PLASMIDS AND THE VIRULENCE OF

PROTEUS MIRABILIS

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ABSTRACT:

The effects of large plasmids on different virulence characteristics of Proteus mirabilis strains mostly from clinical origin were studied. Moreover the inhibitory effect of urea and its derivatives on the swarming property of the strains was investigated.

A. All strains were screened for plasmid detection, antibiotic resistance and swarming ability.

B. Three multiresistant plasmid-carrying strains (PM5, P49 and P991) were cured and two transconjugants (G9pPM5 and G9pP49) were obtained by conjugation between two p+ donors (PM5 and P49) and one p- recipient (G9).

C. By comparing the virulence properties of cured and transconjugant strains with their parental isolates it was found that:

1. Plasmids confer resistance to P. mirabilis strains against one or more antibiotics.

2. The presence of most plasmids reduces the swarming ability of the strains.

3. Plasmids affect the motility and flagellation of P. mirabilis strains.

4. Plasmids enhance the adherence property of their host strains to inert surfaces and uroepithelial cells as well as autoagglutination.

5. Plasmids increase the hydrophobicity of P. mirabilis

strains.

6. The presence of plasmids reduced the growth rate of the strains. This effect was more apparent in iron-restricted medium.

7. Plasmids reduced the growth rate of their host strains in the presence of detergent (SDS).

8. The presence of plasmids reduced the survival of P. mirabilis strains in human and rabbit serum.

9. Plasmids decreased the survival of the strains in aquatic systems.

10. Plasmids reduced the production of urease and increasedsome others such as haemolysin and protease.

D. Urea and some of its relatives inhibited the swarming property of P. mirabilis strains and this effect was concentration dependent.

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INTRODUCTION

I. Introduction

I.1. Properties and pathogenicity of P. mirabilis:

P. mirabilis is a member of the Enterobacteriaceae family, a Gram negative short rod and highly motile bacterium. This non-capsulated pleomorphic bacterium, is a normal inhabitant of the human and animal intestinal tract. It is usually disseminated in human communities, and may function as a primary as well as a secondary pathogen (France and Markham 1967). It can also be readily recovered from sewage, soil, garden vegetables, and many other materials. There are speculations that this organism can cause diarrhoea, and is found frequently in the faeces of patients and less often in healthy people (Muller 1986).

P. mirabilis is the most frequently isolated species in the Proteus genus and a common cause of urinary tract infections (Adler et al. 1971), particularly in long-term catheterized patients (Hawkey et al. 1986). It is second to E. coli in occurrence, but the majority of E. coli infections are confined to the bladder, whereas Proteus strains particularly P. mirabilis have a special predilection to the upper urinary tract where they usually cause much greater kidney damage (Senior 1978), and lead to necrosis of the renal tubular

epithelium (Senior and Sweeny 1983). The symptoms are much more severe in elderly patients (Larson and Olling 1977) and young boys (Bergstrom 1972, Harlett 1976). Moreover, due to production of the enzyme urease, this species can split the urea of the urine and lead to formation of struvite kidney stones, which will be discussed later. This organism is often isolated from the mixed flora of wounds and burns and is a frequent cause of hospital-associated infections, so that during the last two decades several outbreaks of nosocomial infections caused by this organism particularly among neonates have been reported (Kusek and Herman 1981). It is also associated with chronic suppurative otitis, and the heavy discharging indicated that this organism contributes to the pathological process of the infection (Senior and Swenney 1983). It has just recently been suggested that this organism may be a causative agent in rheumatoid arthritis (Ebringer 1991, unpublished data).

P. mirabilis is also reported to be involved in causing pneumonia (Thapa et al. 1987), osteomielitis (Kirkbey and Risoe 1985), and neonatal meningitis (Chou et al. 1988).

P. mirabilis may also protect the body against infections in some situations. During the Napoleonic wars, D.J. Larrey a military surgeon realized that injured soldiers who were infested with blowfly were much more likely to survive. They did not develop blood poisoning and their wounds healed very

quickly. Then he found out that when the flies swarmed on the wounds, they laid eggs and the maggots developed from the eggs. The larva removed the dead and decaying tissues, cleaning and disinfecting wounds (Lewis 1955). In the American civil wars, the method was applied by Zacharias a confederate surgeon to treat wounded soldiers by deliberately placing maggots on their injuries (Erdmann 1987). The maggots were not only being used for this purpuse, but in 1928 William Baer an orthopaedic surgeon successfully used them to treat his patients.

Many scientists tried to discover the nature of this phenomenon, Duncan (1926) and Simmons (1935) discovered that the excreta of blowflies exhibited antibacterial activity, but they were not able to isolate any agent responsible for this effect. Later in 1968 Greenberg reported the disinfecting activity of filtered culture of P. mirabilis isolated from maggots' salivary glands, and named it Mirabilicid. In his early studies, Gary Erdman (1984) discovered the presence of two compounds namely, phenylacetic acid (PAA) and phenylacetaldehyde (PAL), in this extract of P. mirabilis. He suggested that the antibacterial action of these two compounds may be due to their lipophilic property, thus penetrating the cell membrane and interfering with the amino acid transfer reaction of protein synthesis. They were very active in acidic pH (pH 2.5) whereas the pH of the wounds is slightly alkaline. Moreover P. mirabilis itself may increase the pH of the medium

by releasing urease. But in the previous study, Greenburg (1968) found that the pH in maggots gut was 2.9, ideal for PAA and PAL to act as bactericidal agents. In fact maggots work as sterilising filters on the infected wounds, by eating bacteria and killing them in the gut by PAA and PAL derived from Proteus mirabilis.

I.2. Identification:

P. mirabilis grows well in several media and there are a number of widely used formulations. The swarming nature, the fishy odour on nutrient and blood agar, and pale non-swarming colonies on MacConkey and DOC agar are some clues to lead one to guess the presence of Proteus and differentiate it from other Enterobacteria. Moreover, P. mirabilis is lactose and indole negative, and most of the strains gelatine and H_2S positive. This organism is also capable of degrading tyrosine and decarboxylation of valine and leucine.

There are a few selective media for members of this genus. Mac◊Conkey agar has been widely used to differentiate Salmonella and Shigella from Proteus, because Proteus is a non-lactose fermenting bacterium and produces pale gray colonies on this medium. Cysteine lactose electrolyte deficient CLED, Proteeae isolation medium PIM (Hawkey et al. 1983), and Xilinas medium (Xilinas et al. 1975) are also selective media for identification of Proteus species which are discussed in the next chapter.

I.2.a. Typing of P. mirabilis:

Due to the epidemiological importance of P. mirabilis borne infections, numerous methods of typing have been suggested to identify different strains of this species (Kusek and Herman 1981). These methods include serological typing (Perch 1948, Kauffman 1966, Penner and Hennessy 1980), antibiotic sensitivity typing (Kashbur 1974) Dienes reaction (Dienes 1946) biotyping (France and Markham 1967, Burke 1971), bacteriocin typing (Senior 1977a, George 1974, Al-Jomaili 1975), bacteriophage typing (Adler et al 1971, Hickman and Farmer 1976). Each one of these methods can be used for typing of P. mirabilis strains, but usually a combination of some of them is applied for final and definite identification of the strains. The best typing method which specifies almost all of the biological properties of the strain is biotyping.

I.3. Swarming:

The swarming phenomenon is a unique and interesting property in certain members of the genus Proteus especially in P. mirabilis. It has encouraged and provoked scientists to discover the nature and the mechanism of this property since first reported by Hauser in 1885 (Falkinham and Hoffman 1984, Williams 1978). The occurrence of morphological changes on solid medium was the main reason that led Hauser to name this organism as Proteus [a Greek sea god who could change his shape at will(American Heritage Dictionary, second college

edition)]. When Proteus strains are cultured on suitable solid medium growth occurs as a surface film which radiates from the inoculation point and may eventually cover the entire surface of the plate within less than 24 hours. The film often exhibits a wavy appearance which suggests that growth occurred in separate physiological phases. Microscopical examination of the swarming Proteus revealed the morphological events, associated with this phenomenon, which have been described by some investigators (Hoeniger 1964, and 1966, Smith 1972, Williams 1978, Falkinham and Hoffman 1984). The ordinary short rods in broth culture are normally 0.6 μm wide and 1-2 μm long. Although filamentous forms have been reported in broth, on solid medium inoculated with the short rods, initially the cells grow slowly up to 0.8 μm wide and 2-4 μm long, then they start dividing as normal and form the typical bacterial colony. After about 2-3 hours the cells at the edge of the colony stop dividing, they continue to grow and, therefore elongate dramatically up to 40-100 μm. The filaments which have also shown derepression of flagella synthesis, move across the plate more or less concentrically for a distance of about 5-7 mm. The first period of the swarming is now completed, and the migration of the swarmers stops. Then the cells divide along their length into segments and finally form the normal short rods. After a period of growth and consolidation, the cycle starts again with the formation of a new generation of swarmer

cells. The swarmer cells formed in each cycle not only show an increased number of flagella but their average length also increases (Hoeniger 1965), thus the flagellar length of ordinary short rods is 0.8-4 µm, whereas that of the swarmers is 6-12 µm.

Flagella are made of a single protein called flagellin with the molecular weight of 40000, and the highly increased length and number of derepressed flagella indicates significantly enhanced synthesis of this protein (Smith 1972) which may suggest greater nutritional requirements (particularly nitrogen) of swarmer cells on solid media.

In contrast to flagella, fimbriae are numerously present on short cells and with the development of swarmers the number of pili per unit length reduces.

I.3.a. Types of swarming:

According to Belyavin (1951), there are three types of behaviour on agar which are as follows:

A. Zonal type, is the usual periodic zonated growth producing concentric rings around the inoculation point.

B. Non-zonal type, is a type of swarming which does not appear in periodic zones and gives a single thin film across the plate and uniformly covers the surface of the agar.

C. Non-swarming, which represents non-swarming colonies consisting of short and non-motile rods, these strains are also non-motile in nutrient broth.

There is another description of swarming responses reported by Coetzee and Sacks (1960) in which this phenomenon is divided into four types (Y, Z, X, and W) and is basically the same as Belyavin's.

I.3.b. Mechanism of swarming:

The mechanism of this property is not yet clearly understood. Since Hauser (1885) reported the phenomenon there have been many controversies over the regulation of this process. Initially some workers believed that overgrowth of the bacteria in the inoculation point results in the lack of nutrients and stimulates the bacteria to migrate outwards from the colony to where they can find sufficient nutrients (Moltek 1927). Later many investigators proposed that negative chemotaxis from a toxic metabolite which accumulates during bacterial growth, is the main cause of swarming (Lominski and Lendrum 1947, Hughes 1957, Hoeniger 1964, Brogan et al. 1971, Smith 1972). In 1976 Williams and co-workers presented convincing evidence that chemotaxis can not play at least the major role in producing swarmers (Williams et al. 1976). Then in a review in 1978 Williams and Schwarzhoff supported the hypothesis presented by Kvittingen (1949) that swarming is a stage of the normal life cycle of Proteus and its related organisms which is the outcome of the expression of a number of determinants governed by a complex of genetic factors. They also confirmed the three phases in swarming, namely formation

of elongated and highly flagellated cells, migration from the initial colony on the surface of agar, and dividing of the filaments to normal short vegetative rods. Nevertheless, there are still many ambiguities and more work is needed to discover the exact mechanism of swarming.

I.3.c. Swarming inhibitors:

Swarming of Proteus strains often causes problems in laboratory experiments as well as during the isolation of pathogens from clinical specimens. There are numerous papers suggesting diverse methods to prevent swarming. Naylor in 1964 prepared a table of mechanical and chemical methods for swarming inhibition which had already been proposed by several investigators. Then two years later Kopp and his colleagues (Kopp et al. 1966) reported the inhibitory effect of para-nitrophenylglycerol (PNPG) on swarming at a very low concentration which does not affect the growth and motility of bacteria (Senior 1977b). A more complete table was then published by Smith in 1972 (Table 1).

The mechanical way consists of using over-dried or stiff (4-8%) or weak (0.8%) or overlayered agar culturing.

Chemical methods involve the addition of various chemicals to basic agar, each of which supresses swarming through different mechanisms, such as inhibition of growth, repression of flagella formation, etc.

In this study the inhibitory effects of urea, guanidine

Table 1.

List of mechanical and chemical inhibitors of *Proteus* swarming

Mechanical Methods	1- Dried agar surface
	2- Poured or layered plates
	3- Stiff agar (4-8 per cent)
	4- Weak agar (0.8 per cent)
Chemical Methods	
	5- Activated charcoal (1%)
I- Inorganic	6- Aluminium salts
	7- Bismuth sulphate
	8- Boric acid (0.2%)
	9- Electrolyte deficiency
	10- Ferrous salts
	11- Potassium tellurite
	12- Sodium azide
	13- Sodium chloride (10%)
II- Organic	
i- Surfactants	14- Bile salts
	15- Dispersol
	16- Sodium alkyl sulphate
	17- Sodium apocholate
	18- Sodium deoxycholate
	19- sodium ricinoleate
ii- Drugs	20- Barbitone
	21- Cacotheleine
	22- Chloral hydrate
	23- Morphine
iii- Antibiotics	24- Neomycin
	25- Neomycin+polymixin+fucidin
	26- Polymixin+nystatin
	27- Sulphonamides
iv- Others	28- Amino acid deficiency
	29- Ethanol
	30- Ether extraction
	31- Glycopeptide deficiency
	32- Nitrophenylglycerol
	33- Phenyl alcohol
	34- Polyvalent antiserum
	35- Purines (hydrolysed)

Table has been extracted with permission from Smith (1972).

and their derivatives on swarming have been examined.

I. 4. Urea: This compound ($H_2N-CO-NH_2$) is the diamide of carbonic acid and the excretory product in a large number of urolytic animals and man. Urea is produced by several metabolic pathways which are as follows:

1- Urea cycle.

2- Hydrolysis of L-arginine and other guanidine derivatives.

3- Oxidative purine degradation, enzymatic hydrolysis of allantoic acid and glyoxyurea.

4- Various other metabolic pathways eg. by rare oxidative pyrimidine degradation.

In the body, intracellular catabolism of proteins and intestinal absorption both add to overall free amino acids. Some of these amino acids are used in the synthesis of new proteins, but most of them are metabolised for energy production through the Kreb's cycle after deamination. This deamination results in the release of one ammonium ion from removal of each amino group. Ammonium is highly toxic to body tissues and its concentration must be kept very low, therefore most amino groups are transferred from amino acids to specific carrier molecules eg. (alpha-ketoglutarate), by which they are delivered along with free ammonium (released from the rest of the body) to the liver. In the liver urea is produced from carbon dioxide, ammonium and amino groups through the urea

cycle. This process is energy dependent and for the synthesis of one molecule of urea three molecules of ATP are required. Basically the primary function of the urea cycle is to convert the waste and toxic nitrogen into non-toxic , soluble urea which can also be easily excreted by the kidneys.

I.4.a. Properties of urea: Urea is highly soluble in water and extraordinarily non-toxic, therefore in dehydration conditions a large amount of urea can be excreted in a small volume of urine without precipitation or toxication. It is also soluble in ethanol but not in ether. The concentration of urinary urea in human is 0.4-0.5 M, but in some desert mammals it goes as high as 2 M (Griffith et al. 1976). In solid form, urea gives white crystals with molecular weight of 60 and melting point 137°C. Once urea is synthesized in the liver, it accumulates in the plasma to the standard level of 6 mg/ml, and the excess is freely excreted by the kidneys. The load of filtered urea depends upon several factors such as, level of antidiuretic hormone (ADH), normal daily diet, urine flow and normal functioning of kidney.

I.4.b. Urea as an osmotic agent: Urea is a highly penetrating agent and can easily diffuse through the body tissues and cells, and therefore is one of the main factors in balancing the osmolarity of body fluids especially urine. This given balancing function facilitates the filtration of urine through the urinary system (Haldky and Rink 1986). Infusion of

hypertonic urea solution has sometimes been used to reduce cerebral oedema by osmotically removing water from the brain. Red blood cells have also high permeability to urea which protects them from excessive shrinking and deformation during passage through renal capillaries.

I.4.c. Urea as a nitrogen source: Urea represents one of the main nitrogenous excretory products in a wide range of animals as well as in humans. Enormous quantities of this substance are constantly released into the environment through the biological activities of man, animals, birds, reptiles and some types of insects (Harry et al. 1986). The release of urea is associated with the degradation of proteins, amino acids and their derivatives such as, arginine, ornithine, allantoin and allantoic acid (Vogel and Van der Dreft 1976). This urea is then widely utilised by bacteria, plants and some animals as an important nitrogen source in protein synthesis. In humans, almost 20% of the urea produced by the liver is diffused from the blood stream to the intestinal tract where it is hydrolyzed by bacterial urease (Visek 1972). A large number of urea degrading bacteria have been isolated from human faeces which include, Peptostreptococcus productus, Ruminococcus albus, Clostridium innocuum, Fusobacterium prausnitzii, Coprococcus catus, Streptococcus mitis, Eubacterium aerofacience and Eubacterium lentum.

In ruminants, significant amounts of urea endogenously

and exogenously often appear in the rumen. The endogenous urea is transferred directly from the blood stream to saliva and is then swallowed by the animal. Exogenous urea is manually added to the daily diet to enrich the nitrogen source of animal feed. This urea is recycled by symbiotic ruminal bacteria in the rumen, where degradation reactions takes place and ammonia is released as the major source for protein synthesis in these organisms (Bryant 1959). Then the microbial biomass which is produced from these bacteria is digested by the ruminants.

Urea also plays an important role in providing the nitrogen source for plants. The degradative processes of animals and degradation of waste nitrogenous compounds results in the presence of large quantities of urea in soil. On the other hand, urea has long been used as a nitrogenous fertilizer in agriculture. It is not, however, applied as urea itself since due to the activity of bacterial and fungal urease, the sudden release of ammonia would lead to nitrogen loss and increased toxicity of the soil. Therefore, it is applied in the form of urea-aldehyde condensation compounds which slowly release nitrogen for plants.

I.4.d. Urea as an antibacterial agent: Urea and some of its derivatives particularly urethanes have antibacterial properties. Carbamides are a group of these derivatives which are mainly effective on Gram-positive bacteria. They seem to interfere with the functions of the cell membrane.

Diphenylthiourea has been clinically applied in the treatment of tuberculosis and trichlorocarbanilide (TCC) is used in antiseptic soaps. It is believed that the aseptic condition of urine is due to the presence of urea. High concentrations of urea and its related compounds possess denaturing effect on proteins and polynucleotides. Gordon and Jencks (1963) examined the activity of a wide range of such compounds on bovine serum albumin and ovalbumin by measuring the changes in optical rotation which indicated the denaturation of these proteins. The mechanism of the denaturing effect of these compounds is not yet clearly understood, but three factors have been suggested to be involved in this process, the structure of amide groups, hydrogen bond formation and the hydrophobic character of the denaturant. Levine et al (1963) proposed that the denaturation of DNA by urea is mainly due to the hydrophobic property of this agent.

In this study the inhibitory effect of urea, guanidine and some of their analogues on the growth and swarming of P. mirabilis has been observed.

I.5. Plasmids:

Plasmids are extrachromosomal genetic structures which are stably inheritable, and autonomously replicate independent from the chromosome within the bacterial cytoplasm. They are covalently closed circular, double stranded DNA molecules found in a variety of bacterial species. Their molecular weight ranges from 1 to 200 megadaltons, and multiple copies of the same plasmid may be present in one bacterial cell. This copy number may be from 1-2 per cell (stringent plasmids such as F) up to 30-50 copies per cell (relaxed plasmids like Col. E).

Different plasmids can sometimes co-exist in the same cell, these are called compatible, and they are divided into separate groups accordingly. Plasmid incompatibility is defined as the inability of two plasmids to co-exist stably together in the same cell, and this property is used to classify plasmids into a large number of groups on the basis of their mutual incompatibility (Datta 1977). It is suggested that incompatibility is related to the fact that the maintenance of two different plasmids is controlled by the same mechanism. Certainly, incompatible plasmids are closely related.

I.5.a. Conjugation:

Usually the only genetic exchange which occurs between plasmid-free bacteria is that involving free DNA (transformation) or DNA carried by temperate bacteriophage (transduction). In contrast some plasmids may bring about

their own transfer from one cell (the donor) to another (the recipient). Such process is called conjugation, and this type of self transmissible plasmid (eg. Col V) is referred to as a sex factor, which carries the 25-30 kilobase pair transfer operon (tra) and codes for the sex pili which are essential for plasmid transfer. Basically the donor characteristic as originally observed (Glass 1982) is conferred by the presence of the F factor (fertility factor), and such a cell is usually designated as F+ and the recipient which does not possess this genetic element is called F- cell. Now conjugative plasmids distinct from F have deen found in a wide range of Gram-negative bacteria including P. mirabilis.

I.5.b. Mechanism of conjugation:

During conjugation when a proper population of donor and recipient strains are mixed, the cells become united by the sex pili and possibly are pulled together by pilus retraction. Then a breakage occurs at the origin of transfer (oriT) on one strand of double stranded circular plasmid. The broken strand unwinds and with the 5' end leading, moves into the recipient cell through the sex pilus or conjugation tube. In the recipient a complementary strand is synthesised on the given strand, and it will carry a circular double stranded DNA like that of the donor. In the donor synthesis of DNA initiates from the site of oriT which is located in the tra operon, using the intact unbroken strand as template leading to replacement

of the transferred strand. Broda (1979) presented evidence supporting the hypothesis of direct involvement of sex pili in conjugation, but the mechanism of their function is not precisely understood. It may be that the pilus acts as a bridge or tunnel between the two cells, through which the DNA can easily move to the recipient cell(Brinton 1971). There is another idea saying that the contraction of F pilus results in wall to wall contact of the donor and the recipient which eases the transfer of DNA (Curtiss 1969).

Non-transmissible plasmids may be transferred by three methods:

I.5.c. Sex factor mediated transfer: During conjugation induced by a superinfecting sex factor the non-transmissible plasmid can be transferred. Such transfer may involve fusion of the non-conjugative plasmid to the sex factor prior to transfer, but generally it does not occur .

I.5.d. Transduction: The transfer of plasmid by the mediation of a bacteriophage is referred to as transduction, which has been observed in a wide range of bacterial species such as E. coli, Proteus, Pseudomonas, Salmonella and Bacillus. There are two types of transduction, generalized and specialized.

Generalized transduction is a type of transduction in which any plasmid or gene is transduced to recipient, and it has been well demonstrated by Salmonella typhimurium and phage

P22 (Broda 1979). Specialized or restricted transduction involves the transfer of particular genes which occurs only following the lysogenic bacteriophage induction. In this process only the genes which are associated with the integrated prophage can be transduced (Broda 1979).

I.5.e. Transformation: Is a process in which the recipient cell takes up an exogenous DNA which may be either a plasmid or part of a chromosome from the environment. This DNA fragment may incorporate into the host chromosome by homologous recombination or becomes an autonomous replicon staying independent from chromosome. The cells capable of receiving transforming DNA are called competent cells and in natural transformation, this competence is associated with the synthesis of certain proteins in the cell envelope which renders the cell permeable to DNA.

Transformation does not usually occur in vivo, because the conditions are not suitable for a naked DNA to exist in the external environment. But, this process can be carried out in vitro using different methods and during certain growth phases in batch cultures. Under suitable conditions sometimes up to 100% of the cells in a culture may become competent. Such natural transformation commonly occurs in Gram-positives such as Bacillus, Streptococcus, Some Gram-negatives like Neisseria and Haemophilus can also show natural transformation. For other Gram-negatives, treatment with certain chemicals (usually

involving calcium chloride and heat) can induce competence and allow transformation in vitro.

I.5.f. Curing of plasmid DNA:

Most plasmids are stable and autonomously replicate in bacteria, but in certain environmental conditions or in the presence of some chemicals (curing agents) they may be lost during bacterial growth. Curing is in fact the elimination of plasmid from a bacterium without loss of its viability (Singlton 1983). The presence of sublethal dose of intercalating dyes such as acridine orange (Hirota 1960) or ethidium bromide (Bouanchand et al. 1969) and some antibiotics like novobiocin and rifampicin, inhibits the replication of plasmids in a p+ population and gives rise to a number of plasmid-free cells. Growth at high temperature (eg. 40-42°C) in the presence of a detergent such as sodium dodecyl sulphate (SDS) may also eliminate the plasmid from a bacterial population. Plasmid curing has been widely implemented to discover the properties conferred to a bacterial strain by that particular plasmid.

I.5.g. Replication of plasmid DNA:

As mentioned earlier, plasmids are extrachromosomal replicons which can replicate autonomously independent from the bacterial chromosome. Therefore this replication should occur at least once in the cell life cycle for stable maintenance and segregation of plasmid, but it is not controlled by any stage

of the cell cycle. To initiate replication, plasmids must contain an origin of replication (oriR) and may replicate unidirectionally (Col E1) or bidirectionally (F plasmid) and may have more than one oriR region. Plasmid replication requires various host proteins. For example, more than 12 proteins have been reported to be involved in this process in E. coli (Broda 1979). Moreover, the synthesis of RNA is essential for the replication of many plasmids such as F, ColE1 and R6K. This RNA synthesis is suggested to be necessary for the production of RNA primer to allow the subsequent DNA synthesis. Plasmid synthesis must firstly be initiated and then undergo different stages during replication which are, helix destabilization, unwinding, RNA primer synthesis, DNA polymerization and ligation. Plasmids are put into two groups in terms of type of replication, small and large plasmids. Small plasmids with low molecular weight normally have a high copy number (sometimes more than 10 per cell) and their replication is referred to as relaxed replication. In contrast, the replication of large plasmids with molecular weight more than 30 Md seems to be roughly in synchrony with chromosomal replication and is called stringent replication. The copy number of this type of plasmid is not usually more than two per cell.

I.5.h. Mechanism of replication:

The exact mechanism of plasmid replication is not yet clearly

explained. Three models have been suggested for this process:

1. Cairns' model. In this model the supercoiled molecule of DNA unwinds and then replication of both strands starts from a specific origin (ori R) without initial nicking. Then two strands separate and nicking occurs as replication proceeds. A Y- shaped replication fork starts from the origin of replication and moves around the circular molecule of plasmid. If the replication occurs bidirectionally, two Y forks move in opposite directions resulting in the formation of two double stranded DNA molecules; each consists of one old strand and the new replicated daughter strand. When the replication is unidirectional, one fork moves in one direction from the origin of replication (Broda 1979).

2- Gilbert and Dressler model: This type of replication is similar to the Cairns model except that unwinding occurs during replication and the non-replicated section of plasmid remains supercoiled (Gilbert and Dressler 1968).

3- Rolling circle model: A type of asymmetric replication in plasmids and bacteriophages, which starts with initial breakage of one strand. Then the 5' end of this strand attaches to a specific site on the cell membrane, triggering the initiation of plasmid replication. The strand unwinds and replication proceeds by elongation of the 3' hydroxyl end using the unbroken strand as template. Finally, the 5' end is displaced from initiation site on the membrane.

I.6. Cell envelope structure in Gram-negative bacteria:

The application of high resolution electron microscopy revealed fundamental differences between Gram-positive and Gram-negative bacteria. The cytoplasmic membrane of Gram-positives is enclosed in a thick layer of peptidoglycan and in some cases covered by a capsule whereas the structure of the cell envelope in Gram-negative organisms is more complicated, and consists of cytoplasmic membrane and a complex cell wall, as shown in Figure 1.

I.6.a. Cytoplasmic membrane:

The first layer which limits the cellular protoplasm is the cytoplasmic or plasma membrane. The structure of this selectively permeable membrane is generally the same in both Gram-positive and Gram-negative bacteria. The most accepted model for such a cell membrane is the fluid mosaic model suggested by Singer and Nicolson (1972) and based on the unit membrane concept. They proposed that the membrane consists of a double layer of phospholipid molecules with their hydrophobic fatty acid chains (hydrocarbon) pointing inward and the hydrophilic phosphate groups facing outward (Robertson 1981). Moreover, two types of protein molecules have been recognized in this membrane which lie within the two layers or on the surface of one lipid layer and sometimes extended across the width of the membrane (Fig. 2). One type of these proteins *Is* more peripheral and can be easily isolated by

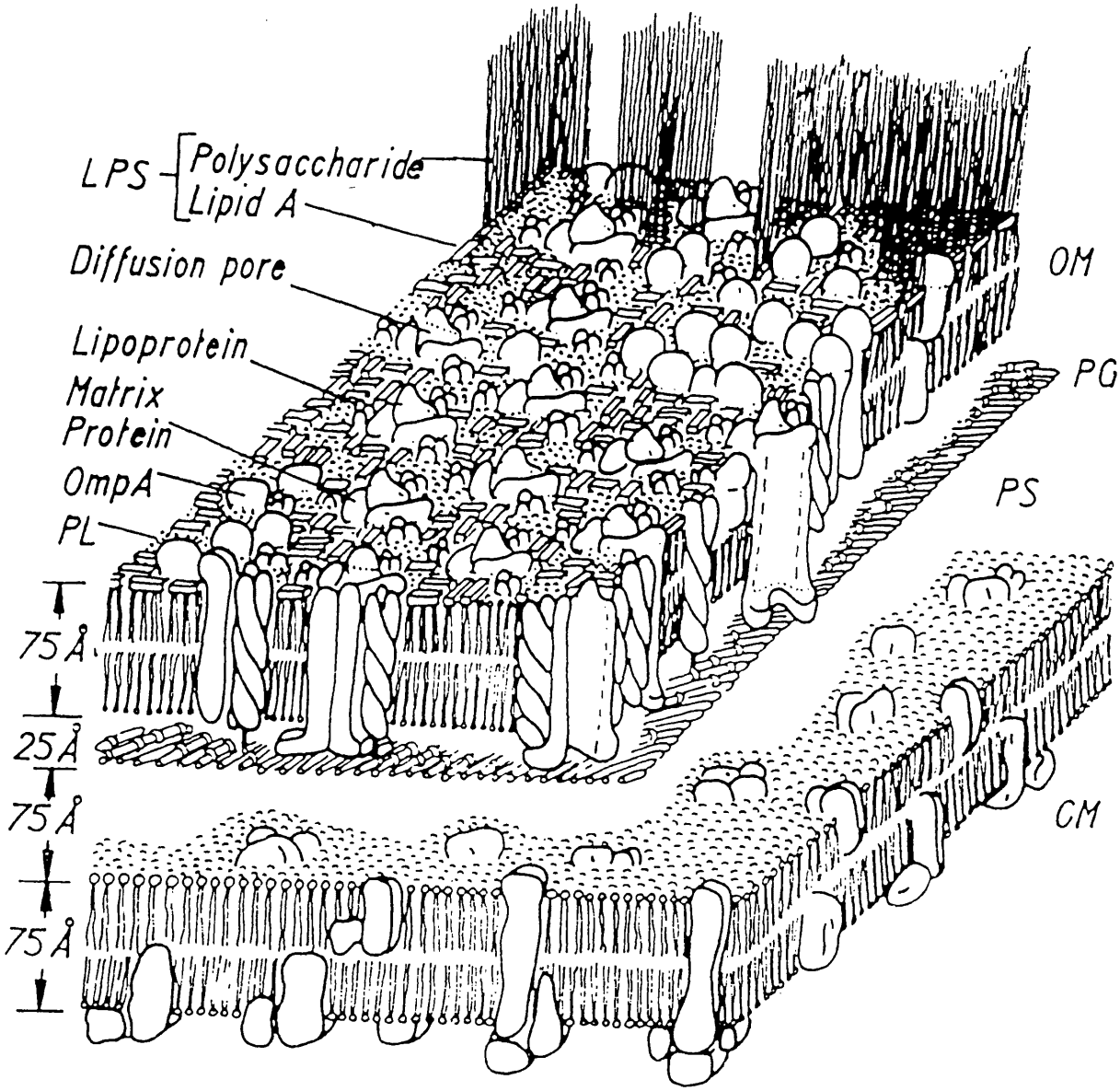

Fig. 1- Model of the Gram-negative cell envelope.

LPS: Lipopolysaccharide; OM: Outer membrane; PG: Peptidoglycan PS: Periplasmic space; CM: Cytoplasmic membrane; OmpA: Outer membrane protein A; PL: Phospholipids.

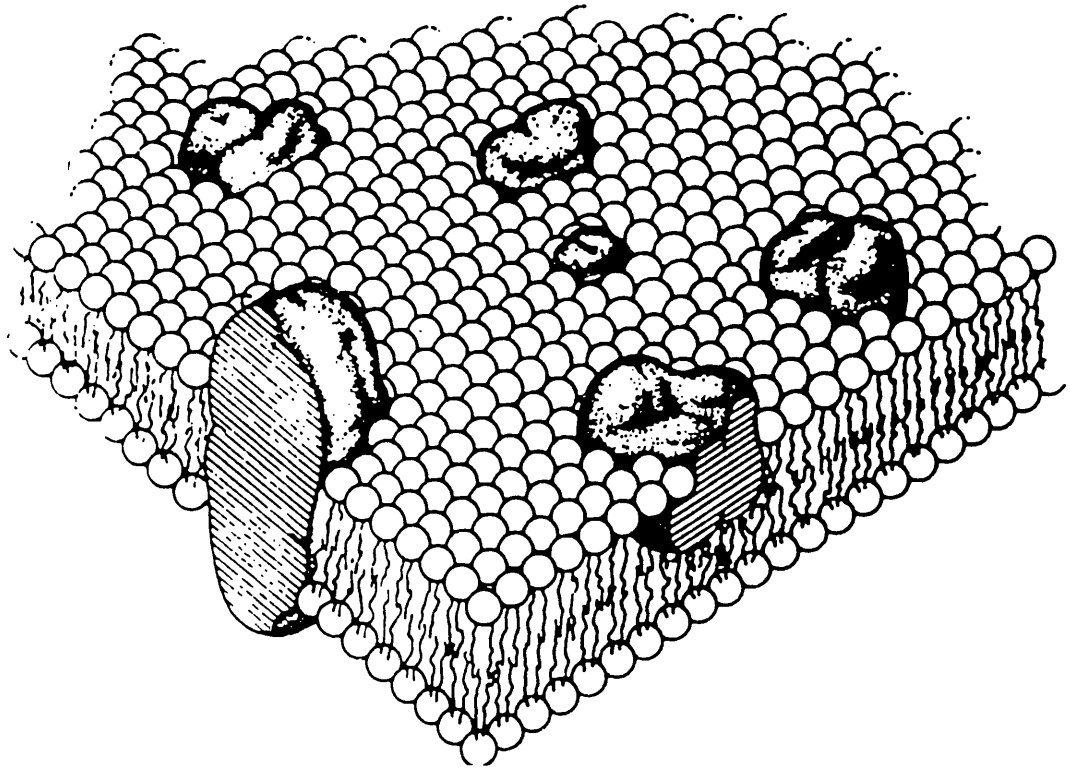

Figure 2. Fluid mosaic model of cytoplasmic membrane from Singer and Nocholson (1972). The empty circles represent the phosphate group with the hydrophobic tails. Proteins are designated by the cross lined particles embeded in the bilayer.

some chelating agents such as EDTA, whereas the other types which are referred to as intrinsic ones are firmly bound to hydrophobic tails and can only be removed by dissociation of the cytoplasmic membrane (Rogers 1983). It is believed that these proteins are bound to phospholipid molecules by divalent cations (eg. Mg^{2+}). The cytoplasmic membrane controls the passage of metabolites in and waste products out and may also selectivly act as a barrier against lethal substances such as toxins and antibiotics. It is also a site for the biosynthesis of extracellular enzymes or toxins as well as for the electron transport chains (Hammond et al 1984). The lipid part regulates the permeability of the cell and provides the lipophilic environment required for membrane enzymes involved in the synthesis of lipopolysaccharides, peptidoglycan and in Gram-positives teichoic acid (Rogers et al 1980).

I.6.b. Cell wall:

The cytoplasmic membrane is surrounded by a structured and mechanically strong layer called cell wall which is responsible for the shape maintenance and integrity of the bacterial cell. The cell wall is formed of the peptidoglycan layer, periplasmic region and the outer membrane.

I.6.b.1. Peptidoglycan:

The first layer of the cell wall which encases the plasma membrane, is the peptidoglycan or murein layer. This rigid layer in Gram-negative bacteria is very thin and constitutes

only 5-10% of the cell wall dry weight, whereas, in Gram-poitives it is thick (50-60% of the wall dry weight) and is the only structure which covers the cytoplasmic membrane (Burge et al. 1977). Its stable complex with crystal violet in Gram staining is an important factor to differentiate between these two main bacterial groups. The basic structure of peptidoglycan layer is made of a network of polyaminosugar chains cross linked by short peptide molecules as shown in Figure 3. The polysaccharide chains or glycan strands sometimes consist of up to 200 disaccharide units which are alternatingly formed by N-actylmuramic acid and N-actylglucosamine molecules (Iterson 1984). The peptidoglycan layer plays a crucial role in physical and physiological maintenance of bacterial cell particularly in Gram-positive organisms. Some antibiotics such as β-lactams are effective on this layer by damaging the peptide cross-link of the peptidoglycan molecules.

I.6.b.2. Periplasmic region:

In Gram-negative bacteria the region between the cytoplasmic and the outer membrane is known as periplasmic region in which the peptidoglycan layer is situated and possibly forms a sponge-like gel in it (Hammond et al 1984). The periplasmic region also contains the aqueous periplasm, a solution of water soluble enzymes and unique series of other proteins and oligosaccharides. The impermeability of the outer

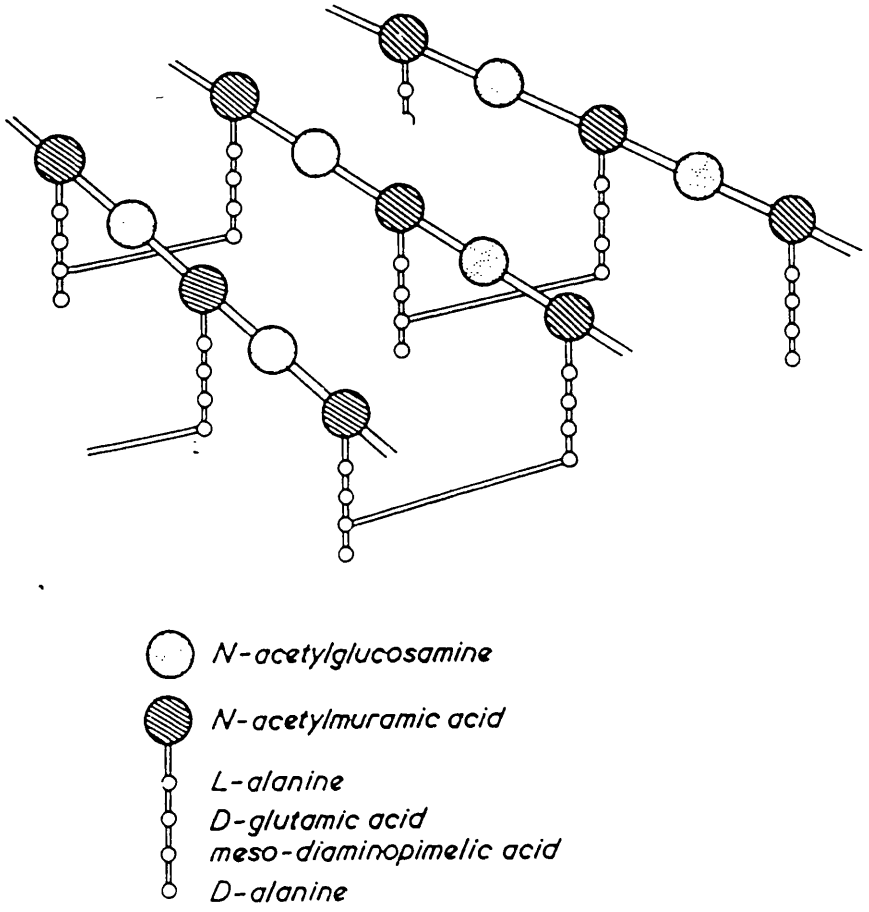

Figure 3. The schematic structure of the peptidoglycan layer of the cell wall.

membrane is the main factor inhibiting the leakage of periplamic contents to the medium. Some of the enzymes present in the periplasmic region are responsible for inactivation of the antibiotics which have been able to penetrate the permeability barrier (Hammond et al 1984). Certain other proteins in this region convert the nutritional compounds to a form which can be transferred through the cytoplasmic membrane.

I. 6. b. 3. Outer membrane:

The presence of the outer membrane in Gram-negative bacteria is physiologically very important for the bacterial cell. In comparison to Gram-positive organisms, the high level of resistance to antibiotics and toxins in Gram-negatives is due to the impermeability of the outer membrane to these compounds. In other words, this membrane constitutes a barrier protecting the bacteria against hydrophobic compounds (eg. antibiotics) as well as large hydrophilic macromolecules such as enzymes and toxins (Hammond et al. 1984).

