INFECTION OF HOMOSEXUALS BY PENICILLINASE AND NON-PENICILLINASE PRODUCING NEISSERIA GONORRHOEAE

A thesis submitted in accordance with the regulations for the degree of Master of Philosophy of the University of London

by

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ABSTRACT

Penicillinase producing Neisseria gonorrhoeae (PPNG) are only occasionally isolated from the homosexual male population although this has a high incidence of gonorrhoea. Colonisation of the rectum is essential for the propagation of the disease amongst homosexuals. PPNG may be unable to infect this site due to an inability to withstand the toxic hydrophobic environment or a deficiency in the means of attaching to the rectal mucosa. This study attempted to identify broad differences between PPNG and non-PPNG in terms of auxotype, plasmid content and surface hydrophobicity. More specifically, the incidence of the envelope phenotype mtr associated with resistance to hydrophobic molecules was determined, as was growth inhibition by the faecal fatty acid oleic acid and two members of faecal flora. The protein II composition of several strains was examined as this protein has been implicated as a possible tissue-specific attachment ligand. In general, the results did not indicate that PPNG were markedly different from non-PPNG, nor were strains causing rectal infection of a particular type. The mtr mutation was found to be most common in PPNG but their sensitivity to oleic acid was far greater than that of non-PPNG. This may indicate that mtr is not involved in resistance to oleic acid and PPNG lack a second mechanism present in non-PPNG, enabling them to survive in the rectum. Alternatively, it may be evidence that the determination of envelope phenotype was equivocal. The relationship between mtr and fatty acid resistance remains obscure. The paucity of PPNG infections in homosexuals might also be due to these strains lacking a pilus

type specific for rectal mucosa or to a propensity for the penicillinase plasmid to be lost in the rectum. Finally, the possibility cannot be excluded that, apart from isolated cases, PPNG have not been introduced into the homosexual population.

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CONTENTS

	PAGE
ABBREVIATIONS	9
LIST OF FIGURES	11
LIST OF TABLES	12
1. INTRODUCTION	14
1.1. THE EPIDEMIOLOGY OF GONORRHOEA	
1.1.1. The Incidence of Gonorrhoea	15
1.1.2. The Transmission of Gonorrhoea	16
1.1.3. Penicillinase Producing Neisseria gonorrhoeae	17
1.1.4. Typing Mechanisms	22
1.2. THE BIOLOGY OF N. GONORRHOEAE	
1.2.1. The Cell Wall of N. gonorrhoeae	26
1.2.2. Protein I	26
1.2.3. Protein II	29
1.2.4. Protein III	32
1.2.5. Pili	33
1.2.6. Lipopolysaccharide and Phospholipids	35
1.2.7. Cell Surface Hydrophobicity	36
1.3. ADHESION OF N. GONORRHOEAE	
1.3.1. Adhesion is a Prerequisite for Infection	40
1.3.2. N. gonorrhoeae Possesses High Avidity Attachment Mechanisms	40
1.3.3. Hydrophobic Interactions in Adhesion	45
1.3.4. Factors Opposing Adhesion	45
1.3.5. Local Antibody	47
1.4. THE HOST ENVIRONMENT	
1.4.1. Fatty Acids are Toxic to N. gonorrhoeae	49
1.4.2. Resistance to Hydrophobic Molecules in N. gonorrhoeae	50
1.4.3. Other Inhibitory Compounds	54
1.4.4. The Effect of Normal Flora	56
1.4.5. Leukocyte Association	59
1.5. AIMS OF THE PROJECT	61
2. MATERIALS AND METHODS	
2.1. BACTERIAL STRAINS	
2.1.1. Sources and Selection of Strains	62
2.1.2. Culture and Storage of Strains	64
2.1.3. Strain Characterisation	65

PAGE

2.2. EXPERIMENTAL PROCEDURES

2.2.1. Envelope Phenotype Determination 66
2.2.2. Oleic Acid Sensitivity Testing 66
2.2.3. Bacteroides spp. Inhibition Assay 68
2.2.4. Outer Membrane Protein Characterisation 68
2.2.5. SDS-PAGE of Protein Extracts 70
2.2.6. Transformation of Envelope Phenotype 71
2.2.7. Measurement of Surface Hydrophobicity 73

3. RESULTS

3.1. STATISTICAL TREATMENT OF DATA 75

3.2. STRAIN CHARACTERISATION

3.2.1. Auxotyping 76
3.2.2. Plasmid Type 78
3.2.3. Linkage of Plasmid Content and Auxotype 78
3.2.4. Penicillin MIC of Non-PPNG 82

3.3. ENVELOPE PHENOTYPE 89

3.3.1. mtr and Site 91
3.3.2. mtr and Sexuality 91
3.3.3. Linkage with Plasmid Type 92
3.3.4. mtr and Auxotype 92
3.3.5. Penicillin MIC and mtr Phenotype 95
3.3.6. Transformation of the mtr Phenotype 97

3.4. OLEIC ACID RESISTANCE

3.4.1. Oleic acid Resistance and Isolation Site 99
3.4.2. Oleic acid Resistance and Sexuality 101
3.4.3. Oleic acid Resistance and mtr Phenotype 102
3.4.4. Oleic acid Resistance and Plasmid Profile 106
3.4.5. Oleic acid Resistance and Auxotype 109

3.5. RESISTANCE TO BACTEROIDES

3.5.1. Resistance to B. fragilis 111
3.5.2. B. fragilis Resistance and Sexuality 113
3.5.3. B. fragilis Resistance and mtr Phenotype 113
3.5.4. Other Linkages 115
3.5.5. Resistance to B. vulgatus 115
3.5.6. Linkage with Sexuality 118
3.5.7. Linkage with Other Phenotypes 119

3.6. PROTEIN II COMPOSITIONS OF N. GONORRHOEAE

3.6.1. Detection of PII 122
3.6.2. Distribution of PII Species in N. gonorrhoeae 126

PAGE

3.7. SURFACE HYDROPHOBICITY OF SPECTINOMYCIN RESISTANT N. GONORRHOEAE 132

3.7.1. The Spectinomycin Resistant PPNG 134
3.7.2. The Spectinomycin Resistant Non-PPNG 134
3.7.3. Comparison of the two Groups 136
3.7.4. Protein Compositions of Spectinomycin Resistant N. gonorrhoeae 136

4. DISCUSSION

4.1. DISCUSSION OF DATA OBTAINED FROM THIS AND OTHER STUDIES

4.1.1. Strain Characterisation 141
4.1.2. Selection of the mtr mutation 143
4.1.3. The Need for Confirmation of the mtr Phenotype 148
4.1.4. The Occurrence of Oleic Acid Resistance in N. gonorrhoeae 150
4.1.5. Fatty Acid Resistance and its Relationship to mtr 152
4.1.6. The Role of Normal Flora in Host Immunity 155
4.1.7. Variation and Absence of any Site-Specific PII Molecules 158

4.2. DISCUSSION OF OTHER FACTORS AFFECTING THE ABILITY OF N. GONORRHOEAE TO INFECT THE RECTUM

4.2.1. Adhesion 161
4.2.2. Permeability of the Cell Wall of N. gonorrhoeae 164
4.2.3. PI as a Marker for Other Phenotypes 165

4.3. THE PENICILLINASE PLASMID AND GROWTH IN THE RECTUM

4.3.1. The Extra Coding Capacity of PPNG 168
4.3.2. Barriers to the Spread of the Penicillinase Plasmid 169
4.3.3. Loss of the Penicillinase Plasmid During Growth 170

4.4. EPIDEMIOLOGICAL REASONS FOR THE SCARCITY OF PPNG IN HOMOSEXUALS 171

4.5. CONCLUSIONS 173

REFERENCES 175

APPENDIX

1. MEDIA SUPPLEMENTS 184

1.1. Composition of IsoVitalex
1.2. Composition of Kellogg's Supplement

2. SDS-PAGE RECIPES 185

2.1. Sample Diluent for SDS-PAGE
2.2. Acrylamide Gel Compositions
2.3. SDS-Tris-Glycine Electrophoresis Buffer

PAGE

3. STATISTICAL DATA

3.1. Formula for Calculation of χ^2 186
3.2. List of Chi-squared Values Cited in Text 186
3.3. Formula for Calculation of Standard Error of Difference in Means 189
3.4. Formula for Calculation of Two Tailed Student's t-Test 189

4. DETERMINATION OF MODE MIC FOR PENCILLIN IN NON-PPNG 190

ABBREVIATIONS

AHU-	Arginine, hypoxanthine and uracil requiring
Arg-	Arginine requiring
ASMW	Apparent subunit molecular weight
BEC	Buccal epithelial cells
CoA	Co-agglutination
CTM	Colony typing medium
DGI	Disseminated gonococcal infection
DST	Direct sensitivity testing
DTT	Dithiothreitol
HEPES	N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid
IUD	Intra-uterine device
KDO	Ketodeoxyoctonate
LPS	Lipopolysaccharide
McAb	Monoclonal antibody
MIC	Minimum inhibitory concentration
MW	Molecular weight
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
Op	Opaque
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PL	Phospholipids
PPNG	Penicillinase producing N. gonorrhoeae
Pro-	Proline requiring
PI	Protein I

PII	Protein II
PIII	Protein III
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STD	Sexually transmitted diseases
TPP	Two phase partitioning
Tr	Transparent
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

LIST OF FIGURES

		PAGE
1.	The Cell Wall of N. gonorrhoeae	27
2.	Penicillin MICs of Non-PPNG	86
3.	Penicillin MICs of Non-PPNG Isolated from Homosexuals and Heterosexuals	87
4.	Penicillin MICs of Non-PPNG: A Comparison of Rectal and Non-Rectal Isolates	88
5.	Penicillin MICs of Non-PPNG According to Envelope Phenotype	96
6.	SDS-PAGE of Sarkosyl Extracts of N. gonorrhoeae	98
7.	SDS-PAGE of Lithium Acetate Extracts of PPNG Isolated from Urethras of Male Homosexuals	123
8.	SDS-PAGE of Lithium Acetate Extracts of Non-PPNG Isolated from Rectums of Male Homosexuals	124
9.	SDS-PAGE of Lithium Acetate Extracts of N. gonorrhoeae Isolated from Urethras of Male Heterosexuals	125

LIST OF TABLES

		PAGE
1.	Incidence of PPNG at St Mary's Hospital 1978 - 1984	20
2.	Inhibition of N. gonorrhoeae by Normal Flora	58
3.	Sources of Strains of N. gonorrhoeae	63
4.	Determination of Envelope Phenotype	67
5.	Results of Auxotyping	77
6.	Results of Plasmid Analysis: Non-PPNG	79
7.	Results of Plasmid Analysis: PPNG	80
8.	Linkage of Plasmid Content and Auxotype	81
9.	Penicillin Sensitivity Testing for Non-PPNG	83
10.	Results of Envelope Phenotype Testing	90
11.	Linkage of mtr Phenotype and Plasmid Content	93
12.	Linkage of mtr Phenotype and Auxotype	94
13.	Results of Oleic Acid Sensitivity Testing	100
14.	Linkage of Oleic Acid Resistance and mtr Phenotype	103
15.	Linkage of Oleic Acid Resistance and mtr Phenotype in PPNG	104
16.	Linkage of Oleic Acid Resistance and mtr Phenotype in Non-PPNG	105
17.	Oleic Acid Resistance in non-mtr Strains	107
18.	Linkage of Oleic Acid Resistance with Plasmid Content	108
19.	Linkage of Oleic Acid Resistance with Auxotype	110
20.	Results of B. fragilis Sensitivity Testing	112
21.	Linkage of Resistance to B. fragilis and mtr Phenotype	114
22.	Linkage of Resistance to B. fragilis with Other Phenotypes	116
23.	Results of B. vulgatus Sensitivity Testing	117
24.	Linkage of Resistance to B. vulgatus with Other Phenotypes in Non-PPNG	120
25.	Linkage of Resistance to B. vulgatus with Other Phenotypes in PPNG	121

PAGE

26. PII Analysis 127

27. The Distribution of PII 128

28. Summary of PII Analysis 129

29. PII Which Occur in More Than One Isolation Group 131

30. Partitioning of Spectinomycin Resistant N. gonorrhoeae in an Aqueous Two Phase System 133

31. Standard Error of the Difference in Means in the Partition of Spectinomycin Resistant N. gonorrhoeae 135

32. Calculation of the Student's t-test 137

33. Standard Error of the Difference in Means in the Partition of Spectinomycin Resistant N. gonorrhoeae 138

34. Major Proteins Present in Whole Cell Lysates of Spectinomycin Resistant N. gonorrhoeae 139

35. The Incidence of the mtr Phenotype in Non-PPNG 147

36. Inhibition by Bacteroides spp. of Non-PPNG 156

37. Serovars Represented in PPNG Isolated from Homosexuals 1982 - 1983 167

38. The Incidence of PPNG in Homosexuals at St Mary's Hospital 172

1. INTRODUCTION

The disease gonorrhoea is prevalent world-wide. It is primarily a disease of the mucosal surfaces of the genito-urinary tract which occurs only in man. In general the infection remains localised and causes little more than discomfort, responding well to antibiotic therapy. However, untreated infections may give rise to complications. In the male, urethral infection may extend to the prostate and epididymis ultimately causing urethral strictures. In women, cervical infection ascends up the genital tract to the fallopian tubes causing pelvic inflammatory disease, one sequela of which is sterility. In a small proportion of cases, the infection invades the bloodstream and disseminates, giving rise to skin lesions, arthritis and tenosynovitis. This condition is known as disseminated gonococcal infection (DGI).

The aetiological agent of gonorrhoea is Neisseria gonorrhoeae. This is a gram-negative bacterium which commonly occurs as kidney-shaped diplococci. It was first described by Neisser in 1879, from whom it derives its name.

1.1. THE EPIDEMIOLOGY OF GONORRHOEA

1.1.1. The Incidence of Gonorrhoea

Gonorrhoea is one of the commonest infectious diseases in the world, with an estimated two hundred and fifty million cases each year (Sandholzer, 1983). In the US there are close to a million cases reported each year (Britigan et al., 1985) and it has been estimated that only half the total number of cases actually get reported (Barnes and Holmes, 1984). In the UK there are over 50 000 reported cases each year (Communicable disease report of the Public Health Laboratory Service, 87/45), over 3 500 of which were seen at the sexually transmitted diseases (STD) clinic, St Mary's hospital, London at the time of this study (Praed Street Clinic annual report, 1984).

Attempts to control the spread of gonorrhoea are impeded by a number of factors. Widespread promiscuity is of primary importance. This not only adds to the total number of cases seen but, due to the often casual nature of such sexual contact, renders the partners of an infected individual impossible to trace. In this way, a reservoir of infection may be set up. The problem may be further compounded by promiscuous individuals if they have asymptomatic infection. The number of cases of gonorrhoea remaining asymptomatic may vary depending on the population being studied (Barnes and Holmes, 1984; Handsfield et al., 1974 and 1980; Janda et al., 1983; Ostrow, 1983;

Robertson et al., 1980.) It is clear that an accurate evaluation of the percentage of cases of gonorrhoea with no symptoms is extremely difficult, particularly as these infections may be largely undetected.

The disease affects both hetero- and homosexuals. The precise number of cases in homosexual men is not known. It can be estimated that between 4 and 10% of all men have homosexual contact in their lifetime and 60% of all cases of gonorrhoea occur in men (Sandholzer, 1983). However, the homosexual population may show a disproportionately high incidence of gonorrhoea; at St Mary's hospital about 40% of all male cases are in homosexuals (McManus et al., 1982). More recently, one report (Weller et al., 1984) has indicated that the rate of gonorrhoea in homosexuals at one STD clinic in London has dropped significantly. However, the rate is still very high when the fact that they represent a minority is taken into consideration. The US has also reported a decrease in the number of homosexuals with gonorrhoea (Judson, 1983).

1.1.2. The Transmission of Gonorrhoea

N. gonorrhoeae infects the mucus membranes of the genitourinary tract, rectum, nasopharynx and eye. Transmission of the organism to any of these sites depends, in general, on the sexual practices of the infected host. In heterosexuals the principal route of transmission is between the urethra and cervix. The rectum may also

be infected in women although this may be as a result of autoinoculation as well as anal sexual intercourse. In homosexuals the infection is transmitted between the urethra and rectum (Willcox, 1981). Thus, in homosexuals rectal infection is essential for propagation of gonorrhoea through the population, whereas this is not the case for heterosexuals. The pharynx may be involved in both populations, although at a low frequency. Infection at this site is presumably due to oral-genital contact. Infection of the conjunctiva is most likely to occur in neonatal babies, who contract the infection (opthalmia neonatorum) during passage through the birth canal of an infected mother.

The infectivity of N. gonorrhoeae is high, with only a small number of organisms (1×10^3) needed to initiate infection (Watt and Ward, 1980). Male to female transmission seems more efficient than vice versa, the incidence of infection for an infected woman after one exposure is 60 to 90%, whilst for a man it is 20 to 35% (Britigan et al., 1985).

1.1.3. Penicillinase Producing N. gonorrhoeae

Penicillin has been the drug most widely used to treat gonorrhoea since 1940. Chromosomally mediated resistance to this drug may result in minimum inhibitory concentrations of penicillin G of 2 - 8 µg/ml and has lead to treatment failures and outbreaks of infection (Barnes and Holmes, 1984). However, within the last decade, strains

of N. gonorrhoeae which carry plasmids encoding a penicillinase enzyme have emerged. Plasmid mediated drug resistance has serious implications because the plasmid may be readily transmitted between strains and so has the potential to spread rapidly through the species (Broda, 1979). Also, further drug resistance genes may be transposed onto these plasmids; in one veterinary example, strains of Salmonella typhimurium acquired resistance genes to six antibiotics in the space of two years (Broda, 1979).

Two closely related penicillinase plasmids occur in N. gonorrhoeae. One plasmid, of 3.2 Md appears to be a 1.2 Md deletion of a larger 4.4 Md plasmid. Both plasmids contain 30 - 40% of the transposable genetic element transposon TN2 which carries a gene encoding a penicillinase enzyme typical of many enteric bacteria (Roberts et al., 1977). Presence of either plasmid in a cell results in an inoculum dependent resistance to penicillin or ampicillin of 10 - 100 µg/ml in vitro (Cannon and Sparling, 1984). The observation that the two plasmids show great homology to the ampicillin resistance plasmids of Haemophilus spp. influenzae and parainfluenzae indicated that they had not arisen through TN2 insertion into an indigenous gonococcal plasmid (Roberts et al., 1977). It is possible that the resistance plasmids were transferred directly between the Haemophilus spp. and N. gonorrhoeae; however it might also be the case that both genera were independent recipients of plasmids from enteric bacteria (Roberts et al., 1977).

The first penicillinase producing N. gonorrhoeae (PPNG) strains were

imported from two foci, South East Asia and West Africa, to both the US and the UK (Ashford et al., 1976; Percival et al., 1976; Phillips, 1976). Strains from the two locations were shown by Perine et al. (1977) to be genotypically dissimilar, Asian strains carried the 4.4 Md plasmid whilst African strains carried the 3.2 Md plasmid. As PPNG became more widespread genetic mixing occurred and the differentiation of strains as African/Asian became artificial as the bacteria became established endemically in many countries (Johnston et al., 1981; van Embden et al., 1981).

In the UK the number of cases reported increased exponentially from the first isolation in 1976. Within the next four years, a significant change in transmission was seen. Up until this time, cases imported from endemic areas were the major cause of the increase in incidence. By 1981 it was obvious that the spread of PPNG had become truly endemic (McCutcheon et al., 1982). In 1983, the isolation rate of PPNG reached its peak at 5% of all isolates. Since then the number isolated each year has decreased. However, the incidence is still very high in developing countries (Chitawarakorn et al., 1985; Obaseiki-Ebor et al., 1985) and in the UK it is still great enough for the STD clinic at St Mary's to have abandoned first-line penicillin therapy in favour of the antibiotic spectinomycin in an attempt to control the spread of PPNG strains. Table 1 shows the number of PPNG isolated from 1978 - 1984 at St Mary's and the percentage of the total number of cases of gonorrhoea that this represents. Resistance to spectinomycin has arisen sporadically but has not become widespread (Ashford et al., 1981; Easmon et al.,

TABLE 1

Incidence of PPNG at St Mary's Hospital
1978 - 1984

	No. of PPNG	Percentage of Total Isolates
1978	3	0.1
1979	12	0.3
1980	25	0.7
1981	57	1.9
1982	212	6.2
1983	277	8.6
1984	192	6.5

1982).

Despite the high numbers of homosexuals infected with gonorrhoea, the incidence of PPNG in this population remains low (Ansink-Schipper et al., 1984; Handsfield et al., 1982a; McManus et al., 1982). At St Mary's, in contrast to the 40% of male infections caused by non-PPNG occurring in homosexuals, only about 5% of male infections caused by PPNG are found in homosexuals. This figure represents a large discrepancy which may be of clinical significance. It may be due to the fact that PPNG originated in the heterosexual population and lack of mixing of the two populations sexually has led to the exclusion of PPNG from the homosexual population. However, PPNG infections have occurred in homosexuals and the high promiscuity of these individuals might have been expected to spread the PPNG strains as seen in the heterosexual population. Also, a sizeable proportion (20%) of homosexual men have been reported as having sexual contact with women (Morse et al., 1982). It appears, therefore, that there are other factors responsible for the paucity of PPNG infections in homosexuals. These may relate to the inability of these strains to set up infection in the rectum, which as mentioned previously is necessary for homosexual but not heterosexual transmission. Whether any basic biological differences exist between PPNG and non-PPNG has not been investigated, but if these exist then they may be responsible for this epidemiological phenomenon.

1.1.4. Typing Mechanisms

The ability to divide isolates of any bacterium into several types on the basis of biological characteristics is a useful tool for the epidemiologist. By identifying individual strains through a number of methods the spread of a clone of bacteria through a population can be monitored. Epidemics can be assessed as to whether they are caused by one strain or several. Studies over a long period of time can monitor temporal changes in the bacterial population and migration of strains between different populations. Hypothetically, it is possible to examine whether strains with defined biological characteristics are limited to a particular biological niche or associated with a clinical syndrome although this correlation may be purely coincidental with no causal link. There are several methods by which N. gonorrhoeae may be typed.

As with other bacterial genera, one of the most effective methods of typing is by use of serological methods. This types strains on the basis of their antigenicity, by agglutination reaction with a panel of antibodies raised against the intact bacterium or its components. No reliable method of serologically typing N. gonorrhoeae existed until 1976 when Johnston et al. first isolated components of the bacterial outer membrane (OM) responsible for serospecificity. Since then several typing schemes have been developed, all based on the antigenicity of the principal outer membrane protein (OMP) of N. gonorrhoeae. The details of how these schemes were developed will be discussed later in section 1.2.2.. The most recently developed and

probably the most effective method is that of Tam et al. (1982). This enables strains to be divided unambiguously into a large number of groups. It has been used widely in epidemiological studies throughout Europe and the US (Ansink-Schipper et al., 1987; Coghill and Young, 1987; Dillon et al., 1987; Kohl et al., 1986).

A second typing system is based on the nutritional requirements of N. gonorrhoeae. Its development succeeded the manufacture of a complete defined gonococcal growth medium (Neisseria defined agar or NEDA) by Catlin in 1973. The medium was designed to give maximal growth of all strains. However, it contained compounds which were not essential for growth of some strains, whilst others had an absolute requirement for these. Due to the precise definition of the NEDA medium, Catlin was able to identify these requirements unambiguously. This method of typing strains of N. gonorrhoeae according to their nutritional requirements is known as auxotyping. This method has the potential to divide strains into many groups, but unfortunately the majority of strains fall into one of two categories; they are either non-requiring (wild type) or proline requiring (pro-). However, auxotyping has been used in epidemiological testing. Notably, strains which cause asymptomatic infection and DGI commonly have requirements for arginine, hypoxanthine and uracil (AHU-) (Crawford et al., 1977). The first PPNG isolates also showed characteristic auxotypes; strains carrying the 4.4 Md plasmid (Asian strains) were pro-, whilst strains carrying the 3.2 Md plasmid (African strains) were arginine requiring (arg-) (Perine et al., 1977).

A further method by which bacteria may be typed is by their susceptibility to a panel of antibiotics, giving a pattern of resistance known as the antibiogram. Each strain will be prevented from growing at a critical concentration (the minimum inhibitory concentration or MIC) for each antibiotic it is presented with. The greater the number of antibiotics tested, the greater the differentiation of the strains. However, this method is time-consuming, usually requiring forty eight hours incubation for reliable results, and labour intensive. It is not particularly suitable for routine screening of large numbers of samples, but it has been used occasionally in epidemiological studies (Barnes and Holmes, 1984).

In spite of there only being four naturally occurring plasmids in N. gonorrhoeae, characterising the plasmid content is almost essential in the epidemiological study of PPNG. The most common is the small 2.6 Md cryptic plasmid which is found in 96% of all isolates and, as such, is of little use to the epidemiologist. The larger 24.5 Md plasmid is of more interest as it is known to mediate conjugation. The presence of this plasmid in PPNG is important as both penicillinase plasmids can be lost spontaneously in the absence of penicillin. A population which contains a sizeable proportion of strains with the conjugative plasmid is therefore at an advantage, as individual bacteria which have lost the penicillinase plasmid can therefore become reinfected with it. Studies of PPNG have shown how these strains have altered since their first isolation. The first apparent difference was the finding that only Asian strains possessed

the conjugative plasmid (Perine et al., 1977). The emergence of the conjugative plasmid in the so-called African strains indicated that genetic mixing had occurred (van Embden et al., 1981).

