashford, W.A., R.G. Golash, and V.G. Hemming. 1976. Penicillinase-producing Neisseria gonorrhoeae. Lancet. 2: 657-658.

16. Barclay, R., and C. Ratledge. 1983. Iron-binding compounds of Mycobacterium avium, M. intracellulare, M. scrofulaceum, and mycobactin-dependent M. paratuberculosis and M. avium. J. Bacteriol. 153: 1138-1146.

17. Barnes, R.C., and K.K. Holmes. 1984. Epidemiology of gonorrhea: Current perspectives. Epidemiologic. Rev. 6: 1-30.

18. Baron, E.S., A.K. Saz, D.J. Kopecko, and J.A. Wohlhieter. 1977. Transfer of plasmid-borne beta-lactamase in Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 12: 270-280.

19. Barrera, O., and J. Swanson. 1984. Proteins IA and IB exhibit different surface exposures and orientations in the outer membranes of Neisseria gonorrhoeae. Infect. Immun. 44: 565-568.

20. Bennett, R.L., and L.I. Rothfield. 1976. Genetic and physiological regulation of intrinsic proteins of the outer membrane of Salmonella typhimurium. J. Bacteriol. 127: 498-504.

21. Bergstrom, S., L. Norlander, A. Norqvist, and S. Normark. 1978. Contribution of a TEM-1-like beta-lactamase to penicillin-resistance in Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 13: 618-623.

22. Bindereiff, A., V. Braun, and K. Hantke. 1982. The cloacin receptor of ColV-bearing Escherichia coli is part of the Fe^{3+}-aerobactin transport system. J. Bacteriol. 150: 1472-1475.

23. Bindereiff, A., and J.B. Neilands. 1983. Cloning of the aerobactin-mediated iron assimilation system of plasmid ColV. J. Bacteriol. 153: 1111-1113.

24. Bindereiff, A., and J.B. Neilands. 1985. Aerobactin genes in clinical isolates of Escherichia coli. J. Bacteriol. 161: 727-735.

25. Biswas, G.D., E.Y. Blackman, and P.F. Sparling. 1980. High-frequency conjugal transfer of a gonococcal penicillinase plasmid. J. Bacteriol. 143: 1318-1324.

26. Biswas, G.D., J.F. Graves, T.E. Sox, F.C. Tenover, and P.F. Sparling. 1982. Marker rescue by a homologous recipient plasmid during transformation of gonococci by a hybrid Pc^r plasmid. J. Bacteriol. 151: 77-82.

27. Biswas, G.D., T. Sox, E. Blackman, and P.F. Sparling. 1977. Factors affecting genetic transformation of Neisseria gonorrhoeae. J. Bacteriol. 129: 983-992.

27. Biswas, G.D., and P.F. Sparling. 1981. Entry of double-stranded deoxyribonucleic acid during transformation of Neisseria gonorrhoeae. J. Bacteriol. 145: 638-640.

28. Bjorin-Rasmussen, L. Hallberg, B. Isaksson, and B. Arvidsson. 1974. Food iron absorption in man: Applications of the two-pool extrinsic tag method to measure heme and nonheme iron absorption from the whole diet. J. Clin. Invest. 53: 247-255.

29. Black, W.J., and J.G. Cannon. 1985. Cloning of the gene for the common pathogenic Neisseria H.8 antigen of Neisseria gonorrhoeae. Infect. Immun. 47: 322-325.

30. Black, W.J., R.S. Schwalbe, I. Nachamkin, and J.G. Cannon. 1984. Characterization of Neisseria gonorrhoeae protein II phase variation by use of monoclonal antibodies. Infect. Immun. 45: 453-457.

31. Blake, M.S., and E.C. Gotschlich. 1982. Purification and partial characterization of the major outer membrane protein of Neisseria gonorrhoeae. Infect. Immun. 36: 277-283.

32. Blake, M.S., and Gotschlich, E.C. 1983. Gonococcal membrane proteins: Speculations on their role in pathogenesis. Prog. Allergy 33: 298-313.

33. Blake, M.S., and E.C. Gotschlich. 1984. Purification and partial characterization of the opacity-associated proteins of Neisseria gonorrhoeae. J. Exp. Med. 159: 452-462.

34. Blake, M.S., E.C. Gotschlich, and J. Swanson. 1981. Effects of proteolytic enzymes on the outer membrane proteins of Neisseria gonorrhoeae. Infect. Immun. 33: 212-222.

35. Blake, M.S., K.K. Holmes, and J. Swanson. 1979. Studies on gonococcus infection. XVII. IgA_1-cleaving protease in vaginal washings from women with gonorrhoea. J. Infect. Dis. 139: 89-92.

36. Blake, M.S., and J. Swanson. 1978. Studies on gonococcus infection. XVI. Purification of Neisseria gonorrhoeae immunoglobulin A_1 protease. Infect. Immun. 22: 350-358.

37. Braun, V. 1981. E. coli cells containing the plasmid ColV produce the iron ionophore aerobactin. FEMS Microbiol. Lett. 11: 225-228.

38. Braun, V., R. Burkhardt, R. Schneider, and L. Zimmerman. 1982. Chromosomal genes for ColV plasmid-determined iron (III)-aerobactin transport in Escherichia coli. J. Bacteriol. 151: 553-559.

39. Brinton, C.C., J. Bryan, J. Dillon, N. Guerina, L.J. Jacobson, A. Labik, S. Lee, A. Levine, S. Lim, J. McMichael, S. Polen, K. Rogers, A.C. To, and C.C. To. 1978. Uses of pili in gonorrhea control: Role of bacterial pili in disease, purification and proterties of gonococcal pili, and progress in the development of a gonococcal pilus vaccine for gonorrhea, pp. 155-178, In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunobiology of Neisseria gonorrhoeae. American Society for Microbiology, Washington, D.C.

40. Britigan, B.E., M.S. Cohen, and P.F. Sparling. 1985. Gonococcal infection: A model of molecular pathogenesis. N. Eng. J. Med. 312: 1683-1694.

41. Brooks, G.F., C.J. Lammel, B.H. Petersen, and D.P. Stites. 1981. Human seminal plasma inhibition of antibody complement-mediated killing and opsonization of Neisseria gonorrhoeae and other Gram-negative organisms. J. Clin. Invest. 67: 1523-1531.

42. Brown, M.R.W., A. Hosmin, and P.A. Lambert. 1984. Evidence that mucoid Pseudomonas aeruginosa in the cystic fibrosis lung grows under iron-restricted conditions. FEMS Microbiol. Lett. 21: 113-117.

43. Buchanan, T.M. 1975. Antigenic heterogeneity of gonococcal pili. J. Exp. Med. 141: 1470-1475.

44. Buchanan, T.M., and J.F. Hildebrandt. 1981. Antigen-specific serotyping of Neisseria gonorrhoeae: Characterization based upon principal outer membrane protein. Infect. Immun. 32: 985-994.

45. Buchanan, T.M., and W.A. Pearce. 1976. Pili as a mediator of the attachment of gonococci to human erythrocytes. Infect. Immun. 13: 1483-1489.

46. Buchanan, T.M., W.A. Pearce, and K.C.S. Chen. 1978. Attachment of Neisseria gonorrhoeae pili to human cells, and investigations of the chemical nature of the receptor for gonococcal pili. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunobiology of Neisseria gonorrhoeae. American Society for Microbiology, Washington, D.C.

47. Buchanan, T.M., J. Swanson, K.K. Holmes, S.J. Kraus, and E.C. Gotschlich. 1973. Quantitative determination of antibody to gonococcal pili. Changes in antibody levels with gonococcal infection. J. Clin. Invest. 52: 2896-2909.

48. Buck, P., and R.F. Rest. 1981. Effects of human neutrophil granule extracts on macromolecular synthesis in Neisseria gonorrhoeae. Infect. Immun. 33: 426-433.

49. Bullen, J.J. 1981. The significance of iron in infection. Rev. Infect. Dis. 3: 1127-1138.

50. Bullen, J.J., H.J. Rogers, and E. Griffiths. 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. 80: 1-35.

51. Bumm, E. 1885. Der mikro-organismus der gonorrhoischem schleimhaut-erkrankungen 'Gonococcus-Neisser', Wiesb., 146, 4 pl.

52. Bundle, D.R. 1979. Antibody to an artificial disaccharide antigen cross-reactive with Neisseria gonorrhoeae lipopolysaccharide. Can. J. Biochem. 57: 367-71.

53. Mills, J., and G.F. Brooks. 1984. Disseminated gonococcal infection, pp. 229-237. In K.K. Holmes, P. Mardh, P.F. Sparling, and P.J. Wiesner (eds.), Sexually Transmitted Diseases. McGraw-Hill Book Company, New York.

54. Campbell, L.A., and R.E. Yasbin. 1984. Mutagenesis of Neisseria gonorrhoeae: Absence of error-prone repair. J. Bacteriol. 160: 288-293.

55. Campbell, L.A., and R.E. Yasbin. 1984. A DNA excision repair system for Neisseria gonorrhoeae. Mol. Gen. Genet. 193: 561-563.

56. Cannon, J.G., W.J. Black, I. Nachamkin, and P.W. Stewart. 1984. Monoclonal antibody that recognizes an outer membrane antigen common to the pathogenic Neisseria but not to most nonpathogenic Neisseria species. Infect. Immun. 43: 994-999.

57. Cannon, J.G., D.G. Klapper, E.Y. Blackman, and P.F. Sparling. 1980. Genetic locus (nmp-1) affecting the principal outer membrane protein of Neisseria gonorrhoeae. J. Bacteriol. 143: 847-851.

58. Cannon, J.G., and P.F. Sparling. 1984. The genetics of the gonococcus. Ann. Rev. Microbiol. 38: 111-133.

59. Casey, S.G., W.M. Schafer, and J.K. Spitznagel. 1985. Anaerobiosis increases resistance of Neisseria gonorrhoeae to O_2-independent antimicrobial proteins from human polynuclear granulocytes. Infect. Immun. 47: 401-407.

60. Casey, S.G., D.R. Veale, and H. Smith. 1979. Demonstration of intracellular growth of gonococci in human phagocytes using spectinomycin to kill extracellular organisms. J. Gen. Microbiol. 113: 395-398.

61. Casey, S.G., D.R. Veale, and H. Smith. 1980. Intracellular survival of Neisseria gonorrhoeae in human urethral exudate. FEMS Microbiol. Lett. 8: 97-100.

62. Casey, S.G., D.R. Veale, and H. Smith. 1983. Cytotoxicity of Neisseria gonorrhoeae for human peripheral blood phagocytes. J. Gen. Microbiol. 129: 1097-1102.

63. Catlin, B.W. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. J. Infect. Dis. 128: 178-194.

64. Cavill, I., and C. Ricketts. 1980. Human iron kinetics, p. 573-604. In A. Jacobs and M. Worwood (eds.), Iron in biochemistry and medicine, Vol. II. Academic Press, Inc., New York.

65. Centers for Disease Control. 1982. Gonorrhea. Sexually transmitted disease (STD) fact sheet no. 35. Atlanta, GA. HHS, CDC publication 81-8195.

66. Cohen, I.R. 1967. Natural and immune human antibodies reactive with antigens of virulent Neisseria gonorrhoeae: Immunoglobulins G, M, and A. J. Bacteriol. 94: 141-148.

67. Collins, M.P.L., and M.R. Salton. 1980. Preparation and crossed immunoelectrophoresis of cytoplasmic and outer membrane fractions from Neisseria gonorrhoeae. Infect. Immun. 30: 281-288.

68. Connelly, M.C., and P.Z. Allen. 1983. Chemical and immunochemical studies on lipopolysaccharides from pyocin 103 sensitive and resistant Neisseria gonorrhoeae. Carbohydr. Res. 120: 171-186.

69. Cooper, M.D., Z.A. McGee, M.H. Mulks, J.M. Koomey, and T.L. Hindman. 1984. Attachment to and invasion of human fallopian tube mucosa by an IgA_1 protease-deficient mutant of Neisseria gonorrhoeae and its wild-type parent. J. Infect. Dis. 150: 737-744.

70. Coulton, J.W., and V. Braun. 1979. Protein II* influences ferrichrome-iron transport in Escherichia coli K12. J. Gen. Microbiol. 110: 211-220

81. Cox, C.D. 1980. Iron reductase from Pseudomonas aeruginosa. J. Bacteriol. 141: 199-204.

82. Cox, C.D. 1980. Iron uptake with ferripyochelin and ferric-citrate by Pseudomonas aeruginosa. J. Bacteriol. 142: 581-587.

83. Cox, C.D., and P. Adams. 1985. Siderophore activity of pyoverdin for Pseudomonas aeruginosa. Infect. Immun. 48: 130-138.

84. Cox, C.D., and R. Graham. 1979. Isolation of an iron-binding compound from Pseudomonas aeruginosa. J. Bacteriol. 137: 357-364.

85. Cox, C.D., K.L. Rinehart, M.L. Moore, and J.C. Cook. Pyochelin: Novel structure of an iron-chelating growth promoter for Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA. 78: 4256-4260.

86. Crawford, G., J.S. Knapp, J. Hale, K.K. Holmes. 1975. Asymptomatic gonorrhea in men caused by gonococci with unique nutritional requirements. Science 196: 1352-1353.

87. Crosa, J.H. 1980. A plasmid associated with virulence in the marine fish Vibrio anguillarum specifies an iron-sequestering system. Nature 283: 566-568.

88. Crosa, J.H. 1984. The relationship of plasmid-mediated iron transport and bacterial virulence. Ann. Rev. Microbiol. 38: 69-89.

89. Crosa, J.H., and L.L. Hodges. 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen Vibrio anguillarum 775. Infect. Immun. 31: 223-227.

90. Crosa, J.H., L.L. Hodges, and M.H. Schiewe. 1980. Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen Vibrio anguillarum. Infect. Immun. 27: 897-902.

91. Crosa, J.H., M.H. Schiewe, and S. Falkow. 1977. Evidence for plasmid contribution to the virulence of the fish pathogen Vibrio anguillarum. Infect. Immun. 18: 509-513.

92. Daly, J.A., T.J. Lee, J.K. Spitznagel, and P.F. Sparling. 1982. Gonococci with mutations to low level pneicillin resistance exhibit increased sensitivity to the oxygen-independent bactericidal activity of human polymorphonuclear leukocyte granule extracts. Infect. Immun. 35: 826-833.

93. De Hormaeche, R.D., M.J. Thornley, and A.M. Glauert. 1978. Demonstration by light and electron microscopy of capsules on gonococci recently grown in vivo. J. Gen. Microbiol. 106: 31-91.

94. Densen, P., and G.L. Mandell. 1978. Gonococcal interactions with polymorphonuclear neutrophils: Importance of the phagosome for bacteriocidal activity. J. Clin. Invest. 62: 1161-1171.

95. Densen, P., L. MacKeen, and R.A. Clark. 1980. Gonococci causing uncomplicated gonorrhea or disseminated gonococcal infection differ in stimulation of neutrophil chemotaxis and phagocytosis, pp. 237-239. In D. Danielsson, S. Normark (eds.), Genetics and Immunobiology of Pathogenic Neisseria, University of Umea, Sweden.

96. Densen, P., L.A. MacKeen, and R.A. Clark. 1982. Dissemination of gonococcal infection is associated with delayed stimulation of complement-dependent neutrophil chemotaxis in vitro. Infect. Immun. 38: 563-572.

97. DeVoe, I.W., and J.E. Gilchrist. 1973. Release of endotoxin in the form of cell wall blebs during in vitro growth of Neisseria meningitidis. J. Exp. Med. 138: 1156-1167.

98. DeVoe, I.W., and J.E. Gilchrist. 1976. Localization of tetramethylphenylenediamine-oxidase in the outer cell wall layer of Neisseria meningitidis. J. Bacteriol. 128: 144-148.

99. Diena, B.B., F.E. Ashton, and M.B. Perry. 1979. Type 14 pneumococcal maccine for prevention of gonorrhea. Lancet 207: 1037.

100. Diena, B.B., F.E. Ashton, A. Ryan, R. Wallace, and M.B. Perry. 1978. The lipopolysaccharide (R-type) as a common antigen of Neisseria gonorrhoeae. I. Immunizing properties. Can. J. Microbiol. 24: 117-123.

101. Dilworth, J.A., J.O. Hendley, and G.L. Mandell. 1975. Attachment and ingestion of gonococci by human neutrophils. Infect. Immun. 11: 512-516.

102. Dougherty, T.J., A. Asmus, and A. Tomaz. 1979. Specificity of DNA uptake in genetic transformation of gonococci. Biochem. Biophys. Res. Comm. 86: 97-104.

103. Douglas, J.T., M.D. Lee, and H. Nikaido. 1981. Protein I of Neisseria gonorrhoeae outer membrane is a porin. FEMS Microbiol. Lett. 12: 305-309.

104. Draper, D.L., J.F. James, G.F. Brooks, and R.L. Sweet. 1980. Comparison of virulence markers of peritoneal and fallopian tube isolates with endocervical Neisseria gonorrhoeae isolates from women with acute salpingitis. Infect. Immun. 27: 882-888.

105. Eaton, J. W., P. Brandt, J.R. Mahoney, and J.T. Lee, Jr. 1982. Haptoglobin: a natural bacteriostat. Science. 215: 691- 693.

106. Eisenstein, B.I., G.D. Biswas, E. Blackman, and P.F. Sparling. 1977. Conjugal transfer of the gonococcal penicillinase plasmid. Science 195: 998-1000.

107. Eisenstein, B.L., and P.F. Sparling. 1978. Mutations to increased antibiotic sensitivity in naturally-occurring gonococci. Nature 271: 242-244.

108. Elin, R.J., and S.M. Wolff. 1976. Biology of endotoxin. Ann. Rev. Med. 27: 127-141.

109. Elwell, L.P., and S. Falkow. 1977. Plasmids of the genus Neisseria, pp. 138-154. In R.B. Roberts (ed.), The Gonococcus. John Wiley & Sons, Inc., New York.

110. Elwell, L.P., M. Roberts, L.W. Mayer, and S. Falkow. Plasmid-mediated beta-lactamase production in Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 11: 528-533.

111. Engelkirk, P.G., and D.E. Schoenhard. 1973. Physical evidence of a plasmid in Neisseria gonorrhoeae. J. Infect. Dis. 127: 197-202.

112. Eschenbach, D.A., T.M. Buchanan, H.M. Pollock, P.S. Forsyth, E.R. Alexander, J. Lin, S. Wang, B. Wentworth, W.M. McCormack, and K.K. Holmes. 1975. Polymicrobial etiology of pelvic inflamatory disease. N. Eng. J. Med. 293: 166-177.

113. Evans, B.A. 1977. Ultrastructural study of cervical gonorrhea. J. Infect. Dis. 136: 248-255.

114. Fecker, L., and V. Braun. 1983. Cloning and expression of the fhu genes involved in iron (III)-hydroxymate uptake by Escherichia coli. J. Bacteriol. 156: 1301-1314.

115. Filip, C., G. Fletcher, J.L. Wulff, and C.F. Earhart. 1973. Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115: 717-722.

116. Finkelstein, R.A., and R.J. Yancey. 1981. Effect of siderophores on virulence of Neisseria gonorrhoeae. Infect. Immun. 32: 609-613.

117. Fiss, E.H., P. Stanley-Samuelson, and J.B. Neilands. 1982. Properties and proteolysis of ferric-enterobactin outer membrane receptors in Escherichia coli K12. Biochemistry 21: 4517-4527.

118. Fitzgerald, T.J., and S.A. Morse. 1976. Alteration of growth, infectivity and viability of Neisseria gonorrhoeae by gonadal steroids. Can. J. Microbiol. 22: 286-294.

119. Fletcher, J., and E.R. Huehns. 1968. Function of transferrin. Nature 218: 1211-1214.

120. Fransson, G.B., and B. Lonnerdal. 1980. Iron in human milk. J. Pediatrics 96: 380-384.

121. Frost, G.E., and H. Rosenberg. 1973. The inducible citrate-dependent iron transport system in Escherichia coli K12. Biochim. Biophys. Acta. 330: 90-101.

122. Gibbs, D.L., and R.B. Roberts. 1975. The interaction in vitro between human polymorphonuclear leukocytes and Neisseria gonorrhoeae cultivated in the chick embryo. J. Exp. Med. 141: 155-171.

123. Gibson, F., and D.J. Magrath. 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by Aerobacter aerogenes 62-1. Biochim. Biophys. Acta 192: 175-184.

124. Glynn, A.A., and M.E. Ward. 1970. Nature and heterogeneity of the antigens of Neisseria gonorrhoeae involved in the serum bactericidal reaction. Infect. Immun. 2: 162-168.

125. Graves. J.F., G.D. Biswas, and P.F. Sparling. 1982. Sequence-specific DNA uptake in transformation of Neisseria gonorrhoeae. J. Bacteriol. 152: 1071-1077.

126. Greco, F., M.S. Blake, E.C. Gotschlich, and A. Mauro. 1980. Major outer membrane protein of N. gonorrhoeae forms channels in lipid bilayer membranes. Fed. Proceed. 39: 1813.

127. Gregg. C.R., M.A. Melly, C.C. Hellerqvist, J.G. Coniglio, and Z.A. McGee. 1981. Toxic activity of purified lipopolysaccharide of Neisseria gonorrhoeae for human fallopian tube mucosa. J. Infect. Dis. 143: 432-439.

128. Grewal, K.K., P.J. Warner, and P.H. Williams. 1982. An inducible outer membrane protein involved in aerobactin-mediated iron transport by ColV strains of Escherichia coli. FEBS Lett. 140: 27-30.

129. Griffiths, E., and J. Humphereys. 1980. Isolation of enterochelin from the peritoneal washings of guinea pigs lethally infected with Escherichia coli. Infect. Immun. 28: 286-289.

130. Griffiths, E., P. Stevenson, T.L. Hale, and S.B. Formal. 1985.

Synthesis of aerobactin and a 76,000-dalton iron-regulated outer membrane protein by Escherichia coli K12-Shigella flexnieri hybrids and by enteroinvasive strains of Escherichia coli. Infect. Immun. 49: 67-71.

131. Griffiths, E., P. Stevenson, and P. Joyce. 1983. Pathogenic Escherichia coli express new outer membrane proteins when growing in vivo. FEMS Microbiol. Lett. 16: 95-99.

133. Guterman, S.K., and S.E. Luria. 1969. Escherichia coli: Strains that excrete an inhibitor of colicin B. Science 164: 1414.

134. Guymon, L.F., M. Esser, and W.M. Schafer. 1982. Pyocin-resistant lipopolysaccharide mutants of Neisseria gonorrhoeae: Alterations in sensitivity to normal human serum and polymyxin B. Infect. Immun. 36: 541-547.

135. Guymon, L.F., D.L. Walstad, and P.F. Sparling. 1978. Cell envelope alterations in antibiotic-sensitive and -resistant strains of Neisseria gonorrhoeae. J. Bacteriol. 136: 391-401.

136. Hagblom, P., E. Segal, E. Billyard, and M. So. 1985. Intragenic recombination leads to pilus antigenic variation in Neisseria gonorrhoeae. Nature 315: 156-158.

137. Hancock, R.E.W., K. Hantke, and V. Braun. 1976. Iron transport in Escherichia coli K-12: Involvement of the colicin B receptor and of a citrate-inducible protein. J. Bacteriol. 127: 1370-1375.

138. Handsfield, H.H. 1984. Gonorrhea and uncomplicated gonococcal infection, pp. 205-220. In K.K. Holmes, P. Mardh, P.F. Sparling, and P.J. Wiesner (eds.), Sexually Transmitted Diseases. McGraw-Hill Book Company, New York.

139. Hantke, K. 1983. Identification of an iron uptake system specific for coprogen and rhodotorulic acid in Escherichia coli K-12. Mol. Gen. Genet. 191: 301-306.

140. Hansen, M.V., and C.E. Wilde III. 1984. Conservation of peptide structure of outer membrane protein-macromolecular complex from Neisseria gonorrhoeae. Infect. Immun. 43: 839-845.

141. Harder, W. and L. Dijkhuizen. 1983. Physiologic responses to nutrient limitation. Ann. Rev. Microbiol. 37: 1-23.

142. Harkness, A.H. 1948. The pathology of gonorrhea. Brit. J. Venereal Dis. 24: 137-147.

143. Harriman, G.R., E.R. Podack, A.J. Braude, L.C. Corbeil, A.F. Esser, and J.G. Curd. 1982. Activation of complement by serum-resistant Neisseria gonorrhoeae: Assembly of the membrane attack complex without subsequent cell death. J. Exp. Med. 156: 1235-1249.

144. Hebeler, B.H., S.A. Morse, W. Wong, and F.E. Young. 1978. Evidence for peptidoglycan-associated protein(s) in Neisseria gonorrhoeae. Biochem. Biophys. Res. Comm. 81: 1011-1017.

145. Hebeler, B.H., W. Wong, S.A. Morse, and F.E. young. 1979. Cell envelope of Neisseria gonorrhoeae CS7: Peptidoglycan-protein complex. Infect. Immun. 23: 353-359.

146. Hebeler, B.H., and F.E. Young. 1975. Autolysis of Neisseria gonorrhoeae. J. Bacteriol. 122: 385-392.

147. Hebeler, B.H., and F.E. Young. 1976. Chemical composition and turnover of peptidoglycan in Neisseria gonorrhoeae. J. Bacteriol. 126: 1180-1185.

148. Hebeler, B.H., and F.E. Young. 1976. Mecnanisms of autolysis of Neisseria gonorrhoeae. J. Bacteriol. 126: 1186-1193.

149. Heckels, J.E. 1977. The surface properties of Neisseria gonorrhoeae. isolation of the major components of the outer membrane. J. Gen. Microbiol. 99: 333-341.

150. Heckels, J.E. 1979. The outer membrane of Neisseria gonorrhoeae: Evidence that protein I is a transmembrane protein. FEMS Microbiol. Lett. 6: 325-327.

160. Heckels, J.E., B. Blackett, J.S. Everson, and M.E. Ward. 1976. The influence of surface charge on the attachment of Neisseria gonorrhoeae to human cells. J. Gen. Microbiol. 96: 359-364.

161. Heckels, J.E., and J.S. Everson. 1978. The isolation of a new outer membrane protein from the parent strain of Neisseria gonorrhoeae P9. J. Gen. Microbiol. 106: 179-182.

162. Heffron, F., C. Rubens, and S. Falkow. 1975. Translocation of a plasmid DNA sequence which mediates ampicillin resistance: Molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. USA. 72: 3623-3627.

163. Hendley, J.O., K.R. Powell, R. Rodewald, H.H. Holzgrefe, and R. Lyles. 1977. Demonstration of a capsule on Neisseria gonorrhoeae. N. Eng. J. Med. 296: 608-611.

164. Hermodson, M.A., K.C.S. Chen, and T.M. Buchanan. 1978. Neisseria pili proteins: Amino-terminal amino acid sequences and identification of an unusual amino acid. Biochemistry 17: 442-445.

165. Hoke, C., and N.A. Vedros. 1982. Taxonomy of the neisseria: Deoxyribonucleic acid base composition, intraspecific transformation,

and deoxyribonucleic acid hybridization. Int. J. Sys. Bacteriol. 32: 57-66.

166. Holbein, B.E., K.W.F. Jericho, and G.C. Likes. 1979. Neisseria meningitidis infection in mice: Influence of iron, variations in virulence among strains, and pathology. Infect. Immun. 24: 545-551.

167. Hollifield, W.C., and J.B. Neilands. 1978. Ferric enterobactin transport system in Escherichia coli K-12. Extraction, assay, and specificity of the outer membrane receptor. Biochemistry 17: 1922-1928.

168. Holmes, K.K., and S.A. Morse. 1983. Gonococcal infection. In P.D. Hoeprich (ed.), Infectious Diseases, 3rd ed. Harper and Row, Inc., Hagerstown, MD.

169. Hoseith, S.K., and B.A.D. Stocker. 1981. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature 291: 238-239.

170. Hook, E.W., D.A. Olsen, and T.M. Buchanan. 1984. Analysis of antigen specificity of the human serum immunoglobin G response to complicated gonococcal infection. Infect. Immun. 43: 706-709.

171. Hussein, S., K. Hantke, and V. Braun. 1981. Citrate-dependent iron transport system in Escherichia coli K-12. Eur. J. Biochem. 117: 431-437.

172. Ison, C., A.A. Glynn, and S. Bascomb. 1982. Acquisition of new genes by oral neisseria. J. Clin. Pathol. 35: 1153-1157.

173. James-Holmquest, A.N., J. Swanson., T.M. Buchanan, R.D. Wende, and R.P. Williams. 1974. Differential attachment by piliated and nonpiliated Neisseria gonorrhoeae to human sperm. Infect. Immun. 9: 897-902.

174. James, A.N., R.D. Wende, and R.P. Williams. 1973. Variation in colonial morphology of Neisseria gonorrhoeae after growth on media containing antimicrobial agents. Appl. Microbiol. 26: 248-251.

175. James, A.N., and R.P. Williams. 1978. Chemotactic effect of Neisseria gonorrhoeae on polymorphonuclear leukocytes, pp. 236-238. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunobiology of Neisseria gonorrhoeae. American Society for Microbiology, Washington, D.C.

176. James, J.F., C.J. Lammel, D.L. Draper, D.A. Brown, R.L. Sweet, and G.F. Brooks. 1983. Gonococcal attachment to eukaryotic cells. Sex. Trans. Dis. 10: 173-179.

177. James, J.F., and J. Swanson. 1977. The capsule of the gonococcus. J. Exp. Med. 145: 1082-1086.

178. James, J.F., and J. Swanson. 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. Infect. Immun. 19: 332-340.

179. James, J.F., and J. Swanson. 1978. Color/opacity variants of Neisseria gonorrhoeae and their relationship to the menstrual cycle, pp. 338-343. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunobiology of Neisseria gonorrhoeae. American Society for Microbiology, Washington, D.C.

180. Jann, B., K. Reski, and K. Jann. 1975. Heterogeneity of lipopolysaccharides. Analysis of polysaccharide chain lengths by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Eur. J. Biochem. 60: 239-246.

181. Jephcott, A.E., A. Reyn, and A. Birch-Anderson. 1971. Neisseria

gonorrhoeae. III. Demonstration of presumed appendages to cells from different colony types. Acta Patho. Microbiol. Scand. Sect. B. 79: 437-439.

182. Johnson, A.P., D. Taylor-Robinson, and Z.A. McGee. 1977. Species specificity of attachment and damage to oviduct mucosa by Neisseria gonorrhoeae. Infect. Immun. 18: 833-839.

183. Johnson, A.P., D. Taylor-Robinson, Z.A. McGee, M.A. Melly, and F.E. Carney. 1977. Preliminary studies on the mechanisms by which Neisseria gonorrhoeae damages host tissue. FEMS Microbiol. Lett. 1: 247-249.

184. Johnson, K.G., I.J. McDonald, and M.B. Perry. 1976. Studies of the cellular and free lipopolysaccharides from Branhamella catarrhalis. Can. J. Microbiol. 22: 460-467.

185. Johnson, K.G., M.B. Perry, and I.J. McDonald. 1976. Studies of the cellular and free lipopolysaccharides from Neisseria canis and N. subflava. Can. J. Microbiol. 22: 189-196.

186. Johnston, K.H., K.K. Holmes, and E.C. Gotschlich. 1976. The serological classification of Neisseria gonorrhoeae. I. Isolation of the outer membrane complex responsible for serotypic specificity. J. Exp. Med. 143: 741-758.

187. Johnston, K.H., and E.C. Gotschlich. 1974. Isolation and characterization of the outer membrane of Neisseria gonorrhoeae. J. Bacteriol. 119: 250-257.

188. Joiner, K.A., K.A. Warren, E.J. Brown, J. Swanson, and M.M. Frank. 1983. Studies on the mechanism of bacterial resistance to complement-mediated killing. IV. C5b-9 forms high molecular weight complexes with bacterial outer membrane constituents on serum-resistant

but not on serum-sensitive Neisseria gonorrhoeae. J. Immunol. 131: 1443-1451.

189. Joiner, K.A., D.A. Warren, M. Tam, and M.M. Frank. 1984. Monoclonal antibodies directed against protein I vary in bactericidal activity. J. Immunol. 134: 3411-3419.

190. Jones, R.B., J.C. Newland, D.A. Olsen, and T.M. Buchanan. 1980. Immune-enhanced phagocytosis of Neisseria gonorrhoeae: Characterization of the major antigens to which opsonins are directed. J. Gen. Microbiol. 121: 365-372.

191. Judd, R.C. 1982. Surface peptide mapping of protein I and protein III of four strains of Neisseria gonorrhoeae. Infect. Immun. 37: 632-641.

192. Judd, R.C. 1982. ^{125}I-Peptide mapping of protein III isolated from four strains of Neisseria gonorrhoeae. Infect. Immun. 37: 622-631.

193. Judd, R.C. 1985. Structure and surface exposure of protein IIs of Neisseria gonorrhoeae JS3. Infect. Immun. 48: 452-457.

194. Kampmeier, R.H. 1978. Identification of the gonococcus by Albert Neisser. Sex. Trans. Dis. 5: 71-72.

195. Kearns, D.H., R.J. O'Reilly, L. Lee, and B.G. Welch. 1973. Secretory IgA antibodies in the urethral exudate of men with uncomplicated urethritis due to Neisseria gonorrhoeae. J. Infect. Dis. 127: 99-101.

196. Kellogg, D.S., Jr., I.R. Cohen, L.C. Norins, A.L. Schroeter, and G. Reising. 1968. Neisseria gonorrhoeae. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. 96: 596-605.

197. Kellogg, D.S., Jr., W.L. Peacock, Jr., W.E. Deacon, L. Brown, and C.I. Pirkle. 1963. Neisseria gonorrhoeae. I. Virulence genetically linked to clonal variation. J. Bacteriol. 85: 1274-1279.

198. Khimji and Miles. 1978. Microbial iron-chelators and their action on Klebsiella infections in the skin of Guinea Pigs. Brit. J. of Exp. Pathol. 59: 137-147.

199. King, G.J., and J. Swanson. 1978. Studies on gonococcus infection. XV. Identification of surface proteins of Neisseria gonorrhoeae correlated with leukocyte association. Infect. Immun. 21: 575-584.

200. Kingsbury, D.T. 1969. Estimate of the genome size of various microorganisms. J. Bacteriol. 98: 1400-1401.

201. Kirven, L.A., and C. Thornsberry. 1977. Transfer of beta-lactamase genes of Neisseria gonorrhoeae by conjugation. Antimicrob. Agents Chemother. 11: 1004-1006.

202. Knapp, J.S., M.R. Tam, R.C. Nowinski, K.K. Holmes, and E.C. Sandstrom. 1984. Serological classification of Neisseria gonorrhoeae using monoclonal antibodies directed against gonococcal outer membrane protein I. J. Infect. Dis. 150: 44-48.

