ASPECTS OF HIGH-LEVEL TRIMETHOPRIM RESISTANCE IN GRAM-NEGATIVE BACTERIA ISOLATED IN SOUTH AFRICA

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ABSTRACT

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Trimethoprim is a broad-spectrum antimicrobial agent frequently used either in combination with sulphamethoxazole (cotrimoxazole) or alone in the treatment of urinary and respiratory tract infections. Since the introduction of this drug in 1969 resistance to it has been monitored in several centres in Europe continuously but only intermittently in the United States of America and developing countries in Africa, Asia, Central and South America. In Europe the incidence of trimethoprim resistance has increased significantly in the last 20 years. In developing countries no trends have been established but the incidence of resistance appears to be greater in these countries than in Europe or the USA.

The mechanisms mediating resistance to trimethoprim have been investigated. Generally alterations of the chromosomal DNA of the organism result in intermediate resistance (trimethoprim minimum inhibitory concentration (MIC) 16 to 1 024 mg/l) while high-level resistance (MIC >1 024 mg/l) most frequently

due to plasmid-mediated dihydrofolate reductases (DHFRs) which are resistant to trimethoprim. High-level resistance is important not only because the organisms are resistant to greater concentrations of trimethoprim than are achievable in body fluids but because the genes mediating resistance are transferable from one organism to another. They have been found on both plasmids and transposons and, more recently, integrated into the bacterial chromosome.

In 1982 a survey was undertaken to determine resistance to cotrimoxazole in isolates from the Johannesburg General Hospital and Baragwanath Hospital. The results showed a higher incidence of resistance than in equivalent hospitals in Europe and the USA at that time. While this gave some indication of the incidence of resistance to trimethoprim the results measured resistance to cotrimoxazole and could not, therefore, be considered accurate for trimethoprim. The present study was undertaken in order to establish the incidence of resistance to trimethoprim alone in gram-negative bacteria and to investigate the genes mediating high-level resistance in these organisms.

A three-month survey of trimethoprim resistance in gram-negative isolates from the Johannesburg, Hillbrow and Baragwanath hospitals was performed. A total of 56.2% of the isolates was resistant to trimethoprim (MIC >8 mg/l) while 42.8% of the resistant isolates was resistant to >1 024 mg/l

of trimethoprim. This was higher than the incidence observed in Europe and the USA but similar to that found in other developing countries. Preliminary hybridization studies using type I and type II DHFR probes indicated that the occurrence of these two genes in South African isolates was less frequent than expected. An investigation of the DHFRs of highly resistant organisms which did not hybridize with either of the probes resulted in the isolation and characterization of a novel enzyme. This was called DHFR type VI. The type VI DHFR gene, which was located on a self-transmissable plasmid, was cloned into the plasmid and bacteriophage vectors pUC18, M13mp18 and mp19. The gene was sequenced and analyzed. Although the type VI DHFR gene was identical in size to both the type I and type V DHFR genes there was an overall amino acid homology between the types I and VI of only 63% and between the types V and VI of 61% . The subunit structure of the type VI DHFR and the incidence of this gene in the bacterial population are currently being investigated.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

B.A.Wyke

7th day of February, 1991.

To my parents with love

and gratitude

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PUBLICATIONS

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1.1 Structure, mechanism of action and properties of trimethoprim

1.1.1 Structure of trimethoprim

Trimethoprim (Tp) is a synthetic antimicrobial agent belonging to the diaminopyrimidines. Its structure is shown in Figure 1.1.

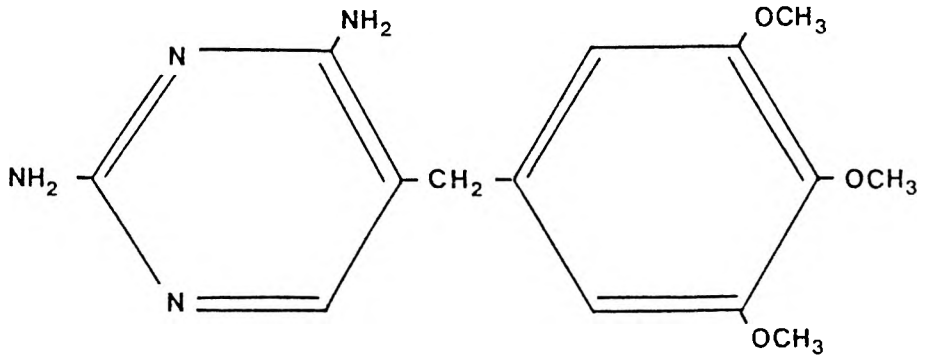


Figure 1.1 Structure of trimethoprim - 2,4-diamino-(3',4',5'-trimethoxybenzyl) pyrimidine (from Bushby & Hitchings, 1968).

1.1.2 Mechanism of action of trimethoprim

The mechanism of action of trimethoprim is based on a general property of all diaminopyrimidines viz. inhibition of folate reductases. In bacteria

dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), derivatives of which are required as catalysts in the manufacture of at least two amino acids (methionine and glycine), for purine synthesis and initiation of protein synthesis. In addition 5,10-methylene tetrahydrofolate is required as a substrate in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by thymidylate synthetase (Hitchings, 1973). These interconversions are detailed in Figure 1.2.

By inhibiting the production of tetrahydrofolate trimethoprim was shown to have a bactericidal effect when an organism (Escherichia coli) was grown in complex medium lacking thymine derivatives (Amyes & Smith, 1974a). A bacteristatic effect was observed, however, when the organism was grown in minimal medium (Then & Angehrn, 1973; Amyes & Smith, 1974b) but the addition of methionine to the minimal medium resulted in a bactericidal effect. The reversal of bacteriostasis was found to be dependent upon continued protein synthesis which deprived the cell of 5,10-methylene THF. Since no dTMP could

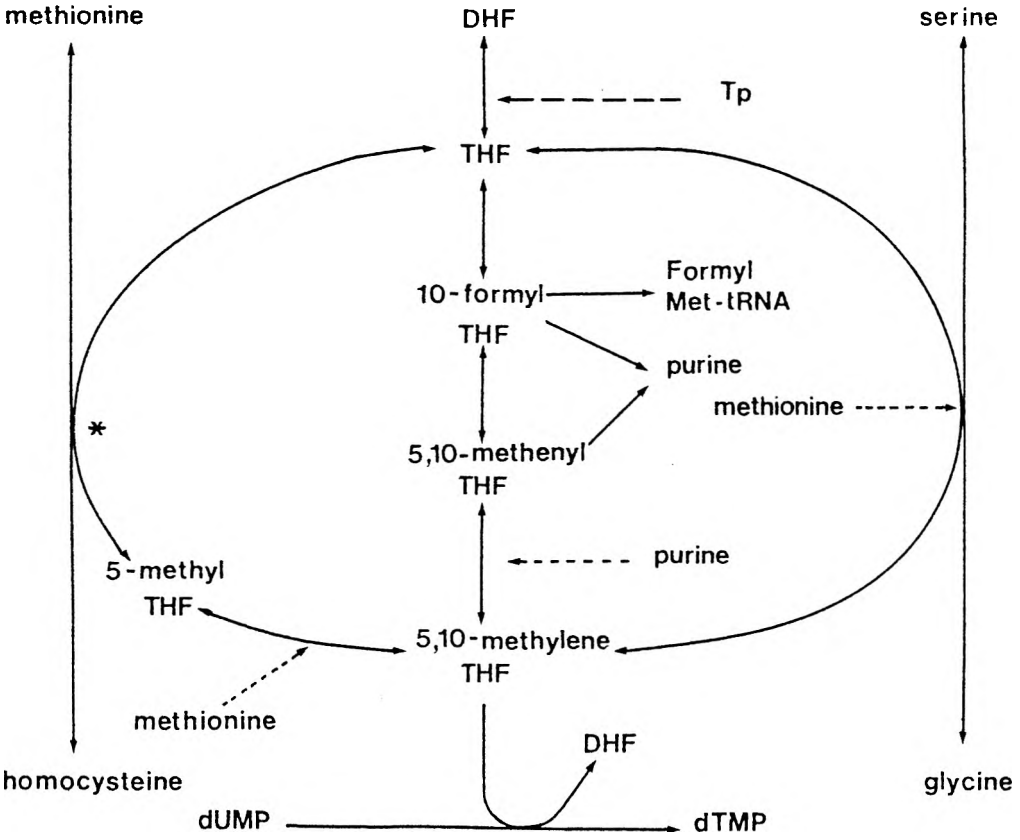


Figure 1.2. Interconversions of THF intermediates and their relationship with purine and protein synthesis. Points of repression are indicated by ------→ and points of inhibition by ——→ . *, this reaction is virtually irreversible with the equilibrium biased towards methionine and THF under normal conditions (from Amyes & Smith, 1974b).

then be synthesized DNA replication ceased. Bacterial growth could be restored to prototrophs grown under these conditions by the addition of thymine and lysine (Amyes & Smith, 1974b).

1.1.3 Properties of trimethoprim

Trimethoprim has been used as an antimicrobial agent for approximately 21 years and has a number of properties which make it suitable for such use:

i) most folate inhibitors e.g. methotrexate and aminopterin bind both bacterial and mammalian DHFRs but trimethoprim selectively binds bacterial DHFRs. Consequently patients experience fewer side effects (Burchall, 1973; Burchall, 1979)

ii) trimethoprim was shown to be active against a broad spectrum of organisms especially selected gram-negative bacilli and streptococci. It was ineffective against *Pseudomonas aeruginosa* and *Clostridium perfringens* (Bushby & Hitchings, 1968).

iii) trimethoprim was shown to be readily absorbed after oral administration with concentrations of the drug in organs and tissues being

several times greater than in serum. It had a half clearance time of approximately 14 hours, concentrating in the urine, and only weak pharmacodynamic effects.

These properties made trimethoprim eminently suitable for therapeutic use. However, it was not introduced as a single antimicrobial agent until 1972 in Finland and 1979 in other European countries.