1.2. THE BIOLOGY OF N. GONORRHOEAE

1.2.1. The Cell Wall of N. gonorrhoeae

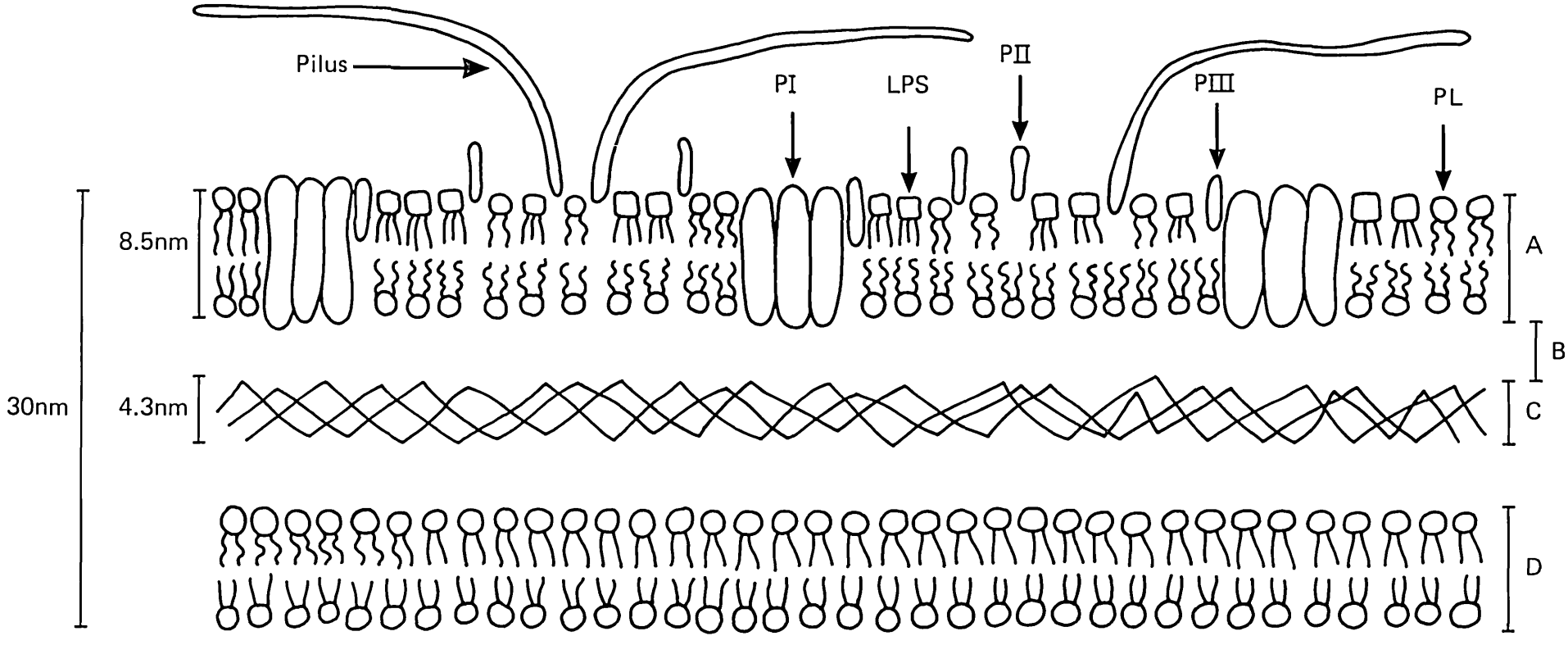
The cell wall of N. gonorrhoeae is similar in its arrangement to that of other gram-negative bacteria (Johnston and Gotschlich, 1974; Wolf-Watz et al., 1975). The OM is composed of phospholipids (PL), lipopolysaccharide (LPS) and a limited number of proteins. The PL and LPS form a bilayer, similar to that of the cytoplasmic plasma membrane with LPS located solely on the outer leaflet. Proteins are inserted into this bilayer and may span the width of it. The structure and location of the OM constituents are discussed in detail in sections 1.2.2. to 1.2.6.. A diagrammatic representation of the gonococcal cell wall is given in figure 1. The measurements are taken from the electron microscope observations of Novotny et al. (1975).

1.2.2. Protein I

This is the major protein of the OM of N. gonorrhoeae which has been designated, variously, principal OMP, major OMP or protein I (PI). Johnston and Gotschlich (1974) first described this protein which they found to account for at least 60% of the total OMP. A subsequent study (Johnston et al., 1976) of several strains established that the apparent subunit molecular weight (ASMW) of PI on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) varied between strains, but not between different colony types of the same strain,

FIGURE 1

THE CELL WALL OF N. GONORRHOEAE



KEY: A = Outer membrane
B = Periplasmic space
C = Peptidoglycan
D = Plasma membrane

from 32 to 39 kd.

The amount of PI present and its location in the OM led Johnston and Gotschlich (1974) to suggest that it might be serologically important. Subsequently, Johnston et al. (1976) immunised rabbits with isolated OMs or crudely purified OM antigens and divided over two hundred strains into sixteen distinct groups by an agar immunodiffusion test. Sandstrom and Danielsson (1980) developed a co-agglutination (CoA) method for serological classification of N. gonorrhoeae. Using selectively absorbed hyperimmune rabbit antigonococcal serum, the sixteen serotypes defined by Johnston et al. (1976) could be referred to three groups WI, WII and WIII. This CoA reaction was found to recognise PI determinants (Sandstrom et al., 1982). The three W antigen groups were found to be analogous to the three groups (A, B and C) obtained by the immunofluorescence technique of Wang et al. (1977) which did not characterise the antigens detected. In 1982, another typing scheme was introduced (Tam et al., 1982). This utilised sixteen monoclonal antibodies (McAbs) raised to purified PI. On the basis of reactions with these sixteen McAbs, strains of N. gonorrhoeae could be divided into two independent serological groups, each recognising a different PI molecule, termed PIA and PIB. Within these two groups many subsets were recognised. PIA and PIB correspond to WI and WII/WIII respectively of the CoA system (Sandstrom et al., 1982).

The structural basis for the division into two groups was elucidated by Barrera and Swanson (1984). When intact cells belonging to the PIB

serogroup were treated with proteinase K, the PI molecules were cleaved at two sites. Two large fragments remained membrane bound whilst one small fragment was lost. PI from strains in group PIA were cleaved at only one site and the portion remaining in the membrane was only slightly smaller than the whole PI molecule. This suggested that the PIA and PIB molecules show a different orientation in the OM, PIB forming a hairpin loop with both ends embedded in the membrane whilst PIA exhibits a loop within the membrane with a small part of one end of the loop exposed at the surface.

PI exists in the membrane of N. gonorrhoeae as a trimer of identical peptides (Blake, 1985). It has also been shown to function as a porin (a transmembrane channel allowing the passage of hydrophilic, polar molecules) by Young et al. (1983). Thus, it shows similarities to the trimeric OMPs of Escherichia coli and S. typhimurium which also function as porins (Nikaido and Vaara, 1985).

1.2.3. Protein II

Protein II comprises a family of related proteins which exhibit heat modifiability of molecular weight (MW) when run on SDS-PAGE. Due to the properties they confer on N. gonorrhoeae they have previously been referred to as leukocyte association proteins or opacity associated proteins.

Minor proteins of MW 25 - 30 kd were noted by Johnston et al. (1976) during their study of the OM of N. gonorrhoeae. Heckels demonstrated that these proteins could be fluorescence or ^{125}-I labelled on intact organisms (Heckels, 1977; Heckels, 1978). This indicated that, like PI, at least a portion of the molecule was surface exposed. He also noted the heat modifiability of these proteins, from fast migrating forms when solubilised at 37°C to slow migrating forms when solubilised at 100°C. A small number of isolates did not possess any heat modifiable proteins. Heckels designated the heat modifiable proteins as protein II (PII).

The PII composition of N. gonorrhoeae is responsible for the differences in opacity seen when colonies grown on solid media are viewed under a stereomicroscope. Variations in opacity range from completely opaque (op) through to completely transparent (tr) (Swanson, 1978a). A comprehensive study of the effect of PII on colony opacity was carried out by Swanson (1982). He found that generally the degree of opacity was correlated with the number and types of PII present. Different PIIs had different effects on opacity; one was not associated with any discernible change in opacity, either singly or in combination with other PIIs. The opacity of a colony is not a stable characteristic; during growth in vitro, variants with different opacity appear, on average, at a rate of 2×10^{-3} per colony forming unit per generation (Mayer, 1982). Changes in opacity were found to concur with the addition or loss of PII which occurred as a single step phenomenon, independent of other PIIs on the same organism (Swanson, 1982).

The effect of PII on colonial opacity is due to its role in cell:cell interactions. Colonies with PII show marked clumping and zones of intercellular adhesion, with the possession of more than one PII type increasing this adhesion (Swanson, 1978b). Aggregation of bacteria affects the light transmission and refraction of the colony, resulting in the observed variations in colony opacity. PII molecules purified from op colonies (PIIop) differ from PII molecules from tr colonies (PIInop) which do not mediate this intercellular binding (Blake, 1985). The observed adhesion in op colonies is not caused by PII:PII interactions as PII does not form aggregates in vitro (Blake and Gotschlich, 1983). Experiments conducted by Blake (1985) in vitro indicate that PIIop binds by a receptor located in its distal third to purified gonococcal LPS, probably to a carbohydrate moiety. Purified PIInop will not bind in this manner.

Opaque, highly aggregated colonies are sensitive to disaggregation and killing by trypsin (Swanson, 1978a). PII is highly susceptible to trypsin cleavage; in intact membranes the protein is cleaved into fragments, one of which remains in the OM (Blake et al., 1981). The surface exposed portion of the molecule shows great antigenic diversity, antibodies raised to whole cells show very little cross-reactivity with other PIIs, even those located on the same cell (Diaz and Heckels, 1984). The membrane portion of the molecule contains epitopes common to all PIIs, thus despite great antigenic heterogeneity, regions of homology exist for all PII (Swanson and Barrera, 1983).

The genetic means of generating a diversity of PII species has been studied. A single cell possesses a repertoire of genes that encode PII (op genes) which enable it to express many serologically different proteins (Stern et al., 1984). Minor sequence alterations appear to be responsible for the variation of PII (Meyer et al., 1985). These alterations are not due to random mutation, as the frequency with which PII variation occurs is too great, but are more likely to be due to recombination or gene conversion events (Stern et al., 1984).

1.2.4. Protein III

Protein III was discovered in 1980 by McDade and Johnston. They demonstrated the presence of this reduction modifiable protein, which migrates on SDS-PAGE with an ASMW of 31 kd in the presence of reducing agents and with an ASMW of 30 kd without reducing agents. They showed that this protein was closely linked to PI in the outer membrane of N. gonorrhoeae, the resulting heteropolymer being clearly visible on 5 - 25% gradient SDS-PAGE where it migrated with a MW of 100 kd. Additional evidence for this arrangement is given by co-precipitation of PI and PIII by antisera to purified PI, as shown by Swanson (1981). Cross-linking of the two proteins has been achieved by chemical means (McDade and Johnston, 1980; Newhall et al., 1980).

PIII appears to be virtually identical for all strains of N. gonorrhoeae (Swanson et al., 1982; Lytton and Blake, 1986). Protease

cleavage studies suggest that a substantial amount of the protein is buried in the OM. However, a portion of the molecule has been shown to be surface exposed, as indicated by the findings of Swanson et al. (1982) that the PIII specific McAb will immunoprecipitate intact organisms.

1.2.5. Pili

Colonies of N. gonorrhoeae grown on solid media do not only exhibit opacity differences, they also occur as morphologically different types. Kellogg et al. (1963) described four colony types which were assigned numbers from 1 - 4 (T1 - 4). On initial isolation from the patient, T1 colonies were the predominant form, with about 10% being T2 and T3. However, repeated subculture gave rise mainly to T4 organisms. Kellogg et al. (1968) established that only bacteria from colony types 1 and 2 were virulent for human volunteers. The basis of the different morphologies and the concurrent difference in virulence was not ascertained until 1971, when two papers appeared simultaneously reporting the presence of pili on N. gonorrhoeae (Jephcott et al., 1971; Swanson et al., 1971). Pili were only observed on Kellogg's colony types 1 and 2. As these types alone were known to be virulent, this suggested that pili played an important part in the pathogenesis of the disease. Swanson et al. (1971) proposed that pili aided the attachment of the bacterium to the host in vivo and this was subsequently demonstrated in vitro by Swanson (1973) and others (for example, Heckels et al., 1976; James-Holmquest et al., 1974; Punsalang and Sawyer, 1973; Trust et al., 1980). The

role of pili in attachment will be discussed in a later section. Pili, therefore, were the first structures on N. gonorrhoeae to be implicated as having a role in the pathogenesis of the disease and, for this reason, they have attracted a great deal of research.

A method of purifying pili with resultant high yields was developed by Brinton et al. (1978) enabling studies of pilus structure to be carried out. On SDS-PAGE the pilus protein, pilin, has a subunit MW varying from 17 - 21 kd, which represents about 160 amino acid residues. Each pilus is made up of identical pilin subunits repeated up to ten thousand times (Schoolnik and Rothbard, 1985).

Antigenically, pili possess both strain specific and common epitopes (Buchanan, 1975). The location of these epitopes has been studied mainly by the use of fragments produced by treatment of purified pili with the chemical cyanogen bromide. This chemical cleaves proteins specifically at methionine residues (Gross and Witkop, 1963). This gives rise to three pilin fragments, CnBr 1 - 3, all of unequal size. The exact location of each cleavage site varies with pilus type (Schoolnik et al., 1982). CnBr1 contains the first seven amino acid residues of the N-terminal and is invariant for all strains, suggesting a functionally important role (Hermodson et al., 1978; Schoolnik et al., 1984). CnBr2 is the region of antigenic cross-reactivity as shown by the ability of anti-CnBr2 antisera to bind both homologous and heterologous CnBr2 fragments (Schoolnik et al., 1982; Rothbard et al., 1984). The region of antigenic diversity is located on CnBr3 (Rothbard et al., 1984 and 1985; Schoolnik et al.,

1982 and 1984). Antibody to this fragment is mainly specific and non cross-reacting, although common determinants have been demonstrated in this region (Rothbard et al., 1985; Virji and Heckels, 1985).

The source of pilus antigenic variation can be traced to the regions of the two pilin genes, pil E1 and E2 which encode the hypervariable carboxy terminal of the protein. Here, amino acid substitutions, in-frame insertions and deletions occur (Hagblom et al., 1985). In addition, there are many silent pilin sequences at other sites on the chromosome (Segal et al., 1985). These silent loci contain truncated pilin sequences which lack the portion of the gene encoding the N-terminal. Their role is probably to act as donors of variant sequences to the expression loci by recombination (Haas and Meyer, 1986). Rearrangements are also responsible for switching pilus expression on and off (Meyer et al., 1982). In most switchings a deletion occurs at a pil gene due to a recombination event. However, in some cases the regulatory event of the switching remains undefined (Segal et al., 1985). During switching, genetic information is not lost, as switching from the non-piliated to the piliated state may result in the expression of a new variant pilin or the re-expression of a former variant (Hagblom et al., 1985).

1.2.6. Lipopolysaccharide and Phospholipid

One of the major functions of the OM in gram-negative bacteria is to act as a strong permeability barrier to a variety of molecules. In

the OMs of gram-negative enteric bacteria this is achieved by the segregation of LPS and PL so that LPS is found exclusively in the outer leaflet and PL are located on the inner leaflet. This gives rise to an asymmetric bilayer with a strongly hydrophilic outer layer. However, in N. gonorrhoeae and also in certain mutants of enteric bacteria, this asymmetry is not complete. Both N. gonorrhoeae and rough mutants of enteric bacteria produce LPS which lacks the long hydrophilic O-polysaccharide side chains (rough or R-type LPS) and the membrane contains regions of PL bilayer (Lysko and Morse, 1981; Nikaido and Vaara, 1985). It is the opinion of Nikaido and Vaara that gram-negative bacteria which do not normally inhabit the gastrointestinal tract do not require a highly impermeable membrane. In species such as N. gonorrhoeae, therefore, regions of PL bilayer are a normal feature of the OM.

1.2.7. Cell Surface Hydrophobicity

The outer membrane of N. gonorrhoeae is believed to contain hydrophobic pockets as witnessed by its ability to undergo hydrophobic interactions in experimental systems (Trust et al., 1980). Intact bacteria were found bound to alkyl substituted sepharose or agarose beads in an assay analagous to that used to study binding to human epithelial cells (Lambden et al., 1979; Trust et al., 1980). The degree of association correlated with the length of the hydrocarbon chain as would be expected for a hydrophobic molecule. However, neither set of experiments utilised a known hydrophilic strain, such as wild type E. coli, as a control to

demonstrate that hydrophilic bacteria would not also interact with the gel matrix in this way.

Hydrophobic interactive chromatography has also been used to study the hydrophobic interactions of N. gonorrhoeae by Magnusson et al. (1979). Labelled bacteria were applied to octyl-sepharose and eluted with increasing concentrations of Triton X-100 which destabilises hydrophobic interactions. However, Trust et al. (1980) reported that this method was not suitable for application to N. gonorrhoeae as the fragile membrane was damaged by the eluant and the eluate from the column consisted of solubilised components of the bacterial cell, and not intact bacteria. Magnusson et al. (1979) also used the technique of measuring the partition of intact bacteria in an aqueous two phase system composed of polyethylene glycol (PEG), which destabilises hydrophobic interactions, and dextran, the covalently linked hydrophobic groups of which interact with like groups on the bacterial cell surface. Wild type enteric organisms with smooth or S-type LPS have been shown to partition in the upper, PEG rich phase indicating that the surface is hydrophilic, whilst the rough mutants of these genera are more hydrophobic and accumulate in the lower dextran rich phase (Magnusson et al., 1977). In common with other bacteria with R-type LPS, N. gonorrhoeae partitions mainly in the lower phase (Magnusson et al., 1979).

The hydrophobicity of a bacterial cell is an indication of the physico-chemical properties of the cell surface. Changes in hydrophobicity will therefore reflect changes in the composition of

the cell surface. The components of the gonococcal OM responsible for hydrophobic interaction have not been established conclusively. There are probably several molecules which together contribute to the overall hydrophobicity of the cell surface. The fact that N. gonorrhoeae LPS is R-type is probably one of the most important determinants. However, the LPS of N. gonorrhoeae is not as rough as some of the rough mutants of enteric bacteria as it possesses a short core oligosaccharide which masks the lipid A portion of the molecule. As the composition of LPS in N. gonorrhoeae does not appear to vary greatly between strains (Wiseman and Caird, 1977), alterations in OMP composition and content may be important in altering surface hydrophobicity.

Several studies have examined the contribution of pili. Purified ^{125}I-labelled pili, which have a high content of non-polar and hydrophobic amino acids bind completely to hydrophobic sepharose. As the area covered by pili and that of the outer membrane of N. gonorrhoeae are apparently of comparable magnitude (Watt and Ward, 1980), pili might be expected to dominate surface hydrophobicity. However, on intact bacteria the advantage conferred by pili on binding to alkyl-sepharose is only about 20%. It may be that through purifying pilin, hydrophobic regions of the molecule normally buried in the membrane or involved in subunit-subunit interactions are exposed (Schoolnik et al., 1984).

The pH at which the bacteria are grown may influence the hydrophobicity of pili. Magnusson et al. (1979) reported that pili

only exerted a hydrophobic effect after growth at pH 6.0 and not when the bacteria had been grown at pH 7.0. No indication of the pH at which the bacteria were grown has been given by Watt and Ward (1980), it is possible that this may have affected their results.

The contribution of PII to the overall hydrophobicity may be negligible. Magnusson et al. (1979) examined differences present in all four colony types, two of which possessed PII (T2 and T3) and two which did not (T1 and T4). They could find no difference in the behaviour of types with and without PII. In contrast to this, Lambden et al. (1979) reported that one PII species actually decreased the tendency to undergo hydrophobic interaction of strain P9. It is of course possible that individual PII species are capable of affecting surface hydrophobicity but the narrowness of Magnusson's investigation precluded this from being discovered. The affects of other membrane proteins have not been assessed. Any alteration in hydrophobicity by OMPs may be either a direct cause or it may be indirect in that the change in OMP composition may affect the amount of LPS/PL in the OM.

1.3. ADHESION OF N. GONORRHOEAE

1.3.1. Adhesion is a Prerequisite for Infection

The events of infection by N. gonorrhoeae have been deduced mainly from electron microscopic studies of urethral mucosa from men with acute gonorrhoea (Ward and Watt, 1972) and in vitro study of the human fallopian tube in organ culture (McGee et al., 1982). Three to eight hours after challenge of the tissue, bacteria are seen to be adherent to the epithelial surface. The initial contact is with microvillous projections from the host cell. The number of bacteria adhering increases up until twenty fours hours after infection, thereafter the number declines. After about twenty hours of infection the microvilli with adherent bacteria become resorbed by the host cell and the bacteria become engulfed by the cell membrane in a manner analogous to phagocytosis. Bacteria within phagocytic vacuoles are transported to the subepithelial connective tissues where they are released and continue to multiply. Without prior adhesion, this engulfment and subsequent setting up of the infection cannot take place. Thus adhesion is a critical step in the pathogenesis of N. gonorrhoeae.

1.3.2. N. gonorrhoeae Possesses High Avidity Attachment Mechanisms

The host specificity and the non-adherence of commensal Neisseriaceae suggests that N. gonorrhoeae possesses highly specific attachment

mechanisms for human epithelial cells (Watt and Ward, 1980). Pili have been implicated as promoters of adhesion by many studies (see section 1.2.5.). The advantage that piliation confers is evident from work showing that piliated organisms adhere in 2 - 10 fold greater numbers than non-piliated (Draper et al., 1980) and at a faster rate (Trust et al., 1980).

The attachment of pili is probably receptor mediated. The system shows high avidity binding for cells of clinical significance in gonorrhoea, whereas for cells not involved in infection or from other mammals and also for surfaces such as polystyrene and glass, non-specific, low-level, weak binding is seen (Watt and Ward, 1980). Binding is also pH-dependent, with piliated and non-piliated bacteria showing different optima; this also points to a specific interaction (Lambden et al., 1980a; Pearce and Buchanan, 1978; Trust et al., 1980).

The underlying mechanism of pilus mediated attachment is little understood. One hypothesis, of Heckels et al. (1976), is that they aid the bacterium in overcoming the repulsive electrostatic barrier described by Curtis (1973) which occurs due to the close approach of the negatively charged surfaces of the bacterium and host cell. The results of experiments carried out by Heckels and his co-workers appeared to reinforce this hypothesis. These experiments showed that by chemically altering the surface charge of the bacterium, the level of attachment to human amnion cells could be increased if the surface charge was abolished or reversed and decreased if the surface was

made more negative. Other work does not, however, corroborate this hypothesis. It has been reported that the major contribution to surface charge in eukaryote cells is by negatively charged sialic acid residues (Grieg and Jones, 1977). The enzyme neuraminidase removes these groups, therefore treatment of cells with this enzyme should alter the charge of the cell, rendering it, to a degree, less negative. Experiments performed by Trust et al. (1980) did not show any enhancement of binding by N. gonorrhoeae to buccal epithelial cells (BEC) treated with neuraminidase, either for piliated or non-piliated bacteria. There is, however, no data to show whether the BEC surface charge was actually reduced. Although no real conclusions can be drawn from these experiments without such data, they may indicate that Heckels' hypothesis is not completely accurate. It may be that the drastic chemical treatments used to modify the bacterial cell surface altered the cell's capacity to attach in a manner which was unrelated to the alteration of surface charge. Also, the attachment substrates used in each experiment are probably far removed from the cells which N. gonorrhoeae attaches to in vivo. This being the case reduces the significance of the results obtained. No other hypothesis as to the nature of the attachment advantage which pili confer on cells has been proposed.

Despite the greater numbers and increased rates at which piliated organisms attach, non-piliated organisms do attach to and invade host cells. Obviously, other components at the surface of N. gonorrhoeae must be important in adhesion. Pili are most likely to assist these interactions by bringing the two surfaces together, thus increasing

the probability of their occurrence. Of the three major proteins exposed at the cell surface, only PII has been shown to have any role in attachment. The first experiments to demonstrate the role of PII were carried out by Lambden et al. (1979). They showed that although piliation was the single most important factor in an attachment assay, non-piliated strains possessing any one of six PII showed enhanced binding when compared to the non-piliated variant which did not have PII. Additional evidence of PII mediated binding was shown by Sugasawara et al. (1983) who demonstrated that a PII specific McAb decreased attachment to HeLa cells for variants containing PII but was without effect for variants lacking PII. A control McAb with no reactivity against PII was without effect. This inhibition of attachment by specific antibody fulfils one of the criteria proposed by Watt et al. (1978) to confirm that a protein is mediating attachment. Two of the remaining criteria (for which data on PII are available) are that the protein is surface located, as PII is known to be (see section 1.2.3.) and that mutants which lack the protein do not adhere. This last criterion is of doubtful significance because pili, widely acknowledged to be the primary mediators of gonococcal attachment, do not fulfil it and neither does PII; strains of N. gonorrhoeae which lack both PII and pili have been shown to adhere to epithelial and tissue culture cells in vitro (Draper et al., 1980; Lambden et al., 1979; James et al., 1983).

Not all studies have reinforced the theory that PII acts as an attachment ligand. In a series of experiments by Draper et al. (1985), it was found that purified OMs without PII could successfully

compete for binding sites on HeLa cells and thus reduce the number of OMs containing PII that adhered. While the authors were unsure of the implications of this result, they suggested that there may have been steric hindrance by OMs lacking PII of OMs containing PII which affected their ability to bind. Despite this result, there is still sufficient data to suggest that PII is important in adhesion.

The mechanism by which PII promotes adhesion is not known. It is possible there is a PII specific receptor on the host cell. Heckels (1982) showed that treatment of BEC with glycosidases reduced attachment of ^{125}I-labelled purified OMs containing PII to levels seen for OMs without PII, suggesting an involvement of a host surface carbohydrate in the adherence of N. gonorrhoeae to BEC. Blake (1985) has some evidence for this in PII molecules from op colonies, as the putative PIIop receptor on the gonococcal membrane is a carbohydrate moiety of LPS. Blake postulates that this carbohydrate moiety is structurally similar to a host cell surface carbohydrate. Expression of PIIop would then allow increased association of the bacterium with the host cell membrane. These data are, however, in direct conflict with those of Bessen and Gotschlich (1987). Their evidence is based on binding studies of purified PII to component proteins of HeLa cells and also to other unrelated proteins, like bovine serum albumen. They found that PII bound to protein sequences which were widely distributed through many proteins and thus postulated that PII may recognise an epitope present on many proteins. It is of course possible that PII binds to both protein and carbohydrate. The conflict may be resolved by using both binding assays in a study of

gonococcal cell:cell and bacterial:host cell interactions.