203. Knapp, J.S., C. Thornsberry, G.K. Schoolnik, P.J. Wiesner, and K.K. Holmes. Phenotypic and epidemiologic correlates of auxotype in Neisseria gonorrhoeae. 1978. J. Infect. Dis. 138: 160-165.

204. Kochan, I. 1973. The role of iron in bacterial infections with special consideration of host-tubercle bacillus interaction. Curr. Top. Microbiol. Immunol. 60: 1-30.

205. Kojima, N., and G.W. Bates. 1979. The reduction and release of iron from Fe^{3+}-transferrin-CO_3^{2-}. J. Biol. Chem. 254: 8847-8854.

206. Konopka, K., A. Bindereiff, and J.B. Neilands. 1982. Aerobactin-mediated utilization of transferrin iron. Biochemistry 21: 6503-6508.

207. Koomey, J.M., R.E. Gill, and S. Falkow. 1982. Genetic and biochemical

analysis of IgA_1-protease: Cloning in Escherichia coli and construction of mutants of gonococci that fail to produce the activity. Proc. Natl. Acad. Sci. USA. 79: 7881-7885.

208. Kovalchik, M.T., and S.J. Kraus. 1972. Neisseria gonorrhoeae: Colonial morphology of rectal isolates. Appl. Microbiol. 23: 986-989.

209. Krone, W.J.A., B. Oudega, F. Stegehius, and F.K. deGraaf. 1983. Cloning and expression of the cloacin DF13/aerobactin receptor of Escherichia coli (ColV-K30). J. Bacteriol. 153: 716-721.

210. Lambden, P.R., J.E. Heckels, L.T. James, and P.J. Watt. 1979. Variations in surface protein composition associated with virulence properties in opacity types of Neisseria gonorrhoeae. J Gen. Microbiol. 114: 305-312.

211. Lambden, P.R., J.N. Robertson, and P.J. Watt. 1980. Biological properties of two distinct pilus types produced by isogenic variants of Neisseria gonorrhoeae P9. J. Bacteriol. 141: 393-396.

212. Landolfo, P.J., T.J. Marrie, N.A. Nelson, and A.R. Ronald. 1981. Cell-mediated immune response in gonococcal infection. Can. J. Microbiol. 27: 76-80.

213. Langman, L., I.G. Young, G.E. Frost, H. Rosenberg, and F. Gibson. 1972. Enterochelin system of iron transport in Escherichia coli: Mutations affecting ferric-enterochelin esterase. J. Bacteriol. 112: 1142-1149.

214. Lankford, C.E. 1973. Bacterial assimilation of iron. CRC Crit. Rev. Microbiol. 2: 273-331.

215. Lawlor, K.M., and S.M. Payne. 1984. Aerobactin genes in Shigella spp. J. Bacteriol. 160: 266-272.

216. Leftik, M.I., J.W. Miller, and J.D. Brown. 1978. Penicillin-resistant gonococcal polyarthritis. JAMA 239: 134.

217. Leith, D.K., and S.A. Morse. 1980. Cross-linking analysis of Neisseria gonorrhoeae outer membrane proteins. J. Bacteriol. 143: 182-187.

218. Leong, J., and J.B. Neilands. 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. 126: 823-830.

219. Luckey, M., R. Wayne, and J.B. Neilands. 1975. In vitro competition between ferrichrome and phage for the outer membrane T5 receptor complex of Escherichia coli. Biochem. Biophys. Res. Comm. 64: 687-693.

220. Lynch, E.C., M.S. Blake, E.C. Gotschlich, and A. Mauro. 1983. Studies of porins: Spontaneously transferred from whole cells and reconstituted from purified proteins of Neisseria gonorrhoeae and Neisseria meningitidis. Biophys. J. 45: 104-107.

221. Lysko, P.G., and S.A. Morse. 1980. Effects of steroid hormones on Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 18: 281-288.

222. Lysko, P.G., and S.A. Morse. 1981. Neisseria gonorrhoeae cell envelope: Permeability to hydrophobic molecules. J. Bacteriol. 145: 946-952.

223. Maeland, J.A. 1968. Antigenic properties of various preparations of Neisseria gonorrhoeae endotoxin. Acta Pathol. Microbiol. Scand. 73: 413-422.

224. Maeland, J.A. 1969. Immunochemical characterization of aqueous ether-extracted endotoxin from Neisseria gonorrhoeae. Acta Pathol. Microbiol. Scand. 76: 484-495.

225. Maeland, J.A., and T. Cristoffersen. 1971. Immunochemical investigations on Neisseria gonorrhoeae endotoxin. Acta Pathol. Microbiol. Scand. Sect. B. 79: 226-232.

226. Magnusson, K.E., E. Kihlstrom, A. Norqvist, J. Davies, and S. Normark. 1979. Effect of iron on surface charge and hydrophobicity of Neisseria gonorrhoeae. Infect. Immun. 26: 402-407.

227. Maier, T.W., L. Zubrzycki, and M.B. Coyle. 1975. Genetic analysis of drug resistance in Neisseria gonorrhoeae: Identification and linkage relationships of loci controlling drug resistance. Antimicrob. Agents Chemother. 7: 676-681.

228. Maier, T.W., L. Zubrzycki, M.A. Coyle, M. Chila, and P. Warner. 1975. Genetic analysis of drug resistance in Neisseria gonorrhoeae: Production of increased resistance by the combination of two antibiotic resistance loci. J. Bacteriol. 124: 834-842.

229. Maness, M.J. and P.F. Sparling. 1973. Multiple antibiotic resistance due to a single mutation in Neisseria gonorrhoeae. J. Infect. Dis. 128: 321-330.

230. Martin, P.M.V., P.V. Patel, N.J. Parsons, and H. Smith. 1981. Induction of phenotypically determined resistance of Neisseria gonorrhoeae to human serum by factors in human serum. J. Gen. Microbiol. 127: 213-217.

231. Mathis, L.S., and J.J. Scocca. 1984. On the role of pili in transformation of Neisseria gonorrhoeae. J. Gen. Microbiol. 130: 3165-3173.

232. Matzanke, B.F., G.I. Muller, and K.N. Raymond. 1984. Hydroxamate siderophore mediated iron-uptake in E. coli: Stereospecific recognition

of ferric rhodotorulic acid (1). Biochem. Biophys. Res. Comm. 121: 922-930.

233. Mayer, L.W. 1982. Rates of *in vitro* changes of gonococcal opacity phenotypes. Infect. Immun. 37: 481-485.

234. Mayer, L.W., K.K. Holmes, and S. Falkow. 1974. Characterization of plasmid deoxyribonucleic acid from *Neisseria gonorrhoeae*. Infect. Immun. 10: 712-717.

235. Mazurier, J., M-H. Metz-Boutique, J. Jolles, G. Spik, J. Montreuil, and P. Jolles. 1983. Human lactotransferrin: molecular, functional and evolutionary comparisons with human serum transferrin and hen ovotransferrin. Experientia 39: 135-141.

236. McCutchan, J.A., D. Katzenstein, D. Norqvist, G. Chikami, A. Wunderlich, and A.L. Braude. 1978. Role of blocking antibody in disseminated gonococcal infection. J. Immunol. 121: 1884-1888.

237. McDade, R.L., and K.H. Johnston. 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. 141: 1183-1191.

238. McFarland, L., T.A. Mietzner, J.S. Knapp, E. Sandstrom, K.K. Holmes, and S.A. Morse. 1983. Gonococcal sensitivity to fecal lipids can be mediated by an Mtr-independent mechanism. J. Clin. Microbiol. 18: 121-127.

239. McGee, Z.A., and R.G. Horn. 1979. Phagocytosis of gonococci by nonprofessional phagocytic cells, pp. 158-161. *In* D. Schlessinger (eds.), Microbiology-1979. American Society for Microbiology, Washington, D.C.

240. McGee, Z.A., A.P. Johnson, D. Taylor-Robinson. 1981. Pathogenic

mechanisms of Neisseria gonorrhoeae: Observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 and type 4. J. Infect. Dis. 143: 413-422.

241. McGee, Z.A., D.S. Stephens, L.H. Hoffman, W.F. Schlech, and R.G. Horn. 1983. Mechanisms of mucosal invasion by pathogenic Neisseria. Rev. Infect. Dis. 5(suppl. 4): s708-s714.

242. McMillan, A., G. McNeillage, and H. Young. 1979. Antibodies to Neisseria gonorrhoeae: A study of the urethral exudates of 232 men. J. Infect. Dis. 140: 89-95.

243. Melly, M.A., Z.A. McGee, R.G. Horn, F. Morris, and A.D. Glick. 1979. An electron microscopic India ink technique for demonstrating capsules on microorganisms: Studies with Streptococcus pneumoniae, Staphylococcus aureus, and Neisseria gonorrhoeae. J. Infect. Dis. 140: 605-609.

244. Melly, M.A., Z.A. McGee, and R.S. Rosenthal. 1984. Ability of monomeric peptidoglycan fragments from Neisseria gonorrhoeae to damage human fallopian-tube mucosa. J. Infect. Dis. 149: 378-386.

245. Meyer, T.F., E. Billyard, R. Haas, S. Storzbach, and M. So. 1984. Pilus genes of Neisseria gonorrhoeae: Chromosomal organization and DNA sequence. Proc. Natl. Acad. Sci. USA 81: 6110-6114.

246. Meyer, T.F., N. Mlawer, and M. So. 1982. Pilus expression in Neisseria gonorrhoeae involves chromosomal rearrangement. Cell. 30: 45-52.

247. Mickelsen, P.A., E. Blackman, and P.F. Sparling. 1982. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from lactoferrin. Infect. Immun. 35: 915-920.

248. Mickelsen, P.A., and P.F. Sparling. 1981. Ability of Neisseria

gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from transferrin and iron compounds. Infect. Immun. 33: 555-564.

249. Mietzner, T.A., G.H. Luginbuhl, E.C. Sandstrom, and S.A. Morse. 1984. Identification of an iron-regulated 37,000-dalton protein in the cell envelope of Neisseria gonorrhoeae. Infect. Immun. 45: 410-416.

250. Miller, M.A., C.S. Scharli, and J. Mills. 1980. Cytoplasmic membrane proteins of spectinomycin-susceptible and -resistant strains of Neisseria gonorrhoeae. J. Bacteriol. 143: 198-204.

251. Miller, R.D., K.E. Brown, and S.A. Morse. 1977. Inhibitory action of fatty acids on the growth of Neisseria gonorrhoeae. Infect. Immun. 17: 303-312.

252. Miller, R.D., and S.A. Morse. 1977. Binding of progesterone to Neisseria gonorrhoeae and other Gram-negative bacteria. Infect. Immun. 16: 115-123.

253. Mintz, C.S., M.A. Apicella, and S.A. Morse. 1984. Electrophoretic and serological characterization of the lipopolysaccharide produced by Neisseria gonorrhoeae. J. Infect. Dis. 149: 544-552.

254. Montgomerie, J.Z., A. Bindereif, J.B. Neilands, G.M. Kalmanson, and L.B. Guze. 1984. Association of hydroxamate siderophore (aerobactin) with Escherichia coli isolated from patients with bacteremia. Infect. Immun. 46: 835-838.

255. Moore, D.G., and C.F. Earhart. 1981. Specific inhibition of Escherichia coli ferri-enterochelin uptake by a normal human serum immunoglobulin. Infect. Immun. 31: 631-635.

256. Morse, S.A. 1979. The biology of the gonococcus. CRC Crit. Rev. Microbiol. 7: 93-139.

257. Morse, S.A., and M.A. Apicella. 1982. Isolation of a lipopolysaccharide mutant of Neisseria gonorrhoeae: An analysis of the antigenic and biologic differences. J. Infect. Dis. 145: 206-216.

258. Morse, S.A., and P.G. Lysko. 1980. The cell envelope of Neisseria gonorrhoeae, pp. 1-6. In D. Danielsson and S. Normark (eds.), Genetics and Immunobiology of Pathogenic Neisseria. University of Umea, Sweden.

259. Morse, S.A., P.G. Lysko, L. McFarland, J.S. Knapp, E. Sandstrom, C. Critchlow, and K.K. Holmes. 1982. Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. Infect. Immun. 37: 432-438.

260. Morse, S.A., C.S. Mintz, S.K. Sarafian, L. Bartenstein, M. Bertram, and M.A. Apicella. 1983. Effect of dilution rate on lipopolysaccharide and serum resistance of Neisseria gonorrhoeae grown in continuous culture. Infect. Immun. 41: 74-82.

261. Negrin, R.S., and J.B. Neilands. 1978. Ferrichrome transport in inner membrane vessicles of Escherichia coli K12. J. Biol. Chem. 253: 2339-2342.

262. Neilands, J.B. 1974. Iron and its role in microbial physiology, pp. 3-34. In J.B. Neilands (ed.), Microbial iron metabolism, a comprehensive treatise. Academic Press, Inc., New York.

263. Neilands, J.B. 1977. Siderophores: Biochemical ecology and mechanism of iron transport in enterobacteria, pp. 3-32. In K.N. Raymond (ed.), Bioinorganic Chemistry II. American Chemical Society, Washington, D.C.

264. Neilands, J.B. 1980. Microbial metabolism of iron, p. 529-572. In A. Jacobs and M. Worwood (eds.), Iron in Biochemistry and Medicine, Vol. II. Academic Press, Inc., New York.

265. Neilands, J.B. 1981. Iron absorption and transport in microorganisms. Ann. Rev. Nutr. 1: 27-46.

266. Neilands, J.B. 1981. Microbial iron compounds. Ann. Rev. Biochem. 50: 715-731.

267. Neilands, J.B. 1982. Microbial envelope proteins related to iron. Ann. Rev. Microbiol. 36: 285-309.

268. Newhall, W.J., W.D. Sawyer, and R.A. Haak. 1980. Cross-linking analysis of the outer membrane proteins of Neisseria gonorrhoeae. Infect. Immun. 28: 785-791.

269. Newhall, W.J., C.E. Wilde III, W.D. Sawyer, and R.A. Haak. 1980. High-molecular-weight antigenic protein complex in the outer membrane of Neisseria gonorrhoeae. Infect. Immun. 27: 475-482.

270. Nikaido, H., and T. Nakae. 1979. The outer membrane of Gram-negative bacteria, pp. 163-250. In A.H. Rose and J.G. Morris (eds.), Advances in Microbial Physiology, Vol 20. Academic Press, Inc. London.

271. Noegel, A., and E.C. Gotschlich. 1983. Isolation of a high molecular weight polyphosphate from Neisseria gonorrhoeae. J. Exp. Med. 157: 2049-2060.

272. Norlander, L., J. Davies, and S. Normark. 1979. Genetic exchange mechanisms in Neisseria gonorrhoeae. J. Bacteriol. 138: 756-761.

273. Normark, S., L. Norlander, J.K. Davies, P. Hagblom, and K. Korch. 1980. Genome organization and genetic exchange in Neisseria gonorrhoeae, pp. 127-130. In D. Danielsson and S. Normark (eds.), Genetics and Immunobiology of Pathogenic Neisseria. University of Umea, Sweden.

274. Norqvist, A., J. Davies, L. Norlander, and S. Normark. 1978. The

effect of iron starvation on the outer membrane protein composition of Neisseria gonorrhoeae. FEMS Microbiol. Lett. 4: 71-75.

275. Norrod, P., and R.P. Williams. 1978. Growth of Neisseria gonorrhoeae in media deficient in iron without detection of siderophores. Curr. Microbiol. 1: 281-284.

276. Novick, A., and L. Szilard. 1950. Description of the chemostat. Science 112: 715-716.

277. Novotny, P., J.A. Short, and P.D. Walker. 1975. An electron microscope study of naturally occuring and cultured cells of Neisseria gonorrhoeae. J. Med. Microbiol. 8: 413-427.

278. Novotny, P., and W.H. Turner. 1975. Immunological heterogeneity of Neisseria gonorrhoeae. J. Gen Microbiol. 89: 87-92.

279. O'Brien, I.G., and F. Gibson. 1970. The structure of enterochelin and related 2,3,-dihydroxy-N-benzoylserine conjugates from Escherichia coli. Biochim. Biophys. Acta 215: 393-402.

280. Odugbemi, T.O., and B. Dean. 1978. Iron contents of different colonial types of Neisseria gonorrhoeae. 104: 161-164.

281. Odugbemi, T.O., and S. Hafiz. 1978. The effect of iron-chelators on the colonial morphology of Neisseria gonorrhoeae. J. Gen. Microbiol. 104: 165-167.

282. Ofek, I., E.H. Beachey, and A.L. Bisno. 1974. Resistance of Neisseria gonorrhoeae to phagocytosis: Relationship to colonial morphology and surface pili. J. Infect. Dis. 129: 310-316.

283. Okada, S., M.D. Rossman, and E.B. Brown. 1978. The effect of acid pH and citrate on the release and exchange of iron on rat transferrin. Biochim. Biophys. Acta 543: 72-81.

284. O'Reilly, R.J., L. Lee, and B.G. Welch. 1976. Secretory IgA antibody responses to Neisseria gonorrhoeae in the genital secretions of infected females. J. Infect. Dis. 133: 113-125.

285. Parez-Casal, J.F., and J.H. Crosa. 1984. Aerobactin iron uptake sequences in plasmid ColV-K30 are flanked by inverted IS1-like elements and replication regions. J. Bacteriol. 160: 256-265.

286. Payne, S.M. 1980. Synthesis and utilization of siderophores by Shigella flexneri. J. Bacteriol. 143: 1420-1424.

287. Payne, S.M., and R.A. Finkelstein. 1975. Pathogenesis and immunology of experimental gonococcal infection: Role of iron in virulence. Infect. Immun. 12: 1313-1313.

288. Payne, S.M., and R.A. Finkelstein. 1978. Siderophore production by Vibrio cholerae. Infect. Immun. 20: 310-311.

289. Payne, S.M., D.W. Niesel, S.S. Peixotto, and K.M. Lawlor. 1983. Expression of hydroxamate and phenolate siderophores by Shigella flexneri. J. Bacteriol. 155: 949-955.

290. Pearce, W.A., and T.M. Buchanan. 1978. Attachment role of gonococcal pili: Optimum conditions and quantitation of adherence of isolated pili to human cells in vitro. J. Clin. Invest. 61: 931-943.

291. Percival, A., J.E. Corkill, O.P. Arya, J. Rowlands, C.D. Alergant, E. Rees, and E.H. Annels. 1976. Penicillinase-producing gonococci in Liverpool. Lancet. 2: 1379-1382.

292. Perry, M.B., V. Dauost, B.B. Diena, E. Ashton, and R. Wallace. 1975. The lipopolysaccharides of Neisseria gonorrhoeae colony types 1 and 4. Can. J. Biochem. 53: 623-629.

293. Perry, M.B., B.B. Diena, and F.E. Ashton. 1977. Lipopolysaccharides of

Neisseria gonorrhoeae, pp. 285-301. In R. Roberts (ed.), The Gonococcus. John Wiley & Sons, New York.

294. Perry, R.D., and R.R. Brubaker. 1979. Accumulation of iron by yersiniae. J. Bacteriol. 137: 1290-1298.

295. Perry, R.D., and C.L. San Clemente. 1979. Sdierophore synthesis in Klebsiella pneumoniae and Shigella sonnei during iron deficiency. J. Bacteriol. 140: 1129-1132.

296. Petersen, B.H., J.A. Graham, and G.F. Brooks. 1976. Human deficiency in the eighth component of complement: The requirement of C8 for serum Neisseria gonorrhoeae bactericidal activity. J. Clin. Invest. 57: 283-290.

297. Petersen, B.H., and R.S. Rosenthal. 1982. Complement consumption by gonococcal peptidoglycan. Infect. Immun. 35: 442-448.

298. Phillips, C.W., Aller, R.D., and S.N. Cohen. 1976. Penicillinase-producing Neisseria gonorrhoeae. Lancet 2: 960.

299. Phillips, I. 1976. Beta-lactamase-producing, penicillinase-resistant gonococcus. Lancet 2: 656-657.

300. Plaut, A.G., J.V. Gilbert, M.S. Artenstein, and J.D. Capra. 1975. Neisseria gonorrhoeae and Neisseria meningitidis: Extracellular enzyme cleaves human immunoglobin A. Science 190: 1103-1105.

301. Pollack, J.R., B.N. Ames, and J.B. Neilands. 1970. Iron transport in Salmonella typhimurium: Mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. 104: 635-639.

302. Pollack, J.R., and J.B. Neilands. 1970. Enterobactin, an iron transport compound from Salmonella typhimurium. Biochem. Biophys. Res. Comm. 38: 989-992.

303. Postle, K., and W. Reznikoff. 1979. Identification of the Escherichia coli tonB gene product in minicells containing ton B hybrid plasmids. J. Mol. Biol. 131: 619-636.

304. Price, R.J., and B. Boettcher. 1979. The presence of complement in human cervical mucus and its possible relevance to infertility in women with complement-dependent sperm-immobilizing antibodies. Fert. Steril. 32: 61-66.

305. Prody, C.A., and J.B. Neilands. 1984. Genetic and biochemical characterization of the Escherichia coli K-12 fhuB mutation. J. Bacteriol. 157: 874-880.

306. Punsalang, A.P., Jr., and W.D. Sawyer. 1973. Role of pili in the virulence of Neisseria gonorrhoeae. Infect. Immun. 8: 255-263.

307. Rank, E.L., and B. Holmes. 1984. Chemotaxis of human polymorphonuclear leukocytes toward Neisseria gonorrhoeae. J. Med. Microbiol. 7: 45-52.

308. Rest, R.F. 1979. Killing of Neisseria gonorrhoeae by human polymorphonuclear neutrophil granule extracts. Infect. Immun. 25: 574-579.

309. Rest, R.F., S.H. Fletcher, Z.Z. Ingham, and J.F. Jones. 1982. Interactions of Neisseria gonorrhoeae with human neutrophils: Effects of serum and gonococcal opacity on phagocyte killing and chemiluminescence. Infect. Immun. 36: 737-744.

310. Rest, R.F., and E. Pretzer. 1981. Degradation of gonococcal outer membrane proteins by human neutrophil lysosomal proteases. Infect. Immun. 34: 62-68.

311. Rice, P.A., and D.L. Kasper. 1977. Characterization of gonococcal antigens responsible for induction of bactericidal antibody in disseminated infection. J. Clin. Invest. 60: 1149-1158.

312. Rice, P.A., and D.L. Kasper. 1982. Characterization of serum resistance of Neisseria gonorrhoeae that disseminate: Roles of blocking antibody and gonococcal outer membrane proteins. J. Clin. Invest. 70: 157-167.

313. Rice, P.A., W.M. McCormack, and D.L. Kasper. 1980. Natural serum bactericidal activity against Neisseria gonorrhoeae isolates from disseminated, locally invasive, and uncomplicated disease. J. Immunol. 124: 2105-2109.

314. Richardson, W.P., and J.C. Sadoff. 1977. Production of a capsule by Neisseria gonorrhoeae. Infect. Immun. 15: 663-664.

315. Roberts, M., L.P. Elwell, and S. Falkow. 1977. Molecular characterization of two beta-lactamase-specifying plasmids isolated from Neisseria gonorrhoeae. J. Bacteriol. 131: 557-563.

316. Roberts, M., and S. Falkow. 1977. Conjugal transfer of R-plasmids in Neisseria gonorrhoeae. Nature. 266: 630-631.

317. Roberts, M., and S. Falkow. 1978. Plasmid-mediated chromosomal gene transfer in Neisseria gonorrhoeae. J. Bacteriol. 134: 66-70.

318. Robertson, J.N., P. Vincent, and M.E. Ward. 1977. The preparation and properties of gonococcal pili. J. Gen. Microbiol. 102: 169-177.

319. Rodriguez, W.J., and A.K. Saz. 1978. Differential binding of penicillin by membrane fractions from penicillin-susceptible and -resistant gonococci. Antimicrob. Agents Chemother. 13: 589-597.

320. Rosenthal, R.S. 1979. Release of soluble peptidoglycan from growing gonococci: Hexamindase and amidase activities. Infect. Immun. 24: 869-878.

321. Rosenthal, R.S., W.J. Folkening, D.R. Miller, and S.C. Swim. 1983.

Resistance of O-acetylated gonococcal peptidoglycan to human peptidoglycan-degrading enzymes. Infect. Immun. 40: 903-911.

322. Rosenthal, R.S., M.A. Gfell, and W.J. Folkening. 1985. Influence of protein synthesis inhibitors on the regulation of extent of O-acetylation of gonococcal peptidoglycan. Infect. Immun. 49: 7-13.

323. Rosenthal, R.S., R.K. Sinha, B.H. Petersen, M.A. Melly, and Z.A. McGee. 1981. Chemical and biological properties of gonococcal peptidoglycan, pp. 7-11. In D. Danielsson and S. Normark (eds.), Genetics and Immunobiology of Pathogenic Neisseria. University of Umea, Sweeden.

324. Rosenthal, R.S., S.C. Swim, W.J. Folkening, B.H. Petersen, R.L. Fouts, and K. Phadke. 1983. Lysozyme-resistant O-acetylated peptidoglycan of Neisseria gonorrhoeae: Strain variation, resistance to human peptidoglycan hydrolases, and pathobiological properties, pp. 311-316. In R. Hakenbeck, J. Holtje, and H. Labischinski (eds.), The target of penicillin: International FEMS symposium on the murein sacculus of bacterial cell walls. Walter de Gruyter and Co., Berlin.

325. Rothbard, J.B., R. Fernandez, and G.K. Schoolnik. 1984. Strain-specific and common epitopes of gonococcal pili. J. Exp. Med. 160: 208-221.

326. Rothbard, J.B., R. Fernandez, L. Wang, N.N.H. Teng, and G.K. Schoolnik. 1985. Antibodies to peptides corresponding to a conserved sequence of gonococcal pilins block bacterial adhesion. Proc. Natl. Acad. Sci. USA 82: 915-919.

327. Russell, and Johnson. 1975. SDS-Polyacrylamide gel electrophoresis of lipopolysaccharides. Can. J. Microbiol. 20: 2013-2018.

328. Salit, I.E., M.S. Blake, and E.C. Gotschlich. 1980. Intrastrain

heterogeneity of gonococcal pili is related to opacity variance. J. Exp. Med. 151: 716-725.

329. Sandstrom, E., and D. Danielsson. 1980. Serology of *Neisseria gonorrhoeae*: Classification with co-agglutination. Acta Pathol. Microbiol, Scan. Sect. B. 88: 27-38.

330. Sandstrom, E.G., J.S. Knapp, and T.M. Buchanan. 1982. Serology of *Neisseria gonorrhoeae*: W-antigen serogrouping by coagglutination and protein I serotyping by enzyme-linked immunosorbent assay detect both protein I antigens. Infect. Immun. 35: 229-239.

331. Sandstrom, E., N. Venizelos, and J. Palmbald. 1983. Chemotactic activity of *Neisseria gonorrhoeae*. Br. J. Vener. Dis. 59: 92-93.

332. Sarubbi, F.A., Jr., E. Blackman, and P.F. Sparling. 1974. Genetic mapping of linked antibiotic resistance loci in *Neisseria gonorrhoeae*. J. Bacteriol. 120: 1284-1292.

333. Sarrubi, F.A., Jr., and P.F. Sparling. 1974. Transfer of antibiotic resistance in mixed cultures of *Neisseria gonorrhoeae*. J. Infect. Dis. 130: 660-663.

334. Sarubbi, F.A., Jr., P.F. Sparling, E. Blackman, and E. Lewis. 1975. Loss of low-level antibiotic resistance in *Neisseria gonorrhoeae* due to *env* mutations. J. Bacteriol. 124: 740-749.

335. Sarafian, S.K., M.R. Tam, and S.A. Morse. 1983. Gonococcal protein I-specific IgG in normal human serum. J. Infect. Dis. 148: 1025-1032.

336. Schade, A.L., and L. Caroline. 1944. Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteria*, *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Science 100: 14-15.

337. Schade, A.L., and L. Caroline. 1946. An iron binding component in

human blood plasma. Science 104: 340-341.

338. Schaefer, R.L., K.F. Keller, and R.J. Doyle. Lectins in diagnostic microbiology: Use of wheat germ agglutinin for laboratory identification of *Neisseria gonorrhoeae*. J. Clin. Microbiol. 10: 669-672.

339. Schiller, N.L. 1980. Interaction of gonococci causing uncomplicated gonococcal infection (UGI) and disseminated gonococcal infection (DGI) with human polymorphonuclear leukocytes (PMNL) and serum, pp. 241-246. In D. Danielsson and S. Normark (eds.), Genetics and Immunobiology of Pathogenic *Neisseria*. University of Umea, Sweden.

340. Schiller, N.L., G.L. Freidman, and R.B. Roberts. 1978. Role of serum factors in the phagocytosis of type 4 gonococci by human polymorphonuclear leukocytes, pp. 207-212. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.B. Sawyer, and F.E. Young (eds.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.

341. Schneider, H., T.L. Hale, W.D. Zollinger, R.C. Seid, C.A. Hannack, and J.M. Griffiss. 1984. Heterogeneity of molecular size and antigenic expression within lipooligosaccharides of individual strains of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Infect. Immun. 45: 544-549.

342. Schoolnik, G.K., T.M. Buchanan, and K.K. Holmes. 1976. Gonococci causing disseminated gonococcal infection are resistant to the bactericidal action of normal human sera. J. Clin. Invest. 58: 1163-1173.

343. Schoolnik, G.K., R. Fernandez, J.Y. Tai, J.B. Rothbard, and E.C. Gotschlich. 1984. Gonococcal pili: Primary structure and receptor binding domain. J. Exp. Med. 159: 1351-1370.

344. Schoolnik, G.K., H.D. Ochs, and T.M. Buchanan. 1979. Immunoglobulin class responsible for gonococcal bactericidal activity of normal human sera. J. Immunol. 122: 1771-1779.

345. Schoolnik, G.K., J.Y. Tai, and E.C. Gotschlich. 1983. A pilus peptide vaccine for the prevention of gonorrhea. Prog. Allergy. 33: 314-331.

346. Schwalbe, R.S., P.F. Sparling, and J.G. Cannon. 1985. Variation of Neisseria gonorrhoeae protein II among isolates from an outbreak caused by a single gonococcal strain. Infect. Immun. 49: 250-252.

347. Sciortino, C.V., and R.A. Finkelstein. 1983. Vibrio cholerae expresses iron-regulated outer membrane proteins in vivo. Infect. Immun. 42: 990-996.

348. Sigel, M., D. Olsen, C. Critchlow, and T.M. Buchanan. 1982. Gonococcal pili: Safety and immunogenicity in humans and antibody function in vitro. J. Infect. Dis. 145: 300-310.

349. Sigel, S.P., and S.M. Payne. 1982. Effect of iron limitation on growth, siderophore, and expression of outer membrane proteins of Vibrio cholerae. J. Bacteriol. 150: 148-155.

350. Sigel, S.P., J.A. Stoebner, and S.M. Payne. 1985. Iron-vibriobactin transport system is not required for virulence of Vibrio cholerae. Infect. Immun. 47: 360-362

351. Sihna, R.K., and R.S. Rosenthal. 1980. Release of soluble peptidoglycan from growing gonococci: Demonstration of anhydro-muramyl-containing fragments. Infect. Immun. 29: 914-925.

352. Simonson, C. 1983. Mechanisms of iron acquisition in Neisseria menigitidis. Doctoral Thesis, Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada.

353. Simonson, C., D. Brener, and I.W. DeVoe. 1982. Expression of a high-affinity mechanism for acquisition of transferrin iron by Neisseria meningitidis. Infect Immun. 36: 107-113.

354. Simonson, C., T. Trivett, and I.W. DeVoe. 1981. Energy-independent uptake of iron from citrate by isolated outer membranes of Neisseria meningitidis. Infect. Immun. 31: 547-553.

355. Sisco, K.L., and H.O. Smith. 1979. Sequence-specific DNA uptake in Haemophillus transformation. Proc. Natl. Acad. Sci. USA 76: 972-976.

356. Slimes, M.A., and P.R. Dallman. 1974. New kinetic role for serum ferritin in iron metabolism. Brit. J. Hematol. 28: 7-18.

357. Smith, C.J., and M.R. Salton. 1977. Crossed immunoelectrophoresis. A new approach to high resolution analysis of gonococcal antigens and antibodies, pp. 303-331. In R.B. Roberts (ed.), The Gonococcus. John Wiley & Sons, New York.

358. Snow, G.A. 1970. Mycobactins: Iron-chelating growth factors from mycobacteria. Bacteriol. Rev. 34: 99-125.

359. Sokol, P.A., and D.E. Woods. 1983. Demonstration of an iron-siderophore-binding protein in the outer membrane of Pseudomonas aeruginosa. Infect. Immun. 40: 665-669.

360. Sox, T.E., W. Mohammed, and P.F. Sparling. 1979. Transformation-derived Neisseria gonorrhoeae plasmids with altered structure and function. J. Bacteriol. 138: 510-518.

361. Sparling, P.F. 1966. Genetic transformation of Neisseria gonorrhoeae to streptomycin resistance. J. Bacteriol. 92: 1364-1371.

362. Sparling, P.F., G.D. Biswas and T.E. Sox. 1977. Transformation of the gonococcus, pp. 155-176. In R.B. Roberts (ed.), The Gonococcus. John Wiley & Sons, New York.

363. Sparling, P.F., L. Guymon, and G. Biswas. 1976. Antibiotic resistance in the gonococcus, pp. 494-500. In D. Schlessinger (ed.), Microbiology-1976. American Society for Microbiology, Washington, D.C.

364. Sparling, P.F., F.A. Sarubbi, Jr., and E. Blackman. 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in Neisseria gonorrhoeae. J. Bacteriol. 124: 740-749.

365. Sparling, P.F., and A.R. Yobs. 1967. Colonial morphology of Neisseria gonorrhoeae isolated from males and females. J. Bacteriol. 98: 513.

266. Stead, A, S.S. Main, M.E. Ward, and P.J. Watt. 1975. Studies on lipopolysaccharide isolated from strains of Neisseria gonorrhoeae. J. Gen. Microbiol. 88: 123-131.

367 Stepheson, M.C., and C. Ratledge. 1980. Specificity of exochelins for iron transport in three species of mycobacteria. J. Gen. Microbiol. 116: 521-523.

368. Stuart, S.J., K.T. Greenwood, R.D.J. Luke. 1982. Iron-suppressible production of hydroxamate by Escherichia coli isolates. Infect. Immun. 36: 870-875.

369. Stein, D.C., L.E. Silver, V.L. Clark, and F.E. Young. 1984. Cloning genes for proline biosynthesis from Neisseria gonorrhoeae: Identification by interspecific complementation of Escherichia coli mutants. J. Bacteriol. 158: 696-700.