1.2 Cotrimoxazole

The sulphonamides were the first group of modern antimicrobial agents to be developed and used in clinical and veterinary practice. By the time trimethoprim was developed sulphonamides had been in use for approximately 34 years and consequently resistance to these agents was common (Hamilton-Miller, 1979). Sulphonamides act within the folic acid biosynthetic pathway by competitively inhibiting dihydropteroate synthetase, the enzyme which catalyzes the condensation of p-aminobenzoic acid with dihydropteridine to form dihydropteroate (Brown, 1962). Dihydropteroic acid is a precursor in the synthesis of dihydrofolic acid. Because both sulphonamides and

trimethoprim act within the same biosynthetic pathway it was felt that the use of both drugs in combination would extend the effectiveness of the sulphonamides. When trimethoprim first came onto the market in 1969, therefore, it was available only in combination with the sulphonamide, sulfamethoxazole (Smx). This combination was known as cotrimoxazole and its use was justified by the following observations:

i) cotrimoxazole was shown to have a synergistic effect *in vitro* (Bushby and Hitchings, 1968; O'Brien et al., 1982; Acar, Goldstein & Chabbert, 1973)

ii) *in vitro* the two drugs in combination were bactericidal as opposed to bacteristatic when used singly (Then & Angehrn, 1973)

iii) trimethoprim was found to be a sulphonamide potentiator, extending the spectrum of these antibiotics to include such species as *Proteus*, *Bordetella*, *Haemophilus* and *Neisseria* (Bushby & Hitchings, 1968; Brumfitt, Hamilton-Miller & Kosmidis, 1973).

Although no data was available it was thought that the administration of the combination would delay the emergence of resistance to trimethoprim (Bushby & Hitchings, 1968). The spectrum of activity of

cotrimoxazole could not be denied but because of the differing pharmacokinetic properties of the two drugs many workers questioned the assumption that the *in vitro* findings would pertain *in vivo* (Greenwood, 1979; Grüneberg, 1979; Lacey, 1979; Amyes, 1982; Lacey, 1982; Salter, 1982). Many also felt that use of the combination would have no effect on delaying the emergence of resistance to trimethoprim. However, despite the arguments for and against the use of cotrimoxazole it has been an effective therapeutic agent and its use has continued despite the subsequent introduction of trimethoprim as a single agent.

1.3 Mechanisms of resistance to trimethoprim

Resistance to antimicrobial agents has always been a problem in clinical practice since the extent of resistance limits the usefulness of the drug in question. A knowledge of the mechanisms involved in resistance is important, therefore, if it is to be both understood and prevented.

An antimicrobial agent can be rendered ineffective by alteration of the drug by the micro-organism. While drug inactivation is an important mechanism of resistance against the β-lactams and aminoglycosides, it does not

appear to be important where trimethoprim is concerned (Hamilton-Miller, 1979). This may be due to the fact that trimethoprim is a synthetic drug and is not as prone to metabolic degradation as are other naturally occurring drugs.

Changes in the environment have been shown to affect susceptibility of micro-organisms to trimethoprim. The presence of thymidine in the medium, for example, has been shown to render trimethoprim ineffective (Then, 1982).

A drug can also be rendered ineffective by a change in the micro-organism. These changes are the most important mechanisms involved in trimethoprim resistance and can be divided into two broad categories viz. intrinsic and acquired resistance (Hamilton-Miller, 1979; Burchall, Elwell & Fling, 1982; Then, 1982).

1.3.1 Intrinsic resistance

Intrinsic resistance to trimethoprim has not been extensively studied but at least two mechanisms have been shown to operate in bacteria which are naturally resistant to trimethoprim. These characteristically give rise to intermediate resistance (minimum inhibitory concentration (MIC)

of trimethoprim 16 to 1 024 mg/l).

i) impermeability of the bacterial cell wall to the drug. This is seen most commonly in Pseudomonas aeruginosa and other Pseudomonas species (Then, 1982).

ii) production of chromosomal dihydrofolate reductases (DHFRs) which have a low affinity for trimethoprim. This occurs in Neisseria spp., Branhamella catarrhalis, Nocardia spp. and anaerobic bacteria such as Bacteroides spp. and Clostridium spp., (Then & Angehrn, 1979; Then, 1982).

1.3.2 Acquired resistance

Acquired resistance may be due either to alterations in the chromosome of the organism or due to the acquisition of plasmids and/or transposons bearing resistance determinants. Chromosomal mutations usually confer intermediate resistance on the organism (MIC 16 to 1 024 mg/l) while plasmid- and/or transposon-mediated resistance is characterized by high-level resistance (MIC >1 024 mg/l).

1.3.2.1 Resistance arising from alterations to the chromosome

Chromosomal mutations may result in any of the following:

i) thymidine auxotrophy. Thy^- mutants lack a functional thymidylate synthetase enzyme which is required to convert dUMP to dTMP. As a result these organisms require exogenous sources of either thymine or thymidine to grow. This allows the organism to function in the presence of trimethoprim as it is not dependent on THF derivatives for the synthesis of thymidylate (Amyes & Smith, 1974b).

ii) cell wall impermeability. Mutations resulting in decreased penetration of the cell wall by the antibiotic have been described in Klebsiella pneumoniae by Smith (1976). Changes in the outer membrane proteins have also been associated with resistance to multiple antimicrobial agents

including trimethoprim in species of Enterobacter, Serratia and Klebsiella (Gutmann et al., 1985).

iii) over-production of normal chromosomal dihydrofolate reductase. This type of resistance occurs as a result of one or more mutations in the regulatory region of the dhfr gene and has been described by several workers (Flensburg & Sköld, 1984; Tennhammar-Ekman, Sundström & Sköld, 1986; Flensburg & Sköld, 1987; De Groot et al., 1988).

iv) production of chromosomal DHFRs less sensitive to trimethoprim. Organisms producing such enzymes have been described by Then & Herman (1981) and Smith & Calvo (1982). A combination of this mechanism with hyperproduction of the enzyme has also been reported by Flensburg & Sköld (1984, 1987) in a clinical isolate of E.coli.

1.3.2.2 Plasmid- and transposon-mediated trimethoprim resistance

Plasmid-mediated resistance to trimethoprim is the most common form of acquired resistance and the most important. Plasmids and transposons bear genes for dihydrofolate reductases (DHFRs) which have little affinity for trimethoprim and are, therefore, resistant to the drug. They usually confer an MIC of trimethoprim >1 024 mg/l. However, two of the enzyme groups confer intermediate resistance (MIC 16 to 1 024 mg/l). The first of the plasmid-mediated enzymes was reported by Fleming, Datta & Gruneberg (1972) three years after the introduction of trimethoprim into clinical use. At this time eight groups of resistant DHFRs have been identified and while the genes coding for all of them have been located in plasmids, some of them have also been found in transposons. By this means they have become integrated into the bacterial chromosome (Towner, Venning & Pinn, 1982). The enzymes have

been identified mainly in Enterobacteriaceae but have spread to other gram-negative organisms e.g. Pseudomonas spp. and Vibrio cholerae (Goldstein, Papadopoulou & Acar, 1986; Young & Amyes, 1986b). They have also been found in staphylococci (Archer, Coughter & Johnston, 1986; Coughter, Johnston & Archer, 1987; Tennant et al., 1988).

1.4 Plasmids and transposable elements

In view of the role that plasmids and transposons play in mediating trimethoprim resistance a brief outline of the essential features of both elements follows. Both plasmids and transposons have been the subject of intensive studies of which the following references are representative (Broda, 1979; Lorian, Ed., 1986; Kleckner, 1981; Shapiro, Ed., 1983).

1.4.1 Plasmids

1.4.1.1 Definition and characteristics of plasmids

Plasmids are extrachromosomal elements of

double stranded (ds) DNA which are found in both eukaryotic and prokaryotic cells. In bacteria, these elements are common and range in size from <1 to >400 Kb. More than one species of plasmid may co-exist and it is not uncommon to find several different plasmids in a single cell. However, the total amount of plasmid DNA which can be stably maintained in a cell is limited and in exponentially growing bacteria the number of each species of plasmid ranges typically from 0.7 to 40 copies per chromosome. Plasmids have characteristic copy numbers and generally this is inversely proportional to the size of the plasmid. They are also able to spontaneously acquire foreign DNA/delete portions of their own DNA.

1.4.1.2 Classification of plasmids

Plasmids are classified broadly by incompatibility grouping. This system of classification is based on the fact that closely related plasmids respond to the

copy number regulating mechanisms of one another and cannot, therefore, co-exist stably. In a cell where there is an established plasmid introduction of a second, related plasmid will result in the elimination of the resident plasmid. Such plasmids belong to the same incompatibility group. This system of classification has been verified by electronmicroscopy of plasmid heteroduplex formations. Heteroduplexes formed between plasmids belonging to the same incompatibility group have been shown to exhibit greater homology than those formed by plasmids from different incompatibility groups.

1.4.1.3 Vertical transfer of plasmids

At the time of cell division, each daughter cell receives at least one copy of each resident plasmid. This is termed vertical transfer and it ensures the maintenance of each plasmid within a single bacterial species. In order for

vertical transfer to occur a basic minimum of plasmid-encoded functions is required. These include determinants for initiation of plasmid replication and control of copy number i.e. regulation of replication initiation. In addition regulation of distribution of plasmid copies at the time of cell division and monomerization of plasmid multimers is required. Although regulation of plasmid replication is autonomous the process is, nevertheless, dependent on host-specified proteins. This dependence (or relative independence) reflects the host range of the plasmid. Where a plasmid has a degree of self-sufficiency in specifying the essential replication functions the host range is broader.

1.4.1.4 Horizontal transfer of plasmids

Apart from the ability of plasmids to transfer vertically they have a propensity for transfer from one species to another and sometimes from one genus to another.

This type of transfer is called horizontal transfer and is effected by conjugation and to a lesser extent by transformation, transduction and protoplast fusion. Apart from the essential genes required for plasmid maintenance many plasmids carry determinants for conjugal transfer to new host cells. The most extensively studied transfer system is that of the F- or fertility factor. Since conjugation is a complex process requiring many genes only large plasmids are capable of this type of transfer. However, smaller, co-resident plasmids may take advantage of another plasmid's conjugation system provided they have their own origins of transfer and can mobilize the conjugation functions.