The outer membrane consists of three major components, lipopolysaccharides, phospholipids and proteins. According to the accepted model of Singer-Nicholson the locations of these molecules are shown in Figure 1. Like cytoplasmic membrane it is made of a bilayer structure of lipids with hydrophobic lipid chain inwards and hydrophilic groups outwards. The difference from the cytoplasmic membrane is that the outer membrane is not a phospholipid bilayer but it contains

phospholipids and low molecular weight lipoproteins in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet.

I. 6. b. 3. a. Lipopolysaccharides.

The main physiologically important structure of the outer membrane is formed by lipopolysaccharide molecules which are located in the exterior layer of the membrane. They are mainly associated with antigenic activity, the endotoxic properties of the bacterial cell and the outer membrane barrier properties.

The basic model of LPS molecules which has been suggested by Luderitz and Westphal (Luderitz et al 1982) consist of three regions; the lipid A region, core polysaccharide and the O-side (O-specific) chain (Fig. 4). The two last parts are sometimes referred to as heteropolysaccharide which are covalently linked to the lipid A. There are two major differences between the lipid structures of LPS and phospholipid molecules; the fatty acids of LPS are always saturated whereas those of the phospholipids are not. Also there are two lipid chains in phospholipids but the LPS molecules usually have up to seven fatty acid chains (Nikaido and Vaara 1985). The LPS molecules are also strongly bound to divalent cations such as Mg^{2+} which gives them a firm position in the outer membrane (Schindler and Osborn 1979).

The lipid A is a glycolipid structure embedded in the

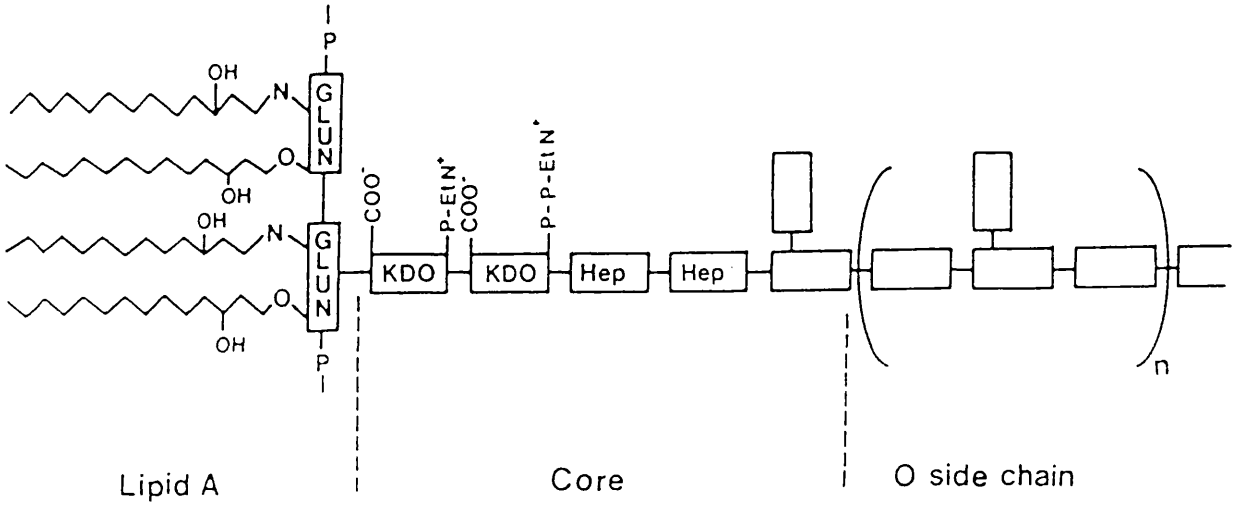

Figure 4. Model of lipopolysaccharide molecules of the outer membrane.

KDO= 2-keto-3-deoxyoctonic acid. Hep= Mannoheptose. GLUN= Glucosamine (disaccharide). P-EtN⁺= Phosphate-ethanolamine. P-P-EtN⁺= Pyrophosphateethanolamine.

membrane and is in fact the bacterial endotoxin. It is the only bacterial lipid which contains hydroxy fatty acid, mainly 3-D-hydroxy acids (Hammond et al 1984). A diversity of lipid chains and D-3-hydroxy fatty acids may be found in Gram-negative bacteria. In enterobacteria these fatty acids are usually linked to glucosamine disaccharide; mainly β-hydroxymyristate (Lüderitz et al 1982).

The core polysaccharide is located outwards between lipid A and O-specific chain. It is made mainly from, glucose, galactose, glucosamine, mannoheptose, KDO and some phosphate residues. The sugars of the core are linked to lipid A by the sugar acid 2-keto-3-deoxyoctonic acid (KDO) or 3-deoxy-D-*manno*-octulosonic acid (Nikaido and Vaara 1985). Although many mutations resulting in the production of defective LPS molecules have been reported (Makela and Stocker 1981), the KDO region seems to be stable and mutation does not affect it (Hammond et al 1984). The core structure is similar in closely related bacterial strains.

The O-side chain extends freely outwards from the core region up to 30 nm. It has a basic structure consisting of a hydrophilic chain of repeating identical oligosaccharide subunits and may be either linear or branched. These are immunologically important structures since they determine the nature of cell surface antigens. The presence of a diverse range of O-serotypes in Gram-negative bacteria (up to one

hundred in Salmonella and E. coli) is due to the variation in sugar composition and the type of glycoside linkage (Lüderitz et al 1982). The synthesis of these polysaccharides is catalyzed by the enzymes associated with the cytoplasmic membrane.

The proteins in the outer membrane are of three types:

I. 6. b. 3. b. Lipoproteins which often extend into the periplasmic space and some of these lipoproteins are covalantly bound to peptidoglycan layer and the others are free in the periplasmic region. These are the most abundant proteins in the bacterial cell and as small in molecular weight as 7200 daltons (Nikaido and Vaara 1985). For example their number in E. coli envelope has been reported to be around $7.5X10^5$ per cell. As mentioned they appear in two forms; about one-third of them are covalently linked to peptidoglycan layer by the amine group of lysine. The rest which are not bound to peptidoglycan contain free lysine. Lipoproteins can be detached from peptidoglycan by enzymatic treatment. Compared to the other outer membrane proteins the function of these proteins is not clearly understood. It is suggested that lipoproteins particularly peptidoglycan-bound ones assist the stability of the outer membrane (Nikaido and Vaara 1985), and mutants lacking these proteins were seen to have unstable membrane and were releasing the periplasmic contents into the environment (Hirota et al 1977).

I.6.b.3.c. Major proteins are a group of proteins always present in the outer membrane and can be readily detected by SDS polyacrylamide gel electrophoresis (PAGE). They may act as binding sites for bacteriophages, bacteriocins and leucocytes or as diffusion pores for hydrophilic molecules which may be used for entry of antibiotics. Certain bacteria demonstrating resistance to antibiotics or which do not bind leucocytes and phages, often possess modified major proteins. The molecular weight of these proteins ranges between 35000 to 38000 in E. coli K12 (Hammond et al 1984).

Based on the extraction conditions, two groups of major proteins have been identified; those which can be extracted in 2% SDS at 60°C such as OmpA (outer membrane protein A) and the other group which are not detectable under these conditions or require higher temperature like matrix proteins or porins.

I.6.b.3.d. OmpA, is a major protein readily extracted from the outer membrane in E. coli. Also its molecular weight changes apparently with increased temperature when detected by SDS polyacrylamide gel electrophoresis. Thus when the preparation in SDS is heated its mobility reduces significantly, and therefore it is referred to as "heat modifiable" protein (Nakamura and Mizushima 1977). The exact function of OmpA is not precisely understood, but this protein acts as phage receptor (Lugtenberg and van Alpen 1983). It is reported that in E. coli the OmpA is the receptor of phage K3 and Tu II (Hammond et al 1984). Some mutants of E. coli

lacking this protein exhibited defective growth rate due to the reduced amino acid transport system (Manning et al 1977).

I.6.b.3.e. Porin protein is a type of outer membrane major protein which can be detected at high temperature in SDS-PAGE. This is a trimer channelled molecule of protein which lies across the outer membrane and is linked to peptidoglycan layer. The channels formed by porins allow the non-specific diffusion of small hydrophilic molecules (less than 700) of nutrients, antibiotics and other inhibitors to cross the membrane (Lutkenhaus 1977). These proteins also serve as receptors for bacteriophages and bacteriocins. Recent electron microscopy studies showed that each of the three subunits of porins have a separate channel which in the middle of their length join to each other and form a single channel at the other side of the outer membrane (Dorset et al 1984). These proteins are coded for by ompC, ompF and phoE determinants in E. coli K12 (Nikaido and Vaara 1985). Nurminen et al (1976) reported the presence of another type of porin in an OmpD mutant of Salmonella typhimurium. Also E. coli mutants lacking OmpC and OmpF proteins which were resistant to specific phages and bacteriocins have been observed (Hammond et al 1984).

I.6.b.3.f. Minor proteins, which are inducible or derepressible proteins and may be induced or altered by genetic determinants such as, plasmids and phages also under specific

environmental or nutritional circumstances. There are different types of these proteins. Most of the specific diffusion channels are in this class. LamB protein in E. coli K12 is a minor protein which is specifically a channel for the passage of maltose and maltodextrins (Nikaido and Vaara 1985). The Tsx protein is a constitutive minor protein with 26000 molecular weight which acts as a receptor for phage T6 and is involved in nucleoside transport.

I. 6. b. 3. g. Phospholipids:

The phospholipids of the outer membrane are almost the same as those of the cytoplasmic membrane, but most of the outer membrane phospholipids are of phosphatidylethanolamine PE and less phosphtidylglycerol PG (Lugtenberg and Peters 1976). Also the ratio of saturated fatty acids particularly in PE is significantly higher than unsaturated ones. This composition is not affected by the conditions of the growth medium. The phospholipid molecules of the outer membrane are predominantly present in the inner leaflet of the membrane (Hammond et al 1984). It is suggested that phospholipids are involved in the permeability barrier of the outer membrane, and the mutants with defective phospholipids were more sensitive to antibiotics (Lugtenberg and Peters 1976).

I. 6. b. 3. h. Properties of the outer membrane:

As mentioned earlier the presence of the cell wall is of great biological importance to Gram-negative bacteria which is

discussed below.

A. Resistance to enzymes. The outer membrane renders the host organism resistant to body defence systems like lysozyme and antibodies. For example many body fluids such as tears and serum contain the enzyme lysozyme which has a significant role in the host defence system. In Gram-positive bacteria this enzyme easily degrades and removes the peptidoglycan layer of the cell followed by lysis (Patterson-Delafield et al 1980). In Gram-negatives, however due to the presence of the outer membrane this enzyme is ineffective on peptidoglycan. Lysozyme can only be active on this group of bacteria if the outer membrane is removed by a chelating agent like EDTA. Moreover, the outer membrane is the main protective structure in enteric bacteria living as normal inhabitants of intestinal tract, against the digestive enzymes and bile salts (Nikaido and Nakae 1977). This protective role is mainly due to the permeability barrier of the outer membrane. As mentioned above, hydrophilic molecules with molecular weight more than 700 can not pass through the porin proteins. Also the strong interaction between the LPS molecules and the segregation of these molecules and phospholipids plays an essential role in survival of bacteria against the lytic effect of enzymes and detergents (Nikaido and Vaara 1985).

B. Resistance to hydrophobic molecules. The outer membrane of Gram-negative bacteria forms an impermeable barrier against

the hydrophobic molecules such as erythromycin, novobiocin, rifampicin and fusidic acid. β-lactam antibiotics are highly effective on peptidoglycan layer of the cell wall, and therefore Gram-positive organisms are much more sensitive to hydrophobic β-lactams than the Gram-negatives. This effect is said to be due to the activity of porin proteins. Although these proteins act as non-specific diffusion channels, most hydrophobic molecules are not able to penetrate through these channels. In addition, by closing the channels, porins can reduce the permeability of the membrane to these molecules (Schindler and Rosenbusch 1981). The high level resistance of Pseudomonas aeruginosa to a variety of antibiotics is suggested to be because of the closed porin channels (Nikaido and Vaara 1985). For β-lactams even when they penetrate through the porins the periplasmic enzyme β-lactamase is often available to inactivate the antibiotic.

C. Resistance to polycationic agents. Polycationic agents such as polymyxins have great affinity towards cytoplasmic membrane. They attach to phospholipid content of this membrane and destroy it (Storm et al 1977). In most enteric bacteria the permeability barriers of the outer membrane particularly the LPS and protein structures were found to be the main factors of resistance to these agents (Vaara et al 1981). The molecule of polymixin B is too large to pass through the porins therefore is less effective than its related agents. It

has been observed that the removal of the outer membrane increased the sensitivity of the organism to polymixins up to 400 times (Teuber 1969).

The presence of different antibacterial inactivating enzymes in the periplasmic region is also an important protective factor for the bacterial cell.

The bacterial surface hydrophobicity is also another function of the outer membrane, protecting the organism against phagocytosis and body immune system and enhancing its invasive property, which will be discussed later.

Putative factors influencing pathogenicity of Gram-negative bacteria are as follows:

I.7. Antibiotic resistance:

I.7.a. Antibiotics:

Antibiotics are microbial products which in very low concentrations (ug/ml) inhibit or kill other microorganisms. Penicillins were the first useful microbial antibiotics developed from Penicillium notatum and P. chrysogenum in the early 1940s. The term antibiotic was first used by Waksman to differentiate penicillins from synthetic drugs such as sulphonamides which had been developed in the mid 1930s. At the present time, the term covers a wide range of antimicrobial agents including antifungal (griseofulvin), antiprotozoal (fumagillin and suramin), antiviral (rifampicin) and a wide variety of bacteriostatic (sulphonamides) and bactericidal (polymixins, aminoglycosides) compounds. Antibiotics attack different physiological and structural features of microorganisms to achieve their antimicrobial goal. In this regard antibacterials can be classified into several groups as follows:

A. Antibacterials acting on synthesis and turnover of the cell wall, eg. penicillins (such as penicillin G or benzylpenicillin, ampicillin and amoxycillin), cephalosporins and cephamycins.

B. Antibacterial agents affecting the cytoplasmic

membrane, like polymixins.

C. Antibacterials acting on nucleic acid metabolism, such as rifampicin, nalidixic acid and novobiocin.

D. Antibacterials acting on folate metabolism which include trimethoprim, sulphonamides and pyrimethamine.

E. Antibacterial drugs interfering in protein synthesis, which include chloramphenicol, erythromycin, lincomycin, tetracyclines, and aminoglycosides (streptomycin, kanamycin, gentamycin, neomycin, etc).

F. Antibacterial agents acting on miscellaneous targets like metronidazole which is effective on obligate anaerobic bacteria, isoniazid (isonicotinic acid hydrazide) which acts only on mycobacteria including M. tuberculosis and ethambutol, which is believed to interfere in the metabolism of RNA, but its mechanism of action is not clearly known.

In recent years the use of antibiotics has been the main way to combat infectious diseases, therefore the synthesis of these drugs has been given particular attention in the pharmaceutical industry. On the other hand the increasing microbial resistance to antibiotics has become a major disadvantage.

I.7.b. Antibiotic resistance in bacteria:

The history of drug resistance is almost as old as the use of chemotherapeutic agents. For the first time in 1907 Ehrlich and his colleagues reported the resistance of

Trypanosoma brucei to p-rosaniline, while they were studying its effectiveness on this group of microorganisms. Soon after, the resistance of pneumococci to ethylhydrocupreine was reported (Morgenroth and Kauffman 1912). After the second World War with the discovery and synthesis of new antibiotics and following the increased application of these agents, developing microbial resistance to them has been frequently observed. This included resistance to sulphonamides (Maclean et al. 1939) penicillin (Abraham et al 1941), and streptomycin (Murray et al. 1964).

The increased appearance of bacterial resistance can be due to the following factors:

1. Widespread use or misuse of a variety of antibiotics in bacterial infections which give rise to the appearance of resistant strains (McDonald 1982).

2. The presence of enormous numbers of microorganisms in nature and rapid multiplication due to short generation time.

3. Autoinfectivity among microorganisms through the mechanism of gene transfer.

The increasing incidence of infections caused by resistant bacterial strains has become a major problem in clinical and pharmaceutical science. Moreover, the biochemical studies have shown that the mechanisms of resistance obtained in vitro are sometimes different from those observed in clinical isolates. For instance, the resistance to penicillin

in clinical strains of Staphylococcus is often found to be due to the production of β-lactamase, whereas, the resistance obtained in laboratory strains does not usually involve this enzyme.

I. 7. c. Mechanisms of antibiotic resistance:

Resistance of a microorganism to a particular antibiotic is normally gained by either the synthesis of an enzyme which inactivates the antibiotic or by biological changes which lead to modification of or inaccessibility of the physiological or structural targets on which that antibiotic is effective. Therefore, the mechanism of resistance varies according to the type of antibiotic as well as the nature of microorganisms. Moreover, bacterial resistance highly depends on its genetic basis, hence it is divided into chromosomal and plasmid encoded resistance.

I. 7. c. 1. Chromosomal resistance. Some Gram-negative organisms without mutation, are inherently resistant to antibiotics. This type of resistance which is sometimes referred to as intrinsic resistance is associated with chromosomal determinants and usually demonstrates a low level and mostly non-enzymatic resistance (Godfrey and Bryan 1984). Some microorganisms exhibit natural intrinsic resistance to antibiotics eg. as Mycoplasmas lack the cell wall they are therefore intrinsically resistant to antibacterial drugs acting on peptidoglycan. The evolutionary or mutational

changes in cellular structure particularly the cell envelope controlled by chromosomal genes also contribute to resistance to antibiotice which will be discussed later.

I.7.c.2. Plasmids and antibiotic resistance:

Most plasmids confer resistance to their host cell against one or more antibiotics (and/or certain metals like mercury). In 1960 Mitsuhashi reported the presence of extrachromosomal drug resistance genes and suggested them to be carried on independent genetic entities called R (resistance) factors (Mitsuhashi 1977). At the same time Watanabe and Nakaya used the terms (RTF) resistance transfer factor (Watanabe and Fukasawa 1960)and (RTA) resistance transfer agent (Nakaya et al. 1960). Later in 1962, Japanese geneticists agreed with the use of the term R-factor. During the last two decades with increased usage of a diversity of antibiotics in treatment of bacterial infections, acquired resistance associated with R-plasmids has been considerably increased in almost all types of pathogenic bacteria and large numbers of R-plasmids have been discovered.

Unlike the chromosomal resistance, the resistance determinants of R-factors usually code for enzymes inactivating or circumventing the effects of antibiotics and other antibacterial agents.

Moreover some R-plasmids reduce cell envelope permeability for particular antibiotic(s) or other inhibitors by mediating

the synthesis of new proteins or increasing the LPS of the cell wall which will be discussed later.

Many R-factors are transmissible and can transfer their resistance to a sensitive cell by conjugation. These are large plasmids and carry genes responsible for transfer by conjugation which are referred to as resistance transfer factor genes (RTF or tra genes), as well as genes coding for drug resistance called resistance determinant genes (RT genes). Large plasmids mostly carry multiple resistance genes. They were first isolated from clinical strains of Shigella and then transferred to E. coli K12. Small non-conjugative plasmids usually carry a single resistance gene and their DNA size is too small to contain a tra operon. These types of plasmids were first found in Staphylococcus aureus and then in other bacteria particularly E. coli, Salmonella, Proteus, and Klebsiella. Sometimes single resistance factors may link to each other and then to a tra gene group and form a transmissible large plasmid carrying multiple resistance determinants. Naturally occurring conjugal R-plasmids are not always stable, therefore they are sometimes spontanously lost after long storage in culture medium or at elevated temperature. Terawaki et al. (1968) reported the loss of an R-plasmid in P. mirabilis in old culture as well as at high temperature. We also found that some of the p+ strains from this bacterium isolated from urinary patients lost their

plasmids after six month storage on nutrient agar slopes. Drug resistance plasmids in strains of P. mirabilis appear to become altered under certain environmental conditions. Thus Rownd and Womble (1977) reported that the density of R-plasmid NR1 (also called R222 and R100) contained in this organism was changed and the RTF was dissociated or reassociated depending on the growth culture conditions. Thus, when the cells were grown for long time in drug-free medium the density of the plasmid was low and the resistance was lost, but after growth in sub-inhibitory concentration of the antibiotic to which resistance was conferred by the R-factor, the density of the plasmid increased and resistance was regained. Curing by different agents such as acridine orange or SDS and high temperature are common laboratory methods for elimination of R-plasmids which will be described later. Table 2 shows some of the R-plasmids found in Proteus. The presence of plasmids RP1, RP4, R6K, R753, N-3, R40a and R1 in clinical and laboratory strains of P. mirabilis has been frequently observed by several researchers. R-plasmid R22K which mediates constitutive production of B-lactamase was originally isolated from P. mirabilis (Kontomichalou et al. 1974).

In this study the effect of large plasmids on the resistance of the urinary and faecal isolates of P. mirabilis to different antibiotics has been investigated.

Table 2. Some R-plasmids of Proteus spp with their incompatibility group and resistance markers.

Plasmid	Inc. group	Resistance markers
R1	FII	Amp, Str, Cap, Sul.
R6K	X	Amp, Str
R22K	N	Str.
R40a	C	Amp, Kan, Sul.
R494	FII	Amp, Tet, Kan.
R701	N	Amp, Str, Kan.
R702	P	Str, Tet, Kan, Sul.
R704a	FII	Amp, Str, Cap, Kan, Sul.
R704b	A-C complex	Tet, Sul.
R705	J	Kan.
R753	V	Amp, Str, Cap, Sul.
R871	A-C complex	Sul.
RP1	P	Kan, Tet, Neo, Kan.
RP4	P	Amp, Kan, Tet.
Rts1	T	Kan.
N3	N	Tet.
P*lac*	A-C complex	Sul.
R768	-	Sul(tra-)

Reference. Hedges (1972).

I. 7. d. Resistance to antibiotics acting on the cell wall:

β-lactam antibiotics. These antibiotics interact with cell wall synthesizing enzymes particularly those involved in the formation of cross link between sub-units of peptidoglycan. Resistance to these agents is usually associated with the hydrolysis of the β-lactam ring in penicillins by secretion of the enzyme β-lactamase. There are also other types of resistance to this group of antibacterials such as permeability barriers or changes in the target sites depending on the genetic basis of resistance i. e. chromosomal or plasmid encoded.

I. 7. d. 1. Chromosomal resistance. The enzymatic degradation of β-lactams is one of the most effective mechanisms of intrinsic resistance (Ogawara 1981). Chromosomally mediated β-lactamase is well demonstrated in many Gram-negatives particularly E. coli, Proteus, Salmonella and Pseudomonas (Godfrey and Bryan 1984). But it is reported that Proteus and Bacteroides produce different types of this enzyme which are equally effective on both penicillins and cephalosporins (Sawai and Tsukamoto 1982). Production of penicillin amidase is another enzymatic activity against penicillins exhibited intrinsically by some Gram-negative bacteria. This enzyme cleaves the side chain of Penicillin G molecule resulting in the formation of 6-aminopenicillinic acid with much less antimicrobial activity.

This type of resistance has been reported in E. coli and Proteus spp. The other mechanism of chromosomal resistance to β-lactams is changes in target affinity towards the antibiotic. Rodriguez-Tebar et al. (1982) reported that the reduced ability of penicillin binding proteins (PBPs) to bind these agents resulted in resistance in Gram-negative bacteria. Alterations of LPS, lipid and porin proteins in the outer membranes of many Gram-negative bacteria have been reported to alter cell permeability and give resistance to β-lactam drugs (Myers et al. 1980). Bacterial tolerance has been reported to be a natural mechanism of resistance to cell wall synthesis inhibitors (Parr and Bryan 1984).

Non-β-lactam antibiotics such as D-cycloserine and phosphomycin are bacteriocidal antibiotics which are active on cell wall synthesis by inhibiting early stages of biosynthesis of peptidoglycan and do not contain the B-lactam ring. Intrinsic resistance to these antibiotics has been reported in Pseudomonas aeruginosa and E. coli (Wargel et al. 1971) which is believed to be due to defective transport system.

I.7.d.2. R-plasmid-encoded resistance. plasmid associated β-lactam resistance in Shigella, Salmonella, E. coli and Proteus has long been established in Europe (Anderson and Lewis 1965) and Japan (Egawa et al. 1966). These strains were carrying R-factors coding for the enzyme β-lactamase. As

mentioned earlier R-plasmid R22K codes for β-lactamase which is more active on cephalosporin (Kontomichalou 1974). This plasmid was first isolated from P. mirabilis. In recent years a number of R-plasmids mediating the production of different types of β-lactamase have been recognized in members of Enterobacteriaceae particularly E. coli and Proteus some of which are listed in Table 2. It has been suggested that β-lactamase resistant antibiotics induce new types of plasmid associated β-lactamases using the previously resistant antibiotic as a substrate (Bush and Sykes 1984). Hence, the administration of many newly introduced antibacterials such as cefoxitin resulted in the high level induction of β-lactamase in some types of enterobacteria (Sanders et al. 1982).

I.7.e. Resistance to antibiotics acting on cytoplasmic membrane.

Polymyxins are a group of antibacterials including polymyxin A, B, D, E, colistin A, circulin A and octopeptins. These agents which are designated as surface acting agents have a high affinity to lipid structures of the bacterial envelope eg. LPS and phospholipid molecules. Polymyxins are more active on Gram-negatives than on Gram-positive bacteria perhaps due to the presence of thick peptidoglycan layer in the latter group (LaPorte et al. 1977). Moreover the absence of outer membrane in Gram-positives may exclude the antibiotics from the primary binding sites on the cell

surface, which are needed for disruption of the cell wall as a mechanical barrier (Storm et al. 1977).

I.7.e.1. Chromosomal resistance. Several bacterial species such as Proteus, Serratia, Providencia, Pseudomonas cepacia, Neisseria meningitidis, and N. gonorhoeae exhibit natural resistance to polymyxins. The resistance of Proteus species to this agent has been well studied, and it is now understood that the structure of the outer membrane (particularly lipopolysaccharide, phospholipid and Mg^{2+} content) plays an important role in this regard. Teuber (1969) reported that the spheroplast of P. mirabilis is 400 times more susceptible to polymixin B (Colistin sulphate) than the intact cell. Norris et al (1985) reported that polymyxin resistant strains of P. mirabilis possess much less phospholipid than their sensitive counterparts. Moreover, their Mg^{2+} level was very low. Recently, Kaca and his co-workers (1990) suggested that the presence of 4-amino-4-deoxy L-arabinose in the lipid A of the LPS molecules can be a major factor of polymyxin resistance in P. mirabilis, and they found that the strains lacking this compound were susceptible to polymyxins. Acquired resistance to polymyxins may be obtained by growing in specific conditions (eg. low concentration of Mg^{2+} in growth medium).

I.7.e.2. Mutational resistance. Resistance may also arise by mutation. A Salmonella typhimurium mutant resistant to polymyxin was shown to have lipopolysaccharides which bound

less to the antibiotic than its parental strain (Vaara 1981). This reduced affinity of the LPS was also found to be due to altered lipid A and the substitution of ester link with 4-amino-4-deoxy-L-arabinose up to 70%. The growth in low concentration of Mg^{2+} resulted in resistance to polymixin and EDTA in Pseudomonas aeruginosa (Brown and Melling 1969). There is no evidence indicating the involvement of R-plasmids in these acquired resistances. It has been suggested that resistant bacteria either do not carry the target sites to which polymixins bind or somehow exclude the antibiotic from cell surface receptors.

I.7.f. Resistance to agents acting on nucleic acids:

Nalidixic acid, one of the most effective antibacterials acting on the bacterial chromosome, is a synthetic product containing 1,8 naphthyridine which acts on (gyrA) subunit of DNA gyrase and inhibits chromosomal replication (Glass 1982). Interestingly, it seems to have no effect on eucaryotic cell DNA. This antibiotic is mainly effective on Gram-negative bacteria including strains of Proteus, E. coli, and Pseudomonas. Goss et al. (1964) reported that nalidixic acid inhibited the synthesis of DNA in E. coli. This inhibition was observed 10 minutes after the exposure of bacteria to the antibiotic.

Novobiocin, a product of Streptomyces spheroides and S. niveus is an antibacterial active on DNA synthesis. It is

mostly effective on Gram-positive bacteria particularly Staphylococcus and certain Streptococcus strains. It also acts on DNA gyrase.

Rifampicin is a semi-synthetic antibiotic derived from natural rifamycins produced by Streptomyces mediterranei. The cellular target for this hydrophobic antibiotic is the DNA-dependent RNA polymarase (Gale et al. 1981), and it is active on Gram-positive bacteria including Mycobacterium tuberculosis and a few Gram-negatives such as Neisseria, Legionella and Haemophilus (Atkinson 1980).

I.7.f.1. Chromosomal resistance. Resistance to nalidixic acid has been developed in Gram-negatives. This resistance is always chromosomally dependent and no R-plasmid resistance has been so far reported. It seems the resistance is due to the reduced affinity of gyrA gene product to this agent. Resistance can also occur by reduced permeability of cell wall to the antibiotic. Gram-positives are naturally resistant to nalidixic acid, and the mechanism of this resistance is not clearly known.

Chromosomal mediated resistance to novobiocin develops easily in Gram-positives which is suggested to be associated with the increased teichoic acid content of the cell wall which results in decreasing permeability of the organism to this antibiotic. The low susceptibility of Gram-negative bacteria is thought to be mainly due to the the presence of

lipopolysaccharide of the outer membrane. Because of the developing resistance, novobiocin has been jointly used with tetracyclines.

Intrinsic resistance to rifampicin in mycobacteria and most Gram-negatives has been suggested to be due to reduced permeability for the antibiotic (Godfrey and Bryan 1984), because the RNA polymerase extracted from resistant strains of Mycobacteria, E. coli and Salmonella was observed to be sensitive to rifampicin (Gale et al. 1981). Changes in hydrophobic side chain of the LPS in a rough mutant Salmonella resulted in 10 fold increased resistance to this agent (Nikaido 1976).

I.7.g. Resistance to antibacterials acting on folic acid metabolism:

Folic acid is a water soluble vitamin which consists of pteroic acid linked to glutamic acid. Its co-enzyme form is tetrahydrofolate (THF) produced from reduction of dihydrofolate by dihydrofolate reductase (DHFR). Tetrahydrofolate is the carrier of one carbon units such as methyl or formyl for the synthesis of methionine, thiamine, purines, thymidylate and formyl methionyl tRNA (Bryan 1982).

Sulphonamides and trimethoprim are two different groups of bacteriostatic antibacterials which interfere in the metabolism of folic acid by different mechanisms i.e. sulphonamides inhibit the synthesis of dihydrofolate whilst

trimethoprim inhibits the activity of DHFR.

In late 1930s when sulphonamides (p-aminobenzensulphonamide) were first introduced, almost all pathogenic bacteria (except P. aeruginosa and Strep. faecalis) were sensitive to them, and because of the quick absorbance and circulation in the blood stream, they were widely used against bacterial diseases especially in treatment and prophylaxis of urinary tract infections . Sulphonamides are only effective on procaryotic cells including both Gram-negative and Gram-positive bacteria because, the metabolic step which is inhibited by these agents is not present in eucaryotic cells. There are different derivatives of sulphonamides such as, sulphadiazine, sulphadimidine, sulphathiazine, sulphapyridine and sulphamethoxazole, which have been synthesized by substitutuion on the amide group, but all of them have the same mechanism of action. Therefore, because of the widespread use of these agents resistance has been developed rapidly in many bacterial species. Today, due to the production of a number of new and effective antibiotics and the increased resistance to sulphonamides, their clinical application is significantly reduced.

Trimethoprim is a synthetic broad spectrum antifolate which is much more effective than sulphonamides. This antibiotic has much higher affinity for bacterial DHFR than the corresponding enzyme in human and animal cells. For

example the concentration of trimethoprim required to inhibit DHFR in man is about 50,000 times more than that in E. coli. Trimethoprim is usually used against respiratory and urinary tract infections. Usually its MIC on sensitive strains is 1 μg/ml but for Proteus strains 2 μg/ml (Grey and Hamilton-Miller 1977). As sulphonamides and trimethoprim are synergistic in action, they are often used in a combination which is called Co-trimoxazole (trimethoprim+sulphamethoxazole). This combination is mostly bactericidal, whereas each one of these agents separately is bacteriostatic. Co-trimoxazole has been mainly effective on brucellosis, typhoid, gonorrhoea as well as on species of Klebsiella and Haemophilus , but has no effect on Pseudomonas aeruginosa, Treponema pallidum and Mycoplasma. Both chromosomal and plasmid mediated resistance to sulphonamides and trimethoprim as well as to co-trimoxazole has been observed in many bacterial species.

I.7.g.1. Chromosomal resistance. Chromosomally acquired resistance to sulphonamides particularly among isolates from urinary tract infections is very common. The minimum inhibitory concentration (MIC) of these agents on 10% of the strains tested by Grey et al. (1979) was 100-1000 μg/ml, and on 90% of them was 5000 μg/ml. Resistance to sulphonamides in hospital isolates is up to 50% (Hamilton-Miller 1984), and among nosocomial infections strains of P. mirabilis are intrinsically resistant to sulphafurazole. O'Brien et al.

(1982) reported that animal strains of Salmonella spp were more likely to be resistant to sulphonamides than the human isolates, thus 55% of strains obtained from animal sources exhibited resistance to these agents whilst this figure in human isolates was only 5%.

As mentioned earlier, trimethoprim has stronger antibacterial properties than sulphonamides. Nevertheless, resistance to this agent in many bacterial species particularly Gram-negatives has been frequently observed. The high incidence of chromosomal resistance to trimethoprim in urinary infection causing strains of E. coli, Proteus spp, Enterobacter spp and K. aerogenes has been reported by several investigators (Grey et al. 1979, Hamilton-Miller et al. 1981, Brumfitt et al. 1983). Acquired ie. mutational resistance to this agent due to high production of DHFR associated with chromosomal determinants has been observed in E. coli, Salmonella and Lactobacillus (Crusberg et al. 1970). Pseudomonas aeruginosa has demonstrated intrinsic resistance to both antibiotics as well as co-trimoxazole. As this organism produces a sensitive DHFR, it is believed that the resistance is due to the activity of the cell membrane as a permeability barrier (Hamilton-Miller 1984).

I.7.g.2. Plasmid resistance. R-plasmid related resistance to these two antifolate agents has been regularly observed. Wise and Abou-Donia (1975) reported high plasmid-mediated

resistance in E. coli, K. pneumonia and Citrobacter to sulphonamides due to production of a resistant dihydropteroate synthetase. A 4.9 megadalton non-conjugative plasmid carrying single resistance to sulphonamides has been observed as a first R-plasmid in Haemophilus and Neisseria (Albritton 1984), which is highly related with the enteric plasmid RSF1010 coding for streptomycin and sulphonamide resistance (Albritton et al. 1982). Although plasmids in enterobacteria mostly code for multiple resistance (Datta 1984) the presence of a number of plasmids carrying single sulphonamide resistance eg. R768, R871, and R470 has been reported in P. mirabilis and P. vulgaris which are listed in Table 2 (Hedges 1975).

Fleming et al (1972) showed that resistance to trimethoprim was transmissible and is conferred by an R-plasmid. Shortly after that Datta and Hedges (1972) reported growing resistance to trimethoprim in London hospitals. In their study, the resistant strains of E. coli and Klebsiella were capable of growing in a medium containing 1 mg/ml of trimethoprim. This resistance was conferred by a plasmid belonging to the W compatibility group. Because of the wide usage of this antibiotic especially in combined form (co-trimoxazole) in urinary tract infections, rapid resistance has been developed in causative organisms particularly P. mirabilis and E. coli (Hamilton-Miller et al. 1981).

The mechanism of resistance to sulphonamides can be due to

A, altered dihydropteroate synthetase (DPS) which has low affinity for sulphonamides, B, hyper production of *p*-aminobenzoic acid which is an antagonist to these drugs (Mitsuhashi 1977), C, decreased permeability of cell envelope (Pato and Brown 1963), D, detoxification of sulphonamide by bacteria is suggested to be another mechanism of resistance (Then 1982). The altered DPS in chromosomal mutants differs from that encoded by R-plasmids, because the latter reduce the affinity of the enzyme to sulphonamides but not for *p*-aminobenzoic acid (Wise and Abou-Donia 1975). White and Woods (1965) reported that sulphonamide resistant strains of Staphylococci from clinical sources produced five times more PABA than the sensitive strains.

Changes in the cell permeability for sulphonamides have been well studied in E. coli strains carrying some R-plasmids which confer resistance to these agents. It was shown that spheroplasts produced from these R+ strains of E. coli are as sensitive as the R- strains to these antibiotics implicating the outer membrane in this resistance(Mitsuhashi 1977).

The mechanism of bacterial resistance to trimethoprim is similar to that to sulphonamides, but they act in different locations. The resistance can be either due to the production of a resistant DHFR or decreased permeability of the cell wall. Intrinsic resistance has been exhibited in Pseudomonas aeruginosa. As this organism produces a sensitive DHFR it is

suggested that its resistance is due to reduced permeability to trimethoprim (Hamilton-Miller et al. 1981). In Neisseria spp a moderately resistant DHFR is involved (Then and Angehrn 1979) and some Clostridium spp have the benefit of both altered permeability and resistant DHFR. At least two types of R-plasmid associated resistant DHFR have been recognized. Fling et al. (1982) in a series of experiments found that 59% of the resistant strains tested contained type 1 DHFR and 26% of them produced type 2 enzyme.

I.7.h. Resistance to antibiotics interfering in protein synthesis:

I.7.h.1. Chloramphenicol (chloromycetin). This antibiotic was originally obtained from Streptomyces venezuelae but is now produced by direct synthesis. Chloramphenicol is a bacteriostatic agent which acts on protein synthesis by binding to the 50S subunit of the 70S type ribosomes and inhibiting the enzyme peptidyl transferase which mediates the peptide bond formation and hence the protein synthesis (Gale et al. 1981). Therefore, it is effective on a wide range of both Gram-negative and Gram-positive organisms and is mainly used against enteric bacilli in intestinal tract infections and is a particularly important agent against Salmonella typhi.

I.7.h.1.a. Chromosomal resistance. To be effective on its targets, chloramphenicol has to pass through the cell envelope

barriers. Therefore permeability of the bacterial cell plays an important role in the resistance against this agent. Chromosomal resistance is not very common compared to plasmid specified cases. The first chromosomal resistance associated with decreased permeability for chloramphenicol was reported in E. coli by Vasquez (1964). Pseudomonas aeruginosa and P. putida exhibit intrinsic resistance to this agent which is suggested to be due to the reduced permeability of their outer membrane because removing of this membrane rendered them sensitive to chloramphenicol (Mitsuhashi et al. 1975). Hickman et al. (1982) reported that the indole-negative Proteus penneri and Flavobacteria are intrinsically resistant to this antibacterial drug and this property has been taken as an identification factor. The mechanism of the resistance is not understood. Some plasmid-free strains of P. mirabilis also show high chromosomal resistance to chloramphenicol (Shaw 1973), which is said to be due to the presence of Tn9 transposon on its chromosome (Shaw 1971). Gram-positive organisms are more sensitive than Gram-negatives, mainly due to the absence of the outer membrane (Atkinson 1980). Nocardia astroides is the only Gram-positive resistant to chloramphenicol and the mechanism of resistance is not known (Gutmann et al. 1983).

I.7.h.1.b. Plasmid resistance. Plasmid mediated resistance to chloramphenicol is currently observed in

bacteria particularly Gram-negatives. Two mechanisms are involved in this type of resistance; production of the enzyme chloramphenicol acetyltransferase (CAT) which causes the acetylation of the antibiotic and forms acetylchloramphenicol. This compound has no antibacterial activity. The other mechanism is the reduced membrane permeability to the antibiotic. As mentioned earlier most of the chloramphenicol resistances are plasmid dependent and can be transmissible. R-plasmids with molecular weight more than 20 Md code for chloramphenicol acetyltransferase (Foster and Shaw 1973) and plasmids belonging to compatibility groups, FII, W, and C have been isolated from Gram-negative strains resistant to this agent. The resistance determinant of the R-plasmid is often located on a transposon called T9 which is a 2500 base pair DNA fragment (Smith and Burns 1984) and specifies a type 1 chloramphenicol acetyltransferase (Bryan 1982). Decreased permeability to the antibiotic in E. coli was reported by Nagai and Mitsuhashi in 1972. They found an R-plasmid containing strain of E. coli resistant to chloramphenicol which was not able to acetylate chloramphenicol. The plasmid was then shown to be associated with the reduced permeability of the organism. In some species of Bacillus the ribosomes fail to bind to chloramphenicol.