370. Stein, D.C., F.E. Young, F.C. Tenover, and V.L. Clark. 1983. Characterization of a chimeric beta-lactamase plasmid of Neisseria

gonorrhoeae which can function in Escherichia coli. Mol. Gen. Genet. 189: 77-84.

371. Steinberg, V.I., and I.D. Goldberg. 1980. On the question of chromosomal gene transfer via conjugation in Neisseria gonorrhoeae. J. Bacteriol. 142: 350-354.

372. Stern, A., P. Nickel, T.F. Meyer, and M. So. 1984. Opacity determinants of Neisseria gonorrhoeae: Gene expression and chromosomal linkage to the gonococcal pilus gene. Cell. 37: 447-456.

373. Stiffler, P.W., S.A. Lerner, M. Bohnhoff, and J.A. Morello. 1975. Plasmid deoxyribonucleic acid in clinical isolates of Neisseria gonorrhoeae. J. Bacteriol. 122: 1293-1300.

374. Sullivan, K.H., and R.P. Williams. 1982. Use of Iodo-Gen and Iodine-125 to label the outer membrane proteins of whole cells of Neisseria gonorrhoeae. Analyt. Biochem. 120: 254-258.

375. Sugasawara, R.J., J.G. Cannon, W.J. Black, I. Nachamkin, R.L. Sweet, G.F. Brooks. 1983. Inhibition of Neisseria gonorrhoeae attachment to HeLa cells with monoclonal antibody directed against Protein II. Infect. Immun. 42: 980-985.

376. Swanson, J. 1973. Studies on gonococcus infection. IV. Pili: Their role in attachment of gonococci to tissue culture cells. J. Exp. Med. 137: 571-589.

377. Swanson, J. 1977. Surface components affecting interactions between Neisseria gonorrhoeae and eukaryotic cells. J. Infect. Dis. 136 (suppl.): s138-s143.

378. Swanson, J. 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. 19: 320-331.

379. Swanson, J. 1978. Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. 21: 292-302.

380. Swanson, J. 1979. Studies on gonococcus infection. XVIII. ^{125}I-Labeled peptide mapping of the major protein of the gonococcal cell wall outer membrane. Infect. Immun. 23: 799-810.

381. Swanson, J. 1980. ^{125}I-labeled peptide mapping of some heat-modifiable proteins of the gonococcal outer membrane. Infect. Immun. 28: 54-64.

382. Swanson, J. 1981. Surface-exposed protein antigens of the gonococcal outer membrane. Infect. Immun. 34: 804-816.

383. Swanson, J. 1982. Colony opacity and protein II compositions of gonococci. Infect. Immun. 37: 359-368.

384. Swanson, J., and O. Barrera. 1983. Immunological characteristics of gonococcal outer membrane protein II assessed by immunoprecipitation, immunoblotting, and coagglutination. J. Exp. Med. 157: 1405-1420.

385. Swanson, J.S., S.J. Kraus, and E.C. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: Their relation to gonococcal growth patterns. J. Exp. Med. 134: 886-906.

386. Swanson, J., and L.W. Mayer. 1984. Biology of Neisseria gonorrhoeae, pp. 187-204. In K.K. Holmes, P. Mardh, P.F. Sparling, and P.J. Weisner (eds.), Sexually Transmitted Diseases. McGraw-Hill Book Company, New York.

387. Swanson, J. L.W. Mayer, and M.R. Tam. 1982. Antigenicity of Neisseria gonorrhoeae outer membrane protein(s) III detected by immunoprecipitation and western blot transfer with a monoclonal antibody. Infect. Immun. 38: 668-672.

388. Swanson, J., E. Sparks, D. Young, and G. King. 1975. Studies on gonococcus infection. X. Pili and leukocyte association factor as mediators of interactions between gonococci and eukaryotic cells _in vitro_. Infect. Immun. _11_: 1352-1361.

389. Tam, M.R., T.M. Buchanan, E.C. Sandstrom, K.K. Holmes, J.S. Knapp, W. Siadak, and R.C. Nowinski. 1982. Serological classification of _Neisseria gonorrhoeae_ with monoclonal antibodies. Infect. Immun. _36_: 1042-1053.

390. Tauber, J., and W. Garson. 1959. Isolation of lipopolysaccharide endotoxin. J. Biol. Chem. _234_: 1391-1393.

391. Tempest, D.W. 1970. The place of continuous culture in microbiological research. Adv. Microb. Phys. _4_: 223-250.

392. Tempest, D.W., and J.T.M. Wouters. 1981. Properties and performance of microorganisms in chemostat culture. Enzyme Microb. Technol. _3_: 283-290.

393. Thongthai, C., and W.D. Sawyer. 1973. Studies on the virulence of _Neisseria gonorrhoeae_. I. Relation of colonial morphology and resistance to phagocytosis by polymorphonuclear leukocytes. Infect. Immun. _7_: 373-379.

394. Tramont, E.C. 1977. Inhibition of adherence of Neisseria gonorrhoeae by human genital secretions. J. Clin. Invest. _59_: 117-124.

395. Tramont, E.C., and J. Ciak. 1978. Antigonococcal antibodies in genital secretions, pp. 274-278. _In_ G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunobiology of _Neisseria gonorrhoeae_. American Society for Microbiology, Washington, D.C.

396. Tramont, E.C., J. Ciak, J. Boslego, C.G. McChesney, C.C. Brinton, and W.

Zollinger. 1980. Antigenic specificity of antibodies in vaginal secretions during infection with Neisseria gonorrhoeae. J. Infect. Dis. 142: 23-31.

397. Trust, T.J., P.R. Lambden, and P.J. Watt. 1980. The cohesive properties of variants of Neisseria gonorrhoeae strain P9: Specific pilus-mediated and non-specific interactions. J. Gen Microbiol. 119: 179-187.

398. Underwood, E. 1977. Iron, pp. 13-25. In E.J. Underwood (ed.), Trace Elements in Human and Animal Nutrition. Academic Press, New York.

399. Valberg, L.S., and P.R. Flanagan. 1983. Intestinal absorption of iron and chemically related metals. In B. Sarkar (ed.), Biological Aspects of Metals and Metal-related Diseases. Raven Press, New York.

400. Valvano, M.A., and J.H. Crosa. 1984. Aerobactin iron transport genes commonly encoded by certain ColV plasmids occur in the chromosome of a human invasive strain of Escherichia coli K1. Infect. Immun. 46: 159-167.

401. Veale, D.R., M. Goldner, C.W. Penn, J. Ward, and H. Smith. 1979. Intracellular survival and growth of gonococci in human phagocytes. J. Gen Microbiol. 113: 383-393.

402. Veale, D.R., C.W. Penn, S. Sutton, and H. Smith. 1978. The effect of specific antiserum on the resistance of Neisseria gonorrhoeae to intracellular killing by phagocytes of human blood. J. Gen. Microbiol. 106: 129-136.

403. Virji, M., and J.E. Heckels. 1984. The role of common and type-specific pilus antigenic domains in adhesion and virulence of gonococci for human epithelial cells. J. Gen . Microbiol. 130: 1089-1095.

404. Wagegg, W., and V. Braun. 1981. Ferric-citrate transport in Escherichia coli requires outer membrane receptor protein FecA. J. Bacteriol. 145: 156-163.

405. Wallace, R. F.E. Ashton, A. Ryan, B.B. Diena, C. Malysheff, and M.B. Perry. 1978. The lipopolysaccharide (R-type) as a common antigen of Neisseria gonorrhoea. II. Use of hen antiserum to gonococcal lipopolysaccharide in a rapid slide test for the identification of Neisseria gonorrhoeae from primary isolates and secondary cultures. Can. J. Microbiol. 24: 124-128.

406. Walstad, D.L., L.F. Guymon, and P.F. Sparling. 1977. Altered outer membrane protein in different colonial types of Neisseria gonorrhoeae. J. Bacteriol. 129: 1623-1627.

407. Walter, M.A., A. Bindereif, J.B. Neilands, and J.H. Crosa. 1984. Lack of homology between the iron transport regions of two virulence-linked bacterial plasmids. Infect. Immun. 43: 765-767.

408. Walter, M.A., S.A. Potter, and J.H. Crosa. 1983. Iron uptake system mediated by Vibrio anguillarum plasmid pJM1. J. Bacteriol. 156: 880-887.

409. Wang, S.P., K.K. Holmes, J.S. Knapp, S. Ott, and D.D. Kyzer. 1977. Immunological classification of Neisseria gonorrhoeae with micro-immunofluorescence. J. Immunol. 119: 797-803.

410. Ward, M.E., and P.J. Watt. 1972. Adherence of Neisseria gonorrhoeae to urethral mucosal cells: An electron-microscopic study of human gonorrhea. J. Infect. Dis. 126: 601-605.

411. Ward, M.E., P.J. Watt, and J.N. Robertson. 1974. The human fallopian tube: A laboratory model for gonococcal infection. J. Infect. Dis. 129: 650-659.

412. Ward, M.E., P.J. Watt, and A.A. Glynn. 1970. Gonococci in urethral exudates possess a virulence factor lost on subculture. Nature 227: 382-384.

413. Warner, P.J., P.H. Williams, A. Bindereif, and J.B. Neilands. 1981. ColV plasmid-specified aerobactin synthesis by invasive strains of Escherichia coli. Infect. Immun. 33: 540-545.

414. Watt, P.J., and A.R. Medlin. Generation of chemotaxis by gonococci, pp. 239-241. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young (eds.), Immunobiology of Neisseria gonorrhoeae. American Society for Microbiology, Washington, D.C.

415. Watt, P.J., and M.E. Ward. 1977. The Interaction of gonococci with human epithelial cells, pp. 356-368. In R.B. Roberts (ed.), The Gonococcus. John Wiley & Sons, New York.

416. Watt, P.J., and M.E. Ward. 1980. Adherence of Neisseria gonorrhoeae and other Neisseria species to mammalian cells, pp. 253-288. In E.H. Beachey (ed.), Bacterial Adherence. Chapman & Hall, London.

417. Wayne, R., and J.B. Neilands. 1975. Evidence for common binding sites for ferrichrome compounds and bacteriophage phi-80 in the cell envelopes of Escherichia coli. J. Bacteriol. 121: 497-503.

418. Weaver, C.A., and J. Konisky. 1980. Ton B-independent ferrichrome-mediated iron transport in Escherichia coli spheroplasts. J. Bacteriol. 143: 1513-1518.

419. Wegener, W.S., B.H. Hebeler, and S.A. Morse. 1977. Cell envelope of Neisseria gonorrhoeae: Relationship between autolysis in buffer and the hydrolysis of peptidoglycan. Infect. Immun. 18: 210-219.

420. Weinberg, E.D. 1974. Iron and susceptibility to infectious disease. Science 184: 952-956.

421. Weinberg, E.D. 1978. Iron and infection. Microbiol. Rev. 42: 45-66.

422. Weinberg, E.D. 1984. Iron withholding: A defense against infection and neoplasia. Physiologic. Rev. 64: 65-102.

423. Weisner, P.J., H.H. Handsfield, and K.K. Holmes. 1973. Low antibiotic resistance of gonococci causing disseminated infection. N. Engl. J. Med. 288: 1221-1222.

424. West, S.E.H., and P.F. Sparling. 1985. Response of *Neisseria gonorrhoeae* to iron limitation: Alterations in expression of membrane proteins without apparent siderophore production. Infect. Immun. 47: 388-394.

425. Westrom, L. 1985. Effect of acute pelvic inflamatory disease on fertility. Am. J. Obstet. Gynecol. 121: 707-713.

426. Williams, P.H. 1979. Novel iron uptake system specified by ColV plasmids: An important component in the virulence of invasive strains of *Escherichia coli*. Infect. Immun. 26: 925-932.

427. Williams, P., M.R.W. Brown, and P.A. Lambert. 1984. Effect of iron deprivation on the production of siderophores and outer membrane proteins in *Klebsiella aerogenes*. J. Gen. Microbiol. 130: 2357-2365.

428. Wiseman, G.M., and J.D. Caird. 1977. Composition of lipopolysaccharide of *Neisseria gonorrhoeae*. Infect. Immun. 16: 550-556.

429. Wolf-Watz, H., T. Elmros, S. Normark, and G.D. Bloom. 1975. Cell envelope of *Neisseria gonorrhoeae*: Outer membrane and peptidoglycan composition of penicillin-sensitive and -resistant strains. Infect. Immun. 11: 1332-1341.

430. Wolf-Watz, H., S. Normark, and G.D. Bloom. 1973. Rapid method for isolation of large quantities of outer membrane from Escherichia coli K12 and its application to the study of envelope mutants. J. Bacteriol. 115: 1191-1197.

431. Wookey, P.J. and H. Rosenberg. 1978. Involvement of inner and outer membrane components in the transport of iron and in colicin B action in Escherichia coli. J. Bacteriol. 133: 661-666.

432. Wright, A., and D.J. Tripper. 1979. The outer membrane of Gram-negative bacteria, pp. 427-480. In I.C. Gunsalus, J.R. Sokatch, and L.N. Ornsten (eds.), The Bacteria. A treatise on structure and function, Vol. VII. Academic Press, New York.

433. Yancey, R.J., S. Breeding, and C.E. Lankford. 1979. Enterochelin (enterobactin): Virulence factor for Salmonella typhimurium. Infect. Immun. 24: 174-180.

434. Yancey, R.J., and R.A. Finkelstein. 1981. Assimilation of iron by pathogenic Neisseria spp. Infect. Immun. 32: 592-599.

435. Yancey, R.J., and R.A. Finkelstein. 1981. Siderophore production by pathogenic Neisseria spp. Infect. Immun. 32: 600-608.

436. Young, J.D., M.S. Blake, A. Mauro, and Z.A. Cohn. 1983. Properties of the major outer membrane protein from Neisseria gonorrhoeae incorporated into model lipid membranes. Proc. Natl. Acad. Sci. USA. 80: 3831-3835.

437. Zak, K., J. Diaz, and J.E. Heckels. 1984. Antigenic variation during infection with Neisseria gonorrhoeae: Detection of antibodies to surface proteins in sera of patients with gonorrhea. J. Infect. Dis. 149: 166-174.

MANUSCRIPT 1

IDENTIFICATION OF AN IRON-REGULATED 37,000-DALTON PROTEIN IN THE CELL ENVELOPE OF *NEISSERIA GONORRHOEAE*

Contributors:

Timothy A. Mietzner

Geraldine H. Luginbuhl

Eric Sandstrom

Stephen A. Morse

ABSTRACT

We examined the outer membrane proteins which appear during the growth of Neisseria gonorrhoeae F62 in complex medium supplemented with 25 uM Desferal mesylate, a potent iron chelator. Outer membranes were prepared by Sarkosyl extraction and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Several higher-molecular-weight (74,000 to >94,000) proteins increased under iron-limiting conditions. In addition we observed the appearance of an iron-regulated protein with an apparent molecular weight of 37,000. This protein comigrated with the gonococcal protein I under normal Laemmli gel conditions. By increasing the ionic strength of the lower gel buffer, separation of protein I and the 37,000-dalton iron-regulated protein occurred. The 37,000-dalton protein stained poorly with Coomassie blue. However, when a silver stain was used, the protein appeared as a major component of the gonococcal outer membrane. Production of this 37,000-dalton protein was suppressed by the addition of iron to the medium. An iron-regulated protein with a similar molecular weight was observed in four clinical isolates and in an additional laboratory strain. Peptide mapping indicated that the 37,000-dalton protein was distinct from protein I and was identical between strains of the WI and WII serogroups.

INTRODUCTION

The gonococcal outer membrane (OM) protein profile resembles that of many Gram-negative bacteria in that there are relatively few proteins, with one to several predominating (12). Several of the OM proteins of *Neisseria gonorrhoeae* have been characterized. The major OM protein, protein I (PI), may account for more than 60% of the total OM protein (12). This protein functions as a porin through which low-molecular-weight, hydrophilic compounds pass (4). The molecular weight of PI is strain specific, ranging between 32,000 and 39,000. The variations in molecular weight have been correlated with structural differences as assessed by peptide mapping (26). These differences have led to the establishment of a serogrouping system based on PI. Using polyclonal antisera, three gonococcal serogroups designated coagglutination (CoA) groups WI, WII, and WIII have been identified (26).

A second major OM protein has been designated protein III (PIII) (30). This protein is surface exposed (31) and is common to all strains of gonococci examined. Chemical cross-linking analysis suggests that PIII is associated *in vivo* with PI (16, 24). The mobility of PIII in sodium dodecyl sulfate (SDS)-polyacrylamide gels is modified by reducing agents. PIII has an apparent molecular weight of 30,000 when electrophoresed under nonreducing conditions and 31,000 when electrophoresed under reducing conditions (10). Another predominate group of OM proteins has been designated protein IIs (PIIs) (30). This group of proteins is expressed variably on the surface of

gonococci, depending on colony phenotype and other incompletely defined parameters. More than one molecular weight species of this protein group may be present in a simgle strain. PIIs exhibit heat-modifiable behavior in SDS-polyacrylamide gels (11). PIIs may be involved in attachment to host cells (15).

Environmental factors can affect OM protein composition. Production of new OM proteins under conditions of iron-limitation occurs in many Gram-negative bacteria (23). In Escherichia coli, iron-limitation affects the synthesis of six envelope proteins with apparent molecular weights of 90,000, 83,000, 81,000, 78,000, 74,000 and 25,000 (13). These proteins have been designated as iron-regulated membrane proteins. The proteins with apparent molecular weights of 81,000 and 78,000 function as receptors for enterobactin- and ferrichrome-bound iron, respectively. These two proteins together with a 74,000-dalton protein are located in the OM (20). Another E. coli OM protein designated as FecA has an apparent molecular weight of 80,500 in SDS-polyacrylamide gels. Induction of this protein requires iron limitation as well as the functional iron chelator citrate (33). A ColV plasmid-encoded OM protein with an apparent molecular weight of 74,000 serves as the receptor for ferri-aerobactin (6). Whether this is the same E. coli OM protein mentioned previously remains to be determined.

Production of iron-regulated OM proteins with molecular weights of ca. 80,000 have been reported in Agrobacterium tumefaciens (17), Vibrio anguillarum (3), and Vibrio cholerae (27). Norqvist et al. (25) observed that gonococci grown in iron-limited medium produced OM proteins with apparent molecular weights of 76,000, 86,000 and 97,000.

In this study, we examined a number of gonococcal strains grown under

iron-limiting conditions. Our results confirm the observation of Norqvist *et al.* regarding production of the high-molecular-weight OM proteins. In addition, we observed a previously undescribed iron-regulated OM protein with an apparent molecular weight of 37,000, which when visualized with a silver stain appears to be a major OM protein. This report describes the environmental conditions under which this protein is produced, the conditions required for the observation of this protein, and the distribution of this protein among different gonococcal strains.

MATERIALS AND METHODS

Organisms. *N. gonorrhoeae* strains were kindly provided as follows: Strain F62 from R. P. Williams (Baylor College of Medicine, Houston, Tex.); strain FA171 from P. F. Sparling (University of North Carolina, Chapel Hill, N.C.); and strains 31481, 32170, 32153, and 32160 from J. Knapp (Seattle Public Health Service Hospital, Seattle, Wash.) The pertinent characteristics of these strains are shown in Table 1. The gonococcal strains were maintained as stock cultures by lyophilization or by freezing at -70°C. Inocula were prepared from stock cultures by growing them on GC agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% (vol/vol) IsoVitale-X (BBL Microbiology Systems, Cockeysville, Md.) and 0.5% (wt/vol) glucose. All cultures were propagated at 37°C in an atmosphere containing 4% CO_2.

Growth conditions. The basal liquid medium used for the growth of *N. gonorrhoeae* contained the following components per liter: proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCL, 5 g; soluble starch, 1 g. After autoclaving, the medium was supplemented with $NaHCO_3$, (420 mg/liter), a supplement similar to IsoVitale-X (1% [vol/vol]), and glucose (0.5% [wt/vol]). Gonococci are not able to remove iron bound to the chelator Desferal (21). To reduce the free iron concentration of the medium, we added 25 uM Desferal mesylate (Ciba-Geigy Corp., Summit, N.J.). An analysis of the complete medium by atomic absorption spectroscopy indicated that it contained 8.0 ± 1.2 uM iron. Theoretically,

the free iron in this growth medium is limited due to the excess Desferal. This medium will be referred to as low-iron medium. Medium in which iron was not limiting was identical in composition except that Desferal was not added. Defined medium was prepared as described previously (22). Iron was made limiting in this medium by omitting ferric nitrate and by adding the iron chelator ethylenediaminedi-(ortho)-hydroxy-phenyl acetate (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 5 ug/liter. This medium will be referred to as low-iron defined medium. Gonococci grown on GC agar for 20 h were suspended in growth medium and used to inoculate 300-ml nephelometer flasks (Bellco Glass Co., Vineland, N.J.) containing 50 ml of the same medium to a density of ca. 25 Klett units (as monitored with a Klett-Summerson colorimeter with a no. 54 filter). The turbidity was measured at intervals during incubation at 37°C in a gyratory shaker. Upon reaching mid-logarithmic phase, the entire suspension was used to inoculate 450 ml of medium. Incubation was continued until the culture reached late-logarithmic phase (100 to 120 Klett units), at which time the cells were harvested by centrifugation.

Preparation of outer membranes. Gonococcal OM were prepared by Sarkosyl extraction (5). Bacteria were washed in a minimal medium and centrifuged. The pellet was suspended in 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.1% (vol/vol) protease inhibitor (10 mM phenylmethylsulfonyl fluoride in isopropanol). The cells were disrupted by sonication and incubated with DNase (40 ug/ml), RNase (40 ug/ml), and 1 mM $MgCl_2$ at 37°C for 15 min. After centrifugation at 3,000 x g for 10 min to remove cell debris, the supernatant was subjected to high-speed centrifugation (48,000 x g for 20 min at 4°C) to sediment the membranes. The membrane pellet was washed once in HEPES buffer plus protease

inhibitor, and the membranes were sedimented (48,000 x g for 20 min at 4°C). The membrane pellet was diluted to 2 mg of total protein per ml and incubated with a final concentration of 0.2% (wt/vol) n-lauroyl sarcosine (Sarkosyl) for 10 min at room temperature. The Sarkosyl-insoluble fraction was sedimented by centrifugation (48,000 x g for 20 min at 4°C), washed once in HEPES buffer containing protease inhibitor, suspended in the same buffer, and stored at -70°C. OM protein concentrations were determined by the method of Bradford (2).

SDS-polyacrylamide gel electrophoresis. OMs (1 mg of protein per ml, final concentration) were solubilized in final sample buffer consisting of 0.0625 M Tris-hydrochloride (pH 6.8), 2.0% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.001% bromophenyl blue, and 5% (vol/vol) 2-mercaptoethanol. Samples were heated at 100°C for 5 min. Unless indicated, 20 ug of protein was added per lane. Gel electrophoresis was carried out on slab gels which were 140 mm long and 2 mm thick with the Laemmli gel and buffer formulations (14) modified by the addition of 70 mM NaCl (final concentration) to the separating gel. Various acrylamide concentrations as well as gradients between concentrations were used and are indicated in the text. Electrophoresis was performed at constant current (16 mA) overnight at room temperature.

Visualization of OM proteins. Gels were stained with 0.1% (wt/vol) Coomassie blue G250 (Miles Laboratories, Elkhart, Ind.) in a water:methanol:acetic acid (5:5:2) solution for 2 to 12 h. Acetic acid (10% [vol/vol]) was used to destain the gels. Alternatively, gels were stained by the silver staining method of Tsai and Frasch (32) modified by omission of the periodate oxidation step. Reagents were freshly prepared before each gel was stained. Development of the stain was terminated with a solution containing

10% (vol/vol) ethanol and 5% (vol/vol) acetic acid.

Peptide mapping. Two-dimensional peptide maps were prepared by the method Sandstrom *et al.* (26). Proteins were isolated by cutting out the appropriate protein band from a 9.5 to 12.5% gradient acrylamide gel. The bands were lyophilized and stored before preparation of their tryptic peptide maps.

RESULTS

Identification of an iron-regulated 37,000-dalton protein. Previous work investigating iron-regulated OM proteins of N. gonorrhoeae examined strain F62 (25). Therefore, this strain was selected as our prototype for much of this study. Inoculation of iron-limited cells of strain F62 into low-iron medium containing various concentrations of Desferal (0 to 100 uM) resulted in a decrease in cell yield with increasing Desferal concentrations (see appendix 1). In another experiment, the inoculum was grown in iron-sufficient medium and used to inoculate both low-iron and regular medium. Growth of strain F62 in low-iron medium consistently resulted in ca. a 5% increase in the mean generation time when compared with growth in regular medium. The Sarkosyl-insoluble OM protein profiles of strain F62 grown under these two conditions are shown in Fig 1. Two high-molecular-weight OM proteins (apparent molecular weights of 103,000 and 79,000) were observed in Coomassie blue-stained gels and were correlated with growth in low-iron medium. The apparent molecular weights of these proteins were similar to those reported by Norqvist et al. (25). By comparison, an identical SDS-polyacrylamide gel stained with the silver stain revealed four iron-regulated proteins. Two of these proteins were the high-molecular-weight species observed in the Coomassie blue-stained gel; the other two were visualized only with the silver stain and had apparent molecular weights of 37,000 and 19,000. No apparent differences were observed in the OM protein profile of cells harvested from

low-iron medium in which the inoculum was grown under iron-limiting or iron-sufficient conditions (data not shown).

The 37,000-dalton (37K) protein was especially interesting because of several unique properties. This protein stained heavily with the silver stain when 20 ug of protein was electrophoresed (Fig 1). With 20 ug of protein, the 37K protein was not observed in gels stained with Coomassie blue. However, with 40 ug of protein, a faint band was observed in Coomassie blue-stained gels. Separation of this protein from PI was dependent upon the ionic strength of the separating gel. Incorporation of 70 mM NaCl (final concentration) in the lower gel resulted in optimal separation of the 37K protein from PI; however, in the absence of an increased NaCl concentration, the 37K protein comigrated with PI. This phenomenon was demonstrated in the following experiment. Sarkosyl-insoluble OM proteins from strain F62 were electrophoresed under standard Laemmli gel conditions (14) (no NaCl added) on a 9.5 to 12.5% acrylamide gradient gel. A protein with an apparent molecular weight of 37,000 was not observed (data not shown). When the band containing PI was cut out and electrophoresed on a 9.5 to 12.5% acrylamide gradient gel containing 70 mM NaCl, separation of the 37K protein and the lower-molecular-weight PI was observed (data not shown). A similar phenomenon was observed in N. gonorrhoeae FA171, which possesses a PI with a lower apparent molecular weight (34,000) than the PI of strain F62 (36,000).

Separation of the 37K protein from PI was also dependent upon the concentration of acrylamide in the gel (Fig 2). At an acrylamide concentration of 9.5%, two bands were clearly observed by silver staining. The 37K protein stained faintly with Coomassie blue. At an acrylamide concentration of 12.5%, the proteins (37K and PI) did not appear to migrate as

separate bands when gels were stained with Coomassie blue. Silver staining of the 12.5% gel revealed that the two proteins, PI and 37K, migrated close together. We did not observe any change in the migration of the 37K protein when the OM preparation was solubilized at room temperature for 30 min or if 2-mercaptoethanol was omitted from the final sample buffer (data not shown).

Growth at pH 6.3 increased the mean generation time by 22% with respect to growth in medium at pH 7.4 (see appendix 2). The 37K protein was not observed in OMs of cells grown at pH 6.3. If Desferal (25 uM) was added to this medium there was a further increase in the mean generation time of ca. 2%; the 37K protein was observed in Sarkosyl-extracted OMs from these cells (Fig 3). Thus, the expression of the 37K protein was not due to a decreased growth rate. In addition, the appearance of the 37K protein was not growth-phase dependent. It was observed in OM preparations of strain F62 taken from early-, middle-, and late-logarithmic-phase cultures grown in low-iron medium. The 37K protein was also observed in cells grown in low-iron defined medium (Fig 5). Thus, the production of this protein was probably not an artifact of the medium or the iron chelator used.

Synthesis of the 37K protein was influenced by the concentration of available iron. Strain F62 was grown in low-iron medium (100 Klett units) to permit expression of the 37K protein. These cells were then used to inoculate four Klett flasks containing 50 ml of low-iron medium supplemented with 0, 1, 10, or 100 uM ferric nitrate. The bacteria were grown to mid-logarithmic phase (80 Klett units) and harvested, and OMs were prepared as previously described. The results (Fig 4) show that the amount of the 37K protein as well as the higher-molecular-weight proteins (103,000, 79,000) decreased with increasing concentrations of iron. With low-iron defined medium, the

production of the 37K protein was suppressed by the addition of 100 uM ferric nitrate (Fig 5). Therefore, it seems likely that the synthesis of the 37K protein is regulated by the available iron in the environment.

Occurrence of the 37K protein. Several clinical isolates and an additional laboratory strain of *N. gonorrhoeae* were grown under iron-limited conditions and examined for the presence of iron-regulated proteins. The results are shown in Table 1. The gonococcal strains varied with regard to CoA group, auxotype, ability to utilize lactoferrin-bound iron, and the production of higher-molecular-weight iron-regulated OM proteins. An iron-regulated protein with an apparent molecular weight of 37,000 was observed in OM preparations from all strains examined.

Peptide mapping. Since the 37K protein and PI comigrate under normal Laemmli gel conditions it was possible that the 37K protein was a modified PI. Tryptic maps of these two proteins indicated that the 37K protein was distinct from PI (Fig 6). The two proteins (PI and 37K) did not appear to share any common tryptic peptides. Furthermore, the 37K proteins isolated from strains F62 (CoA group WII) and FA171 (CoA group WI) were nearly identical.

DISCUSSION

Production of OM proteins under conditions of iron limitation is a well-recognized phenomenon among Gram-negative organisms (23). The human host presents an environment which is limited with respect to free iron, most of the available iron being bound to the proteins transferrin and lactoferrin (34). Consequently, expression of iron-regulated proteins is likely to occur *in vivo* (7).

The mechanism(s) by which *N. gonorrhoeae* obtains iron from its environment is incompletely understood. Some pathogens utilize siderophores, soluble low-molecular-weight compounds which have a high-affinity for iron (23). These siderophores compete with the host for iron and render it available to the bacterium. In some cases, iron-regulated OM proteins function as bacterial receptors for ferric-siderophore complexes (6, 13, 23). The existence of a gonococcal siderophore has been reported (35) but not confirmed (S. West, E. Blackman, P. Mickelsen, and F. Sparling, Abstr. Int. Soc. Sexually Transmitted Disease Res. 1983, abstract no. 8, p. 40). Direct association between host iron-binding proteins and the surface of *N. meningitidis* has been shown to be a prerequisite for utilization of transferrin-bround iron (1). A similar interaction has been observed in this laboratory between *N. gonorrhoeae* and lactoferrin (see appendix 3). The acquisition of iron from the host probably involves the interaction of iron or some iron-binding component with a cell surface structure. The iron-regulated

gonococcal OM proteins may be involved in such a surface-associated mechanism.

A unique 37,000-dalton OM protein is produced under iron-limited conditions by N. gonorrhoeae. This protein is only resolved in SDS-polyacrylamide gels under stringent conditions. One of these conditions appears to be an increased ionic strength within the resolving gel. An increase in ionic strength has been reported to sharpen protein bands in SDS-polyacrylamide gels (29). This increased resolution may permit PI and the 37K protein to migrate as two distinct bands. However, if a PI with a lower apparent molecular weight (from strain FA171) is run at low ionic strength, the same comigration of 37K and PI occurs. Presumably, the two proteins would separate due to the increased difference between their respective apparent molecular weights (37,000 and 34,000). Therefore, an alternative hypothesis that a subtle alteration in the migration of the 37K protein occurs at increased ionic strength cannot be ruled out at this time. It is also of interest that the 37K protein stains poorly with Coomassie blue but stains well with the silver stain. For the silver stain, the relationship between concentration and staining intensity is approximately linear for certain proteins (e.g., phosphorylase); with other proteins (e.g., bovine serum albumin) this relationship is not linear (9). If the former is true of the 37K protein, it may constitute a major OM component of gonococci grown under iron-limiting conditions.

It has been suggested recently that the silver stain reacts with negatively charged groups on proteins (8). Magnusson et al. (18) observed that the production of new OM proteins in gonococci grown under iron-limited conditions was accompanied by an increase in the negative surface charge of the organism. It is tempting to speculate that the 37K protein may contribute

to this increased negative charge.

Production of some OM proteins of Pseudomonas aeruginosa is dependent upon the growth medium used (10). We observed similar amounts of the 37K protein from gonococci grown in both defined and complex medium. In addition, there was no apparent growth phase-dependent production of the 37K protein. Detectable amounts of the 37K protein were present in OMs of cells grown to stationary phase in normal medium (data not shown). However, we attribute this to the decrease in iron available from the medium by the increased number of cells.

The OM composition of E. coli can be altered by changing its growth rate (25). Growth of gonococci under conditions of iron limitation increased the mean generation time by ca. 5%. Therefore, we examined whether production of the 37K protein occurred as a consequence of a slower growth rate. Growth at pH 6.3 increased the mean generation time by 22%, yet no increase in the 37K protein was observed. When fully expressed, the continued production of the 37K protein was reduced by the addition of iron in a concentration-dependent fashion. Furthermore, addition of iron to low-iron defined medium before growth of gonococci completely suppressed production of the 37K protein. Thus, it seems likely that the 37K protein is, in some way, regulated by the concentration of free iron.

Several high-molecular-weight iron-regulated OM proteins have been reported in various strains of N. gonorrhoeae (25). We observed that a 37K protein was present in six strains examined and that its presence was independent of CoA group, auxotype, ability to utilize lactoferrin, and production of the various higher-molecular-weight iron-regulated proteins. Migration of the 37K protein in SDS-polyacrylamide gels appeared identical

among all strains examined. Tryptic peptide maps of the 37K protein from two strains (F62 and FA171) which differed in many characteristics (see Table 1) were nearly identical. However, some subtle differences were observed. Whether these differences are structurally relevant or merely methodological artifacts remains to be seen. PI and the 37K protein shared no common peptides. This negates the possibility that the 37K protein is either a modified or an unprocessed PI and further suggests that it is a unique protein. Additional studies will examine the variability of the 37K protein among gonococcal strains.