1.4.1.5 Role of plasmids in chromosomal gene transfer

During conjugation plasmids may also effect the transfer of chromosomal genes from one bacterium to another. Although the bacterial chromosome and plasmid DNA

are physically and functionally discrete physical interaction between these two entities can and does occur. Integration of the F-factor into the chromosome, for example, yields a high frequency recombinant strain which, when transferring F-factor genes to a recipient strain, transfers the chromosomal genes adjacent to the F-factor at the same time. A second means by which plasmids may acquire and transfer chromosomal genes occurs as a result of imprecise excision of an integrated plasmid from the chromosome. Chromosomal genes adjacent to the plasmid may be incorporated into the plasmid in this manner.

1.4.1.6 Plasmid-encoded functions

The bacterial properties specified by plasmids vary considerably and range from resistance to noxious agents to factors which enhance pathogenicity. These properties can be divided into four broad categories:

i) Resistance to noxious agents. Bacteria are continually exposed to noxious substances in their environment e.g. mutagenic chemicals, antibiotics, toxic heavy metal ions, ultraviolet irradiation and lytic viruses. Resistance to some of these challenges, most notably to heavy metal ions and antibiotics, have been found to be plasmid-mediated.

ii) Enhanced bacterial competitiveness. Some bacteria are known to produce proteins and oligopeptides which enhance their own competitiveness by inhibiting the growth of other competing organisms. Examples of such substances are bacteriocins and microcins and these are generally plasmid-mediated.

Ferric iron is essential for bacterial growth and is present in the environment in very small quantities. Some bacteria produce siderophores which have a high affinity for iron and thereby deplete

the environment thus enhancing their own competitiveness. The genes for production of the siderophore and the siderophore-iron complex uptake system are plasmid-mediated.

The capacity of some bacteria to adhere to cell surfaces enhances their ability to colonize particular tissues (e.g. intestinal mucosa). Such adhesins are plasmid-mediated.

iii) Adaptation to specialized ecosystems.

In clinical situations there are many examples of such adaptations. The hospital environment is particularly hostile to bacteria but many plasmid-mediated functions assist bacteria to survive. These include resistance to many antibiotics, mutagenic compounds such as acriflavin and noxious chemicals e.g. mercury and hexachlorophenes which are common components of disinfectants.

In the human body invading bacteria are subjected to a battery of host

defences. Some bacteria have developed their own defences to help them colonize and multiply in this hostile environment. These mechanisms include the production of siderophores, adhesins, complement resistance factors and antiphagocytic factors. The acquisition of multiple antibiotic resistance factors also enhances their ability to survive.

iv) Increased pathogenicity. In some cases the ability of an organism to cause severe disease is related to the presence of one or more plasmids. A well known example is that of enterotoxigenic E.coli. E.coli is part of the normal flora of the large intestine and does not cause disease under normal circumstances. However, if this organism acquires plasmids encoding an enterotoxin and an adhesin and colonizes the small intestine, symptoms similar to cholera are produced. Other examples of enhanced pathogenicity due to the

presence of plasmid-mediated genes can be seen in shigellae and enteroinvasive E.coli. In both organisms their ability to penetrate and multiply within the intestinal mucosa is due to the expression of genes located on a large plasmid.

Because of the mobility of plasmids and their facility for acquisition, deletion and exchange of genetic material plasmids are important factors in the successful adaptation of bacteria to changing environmental conditions.

1.4.2 Transposable elements

Transposable elements are discrete units of double-stranded (ds) DNA capable of translocating to new sites in the bacterial chromosome and plasmids without requiring extensive DNA homology.

1.4.2.1 Classification of transposable elements

i) Insertion sequences. The smallest, simplest transposable elements are insertion sequences. These consist of

short inverted repeat sequences (10- to 40-bp in length) on either end of a central sequence which contains the genes necessary for transposition. In addition short, direct repeat sequences (4- to 11-bp) of target DNA can be found on both sides of the inserted element (Figure 1.3.).

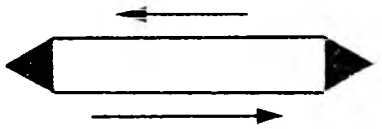


Figure 1.3. Structure of an insertion sequence. Black triangles indicate inverted repeat sequences and arrows indicate direction of transcripts (from Schmitt, 1986).

Insertion sequences are commonly found in bacterial DNA and range in size from 700- to 1 800-bp. The terminal repeats serve as recognition sequences for the transposases that catalyze the fusion of these ends

with the target DNA. The intervening DNA sequence usually contains a single, large open reading frame (ORF) encoding a polypeptide of 300 to 400 amino acids. Frequently there is a smaller ORF within the larger one but in the anti-sense orientation.

ii) Transposons. These are more complex in structure than insertion sequences and are divided into three classes:

a) Class I transposons (compound transposons) contain antibiotic resistance genes flanked on either side by two copies of an insertion sequence in direct or inverted orientation (Figure 1.4.). Examples of this type are Tn5 and Tn10.

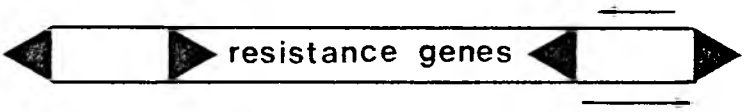


Figure 1.4. Structure of Class I transposons (from Schmitt, 1986).

b) Class II or complex transposons are flanked on either end by an inverted repeat sequence. The central sequence encodes genes for transposition and frequently antibiotic resistance. The inverted repeat sequences serve as recognition sites for the transposases (Figure 1.5.). An example of this class of transposon is Tn3.

Figure 1.5. Structure of Class II transposons (from Schmitt, 1986).

c) Class III transposable elements consist of transposing bacteriophages such as Mu and D108. The structure of these elements is different from Class I and Class II transposons in that their ends do not have inverted repeat sequences. Homologies do occur, however, but they are not symmetrical. Sequences at the beginning of the bacteriophage have been shown to be homologous with sequences occurring approximately 80-bp in from the other end. In addition a seven to nine base pair sequence at one end has been found to be repeated

several times at the other end of the element and is homologous with the insertion sequence IS5.

1.4.2.2 Transposition

The most important property of transposable elements is their ability to integrate into DNA. This process involves non-homologous recombination catalyzed by transposon-encoded transposases. It is entirely independent of homologous recombination mediated by the recA system of the bacterium. The mechanisms involved in transposition are not well defined owing to a lack of suitable in vitro systems. However, there is evidence supporting at least two different mechanisms of transposition viz. conservative and replicative. In conservative transposition (exhibited by Class I elements) the donor replicon is lost whereas in replicative transposition (exhibited by Class I and Class II elements) both the donor and target

replicons retain copies of the transposon. Although transposons insert into various loci of the target DNA most elements show a preference for specific sites. It is thought that this is determined by the preference of the transposase enzymes for certain sequences.

1.4.2.3 Transposons and trimethoprim resistance

Although numerous transposons mediating antibiotic resistance have been identified only five have been associated with trimethoprim resistance. The most common of these is the element Tn7. This encodes resistance to mercury, streptomycin and spectinomycin as well as trimethoprim (Barth et al., 1976). Tn402 contains the gene dhfrIIc and codes for trimethoprim resistance only (Shapiro & Sporn, 1977). The gene encoding DHFR type Ib resides in Tn4132 (Young & Amyes, 1985) and recently a Tn21-like transposon was found to harbour three different trimethoprim resistance genes, dhfrIa, dhfrII and

dhfrV, in addition to a sulphonamide resistance gene, sulI (Sundstrom & Skold, 1990). The fifth transposon, Tn4003, bears the gene for DHFR S1 which was found in staphylococci (Young, Skurray & Amyes, 1987).

The ability of transposons to insert into any DNA replicon has greatly increased the spread of antibiotic resistance genes which, in turn, has resulted in marked changes in the epidemiology of antibiotic resistance.

1.5 Plasmid-mediated trimethoprim-resistant dihydrofolate reductase enzymes

The plasmid- and/or transposon-mediated resistant DHFRs have been classified according to their biochemical and physical properties. These are summarized in Table 1.1.

1.5.1 Resistant DHFRs in gram-negative bacteria

i) There are two type I DHFRs: the prototype type Ia (Skold & Widh, 1974; Pattishall et al., 1977; Fling & Elwell, 1980) and the type Ib (Young & Amyes, 1985). The type Ia is

the most ubiquitous of all DHFRs, having been found on all continents (Amyes, 1989; Amyes & Towner, 1990). It is larger than the chromosomal DHFR - 35 Kda vs 17.7 Kda (Smith & Calvo, 1980) - and has been shown to consist of two subunits each approximately 18 Kda (Fling & Elwell, 1980). The type Ib has similar biochemical properties but is smaller than the type Ia - 24.5 Kda. It is not known whether this enzyme functions as a monomer or a dimer. It is not as commonly found as the type Ia and to date has only been reported in the United Kingdom (Young & Amyes, 1985). Both enzymes confer trimethoprim minimum inhibitory concentrations (MIC) >1 000 mg/l on their host organisms. The type Ia gene is located most commonly in transposon Tn7 which also mediates resistance to streptomycin and spectinomycin. However, it has recently been found in a Tn21-like transposon which also mediates sulphonamide resistance (Sundström & Sköld, 1990).

ii) The type II group of DHFRs has three subtype enzymes: the prototype type IIa (R67), the

type IIb (R388) and the type IIc (R571:Tn402) (Shapiro and Sporn, 1977; Pattishall et al., 1977; Amyes & Smith, 1974c; Amyes and Smith 1976; Amyes & Smith, 1978; Fling & Elwell, 1980; Broad & Smith, 1982). These enzymes differ in their affinity for trimethoprim but for practical purposes they are all insensitive to the drug. They confer MICs >1 000 mg/l on their hosts. The type IIa and IIb enzymes are both 35 Kda in size while the type IIc is slightly smaller - 34 Kda. The type IIa has been shown to function as a tetramer (Smith et al., 1979), each subunit being 8.5 Kda in size. The types IIa and IIb have been identified in Europe, America and Asia but the type IIc has only been found in the United Kingdom and possibly Finland (Amyes & Towner, 1990; Huovinen, Pulkkinen & Toivanen, 1983).

iii) The type III group of DHFRs also contains three subtypes, the prototype having been isolated from a strain of Salmonella typhimurium in New Zealand (Anderson, 1980; Fling, Elwell & Walton, 1982; Joyner et al., 1984). This enzyme has subsequently also been

found in the United Kingdom (Thomson, Barg & Amyes, 1990). It is distinct from the types I and II DHFRs in all its biochemical and physical properties but most notably in the trimethoprim MIC it confers on its host bacterium (MIC = 64 mg/l). This was the first example of plasmid-mediated, intermediate trimethoprim resistance. More recently type IIIb and IIIc enzymes have been identified in the United States (Barg et al., 1990). They differ from the prototype and one another in their pI values. Comparison of the first 47 amino acid residues of the type IIIa and IIIb enzymes shows that they differ significantly in this respect as well (Thomson, Barg & Amyes, 1990).

iv) The type IV DHFR has only been found in bacteria isolated in South India (Young & Amyes, 1986a). This enzyme also confers intermediate resistance on its host organism (MIC 10 to 160 mg/l) but has the unique property of inducibility. This enzyme is the largest of the DHFRs having a molecular weight of 46.7 Kda. Recently Thomson, Young & Amyes (1990) have shown that

Table 1.1. Properties of plasmid-encoded dihydrofolate reductases.