1.3.3. Hydrophobic Interactions in Adhesion

Adhesion may be promoted by non-specific hydrophobic interactions between like groups on both bacterial and host cell surfaces. This has been seen in other systems (Magnusson et al., 1977). However, the extent to which hydrophobicity contributes to attachment may be minimal when compared to other attachment mechanisms. Watt and Ward (1980) found that one P9 variant which was relatively less hydrophobic than a second P9 variant had a two-fold attachment advantage to BEC. Also, strains with equal hydrophobicity did not show equal attachment to BEC (Lambden et al., 1979). It is possible that PII binding to host cells is not promoted by hydrophobic interactions. The studies of PII interaction with HeLa cells conducted by Bessen and Gotschlich (1987) found that binding took place in the presence of Tween, which minimises hydrophobic interactions, and was unaffected by increasing salt concentration, which promotes hydrophobic interactions.

1.3.4. Factors Opposing Adhesion

There are several forces which may oppose the adhesion of N. gonorrhoeae to the host. The flow of mucus secretions and urine in the cervix and urethra are both potential methods by which bacteria might be removed, as is the passage of faeces through the rectum. The

low level colonisation of the cervix and urethra by normal flora may reflect this flushing action. The rapidity with which N. gonorrhoeae attaches may help prevent its removal; one report claimed that a male patient who urinated two minutes after intercourse with an infected female still developed gonococcal urethritis (Bernfield, 1972). However, opinions vary as to whether this was an exceptional case (Felton, 1972; Wigfield, 1972). James and Swanson (1978) have also suggested that the clumping shown by opaque organisms may protect them from the flushing forces of urine and faeces; it allows a large number of organisms to be held on the host cell surface by relatively few pili (Blake, 1985). This may in part account for the fact that op organisms are frequently isolated from the urethra and rectum whilst tr forms are not. In contrast, tr organisms may be at an advantage at the cervix. Cervical mucus may present a physical barrier through which the bacteria have to penetrate before they can adhere to the receptors on the epithelial surface. The lesser amount of clumping seen in tr organisms may aid the bacteria to penetrate through the thick gel matrix. Cervical mucus may also contain glycoproteins which are antigenically similar to, and indeed may even be derived from the tissue antigens at this site (Pigman and Moschera, 1973) raising the possibility that the host cell receptor for N. gonorrhoeae is mimicked by the mucus. If this were the case then the bacterium might bind erroneously to the mucus and in so doing, be prevented from setting up infection.

1.3.5. Local Antibody

Local and serum antibodies are provoked by local infection with N. gonorrhoeae. Serum antibodies are of classes IgA and IgG (Lammel et al., 1985) and presumably reflect local secretions. Local antibodies of IgA and IgG classes have been shown to block bacterial adherence in vitro (Tramont, 1977).

All strains of N. gonorrhoeae produce an extracellular enzyme which cleaves IgA molecules. The proteolytic action of this enzyme may disrupt antibody function, preventing antibody blocking of attachment and bacterial agglutination. The production of IgA protease may be an important factor in virulence as it is produced by all pathogenic but not commensal Neisseriaceae (Mulks and Plaut, 1978).

The ability of N. gonorrhoeae to vary its surface antigenic determinants may be crucial in avoiding host antibody blocking of adherence. The principal determinants involved in attachment, pili and PII, are both capable of extensive variation.

The expression of PII in vitro is subject to rates of change at an average of 2×10^{-3} colony forming units per generation as determined by monitoring transition from tr to op colony type (Mayer, 1982). This variation in vitro is presumably due to random recombination events in the absence of any selection pressure. High rates of transition are also seen in vivo in isolates from the same patient at

different times (Schwalbe et al., 1985) and in isolates from contact pairs (Zak et al., 1984). This in vivo transition, which is probably in response to the host immune system, may be non-random. When variants of laboratory strain FA1090 expressing a known PII molecule (PIIb) were exposed to a bactericidal McAb specific for PIIb, the resultant surviving population had not undergone totally random changes in PII complement. Rather, they had simply switched off the expression of the susceptible PIIb. Although a few survivors now expressed a totally new PII type, the majority did not (Black et al., 1984). This does not, however, explain why such high rates of transition should occur during in vitro culture or the mechanism by which the transition becomes non-random.

Variation in pilin subunit MW between contact pairs (Zak et al., 1984) and different isolation sites on the same host (Duckworth et al., 1983) is common. The observation of Duckworth and co-workers that, along with the principal pilin band, minor bands of a different MW were also present might indicate the existence of a minority population expressing a different pilus type. Whether these variants arise specifically to escape the host immune system or by random switching events has not been assessed. In either case, this variation and that seen for PII may well be an important factor as to whether the bacterium infects the host or is cleared by the host immune system.

1.4. THE HOST ENVIRONMENT

N. gonorrhoeae colonises a number of mucosal surfaces which vary greatly with respect to the secretions they produce and the normal flora they are colonised by. These differences may have a profound effect on the ability of the bacterium to establish disease through their effects on the bacterium and its attachment to the host.

1.4.1. Fatty Acids are Toxic to N. gonorrhoeae

The ability to withstand the toxic effects of fatty acids may be of primary importance for organisms infecting the rectum and to a lesser extent for organisms infecting the cervix. The rectum is a rich source of fatty acids with an average of 4g being excreted by an adult over twenty four hours (Geigy Scientific Tables). The composition of faecal fats depends on the diet, but in the Western world there are generally four that are preponderant, stearic, palmitic, oleic and linoleic acid (Gurr and James, 1971). The latter three are known to be inhibitory to *N. gonorrhoeae* (Miller *et al.*, 1977). The precise effects of fatty acids have not been elucidated, but it is likely that inhibition of electron transport is at least partially responsible for the observed growth inhibition (Miller *et al.*, 1977). There may be other metabolic effects, as yet uncharacterised. Gram-negative bacteria which habitually inhabit the rectum are resistant to the toxic action of fatty acids by virtue of the fact that they exclude them from the cell. The cell surface of

many enteric bacteria is hydrophilic (see section 1.2.7.). This provides an effective barrier against the hydrophobic fatty acids. Strong lateral interactions between LPS molecules may also contribute to the impermeability of the cell (Nikaido and Vaara, 1985).

Deep rough mutants of enteric bacteria and N. gonorrhoeae are by contrast highly susceptible to fatty acids. The presence of PL bilayers in the OM provides a channel through which hydrophobic molecules can diffuse and enter the cell (Lysko and Morse, 1981). Another possible method by which hydrophobic molecules enter the cells of bacteria with R-type LPS has been proposed by Nikaido and Vaara (1985). They believe that the lateral interactions between LPS molecules may be decreased in R-type LPS through loss of negatively charged phosphates groups, bridging of which is important in LPS:LPS interactions. This then renders the membrane more permeable to hydrophobic molecules.

1.4.2. Resistance to Hydrophobic Molecules in N. gonorrhoeae

Mutants of N. gonorrhoeae arise with decreased OM permeability. These mutants are termed mtr (multiple transformable resistance) (Cannon and Sparling, 1984) and are resistant at a low level to a number of hydrophobic antibiotics, detergents and dyes. Maness and Sparling (1974) first recognised that low level resistance to a number of agents in clinical isolates could be due to one mechanism, rather than several individual mechanisms, on the basis that resistance was

lost or restored by one single mutational event. They predicted that the gene product of the locus would affect some part of the cell wall or membrane and would thus affect permeability. Corresponding hypersensitive mutants have a mutation at a locus known as env (Sarubbi et al., 1975). Studies on the uptake of crystal violet by mtr and env mutants confirmed that the observed resistance/ sensitivity was due to alterations in membrane permeability (Guymon and Sparling, 1975). The env mutation is dominant to mtr when they occur in the same cell (Eisenstein and Sparling, 1978; Sarubbi et al., 1975).

When the OMs from these two mutants were analysed they were found to be compositionally different. Guymon et al. (1978) showed that OMs from strains with the mtr envelope phenotype contained seven times the amount of a minor OMP with a subunit MW of 52 kd when compared to OMs from wild type strains. In env mutants, the amount of this protein present was correspondingly decreased. The means by which such a difference alters the permeability of the cell is unclear, but there may be one explanation. In other gram-negative genera, fluctuations in the total amount of OMP may affect the amount of PL found in the outer leaflet of the membrane; where the total amount of OMP was decreased, more PL were found in the outer membrane (Koplow and Goldfine, 1974). Although not reported, it is possible that the opposite is true where the total amount of OMP is increased. By this reckoning, the extent of the PL bilayer in the membrane may be reduced in mtr strains. Although Nikaido and Vaara (1985) in their review paper have suggested that increasing the PL bilayer, as might

happen in *env* strains, would not render the OM more permeable than that of the wild type, they have not stated the converse. This theory makes the assumption that the sevenfold increase in the 52 kd protein is not accompanied by a concomitant decrease in other OMPs, this is of course possible and may not have been detected by Guymon *et al*. (1978).

A second difference exists between the OMs of *mtr* and *env* strains, although its contribution to membrane permeability is even more obscure than that of the 52 kd protein. Lysko and Morse (1981) examined the phospholipase sensitivities of PL on whole cells of either *mtr*, wild type or *env* phenotype. The PL of *env* strains were degraded by the enzyme, while those of *mtr* and wild type strains remained intact. Pretreatment of *mtr* and wild type bacteria with exoglycosidase rendered their PL susceptible to phospholipase but was without additional effect for *env* strains. Lysko and Morse concluded that wild type and *mtr* strains possessed a glycosylated component in their OMs which masked the PL head groups at the cell surface and thereby protected them from the phospholipase. This masking of the head groups might decrease the permeability of the membrane by reducing the extent to which hydrophobic molecules could pass through the PL bilayers. However, this does not account for the increased resistance of *mtr* strains over wild type, unless the effects of the 52 kd protein and PL glycosylation are synergistic. Whether there is any change in LPS composition in *mtr* strains which might increase lateral interactions and thus decrease permeability as described in section 1.4.1. has not been investigated.

Because of the role of the mtr mutation in resistance to hydrophobic molecules, Morse et al. (1982) postulated that mtr strains would predominate in environments rich in hydrophobic molecules, i.e. the rectum. In order to investigate this hypothesis, they determined the envelope phenotypes of a large number of clinical isolates by means of their MIC to four hydrophobic agents; Triton X-100, crystal violet, fusidic acid and erythromycin. Strains with an MIC for each above a critical level were termed mtr and this was confirmed in genetic transformation studies, where DNA from a resistant strain was capable of transforming a sensitive strain to the resistant phenotype. After this analysis, they tested for resistance to faecal lipids. Their results showed, as expected, that mtr strains were isolated most frequently from homosexuals. mtr strains were also more resistant to faecal lipids than non-mtr strains.

A second study (McFarland et al., 1983) indicated that, although the mtr phenotype was selected for by the rectal environment and was an important factor in resistance to faecal lipids, other factors were also important. Strains which were AHU- auxotype and had the wild type envelope phenotype were as uniformly hypersensitive to faecal lipids as were strains with the env mutation. Rectal isolates from women and homosexual men with the wild type envelope phenotype were more resistant than wild type isolates from the heterosexual male urethra and female cervix, suggesting that in these strains a second mechanism was operating. This mechanism is not thought to be due to exclusion of the faecal lipids from the cell, but rather to an alteration within the cell of the target of fatty acid inhibition.

The level of resistance in the bacteria with this putative second resistance mechanism was not as high as that of mtr strains; it is possible that in the mtr strains both resistance mechanisms were operating. No further studies have been carried out as to the nature of the mtr-independent resistance mechanism.

1.4.3. Other Inhibitory Compounds

Fatty acids are probably the most important inhibitory compounds found in the rectal environment but there are many other compounds present at the urethra and cervix which are potentially harmful to N. gonorrhoeae.

The urethra is flushed with urine, the pH of which may fluctuate between 4.8 and 7.5 (Geigy Scientific Tables). McCutcheon et al. (1977) demonstrated that N. gonorrhoeae was killed rapidly by urine with a pH of less than 6.0. Compounds dissolved in urine may also affect the organisms ability to survive; these include urea, which is bacteriostatic and NaCl, high concentrations of which may exert a powerful osmotic effect on the bacterium, leading to damage and eventual death of the cell (McCutcheon et al., 1977). In the male, the urethra is also flushed by semen which contains significant amounts of zinc ions (1 mg/ml) which have been shown to be inhibitory by Lambden et al. (1979). Their study suggested that possession of certain PII types increased the resistance of P9 to this metal ion. This, however, is unlikely to be of in vivo significance since the

most resistant strain possessed an MIC of only 0.1 mg/ml.

The composition of cervical mucus undergoes cyclical changes which correspond to the stage of the oestrous cycle. The recovery of bacteria of varying opacity according to the stage of the cycle is evidence that different selection pressures are operating as the composition of the mucus alters. These changes do not simply reflect spontaneous changes in PII composition because isolates from the urethra and rectum are consistently op. The inference is that there are selection pressures operating at the cervix that do not occur at other sites. It may be that one of the most important factors is the presence of proteolytic activity at the cervix; this may be greatest at the time of menstruation as menstrual blood itself is proteolytic (Beller, 1971). Tr organisms which are isolated at or near menstruation are less sensitive to protease growth inhibition than op forms (Swanson, 1978a) and thus are presumably at an advantage during this phase of the cycle.

The steroid hormone progesterone is a potent growth inhibitor *in vitro* for *N. gonorrhoeae*; it is either bacteriostatic or bactericidal, depending on the concentration it is present at (Morse and Fitzgerald, 1974). Salit (1982) demonstrated *in vitro* that the presence of progesterone in the growth medium selected for tr organisms which were less sensitive to inhibition than op. However, the levels of progesterone in cervical mucus are decreased prior to, at and immediately after menstruation whilst op bacteria are isolated at the time of and during ovulation (James and Swanson, 1978).

Therefore the drop in progesterone levels does not correspond with an emergence of previously inhibited op forms and so it does not appear that progesterone levels exert any selection pressure on N. gonorrhoeae.

Minor compounds present in cervical mucus which may also affect the growth of N. gonorrhoeae include numerous enzymes, glycogen, which may support the growth of inhibitory lactobacilli (see section 1.4.4.), electrolytes such as NaCl and Cu^{++} ions (Schumacher, 1973). Cu^{++} are also released from certain intra-uterine devices (IUDs) and are known to be inhibitory to N. gonorrhoeae (Cohen and Thomas, 1974). As with zinc ions, Lambden et al. (1979) found that some variants of P9 were slightly more resistant to Cu^{++} and attributed this to their PII composition. However, no real in vivo effect of copper ions has been demonstrated as no decrease in the incidence of gonococcal salpingitis has been seen in women fitted with IUDs (Westrom et al., 1976).

1.4.4. The Effect of Normal Flora

The sites infected by N. gonorrhoeae vary with respect to the sheer number of bacteria as well as the bacterial species found there. The urethra contains only small numbers of organisms representative of those found on the skin and perineum; diphtheroids, staphylococci, bacilli, streptococci and coliforms (Jawetz et al., 1982). The cervix is colonised by a mixed flora of aerobes and anaerobes, including

lactobacilli, staphylococci, streptococci, peptococci and peptostreptococci (Ohn and Golask, 1975). The colonic and faecal flora contain at least four hundred species of bacteria. Of these, the genus Bacteroides accounts for thirty percent, in particular the species vulgatus, distasonis, thetaiotamicron, fragilis and ovatus (Salyers, 1984). Other genera present at this site include lactobacilli, clostridia and coliforms (Jawetz et al., 1982).

Many bacterial species, both anaerobic and aerobic, have been shown to inhibit growth of N. gonorrhoeae, in vitro (table 2). The ability to inhibit growth appears to be strain dependent, as not all strains of a given species show the same degree of inhibition. The nature of the inhibition is unclear; Morin et al. (1980) have shown that inhibition of N. gonorrhoeae by anaerobes is not due to pH change or depletion of the media. One group's results indicated that the release of large amounts of fatty acids by some bacteria during growth may inhibit N. gonorrhoeae (Ingraham et al., 1978). Another study purified a growth inhibitor from Staphylococcus haemolyticus which appeared to affect the gonococcal membrane (Frenette et al., 1984).

One study has correlated the presence or absence of infection with N. gonorrhoeae with the carriage of inhibitory lactobacilli in the cervix. Saigh et al. (1978) found that in women exposed to N. gonorrhoeae, those that were carrying inhibitory lactobacilli as normal flora of the cervix were more likely to avoid infection. This may indicate that the lactobacilli themselves exert a protective

TABLE 2

Inhibition of *N. gonorrhoeae* by Normal Flora

Bacterial Group Shown to Inhibit *N. gonorrhoeae*	Reference
Gram-negative enteric rods	
e.g. *E.coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *B. fragilis*, *B.vulgatus*	Kraus *et al*., 1976 Morin *et al*., 1980
Gram-positive cocci	
e.g. *Staphylococcus aureus*, *Peptostreptococcus anaerobius*	Kraus *et al*., 1976 Morin *et al*., 1980
Gram-positive rods	
e.g. Lactobacilli (uncharacterised)	Saigh *et al*., 1978

effect, or else that they are indicative of other conditions in the host that might affect the acquisition of N. gonorrhoeae. Carriage of streptococci and staphylococci, which also inhibited N. gonorrhoeae in vitro could not be correlated with any protective effect. Separate investigations have found that carriage of S. epidermidis and N. meningitidis in the male urethra protects against infection with N. gonorrhoeae, despite repeated exposure to the bacterium (Kraus and Ellison, 1974; Volk and Kraus, 1973).

1.4.5. Leukocyte Association

The ability of any pathogenic bacterium to avoid destruction is an essential prerequisite of life within the host (Watt and Ward, 1980). In both localised and disseminated infection N. gonorrhoeae will come into contact with neutrophils, monocytes and macrophages (Rest et al., 1985). Other micro-organisms, such as enteric bacteria, evade phagocytosis through the hydrophilicity of their cell surface, with phagocytosis of rough mutants being enhanced (Magnusson et al., 1977). In N. meningitidis, the hydrophobicity of R-type LPS is offset by the production of a hydrophilic capsule (Watt and Ward, 1980). The production of a capsule by N. gonorrhoeae has not been well established and thus the cell surface is hydrophobic and more susceptible to phagocytosis. Therefore N. gonorrhoeae must possess other methods of evading phagocytosis.

Several experiments have shown that pili are anti-phagocytic

(Dilworth et al., 1975; Punsalang and Sawyer, 1973; Thongtai and Sawyer, 1973). Pili may block phagocytosis by binding to the phagocytic cell surface and disrupting membrane mobility, thus preventing engulfment (Watt and Ward, 1980). However, there is evidence that PII might be even more important in leukocyte association and could possibly counter the anti-phagocytic effects of pili. The importance of PII in this field was first discovered by Swanson and his co-workers in a series of studies of gonococcal:leukocyte association (King and Swanson, 1978; Swanson et al., 1974; Swanson et al., 1975). More recent studies have reinforced the concept that PII is a more important determinant of gonococcal:leukocyte association than pili. In phagocytic assays, PII containing variants are killed regardless of their state of piliation and pili have no effect on the stimulation of chemiluminescence which is an index of the association between phagocytes and bacteria (Virji and Heckels, 1986). Not all PII appear to promote this leukocyte association (Rest et al., 1985). In agreement with this, Lambden et al. (1979) found that, for P9, possession of one particular PII species decreased the leukocyte association of that variant. Variation of PII composition in N. gonorrhoeae may thus affect the extent of leukocyte association and hence phagocytic killing and therefore may be important in survival in vivo.

1.5. AIMS OF THE PROJECT

The ability of N. gonorrhoeae to adapt in order to survive in the sometimes hostile host environment may be a crucial determinant in the pathogenesis of gonorrhoea. Strains deficient in survival mechanisms or unable to vary their surface structures would then be at a disadvantage and their host range may be restricted. The observation that PPNG do not infect homosexuals may indicate that these strains are unable to colonise the rectum due to a deficiency of this kind.

Thus far, detailed knowledge of the biology of N. gonorrhoeae is restricted to a few laboratory adapted strains; findings for these strains may not parallel those of clinical isolates and none of the laboratory strains produce penicillinase. Few studies have concentrated on PPNG. This project has examined some phenotypic characteristics of both non-PPNG and PPNG from different isolation sites of hetero- and homosexuals. The phenotypes studied may relate directly to the ability to survive in the rectal environment or else they may simply illustrate fundamental differences between PPNG and non-PPNG.

2. MATERIALS AND METHODS

2.1. BACTERIAL STRAINS

2.1.1. Sources and Selection of Strains

All strains of N. gonorrhoeae were isolated from patients attending the STD clinic at St Mary's Hospital, London. PPNG and non-PPNG strains were selected as shown in table 3. Spectinomycin resistant PPNG and non-PPNG were also selected as these appear to represent a single clone, with all isolates showing an identical serotype, auxotype and MIC for several antibiotics. The strains differ only in the carriage of the 4.4 Md plasmid, therefore the non-PPNG spectinomycin resistant strains represent isolates that have been cured of the penicillinase plasmid. The mtr control strain FA171 was obtained from P.F. Sparling, Department of Medicine, University of Carolina and has been described in detail elsewhere (Sparling et al., 1975; Guymon et al., 1978). Strain H1 was originally a female genital isolate which has been maintained in vitro at the Bacteriology Department, St Mary's Hospital Medical School, London since 1976. Bacteroides spp. fragilis and vulgatus were originally obtained from the Department of Bacteriology, St Thomas' Hospital, London. E. coli J5 and S. typhimurium Re mutant were obtained from Dr. B. Wilson, Department of Bacteriology, St Mary's Hospital Medical School, London. J5 is a wild type strain of E. coli K12. S. typhimurium strain Re is a deep rough mutant, the LPS of which contains only

TABLE 3

Sources of Strains of N. gonorrhoeae

Non-PPNG

Sex	Sexuality	Isolation Site	Number of Isolates
Female	Heterosexual	Cervix	26
Female	Heterosexual	Rectum	25
Male	Heterosexual	Urethra	27
Male	Homosexual	Urethra	25
Male	Homosexual	Rectum	26

PPNG

Sex	Sexuality	Isolation Site	Number of Isolates with plasmid type	
			3.2 Md	4.4 Md
Female	Heterosexual	Cervix	12	26
Female	Heterosexual	Rectum	0	3
Male	Heterosexual	Urethra	13	23
Male	Homosexual	Urethra	3	13
Male	Homosexual	Rectum	3	8

Spectinomycin Resistant N. gonorrhoeae

Sex	Sexuality	Isolation Site	Number of Isolates	
			PPNG	non-PPNG
Female	Heterosexual	Cervix	3	3
Male	Heterosexual	Urethra	3	3

ketodeoxyoctonate (KDO) and lipid A.

2.1.2. Culture and Storage of Strains

N. gonorrhoeae was primarily isolated on GC base agar (Difco Laboratories, East Molesey, Surrey) 18g/l supplemented with 1% IsoVitalex (see appendix 1.1.) and antibiotics (vancomycin, 3 mg/l; amphotericin B, 1.5 mg/l; colistin 10^5 units/l and trimethoprim 5 mg/l) to inhibit the growth of normal flora (Phillips et al., 1972). Subsequently, strains were cultured on non-selective GC base agar with 2% Kellogg's supplement (see appendix 1.2.). 1 mg/l of benzyl penicillin (Crystapen, Glaxo, Greenford, Middlesex) was added to select PPNG and to maintain the penicillinase plasmid. Where maintenance of colony phenotype was necessary, strains were cultured on the modified colony typing medium (CTM) of James et al., (1982) with 2% Kellogg's supplement. Where a starchless medium was required, direct sensitivity testing (DST) agar (Oxoid Ltd., Basingstoke, Hampshire) 20 g/l with 5% lysed horse blood (Tissue Culture Services Ltd., Buckingham, Buckinghamshire) with or without 1% IsoVitalex supplement was used. Growth of N. gonorrhoeae was always performed at 37°C in air containing 6% CO_2. Strains were subcultured a minimum number of times either by taking a sweep of confluent growth from a pure culture or by transfer of several individual colonies if cultures were contaminated. Bacteroides strains were grown on DST agar with lysed blood supplement in an Mark 2 anaerobic cabinet (Don Whitley, Shipley, West Yorkshire) at 37°C. E. coli and S. typhimurium were grown on nutrient agar (Oxoid) 20 g/l in air at 37°C. Between

experiments, all strains were stored in 15% glycerol (Sigma Chemical Company Ltd, Poole, Dorset) in glucose broth (Oxoid) 23 g/l under liquid nitrogen.

2.1.3. Strain Characterisation

All strains of N. gonorrhoeae were screened for the presence of plasmids by the rapid alkaline lysis method of Birnboim and Doly (1979) and auxotyped by the system of Carifo and Catlin (1973). For non-PPNG strains the MIC of penicillin was determined by an agar dilution technique using DST agar supplemented with lysed blood and IsoVitalex. Doubling dilutions of penicillin were made using penicillin Adatabs supplied by Mast Diagnostics, Bootle, Merseyside at a concentration of 100 µg/ tablet. Penicillin concentrations from 2 - 0.0075 mg/l were tested. Test strains were grown overnight and inoculated onto plates at an optical density $(OD)_{540nm}$ of 1.0 (approximately 10^8 colony forming units/ml) with a multipoint inoculator (A400, Denley Instruments Ltd., Billinghurst, Sussex). Plates were incubated for forty eight hours before reading the results. Strains H1 and FA171 were used as controls.

2.2 EXPERIMENTAL PROCEDURES

2.2.1. Envelope Phenotype Determination

Envelope phenotype was determined by the method of Morse et al. (1982). The MICs of erythromycin and fusidic acid were ascertained by making doubling dilutions of erythromycin and fusidic acid Adatabs supplied by Mast at 100 µg/tablet for both antibiotics in GC base agar with IsoVitalex supplement. This same dilution technique was used for Triton X-100 (BDH Ltd, Poole, Dorset) and 0.5% aqueous 'Gurr' crystal violet (BDH). Inoculation and incubation were carried out as in section 2.1.3. The concentrations that each compound were tested at and the MIC values characteristic of the mtr and env phenotypes are shown in table 4.