To our knowledge, this is the first report of a gonococcal iron-regulated protein with a molecular weight of 37,000. Recently, we observed a protein with the same apparent molecular weight that was produced under iron-limiting conditions by three strains of *Neisseria meningitidis*. The meningococcal and gonococcal 37K proteins exhibit similar staining characteristics on SDS-polyacrylamide gels. No iron-regulated protein with an apparent molecular weight of 37,000 was observed in a strain of *Neisseria flavescens*. We are presently examining the immunological and structural similarities between the gonococcal and meningococcal 37K proteins (see appendix 4). It is tempting to speculate that there is a correlation between production of this protein and pathogenicity among the neisseria. Gonococci and meningococci exhibit similar iron uptake mechanisms; commensal neisseria are apparently different (21, 28). Therefore, the 37K protein may have a role in the acquisition of iron from an iron-limited environment.

LITERATURE CITED

1. Archibald, F. S., and I. W. DeVoe. 1979. Removal of iron from human transferrin by *Neisseria meningitidis*. FEMS Microbiol. Lett. 66:159-162.

2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

3. Crosa, J. H., and L. L. Hodges. 1981. Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum* 775. Infect. Immun. 31: 223-227.

4. Douglas, J. T., M. D. Lee, and H. Nikaido. 1981. Protein I of *Neisseria gonorrhoeae* is a porin. FEMS Microbiol. Lett. 12: 305-309.

5. Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115: 717-722.

6. Grewal, K. K., P. J. Warner, and P. H. Williams. 1982. An inducible outer membrane protein involved in aerobactin-mediated iron transport by ColV strains of *Escherichia coli*. FEBS Lett. 140: 27-30.

7. Griffiths, E., P. Stevenson, and P. Joyce. 1983. Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*. FEMS Microbiol. Lett. 16: 95-99.

8. Guevara, J. Jr., D. A. Johnston, L. S. Ramagali, B. A. Martin, S. Capetillo, and L. V. Rodriguez. 1982. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels. Electrophoresis. 3: 197-205.

9. Hames, B. D. 1981. Introduction to PAGE, p. 1-91. *In* B. D. Hames and C. Ricketts (eds.), Gel electrophoresis of proteins: a practical approach. IRL Press Ltd., London.

10. Hancock, R. E. W., and A. M. Carey. 1979. Protein D1- a glucose inducible, pore-forming, pore-forming protein from the outer membrane of Pseudomonas aeruginosa. FEMS Microbiol. Lett. 8: 105-109.

11. Heckels, J. E. 1977. The surface properties of Neisseria gonorrhoeae: Isolation of the major components of the outer membrane. J. Gen. Microbiol. 99: 333-341.

12. Johnston, K. H., and E. C. Gotschlich. 1974. Isolation and characterization of the outer membrane of Neisseria gonorrhoeae. J. Bacteriol. 119: 250-257.

13. Klebba, P. E., M. A. McIntosh, and J. B. Neilands. 1982. Kinetics of biosynthesis of iron-regulated membrane proteins in Eschericnia coli. J. Bacteriol. 149: 880-888.

14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

15. Lambden, P. R., J. E. Heckels, L. T. James, and P. J. Watt. 1979. Variations in surface protein composition associated with virulence properties in opacity type of Neisseria gonorrhoeae. J. Gen. Microbiol. 114: 305-312.

15. Lambden, P. R., J. E. Heckels, L. T. James, and P. J. Watt. 1979. Variations in surface protein composition associated with virulence properties in opacity type of Neisseria gonorrhoeae. J. Gen. Microbiol. 114: 305-312.

16. Leith, D. K., and S. A. Morse. 1980. Cross-linking analysis of Neisseria gonorrhoeae outer membrane proteins. J. Bacteriol. 143: 182-187.

17. Leon, S. A., and J. B. Neilands. 1981. Relationship of sidero-phore-mediated iron assimilation to virulence in crown gall disease. J. Bacteriol. 147: 482-491.

18. Magnusson, K. E., K. E. Kihlstrom, A. Norqvist, J. Davies, and S. Normark. 1979. Effect of iron on surface charge and hydrophobicity of *Neisseria gonorrhoeae*. Infect. Immun. 26: 402-407.

19. McDade, R. L., Jr., and K. H. Johnson. 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. 141: 1183-1191.

20. McIntosh, M. A., and C. F. Earhart. 1977. Coordinate regulation by iron of the synthesis of phenolate compounds and three outer membrane proteins in *Escherichia coli*. J. Bacteriol. 131: 331-339.

21. Mickelsen, P. A., E. Blackman, and P. F. Sparling. 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis* and commensal *Neisseria* species to obtain iron from lactoferrin. Infect. Immun. 35: 915-920.

22. Morse, S. A., and L. Bartenstein. 1980. Purine metabolism in *Neisseria gonorrhoeae*: The requirements for hypoxanthine. Can. J. Microbiol. 26: 13-20.

23. Neilands, J. B. 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. 36: 285-309.

24. Newhall, W. J., W. D. Sawyer, and R. A. Haak. 1980. Cross-linking analysis of the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. 28: 785-791.

25. Norqvist, A., J. Davis, L. Norlander, and S. Normark. 1978. The effect of iron starvation on the outer membrane protein composition of *Neisseria gonorrhoeae*. FEMS Microbiol. Lett. 4: 71-75.

26. Sandstrom, E. C., K. C. S. Chen, and T. M. Buchanan. 1982. Serology of Neisseria gonorrhoeae: Coagglutination groups WI and WII/WIII correspond to different outer membrane protein I molecules. Infect. Immun. 38: 462-470.

27. Sigel, S.P., and S. M. Payne. 1982. Effect of iron-limitation on growth, siderophore production, and expression of outer membrane proteins of Vibrio cholerae. J. Bacteriol. 150: 148-155.

28. Simonson, C., D. Brenner, and I. W. DeVoe. 1982. Expression of a high-affinity mechanism for the acquisition of transferrin iron by Neisseria meningitidis. Infect. Immun. 36: 107-113.

29. Steck, T. L., and C. F. Fox. 1972. Membrane proteins, p. 27-75. In C. F. Fox and A. D. Keith (eds.), Membrane molecular biology. Sinauer Associates Inc., Stamford, Conn.

30. Swanson, J., and J. Heckels. 1980. Proposal: nomenclature of gonococcal outer membrane proteins, p. 21-26. In S. Normark and D. Danielsson (eds.), Genetics and immunobiology of pathogenic neisseria. EMBO Workshop, Hemavan, Sweden.

31. Swanson, J., L. W. Mayer, and M. R. Tam. 1982. Antigenicity of Neisseria gonorrhoeae outer membrane protein(s) III detected by immunoprecipitation and Western blot transfer with a monoclonal antibody. Infect. Immun. 38: 668-672.

32. Tsai, L. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119: 115-119.

33. Wagegg, W., and V. Braun. 1981. Ferric citrate transport in Escherichia

coli requires outer membrane receptor protein Fec A. J. Bacteriol. 145: 156-163.

34. Weinberg, E. D. 1978. Iron and Infection. Microbiol. Rev. 42: 45-66.

35. Yancey, R. J., and R. A. Finkelstein. 1981. Siderophore production by pathogenic Neisseria spp. Infect. Immun. 32: 600-608.

Fig. 1. OM protein profile (9.5 to 12.5% acrylamide gradient gel) of *N. gonorrhoeae* F62 grown in low-iron medium (+DF) and in regular medium (-DF). Each lane was loaded with 20 ug of protein, and the gel was electrophoresed as described in the text. The gel was stained with either silver or Coomassie blue. Molecular weight standards were phosphorylase (94K), albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), trypsin inhibitor (20K), and lactalbumin (14K). The iron-regulated proteins were observed when strain F62 was grown in low-iron medium. Two of these proteins are present in the Coomassie blue-stained gel (103K and 79K); four are present in the silver stained gel (103K, 79K, 37K, and 19K).

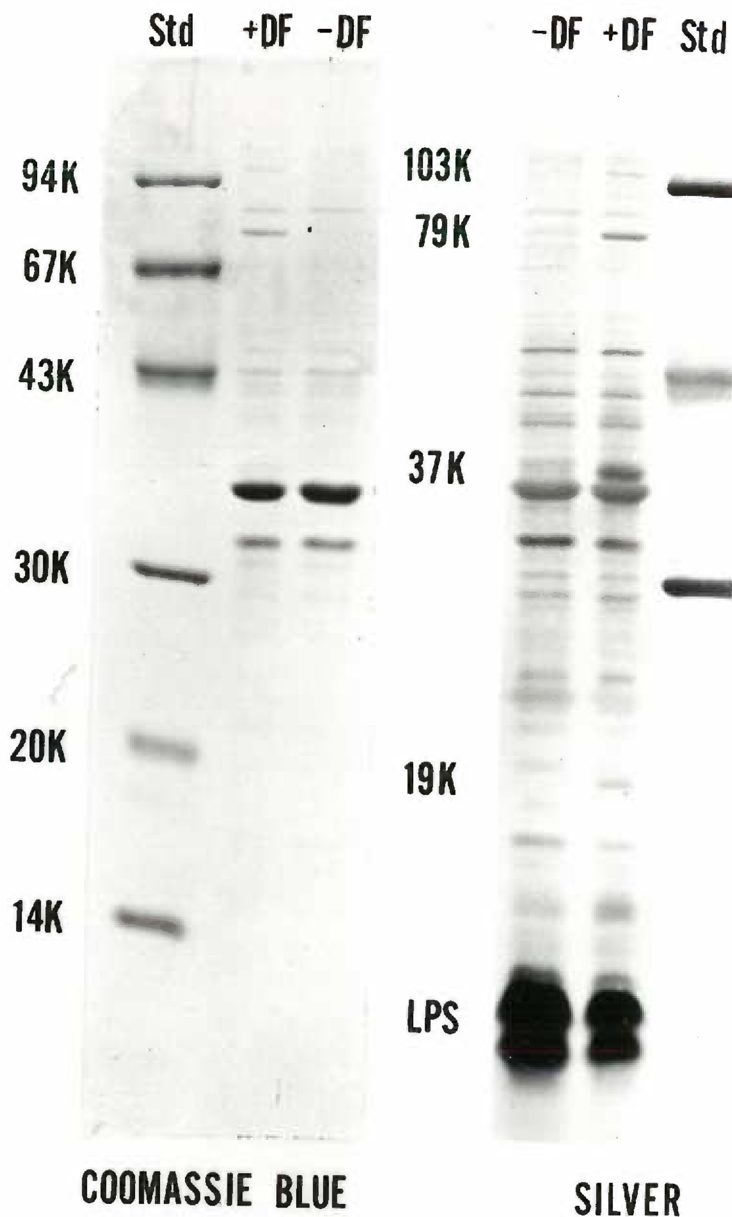

Std +DF -DF
-DF +DF Std
94K
67K
43K
30K
20K
14K
103K
79K
37K
19K
LPS
COOMASSIE BLUE
SILVER

Fig. 2. Effect of acrylamide concentration on the separation of PI and the 37K protein. OMs (20 ug of protein) from N. gonorrhoeae F62 grown in low-iron medium was electrophoresed as described in the text. At acrylamide concentrations of 9.5%, separation of the 37K protein and PI occurred when visualized by the silver stain. At acrylamide concentrations of 12.5%, the 37K protein and PI did not separate and could only be seen as two bands by virtue of their different silver staining characteristics. Ag, Silver stain; CB, Coomassie blue stain.

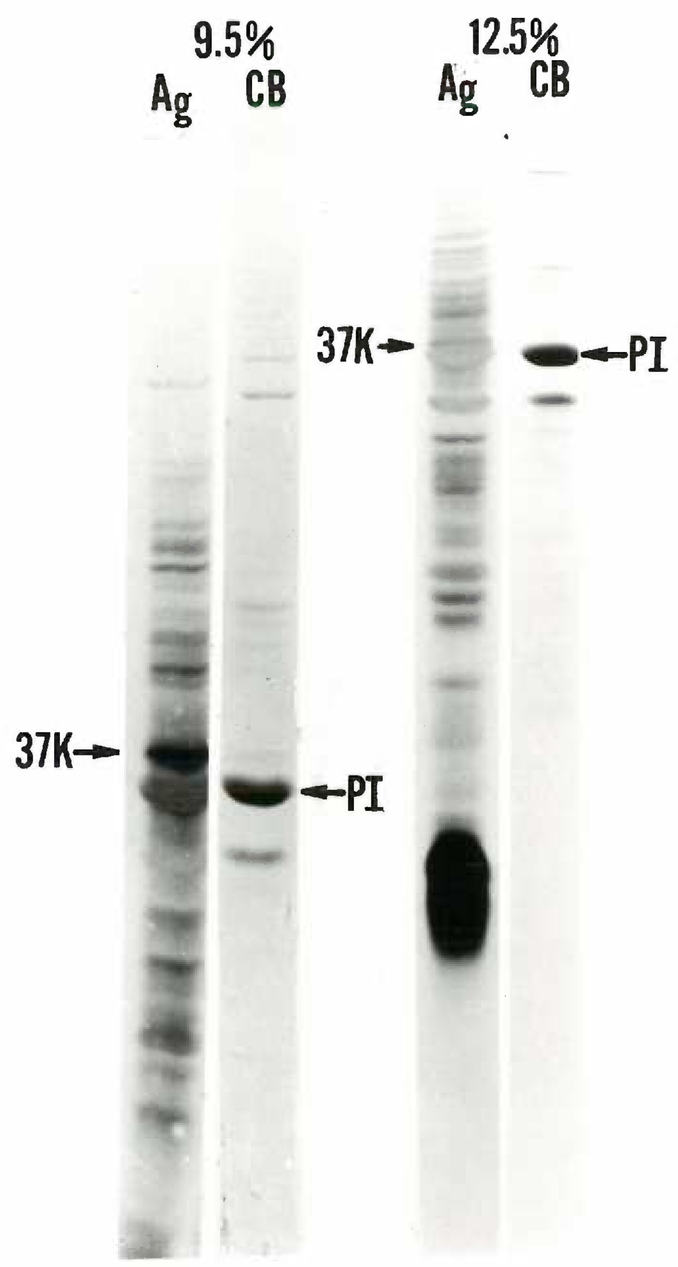

9.5%
Ag
CB
37K
PI
12.5%
Ag
CB
37K
PI

Fig. 3. OM protein profiles of *N. gonorrhoeae* F62 grown at pH 6.3 in low-iron medium (+DF) and regular medium (-DF). The growth medium contained 50 mM HEPES adjusted to a final pH of 6.3. OMs (20 ug of protein) was electrophoresed as described in the text, and the gels were stained with silver stain.

-DF +DF
37K
pH 6.3

Fig. 4. Coomassie blue-stained gradient (9.5 to 12.5% acrylamide) gel of OMs (40 ug of protein) prepared from *N. gonorrhoeae* F62 grown as described in the text.

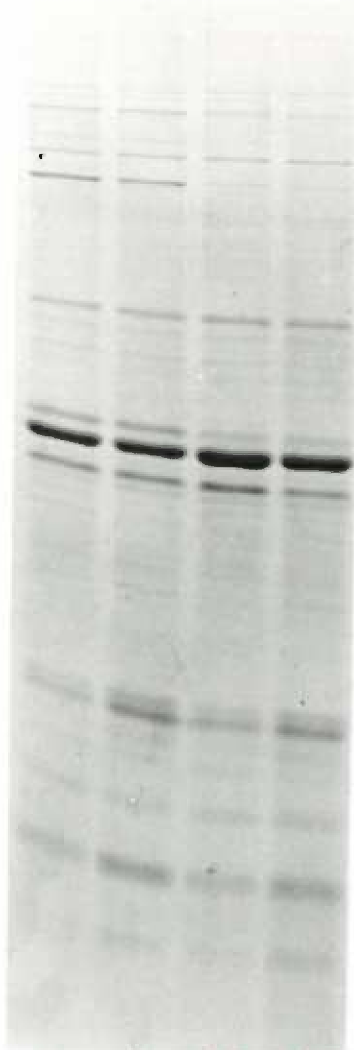

A B C D
37K
0 1 10 100
μM Fe Added

Fig. 5. Coomassie blue-stained gel (9.5 to 12.5% acrylamide) of) OM preparations (40 ug of protein per lane) from N. gonorrhoeae F62 grown in regular defined medium (A), low-iron defined medium supplemented with 100 uM ferric nitrate (B), and low-iron defined medium without additional iron (C).

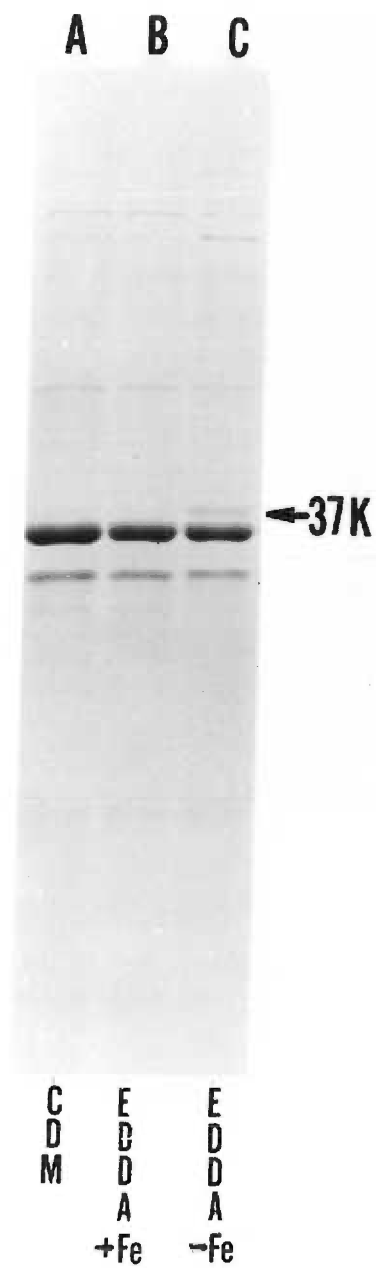
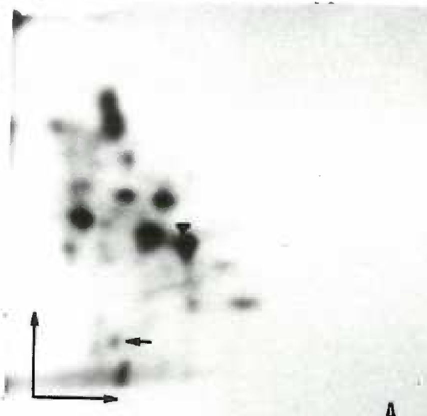
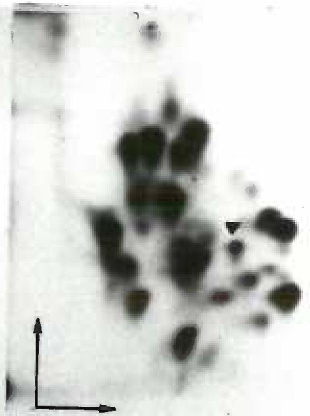
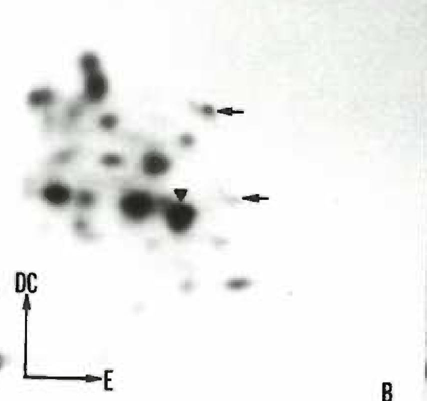

A
B
C
37K
CDM
EDDA +Fe
EDDA -Fe

Fig. 6. Autoradiographs of tryptic peptide maps of ^{125}I-labeled 37K and PI molecules. Peptides were separated by paper electrophoresis (E) in the first dimension and descending chromatography (DC) in the second dimension. Panels A and B are tryptic maps of the 37K protein from _N. gonorrhoeae_ strains FA171 and F62, respectively. Panels C and D are tryptic maps of the PI molecules from strains FA171 (WI) and F62 (WII), respectively. The triangle denotes the internal marker valine. The arrows seen in panels A and B point out minor peptides which appear to be unique to the 37K protein of the respective strain.


A


C


DC
E
B

D

Table 1. N. gonorrhoeae iron-regulated OM proteins.

Strain	Auxotype	Protein I serogroup	Lf[a]	Presence of iron-regulated proteins (kilodaltons)						
				103	89	85	79	74	37	18
31481	Prototrophic	WI	ND	+	–	–	+	–	+	0[c]
32170	Prototrophic	WII	ND	+	–	+	+	–	+	0
32153	AHU⁻	WI	ND	+	–	–	–	+	+	0
32160	Prototrophic	WI	ND	+	+	–	–	–	+	0
F62	Pro⁻	WII	–	+	–	–	+	–	+	+
FA171	Protrophic	WI	+	+	–	–	+	–	+	–

a. Ability of the strain to utilize the iron from lactoferrin.

b. Phenotype not determined

c. Protein was not detected because the gel with that sample was not silver-stained or lower-molecular-weight proteins were run off the gel to better separate the higher-molecular-weight iron-regulated proteins.

MANUSCRIPT 2

PURIFICATION AND CHARACTERIZATION OF THE MAJOR IRON-REGULATED PROTEIN OF *NEISSERIA GONORRHOEAE*

Contributors:

Timothy A. Mietzner

Gary K. Schoolnik

Stephen A. Morse

INTRODUCTION

The cellular composition and many phenotypic properties of *Neisseria gonorrhoeae* can be influenced by the nature of its growth environment (18,21). The majority of the extracellular iron in the human host is bound to the proteins transferrin and lactoferrin (30). The amount of free iron which remains in equilibrium with these proteins is on the order of 10^{-18} M (3), far too low to support bacterial growth. During the course of a natural infection, gonococci must encounter and overcome this iron-restricted environment. Many pathogenic microorganisms have been shown to respond *in vitro* to an iron-limited environment by expressing new proteins (for review see ref. 19). Many of these proteins are functional components of the microorganism's iron-uptake system. Recent studies have determined that iron-regulated proteins of *Escherichia coli* and *Vibrio cholerae* are expressed during experimental infections *in vivo* (9,24). Therefore, proteins expressed during iron-limitation are most likely present and functional during natural infection.

The membrane proteins expressed by *N. gonorrhoeae* when grown under iron-limitation have been examined (17,20,31). Norqvist *et al.*(20) described the presence of several high-molecular-weight (70,000 to 100,000) membrane iron-regulated proteins. The number and apparent molecular weights of these proteins varied among gonococcal isolates. Subsequent studies have confirmed the presence of these high-molecular-weight iron-regulated proteins (17,31). In addition, these reports described a previously unidentified iron-regulated

protein with an apparent molecular weight of *ca*. 37,000. Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis visualized with a sensitive silver stain indicated that this protein represented a major component of Sarkosyl-insoluble membrane preparations from gonococci grown under iron-limited conditions (17). For this reason, this protein is referred to as the major iron-regulated protein (MIRP). To date no function has been identified for the gonococcal MIRP.

In order to facilitate study of the MIRP of *N. gonorrhoeae*, a method to purify this protein in quantities sufficient to permit biological and chemical analysis was developed. This procedure and several of the biochemical characteristics of the MIRP are described in this report.

MATERIALS AND METHODS

Media and reagents. GC agar medium (Difco Laboratories, Detroit, Michigan) supplemented with 1% (v/v) Iso Vitale-X (BBL Microbiology Systems, Cockeysville, Maryland) and 0.5% (w/v) glucose was used for the routine maintainance of gonococci. Cultures were grown at 37° C in a humidified atmosphere containing 4% CO_2. The basal liquid medium used for the growth of the organisms has been previously described (17). Analysis by atomic absorption spectroscopy indicated that this medium contained *ca.* 8.0 uM iron. In order to reduce the amount of free iron in the medium, 25 uM Desferal mesylate (Ciba-Geigy Corp., Summit, New Jersey) was added. This medium is referred to as low-iron liquid medium. Gonococci are not able to remove iron bound to the chelator Desferal (29). Theoretically, the free iron in this growth medium is limited due to the excess Desferal. This medium is referred to as low-iron medium.

Sodium dodecyl sulfate (SDS), acrylamide, 2-mercaptoethanol, urea, molecular weight standards for SDS-polyacrylamide gels, and bromphenyl blue were obtained from BioRad Laboratories, Richmond, California; Tris(hydroxymethyl)-aminomethane base (Tris buffer), hexadecyl-trimethylammonium bromide (CTB), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA) from Sigma Chemical Co., St. Louis, Missouri; N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES buffer) from Research Organics Inc., Cleveland, Ohio; Coomassie brilliant blue R-250 (Coomassie blue) from Bethesda Research Laboratories, Bethesda, Maryland; Zwittergent 3-14 from Calbiochem, La Jolla, California;

carboxymethyl (CM)-Sepharose CL-6B, Sephacryl S-300, gel filtration molecular weight standards, and pI standards from Pharmacia fine chemicals, Piscataway, New Jersey. All other chemicals were of reagent grade.

Bacteria and growth conditions. *N. gonorrhoeae* strain F62 was kindly provided by R.P. Williams (Baylor College of Medicine, Houston, Texas). All purification procedures and subsequent experiments utilized this strain as the source of the MIRP. Transparent (Op^-), non-piliated (P^-) variants of this strain were selected and inocula prepared following growth on GC agar medium for 20 h. Gonococci from these plates were harvested and suspended in low-iron medium. This suspension was used to inoculate 300 ml Nephelometer flasks (Bellco Glass Co., Vineland, New Jersey) containing 50 ml of the same medium to a density of *ca.* 25 Klett units (as monitored using a Klett-Summerson colorimeter with a #54 filter). The turbidity was measured at intervals during incubation at 37° C in a gyratory shaker. Upon reaching mid-logarithmic phase, these suspensions were diluted 1:10 in low-iron medium. Incubation was continued until the culture reached late-logarithmic phase (100 to 120 Klett units), at which time the cells were harvested by centrifugation (10,000 x g for 10 min at 4° C).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using the gel and buffer formulations described by Laemmli (13). The resolving gel consisted of 10% acrylamide (w/v) and contained 70 mM NaCl as described previously (17). All protein determinations were performed using the method of Markwell *et al.* (15). Protein concentrations were adjusted to 1 mg/ml in a final sample buffer consisting of 62.5 mM Tris buffer, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v)bromphenyl blue, and 5% (v/v) 2-mercaptoethanol. After dilution, samples

were heated to 100° C and held for 5 min. Electrophoresis was carried out using slab gels which were 140 mm long and 2 mm thick. A constant current (16 mA) was applied to the gel overnight at room temperature, and electrophoresis was terminated when the dye front reached the bottom of the gel. The gels were stained for 2-12 hours in a solution of 0.1% (w/v) Coomassie blue in water:methanol:acetic acid (5:5:2). A 10% (v/v) acetic acid solution was used to destain the gels. Alternatively, gels were stained by the silver staining method of Tsai and Frasch (29) modified by the omission of the periodate oxidation step. Development of the stain was terminated using a solution containing 10% (v/v) ethanol and 5% (v/v) acetic acid.

Cell fractionation. Bacteria harvested from 10 l of medium were washed once in Davis A defined medium (Difco). The cell pellet was suspended in 100 ml of 10 mM HEPES buffer, pH 7.4, containing 0.1% (v/v) serine-protease inhibitor (10 mM PMSF in isopropanol). Aliquots of 5 ml were subjected to sonication for a total of 1 min using a high intensity sonifier (Branson Instruments Inc., Stamford, Conn.). This suspension was centrifuged at 48,000 x g for 60 min. The pellet was enriched for cellular membranes, while the supernatant contained primarily soluble proteins. Both the membrane and supernatant fractions were analyzed by SDS-PAGE for the proportion of the MIRP contained in each.

Selective solubilization of the MIRP from gonococcal membranes. The MIRP associated with the membrane fraction was selectively solubilized using the detergent CTB. Optimum ratios of detergent to membrane protein were determined as follows: Crude membranes were washed once in 10 mM HEPES buffer, pH 7.4, containing protease inhibitor and pelleted by centrifugation (48,000 x g for 60 min at 4° C). The pellet was suspended in 10 mM Tris

buffer, pH 8.0, to a final crude membrane protein concentration of 1 mg/ml. Increasing amounts of CTB, up to a final concentration of 0.8% (w/v), were added to 1 ml aliquots of the crude membrane suspensions. The detergent-membrane mixtures were incubated at room temperature for 20 min, at which time the insoluble material was removed by centrifugation at 48,000 x g for 60 min (room temperature). Equal volumes of the supernatants were analyzed by SDS-PAGE for the solubilized MIRP. The optimum detergent-to-protein ratio was defined as the lowest concentration of CTB which solubilized all of the membrane-associated MIRP.

Isolation of crude MIRP preparations. The pellet from sonicated whole cells was diluted to a final protein concentration of 1 mg/ml and CTB added to the optimum detergent:protein ratio. After incubation for 20 min at room temperature, the solubilized proteins were separated by centrifugation (48,000 x g for 60 min at room temperature). The supernatant was enriched for the MIRP and was referred to as the crude preparation of the membrane-associated MIRP.

A similar method was used to obtain a preparation enriched for the MIRP associated with the supernatant fraction of sonicated whole cells. The supernatant fraction was diluted to a final protein concentration of 1 mg/ml in 10 mM Tris buffer, pH 8.0. To this suspension, CTB (0.05% [w/v] final concentration) was added which resulted in the formation of a white precipitate. This mixture was incubated at room temperature for 20 min and the precipitate removed by centrifugation at 48,000 x g for 60 min (room temperature). The resulting supernatant was enriched for the MIRP and was referred to as the crude preparation of the soluble MIRP.

Ion-exchange chromatography. Separation of proteins by ion-exchange

chromatography was accomplished using the cation-exchange gel matrix CM-Sepharose 6B-CL. A 250 mm x 20 mm column was prepared by washing the CM-Sepharose 6B-CL with two bed-volumes of 0.1 N NaOH followed by two bed-volumes of 1 M NaCl in 10 mM Tris buffer (pH 8.0) containing 0.05% (w/v) CTB. The column was then equilibrated with 5 bed-volumes of 10 mM Tris buffer containing 0.05% (w/v) CTB (final equilibration buffer). The crude MIRP preparations were applied to the column and washed with the final equilibration buffer until all material not interacting with the column had eluted. A 0 to 1 M NaCl gradient in a total volume of 500 ml of the final equilibration buffer was used to elute proteins from the column. Fresh column packing was used for each purification. The column flow rate was maintained at 20 ml/h, and the elution profile was followed by absorption at 280 nm using an in-line UV-2 monitor (Pharmacia). Fractions of 4 ml were collected.

Molecular seive chromatography. Sephacryl S-300 was used for gel filtration chromatography. A 850 mm x 20 mm column was equilibrated and proteins were eluted with 10 mM Tris, pH 8.0, containing 0.05% (w/v) CTB and 0.3 M NaCl. Alternatively, gel filtration in a Zwittergent 3-14-containing buffer as described by Blake *et al.* (2) was used. Samples for molecular seive chromatography were dialyzed overnight in the respective elution buffer. The dialyzed protein in a 2 ml volume was applied to the column and the flow rate was maintained at 30 ml/h. Fractions of 2.0 ml were collected and the protein absorption at 280 nm was followed. The molecular weight standards used were ferritin (440,000 daltons), catalase (232,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen A (25,000 daltons), and ribonuclease A (13,700 daltons). Protein I from *N. gonorrhoeae* was purified as previously described (2).

Isoelectric focusing. Isoelectric focusing was performed using previously described methods (12). Protein samples were dialyzed against 10 mM Tris buffer, pH 7.2, containing 6 M urea and 0.01% Triton X-100. Prepoured native polyacrylamide gels containing an ampholine mixture of 3.5 to 9.5 (LKB, Bromma, Sweden) were used. Determination of isoelectric points were extrapolated from mixture of standard proteins with known pI values.

Amino acid composition and N-terminal sequence analysis. Samples used for the amino acid composition and N-terminal sequence analysis were prepared from the membrane-associated fraction of the MIRP. The purified preparation from the gel filtration column was concentrated and dialyzed for 48 h at 4° C against two changes of distilled water. This step resulted in the formation of a precipitate which was removed by centrifugation. The supernatant fraction retained 60% of the total protein in the original sample. The amino acid composition of the MIRP was determined by hydrolysis in 4N methane-sulfonic acid (26) in evacuated, sealed tubes at 115° C for 22, 48, 72, and 96 h. The values for serine and threonine were corrected for destruction during hydrolysis by extrapolation to 0 time. The values for leucine, isoleucine, and valine were corrected for slow hydrolysis of the peptide bond by extrapolation to infinite time. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic oxidation.

Automated Edman degradation was performed with a Beckman 890C sequencer (Beckman Instruments, Palo Alto, California), using a modified Quadrol program (No. 0011576) of Beckman Instruments in combination with polybrene. Thiazolinone deivatives of amino acids were converted to Pth-derivatives with aqueous 1.0 N HCl at 80° C for 10 min. Pth-amino acids were identified by HPLC and confirmed by gas chromatography and/or thin-layer chromatography.

RESULTS

Cellular disruption and fractionation. Iron-starved gonococci were harvested and washed as described previously. These cells were subjected to sonication followed by centrifugation. Analysis of the pellet and supernatant fractions by SDS-PAGE indicated that the MIRP was approximately equally distributed in each fraction (Fig 1, lanes b and e). The MIRP associated with the supernatant fraction was resistant to centrifugation at 60,000 x g for 90 min (data not shown). The MIRP associated with the particulate fraction appeared to be tightly bound as subsequent washings of the pellet did not remove detectable amounts of the protein.

Preparation of crude detergent fractions. Initial observations indicated that the MIRP was completely solubilized from the MIRP-containing particulate fraction in the presence of 2% (w/v) CTB. Other proteins in this crude membrane fraction were only partially solubilized at this CTB concentration. Therefore, the ability of lower concentrations of CTB to selectively solubilize the MIRP from the particulate fraction was examined. Fig 2 demonstrates the effect of increasing the concentration of CTB (0% to 0.8% [w/v]) on the release of the MIRP from the particulate fraction. Nearly all of the MIRP was released at a concentration of 0.05% (w/v) CTB. Therefore, a detergent:protein ratio of 0.5 (w:w) was used to extract the crude membrane-associated MIRP (Fig 1, lane c).

Using the same detergent:protein ratio, an extract which was enriched for the MIRP could be obtained from the supernatant fraction of sonicated whole

cells. The addition of the CTB resulted in the formation of a white precipitate which could be removed by centrifugation. The resulting supernatant was enriched for MIRP (Fig 1, lane F).