DHFR type	DHFR sub-type	Plasmid	MIC for Tp (mg/l)	ID50 Tp (μM)	ID50 Mtx (μM)	Km DHF (μM)	Ki Tp (μM)	TD50 (min)	Mr (Kda)
I	a	R483(Tn7)	>1 000	57	4.4	5.6	7.4	0.5	35
	b	pUK163(Tn4132)	>1 000	32	2.8	11	41	1.2	24.5
II	a	R67bis	>1 000	70 000	1 100	4.6	6 100	>12	35
	b	R388	>1 000	80 000	7.5	8.3	150	>12	35
	c	R751(Tn402)	>1 000	20 000	1 000	4.2	400	>12	34
III	a	pAZ1	64	2.1	ND	0.4	0.019	ND	16.9
	b	pBH600	128	2.0	0.02	9.52	0.4	>12	17.0
	c	pBH700	256	3.0	0.007	3.12	0.52	> 8	22.0
IV		pUK1123	10	0.2	0.02	37	0.063	>12	46.7
V		pLM028(Tn21-like)	>1 000	23	3.5	15.5	3.2	3.0	35
VI		pUK672	>1 000	200	7.25	31.25	75	0.4	10.0*
VII		pUN835	>1 000	30	3.0	20.0	7.0	1.5	11.5
SI		pSK100(Tn4003)	>1 000	50	0.002	10.8	11.6	>12	19.7

Tp = trimethoprim; Mtx = methotrexate; DHF = dihydrofolate; DHFR = dihydrofolate reducatase
ID50 = concentration of drug required to inhibit enzyme activity by 50%
TD50 = time taken for the enzyme to lose half its activity at 45 °C
MIC = minimum inhibitory concentration; ND = not done; *, as determined by column chromatography.
References: Sköld & Widh, 1974; Pattishall et al., 1977; Young & Amyes, 1985; Smith et al., 1979; Broad & Smith, 1982; Amyes, 1986; Amyes & Smith, 1974c; Amyes & Smith, 1976; Joyner et al., 1984; Amyes & Towner, 1990; Barg et al., 1990; Young & Amyes, 1986a; Sundström et al., 1987; Wylie et al., 1988; Amyes et al., 1989; Young, et al., 1987).

this enzyme comprises two dissimilar subunits: the native DHFR protein - 33 Kda, and the DNA-binding protein NS1 - *c.* 10 Kda. The function of the latter subunit has not been clarified but it has been suggested that it performs some role in the induction of the enzyme.

v) The type V DHFR has been found in isolates from Sri Lanka (Sundström, Vinayagamoorthy & Sköld, 1987) and more recently in the United Kingdom (Towner et al., 1990), Finland (Heikkila et al., 1990) and Sicily (Agodi et al., 1990). This enzyme is very similar to the type I DHFRs but was classified in a separate category on account of its small molecular weight - *c.* 5 Kda - and the lack of hybridization with a type I gene probe (Thomson & Amyes, 1988). Sequence analysis (Sundström et al., 1988) together with analysis of the molecular weight by native polyacrylamide gel electrophoresis (PAGE) (Thomson, 1990) has shown that it has a molecular weight of 35 Kda and functions as a dimer. Although there is 75% sequence homology between the type I and type V DHFR

genes the lack of hybridization between the two genes indicates that the type V should remain in a separate class. Like the type Ia DHFR the type V DHFR gene has also been located in a Tn21-like transposon mediating sulphonamide resistance (Sundström et al., 1988; Sundström & Sköld, 1990).

vi) The type VI DHFR has only been described in isolates from South Africa (Wylie et al., 1988). It has unique biochemical properties and confers an MIC >1 000 mg/l on its host organism. This enzyme will be discussed more fully in a later chapter.

vii) After the isolation of the type VI DHFR Amyes et al. (1989) isolated another type I-like enzyme mediating high-level resistance (MIC >1 000 mg/l). Because it had a different molecular weight - 11.5 Kda - and did not hybridize with probes of other DHFRs it was classified as DHFR type VII.

The dihydrofolate reductases described above have all been isolated in various species of enterobacteria, the type Ia having been found in other gram-negative bacteria as well (Young & Amyes, 1986b).

1.5.2 Resistance DHFRs in gram-positive bacteria

The type S1 DHFR, located on transposon Tn4003, is unique to staphylococci and is the only such enzyme reported in gram-positive bacteria at this time (Young, Skurray & Amyes, 1987; Tennant et al., 1988). The S1 DHFR is biochemically distinct from all other plasmid-mediated DHFRs found in gram-negative bacilli and confers high-level resistance on its host organism (MIC >1 000 mg/l). It was first described in an Australian isolate but has been identified in the USA, United Kingdom and more recently in Switzerland (Archer, Coughter & Johnston, 1986; Coughter, Johnston & Archer, 1987; Amyes & Towner, 1990; Burdeska et al., 1990).

1.6 Epidemiology of trimethoprim resistance

Comparison of the prevalence rates of resistance to trimethoprim is difficult as many factors may influence the percentage of resistant strains reported. These factors include:

i) Choice of organism and specimen. Urinary isolates are subjected to much higher concentrations of

trimethoprim than those from sputum (Brumfitt, Hamilton-Miller & Kosmidis, 1973) hence a greater selective pressure on urinary organisms for acquired resistance. A greater percentage of Klebsiella are intrinsically resistant to trimethoprim than E.coli and this could also affect the reported resistance rate.

ii) Methodology. Some media have been reported to be antagonistic to trimethoprim activity (Amyes & Smith, 1974a) due to the presence of thymine and/or thymine derivatives. The inoculum size is important as apparent resistance occurs where the number of colony forming units per millilitre is too great (Bach et al., 1973).

iii) Type of patient and hospital. It has been shown that trimethoprim-resistant organisms are isolated more frequently from elderly patients (Huovinen, 1984; Amyes Doherty & Young, 1986; Huovinen & Toivanen, 1986) and from patients in so called "long stay" hospitals (Amyes et al., 1980; Young & Amyes, 1983; Huovinen & Toivanen, 1986). There is also a difference in the number of resistant organisms isolated from in-patients compared to those visiting general practitioners (Towner, Smith & Cowlishaw, 1983), resistant strains being

isolated more frequently from in-patients. This has been presumed to be due to the greater exposure of patients and organisms to antibiotics in a hospital environment.

iv) Occurrence of local epidemics. An epidemic of one or more resistant organisms has obvious problems in epidemiology but such a situation is more easily recognisable than an epidemic of a trimethoprim-resistant plasmid. Many such epidemics have been reported (Goldstein, Papadopoulou & Acar, 1986) and are only recognised when extensive plasmid analyses are performed.

v) Use of cotrimoxazole discs. The use of combination discs does not allow an accurate assessment of trimethoprim resistance. Cotrimoxazole is active against organisms which are trimethoprim ^{r} Smx^{s} (Acar, Goldstein & Chabbert, 1973; Amyes, 1981).

For these reasons assessment of changes in trimethoprim resistance is really only significant when long-term, follow-up studies are performed by the same laboratory. Since the introduction of trimethoprim as a chemotherapeutic agent resistance to the drug has been monitored on a continuous basis in several centres. These include Edinburgh, Nottingham and London (United Kingdom), Paris (France) and Turku (Finland). Single

surveys have been performed in many other areas in Europe, South, Central and North America, Asia, the Middle East and Africa but there has been no follow-up data published from these areas so far.

Most studies have included all members of Enterobacteriaceae although a number of studies have been performed in which resistance to trimethoprim in a single member (E.coli) or group (shigellae) has been monitored. The following discussion of trimethoprim resistance will, unless specified, be confined to the family of Enterobacteriaceae.

1.6.1 Trimethoprim resistance in London

The changing pattern of trimethoprim resistance has been monitored in various London hospitals since the introduction of trimethoprim into clinical practice. In the Whittingdon Hospital a survey performed in 1970 by Anderson, Datta & Shaw (1972) showed that resistance to trimethoprim occurred in 5.6% of all Enterobacteriaceae causing infections. Surveys were repeated five and seven years later in 1975 and 1977 by Amyes, Emmerson & Smith (1978) and nine, eleven and thirteen years later in 1979, 1981 and 1983 by Chirnside, Emmerson & Smith (1985).

Over this period of time the incidence of trimethoprim resistance in urinary tract isolates rose from 9.8% in 1975 to 13.1% in 1979 and then decreased to 11.9% in 1983. Comparison of trimethoprim resistance in isolates from in-patients with those from out-patients showed a slightly different pattern. While resistance in organisms isolated from in-patients followed the general trend described, resistance in organisms isolated from out-patients increased steadily from 0.5% in 1975 to 6.5% in 1983. Transferable R-plasmids were seen in 10.3% of all resistant isolates in 1975 and this increased by 27.8% to 38.1% in 1983. During this period the number of isolates with transferable R-plasmids from out-patients was greater than those from in-patients.

In 1981 the first trimethoprim R-plasmids transferring resistance to trimethoprim alone were isolated and these were more numerous in out-patient than in-patient isolates. Prior to this trimethoprim resistance genes most frequently co-transferred with streptomycin/spectinomycin and sulphonamide resistance.