2.2.2. Oleic Acid Sensitivity Testing

Sensitivity to oleic acid was determined by an agar dilution technique. A starchless medium was required as starch can bind and detoxify lipids (McFarland et al., 1983). Therefore, as GC base agar contains starch, DST agar with lysed blood supplement was used. Final concentrations of 10, 2 and 1.0 g/l of oleic acid (obtained from Sigma) were tested. Inoculation and incubation were carried out as in section 2.1.3.. Strains with an MIC greater or equal to 10 g/l were classified as resistant.

TABLE 4

Determination of Envelope Phenotype

Antibiotic Concentrations Tested for Envelope Phenotype Determination

Doubling dilutions of erythromycin from	2.0 - 0.06 mg/l
fusidic acid from	0.5 - 0.03 mg/l
Triton X-100 from	2.0 - 0.06 g/l
crystal violet from	1.0 - 0.25 mg/l

MIC Values Characterisitic of mtr Phenotype

≥2.0 mg/l erythromycin

≥0.5 mg/l fusidic acid

≥2.0 g/l Triton X-100

≥1.0 mg/l crystal violet

MIC Values Characterisitic of env Phenotype

≤0.06 mg/l erythromycin

≤0.03 mg/l fusidic acid

≤0.06 g/l Triton X-100

≤0.25 mg/l crystal violet

2.2.3. Bacteroides spp. Inhibition Assay

Overnight cultures of Bacteroides species fragilis or vulgatus grown on DST agar with lysed blood supplement were inoculated at an OD_{540nm} of 2.0 as a single streak onto fresh plates of the same agar. These were incubated for forty eight hours under anaerobic conditions. The resultant confluent growth was completely removed from the plate with a sterile cotton swab. No further growth of Bacteroides occurred after this treatment. Test strains of N. gonorrhoeae at OD_{540nm} 1.0 were streaked onto the plate perpendicular to and across the region of the Bacteroides streak. The plates were re-incubated for 24 hours. Zones of inhibition of gonococcal growth were detected under a dissecting microscope.

2.2.4. Outer Membrane Protein Characterisation

Total cell protein was prepared from an overnight culture of N. gonorrhoeae grown on CTM. Growth was suspended thickly in saline and the amount of protein present was measured by the method of Lowry using bovine serum albumen as a standard (Lowry et al., 1951). Samples were diluted to 1 mg of protein/ml in 1 x SDS-PAGE sample diluent (see appendix 2.1.) containing 10% dithiothreitol (DTT) (Sigma). The samples were then heated at either 37 or 100°C to solubilise the proteins.

Two methods were used to purify the OM from the total gonococcal cell

protein. OMs were extracted using sodium lauryl sarcosinate (Sarkosyl) by a modification of the method of Walstad et al. (1977) in an attempt to visualise the 52 kd OMP associated with the mtr phenotype. Overnight cultures of N. gonorrhoeae grown on GC base agar were suspended in phosphate buffered saline (PBS) (Oxoid). The cells were pelleted by centrifugation at 4°C and washed once in the same manner with ice-cold PBS. The pelleted cells were resuspended in 40 mls of 10 mM N-2-Hydroxyethyl piperazine-N'-2-ethane sulphonic acid (HEPES) buffer (Gibco Ltd., Paisley, Scotland) and ruptured by three cycles of rapid freezing at -70°C and thawing at 50°C. Rupture was confirmed by gram staining. The ruptured cells were stored overnight at -70°C, then thawed and incubated for 15 minutes in 1 mM $MgCl_2.6H_2O$ (BDH) containing 40 µg/ml deoxyribonuclease from bovine spleen (Koch-Light Ltd, Haverhill, Suffolk). Unbroken cells were removed by centrifugation at 3 000 x g for 5 minutes and the resulting clear supernatant was spun at 48 000 x g for 20 minutes at 4°C. The resultant pellet, which contained crude cell envelopes, was resuspended in PBS and the protein content measured before being washed during a second spin at 48 000 x g for 60 minutes at 4°C. The pellet was resuspended at a concentration of 2 mg protein/ml in 10 mM HEPES containing 0.2% Sarkosyl (Sigma) and left at room temperature for 10 minutes in order for the Sarkosyl to remove cytoplasmic protein from the crude envelope preparation. At the end of the incubation, the suspension was diluted to 40 mls with distilled water, then spun at 48 000 x g for 20 minutes at 4°C in order to pellet the OM whilst leaving cytoplasmic protein in the supernatant. The final pellet was resuspended in a small amount of PBS for estimation of its protein content. Samples were prepared for

electrophoresis in the absence of DTT by solubilising at 100°C.

OMs were also purified by the modified method of Heckels (1977) in order to examine their PII composition. Strains of N. gonorrhoeae were grown up on CTM overnight. The growth was removed from the plate, washed twice by centrifugation in PBS and then resuspended at a concentration of 2% w/v in 0.3 M lithium acetate (BDH) pH 6.0. These suspensions were shaken at 45°C for 2.5 hours, during which time large amounts of OM were budded off in the form of blebs. Intact cells and other debris were removed by three centrifugations at 15 000 x g at 4°C. After the third spin, the resulting supernatant was centrifuged at 100 000 x g for 2.5 hours at 4°C. The pellet containing crude OM was washed twice in 0.2 M lithium acetate pH 5.8 by centrifugation at 100 000 x g at 4°C and finally resuspended in a small volume of the same buffer. Protein estimation and sample preparation for electrophoresis was carried out as before in the absence of DTT at solubilisation temperatures of 37 and 100°C.

2.2.5. SDS-PAGE of Protein Extracts

The total cell protein and lithium acetate OM preparations described in section 2.2.4. were analysed by SDS-PAGE using the discontinuous system of Laemmli (1970). 10 µg total of protein was applied to a vertical slab gel which consisted of a 5% total acrylamide concentration stacking gel and a 15% total acrylamide concentration separating gel (see appendix 2.2.). The gel was run in SDS-tris-

glycine buffer (see appendix 2.3.) using a constant current supply at 20 mA. The run was stopped when the tracking dye was at least 7 cm into the separating gel. After removal of the stacking gel, the separating gel was stained with 0.1% Coomassie brilliant blue R250 (Sigma) dissolved in 50% methanol, 10% acetic acid for one hour and subsequently destained in several changes of 10% methanol, 10% acetic acid. Protein molecular weights were estimated by reference to the migration of protein standards of known molecular weight in the range of 12 to 93 kd (SDS-PAGE standards supplied by BioRad Laboratories Ltd, Watford, Hertfordshire or 'Electran' molecular weight markers obtained from BDH).

Sarkosyl extracts were run on a 5 - 15% linear gradient gel in an attempt to optimise separation in the region of 50 kd. Gradient gels were formed using a BioRad gradient gel former (model 385). A linear gradient of acrylamide was achieved by gradual mixing of 5 and 15% acrylamide solutions (see appendix 2.2.) whilst pumping the mixture into the gel-casting apparatus. Following polymerisation of the acrylamide gradient, a 5% stacking gel was added. Gel loading, running and staining were performed as for non-gradient gels.

2.2.6. Transformation of Envelope Phenotype

In order to confirm that strains with MIC values indicative of the *mtr* envelope phenotype were *mtr* as originally defined by Guymon *et al*. (1978), the non-*mtr* control strain H1 was used as the recipient

of DNA from presumptive mtr strains in a transformation experiment based on the method of Janik et al. (1976). One PPNG, one non-PPNG and the FA171 mtr control strain were used as donors.

A heavy suspension of an overnight culture of the DNA donor strain was made up in saline citrate buffer (0.015 M NaCl, 0.015 M trisodium citrate; BDH). The OD_{540} of the suspension was adjusted to 4.0 and an equal volume of saline citrate buffer containing 0.1% SDS (lysis buffer) was added. The bacteria were lysed and their DNA released by heating the suspension at 60°C for one hour. This DNA preparation was then cooled and diluted 1:10 in saline citrate buffer prior to use. A suspension of T1 and T2 colonies from the recipient strain was made up in saline and the OD_{540nm} was adjusted to 1.0. These colony types were used as their genetic competence is much greater than those that are non-piliated (Cannon and Sparling, 1984). Six 20 µl drops of the recipient suspension were mixed separately with six 20 µl drops of the donor DNA on one GC base agar plate. This plate was incubated at 37°C for six hours to allow the transformants to grow. This was a slightly longer incubation time than that used by Janik et al. (1976) and may have been required because mtr strains have slower growth rates than wild type strains (Guymon et al., 1978). After incubation, the growth from all six drops was removed from the plate and suspended in 1 ml of saline. This suspension was diluted ten-fold from 10^{-1} to 10^{-7} in saline. 100 µl of each dilution were spread onto selective plates containing 2 mg/l erythromycin as described in section 2.2.1.. Untransformed recipients were plated onto this selective medium to ensure that spontaneous mutation to erythromycin

resistance had not occurred and the donor DNA preparation was plated out onto non-selective media to check it did not contain any viable bacteria. All plates were left to incubate for 72 hours. If no growth was seen on either of the control plates, then colonies on the test plates were assumed to be mtr transformants. This was confirmed by further testing for resistance to fusidic acid 0.5 mg/l, Triton X-100 2g/l and crystal violet 1 mg/l in GC base agar as in section 2.2.1..

Sarkosyl extracts of donor, recipient and transformant were prepared and analysed by SDS-PAGE on 5 - 15% linear gradient acrylamide gels in order to determine the amount of the 52 kd protein associated with the mtr phenotype present in the OM.

2.2.7. Measurement of Surface Hydrophobicity

Surface hydrophobicity was determined by a modification of the two phase partitioning (TPP) method of Magnusson et al. (1977). Each test strain was streaked onto quarter of a CTM plate previously spread with 150 µCi of tritiated (^{3}H) glucose (specific activity of 500 mCi/mmole, Amersham International plc, Little Chalfont, Buckinghamshire) and incubated overnight to allow the bacteria to incorporate the label. The labelled bacteria were suspended in saline to an OD_{540nm} of 1.0 for testing. The hydrophilic E. coli wild type J5 strain and the hydrophobic Re mutant of S. typhimurium were used as controls.

A solution containing 4.4% w/v PEG (Sigma), 6.2% w/v dextran T500 (Pharmacia Ltd, Milton Keynes, Buckinghamshire) in 0.03 M Tris-HCl pH 7.0 was made up and left overnight at 4°C prior to use. During this time the mixture separated into two phases, the top containing hydrophilic PEG and the bottom containing hydrophobic dextran. 2 mls of each phase were placed in a single test tube. 100 µl of the labelled test strain were added to the tube and mixed by inverting twenty times. The tube was then left at room temperature for one hour to allow the bacteria to partition according to their surface hydrophobicity.

Duplicate 500 µl samples were taken from both the upper and lower phases, the remaining 2 mls was mixed by vortexing and then a further two 500 µl samples were taken to represent the interphase of the system. The samples were counted using Pico-fluor 30 scintillation fluid (Canberra Packard, Pangbourne, Berkshire) in a TriCarb liquid scintillation counter (Canberra Packard). The total amount of radioactivity added to each tube was assessed by counting 100 µl each of the original labelled bacterial suspension, thus the percentage of counts and by analogy, the percentage of bacteria in each phase could be calculated.

RESULTS

3.1. STATISTICAL TREATMENT OF DATA

The results of the auxotype and plasmid analysis, envelope phenotype determination and resistance to oleic acid, *B. fragilis* and *B. vulgatus* were all compared by the chi-squared test of the independence of categorical variables in a 2 x 2 contingency table according to the formula given in appendix 3.1. (Campbell, 1967). Any chi-squared value greater than 3.84 ($p < 0.05$) was considered significant. A list of all chi-squared values cited in the text is given in appendix 3.2..

The results of the TPP experiment were analysed by two statistical methods. The mean ratios were compared by assessing the standard error of the difference between means using the formula in appendix 3.3. (Moroney, 1980). Where the difference between two means was equal to or greater than three times the standard error of the difference, the difference was regarded as statistically highly significant. Secondly, the difference between the total mean ratios of each group was assessed by the two tailed Student's t-test, the formula for which is given in appendix 3.4. (Haber and Runyon, 1973).

3.2. STRAIN CHARACTERISATION

3.2.1. Auxotyping

The results of the auxotyping analysis are presented in table 5. The most prevalent auxotype was wild type (non-requiring). 54% of all isolates belonged to this auxotype, although it was significantly more common in non-PPNG (61%) than PPNG (46%) ($p < 0.05$). In PPNG, pro- was almost as common, with 45% of PPNG strains belonging to this auxotype. The incidence of pro- in non-PPNG was 28%, which was significantly lower than the incidence of pro- in PPNG ($p < 0.01$). The incidence of arg- strains was low in both groups, 9% and 6% for PPNG and non-PPNG, respectively. The auxotype AHU- was only found in non-PPNG where it occurred in 5% of the strains tested.

The occurrence of auxotype did not correlate with site of isolation. However, the sexuality of the patient did appear to be related to the auxotype as wild type strains were isolated significantly more frequently from homosexuals than heterosexuals ($p < 0.001$). The auxotype AHU- did not occur in homosexuals but this was not significant as the incidence of AHU- was so low for all groups. The scarcity of AHU- strains in homosexuals may be due to their hypersensitivity to faecal lipids (McFarland et al., 1983). Correspondingly, only one out of twenty five non-PPNG isolates from the female rectum was of this auxotype.

TABLE 5

Results of Auxotyping

	Number tested	Percentage of Strains with Auxotype Wild	Pro-	Arg-	AHU-
Non-PPNG					
Female cervix (heterosexual)	26	54	35	4	7
Female rectum (heterosexual)	25	52	36	8	4
Male urethra (heterosexual)	26*	58	27	4	11
Male urethra (homosexual)	25	72	24	4	0
Male rectum (homosexual)	26	72	20	8	0
PPNG					
Female cervix (heterosexual)	38	34	50	16	0
Female rectum (heterosexual)	3	0	100	0	0
Male urethra (heterosexual)	36	47	47	6	0
Male urethra (homosexual)	16	69	31	0	0
Male rectum (homosexual)	11	64	27	9	0

Notes : Wild = wild type
Pro- = proline requiring
Arg- = arginine requiring
AHU- = arginine, hypoxanthine and uracil requiring
* = one isolate failed to grow on NEDA

3.2.2. Plasmid Type

The results of plasmid screening are shown in tables 6 and 7. All isolates, bar two non-PPNG strains isolated from heterosexuals, carried the cryptic 2.6 Md plasmid. 21% of all isolates tested carried the 24.5 Md conjugal plasmid but the distribution of this plasmid was not uniform amongst different isolation groups. In PPNG, carriage of the 24.5 Md plasmid was correlated with possession of the 4.4 Md penicillinase plasmid ($p < 0.01$) and rarely occurred with the 3.2 Md penicillinase plasmid (one out of thirty one strains). In PPNG strains carrying the 4.4 Md plasmid, the conjugal plasmid occurred with equal frequency in both heterosexuals and homosexuals. Non-PPNG also showed no difference in the incidence of the conjugal plasmid in hetero- and homosexuals.

3.2.3. Linkage of Plasmid Content and Auxotype

The data used to establish the existence of any links between plasmid content and auxotype are shown in table 8. For PPNG strains, auxotype and plasmid type were closely linked; the auxotype pro- was correlated strongly with carriage of the 4.4 Md plasmid ($p < 0.01$) and the auxotype arg- was correlated with the 3.2 Md plasmid ($p < 0.001$). In PPNG, carriage of the 24.5 Md plasmid was correlated with the wild type auxotype ($p < 0.05$). In view of the correlation between the incidence of the 4.4 Md plasmid and the conjugal plasmid and also the correlation between the 4.4 Md plasmid and the pro- auxotype, it was

TABLE 6

Results of Plasmid Analysis : Non-PPNG

	Number tested	Plasmid Content of Strains (%) 2.6 Md	2.6 + 24.5 Md	None
Female cervix (heterosexual)	26	76	20	4
Female rectum (heterosexual)	25	80	20	0
Male urethra (heterosexual)	27	81	15	4
Male urethra (homosexual)	25	72	28	0
Male rectum (homosexual)	26	82	18	0

TABLE 7

Results of Plasmid Analysis : PPNG

	Number tested	Plasmid Content of Strains (%) 4.4 Md	4.4 + 24.5 Md	3.2 Md	3.2 + 24.5 Md
Female cervix (heterosexual)	38	58	10	32	0
Female rectum (heterosexual)	3	67	33	0	0
Male urethra (heterosexual)	36	39	25	36	0
Male urethra (homosexual)	16	50	31	19	0
Male rectum (homosexual)	11	55	18	18	9

Notes : All PPNG had the 2.6 Md plasmid

TABLE 8

Linkage of Plasmid Content and Auxotype

Non-PPNG

Auxotype	Plasmid Content of Strains		
	2.6 Md	2.6 + 24.5 Md	None
Wild	55	18	1
Pro-	29	6	0
Arg-	6	1	0
AHU-	6	0	0

PPNG

Auxotype	Plasmid Content of Strains			
	4.4 Md	4.4 + 24.5 Md	3.2 Md	3.2 + 24.5 Md
Wild	17	14	16	1
Pro-	34	7	6	0
Arg-	1	0	8	0

Notes : All PPNG had the 2.6 Md plasmid

perhaps surprising that it was the wild type rather than the pro- auxotype that was correlated with the presence of the conjugal plasmid. However this may be easily explained. Only a proportion of strains harbouring the 4.4 Md plasmid also had the conjugal plasmid. Of these fourteen out of twenty five were wild type auxotype. The remainder of strains which did not possess the conjugal plasmid were largely pro- (thirty two out of forty nine). Thus, despite the linkage of 4.4 Md plasmid incidence with both the 24.5 Md plasmid and pro- auxotype, very few pro- strains actually contained both plasmids. No linkage of plasmid and auxotype was observed in non-PPNG.

3.2.4. Penicillin MIC of Non-PPNG

The mode MIC of penicillin for each group is shown in table 9. Determination of these averages is shown in appendix 4. Overall, non-PPNG strains were fairly resistant to penicillin, with 61% of all strains tested showing an MIC $\geq$0.125 mg/l of penicillin. Female cervical isolates showed the greatest incidence of sensitive strains (mode MIC 0.015 mg/l) whilst rectal isolates from male homosexuals were the most frequently resistant (mode MIC 0.25 mg/l). All other groups possessed the same mode MIC (0.125 mg/l). AHU- strains are characteristically sensitive to penicillin (Handsfield et al., 1980). In this study, five out of six AHU- strains were inhibited by penicillin at a concentration of $\leq$0.06 mg/l.

TABLE 9

Penicillin Sensitivity Testing for Non-PPNG

	Number tested	Mode MIC (mg/l)
Female cervix (heterosexual)	26	0.015
Female rectum (heterosexual)	25	0.125
Male urethra (heterosexual)	27	0.125
Male urethra (homosexual)	25	0.125
Male rectum (homosexual)	26	0.25

A plot of penicillin concentration versus number of strains with an MIC of that concentration showed that there were two concentrations at which peaks occurred; at 0.015 mg/l and 0.125 mg/l (figure 2). These two peaks occurred, to some extent in strains from all isolation sites, although in rectal isolates from male homosexuals, the second peak was misplaced slightly to 0.25 mg/l and rectal isolates from females showed a third small peak at 1.0 mg/ml. A comparison of the two individual plots of strains from hetero- and homosexuals showed the two groups did not differ substantially in their level of resistance to penicillin (figure 3). The same could be said when rectal isolates were compared to isolates from the urethra and cervix (figure 4). Here the shape of the two plots differed only in the small peak in the incidence of resistance at the penicillin concentration 1.0 mg/l.

NOTES TO FIGURE 2

x axis = Concentration of Penicillin-G in mg/l

A = 2.0
B = 1.0
C = 0.5
D = 0.25
E = 0.125
F = 0.06
G = 0.03
H = 0.015
I = 0.0075

y axis = Number of strains with an MIC of that concentration

FIGURE 2

PENICILLIN MICs OF NON-PPNG

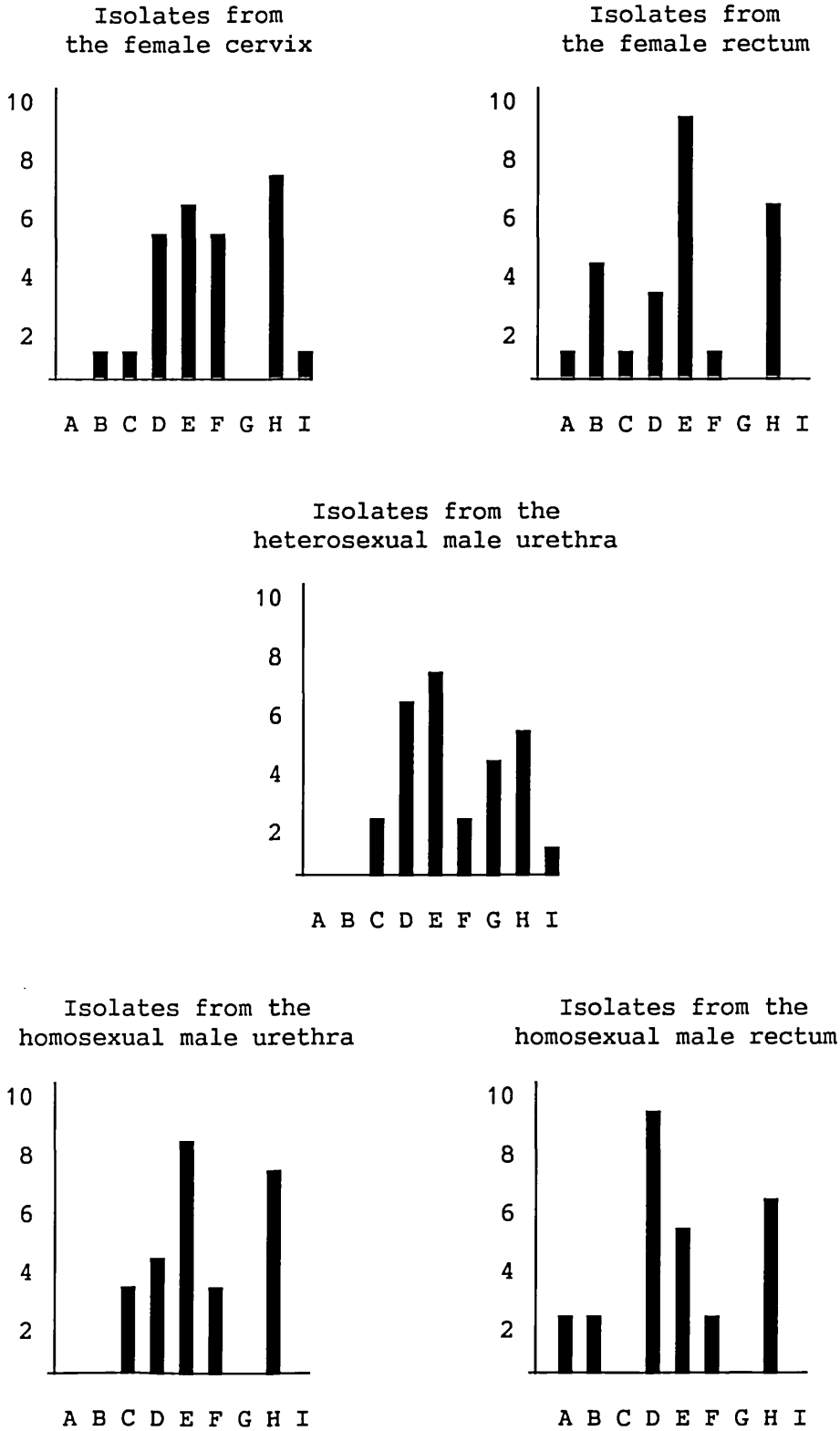
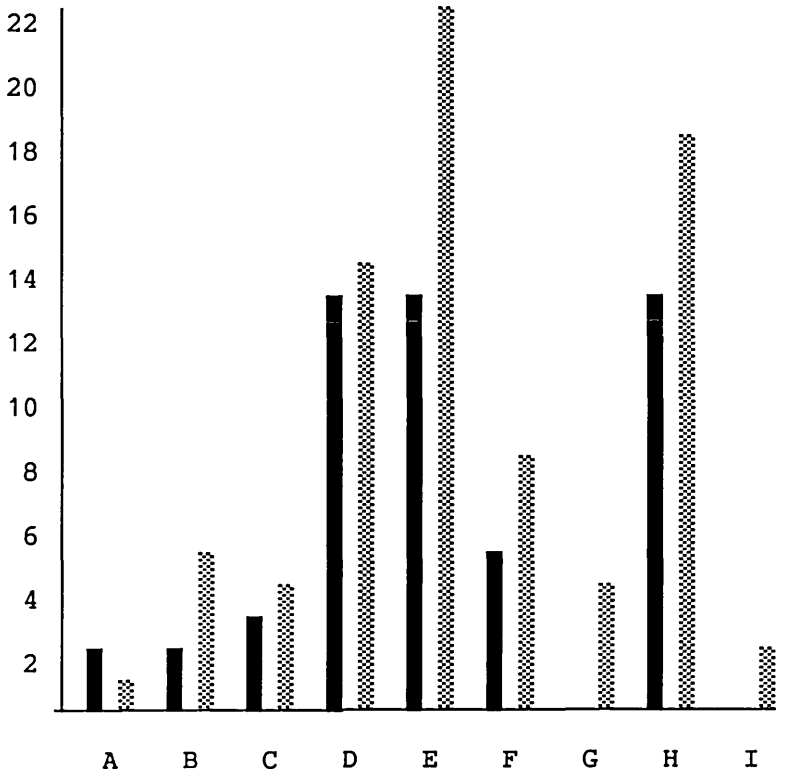


FIGURE 3

PENICILLIN MICs OF NON-PPNG ISOLATED FROM HOMOSEXUALS AND HETEROSEXUALS



Notes : x axis = Concentration of Penicillin-G in mg/l

A = 2.0
B = 1.0
C = 0.5
D = 0.25
E = 0.125
F = 0.06
G = 0.03
H = 0.015
I = 0.0075

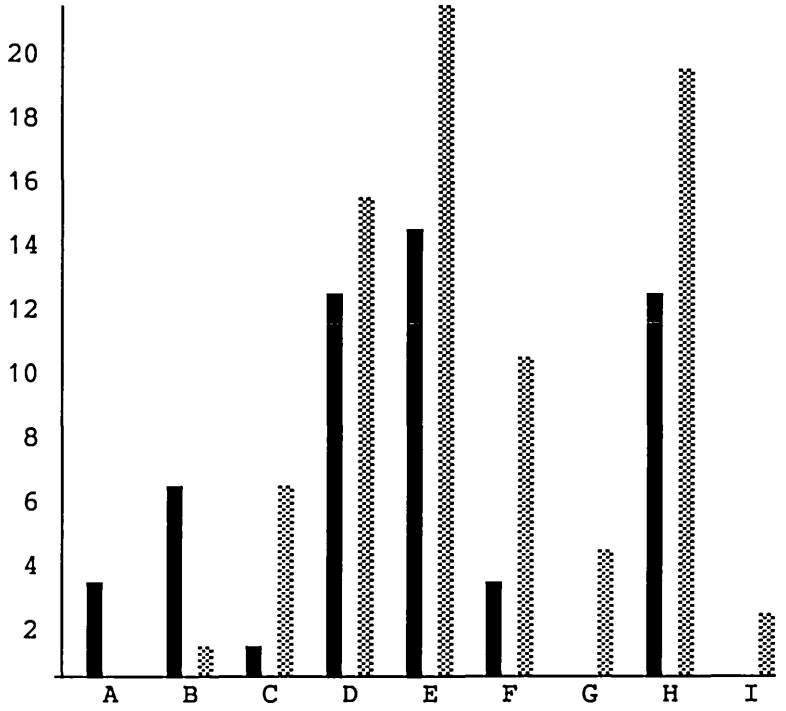
y axis = Number of strains with an MIC of that concentration

■ Strains from homosexuals

▒ Strains from heterosexuals

FIGURE 4

PENICILLIN MICs OF NON-PPNG:
A COMPARISON OF RECTAL AND NON-RECTAL ISOLATES



Notes : x axis = Concentration of Penicillin-G in mg/l

A = 2.0
B = 1.0
C = 0.5
D = 0.25
E = 0.125
F = 0.06
G = 0.03
H = 0.015
I = 0.0075

y axis = Number of strains with an MIC of that concentration

Strains from the rectum

Strains from other sites

3.3. ENVELOPE PHENOTYPE

The role of the mtr mutation in reducing the permeability of N. gonorrhoeae to hydrophobic molecules has been mentioned (see section 1.4.2.). The observation by Morse et al. (1982) that this mutation was more common in isolates from homosexuals might indicate that the mutation is selected by and may help to protect the bacterium from the hydrophobic environment of the rectum. It has also been suggested that the mutation is uncommon in PPNG, hence the scarcity of PPNG infections in homosexuals (Handsfield et al., 1982b). In view of this, the envelope phenotypes of all PPNG and non-PPNG strains were determined.