Isolation of the MIRP from CTB-extracts of the pellet and supernatant whole cell sonicate fractions. The CTB-soluble extracts enriched for the MIRP were further purified by ion-exchange chromatography using CM-Sepharose 6B-CL. The crude preparations were applied in the same buffer used to extract the MIRP (10 mM Tris buffer, pH 8.0, containing 0.05% [w/v] CTB). At pH 8.0, the majority of the proteins did not bind to the column matrix. One major peak was resolved when a NaCl gradient of 0 to 1M was applied to the column (Fig 3). A pink color was associated with the fractions comprising this major peak (appendix 5). When analyzed by SDS-PAGE and visualized by a sensitive silver stain, it was determined that this peak contained the MIRP as the predominant protein (Fig 1, lanes d and g). The MIRP-containing peak was pooled and dialyzed against buffer (0.05% CTB [w/v] in Tris, pH 8.0). Dialysis against this buffer did not remove or reduce the pink color associated with the fraction. No differences in the elution profile were observed between the MIRP isolated from the supernatant or particulate fractions of the whole cell sonicates (data not shown). In subsequent isolations, the crude extracts from both fractions of the whole cell sonicates were pooled for the ion-exchange chromatography step. A diffuse peak associated with a component of the CTB resolved much later in the gradient (data not shown). Therefore, fractions were only collected through the first half of the NaCl gradient.

Contaminating proteins were removed by subjecting the pooled MIRP-containing fractions to gel filtration chromatography using Sephacryl S-300. The MIRP-containing peak had a Kav of 0.6 when the elution buffer

consisted of 10 mM Tris (pH 8.0), 0.05% CTB (w/v), and 300 mM NaCl (Fig 4). This corresponded to a molecular weight of between 26,000 and 32,000. After gel filtration, the MIRP-containing fraction appeared to be pure by the criterion of a single band when analyzed by SDS-PAGE and visualized using a sensitive silver stain (Fig 5). Protein I has been reported to elute as a trimer in association with detergent micelles when an elution buffer consisting of 50 mM Tris (pH 8.0), 0.05% (w/v) Zwittergent 3-14, 10 mM EDTA, and 200 mM NaCl was used (2). For comparison, the MIRP-containing fraction was dialyzed against this buffer and analyzed by gel filtration. The Kav of the MIRP-containing fraction under these conditions was identical to that obtained using the CTB-containing buffer. Protein I eluted with a Kav which corresponded to a molecular weight of 188,000 under these conditions, similar to the value previously reported (2).

Yeild. Typically 1-2 mg of the MIRP could be isolated from gonococci propagated as described in 1 liter of low-iron medium. The amount of MIRP which could be isolated using these methods was dependent upon the degree to which gonococci had been iron-stressed. When maximal expression of the MIRP was acheived, the amount of the purified MIRP recovered represented between 1 and 3% of the total protein content of gonococcal cells.

Isoelectric focusing. Purified MIRP preparations were analyzed by isoelectric focusing using an ampholyte gradient of 3.5 to 9.5 (Fig 6). Preparations purified from either the soluble or particulate fractions of whole cell sonicates migrated with identical pI values which corresponded to an isoelectric point just slightly greater than trypsinogen (pI 9.35). The possibility that this basic pI value was an artifact of the isolation procedure or that the cationic detergent CTB was contributing to this positive

isoelectric point was negated since Triton X100-solubilized whole gonococci propagated in low-iron medium contained a protein which migrated with an identical pI as the purified MIRP preparations (data not shown).

Amino acid composition. The amino acid composition of the gonococcal MIRP is shown in Table 1. Also included in Table 1 are previously published amino acid compositions of two different Protein I molecules (2) and a Protein II molecule (1). The integral amino acid composition of the MIRP was based on an estimated molecular weight of 36,000 from SDS-PAGE analysis. Although the amino acid composition of the MIRP was similar in character to the compositions of Protein(s) I and Protein II, the distribution of individual amino acids was unique. Notably, the MIRP contained comparatively fewer aromatic amino acids and a greater number of proline residues than the Protein(s) I and Protein II. Furthermore, the MIRP contained a higher proportion of basic amino acids than the Protein I molecules but a lower proportion than Protein II. Given the basic isoelectric point of the MIRP and the prediction of 76 dicarboxylic acid residues versus 48 basic amino acids, it is likely that a substantial number of the aspartates and glutamates exist as amides.

N-terminal amino acid sequence. The N-terminal amino acid sequence of the gonococcal MIRP is shown in Fig 7. Analysis of the N-terminal residue of the MIRP detected aspartic acid and lysine in a molar ratio of 2.2:1, respectively. All subsequent residues were detected as single amino acids. This sequence is compared to the N-terminal amino acid sequences of other published gonococcal outer membrane proteins. The first five residues from the N-terminus of the MIRP and Protein I were nearly identical; of the five, three were identical and the two mismatched amino acids represented conservative substitutions. No other apparent homology between the gonococcal MIRP, Protein I, or Protein II was observed.

DISCUSSION

The protein composition of gonococci cultivated *in vitro* has been the subject of several reports. Among the proteins which have been purified and characterized with respect to their biochemical properties are pili (11,22,23), protein(s) I (2), protein(s) II (1), and a high-molecular weight outer membrane protein-macromolecular complex (OMP-MC) (10). Of these, only the OMP-MC has been reported to be structurally conserved between diverse gonococcal isolates (10). Like the latter, the MIRP is common to all gonococcal strains examined (16,17,31). MIRP-specific rabbit antiserum and murine monoclonal antibodies have been used to analyze the antigenic conservation of this protein among members of the genus *Neisseria*. An antigenically related iron-regulated protein with an apparent molecular weight similar to the gonococcal MIRP could be detected in all strains of *N. meningitidis*, *N. lactamica*, and *N. cinerea* examined (16). The remaining species of the genus *Neisseria* did not express a similar protein. The implication of the highly conserved nature of the gonococcal MIRP suggests that this protein may play some indespensable functional role. Along these lines, West *et al*. (31) examined the expression of the gonococcal iron-regulated proteins and determined that they were not coordinately regulated. In the presence of various iron-containing proteins as iron sources, gonococci expressed different combinations of iron-regulated proteins. The MIRP was expressed in the presence of all of the iron-containing molecules tested. The consistent expression of this protein under all conditions of iron limitation and its conservation among gonococci

prompted speculation that this protein may play a central role in iron acquisition by the gonococcus (17, 31). However, to date no function for this protein has been determined. Understanding the biochemical properties of the MIRP will certainly aid in elucidating this function.

The gonococcal MIRP has been previously reported to be associated with crude membranes (31) and Sarkosyl-insoluble membrane fractions (17,20). This report suggests that in addition to the MIRP associated with the particulate fraction of sonicated gonococci grown under iron-limited conditions, a substantial proportion (*ca*. 50%) of the MIRP was resistant to centrifugation at forces which normally pellet membranes. Under these conditions, nearly all of the Protein I (*ca*. 95 %) was associated with the pellet. The chromatographic properties of the non-membrane associated MIRP-containing fraction have been examined by gel filtration in the absence of detergents (Fig 8). The results indicate that MIRP associated with this fraction elutes at an identical position to that obtained when the purified MIRP is chromatographed in detergent-containing buffers; both having Kav values of 0.6 which correspond to a molecular weight of *ca*. 29,000. These observations suggest that after cellular disruption by sonication the MIRP may exist in both a membrane associated and non-membrane associated form. Proteins which are both membrane-bound and free have been observed in *E. coli*. These are classified as binding proteins and are found within the periplasmic space (14). We have analyzed the gonococcal periplasmic proteins using osmotic shock treatment. Initial results indicated that the gonococcal MIRP was not found in the osmotic shock fluid, suggesting that the MIRP was not free within the periplasmic fluid (data not shown). Another possibility is that the MIRP

loosely associates with membranes and is released upon sonication. Alternatively, the MIRP might be a soluble cellular protein which, due to its highly charged character, associates with membranes during the separation of the particulate and soluble fractions. The nature of the interaction between the MIRP and the gonococcal membrane as well as the precise cellular location of this protein have not yet been determined.

We have selectively solubilized the MIRP from gonococcal membranes using the cationic detergent CTB. Blake et al. (2) have previously utilized this detergent at low-ionic strength to precipitate a fraction enriched for Protein I from whole gonococci. We observed that nearly all the MIRP could be solubilized using a similar procedure. Under stringent conditions a fraction enriched for the MIRP could be obtained at a CTB:protein ratio of 0.5 (w:w). Low concentrations of other detergents such as Triton X-100 and Sarkosyl did not selectively solubilize the MIRP from membranes (data not shown). Related work in this laboratory has indicated that CTB at similar detergent to membrane protein ratios efficiently solubilizes gonococcal membrane phospholipids. Whether or not this results in the release of the MIRP from the gonococcal membranes is speculative.

Addition of CTB to the non-sedimental whole cell sonicates in the same proportions as described above resulted in formation of a precipitate. The predominant protein species remaining in the cleared supernatants was the MIRP. CTB precipitates acidic polysaccharides under conditions of low-ionic strength (27). Presumably, it is this property which precipitates the nucleic acids, carbohydrates, and associated proteins which are found in the non-sedimental fraction of sonicated whole cells. The highly soluble nature

of the MIRP in CTB apparently results in the retention of this protein in the cleared supernatant.

The CTB-solubilized MIRP bound to CM-Sepharose and could be eluted using a gradient of NaCl. Binding of the MIRP to the cation-exchange matrix occurred in the presence of the cationic detergent CTB. The protein eluted as a single peak at ca. 150 mM NaCl. This fraction appeared to be substantially enriched for the MIRP by the criterion of a single major band after analysis by SDS-PAGE. However, minor contaminating proteins with apparent molecular weights between 20,000 and 32,000 were associated with the MIRP-containing fraction. The presence and quantities of these proteins were variable from preparation to preparation. Blake et al. isolated Protein II using the same column matrix as was used for the purification of the MIRP (1). Protein(s) II have been reported to have molecular weights of between 24,000 and 30,000 (28). Therefore, contaminating Protein(s) II may pose a problem in this purification scheme. To limit potential contamination with Protein II molecules transparent organisms were used for the original inoculum. Contaminating proteins could usually be removed by molecular seive chromatography.

Molecular weight estimates obtained by gel filtration (ca. 29,000) were slightly lower but in similar agreement to those obtained by SDS-PAGE analysis (36,000 to 37,000) (17,31). The low molecular weight estimates obtained by gel filtration suggest that the MIRP elutes as a monomeric species in aqueous and detergent containing buffers (CTB and Zwittergent 3-14). In a Zwittergent 3-14-containing buffer, Protein I elutes as a trimer interacting with detergent micelles which increase the estimated molecular weight of this

complex by ca. 60,000 daltons (2). Under similar conditions, Protein II also has an increased molecular weight due to micellular interaction; however this protein behaves as a monomeric species (1). As judged by an estimated molecular weight lower than predicted from SDS-PAGE analysis, the gonococcal MIRP did not appear to interact with micelles of either CTB or Zwittergent 3-14.

The highly cationic nature of the MIRP was analyzed by isoelectric focusing. Difficulties in determining the isoelectric points for basic proteins are well-documented (6,7). In our system, the MIRP had a pI slightly greater than 9.35. However, the exact isoelectric point was not determined. From the predicted amino acid composition of MIRP, it can be inferred that a substantial number of dicarboxylic acid residues contribute to this basic isoelectric point in their amide form. The amino acid composition of the MIRP was similar to that of Protein II which has a pI of between 9.0 to 10.0 (1). Moreover, the ability of the MIRP to interact with the CM-sepharose matrix at pH 8.0 is further evidence for the highly cationic nature of this protein.

The amino acid composition of the MIRP predicted only one cysteine residue, similar to that found for Protein I. SDS-PAGE analysis of this protein have suggested that it was not modifiable by reducing agents; thus, it is not clear whether or not this residue participates in quarternary protein structure. Cyanogen bromide cleavage at methionine residues has been used by Schoolnik et al. (23) to disect the structure/function relationships of pilin. The presence of three predicted methionine residues from the amino acid composition of the MIRP suggests that four individual peptides can be generated by cyanogen bromide cleavage. These may be useful in future studies

for disecting the structure/function relationships of the MIRP.

It was interesting that the first five amino acids of the MIRP were similar to the first five residues of Protein I. The significance of this conserved sequence is not apparent. However, it is tempting to speculate that this sequence may imply some function or cellular destination common to Protein I and the MIRP. Alternatively, the occurrence of this common sequence may be an evolutionary artifact; suggesting that the MIRP and Protein I are descendents of a common progenitor protein. Hydropathic analysis of the 63 N-terminal amino acid residues of the MIRP indicated that it is a relatively hydrophilic segment (Fig 9). The major hydrophobic segment of the N-terminus appears to lie between the residues 15 and 30. Data from the amino acid composition indicated that this protein was lower in the proportion of aromatic amino acids than both Protein I and Protein II. Given the distribution of the MIRP in both the particulate and soluble fractions of the cell sonicates, it might suggest that this protein does not require a strict membrane environment in order to function.

The relationship between the ability to obtain iron from the host environment and microbial survival and virulence during infection has recently been reviewed (5,8). The conservation of the MIRP, particularly among the pathogens _N. gonorrhoeae_ and _N. meningitidis_ may indicate that this protein is a necessary determinant of pathogenicity. However, the expression of a protein which is antigenically related to the gonococcal MIRP in the non-pathogenic species of _N. lactamica_ and _N. cinerea_ suggests that the presence of this protein is not in itself a lone virulence determinant. Since the MIRP is apparently produced by gonococci in response to the lack of

available iron in the environment, a plausible prediction might be that this protein is a functional component of a high-affinity iron-uptake system which is required for growth of gonococci on mucosal surfaces. Simonson _et al._ (25), in examining iron uptake by _N. meningitidis_, determined that when membranes from iron-starved meningococci were incubated with ^{59}Fe-citrate a large proportion of the iron remained associated with a protein complex which had an apparent molecular weight of 36,500. Whether or not the iron-regulated protein of meningococci, which is antigenically-related to the gonococcal MIRP, is involved in this association has not been analyzed. Experiments similar to those performed by Simonson _et al._ have not been attempted for the gonococcus. However, it seems possible that both gonococci and meningococci may possess similar iron-uptake systems in which a conserved protein such as the MIRP may play a role. These suggestions warrant further investigation which should be facilitated by the availability of purified preparations of the MIRP.

SUMMARY

The major iron-regulated protein (MIRP) of *N. gonorrhoeae* has been purified by extraction of whole cell sonicates with the detergent cetyltrimethylammonium bromide (CTB) at low ionic strength and under stringent detergent to protein ratios. This crude preparation was further purified by ion-exchange chromatography followed by gel filtration. The final preparation appeared to be pure by the criterion of a single band on SDS-polyacrylamide gels which were visualized using a sensitive silver stain. The purified MIRP displayed an unusually basic isoelectric point, this value being slightly greater than 9.35. The amino acid composition supported the finding that MIRP was a cationic protein. Furthermore, the MIRP shared a similar but unique distribution of amino acid residues as the gonococcal Protein(s) I and II. The N-terminal amino acid sequence revealed remarkable homology among the first five residues of the MIRP and the previously published sequence of Protein I. No other homology within the N-terminal sequences of Protein(s) I, II, and the MIRP was apparent. Presently, the function of the gonococcal MIRP has remained elusive. The ability to obtain reasonable quantities of this protein in a pure form should facilitate further studies of this protein and aid in determining its role in pathogenesis.

REFERENCES

1. Blake, M.S. and E.C. Gotschlich. 1984. Purification and partial characterization of the opacity associated proteins of *Neisseria gonorrhoeae*. J. Exp. Med. 159: 452-462.

2. Blake, M.S., and E.C. Gotschlich. 1982. Purification and partial characterization of the major outer membrane protein of *Neisseria gonorrhoeae*. Infect. Immun. 36: 277-283.

3. Bullen, J., H.J. Rogers, E. Griffiths. 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. 80: 1-35.

4. Chen, C.Y., C.S. Parsons, and S.A. Morse. 1984. Membrane proteolipids of *Neisseria gonorrhoeaea*. In. G. Schoolnik, G.F. Brooks, S. Falkow, J.S. Knapp, A. McCutchen, and S.A. Morse (ed). The Pathogenic *Neisseria*. American Society for Microbiology, Washington, D.C. In press.

5. Crosa, J.H. 1984. The relationship of plasmid-mediated iron transport and bacterial virulence. Ann. Rev. Microbiol. 38: 69-89.

6. Delincee, H., and B.J. Radola. 1978. Determination of isoelectric points in thin layer isoelectric focusing: The importance of attaining the steady state and the role of CO_2 interference. Analyt. Biochem. 90: 609-623.

7. Fredriksson, S. 1978. Temperature dependence of ampholine pH gradients used in isoelectric focusing. J. Chromatog. 151: 347-355.

8. Griffiths, E. 1983. Availability of iron and survival of bacteria in infection. *In* Medical Microbiology, Volume 3, Role of the cell envelope

in survival of bacteria in infection, ed. Easmon, C.S.F., J. Jeljaszewicz, M.R.W. Brown, and P.A. Lambert, pp. 151-177. Academic Press Inc. Ltd., London.

9. Griffiths, E., P. Stevenson, and P. Joyce. 1983. Pathogenic Eschericia coli express new outer membrane proteins when growing in vivo. FEMS Microbiol. Lett. 16: 95-99.

10. Hansen, M.V., and C.E. Wilde III. 1984. Conservation of peptide structure of outer membrane protein-macromolecular complex from Neisseria gonorrhoeae. Infect. Immun. 43: 839-845.

11. Hermodson, M.A., K.C.S. Chen, and T.M. Buchanan. 1978. Neisseria pili proteins: Amino-terminal amino acid sequences and identification of an unusual amino acid. Biochemistry 17: 442-445.

12. Laas, T., I. Olsson, and L. Soderberg. 1980. High voltage isoelectric focusing with Pharmalyte: Field strength and temperature distribution, zone sharpening, isoelectric spectra, and pI determinations. Analyt. Biochem. 101: 449-461.

13. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

14. Lo, T.C.Y. 1979. The molecular mechanisms of substrate transport in Gram-negative bacteria. Can. J. Biochem. 57: 289-301.

15. Markwell, M.K., S.M. Haas, L.L. Bieber and N.E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87: 206-210.

16. Mietzner, T.A., R.C. Barnes, Y.A. JeanLouis, and S.A. Morse. 1985. Distribution of an antigenically related iron-regulated protein among the Neisseria species. Submitted for publication.

17. Mietzner, T.A., G.H. Luginbuhl, E.C. Sandstrom, and S.A. Morse. 1984. Identification of an iron-regulated 37,000 dalton protein in the cell envelope of Neisseria gonorrhoeae. Infect. Immun. 45: 410-416.

18. Morse, S.A., C.S. Mintz, S.K. Sarafian, L. Bartenstein, M. Bertram, and M.A. Apicella. 1983. Effect of dilution rate on lipopolysaccharide and serum resistance of Neisseria gonorrhoeae grown in continuous culture. Infect. Immun. 41: 74-82.

19. Neilands, J.B. 1982. Microbial envelope proteins related to iron. Ann. Rev. Microbiol. 36: 285-309.

20. Norqvist, A., J. Davies, L. Norlander, and S. Normark. 1978. The effect of iron-starvation on the outer membrane protein composition of Neisseria gonorrhoeae. FEMS Microbiol. Lett. 4: 71-75.

21. Penn, C.W., N.J. Parsons, S.C. Rittenberg, S.C. Sanyal, D.R. Veale, and H. Smith. 1978. Gonococci grown in vivo and in vitro: Selection and phenotypic change in relationship to pathogenesis and immunity. In Immunobiology of Neisseria gonorrhoeae, ed. G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer, and F.E. Young, pp.356-359. American Society for Microbiology, Washington, D.C.

22. Robertson, J.N., P. Vincent, and M.E. Ward. 1977. The preparation and properties of gonococcal pili. J. Gen. Microbiol. 102: 169-177.

23 Schoolnik, G.K., J.Y. Tai, J. Rothbard, and E.C. Gotschlich. 1983. A pilus peptide vaccine for the prevention of gonorrhea. Prog. Allerg. 33: 314-331.

24 Sciortino, C.V., and R.A. Finkelstein. 1983. Vibrio Cholerae expresses iron-regulated outer membrane proteins in vivo. Infect. Immun. 42: 990-996.

25. Simonson, C., D. Brener, and I.W. DeVoe. 1982. Expression of a high-affinity mechanism for acquisition of transferrin iron by Neisseria meningitidis. Infect. Immun. 36: 107-113.

26. Simpson, R.J., M.R. Neuberger, and T.Y. Liu. 1976. Complete amino acid analysis of proteins from a single hydrolysate. J. Biol. Chem. 251: 1936-1940.

27. Stacey, M. and S.A. Barker. 1960. The isolation and homogeneity of bacterial polysaccharides and their comlexes. In Polysaccharides of microorganisms, ed. M. Stacey and S.A. Barker, p. 35. Clarendon Press, Oxford.

28. Swanson, J. 1978. Studies on gonococcus infection XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. Infect. Immun. 21: 292-302.

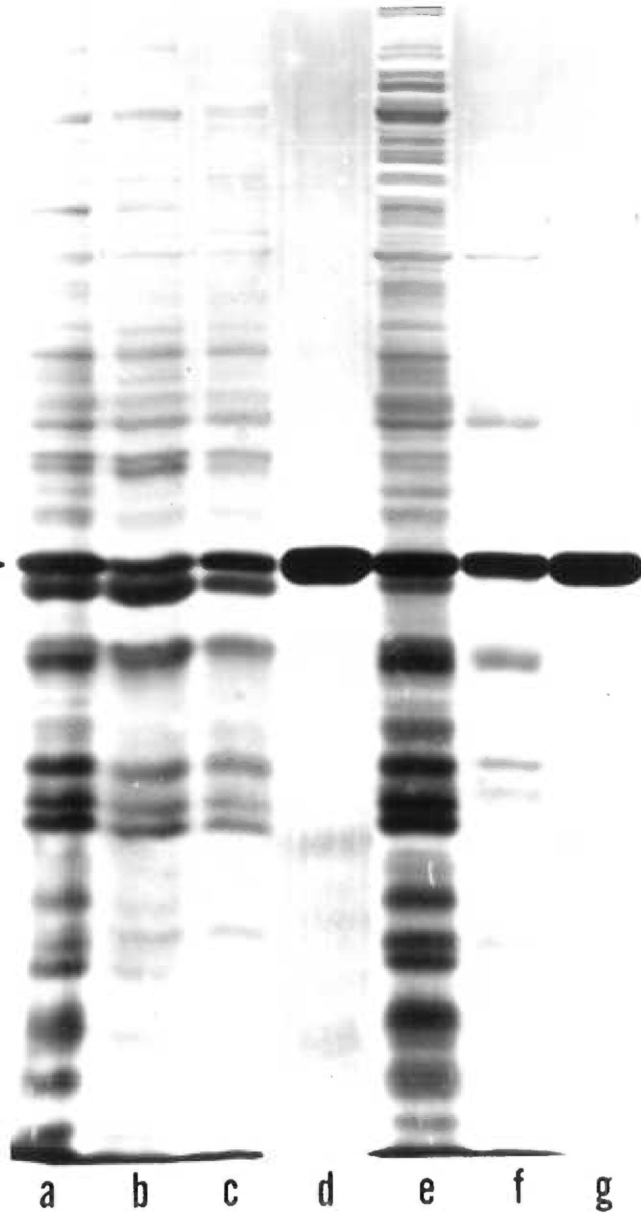
29. Tsai, L.M. and C.E. Frasch. 1982. A sensitive silver stain for detecting lipopolysacharide in polyacrylamide gels. Analyt. Biochem. 119: 115-119.

30. Weinberg, E.D. 1978. Iron and infection. Micrbiol. Rev. 42: 45-66.

31. West, S.E.H., and P.F. Sparling. 1985. Response of Neisseria gonorrhoeae to iron limitation: Alterations in expression of membrane proteins without apparent siderophore production. Infect. Immun. 47: 388-394.

32. Yancey, R.J., and R.A. Finkelstein. 1981. Assimilation of iron by pathogenic Neisseria spp. Infect. Immun. 32: 592-599.

Fig 1. A silver-stained SDS-PAGE analysis of the purification steps used for the isolation of the gonococcal MIRP. Lane a represents the protein profile obtained from 5 ul of the whole cell sonicate. Lanes b and e are the protein profiles of the particulate and the soluble fractions, 5 ul each respectively, from sonicated whole cells (the pellet obtained from the centrifugation was suspended in a volume identical to that of the soluble fraction). Lanes c and f are protein profiles of the crude preparations from the membrane-associated and the soluble MIRP, respectively. These preparations were obtained after the soluble and particulate fractions from sonicated whole cells were diluted to 1 mg per ml in the extraction buffer (0.05% CTB in Tris, pH 8.0); 30 ul volumes of each extract were analyzed. Lanes d and g are the pooled fractions of the crude membrane-associated MIRP and the crude soluble MIRP, respectively, after ion-exchange chromatography; a protein concentration of 15 ug was loaded in each well.

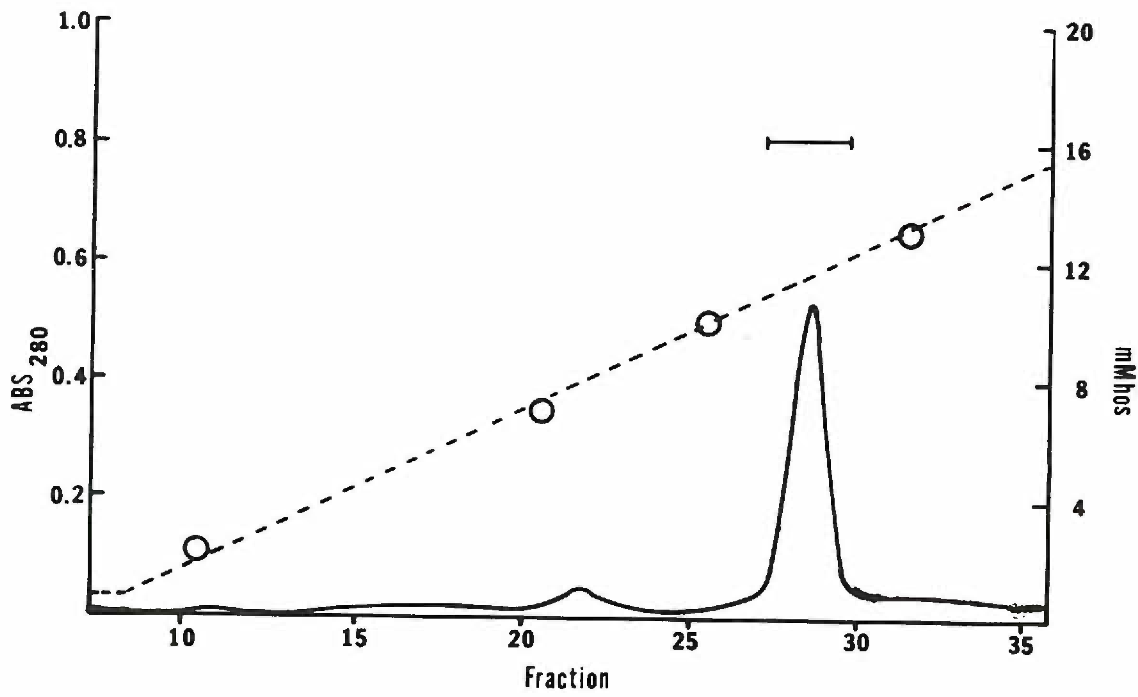


MIRP
a
b
c
d
e
f
g

Fig 2. SDS-PAGE analysis of the selective solubilization of the gonococcal MIRP by CTB (stained with Coomassie blue). Crude membranes were incubated in the presence of CTB as described in the text at concentrations (% w/v) of 0 (lane a), 0.0125 (lane b), 0.025 (lane c), 0.05 (lane d), 0.1 (lane e), 0.2 (lane f), 0.4 (lane g), and 0.8 (lane h). Equal volumes (50 ul) were analyzed after separation of the insoluble material by centrifugation. At CTB concentrations of 0.0125% and 0.025%, the MIRP appeared to be selectively solubilized. Nearly all of the membrane-associated MIRP was solubilized at a CTB concentration of 0.05% (w/v). Under the conditions used, this concentration corresponded to a detergent:protein ratio of 0.5 (w/w). Subsequent purification steps utilized this ratio.

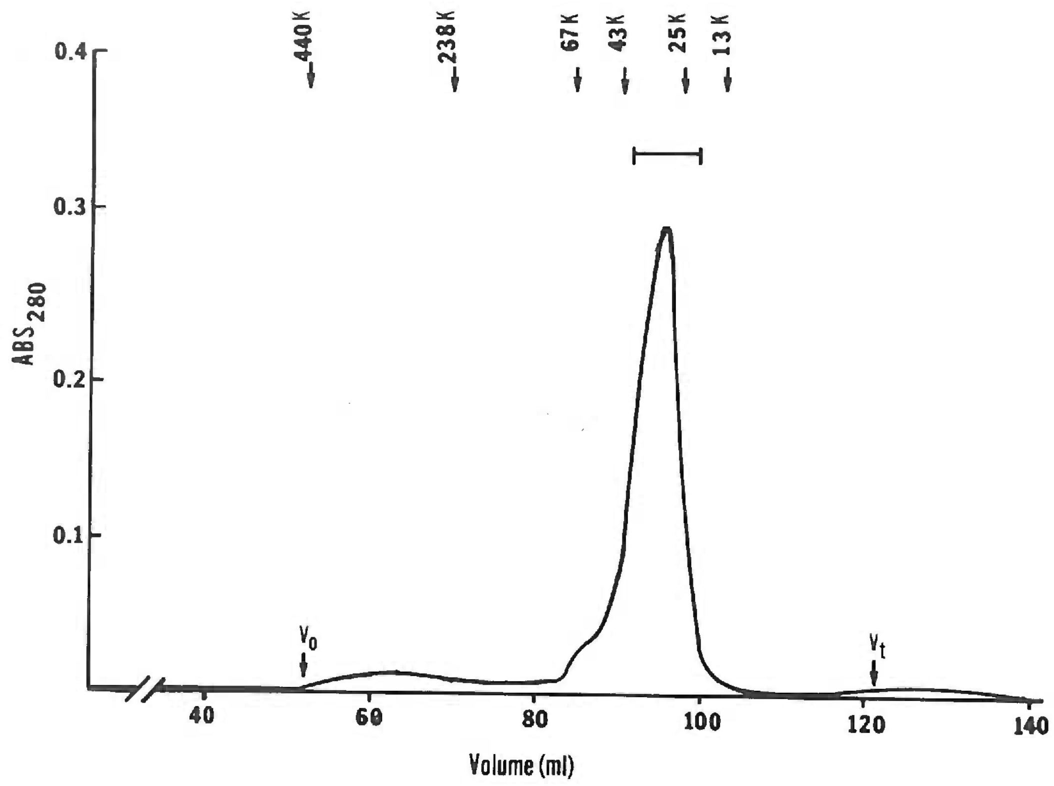
MIRP
a
b
c
d
e
f
g
h

Fig 3. Elution profile of a CM-Sepharose column used to purify the MIRP from crude preparations. After the sample was applied, the column was eluted with a NaCl gradient of 0 to 1 M in a total buffer volume of 500 ml. Fractions (4 ml) were collected only through the first half of the gradient. Conductivity of the elution buffer was followed and is depicted by the dashed line. The bar (⊢—⊣) indicates the fractions which were pooled. The MIRP eluted as a single peak at a conductivity of 11 mMhos which corresponded to a NaCl concentration of 150 mM.



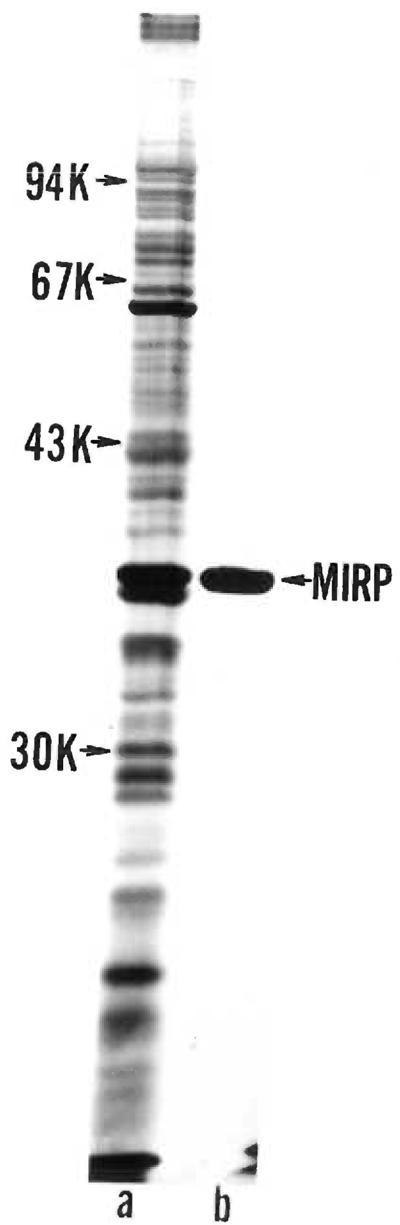
1.0
0.8
0.6
0.4
0.2
ABS280
20
16
12
8
4
mMhos
10
15
20
25
30
35
Fraction

Fig 4. Gel filtration of the pooled MIRP fractions obtained by ion-exchange chromatography (Fig 3). This sample was lyophilized, suspended in a volume of 2 ml, and dialyzed against an elution buffer consisting of 10 mM Tris buffer (pH 8.0), 0.05% CTB, and 300 mM NaCl. The dialyzed preparation was applied to a Sephacryl S-300 column and chromatographed as described in the text. The following molecular weight standards were used for the calabration of the column: Ferritin, 440,000 daltons (440K); catalase, 238,000 daltons (232K); bovine serum albumin, 67,000 daltons (67K); ovalbumin, 43,000 daltons (43K); chymotrypsinogen A, 25,000 daltons (25K); and ribonuclease A, 13,700 daltons (13K). V_o and V_t were determined using blue dextran and vitamin B_{12}, respectively. The MIRP-containing fraction eluted with a Kav of 0.6, which corresponded to a molecular weight of between 26,000 and 32,000. The bar (⟼) indicates the MIRP-containing fractions which were pooled . Analysis of this preparation by SDS-PAGE is shown in Fig 5.



440 K
238 K
67 K
43 K
25 K
13 K
0.4
0.3
0.2
0.1
ABS280
V0
Vt
40
60
80
100
120
140
Volume (ml)

Fig 5. A silver-stained SDS-polyacrylamide gel of the purified MIRP-containing fraction after gel filtration (Fig 4). Lane a is a protein profile of whole gonococci representing the starting material used for the isolation of the MIRP. Lane b represents 10 ug of the final purified protein. No contaminating proteins were detected in the preparation.



94K
67K
43K
30K
MIRP
a
b

Fig 6. Isoelectric focusing of the purified MIRP isolated from the soluble (lane a) and the particulate fractions (lane b) of sonicated whole cells. Both purified preparations appeared to migrate at identical positions near the cathode. Under the conditions used, a pI of slightly greater than trypsinogen (pI 9.35) was observed. For this analysis, 20 ug of each purified protein preparation was focused.