I.7.h.2. Tetracyclines. This group of antibiotics was first introduced in late 1940s (Levy 1984). The basic

structure of all types of natural and semi-synthetic tetracyclines is a four ring formula called naphthacene. Different types of these agents which have modified side chains on carbons 5, 6 or 7 (Levy 1984) are; chlortetracycline, oxytetracycline, demethylchlortetracycline, doxycycline, methacycline, minocycline and tetracycline. The first one was chlortetracycline which was isolated from Streptomyces aureofaciens and then oxytetracycline was discovered in S. rimosus. A few years later scientists succeeded in producing other semi-synthetic derivatives from tetracycline and chlortetracycline. These are bacteriostatic and all have broad spectrum activity on susceptible bacteria particularly, Brucella, Bacterioides, Corynebacterium and Chlamydia. Tetracyclines are usually used in hydrochloride form to increase water solubility (Bryan 1982), and their lipophilicity plays an important role in their absorbance in the gastrointestinal tract (Levy 1984). Tetracyclines prevent the enzymatic and non-enzymatic binding of aminoacyl-tRNA to A and B sites of 70S ribosomes. The major binding is on A site and only one molecule of the antibiotic per ribosome is sufficient to inhibit protein synthesis (Tritton 1977).

Bacterial resistance to tetracyclines is common but develops slowly, and the genetic basis of this resistance can be either chromosomal and/or plasmid determined.

I.7.h.2.a. Chromosomal resistance. Pseudomonas aeruginosa

exhibits intrinsic resistance to tetracyclines (Tseng and Bryan 1973) which is believed to be due to the decreased permeability of the outer membrane (Tseng and Bryan 1974). Proteus species demonstrate both chromosomal and plasmid resistance. Hughes and Datta (1972) reported that among 437 strains of different members of the Enterobacteriaceae which have been isolated between years 1917-1954, only 9 strains of Proteus spp showed non- transmissible resistance. But along with the increased use of these agents the number of the resistant strains also increased, and according to Levy (1984) in a survey more than 90% of clinical isolates of P. mirabilis exhibited apparent chromosomal resistance to tetracyclines.

I.7.h.2.b. Plasmid resistance. Plasmid mediated resistance to tetracyclines was first reported in Shigella dysenteriae in the mid 1950s by Akiba et al. (1960). The resistance was due to the presence of a transmissible multiresistant plasmid coding for resistance to tetracycline, chloramphenicol, streptomycin and sulphonamide. Today the majority of tetracycline resistance in Enterobacteria is determined by plasmids (Levy 1984). Such plasmid induced resistance is always associated with the synthesis of new proteins which are mostly located on the outer and inner membranes. These proteins seem to be involved in the reduced permeability for tetracyclines and probably catalyze tetracycline efflux. R-plasmid R100-1 which carries the Tn10 transposon in E. coli

codes for two proteins of molecular weights 15000 and 36000 Md, and about 400 molecules of 15000 Md protein have been detected on the inner membrane of this bacterium. Levy and McMurry (1974) observed the presence of a new protein in E. coli strains containing R-plasmid which were grown in the presence of tetracycline and called it TET protein. Izaki and Arima (1965) reported R-plasmid carrying E. coli was able to grow in medium containing 400 µg/ml tetracycline, after being exposed to 20 µg/ml concentration of this agent. There is another tetracycline resistance mechanism based on an alteration of the 30S ribosomal subunit which then does not bind to the antibiotic (Scavizzi 1972).

I.7.i. Aminoglycosides:

These are a large group of antibiotics with broad spectrum activity which contain a cyclic alcohol (aminocyclitol) in glycoside linkage with one or more aminosubstituted sugars. Aminoglycosides which include streptomycin, kanamycin, gentamycin, spectinomycin, neomycin, amikacin, framycin, paromomycin and apramycin, have bactericidal activity (except spectinomycin which is only made of an aminocyclitol and lacks the aminosugar). They are effective on both Gram-negative and Gram-positive bacteria particularly Pseudomonas aeruginosa but ineffective on obligate anaerobes. They are all therefore, of clinical importance being widely used against serious infections, but

toxicity limits their usage. Aminoglycoside antibiotics appear to inhibit polypeptide chain initiation by binding to a protein designated as P10 or S12 in the 30S subunit of the 70S ribosome and eventually prevent protein biosynthesis. The antibiotic does not directly bind to S12 protein and how this interaction leads to bacterial death is not clearly known. Sublethal concentrations of aminoglycosides specially streptomycin cause misreading of certain codons in the mRNA, possibly due to changes within the ribosome, caused by antibiotic binding (Brayn and Kwan 1983).

Both chromosomal and plasmid associated resistance to these antibiotics has been developed in many bacterial species. Different mechanisms are involved in bacterial resistance to aminoglycosides such as reduced permeability of the outer and the cytoplasmic membrane, enzymatic modification of the antibiotic, changes in the cellular energy mechanism involved in the accumulation of these agents and cationic antagonism in the growth medium (Holtje 1978).

I.7.1.1. Chromosomal resistance. As mentioned anaerobic and fermentative bacteria are intrinsically resistant to aminoglycosides, because they lack the negatively charged transporter to which the antibiotic binds and which provides the conditions for antibiotic accumulation. Moreover, in anaerobic conditions their activity is significantly reduced on facultative organisms. Chromosomal resistance to

aminoglycosides has been observed in clinical isolates of Pseudomonas aeruginosa which failed to accumulate these agents due to mutational changes in cellular energy mechanism (Miller et al. 1980). As aminoglycosides have cationic properties at neutral pH (Holtje 1978) and bind to anionic sites ditributed on the cell surface, they can be antagonized by cations. Hence, it is shown that strains of Pseudomonas aeruginosa exhibit unstable mutational resistance to these agents in the presence of increased concentrations of cationic compounds (Bryan 1984). Hancock et al. (1981) reported a Mg^{2+} binding site in this organism associated with cation antagonism with aminoglycoside. The main mechanism of chromosomal resistance to aminoglycosides is thought to be the reduced permeability of the cytoplasmic and the outer membrane.

I.7.i.2. Plasmid resistance. Plasmid-borne resistance to aminoglycosides is widespread particularly in Gram-negatives (Bryan 1984) and most of the aminoglycoside inactivating enzymes are plasmid-specified. Such plasmids code for enzymes like streptomycin phosphotransferase (Kida et al. 1975), kanamycin acetyltransferase (Benveniste and Davies 1971), and gentamicin adenylyltransferase (Daniels et al. 1974) which leads to acetylation, phosphorylation and adenylylation of the hydroxyl group of aminoglycosides. These enzymes convert the antibiotic to a compound with no antibacterial activity. Single plasmids sometimes specify multi-enzymatic resistance

in a bacterial strain. R-plasmids are also reported to be capable of coding for modification of ribosomes which then fail to bind aminoglycosides (Kono and O'Hara 1977). Tanaka (1970) observed R-plasmid carrying strains of Pseudomonas aeruginosa resistant to gentamicin which did not produce inactivating enzymes. Then he found that the ribosomes of these strains do not bind this antibiotic. A mutational resistance in the str gene due to the presence of R-plasmid gives rise to a modified P10 protein in the ribosome which fails to bind streptomycin or will not be affected by antibiotic bound.

R-plasmid resistance to aminoglycosides has been reported in clinical isolates of P. mirabilis. Hedges (1975) showed that the presence of plasmid R772 in urinary strains of P. mirabilis conferred resistance to kanamycin. This plasmid is conjugative but it is also capable of mobilizing non-selftransmissible DNAs (Coetzee 1978). Streptomycin resistance has been repeatedly observed in P. mirabilis. This resistance is not always plasmid induced, and it seems that the chromosome was somehow mutated. The most effective aminoglycoside on this bacterium is said to be gentamicin with the minimum inhibitory concentration of 25 µg/l (Torres et al. 1986).

I.7.j. Resistance to Nitrofurans:

These are a group of synthetic antibiotics active against

a wide range of Gram-positive and Gram-negative bacteria as well as some fungi and protozoa. There are different types of nitrofurans such as nitrofurantoin, nitrofurazone, furazolidone (furoxone), nifuratel and furylfuramide, all derivatives of a common structure which consists of a monocyclic 2-substituted 5-nitrofuran ring (Grunberg and Titsworth 1973). They are bacteriostatic at low concentration and most of them bactericidal at high concentration. Nitrofurazone or furacin (5-nitro-2-furaldehyde semicarbazone) was the first agent of this group used against clinical infections, but because of its side effects (it causes arthritis and peripheral neuritis) it is now only used topically to treat infected wounds, burns and mucosa (Leopold 1972). Nitrofurantoin or Furadantin, (N-5-nitro-2-furfurylidin-1-aminohydantoin) is a broad spectrum bactericidal agent the most widely used nitrofuran especially against urinary pathogens such as P. mirabilis and E. coli (Rabin and Lockerby 1984). Its high usage in urinary tract infections depends on complete absorption as well as on quick dissemination in kidney tissues. Nitrofurantoin is generally effective on coliforms and enterococci but some strains of Proteus and Klebsiella have shown resistance and Pseudomonas aeruginosa is commonly resistant. Furazolidone (furoxone) is used as a gastrointestinal disinfectant particularly in veterinary medicine and is mainly effective on Salmonella and

Shigella. The mechanism of antibacterial activity of nitrofurans is not completely understood, but it has been suggested that they function as electron acceptors and therefore interfere in some physiological activity of bacteria eg. the respiratory system. They undergo a nitroreduction process and turn to amino or hydroxylamino derivatives which are later reduced to open chain nitrile compounds. These compounds are sometimes much more toxic than the original agents and possibly result in cytotoxicity (McCalla et al. 1975). In fact the reduced nitro group is a vital intermediate metabolite for these compounds to confer on them antibacterial and sometimes mutagenic activities for which their first target is said to be the bacterial DNA (Rabin and Lockerby 1984).

Resistance to nitrofurans has been observed in some bacterial species, but compared to other antibiotics used against clinical pathogens, the percentage of resistance to these agents has been significantly low. According to Shaw (1974) all E. coli strain tested were sensitive to nitrofurans. In a study by Bush et al. (1974) between years 1968 to 1972 the antimicrobial effectiveness of nitrofurantoin and ampicillin on urinary and respiratory infections was compared. They found that nearly all strains of E. coli, Proteus and Klebsiella were sensitive to nitrofurantoin, and that throughout these years there were no significant changes in their

susceptibility although many isolates were resistant to ampicillin at first and their resistance significantly increased after four years.

I.7.j.1. Chromosomal resistance. Mutational resistance to nitrofurans associated with the chromosomal genes has been observed in E. coli strains which in one study exhibited a 10 fold resistance to these agents (Asnis et al. 1952). In another study, exposure of this organism to 10-20 µg/ml of nitrofurazone resuted in the appearence of mutant strains showing up to a sevenfold increase in resistance (McCalla et al. 1970). The main mechanism of this type of resistance was the cellular deficiency of nitrofuran reductase (NR) activity of the strains (Rabin and Lockerby 1984). Sometimes cross resistance occurs between nitrofurans and other antibiotics particularly sulphonamides. Cross resistance has been also observed between nitrofuran derivatives like, nitrofurazon furazolidon and nitrofurantoin (Paul and Paul 1964). Dastidar et al. (1979) showed that loss of NR enzyme in a biotype of a Vibrio cholera strain by chromosomal mutation conferred resistance to this organism. Pseudomonas aeruginosa is said to readily gain nitrofuran resistance compared to many other bacteria.

I.7.j.2. Plasmid resistance. There are also reports indicating nitrofuran resistance mediated by genes carried on R-plasmids. The transfer of drug resistance factor R46 to a

rec A strain of E. coli rendered it resistant to nitrofurans (Oliver 1981). This plasmid which was transferred by transduction with phage P1, neither effected the DNA repair mechanisms of the strain, nor changed the cell permeability for these agents. Some workers are reported to have been unable to transfer nitrofuran resistance plasmids among strains of E. coli and Klebsiella (Dulaney et al. 1968, Cooke et al. 1971, Chadwick and Neill 1973). In a study by Arai et al. (1975) a weak transferable resistance to nitrofurans especially nitrofurazone mediated by R-plasmids was observed in some bacterial strains. The plasmid responsible was transferred by conjugation from a resistance plasmid-carrying strain which seemed to have decreased NR activity.

I.8. Bacterial attachment:

Pathogenesis of bacterial infections involves a number of factors, but the attachment of bacteria to the surface of animal and human cells is considered to be the major step in colonization (Ofek and Beachey 1980) which plays an important role in the initiation of infection. This colonization is followed by invasion, dissemination and tissue damage. Two types of factors are involved in bacterial adherence, virulence properties of the organism and the host response.

The surface of procaryotic cells including bacteria has a net negative charge (Read 1989), which apparently acts against the bacterial attachment to surfaces as well as to mammalian cells. But there are other attractive forces on the bacterial surface which can overcome the repulsive electrostatic force between two negatively charged surfaces. It is suggested that the presence of hydrophobic molecules on the bacterial surface significantly enhances the bacterial adherence to inert surfaces (Ward et al. 1977). There are also other means to facilitate the bacterial adhesion, such as surface adhesins and pili.

Bacteria can adhere to specific tissues by producing molecules called adhesins which project through their own cell wall and interact with complementary receptors on the surface of the host cells (Ofek et al. 1978). Bacterial hydrophobicity and other properties may reduce the repelling property of the

two surfaces and may allow them to approach the epithelial cells enabling the bacterial adhesins to bind to the cell receptors. Bacterial adhesins are usually made of protein, whereas the host receptors are molecules of glycoprotein or glycolipid. The interaction between these two molecules is similar to the immunological reaction of antigen-antibody.

I.8.a. Pili: or fimbriae are the most important surface adhesins which play a crucial role in bacterial attachment (Schaefer et al. 1979, Svanborg-Eden et al. 1987, Wray et al. 1986). They are short, rigid and straight appendages emerging from bacterial envelope and quite distinct from flagella. Wray and co-workers reported some types of pili in E. coli which consist of one or two strands, each of which in fact was a chain of a single protein called pilin of 16000 daltons molecular weight. The two strands were usually wound helically around each other and formed a tube like structure with 8-10 nm diameter and up to several micrometer length (Hagberg et al. 1983). Pili are commonly found among Gram-negatives particularly Enterobacteriaceae and species of Pseudomonas, Neisseria and Vibrio (Rogers 1983).

I.8.b. Types of pili: Two main classes of pili are proposed, fimbriae and sex pili. Fimbriae are common pili which are present on the surface of a large number of bacteria. They are divided into at least four types (type 1, 2, 3 and 4) on the basis of structure and receptors they attach to.

Type 1 fimbriae are a well recognized group of pili with an axial hole and 6-8 nm diameter which are very common in the Enterobactericeea. They are several hundred in number distributed on the bacterial surface and can easily be removed by blending or growing in a high speed shaker. The removed pili sometimes adhere to each other and form a pellicle on the surface of liquid medium. In some cases they appear in a polar position like those of Pseudomonas aeruginosa. Bacteria containing type 1 fimbriae are designated by their ability to mediate erythrocyte agglutination. They also adhere strongly to each other and form bacterial clumps in liquid medium. Both bacterial adhesion and agglutination can be inhibited in the presence of D-mannose and alpha-D-mannosides. It is suggested that mannose acts at surface receptors blocking the interaction of pili with the red blood cells (Read 1989). Therefore type 1 fimbriae are called mannose sensitive (MS). It is believed that these fimbriae help the bacteria to attach to those glycoproteins on the surface of epithelial cells of the bladder which is well demonstrated in E. coli.

Type 2 fimbriae which have been described in Salmonella morphologically resemble type 1, but these are non-haemagglutinating and non-adhesive (Old and Payne 1971). Because of close antigenic relationships of type 1 and type 2 fimbriae, it is suggested that type 2 is the mutagenic variant of type 1 which has lost its adhesive property (Clegg and

Gerlach 1987). In other words genetically the receptor binding activity of these fimbriae is eliminated.

Type 3 fimbriae are both morphologically and physiologically different from the other two types (1 and 2). These are thin organelles with 4-5 nm diameter without an axial hole and are made of a polymer of a pilin with the molecular weight of 19500-24000 daltons (Old and Adegbola 1983). They do not agglutinate erythrocytes unless in the presence of tannic acid (Duguid et al. 1966) and are antigenically distinct from type 1 fimbriae (Korhonen et al. 1983). Mannose and its derivatives can not bind to adhesins of type 3 fimbriae and do not affect their adhesive property, therefore, they are referred to as mannose resistant (MR) pili. Bacteria demonstrating this type of pili adhere strongly to non-living surfaces such as glass, plastics or cellular fibres. They are commonly found on the surface of Klebsiella and species of Proteus. There is another type of fimbriae (type 4) found in Proteus species particularly P. mirabilis which will be discussed later.

I.8.c. Bacterial adherence as a virulence factor in urinary tract infections:

A number of bacteria are capable of invading and causing infection in the urinary system which mainly include strains of E. coli and P. mirabilis. The attachment of these organisms to uroepithelial cells is believed to be a major factor in

their pathogenicity (Reid and Sobel 1987). The origin of these infections is mostly the intestinal tract (Senior 1987) where the causative bacteria are predominantly living in symbiosis with the digestion system. Much work has been done on the critical role of adhesion of bacterial species which are selectively pathogenic to the urinary tract out of the large number of microorganisms present in faecal flora (Swanborg and Hagberg 1981). They colonize the preurethral region and ascend towards bladder. This form of infection is more common in women due to the anatomy of their urinary system. During the day the continuous urine flow does not allow bacteria to settle easily on the bladder epithelium. but in the night or in people with disorders of the bladder, when the urination is very slow or obstructed, conditions are suitable for bacteria to adhere to the epithelial cells resulting in symptomatic or asymptomatic bacteriurea. Then depending on the virulence of the organisms as well as host factors they may eventually ascend towards the upper urinary tract and cause pyelonephritis. This type of infection is frequently observed in catheterized patients (Daifuker and Stamn 1986). Therefore, the adhesion of uropathogenic bacteria is a prerequisite condition for colonization, followed by invasion, dissemination and tissue damage. Sometimes this attachment is prevented by mechanical and non-specific defence mechanisms (Reid and Sobel 1987). For example the mucosal epithelium of

the bladder continuously releases a type of mucoprotein into the urine which is called Tamm Horsfall Protein (THP) (Read 1989). This compound is a glycoprotein and contains mannose which sticks to mannose sensitive (MS) fimbriae of bacteria like E. coli and inhibits their adherence to the epithelial cell receptors. Then the bacteria are eliminated from the bladder by urine flow. The significance of bacterial adhesion to uroepithelium encouraged the search to find ways to control this phenomenon and thereby prevent urinary tract infections, such as; non-immune inhibition of attachment, vaccine production, usage of antibacterial agents which reduce the adhesion e.g. Taorolin (Gorman et al. 1987) and use of non-pathogenic organisms to exclude pathogens from urinary system.

I.8.d. Adherence of P. mirabilis strains to surfaces:

P. mirabilis strains attach readily to mammalian cells as well as non-living surfaces such as, plastic, glass and cellulose. It has been shown that members of Proteeae particularly P. mirabilis adhere to the desquamated epithelium of the bladder. This adherence is mediated by fimbrial structures present on the cell surface. Silverblatt (1974) studied the attachment of these bacteria to rat renal epithelium. Electron microscopical examination showed a type of surface ligand by which P. mirabilis attaches to the renal cells. These adhesins were in many respects different from the other three types described earlier. Duguid and Gillies (1958)

reported the presence of a kind of mannose-resistant (MR) fimbriae in P. mirabilis with haemagglutinating property but different from those found in other Enterobacteria. These fimbriae were called mannose-resistant proteus-like (MR/P) or type 4 fimbriae (Duguid et al. 1966) and were capable of agglutinating all types (fowl, guinea pig, horse, sheep and human) of fresh erythrocytes. It was shown that fimbriated Proteus lost its adhesins and haemagglutinating property when cultured 3-5 times on agar plates and then again gained them after several passages in broth (Coetzee 1962). Silverblatt (1974) reported the appearence of two types of fimbriae in Proteus mirabilis. After 2-3 hours of growing in shaken broth or on agar thin fimbriae (4 nm) were developed, but after 2-6 serial stationary passages in broth culture for 48 hours, thick pili of about 7 nm (which were in fact type 4 MR/P fimbriae) appeared on the surface of the cells. It is believed that thin fimbriae carry mannose-resistant Klebsiella-like adhesins (MR/K) by which tanned haemagglutination (in the presence of tannic acid) occurs (Old and Adegbola 1982). Later Adegbola et al. (1983) in an electron microscopy study reported the presence of both types of fimbriae in 48 urinary strains of P. mirabilis and described them as, thin non-channelled fimbriae with 4 nm diameter associated with MR/K haemagglutinin and the other type, channelled thick (7-8 nm) pili which cause MR/P haemagglutination and peritrichously

covered the bacterial surface. Wray and co-workers (1986) succeeded in purifying a protein from the surface of a uropathogenic strain of P. mirabilis with high affinity for epithelial cells. This protein was found to be like a flexible rod of molecular weight 17500 daltons close to that of the other fimbriae. They suggested this protein, which they called UCA (uroepithelial cell adhesin), was directly responsible for binding P. mirabilis to uroepithelium. It was also shown that UCA itself (separated from cell surface) was capable of binding to epithelial cells. Another adhesive protein was purified from the cell surface with molecular weight of 30000 daltons which was suggested to be MR/P thick fimbriae (Wray et al. 1986). Therefore, these are convincing lines of evidence indicating the direct involvement of fimbriae in the attachment of P. mirabilis to uroepithelium and that the fimbriated strains demonstrating firm adhesion are more virulent than the non-piliated ones which exhibit poor adhesive property with relativly low infectivity (Silverblatt 1978).

Adherence is not always beneficial to this organism, because the fimbriae also mediate its attachment to phagocytic cells (Silverblatt 1974). Thus adherence of Proteus fimbriae to host cells aids infection whereas adherence to phagocytes followed by bacterial ingestion reduces infection. Silverblatt and Ofek (1978) studied changes in piliation of P. mirabilis

during an experimental kidney infection in the rat. Electron microscopical observations showed heavily fimbriated strains adhering strongly to the bladder as well as the mucosal surface of the pelvis, whereas almost all strains reaching the renal cortex were lightly or non-fimbriated. This adaptibility to environmental changes is believed to be a mechanism of survival. In the bladder and pelvis a high level of fimbriation is required; firm attachment is needed for persistence in continuous urine flow. However, in the cortex and the blood stream, to survive against phagocytosis the organism undergoes a transitional phase from highly piliated to non-fimbriated type. In another experiment both fimbriated and non-fimbriated P. mirabilis strains were directly injected into the blood and renal tissues to avoid the bacterial attachment to pelvis. After one hour almost equal numbers of both types were present in cortex as well as in blood, but five hours later the heavily piliated strains were nearly removed from the kidney and did not cause any damage whereas non-fimbriated isolates persisted in these tissues which developed renal cortical abscesses. This suggests that in the kidney and blood stream highly fimbriated strains are much more vulnerable to phagocytosis.

I.8.e. The effects of plasmids on bacterial adherence:

The role of plasmids in adhesion of human pathogens as well as plant parasites has been extensively studied by

several investigators (Kenward et al. 1978, Williams et al. 1978, Onaolapo et al. 1987). Although bacterial adhesion and formation of pili are coded by genetic determinants (fim A, B and C) carried by the bacterial chromosome, there is clear evidence indicating the direct involvement of plasmids of different incompatibility groups in production and functions of pili in certain bacteria. In an extensive investigation, Bradley suggested that thin and thick pili are determined by plasmids belonging to incompatibility groups W (Bradley 1978), N_3 (Bradley 1979), I_1 (Bradley 1980a), B, C, H, J, M, V and X (Bradley 1980b), I_2, I_5, and Z (Bradley 1984). In his experiments on an E. coli strain BW86, he discovered that plasmids I, B, K, and Z determined the construction of both thin flexible and thick pili. These are transmissible plasmids and can be transferred to other strains in liquid and solid medium, whereas plasmids coding thick fimbriae are transferable only on solid agar (Bradley 1984). R-plasmid RP1 and RP4 have been found to mediate changes in the outer membrane as well as the surface properties (including adhesion) of many bacteria especially Enterobacteriaceae. Onaolapo et al. (1987) reported that plasmid RP1 which carries resistance determinants to ampicillin, tetracycline, neomycin, kanamycin and carbenicillin (Grinsted et al. 1972), increased the adhesion of P. mirabilis to inert surfaces such as glass and medical

prostheses. This adhesion was 10-100 times stronger than for plasmid-free strains in a mixed culture. In pure culture also plasmid RP1 enhanced the adherence of P. mirabilis and this difference was greater in iron-limited medium.

The presence of plasmids may also affect other surface components such as specific receptors, adhesins, capsules and cell wall proteins which are directly or indirectly involved in bacterial adhesion. For example, the production of K88 antigen in an animal strain of E. coli was transferred to a non-producing strain by plasmid DNA (Ofek and Beachey 1980). Sometimes the presence of R-plasmids adversely affect bacterial adherence. Denoya et al. (1985) reported that a multiresistance plasmid decreased the attachment of a nosocomial strain of Klebsiella pneumoniae to cerebrospinal fluid shunts. This may be due to the activity of blocking molecules such as capsular mucoproteins which mask the surface ligands therefore reduce the adhesive property of the organism (Selinger and Reed 1979).

In this study the influence of large plasmids on the adherence of clinical isolates of P. mirabilis to glass beads cover slips and uroepithelial cells was investigated.

I.9. Bacterial hydrophobicity:

It is believed that the solid-liquid interface has a significant effect on bacterial physiology (Loosdrecht et al. 1987). The study of bacterial surface properties such as

electrostatic charge and hydrophobic activity has revealed the nature of the molecular mechanisms of bacterial interaction with the host cell as well as the environment. The influence of inorganic colloids on a variety of microbial processes in soil such as denitrification and nitrogen fixation has been studied as early as 1913 by Sohngen. Although bacterial and mammalian cell surfaces are negatively charged, the hydrophobic (water resisting) molecules present on the cell surface overcome this repulsive charge and facilitate the bacterial adherence to surfaces as well as each other. In 1965 Stotzky and Ren examined the stimulating effect of clay minerals on different microorganisms. Robertson et al. (1977) suggested that fimbriae can act as non-specific adherents and provide a bridge over the electrostatic barrier between bacteria and the cell surface. In other words, the tip of the pilus is the point for initial contact which penetrates the negative charged barrier because of having small surface and may be enhanced by uncharged hydrophobic molecules and finally neutralize the surface charge repulsion (Ward et al 1977). This hydrophobic property in bacteria combined with specific surface receptors on the host cells provides strong attachment to mucoepithelial cells. Therefore, hydrophobic interactions have been implicated in bacterial adherence to host tissues, phagocytes, inert surfaces and water-air interfaces.

I.9.a. Plasmids and bacterial hydrophobicity:

As mentioned earlier, many plasmids mediate alterations in the outer membrane including surface molecules of microorganisms. The role of plasmids in controlling the formation of fimbriae has already been discussed. There are reports suggesting the direct influence of plasmids on bacterial hydrophobicity. Plasmid RP4 was reported to enhance hydrophobicity of laboratory strains of E. coli (Ferreiro and Criado 1984). Later Tewari et al. (1985) examined the role of plasmid ColV-K30 and ColV-IK94 on the hydrophobicity of E. coli strain 1829 using the HIC (hydrophobic interaction chromatography) method. It was found that ColV plasmids significantly increased the hydrophobicity of this organism. An effect of plasmid RP1 on surface properties of a clinical strain of P. mirabilis has also been observed (Onaopalo and Klemperer 1986). Its effect varied with growth conditions and the method used for measuring hydrophobicity. In stationary phase plasmid bearing strains were more hydrophobic when measured with HIC method.

In the work reported here, the influence of large plasmids on the surface hydrophobicity of urinary isolates of P. mirabilis was also studied.

I.10. Factors influencing bacterial growth:

Bacterial growth is defined as the coordinated increase in the number of bacteria in a population, which is in fact

dependent on the multiplication of single cells occurring by binary fission. The growth of bacteria is highly correlated with the structural and physiological capability of the microorganism as well as the physicochemical conditions of the growth medium. In normal conditions (eg. at suitable temperatures and in the absence of inhibitiors such as antibiotics) bacteria are able to grow in any medium which contains:

A- Diffusible molecules which are able to pass passively through cell membranes like, oxygen, carbon dioxide and certain amino acids.

B- Transportable substances which can be introduced into the cell via specific transport systems. These systems require metabolic energy and in many cases specific protein carriers located within the cell membranes.

This assumes that the molecules entering the cell by A and B can provide energy, nitrogen, a utilizable carbon source, trace elements and any specific growth requirements.

Although the complete nutritional requirements of some types of bacteria are not yet determined, almost all bacteria are able to grow in the presently available media. In recent years, the critical role of growth media particularly in relation to phenotypic variation and changes in the cell envelope has been given special attention (Klemperer et al. 1979). There are numbers of chemically defined and non-defined

media being used for bacterial growth but the traditional non-defined media, which are mainly based on natural protein digests and are the soluble product of enzymatic hydrolysis of meat or fish (eg. pepton-tryptic digest nutrient broth or agar) are widely used. These media are cheap and bacterial growth even with small inocula is reliable. Bacteria which usually grow in animal tissues or in their products (eg. milk or egg) often require extra amino acids, nucleic acids and vitamins. Therefore, if these are grown in vitro, meat or yeast extract is added to the medium to provide more protein or vitamins. But it has been observed that pathogenic bacteria which are grown in vitro may lose some of their virulence determinants because the expression of virulence genes is complete only in natural conditions of infection in vivo (Smith 1977). Complete in vitro growth conditions corresponding to the in vivo situation can only be provided when complete information about the structure and physiology of bacteria (directly isolated from infection) is obtained. There are also other factors affecting bacterial growth such as pH, temperature and osmotic pressure of the medium (Brown and Williams 1985). Growth conditions often influence the cell envelope, therefore lack of substantial nutrients in a growing culture results in significant changes in physiology and biochemical structure of the bacterial envelope. Smith (1983) reported the loss of some virulence properties in bacteria by

in vitro growth. The virulence was then restored by animal passage and he suggested, this may be due to phenotypic variation in the cell envelope. Phosphate, sulphate and iron deficient growth medium resulted in synthesis of new outer membrane proteins in *E. coli* (Lugtenberg and Alpen 1983) and such deficiencies might occur *in vivo*.

I. 10. a. *The significance of iron in bacterial growth*:

Iron is an essential nutrient which plays an important role in bacterial metabolism (Neilands 1974). In the laboratory, it is usually available to bacteria in sufficient amounts, but although there is plenty of iron in the animal and human body, it is mostly found intracellularly in combined form with organic substrates (eg. haemoglobin) and the amount of free iron in body liquids which can be used by bacteria is very low (Griffiths 1987). Therefore many bacteria especially Gram-negatives, synthesize iron chelation and transport systems (Brown and Williams 1985), which involve production of chelating agents called siderophores. The uptake proteins of these systems are usually present in the cell envelope, but they are induced only when bacteria are deprived of iron in an iron limited-medium. Iron depletion in the growth medium causes changes in the cell envelope as well as in cell metabolism (Brown and Melling 1969). It is reported that the limitation of iron mediates changes in tRNA species in *E. coli* (Griffiths 1983). Iorio and Plocke (1981) observed that the

activity of ribosomes was increased in the absence of iron.

Lack of other nutrients such as phosphates, sulphates, magnesium and zinc also affects bacterial physiology (Weinberg 1978). The growth and nutritional requirements of many bacteria particularly members of the Enterobacteriaceae including E. coli (Williams 1979, Klemperer 1979), Salmonella (Neidenhardt et al. 1974), Klebsiella pneumonia (Sterkenberg 1984) and Pseudomonas aeruginosa (Kenward 1979) have been extensively investigated.

I. 10. b. Growth and nutritional requirements of P. mirabilis:

P. mirabilis grows readily in nature and in all types of media. It is a saprophytic organism and due to production of urease is capable of metabolising urea as a nitrogen source to synthesize proteins. Among Enterobacteria only Proteus species especially P. mirabilis are able to decarboxylate valine and leucine (Naylor 1964). This organism also degrades tyrosine and gelatine but needs nicotinic acid for its growth. In comparison to E. coli and Pseudomonas not much work has been done on the growth of P. mirabilis. The swarming phenomenon which is one of the main characteristics of this group of bacteria has been well studied by many microbiologists, and most of their work is concerned with the nutritional requirements for this phenomenon (Naylor 1964, Smith 1972, Armitage 1981, Jin and Murray 1988). The role of 22 different

amino acids (L-isomers) in the growth rate and their ability to stimulate swarming also whether they serve as nitrogen, carbon or energy sources in P. mirabilis has been examined by Jones and Park (1967). It was found that although some of these amino acids such as proline and alanine can be used in energy producing proceses by this bacterium, the main role of these amino acids is to act as nitrogen sources for protein synthesis. They also observed that most of the amino acids particularly glutamic acid, glutamine, proline and serine reduce the generation time and increased the growth rate in liquid medium as well as stimulating the swarming of P. mirabilis on corresponding solid medium. Jin and Murray (1988) found that the two cations, iron and zinc have a vital effect on the life cycle of P. mirabilis. These two elements even protect the cell from the inhibitory effect of EDTA on the growth and swarming of this organism. The role of other elements such as Ca, Mg, and Cu was also studied. They suggested that the presence of these cations is needed for the formation of macromolecules like bacterial enzymes and toxins. Part of the requirement for trace elements may be due to the metal binding property of some surface ligands and molecules (Beveridge and Koval 1981). Finch and Brown (1978) reported that limitation of Mg in growth medium resulted in the loss of surface antigens. Growth of P. mirabilis in iron-deficient medium increased the sensitivity of the strains to human and

guinea-pig serum (Onaolapo and Klemperer 1987). The sensitivity of Pseudomonas to serum was also increased when depleted of carbon (Anwar et al. 1983). The effect of different electrolytes and carbohydrates on the life cycle of Proteus has been studied by Naylor (1964). He found that the presence of some electrolytes such as NaCl, KCl, NH_4Cl and $(NH_4)_2SO_4$ enhanced the growth and swarming of this organism. The maximal growth in broth occurred at the concentration of 0.5% of NaCl, and at this concentration in solid agar complete swarming was observed. Among 10 different genera tested by Naylor, the growth response and turbidity of P. mirabilis cultures in broth was at the highest level. He suggested that the swarming phenomenon is a kind of growth response to the high nutritional value of the medium particularly in respect of electrolytes and carbohydrates. But there is still a lot to be done to find out exact nutritional requirements and growth conditions of P. mirabilis and their relation with swarming.

I.10.c. Effect of plasmids on the growth and nutrition of bacteria:

There is evidence indicating that the presence of plasmids originating from clinical sources affects the growth and nutritional requirements of bacteria such as E. coli (Melling et al. 1977, Klemperer 1978), Pseudomonas aeruginosa (Kenward 1978) and Klebsiella pneumoniae (Denoya 1986). Some of the plasmids mediate iron uptake especially in iron

deficient media (Griffiths et al. 1983). Williams (1979) reported a new iron uptake system in E. coli specified by some ColV plasmids. In contrast, E. coli strains carrying R-plasmid RP1 were shown to have greater nutritional requirements particularly for iron than their plasmid-free counterparts (Klemperer 1979). This difference was significant in batch cultures. Since the level of iron in body tissues is lower than in culture media, the virulence of some plasmid-bearing strains is reduced (although virulence of those that carry plasmid-encoded aerobactin production would be increased) and the stability of the plasmid itself may be affected (Williams 1979) The presence of plasmids pRR12 and ColE increased the thymine requirement in P. mirabilis (Womble et al. 1978). These strains needed nicotinic acid, thymine, leucine and tryptophan for their complete growth. In strains grown in thymine-limited medium, the replication of plasmids was incomplete and they formed partially supercoiled intermediates with densities between covalently closed circular (CCC) and open circular plasmid DNA. The replicating intermediates remained partially supercoiled at pH 11 and then yielded the non-replicating plasmid DNA in nicked (relaxed) structure.

Here the role of plasmids in the growth of clinical isolates of P. mirabilis in iron containing and iron-limited nutrient broth has been studied.

I. 10. d. The effect of anionic detergents on bacterial growth:

Anionic detergents are a group of antiseptic compounds which at low concentrations are bacteriostatic and at high concentrations have bactericidal activity. These are surfactant detergents active on the bacterial envelope and their mechanism of action is believed to be the disintegration of the cytoplasmic membrane (Hirota et al. 1970). Gram-positive bacteria are more sensitive to these agents than Gram-negatives (Russell and Gould 1988). This increased resistance in Gram-negatives is due to the presence of the outer membrane which is often impermeable to such agents. Sodium dodecyl sulphate (SDS) or sodium lauryl sulphate (SLS) and sodium deoxycholate (DOC) are the best known anionic detergents which are extensively applied in microbiology and molecular biology laboratories. Their effects may be reversed by eg. cationic detergents like quats (quaternary ammonium compounds) or by certain metals which may compete for anionic sites on the cell surface. The effect of these agents on Gram-negative bacteria has been well studied by several investigators (Shafa and Salton 1960, Abram and Gibson 1961, Kopp and Muller 1965, Filip et al. 1973). Sanderson et al (1974) studied the permeability of the outer membrane in lipopolysaccharide mutant strains of Salmonella typhimurium and they found that these mutants were more sensitive to

detergents than their parental ones. The same finding was reported in E. coli K12 by Benson and Decloux (1985). SDS was shown to be more active on E. coli and Pseudomonas than DOC (El-falaha et al. 1989).

Here the effect of SDS on the growth rate of p+ and p- strains of P. mirabilis has been studied.

I.11. Plasmids and the motility of P. mirabilis:

Motility is always considered to be an important virulence property in pathogenic bacteria by which they disseminate in the body as well as the environment. Motility enables the organism to respond to certain environmental conditions by negative chemotaxis from toxic substances or positive chemotaxis towards nutrients. Swarming of Proteus (which has been described earlier) is a clear feature of bacterial motility on the surface of solid media. Motility is also one of the most useful characteristics for bacterial identification. Most of the Gram-negative bacteria, particularly members of the Enterobacteriaceae have been observed to be highly motile organisms. In a study by Latham and Johnston (1972) on the motility of 89 strains of 14 different species of this family, P. mirabilis was found to be one of the most motile species. Douglas (1978) reported a direct relation between migration speed and swarming zonation of this organism on agar and the motility in broth culture. If swarming movement on the leading edge of the colony was slow

or stopped, the motility in liquid suspension was also very slow and vice versa. Therefore he described the periodic growth and zone formation in Proteus as a result of frequent changes in the velocity of cellular motility.

I.11.a. Proteus flagella. Bacterial motility depends upon the activity of long filamentous appendages called flagella. These are several times longer than the bacterial cell (10-40 μm) and their diameter is 20-30 nm. Leifson et al (1954) investigated the development and morphology of Proteus flagella in liquid medium, and reported that although the flagella of all type of bacteria have a spiral shape, Proteus flagella seemed to be unique in their type of curvature. They appear in at least four shapes which are referred to as curly, coiled, semicoiled and normal. Under environmental conditions particularly pH, each type of curvature can be changed to another. Individual strains of Proteus may exhibit one or more types of flagella but the normal and curly types are most common. Flagella in Proteus strains mainly consist of a single protein called flagellin with molecular weight 20,000 daltons (Hoeniger 1965).

Plasmids sometimes mediate changes in bacterial motility and flagella formation. Some plasmids depress flagellation and eliminate swarming in P. mirabilis strains, whereas some others induce flagella formation and initiate swarming (Hesselwood and Smith 1974). ColV plasmids are reported to

have reduced the motility of strains of E. coli (Tewari 1986).

I. 12. Survival of P. mirabilis in different environments:

I. 12. a. Survival in serum. Serum is the fluid fraction of coagulated blood which normally contains, various nutrients, antibodies, lysozyme, complement proteins, electrolytes, albumins, globulins, waste products of the cells and phagocytes. Some of these components specifically (eg. antibodies and lymphocytes) or non-specifically (eg. complement and lysozyme) are involved in body defence mechanisms and exhibit bactericidal activities which lyse, kill or inhibit invading pathogens present in the serum. For example, monoclonal antibodies (MAbs) protect the host against infectious diseases by binding to specific antigenic sites on the bacterial surface and affecting their properties. These structures mainly include different regions of the lipopolysaccharide of the cell wall such as side chains (O specific chain or O antigen) (Kirkland et al. 1984) and lipid A region (Braude and Siemienski 1960). Moreover, the outer membrane proteins, capsular polysaccharides (Cross et al. 1983), and fimbrial adhesins (Abraham et al. 1985, Read 1989) can also be involved in such interactions. The effects of monoclonal antibodies present in human serum on a wound isolate of P. mirabilis were examined by Harmon et al. (1989). They found that MAbs 4F [immunoglobuline GI (IgGI)] inhibited the motility of P. mirabilis and was strongly protective in a

burned wound sepsis caused by this organism.