The envelope phenotypes of all isolates are shown in table 10. The incidence of the mtr envelope phenotype varied greatly between PPNG and non-PPNG strains. 47% of PPNG were of this phenotype as compared to 22% of non-PPNG. This difference was statistically significant ($p < 0.001$) and remained if the incidence of the mtr phenotype was compared between PPNG and non-PPNG isolated from the same site. In this way, the percentage of penicillinase producing mtr strains isolated from the female cervix (45%) was significantly different ($p < 0.05$) from the number in non-PPNG strains isolated from the same site (19%). Urethral isolates from both homo- and heterosexuals also showed this difference; 88% of PPNG from homosexual male urethras were mtr compared with 16% of non-PPNG from this site ($p < 0.001$) and for urethral isolates from heterosexuals, 33% of PPNG were mtr compared with 11% of non-PPNG

TABLE 10

Results of Envelope Phenotype Testing

	Number tested	Percentage of Strains of Phenotype mtr	non-mtr
Non-PPNG			
Female cervix (heterosexual)	26	19	81
Female rectum (heterosexual)	25	32	68
Male urethra (heterosexual)	27	11	89
Male urethra (homosexual)	25	16	84
Male rectum (homosexual)	26	31	69
PPNG			
Female cervix (heterosexual)	38	45	55
Female rectum (heterosexual)	3	33	67
Male urethra (heterosexual)	36	33	67
Male urethra (homosexual)	16	88	12
Male rectum (homosexual)	11	45	55

($p < 0.05$).

3.3.1. mtr and Site

In PPNG the greatest number of mtr strains was found in homosexual male urethral isolates (fourteen out of sixteen). Rectal isolates from homosexual men and isolates from female cervices had the same incidence of mtr (45%), whilst isolates from heterosexual men showed the fewest (33%). Thus, there was no obvious correlation of the incidence of mtr and site of isolation. By contrast, in non-PPNG the mtr phenotype was obviously correlated with the site of isolation. The phenotype was most prevalent in rectal isolates (male 31%, female 32%) which was significantly different from the incidence in the urethra ($p < 0.05$). Urethral isolates, together with those from the cervix had a similar frequency of the mtr phenotype (female cervix 19%, heterosexual and homosexual male urethra 13%). Thus in non-PPNG, the incidence of the mtr phenotype was correlated with isolation from the rectum.

3.3.2. mtr and Sexuality

In PPNG, the incidence of the mtr phenotype was correlated with the sexuality of the patient. 70% of isolates from homosexuals, compared with 39% from heterosexuals were mtr ($p < 0.01$). However, the incidence of mtr in homosexual male rectal isolates (45%) did not differ significantly from that of all isolates from heterosexuals

(39%), whereas the homosexual male urethral isolates were significantly different from all others ($p < 0.001$ for all isolates from heterosexuals, $p < 0.05$ for homosexual male rectal isolates). Non-PPNG showed no relationship between patient sexuality and mtr phenotype. The percentage of mtr strains isolated from the urethras of male homosexuals was almost half that seen for rectal isolates from homosexuals (16 and 31% respectively). This, together with the high incidence of mtr seen in strains isolated from female rectums is an indication that, for non-PPNG, patient sexuality was not an important determinant of envelope phenotype.

3.3.3. Linkage with Plasmid Type

The data used to analyse the linkage of mtr with plasmid carriage are shown in table 11. The mtr phenotype was positively correlated with the carriage of the 4.4 Md plasmid. 60% of all strains carrying the 4.4 Md plasmid were mtr as compared to only 16% of strains carrying the 3.2 Md plasmid ($p < 0.001$). It was not linked with any other plasmid combination.

3.3.4. mtr and Auxotype

The data used to analyse the linkage of mtr with auxotype are shown in table 12. For non-PPNG strains, the mtr phenotype occurred significantly more frequently in strains that had pro- auxotype, 53% of all mtr strains had this auxotype. Owing to the small total number

TABLE 11

Linkage of mtr Phenotype and Plasmid Content

Non-PPNG

Plasmid Content	Envelope Phenotype of Strains: mtr	non-mtr
2.6 Md	21	76
2.6 + 24.5 Md	5	20
None	0	2

PPNG

Plasmid Content	Envelope Phenotype of Strains: mtr	non-mtr
4.4 Md	32	20
4.4 + 24.5 Md	12	9
3.2 Md	5	25
3.2 + 24.5 Md	0	1

Notes : All PPNG had the 2.6 Md plasmid

TABLE 12

Linkage of mtr Phenotype and Auxotype

Non-PPNG

Auxotype	Envelope Phenotype of Strains	
	mtr	non-mtr
Wild	10	68
Pro-	15	21
Arg-	3	4
AHU-	0	6

PPNG

Auxotype	Envelope Phenotype of Strains	
	mtr	non-mtr
Wild	24	24
Pro-	25	22
Arg-	0	9

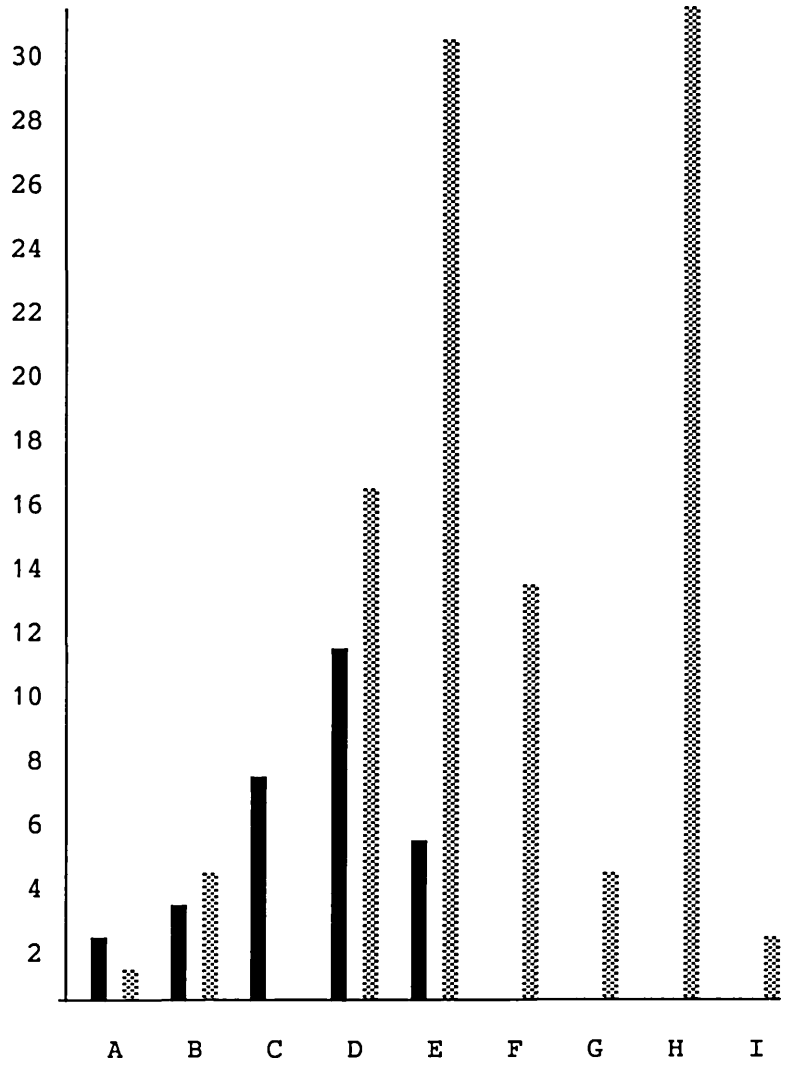
of non-PPNG strains that had the pro- auxotype (28%), this result was highly significant ($p < 0.001$). No linkage of auxotype and mtr was seen in PPNG, where 50% of mtr strains and 40% of all PPNG were proline requiring. None of nine arg- PPNG strains had the mtr phenotype; this showed reverse linkage i.e., that the concurrence of the arg- auxotype and mtr phenotype was significantly rare ($p < 0.01$). This was probably coincidental and not causal, as the arg- auxotype was associated with carriage of the 3.2 plasmid (see section 3.2.3.) and the mtr phenotype was found rarely in strains carrying this plasmid (see section 3.3.3.) and furthermore, it was not seen in non-PPNG, where three out of four arg- strains were mtr.

3.3.5. Penicillin MIC and mtr Phenotype

The mtr mutation causes an elevation in resistance to penicillin, by 2 - 4 fold over the wild type. In these tests, none of the mtr strains had a penicillin MIC below 0.125 mg/l, therefore they all fell into the resistant or intermediate resistance class, except the control strain FA171, which had an MIC of 0.015 mg/l (this value is identical to that found by Sparling et al., 1975). For non-mtr strains, 49% had an MIC $\leq$0.06 mg/l which is classed as sensitive. A plot of the data from the two groups showed that whilst wild type strains retained the two peak plot described in section 3.2.4., the mtr strains showed only one peak at 0.25 mg/l (figure 5).

FIGURE 5

PENICILLIN MICs OF NON-PPNG ACCORDING TO ENVELOPE PHENOTYPE



Notes : x axis = Concentration of Penicillin-G in mg/l

A = 2.0
B = 1.0
C = 0.5
D = 0.25
E = 0.125
F = 0.06
G = 0.03
H = 0.015
I = 0.0075

y axis = Number of strains with an MIC of that concentration

■ Strains with mtr phenotype

▒ Strains without mtr phenotype

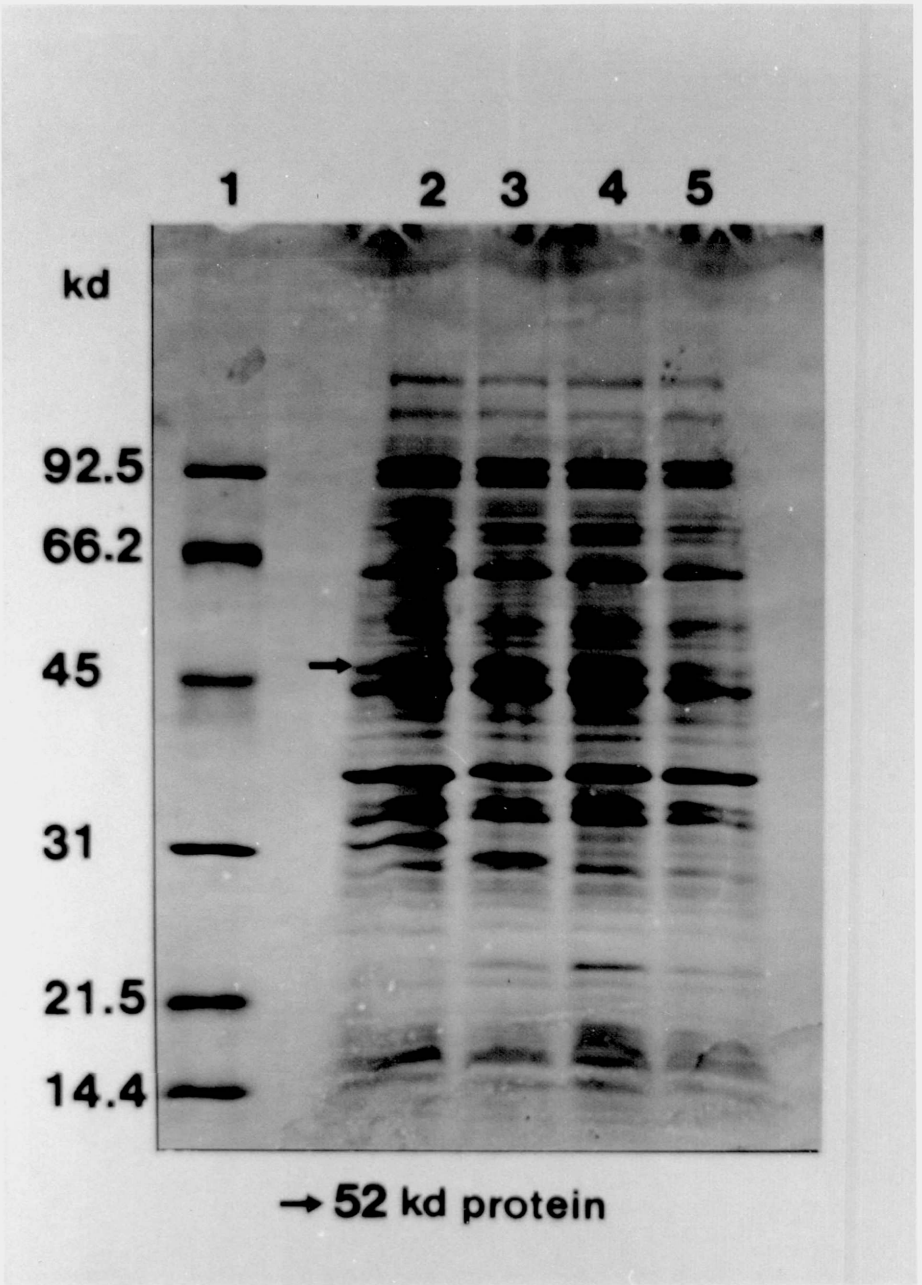
3.3.6. Transformation of the mtr Phenotype

The mtr phenotype was shown to be transformable. mtr control strain FA171, non-PPNG strain no. 37 and PPNG strain no. 35 were all capable of transforming non-mtr control strain H1 to resistance to the four hydrophobic compounds used to determine envelope phenotype. However, SDS-PAGE of sarkosyl extracts of the OMs from the untransformed control strains H1 and FA171 did not show any differences in the amount of the 52 Kd protein present, nor could any increase in the amount of this protein be detected in the H1 transformants (figure 6). Thus the mtr status of these strains could not be proven.

It is possible the reason that no difference is seen between the amounts of the 52 kd protein present in the OMs of FA171 and H1 is that this protein is independent of the mtr phenotype. Analysis of more strains, both mtr and wild type, is required to clarify the relationship between the mtr phenotype and the 52 kd protein.

FIGURE 6

SDS-PAGE OF SARKOSYL EXTRACTS OF N. GONORRHOEAE



Lane 1 : BioRad low MW markers
2 : FA171 (mtr control strain)
3 : H1 (non-mtr control strain)
4 : Transformant (H1 x PPNG mtr)
5 : Transformant (H1 x non-PPNG mtr)

3.4. OLEIC ACID RESISTANCE

The mtr phenotype decreases the permeability of N. gonorrhoeae to hydrophobic molecules; however, another mechanism may operate specifically in resistance to fatty acids, rendering the possession of an mtr mutation less important (see section 1.4.2.). Fatty acids are present at both the cervix and the rectum and their inhibitory properties may affect the ability of N. gonorrhoeae to infect either of these sites. The resistance of all strains to growth inhibition by oleic acid, which is present at both sites, was determined. The results are shown in table 13. Although no significant difference was seen in the total incidence of resistance between non-PPNG and PPNG (36% and 29%, respectively), there was variation in the incidence of resistance both between and within the two groups. This will be outlined in the following section.

3.4.1. Oleic Acid Resistance and Isolation Site

As oleic acid is present at both the cervix and rectum, it was expected that bacteria isolated from these sites would have been exposed to this molecule in vivo and consequently would be more resistant to its inhibitory action than organisms from the urethra. Examination of the results obtained shows clearly that this was not the case. For both PPNG and non-PPNG, perhaps surprisingly, the highest percentage of resistant organisms isolated were from the urethras of male homosexuals (63% and 48%, respectively).

TABLE 13

Results of Oleic Acid Sensitivity Testing

	Number tested	Percentage of Strains Oleic Acid Resistant	Sensitive
Non-PPNG			
Female cervix (heterosexual)	25	44	56
Female rectum (heterosexual)	25	28	72
Male urethra (heterosexual)	25	24	76
Male urethra (homosexual)	25	48	52
Male rectum (homosexual)	21	38	62
PPNG			
Female cervix (heterosexual)	38	18	82
Female rectum (heterosexual)	3	67	33
Male urethra (heterosexual)	36	17	83
Male urethra (homosexual)	16	63	37
Male rectum (homosexual)	11	45	55

Penicillinase producing rectal isolates showed an incidence of resistance elevated above that of penicillinase producing cervical and urethral isolates from heterosexuals. The incidence of resistance in the latter two groups was low and virtually identical which was not as predicted. The situation was reversed in non-PPNG; cervical isolates were only slightly less resistant than those from homosexual male urethras (44% compared with 48%) and both were considerably more often resistant than rectal isolates (33%). As seen for PPNG, heterosexual male urethral isolates showed the lowest level of resistance. Comparing PPNG and non-PPNG, the most striking and also the only significant difference was seen in the resistance of cervical isolates (18% and 44% respectively, $p < 0.05$).

3.4.2. Oleic Acid Resistance and Sexuality

There was a definite correlation between oleic acid resistance and homosexuality. Combined results of PPNG and non-PPNG strains showed that 48% of strains from homosexuals and 26% of strains from heterosexuals were oleic acid resistant ($p < 0.001$). This correlation was absent for non-PPNG alone, where comparison of strains according to the sexuality of the patient they were isolated from gave no significant differences. In PPNG, however, the correlation remained, with total homosexual differing from total heterosexual isolates ($p < 0.001$) and homosexual urethral differing from heterosexual urethral isolates ($p < 0.01$). As with the *mtr* data, isolates from homosexual male urethras had a greater proportion of resistant strains (63%) than all other PPNG (23%) ($p < 0.01$).

3.4.3. Oleic Acid Resistance and mtr Phenotype

The possibility that the mtr phenotype and resistance to oleic acid were linked was examined. As the mtr mutation confers low level resistance to hydrophobic molecules, it was possible that strains with the mtr envelope phenotype would be more likely to be resistant to oleic acid. The data used to analyse whether such a link existed are shown in tables 14 - 16. In this study, 54% of all mtr strains were oleic acid resistant, whilst in non-mtr strains the proportion was only 22%. This difference was significant ($p < 0.001$) indicating that the two phenotypes were in some way linked (table 14). Analysis of non-PPNG and PPNG (table 14) showed that this was also the case when the two groups were assessed separately ($p < 0.001$ for both non-PPNG and PPNG). However, if the PPNG strains were split according to the sexuality of the patient from which they were isolated (homosexual and heterosexual), the strains from heterosexuals no longer showed any linkage ($p < 0.5$), whilst the strains from homosexuals retained it ($p < 0.01$) (table 15). In non-PPNG, both isolates from heterosexuals and homosexuals showed a positive correlation between the coincidence of the phenotypes ($p < 0.01$ and $p < 0.001$, respectively) (table 16). Also, in non-PPNG isolated from homosexuals it was perhaps notable that the correlation between oleic acid resistance and mtr phenotype was absolute, with zero out of nine mtr strains being oleic acid sensitive; whilst in PPNG from homosexuals, over a quarter (five out of nineteen) mtr strains were sensitive.

TABLE 14

Linkage of Oleic Acid Resistance and mtr Phenotype

All Isolates

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	40	34
non-*mtr*	32	115

Non-PPNG

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	19	7
non-*mtr*	25	70

PPNG

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	21	27
non-*mtr*	7	45

TABLE 15

Linkage of Oleic Acid Resistance and mtr Phenotype in PPNG

Isolates from Heterosexuals

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	7	22
non-mtr	6	38

Isolates from Homosexuals

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	14	5
non-mtr	1	7

TABLE 16

Linkage of Oleic Acid Resistance and mtr Phenotype in Non-PPNG

Isolates from Heterosexuals

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	10	7
non-mtr	14	44

Isolates from Homosexuals

Envelope Phenotype	Number of Strains Oleic Acid Resistant	Sensitive
mtr	9	0
non-mtr	11	26

The occurrence of fatty acid resistance in wild type strains led McFarland to propose that an mtr-independent resistance mechanism existed (McFarland et al., 1983). Of the non-mtr strains, 26% of those that were non-PPNG were also oleic acid resistant. This figure was greater than that seen for PPNG (13%). However, analysis of the results did not suggest that wild type isolates from any one site were more likely to oleic acid resistant, nor did resistance follow the same pattern within the two groups (table 17). Thus, apart from the fact that non-mtr mediated oleic acid resistance was more common in non-PPNG than PPNG, no conclusions can be reached.

3.4.4. Oleic Acid Resistance and Plasmid Profile

The data used to determine whether resistance to oleic acid was associated with carriage of any one plasmid are shown in table 18. In PPNG strains from homosexuals there was a concurrence of the 4.4 Md plasmid and oleic acid resistance which was significant ($p < 0.05$). This could be accounted for by the linkage of the 4.4 Md plasmid with the mtr phenotype (see section 3.3.3.) and, in turn, by the linkage of the mtr phenotype with oleic acid resistance (see section 3.4.3.). However, there was no correlation of oleic acid resistance with carriage of the 4.4 Md plasmid for PPNG isolates from heterosexuals, this was probably due to the absence of linkage between mtr and oleic acid resistance in these strains (see section 3.4.3.). No further linkages of plasmid type and oleic acid resistance were seen.

TABLE 17

Oleic Acid Resistance in non-mtr Strains

	Number of Strains Resistant	Sensitive	Percentage Resistant
Non-PPNG			
Female cervix (heterosexual)	7	13	35
Female rectum (heterosexual)	2	14	12
Male urethra (heterosexual)	5	17	23
Male urethra (homosexual)	8	13	38
Male rectum (homosexual)	3	13	19
PPNG			
Female cervix (heterosexual)	3	18	14
Male urethra (heterosexual)	3	20	13
Male urethra (homosexual)	0	2	0
Male rectum (homosexual)	1	5	17

TABLE 18

Linkage of Oleic Acid Resistance with Plasmid Content

Non-PPNG

Plasmid Content	Number of Strains Oleic Acid Resistant	Sensitive
2.6 Md	33	61
2.6 + 24.5 Md	10	14
None	0	2

PPNG

Plasmid Content	Number of Strains Oleic Acid Resistant	Sensitive
4.4 Md	17	34
4.4 + 24.5 Md	7	14
3.2 Md	6	24
3.2 + 24.5 Md	0	1

Notes : All PPNG had the 2.6 Md plasmid

3.4.5. Oleic Acid Resistance and Auxotype

The data used to assess linkage between oleic acid resistance and auxotype are shown in table 19. In non-PPNG strains oleic acid resistance was linked to the pro- auxotype ($p < 0.001$). This was presumably due to the linkage of *mtr* with proline requirement and oleic acid resistance. In support of this, no such link was found for PPNG strains which do not show linkage of *mtr* with either the pro- auxotype or, in the case of those isolated from heterosexuals, oleic acid resistance (see sections 3.3.4. and 3.4.3.). In non-PPNG, oleic acid resistance occurred infrequently with the wild type auxotype, this was significant ($p < 0.05$) due to the large percentage of these strains which were this auxotype.