−
+
A
B
5.5
7.5
9.5
pI

Fig 7. Amino terminal 63 residues of the gonococcal MIRP. Also included are the published N-terminal amino acid sequences of Protein I (PI) from strain R10 (2) and Protein II (PII) molecules from strain R10 (a) and MS11 (b) (1). The analysis detected a ratio of 2.2:1, aspartic acid to lysine, for the N-terminal residue. Therefore, aspartic acid is listed as this amino acid. All other residues were detected as pure compounds. The first five residues of the MIRP and Protein I show a great deal of homology (solid lines represent a perfect match, dashed lines represent a conservative substitution). The amino acids which are in brackets indicate that their identity was determined by HPLC analysis but not confirmed by either GLC or TLC. The asterick (*) indicates that the residue may be either cystine or cysteine.

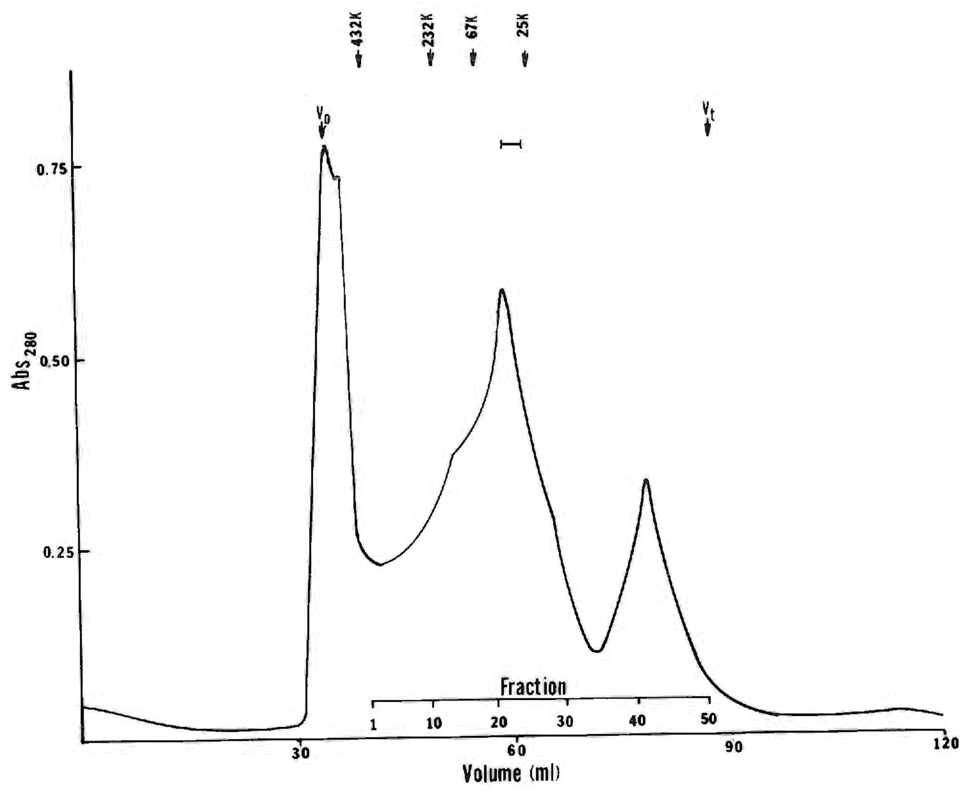
	1									10								
MIRP	Asp	Ile	Thr	Val	Tyr	Asn	Gly	Gln	His	Lys	Glu	Ala	Ala	Gln	Ala	Val	Ala	Asp
	|	¦	|	¦	|													
PI	Asp	Val	Thr	Leu	Tyr	Gly	Ala	Ile	Lys	Ala	Gly	Val						
PII[a]	Ala	Gly	Glu	Asp	Glu													
PII[b]	Ala	Ser	Glu	Glu	Gly	Arg	Gly	Pro	Tyr									

		20										30						
MIRP	Ala	Phe	Thr	Cys	Ala	Thr	Lys	Ile	Lys	Val	Lys	Leu	Asn	Ser	Ala	Lys	Gly	Asp

				40										50				
MIRP	Gln	Leu	Ala	Gly	Gln	Ile	Lys	Glu	Glu	Gly	Ser	Arg	Ser	Pro	Ala	Asp	Val	Phe

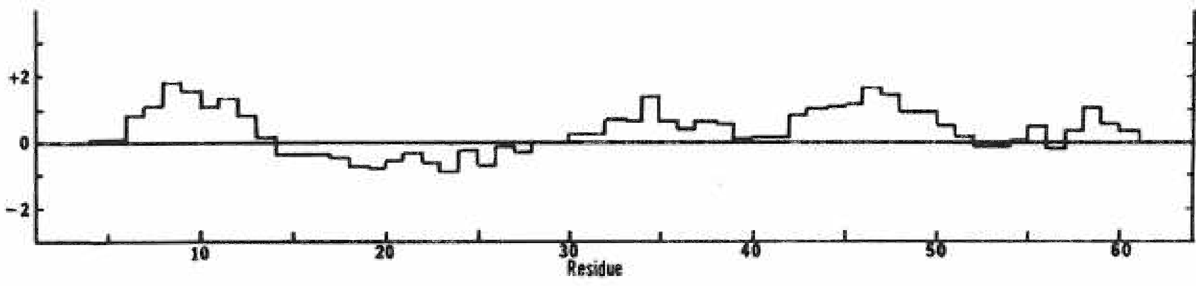
						60			
MIRP	Tyr	Ser	Glu	His	Ile	Pro	Arg	Leu	Ala

Fig 8. Elution profile of the non-sedimentable fraction of gonococcal whole cell lysates. Gonococci propatated in low-iron medium were disrupted by sonication and the pellet and supernatant fractions separated as described. A volume of 1 ml (4.4 mg total protein) of the supernatant fraction was passed over a Sephacryl S-300 column in the presence of a elution buffer cosisting of 10 mM Tris, pH 8.0, and 0.3 M NaCl. The elution profile was followed by absorbance at 280 nm using an in-line UV monitor and fractions of 0.5 ml were collected. The presence of the MIRP was determined by analysis of 50 ul of each fraction by SDS-PAGE. The fraction containing the highest concentration of the MIRP eluted with a molecular weight corresponding to 29,000.



432K
232K
67K
25K
V_o
V_t
0.75
0.50
0.25
Abs_{280}
Fraction
1
10
20
30
40
50
30
60
90
120
Volume (ml)

Fig 9. Local average hydrophobicity of the N-terminal 63 amino acids of the gonococcal MIRP. The hydrophilicity values are derived from the hexapeptide averages along the length of the sequence plotted at the midpoint of the averaged group of residues.



+2
0
-2
10
20
30
40
50
60
Residue

Table 1. Comparison of amino acid composition of purified gonococcal proteins.

AMINO ACIDS	MIRP	PI(MS11)	PI(R10)	PII(R10)
Neutral	216 (.64)	194 (.67)	206 (.67)	163 (.62)
Aliphatic	176 (.52)	156 (.52)	162 (.53)	147 (.56)
glycine	27	33	42	32
alanine	54	27	27	26
valine	34	27	27	29
leucine	34	21	18	15
isoleucine	14	12	6	11
serine	4	21	27	21
threonine	9	15	15	13
Aromatic	19 (.03)	24 (.08)	27 (.09)	10 (.04)
phenylalanine	10	12	12	5
tyrosine	9	12	15	5
Sulfer-containing	4 (.01)	5 (.02)	5 (.02)	5 (.02)
1/2 cystine	1	1	1	-
methionine	3	4	4	5
Imino acids/proline	17 (.05)	9 (.03)	12 (.04)	1(.003)
Dicarboxylic amino acids	76 (.22)	69 (.23)	69 (.23)	58 (.22)
aspartic/asparagine	33	33	33	42
glutamic/glutamine	43	36	36	16
Basic amino acids	48 (.14)	33 (.11)	30 (.10)	41 (.16)
histidine	6	6	6	4
arginine	15	9	6	22
lysine	27	18	18	15
TOTAL	339	296	305	262

a) Data from Blake *et al.*(2) and Blake *et al.* (1). Designation in parenthesis indicates the strain from which the protein was isolated.

b) Values in parenthesis represent the proportion of residues which belong to the respective group of amino acids.

MANUSCRIPT 3

THE DISTRIBUTION OF AN ANTIGENICALLY RELATED IRON-REGULATED PROTEIN AMONG THE *NEISSERIA SPP.*

Contributors:

Timothy A. Mietzner

Robert C. Barnes

Stephen A. Morse

ABSTRACT

Several iron-regulated proteins of *Neisseria gonorrhoeae* have been reported. One of these, a 37,000 dalton (37K) protein, appears to be common to all gonococcal isolates. Recently, the occurrence of a similar protein has also been noted in *Neisseria meningitidis*. The gonococcal 37K protein has been purified and used to produce both rabbit monospecific antiserum and murine monoclonal antibodies. Using these antibody reagents, we analyzed 57 strains from nine species of *Neisseria* and the closely-related organism *Branhamella catarrhalis* for the presence of proteins antigenically-related to the gonococcal 37K protein. Strains grown on low-iron medium were probed for antigenic reactivity using western blot techniques and an enzyme-linked immunosorbant assay. Proteins which cross-reacted with the rabbit monospecific antiserum were designated as AgR-37K proteins. The data indicated that the AgR-37K proteins were conserved among the 40 strains of *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, and *N. cinerea* tested. Seventeen strains from other species of *Neisseria* and *Branhamella* did not express AgR-37K proteins with the exception of one *N. subflava* isolate. All AgR-37K proteins appeared to be regulated by the amount of available iron in the growth medium. Murine monoclonal antibodies were used to probe the antigenic heterogeneity of the AgR-37K proteins from different *Neisseria* spp. Two of 7 monoclonal antibodies were broadly cross-reactive, recognizing the AgR-37K proteins from all species examined. The remaining 5 monoclonal antibodies were more discriminating, recognizing the AgR-37K proteins from certain species. The antigenic conservation of these AgR-37K proteins, particularly among the pathogenic members of the genus *Neisseria*, may imply that these proteins serve a common function in pathogenicity.

INTRODUCTION

The genus Neisseria is a group of closely-related organisms which inhabit the mucosal surfaces of mammals. Bergey's manual of systematic bacteriology currently lists eleven species which belong to this genus (3). Within the genus Neisseria, only N. gonorrhoeae and N. meningitidis are considered to be primary pathogens of man (3). The remaining members are generally regarded as commensal organisms existing as normal inhabitants of healthy mucosal flora. A number of reports have associated these commensal Neisseria spp. with various infections (for recent reviews see ref. 10,12). However, the infrequency of these reports suggest that these species are oportunistic pathogens. The properties which set N. meningitidis and N. gonorrhoeae apart as pathogens are incompletely understood and are actively being studied.

The ability of N. gonorrhoeae to alter its antigenic composition may be an important factor in pathogenesis. For example, the major outer membrane protein, protein I (PI), is antigenically and structurally distinct among different strains (28). Another class of gonococcal outer membrane proteins are designated protein II (PII). This group is defined by their molecular weight and by the heat-modifiable behavior they exhibit when analyzed by SDS-polyacrylamide gel electorphoresis (11,33). None, one, or several of the proteins belonging to this group may be expressed at any one time by a given gonococcal clone (17). Gonococcal pili are also able to exhibit intrastrain heterogeneity (4). This heterogeneity has been linked to a chromosomal rearrangement (20). Lipopolysacharides (LPS) of gonococci exhibit

compositional variation between strains (37) as well as intrastrain variation (37). Furthermore, the LPS composition may be phenotypically influenced by the growth environment (24).

Several conserved antigenic components of gonococcal outer membranes have been described. Newhall et al. (26) reported a high-molecular-weight outer membrane protein complex (OMP-MC) which was common to all gonococci examined. Pili, while antigenically different, contain a highly conserved region within the pilus subunit primary structure. This region may play a functional role in mucosal attachment (30). Protein III (PIII) is a major outer membrane protein common to all strains of N. gonorrhoeae. Peptide mapping has indicated that the structure of PIII is highly conserved among gonococcal isolates (15). Zak et al. (39) described an immunogenic surface protein with an apparent molecular weight of 43,000 which was antigenically common to all isolates examined. Recently, Cannon et al. (5) reported an antigen (H.8) which was conserved among all strains of gonococci and meningococci but absent from most commensal Neisseria spp.

The presence of a 37,000 dalton (37K) iron-regulated protein in N. gonorrhoeae has been reported previously (22,35). This protein was common to all gonococci examined. Peptide maps of the 37K proteins from two unrelated strains were nearly identical (22). More recently, an antigenically related protein, with a molecular weight identical to that of the gonococcal iron-regulated 37K protein, was detected in several strains of N. meningitidis (23). The antigenic conservation of this protein among the pathogenic Neisseria spp. prompted us to extend this analysis to other members of the genus. This report describes the distribution of these antigenically related proteins among species comprising the genus Neisseria and their regulation by available iron. Finally, murine monoclonal antibodies were used to probe the intragenic heterogeneity of the antigenically related iron-regulated proteins

MATERIALS AND METHODS

Bacterial strains. The strains used for this study are listed in Table 1. These strains were kindly provided from the following collections: R.P. Williams (Baylor College of Medicine, Houston, Tex.); J.W. Biddle, D. Kellogg, and R.J. Arko (STDLP, Centers for Disease Control, Atlanta, Ga.); R. Rice (CPS, Centers for Disease Control); R. Weaver and D. Hollis (Bacterial Diseases Division, Centers for Disease Control); J. Knapp (*Neisseria* Reference Laboratory, Seattle, Wash.); H. Schneider (Walter Reed Army Institute of Research, Washington, D.C.); the American Type Culture Collection (Rockville, Md.); and from a collection in this laboratory. All strains were maintained on plates as stock cultures or by freezing at -70° C in a solution of 1.5% (w/v) trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), 1.25% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), and 6.25% (v/v) glucose. Inocula were prepared from stock cultures grown on GC medium base agar (lot# 0289-01-1; Difco Laboratoriex, Detroit, Mich.) which contained a supplement (1% v/v) similar to Iso Vitale X (BBL) except that the glucose and $Fe(NO_3)_3$ were omitted. This medium was further supplemented with a final concentration of 0.5% glucose (w/v). All cultures were incubated at 37° C in a humidified atmosphere containing 4% CO_2.

Growth conditions. Agar medium identical in composition to the GC agar medium described above was used for the growth of organisms under conditions in which iron was not limiting. Liquid medium of similar composition (not including the agar) contained ca. 8.0 uM iron (22). In order to limit the

amount of iron available, Desferal mesylate (Ciba-Geigy Corp., Summit, N.J.), a potent iron-chelator, was added at final concentrations up to 25 uM. Medium containing Desferal at concentrations which allowed expression of the iron-regulated proteins was referred to as low-iron agar medium. Gonococci and meningococci do not utilize the iron bound to Desferal (38). Commensal Neisseria spp. also were unable to grow in the presence of Desferal without an alternative iron source (21). This iron-chelator has been successfully used with gonococci to study the proteins expressed under conditions of iron-limitation (22,27,36). Organisms were grown on regular and low-iron agar medium by streaking inocula with a cotton swab onto a petri dish (100 X 25 mm) containing 20 ml of agar. Cultures were incubated for 16-20 h at 37° C in a humidified atmosphere containing 4% CO_2.

Preparation of crude bacterial membranes. Cells were harvested by suspending the growth from the agar plates in Davis A minimal medium (Difco Laboratories). The suspensions were centrifuged and the pellets washed once in 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer (pH 7.4) containing 0.1% (v/v) proteinase inhibitor (10 mM phenylmethylsulfonyl fluoride in isopropanol). The pellet was suspended in the HEPES buffer, and the cells were disrupted by sonication using a high intensity sonifier (Branson Instruments Incorporated, Stamford, Conn.) for a total of 1 min. The sonicated cell suspensions were centrifuged at 48,000 x g for 60 min. The pellets are enriched for total membrane proteins and are referred to as crude membrane preparations. Pellets were suspended in HEPES buffer with proteinase inhibitor to a final protein concentration of 2 mg/ml as determined by the method of Lowry et al. (18) as modified by Markwell et al. (19). The suspensions were then aliquoted and stored at -20° C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Crude membrane preparations (1 mg of total membrane protein per ml) were solubilized in the final sample buffer as described previously (22). After dilution, samples were heated to 100° C and held for 5 min. Electrophoresis was carried out using 10% acrylamide slab gels which were 140-mm long and 2-mm thick in conjunction with Laemmli gel and buffer formulations (16) modified by the addition of 70 mM NaCl (final concentration) to the separating gel. Electrophoresis was performed at constant current (16 mA) overnight at room temperature.

For Coomassie blue staining, 35 ug of crude-membrane protein preparation was added per lane. Gels were stained for 2-12 h in a solution of 0.1% (w/v) Coomassie blue G250 (Miles, Elkhart, Ind.) in water:methanol:acetic acid (5:5:2). Acetic acid (10% v/v) was used to destain the gels. Alternatively, gels were stained by the silver staining method of Tsai and Frasch (35) modified by the omission of the periodate oxidation step. A total of 20 ug of crude membrane protein preparation was loaded per lane for visualization by this method. Reagents were freshly prepared prior to staining each gel. Development of the stain was terminated using a solution containing 10% (v/v) ethanol and 5% (v/v) acetic acid.

Preparation of rabbit antiserum specific for the 37K protein. Purification of the 37K protein from *N. gonorrhoeae* strain F62 has been described elsewhere (23). Rabbit antiserum against the purified 37K protein was prepared as described by Blake and Gotschlich (2). Purified 37K protein (200 ug) in complete Freund's adjuvant (Difco) was injected subcutaneously into female New Zealand White rabbits. After 3 weeks, the rabbits were injected with 200 ug of purified 37K protein in incomplete Freund's adjuvant (Difco). A final injection of purified 37K protein alone was given 3 weeks later. Control sera were obtained from these rabbits prior to immunization.

Preparation of monoclonal antibodies specific for the 37K protein. Monoclonal antibodies (MAbs) were prepared by injecting BALB/c mice subcutaneously on day 1 with 80 ug of purified 37K protein (prepared from N. gonorrhoeae strain F62) in 0.1 ml of incomplete Freund's adjuvant. On days 14 and 21, 50 ug of 37K protein in phosphate buffered saline was injected intravenously. On day 24, fusions of spleen cells with NS-0 myeloma cells was performed as previously described (9). Hybridoma cells producing antibody to the 37K protein were identified using the enzyme-linked immunosorbent assay (ELISA) described below. Hybridomas producing antibody to the 37K protein were cloned by limiting dilution. ELISA-positive independent clones were expanded and tissue culture supernatants were collected for antigenic analysis.

Enzyme-linked immunosorbent assay. The ELISA used for the identification of monoclonal antibodies specific for the 37K protein utilized methods similar to those described by Sarafian et al. (29). Purified 37K protein was diluted in antigen diluent (50 mM carbonate buffer pH 9.6, containing 0.02% sodium azide) to a protein concentration of 10 ug/ml. To coat the wells of a flat bottom microtiter plate (Costar 96 well tissue culture clusters, catalogue number 3596, Costar, Cambridge, Mass.), 50 ul of the antigen dilution was deposited in each well and the plate incubated overnight at 37° C. Unbound protein sites were blocked by adding 100 ul of 1% (w/v) bovine serum albumin (Sigma) in phosphate-buffered saline pH 7.6 (PBS) followed by incubation for 2 h at 37° C. At this time, the blocking solution was removed. Fifty ul of tissue culture supernatant was added to the coated wells and the plates incubated at 37° C for 1 h. The plates were washed three times in PBS containing 0.05% Tween 20 (Sigma) as previously described (29). Antibody to mouse immunoglobulin conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersberg, Md.), diluted 1:400 in PBS, was added to each well

and the plate incubated at 37° C for 1 h. After washing, p-nitrophenyl phosphate (1 mg/ml w/v; Sigma) in substrate diluent (5 mM carbonate buffer, pH 9.8, containing 1 mM $MgCl_2$) was added to each well. After incubation, the reaction was stopped by the addition of NaOH and the extinction values determined at 405 nm using a microELISA auto reader (MR580, Dynetech, Alexandria, Va.) to ascertain the presence or absence of specific antibody.

A similar assay was used to detect the presence or absence of an antigenically related 37K protein in crude membranes from organisms grown on low-iron agar medium. In this assay, 50 ul of antigen diluent containing 2.5 ug of crude membrane protein was added to wells of a flat bottom microtiter plate. The plates were incubated overnight at 37° C and the unbound protein sites blocked. Rabbit antiserum specific for the 37K protein of _N. gonorrhoeae_ strain F62 was diluted 1:500 in PBS, and 50-ul volumes were deposited in each well and incubated at 37° C for 1 h. Antibody to rabbit immunoglobulin conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories) diluted 1:400 in PBS was added to each well and incubated at 37° C for 1 h. The wells were washed, and 50 ul of the substrate solution was added. The reaction was terminated after 1 h at 37° C and the extinction values at 405 nm were determined using a microELISA auto reader. Binding of 37K protein-specific MAb to crude membranes was also examined using the following modification of this assay. Tissue culture supernatants (50 ul) were added in place of the 37K protein-specific rabbit antiserum. Antibody to mouse immunoglobin conjugated to alkaline phosphatase was utilized to recognize bound monoclonal antibody.

Western blot analysis. SDS-PAGE was performed using 10% acrylamide gels loaded with 40 ug of crude membrane protein per lane. Western blots were prepared in a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Richmond Calf.) as described by Towbin et al. (34). The nitrocellulose membranes (0.45 um; Schleicher and Schuell, Keene, N.H.) onto which the protein profiles were blotted were incubated with 50 mM Tris(hydroxymethyl)-aminomethane (Tris) hydrochloride (Sigma) buffer, pH 7.4, containing 0.9% (w/v) NaCl, 0.02% sodium azide and 5% ovalbumin (Sigma) to block free protein-binding sites. Rabbit antiserum diluted 1:1000 in the blocking buffer was incubated with the nitrocellulose membranes for 5 h at room temperature. After extensive rinsing in the blocking buffer, ca. 10^7 cpm of ^{125}I-labeled protein A (New England Nuclear, Boston, Mass.) was added to the same buffer and incubation continued at room temperature for 2 h to detect bound antibody. The blots were washed extensively, dried, and visualized by autoradiography at -70° C using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

For the analysis of the MAb on western blots, the method of Blake et al. (1) was used. A 1:100 dilution of tissue culture supernatant was used. Detection of bound antibody was accomplished using antibody to mouse immunoglobulin conjugated to alkaline phosphatase. The developing reagent was 5-bromo-4-chloroindoxyl phosphate (Sigma) in the presence of nitro blue tetrazolium (Sigma).

RESULTS

Expression of the gonococcal 37K protein on low-iron agar medium. We have previously reported the expression of an iron-regulated 37K protein from *N. gonorrhoeae* grown in GC broth medium containing 25 uM Desferal (22). This protein appeared to be under iron-regulation as iron added back to this medium suppressed its synthesis (22). In order to facilitate the screening of a large number of *Neisseria* spp., the conditions for expression of the 37K protein on GC agar medium were examined. Desferal was added to GC medium base agar at concentrations of 6.25, 12.5, and 25 uM. *N. gonorrhoeae* strain F62 was inoculated onto these plates and after overnight incubation, the cells were harvested and analyzed by SDS-PAGE for the expression of the 37K protein. This protein was only detected in substantial amounts in cells grown on medium containing 25 uM Desferal (Fig. 1). Furthermore, it was noted that considerably less growth occurred on the medium containing this concentration of Desferal compared with the medium containing none or lower concentrations of the iron-chelator. This observation was noted for all *Neisseria* spp. examined. Presumably, this was a consequence of iron-limitation and was analogous to previous observations which used Desferal in GC broth medium (22,27,36). Therefore, the medium containing 25 uM Desferal was referred to as low-iron GC agar medium. This medium was used to screen all strains of the *Neisseria* spp. which were examined.

Identification of Neisseria spp. expressing a protein which was antigenically related to the 37K protein (AgR-37K). The SDS-PAGE analysis of crude membranes from nine species of *Neisseria* and one species of *Branhamella*

grown on low-iron medium is shown in Fig. 2A. Most of the strains examined by this method had proteins migrating at or near 37,000 daltons. Therefore, we used rabbit antiserum prepared against the purified 37K protein from *N. gonorrhoeae* (strain F62) to probe for an antigenically related protein in the different species. Fig. 2B shows an autoradiograph of a western blot of the gel shown in Fig. 2A, which had been probed with the 37K protein-specific rabbit antiserum and ^{125}I-protein A. When grown under the conditions necessary for expression of the iron-regulated 37K protein in *N. gonorrhoeae*, only *N. meningitidis*, *N. lactamica*, and *N. cinerea* expressed cross-reacting proteins which had apparent molecular weights similar to that of the gonococcal 37K protein. Using this criterion we have designated these proteins as AgR-37K. A higher molecular weight band (*ca.* 41,000, 41K) also reacted with this antiserum in preparations from *N. meningitidis*. However, the presence or absence of this 41K protein was inconsistant among meningococcal strains examined. Variations in the expression of this protein was also observed within identically prepared samples from the same strain (data not shown). Similarly, the gonococcus produced a protein with an apparent molecular weight of 41,000; the expression of which appeared to vary to the same degree as observed for meningococci (data not shown). The presence of this higher molecular weight protein has not been noted for *N. lactamica* or *N. cinerea*; however, only a limited number of preparations from these strains have been examined by western blotting.

Survey of Neisseria spp. using an ELISA specific for AgR-37K proteins. We utilized an ELISA to screen a large number of *Neisseria* spp. for their reactivity with the rabbit antiserum prepared against the gonococcal 37K protein. Results of the ELISA performed on the 10 different species previously analyzed in Fig. 2A and 2B are presented in Table 2. The results of the ELISA were in good agreement with the data obtained by western

bloting, i.e., all preparations in which an AgR-37K protein was detected by western blotting gave ELISA values greater than 0.2; those preparations which did not show western blot reactivity gave ELISA values of less than 0.04. Using this ELISA, we examined 22 strains of Neisseria spp. which were positive and 8 strains of Neisseria and Branhamella spp. which were negative for AgR-37K proteins by western blotting (data not shown). ELISA results similar to those described above were obtained in all cases.

A total of 57 different strains from 9 Neisseria spp. and B. catarrhalis were screened by this ELISA. The results are presented in Table 3. All strains of N. gonorrhoeae, N. meningitidis, N. lactamica and N. cinerea examined were positive for an AgR-37K protein. The remaining Neisseria and Branhamella spp. did not react in this ELISA with the exception of one strain of N. subflava (CDC 8886). Western blot analysis of this strain using the rabbit antiserum specific for the gonococcal 37K protein recognized a protein with an apparent molecular weight of approximately 37,000 daltons. The identity of this strain as N. subflava was comfirmed by standard identification methods (D. Hollis, personal communication). Four other strains of N. subflava did not react in the AgR-37K protein-specific ELISA.

Effect of iron on the expression of the AgR-37K proteins. Strains belonging to each of the Neisseria spp. in which an AgR-37K protein was identified were used to test the effect of iron on the expression of the AgR-37K protein. Each of the strains was grown on regular and low-iron GC medium agar. Crude membranes were prepared and analyzed by SDS-PAGE. In all cases, the membrane preparations from cells grown on low-iron GC medium agar contained increased amounts of a protein with a molecular weight of ca. 37,000 when compared with cells grown on regular GC medium agar (Fig 3). N.

meningitidis exhibited only modest increases in the protein band migrating at 37,000 daltons. A previous report has suggested that this band may be a complex of comigrating proteins (32). Therefore, lack of a substantial increase in the relative proportion of this band from preparations of N. meningitidis grown on low-iron medium may not be apparent due to constitutively produced proteins. It was of interest that the iron-regulated proteins from N. subflava, and some strains of N. cinerea had slightly lower apparent molecular weights than the gonococcal 37K protein.

Reactivity of monoclonal antibodies with the AgR-37K proteins. Murine monoclonal antibodies were utilized in conjunction with western blot and ELISA techniques to probe the antigenic heterogeneity of the AgR-37K iron-regulated proteins. Of the seven MAbs analyzed by ELISA only one, RT 36, recognized the 37K protein in crude membranes of N. gonorrhoeae (strain F62) grown on low-iron medium. We extended this analysis to the membrane preparations from strains of the species of Neisseria which express an AgR-37K protein. MAb RT 36 gave a positive ELISA value with the other four species (Table 4). The remaining MAbs were analayzed using western blotting techniques. All the MAbs used in this analysis detected a single band which had an apparent molecular weight of 37,000 in membrane protein profiles of N. gonorrhoeae strain F62. These MAbs were further used to probe strains from the Neisseria spp. identified as containing iron-regulated AgR-37K proteins. Fig. 4 demonstrates the reactivity of five of the McAbs with western blots of membrane protein profiles of N. gonorrhoeae, N. meningitidis, N. lactamica, and N. cinerea. The McAbs exhibited differences in their reactivity. MAb RT 26 appeared to exhibit broad specificity, reacting with all four species . MAb RT 14 also reacted with all species except N. subflava (Table 4). MAb RT 7 had an

interesting pattern of reactivity; it did not recognize the AgR-37K proteins from _N. cinerea_ or _N. subflava_. In addition, this antibody recognized the 41K protein of _N. meningitidis_. MAb RT 27 reacted similarly to RT 7. MAb RT 29 also did not react with the AgR-37K proteins of _N. cinerea_ and _N. subflava_. The cross-reacting 41K protein of _N. meningitidis_ was poorly recognized by this antibody. A summary of the monoclonal reactivity patterns with the _Neisseria_ spp. is given in Table 4.

DISCUSSION

Proteins which are expressed under conditions of iron-limitation have been reported in a number of microorganisms (25). Many of these proteins have been implicated as functional components of the bacterium's iron-uptake system. Norqvist et al. (27) first reported that gonococci expressed new membrane proteins when grown under iron-limiting conditions. The molecular weights of these Sarkosyl-insoluble membrane proteins ranged from 70,000 to 100,000 daltons. The number and the apparent molecular weights of these iron-regulated proteins varied among gonococcal isolates . Mietzner et al. (22) confirmed these findings and reported an additional iron-regulated protein with an apparent molecular weight of 37,000 which was common to all gonococcal strains examined. More recently, West et al. (36) examined crude membrane preparations of gonococci and reported similar findings with respect to both the higher molecular weight iron-regulated proteins and a protein with an apparent molecular weight similar to 37,000. Several other proteins, in addition to those reported previously, appeared to be iron-regulated; presumably, these were Sarkosyl-soluble membrane proteins which were not previously observed in the earlier reports (22,27). When gonococci were grown in a low-iron environment supplemented with different iron-containing compounds (e.g., transferrin or lactoferrin) various combinations of iron-regulated proteins were expressed (36). The 37K protein was unique in that it was expressed in all strains examined and in the presence of all iron-containing molecules tested. The consistent expression of this protein under all conditions of iron-limitation and its conservation among gonococci

prompted West to speculate that this protein may play a central role in iron-acquisition by the gonococcus.

We have purified the 37K protein from N. gonorrhoeae strain F62 (23). Using this purified preparation, both monospecific rabbit antiserum and murine monoclonal antibodies were prepared. These reagents were used to probe the Neisseria spp. for the presence of a protein which was antigenically related to the gonococcal 37K protein. Fifty-seven strains of Neisseria and Branhamella spp. were analyzed in an AgR-37K protein-specific ELISA. The specificity of this ELISA was confirmed by western blot analysis of 30 of these strains. In all cases there was good agreement between results obtained by western blot analysis and the AgR-37K protein-specific ELISA. The results suggested that this protein was conserved among strains of N. meningitidis, N. lactamica, N. cinerea, and N. gonorrhoeae. There appeared to be subtle variations in the apparent molecular weights of the AgR-37K protein in some strains of N. cinerea and in the single N. subflava isolate which reacted in the ELISA. The AgR-37K protein of N. meningitidis appeared to comigrate with the gonococcal 37K protein in all cases. A 41K protein which cross-reacted with the rabbit antiserum specific for the gonococcal 37K protein was observed in preparations from both N. meningitidis and N. gonorrhoeae. The expression of this protein was not consistant in different preparations from the same strains prepared under identical conditions. An iron-regulated protein with an apparent molecular weight of 41,000 has been previously reported (36) in crude membranes of N. gonorrhoeae. A similar protein was not observed in Sarkosyl-extracted membrane preparations (22,27). One explanation for the cross-reactivity of these two proteins may be that the 41K protein is the unprocessed form of the 37K protein. Alternatively, these two proteins may be unrelated and simply share a cross-reacting epitope.

Cannon et al. (5) have reported that the H.8 antigen was conserved among strains of N. gonorrhoeae, N. meningitidis, N. lactamica (4 of 5 strains tested), and N. cinerea (1 of 1 strain tested). However, the H.8 antigen is different from the 37K protein. It has an apparent molecular weight of 20,000 which can be modified by heat to migrate at 30,000 daltons on SDS-PAGE. It is interesting that these two antigens are conserved among the four species of Neisseria. Studies which employ thermal renaturation techniques to analyze DNA hybridization (13) have indicated that the gonococcus and the meningococcus are closely related (93.17%). DNA from N. lactamica hybridized with DNA from N. meningitidis (79.04%) and N. gonorrhoeae (80.43%) to a lesser degree. All members of the genus Neisseria hybridized to the other members at levels greater than or equal to 62%. More restrictive analysis have shown lower but similar values (8). However, it follows that the degree of relatedness of the Neisseria spp. may favor antigenic conservation. The consistent expression of both the H.8 antigen and the AgR-37K proteins in N. gonorrhoeae, N. meningitidis, N. lactamica, and N. cinerea may reflect evolutionary divergence away from the other members of this genus. Furthermore, the conservation of these antigens among the pathogenic Neisseria spp. may implicate them as important factors in pathogenesis.

We observed the expression of an AgR-37K protein in one strain of N. subflava (of 5 tested). This was the only exception among the 17 commensal species (non-lactamica and non-cinerea) which were tested in the AgR-37K protein-specific ELISA. This result is similar to the study of Cannon et al. (5) which described the occurrence of one strain of N. sicca (of 9 tested) that reacted with the monoclonal antibody specific for the H.8 antigen. The ability of Neisseria spp. to genetically transform other members of the genus has been the subject of numerous reports (6,13,31). Ison et al. (14)

presented preliminary evidence for intragenic transformation of gonococci by normal throat flora. A similar explanation could apply to the atypical expression of antigens in *Neisseria* spp. which do not normally contain them.