At the Hammersmith Hospital, London, the number of

plasmid incompatibility groups associated with trimethoprim resistance was found to have increased by 1977. Initially trimethoprim resistance determinants were only found on IncW plasmids (Jobanputra & Datta, 1974) but by 1977 trimethoprim R-plasmids belonging to seven different incompatibility groups were found (Datta et al., 1980).

The first survey of trimethoprim resistance at the Royal Free Hospital, London, took place between 1973 and 1975. In this study isolates from infected urines were studied (Grey, Hamilton-Miller & Brumfitt, 1979). The total trimethoprim resistance was only 3.2% when a breakpoint of 2 mg/l was used. Of these resistant organisms 11.3% were highly resistant (MIC >1 000 mg/l) and trimethoprim R-factors were demonstrated in only 9%. These plasmids were all of the IncW incompatibility group. A second survey in the same hospital followed immediately from 1975 to 1977 (Brumfitt, Hamilton-Miller & Gooding, 1980). This showed that the incidence of trimethoprim resistance in hospital isolates had increased by 9.2% to 12.4% and that high-level resistance had increased by 7.1% to 18.4%. Resistance in out-patient isolates

was determined to be 7.5% of which 20% was high-level resistance. A third survey from October 1978 to December 1979 (Hamilton-Miller, Gooding & Brumfitt, 1981) showed that the incidence of trimethoprim resistance had remained constant at 11.5%. Of the resistant gram-negative bacilli 60% had MICs >1 000 mg/l, which was a substantial increase (41.6%) over the previous survey. Of the resistant isolates 14.5% transferred their determinants. A fourth survey of urinary pathogens collected at this hospital two years later in 1981 (Brumfitt, Hamilton-Miller, & Wood, 1983) showed that trimethoprim resistance had remained stable at 13% and high-level resistance was now present in 75% to 90% of the resistant strains, an increase of 15% to 30%. Resistance in isolates from general practice was only 5.8% at this time. The statistics of these surveys suggested that the rate at which resistance was being acquired was slowing down and that the introduction of trimethoprim as a single agent in 1979 had had no discernible effect on trimethoprim resistance as a whole. Four years later in 1985, however, trimethoprim resistance in urinary isolates from this hospital increased by 12.6% to 25.6% (Hamilton-Miller & Purves, 1986)

while the incidence in isolates from general practice increased dramatically from 5.8% to 21%. The proportion of gram-negative resistant strains with MICs >1 000 mg/l was 81%, a figure comparable to the 1981 survey.

At the University Hospital, London, Gruneberg (1976) surveyed resistance in all urinary pathogens from 1971 to 1974 from both the hospital and general practice. In the community isolates trimethoprim resistance rose gradually from 6% in 1971 to 10.5% in 1974 while in hospital strains resistance increased from 20.1% to 28.5%. There was little change in the percentage of E.coli which acquired resistance over the four years indicating that, up until that time, R-plasmids had not spread to any great extent in this environment.

1.6.2 Trimethoprim resistance in Nottingham and other areas in England

In Nottingham trimethoprim resistance has been monitored consistently from 1978 to 1985 (Towner & Slack, 1986). From 1978 to 1983 resistance in urinary isolates from in-patients gradually rose from 9.7% to 13.6%. In 1984, however, there was a

sharp increase of 5.9% to 19.3% and thereafter a levelling off to 18.9%.

The trend in isolates from general practice paralleled this but at a lower level. Initially resistance was only 0.5% in 1978 but increased to 11.6% in 1983. In 1984 and 1985 the percentage increased to 16.9% and 15.8% respectively. Although the incidence of resistance fell slightly in 1979 the number of isolates owing their resistance to transferable R-plasmids trebled. There was also a large increase in the proportion of high-level resistance which was not transferable (Towner et al., 1980). Further study of these strains resulted in the first report of chromosomally-located transposon Tn7 (Towner, 1981). This type of resistance was found to be more common than first anticipated as 12 out of 50 isolates were shown to possess transposons integrated into the chromosome (Towner, Venning & Pinn, 1982).

Trimethoprim resistance plasmids in this area were first reported to belong to the IncP and IncI incompatibility groups (Towner et al., 1979) but later a greater variety of groups was implicated (Towner & Wise, 1983). The first R-plasmids mediating resistance to trimethoprim alone were

reported in 1980 and an increasing number of trimethoprim-resistant strains was found to be susceptible to sulphonamides. This was attributed to the changed selection pressure when trimethoprim was introduced on its own in 1979.

A survey of trimethoprim-resistant Shigella dysenteriae, S. boydii and S. flexneri isolated in England and Wales between 1979 and 1983 (Gross, Threlfall & Ward, 1984) showed an increase in trimethoprim resistance from 1.3% to 16.8%. Contrary to expectation the proportion of patients with recent foreign contact was smaller amongst those with resistant strains than among those with susceptible strains. This indicated that resistance factors were being acquired locally by these bacteria.

1.6.3 Trimethoprim resistance in Scotland

The incidence of trimethoprim resistance in Edinburgh, Scotland, has been monitored in various types of hospitals and in general practice since 1978. From 1978 to 1982 the percentage of trimethoprim-resistant isolates (MIC >10 mg/l) in general hospitals ranged from 12.5% to 20.4%. Five

percent of the isolates had MICs >1 000 mg/l and trimethoprim R-plasmids were found in 5% of the resistant isolates (Amyes et al., 1980; Amyes, 1983; Amyes, 1986).

During the period October 1978 to April 1979 the incidence of resistance in a convalescent hospital was much greater (46%) with 13.4% of the isolates possessing R-plasmids (Amyes et al., 1980). Later, in 1981, the incidence of trimethoprim resistance in urinary isolates from patients in the orthopaedic unit of the Edenhall Hospital (a "long stay" situation) was reported to be an unprecedented 64%. A total of 25.5% of these isolates was resistant to >1 000 mg/l trimethoprim (Amyes et al., 1982). Of 100 representative isolates 11 were shown to possess trimethoprim R-plasmids while 12 were unable to transfer resistance and did not appear to possess any plasmids. Resistance in the latter isolates was shown to be due to the insertion into the chromosome of two different transposons bearing resistant DHFR genes (Young & Amyes, 1983).

The incidence of resistance in general hospitals between 1982 and 1984 was seen to decline from 18.3% to 12.3%. There was also a decrease in

high-level resistance from 13.6% to 8.0%. In 1982 the proportion of highly resistant isolates was 74% but this decreased in 1984 to approximately 65%. The number of strains possessing R-plasmids also decreased from 6.9% in 1982 to 2.7% in 1984 and not all of these were transmissible (Amyes, Doherty & Young, 1986). In 1982 half of the highly resistant strains were unable to transfer resistance but by 1984 this had increased to two thirds. It was suggested that this trend was due to the integration of transposons bearing resistant DHFR genes into the chromosome.

A study of urinary isolates obtained from patients attending general practitioners was performed from October 1981 to October 1982 (Amyes, 1987). A total of 11.4% of the isolates was resistant to trimethoprim (MIC >10 mg/l) and of these 69.8% were resistant to >1 000 mg/l trimethoprim. This represents 8% of all isolates which is a little over half that seen in hospital isolates at the same time. Transferable resistance was seen in 45% of the highly resistant isolates and most of these carried streptomycin/spectinomycin resistance determinants linked to trimethoprim. This suggested the presence of the transposon Tn7. In the

remaining 55% of the high-level resistant isolates resistance was not transferable. Only 5 of these lacked visible plasmids.

In Glasgow, Scotland, resistance has been monitored but less frequently than in the centres reported above. From July 1979 to February 1980 192 resistant urinary isolates were collected and of these 56% were highly resistant (Kraft, Platt & Timbury, 1983). One hundred and twenty-three isolates were E.coli of which 71% had MICs >1 000 mg/l. Resistance was transferable in 50% of the E.coli, the balance of the resistance being presumed to be due to the presence of transposons or tra plasmids. In a follow-up survey two years later in 1982 (Kraft, Platt & Timbury, 1984) an increase of 10% in the number of highly resistant E.coli was seen - 71% to 81%. However, only 35% of these highly resistant isolates transferred their trimethoprim resistance markers. Plasmids which transferred trimethoprim resistance independently of Su resistance were more common in the second series although the majority of the strains were still resistant to sulphonamides. This suggested that although cotrimoxazole was still prescribed almost exclusively the genetic location and linkage

of resistance markers was changing.

A similar study of trimethoprim-resistant coliform urinary isolates from out-patients was performed between 1981 and 1983 (Kraft, Platt & Timbury, 1985). Only 10.2% of all isolates were resistant to trimethoprim (MIC >10 mg/l). Of the 44 trimethoprim-resistant E.coli studied 73% were highly resistant, which was similar to the percentage in hospital isolates. Resistance was transferable in 34% and the percentage of co-transferred trimethoprim and Su and of Su resistance without trimethoprim was similar to that in the 1982 hospital study.

1.6.4 Trimethoprim resistance in Europe and Scandinavia

In Europe trimethoprim resistance has been studied primarily in two centres (Paris, France and Turku, Finland) while single reports have emanated from other countries.

Trimethoprim resistance in Paris has been monitored since 1972 (Acar & Goldstein, 1982; Goldstein, Papadopoulou & Acar, 1986). All Enterobacteriaceae isolated from clinical specimens at the St Joseph Hospital, Paris, from 1972 to 1984 were collected

and tested for trimethoprim resistance. The majority of these isolates were from urine specimens (c. 70%) and c. 25% were from out-patients.

Over a ten-year period there was an increase in trimethoprim resistance from 17.9% in 1972 to a peak of 35.4% in 1982/3. In 1984 the incidence of resistance fell by 11.1% to 24.3%. The percentage of resistant strains which were highly resistant rose by 50.5% from 44.9% in 1974/5 to 95.4% in 1984. The pattern of transferability, however, was different. In 1972/3 31.2% of high-level resistance was transferable. Ten years later this reached a peak of 62.6% and then decreased by 12.6% to 50% in 1984. This pattern is consistent with that described in the United Kingdom at the same time.

The most common incompatibility group of plasmids transferring trimethoprim resistance was IncC, although a variety of other groups was represented. These plasmids frequently transferred trimethoprim and Su as well as other antibiotic markers (Papadopoulou et al., 1986).