TABLE 19

Linkage of Oleic Acid Resistance with Auxotype

Non-PPNG

Auxotype	Number of Strains Oleic Acid Resistant	Sensitive
Wild	21	53
Pro-	20	13
Arg-	3	3
AHU-	0	6

PPNG

Auxotype	Number of Strains Oleic Acid Resistant	Sensitive
Wild	13	34
Pro-	15	32
Arg-	2	7

Notes : All PPNG had the 2.6 Md plasmid

3.5. RESISTANCE TO BACTEROIDES

It is thought that, in some cases, the normal flora of the host may aid host immunity to gonorrhoea by preventing the gonococcus from growing. Inhibition of N. gonorrhoeae by a number of bacterial species has been shown in vitro (see section 1.4.4.). One study found a reduced incidence of gonorrhoea in women who carried inhibitory lactobacilli amongst their normal cervical flora (Saigh et al., 1978). The rectal environment is heavily colonised by bacteria, some of which may compromise infection by N. gonorrhoeae. In order to determine whether sensitivity to normal flora might affect rectal colonisation, the sensitivity of all strains of N. gonorrhoeae to members of the genera Bacteroides, which represent a large part of the normal bowel flora, was examined.

3.5.1. Resistance to B. fragilis

The sensitivities of all strains of N. gonorrhoeae to B. fragilis are shown in table 20. The majority tested were sensitive to inhibition by B. fragilis. As a group, non-PPNG were significantly more often resistant than PPNG ($p < 0.05$). The greatest frequency of resistance was shown by non-PPNG strains from homosexual male rectums (14%), with isolates from all other sites (apart from PPNG from female rectums) showing incidences of <10 % and in the case of PPNG from female cervices and male homosexuals, 0%. No significant differences existed for isolates from different sites within the two groups or

TABLE 20

Results of B. fragilis Sensitivity Testing

	Number tested	Percentage of Strains B. fragilis Resistant	Sensitive
Non-PPNG			
Female cervix (heterosexual)	25	4	96
Female rectum (heterosexual)	24	8	92
Male urethra (heterosexual)	26	8	92
Male urethra (homosexual)	24	8	92
Male rectum (homosexual)	21	14	86
PPNG			
Female cervix (heterosexual)	37	0	100
Female rectum (heterosexual)	3	33	67
Male urethra (heterosexual)	34	3	97
Male urethra (homosexual)	16	0	100
Male rectum (homosexual)	11	0	100

between the two groups when isolates from the same site were compared. Combined results from all rectal isolates showed the highest frequency of resistance (10%), compared with combined urethral (5%) and cervical (2%), but none of these differences were significant. Thus, no correlation of resistance and site of isolation was indicated.

3.5.2. B. fragilis Resistance and Sexuality

In non-PPNG resistance to B. fragilis was slightly more common in isolates from homosexuals (11%) compared to isolates from heterosexuals (7%). However, this was not significant. Similarly, for PPNG, even the fact that all of the isolates from homosexuals were sensitive was insignificant due to the fact that only 3% of isolates from heterosexuals were resistant. Thus, it does not appear that the sexuality of the patient was important in the selection of N. gonorrhoeae resistant to B. fragilis.

3.5.3. B. fragilis Resistance and mtr Phenotype

The data used to establish whether resistance to B. fragilis was linked to the mtr phenotype are shown in table 21. In non-PPNG, resistance to B. fragilis was correlated with the mtr phenotype ($p < 0.05$). However, this is probably just an indication that resistance was more common in mtr strains and does not necessarily suggest a direct causal linkage as, in spite of the statistical

TABLE 21

Linkage of Resistance to B. fragilis and mtr Phenotype

Non-PPNG

Envelope Phenotype	Number of Strains B. fragilis Resistant	Sensitive
mtr	5	20
non-mtr	5	90

PPNG

Envelope Phenotype	Number of Strains B. fragilis Resistant	Sensitive
mtr	1	48
non-mtr	1	51

significance, only 20% of mtr were resistant. No such correlation existed for PPNG.

3.5.4. Other Linkages

No further linkage of resistance to B. fragilis to any other phenotype was observed (table 22).

3.5.5. Resistance to B. vulgatus

The results of the B. vulgatus sensitivity testing are shown in table 23. The incidence of resistance to inhibition by B. vulgatus varied greatly between PPNG and non-PPNG strains but for both groups it was always more common than resistance to B. fragilis. 52% of all non-PPNG were B. vulgatus resistant, this was significantly different ($p < 0.01$) from the incidence in PPNG (32%).

In non-PPNG the highest incidence of resistance was in isolates from homosexual male rectums (71%). All other sites showed similar rates, with between 43 and 54% of strains being resistant. In PPNG the greatest number of resistant organisms were isolated from the urethras of male heterosexuals (44%). 30% of isolates from female cervices were resistant and the lowest incidence of all was seen in strains from homosexuals, where only 18% were resistant. These data showed no significant differences between the rates of isolation of

TABLE 22

Linkage of Resistance to B. fragilis with Other Phenotypes

Non-PPNG

		Number of Strains B. fragilis	
		Resistant	Sensitive
Auxotype	Wild	7	65
	Pro-	3	32
	Arg-	0	6
	AHU-	0	6
Plasmid	2.6 Md	10	84
Content	2.6 + 24.5 Md	0	24
	None	0	2
Oleic Acid	Resistant	6	36
	Sensitive	4	73

PPNG

		Number of Strains B. fragilis	
		Resistant	Sensitive
Auxotype	Wild	0	48
	Pro-	2	43
	Arg-	0	8
Plasmid	4.4 Md	1	50
Content	4.4 + 24.5 Md	1	19
	3.2 Md	0	29
	3.2 + 24.5 Md	0	1
Oleic Acid	Resistant	1	31
	Sensitive	1	68

Notes : All PPNG had 2.6 Md plasmid

TABLE 23

Results of B. vulgatus Sensitivity Testing

	Number tested	Percentage of Strains B. vulgatus Resistant	Sensitive
Non-PPNG			
Female cervix (heterosexual)	23	43	57
Female rectum (heterosexual)	23	49	51
Male urethra (heterosexual)	26	54	46
Male urethra (homosexual)	24	46	54
Male rectum (homosexual)	21	71	29
PPNG			
Female cervix (heterosexual)	37	30	70
Female rectum (heterosexual)	3	33	67
Male urethra (heterosexual)	34	44	56
Male urethra (homosexual)	16	19	81
Male rectum (homosexual)	11	18	82

resistant organisms. Comparison of non-PPNG and PPNG from the same isolation site showed that, for each site, non-PPNG isolates were more likely to be resistant than the corresponding PPNG strains, although the only significant difference was between non-PPNG and PPNG isolated from the rectum ($p < 0.05$).

Combined data from PPNG and non-PPNG showed that rectal isolates had the highest incidence of resistance (50%), with urethral and cervical isolates possessing much lower rates of resistance (43 and 35% respectively). None of these differences were statistically significant. Considering the data, it does not appear that there was any site specific selection of resistance.

3.5.6. Linkage with Sexuality

The combined results for all isolates from heterosexuals and homosexuals were scarcely any different, with incidences of resistance of 42% for heterosexuals and 43% for homosexuals. In PPNG, strains from homosexuals had a low incidence of resistance (18%). This was a significantly lower frequency of resistance compared to non-PPNG from the same patient group ($p < 0.01$). 44% of PPNG isolates from heterosexual male urethras were resistant and this figure was significantly different from that of urethral isolates from homosexuals ($p < 0.05$). However, when data from males and females were combined, no significance remained, and thus there was no apparent correlation of heterosexuality with resistance.

In non-PPNG there was no significant difference between strains from homosexuals and heterosexuals, but those from homosexuals were more likely to be resistant than those from heterosexuals (58% versus 49%). Thus, no obvious linkage of resistance with sexual preference was found in either PPNG or non-PPNG.

3.5.7. Linkage with other Phenotypes

No linkage with any of the phenotypes examined was seen (see tables 24 and 25).

TABLE 24

Linkage of Resistance to B. vulgatus with Other Phenotypes in non-PPNG

		Number of Strains B. vulgatus Resistant	Sensitive
Envelope Phenotype	mtr	13	11
	non-mtr	48	45
Auxotype	Wild	39	31
	Pro-	19	16
	Arg-	2	3
	AHU-	1	5
Plasmid Content	2.6 Md	48	42
	2.6 + 24.5 Md	12	11
	None	1	1
Oleic Acid	Resistant	22	19
	Sensitive	38	37
B. fragilis	Resistant	7	3
	Sensitive	54	53

TABLE 25

Linkage of Resistance to B. vulgatus with Other Phenotypes in PPNG

		Number of Strains B. vulgatus	
		Resistant	Sensitive
Envelope Phenotype	mtr	15	34
	non-mtr	18	35
Auxotype	Wild	11	37
	Pro-	18	27
	Arg-	3	5
Plasmid Content	4.4 Md	18	33
	4.4 + 24.5 Md	6	14
	3.2 Md	8	21
	3.2 + 24.5 Md	0	1
Oleic Acid	Resistant	13	17
	Sensitive	18	52
B. fragilis	Resistant	1	1
	Sensitive	31	68

Notes : All PPNG had the 2.6 Md plasmid

3.6. PROTEIN II COMPOSITIONS OF N. GONORRHOEAE

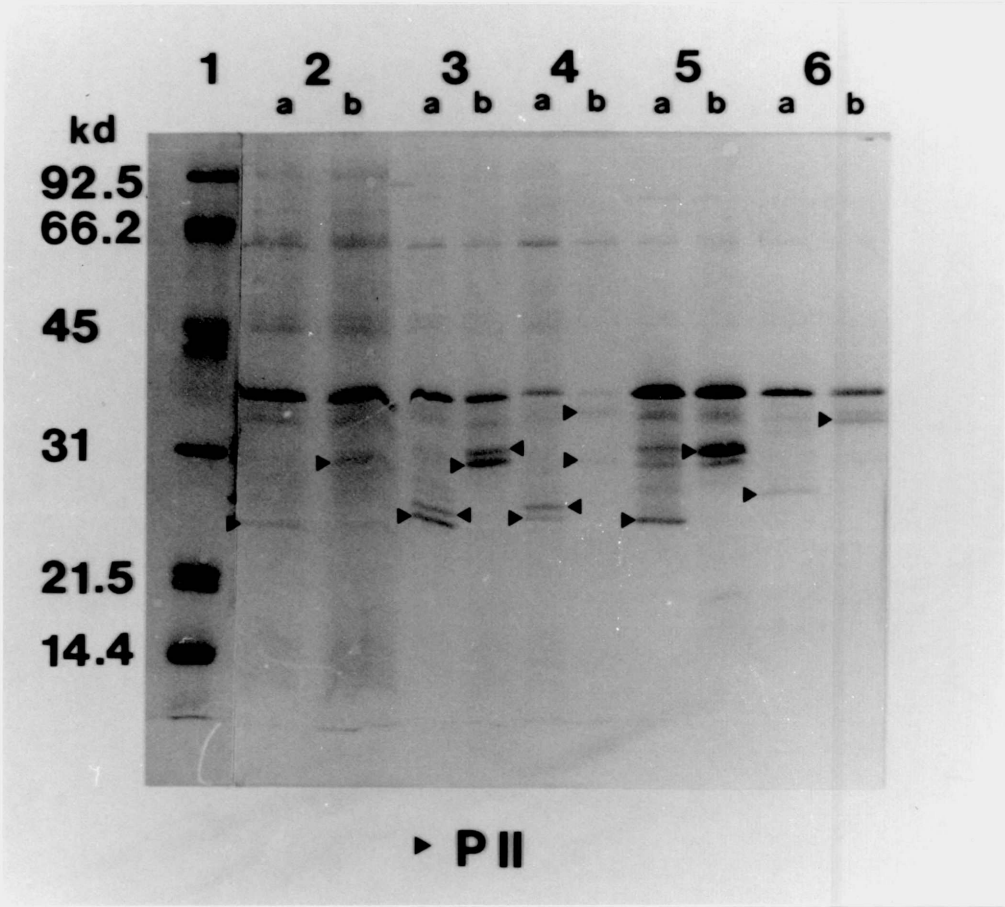
The importance of PII as a mediator of gonococcal attachment and also as possible aid to survival *in vivo* (see sections 1.3.2. and 1.4.3.) prompted a study of the PII compositions of both PPNG and non-PPNG from homosexuals and heterosexuals in order to ascertain whether any obvious differences existed which might be related either to the site of isolation or the sexuality of the patient.

3.6.1. Detection of PII

Strains from five isolation groups were chosen. These were PPNG from heterosexual male urethras, homosexual male urethras, homosexual male rectums and non-PPNG from heterosexual male urethras and homosexual male rectums. Lithium acetate extraction of the OM of *N. gonorrhoeae* yielded preparations of varying purity; although the principal proteins present in these were the major OMPs PI and PII, all samples showed some contamination with other proteins, particularly those of higher MW. PII was detected by solubilising the lithium acetate extracted membranes at two temperatures, 37°C and 100°C, and observing modifications of MW of protein bands in the range 20 - 30 kd. Figures 7 - 9 represent photographs of SDS-PAGE of lithium acetate extracts of several strains showing clearly the heat-modification of PII MW. Incomplete conversion from slow to fast migrating forms has been reported previously by Swanson (1982); in this study, even treatment at 100°C for one hour did not completely

FIGURE 7

SDS-PAGE OF LITHIUM ACETATE EXTRACTS OF PPNG ISOLATED FROM URETHRAS OF MALE HOMOSEXUALS

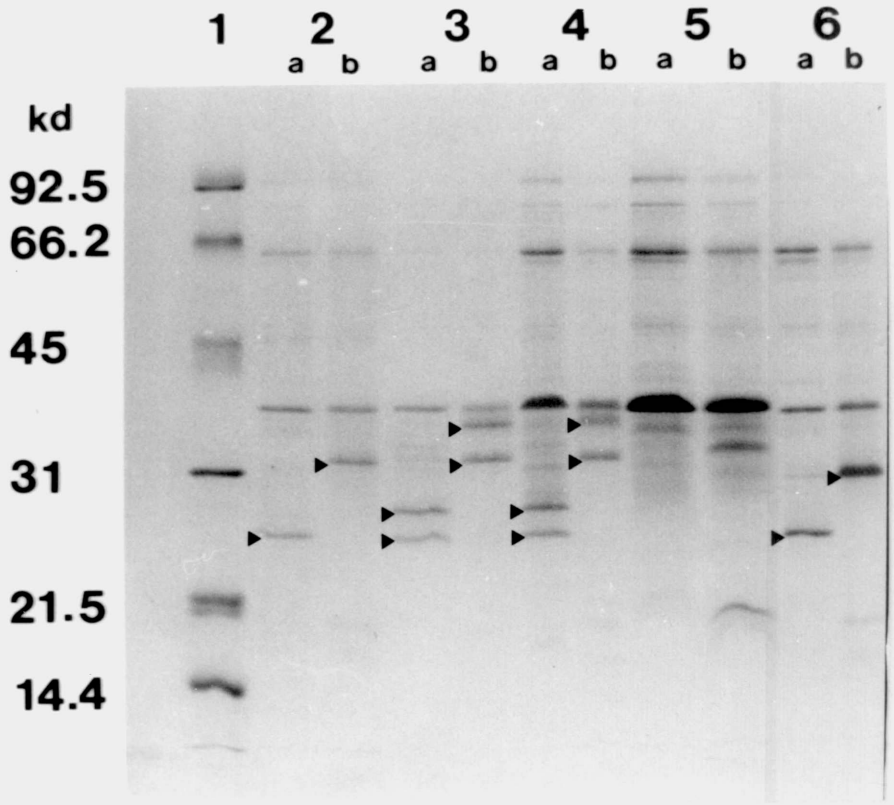


Lane 1: BioRad low MW markers
2: PPNG no. H10
3: PPNG no. H12
4: PPNG no. H13
5: PPNG no. H14
6: PPNG no. H16

Lane a = solubilisation at 37°C
Lane b = solubilisation at 100°C

FIGURE 8

SDS-PAGE OF LITHIUM ACETATE EXTRACTS OF NON-PPNG ISOLATED FROM RECTUMS OF MALE HOMOSEXUALS

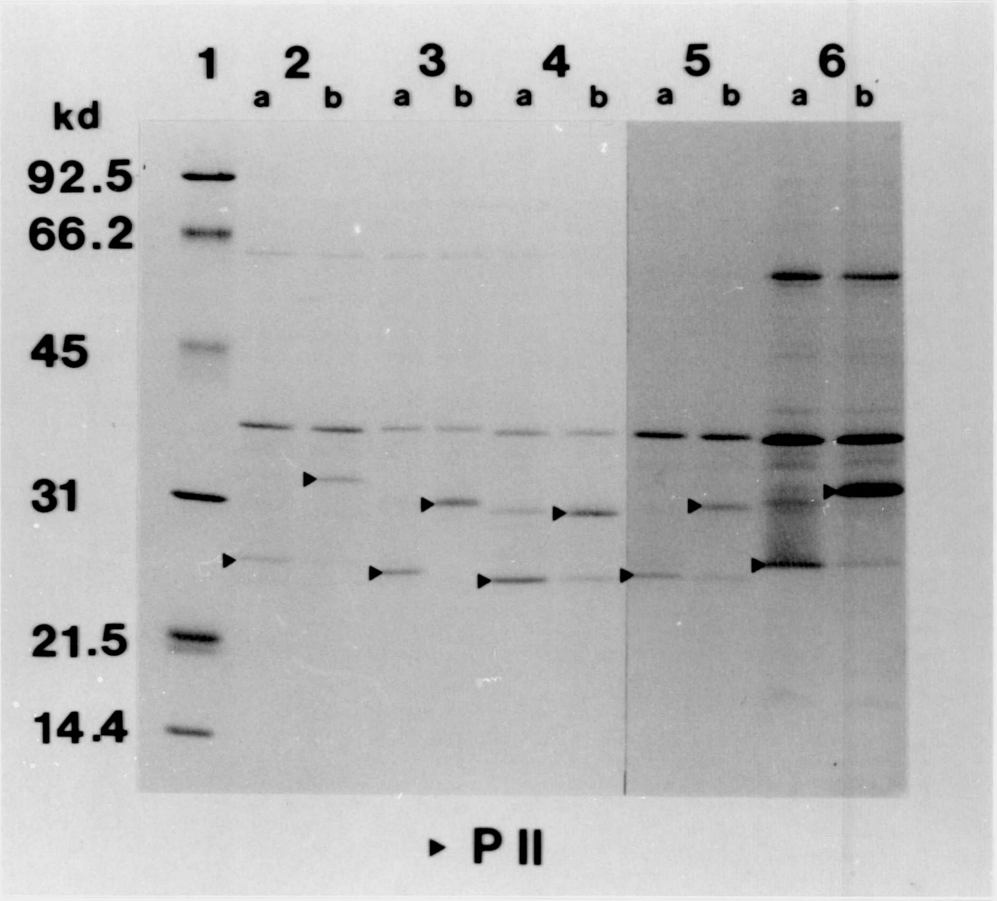


Lane 1: BioRad low MW markers
2: Non-PPNG no. 117
3: Non-PPNG no. 133
4: Non-PPNG no. 135
5: Non-PPNG no. 136
6: Non-PPNG no. 137

Lane a = solubilisation at 37°C
Lane b = solubilisation at 100°C

FIGURE 9

SDS-PAGE OF LITHIUM ACETATE EXTRACTS OF N. GONORRHOEAE ISOLATED FROM URETHRAS OF MALE HETEROSEXUALS



Lane 1: BioRad low MW markers
2: Non-PPNG no. 17
3: Non-PPNG no. 31
4: Non-PPNG no. 49
5: PPNG no. 478
6: PPNG no. 581

Lane a = solubilisation at 37°C
Lane b = solubilisation at 100°C

convert some of the PIIs from the slow to the fast migrating form (see figure 9, lane 4, strain no. 49). Due to this incomplete MW conversion of some PIIs and the presence of contaminating protein bands of around 30 kd, all MWs cited in the text refer to the observed MW of the protein when solubilised at 37°C. Also, for the purpose of this text and in the absence of any other means of judging, PIIs with the same MW were classified as being the same species.

3.6.2. Distribution of PII Species in N. gonorrhoeae

All twenty seven strains of N. gonorrhoeae examined in this study possessed one or two PIIs with MWs which varied from 22 - 29 Kd. Twenty one PIIs with different MW were observed. The MWs and strains from which these were isolated are listed in table 26. Table 27 is a list of PIIs present in strains divided according to whether they produce penicillinase, the site they were isolated from and the sexuality of the patient from which they were isolated. Table 28 shows a summary of all the data collected. It illustrates the great variability of PII and demonstrates that isolates from the same site, from patients of the same sexuality or from PPNG and non-PPNG do not show much similarity in their PII compositions. All these data reinforce the finding of other studies that the expression of PII is subject to considerable variation (Zak et al., 1984; Schwalbe et al., 1985).

TABLE 26

PII Analysis

PPNG from homosexual male rectums

Strain No.	MW PII (Kd)
H7	27.2, 24.5
H11	26.9
H15	27.0
H17	26.0, 25.0
H18	27.0, 26.0
H19	27.8, 26.6

PPNG from heterosexual male urethras

Strain No.	MW PII (Kd)
478	24.0
506	27.0, 25.0
536	27.0, 25.0
581	25.4
645	26.6, 25.0

PPNG from homosexual male urethras

Strain No.	MW PII (Kd)
H10	24.0
H12	26.6, 25.0
H13	26.3, 25.7
H14	25.0
H16	28.2

Non-PPNG from heterosexual male urethras

Strain No.	MW PII (Kd)
17	25.0, 23.7
31	24.0
49	22.9
52	24.0, 22.0
56	23.7, 22.9
59	24.0

Non-PPNG from homosexual male rectums

Strain No.	MW PII (Kd)
117	25.7
133	27.2, 25.0
135	27.2, 25.0
136	22.9
137	24.5

TABLE 27

The Distribution of PII

MW (Kd) of PII in PPNG		MW (Kd) of PII in non-PPNG
28.2	26.0	27.2
27.8	25.7	25.7
27.2	25.4	25.0
27.0	25.0	24.5
26.9	24.5	24.0
26.6	24.0	23.7
26.3		22.9
		22.0

MW (Kd) of PII in N. gonorrhoeae from the urethra		MW (Kd) of PII in N. gonorrhoeae from the rectum	
28.2	25.0	27.8	26.0
27.0	24.0	27.2	25.7
26.6	23.7	27.0	25.0
26.3	22.9	26.9	24.5
25.7	22.0	26.6	22.9
25.4			

MW (Kd) of PII in N. gonorrhoeae from heterosexuals	MW (Kd) of PII in N. gonorrhoeae from homosexuals	
27.0	28.2	26.0
26.6	27.8	25.7
25.4	27.2	25.0
25.0	27.0	24.5
24.0	26.9	24.0
23.7	26.6	22.9
22.9	26.3	
22.0		

TABLE 28

Summary of PII Analysis

	No. of isolates	No. of different PII	No. of PII /isolate	No. of combinations of PII
PPNG isolates	16	13	1 - 2	13
Non-PPNG isolates	11	8	1 - 2	8
Isolates from heterosexuals	11	8	1 - 2	8
Isolates from homosexuals	16	13	1 - 2	15
Urethral isolates	16	11	1 - 2	11
Rectal isolates	11	10	1 - 2	10

Eight PII species occurred in strains from more than one isolation group (see table 29). One PII (25 Kd) was seen in at least one isolate from all five groups. Five other PIIs were restricted as to the groups in which they occurred, i.e., they occurred only in isolates from one site or only in PPNG or non-PPNG. Two PIIs (MW 27.2 and 24.5 Kd) were only seen in rectal isolates. The 27.2 Kd MW protein occurred in three strains (two non-PPNG and one PPNG) whilst the 24.5 Kd MW PII was observed in one PPNG and one non-PPNG strain. One PII (24 Kd) occurred in three non-PPNG and two PPNG strains isolated from the urethra. One isolate from each of the PPNG isolation groups possessed a PII with MW 26.6 Kd which was not present in any non-PPNG. One PII was exclusive to non-PPNG; three strains (two urethral and one rectal isolate) expressed a PII with MW 22.9 Kd.

In this limited analysis, PII specific to both site and PPNG/non-PPNG were identified. However, no PII was present in every single PPNG or non-PPNG examined, nor was any one PII present in every single strain from one site, neither was any PII confined to homosexuals or heterosexuals. Furthermore, it is likely that an extension of this PII analysis would show that even the PII shown to be restricted by site or otherwise in their occurrence are more widespread than has been demonstrated.

TABLE 29

PII Which Occur in More Than One Isolation Group

MW of PII (Kd)	Present in Group No.
27.2	2 and 5
27.0	3 and 5
26.6	3, 4 and 5
25.7	2 and 4
25.0	1, 2, 3, 4 and 5
24.5	2 and 5
24.0	1, 3 and 4
22.9	1 and 2

Group numbers : 1 = Non-PPNG from heterosexual male urethras

2 = Non-PPNG from homosexual male rectums

3 = PPNG from heterosexual male urethras

4 = PPNG from homosexual male urethras

5 = PPNG from homosexual male rectums

3.7. SURFACE HYDROPHOBICITY OF SPECTINOMYCIN RESISTANT N. GONORRHOEAE

The hydrophobicity of the bacterial cell surface is determined principally by LPS and OMPs (see section 1.2.7.) and changes in these molecules may manifest themselves in changes in hydrophobicity. As the composition of the OM may be an important factor in gonococcal survival *in vivo*, alterations in hydrophobicity may indicate that potentially significant changes in the composition of the OM have taken place. The existence of a number of phenotypically identical spectinomycin resistant PPNG and non-PPNG afforded an excellent opportunity to examine any effect of the acquisition or loss of the 4.4 Md plasmid on the hydrophobicity of the bacterial cell surface, assuming the non-PPNG to represent isolates cured of the penicillinase plasmid by natural means. Thus, the TPP experiments were not carried out to examine the hydrophobicity of *N. gonorrhoeae per se* but rather as a means of detecting any gross differences existing between the PPNG and the 'cured' non-PPNG. Whilst differences would not necessarily infer that alterations of important surface structures had taken place, there is no doubt that such changes could affect surface hydrophobicity.