Polymorphonuclear leukocytes (PMNs) present in serum have potent bactericidal activity against invasive pathogens. These cells which comprise an important part of the body's defence mechanism, destroy the bacteria after adhering to and phagocytosing them. Shafer et al. (1988) reported the purification of two antimicrobial proteins extracted from human PMNs from the serum. These proteins which are referred to as CAP37 and CAP57, seem to have a strong bactericidal effect against Gram-negative bacteria particularly Enterobacteria. These authors suggested that the negatively charged residues (eg. phosphates) on lipid A in LPS, determine the susceptibility of bacteria to such cationic antimicrobial proteins. They also studied the effect of these proteins on rough and smooth strains of P. mirabilis and it was shown that some strains demonstrate resistance against the antibacterial activity of CAP37 and CAP57.

Adherence properties and surface adhesins have a significant role in the interaction between bacteria and defence factors particularly leukocytes, antibodies and complement proteins. Fimbriae increase the virulence of bacteria by enabling them to adhere to host cells and develop the infection, but when this attachment occurs with phagocytes fimbriae are disadvantageous. The antigenicity of pili has been well studied by Pearce and Buchanan (1980). They were

able to eliminate the adhesive property of fimbriae by using relevent antisera. Homologous antibody to these fimbriae abolished their colonization activity *in vivo* (Evans 1978), and their attachment to surfaces *in vitro*. These studies showed that fimbriae have immunogenic properties even in natural infections. This may be the reason why highly fimbriated strains of *P. mirabilis* in the bladder and urethra, lose their pili or reduce them to a very small number when entering the renal cortex or blood stream.

Therefore to survive and grow in the host body, bacteria must resist humoral and cellular defence mechanisms (Smith 1984). On the other hand, serum contains plenty of nutrients such as serum albumin and globulin, glucose and electrolytes which can be used by invading bacteria. Therefore, the survival of an organism in serum depends on the outcome of its challenge with the defence systems and the usage of these nutrients. Bacterial resistance to the antibacterial actions of serum is very important in the pathogenesis of infections. In other words, resistant strains seem to be more virulent and survive longer in serum which gives them the chance to multiply and disseminate throughout the host body. Several factors are involved in bacterial serum resistance such as synthesis of new proteins in the outer membrane, alteration of O or K antigen, production of intact LPS and reduction of surface adhesins. The outer membranes of bacteria which

initially interact with defence factors play an essential role in determining resistance to these factors. Penn et al. (1977) reported the increased resistance of N. gonorrhoeae to complement mediated antimicrobial activity of serum and phagocytosis by several passages of growth in an implanted chamber. This resistance was lost after a few generations in broth culture. It was suggested that this adaptibility was due to changes in the LPS content of the outer membrane. Induced resistance to serum factors has been shown in E. coli K12 (Finn 1982). Strains grown in vivo were more resistant to serum than the strains grown in nutrient broth. The loss of Proteus fimbriae in kidney and blood also indicates the environmental adaptibility of surface properties to bactericidal effects of serum.

I. 12. a. 1. Plasmids and serum resistance:

Plasmids are found to be involved in survival of their host organism in serum. The presence of plasmids in bacteria, depending on the type of that plasmid as well as on the characteristics of the host cell, increases or reduces the resistance to serum factors. E. coli strains containing the ColV plasmid demonstrated increased resistance to human and rabbit serum (Binns et al. 1977), and the resistance was reduced after curing the strains. Smith (1974) observed that injection of ColV+ and ColV- strains of E. coli K12 into chickens resulted in significantly increased numbers of

plasmid-carrying strains compared to the plasmid-free cells after 24 hours. It was shown that serum components were not able to lyse ColV+ cells. The mechanism of ColV mediated resistance to the bactericidal effect of serum is not yet clearly understood. ColV plasmids carry the traT gene which codes for a protein of molecular weight 25000 which is one of the major proteins of the outer membrane. It is suggested that this protein may be involved in the serum resistance conferred by ColV and some R-plasmids such as R100. For ColV, the ISS gene which does not encode traT protein is also involved (Binns et al. 1979). On the contrary, some types of plasmids reduce the resistance of bacteria to serum. The presence of R-plasmids RP1 and RP68 were reported to increase the sensitivity of Pseudomonas aeruginosa strain PAO1 to serum in an experimental burn infection in mouse (Wretlind et al. 1985). It was found that the mortality of the plasmid-containing strains was 10-400 times greater than their plasmid-free counterparts in the mouse body, and they were more sensitive to human serum. The resistance was restored by curing. Plasmid FP93 also rendered P. aeruginosa susceptible to serum and reduced its virulence (Pemberton and Holloway 1973). In P. mirabilis the presence of R-plasmid RP1 led to increased sensitivity to bactericidal action of serum and the results were nearly the same with human and guinea pig serum (Onaopolo and Klemperer 1987). This loss of resistance was

mainly attributed to increased phagocytosis, because plasmid RP1 is reported to be associated with enhanced hydrophobicity in P. mirabilis (Onaolapo and Klemperer 1986) and the hydrophobic strains were said to be more susceptible to phagocytosis (Xiu et al. 1983). The effects of plasmids on serum sensitivity of bacteria may also involve specific receptors on target cells which are recognized by surface molecules of the organism (Stendahl 1983) or increased antigenic properties of O-specific antigen by amino acid phosphate substitution (Vinogradov 1990).

In this study, the growth and survival of plasmid-bearing and plasmid-free isolates of P. mirabilis (of clinical origin) in human and rabbit serum have been examined.

I. 12. b. Survival and growth of bacteria in sewage:

Sewage consists of household and industrial effluent containing organic and inorganic substances in three forms, suspensions, solutions and colloidal. More than 90% of these substances are organic compounds which mainly consist of :

Organic acids 40%, amino acids 11%, creatinine 4%, carbohydrates 29% and anionic surfactants 15%. The inorganic compounds include different kind of electrolytes such as, sodium and potassium chloride, calcium chloride, calcium sulphate and calcium carbonate. The frequent addition of industrial effluents may sometimes alter this composition. The average amount of sewage from a population of 20000-30000 will

depend on such factors as rainfall, but will be around one million gallons per day (Hanel 1988), a huge volume of medium suitable for the growth of a diverse range of microorganisms including, viruses, bacteria, protozoa, fungi, nematodes and insect larvae. These organisms are always in competition with each other for their growth and survival, and ability to produce inhibitors or to predate on other organisms will aid competition. For example the production of bacteriocins by some microorganisms was shown to inhibit the growth of Salmonella typhi and S. typhimurium in a sewage sediment (Padilla et al. 1990). During the treatment of sewage by activated sludge in which there are numbers of protozoa, the viable count of bacteria was significantly decreased (90-98%) and the pathogenicity of diarrhoea-causing organisms such as Salmonella and E. coli was reduced down 5-10 fold (Hanel 1988). Lim and Flint (1989) reported the survival of E. coli in sterilised lake water for 12 days. The survival time was reduced in unfiltered water because of the presence of other microorganisms, but when synthetic sewage was added the growth rate of E. coli increased significantly, which indicates the high nutritional value of sewage for microbial growth in the absence of inhibitors. Klebsiella pneumoniae was shown to reduce to a tiny coccoid form (0.25-0.50 µm diameter) due to lack of nutrients in well water (Lappin-Scott et al. 1988). Its size was restored when grown in rich medium. Moreover the

presence of bacteriophages in sewage may have considerable inhibitory effect on the growth and survival of some bacteria. There are also certain bacteria and fungi antagonistic to pathogenic organisms. For instance, saprophytic bacteria are always in competition with most pathogens for substrates and organic nutrients in sewage, which may eventually limit the population and spread of these bacteria. Therefore, in the study of survival of a particular organism in sewage the protective role of nutritional factors as well as the effect of biological and chemical inhibitors must be considered.

Among bacteria, some types are frequently encountered in sewage and activated sludge such as, Pseudomonas spp, E. coli, Proteus, Aerobacter and Streptococcus. Most of these bacteria are normal inhabitants of human and animal intestines, and the presence of coliforms is considered as an indicator of water contamination. Table 3 which has been extracted from Hanel (1988) shows some of the important pathogens found in sewage.

I.12.c. Survival of bacteria in effluent:

The presence of large numbers of microorganisms as well as other organic materials in sewage, greatly increases the BOD (biochemical oxygen demand), which environmentally is very harmful. Moreover the existence of pathogenic bacteria is a big threat to human populations and enhances the spread of water-borne diseases. Therefore, sewage is treated to reduce its BOD and remove the microbial pathogens.

After primary treatment, which consists only of passage through a metal net or a rotary cutting device, sewage is piped into a pool containing activated sludge which is a biomass of saprophytic bacteria and protozoa. The mixture is agitated for several hours or one day and then transferred to settling tanks, from which the final effluent is ultimately discharged. There are also other methods of treatment such as using biological filters which are basically the same as activated sludge. Final effluent still contains particles of organic matter and considerable number of microbes and it can be a source of infection for the community (Jones and Watkins 1985). Therefore, when it is to be discharged into the rivers, further treatment (tertiary treatment) is carried out.

Survival of different type of bacteria in effluent has been examined by some investigators. Araujo et al (1990) reported the survival and growth of Aeromonas hydrophila in effluent pouring into the sea over a one year study. The number of bacteria in samples taken from near the sea shore was as high as faecal coliforms. but when the offshore sea water was tested this number was very low. There was a direct correlation between the survival of strains of Pseudomonas aeruginosa in sea water receiving effluent and freshwater with resistance to antibiotics and heavy metals (Vicente et al. 1990). The strains which were isolated from more polluted water were demonstrated to have higher resistance to heavy

metals. Jones et al. (1990) monitored the epidemiological relation between the presence of Campylobacter in effluent with the incidence of enteritis in the community. The number of bacteria as well as incidence of infection was season dependent and the peak was in May and June.

Table 3- Pathogenic bacteria in sewage originating from faeces and urine.

Bacterial species	Infection caused
Salmonella typhimurium	Enteritis
Salmonella typhi	Typhoid
Salmonella paratyphi B	Paratyphoid
Escherichia coli	Enteritis
Shigella spp	Dysentery
Proteus spp	Urinary infection
Vibrio cholerae	Cholera
Yersinia enterocolitica	Gastroenteritis
Yersinia pseudotuberculosis	Paratuberculosis
Mycobacterium tuberculosis	Tuberculosis

I. 12. d. The growth and survival of bacteria in river water:

Rivers which are passing through big and industrial cities persistently receive discharges of organic pollutants and contain large numbers of microorganisms. Dilution of wastewaters into large bodies of water such as rivers, lakes and the sea is the most common and easiest method of disposal (Tchobanoglous 1979), but in industrial countries where the wastewaters are highly polluted, complete treatment of sewage is considered to be essential before its discharge into the rivers. But, in some cases, especially when wastewater is diluted into a large river, only the primary treatment is carried out. In this case polluted river water is similar to diluted sewage, and the number of faecal coliforms can be use as an indicator of pollution (Feresu and Van Sickle 1990). Sokari et al. (1988) studied the survival of Pseudomonas aeruginosa and E. coli in river water. The strains which survived best were those resistant to one or more antibiotics. The relation between survival in water and antibiotic resistance has also been reported in Salmonella typhimurium (Morinigo et al 1990) where more than 90% of the isolated strains were resistant to tetracyclines. E. coli and Salmonella were also observed to grow readily in tropical fresh water (in a rain forest river). This may be due to the increased attachment ability of p+ strains (Hicks and Rowbury

1986 and 1987) which will be discussed later. The number of both of them increased rapidly, but the cellular activities of E. coli such as motility and respiration were greater than Salmonella (Jimenez et al. 1989). The seasonal distribution of Aeromonas hydrophila was reported by Pathak et al. (1988).

I. 12. e. Effect of plasmids on the survival of bacteria in aquatic systems:

The presence of plasmids in bacteria growing in aquatic media such as sewage, effluent and river water has been observed by several researchers (Burton et al. 1982, Mach and Grimes 1982, Saye et al. 1987). Day et al. (1988a) reported the presence of plasmids in more than 11.4% of Pseudomonas species isolated from effluent. In one case, fifteen strains out of thirty six isolates of this organism collected from one site, contained plasmid. These were mostly large plasmids (35-312 kb) and it was believed that some of them contribute to naphthalene utilization, and this property was not transferable. This may be due to adaptation of bacteria to the effluent which stabilized the gene reponsible for naphthalene catabolism (Day et al. 1988b). It has previously been reported that naphthalene utilization is both chromosomal and plasmid encoded (Dunn and Gunsalus 1973), and in the latter case, it is transmissible (Cain and Williams 1982). Baya et al. (1986) reported that the incidence of multiple plasmid-carrying bacteria in waters exposed to toxic wastes is higher than in

polluted sewage. The survival of E. coli strain HB 101 containing different sizes of R-plasmids (3.9-48 Kb) and coding for resistance to various antibiotics in river water has been observed by Chao and Feng (1990). The strains did not survive more than 3 days in unfiltered water but they maintained their numbers in sterilized river water for seven days.

The increased attachment property of bacteria coded by plasmids can be one of the main factor of survival in waters. In an extensive study, Hicks and Rowbury found out that plasmid-encoded attachment increased the resistance of E. coli strains to chlorine (Hicks and Rowbury 1986a), acids (Hicks and Rowbury 1986b and 1987a), bacteriophages (Hicks and Rowbury 1987b) and metals (Hicks and Rowbury 1988).

We also examined the role of large plasmids in the survival and growth of clinical strains of P. mirabilis.

I. 13. Enzymes in P. mirabilis.

Production of different enzymes is very common among Proteeae and plays a significant role in the growth and pathogenicity of these bacteria. Also members of this group of bacteria, especially P. mirabilis, are a major source of some types of enzymes such as urease and tryptophan deaminase (TDA). The most important enzymes produced by P. mirabilis are urease, haemolysin, protease and lecithinase.

I. 13. a Urease. An important virulence factor in P. mirabilis is the production of the enzyme urease. Enormous quantities of urea are constantly released into the environment through the biological activities of living beings. It is one of the main nitrogen sources for many bacteria (Mobly and Hausinger 1989), and the main factor in degradation and utilization of urea is the enzyme urease.

Urease (urea amidohydrolase) catalyzes the hydrolysis of urea to yield ammonia and carbamate, which then spontaneously hydrolyses to carbonic acid and a second molecule of ammonia (Andrews et al. 1984). Then at physiological pH the carbonic acid dissociates to carbon dioxide and water while the ammonia molecules associate with water resulting in the increased pH of the medium.

Urease is the best studied enzyme which has been obtained in pure form. It was first isolated from jack beans (Canavalia

ensiformis) by Sumner in 1926. Many plants, some invertebrates and many eucaryotic microorganisms synthesize urease. Moreover, urease activity is widely observed in enormous numbers of procaryotes and more than 200 distinct urolytic microorganisms had been reported by 1947 (Sumner and Somers 1947). This urolytic property is often used as a criterion for classification and identification of certain genera or species (Booth and Vishniac 1987). The high level of urease activity in Proteus spp is used to distinguish them from many other members of the Enterobacteriaceae family (Christensen 1946). Microbial ureases are important in the metabolism of ruminants, the transformation of certain nitrogenous compounds and the pathogenesis of some animal and human infections. This enzyme is directly involved in the formation of infectious kidney stones following pyelonephritis. It is also associated with the pathogenesis of ammonia encephalopathy, hepatic coma, urinary catheter encrustation and hepatic ulceration (Mobley and Hausinger 1989). There are suggestions that urease may also play a role in the inactivation of complement proteins in renal tissues (McLaren 1968).

P. mirabilis is the primary urease producing uropathogen in humans (Rubin et al. 1986), and the most common organism implicated in production of kidney stones (Rosenstein 1986). Infection induced stones constitute 15-20% of all urinary stones (Griffith and Osborne 1987), which are usually a mixture

of struvite ($MgNH_4PO_4.6H_2O$) and carbonate apatite [$Ca_{10}(PO_4)_6.CO_3$]. In the kidney the ammonia released from urea hydrolysis, causes alkalinization of urine, and between pHs 6.5 and 9 the supersaturation and crystallization of polyvalent ions which are normally soluble occurs folowed by formation of stones (Griffith 1978). Stegmayr and Stegmayr (1983) reported the loss of calcium and magnesium ions during stone formation in an experimental urine infection by P. mirabilis. The role of Proteus urease in causing pyelonephritis in a rat model and tissue culture experiment was examined by Braude and Siemienski (1960). They showed a direct effect of urease toxicity on renal tissue by using killed Proteus suspensions with active and inactivated urease. Only the active enzyme was able to cause necrosis in renal tubular cells. In tissue culture also the intracellular infection by P. mirabilis developed as the concentration of urea in the medium increased. It is suggested that different stages of cellular growth and changes in cell membranes are involved in the elaboration of this enzyme. For example in P. mirabilis the enzyme level in elongated swarmer cells is reported to be significantly higher than that of the ordinary short rods (Falkinham and Hoffman 1984). Mclean et al. (1986) by precipitating the ammonia with sodium tetraphenylboron and silver staining showed that the urease activity in P. mirabilis is associated with the periplasm and outer membrane.

Urease can be inhibited by a group of chemicals most of which act competitively on this enzyme. Several urea analogues are among these inhibitors, such as, methylurea, hydroxyurea, thiourea and hydroxamic acids. Other inhibitors include phosphoric acid, phenylphosphorodiamidate, boric acid, mercaptoethanol, fluoride and iodoacetic acid.

I. 13. a. 1. Plasmids and urease. Most bacterial ureases are controlled by chromosomal genes. Walz et al. (1988) inserted fragments of chromosomal DNA from a urinary strain of P. mirabilis into phage genome pHC79 and then transduced to E. coli strain HB101. A urease-positive DNA fragment of 10.2 Kb molecular weight was isolated which conferred urease activity to E. coli. Recently, Jones and Mobley (1990) reported that urease genes are localized on a 7.6 Kb region of chromosomal DNA in P. mirabilis which can be transferred to a urease-free recipient cell. Plasmid-encoded urease has also been reported in some Proteus species (Mobley and Hausinger 1989). The transfer of urease gene(s) by a 140 Kb plasmid DNA from Providencia stuartii to E. coli was reported by Grant et al. (1981). Cook (1976) demonstrated urease production induced by a plasmid in Enterococcus faecium which is in the normal flora of the sheep rumen. A rare plasmid-borne urease in E. coli has been reported by Wachsmuth et al. in 1979.

I. 13. b. Haemolysin:

Haemolysin is an extracellular enzyme secreted from many Gram-positive and Gram-negative bacteria, and lyses the erythrocytes of man and most animals. In many bacterial species, production of haemolysin has been associated with their virulence (Kinyon et al. 1977) such as that of E. coli (Knapp et al. 1984), Pseudomonas aeruginosa (Pritchard and Vasil 1986) and P. mirabilis (Peerbooms et al. 1983). It is reported that secretion of haemolysin protein in E. coli requires translocation across both cytoplasmic and outer membrane and it does not involve the conventional N-terminal signal sequence (Felmlee et al. 1985). Haemolytic activity in P. mirabilis has been reported by several workers (Koronakis et al 1987, Senior and Hughes 1987, Mobley and Chippendale 1990). Some did not find any haemolysin in this organism (Werner and Rettger 1919), but most of them observed high level production of the enzyme in all strains tested (Taylor 1928, Philips 1955, Peerbooms et al. 1983). It has been suggested that haemolytic activity in clinical isolates of P. mirabilis is an extracellular but cell dependent function (Senior and Hughes 1987). Peerbooms and co-workers tested haemolytic activity ofP. mirabilis in both broth culture and on blood agar. They found that in young broth culture this property was strongly cell associated and the enzyme level was very low in cell-free supernatant. The growth phases seemed to

be significantly important in haemolysin production, because the activity of young exponential growing culture was very high whereas it was absent or low in stationary phase. Mobley and Chippendale (1990) proposed a correlation between MR/P haemagglutinin fimbriae and haemolytic property of P. mirabilis, with adherence of bacteria to epithelial cells potentiating their enzyme secretion. Berger et al (1982) believe that in E. coli the determinants coding for these two properties can be carried by the same genes. The nature of Proteus haemolysin is not clearly understood. The strong and fast effect of chloramphenicol and trypsin on the production of extracellular haemolysin of Proteus has been attributed to their effect on protein synthesis (Peerbooms et al 1983). Koronakis et al (1987) reported the production of two types of haemolysin in Proteus species; one was an extracellular and cell-free haemolytic factor, which was found in 40% of P. vulgaris and all strains of M. morganii, and the other which was produced by almost all strains of P. mirabilis and the other 60% of P. vulgaris strains tested, which seemed to be highly associated with the bacterial cell. This idea was supported by hybridization of total chromosomal DNA with regions of E. coli alpha-haemolysin (hly) which showed homology between genes coding for haemolytic activity in three strains of Proteeae tested as well as E. coli. But this homology was not complete in P. mirabilis because one of the two gene

determinants of E. coli (hly D) did not hybridize with DNA from this organism which produced only cell-dependent haemolysin. Experiments with dialysed and membrane filtered culture of P. mirabilis showed that this haemolysin is not of low molecular weight and it can not be detected in culture supernatant. There is a possibility that the active haemolysin first appears on the surface of organisms and while it is bound to the surface remains active, but when it dissociates from the cell is unstable (Peerbooms et al. 1983).

I. 13. b. 1. Plasmids and haemolysin activity of bacteria:

Haemolytic activity associated with plasmid DNA has been observed in some bacterial species particularly those originated from the animal intestine. De la Cruz et al. (1980) reported the presence of plasmid determinants coding for haemolysin production in E. coli. These are transmissible plasmids belonging to different incompatibility groups, but it has been shown that they code for the same (Hly) determinant (Goebel et al 1981). These determinants mainly consist of three cistrons, HlyA, HlyB and HlyC which are usually responsible for synthesis of haemolysin as well as its export (Goebel and Hedgpeth 1982). In the human, E.coli strains containing plasmid-borne haemolysin are mostly isolated from urinary infections and less in faecal strains. Studies on faecal strains from animal origin have shown that haemolysin determinants are mostly carried on large plasmids. Muller et

al (1983) isolated a DNA fragment of molecular weight 7.5 Kb from one of these plasmids designated as pHly152. This fragment was found to be responsible for haemolysin production and carries the three cistrons HlyA, HlyB and HlyC. They also found that there is close analogy between chromosomal Hly determinants and those of plasmids; and the only difference was in HlyA. Pritchard and Vasil (1986) were able to sequence a 3.3 Kb fragment of Pseudomonas aeruginosa DNA coding for heat labile haemolysin. The plasmid was then used to transform E. coli and the resulting clones produced the same enzyme.

Here we studied the role of large plasmids on the haemolytic activity of clinical isolates of P. mirabilis.

I.13.c. Protease

Secretion of proteolytic enzymes particularly extracellular protease is very common among bacteria. In 1947 the first bacterial protease was discovered by Lang and Otteston from Bacillus licheniformis which at that time was thought to be Bacillus subtilis. Microbial proteases depending on their properties and active site, are divided into three groups, serine, metallo and acid proteases. Their molecular weight, according to the type of the enzyme ranges from 25000 to 30000 dalton. They are widely used for commercial purposes, for example serine proteases have long been used in washing powders as well as in the leather industry, and the acid types have important application in dairy products as a substitute

to calf rennet. Protease production by human and animal pathogens has also been reported to be a major virulence factor (Suzuki *et al.* 1987). A type of IgA degrading protease has been detected in mucoid strains of *Pseudomonas aeruginosa* which are frequently isolated from the lungs of patients affected with cystic fibrosis (CF) (Ombaka *et al.* 1983). As an important role of IgA is to protect mucus membranes from bacterial colonization, therefore this type of protease is considered to be important in the pathogenicity of this organism. The enzyme was seen to be more stable in iron-restricted conditions as occur *in vivo* (Boyce and Miller 1982). Protease production in *Vibrio cholera* has been observed by Wiersma *et al*. (1978) who also realized that proteolytic activity of this organism increased in iron-limited medium. In *E. coli*, the enzyme protease was found to be coded by a gene marked as (SppA) (Suzuki *et al.* 1987). By using the gene cloned on a plasmid and constructing an *E. coli* strain carrying an ampicillin resistance gene, they were able to map the SppA gene and suggested that the production of protease is an envelope property and is involved in the virulence of this bacterial species. The presence of another type of enzyme in *E. coli* was reported by Maurizi (1987) which is capable of degrading bacteriophage lambda N-protein. Senior *et al.* (1987) reported that urinary strains of *P. mirabilis* also produced an EDTA sensitive IgA protease which cleaved IgA into two

sections at different sites from other microbial proteases. They suggested that this protease may be a virulence factor in P. mirabilis. The properties of IgA protease in this organism was later studied in detail by Looms et al. (1990). The secretion of a type of prochymosine from a L-form variant of P. mirabilis carrying a large plasmid has been repoted by Klessen et. al. (1989). This enzyme was capable of clotting milk proteins.

We have also examined the production of protease in plasmid- free and plasmid-bearing strains of P. mirabilis of clinical and faecal origin.

I. 14. The aims of this work

The aims of this study are to find out whether:

1. The presence of plasmids influence characteristics of *P. mirabilis* strains, changes in which may affect virulence or virulence-related properties ie. such characteristics as:

A. Antibiotic resistance.

B. Swarming ability.

C. Growth rate.

D. Adherence property and autoagglutination.

E. Motility and flagellation.

F. Survival in human and rabbit serum.

G. Survival in aquatic systems.

H. Enzyme production (urease, haemolysin, protease, lecithinase, DNase and amylase).

2. The elimination of plasmids changes or removes these effects.

3. The presence of urea and its derivatives affect the swarming property of *P. mirabilis* strains.

MATERIALS AND METHODS

II. Materials and Methods

II. 1. Bacterial strains:

Clinical isolates (137 strains) of *P. mirabilis* were obtained from the Microbiology laboratory of U. C. H. where they had been isolated from patients suffering from urinary tract infections. Moreover, some faecal strains were collected from Animal Disease Diagnostic Laboratory, Razi Institute, Tehran, Iran. and the P33 strain, a faecal isolate was obtained from NCTC (Colindale).

II. 2. Media:

The media used were as follows:

Nutrient broth No. 2 Oxoid 25 g/1000ml

Nutrient agar Oxoid 28 g/1000ml

Sensitest agar Oxoid 32 g/1000ml a suitable medium for antibiotic sensitivity tests which produces clear and measurable zones of sensitivity around antibiotic discs

Cysteine, lactose, electrolyte, deficient (CLED) Oxoid.

A selective medium to inhibit swarming, due to lack of salts, used for isolation of *Proteus* species (36. 2 g/1000 ml)

Proteae isolation medium (PIM):

PIM is one of the best selective media for identification of *Proteus* strains (Hawkey, 1983) and its formula is as follows:

L-Tyrosine: 4 g. DL-Tryptophan: 5 g. Tryptophan soy agar: 40g (instead of trypticase agar). Agar to inhibit swarming: 8 g.

Distilled water; 1000 ml. After autoclaving and cooling down to 45-50°C, 5 mg Clindamycin and 100 mg Colistin sulphate were aseptically added. The colonies of Proteus strains grew well on this medium and appeared brown in colour while the other bacteria (resistant to added antibiotics) were colourless.

Xilinas medium:

This was another selective medium used for isolation of Proteus strains (Xilinas et al 1975) and it is made of:

Brain heart infusion agar.	40 grams
Bile salt (Oxoid) NO. 3.	5 "
Lithium chloride (LiCl).	5 "
Sodium thiosulphate ($Na_2S_2O_3$, $5H_2O$).	6 "
Tri sodium citrate ($Na_3C_6H_5O_7$, $2H_2O$).	6 "
Distilled water	1000 ml (pH = 7)

II.3. Methods:

II.3.a. Identification of Proteus strains;

All strains were identified by using the API method (API system S.A France) which was as follows:
Bacteria were streaked on selective medium (e.g. PIM or CLED) to give single colonies and incubated at 37°C overnight. One single colony was homogeneously suspended in 5 ml sterile distilled water, then API 20 E strip was removed from its cover, and the proper amount of bacterial suspension was pipetted into the tubes provided on the strip. The tubes ADH, LDC, ODC, URE, and H_2S, were covered with mineral oil to

provide anaerobic conditions. Five ml of water was poured into the wells of the incubation box (tray and lid), to give sufficient humidity. The strip was then placed into the incubation box and incubated at 37°C for 18-24 hours. After incubation, the necessary reagents were added to the relevent tubes and the reactions were observed and recorded on the report sheet by referring to the intepretation table. A 7-digit profile number was obtained from the 20 tests on the strip by adding the positive reactions in each group. The strain was identified by checking the profile number in Analytical Profile Index or using APILAB software.

II.3.b. Plasmid detection:

Three methods were applied for plasmid extraction which are as follows:

1-Birnboim and Doly (1979).

2-Hansen and Olson (1978).

3-Kado and Liu (1981).

The Kado and Liu method was found to work much better for plasmid detection in P. mirabilis strains, and was applied, with some modifications, as follows:

One ml of overnight culture was spun down in an Eppendorf tube for 2 min in microcentrifuge (12000r/min) and resuspended in 3 µl T.E. buffer(50mM Tris+1mM EDTA). Then 120 µl of lysis solution (0.05M Tris+3% SDS, pH=12.52-12.56) was added and mixed gently and then incubated in a 55°C water bath for 30-40

min. After incubation 120 µl phenol/chloroform (5g phenol crystal, 5ml chloroform, 1 ml TE, freshly made) was added and shaken briefly, then spun for 5-10 minutes in a microcentrifuge (12000r/min). Then 60-90 µl of the aqueous phase was transferred to another tube and 10-15 µl of bromo phenol blue (BPB) was added and mixed gently. Finally 40-50 µl was loaded on 0.7% agarose gel and electrophoresed at 140 V for 4 hours. After electrophoresis the gel was stained with 0.5 µg/ml ethidium bromide (ET) in TBE buffer for 60 minutes, then washed with distilled water and checked under a UV box.

II.3.c. Sizing of plasmid DNA:

Samples were loaded on the gel along with DNAs from a bacterium carrying several plasmids of known molecular weight and mobility. In this study the marker was E.coli (39R861) a K12 derivative which is (F^- lac^+ and Nal^r) and was kindly provided by Dr. Threlfall of the Drug Resistance Section, Central Public Health Laboratory Colindale. The marker carries four plasmids with the following specifications.

Table 4: Marker plasmids and their resistance determinants.

	M.W.	Mobility	Resistances (see Table 7)
1.	$98x10^6$	5.9	Cap, Suf, Tet
2.	$42x10^6$	8.6	-------
3.	$23.9x10^6$	10	Cap, Kan, Str, Suf
4.	$4.2x10^6$	17.6	Suf, Tet

Mobilities of the plasmids have already been worked out from the standard equation (next page).

After completion of electrophoresis the gel was stained and washed as explained above and photographed (Figures 5 and 6). The travelling distance of the marker and the sample plasmids were measured, then the mobilities of the samples were calculated using the following equation:

$$\text{Mobility} = \frac{\text{Distance travelled by sample X Mobility of marker}}{\text{Distance travelled by marker plasmid}}$$

The molecular weight of the plasmid was worked out by comparing with the mobilities of the marker plasmids (Table 4) of which the molecular weights are known, as shown in Fig 7.

II.3.d. Antibiotic resistance test:

A 0.1 ml sample of overnight culture of the bacteria was poured onto Sensitest agar plates and spread with the help of a glass spreader, then the antibiotic multodiscs or single discs were placed on the agar and after a few minutes which was given for drying, the plates were incubated overnight at 37°C. After incubation the sensitivity of the organism was measured by sizing the clear zones around each antibiotic.

II.3.e. Conjugation:

The transfer of plasmid from a p+ strain (Donor) to a p- strain (Recipient) was carried out by growing the overnight culture of each one in nutrient broth for 2 hours separately to 5×10^4 cell/ml. Then 1 ml of each culture was added to 10 ml

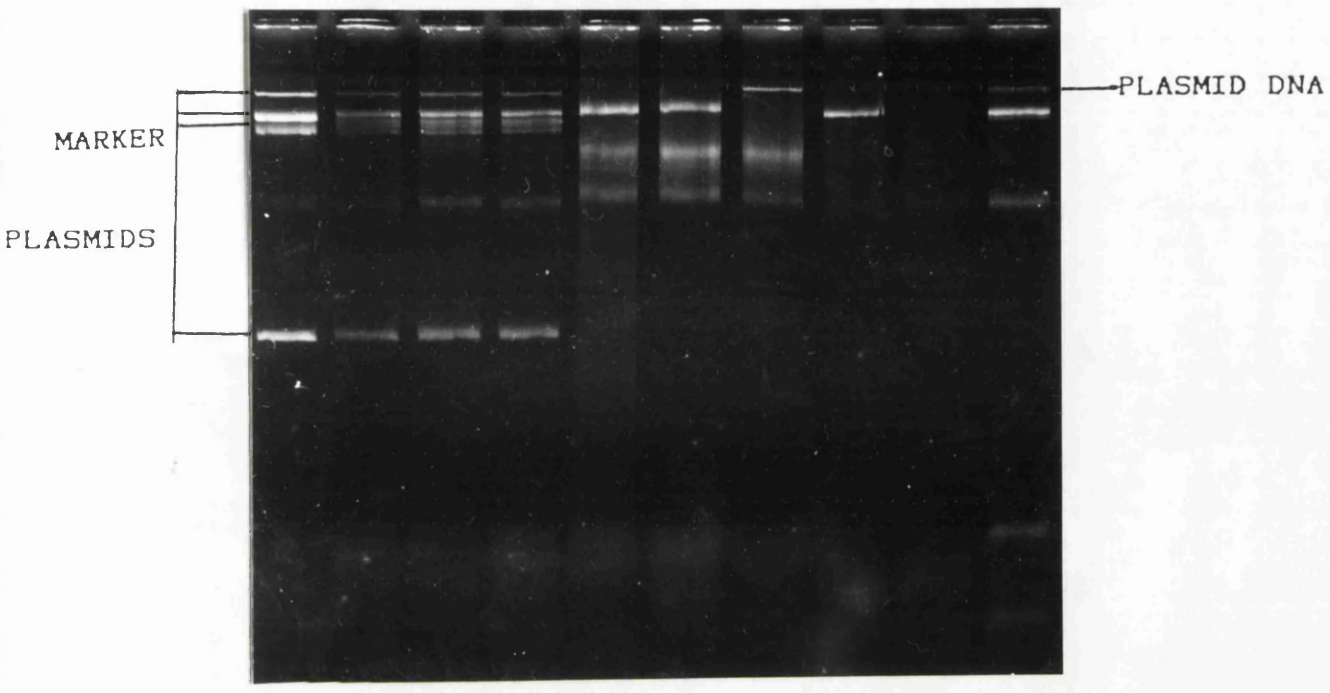

Figure 5. Extraction of plasmid DNA from clinical strains of P. mirabilis by agarose gel electrophoresis at 140 V for 3 hours.

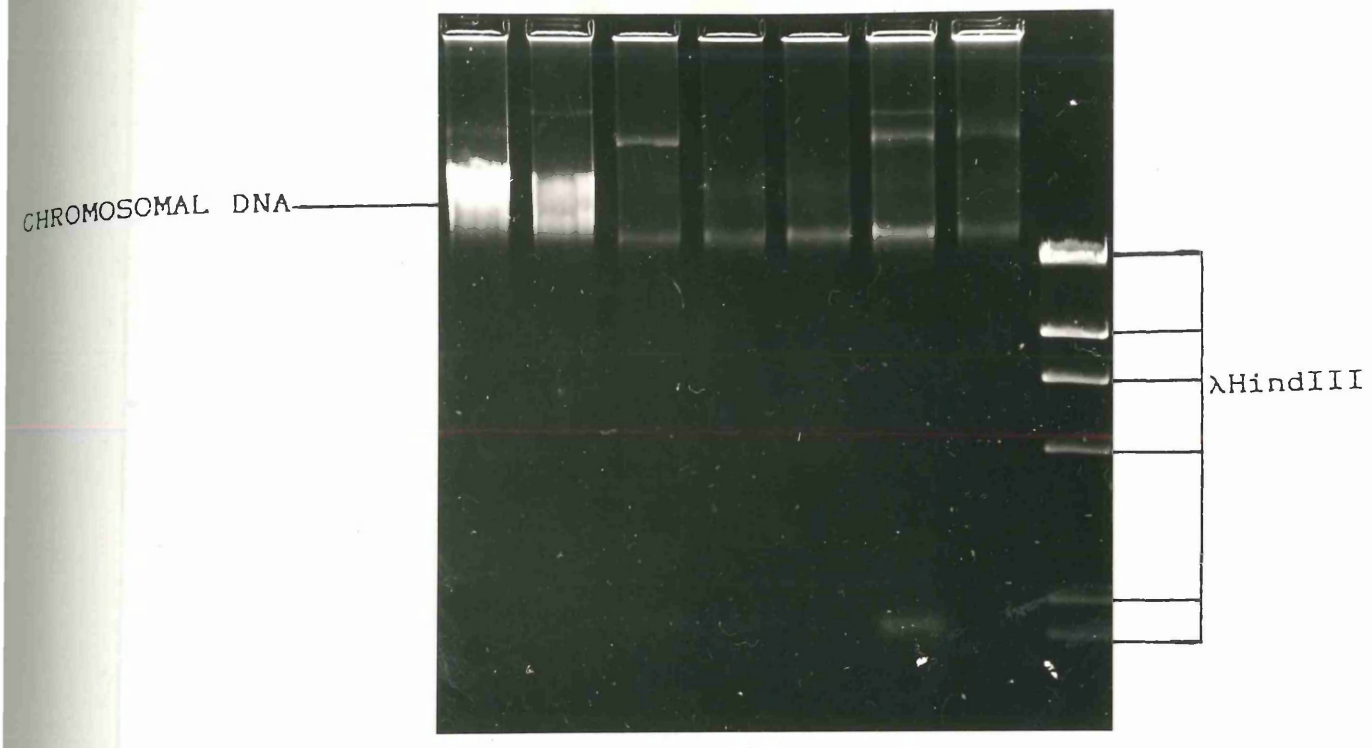

Figure 6. Detection and sizing of plasmid DNA from stains of P. mirabilis by agarose gel electrophoresis.

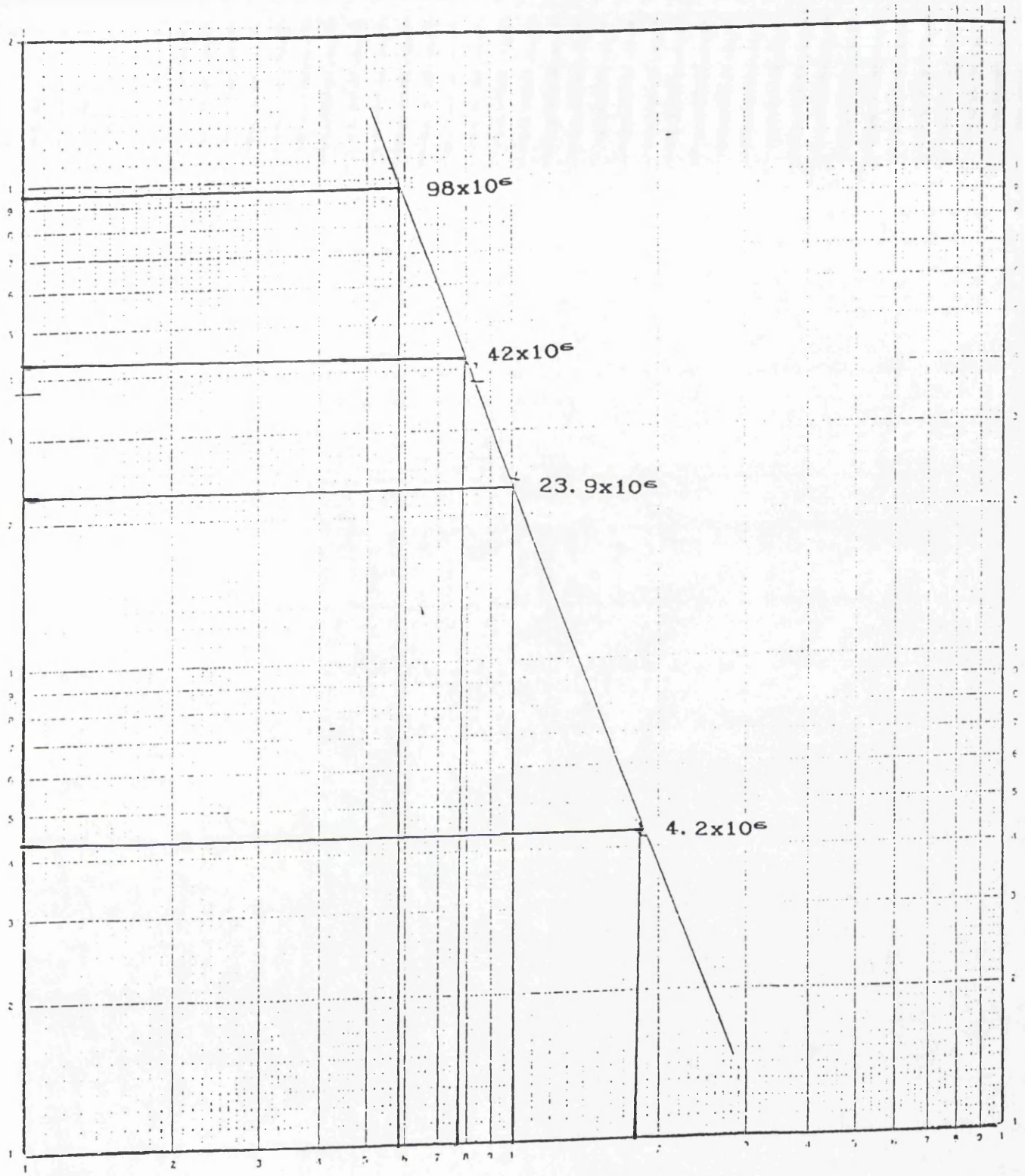

Figure 7. Sizing of plasmid DNA by comparing its mobility with the marker plasmids.

nutrient broth and incubated for 4 hours or overnight. The mixture was diluted (10^{-2}-10^{-9}) and plated on selective medium plus the antibiotic to which the donor was sensitive and the recipient was resistant, then incubated at 37°C, overnight. Five colonies from each plate were taken and grown in nutrient broth for 2 hours and tested for antibiotic resistance. The transfer of the plasmid was also checked by agarose gel electrophoresis.