Other countries in Europe have reported on trimethoprim resistance at various times. In Italy Romero & Perducca (1977) reported an increase in

resistance of 16.8% from 13.5% in 1973 to 30.3% in 1975. There was also an increase in the proportion of highly resistant organisms from 12% to 23.3%. By 1975 20% of the resistance was R-factor mediated and a number of different plasmid incompatibility groups was involved.

Trimethoprim resistance in Spain is apparently common in enterobacteria although no surveys have been published (Delgado & Otero, 1988). Resistance in Shigella, a common pathogen in Madrid, has been shown to occur in 89% to 97% of isolates (Lopez-Brea et al., 1983; Palenque, Otero & Noriega, 1983). The majority of these highly resistant organisms contained the type I DHFR gene (Delgado & Otero, 1988).

In Bulgaria the first trimethoprim-resistant Shigella sonnei were isolated in 1980 (Bratoeva & John, 1989). In the period 1983 to 1987 20% of all shigellae isolated were trimethoprim resistant. The type I DHFR was present and all of the strains tested transferred their resistance.

In Finland cotrimoxazole was introduced in 1969 as in other European countries, but trimethoprim on its own came into clinical use in 1972, seven years earlier than in most other countries (Kasanen &

Sundquist, 1982; Huovinen, 1987). Since 1981 the amount of trimethoprim prescribed to out-patients has exceeded the amount of cotrimoxazole prescribed (Huovinen et al., 1985). However, the levels of resistance are comparable with those reported in Europe and some areas of the United Kingdom. This suggests that the use of trimethoprim alone does not adversely affect the development of resistance (Kasanen & Sundquist, 1982).

In 1978 enterobacteria isolated from in- and out-patients at the Turku Unversity Hospital (Huovinen & Toivanen, 1980) were tested for resistance to trimethoprim. Out-patient isolates were less resistant to trimethoprim than in-patient isolates (20.3% vs 39.8% respectively). Three years later in 1981 this survey was repeated using in-patient isolates (Huovinen, Pulkkinen & Toivanen, 1983) and only 9% were found to be resistant to trimethoprim, 38.6% of these being highly resistant. Resistance was transferable in 19% of these isolates.

A survey in 1979 of trimethoprim resistance in enterobacteria isolated from urine specimens at the Turku City Hospital - a "long stay" hospital - (Huovinen, Pulkkinen & Toivanen, 1983) showed that

41% of the isolates were resistant (MIC >8 mg/l). The proportion of resistant isolates with MICs >1 000 mg/l was 60.4%. Transferable resistance occurred in 13% of these isolates. The survey was repeated approximately one year later in 1980/1 and showed a similar situation. Resistance was now found in 35.4% of the isolates, compared to the previous 41%, and 68.7% of these, an increase of 8.3%, were highly resistant. The incidence of transferable plasmids had also increased by 7% to 20%.

Analysis of isolates collected in the Turku City Hospital in 1981/2 from patients more than 65 years of age in the geriatric wards showed an overall resistance rate of 22%. However, 38% of isolates from those patients who were catheterized were resistant to trimethoprim while only 13.4% of the strains from the non-catheterized patients were resistant (Huovinen, 1984).

In 1983/4 trimethoprim resistance at Turku City Hospital remained between 32% and 35% (Huovinen et al., 1986). A high proportion of these isolates (47.2%) were shown to possess Tn7-like sequences suggesting the presence of DHFR type I in these isolates.

Two large surveys of trimethoprim resistance in out-patient isolates were undertaken in the Turku, Helsinki and Rovaniemi districts from 1978 to 1984 and 1986 to 1988 (Huovinen, et al., 1985; Heikkila et al., 1990a). In the Turku area resistance virtually trebled from 5.4% in 1978 through 9.2% in 1984 to 16% in 1988. Of the resistant isolates studied in the 1978 survey 83% were highly resistant and 39% carried Tn7-like sequences. Nine years later in 1987 93% of the resistant isolates from this area had MICs >1 000 mg/l, a 10% increase.

In the Helsinki district resistance was reported to be 1.0% from 1972 to 1977 (Kasanen & Sundquist, 1982) and then increased in 1980 to between 2.9% and 4.2%. By 1984 resistance had increased to 11.1% of which 84% were highly resistant. Tn7-like sequences were demonstrated in 66% of the strains with MICs >1 000 mg/l (Huovinen et al., 1985). Four years later in 1988 the incidence of resistance had increased by 7.9% to 19%, high-level resistance being present in 94% to 99% of the resistant strains.

In the Rovaniemi district resistance remained constant initially, being reported as 3.1% in 1980

and 5.7% in 1984. Only 76% of the resistant isolates in 1984 had MICs >1 000 mg/l and 49% were shown to contain Tn7-like sequences. This situation changed between 1986 and 1988 when 14% of all isolates were found to be resistant to >8 mg/l. In 1986 and 1987 all of the resistant isolates had MICs >1 000 mg/l but in 1988 this decreased to 89%. The percentage of E.coli containing the type I DHFR increased steadily in all three regions from 1986 to 1988, the largest increase occurring in the Helsinki district (7.3% to 13.9%).

Heikkila et al. (1990b) performed a study of trimethoprim resistance in Shigella species isolated in Finland between 1975 and 1988. Their results showed that resistance in these organisms was low (3.0%) between 1975 and 1982 but increased dramatically in the ensuing years to 42% in 1988. High-level resistance in these organisms predominated (98%) and the type I DHFR gene was found in 85% of the isolates studied. The type V DHFR was present in those strains isolated from travellers to Sri Lanka.

In Stockholm, Sweden, the level of resistance in urinary isolates from in-patients varied between 1.6% and 3.6% in 1977/8 (Dornbusch & Toivanen,

1981). Transfer of resistance was achieved in 25% of resistant donors but a Tn7-like sequence was demonstrated in only one of the donor organisms (Dornbusch & Hagelberg, 1983).

In a remote area of Sweden (Jamtland) only 153 resistant isolates were recovered from about 2 000 specimens. Of these isolates 28% were highly resistant, 34.9% of which contained Tn7-like sequences. These were transferable in some strains but not in others (Steen & Sköld, 1985). There was good correlation between the use of trimethoprim and the occurrence of resistant bacteria in the community. However, 33% of the patients who had not been exposed to trimethoprim before harboured resistant flora (Sköld, Boethius & Steen, 1986). This was thought to be due to selective pressure exerted by other antibiotics on bacteria harbouring multiple resistance plasmids or the spread of Tn7 in enterobacteria.

1.6.5 Trimethoprim resistance: overview of Europe

Trimethoprim resistance in the United Kingdom, Europe and Scandinavia developed in much the same way although percentages varied from centre to

centre. Initially trimethoprim resistance was low in most areas and was due largely to intrinsic mechanisms of resistance. There was a gradual but steady increase in resistance in the seventies but a marked rise in the proportion mediated by transferable R-plasmids. The R-plasmids also belonged to an increasing number of incompatibilty groups. In the early eighties a surge of resistance occurred with the majority of resistant isolates being highly resistant. A decrease in the number of transferable R-plasmids was observed and the first chromosomally integrated transposons bearing resistant DHFR genes were reported. The proportion of non-transferable, high-level resistance increased steadily while there was an apparent levelling off of the number of trimethoprim-resistant organisms isolated. Some centres such as Edinburgh and Portsmouth even showed a decrease in the incidence of trimethoprim resistance (Maskell, 1985; Amyes, Doherty & Young, 1986).

1.6.6 Trimethoprim resistance in the USA

Trimethoprim resistance trends in the United States

have not been as closely monitored as in Europe and the UK. Additionally, where surveys have been undertaken, resistance was tested using Tp/Smx combination discs and, therefore, the percentage reported cannot be considered an accurate indication of the incidence of trimethoprim resistance. Individual reports emanating from the USA suggest, however, that there has not been the same surge in resistance as was seen in Europe and the UK. In a worldwide survey of Tp/Smx resistance O'Brien et al. (1982) reported that resistance to cotrimoxazole in isolates collected between 1977 and 1980 in 4 hospitals in the USA ranged from 3% to 9.5%. In a later report Mayer et al. (1985) further elaborated that in the Brigham and Woman's Hospital, Boston, resistance in Enterobacteriaceae from 1977 to 1982 had remained stable except in E.coli and Klebsiella. In E.coli resistance had increased from 2% to 5% and in Klebsiella from 7% to 14%. In 1983 resistance to cotrimoxazole was reported to range from 4% to 6% in E.coli isolated at three hospitals (Murray et al., 1985).

1.6.7 Trimethoprim resistance in developing countries

Although trimethoprim resistance has not been surveyed in Mexico, reports on its emergence in faecal organisms isolated from students receiving prophylactic cotrimoxazole therapy while staying in Mexico indicated that high-level resistance determinants were not only present in that area but were probably fairly common (Murray et al., 1982). From a sample of 100 trimethoprim-resistant faecal E.coli 40 were found to transfer their trimethoprim resistance determinants. The type I DHFR gene was shown to be present in 12 of 20 tra^+ isolates (Rudy & Murray, 1984) and frequently co-transferred with ampicillin and streptomycin resistance markers (Murray & Rensimer, 1983).

In Santiago, Chile, Murray et al. (1985) reported that the incidence of resistance to Tp/Smx in E.coli isolated during February and July 1983 was 44%. High-level trimethoprim resistance occurred in all of the 15 isolates tested and seven of these isolates transferred this marker. There was no difference in the incidence of trimethoprim resistance in the out-patient strains compared to the in-patient strains. In a more comprehensive

study performed in the same area (Urbina et al., 1989) 34% of all enterobacteria collected between 1983 and 1984 were resistant to trimethoprim. In this survey resistance to trimethoprim and Smx was determined separately and, therefore, these statistics reflect trimethoprim resistance more accurately than those of Murray et al. (1985). Of the resistant bacteria 52% were able to transfer their resistance by conjugation. In the majority of cases (87%) trimethoprim resistance was linked to Smx resistance. The linkage of trimethoprim with streptomycin resistance markers suggested the presence of the transposon Tn7 in this area and hence the type I DHFR.