The results of the experiments are shown in table 30. The partition of the hydrophobic control strain, *S. typhimurium* Re mutant as determined by the ratio of counts in the top hydrophilic phase to those in the bottom hydrophobic phase often approached or exceeded unity. Ideally, as the LPS of the Re mutant comprises only lipid A and KDO and is, therefore, the roughest and most hydrophobic LPS

TABLE 30

Partitioning of Spectinomycin Resistant
N. gonorrhoeae in an Aqueous Two Phase System

	Strain No.	Counts in top phase / Counts in bottom phase
PPNG	396	3.8
	411	0.85
	412	0.55
	416	7.55
	449	1.4
Non-PPNG	520	2.55
	534	3.15
	644	3.05
	656	4.5
	663	6.5
	684	2.4
Controls*	Hydrophobic	0.77
	Hydrophilic	14.0

Notes : All results are average of two experiments
except * average of eight experiments

mutant possible, it should always show the smallest ratio and not reach unity. However, the ratio of the Re mutant was always less than that of the hydrophilic wild type E. coli control. As this possesses complete LPS it is therefore very unlikely to undergo hydrophobic interactions and, accordingly, always showed the largest ratio. The LPS of N. gonorrhoeae contains a short core chain and therefore, this organism will undergo hydrophobic interactions but to a lesser extent than the Re mutant. As expected, all the isolates of N. gonorrhoeae apart from one PPNG (no. 412) had ratios higher than the Re mutant and all were lower than that of the E. coli control. Therefore, as a qualitative analysis, the system was working adequately well.

3.7.1. The Spectinomycin Resistant PPNG

Although six isolates were tested in these experiments, one (no. 409) behaved anomalously and was excluded from subsequent analysis. The difference between the mean ratios, the standard error of this difference and its significance are all given in table 31. Two of the five remaining isolates (411 and 449) did not differ significantly when the mean ratios were compared. In spite of the small differences in the mean ratios, the other three isolates all varied significantly from each other and also from 411 and 449.

3.7.2 The Spectinomycin Resistant Non-PPNG

The non-PPNG were more homogeneous as a group than the PPNG. The

TABLE 31

Standard Error of the Difference in Means
in the Partition of Spectinomycin Resistant N. gonorrhoeae

PPNG and non-PPNG

Isolate Nos.	Difference between means ($\bar{x}_1 - \bar{x}_2$)	Standard Error of the difference between means	Significance
396 + 520	1.25	0.61	None
396 + 534	0.65	0.37	None
396 + 644	0.75	0.37	None
396 + 656	0.7	0.4	None
396 + 663	2.7	0.45	x3
396 + 684	1.4	0.45	x3
411 + 520	1.7	0.18	x3
411 + 534	2.3	0.1	x3
411 + 644	2.2	0.1	x3
411 + 656	3.65	0.2	x3
411 + 663	5.65	0.28	x3
411 + 684	1.55	0.28	x3
412 + 520	2.0	0.18	x3
412 + 534	2.6	0.1	x3
412 + 644	2.5	0.1	x3
412 + 656	3.95	0.2	x3
412 + 663	5.95	0.28	x3
412 + 684	1.85	0.28	x3
416 + 520	5.0	0.7	x3
416 + 534	4.4	0.68	x3
416 + 644	4.5	0.68	x3
416 + 656	3.05	0.7	x3
416 + 663	1.05	0.73	None
416 + 684	5.15	0.73	x3
449 + 520	1.15	0.27	x3
449 + 534	1.75	0.23	x3
449 + 644	1.65	0.23	x3
449 + 656	3.1	0.3	x3
449 + 663	5.1	0.35	x3
449 + 684	1.0	0.35	None

results are shown in table 31. Four isolates (520, 534, 644 and 684) gave similar ratios. Isolates 656 and 663 differed significantly from each other and all the other non-PPNG.

3.7.3. Comparison of the two Groups

Considering the mean ratio of each by the Student's t-test, the two groups were significantly different ($p < 0.05$). Calculations are shown in table 32. Differences between the mean ratios of individual isolates were also assessed (see table 33). The majority of isolates were significantly different from each other, however there were a few exceptions; PPNG no. 396 was not significantly different from four of the non-PPNG isolates (520, 534, 644, 656), neither was there was any significant difference in the mean ratios of 416 and 663 or 449 and 684. Inspite of the significant difference between the two groups, the heterogeneity within both groups, particularly the PPNG, makes it difficult to believe that the observed differences in the hydrophobicity of the cell surface of PPNG and non-PPNG were attributable to the presence or absence of the penicillinase plasmid.

3.7.4. Protein Compositions of Spectinomycin Resistant N. gonorrhoeae

Total cell protein of nine isolates was examined by SDS-PAGE and the major bands present in each lane were measured (results not shown). Minor bands were ignored. All nine isolates were basically very similar with ten major bands occuring in each (see table 34 for MW).

TABLE 32

Calculation of the Student's t-test

Null hypothesis : the two population means are equal
i.e., $\mu_1 = \mu_2$ or $\mu_1 - \mu_2 = 0$

Population 1 PPNG	Population 2 Non-PPNG
$n_1 = 5$	$n_2 = 6$
$\bar{x}_1 = 2.8$	$\bar{x}_2 = 3.7$
$\Sigma x_1^2 = 34.6$	$\Sigma x_2^2 = 12.5$

$$t = \frac{(2.8 - 3.7) - 0}{\sqrt{\left(\frac{34.6 + 12.5}{5 + 6 - 2}\right)\left(\frac{1}{5} + \frac{1}{6}\right)}} = 0.65$$

With nine degrees of freedom, t = 2.26 p = 0.05

Observed value of t lies between ≥2.26 and ≤2.11

Therefore, the null hypothesis is rejected,
the two population means are not equal.

TABLE 33

Standard Error of the Difference in Means
in the Partition of Spectinomycin Resistant N. gonorrhoeae

Isolate Nos.	Difference between means ($\bar{x}_1 - \bar{x}_2$)	Standard Error of the difference between means	Significance
	PPNG		
396 + 411	2.95	0.3	x3
396 + 412	3.25	0.3	x3
396 + 416	3.75	0.76	x3
396 + 449	2.4	0.4	x3
411 + 412	0.3	0.05	x3
411 + 416	6.7	0.67	x3
411 + 449	0.55	0.2	None
412 + 416	7.0	0.67	x3
412 + 449	0.85	0.2	x3
416 + 449	6.15	0.7	x3
	Non-PPNG		
520 + 534	0.6	0.2	None
520 + 644	0.5	0.2	None
520 + 656	1.95	0.27	x3
520 + 663	3.95	0.33	x3
520 + 684	0.15	0.33	None
534 + 644	0.1	0.14	None
534 + 656	1.35	0.23	x3
534 + 663	3.35	0.3	x3
534 + 684	0.75	0.3	None
644 + 656	1.45	0.23	x3
644 + 663	3.45	0.3	x3
644 + 684	0.65	0.3	None
656 + 663	2.0	0.35	x3
656 + 684	2.1	0.35	x3
663 + 684	4.1	0.35	x3

TABLE 34

Major Proteins Present in Whole Cell Lysates of Spectinomycin Resistant N. gonorrhoeae

Strains examined : 396, 411, 416, 449, 534, 644, 656, 663, 684

MW (Kd) of Bands Present in all Strains :	72.4	
	70.0	
	51.9	
	47.9	
	44.7	
	38.0	(PI)
	34.3	
	30.2	(PIII)
	29.5	(PII)
	29.2	(PII)
MW (Kd) of Extra Bands (present in strain no.) :	34.7	(396, 411, 656)
	33.5	(644)
	31.6	(663)

Five isolates possessed extra proteins (listed in table 34). These bands were not PII as no heat-modification of MW was seen. The presence or absence of these extra bands did not appear to be related in any way to the manner in which the bacteria partitioned. Therefore there was no observable effect of alterations in total cell protein on surface hydrophobicity as determined by TPP and also no perceiveable effect of the penicillinase plasmid on the composition of total cell protein.

4. DISCUSSION

4.1. DISCUSSION OF DATA OBTAINED FROM THIS AND OTHER STUDIES

4.1.1. Strain Characterisation

Auxotyping and plasmid screening are two methods of classifying strains of N. gonorrhoeae (see section 1.1.4.). These two analyses were carried out primarily to ascertain whether possession of a particular strain characteristic was correlated with isolation from any site or patient group.

Certain auxotypes have previously been correlated with strain characteristics. Thus, as mentioned in section 1.1.4., strains with the auxotype AHU- often do not produce symptoms in the host and frequently cause disseminated disease. The prevalence of these strains varies according to the population studied; in one report from Seattle, AHU- was the predominant auxotype (Handsfield et al., 1980). These strains are rarely isolated from homosexuals, this is probably due to their high sensitivity to the inhibitory effects of faecal fats (see section 3.2.1.). Thus, the number of AHU- strains present in a population may be important in determining the incidence of gonorrhoea in the homosexuals of that population. However, the auxotype was not found in any PPNG included in this study and so it cannot be used as an explanation for the observed scarcity of PPNG in homosexuals. The inadequacy of auxotyping as a typing mechanism is

underlined by the results obtained in this project, as the majority of strains were either non-requiring or pro- and only two other types, arg- and AHU-, were represented. Therefore any correlations seen, such as that between the wild type auxotype and homosexual infection (see section 3.2.1.), are unlikely to be significant except in a statistical sense.

The plasmid analysis revealed that many PPNG isolated from 1976 - 1985 could still be classified as Asian or African; thus the conjugal plasmid was rarely found with the 3.2 Md plasmid, and the auxotype arg- was commonly found in strains with this plasmid. The pro- auxotype was correlated with strains carrying the 4.4 Md plasmid. Thus, there was nothing unusual in the PPNG strains included in this study. Virtually all strains possessed the 2.6 Md cryptic plasmid. Strains without this plasmid show certain traits; they grow poorly in vitro, they differ in their susceptibility to antimicrobial agents when compared to all other strains and they have growth requirements for proline, citrulline and uracil (Dillon and Pauzé, 1981). Unusual strains such as these may well show a restricted host range; however, this was not a significant factor as there were only two non-PPNG strains that did not carry the 2.6 Md plasmid.

The results obtained do not suggest that there was anything atypical in any of the strains included in this study in terms of their auxotype or plasmid content. There is no indication, apart from the already observed scarcity of AHU- strains in homosexuals, that strains of a particular type are concentrated within or excluded from

a patient group. However, it is possible that with more detailed serological analysis, evidence for this would emerge. With the available data, it is not possible to comment on the likelihood of this being the case.

4.1.2. Selection of the mtr Mutation

The mtr mutation has been the subject of several studies and hypotheses. Initial studies in vitro indicated that the mutation was selected for in homosexual infection by exposure to the hydrophobic environment of the rectum, to which mtr was believed to afford a degree of protection (see section 1.4.2.). In support of this, a preliminary study of PPNG isolated from homosexuals (Handsfield et al., 1982b) showed that only a small percentage (one out of twenty four) of these strains were mtr, suggesting the existence of a causal link between the low incidence of mtr in PPNG and their inability to become established in the homosexual population (Handsfield et al., 1982a). However, later work claimed to show that the mtr mutation was not the important factor in more specific resistance to faecal fatty acids which are potent inhibitory molecules present in the rectum. Resistance to these was proposed to be due to an mtr-independent mechanism, as yet uncharacterised (see section 1.4.2.).

The role of the mtr mutation thus being questioned, led Reid et al. (1985) to propose an alternative. They believed that their results, obtained from a comprehensive survey of non-PPNG isolates, indicated

that the mutation was selected by increased antibiotic levels within the host. Thus homosexuals, who typically overuse antibiotics, would exert a greater selection pressure on N. gonorrhoeae than would heterosexuals, this then accounting for the larger number of mtr strains isolated from homosexuals. The foundation of this hypothesis was the observation that the isolation rate of mtr strains from homosexuals was higher in urethral isolates than it was in rectal isolates. They argued that the reverse would be the case if the hydrophobic environment of the rectum was the significant factor. However, the hypothesis omits to explain why the rate of isolation of mtr strains was nearly five times greater for urethral than rectal isolates from homosexuals. By their own admission, the selection pressures (in terms of antibiotics) should be equal at both sites, therefore it would be predicted that the percentage of strains with the mtr phenotype from both sites would be approximately equal. It is possible, although not discussed by Reid and her colleagues, that antibiotic selection pressures are actually decreased in the rectum. This could be caused, in the case of penicillin, by organisms of the normal faecal flora producing penicillinase and so inactivating the penicillin (Owen and Hill, 1972) or else N. gonorrhoeae trapped in faeces may be physically protected from the antibiotic (John and Jefferis, 1973). The work of Morse et al. (1982) cannot be used either to support or refute Reid's theory, as the results of isolates from homosexuals were not divided according to site.

The results obtained from this project do little to strengthen any hypotheses which exist as to the role and selection of the mtr

phenotype, indeed they may serve to worsen the situation. Firstly, and perhaps most significantly, the results showed that the phenotype was present in a high percentage of PPNG and further concentrated in PPNG isolated from homosexuals. This contradicts what was previously reported by Handsfield et al. (1982b) in the only other study to include PPNG. It may be evidence that the premise that PPNG do not infect homosexuals because they are only rarely mtr is false. The omission of any PPNG isolated from heterosexuals from the study of Handsfield et al. (1982b) was unfortunate, as there is no way of confirming whether the results obtained were simply the product of an exceptionally low occurrence of mtr in the local population of PPNG. Otherwise, the results could indicate that there has been expansion of a single clone with an alternative resistance mechanism amongst the homosexual population studied. There is insufficient data included in the brief report to judge whether this is the case; although the results of auxotyping showed that two types were represented, in the absence of any indication as to the number of isolates belonging to each type, the possibility that the majority of the bacteria tested belonged to a single clone cannot be discounted. At the most extreme, it may be that the single mtr strain was the only isolate with the second auxotype. Alternatively, the results may be contradictory because of the expansion of one clone of PPNG amongst the homosexual patient population seen at the Praed Street Clinic. However, the results of the strain characterisation do not suggest this (see section 4.1.1.) and in addition, the bacteria were isolated over a period of four years and represent infections acquired in the UK and abroad.

It is also interesting to examine the nature of the selection mechanism as suggested by these results. A direct comparison may be made between the data obtained from non-PPNG by Reid et al. (1985) and Morse et al. (1982) with those obtained from non-PPNG in this study (table 35). Whilst the data from the experiments of the former two groups show an association of the mtr phenotype with homosexual infection in general, the results of this project indicate that the phenotype was selected for in strains infecting the rectum in both homosexuals and heterosexuals. The incidence of mtr observed amongst female rectal isolates was surprisingly high in comparison to that seen in either of the other studies. In the case of Morse and his colleagues, the isolates from female patients were not divided according to site, but the total incidence for the two sites was still very low (5%). Even assuming that all the mtr strains were isolated from the rectum, the percentage would still be below ten. Isolation of N. gonorrhoeae from the rectum of a female is often supposed to not be due to active infection, but rather to autoinoculation by contaminated discharge where there is cervical infection. Morse et al. (1982) used this as an explanation for the low incidence of mtr in female rectal isolates. In this study an alternative explanation must be sought as it is unlikely that all the female rectal isolates included were cultured as a result of contamination of rectal swabs. If this were the case, then the incidence of mtr would be expected to be approximately equal in the two groups. The results obtained from both female rectal and male rectal isolates are consistent with the original hypothesis that the rectal environment is directly responsible for the higher incidence of mtr in isolates from this site.

TABLE 35

The Incidence of the mtr Phenotype in Non-PPNG

	Percentage of strains with mtr phenotype		
	This project	Reid et al. (1985)	Morse et al. (1982)
Homosexual male urethra	16	50	*
Homosexual male rectum	31	11	*
Heterosexual male urethra	11	4	5
Female cervix	19	3	*
Female rectum	32	4	*
Female cervix and rectum	25	3	5
Homosexual male urethra and rectum	23	27	24

* Results not divided according to site

Whilst the data obtained from non-PPNG in this project suggest that the selection pressures at the urethra and rectum are not equal, the data obtained from PPNG are in marked contrast. The figures of incidence of mtr in homosexuals show, in agreement with the data of Reid et al. (1985), that the mtr phenotype was more common in homosexual urethral isolates than rectal isolates (87 and 45%, respectively), with the incidence in both being elevated above that of isolates from heterosexuals (33%). It is unfortunate that only three PPNG were isolated from female rectums, as data from these strains could aid to determine how important the site of infection is in selection of the mtr phenotype in PPNG; neither can any comparison be made with the corresponding non-PPNG isolates from the same site. It may be that the dissimilarity between the mtr incidence in non-PPNG and PPNG isolates from homosexuals is an indication that mtr was selected by a different process in each group. Otherwise, and in the light of work carried out by Bygdeman et al. (1982), it may be that two separate mutations selected in response to different stimuli but with identical phenotypes were present. This will be discussed in the next section.

4.1.3. The Need for Confirmation of the mtr Phenotype

The mtr envelope phenotype was originally defined by Guymon et al. (1978) as a mutant phenotype with elevated resistance to penicillin and a number of hydrophobic dyes, detergents and antibiotics and also a seven-fold increase in the amount of a minor protein (MW 52 Kd) present in the OM. The determination of envelope phenotype by MIC

testing, as developed by Morse et al. (1982), was a convenient way of screening a large number of isolates; however they subsequently confirmed that strains with MIC values indicative of the mtr phenotype were actually genotypically mtr by demonstrating that each possessed an increased amount of the 52 Kd protein and was capable of transforming a sensitive strain to the mtr phenotype.

In 1982, following a study of the genetic linkage of antibiotic resistance and serogroup, Bygdeman et al. described a strain which possessed a similar pattern of resistance to that caused by the mtr locus. However, when this strain was used as a genetic donor, the resultant transformants not only showed the mtr pattern of resistance but also the donor serogroup (WII). This co-transformation of serogroup with resistance was not seen in the study of Morse et al. (1982) which suggested that a second locus, closely linked to PI and genetically distinct from mtr might be responsible for the resistant phenotype. Furthermore, analysis of the OMs of the donor and its transformants failed to detect any increase in the amount of the 52 Kd protein present. No further characterisation of this putative second locus has been published. In spite of this, Cannon and Sparling (1984) named the locus nsr (for non-specific resistance) and have stated that it should be regarded as a locus separate from mtr because of its close linkage with serogroup WII and its lack of effect on the 52 Kd protein.

This study and also that of Reid et al. (1985) did not confirm that all strains were mtr either by transformation or OMP analysis and

therefore it is possible that any number of strains from either study might not truly be mtr. The limited number of transformations carried out in this study showed that the resistant phenotype was easily transformable and whilst no increase in the amount of the 52 Kd protein was seen in the OMs of the transformants, neither could it be seen in the control strain FA171 previously demonstrated to have the band by Morse et al. (1982). It is possible that Bygdeman and her colleagues failed to detect the increase for a technical reason similar to that experienced in this study. It is obvious that further study is required to definitely identify nsr as a separate locus. In addition, a more reliable and rapid way of screening for the mtr phenotype would be desirable. An ideal method would be by quantitatively measuring the amount of the 52 Kd protein present using an enzyme-linked, immunosorbent assay (ELISA), providing that an antibody to this minor OMP could be raised.

4.1.4. The Occurrence of Oleic Acid Resistance in N. gonorrhoeae

The toxicity of fatty acids for N. gonorrhoeae has been documented (Miller et al., 1977). The ability of N. gonorrhoeae to resist the toxic effects of these molecules in vivo may be critical in order for infection to become established. This is especially important in the rectum where fatty acid concentrations are high (4 - 5% w/w wet faeces; McFarland et al., 1983). Accordingly, strains infecting homosexuals, which must be able to infect both the rectum and the urethra, have been found to be more resistant to total faecal fat extracts than isolates from heterosexuals (McFarland et al., 1983;

Morse et al., 1982).

This project examined the sensitivity of N. gonorrhoeae to oleic acid which is one of the four major dietary fatty acids found in faeces (section 1.4.1.). It is probably the only study to have included PPNG amongst those strains tested. Significantly, the results showed that oleic acid resistance was not widespread amongst PPNG which had been isolated from heterosexuals; this is consistent with the theory that these strains are at a disadvantage in the rectum. Correspondingly, isolates from homosexuals exhibited a high incidence of resistance to oleic acid. Overall, non-PPNG showed a much higher percentage of strains which were oleic acid resistant, with the greatest number of resistant strains having been isolated from homosexuals. However, a number of anomalies exist within the results. In non-PPNG the number of resistant strains from both male and female rectums was smaller than the number isolated from the cervix. Oleic acid is present at both sites, although data on the abundance of this molecule at the cervix are not available. It is unlikely that oleic acid is present at the cervix in larger amounts than in the rectum and it is also unlikely that a high level of resistance is needed to survive in the cervix. PPNG from female cervices did not show such an elevated resistance and there is no epidemiological evidence to suggest that they are at a disadvantage at this site. Thus, the reason underlying this anomalous result remains unknown.

It is of course possible that oleic acid resistance per se is of little significance in survival in the rectum or cervix. There is

virtually no data to judge whether other compounds present at either site are inhibitors of N. gonorrhoeae, whether in vitro or in vivo. The work of McFarland et al. (1983) indicated that the most inhibitory components of faecal fat extracts were three of the four major dietary fatty acids, palmitic, oleic and linoleic acids. Thus resistance to either palmitic or linoleic acid may be of greater importance. Preliminary studies in the course of this project were made with linoleic acid which appears to be inhibitory at a level of as little as 100 mg/L. The high sensitivity of N. gonorrhoeae to this molecule rendered the results poorly reproducible due to the technical difficulties experienced in measuring milligram amounts of such a viscous compound and the study was abandoned. Palmitic acid is similarly intractable, being virtually insoluble in water and requiring organic solvents such as ether, chloroform and benzene (Merck Index), thus proving to be impossible to use in MIC testing.

4.1.5. Fatty Acid Resistance and its Relationship to mtr

The precise mechanism or mechanisms conferring fatty acid resistance on N. gonorrhoeae are not known. Resistance was formerly attributed to the possession of mtr but subsequently has been proposed to be mtr-independent (section 1.4.2.). It is unlikely that mtr has no role in resistance to fatty acids owing to its overall effect of decreasing the permeability of the OM to hydrophobic molecules, as it is thought that both fatty acids and the compounds used to determine envelope phenotype enter the cell by the same route (McFarland et al., 1983). Accordingly, in both this project and those of Morse et

al. (1982) and McFarland et al. (1983), mtr strains were the most resistant of all envelope phenotypes to fatty acids. The proposal that an mtr-independent mechanism existed was based on the observation of McFarland et al. (1983) that the resistance of wild type envelope phenotype strains from homosexuals and heterosexual women to faecal fats was elevated relative to that of wild type strains from male heterosexuals. Also, wild type strains from both male and female rectums were less sensitive than those from other sites. This second mechanism was not thought to be due to a decrease in the permeability of the cell wall of N. gonorrhoeae but more likely to be due to an alteration in the cellular target for fatty acids.

Examination of the linkage of the mtr phenotype and oleic acid resistance in this project raises some interesting questions. The data showed clearly that, for non-PPNG, mtr and oleic acid resistance were linked, i.e. that if an isolate was mtr then it was highly likely to also be oleic acid resistant. This was also true for PPNG isolated from homosexuals, but the link was absent in PPNG from heterosexuals. Assuming the linkage between the two phenotypes to be causal, this dichotomy in PPNG might be an indication that those from heterosexuals were susceptible to oleic acid because they were not true mtr. The possibility that misclassification of envelope phenotype may have occurred has already been discussed (section 4.1.3.) and it is tempting to suggest that this was the case for all those strains with an apparent mtr phenotype which were sensitive to oleic acid. Thus, it may be as originally stated by Handsfield et al.

(1982b) that the mtr phenotype is rare in PPNG leaving them at a physical disadvantage in the rectum. The minority of PPNG strains with the mtr phenotype would therefore be concentrated in homosexuals, as indicated by the retention of linkage in this group. The true mtr phenotype is apparently more common in non-PPNG, hence the greater incidence of these strains in homosexuals.

The mtr status of the bacterium is not the only factor in oleic acid resistance. Whilst a strain which was mtr was highly likely to be oleic acid resistant, the converse, i.e. that an non-mtr strain was likely to be sensitive, was not true. It was on similar evidence that McFarland et al. (1983) proposed the existence of an mtr-independent mechanism. The incidence of oleic acid resistance in non-mtr strains was much more common in non-PPNG than PPNG (26 and 13% respectively) and this extended to non-mtr PPNG isolates from homosexuals.

The situation may be envisaged where the majority of PPNG strains have neither the mtr or the second mechanism to protect them against the toxic effects of fatty acids in the rectum. Those few that do possess the mtr mutation are able to infect homosexuals. Otherwise, it may be that the mtr mutation is unimportant and the observed resistance/sensitivity to oleic acid is wholly due to the second resistance mechanism. As this appears to be rare in PPNG, this Finally, it must be remembered that there are still strains which are neither mtr or resistant to oleic acid which have infected homosexuals thus there are obviously other factors to consider.

However, it is probably true to say that the possession of one or other mechanism will increase the chance of survival in the rectum.

4.1.6. The Role of Normal Flora in Host Immunity to Infection

The *in vitro* inhibition of *N. gonorrhoeae* by normal flora has been studied by several groups (Kraus *et al.*, 1976; Morin *et al.*, 1980; Saigh *et al.*, 1978). These concentrated mainly on the inhibitory powers of members of the urogenital tract rather than faecal flora. The study of Morin *et al.* (1980) is the exception; this study tested thirty two strains of anaerobes representing eighteen species for antigonococcal activity by a method virtually identical to that used in this project. Both *B. fragilis* and *vulgatus* were included in Morin's study but none of the test strains of *N. gonorrhoeae* were penicillinase producers. Therefore the possibility that PPNG might be highly susceptible to inhibition by faecal flora, thus lessening their chances of survival in the rectum, had not been previously investigated.