II. 3. f. Curing the Plasmid-bearing strains of P. mirabilis:

Two methods were applied for curing the p+ strains which are listed below:

A- SDS+heat treatment (Salisbury et al. 1972).

B- Acridine orange treatment (Hirota 1960).

The first method did not work with strains of P. mirabilis, but the method of Hirota seemed to be effective on some strains, and was applied with some modifications , as follows:

A volume of 0.8 ml overnight culture in nutrient broth was added to 20 ml of nutrient broth containing 0.05 mg /ml acridine orange. Then 0.6 ml Tris (1 mM) was added to provide the required pH 7.6 which causes more efficient curing (Rowbury and Goodson 1978). The mixture was incubated overnight at 37°C statically. Then the culture was diluted to 10^{-2}-10^{-9} and plated on selective medium and incubated at 37°C overnight. The antibiotic resistance was tested by replica plating on selective medium containing antibiotic, and the loss of plasmid

was checked by agarose gel electrophoresis.

II. 3. g. Swarming test:

Twenty ml of nutrient agar was poured into petri plates and after setting, these were dried in a 60°C oven for 10 minutes and inoculated centrally or marginally using a loopful of overnight culture. The plates were then incubated at 30°C for 24 hours. The swarming distance was measured from the inoculation point to the leading edge.

II. 3. h. Attachment to glass beads:

Strains were grown overnight in 20 ml of nutrient broth at 37°C static, and then spun down and resuspended in 1 ml 0. 065 M phosphate buffer. A 0. 3 ml sample of the suspension was added to a pipette containing 5 ml bed volume of glass beads(150-212 micron Sigma England) and plugged with 3-4mm glass beads, and another 0. 3 ml of the culture was transferred to a Hilger tube as control. The column was eluted with 3 ml of 0. 065M phosphate buffer and the effluent was collected in another Hilger tube. The volume of the control was adjusted to that of the sample by adding the same concentration of phosphate buffer. The optical density was measured by Hilger colorimeter using a 490 nm filter, and the attachment of the organism was worked out by the following equation:

$$\text{Adherence} = \frac{\text{O/D of the control} - \text{O/D of the sample} \times 100}{\text{O/D of the control}}$$

II.3.i. Attachment to glass coverslip:

The cells were grown in 50 ml nutrient broth at 37°C for 4 hours without shaking. Twenty ml of the culture was transferred into a sterile plate, and five coverslips (acid washed and rinsed with distilled water) were placed into the broth culture for 30 minutes. The coverslips were then gently washed with sterile distilled water to remove non adherent bacteria, then stained with Gram's crystal violet. The number of attached cells per field were counted under light microscope.

II.3.j. Adherence to epithelial cells:

The method of Harber was applied (Harber et al, 1985). Bacteria were grown in 20 ml N/B at 37°C for 5 hours static, they were then spun down and resuspended in phosphate buffer saline [PBS (KH_2PO_4, 3.45 g. Na_2HPO_4, 4.45 g. NaCl, 5 g. KCl, 0.2 g. D.W. 1000 ml) pH= 6.8] to give 10^8 cells/ml. The epithelial cells were collected from urine or tissue culture medium by centrifuging and then resuspended in PBS with the final concentration of 10^5 cells/ml. One ml of this suspension was added to 1 ml of bacterial suspension and incubated for 1 hour at 37°C with continuous rotation. The mixture was then filtered through a 5 µm Nucleopore polycarbonate membrane filter, and washed with 15 ml PBS. The filter was then pressed onto a glass microscope slide to give an imprint containing epithelial cells and bacteria. The filter was discarded and the slide was stained with 1% methylene blue for 1 minute and

examined under a phase contrast microscope.

II.3.k. Hydrophobicity:

Different methods have been proposed by several investigators to measure the hydrophobicity of bacteria such as bacterial adherence to hydrocarbons (BATH), salt aggregation (SA), latex particle agglutination (LPA) and adhesion to polystyrene (Dillon et al. 1986), direction of spreading DOS (Sar 1986), contact angle measurement (CAM) phase distribution (Mozes and Rouxhet 1987). The best method which was found to be quantitatively accurate and quick was hydrophobic interaction chromatography (HIC) method (Smyth et al 1978) and used with slight modifications.

Pasteur pipettes were plugged with glass beads size 3 mm and filled with octyl sepharose (CL Pharmacia, Sweden) to bed volume of 1 ml. The column was equilibrated with required concentration of ammonium sulphate (i.e. 0.01, 0.05, 0.10, 0.25, 0.50, and 1.0 M) in 0.01 M phosphate buffer (pH=7.2). Strains were grown O/N at 37°C without shaking, and spun down and resuspended in 0.01 M phosphate buffer to give 10^{9}-10^{10} cells/ml. A 100 µl sample of the suspension was then poured onto the sepharose, and eluted with the same concentration of ammonium sulphate to which it was equilibrated. The discharge of the elution was collected in a cuvette, and the hydrophobicity of the samples was observed by measuring absorbance at 600 nm by a spectrophotometer.

II.3.1. Measurement of growth:

II.3.1.1. Growth in nutrient broth.

One ml of overnight culture of bacteria was added to 20 ml nutrient broth in 50 ml screw cap bottles and incubated at 37°C static and shaking. Samples were taken at intervals (i.e. 1, 2, 3, 4, 5 hours, and O/N), and the optical density was measured at 550 nm by Hilger colorimeter.

II.3.1.2. Measurement of growth rate in iron depleted nutrient broth:

The method was basically the same as above but in this experiment iron was removed from the medium.

Elimination of iron: 10 g of sodium chelex resin (Sigma) and 30 g of nutrient broth powder were added to 100 ml of double distilled water. The mixture was stirred at room temperature for 6 hours, and dialysed in 900 ml deionized water at 4°C for 12 hours. The iron content of the dialysate was measured by atomic absorption spectrophotometer (PYE Unicam sp 90) and the standard curve was made with three following concentrations of ferric chloride:

	Con. of $FeCl_3$	Absorption
Standard 1	0.1 mg/l	0.001
" 2	0.5 mg/l	0.044
" 3	1.0 mg/l	0.088
Whole broth	x =3.6 mg/l	0.316
Dialysate	x'=0.5 mg/l	0.045

II. 3. 1. 3. Growth in the presence of detergents:

Appropriate concentrations (50, 100, 150, and 200 µg /ml) of detergent (eg. SDS) were incorporated into nutrient broth. One ml of overnight culture was added to 20 ml of this medium in 50 ml conical flasks and incubated at 37°C. Samples were taken every 2 hours and the optical density was checked using Hilger colorimeter at 490 nm.

II. 3. m. Measurement of viable count:

The number of viable bacteria was calculated by using the Miles and Misra method with a small modification. The cells were serially diluted in saline in 10 fold dilution. A 0.02 ml sample of appropriate dilution was pipetted onto the surface of a plate containing selective medium (e.g., C. L. E. D. for Proteus spp) from an approximate height of 2.5 cm. The plates had already been dried in a 60°C oven for 10 min, to allow them subsequently to absorb the drops of the sample. Several drops of each dilution were placed on the plate which was marked for that dilution. To determine the number of viable cells/ ml of the culture, the total number of colonies on the plate of each dilution was divided by the number of the drops and multiplied by 50, then multiplied by dilution.

II. 3. n. Measurement of optical density:

The optical density of the cultures was estimated with a Hilger photoelectric colorimeter using 550 nm filter for N/B cultures and 490 nm for phosphate buffer.

II. 3. o. Clump formation test:

The bacteria were grown overnight at 37°C in static conditions. The culture was shaken gently to give a homogeneous suspension, and 100 µl was placed between a haemocytometer slide and a glass coverslip. The clumps were observed under phase contrast microscope (P. C. M.), and the number of clumps were counted in five squares then multiplied by 10^4 to find the number of clumps per ml of the culture.

II. 3. p. Motility test:

One drop of O/N culture was placed on a glass slide and covered with a glass coverslip, then checked for motility under P. C. M. The rate of motility was measured by attaching a plastic sheet (divided into 1 cm squares) on the video screen of a videorecorder microscope, and the distance travelled by the bacteria was measured.

The percentage of motility was also checked by counting the motile bacteria out of at least 100 cells.

II. 3. q. Effects of detergent on clump formation and motility:

Different concentrations of detergent were added to nutrient broth, and bacteria were grown overnight static. The clumps were observed under (PCM) using haemocytometer slide, and the percentage of motility was examined as described earlier.

II. 3. r. Inhibition of swarming:

Most of the Proteus strains are swarmers on the surface of ordinary media (eg. nutrient agar) and always cause problems in separating mixed cultures in diagnostic laboratories or in experiments which need to have single colonies. CLED is a selective medium to prevent swarming, but because of the blue colour, the colonies are not clearly visible. Also it can not be used in the enzyme production experiments particularly in Haemolysin test which requires salt for the protection of red blood cells. There are number of other methods to stop Proteus swarming. A mechanical way is to grow the organism on the high concentration (6%) of agar. Moreover a wide range of chemicals such as: Sodium azide, Boric acid, Bile salts (Smith 1972), paranitrophenyl glycerol PNPG (Senior 1977b), calcium ions (Cole and Smith 1981), etc, are used to inhibit swarming (Table 1). The best one which was found to be effective was 0.2% Boric acid in nutrient agar.

II.3.s. The inhibitory effect of urea and its analogues on swarming:

Different concentrations of urea or other chemicals were incorporated into nutrient agar and precisely 20 ml was poured into each plate. The plates were then dried in 60°C oven for 10 minutes, and inoculated marginally with sterile wireloop and incubated at 30°C for 24 hours. The swarming inhibition was observed by measuring and comparing the swarming distance of the strains in different concentrations of the chemical.

II.3.t. Checking effects of urea on flagellation:

A. Flagellar staining. The two following methods were used:

1. Kodaka's staining, which ivolves the use of two solutions.

Solution 1. 5% phenol, 10 ml. Tannic acid, 2 g. Saturated aluminium potassium sulphate(SAPS), 10 ml.

Solution 2. Saturated crystal violet in ethanol (120 g/l). Both solutions were filtered and kept in the dark.

A thin smear of bacterial suspension in distilled water was prepared and dried at room temprature. Ten parts of solution 1 were mixed with one part of solution 2, and applied on the slide for 1 minute, then washed with water and blotted gently. The flagella were observed under phase contrast microscope using immersion lens.

2. Silver nitrate staining. In this method also two reagents were used, mordant and stain.

Mordant:	Saturated aluminium sulphate	5 ml
	10% Tannic acid	10 ml
	5% Ferric chloride	1 ml

The solutions were mixed and kept at 5°C.

Stain:	5% silver nitrate ($AgNO_3$)	100 ml
	880 (s,g) ammonia (NH_3)	2-4 ml

Ammonia was added to 80 ml of silver nitrate dropwise, and with the first few drops a brown precipitate appeared. The addition of ammonia continued until the precipitate

disappeared. Then more ammonia was added until a faint cloudiness persisted. The stain was covered with aluminium foil and kept at 5°C. The preparation of the slide was as above, then a few drops of mordant was poured on the slide and left for 4 minutes. It was then washed gently with water and covered with silver stain (which was previously heated in boiling tube) and left for 5 minutes. The slide was washed and blotted gently and the excessive stain was removed, then examined under (PCM) using X100 lens.

Comparatively, flagella were much more visible and clear in silver staining than that of Kodaka's method, therefore it was applied for the relevant experiments.

B. To find the effect of urea on flagellation of Proteus strains, nutrient agar plates were prepared containing different concentrations of urea. The plates were inoculated marginally and incubated at 30°C for 18 hours, and the flagellation was checked by comparing at least 10 cells in each concentration.

II.3.u. Checking effects of urea on long cell formation:

The preparation of the medium and inoculation was the same as above, but after incubation smears were made from different concentrations and stained with Gram crystal violet. The slides were examined under PCM and the length of at least 20 cells was measured using graticule lens and the mean was taken as the result of the experiment.

II. 3. v. Haemolysin assay:

The haemolytic activity of plasmid-bearing and plasmid-free strains of P. mirabilis was assayed by adding 70 ml of defibrinated horse or sheep blood and the proper concentration of a swarming inhibitor such as, paranitrophenolglycerin, bile salt or boric acid(in this experiment 0.02% of boric acid was used) to 1000 ml presterilised nutrient agar or blood agar base (Oxoid) at 50°C. A 25 ml volume was poured into petri plates and after setting these were dried in 60°C oven for 10 mins. Samples were taken from single colonies on nutrient agar plate using a sterile wireloop and streaked on blood agar plates. The plates then were incubated at 37°C for 24 and 48 hours. The haemolysin production of the strains was checked by measuring the average size of the clear zones around at least 10 single colonies.

To demonstrate and quantify haemolysin in bacterial supernatant, 1ml of overnight culture was spun down in an eppendorf tube for 10 mins. Wells were dug aseptically in the blood agar and 100 µl of supernatant containing 0.3% sodium azide as a growth inhibitor was poured into the wells. The plates were incubated at 37°C for 24 and 48 hours, and the size of the clear zones surrounding the wells indicated the haemolytic activity of the strains.

II. 3. w. Protease assay:

One hundred ml of sterilised skimmed milk and 0.02% of

boric acid was added to 900 ml of nutrient agar at 50°C and 25 ml was poured into each plate. The production of protease by bacteria and its presence in the supernatant was observed as described for haemolysin.

II. 3. x. Lecithinase production test:

Fifty ml of Egg yolk emulsion (Oxoid) was added to 950 ml of nutrient agar containing 0.02% boric acid, and 25 ml was poured into the plates. After setting and drying the production of lecithinase by the bacterial colony and supernatant was checked as explained in haemolysin assay.

II. 3. y. Amylase secretion assay:

Twenty five ml of nutrient agar containing 1% soluble starch was poured into petri plates and after setting the production of amylase was observed by measuring the size of clear zones surrounding the colonies and the wells.

II. 3. z. DNase production test:

To detect the DNase activity of the bacteria 39 g of DNase agar (Oxoid) and 0.02% boric acid (swarming inhibitor) w suspended in 1000 ml distilled water and after autoclaving 25 ml was poured into the plates. The plates were then dried and inoculated with a wireloop by diluting method to give single colonies. Also, wells were dug in the agar and 100 µl of bacterial spernatant was poured into the wells, and both were incubated at 37°C for 24 and 48 hours. After incubation the agar was flooded with 5 ml of 1M hydrochloric acid. The

acid reacts with DNA of the medium and forms a type of cloudy precipitate. DNase producing colonies and supernatants were surrounded by a clear area containing nucleotide fractions which are the result of DNase activity of the organism.

II.3.a'. Qualitative test for production of urease:

Twenty four g of urea base agar was added to 950 ml distilled water, autoclaved and cooled down to 50°C. Then 50 ml of 40% filtered urea solution was added and 10 ml was poured into sterilised universals and the agar was set as slopes. Samples were taken from single colonies on nutrient agar by wireloop and streaked on the slopes and incubated at 37°C for 24 hours. The production of urease was indicated by the appearance of pink colour in the medium.

II.3.b'. Comparative assay for urease secretion on solid medium:

The preparation of the medium was the same as above but, after cooling 20 ml of the agar was poured into petri plates. Then after setting the plates were dried in 60°C oven, inoculated centrally and incubated at 37°C. The progressive appearance of pink zones around the inoculation point was measured at intervals e.g. (0, 2, 4, 6 hours and overnight).

II.3.c'. Production of urease in liquid medium:

Twenty five g of nutrient broth No.2 was added to 950 ml distilled water and autoclaved. The medium was then cooled down to 50°C and 50 ml of 40% prefiltered urea solution was

added to it, and distributed in 20 ml volumes into 50 ml sterilised conical flasks. The overnight cultures of the strains were brought to the same optical density by adding sterile nutrient broth and 1 ml was added to the flasks. The flasks were then incubated in a 37°C shaking water bath, and samples were taken at time intervals as in the above experiment, and the increased pH of the medium was observed.

II.3.d'. Survival in human serum:

The bacteria were grown overnight and brought to the same optical density. A 1 ml sample was added to 20 ml of serum which had already been diluted down to 30% by adding 0.01 M phosphate buffer in 50 ml conical flasks. The flasks then were incubated at 37°C without shaking. Samples were taken at intervals (e.g. 0, 2, 4, 6, hours and O/N), and plated on selective medium (CLED) using the method of Miles and Misra for viable count.

II.3.e'. Survival in sewage effluent and river water:

The overnight culture was brought to the same optical density and 1 ml was added to 20 ml of effluent or river water in 50 ml conical flasks and incubated in a 37°C shaking water bath for 5 days. Samples were taken every day and plated on selective medium (PIM or CLED) for viable countusing the Miles and Misra method.

II.3.f' Survival in sewage :

The method was similar to that with effluent, except that

prior to incubation, the sewage was diluted 4 times by adding distilled water and used without sterilization.

II.3.g'. Transmission electron microscopy (TEM):

Preparation of sample for negative staining. Bacteria were grown in nutrient broth at 37°C without shaking for 4-5 hours. The cells were spun down (3000r/min) and resuspended gently in the same volume of prefiltered distilled water. One drop of suspension was placed on a carbon coated copper grid, and after 30 seconds it was blotted with filter paper. Then one drop of prefiltered solution of 10% potassium phosphotungstate was put on the grid and after 30 seconds excessive stain was blotted and the organisms were observed under a JEOL electron microscope.

RESULTS

III. Results.

In this study, 137 clinical strains of P. mirabilis collected from patients with urinary tract infection and 8 faecal strains have been examined.

III. 1. Identification.

Initially 145 strains were tested for identification using the API20E method. Eight strains of urinary isolates were identified as P. vulgaris, Providencia stuartii and Morganella morganii, but the 137 others were P. mirabilis, which were later used for the experiments. The faecal strains were all P. mirabilis and marked as RM1-RM6, RM8 and P33. All faecal and urinary strains of P. mirabils (which are listed in Table 5) were urease positive by testing on Christensen's urea agar slopes inoculated at 37°C for 24 hours (qualitative test).

III. 2. Plasmid extraction.

All 145 strains of P. mirabilis were examined for plasmids by extraction using an alkaline lysis method (Kado and Liu 1981). The experiment was conducted at least twice for each strain and the results are shown in Table 5. A total of 44 strains were carrying plasmids of which 32 isolates contained one, and the rest had two or more plasmids. Four of the faecal strains (RM1, RM2, RM3 and RM5) were plasmid-positive. Some of the plasmids carried in strains which were used in further experiments were sized by running along with plasmids of known molecular weight, and the results are shown in Table 6.

Table 5. Antibiotic resistance and swarming distance (in 18 h) of plasmid-bearing and plasmid-free strains of P. mirabilis.

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
PM1	-	3.8	S	S	S	S	S	R	S	S	R
PM2	-	3.4	S	S	S	S	S	S	S	S	S
PM3	-	4.1	S	S	S	S	S	S	S	S	S
PM4	1	3.2	S	R	S	R	S	S	S	S	R
PM5	1	0	R	R	S	S	R	S	S	R	R
PM6	-	4.0	S	S	S	S	S	S	S	S	S
PM7	-	0	S	S	S	S	S	S	S	S	R
PM8	-	1.6	S	S	S	S	S	S	S	S	S
PM9	-	2.8	S	S	S	S	S	S	S	S	R
PM10	-	2.2	S	S	S	S	S	S	S	S	R
PM11	-	0	S	S	S	S	S	R	S	S	S
PM12	-	3.2	S	S	S	S	S	S	S	S	R
PM13	1	0.8	S	S	S	S	S	S	S	S	R
PM14	1	0.6	R	S	S	S	S	S	R	S	R
PM15	1	0	S	S	S	S	S	S	S	S	S
G7	2	4.3	S	S	S	S	S	S	R	S	R
G8	2	0	S	R	S	S	S	S	S	S	R
G9	-	7.8	S	S	S	S	S	R	S	S	R
G10	-	5.3	S	S	S	S	S	R	S	S	R
G11	2s	6.8	S	S	S	S	S	S	S	S	S
G12	1	1.1	R	S	S	S	S	R	R	R	R
G13	-	4.8	S	S	S	S	S	S	S	S	R

Table 5 (continued)

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
G14	–	3.5	R	R	S	S	S	S	S	S	R
G15	1	0.9	R	R	S	S	S	S	S	S	R
G16	–	6.7	S	S	S	S	S	S	S	S	S
G17	1	3.3	S	R	S	S	S	R	S	S	R
G18	–	4.3	S	S	S	S	S	R	S	S	R
G19	–	4.7	S	S	S	S	S	S	S	S	S
G20	–	5.2	S	S	S	S	S	S	S	S	S
G21	–	3.6	S	S	S	S	S	S	S	S	S
G22	–	4.4	R	S	S	S	S	S	S	S	S
G23	–	3.8	R	S	S	S	S	S	S	S	S
G24	1	1.3	S	S	S	S	S	R	S	S	R
G25	–	2.9	S	S	S	S	S	S	S	S	S
G26	–	5.5	R	S	S	S	S	S	S	S	S
G27	–	5.3	S	S	S	S	S	S	S	S	S
G28	–	3.7	S	S	S	S	S	S	S	S	S
G29	–	4.6	S	S	S	S	S	S	S	S	S
G30	1	2.8	S	R	S	S	S	S	S	S	R
G31	–	6.1	R	S	S	S	S	S	S	S	S
G32	2	4.6	S	R	R	R	S	S	S	R	R
G33	–	3.0	S	R	S	S	S	S	S	S	S
G34	–	5.2	S	S	S	S	S	S	S	S	S
G35	–	3.9	S	S	S	S	S	S	S	S	S
G36	–	4.8	S	S	S	S	S	S	S	S	S

Table 5 (continued)

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
G37	1	1.7	R	S	S	S	S	S	S	S	S
G38	-	0.9	S	S	S	S	S	S	S	S	S
G39	-	7.4	S	S	S	S	S	S	S	S	S
G40	-	6.7	S	S	S	S	S	S	S	S	S
G41	-	4.3	S	S	S	S	S	S	S	S	S
G42	1	1.2	S	R	S	S	S	R	S	S	R
G43	-	4.5	S	S	S	S	S	S	S	S	R
P4	2	0	S	R	S	S	S	S	S	S	R
P8	1	3.1	S	S	S	S	S	S	S	S	S
P33	-	5.2	S	S	S	S	S	S	S	R	S
P49	1	0	R	R	S	S	S	S	S	S	R
P162	1	0	S	S	S	S	S	S	R	S	S
P200	2s	6.1	S	S	S	S	S	S	S	S	S
P263	2	2.6	S	R	S	S	S	S	S	S	S
P405	-	4.1	S	S	S	S	S	S	S	S	S
P406	-	5.5	S	S	S	S	S	S	S	S	S
P407	-	4.7	R	R	S	S	S	S	S	S	S
P538	-	5.1	S	S	S	S	S	S	S	S	S
P920	-	3.3	S	S	S	S	S	S	R	S	R
P991	1	0	R	R	S	R	S	R	R	R	R
41	1	0.8	S	S	S	S	S	R	S	S	R
77	-	3.4	S	S	S	S	S	S	S	S	S

Table 5 (continued)

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
82	-	6.1	S	S	S	S	S	S	S	S	S
89	1	1.3	S	R	S	S	S	R	S	S	R
112	-	6.7	S	S	S	S	S	S	S	S	S
115	1	1.2	S	R	S	S	S	S	S	S	R
119	-	4.3	S	S	S	S	S	S	S	S	S
128	-	3.9	S	S	S	S	S	S	S	S	R
132	-	4.4	S	S	S	S	S	S	S	S	S
133	-	4.8	S	S	S	S	S	S	S	S	S
142	-	2.6	S	S	S	S	S	S	S	R	S
155	-	7.2	S	S	S	S	S	S	S	S	S
166	3s	4.8	S	S	S	S	S	S	S	S	S
169	-	3.2	S	S	S	S	S	S	S	S	S
180	2	4.2	S	R	S	S	S	S	S	S	R
192	-	1.8	S	R	S	S	S	R	S	S	R
196	-	4.7	S	S	S	S	S	S	S	S	S
202	-	5.3	S	S	S	S	S	S	S	S	S
226	1	3.1	S	S	S	S	S	R	S	R	S
250	-	5.3	R	S	S	S	S	S	S	S	S
254	-	2.9	S	S	S	S	S	R	S	S	S
263	1	0.5	S	S	S	S	S	S	S	S	S
274	2	0	R	R	S	R	S	R	R	R	R
296	-	3.2	S	S	S	S	S	S	S	S	R

Table 5 (continued)

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
314	–	5.2	S	S	S	S	S	S	S	S	S
315	–	3.7	R	S	S	S	S	S	S	S	S
322	5s	3.7	S	S	S	S	S	S	S	S	S
327	–	2.8	S	S	S	S	S	S	S	S	S
351	–	5.6	S	S	S	S	S	S	S	S	S
401	–	2.9	S	S	S	S	S	S	S	S	S
434	–	5.4	S	S	S	S	S	S	S	S	S
436	–	3.9	S	S	S	S	S	S	S	S	S
443	1	0	S	R	S	S	S	S	S	S	R
467	–	3.9	S	S	S	S	S	S	S	S	S
481	–	4.8	S	S	S	S	S	S	S	S	S
504	–	6.0	S	S	S	S	S	S	S	S	S
507	–	4.7	S	S	S	S	S	S	S	S	R
509	–	4.8	S	S	S	S	S	S	S	S	S
510	–	1.7	S	S	S	S	S	R	S	S	R
512	1	1.2	S	S	S	S	S	R	S	S	R
522	–	3.4	S	S	S	S	S	S	S	S	R
531	1	2.3	S	S	S	S	S	S	S	S	R
545	1	4.1	S	S	S	S	S	S	S	S	S
568	–	4.0	S	S	S	S	S	S	S	S	R
580	2	6.1	R	R	S	S	S	S	S	S	S
626	–	2.7	S	S	S	S	S	S	S	S	R

Table 5 (continued)

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
653	-	3.3	S	S	S	S	S	S	S	S	S
673	-	4.1	S	S	S	S	S	R	S	S	S
704	-	0.8	S	S	S	S	S	S	S	S	S
705	-	4.3	S	S	S	S	S	S	S	S	S
710	1	0.6	S	S	S	S	S	S	S	S	S
717	-	6.1	S	S	S	S	S	S	S	S	S
719	-	4.1	S	S	S	S	S	S	S	S	S
741	-	7.3	S	S	S	S	S	S	S	S	S
783	-	3.8	S	S	S	S	S	S	S	S	S
789	-	4.5	S	S	S	S	S	S	S	S	S
827	-	6.1	S	S	S	S	S	S	S	S	S
844	-	5.1	S	S	S	S	S	S	S	S	R
864	-	1.9	S	S	S	S	S	R	S	S	S
873	-	7.2	S	S	S	S	S	S	S	S	S
912	-	0	S	S	S	S	S	S	S	S	S
914	-	3.0	S	S	S	S	S	S	S	S	R
925	-	4.5	S	S	S	S	S	S	S	S	S
928	-	5.1	S	S	S	S	S	S	S	S	S
946	-	5.7	S	S	S	S	S	S	S	S	S
948	-	1.8	S	S	S	S	S	S	S	S	S
954	-	5.4	S	S	S	S	S	S	S	S	S
956	-	5.3	S	S	S	S	S	S	S	S	S

Table 5 (continued)

Strains	Number of plasmids	Swarming distance in cm	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
979	-	2.6	S	S	S	S	S	S	S	S	S
985	-	5.1	S	S	S	S	S	S	S	S	S
997	-	4.5	S	S	S	S	S	S	S	S	S
RM1	1	2.5	S	S	S	R	S	S	S	R	S
RM2	1	1.2	S	S	S	R	S	R	S	R	S
RM3	1	2.1	S	S	S	S	S	S	S	R	S
RM4	-	4.0	S	S	S	S	S	S	S	S	S
RM5	1	2.1	S	S	S	S	S	S	S	R	S
RM6	-	4.5	S	S	S	S	S	S	S	S	S
RM8	-	4.2	S	S	S	S	S	S	S	S	S

S = Small

Table 6.

The size of some of the plasmids from strains of *P. mirabilis*.

Strain	plasmid	No. of plasmid	size of plasmid
PM4	pPM4	1	42 Md
PM5	pPM5	1	105 Md
PM14	pPM14	1	68 Md
P49	pP49	1	32 Md
P991	pP991	1	78 Md

Table 7. List of antibiotics used in the experiments.

Antibiotics	Abbreviations	Concentrations
Ampicillin	Amp	25 ug
Cephaloridine	Cep	25 "
Chloramphenicol	Cap	50 "
Colistine sulphate	CT	10 " (polymyxin B)
Co-trimoxazole	Sxt	25 "
Gentamicin	Cn	10 "
Kanamycin	Kan	30 "
Nalidixic acid	Nal	30 "
Nitrofurantoin	Nit	200 "
Streptomycin	Str	25 "
Sulphafurazole	Suf	500 "
Tetracycline	Tet	50 "

III.3 Antibiotic resistance:

The antibiotic sensitivity of strains of P. mirabilis was tested against different antibiotics which are listed in Table 7, with stated concentrations. All strains were resistant against sulphafurazole and colistin sulphate, and sensitive to gentamicin, therefore the results with these are not shown in Table 5. Among the other antibiotics the greatest incidence of resistance was exhibited against ampicillin and the lowest to nalidixic acid and nitrofurantoin. The involvement of plasmids in antibiotic resistance of P. mirabilis is clearly demonstrated. More than 75% of plasmid-bearing strains were resistant to one or more antibiotics and the mean number of resistance per p+ strain was 1.91, whereas only 35% of plasmid-free strains showed resistance with the mean number of resistance being 0.45 and most of the p- strains that did show resistance to the antibiotics of Table 5 had only a single resistance. A comparative abstract of the resistance figures of Table 5 is given in Table 8.

Most of plasmid-bearing strains were multi-resistant and only 27% showed single resistance. Strain P.991 which carries a 78 Md plasmid exhibited a seven antibiotic resistance pattern and strain PM.5 containing a 105 Md plasmid DNA was resistant to five broad spectrum antibiotics. Interestingly, all of the p- strains were sensitive to kanamycin and only one of them showed resistance to

Table No. 8. Distribution of antibiotic resistance in p- and p+ strains of *P. mirabilis*.

	Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Am
Strains resistant.	17	22	2	6	2	21	7	12	48
% of resistance.	11.7	14.5	0.7	4.1	0.7	14.5	4.8	8.3	33
p+ Strains resistant.	9	18	1	6	1	11	6	10	26
% of p+ resistance.	21	42	2.3	14	2.3	26	14	23	60
p- Strains resistant.	8	4	1	0	1	10	1	2	22
% of p- resistance.	8	4	1	0	1	10	1	2	22

streptomycin. Resistance to co-trimoxazole and cephaloridin was also very low among p- isolates and although the highest resistance was shown towards ampicillin, all of the faecal strains (p+ and p-) were susceptible to this antibiotic.

III. 4. Conjugation.

To find out whether the plasmids present in p+ strains of P. mirabilis are associated with antibiotic resistance as well as other virulence properties of this organism, conjugation was carried out. These plasmids had to be transferred to a p- strain (recipient) which was sensitive to those antibiotics to which the donor was resistant. Therefore in the first experiment the recipient was E. coli K12 F- Lac^+ Nal^r, strain number 14R525 (Threlfall et al 1986) and here is marked as M2 which was obtained from the Drug Resistance Section of the Central Public Health Laboratory, Colindale. The strains, G12, PM13, PM14 and P49 of P. mirabilis were taken as donors. The experiment was carried out three times and conjugation with strains G. 12 and P49 was successful but not with PM13 and PM14. Antibiotic sensitivity of the transconjugants was tested and the results are shown in Table 9. The transconjugant M2pG12 gained resistance to Amp, Cap and Sxt whilst strain M2pP49 was rendered resistant to Amp, Tet and Cep. The transfer of the plasmids was confirmed by agarose gel electrophoresis (AGE) of the transconjugants.

In another experiment, the conjugation was carried out

Table 9. The transfer of plasmids from strains of *P. mirabils* to *E. coli K12* (M2) strain.

Strains	Plasmid	Antibiotic sensitivity								
		Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
G12 (Don)	pG12	R	S	S	S	S	R	R	R	R
P49 "	pP49	R	R	S	S	S	S	S	S	R
M2 (Rec)	-	S	S	S	S	R	S	S	S	S
M2pG12	pG12	S	S	S	S	R	R	S	R	R
M2pP49	pP49	R	R	S	S	R	S	S	S	R

Rec= Recipient Don= Donor

between pairs of strains of P. mirabilis. For these tests plasmid-free strains were used as recipients. Thus strains G9 and G10 which were resistant to chloramphenicol were used as recipients and four multi-resistant isolates PM4, PM5, PM14 and P49 carrying plasmids of molecular weights 42, 105, 68 and 32 megadalton respectively, and all sensitive to Cap were used as donors and the experiment was carried out several times. Conjugation did not seem to work with strain G10 as recipient or with strain PM14 as donor, but transconjugants were obtained with G9 and three other donors. Thus as shown in Table 10, the transconjugants G9pPM4, G9pPM5 and G9pP49 gained antibiotic resistance. The presence of plasmids in the transconjugants was confirmed by agarose gel electrophoresis, indicating that plasmid pPM4 carries resistance determinants to Cep and Kan, plasmid pPM5 to Tet, Cep and Sxt, and plasmid pP49 confers resistance to Tet and Cep. As the strain G9 was intrinsically resistant to ampicillin it was not possible to find out whether pPM4, pPM5 or pP49 carry resistance to this antibiotic but in the previous experiment with E. coli (M2) as recipient it was shown that Amp resistance was transferred to transconjugant M2pP49 by the plasmid.

III.5. Curing of plasmid-bearing strains of P. mirabilis:

Some plasmid-containing strains were cured by acridine orange using the Hirota (1960) method as modified by Rowbury and Goodson (1978). The aim was then to compare the p+ and p-

Table 10.

Conjugation between p- and p+ strains of *P. mirabilis*.

Strains	Plasmid	Plasmid size	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
PM4 (Don)	pPM4	42 md	S	R	S	R	S	S	S	S	R
PM5 "	pPM5	105 "	R	R	S	S	R	S	S	R	R
P49 "	pP49	32 "	R	R	S	S	S	S	S	S	R
G9 (Rec)	-	-	S	S	S	S	S	R	S	S	R
G9pPM4	pPM4	42 "	S	R	S	R	S	R	S	S	R
G9pPM5	pPM5	105 "	R	R	S	S	S	R	S	R	R
G9pP49	pP49	32 "	R	R	S	S	S	R	S	S	R

Rec=Recipient Don=Donor

strains with respect to potential virulence properties. Five multi-resistant isolates carrying one plasmid were selected for this purpose which are as follows, PM5, PM14, G12, P49 and P991. Three of these strains were cured after several experiments but the other two were not. The antibiotic sensitivities of the cured strains were then checked (Fig8). The results are shown in Table 11 and the loss of plasmid from cured derivatives was confirmed by agarose gel electrophoresis. As is shown, the loss of plasmid pPM5 rendered its host strain PM5c sensitive to tetracycline, cephaloridin and ampicillin. Interestingly, the cured derivative was still resistant to co-trimoxazol whereas this resistance was also transferrable by conjugation. This may indicate that resistance determinants to Sxt were carried by both chromosome and plasmid in PM5 strain. As was expected, curing of plasmid pP49 was associated with loss of resistance to Amp, Tet and Cep, which confirmed the results obtained from conjugation. Also loss of plasmid pP991 rendered its host sensitive to all antibiotics to which the parent was resistant except tetracycline.

III.6. Effect of plasmids on the swarming property of P. mirabilis:

The swarming ability of all 145 strains of P. mirabilis was tested and the results are shown in Table 5. A comparison between the swarming zone sizes of plasmid-free and plasmid-

Table 11. Antibiotic sensitivity of cured strains of *P. mirabilis*.

Strains	Plasmid	plasmid size	Antibiotic sensitivity								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
PM5	pPM5	105 md	R	R	S	S	R	S	S	R	R
PM5c	-	-	S	S	S	S	R	S	S	R	S
P49	pP49	32 "	R	R	S	S	S	S	S	S	R
P49c	-	-	S	S	S	S	S	S	S	S	S
P991	pP991	78 "	R	R	S	R	S	R	R	R	R
P991c	-	-	R	S	S	S	S	S	S	S	S

The experiment was carried out several times and the loss of plasmid was confirmed by gel electrophoresis. C = Cured

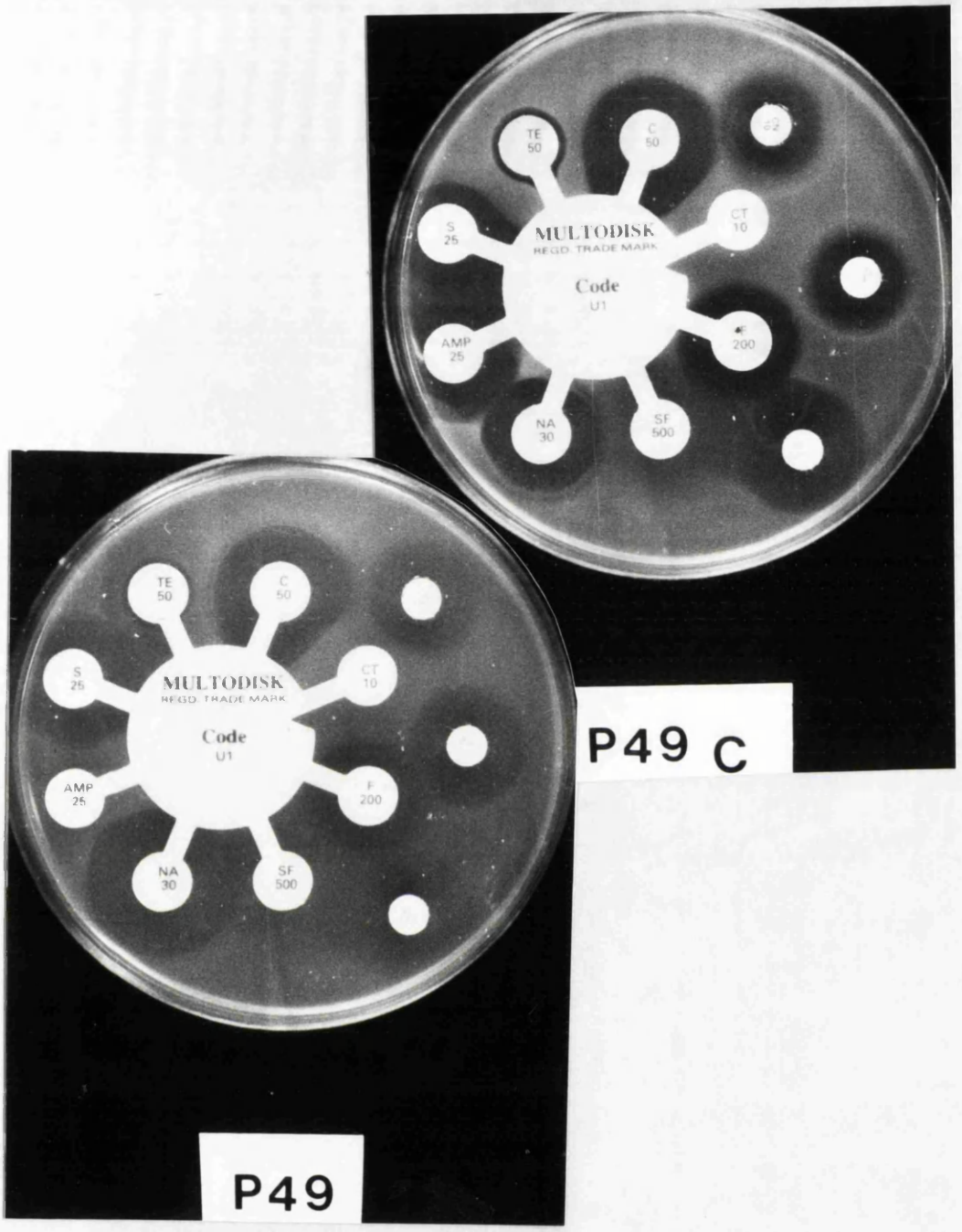

Figure 8. Antibiotic resistance of P. mirabilis strain P49 and its cured derivative.