Murray et al. (1985) performed a study of Tp/Smx resistance in enteric pathogens (E.coli, Shigella spp. and Salmonella spp.) in Brazil and showed that 44.4% were resistant to the combination. In Honduras the same authors reported Tp/Smx resistance in E.coli isolated in 1983 to be 38% while a survey of a smaller number in Costa Rica showed that between 25% and 48% of the E.coli were resistant.

A study similar to that performed in Chile by Murray et al. (1985) was undertaken in Bangkok,

Thailand, and this showed that 40% of all E.coli isolated were resistant to Tp/Smx. The incidence of resistance was the same when out-patient and in-patient isolates were compared.

In Israel Alon et al. (1987) reported that resistance to Tp/Smx in urinary pathogens had increased steadily from 1980 to 1985. It is probable, therefore, that resistance to trimethoprim alone increased in a similar fashion. In two hospitals (B & C) resistance in 1980 was 46% and 28% respectively. This increased to 63% in hospital B and 54% in hospital C in 1984. In an out-patient study resistance to Tp/Smx increased from 27% in 1981 to 51% in the first half of 1985. The slower development of resistance in community isolates correlates with that experienced in the UK.

In Kuwait Chugh (1985) collected enteric pathogens (E.coli, Shigella spp. and Salmonella spp.) from 1980 to 1983 and found that a total of 22.9% were resistant to trimethoprim. Of the highly resistant strains 98% transferred their determinants.

A study of trimethoprim resistance in urinary pathogens isolated at a hospital in Vellore, South India (Young et al., 1986) showed that 64% of the

isolates were resistant to 10 mg/l of trimethoprim. High-level resistance dominated, occurring in 57.3% of all isolates studied. More than half of the resistant isolates (58.2%) transferred their resistance genes. A high proportion of trimethoprim R-plasmids also carried Smx resistance determinants. A more recent report on trimethoprim resistance in Vibrio cholerae 01 isolated in this hospital (Jesudason & John, 1990) indicated that prior to 1987 these bacteria were susceptible to cotrimoxazole. However, from July 1987 to October 1987 resistance increased at an alarming rate from 4.5% to 81.5%. All of the isolates with trimethoprim MICs >1 000 mg/l which were selected for further analysis were shown to be tra^+. Plasmids with five different antibiograms were identified although one, which conferred resistance to Smx and chloramphenicol in addition to trimethoprim, predominated. It was not determined which high-level resistance gene or genes were responsible for this resistance. However, the type I DHFR has been reported in Shigella dysenteriae 1 in other centres in India (Haider et al., 1990).

In Africa Amyes & Young (1987) reported that resistance in enterobacteria isolated in Dar es

Salaam, Tanzania, was 36%, high-level resistance occurring in 22% of the isolates. The type Ia DHFR was identified in a strain of Vibrio cholerae, this being the the first report of the presence of this gene outside of the Enterobacteriaceae.

In Nigeria Lamikrana & Ndep (1989) reported that 63.3% of the gram-negative bacilli causing urinary tract infections were resistant to trimethoprim. The majority of resistant isolates (86%) were highly resistant and 50.8% were capable of transferring their resistance. Trimethoprim and Smx resistance co-transferred in 93.4% of the isolates and trimethoprim and streptomycin resistance only occurred in 7.5% of isolates. This suggested that Tn7 is not as widely spread in Nigeria as in other areas.

A survey of trimethoprim resistance in South Africa was undertaken from November 1986 to January 1987. The results of this will be discussed fully in the following chapter.

1.6.8 Trimethoprim resistance: developed vs developing countries

Comparison of the available data on trimethoprim

resistance from first-world and developing countries shows that the incidence of resistance is much greater in developing countries. It has been postulated that this could be due to the fact that trimethoprim is freely available in some of these countries without prescription and its use, therefore, is not as well controlled as in western countries (Farrar, 1985). In addition, trimethoprim is commonly used for the treatment and prevention of diarrhoeal disease in third-world countries and because these agents are relatively inexpensive and have broad spectrum activity, they have been over-utilised by practitioners more extensively in third-world countries. Inefficient control of hospital cross-infection may also play a role in the high frequencies of antimicrobial resistance in organisms isolated from hospital patients. Poor sanitary conditions, which are widespread in the third-world, also allow constant exposure of the population to enteric pathogens. This in turn probably assists in the spread of many antibiotic resistance determinants. Although a large proportion of trimethoprim resistance in developing countries is high-level (MIC >1 000 mg/l) most of the determinants have

been shown to be transferable. This suggests that, unlike trends in Europe and the UK, resistance has remained plasmid-associated in third-world countries. Changes in this regard may yet become evident as more data are gathered from these countries.

CHAPTER 2 EPIDEMIOLOGY OF TRIMETHOPRIM RESISTANCE IN SOUTH AFRICA.

2.1 INTRODUCTION

The incidence of trimethoprim resistance in South Africa had not been investigated prior to 1987. However, data on the incidence of resistance to cotrimoxazole in isolates from the Johannesburg General Hospital (now the Hillbrow Hospital) and Baragwanath Hospital were published as part of the worldwide survey of O'Brien et al. in 1982. Resistance to cotrimoxazole was greater in the South African hospitals than in most other participating hospitals which indicated that resistance to trimethoprim would also be high.

A three-month survey (November 1986 to January 1987) was performed in which resistance to trimethoprim alone was determined in gram-negative bacteria. Isolates were collected from the Johannesburg, Hillbrow and Baragwnanth Hospitals. In a preliminary study a number of isolates (36) were also tested for the presence of the type I and/or type II DHFR genes by DNA hybridization.

2.2 MATERIALS AND METHODS

2.2.1 Bacteria

Gram-negative bacteria isolated in the routine diagnostic laboratories of the Baragwanath, Hillbrow and Johannesburg Hospitals were collected and identified to genus level by standard biochemical tests. Twenty-seven highly resistant isolates from this survey together with nine other gram-negative organisms (MIC >1 024 mg/l) which were isolated in 1983 were selected for hybridization studies.

2.2.2 Minimum inhibitory concentration (MIC) determination

The MIC of trimethoprim for each organism was determined by the method advocated by the National Committee for Clinical Laboratory Standards (1985). A small volume (1 ml) of Davis-Mingioli (DM) minimal medium (Davis & Mingioli, 1950) containing 2.8% glucose was inoculated with the isolate and incubated at 37 °C until the medium was

turbid (c. 2 to 2.5 h). The log phase culture was diluted to c. 5.10^4 cfu/ml and inoculated onto DM minimal agar plates containing 2.8 g/l glucose and appropriate concentrations of trimethoprim lactate (Wellcome). A multipoint inoculator (Mast) was used for this purpose. The plates were incubated at 37 °C and read at 42 h. Isolates that did not grow on this medium were then tested on DM minimal medium plus thymidine (25 mg/l). Organisms that failed to grow on the supplemented minimal medium were retested on Iso-Sensitest agar (Oxoid). High-level resistance was defined as MIC >1 024 mg/l and low-level (intermediate) resistance as MIC 16 to 1 024 mg/l.

2.2.3 Gene probes

A 500-bp HpaI fragment containing the type Ia DHFR gene was isolated from pFE506 (Fling & Elwell, 1980) and used as the type I probe. The type II DHFR probe used originated from pFE364 and consisted of an 800-bp fragment cloned into the EcoRI site of pUC4 (a gift from M.E. Fling).

2.2.4 Preparation and purification of gene probes

Plasmid DNA was extracted by the method of Clewell and Helinski (1969) with the following modifications. Bacterial cultures were grown overnight in Luria Broth (10% tryptone, 5% yeast extract, 5% NaCl) and 100 ml volumes were used for the preparation of plasmid. The pelleted bacteria were resuspended in 2 ml 20% sucrose and 0.25 ml of 10 mg/l lysozyme (Sigma) was added. Following this 2.5 ml of both the 0.5 M EDTA solution (pH 8.5) and the lysing solution (10% Triton X-100, 0.0625 M EDTA pH 8.5, 0.05 M Tris pH 8.5) were added. The lysates were cleared by centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments Inc., Palo Alto, California, USA) at 29 000 g for 45 m. The plasmids were purified by cesium chloride density centrifugation as described by Maniatis et al. (1982). The plasmids were then restricted with the appropriate enzymes according to the manufacturer's specifications (Boehringer Mannheim GmbH, Penzberg, West Germany) and the fragments separated by a minisub gel electrophoresis apparatus (Biorad Laboratories, Munich, West Germany) with 2% NuSieve agarose (FMC

Corporation, Rockland, M.E. 04841, USA) for the pFE506 digestion and 1% SeaPlaque agarose (FMC Corporation) for the pUC4-12 digestion. The appropriate fragments were cut out of the gels and placed in 1.5 ml reaction tubes (Eppendorf Geratebau Netheler + Hinz GmbH, Hamburg, West Germany) with 0.2 ml Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8). The tubes were placed in a waterbath at 60 °C until the gel was liquified (approximately 10 m). The DNA was extracted from the gel using 2x the volume phenol saturated with TE buffer. The tubes were vortexed briefly and centrifuged in a benchtop microfuge (Eppendorf) at 14 000 rpm for 7 m. The supernatants were removed, reserved and a further 0.1 ml TE buffer added to the phenol/gel mix. The extraction procedure was repeated. The supernatants were combined and extracted twice with ether. The DNA was precipitated with 2x volume of absolute ethanol/300 mM sodium acetate, centrifuged, washed with 70% ethanol and dried. The pellets were resuspended in TE buffer to an approximate concentration of 1 µg/5 µl.

2.2.5 Nick translation and hybridization

Nick translation was performed with ^{32}P-dCTP and a nick translation kit (Amersham International PLC, Bucks., UK) following the manufacturer's instructions (Appendix A). Plasmid and chromosomal DNA were extracted from each of the 36 isolates and the appropriate controls by the method of Kado & Liu (1981), electrophoresed on a horizontal 0.7% agarose gel and transferred onto Biodyne nylon membrane (Pall Ultrafine Filtration Corporation, Glen Cove, N.Y., USA). This procedure and subsequent hybridization of the labelled probes were performed according to the manufacturer's instructions (Appendix B). The washed blots were autoradiographed, stripped of the first probe and re-hybridized with the second probe.