We have previously defined the iron-regulated proteins in functional terms as those proteins which increase or are unique to cells grown under iron-limited conditions (23). The *Neisseria* spp. were examined for the expression of an iron-regulated protein which migrated at or near 37,000 daltons. In all cases, the relative amount of a protein migrating near this molecular weight increased when grown on low-iron GC medium agar. Presumably, these proteins were the AgR-37K proteins previously identified. The conserved antigenic structure of the AgR-37K proteins and their ability to be modulated by iron may imply a common function. Since many iron-regulated proteins have been shown to have a role in iron-uptake, it is tempting to speculate that the AgR-37K proteins of the *Neisseria* spp. may serve a similar function. Crosa (7) has recently reviewed the relationship between iron-transport and bacterial virulence for the plasmid-mediated iron-uptake systems of *Vibrio anguillarum* and *Escherichia coli*. For both of these organisms, virulence was associated with a functional iron-transport system. To date, no direct function in iron-uptake has been ascribed to the gonococcal 37K protein. However, presuming that this protein does function in iron-aquisition, a similar correlate as that described by Crosa may also apply to the *Neisseria* spp.

Monoclonal antibodies specific for the gonococcal 37K protein were used to probe the antigenic heterogeneity of the AgR-37K proteins. Of the 7 MAbs tested, only two (RT 26 and RT 36) exhibited broad cross-reactivity. The other MAb appeared to be more stringent in their reactivity. RT 14 did not recognize the AgR-37K protein of *N. subflava* but reacted well with the other

3 nongonococcal species examined. RT 7, RT 16, and RT 29 did not recognize the AgR-37K protein of N. cinerea or N. subflava. Furthermore, differences in the degree of reactivity of the MAbs to the AgR-37K proteins of N. lactamica and N. meningitidis suggests that there might be subtle variations in the antigenic composition of these proteins. Whether or not this variation represents evolutionary diversity or important functional differences remains to be seen.

Of the 7 MAbs analyzed only one, RT 36 (IgM), recognized the gonococcal 37K protein in crude membranes as analyzed by ELISA. Another IgM monoclonal antibody specific for the gonococcal 37K protein did not react against the membrane-presented protein in the ELISA (data not shown). This suggests that MAb RT 36 was not non-specifically binding to the membranes of cells grown in low-iron medium. MAb RT 36 was broadly reactive with all AgR-37K proteins. It was not surprising that most of the MAbs did not react with membrane-presented determinants since the antibodies were prepared to the purified 37K protein. We intend to expand the bank of MAbs to include several which recognize membrane-presented epitopes in order to examine the antigenic heterogeneity of these determinants among the Neisseria spp. These MAbs may also be useful in understanding the function of this conserved protein. Furthermore, we have yet to extend our analysis of the 37K-protein specific MAbs to large numbers of strains within the same species. Initial studies analyzing a limited number of strains indicated that the antigenic heterogeneity (as assayed by the MAbs) is conserved within each Neisseria spp.

Many of the physical and biologic properties of the gonococcal 37K protein have been reported (23) or are presently being analyzed, however its function remains illusive. Analysis of sera from patients convalescing from gonococcal infections indicated that antibodies specific for the 37K protein were present

(S.A. Morse, personal observation). This suggests that the 37K protein is both expressed and antigenic *in vivo*. Further studies are underway to determine the extent and nature of this response. However, it seems likely that this protein may play some essential function *in vivo*. The conservation of this protein both within the species and among the pathogenic *Neisseria* spp. may suggest that this function is essential for the pathogenesis of these organisms

References

1. Blake, M.S., K.H. Johnston, G.J. Russell-Jones, and E.C. Gotschlich. 1983. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Analyt. Biochem. 136: 175-179.

2. Blake, M.S. and E.C. Gotschlich. 1984. Purification and partial characterization of the opacity associated proteins of *Neisseria gonorrhoeae*. J. Exp. Med. 159: 452-462.

3. Bovre, K. 1984. Family VIII. *Neisseriaceae*. p. 288-310. In N.R. Kreig and J.G. Holt (ed.). Bergey's manual of systematic bacteriology, Vol. 1. Williams and Wilkens, Baltimore/London.

4. Buchanan, T.M. 1975. Antigenic heterogeneity of gonococcal pili. J. Exp. Med. 141: 1470-1475.

5. Cannon, J.G., W.J. Black, I. Nachamkin, and P.W. Stewart. 1984. Monoclonal antibody which recognizes an outer membrane antigen common to the pathogenic *Neisseria* species but not to most nonpathogenic Neisseria species. Infect. Immun. 43: 994-999.

6. Catlin, B.W., and L.S. Cunningham. 1961. Transforming activities and base contents of deoxyribonucleate prepatations from various *Neisseria*. J. Gen. Microbiol. 26: 303-312.

7. Crosa, J.H. 1984. The relationship of plasmid-mediated iron transport and bacterial virulence. Ann. Rev. Microbiol. 38: 69-89.

8. Elwell, L.P., and S. Falkow. 1977. Plasmids of the genus *Neisseria*. p. 138-154. In R.B. Roberts (ed.). The gonococcus. John Wiley & Sons, Inc., New York.

9. Fazekas De St. Groth, S., and D. Scheidegger. 1980. Production of monoclonal antibodies: Strategy and Tactics. J. Immunol. Meth. 35: 1-21.

10. Feder, H.M., and R.A. Garibaldi. 1984. The significance of nongonococcal, nonmeningococcal Neisseria isolates from blood cultures. Rev Infect. Dis. 6: 181-188.

11. Heckels, J.E. 1977. The surface properties of Neisseria gonorrhoeae: Isolation of the major components of the outer membrane. J. Gen. Microbiol. 99: 333-341.

12. Herbert, D.A., and J. Ruskin. 1981. Are "nonpathogenic" Neisseria pathogenic? American J. Clin. Path. 75: 739-743.

13. Hoke, C., and N.A. Vedros. 1982. Taxonomy of the neisseria: Deoxyribonucleic acid base composition, intraspecific transformation, and deoxyribonucleic acid hybridization. Int. J. Sys. Bacteriol. 32: 57-66.

14. Ison, C., A.A. Glynn, and S. Bascomb. 1982. Acquisition of new genes by oral Neisseria. J. Clin. Pathol. 35: 1153-1157.

15. Judd, R.C. 1982. ^{125}I-Peptide mapping of protein III isolated from 4 strains of Neisseria gonorrhoeae. Infect. Immun. 37: 622-631.

16. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

17. Lambden, P.R., and J.E. Heckels. 1979. Outer membrane protein composition and colonial morphology of Neisseria gonorrhoeae strain P9. FEMS Microbiol. Lett. 5: 263-265.

18. Lowry, D.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

19. Markwell, M.K., S.M. Haas, L.L. Bieber and N.E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87: 206-210.

20. Meyer, T.F., N. Mlawer, and M. So. 1982. Pilus expression in Neisseria gonorrhoeae involves chromosomal rearrangement. Cell 30: 45-52.

21. Mickelsen, P.A., E. Blackman and P.F. Sparling. 1982. Ability of N. gonorrhoeae, N. meningitidis and commensal Neisseria species to obtain iron from lactoferrin. Infect. Immun. 35: 915-920.

22. Mietzner, T.A., G.H. Luginbuhl, E.C. Sandstrom and S.A. Morse. 1984. Identification of an iron-regulated 37,000 dalton protein in the cell envelope of Neisseria gonorrhoeae. Infect. Immun. 45: 410-416.

23. Mietzner, T.A., and S.A. Morse. 1985. Iron-regulated membrane proteins of Neisseria gonorrhoeae: Isolation and partial characterization of a 37,000 dalton iron-regulated protein. In. G. Schoolnik, G.F. Brooks, S. Falkow, J.S. Knapp, A. McCutchen, and S.A. Morse (ed). The Pathogenic Neisseriae. American Society for Microbiology, Washington, D.C. In press.

24. Morse, S.A., C.S. Mintz, S.K. Sarafian, L. Bartenstein, M. Bertram, M.A. Apicella. 1983. Effect of dilution rate on lipopolysaccharide and serum resistance of Neisseria gonorrhoeae grown in continuous culture. Infect. Immun. 41: 74-82.

25. Neilands, J.B. 1982. Microbial envelope proteins related to iron. Ann. Rev. Microbiol. 36: 285-309.

26. Newhall, W.J., C.E. Wilde, W.D. Sawyer, and R.A. Haak. 1980. Higher-molecular-weight antigenic protein complex in the outer membrane of Neisseria gonorrhoeae. Infect. Immun. 27: 475-482.

27. Norqvist, A., J. Davies, L. Norlander, and S. Normark. 1978. The effect of iron-starvation on the outer membrane protein composition of Neisseria gonorrhoeae. FEMS Microbiol. Lett. 4: 71-75.

28. Sandstrom, E.C., K.C.S. Chen and T.M. Buchanan. 1982. Serology of Neisseria gonorrhoeae: CoAgglutination groups WI and WII/WIII correspond to different outer membrane protein I molecules. Infect. Immun. 38: 462-470.

29. Sarafian, S.K., M.R. Tam and S.A. Morse. 1983. Gonococcal protein I-specific opsonic IgG in normal human serum. J. Infect. Dis. 148: 1025-1032.

30. Schoolnik, G.K., J.Y. Tai, J. Rothbard and E.C. Gotschlich. 1983. A pilus peptide vaccine for the prevention of gonorrhea. Prog. Alleg. 33: 314-331

31. Siddiqui, A., and I.D. Goldberg. 1975. Intragenic transformation of Neisseria gonorrhoeae and Neisseria perflava to streptomycin resistance and nutritional independence. J. Bacteriol. 124: 1359-1365.

32. Simonson, C., D. Brener, and I.W. DeVoe. 1982. Expression of a high-affinity mechanism for acquisition of transferrin iron by Neisseria meningitidis. Infect. Immun. 36: 107-113.

33. Swanson, J. 1978. Studies on gonococcus infection XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. Infect. Immun. 21: 292-302.

34. Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4354.

35. Tsai, L.M. and C.E. Frasch. 1982. A sensitive silver stain for detecting lipopolysacharide in polyacrylamide gels. Analyt. Biochem. 119: 115-119.

36. West, S.E.H., and P.F. Sparling. 1985. Response of Neisseria gonorrhoeae to iron limitation: Alterations in expression of membrane proteins

without apparent siderophore production. Infect. Immun. 47: 388-394.

37. Wiseman, G.M., and J.D. Caird. 1977. Composition of the lipopolysacharide of Neisseria gonorrhoeae. Infect. Immun. 16: 550-556.

38. Yancey, R.J., and R.A. Finkelstein. 1981. Assimilation of iron by pathogenic Neisseria spp. Infect. Immun. 32: 592-599.

39. Zak, K., J.-L. Diaz, D. Jackson, and J.E. Heckels. 1984. Antigenic variation during infection with Neisseria gonorrhoeae: Detection of antibodies to surface proteins in sera of patients with gonorrhea. J. Infect. Dis. 149: 166-174.

Fig. 1. SDS-PAGE analysis of crude membrane preparations from *N. gonorrhoeae* strain F62 grown on GC base medium agar supplemented with (A) 0 uM, (B) 6.25 uM, (C) 12.5 uM, and (D) 25 uM Desferal. Proteins were visualized by staining with Coomassie blue. Only preparations from cells grown on GC base medium agar containing 25 uM Desferal expressed substantial amounts of the 37K protein.

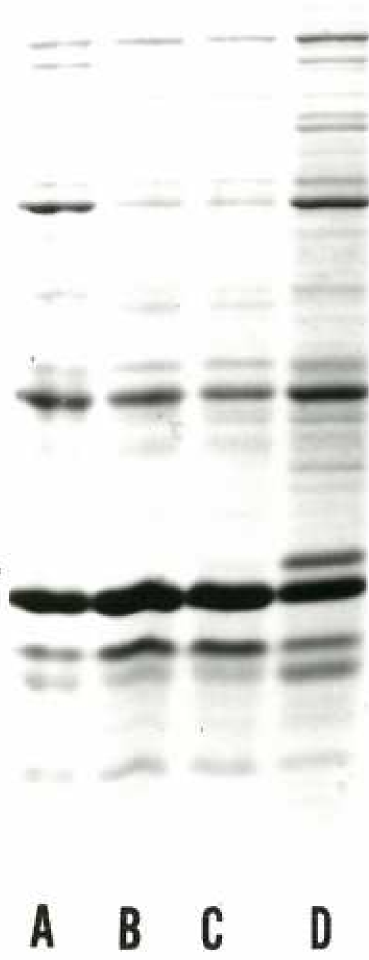
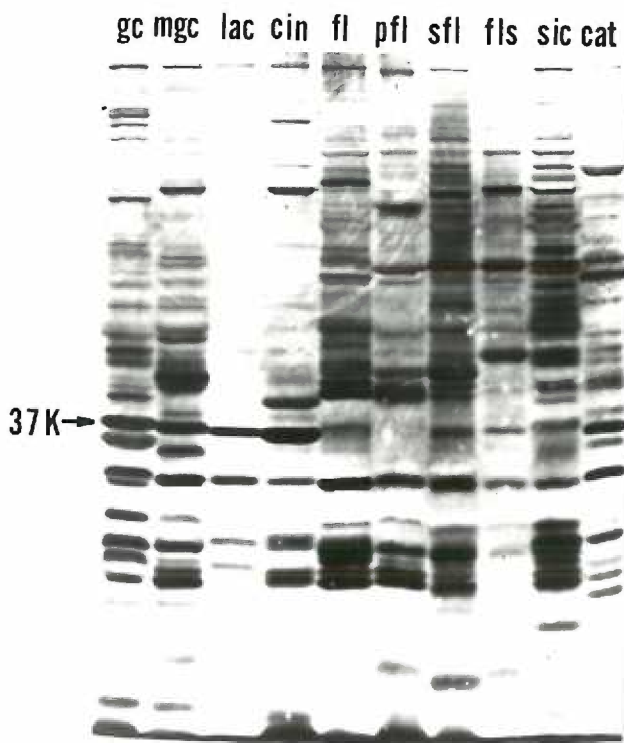

37K
A
B
C
D

Fig. 2. Analysis of 9 different strains of Neisseria spp. and B. catarrhalis for the production of AgR-37K proteins. Fig. 2A shows an SDS-PAGE separation of crude membranes from strains grown on low-iron medium. The proteins were visualized using the silver stain. Fig. 2B is an autoradiograph of a western blot which was probed using rabbit antiserum specific for the gonococcal 37K protein and ^{125}I-protein A. The samples used were the identical preparations in the silver-stained gel shown in Fig. 2A. The strains used for this analysis were N. gonorrhoeae strain F62 (gc), N. meningitidis strain 80050756 (mgc), N. lactamica strain 1519 (lac), N. cinerea strain 33683 (cin), N. flava strain NS-6 (fl), N. perflava strain 4326 (pfl), N. subflava strain D9657 (sfl), N. flavescens strain NS-10 (fls), N. sicca strain 30016 (sic), and B. catarrhalis strain 30018 (cat). N. meningitidis, N. lactamica, and N. cinerea expressed proteins which cross-reacted with the gonococcal 37K protein-specific antiserum. These proteins all had approximate molecular weights of 37,000. In addition, a cross-reacting band was observed in the preparation from N. meningitidis (designated by an arrow) which had an apparent molecular weight of 41,000.


gc mgc lac cin fl pfl sfl fls sic cat
37K

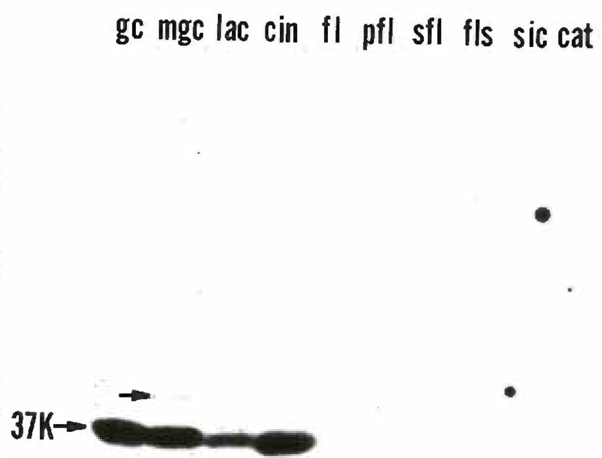
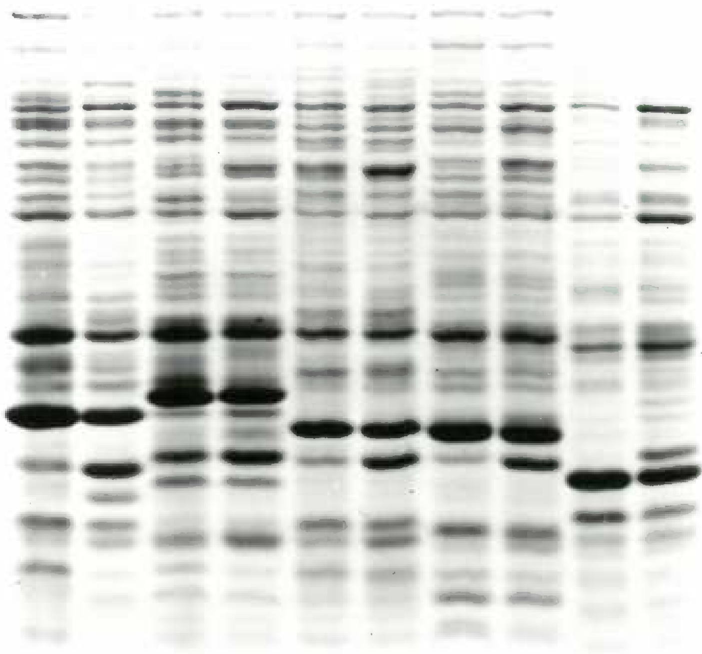

gc mgc lac cin fl pfl sfl fls sic cat
37K

Fig. 3. SDS-PAGE comparison of membrane preparations from cells grown on GC base medium agar containing 25 uM Desferal (+; low-iron medium) and on medium which was not supplemented with this chelator (-; iron was not limiting). Strains from Neisseria spp. which express AgR-37K proteins contain a protein with an apparent molecular weight of ca. 37,000 which increases in relative proportion in preparations grown in the presence of Desferal when compared with preparations from cells grown on regular GC agar medium. Equal amounts of crude membrane protein (35 ug) were added to each lane. The proteins were visualized by Coomassie blue staining. Strains used for this analysis were N. subflava strain B886 (sfl), N. meningitidis strain 80050756 (mgc), N. cinerea strain 33683 (cin), N. lactamica strain 1519 (lac), and N. gonorrhoeae strain F62 (gc).



sfl
mgc
cin
lac
gc
–
+
–
+
–
+
–
+
–
+
←37K

Fig. 4. Western blot reactivity of MAbs to strains of *Neisseria* spp. which express AgR-37K proteins. Identical concentrations of crude membrane proteins from single preparations of cells grown on low-iron medium were blotted onto nitrocellulose and reacted with each MAb. Antibody/antigen complexes were detected using alkaline phosphatase conjugated to anti-mouse immunoglubulin and visualized using an immunochemical stain. For the purposes of illustration, the region of the western blot which reacted with the McAbs was cut out and arranged in a column in order to compare the reactivity to AgR-37K proteins. The MAb used to probe each blot is listed in the left margin. For each MAb, preparations from the following strains were analyzed: *N. lactamica* strain 1341 (lac), *N. cinerea* strain 30073 (cin), *N. meningitidis* strain 80073116 (mgc), and *N. gonorrhoeae* strain F62 (gc). A single band corresponding to the 37K protein was detected in crude membrane preparations from gonococci by all the MAbs. The AgR-37K proteins in the nongonococcal crude membranes were either not detected or reacted to the same, or a lesser, degree as the gonococcal 37K protein. RT 7, RT 27, and RT 29 recognized a band with an apparent molecular weight of 41,000 in the crude membrane preparations of *N. meningitidis*.

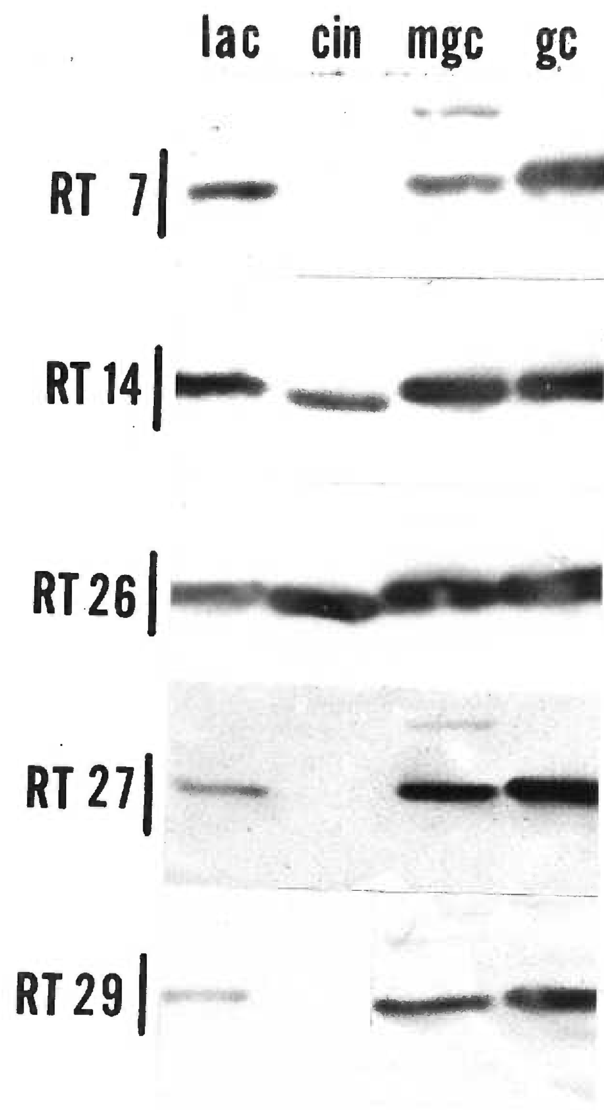

lac
cin
mgc
gc
RT 7
RT 14
RT 26
RT 27
RT 29

Table 1. Designations and sources of strains used in this study

Species		Strain designation	Source
N. gonorrhoeae		F62	R.P. Williams
		F18, F28, F29, F45	J.W. Biddle
		84021704, 84021705, 84021706, 84033829	R. Rice
N. meningiditis	(serogroup A)	80084313	R.J. Arko
	(B)	82010805, 100, E6942, 79118417	''
	(C)	80073116	''
	(W135)	80084822	''
	(Y)	80050756, 8438	''
	(Z)	8530, 80046175, 200	''
	(L)	1721	R. Rice
N. lactamica		C1189, C9262, D9238, D9292, D1748, D7059, D7256, E6040	D. Hollis
		85021174	J.W. Biddle
		NS-19	D. Kellogg
		1341	S.A. Morse
N. cinerea		33683, 30073, 33720, 32165, 33837, 34382, 33295	J. Knapp
N. subflava	(biovar flava)	NS-6, NS-17	D. Kellogg
		4324	S.A. Morse
	(biovar subflava)	B886, D9657, D8822, D9488, E113	D. Hollis
	(biovar perflava)	4326	H. Schneider
N. flavescens		NS-10	D. Kellogg
		13120	ATCC
N. mucosa		A7895	D. Hollis
N. sicca		30016	J. Knapp
N. ovis		T2B	D. Kellog
B. catarrhalis		30018	J. Knapp
		1604, 1165	S.A. Morse

Table 2. Comparison of ELISA values with western blot reactivity of different strains of *Neisseria* spp. and *B. catarrhalis*

Species	Strain	ABS(405nm)	Western Blot Reactivity[a]
N. gonorrhoeae	F62	0.237	+
N. meningitidis	80050756	0.293	+
N. lactamica	1519	0.295	+
N. cinerea	33683	0.256	+
N. flava	NS-6	0.018	-
N. perflava	4326	0.011	-
N. subflava	D9657	0.020	-
N. flavescens	NS-10	0.032	-
N. sicca	30016	0.014	-
B. catarrhalis	30018	0.002	-

a) + or - reactivity indicates the presence or absence of a band with an approximate apparent molecular weight of 37,000 on western blots probed with rabbit antiserum specific for the gonococcal 37K protein and ^{125}I-protein A.

Table 3. Survey of strains from *Neisseria* spp. and *B. catarrhalis* for the expression of an AgR-37K protein

Species	Positive reactivity/number tested	
	Western Blot[a]	ELISA[b]
N. gonorrhoeae	1/1	9/9
N. meningitidis	13/13	13/13
N. lactamica	1/1	11/11
N. cinerea	6/6	7/7
N. subflava	1/2	1/5
N. flava	0/1	0/3
N. perflava	0/1	0/1
N. flavascens	0/1	0/2
N. sicca	0/1	0/1
N. mucosa	0/1	0/1
N. ovis	0/1	0/1
B. catarrhalis	0/1	0/3

a) Positive reactivity refers to the presence of a band for the strain tested with an approximate apparent molecular weight of 37,000 on western blots probed with rabbit antiserum specific for the gonococcal 37K protein and ^{125}I-protein A.

b) Positive reactivity indicates that an ELISA value of greater than 4 S.D. above background was obtained for the strain tested.

Table 4. Reactivity of MAbs to the different strains of *Neisseria* spp. producing AgR-37K proteins[a]

MAb	Isotype	Reactivity				
		N.gonorrhoeae	N.meningitidis	N.lactamica	N.cinerea	N.subflava
RT 7	IgG_1	+	+	+	-	-
RT14	IgG_1	+	+	+	+	-
RT16	IgG_1	+	+	+	-	-
RT26	IgG_1	+	+	+	+	+
RT27	IgG_1	+	+	w[b]	-	nd[c]
RT29	IgG_1	+	+	w	-	-
RT36[d]	IgM	+	+	+	+	nd

a) Strains used for this analysis were *N. gonorrhoeae* strain F62, *N. meningitidis* strain 80073116, *N. lactamica* strain 1341, *N. cinerea* strain 30073, and *N. subflava* strain B886.

b) A qualitatively weak response by the MAb to the AgR-37K protein of this species as determined by western blotting.

c) Reactivity has not been determined.

d) Reactivity for this MAb was determined by ELISA using wells coated with membranes containing AgR-37K proteins. All other reactivities were determined by western blot analysis.

SUMMARY OF DISSERTATION WORK

Discussion of the results reported in this thesis are found at the end of each manuscript and will not be reiterated in this section. A summary of the relevant findings and their potential significance are as follows:

1. Growth of gonococci under iron-limiting conditions results in the expression of several proteins in the cell envelope (iron-regulated proteins); among these are a major protein with an apparent molecular weight of 37,000. Recently West _et al_. (see literature review reference 424) have confirmed the existence of this protein in similar preparations of _N. gonorrhoeae_. They report this protein to have an apparent molecular weight of 36,000 by SDS-PAGE analysis. The subtle discrepencies in the molecular weights of this protein likely occur due to differences in electrophoretic conditions. The studies reported in this thesis employ gradient gels with increased ionicity in the resolving gel buffer whereas, West _et al_. do not.

2. Expression of the 37,000 dalton protein was influenced by the amount of iron available to the gonococcus. Based upon silver staining, this protein appeared to be the most abundant iron-regulated protein present in Sarkosyl-insoluble membrane preparations. Therefore, we have referred to this protein as the major iron-regulated protein (MIRP)

3. The MIRP was common to all gonococci examined. Peptide mapping indicated that this protein was not a modified Protein I. This analysis also indicated that the MIRP was structurally conserved between two unrelated strains of gonococci. This conservation may imply that the MIRP plays an

essential role for the survival of gonococci.

4. The occurrence of substantial quantities of the MIRP in the non-sedimentable fractions of sonicated whole cells was observed; this in addition to a significant amount of membrane-associated MIRP. This finding is interesting in that most proteins are either membrane-bound or soluble. It is not clear whether or not the non-sedimentable MIRP actually is "soluble" monomeric MIRP or if it is in some way associated with a complex which is resistant to centrifugation at high speeds.

5. The fortuitous finding that the gonococcal MIRP could be extracted at low-concentrations of the cationic detergent CTB greatly facilitated the purification of this protein. Low-concentrations of CTB were effective in solubilizing the MIRP from particulate fractions. Under similar conditions, CTB precipitated components of the non-sedimentable fraction, leaving a supernatant enriched with the MIRP.

6. The MIRP from the CTB-containing fractions bound to a cation exchange gel matrix (CM-Sepharose) at a relatively high pH (8.0). Most of the contaminating proteins did not interact with the column matrix at this pH. The bound MIRP could be eluted by a linear NaCl gradient as a single peak.

7. The isolated MIRP-containing fractions exhibited a pink-to-red color; the nature of which is not understood. This is interesting in light of the fact that some proteins (e.g., lactoferrin), when complexed with iron, are red. Since the MIRP is produced by gonococci in response to the lack of available iron, a plausible prediction might be that this protein is a component of the gonococcal iron-uptake system. Studies have not yet examined the capacity of this protein to bind metal ions or to participate in the uptake of iron by _N. gonorrhoeae_.

8. Further purification of the MIRP could be accomplished by gel filtration. Estimates from molecular seive chromatography predicted that the MIRP had a slightly lower-molecular-weight (26,000 to 32,000) than determined by denaturing SDS-PAGE analysis. Under similar conditions, Protein I (which has a monomeric molecular weight of 36,000 by SDS-PAGE analysis) eluted as a multimeric species in association with a 60,000 dalton detergent micelle; the molecular weight of this complex corresponded to 188,000. From this analysis, no evidence was found for the association of the MIRP as a polymeric complex. However, this analysis can not rule out the possibility that some type of association occurs under native cellular conditions. The gonococcal MIRP did not associate with Zwittergent-3,14 micelles; a property displayed by purified preparations of both Protein I and Protein II.

8. The biochemical properties of the purified gonococcal MIRP were examined. The the isoelectric point of this protein was greater than 9.35. The highly basic nature of this protein was predicted by its interaction with the cation exchange matrix at high pH values. Moreover, the predicted amino acid composition of this protein was consistent with its highly cationic character; in addition to the basic amino acid residues, a large proportion of dicarboxylic acids were present. It is likely that a significant number of these residues exist in their amide form and contribute to the high isoelectric point.

9. Of interest was the N-terminal homology shared between MIRP and Protein I. The significance of this observation is not readily apparent. However, this homology may have some functional significance common to both proteins. Alternatively, this homology may suggest some evolutionary

relationship between the MIRP and Protein I of N. gonorrhoeae.

10. Purified preparations of the MIRP have been used to prepare specific antiserum and murine monoclonal antibodies. These immunological reagents were used to probe the Neisseria spp. for the distribution of proteins which were antigenically related to the major iron-regulated protein of N. gonorrhoeae (AgR-37K). This study found that N. meningitidis, N. lactamica, and N. cinerea all express a AgR-37K protein with an apparent molecular weight similar to the gonococcal MIRP. In addition to its potential phylogeneic significance, the conservation of this protein among the pathogens N. meningitidis and N. gonorrhoeae may indicate that it is a necessary determinant of pathogenicity. However, the expression of an AgR-37K protein among the non-pathogenic species of N. lactamica and N. cinerea suggests that the presence of this protein is not in itself a lone determinant of virulence.

11. The AgR-37K proteins from all Neisseria spp. were iron-regulated. This finding may reflect a common role for AgR-37K proteins in iron-acquisition.

12. Monoclonal antibodies prepared against the gonococcal MIRP reacted variably with the AgR-37K proteins from the different Neisseria spp. This suggests that there may be some evolutionary divergence in the structure of this protein. The implications of this finding may be clinically relevent. Presently, identification of Neisseria spp. rely on time-consuming and costly bacteriological methods. Production of monoclonal antibodies which react specifically with AgR-37K protein epitopes unique to each species of Neisseria may provide a rapid test for the especiation of medically important Neisseria.

To date, the function of the gonococcal MIRP has not been determined; although there is some circumstantial evidence which suggests a role for this

protein in iron-acquisition. These studies have laid the foundation for analyzing the functional role that MIRP plays during the course of natural infection. Understanding the role of this protein may uncover new physiologic and metabolic mechanisms which are used by gonococci. This knowledge may lead to new strategies toward the development of safer and more effective treatment regimens. Moreover, the exploitation of MIRP as a component of a gonococcal vaccine should also be evaluated. At the very least, continuing studies of the gonococcal MIRP should lead to a better understanding of the immunopathology of gonococcal infection and underscore the relationship between iron and virulence.

APPENDIX

Appendix 1. Effect of desferal on the growth of *N. gonorrhoeae*.

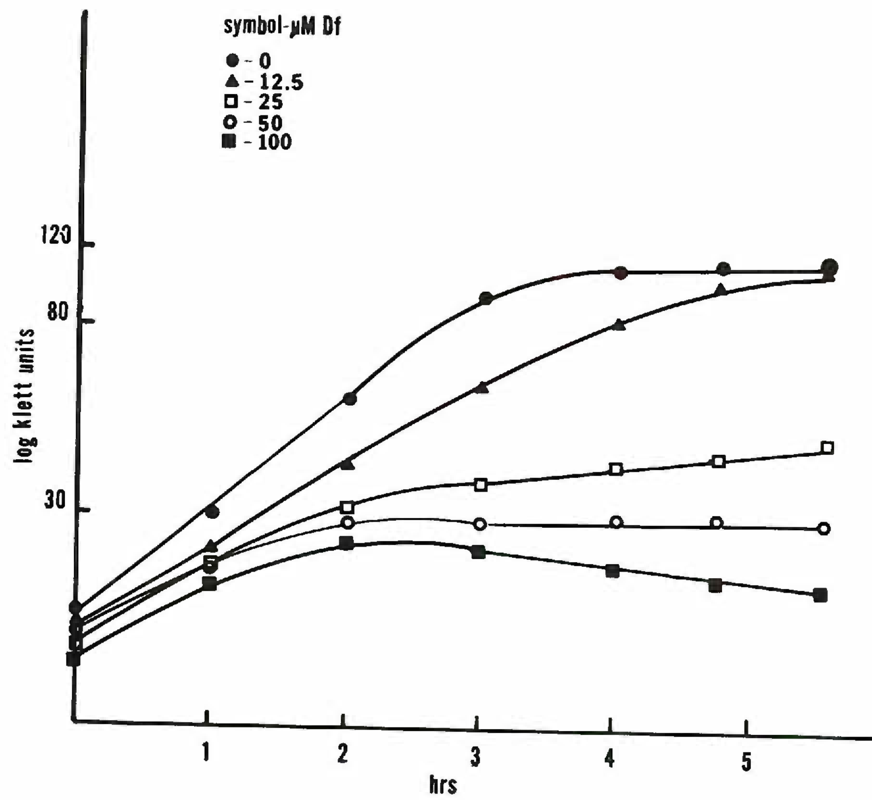
N. gonorrhoeae strain F62 was grown in 50 ml of GC broth medium as described in manuscript 1. To this medium 0, 12.5, 25, 50, and 100 uM desferal was added. The initial inoculum was taken from cells previously grown in iron-limited medium. These cells were harvested by centrifugation, washed in GC broth medium without desferal, and suspended to a high optical density in GC broth medium without desferal. This suspension was used to inoculate flasks containing different concentrations of desferal. Growth was monitored by optical density. These growth curves are shown in Figure 1.