Amp= Ampicillin. C= Chloramphenicol. CN= Gentamycin.

CT= Polymyxin B. F= Nitrofurantoin. K= Kanamycin.

NA= Nalidixic acid. S= Streptomycin. SF= Sulphafurazole.

SXT= Co-trimoxazole. TE= Tetracycline.

positive strains showed what appeared to be significant effects of plasmids on this property. Many plasmid-bearing isolates failed to swarm or exhibited very small swarming distances and the mean swarming zone size was 2.28 cm. In contrast almost all the highly swarming strains were plasmid-less with the mean size of swarming zones 4.17. Strikingly, when three non-swarming strains (PM5, P49 and P991) were cured they started swarming; and when the high swarmer strain G.9 took up plasmids by conjugation its swarming distance was significantly reduced (Table 12). The size of the inoculum did not seem to affect the swarming distance.

III.7. The inhibitory effect of urea on swarming of P. mirabilis strains:

The effect of urea on the swarming property of P. mirabilis was observed by incorporating different concentrations into the nutrient agar plates. For this purpose 16 highly swarming strains were selected and the effect of this agent was tested. As is indicated from the results in Table 13, the effect was concentration dependent; at the concentration of 3.5% of urea none of the strains were able to swarm, and some of them like strain G9 even stopped swarming at 1.5% concentration of urea.

III.7.a. Swarming inhibition by urea+urease inhibitor:

To find out whether the inhibitory effect was due to urea itself or as a result of urease activity of the bacteria, a

Table 12.

Effects of plasmids on swarming of *P. mirabilis* strains.

Strains	Plasmid	Size of plasmid	swarming zone size
PM4	pPM4	42 Md	3.2 Cm
PM5	pPM5	105 "	0
PM5c	none	N. A.	5.1 "
P49	pP49	32 "	0
P49c	none	N. A.	5.3 "
P991	pP991	78 "	0
P991c	none	N. A.	4.2 "
G9	none	N. A.	7.8 "
G9pPM4	pPM4	42 "	4.0 "
G9pPM5	pPM5	105 "	3.2 "
G9pP49	pP49	32 "	4.6

C = Cured N.A. = Not applicable

Table 13. The inhibitory effect of urea on swarming of *P. mirabilis* strains.

Strains	Concentrations of urea in nutrient agar (%)							
	0.0	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PM1	5.8	1.7	0.8	0.5	0.2	0.1	0	0
PM2	5.2	1.5	1.0	0.6	0.3	0.1	0	0
PM5c	3.9	1.1	0.5	0.2	0	0	0	0
P49c	3.6	0.9	0.4	0.1	0	0	0	0
P991c	4.1	1.1	0.6	0.2	0	0	0	0
G9	4.8	0.3	0	0	0	0	0	0
G13	5.9	2.0	1.3	0.9	0.6	0.3	0	0
G16	2.8	0.8	0.4	0.4	0.1	0	0	0
P33	6.1	1.9	1.2	0.8	0.3	0.1	0	0
133	5.7	1.2	0.7	0.4	0.2	0	0	0
196	5.4	0.8	0.6	0.3	0.1	0	0	0
202	3.9	1.2	0.9	0.6	0.3	0.1	0	0
250	5.2	1.6	0.8	0.2	0	0	0	0
P405	5.3	1.5	0.9	0.7	0.4	0.1	0	0
P406	6.2	1.9	1.2	0.8	0.5	0.2	0	0
705	5.8	1.3	0.8	0.5	0.3	0.1	0	0

The experiment was conducted several times and the results were consistent. Figures indicate the swarming distances of the strains in centimeters.

urease inhibitor, acetohydroxamic acid (25 mM), was added. As is shown in Table 14 on this medium almost all strains failed to swarm at 3% urea and even at the concentration of 2.5% only a few strains demonstrated weak swarming ability. Therefore, the elimination of urease in the presence of inhibitor led to a slightly increased antiswarming effect of urea indicating that it is probably urea itself rather than a urease-produced product which inhibited swarming. In another experiment the effect of urease inhibitor alone (acetohydroxamic acid 25 mM) on swarming was studied and the results obtained showed (Table 15) a slight reduction of swarming by this compound.

III.7.b. The effect of alkaline pH on swarming of P. mirabilis:

The above results suggested that urease activity was not necessary for the antiswarming effect of urea. But nonetheless, to check whether increased medium pH due to ammonia released from urea degradation was responsible for reduced swarming of bacteria, the pH value of nutrient agar plates (without urea) was increased up to 9 by sodium hydroxide. The plates were inoculated with the same strains and incubated at 30°C. The results showed (Table 16) that, although the increased pH had a slight effect on swarming, it could not account for the antiswarming ability of urea particularly in view of the increased inhibitory effect of urea in the absence of urease (Table 14).

Table 14. The inhibitory effect of urea+urease inhibitor (acetohydroxamic acid) on swarming of P. mirabilis.

Strains	Concentrations of urea in nutrient agar (%)							
	0.0	A.H.	1.0	1.5	2.0	2.5	3.0	3.5
PM1	6.0	4.9	1.8	0.9	0.3	0	0	0
PM2	5.2	4.1	1.4	0.8	0.5	0.2	0	0
PM5c	4.9	3.7	0.9	0.4	0.2	0	0	0
P49c	4.5	3.4	0.8	0.4	0.1	0	0	0
P991c	4.3	3.8	0.6	0.3	0	0	0	0
G9	4.2	3.3	1.2	0	0	0	0	0
G13	5.9	4.8	1.6	0.9	0.5	0.2	0	0
G16	2.3	2.2	0.5	0.2	0	0	0	0
P33	5.8	4.6	1.6	1.3	0.7	0.3	0	0
133	6.1	5.2	1.1	0.6	0.4	0.1	0	0
196	4.7	3.7	0.7	0.5	0.2	0	0	0
202	4.2	3.5	0.9	0.4	0.2	0	0	0
250	4.9	4.3	1.6	0.8	0.5	0.2	0	0
P405	5.7	4.1	1.4	0.9	0.4	0.3	0.1	0
P406	6.2	5.6	1.8	1.2	0.7	0.4	0.1	0
705	5.8	3.9	1.5	0.9	0.5	0.2	0	0

The experiment was conducted five times and the results were consistent.

Figures are swarming zone sizes of the strains in centimeters.

A.H. = Acetohydroxamic acid 25 mM.

Table 15. The effect of acetohydroxamic acid on the swarming ability of P. mirabilis strains.

Strains	Concentrations of acetohydroxamic acid in agar (in mM)						
	0.0	15	20	25	30	35	40
PM1	5.2	4.9	4.2	3.5	2.1	0.3	0
PM2	4.6	3.6	3.0	2.3	1.4	0.7	0.2
PM5c	3.4	2.8	2.1	1.2	0	0	0
P49c	3.2	2.2	1.8	0.6	0.3	0	0
P991c	4.2	3.1	2.4	1.9	0.9	0.3	0
G9	3.1	2.7	1.8	1.2	0.3	0	0
G13	6.2	5.3	3.6	1.7	0.6	0	0
G16	2.9	1.1	0.5	0	0	0	0
P33	6.4	4.5	3.8	2.6	1.4	0.9	0.4
133	4.9	3.8	2.6	1.9	1.3	0.8	0.5
196	4.6	3.7	2.8	2.2	1.6	0.7	0.6
202	3.5	2.4	2.1	1.5	1.2	1.2	0.9
250	4.4	2.9	1.6	0.9	0.5	0.4	0.2
P405	4.8	3.2	2.6	1.9	1.2	0.7	0.3
P406	4.7	4.6	3.9	2.9	2.1	1.6	1.2
705	3.8	2.7	1.9	1.1	0.5	0.3	0.2

The experiment was repeated four times and the results were consistent. Figures are the swarming zone size of the strains in centimeters.

Table 16. The effect of alkaline pH (pH, 9) on the swarming property of P. mirabilis strains.

Strains	The pH values of nutrient agar	
	7.5	9
PM1	6.2	3.8
PM2	5.8	3.1
PM5c	5.1	3.2
P49c	4.7	2.6
P991c	4.8	3.2
G9	5.2	4.1
G13	5.9	4.3
G16	2.9	1.9
P33	6.3	5.7
133	5.7	3.9
196	4.9	2.2
202	4.0	2.8
250	5.1	3.3
P405	5.5	3.4
P406	6.3	4.9
705	5.6	4.5

The experiment was repeated several times in duplicate and the results were almost consistent. Figures are the swarming distance of the strains in centimeters.

III.7.c. The effect of urea derivatives on swarming of P. mirabilis strains:

The inhibitory effect of several urea analogues on swarming was also examined to find out if there is any correlation between the structure and activity of these compounds, which are as follows:

1. Methylurea $CH_3-NH-CO-NH_2$
2. Thiourea $NH_2-CS-NH_2$
3. Methylthiourea $CH_3-NH-CS-NH_2$
4. 1-1, Dimethylurea $(CH_3)_2-N-CO-NH_2$
5. 1-3, Dimethylurea $CH_3-NH-CO-NH-CH_3$
6. S-Methylisothiourea $CH_3-S-C(=NH)NH_2$
7. O-Methylisourea $CH_3-O-C(=NH)NH_2$

1. Methylurea. This was the first compound of this group to be tested and the results showed (Table 17) that it is also capable of inhibiting the swarming property of P. mirabilis, but its effect was not as strong as urea itself. For example at the concentration of 3% of methylurea all strains tested still exhibited swarming and at the concentration of 1% it only reduced swarming distance of the strains by 38% whereas at this concentration, urea inhibited 74% of swarming ability of the strains.

2. Thiourea. The effect of thiourea was also examined and the results which are shown in Table 18 indicated that on the contrary to methylurea, thiourea was more effective than urea

Table 17. The inhibitory effect of methylurea on the swarming property of *P. mirabilis* strains.

Strains	Concentrations of methylurea in nutrient agar (%)							
	0.0	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PM1	5.9	2.9	2.5	1.7	1.3	0.9	0.7	0.3
PM2	5.6	3.1	2.7	1.6	1.1	0.8	0.4	0
PM5c	3.7	2.3	1.9	1.3	0.8	0.5	0.2	0
P49c	3.8	2.4	2.1	1.7	1.1	0.7	0.4	0
P991c	3.6	2.2	1.8	1.4	0.9	0.7	0.3	0
G9	4.1	2.7	2.1	1.2	0.8	0.6	0.4	0
G13	6.1	4.8	3.7	3.1	2.3	1.8	1.2	0.5
G16	3.4	1.9	1.6	1.1	0.7	0.4	0.1	0
P33	6.2	4.1	3.2	2.7	1.9	1.1	0.7	0
133	5.8	3.9	2.8	2.3	1.9	1.4	0.9	0.4
196	4.9	3.7	2.9	2.1	1.5	0.9	0.2	0
202	3.9	3.2	2.5	1.7	1.1	0.8	0.3	0
250	4.7	3.8	2.8	1.9	1.1	0.6	0.4	0
P405	5.2	4.9	4.2	3.1	2.6	1.8	0.8	0
P406	6.2	4.8	4.1	3.7	2.9	2.2	1.7	0.8
705	5.7	4.2	3.3	2.4	1.7	1.1	0.6	0.3

The experiment was repeated four times and the results were consistent. Figures are the swarming distance of the strains in centimeters.

Table 18. The inhibitory effect of thiourea on the swarming property of *P. mirabilis* strains.

Strains	Concentrations of thiourea in nutrient agar (%)								
	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PM1	5.4	1.1	0.7	0.2	0	0	0	N.G.	N.G.
PM2	5.2	0.7	0.3	0	0	0	0	0	N.G.
PM5c	4.2	0.5	0.1	0	0	0	0	N.G.	N.G.
P49c	3.8	0.6	0.1	0	0	0	0	N.G	N.G.
P991c	3.3	0.4	0	0	0	0	0	N.G.	N.G.
G9	4.3	0.5	0.2	0	0	0	0	0	N.G.
G13	5.6	1.2	0.8	0.1	0	0	0	0	N.G.
G16	3.5	0.5	0	0	0	0	0	0	N.G.
P33	5.9	1.2	0.7	0.2	0	0	0	N.G.	N.G.
133	5.7	0.7	0.4	0	0	0	0	N.G.	N.G.
196	4.4	0.8	0.3	0	0	0	0	N.G.	N.G.
202	4.1	0.3	0	0	0	0	0	N.G.	N.G.
250	4.2	0.6	0.1	0	0	0	0	0	N.G.
P405	4.5	0.8	0.2	0	0	0	0	0	N.G.
P406	6.2	1.8	0.9	0.3	0	0	0	0	N.G.
705	5.8	0.4	0.2	0	0	0	0	0	N.G.

The experiment was repeated five times and the results were consistent. Figures are the swarming zone size of the strains in centimeters. N.G. = No growth.

in inhibiting the swarming of P. mirabilis, so that a concentration of 1% caused 95% reduction in average swarming distance of the strains tested.

3. Methylthiourea, was another analogue of urea tested for the effect of different concentrations on swarming of P. mirabilis. The results showed (Table 19) that this chemical inhibited 87% of average swarming of the strains at the concentration of 1%, which was more effective than methylurea but weaker than thiourea. Complete inhibition of swarming in all strains occurred at 2% concentration and at 4% none of the strains were able to grow.

4. As has been mentioned the antiswarming property of some other urea derivatives was also tested and the results are given in Tables 20 (for 1,1-dimethylurea), 21(for 1,3-dimethylurea), 22 (for O-methylisourea), 23 (for S-methylisothiourea). The strongest effect on swarming and growth was exhibited by O-methylisourea; concentration of 0.5% stopped swarming for all strains.

Interestingly, subculturing of the strains of P. mirabilis on a medium containing urea or its analogues reduced the subsequent swarming distance of those strains even when cultured on plain agar. Thus, the colonies taken from nutrient agar plates with 4% thiourea exhibited less swarming on urea agar (Table 24) as well as its analogues like S-methylisothiourea (Table 25) than the original cultures.

Table 19. The inhibitory effect of methylthiourea on the swarming ability of P. mirabilis srtains.

Strains	Concentrations of methylthiourea in nutrient agar (%)								
	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PM1	6.6	1.5	1.1	0.7	0	0	0	0	N.G.
PM2	5.7	1.2	0.9	0.3	0	0	0	0	N.G.
PM5c	4.8	0.5	0.2	0	0	0	0	N.G.	N.G.
P49c	4.9	0.6	0.3	0	0	0	0	N.G.	N.G.
P991c	4.6	0.4	0.2	0	0	0	0	N.G.	N.G.
G9	5.2	1.1	0.8	0	0	0	0	0	N.G.
G16	4.1	0.5	0	0	0	0	N.G.	N.G.	N.G.
P33	6.2	1.3	1.1	0.7	0	0	0	0	N.G.
133	5.5	1.1	0.8	0.2	0	0	0	0	N.G.
196	5.7	0.6	0.3	0	0	0	0	0	N.G.
202	4.6	0.8	0.4	0	0	0	0	N.G.	N.G.
250	5.3	1.1	0.7	0	0	0	0	0	N.G.
P405	6.1	1.5	1.1	0.5	0	0	0	0	N.G.
P406	6.8	1.8	1.2	0.6	0	0	0	0	N.G.
705	5.4	1.3	0.9	0.2	0	0	0	0	N.G.

The experiment was carried out four times and the results were consistent. Figures are the swarming zone size of the strains in centimeters. N.G. = No growth.

Table 20. The inhibitory effect of 1,1-dimethylurea on the swarming property of P. mirabilis strains.

Strains	Concentrations of 1,1-dimethylurea in agar (%)						
	0.0	0.1	0.2	0.5	1.0	1.5	2.0
PM1	4.2	1.2	0.9	0.5	0	N.G.	N.G.
PM2	4.5	1.0	0.8	0.3	0	0	N.G.
PM5c	3.7	0.9	0.7	0.3	0	N.G.	N.G.
P49c	3.2	0.8	0.7	0.1	0	N.G.	N.G.
P991c	2.6	0.7	0.5	0.2	0	N.G.	N.G.
G9	3.9	0.8	0	0	0	0	N.G.
G13	6.7	0	0	0	0	N.G.	N.G.
G16	1.9	0	0	0	0	N.G.	N.G.
P33	4.3	1.2	0.2	0	0	N.G.	N.G.
133	7.1	0.9	0.2	0	0	N.G.	N.G.
196	6.2	0.2	0	0	0	N.G.	N.G.
202	5.1	0.8	0.1	0	0	N.G.	N.G.
250	4.7	0.1	0	0	0	N.G.	N.G.
P405	6.4	0.3	0	0	0	N.G.	N.G.
P406	5.8	0.5	0.1	0.1	0	N.G.	N.G.
705	4.6	0.1	0	0	0	N.G.	N.G.

The experiment was carried out four times and the results were consistent. Figures are the swarming distance of the strains in centimeters.

Table 21. The inhibitory effect of 1,3-dimethylurea on the swarming ability of *P. mirabilis* strains.

Strains	Concentration of 1,3-dimethylurea in agar (%)								
	0.0	0.1	0.2	0.5	1.0	1.5	2.0	2.5	3.0
PM1	7.1	3.2	1.9	1.5	0.5	0.1	0	N.G.	N.G.
PM2	6.4	3.2	0.9	0	N.G.	N.G.	N.G.	N.G.	N.G.
PM5c	5.2	0.9	0.3	0	N.G.	N.G.	N.G.	N.G.	N.G.
P49c	5.1	1.2	0.4	0	0	0	0	N.G.	N.G.
P991c	4.7	1.8	1.3	0.9	0.4	0	0	N.G.	N.G.
G9	3.8	0.9	0.5	0	0	N.G.	N.G.	N.G.	N.G.
G13	7.2	3.4	1.6	0.8	0.6	0.4	0	0	N.G.
G16	3.2	0.7	0.2	0	0	N.G.	N.G.	N.G.	N.G.
P33	5.9	3.4	2.9	2.7	1.5	0.6	0	0	N.G.
133	6.1	3.1	2.2	1.8	0.9	0.5	0	0	N.G.
202	5.2	2.1	1.6	1.1	0.4	0	0	0	N.G.
250	5.6	2.9	1.8	1.5	0.6	0	0	0	N.G.
P405	5.9	3.1	1.7	1.2	0.7	0.3	0	0	N.G.
P406	7.3	4.2	3.2	2.5	0.9	0.4	0	0	N.G.
705	5.5	2.3	1.7	1.4	0.8	0.5	0	0	N.G.

The experiment was carried out four times and the results were consistent. Figures are the swarming distance of the strains in centimeters. N.G. = No growth.

Table 22. The effect of O-methylisourea on the swarming ability of *P. mirabilis* strains.

Strains	Concentrations of O-methylisourea in agar (%)				
	0.0	0.1	0.2	0.5	1.0
PM1	5.7	4.9	0	N.G.	N.G.
PM2	4.9	2.6	0	0	N.G.
PM5c	3.6	2.9	0	0	N.G.
P49c	3.9	2.3	0	0	N.G.
P991c	2.9	1.4	0.3	0	N.G.
G9	3.5	1.3	0	0	N.G.
G13	5.8	4.2	0.5	0	N.G.
G16	2.8	0.7	0	0	N.G.
P33	5.9	3.8	0	0	N.G.
133	5.1	3.3	0	0	N.G.
196	4.8	2.8	0	0	N.G.
202	3.1	0.8	0	0	N.G.
250	3.6	3.1	0	0	N.G.
P405	4.4	2.6	0	0	N.G.
P406	6.3	4.9	0	0	N.G.
705	4.3	2.2	0	0	N.G.

The experiment was conducted five times and the results were consistent. Figures are the swarming distance of the strains in centimeters. N.G. = No growth.

Table 23. The effect of S-methylisothiourea on the swarming property of *P. mirabilis* strains.

Strains	Concentrations of S-methylisothiourea in agar (%)								
	0.0	0.1	0.2	0.5	1.0	1.5	2.0	2.5	3.0
PM1	7.3	4.2	3.4	0.5	0.2	0	0	0	0
PM2	6.4	3.8	2.5	0.5	0	0	0	0	0
PM5c	5.7	2.2	1.3	0.4	0.1	0	0	0	N.G.
P49c	5.5	2.1	1.3	0.3	0	0	0	0	0
P991c	5.2	1.9	1.4	0.4	0	0	0	0	0
G9	5.6	1.8	1.2	0.3	0	0	0	0	N.G.
G13	6.6	4.1	3.2	0.9	0.5	0	0	0	0
G16	3.1	1.2	0.9	0	0	0	0	0	N.G.
P33	6.5	3.4	1.2	0.4	0	0	0	0	0
133	6.1	3.2	1.2	0.2	0	0	0	0	N.G.
196	7.2	4.9	3.5	2.4	1.5	0.9	0.5	0.3	0
202	4.8	0.9	0.4	0	0	0	0	0	N.G.
250	5.8	1.1	0.5	0.2	0	0	0	0	0
P405	5.9	1.9	0.7	0	0	0	0	0	0
P406	7.4	5.1	3.6	2.8	1.2	0.6	0.2	0	0
705	5.2	2.8	1.7	0.5	0	0	0	0	0

The experiment was carried out four times and the results were consistent. Figures are the swarming distance of the strains in centimeters. N.G. = No growth.

Table 24. The effect of urea on the swarming of P. mirabilis strains which have been previously subcultured on nutrient agar+4% thiourea for 48 hours.

Strains	Concentrations of urea in nutrient agar plates (%)							
	0.0	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PM1	3.8	1.3	0.6	0.3	0.1	0	0	0
PM2	3.3	1.1	0.4	0.2	0	0	0	0
PM5c	2.7	0.3	0	0	0	0	0	0
P49c	2.4	0.5	0	0	0	0	0	0
P991c	2.6	0.6	0	0	0	0	0	0
G9	2.5	0.2	0	0	0	0	0	0
G13	4.1	1.7	1.2	0.6	0.4	0.2	0	0
G16	0.9	0.2	0	0	0	0	0	0
P33	3.1	1.4	1.0	0.7	0.2	0	0	0
133	3.8	1.1	0.8	0.3	0.1	0	0	0
196	3.5	0.7	0.6	0.2	0	0	0	0
202	1.7	1.1	0.7	0.4	0	0	0	0
250	4.1	1.2	0.5	0.1	0	0	0	0
P405	3.4	1.3	0.8	0.2	0	0	0	0
P406	3.9	1.5	0.9	0.6	0.2	0	0	0
705	2.8	0.8	0.5	0.2	0.1	0	0	0

The experiment was conducted five times and the results were consistent. Figures are the swarming distance of the strains in centimeters.

Table 25. The effect of S-methylisothiourea on the swarming ability of P. mirabilis strains previously subcultured on 0.5% thiourea.

Strains	Concentrations of S-methylisothiourea in agar (%)								
	0.0	0.1	0.2	0.5	1.0	1.5	2.0	2.5	3.0
PM1	1.9	0.8	0	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
PM2	0.9	0.3	0	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
PM5c	0.5	0	0	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
P49c	0.6	0.2	0	0	0	0	N.G.	N.G.	N.G.
P991c	0.8	0.3	0	0	0	0	N.G.	N.G.	N.G.
G9	0.3	0	0	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
G13	1.8	0.5	0	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
G16	0	0	0	N.G.	N.G.	N.G.	N.G.	N.G.	N.G.
P33	2.7	1.4	0.7	0	0	0	0	0	N.G.
133	1.9	0.9	0.5	0.3	0.2	0	N.G.	N.G.	N.G.
196	1.7	1.5	1.2	0.9	0.5	0.3	0	0	0
202	1.2	0.8	0.4	0	N.G.	N.G.	N.G.	N.G.	N.G.
250	2.1	1.3	0.5	0	0	0	0	0	N.G.
P405	1.6	0.9	0.3	0	0	0	0	0	N.G.
P406	3.2	1.3	1.4	0	0	0	0	0	0
705	1.7	1.1	0.7	0.3	0	0	0	0	N.G.

The colonies were taken from five days old cultures on nutrient agar containing 0.5% thiourea.

Figures are the swarming distances in centimeters.

III.7.d. The effect of guanidine on swarming:

Guanidine [$(H_2N_2)_2$-C=NH] is an antiviral agent. The antiswarming activity of this compound was also observed on 16 highly swarmer strains of P. mirabilis. As in the previous experiments, different concentrations of guanidine were incorporated into the agar and after inoculation plates were incubated at 30°C for 18 hours. The results obtained showed (Table 26) that this agent inhibited swarming at 2% and growth of the strains at 4% concentration. In contrast to urea, the methyl derivative of guanidine seemed to be slightly more inhibitory than itself; at 1.5% concentration it stopped swarming of all of the test strains (Table 27). The swarming and growth inhibitory concentrations of all of the above chemicals are shown in Tables 28 and 29.

III.7.e. Effect of urea on the motility, flagellation and size of agar grown strains of P. mirabilis:

Urea was shown to greatly reduce the motility and formation of flagella as well as the length of this organism grown on agar plates containing different concentrations of urea. The motility of the strains was observed in hanging drops and in microtubes, the flagellation was checked by negative staining under a phase contrast microscope, and the size of the cells was measured by a graticule lens. The experiments were conducted 8-10 times and the means of the readings are shown in Table 30. As is indicated, the effects

Table 26. The inhibitory effect of guanidine on the swarming property of *P. mirabilis* strains.

Strains	Concentrations of guanidine in nutrient agar (%)								
	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
PM1	5.6	3.9	1.8	0.5	0	0	0	0	N.G.
PM2	5.1	3.4	1.1	0	0	0	0	0	N.G.
PM5c	4.3	3.1	2.2	0.8	0	0	0	0	N.G.
P49c	3.9	2.8	1.3	0.4	0	0	0	0	N.G.
P991c	4.4	3.2	1.8	0.5	0	0	0	0	N.G.
G9	4.2	2.7	1.4	1.1	0	0	0	0	N.G.
G13	6.3	4.1	2.9	0.8	0	0	0	0	N.G.
G16	2.7	0.9	0	0	0	0	0	0	N.G.
P33	6.1	3.8	3.1	0.9	0	0	0	0	N.G.
133	5.5	2.9	1.9	1.2	0.3	0	0	0	N.G.
196	4.8	3.6	2.2	0.8	0	0	0	0	N.G.
202	3.8	3.1	1.7	0.3	0	0	0	0	N.G.
250	4.7	2.8	1.2	0	0	0	0	0	N.G.
P405	5.2	2.6	1.6	0	0	0	0	0	N.G.
P406	6.4	4.2	2.9	0.7	0	0	0	0	N.G.
705	4.7	2.1	1.6	0.5	0	0	0	0	N.G.

The experiment was conducted five times and the results were consistent. Figures are the swarming zone size of the strains in centimeters. N.G. = No growth.

Table 27. The inhibitory effect of 1-methylguanidine on the swarming ability of *P. mirabilis* strains.

Strains	Concentrations of 1-methylguanidine in agar (%)							
	0.0	0.1	0.2	0.5	1.0	1.5	2.0	2.5
PM1	6.8	4.8	4.5	3.8	3.1	0	0	N.G.
PM2	6.7	3.6	3.1	2.1	1.5	0	0	N.G.
PM5c	4.2	2.1	1.8	1.2	0.9	0	0	N.G.
P49c	5.1	2.9	2.1	1.3	1.1	0	0	N.G.
P991c	4.8	2.7	2.2	1.9	1.2	0	0	N.G.
G9	4.9	2.4	1.8	1.3	0.8	0	0	N.G.
G13	6.5	5.6	5.1	4.5	3.7	0	0	N.G.
G16	2.1	1.2	0.6	0	0	0	N.G.	N.G.
P33	5.3	4.1	3.1	2.9	2.3	0	0	N.G.
133	6.1	4.2	3.4	3.1	2.8	0	0	N.G.
196	6.4	5.3	4.7	4.2	3.9	0	0	N.G.
202	3.5	2.2	1.6	1.1	1.1	0	0	N.G.
250	4.4	4.1	3.8	3.2	1.8	0	0	N.G.
P405	5.9	5.1	4.6	3.9	2.9	0	0	N.G.
P406	7.6	6.2	5.7	4.6	3.8	0	0	N.G.
705	5.2	4.7	4.1	3.6	2.6	0	0	N.G.

The experiment was carried out four times and the results were consistent. Figures are the swarming distance of the strains in centimeters. N.G. = No growth.

Table 28. The inhibitory concentrations of urea and its relatives on swarming and growth of *P. mirabilis* strains.

Compounds	Percentage of swarming inhibited at 1% concentration	Complete swarming inhibition of all strains at concentration:	Growth inhibition of all strains at concentration:
Urea	80%	3.5%	N. T.
Urea+acetohydroxamic acid	76%	3%	N. T.
Acetohydroxamic acid	24%	N. T.	N. T.
Methylurea	38%	5%	N. T.
Thiourea	93%	2%	4%
Methylthioura	88%	2%	4%
1,1-dimethylurea	100%	1%	1.5%
1,3-dimethylurea	92%	2%	3%
O-methylisourea	100%	0.5%	1%
S-methylisothiourea	98%	3%	N. T.
Guanidine	74%	2.5%	4%
1-methylguanidine	46%	1.5%	2.5%

N. T= Not tested.

Table 29. Order of average of effectiveness of urea and analogues on the swarming property of P. mirabilis strains.

Analogue	Order of effectiveness	Inhibitory concentration
O-methylisourea	1	0.23%
1, 1-dimethylurea	2	0.54%
S-methylisothiourea	3	1.21%
1, 3-dimethylurea	4	1.31%
Thiourea	5	1.43%
Methylthiourea	6	1.64%
Urea	7	2.59%
Urea+acetohydroxamic acid (urease inhibitor)	8	2.68%
Methylurea	9	4%
Acetohydroxamic acid	-	N.T.

N.T. = Not tested.

Table 30. The effects of urea on the motility, flagellation and long cell formation of cells from swarming plates of P. mirabilis.

Strains	Concentrations of urea in nutrient agar (%)											
	0.0			0.5			2.0			4.0		
	M	S	F	M	S	F	M	S	F	M	S	F
PM1	90	58	+++	70	29	++	37	7	++	20	2	+
PM2	86	49	+++	75	32	++	35	6	++	24	1	+
G13	87	66	+++	69	43	++	38	12	+	27	1	+
P33	92	74	+++	78	48	+++	44	8	++	30	1	+
133	85	55	+++	64	30	++	32	5	++	21	1	++
196	78	62	+++	57	56	++	36	10	++	18	1	+
P405	86	53	+++	59	42	++	32	11	++	17	1	+
P406	93	78	+++	77	54	+++	41	18	++	28	2	++

M= Percentage of motile cells.

S= Size of swarmers (long cells) in micrometers.

F= Flagella numbers per cell ; 1-10=+, 10-100=++, 100 and over=+++.

Figures are the mean of 8-10 readings.

of urea on these characteristics were concentration dependent and at 4% concentration there was the greatest effect on motility, flagellation and size for the strains of P. mirabilis.

III.8. Electron microscopy study of p+ and p- strains of P. mirabilis:

The possible effect of plasmids on the morphological characteristics of this organism such as the size of the cells and/or the number of appendages was studied by transmission electron microscope (TEM, JEOL 100 B)

In samples which were taken from overnight cultures in nutrient broth, there was no difference between the size of p- and p+ cells but the p- cells were shown to be slightly more flagellated. In exponential broth cultures (after 3-4 hours of incubation) a number of long cells were observed among p- cells. Also on nutrient agar even on those containing swarming inhibitor, the average size for cells of plasmid-free strains was more than for plasmid-bearing isolates.

Flagellar study of strains of P. mirabilis in broth culture by both TEM and flagella staining revealed that p- strains, especially long cells, were highly flagellated whereas most of the cells from p+ strains had small numbers of sparsely located flagella.

III.9. Attachment:

III.9.a. The effect of plasmids on adhesion of strains of P. mirabilis to cover slips and glass beads (static):

The adherence of three p+ strains (PM5, P49, P991) and their cured derivatives as well as the p- strain G9 with the two transconjugants G9pPM5 and G9pP49 to glass cover slips, glass beads and uroepithelial cells was examined. The clump formation of these strains was also observed using a haemocytometer. The results with the glass cover slips are shown in Table 31, and the attachment to glass beads as well as clumping with the cells grown in static conditions are exhibited in Table 32. The results in Tables 31 and 32 indicate that the presence of a plasmid significantly increased the attachment of their host cells to inert surfaces like glass plates or beads. Moreover, these plasmids increased auto-agglutination (clumping) of the strains. These two properties were much weaker in plasmid-less isolates. The average number of cells attached to one cover slip in plasmid-bearing strains was 56 whereas in plasmid-free strains was 32 and the difference was highly significant. Also the mean percentage of adherence to glass beads in p+ strains of P. mirabilis was 65% and this figure in their p- counterparts was 45%, again the difference was significant.

III.9.b. Attachment of shaking culture to glass beads:

To find out whether the presence of pili has any effect

Table 31.

Attachment of p+ and p- strains of P. mirabilis grown at 37°C in static conditions to glass cover slips.

Strains	Plasmids	No. of attached cells/cover slip
PM5	1	63
PM5c	none	41
P49	1	48
P49c	none	33
P991	1	82
P991c	none	29
G9	none	26
G9pPM5	1	51
G9pP49	1	38

Experiment was carried out several times in duplicate. Figures are the average number of cells attached to 8-10 cover slips.

Table 32.

Adherence of p+ and p- strains of P. mirabilis (grown in static conditions) to glass beads.

Strains	Control	Samples	Adherence	Clumping
PM5	0.68	0.17	75%	+++
PM5c	0.85	0.49	42%	+
P49	0.63	0.20	68%	++
P49c	0.71	0.46	45%	+
P991	0.59	0.13	77%	+++
P991c	0.76	0.49	36%	++
G9	0.72	0.56	22%	+
G9pPM5	0.66	0.34	48%	++
G9pP49	0.54	0.37	32%	+

Experiment was conducted several times in duplicate with consistent results.

The results are the mean of all duplicate tests. The test figures are optical density of the cultures measured by Hilger colorimeter at 490 nm after passage through a glass bed column.

on the adhesion of P. mirabilis strains to surfaces, the experiment was repeated with glass beads using the same strains grown overnight at 37°C in a high speed linear shaker which is believed to remove or inhibit the growth of pili. Results showed a reduction in the adherence property of the tested strains particularly plasmid-containing ones to glass beads. The clumping of the cells was also reduced. Results are shown in Table 33.

III. 9. c. Adherence to uroepithelial cells:

Attachment of all p- and p+ strains which have been used in the previous experiments (cured, transconjugants and their parents) to uroepithlial cells was studied using the method of Harber et. al. (1985). The epithelial cells were collected from a healthy female and freshly used for the experiment. For each strain, at least ten epithelial cells were checked under phase contrast microscope. This microscopical examination of the cells showed a large differences beween the numbers of p+ and p- cells attached to the uroepithelial cells indicating that the presence of plasmids enhances the ability of host strains to adhere to these cells. The number of attached bacteria was not countable.

III. 10. Hydrophobicity of p- and p+ strains of P. mirabilis:

The hydrophobicity of the strains was determined by measuring the retention of bacteria on octyl-sepharose in the

Table 33.

Adherence of p+ and p- strains of P. mirabilis grown in shaking condition to glass beads.

Strains	Control	Samples	Adherence	Clumping
PM5	0.79	0.53	43%	++
PM5c	0.95	0.66	36%	+
P49	0.73	0.34	53%	++
P49c	0.89	0.67	24%	+
P991	0.67	0.32	43%	++
P991c	0.86	0.44	35%	+
G9	0.82	0.67	18%	-
G9pPM5	0.77	0.53	31%	+
G9pP49	0.81	0.59	27%	+

The experiment was repeated several times in duplicate with consistent results.

The results are the mean of two readings of all experiments.

Figures are optical density of the cultures measured by Hilger colorimeter at 490 nm after passage through a glass bed column.

presence of different concentrations of ammonium sulphate. The experiment was carried out several times in duplicate . The results showed (Table 34) that the presence of a plasmid positively affects the bacterial hydrophobicity. The strains containing plasmid exhibited more affinity to octyl-sepharose than their p- derivatives. The mean per cent of hydrophobic cells which adhered to octyl-sepharose column at 1M concentration of amonium sulphate was 70.2 for p+ strains and 52.5 for p- isolates. The increased concentration of ammonium sulphate enhanced the hydrophobic property of both p- and p+ cells.

III. 11. Growth:

III. 11. a. The influence of plasmids on the growth rate of strains of P. mirabilis:

The growth of plasmid-containing and plasmid-free strains was observed by growing them in nutrient broth at 37°C in static and shaking conditions. The results which are the optical densities of the strains are shown in Tables 35 and 36. As is shown, the plasmid-free strains gave higher growth than the plasmid-bearing isolates. The difference was greater in stationary phase and the O.D. of static growth was much lower than the strain grown under shaken conditions. Moreover under shaken conditions the difference between the final optical densities of p+ and p- strains was less than that in static grown especially for stationary phase cells. The final

Table 34.

Hydrophobicity of p+ and p- strains of P. mirabilis in the presence of ammonium sulphate.

Strains	Concentrations of ammonium sulphate (in Molarity)					
	0.01	0.05	0.10	0.25	0.50	1.0
PM5	37.2	43.1	46.7	55.4	62.2	71.5
PM5c	22.6	30.9	34.8	41.5	48.4	53.7
P49	18.4	25.0	39.2	53.1	58.6	64.5
P49c	12.3	14.7	31.7	38.8	42.5	49.8
P991	42.3	47.5	57.2	64.4	73.6	80.8
P991c	17.6	29.2	37.9	41.3	45.1	54.2

The experiment was carried out several times in duplicate with consistent results.

The results are the mean of two readings of all experiment.

Figures are the percentage of the cells retained on the octyl-sepharose column.

Table 35. The effect of plasmids on the growth rate of *P. mirabilis* strains in static conditions.

Strains	Plasmids	Incubation times (in hours)						
		0h	1h	2h	3h	4h	5h	O/N
PM5	1	0.01	0.01	0.04	0.08	0.11	0.17	0.37
PM5c	-	0.01	0.02	0.06	0.14	0.21	0.32	0.56
P49	1	0.01	0.02	0.05	0.07	0.12	0.19	0.26
P49c	-	0.01	0.05	0.14	0.19	0.27	0.34	0.48
P991	1	0.00	0.01	0.04	0.09	0.15	0.19	0.22
P991c	-	0.01	0.03	0.08	0.17	0.28	0.36	0.47
G9	-	0.01	0.02	0.06	0.11	0.21	0.39	0.52
G9pPM5	1	0.00	0.01	0.03	0.07	0.12	0.20	0.35
G9P49	1	0.01	0.02	0.05	0.09	0.11	0.26	0.32

The experiment was repeated five times with consistent results. The results are the mean of two readings of all experiments. Figures are the optical density of the cultures measured by Hilger colorimeter at 490 nm.

Table 36. The effect of plasmids on the growth rate of *P. mirabilis* strains grown in shaking conditions.

Strains	Plasmids	Incubation times (in hours)						
		0h	1h	2h	3h	4h	5h	O/N
PM5	1	0.01	0.18	0.25	0.34	0.49	0.56	0.69
PM5c	-	0.01	0.20	0.39	0.49	0.63	0.87	0.98
P49	1	0.01	0.14	0.30	0.43	0.48	0.56	0.68
P49c	-	0.01	0.19	0.39	0.55	0.62	0.81	0.90
P991	1	0.00	0.17	0.31	0.47	0.59	0.66	0.82
P991c	-	0.00	0.25	0.40	0.54	0.61	0.77	0.94
G9	-	0.01	0.24	0.57	0.71	0.79	0.83	1.04
G9pPM5	1	0.01	0.17	0.28	0.42	0.53	0.69	0.78
G9pP49	1	0.00	0.15	0.35	0.50	0.61	0.74	0.80

The experiment was repeated five times, the results are the mean of two reading of each experiment and the standard error was not significant. Figures are optical densities of the cultures mrasured by Hilger colourimeter at 4900 nm.

mean optical densities for p+ isolates of P. mirabilis in static and shaken cultures in stationary phase were 0.30 and 0.74 respectively, whereas these figures for p- strains were 0.51 and 0.95.