A direct comparison of the data from non-PPNG in this project and those of Morin *et al.* (1980) can be made (table 36) and this shows that large differences exist in the potency of gonococcal inhibition by different strains of one species. Whether these differences are due to the site from which the strain was isolated is not clear as Morin did not indicate the source of his test strains. Kraus *et al.* (1976) had previously found no obvious differences in the inhibitory

TABLE 36

Inhibition by Bacteroides spp. of Non-PPNG

Inhibitory strain (strain no.)	Study	Percentage of total inhibited
B. fragilis (B1A)	Morin *et al.*	86
B. fragilis (B15)	Morin *et al.*	9
B. fragilis	This project	84
B. vulgatus (B4)	Morin *et al.*	5
B. vulgatus	This project	52

powers of coliforms, staphylococci and streptococci from sites as diverse as the vagina, the urinary tract and the upper respiratory tract. Certainly, the results obtained in this project did not give any reason to believe that a site specific selection process was operating; isolates of N. gonorrhoeae from the rectum were no more resistant than those from the urogenital tract, neither was any link with patient sexuality obvious.

Considering the data obtained in this project and from the studies of Saigh et al. (1978) and Morin et al. (1980) it seems very likely that the inhibitory powers of most bacterial species are strain dependent and are not uniform throughout the species. Thus, it is not surprising that the experiments carried out in this project using one strain from each of two species were inconclusive and possibly misleading. More conclusive results would probably be obtained using an approach similar to that of Saigh et al. (1978). In their study, normal flora from individual female patients attending an STD clinic were collected and tested for their ability to inhibit N. gonorrhoeae. Each patient was also screened for the presence of N. gonorrhoeae at the same site, with a view to later correlating the isolation of inhibitory normal flora with the incidence of a positive culture for N. gonorrhoeae. From a practical point of view, carrying out this type of screening with rectal isolates would be difficult and time-consuming, as the flora of this site are largely anaerobic and represent numerous species. It is unlikely that the role of normal flora in host immunity to gonorrhoea is absolute; if they play any part, it is probably to

determine whether the patient will succumb to the disease at all, rather than acting selectively against either PPNG or non-PPNG.

4.1.7. Variation and Absence of any Site-Specific PII Molecules

The studies of OMPII undertaken so far have been numerous and varied in their approach. As discussed in sections 1.3.2. and 1.4.3., investigations of the properties of isogenic laboratory strains with different PII compositions suggest strongly that PII is an important factor in virulence, playing a role in adhesion and possibly enhancing the chances of survival in different niches within the host. Selection of PII occurs *in vivo* at different sites on the host and also at different times of the oestrous cycle in women as manifested by the recovery of organisms which vary with respect to their opacity. This is presumably due to the influences of the host although the underlying selection factor is not always obvious.

Despite the extensive variation of PII which has been shown to occur *in vivo*, it does not appear that one specific PII is selected for at any site (Schwalbe *et al.*, 1985; Zak *et al.*, 1984). However, attention so far has focused exclusively on male and female genitourinary isolates and it is possible that selective forces do operate in the rectum, favouring strains expressing one or more particular PII types. Additionally, no clinical isolates of PPNG from any site have been studied for their PII compositions and therefore the possibility that PPNG are deficient in a particular PII

associated with rectal infection remains.

Preliminary investigations into the existence of any PII molecules which were exclusive to non-PPNG or associated with rectal infection were carried out. Analysis of the PII compositions of a small number of N. gonorrhoeae strains did not give any cause to believe that any PII were specific either to non-PPNG or isolates from the rectum. However this cannot be stated categorically for several reasons. The rate of change of PII composition in N. gonorrhoeae is very high (as great as 4×10^{3} per colony forming unit per generation; Mayer 1982), thus repeated subculture may completely change the PII composition of any isolate. In the course of these experiments subculturing was kept to a minimum but this was not always possible; this was especially true for a number of PPNG from homosexuals which were, nonetheless, included in this study due to their scarcity even though they have been passaged non-selectively several times. Additionally, classifying PII as 'alike' or 'dissimilar' merely on the basis of their ASMW on SDS-PAGE would not necessarily distinguish between very small variations in MW, neither does an identical MW definitely indicate an identical protein.

A complete and thorough study of PII as it occurs in vivo in both urogenital and rectal isolates would be extremely difficult to carry out but would be necessary to obtain unambiguous results. This would entail initial confirmation that each infection was caused by only one strain (by serotyping, auxotyping, antibiogram, plasmid content) and the examination of PII composition as near as possible to the

first subculture with continual monitoring of colony opacity to ensure no shift in PII composition had taken place. Even with these precautions, transition from one type to another would not be eliminated, merely kept to a minimum. Classification of PII as alike or dissimilar would require not only MW determination, but also more detailed study such as two-dimensional electrophoresis, peptide mapping and antigenicity testing.

Consideration of this work and other studies leads to the conclusion that PII is a hypervariable antigen with a small probability that there is one or even several PII types which are required for rectal colonisation. The frequency of the occurrence of structurally and antigenically identical PII amongst strains of N. gonorrhoeae may be so low as to preclude this from being the case.

4.2. DISCUSSION OF OTHER FACTORS AFFECTING THE ABILITY OF N. GONORRHOEAE TO INFECT THE RECTUM

In addition to those factors investigated in this project, there are several others which may be of great importance. None of these have previously been investigated in the context of either infection by PPNG or infection of the rectum.

4.2.1. Adhesion

If a barrier to infection of the rectal mucosa by PPNG does exist, then it is most likely that it operates at the first critical step of the infection process, adhesion. As described in section 1.3.1., once adhesion to host cells has occurred, invasion of these cells is an inevitable consequence. Studies of the adhesion of N. gonorrhoeae have concentrated on the adhesive properties of laboratory adapted strains or their purified and radioiodinated pili using substrates which either have no clinical significance (erythrocytes and BEC), represent transformed cell lines (HeLa) or relate mainly to heterosexual infection (sperm, endocervical cells and fallopian tube mucosa). No experiments have been carried out using cells originating from rectal tissue, therefore nothing is known as to the nature of the gonococcal receptor or the kinetics of binding of organisms at this site. In addition, the attachment of PPNG to cells of any origin has not been investigated. Therefore there is no evidence to judge whether or not PPNG have a reduced ability to adhere to the cells of rectal mucosa.

In vitro experiments have shown that under certain conditions, different pilus types exhibit tissue tropisms but this has been shown solely for four pilus types of laboratory strain P9 and may not be generally applicable. In experiments using both purified, radioiodinated pili (Heckels, 1982; Lambden *et al*., 1980a and b) and piliated whole cells (Virji and Everson, 1981), the four pilus types showed different binding affinities for erythrocytes, BEC, HEp-2 and Chang conjunctival cells. The significance of these results is questionable as all four cell types are either clinically unimportant or represent transformed lines; additionally comparison of data obtained from purified, iodinated pili and piliated whole organisms has shown a discrepancy. The experiments of Lambden *et al*. (1980b) showed that neuraminidase treatment of BEC reduced the level of binding of radioiodinated α pili isolated from strain P9. In contrast, Trust *et al*. (1980) found the extent of binding of intact α piliated P9 to BEC unaffected by neuraminidase treatment. The anomaly of the results may be attributable to the iodination of pili altering their binding capacity, or it may underline the fact that purified pili do not behave in the same manner as those *in situ*. The work of Pearce and Buchanan (1978), albeit using purified pili, provides evidence questioning the existence of tissue tropisms. They showed that four antigenically distinct pilus types from four strains bound equally to both clinically important and unimportant cell types, including cervical-vaginal mucosa, fallopian tube mucosa and sperm. Thus, it has not been proven that *in vitro* tissue tropisms exist either in intact, piliated, clinical isolates of *N. gonorrhoeae* or for cells of clinical significance. Indeed, a study of clinical isolates showed pili to be hypervariable surface structures, with no

apparent correlation between pilus type and site of isolation (Zak et al., 1984). In spite of this, it is conceivable that variation in pilus type may provide selective adherence to different mucosal surfaces. One or more pilus types may be associated with enhanced adhesion to rectal mucosa. Any gonococcus unable to express these pili would then be more likely to be swept away by faeces or more vulnerable to the harmful effects of normal flora and inhibitory compounds as a direct cause of their reduced ability to adhere. It may be hypothesised that such a deficiency exists in the majority of PPNG, this then being a major reason underlying their scarcity in homosexuals.

Exactly what would cause such a deficiency or why it should exist is not immediately apparent. The genetic controls operating on pilus expression involve extensive and complex rearrangements of genomic DNA (Haas and Meyer, 1986; Hagblom et al., 1985; Segal et al., 1985). The mechanisms by which these rearrangements are regulated are not known, but it may be that pilus production is affected by external conditions and it may be impaired by the prevailing conditions in the rectum in terms of nutrient availability, oxygen concentration and presence of inhibitory molecules. Such factors would, however, be unlikely to act selectively on PPNG and the reason for the lack of production of a particular pilus type may simply be that the organism lacks the genetic information required. Finally, it must be remembered that tissue tropisms may not exist at all for any pilus types and therefore the complement of pilus types possessed by an individual organism would not affect its ability to adhere at any

site.

4.2.2. Permeability of the Cell Wall of N. gonorrhoeae

The permeability of N. gonorrhoeae to fatty acids and bile salts may be a critical factor as to whether or not the bacterium can survive in the rectum. Whilst the mtr mutation may well have some effect in decreasing the cell's permeability, it has not been shown unequivocally to protect the bacterium from fatty acids. Resistance to these molecules has been proposed to be through an alteration of the cellular target but this is by no means proven (section 4.1.5.). Other structures of the gonococcal OM may be important in reducing its permeability.

The importance of glycosylation of PL head groups on the outer leaflet of the OM has been mentioned (section 1.4.2.). The composition of N. gonorrhoeae PL has not been extensively studied, if at all. It maybe that PPNG lack these glycosylated residues or may have a reduced number, thus their permeability is increased. Such a difference could even counteract the protective effect of mtr as the principal route into the cell of hydrophobic molecules is via PL bilayers.

The nature of the LPS in the OM may also be important. The role of lateral LPS-LPS interactions in permeability and the lessening of these in deep rough mutants has been mentioned previously (section

1.4.1.). As N. gonorrhoeae produces only R-type LPS, this is one explanation for its overall sensitivity; however, it could also be that differences in LPS composition may effect an even greater reduction in LPS-LPS bond strength. A detailed study of gonococcal LPS has not been carried out and PPNG have not been studied at all; the few strains so far examined have not shown very different LPS compositions. It may be that only a slight difference is enough to lower the LPS-LPS bond strength, as Nikaido and Vaara (1985) have shown that the loss of charged phosphate groups rather than entire sugar residues is responsible for weakening the bonds.

4.2.3. Protein I as a Marker for Other Phenotypes

Although PI apparently plays little part in the survival or adhesion of N. gonorrhoeae, different PI molecules have been found to be associated with various clinical syndromes, e.g. serogroup WII with homosexual infection (Bygdeman, 1981) and serogroup PIA with DGI (Britigan et al., 1985). With the relatively recent development of serological classification of PI using McAbs (see section 1.2.2.) there is the potential to subdivide N. gonorrhoeae into many serovars and this may reveal other linkages of PI types with clinical syndromes. One preliminary study of prostitutes in Germany gave some evidence that one serovar (1B24) was being selected for in this population (Kohl et al., 1985). Thus the close linkage of PI serovars with other phenotypes may be a useful tool in research. It is not the case, however, that all PPNG isolated from homosexuals at St Mary's Hospital belong to the same serovar. Dr S. Bygdeman, Department of

Clinical Bacteriology, Sodersjukhuset, Stockholm has kindly serotyped fourteen of these strains and they were found to belong to several serovars (table 37).

TABLE 37

Serovars Represented in PPNG
Isolated from Homosexuals 1982 - 1983

Serovar	No. of Isolates
Ae	2
Back	8*
Bcjk	1
Bcgjk	2
Abedgkih	1

* Includes two contact pairs

4.3. THE PENICILLINASE PLASMID AND GROWTH IN THE RECTUM

4.3.1. The Extra Coding Capacity of PPNG

Apart from phenotypic traits shown by PPNG and non-PPNG as separate groups, the sole fundamental difference between the two is the possession of a penicillinase encoding plasmid. Whether and how these relatively small pieces of extrachromosomal DNA would influence the bacterium so much as to alter its infective potential is not known. Both penicillinase plasmids have been studied extensively (Dickgeisser et al., 1982; Fayet et al., 1982; Roberts et al., 1977). The ß-lactamase gene is encoded on a portion of a Tn-3 like transposon (Tn 2301). This transposon is incapable of self transposition as both the gene encoding the transposase and the left handed inverted repeat sequence are deleted from it. This truncated transposon makes up 1.6 Kb of both plasmids, leaving 5.5 and 3.5 Kb of DNA not involved in ß-lactamase production. No further genes have been identified on either plasmid, but this amount of DNA could potentially code for three to five proteins of about 50 Kd. However, even with this additional coding capacity that PPNG possess, the plasmid has a different G + C ratio to the gonococcal chromosome and shows extensive homology with the Haemophilus plasmid and virtually none with the gonococcal genomic DNA. Therefore it appears that there is no duplication of chromosomal genes and so it is unlikely that any gene products derived from the plasmid would affect the cellular components important in the pathogenesis of this organism. A limited study of six spectinomycin resistant PPNG and six spectinomycin

resistant non-PPNG, all of which probably belonged to one strain (see section 2.1.1.), did not show any significant differences between those strains which possessed the plasmid and those which did not, as manifested by alterations in surface hydrophobicity and total cell protein compositions.

4.3.2. Barriers to the Spread of the Penicillinase Plasmid

As it seems unlikely that the penicillinase plasmid *per se* modifies the infective potential of *N. gonorrhoeae* rendering it unable to infect the rectum, an alternative explanation must be sought. It may be that some type of barrier to the spread of the plasmid exists whereby only certain strains can accept the plasmid. The mechanism of conjugation in *N. gonorrhoeae* is not well understood and may be very different from that of widely studied enteric bacteria such as *E. coli*. If sex pili are involved, which has not been proven, it may be that only certain strains possess a receptor for this pilus. If the conjugation system does not involve pili, some other component of the OM may be specifically required for conjugation. The existence of such a barrier to the spread of the penicillinase plasmid could result in a conservation of phenotypic traits amongst the PPNG population. One such trait could be the underlying reason for the inability to infect homosexuals. However, PPNG are a diverse group by all accounts and there is no evidence that either of the two penicillinase plasmids are excluded from any bacterial cells.

4.3.3. Loss of the Penicillinase Plasmid During Rectal Growth

A second hypothesis might be that the resistance plasmid is somehow cured by the environment of the rectum. In support of this, more PPNG have been isolated from the urethras of homosexual men than rectums, although this is also seen for non-PPNG and may just be a general indication that all strains of N. gonorrhoeae have a reduced ability to infect the rectum. It is perhaps naive to propose that some compound found in the environment would act directly to cure the plasmid, as in other bacterial genera curing agents are powerful DNA intercalaters such as ethidium bromide and acridine orange and comparable molecules would not be found in vivo. If curing of the plasmid does occur, then it is more likely to be attributable to a lifting of antibiotic pressure in the rectum in the manner discussed in section 4.1.2.. Decreasing the external penicillin concentration profoundly affects the stability of the plasmid and with a lack of selective pressure to keep the plasmid, it would be eventually diluted out of the population. There is some evidence that the anaerobic rectal environment may affect conjugation in enteric bacteria (Burman, 1977) and therefore reinfection of cured organisms with the plasmid may be less likely to occur. However, as stated previously (section 4.3.2.) the conjugation system of N. gonorrhoeae may be completely different to that seen in enterics.

4.4. EPIDEMIOLOGICAL REASONS FOR THE SCARCITY OF PPNG IN HOMOSEXUALS

The low incidence of PPNG infections in homosexual men may be accounted for wholly by epidemiological factors. Homosexuals do not mix sexually with the heterosexual population in which PPNG arose and this sexual segregation would exclude these organisms from their population. However, a significant proportion of homosexuals are reported to be bisexual and also in spite of a few isolated cases of PPNG infection in homosexuals, the resistant organisms have not spread and no significant increase in their incidence has been seen at St Mary's Hospital (table 38) or reported elsewhere. One report stated that it would be expected that, once introduced, PPNG would spread rapidly through the homosexual population (Janda et al., 1983) Further evidence that biological factors may be responsible is shown by the phenotypic differences exhibited by the two groups which may affect their ability to survive in the rectum. Conclusive evidence that epidemiological reasons are not important in this phenomenon could be obtained by identification of bisexual men exposed to PPNG and rigorous contact tracing to demonstrate that PPNG have had ample opportunity to enter and become endemic in the homosexual community.

TABLE 38

The Incidence of PPNG in Homosexuals at St Mary's Hospital

	PPNG from Homosexuals	Total No. of PPNG
1981	0	57
1982	3	212
1983	11	277
1984	12	192

4.5. CONCLUSIONS

The factors which could potentially affect the ability of N. gonorrhoeae to infect any site within the host are numerous. The reasons for the apparent inability of PPNG to infect homosexuals present a very complex picture, with no simple explanation. It is almost certain that many factors add up to render these strains inefficient at colonising homosexuals. It seems likely that PPNG are more susceptible to hydrophobic molecules that are found in the rectum, although whether this is because the permeability of these strains is increased or they lack another resistance mechanism is not clear. They may lack pili with an increased affinity for rectal mucosa cells, thus their rate of adhesion to these cells will be slow. Subsequently, they may be exposed to toxic compounds (to which they are already highly sensitive) for an extended length of time or they may be more susceptible to the flushing action of faeces. All of these factors would act to control the spread of PPNG amongst homosexuals to such an extent that, as observed, recovery of these strains from this group is extremely rare. It is unlikely that resistance to normal flora plays a major role in the survival of PPNG in the rectum.

There may be an inherent tendency for the penicillinase plasmid to be lost due to the conditions of the rectum and eventually diluted out of the population, thus these infections would be characterised as non-penicillinase producing. Epidemiological factors may have helped to limit the access of PPNG strains to the homosexual

population, but it is unlikely that these have been the only reason for the observed scarcity of homosexual infection by PPNG.

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APPENDIX

1. MEDIA SUPPLEMENTS

1.1. Composition of IsoVitalex

	Percentage (w/v)
D-glucose	10.0
Cysteine hydrochloride	2.5
L-glutamine	1.0
L-cysteine	0.11
Adenine	0.1
Guanine hydrochloride	0.003
Vitamin B12	0.001
Thiamine hydrochloride	0.0003
Nicotinamide adenine dinucleotide	0.025
Cocarboxylase	0.01
Ferric nitrate	0.002
p-Aminobenzoic acid	0.0013

1.2. Composition of Kellogg's Supplement

	Percentage (w/v)
D-glucose	20.0
L-glutamine	0.25
Cocarboxylase	0.002
Ferric nitrate	0.05

2. SDS-PAGE RECIPES

2.1. Sample Diluent for SDS-PAGE

SDS	2% (w/v)
Glycerol	10% (v/v)
Tris pH 6.8	0.08 M
Bromophenol blue	0.001% (w/v)

2.2. Acrylamide Gel Compositions

Separating gel	5%	15%
Acrylamide	5.0% (w/v)	15.0% (w/v)
Bis-acrylamide	0.067% (w/v)	0.09% (w/v)
Tris pH 8.7	0.37 M	0.37 M
SDS	1.0% (w/v)	1.0% (w/v)
Ammonium persulphate	0.03% (w/v)	0.03% (w/v)
TEMED	0.003% (v/v)	0.003% (v/v)
Stacking gel		
Acrylamide	5.0% (w/v)	
Bis-acrylamide	0.13% (w/v)	
Tris pH 6.8	0.125 M	
SDS	1.0% (w/v)	
Ammonium persulphate	0.15% (w/v)	
TEMED	0.1% (v/v)	

2.3. SDS-Tris-Glycine Electrophoresis Buffer

SDS	0.1% (w/v)
Tris	0.025 M
Glycine	0.013 M

3. STATISTICAL DATA

3.1. Formula for Calculation of χ^2

$$\chi^2 = \frac{\Sigma(o - e)^2}{e}$$

where o = observed value

e = expected value

3.2. List of Chi-squared Values Cited in Text

Group	Group	Characteristic	Chi^2
Non-PPNG	PPNG	Wild type auxotype	5.37
Non-PPNG	PPNG	Pro- auxotype	7.05
		Wild type auxotype	11.71
PPNG with 4.4 Md plasmid	PPNG with 3.2 Md plasmid	Presence of 24.5 Md plasmid	8.51
		Pro- auxotype	10.66
		Arg- auxotype	15.73
PPNG with 24.5 Md plasmid	PPNG without 24.5 Md plasmid	Wild type auxotype	6.07
Non-PPNG	PPNG	*mtr* phenotype	16.8
Non-PPNG from female cervices	PPNG from female cervices	*mtr* phenotype	4.45
Non-PPNG from homosexual male urethras	PPNG from homosexual male urethras	*mtr* phenotype	20.25
Non-PPNG from heterosexual male urethras	PPNG from heterosexuals male urethras	*mtr* phenotype	4.2
Non-PPNG from the rectum	PPNG from the urethra	*mtr* phenotype	5.42

Group	Group	Characteristic	Chi^2
PPNG from homosexuals	PPNG from heterosexuals	*mtr* phenotype	7.91
PPNG from homosexual male urethras	PPNG from heterosexuals	*mtr* phenotype	12.52
PPNG from homosexual male urethras	PPNG from homosexual male rectums	*mtr* phenotype	5.53
PPNG with 4.4 Md plasmid	PPNG with 3.2 Md plasmid	*mtr* phenotype	17.7
Non-PPNG with pro- auxotype	Non-PPNG without pro- auxotype	*mtr* phenotype	11.25
PPNG with arg- auxotype	PPNG without arg- auxotype	*mtr* phenotype	8.95
Non-PPNG from female cervices	PPNG from female cervices	Resistance to oleic acid	4.83
Isolates from homosexuals	Isolates from heterosexuals	Resistance to oleic acid	10.88
PPNG from homosexuals	PPNG from heterosexuals	Resistance to oleic acid	12.38
PPNG from homosexual male urethras	All other PPNG	Resistance to oleic acid	10.22
Isolates with *mtr* phenotype	Isolates without *mtr* phenotype	Resistance to oleic acid	23.36
Non-PPNG with *mtr* phenotype	Non-PPNG without *mtr* phenotype	Resistance to oleic acid	19.29
PPNG with *mtr* phenotype	PPNG without *mtr* phenotype	Resistance to oleic acid	11.4
PPNG from heterosexuals with *mtr* phenotype	PPNG from heterosexuals without *mtr* phenotype	Resistance to oleic acid	1.32
PPNG from homosexuals with *mtr* phenotype	PPNG from homosexuals without *mtr* phenotype	Resistance to oleic acid	8.86

Group	Group	Characteristic	Chi^2
Non-PPNG from heterosexuals with *mtr* phenotype	Non-PPNG from heterosexuals without *mtr* phenotype	Resistance to oleic acid	7.27
Non-PPNG from homosexuals with *mtr* phenotype	Non-PPNG from homosexuals without *mtr* phenotype	Resistance to oleic acid	14.55
PPNG from homosexuals with 4.4 Md plasmid	PPNG from heterosexuals with 4.4 Md plasmid	Resistance to oleic acid	4.56
Non-PPNG with pro- auxotype	Non-PPNG without pro- auxotype	Resistance to oleic acid	10.9
Non-PPNG with Wild type auxotype	Non-PPNG without Wild type auxotype	Resistance to oleic acid	6.3
Non-PPNG	PPNG	Resistance to *B. fragilis*	4.31
Non-PPNG with *mtr* phenotype	Non-PPNG without *mtr* phenotype	Resistance to *B. fragilis*	5.6
Non-PPNG	PPNG	Resistance to *B. vulgatus*	9.27
Non-PPNG from rectums	PPNG from rectums	Resistance to *B. vulgatus*	6.03
PPNG from homosexual male urethras	PPNG from heterosexual male urethras	Resistance to *B. vulgatus*	4.48
Non-PPNG from homosexuals	PPNG from homosexuals	Resistance to *B. vulgatus*	10.61

3.3. Formula for Calculation of Standard Error of Difference in Means

$$\text{Standard error of the difference between means} = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}$$

where σ^2 is the standard deviation given by the formula

$$\frac{\Sigma(x - \bar{x})^2}{n}$$

and x = the observed value and $\bar{x}$ = the average of n observations

3.4. Formula for Calculation of Two Tailed Student's t-Test

To compare the means of two populations with $n_1 + n_2 - 2$ degrees of freedom

$$t = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)}{\sqrt{\left(\frac{\Sigma x_1^2 + \Sigma x_2^2}{n_1 + n_2 - 2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where $\bar{x}$ = population mean

$\mu_1 - \mu_2$ = expected value stated in null hypothesis

Σx^2 = sum of squares

n = number in the sample size

4. DETERMINATION OF MODE MIC FOR PENICILLIN IN NON-PPNG

	Number tested	Penicillin concentration (mg/l)	Number of strains inhibited
Female cervix (heterosexual)	26	1.0	1
		0.5	1
		0.25	5
		0.125	6
		0.06	5
		0.03	0
		0.015	7
		0.0075	1
		Mode MIC = 0.015 mg/l	
Female rectum (heterosexual)	25	2.0	1
		1.0	4
		0.5	1
		0.25	3
		0.125	9
		0.06	1
		0.03	0
		0.015	6
		Mode MIC = 0.125 mg/l	
Male urethral (heterosexual)	27	0.5	2
		0.25	6
		0.125	7
		0.06	2
		0.03	4
		0.015	5
		0.0075	1
		Mode MIC = 0.125 mg/ml	
Male urethral (homosexual)	25	0.5	3
		0.25	4
		0.125	8
		0.06	3
		0.03	0
		0.015	7
		Mode MIC = 0.125 mg/l	

	Number tested	Penicillin concentration (mg/l)	Number of strains inhibited
Male rectal (homosexual)	26	2.0	2
		1.0	2
		0.5	0
		0.25	9
		0.125	5
		0.06	2
		0.03	0
		0.015	6

Mode MIC = 0.25 mg/l