Final cell yeild was determined by dry weight after each culture had grown into stationary phase. The yield of each culture (expressed as mg dry weight per 40 ml of medium) was determined to be as follows:

Desferal Concentration	Dry Weight (mg/40 ml)
0	29.3
12.5	25.0
25.0	13.9
50.0	11.5
100.0	9.2

Thus, the total cellular yield appears to be related to the concentration of desferal used in the medium, suggesting that it is limiting a growth essential nutrient.

Fig. 1. Growth curves for *N. gonorrhoeae* strain F62 grown in medium containing increasing concentrations of desferal.

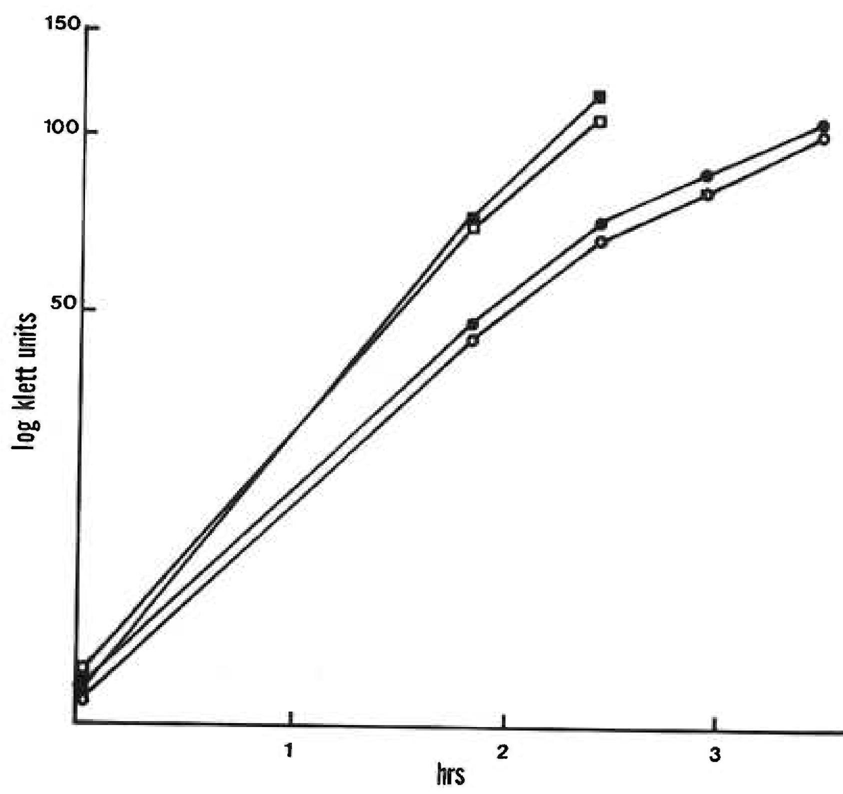


symbol-μM Df
● - 0
▲ - 12.5
□ - 25
○ - 50
■ - 100
120
80
30
log klett units
1
2
3
4
5
hrs

Appendix 2. Effect of growth at pH 6.3 and 7.2 in the presence and absence of desferal.

N. gonorrhoeae strain F62 was grown in low-iron GC broth medium (containing 25 uM desferal) as described in manuscript 1. For growth of gonococci at a lower pH value, HEPES buffer was added to the growth medium and the pH was adjusted to pH 6.3. Growth of gonococci was monitored by optical density. Growth curves under each condition are shown in Figure 1. The mean generation times for each condition were as follows: pH 7.2 without desferal, 42 min; pH 7.2 with desferal, 44 min; pH 6.3 without desferal, 54 min; and pH 6.3 with desferal, 55 min. Analysis of the Sarkosyl-insoluble membrane preparations by SDS-PAGE indicated that the 37K protein was only expressed in the presence of desferal. Thus, expression of the 37K protein was not the consequence of a decreased growth rate.

Fig. 1. Growth curves of N. gonorrhoeae strain F62 grown at different pH values and in the presence and absence of desferal. Symbols: Growth at pH 7.2 in the absence of desferal, ■ ; pH 7.2 in the presence of desferal, □ ; pH 6.3 in the absence of desferal, ● ; and pH 6.3 in the presence of desferal, ○ .



150
100
50
log klett units
1
2
3
hrs

Appendix 3: Utilization of lactoferrin-bound iron by gonococci: Requirements for a specific receptor.

(These results were presented at the fifth meeting of the International Society for Sexually Transmitted Diseases Research, Seattle, WA, 1983, and the abstract is published in the proceedings of this meeting by Mietzner, T.A. and S.A. Morse,
p. 100)

INTRODUCTION

Bacteria must overcome a host-imposed nutritional immunity to become a successful pathogen. Many bacteria have evolved complex systems which employ low-molecular weight siderophores to compete with host iron-binding proteins for available iron. These siderophores sequester iron making it available to the microorganism; in some cases by way of specific receptors (1). Convincing evidence that siderophores are produced by N. gonorrhoeae has not been found. However, it is known that all gonococci utilize transferrin-bound iron while ca. half of the strains can utilize lactoferrin-bound iron (2,3). Therefore, gonococci must have some mechanism by which they remove the iron from these proteins. All meningococcal strains examined remove iron from both

transferrin and lactoferrin (3,4). Utilization of transferrin-bound iron requires contact between the meningococcal cell surface and the transferrin molecule (5). An analogous system may exist for the gonococcus with respect to the utilization of iron from transferrin and lactoferrin. The inability of certain strains of gonococci to utilize lactoferrin-bound iron provides a means to examine the mechanism by which iron is obtained from lactoferrin. This study analyzes the binding of lactoferrin to strains which can and cannot utilize lactoferrin-bound iron.

METHODS AND RESULTS

Identification of strains which utilize lactoferrin-bound iron. The ability of gonococcal strains to utilize iron from lactoferrin was assessed by a modification of the method described by Mickelsen et al. (3). Chemostat defined medium (CDM) (6) was prepared as a five-fold concentrated solution and deferrated by treatment with Chelex-100 (Biorad) as described (3). This medium did not support the growth of gonococci without the addition of iron; it will be referred to as deferrated CDM. Addition of 1.4% agarose (Sigma, Type 1) to the deferrated CDM allowed the growth of gonococci on solid medium, presumably due to the contaminating iron present in the agarose. To render this iron unavailable for use by the gonococcus, 25 uM desferal mesylate (Ciba-Geigy) was added. This medium is referred to as deferrated CDM agar. No growth occurred on the medium in the presence of this concentration of desferal. Gonococcal strains to be tested were grown to 100 Klett units in GC broth containing 25 uM desferal. A 100 ul volume of the culture was added to 20 ml of molten deferrated CDM agar at 42°C, mixed well and allowed to harden in a petri dish. Wells (1 cm in diameter) were cut in the hardened agar and the iron source to be tested was added. The iron sources tested were 100% saturated lactoferrin purified from human breast milk, ferric-citrate which served as a positive control, and bovine serum albumin (Sigma) which served as a negative control.

Using this bioassay we confirmed a previous report (2) that strain F62 could not utilize the iron bound to lactoferrin (LF^-). We identified strain FA171 as being able to utilize the iron from lactoferrin (Lf^+). The results are shown in Figure 1.

Lactoferrin-binding assay. Binding of human lactoferrin by strains F62 and FA171 was examined. Lactoferrin (7 mg in 200 ul phosphate buffered saline, pH 7.4) was labeled with ^{125}I by reacting the solution with 250 uCi of ^{125}I (New England Nuclear) in the presence of three iodobeads (Pierce). After 3 min the reaction was stopped by removing the solution from the iodobeads and passing it over a Sephadex G25 column to separate the free ^{125}I from the labeled protein. The specific activity of the protein was determined to be 1.3×10^5 cpm/ug. Bacteria (2×10^{10} colony forming units) were removed from a mid-log phase culture growing in GC broth containing 25 uM desferral and harvested by centrifugation. The cells were resuspended in 5 ml of deferrated CDM supplemented with 0.5% (w/v) bovine serum albumin. ^{125}I-lactoferrin (1×10^5 cpm) was added and the suspension was agitated for 30 min at 37° C. The cells were centrifuged, washed, and the final pellet counted in a gamma counter (Beckman, Gamma 4000) to determine the amount of ^{125}I-lactoferrin bound. The results were expressed as a percentage of the total ^{125}I-lactoferrin added.

Under the conditions of this assay, strain FA171 (Lf^+) bound 36% of the total lactoferrin added while strain F62 (Lf^-) bound only 8%. These results are shown in Figure 2.

The specificity of binding was examined by adding a 50-fold excess (by weight) of unlabeled bovine serum albumin, human transferrin, or human lactoferrin (in addition to the labeled lactoferrin)to the binding assay

described previously. This addition was concominant with the addition of the labeled-lactoferrin. The results are shown in the histograph in Figure 3. The control represents the amount of lactoferrin bound (expressed as the percentage of the total ^{125}I-lactoferrin) when no additional unlabeled protein was added. Unlabeled human transferrin and bovine serum albumin did not inhibit the binding of ^{125}I-lactoferrin to strains FA171 or F62. However, a 50-fold excess of unlabeled lactoferrin reduced the binding of ^{125}I-lactoferrin by strain FA171 to the level observed with strain F62. In no case did the addition of unlabeled protein decrease the amount of ^{125}I-lactoferrin bound to strain F62. We concluded that the radioactivity associated with strain F62 represented background inherent in the assay.

Kinetic studies using radiolabeled lactoferrin. The results from these experiments suggested that there may be a specific receptor for lactoferrin on the surface of Lf^+ strains. Such a receptor would be expected to exhibit typical receptor/ligand kinetics such as time- and concentration-dependent binding. To control for any interstrain differences we constructed an isogenic set of strains differing only in their ability to utilize lactoferrin-bound iron. Lactoferrin utilization is a transformable phenotype (Dr. P.F. Sparling, personal communication). Transformation was accomplished by the procedure of Janik *et al*. (7). Crude DNA was isolated from the donor strain FA171 (Lf^+) by suspending bacteria in a solution of standard saline citrate containing 0.25% sodium dodecyl sulfate. This solution was heated for 60 min at 65°C. A 16 hr culture of F62 (T2) colonies were incubated with a small amount of the crude FA171 DNA extract for 4 hrs on regular GC agar. The bacteria were transferred to (and transformanta selected for growth on) deferrated CDM agar containing 30 ug lactoferrin/ml. The plates were

incubated for 24 hrs and several individual colonies were picked and streaked on GC agar. The Lf^+ phenotype of one transformant, 171/62L, was confirmed using the bioassay previously described (Fig. 4). The identity of this strain as a true transformant of the parental strain (F62) was confirmed by ELISA using a monoclonal antibody directed against protein I and by auxotyping. The properties of FA171, F62, and the transformant, 171/62L, are summarized in Table 1. This data indicated that 171/62L retained phenotypic characteristics of the recipient strain (F62) with the exception of the Lf^+ phenotype encoded for by the donor DNA. This transformant was used in the subsequent kinetic studies.

In order to analyze time- and concentration-dependent binding of lactoferrin, the previously described binding assay was modified in the following way. Gonococci (5 X 10^9 colony forming units) were grown in CDM. The cells were incubated for different times in the presence of ^{125}I-lactoferrin. The extent of binding was expressed as a percentage of the total ^{125}I-lactoferrin added. The results are shown in Figure 5. Increasing amounts of lactoferrin were bound by strain 171/62L over time; maximal binding occurred at 30 min. No time-dependent binding of ^{125}I-lactoferrin was observed for strain F62.

Using the modified binding assay described previously, different concentrations of ^{125}I-lactoferrin were added to the cell suspension and incubated with the cells for 30 min. The extent of binding was determined and expressed as picomoles bound versus picomoles of lactoferrin added. The results are shown in Figure 6. The transformant, 171/62L, exhibited saturable binding while F62 did not.

DISCUSSION

Simonson *et al*. described a high-affinity mechanism for the utilization of transferrin-bound iron by the meningococcus (5). This mechanism involved energy-independent binding of transferrin followed by an energy-dependent removal of iron from the transferrin molecule. Since meningococci also utilize iron from lactoferrin, it is possible that they possess a common mechanism by which they remove iron from both transferrin and lactoferrin. These results indicated that binding of lactoferrin is required for gonococci to utilize the iron from lactoferrin. Furthermore, the inability of Lf^- strains to utilize lactoferrin-bound iron is due to the absence of a lactoferrin receptor. This is consistant with what is observed in the meningococcus.

The binding of lactoferrin by way of a specific receptor suggested that a surface component(s) may be involved. Efforts to identify this receptor were unsuccessful. Scatchard plot analysis of the binding data indicated that the binding of lactoferrin to gonococci was not directed to only one receptor, suggesting that there might be an interfering component which affects lactoferrin binding. Since it is known that DNA binds human lactoferrin (8), one possible component might be extracellular DNA from lysing gonococcal cells. Preliminary studies have shown that the incorporation of DNase into the bioassay results in an increase in the zone size around the wells containing lactoferrin. The increased zone size was proportional to the

amount of DNase added to the assay. One interpretation is that the DNA from lysing cells is binding the lactoferrin and rendering it inaccessible to the gonococcal receptor. Further studies will be required to understand this heterologous binding.

REFERENCES

1. Neilands, J.B. 1981. Iron absorption and transport in microorganisms. Ann. Rev. Nutr. 1: 27-46.

2. Mickelsen, P.A., E. Blackman and P.F. Sparling. 1982. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from lacteferrin. Infect. Immun. 35: 915-920.

3. Mickelsen, P.A. and P.F. Sparling. (1981). Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from transferrin and iron compounds. Infect. Immun. 33: 555-564.

4. Archibald, F.S. and I.W. DeVoe. 1979. Removal of iron from human transferrin by Neisseria meningitidis. FEMS Microbiol. Lett. 6: 159-162.

5. Simonson, C., D. Brener and I.W. DeVoe. 1982. Expression of a high-affinity mechanism for acquisition of transferrin-iron by Neisseria meningitidis. Infect. Immun. 36: 107-113.

6. Morse, S.A.and L. Bartenstein. (1980). Purine metabolism in Neisseria gonorrhoeae: The requirements for hypoxanthine. Can. J. Microbiol. 26: 13-20.

7. Janik, A., E. Juni and G.A. Heym. 1976. Genetic transformation as a tool for detection of Neisseria gonorrhoeae. J. Clin. Microbiol. 4: 71-81.

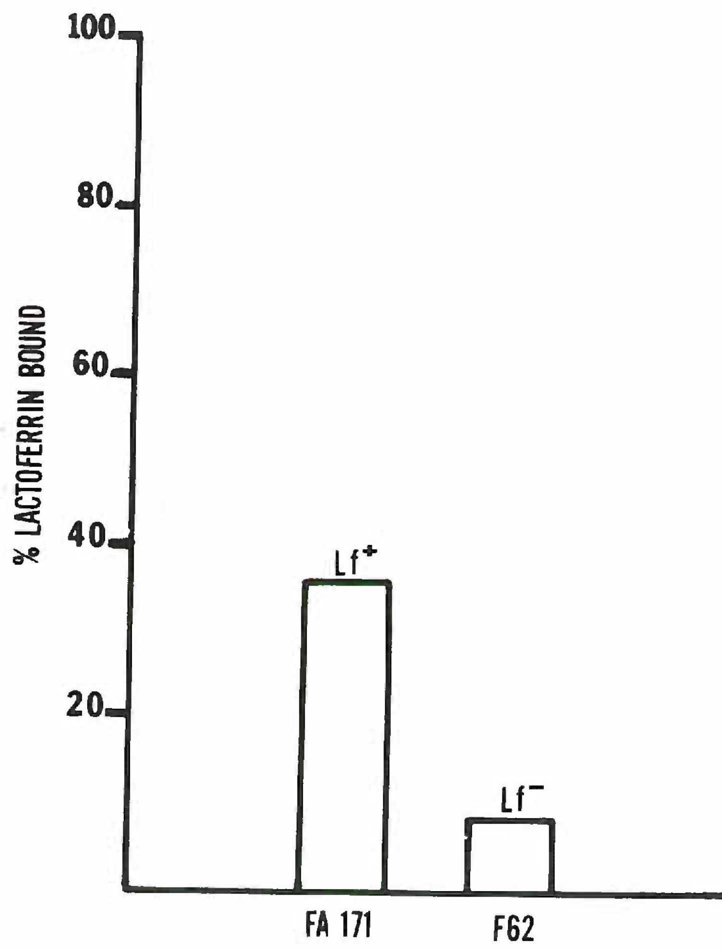
8. Bennet, R.M., J. Davis, S. Cambell, and S. Portnoff. (1983). Lactoferrin binds cell membrane DNA. J. Clin. Invest. 71: 611-618.

Figure 1. Bioassay used to differentiate between those gonococci which utilize the iron from lactoferrin (Lf^+) and those which cannot (Lf^-). Iron sources tested were 100% saturated lactoferrin (30 ug/well), ferric citrate (0.5 ug/well) and bovine serum albumin (30 ug/well). A large confluent ring of growth around the well after 24 hrs was taken as a positive result.

F 62
Fe-Cit
BSA
Lf

FA 171
Fe-Cit
BSA
Lf

Figure 2. Binding of ^{125}I-lactoferrin to strains FA171 and F62. Results are expressed as the amount of ^{125}I-lactoferrin bound as a percentage of the total ^{125}I-lactoferrin added.



100
80
60
40
20
% LACTOFERRIN BOUND
Lf+
Lf−
FA 171
F62

Figure 3. Binding of ^{125}I-lactoferrin to strains FA171 and F62 in the presence of unlabeled lactoferrin (50 ug), unlabeled transferrin (50 ug) and unlabeled bovine serum albumin (50 ug). The control is the extent of binding without the addition of any unlabeled protein. Results are expressed as a percentage of the total ^{125}I-lactoferrin added.

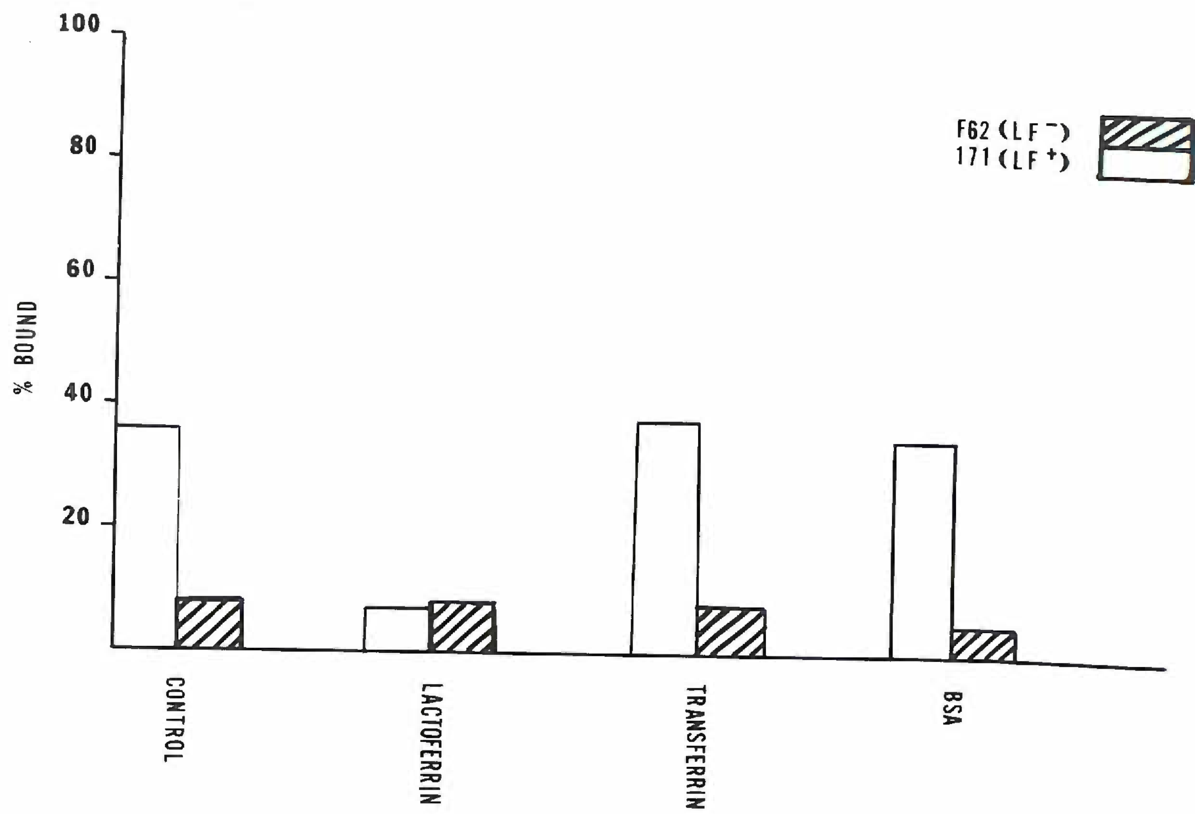
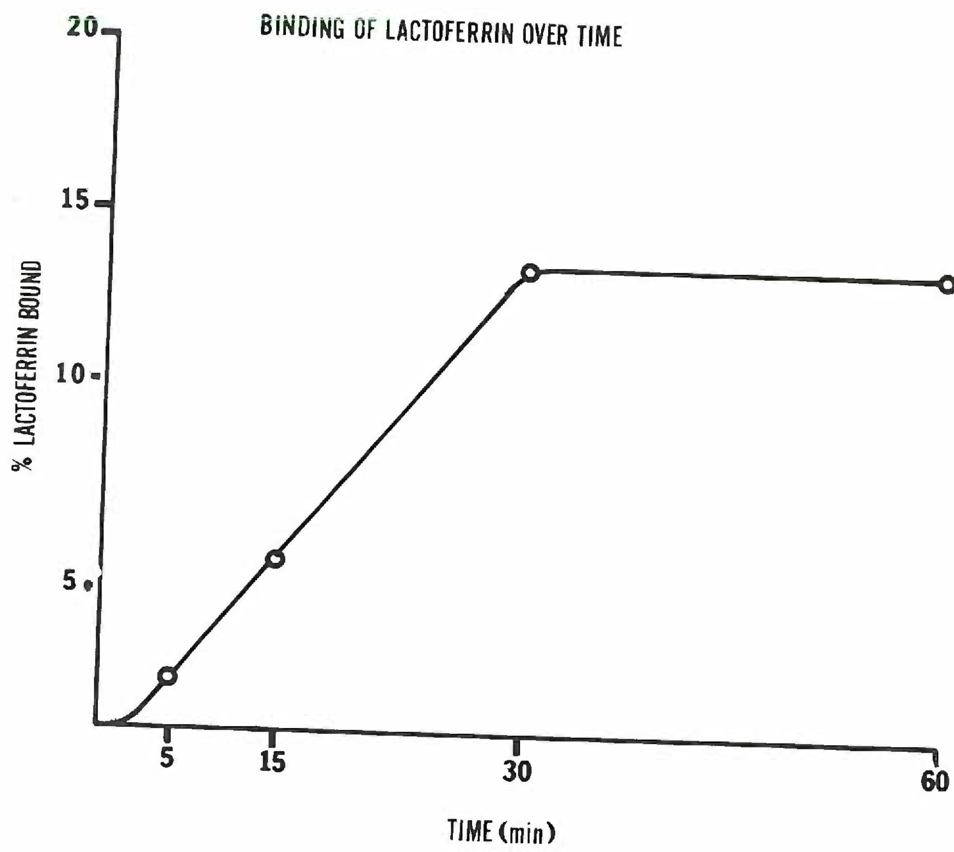

100
80
60
40
20
% BOUND
F62 (LF⁻)
171 (LF⁺)
CONTROL
LACTOFERRIN
TRANSFERRIN
BSA

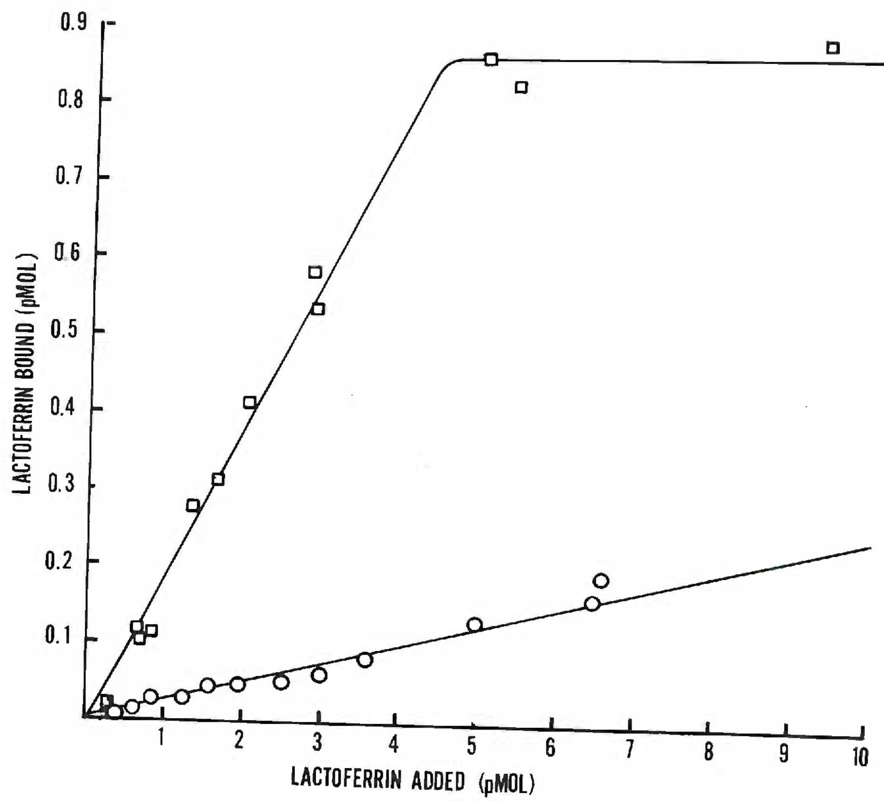
Figure 4. Bioassay used to differentiate those strains of gonococci which utilize iron from lactoferrin (Lf^+) and those which cannot (Lf^-)(see Figure 1 legend). This assay was used to confirm the ability of the transformant, 171/62L, to express the Lf^+ phenotype.

Figure 5. Time-dependent binding of ^{125}I-lactoferrin to strain 171/62L. Binding was carried out by incubating gonococci with ^{125}I-lactoferrin for 5, 15, 30 and 60 min. The extent of binding was determined and the binding at 0 min subtracted.



BINDING OF LACTOFERRIN OVER TIME
% LACTOFERRIN BOUND
20
15
10
5
5
15
30
60
TIME (min)

Figure 6. Concentration-dependent binding of ^{125}I-lactoferrin to strains 171/62L and F62. Varying concentrations of ^{125}I-lactoferrin was added to the binding assay described above. Results are given as pMoles bound versus pMoles added. 171/62L □ , F62 ○ .



0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
LACTOFERRIN BOUND (pMOL)
1
2
3
4
5
6
7
8
9
10
LACTOFERRIN ADDED (pMOL)

Table 1: Characteristics of strains FA171, F62 and 171/62L

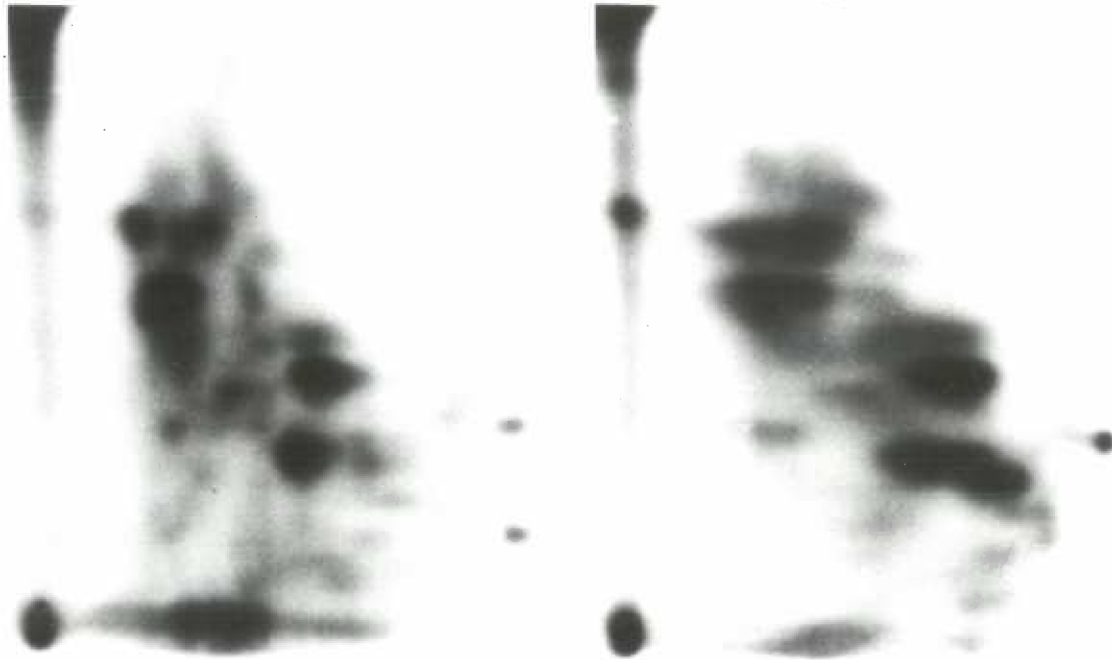
	Strain		
Characteristic	F62	FA171	171/62L
Auxotype	Pro^-	Proto	Pro^-
Serogroup*	WII	WI	WII
Lf Phenotype	Lf^-	Lf^+	Lf^+

*Serogroup determined in an ELISA using the monoclonal GC3C8 directed against protein I serogroup WII.

Appendix 4. Comparison of peptide maps prepared from the gonococcal 37K protein and an antigenically related iron-regulated 37,000 dalton protein of *N. meningitidis*.

The structure of the gonococcal 37K protein from strain F62 was compared to a meningococcal protein (from strain C-11) which had an identical molecular and which cross-reacted with antiserum specific for the gonococcal 37K protein (Manuscript 3). These two proteins were cut from an SDS-polyacrylamide gel and peptide maps were prepared (Fig. 1). Several peptides common to both of these proteins can be observed, suggesting that they share structural homology. The peptide map of the meningococcal protein showed several additional peptides. These peptides were probably the result of a constitutively expressed unrelated protein which comigrated with the meningococcal iron-regulated protein.

Fig. 1. Autoradiograph of tryptic peptide maps of ^{125}I-labeled 37K protein from *N. gonorrhoeae* strain F62 and a related protein from *N. meningitidis* strain C-11. Proteins were isolalated by cutting the band from a 10% SDS-polyacrylamide gel. Peptides were separated by paper electrophoresis (E) in the first dimension and by descending chromatography (DC) in the second dimension.


N. gonorrhoeae F62
N. meningitidis C-11
DC
E

Appendix 5. Pink-to-red color exhibited by a purified, concentrated preparation of the gonococcal MIRP.

See figure 1.

Fig. 1. Color photograph demonstrating the pink-to-red color of the purified gonococcal MIRP (manuscript 2). The protein concentration of this suspension was 2.4 mg per ml.

Appendix 6. Studies analyzing the iron-binding potential of the gonococcal MIRP.

Previous studies by Simonson *et al.* have analyzed the meningococcal iron-uptake system (see literature review, p. 97). Some notable observations made in these studies were that:

a) Iron complexed to citrate was efficiently assimilated *N. meningitidis*;

b) Iron acquisition occurred as a biphasic process involving energy-independent binding of the ferric-citrate complex, followed by an energy-dependent uptake of the iron moiety;

c) Cell membrane associated iron appeared to be bound to a protein complex with an apparent molecular weight of 36,500.

Since *N. gonorrhoeae* and *N. meningitidis* share extensive DNA homology (see literature review reference 164) and express iron-regulated 37,000 dalton proteins which are antigenically related, experiments were designed to examine the ability of the gonococcal MIRP to directly bind iron.

N. gonorrhoeae strain F62 was grown in defined medium supplemented with 50 uM desferal. At mid-logarithmic phase, 25 ml of cells were harvested by centrifugation, suspended in 10 ml of defined medium containing 2 mM KCN, and incubated at 37° C for 10 min. ^{55}Fe-citrate (10 ul, 7×10^{6} cpm) was then added and the cells incubated for an additional 15 min. The gonococci

were harvested by centrifugation and washed with 50 mM HEPES buffer (pH 7.4) containing 0.36 M sodium citrate, and 2mM KCN (HCC buffer). Cells were suspended in a 2 ml volume of HCC buffer containing 2% (v/v) Triton X100 and incubated at room temperature for 15 min to solubilize cellular proteins. The insoluble cellular debris was removed by centrifugation (48,000 x g for 20 min) and the supernatant diluted to a final concentration of 0.5% Triton X100 (v/v), 0.15 M NaCl in HCC buffer. Aliquots (0.5 ml) of this suspension were preabsorbed with formalin-fixed protein A bearing staphylococci (SPA). After removal of the SPA by centrifugation, rabbit anti-MIRP or preimmune rabbit serum diluted 1:10 (final dilution) was added to the absorbed, solubilized aliquots. Incubation was carried out at 37° C for 30 min, at which time SPA was added and incubation continued for an additional hour. The SPA was removed by centrifugation, washed, and the final amount of labeled iron associated with the pellet determined by standard scintilation techniques.

The results of these experiments suggested that the cells bound _ca._ 10 % of the total ^{55}Fe-citrate added to the system. Of these cell-associated counts, 64% were solubilized by the addition of 2% Triton X100. Immunoprecipitation of the detergent solubilized cells with MIRP-specific antibody recovered 1.3% of the detergen soluble counts. Immunoprecipitation with preimmune rabbit serum precipitated less than 0.3% of the detergent soluble counts.

The results of these experiments are difficult to interpret. There was four-fold greater amount of ^{55}Fe associated with the anti-MIRP immunoprecipitate when compared with the preimmune control. However, the small proportion of total label associated with the anti-MIRP

immunoprecipitate does not suggest that MIRP is the major component which binds the labeled iron. On the other hand, the efficiency of immunoprecipitation or the effect of KCN was never assessed in these experiments. While the data _may_ infer that MIRP binds iron, the numbers are less than convincing. Further studies which optimize this or a similar assay will be required before reasonable conclusions can be reached regarding the iron-binding potential of the gonococcal MIRP.