III.11.b. The growth of p+ and p- strains of P. mirabilis in iron depleted nutrient broth:

The iron content of nutrient broth No. 2 (Oxoid) was greatly reduced as described in Materials and Mthods, and the growth of plasmid-free and plasmid-carrying strains of P. mirabilis in this medium was observed at 37°C in static and shaken conditions. The results are shown in Tables 37 and 38, and as was expected the growth of both p- and p+ strains was reduced compared to that in ordinary nutrient broth. Moreover the p- strains grew better than p+ counterparts in both static and shaken conditions, but their growth was much higher in shaken cultures. The final mean optical densities of p+ cultures after overnight growth in static and shaken cultures were 0.13 and 0.18 respectively while for the p- strains these values were 0.28 and 0.43 with significant differences.

Table 37.

The growth of P+ and P- strains of P. mirabilis in Iron depleted nutrient broth at 37° C (static).

Strains	Plasmids	Incubation time(in hours)						
		0	1	2	3	4	5	O/N
PM5	1	0.02	0.02	0.03	0.03	0.05	0.08	0.11
PM5c	0	0.02	0.03	0.06	0.09	0.16	0.21	0.32
P49	1	0.03	0.03	0.03	0.04	0.06	0.06	0.09
P49c	0	0.03	0.03	0.04	0.08	0.14	0.19	0.24
P991	1	0.03	0.04	0.04	0.05	0.07	0.10	0.12
P991c	0	0.04	0.05	0.07	0.08	0.14	0.18	0.26
G9	0	0.04	0.05	0.06	0.09	0.12	0.17	0.28
G9pPM5	1	0.03	0.04	0.05	0.08	0.10	0.13	0.16
G9pP49	1	0.04	0.04	0.06	0.08	0.11	0.14	0.17

Figures are the mean of two readings of optical density of the cultures measured at 490 nm by Hilger colorimeter.

Table 38.

The growth of P+ and P- strains of P. mirabilis in Iron depleted nutrient broth at 37° C (shaking).

Strains	Plasmids	Incubation time(in hours)						
		0	1	2	3	4	5	O/N
PM5	1	0.02	0.03	0.06	0.09	0.14	0.18	0.21
PM5c	0	0.02	0.04	0.09	0.12	0.23	0.35	0.48
P49	1	0.03	0.04	0.06	0.07	0.09	0.11	0.15
P49c	0	0.03	0.06	0.09	0.15	0.28	0.38	0.46
P991	1	0.03	0.05	0.08	0.11	0.14	0.17	0.23
P991c	0	0.04	0.07	0.10	0.18	0.27	0.39	0.44
G9	0	0.04	0.07	0.09	0.12	0.18	0.24	0.35
G9pPM5	1	0.04	0.05	0.06	0.07	0.09	0.13	0.16
G9pP49	1	0.03	0.06	0.08	0.08	0.11	0.15	0.18

Figures are the mean of two readings of optical density of the cultures measured by Hilger colorimeter at 490 nm.

III. 11. c. The effect of SDS on the growth of p- and p+ strains of P. mirabilis:

Different concentrations of SDS (sodium dodecyl sulphate) were added to nutrient broth and the growth rates of plasmid-containing isolates of P. mirabilis and their cured and transconjugant derivatives were checked at time intervals at 37°C in static cultures. The results are shown in Tables 39, 40, 41 and 42. SDS decreased the growth of both p- and p+ strains and the effect was concentration dependent. The growth of p+ strains was again lower than that of p- ones, but with the longer incubation time and higher concentrations, the p- strains seemed to be slightly more sensitive to the inhibitory effect of the detergent.

III. 12. Motility and clumping of plasmid-bearing and plasmid-free strains of P. mirabilis:

Motility of 37°C grown broth cultures of P. mirabilis strains was observed under phase contrast microscope (PCM) and the results are shown in Table 43. Plasmid-containing isolates appeared to be less motile than their p- derivatives and this reduced motility was time dependent, for example the motility of strain PM5 after 2 hours of incubation was 75%, after 6 hours 35% and the overnight culture contained only 20% motile bacteria. However, the motility of its p- derivative in the mentioned intervals was 90, 70 and 45 per cent respectively. The average motility after 6 hours of incubation in p- strains

Table 39.

The effect of SDS on the growth rate of P. mirabilis strain PM5 and its p- derivative.

Incubation times.	State of plasmid	Concentrations of SDS in broth (µg/ml)			
		0	50	100	200
0 hour	p+	0.01	0.01	0.01	0.01
	p-	0.02	0.02	0.02	0.02
2 hours	p+	0.02	0.01	0.01	0.01
	p-	0.08	0.08	0.07	0.05
4 hours	p+	0.04	0.03	0.02	0.02
	p-	0.11	0.08	0.06	0.05
6 hours	p+	0.16	0.11	0.08	0.06
	p-	0.24	0.18	0.09	0.05
O/N	p+	0.31	0.29	0.18	0.16
	p-	0.54	0.51	0.36	0.12

The experiment was carried out several times with consistent results. The results are the avarge of two readings of all experiments.

Figures are the optical densitis of the cultures measured by Hilger colorimeter at 490 nm. O/N= Overnight.

Table 40.

The effect of SDS on the growth rate of P. mirabilis strain P49 and its cured derivative.

Incubation times	State of plasmid	Concentrations of SDS in broth (µg/ml)			
		0	50	100	200
0 hour	p+	0.01	0.01	0.01	0.01
	p-	0.01	0.01	0.01	0.01
2 hours	p+	0.03	0.02	0.01	0.01
	p-	0.06	0.06	0.05	0.03
4 hours	p+	0.07	0.06	0.04	0.03
	p-	0.12	0.11	0.08	0.05
6 hours	p+	0.20	0.18	0.09	0.07
	p-	0.36	0.32	0.19	0.08
O/N	p+	0.32	0.30	0.24	0.17
	p-	0.55	0.46	0.29	0.11

The experiment was carried out several times with consistent results. The results are the mean of two readings of all experiments.

Figures are the optical densities of the cultures measured by Hilger colorimeter at 490 nm.

O/N= Over night.

Table 41.

The effect of SDS on the growth rate of P. mirabilis strain P991 and its p- derivative.

Incubation times	State of plasmid	Concentrations of SDS in broth (µg/ml)			
		0	50	100	200
0 hour	p+	0.01	0.01	0.01	0.01
	p-	0.01	0.01	0.01	0.01
2 hours	p+	0.02	0.02	0.01	0.01
	p-	0.04	0.03	0.03	0.01
4 hours	p+	0.05	0.04	0.03	0.02
	p-	0.10	0.10	0.05	0.03
6 hours	p+	0.19	0.16	0.12	0.06
	p-	0.31	0.24	0.14	0.09
O/N	p+	0.27	0.25	0.21	0.15
	p-	0.49	0.46	0.23	0.14

The experiment was carried out several times with consistent results. The results are the mean of two readings of all experiments.

Figures are optical densities of the cultures measured by Hilger colorimeter at 490 nm.

O/N= Over night.

Table 42.

The effect of SDS on the growth rate of *P. mirabilis* strain G9 and its transconjugant derivatives G9pPM5 and G9pP49

Incubation times	Plasmids	Concentrations of SDS in broth (µg/ml)			
		0	50	100	200
0 hour	-	0.01	0.01	0.01	0.01
	pPM5	0.01	0.01	0.01	0.01
	pP49	0.01	0.01	0.01	0.01
2 hours	-	0.05	0.05	0.04	0.03
	pPM5	0.02	0.02	0.01	0.01
	pP49	0.03	0.02	0.02	0.01
4 hours	-	0.14	0.12	0.08	0.04
	pPM5	0.06	0.05	0.03	0.02
	pP49	0.07	0.07	0.04	0.02
6 hours	-	0.39	0.30	0.23	0.09
	pPM5	0.18	0.17	0.13	0.10
	pP49	0.22	0.20	0.12	0.09
O/N	-	0.52	0.41	0.26	0.11
	pPM5	0.33	0.30	0.17	0.14
	pP49	0.41	0.38	0.21	0.12

The experiment was repeated several times with consistent results. The results are the mean of two readings of all experiments. Figures are optical densities of the cultures measured by Hilger colorimeter at 490 nm. O/N= Over night.

was 67.5% and that of the p+ isolates was 45%.

By contrast to the motility, the presence of plasmid led to the increased number and size of clumps of cells and this property was also time dependent. The mean number of clumps formed per square millimeter in overnight cultures of p- strains was 25.75 and by their p+ counterparts was 102.2.

III.12.a. Effects of detergent (SDS) on the motility and clumping of p+ and p- strains of P. mirabilis:

The effect of SDS on the motility and clumping of the strains of P. mirabilis was tested by adding different concentrations of detergent into the nutrient broth and incubating at 37°C static and then sampling at intervals. The results which are shown in Table 44 indicated that the increased concentration of SDS reduced the number and the size of clumps particularly in p+ strains and at the concentration of 200µg/ml no clumps were found in almost all strains. But its effect on the motility of the strains was different from clumping. Thus at low concentrations of SDS the percentage of motility was increased but at higher concentrations (eg. 200 µg/ml) it was significantly reduced. The experiment was repeated several times and every strain was tested separately.

III.13. Plasmids and the survival of strains of P. mirabilis in different environments:

III.13.a. Survival in human and rabbit serum:

The antibacterial activity of human and rabbit serum was

Table 43. The effect of plasmids on the motility and clump formation of P. mirabilis strains grown static in nutrient broth at 37°C.

Strains	Property tested	Incubation times (in hours)				
		0 h	2 h	4 h	6 h	O/N
PM5	Cl	0	14	32	73	115
	Mot	75	70	60	35	20
PM5c	Cl	0	0	8	12	18
	Mot	90	90	85	70	45
P49	Cl	0	18	38	85	124
	Mot	80	80	65	40	30
P49c	Cl	0	4	11	21	33
	Mot	90	90	75	60	50
P991	Cl	0	5	18	73	106
	Mot	75	75	65	40	15
P991c	Cl	0	0	7	17	24
	Mot	90	80	80	70	30
G9	Cl	0	2	6	19	28
	Mot	95	90	85	70	40
G9pPM5	Cl	0	5	17	34	88
	Mot	85	85	65	50	25
G9pP49	Cl	0	7	18	49	78
	Mot	90	80	60	40	15

Cl.= Mean number of clumps in at least five squares.

Mot.= Percentage of motile cells. O/N= Overnight.

Table 44. The effect of SDS on the motility and clumping of overnight cultures of P. mirabilis strains.

Strains	Property tested	Concentration of SDS in broth (µg/ml)			
		0	50	100	200
PM5	Cl	110	102	43	9
	Mot	20	25	15	10
PM5c	Cl	17	11	6	0
	Mot	50	60	40	20
P49	Cl	118	105	53	17
	Mot	25	35	20	15
P49c	Cl	21	16	7	3
	Mot	40	45	20	5
P991	Cl	98	72	36	12
	Mot	20	15	15	10
P991c	Cl	32	30	18	4
	Mot	45	45	15	10
G9	Cl	27	21	12	8
	Mot	50	60	40	15
G9pPM5	Cl	104	77	56	23
	Mot	15	20	15	5
G9pP49	Cl	82	54	46	19
	Mot	30	35	20	10

Cl.= The mean number of clumps in at least five squares.

Mot.= Percentage of motile cells.

studied on strains PM5, P49, P991 and their p- derivatives. To reduce the rapid bactericidal activity of the two sera, they were diluted down to 30% of the normal concentration by adding 0.01M phosphate buffer. The results which are shown in Tables 45 and 46 indicated that plasmid-bearing strains of P. mirabilis were more sensitive to the antibacterial activity of both human and rabbit serum. The mean number of viable cells in p+ strains after 5 hours incubation were 2.3 and 38.3 in human and rabbit serum respectively, whereas these figures for p- isolates were 3.3 and 45.6 (all $x10^5$). In both human and rabbit serum the number of bacteria increased in the first 2-3 hours and then it decreased quickly so that with some strains there were no bacteria in overnight culture.

III.13.b. Survival in sewage:

The strains PM5, P49 and P991 as well as their cured derivatives were grown for five days in natural sewage (without sterilization) which was four times diluted. As is shown in Table 47, in the first two days the plasmid-free strains grew and gave higher growth rate than the p+ counterparts but from the third day their number decreased rapidly so that in the samples from the fifth day no p- bacteria were found. Whereas plasmid-bearing strains grew slowly initially with a lower growth rate but survived slightly longer in sewage.

Table 45.

Survival of plasmid-free and plasmid-bearing strains of P. mirabilis in human serum.

Strains	Plasmids	Incubation time (in hours)						
		0	1	2	3	4	5	O/N
PM5	1	2.2	7.3	14.1	14.9	9.7	1.8	0.8
PM5c	0	2.1	9.4	19.2	18.3	10.4	3.2	1.3
P49	1	2.4	5.9	9.1	10.7	6.4	3.1	0
P49c	0	2.6	8.7	13.5	16.2	7.2	2.5	1.1
P991	1	3.1	6.3	8	12.2	9.1	1.9	0.4
P991c	0	3.2	8.2	11.8	18.7	14.2	4.1	1.5

Figures are the viable count ($x10^5$) and the mean of three experiments.

The results of each experiment were consistent.

The serum concentration was 30%.

Table 46.

Survival of p+ and p- strains of P. mirabilis in rabbit serum.

Strains	Plasmids	Incubation time (in hours)						
		0	1	2	3	4	5	O/N
PM5	1	3.00	6.6	24.5	71	43.3	29	5.2
PM5c	0	2.9	11.7	37.8	95	108	44	17
P49	1	2.3	7.5	22	68	73	41	6.3
P49c	0	2.4	18.2	56.9	124	110	35	14
P991	1	2.8	9.7	28	76	61	45	7
P991c	0	2.6	36	84	112	101	58	16

Figures are viable count of the strains (x 10^5).

Experiment was carried out three times in duplicates and the results were consistent..

The serum concentration was 30% .

Table 47.

Survival of P+ and P- strains of *P. mirabilis* in sewage.

Strains	Plasmids	Incubation time (in days)					
		0	1	2	3	4	5
PM5	1	4.8	20	22	14	1.5	1
PM5c	0	5.1	28	43	6	1	0
P49	1	5.2	32	30	21	5	1
P49c	0	5.6	45	58	8	2	0
P991	1	6.1	14	29	27	6	1
P991c	0	5.8	23	42	38	4	0

The experiment was carried out three times and the results were consistent.

Figures are for the viable count ($x10^4$) of the cells.

The sewage was 4 times diluted.

III. 13. c. Survival in sewage effluent:

The ability of the above strains to survive in sewage effluent which has been collected from final treatment tank was also checked by the same method as with sewage but without dilution. The results showed (Table 48) considerable difference between the growth rates of p- and p+ strains of P. mirabilis; the cured strains showed slightly better growth and survival in the sewage effluent. The mean number of viable cells on the second day of incubation in p+ was 34.6×10^4 while that of the p- strains was 65.3×10^4. The peak of the growth of both p- and p+ isolates was on the second day then on the following days their number decreased quickly so that by the sixth day there were almost no live bacteria in the medium.

III. 13. d. Survival in river water:

The growth of plasmid-bearing strains PM5, P49 and P991 was compared with their cured derivatives in river water (collected from the Thames) and the results are shown in Table 49. The growth of both p- and p+ strains was very weak and after four days of incubation all the bacteria were dead. However the effect of plasmids was clear and plasmid-free cells gave better growth.

III. 14. Plasmids and the production of enzymes by strains of P. mirabilis:

The production of extracellular enzymes which are believed to be associated with the virulence of P. mirabilis

Table 48.

Survival of P+ and P- strains of *P. mirabilis* in sewage effluent.

Strains	Plasmids	Incubation time (in days)					
		0	1	2	3	4	5
PM5	1	4.1	12	30	17	1	0.2
PM5c	0	4.2	32	56	27	4	0.7
P49	1	4.00	22	24	22	13	0.01
P49c	0	4.3	38	45	28	5	0.02
P991	1	5	16	50	21	12	0.4
P991c	0	5.6	60	95	58	16	0.3

The experiment was conducted three times and the results were consistent.

Figures are given as viable count ($x10^4$) and the mean of three experiments.

Table. 49.

Survival of p+ and p- strains of P. mirabilis in river water.

Strains	Plasmids	Incubation times (in days)					
		0	1	2	3	4	5
PM5	1	3.7	18	11	2.3	0	0
PM5c	-	3.9	31	17	4.2	1.3	0
P49	1	4.5	14	6	1.5	1.2	0
P49c	-	4.6	26	13	3.3	0	0
P991	1	4.8	22	9	0.8	0	0
P991c	-	4.4	38	19	2.1	0.6	0

The experiment was repeated three times in duplicate and the results were consistent.

Figures are the viable count ($X10^4$) of the cells.

(Peerbooms et. al. 1983, Senior 1987) was investigated. These enzymes included, urease, haemolysin, protease, lecithinase. DNase and amylase.

III. 14. a. Plasmids and urease secretion. Urease is one of the most important enzymes produced by the genus Proteus by which they can be differentiated from other members of Enterobacteriaceae and it is suggested to play a major role in the pathogenicity of this group of bacteria (Senior 1983). Qualitative study of production of this enzyme was carried out on all 145 strains using Christensen's urea agar slopes and they were all urease positive. Subsequently, 16 p+ and 16 p- strains were selected and the production of urease was quantified as explained in Materials and Methods. Sixteen strains contained plasmids and the others were plasmid-free and the results are shown in Tables 50 (urease on solid agar) and 51 (urease in liquid medium). The size of the pink zones on urea agar plates indicated that the presence of plasmids in P. mirabilis strains seemed to have reduced release of urease into the medium. The mean zone size for p- strains after six hours of incubation was 2.64 whereas that for the p+ isolates was 0.98 cm. The production of urease in nutrient broth containing 2% urea was determined by the measurement of the pH values of the medium in different time intervals. The pH change of cultures of p- strains especially after 4 hours of incubation was significantly higher than the pH change

Table 50. Ammonia production by plasmid-free and plasmid-bearing strains of P. mirabilis on urea agar.

Strains	Number of plasmids	Incubation time (in hours)				
		2 h	4 h	6 h	O/N	48 h
PM1	-	0.5	1.1	2.4	W/P	W/P
PM2	-	0.5	1.2	2.6	W/P	W/P
PM4	1	0.2	0.4	1.6	3.0	W/P
P4	2s	0.4	0.7	2.2	W/P	W/P
PM5	1	0	0	0	1.6	2.1
PM5c	-	0.9	1.5	2.8	W/P	W/P
P49	1	0	0	0	2.2	3.5
P49c	-	0.8	1.3	2.3	W/P	W/P
P991	1	0	0	0	1.4	2.9
P991c	-	o.6	1.0	2.5	W/P	W/P
RM1	1	0.5	1.1	2.0	3.4	"
RM2	1	0.3	0.8	1.2	3.5	"
RM3	1	0.3	0.6	1.0	2.6	"
RM4	-	0.9	1.5	3.1	W/P	"
RM6	-	0.8	1.2	2.8	W/P	"
RM8	-	0.5	0.9	2.1	W/P	"

The experiment was conducted several times and the results were consistent.

Figures are the zone sizes of the strains in centimeters.

W/P= Whole plate. O/N= Overnight.

Table 50 (continued)

Strains	Number of plasmids	Incubation time (in hours)				
		2 h	4 h	6 h	O/N	48 h
G7	1	0.6	0.9	1.5	3.3	W/P
G8	1	0.2	0.4	0.9	2.6	"
G9	-	0.9	1.5	3.3	W/P	"
G10	-	0.7	1.3	3.4	W/P	"
G12	1	0.2	0.5	1.1	2.2	"
G13	-	0.6	1.3	2.9	W/P	"
G16	-	0.3	0.9	1.8	3.6	"
G17	1	0	0.2	1.2	2.8	"
G30	1	0.3	0.8	1.6	3.1	"
G32	2s	0.3	0.7	1.4	3.2	"
P33	-	0.7	0.9	2.6	W/P	"
133	-	0.4	1.2	2.5	W/P	"
P406	-	0.8	1.3	3.1	W/P	"
202	-	0.4	0.9	2.7	W/P	"
G9pPM5	1	0	0	0.3	1.8	2.9
G9pP49	1	0.2	0.9	2.8	W/P	W/P

The experiment was conducted several times and the results were consistent.

Figures are the zone sizes of the strains in centimeters.

W/P= Whole plate. O/N= Overnight.

Table 51. The production of ammonia by p- and p+ strains of *P. mirabilis* in nutrient broth containing 2% urea.

Strains	Number of plasmids	Incubation time (in hours)				
		0	2 h	4 h	6 h	O/N
PM1	-	7.36	9.27	9.46	9.55	9.62
PM2	-	7.30	8.92	9.38	9.51	9.77
PM4	1	7.32	8.11	8.47	9.14	9.43
P4	2s	7.34	8.85	9.47	9.62	9.88
PM5	1	7.31	7.34	7.42	7.44	8.12
PM5c	-	7.36	8.05	9.51	9.53	9.81
P49	1	7.35	7.70	8.31	8.81	9.05
P49c	-	7.34	9.27	9.54	9.56	9.80
P991	1	7.34	8.25	8.93	9.32	9.42
P991c	-	7.32	9.41	9.58	9.61	9.89
RM1	1	7.37	9.18	9.25	9.31	9.55
RM2	1	7.35	8.52	8.95	9.22	9.67
RM3	1	7.34	8.88	9.42	9.65	9.85
RM4	-	7.37	9.15	9.58	9.74	9.93
RM6	-	7.36	9.23	9.55	9.84	9.86
RM8	-	7.38	9.32	9.62	9.81	9.95

The experiment was carried out four times and the results were consistent.

Figures are the pH values of the medium.

Table 51 (continued)

Strains	Number of plasmids	Incubation times (in hours)				
		0	2. h	4. h	6. h	O/N
G7	1	7.32	8.24	8.92	9.42	9.91
G8	1	7.34	8.42	8.71	8.84	9.36
G9	-	7.35	9.27	9.61	9.69	9.85
G10	-	7.35	9.37	9.65	9.79	9.93
G12	1	7.39	8.18	8.31	8.45	9.71
G13	-	7.35	9.42	9.63	9.81	9.92
G16	-	7.33	8.48	9.27	9.84	9.96
G17	1	7.31	8.11	8.87	9.44	9.74
G30	1	7.35	8.05	8.51	9.12	9.65
G32	2s	7.36	8.48	8.92	9.57	9.81
P33	-	7.35	8.68	9.46	9.71	9.88
133	-	7.38	8.41	9.54	9.80	9.89
202	-	7.30	8.23	9.12	9.37	9.85
P406	-	7.33	9.27	9.73	9.88	9.94
G9pPM5	1	7.33	7.54	7.92	8.21	8.72
G9pP49	1	7.36	8.05	8.44	9.52	9.62

The experiment was carried out four times and the results were consistent.

Figures are the pH values of the medium.

produced by p+ strains which corresponded with the results obtained from solid medium experiments. The involvement of plasmids in reducing the urease activity of P. mirabilis was clearly demonstrated for cured and transconjugant derivatives compared to their parental strains. No difference was found between the urease activity of faecal and urinary isolates in both liquid and solid medium.

III. 14. b. Plasmids and haemolysin production. The secretion of haemolysin by colonies of p- and p+ strains of P. mirabilis as well as its presence in supernatants from both cultures was investigated; the size of haemolytic zones around the colonies and the wells of supernatant was measured after 24 and 48 hours (see Materials and Methods). The experiment was carried out several times with thirty two strains of which 16 were carrying plasmids. The results were consistent and are shown in Tables 52 and 53 with horse and sheep blood respectively. The results with both sheep and horse blood indicated that the haemolytic activity of plasmid-containing strains is higher than plasmid-less isolates. The mean of haemolytic zone sizes produced by colonies of p+ strains was 12.9 mm with horse blood and 11.7 mm with sheep blood whereas these results for p- isolates were 7.7 and 7.9. The zones produced by bacterial colonies of all strains were larger than those formed by bacterial supernatant. With the prolonged incubation time up to 48 hours the haemolysis zones spread

Table 52.

Production of Haemolysin by p+ and p- strains of P. mirabilis on horse blood.

Strains	Plasmids	Bacterial colony		Bacterial supernatant	
		24h	48h	24h	48h
PM1	0	1	8	2	7
PM2	0	1	6	-	5
P4	2	2	8	2	7
PM4	1	4	9	3	10
PM5	1	7	18	2	8
PM5c	0	2	10	-	4
P49	1	5	14	3	11
P49c	0	-	7	-	5
P991	1	4	12	-	6
P991c	0	-	8	-	2
RM1	1	5	21	2	7
RM2	1	4	12	2	8
RM3	1	5	18	3	10
RM4	0	1	10	-	4
RM6	0	2	7	2	8
RM8	0	3	9	5	12

Figures are the haemolytic zone sizes of the strains in millimeters and the mean of five experiments.

The results were consistent and the standard error was not significant.

Table 52 (continued).

Production of haemolysin by p+ and p- strains of P. mirabilis on horse blood:

Strains	Plasmids	Bacterial colony		Bacterial supernatant	
		24 h	48 h	24 h	48 h
G7	1	3	10	2	11
G8	1	5	12	3	8
G9	-	-	3	-	4
G10	-	1	4	-	2
G12	1	4	11	2	7
G13	-	2	9	1	10
G16	-	-	6	-	5
G17	1	3	12	3	10
G30	1	5	13	2	15
G32	2	4	10	2	7
P33	-	3	11	6	14
133	-	2	10	2	8
202	-	1	8	1	6
P496	-	3	7	2	9
G9pPM5	1	3	14	3	8
G9pP49	1	4	12	3	9

Figures are the haemolytic zone sizes of the strains in millimeters and the mean of five experiments.

The results were consistent.

Table 53.

Production of haemolysin by p+ and p- strains of P. mirabilis on sheep blood agar.

Strains	Plasmid	Bacterial colony		bacterial supernatant	
		24 h	48 h	24 h	48 h
PM1	-	2	7	-	5
PM2	-	1	4	-	4
P4	2	3	8	4	10
PM4	1	3	10	2	7
PM5	1	6	19	3	9
PM5c	-	2	7	-	4
P49	1	4	16	2	10
P49c	-	2	7	-	-
P991	1	5	17	2	6
P991c	-	-	5	-	2
RM1	1	5	18	3	11
RM2	1	3	14	2	8
RM3	1	4	12	1	7
RM4	-	-	7	-	5
RM6	-	1	10	-	6
RM8	-	-	5	-	3

Figures are the haemolytic zone sizes of the strains in millimeters and the mean of five experiments.

The results of each experiment were consistent.

Table No. 53 (continued).

Production of haemolysin by p+ and p- strains of P. mirabilis on sheep blood agar.

Strains	Plasmids	Bacterial colony		Bacterial supernatant	
		24 h	48 h	24 h	48 h
G7	1	5	12	3	7
G8	1	2	8	2	5
G9	-	-	4	-	2
G10	-	1	5	1	4
G12	1	2	10	3	7
G13	-	2	9	2	10
G16	-	1	8	-	5
G17	1	1	5	-	4
G30	1	2	5	2	6
G32	2	3	11	2	10
P33	-	2	12	2	9
133	-	1	6	-	5
203	-	1	5	1	4
P406	-	2	8	2	3
G9pPM5	1	3	13	1	8
G9pPM49	1	2	6	1	4

Figures are the haemolytic zone sizes of the strains in millimeter and the mean of five experiments.

The results of each experiment were consistent.

diffusely over the plate and strains which did not produce haemolysin within 24 hours, after another 24 hours of incubation demonstrated clear cut zones of haemolysis.

There was no difference between the haemolytic activity of faecal and urinary strains.

III. 14. c. Plasmid and secretion of protease. The production of protease in 16 plasmid-bearing and 15 plasmid-free strains of P. mirabilis as well as its presence in bacterial supernatant was observed. The results which are shown in Table 54 indicated that the proteolytic activity of plasmid-carrying strains was higher than their plasmid-less counterparts (Fig 9). Here also the prolonged incubation time gave better chance to the colonies or supernatant with low production of the enzyme to demonstrate this difference. The mean size of the proteolytic zones by bacterial colonies was 8.5 mm.

III. 14. d. Plasmids and lecithinase production. The above strains were also tested for lecithinase activity by both bacterial colony and supernatant within 24 and 48 hours of incubation. The results obtained showed (Table 55) that P. mirabilis did not seem to be very active in decomposing lecithin, but in some cases the difference between p+ and p- strains was clearly demonstrated. The mean size of clear zones produced by colonies of p+ strains after 48 hours was 4.67 and that in the p- isolates was 1.86 mm.

Table 54. The production of protease by plasmid-bearing and plasmid-free strains of P. mirabilis.

Strains	Plasmids	Bacterial colony		Bacterial supernatant	
		24 hours	48 hours	24 hours	48 hours
PM1	0	3	5	3	7
PM2	0	0	2	4	8
PM4	1	3	6	5	12
P4	2s	4	7	6	9
PM5	1	2	6	5	8
PM5c	0	0	3	0	2
P49	1	3	5	8	11
P49c	0	0	0	2	5
P991	1	0	6	7	13
P991c	0	0	2	2	6
RM1	1	4	7	6	14
RM2	1	4	9	4	13
RM3	1	2	8	5	16
RM4	0	0	4	2	3
RM6	0	1	3	0	3
RM8	0	0	2	0	6

The experiment was carried out several times and the results were very close. Figures are the proteolytic zone size in millimeters.

Table 54. (continued)

Strains	PLasmids	Bacterial colony		bacterial supernatant	
		24 hours	48 hours	24 hours	48 hours
G7	1	5	9	14	18
G8	1	1	4	5	12
G9	0	0	3	0	4
G10	0	0	0	0	3
G12	1	8	12	11	20
G13	0	1	2	1	5
G16	0	0	3	0	4
G17	1	2	5	3	7
G30	1	3	3	5	16
G32	2s	4	6	7	14
P33	0	0	3	0	5
133	0	0	0	0	3
202	0	1	3	2	4
P406	0	1	5	0	4
G9pPm5	1	3	7	6	10
G9pP49	1	2	3	3	6

The experiment was carried out several times and the results were very close. Figures are the proteolytic zone size of the strains in millimeters.

Table 55. The effect of plasmids on the production of lecithinase in *P. mirabilis* strains.

Strains	Plasmids	Bacterial colony		Bacterial supernatant	
		24 hours	48 hours	24 hours	48 hours
PM1	0	0	3	0	2
PM2	0	0	2	0	0
PM4	1	1	2	0	3
P4	2	2	3	2	4
PM5	1	2	4	3	4
PM5c	0	0	2	0	3
P49	1	3	4	5	6
P49c	0	2	2	3	4
P991	1	2	5	4	7
P991c	0	0	0	1	4
RM1	1	4	7	3	5
RM2	1	3	4	2	2
RM3	1	4	5	1	6
RM4	0	2	3	0	3
RM6	0	0	2	1	5
RM8	0	0	0	2	3

The experiment was repeated several times and the results were very close. Figures are the zone size of the lecithinase activity of the strains in millimeters.

Table 55. (continued).

Strains	Plasmids	Bacterial colony		Bacterial supernatant	
		24 hours	48 hours	24 hours	48 hours
G7	1	3	6	2	3
G8	1	4	5	3	5
G9	0	0	2	0	3
G10	0	0	0	2	3
G12	1	5	6	3	7
G13	0	2	3	0	0
G16	0	0	2	2	4
G17	1	3	3	2	6
G30	1	0	4	2	4
G32	2s	5	7	4	6
P33	0	2	2	2	3
133	0	0	2	1	3
202	0	1	4	1	5
P406	0	2	3	3	5
G9pPM5	1	2	4	0	3
G9pP49	1	3	6	2	7

The experiment was repeated several times and the results were very close. Figures are the zone size of the lecithinase activity of the strains in millimeters.

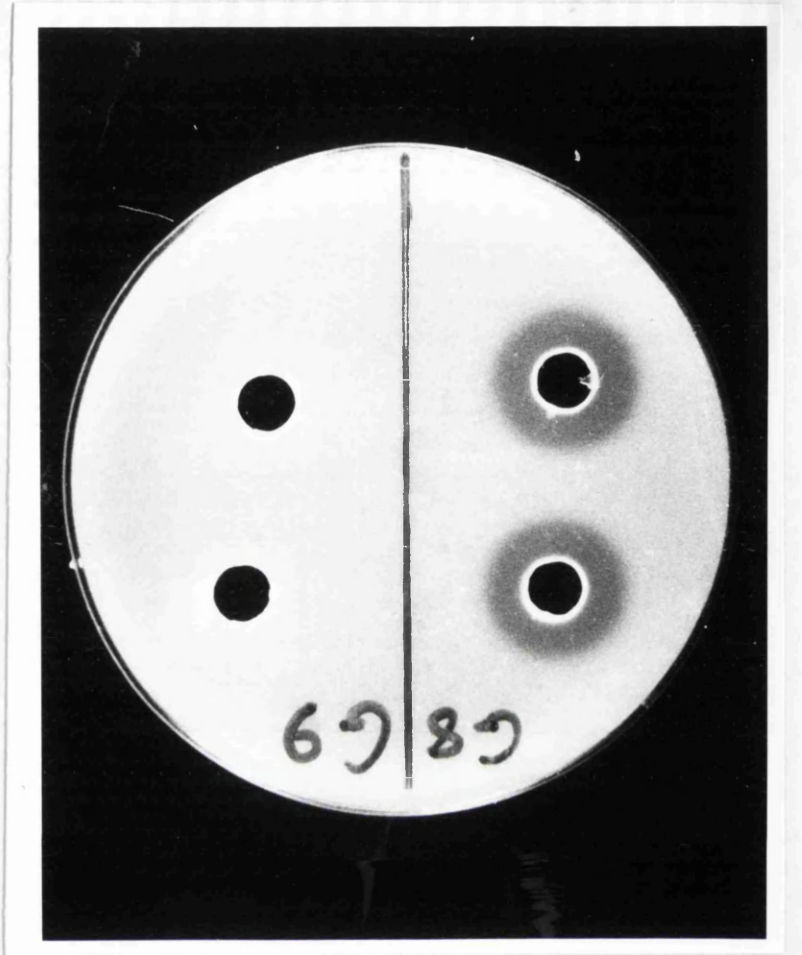

Figure 9. Protease production by p+ (G8) and p- (G9) strains of P. mirabilis on milk agar.

III. 14. e. Plasmids and production of DNase and amylase:

Plasmids were seen to have no specific effect on production of DNase and amylase. Some strains of P. mirabilis (irrespective of the presence or absence of plasmids) exhibited good DNase activity and others not, on the contrary amylase production was not significant in either plasmid-free and plasmid-containing strains of this organism.

DISCUSSION.

IIII. Discussion

The effect of plasmids on different potential virulence characteristics of P. mirabilis has been studied and 145 urinary and faecal strains of which 32 carried one and 12 contained two or more plasmids, were examined.

IIII. 1. Curing:

As was mentioned earlier, two methods were applied to cure some of the plasmid-bearing strains namely.

A. SDS+Heat treatment.

B. Acridine orange+Tris curing.

At least six strains were subjected to curing by using both methods. The experiment with SDS+heat was repeated several times and it was not successful, but three strains (PM5, P49 and P991) were successfully cured using acridine orange method.

The failure of the SDS method in curing the plasmid-containing strains of P. mirabilis used in this study, may be due to their increased resistance to this agent. The presence of these plasmids may have induced changes in the cell envelope like coding for new proteins in the outer membrane and consequently reduce the cell permeability for detergents. Subsequent tests regarding the effect of SDS on the growth of p+ and p- strains of P. mirabilis showed that although the plasmid-bearing isolates grow slower than their plasmid-free counterparts in the presence of SDS, with prolonged incubation

time these are more resistant to this agent (Tables 39-42) . Therefore they may also be resistant to the selective effect of SDS on plasmid-containing organisms which is the main step in the curing process.

All six strains were also tested for curing by growing them in nutrient broth containing 50 µg/ml acridine orange supplemented with 0.01 M Tris at slight alkaline pH ie. 7.6, which increases curing efficiencies up to 99% (Rowbury and Goodson 1978). Although three strains were cured, the other three still maintained their plasmids. Acridine orange is only effective on strains carrying sex factors like F or some related plasmids (Hirota 1960), of which the agent inhibits replication by interfering with the synthesis of RNA which is required for initiation of this process (Rowbury 1977). Therefore it is possible that those strains which were not cured (eg. G12, P13 and P14) were carrying elements unrelated to F plasmids. These plasmids may inhibit the penetration of acridine into their host cell by changing the permeability of the cell envelope or may have an initiation process for their replication which is insensitive to acridines. The other possibility is that the pH of the medium during the overnight growth does not remain alkaline (7.6) for these strains and therefore this decreases the effectiveness of acridine (Rowbury and Goodson 1978).

IIII.2. Conjugation:

Conjugation was attempted between different p- and p+ strains of P. mirabilis, and after several experiments two transconjugants (G9pPM5 and G9pP49) were obtained by conjugal transfer between strain G9 as recipient and strains PM5 and P49 as donors.

The reason that only two transconjugants were obtained was that firstly, finding a p- strain resistant to one antibiotic to which the donors were sensitive was a limiting factor.

Secondly, many of the plasmids failed to transfer to recipients by conjugation possibly because they were non-conjugative. This may be due to the lack of conjugal transfer determinants or to strong repression of their transfer properties. In this case they could have been transferred by other plasmid transfer systems such as F mediated conjugation or by transformation which was not checked in this study.

The reason that plasmids pPM5 and pP49 were transferred to recipient G9 may be because they are related to F as suggested by the finding that they could also be cured by acridine orange.

IIII.3. Plasmids and antibiotic resistance in P. mirabilis strains.

The antibiotic sensitivity pattern of all strains and the involvement of plasmids in resistance were the first potential

virulence properties studied. The results showed (Table 5) that almost all plasmid-carrying strains were resistant to one or more broad spectrum antibiotics and some of them exhibited resistance to up to seven of the tested antibiotics. A comparison between the resistance pattern of the strains is given in Table 8. As is indicated, 100% of kanamycin resistant strains were plasmid-carriers, also 86% of streptomycin, 82% cephaloridine and 83% co-trimoxazole resistant isolates contained a plasmid. Whereas plasmid-free isolates which constitute 70% of the total strains, exhibit a low level of resistance. These figures suggest that drug resistance in clinical isolates of P. mirabilis is mostly plasmid-mediated. The effect of some plasmids on this property of the strains became more clear when it was possible to correlate (a), loss of a specific plasmid with the absence of resistance (b), plasmid gain with the appearance of resistance to all or some antibiotics.

The involvement of plasmid carriage in resistance to antibacterial agents in Gram-negatives is well recorded (Anderson 1968). Mitsuhashi (1977) reported the predominant multiple resistance in clinical strains of Proteus and E. coli mediated by R-plasmids. The largest R-plasmid with molecular weight of 120 megadalton is Rts1 which has originally been isolated from P. mirabilis (Terawaki and Rownd 1972). Several mechanisms may be involved in the resistances observed here.

For instance these plasmids may possibly change the envelope permeability of strains of P. mirabilis for the test antibiotics due to alteration of cell wall proteins. Norris et al. (1985) reported strains of P. mirabilis which were resistant to several antibiotics, contained the greatest protein content of the outer membrane among the strains tested. These R-plasmids may also code for the synthesis of enzymes which specifically inactivate some types of antibiotics.

An interesting finding was where resistance to an antibacterial was transferred by conjugation but it was not eliminated by curing. Like resistance to co-trimoxazole (Sxt) in strain PM5 which suggests that resistance to this antibiotic is carried by both chromosoml and plasmid determinants.

It would be interesting to examine PM5 and PM5c more quantitatively to see whether the cured strain was less resistant.

IIII.4. Plasmids and swarming property of P. mirabilis strains:

The swarming ability of all 145 strains as well as three cured strains and transconjugants were tested by incubation of marginally inoculated nutrient agar plates at 30°C for 18 hours. The results obtained showed (Tables 5 and 12) that the presence of plasmids reduced the swarming distance of the majority of P. mirabilis strains. In other words almost all plasmid-free isolates seemed to be highly swarming whereas some of plasmid-positive strains such as PM5, PM15, P49, 443 and 912 failed to swarm and the others exhibited low swarming ability. The effect of some plasmids became more clear when cured and transconjugant strains were studied. Curing of non-swarming plasmid-bearing strains initiated swarming and the gain of a plasmid by transconjugants reduced this property. Therefore the involvement of plasmids in the swarming property of P. mirabilis strains was confirmed but the possible mechanism of their effect is yet to be clarified.

For many years the nature of swarming and the factors involved in this phenomenon such as negative or positive chemotaxis (Adler 1966, Smith 1972, Armitage 1974) and the composition of growth medium (Hoeniger 1963) have been investigated as has the possibility that it is a stage of the normal life cycle (Kvettingen 1949, Williams et al. 1976). The differential study of long swarmer cells and ordinary short

rods showed that the nutritional requirements and the respiratory activity of swarming cells is significantly lower than that of the normal non-swarmer cells (Armitage 1981, Falkinham and Hoffman 1984). In fact swarmers are a type of long non-septate and multinucleated cells whose growth phase is incomplete and the last stage of growth, which is the dividing into short cells, has not taken place. In other words the swarming strains of Proteus have completed the DNA synthesis and segregation stages (Armitage et al. 1974), but are unable to go on to division and cell envelope formation. Also significant differences between the outer membrane proteins and LPS of swarmer and short cells in P. mirabilis and P. vulgaris have been observed (Armitage et al. 1975 and 1979, Falkinham and Hoffman 1984). This may be the reason for less nutrient and oxygen uptake by the swarmers (Armitage 1981).

Plasmids are well known to be able to cause envelope changes in enterobacteria. They can lead to synthesis of new envelope (eg. outer membrane) proteins and to the modification of envelope properties due to alteration of proteins or lipopolysaccharides (Hesslewood and Smith 1974, Denoya et al. 1986). Therefore it is possible that the presence of these plasmids is associated with envelope changes which may allow the increased uptake of nutrients followed by increased synthesis of components of the cell wall which may eventually promote cell division and formation of ordinary short rods.

Hesslewood and Smith (1974) found that the introduction of R-plasmid R6K into a strain of P. mirabilis abolished its swarming ability whereas R64 increased it.

It is clear that envelope changes can alter swarming and it is therefore no surprise that the major envelope changes produced by the plasmids studied here (eg. indicated by altered adhesion and surface hydrophobicity) reduce swarming. There are several possible mechanisms.

Firstly, for swarming to occur, aside from any other envelope changes, cells will need to have numerous flagella inserted. The presence of plasmid-encoded proteins or the occurrence of plasmid-associated changes eg. to the LPS might make such insertion difficult and hence reduce flagellation and swarming zone size (Table 14).

Secondly, plasmid presence in the bacterial cell may somehow interact with genetic determinants of swarming and flagella formation on the chromosomal DNA. In most cases this interaction may be associated with suppression of these determinants resulting in reduced flagellation as well as the swarming ability of P. mirabilis.

Thirdly, the envelope changes may affect nutritional uptake and inhibitor efflux eg. if swarming is potentiated by intracellular accummulation of an inhibitor or toxin, the plasmid might allow this to leak out and hence induction of swarming be inhibited.

It was also observed that some plasmid-containing strains such as G11, P200, 580 and 166 demonstrated high swarming property. This was similar to the finding by Hesslewood and Smith (1974) that the introduction of plasmid R1818 (R46) to a plasmid-minus strain of P. mirabilis increased swarming distance of the strain. It is suggested that the presence of this plasmid enhanced the mucopeptide bonding followed by formation of macromolecules which may be associated with the increased swarming ability of this organism. Alternatively, the envelope changes in these p+ strains might prevent any leakage of a toxic swarming initiator and therefore, hasten the process.

As a conclusion, the swarming phenomenon can be described as a function of the cell envelope which is influenced by external and internal factors. The external factors are the physicochemical condition of the growth medium and environment, and the internal factors are mainly the chromosomal and plasmid determinants.

IIII.5. Effects of urea and its derivatives on the swarming property of P. mirabilis:

Swarming always causes problems in identification and isolation of mixed cultures on the surface of solid media. Therefore, so far many mechanical and chemical methods (which are listed in Table 1) have been applied by microbiologists to inhibit this phenomenon.

In this study the effect of urea and some related compounds on the swarming ability of sixteen highly swarming strains of P. mirabilis has been examined. The results obtained showed (Table 13) that urea has considerable inhibitory effect on swarming so that at the concentration of 4% complete inhibition of all test strains occurred.

There appear to be several mechanisms by which urea could act. Firstly, it could act as a denaturing agent. Secondly, its osmotic property might be involved in this process since the concentration needed for its effect was quite high and urea penetrates poorly into P. mirabilis. Thirdly, urea might act via its degradation by urease with either ammonia or the concomitant alkaline pH being the inhibitory factors. Therefore any of these effects or a combination of them might be involved in this process.

One possible mechanism may be attributed to the denaturing effect of urea and related analogues on the external proteins of the organism. This effect may be due to its ability to break the hydrogen-bonds between some components of the cell wall such as proteins or LPS (Abram and Gibbson 1961). But the question is how the denaturing property might affect the swarming of Proteus. As urea is likely to be effective on the surface of these bacteria because of low penetration, then flagella which are needed for swarming, might be impaired or their expression inhibited by this

agent. Therefore either the swarming cells will not be produced or as they are not able to move away from the initial colony, they may immediately divide into short rods. The results in this study showed many derivatives of urea are more effective than urea itself (Table 28) which may be due to increased hydrogen-bonding effect of these compounds.

The denaturing effect of urea and a number of its analogues on bovine serum albumin and ovalbumin has been studied by Gordon and Jencks (1963). It was observed that urea has a great denaturing effect on both serum albumin and ovalbumin and the effect was concentration dependent. Methyl substitution reduced the denaturing property of urea; on the contrary the derivatives containing a sulphur atom such as thiourea exhibited stronger effects on test proteins. This is similar to the results obtained here that is, methylurea had less inhibitory effect on swarming Proteus but thiourea was much more effective than urea in this respect. The denaturing effect of urea on DNA has been attributed to its hydrophobic property (Levine et al. 1963), but because of poor penetration of these compounds into the bacterial cell, a denaturing effect on chromosomal DNA looks rather unlikely

The denaturing effect of urea may also damage tRNA molecules involved in protein synthesis. This effect has recently been studied by Hegg and Thurlow (1990). They treated fifteen species of tRNA with diethylpyrocarbonate and then ran

the samples on polyacrylamide gel containing 8 molar urea. The results obtained showed several minor bands in addition to the main bands in 12 samples. Characterization of the bands indicated that these extra bands were denatured tRNAs which had been obtained from the intact tRNA molecules.

Another possible mechanism for the inhibitory effect of urea and its analogues may be due to increased osmolarity produced by these agents. The internal disruption may be caused by the plasmolysis due to high external urea concentration and its low penetrability; water will flood out of the cells which could prevent the synthetic processes (eg. flagellar synthesis) needed for swarming. If this was the reason for the inhibition, equal molar concentration of the agents would produce equal inhibitory effects unless some penetrate better. In fact there is a 7 fold difference between the least (3.5%) and most effective (0.5%) agents (Tables 28 and 29) and so this is unlikely to be the basis for the urea effects on swarming.

Degradation of urea and its derivatives by urease and changes in the pH of the medium due to ammonia release might also be considered as an inhibitory mechanism on swarming of P. mirabilis. But this does not seem to be the likely factor in the present study. Since firstly, the effect of urea plus urease inhibitor (Table 14) was stronger than urea itself (Table 13), indicating that in the presence of urease part of

the urea is degraded into carbon dioxide and ammonia and consequently reduces the inhibitory effect of this agent. Secondly, most of urea analogues which are even more effective than urea are not necessarily degraded by urease. Thirdly, increasing the pH of the medium up to pH, 9 did not significantly inhibit swarming (Table 16).

The other possible mechanism of inhibitory effect of urea and its derivatives on swarming may be attributed to the nutritional utility of these compounds as a nitrogen source in the biosynthesis of cellular proteins. As was mentioned there are speculations that the protein synthesis of swarmer cells of P. mirabilis is incomplete compared to ordinary short rods (Armitage et al. 1976). Therefore the presence of urea or some of its analogues may enhance the nitrogen content of the medium and contribute to the process of protein synthesis in the cell envelope which consequently promotes the division of swarmer cells and production of non-swarming short rods. This seems however unlikely to be the case since most of the urea analogues would not be degraded and provide nitrogen.

An interesting finding in this study was that the bacteria which have previously been grown on nutrient agar containing thiourea exhibited much less swarming ability than the original cultures when they were cultured on urea agar (Table 24). This also happened with strains grown on S-methylthiourea agar (Table 25). These results are in contrast

with the habituation phenomenon and indicate that the denaturing effect of the may have injured the organism which is not able to demonstrate its normal properties or that a mutant has been selected.

Guanidine (hydrochloride) also exhibited an inhibitory effect on swarming strains of P. mirabilis. Its effect seemed to be stronger than urea and complete swarming inhibition occurred at the concentration of %2.5 (Table 27). This property of guanidine may also be due to its denaturing activity on cellular proteins which has been reported to be stronger than urea (Greenstein 1939, Greenstein and Edsall 1940). Guanidine is normally used as an antiviral agent against RNA viruses such as Picornaviruses. It is believed that guanidin prevents the multiplication of these viruses by denaturing the RNA molecule of the virus which is similar to the effect of urea on tRNA reported by Hegg and Thurlow (1990).

Unlike urea the methyl derivative of guanidine seemed to be slightly more effective on swarming of P. mirabilis strains (Table 27).

IIII.6. The effect of urea on the motility, flagellation and size of P. mirabilis strains:

The influence of urea on the motility of P. mirabilis strains was observed using hanging drop and microslide test. The size (filamentation) and flagellation was also checked by flagella staining and electron microscopy. The results showed

that urea exhibited significant reducing effects on motility, size and flagellation of the strains tested and the effect was concentration dependent (Table 30). These results indicate that urea not only inhibits swarming ability of the strains, but the formation of long swarmer cells is also affected ie that urea does not simply stop swarmers moving outwards. It seems however that swarming is the first (among these three properties) to be impaired by urea, because at low concentrations of urea when the swarming was significantly reduced (but not stopped) the swarming cells were present and highly flagellated. Accordingly part of the urea effect but not all is simply on the concerted movement of the swarmers.

As mentioned earlier, since urea penetrates poorly into the bacterial cell, therefore its denaturing property is most likely effective on outer membrane components such as pili and flagella. The decreased number of flagella may occur due to repressing of new flagella formation, impairing the functions of already produced flagella and inducing the production of damaged flagella. Electron microscopy of the samples showed that at the concentration where the cells were still motile, flagella were apparently intact and normal, but with the increased concentration completely immobilized cells appeared which did not possess any flagella. Therefore it seems likely that urea affects the cell envelope by impairing flagellar formation through its denaturing effects.

IIII.7. Plasmids and adherence of P. mirabilis strains:

The attachment of plasmid-carrying and plasmid-free isolates of P. mirabilis to glass cover slips, glass beads and uroepithelial cells was studied. In all cases plasmid-bearing strains were more adherent to the surfaces tested than their plasmid-minus counterparts. Loss of plasmid reduced the adherence property of the strains and plasmid-gain by transconjugants enhanced it (Tables 31 and 32).

There are several ways in which attachment could be enhanced by plasmids. Firstly, the negative surface charge which repels the close approach of cells to each other or surfaces could be reduced by the presence of a plasmid. This might be examined by comparing electrophoresis of p+ and p- strains as has been done for F+ and F- ones (Maccacaro 1961). Secondly, increased hydrophobicity can enhance attachment and since each tested plasmid enhanced hydrophobicity this may be a more likely factor in the plasmid effect. Thirdly, these plasmids might confer specific attachment organelles (eg. fimbriae) or surface substances which are "sticky" like polysaccharides.

There are reports suggesting that R-plasmids are associated with the expression of different types of fimbriae (Williams et al. 1978, Ofek and Beachey 1980) which are believed to be involved in bacterial adhesion of E. coli (Clegg and Gerlach 1987), Salmonella spp (Old and Tavendale

1986) and P. mirabilis (Wray et al. 1986) to surfaces. The measurement of bacterial attachment to inert surfaces such as cover slips or glass beads has been considered as a model for their adhesion to body tissue (Harber et al. 1983). Hicks and Rowbury (1986 and 1987) carried out extensive studies on the involvement of Col V plasmids in the adherence of E. coli to sands and glass beads. They observed that ColV+ strains attach to a greater extent to glass beads than the ColV- counterparts and suggested that surface fimbriae play an important role in this property.

R-plasmid RP1 was reported to promote the adhesion of clinical strains of P. mirabilis to medical prostheses (Onaolapo et. al. 1987). This enhanced adherence was attributed to the increased negative charge on the surface of plasmid-containing isolates. In the present study electron microscopy showed that p+ strains seemed to be more piliated than p- ones. The presence of specific organelles or new polysaccharides could be examined in more detail by isolating and anlayzing the envelope for new proteins or polysaccharides or examining the growth medium of vigorously shaken cultures since organelles are frequently removed by shaking and surface polysaccharides often appear in the medium. If any of the tested p+ strains produce enhanced attachment due to pili or fimbriae, bacteriophages which use these for entry, and cannot attach to cured strains , could be isolated eg. from

sewage and used to show up the organelles (if they coat their surface) as was done for F pili (Brinton 1971). In fact as shown in Table 33 the percentage of potentially adherent cells particularly from plasmid-bearing strains was markedly reduced in overnight shaking grown culture. These results indicate that fimbriae may be effectively involved in the adhesion of P. mirabilis to surfaces. In a study by Wray et al. (1986) on the adherence of P. mirabilis strains to uroepithelial cells, a single protein with molecular weight 17500 was isolated which was suggested to be the main factor in bacterial adhesion. Molecules of this adhesin were seen to assemble together on the surface of the bacterial cell and form a flexible rod shaped appendage.

R-plasmids may also code for surface compounds such as specific receptors or hydrophobic molecules (Lomberg et al. 1982) which are said to be associated with bacterial adhesion to body tissues.

Ellwood et al. (1982) speculated that attachment of bacteria to surfaces is energetically beneficial to them especially in nutrient deficient medium. Therefore as the presence of plasmids increases the nutritional requirement of bacteria (Melling et al. 1977, Klemperer et al. 1979), the increased attachment of plasmid-carrying strains may have evolved as a solution to supplying the extra energy requirements of the p+ cell.

It has been reported that multiresistant clinical isolates of P. mirabilis are more adherent to uroepithelial cells than the sensitive or less resistant strains (Cellini et al. 1987). This is in accord with the results obtained here that multiresistant plasmid-bearing strains (PM5, P49 and P991) exhibited higher adhesive property than their plasmid-minus derivatives. Possibly factors enhancing attachment are encoded by genes closely linked to resistance genes and therefore tend to be carried together.

IIII.8. Effect of plasmids on hydrophobicity of P. mirabilis:

Hydrophobicity of plasmid-containing and plasmid-free strains of P. mirabilis from clinical origin were tested using the hydrophobic interaction chromatography (HIC) method in the presence of different concentrations of ammonium sulphate. Results showed (Table 34) that plasmid carriage confers greater hydrophobic property to the host cell. With the increased concentration of ammonium sulphate the hydrophobicity of both p+ and p- strains was increased, but this salt was more effective on p+ isolates. Many surface properties such as autoagglutination and bacterial adherence to epithelial cells, phagocytes, plastics, and hydrocarbons have been attributed to surface hydrophobicity (Jann et al. 1981, Rosenberg et al. 1982). Hence, it is considered to be an important factor influencing phagocytosis, microbial invasion

and pathogenicity (Loosdrecht et al. 1987).

The increased hydrophobicity of p+ P. mirabilis strains tested in this study and particularly the increase in this property in p+ transconjugants and its reduction in cured strains indicates the important role of these elements in hydrophobicity. This effect could be due to changes in surface molecules and ligands on the cell wall. For example, the plasmid may introduce envelope molecules which have hydrophobic regions presented at the cell surface. It has been suggested that sex pili may also be involved in the increased hydrophobicity (Tewari et al. 1985) and this could occur if the exposed amino acids on the pilus surface were predominantly hydrophobic ones. Rosenberg et al. (1982) studied the hydrophobicity of smooth strains of P. mirabilis and their rough mutants R45 and R51. They observed that rough mutants exhibited a higher hydrophobicity than their smooth parental strains. This difference was greater when hydrophobicity was measured by adherence to xylene. It was suggested that the increased hydrophobicity in rough mutants of P. mirabilis was due to differences in localization of phospholipid molecules in the outer and inner layer of the cell wall. In other words, the phospholipid content of the outer membrane in smooth strains is mostly located in the inner leaflet and is not exposed to the environment, whereas in deep rough mutants phospholipid molecules are present in

both layers of this membrane (Nikaido and Nakae 1979). It is also suggested that the carbohydrate chains of lipopolysaccharide molecules may shield the surface of smooth strains of P. mirabilis and reduce the hydrophobic property of the cells (Hassin et al. 1976).

The involvement of pili is considered to be a major factor in bacterial surface hydrophobicity. Smyth et al. (1978) found that strains of E. coli possessing such pili as the K88 antigen adsorbed to a greater extent to octyl and phenyl-sepharose than their corresponding K88- strains. Bradley (1983) reported that plasmid RP1 codes for a type of rigid constitutive pilus which is suggested to increase surface hydrophobicity in P. mirabilis (Onaolapo and Klemperer 1986).

As bacterial surface hydrophobicity to hydrocarbons has been taken as a likely indicator of their adherence to mammalian cells, a role for plasmids in the virulence of P. mirabilis is suggested by the results obtained in this study.

IIII.9. Plasmids and the growth of P. mirabilis:

The growth of plasmid-bearing and plasmid-free strains of P. mirabilis in batch cultures of nutrient broth at 37°C in static and shaking conditions was examined by testing their optical densities at intervals. A total of nine strains consisting of three p+ isolates (PM5, P49, P991) and their cured derivatives as well as the p- recipient G9 and two

transconjugants (G9pPM5, G9pP49) were tested in this experiment.

As mentioned earlier, the presence of plasmids reduced the growth of test strains in both static and shaking conditions (Tables 35 and 36). This was indicated by the difference in optical densities of the cultures. In other words, in a limited amount of nutrient and oxygen plasmid-bearing strains grew slower than plasmid-free isolates and in the stationary phase gave less yield.

The in vitro conditions for bacterial growth are always different from those of the body and mostly insufficient for the expression of all virulence determinants. This is more apparent when bacteria are grown in batch cultures in which the nutritional factors are limited. Therefore bacteria which are grown in standard medium for some generations exhibit immunogenic and virulence properties potentially much different from those grown in vivo (Brown and Williams 1985). They often lose virulence by growing in vitro which can be restored after several passages through the animal body (Smith 1983). Growth conditions significantly affect both the structure and physiology of the cell envelope hence lack of essential nutrients induces dramatic changes in this region of the bacterial cell particularly the outer membrane which is considered to be the permeability barrier of Gram-negative bacteria (Hancock 1984).

Effect of R-plasmids on the growth rate and on the structure of the Gram-negative bacterial cell wall has been reported by several workers. Klemperer et al. (1979) reported that strains of E. coli harbouring R-plasmid RP1 have greater nutritional requirement for several nutrients like PO_4^{3-} and Mg^{2+} than their corresponding plasmid-less strains. This may be because such cells have differences in phospholipid content of the outer membrane rather as rough and smooth strains of P. mirabilis do (Rosenberg et al. 1981). It is suggested that E. coli strains harbouring R-plasmids have longer generation time than plasmid-minus ones, and this prolonged time was dependent on the size of the plasmid carried (Zund and Lebek 1980). The results obtained here showed (Table 35 and 36) that the transconjugant G9pPm5 which carries a 105 Md plasmid grows more slowly than strain G9pP49 with a smaller plasmid of 32 Md molecular weight.

As mentioned earlier the three p+ strains used in these experiment were originally non-swarmer, whereas the p- derivatives and the recipient were of swarming type. Therefore the significant difference between the growth of p+ strains in static and shaking conditions may indicate the high oxygen requirement in these strains compared with p- isolates. This may be in fact related to the swarming nature of p- strains which is similar to the finding by Armitage (1981) with swarming and non-swarming strains of P. mirabilis. This was

also later observed by Falkinham and Hoffman (1984).

IIII.9.a Plasmids and growth of P. mirabilis strains in iron depleted nutrient broth:

One important nutrient which plays an essential role in bacterial growth is iron (Neiland 1974). Usually in laboratory media the iron level provided is enough to meet the bacterial requirement, but in the animal and human body it is typically unavailable to them. In this study the growth of plasmid-carrying and plasmid-less strains of P. mirabilis in iron deficient nutrient broth has been investigated. The results showed that the growth rate of both p+ and p- strains in this medium was much lower than that in ordinary nutrient broth in which the iron content was approximately 3.6 mg/l. This shows that both the p- and p+ organism have a high requirement for iron. Moreover, plasmid-free strains grew better than plasmid-bearing isolates which indicates that the presence of plasmids may increase the iron requirement of the host cell. This effect was greater in static growth conditions. Some plasmids are said to be involved in the iron uptake system of bacteria. For example the presence of ColV plasmids in bacteraemic strains of E. coli is reported to increase the uptake of iron which enhances the virulence of the organism in the host body (William 1979). Therefore even if ColV+ strains of E. coli have a higher iron requirement than ColV- isolates, their growth would not be greatly affected when grown in iron

limited medium because of their ability to take up more iron. In contrast, plasmid RP1 was observed to have increased the iron need of E. coli compared to strains lacking this plasmid and radioactive iron was taken up rapidly by non-growing plasmid-carrying strains (Klemperer et al. 1979).

Iron deficient conditions induce the expression of new outer membrane proteins (Lugtenberg and Van Alpen 1983) with corresponding changes in drug resistance of bacteria (Gilbert and Brown 1978).

IIII.9.b. Effect of detergents on the growth of p+ and p- strains of P. mirabilis:

The effect of different concentrations of sodium dodecyl sulphate (SDS), a surfactant detergent, on the growth of three plasmid-containing strains and their cured derivatives as well as on transconjugants was studied. SDS was shown to inhibit the growth of both p- and p+ strains of P. mirabilis (Tables 39-42) and the effect was concentration dependent. Initially p+ strains grew slower than p- isolates, but with the increased concentration and longer incubation time they grew potentially better in the medium. For example the optical density of PM5 at 50 µg/ml concentration of SDS was 0.44 and that of the PM5c was 0.85, whereas the O.D. of these strains at concentration of 200µg/ml was 0.36 and 0.23 respectively.

SDS is reported to have been the most effective detergent as an inhibitor of the growth of E. coli comparing with sodium

deoxycholate (DOC) and sarkosyl and the wild type of this organism was shown to be more resistant than its envelope mutant DC2 (El-Falaha 1989). This sensitivity was suggested to be due to changes in cytoplasmic or outer membrane. Clark (1984) reported increased content of ethanolamine molecules in the lipopolysaccharide of the cell wall in SDS resistant strains of E. coli.

The effect of SDS on the growth and motility of P. mirabilis was observed by Kopp and Muller in 1965. They reported that at the concentration 0.5 millimolar of SDS the growth of P. mirabilis was impaired and it was completely stopped at 1 millimolar concentration. They also stated that of the three tested anionic detergents (sodium butyl, sodium hexadecyl and sodium dodecyl sulphate), SDS was the most active agent on the growth and motility of P. mirabilis. Norris et. al. (1985) reported that the minimum inhibitory concentration (MIC) of SDS for P. mirabilis strains tested was ranging between 160 to 400 µg/ml. Results obtained here show significant reducing effect of SDS on the test strains particularly at concentration of 200 µg/ml in nutrient broth. The more prolonged growth of plasmid-bearing strains may be due to reduced permeability of the cell envelope for detergent. Armitage et al. (1975) observed that the minimum concentration of DOC required for complete growth inhibition in swarmer cells was half that needed for inhibition of

ordinary short forms of this organism. Therefore more sensitivity of p- strains to SDS could be because of their swarming nature. This difference may be due to stronger binding between the cell wall components such as phospholipid molecules (Hirota 1970) in short rods which could also be associated with the presence of R-plasmids (Kenward et al. 1978).

Some plasmids are reported to have increased the susceptibility of P. mirabilis to DOC (Hesslewood and Smith 1974). This reduced resistance was attributed to the decreased level of mucopeptide bonds of the outer membrane.

IIII. 10. Plasmids and motility of P. mirabilis:

The motility and flagellation of p+ and p- strains of P. mirabilis was observed by hanging drop test, flagella staining and electron microscopy after growth in nutrient broth at 37°C. The results obtained indicated that the wild type plasmid-containing cells were less motile and less flagellated than their p- derivatives (Table 43). Likewise, the motility and flagellation of recipient strain G9 seemed to have decreased significantly by introduction of the plasmids, and this difference was more marked in transconjugant G9pPM5 possibly due to the larger size of the plasmid. Moreover, the number and size of clumps was greater in p+ isolates. This reduced motility and increased clumping was time dependent so that in overnight cultures the highest number and size of

clumps and the lowest rate of motility was observed. But there was no considerable change in the flagellation of free cells during incubation. Therefore the reduced motility can be due to autoagglutination and decreased number of free cells. This effect of plasmids on motility and flagellation may be associated with the changes which occur in the envelope properties. These are indicated by increased adherence and formation of clumps (Tables 31 and 32), increased hydrophobicity (Table 34) and reduced swarming ability on solid media (Table 5 and 12).

IIII.11. Effects of SDS on the motility and clumping of p+ and p- strains of P. mirabilis:

The activity of the surfactant detergent SDS on the motility and autoagglutination of plasmid-bearing and plasmid-free strains of P. mirabilis was observed by examining overnight cultures in nutrient broth containing different concentrations of SDS. The results showed that at low concentration of SDS (eg. 50 µg/ml) the motility of both p+ and p- strains was slightly increased and the number of clumps was decreased and they were dissociated (particularly in p+), but at higher concentrations both clumping and motility were greatly reduced (Table 44). The effect was significant on the motility of p- strains which were originally more motile than p+ isolates.

The initial increase in motility may be due to the

dissociation of clumps presumably because SDS reduces attachment, which releases large numbers of free motile cells into the medium.

The inhibitory effect of SDS on the motility and autoagglutination of P. mirabilis is related to its activity on cell envelope compounds particularly protein complexes of the outer membrane (El-Falaha et al. 1989). Therefore, surface appendages such as pili and flagella are the first structures to be impaired by the biological activity of surfactant agents. This could be the reason why the concentration of SDS required to abolish motility is much lower than that needed for growth inhibition. SDS is reported to be the most active agent on the motility of P. mirabilis, as at a concentration of 0.05 mM it inhibited motility up to 50% and complete inhibition occurred at 0.1-0.2 mM concentration of this agent (Kopp and Muller 1965). Other detergents such as sodium butyl and sodium hexadecyl sulphate were not able to exhibit such activity. It has been reported that the addition of casein peptone to the medium reduced the impairing effect of SDS on motility of P. mirabilis. This suggests that peptone caseinate might be utilized by bacteria to repair the damaged proteins on the cell wall or to prevent the damage which may be caused by the agent.

Electron microscopy of the samples after SDS treatment showed that non-motile cells were sparsely flagellated but the

flagella present did not seem to be damaged. Koffler et al. (1957) reported the digestion of mesophile flagella in the presence of 3.5 mM concentration of SDS. Almost similar observations were reported in Salmonella typhimurium by Kerridge et al. (1962).

IIII.12. Plasmids and survival of P. mirabilis strains in serum:

Survival of plasmid-carrying and plasmid-less strains of P. mirabilis in human and rabbit serum was tested using serum diluted down to 30% of normal concentration. It was shown that plasmid-free strains survived better in human as well as rabbit serum (Tables 45 and 46).

Some drug resistance plasmids are reported to have increased the sensitivity of their host cells to the bactericidal action of human and animal serum (Wretlind et al. 1985, Onaolapo and Klemperer 1987). Thus R-plasmid RP1 decreased the resistance of a clinical strain of P. mirabilis to guinea pig and human serum and the effect was more significant in the absence of iron. This increased sensitivity was attributed to enhanced phagocytosis. The outer membrane of R-factor-containing strains is different from that of plasmid-minus isolates especially when grown in iron-depleted medium and this difference which is partly in fatty acid content of phospholipid molecules (Onaolapo 1986) may explain the serum resistance effect of plasmids. Serum complement proteins are

suggested to attack the hydrophilic sites of the outer membrane which are believed to be associated with lipid structures of this membrane (Taylor 1983) and this attack might be affected by plasmid changes in the cell envelope. It is also reported that ammonia released by urease activity of P. mirabilis strains in kidney tissue inactivates the complement system (Maclaren 1968). In this study, the semi-quantitative test of urease production by P. mirabilis strains showed that p- strains release more ammonia in the medium which can be more protective in the presence of serum complements.

R-plasmids also decreased resistance of Pseudomonas aeruginosa to human and rat serum (Wright et al. 1977) which was suggested to be due to changes in the cell envelope (Wretlind et al. 1985).

Serum antibodies are said to affect the iron uptake system of the bacteria (Brown and Williams 1985). As mentioned earlier the iron requirement of plasmid-bearing strains of P. mirabilis is greater than that of plasmid-free counterparts, which could be a reason for their lower growth in the presence of serum antibodies, since iron is not usually readily available in the body fluids.

Monoclonal antibodies have also been shown to be highly reactive with P. mirabilis strains isolated from different sources (Harmon et al. 1989). Most of these antibodies were

active against the LPS of the outer membrane. This may explain the better survival of the test strains in rabbit serum, since the rabbit serum was taken from a rabbit kept in an animal house where it may have been exposed to relatively little bacterial challenge. Phenotypic changes in surface hydrophobicity have been reported to be associated with serum resistance in mutant strains of E. coli (Svanborg-Eden et al. 1987). Some plasmids are reported to increase the host resistance to antibacterial action of serum, like ColV plasmids which increased the resistance of E. coli in human serum (Binns et al. 1979) and allowed strains to grow better in intraperitoneal chambers (Finn et al. 1982).

IIII. 13. Plasmids and survival of P. mirabilis strains in aquatic environments:

Survival of p+ and p- isolates of P. mirabilis in sewage, sewage effluent and river water was examined by growing them for at least five days in a 37°C shaking water bath. The results showed that plasmid carriage reduced survival in all three tested environments. There are controversies over the effect of plasmids on the survival of the host in media in which growth is under stress eg. in starvation conditions, at extreme temperature or in the presence of competing organisms. Godwin and Slater (1979) studied the effect of growth environments on E. coli K12 strain carrying the drug resistance plasmid Tp120. They suggested that in a non-

selective medium the presence of a plasmid is not only useless but it becomes a burden to the host, because it uses for unnecessary plasmid functions, nutritional sources which could be utilized in more vital physiological processes associated with survival of the microorganism in that particular environment. By contrast it seems that in selective media, especially those containing growth inhibitors such as antibiotics, the persistence of drug resistance plasmids is required to protect the host cell and the strains lacking these elements are eliminated. But in a drug-free environment with nutritional limitation these plasmids appear to be redundant and the environmental conditions can be against the plasmid-carrying strains, because the replication of plasmid DNA and the production of its gene products (proteins and other cell components coded by them) requires nitrogen and energy sources which otherwise can be used in bacterial multiplication and growth. Melling et al. (1977) showed that in chemostat continuous culture, an E. coli strain carrying plasmid RP1 was not able to compete with an isogenic plasmid-free strain and gave a lower growth rate. This indicates that p- strains grow more freely than their p+ counterparts. Zund and Lebek (1980) reported that the presence of plasmids in E. coli strains increased the generation time. There was a correlation between the molecular size of the plasmid and the increased generation time and plasmids larger than 80 Kb of

molecular weight were more effective in this process. It is also believed that increased copy number of plasmids may be involved in increasing the generation time of bacterial cells (Nordstrom et al. 1977). The effects of plasmids on the surface properties of the cell envelope may also influence the survival of bacteria in environments containing other growth competitors such as protozoa (Wijsin and Alexander 1988). In such environments due to an enhanced adherence property, plasmid-bearing strains may exhibit more affinity towards phagocytic organisms resulting in their phagocytosis and ingestion. Chao and Feng (1990) reported that the transfer of R-plasmids to E. coli strains reduced their subsequent survival time in river water. But when the water was filter-sterilized they survived longer than in non-sterile water. The sewage, effluent and river water used in this study were not sterilized and as mentioned earlier these media can contain a wide range of other organisms including protozoa which are also deliberately added for sewage treatment. Sinclair and Alexander (1984) studied the significance of resistance to starvation in the ability of bacterial species to survive in sewage and lake water. Most of the test bacteria declined by up to four times in non-sterile conditions. In sterile lake water due to poor nutritional value they failed to survive long, but in sterilized sewage they managed to survive much longer. This was attributed to their ability of utilizing

organic compounds present in sewage in the absence of competitor organisms. The role of starvation resistance has also been studied by many other investigators (Postgate and Hunter 1962, Strange 1968, Novitsky and Morita 1977). The plasmid-mediated changes in envelope permeability might also reduce the diffusion of nutritional factors into the cell (Zund and Lebek 1980). Therefore, although the presence of R-plasmids in bacterial species increases their resistance to antimicrobial agents, in nutrient-deficient environments they may render the host more susceptible to starvation resulting in weakened survival ability in such media. This may be the reason why, for plasmid-containing organisms grown in non-selective nutrient limited environments, the plasmid is lost after a few generations from all or part of the population (Freter et al. 1983, Devanas et al. 1986).

The long survival times of a small number of organisms of plasmid-bearing strains of P. mirabilis tested in this study may be due to their ability to attach to surfaces (Tables 31 and 32) and therefore show increased nutrient uptake and reduced permeability to toxic wastes present in sewage and its effluent (Saz et al. 1963, Liang et al. 1982). Some plasmids are reported to confer increased survival on their host cells in some environments such as soil (Devanas et al. 1986) and mammalian intestine (Levy et al. 1980).

IIII. 14. The significance of plasmids in the production of extracellular enzymes in P. mirabilis strains:

The role of plasmids in secretion of some extracellular enzymes by urinary and faecal strains of P. mirabilis as well as their presence in bacterial supernatant was studied. Thirty-two plasmid-carrying and plasmid-free isolates were tested in each experiment.

IIII. 14. a. Plasmids and production of urease:

As was mentioned earlier, the qualitative 24 hours urease test of all 145 strains was positive indicating the involvement of chromosomal determinants in the production of this enzyme. Further quantitative experiments on urea agar showed that the presence of plasmids greatly reduced either the production or the release of extracellular urease in these strains. The presence of urease in liquid medium (nutrient broth containing 2% urea) was determined by measuring the increased pH of the medium due to the release of ammonia from urea degradation. The results obtained by this method confirmed the reducing effect of plasmids on urease activity of the strains. This effect was shown in more detail by the findings that the loss of a plasmid by curing gave a strain which showed increased pH of the medium compared to its parent whereas in transconjugants, the pH of urea broth was less than that for the p- parent.

It has been reported that urease in P. mirabilis is urea

inducible (Rosenstein et al. 1986) and is closely associated with the periplasmic components particularly peptidoglycan fraction (McLean et al. 1986). On the other hand it is well documented that R-plasmids mediate changes in the physiology and structure of the cell envelope (Hirota 1970, Hesslewood and Smith 1974, Kenward et al 1978). One type of change is to reduce the cell permeability for uptake of external inhibitory agents such as antibiotics and toxins. This decreased envelope permeability might also affect the release of some macromolecules like enzymes with high molecular weight into the environment, by inhibiting their passage through the cell membranes. Urease is one of largest bacterial enzymes with a molecular weight up to 800,000 (Wals et al. 1988). This may explain the results obtained here. Falkinham and Hoffman (1984) reported that the urease activity of swarmer cells of P. mirabilis was constitutive and significantly higher than the ordinary short form of this organism. This might be due to increased permeability of the envelope of the swarmers (Armitage et al. 1979).

IIII.14.b. Plasmids and haemolysin production:

The study of haemolytic activity of 32 plasmid-bearing and plasmid-minus strains of P. mirabilis on horse and sheep blood indicated the enhancing effect of plasmids on the secretion of haemolysin. This effect was more clear when three p+ pairs (PM5, P49, P991) and their cured derivatives as well

as recipient strain G9 and its transconjugants (G9pPM5 and G9pP49) were studied. For instance the mean size of haemolytic zones in cured strains was 5.1 mm whereas this figure in their parental p+ isolates was 12.1 mm. There was no difference between the haemolytic activity of faecal and urinary strains. There is evidence that in a large majority of urinary and faecal bacteria the production of haemolysin is determined by chromosomal genes (Muller et al1983). But it has been shown that the genetic determinants for haemolysin production can also be carried by extrachromosomal elements (de la Cruz et al. 1979). Noegel et al. (1981) reported the presence of three transmissible plasmids in an alpha-haemolytic strain of E. coli with the molecular weight of 65, 41 and 32 megadaltons belonging to incompatibility groups J2 and N. By separately transforming these plasmids to E. coli K12 it was found that the 41 megadalton plasmid (pHly152) was responsible for haemolytic activity of the organism. De la Cruz et al. (1980) by using DNA fragments of this plasmid as hybridization probes found that a section of 3.8 megadalton was common in several haemolytic plasmids belonging to four different incompatibility groups. Koronakis et al. (1987) reported clear homology between genetic determinants coding for haemolysin production in strains of Proteaee including P. mirabilis and alpha-haemolysin in E. coli. The data presented here strongly indicates that the production of haemolysin in test strains of

P. mirabilis is chromosomally determined but its transport or secretion may be affected by plasmids; it is however, possible that, for some of the p+ strains, there is plasmid-encoded as well as chromosomally-encoded enzyme. Studies of the properties of this enzyme would be needed to establish or disprove this possibility. The decreased haemolytic property of bacterial supernatant suggests that even after secretion this enzyme remains highly cell associated.

IIII. 14. c. Plasmids and protease production:

The proteolytic activity of 32 plasmid-carrying and plasmid-free strains of P. mirabilis was studied by degrading milk proteins in nutrient agar plates containing 10% skimmed milk. The results obtained showed that although all strains exhibited proteolytic activity the production of protease in p+ strains was significantly higher than that of p- isolates (Table 54). There was no appreciable difference between the extent of protease secretion by a bacterial colony and the corresponding supernatant. The production of protease by strains of P. mirabilis has been reported by several investigators (Hampson et al. 1963, Senior et al. 1987 and 1988, Loomes et al. 1990). These reports indicate the capability of this protease of degrading immunoglobulins. But another type of proteinase which is able to clot milk proteins has been observed in P. mirabilis (Klessen et al. 1989). Suzuki et al. (1987) suggested that production of protease is

an envelope property and involved in bacterial virulence. The involvement of plasmids in production of this enzyme was shown by Hesslewood and Smith (1974). They reported that introduction of plasmid R6K to a laboratory strain of P. mirabilis reduced swarming and increased proteolytic enzyme secretion of this organism, whereas plasmid R46 reduced the enzymatic activity and enhanced swarming. It was suggested that these plasmids affect the mucopeptide bonding of the cell envelope in different ways. It was mentioned earlier that the plasmids tested here decreased swarming distance of the strains and increased the secretion of protease. The plasmids may simply aid secretion or there may be both chromosomally and plasmid-encoded protease.

IIII.14.d. Plasmids and lecithinase production:

The secretion of lecithinase was also observed in 32 p+ and p- clinical and faecal strains of P. mirabilis using egg yolk agar. The lecithinase activity of some plasmid-bearing strains such as G12 and G32 seemed to be relatively high, but the results obtained did not indicate that plasmids play a particularly significant role in the production or secretion of this enzyme (Table 55).

IIII.14.e. Plasmids and DNase and Amylase production:

None of the test strains exhibited amylase activity and for the few strains which were seen to produce DNase, it did not seem to be associated with the presence of plasmids.

Chambers (1975) reported that DNase was found to be released from swarmer cells of Proteus strains, but only when they are killed.

IIII. 15. Conclusion and future works:

The results obtained in this study showed that plasmids have significant effects on different potential virulence properties of P. mirabilis. Most of these effects are suggested to be due to alteration of envelope properties. Although the plasmids studied here have not been shown to alter specific outer membrane components, there is much indirect evidence for envelope changes. Thus each tested plasmid enhanced attachment, surface hydrophobicity and clumping, all betokening envelope changes.

For the future, it would be of interest to examine:

A. Outer membrane protein composition of the p+ and p- pairs by SDS PAGE to acertain whther specific plasmid-encoded proteins and/or lipopolysaccharides are inserted into the outer membrane.

B. The possibility that any of the plasmids cause increased antibiotic sensitivity by enhancing outer membrane permeability.

C. Whether the presence of large amounts of transfer components is linked to failure of swarming ie. whether drd plasmids are particularly effective in inhibiting swarming. This may not be the case since plasmids pPM13 and pPM14 were

ineffective donors but showed very poor swarming.

D. The exact mechanism of the inhibitory effect of urea and its derivatives on swarming.

E. The effects of plasmids by which they enhance the synthesis or secretion of some extracellular enzymes and inhibit that of others.

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