2.3 RESULTS

2.3.1 Trimethoprim resistance in all hospital strains

The results of the survey are summarized in Table 2.1. The total number of isolates collected was 2 914 and of these 1 141 (39.2%) were Escherichia coli, 540 (18.5%) Klebsiella spp., 438 (15%) Pseudomonas spp., 346 (11.9%) Proteus spp., 289 (9.9%) Enterobacter spp. and 160 (5.5%) various other gram-negative bacilli. The number of bacteria resistant to trimethoprim (MIC >8 mg/l) was 1 637 (representing 56.2%) and 700 (24%) had high-level resistance (MIC >1 024 mg/l). Therefore, 42.8% of the resistant strains were resistant to >1 024 mg/l trimethoprim.

The overall resistance rate in Enterobacteriaceae (MIC >8 mg/l) was 48.5% (1 196/2 464) and amongst these the highest percentage resistance occurred in Klebsiella spp. (75.4%). The Klebsiella spp. isolated in the Hillbrow Hospital were particularly resistant to trimethoprim as a total of 90.1% (136/151) were resistant to 16 mg/l or more of trimethoprim. However, the majority of these resistant strains

Table 2.1. Trimethoprim resistance in gram-negative bacteria.

ISOLATE	BARAGWANATH			JOHANNESBURG			HILLBROW			TOTAL		
	Total	HLR	LLR	Total	HLR	LLR	Total	HLR	LLR	Total	HLR	LLR
ENTEROBACTERIACEAE												
E.coli	429	159	11	475	119	15	237	71	15	1 141	349	41
Klebsiella spp.	275	104	101	114	27	39	151	18	118	540	149	258
Proteus spp.	23	2	17	125	20	40	198	44	109	346	66	166
Enterobacter spp.	97	17	23	95	12	37	97	6	24	289	35	84
Other entero-bacteria	48	7	1	62	6	13	38	7	14	148	20	28
A: TOTAL	872	289	153	871	184	144	721	146	280	2 464	619	577
OTHER STRAINS												
Pseudomonas spp.	129	31	98	129	12	115	180	37	143	438	80	356
Aeromonas spp.	1	0	0	-	-	-	3	0	1	4	0	1
Alkaligenes spp.	-	-	-	4	1	1	4	0	2	8	1	3
B: TOTAL	130	31	98	133	13	116	187	37	146	450	81	360
A+B: TOTAL	1 002	320	251	1 004	197	260	908	183	426	2 914	700	937

HLR = High-level resistance (MIC >1 024 mg/l)

LLR = Low-level resistance (MIC 16 to 1 024 mg/l)

(118/136, 86.8%) fell into the low-level resistance category. Of the E.coli isolates only 34.2% (390/1 141) were resistant to trimethoprim (MIC >8 mg/l). However, 89.5% (349/390) of the resistant strains were highly resistant (MIC >1 024 mg/l). This was a surprisingly high proportion especially when this figure is compared with those of other genera; 36.6% of resistant Klebsiella spp., 28.4% of resistant Proteus spp. and 29.4% of resistant Enterobacter spp. had MICs of >1 024 mg/l.

The proportion of high-level resistant isolates in each genus varied considerably. In Enterobacter spp. only 12.1% (35/289) of all isolates were highly resistant, in Proteus spp. 19.1% (66/346), in Klebsiella spp. 27.6% (149/540) and in E.coli 30.6% (349/1 141). Since E.coli represents 46.3% of the enterobacteria isolated, this affects the final percentage of high-level resistance in the whole enterobacterial population. In spite of this, however, the final percentage of 25.1% (619/2 464) is not remarkably high.

Significant resistance to trimethoprim was observed in Pseudomonas spp. as only two out of 438 strains were sensitive to trimethoprim. Amongst the 436 resistant pseudomonads, however, only 18.3% were

resistant to >1 024 mg/l of trimethoprim.

2.3.2 Trimethoprim resistance in urinary isolates

Since trimethoprim is used frequently and is particularly indicated in the treatment of urinary tract infections, an analysis of bacteria isolated from urine specimens was performed. These results are presented in Table 2.2.

There were 2 914 strains isolated in the original survey and 1 218 (41.8%) of these were obtained from urine specimens. Amongst the urinary strains 771 (63.3%) were *E.coli*, 168 (13.8%) *Klebsiella spp.*, 115 (9.4%) *Proteus spp.*, 68 (5.6%) *Enterobacter spp.* and 96 (7.9%) various gram-negative bacilli. Overall resistance to trimethoprim (MIC >8 mg/l) was 49.1% (598/1 218). However, this proportion included the *Pseudomonas spp.*. When the *Pseudomonas spp.* were excluded 46% (529/1 149) were resistant to trimethoprim.

When all the resistant strains were examined for high-level resistance (MIC >1 024 mg/l) 66.6% (398/598) fell into this category. When the pseudomonads were excluded an even higher percentage of 71.8% (380/529) of the resistant

Table 2.2. Trimethoprim resistance in urinary isolates.

ISOLATE	BARAGWANATH			JOHANNESBURG			HILLBROW			TOTAL		
	Total	HLR	LLR	Total	HLR	LLR	Total	HLR	LLR	Total	HLR	LLR
ENTEROBACTERIACEAE												
E.coli	275	114	9	384	102	12	112	44	9	771	260	30
Klebsiella spp.	82	34	28	64	18	20	22	5	13	168	57	61
Proteus spp.	4	1	1	65	7	23	46	23	14	115	31	38
Enterobacter spp.	28	11	6	33	9	8	7	1	1	68	21	15
Other entero-bacteria	3	3	0	17	3	4	7	5	1	27	11	5
TOTAL	392	163	44	563	139	67	194	78	38	1 149	380	149
OTHER STRAINS												
Pseudomonas spp.	6	4	2	43	6	37	20	8	12	69	18	51
TOTAL	398	167	46	606	145	104	214	86	50	1 218	398	200

HLR = High-level resistance (MIC >1 024 mg/l)

LLR = Low-level resistance (MIC 16 to 1 024 mg/l)

isolates grew in the presence of trimethoprim at 1 024 mg/l.

The highest percentage of high-level resistance was in those isolates from Baragwanath Hospital where 78.4% (167/213) of the resistant isolates had trimethoprim MICs >1 024 mg/l. Thus, 42% of all urine isolates from this hospital were highly resistant to trimethoprim.

The Klebsiella spp. were again the most resistant organisms amongst the Enterobacteriaceae. The overall rate of trimethoprim resistance in this genus was 70.2% (118/168). However, only 48.3% (57/118) of these were highly resistant to trimethoprim.

E.coli isolates represented 63.3% (771/1 218) of all the urinary strains examined and of these 37.6% (290/771) were resistant to >8 mg/l trimethoprim. Moreover, 89.7% (260/290) of these resistant bacteria had MICs >1 024 mg/l. The resistant E.coli strains isolated at Baragwanath Hospital had the highest percentage at 92.7% (114/123) of high-level resistance. Other genera in the three hospitals had lower proportions of high-level resistance in Tp-resistant bacteria; Klebsiella spp. 48.3% (57/118), Proteus spp.

45% (31/69), Enterobacter spp. 58% (21/36) and Pseudomonas spp. 26% (18/69).

2.3.3 Comparison of the incidence of trimethoprim resistance in urinary and non-urinary isolates

A comparison of the prevalence rates of resistance in urinary and non-urinary isolates is presented in Table 2.3. Overall there were more resistant isolates in the non-urinary group (61.3%, 1 039/1 696) than in the urinary group (49.1%, 598/1 218). Analysis of these figures shows, however, that 70.9% (737/1 039) of the resistance in non-urinary isolates was low-level (MIC 16 to 1 024 mg/l) compared to 33.4% (200/598) in urinary isolates. This difference is statistically significant ($X^2 = 216.41$, $p < 0.0001$).

Analysis of resistance in the four main genera shows that amongst both E.coli and Enterobacter spp. isolated from urine specimens a greater proportion were resistant to trimethoprim than their non-urinary counterparts. High-level resistance accounted for 89.7% and 58.3% in urinary isolates of E.coli and Enterobacter spp. respectively. Klebsiella spp. and Proteus spp.

Table 2.3. Comparison of the percentage of trimethoprim resistance in urinary and non-urinary isolates.

	URINARY		NON-URINARY	
Total Tp Resistance (%)	49.1		61.3	
% HLR in resistant isolates	66.6		29.1	
% HLR in all isolates	32.7		17.8	
Resistance in:	Total(%)	HLR(%)	Total(%)	HLR(%)
E.coli	37.6	89.7	27.0	89.0
Klebsiella spp.	70.2	48.3	77.7	31.8
Proteus spp.	60.0	44.9	70.6	21.5
Enterobacter spp.	52.9	58.3	37.6	16.9

HLR = High-level resistance (MIC >1 024 mg/l)

isolated from urine were less resistant than those from other specimens. However, the greater proportion of the resistant urinary strains had trimethoprim MICs >1 024 mg/l. Overall, therefore,

a much greater proportion of urinary isolates were highly resistant to trimethoprim than non-urinary isolates (66.6% vs 29.1%).

2.3.4 Comparison of trimethoprim resistance in different hospitals

The frequency of trimethoprim resistance (MIC >8 mg/l) in the three hospitals varied considerably. When only the enterobacterial strains were examined 59% (426/721) of the Hillbrow Hospital isolates were resistant compared with 50.6% (442/872) for Baragwanath Hospital and 37.6% (328/871) for the Johannesburg Hospital. These differences are statistically significant (X^2 = 26.36, $p < 0.001$).

2.3.5 DNA hybridization

In the preliminary study a small number of *E.coli*, *Klebsiella spp.* and *Proteus spp.* isolates were investigated by DNA hybridization for the presence of the type I and type II genes. The only criterion used for their selection was an MIC >1 024 mg/l. Twenty-seven isolates from the 1986/7 survey were

investigated together with nine others isolated in 1983. There were twelve each of E.coli, Klebsiella spp. and Proteus spp.. None of the isolates from the 1983 group and none of the Proteus spp. from the 1986/7 group hybridized with either probe. Of the E.coli isolates only two hybridized positively with the type I probe but none with the type II probe. Similarly, only six of the Klebsiella spp. hybridized positively with the type I probe but none with the type II probe. Thus, a total of eight of the 36 isolates hybridized positively with the type I probe but none with the type II probe. Figures 2.1. and 2.2. illustrate some of these results.

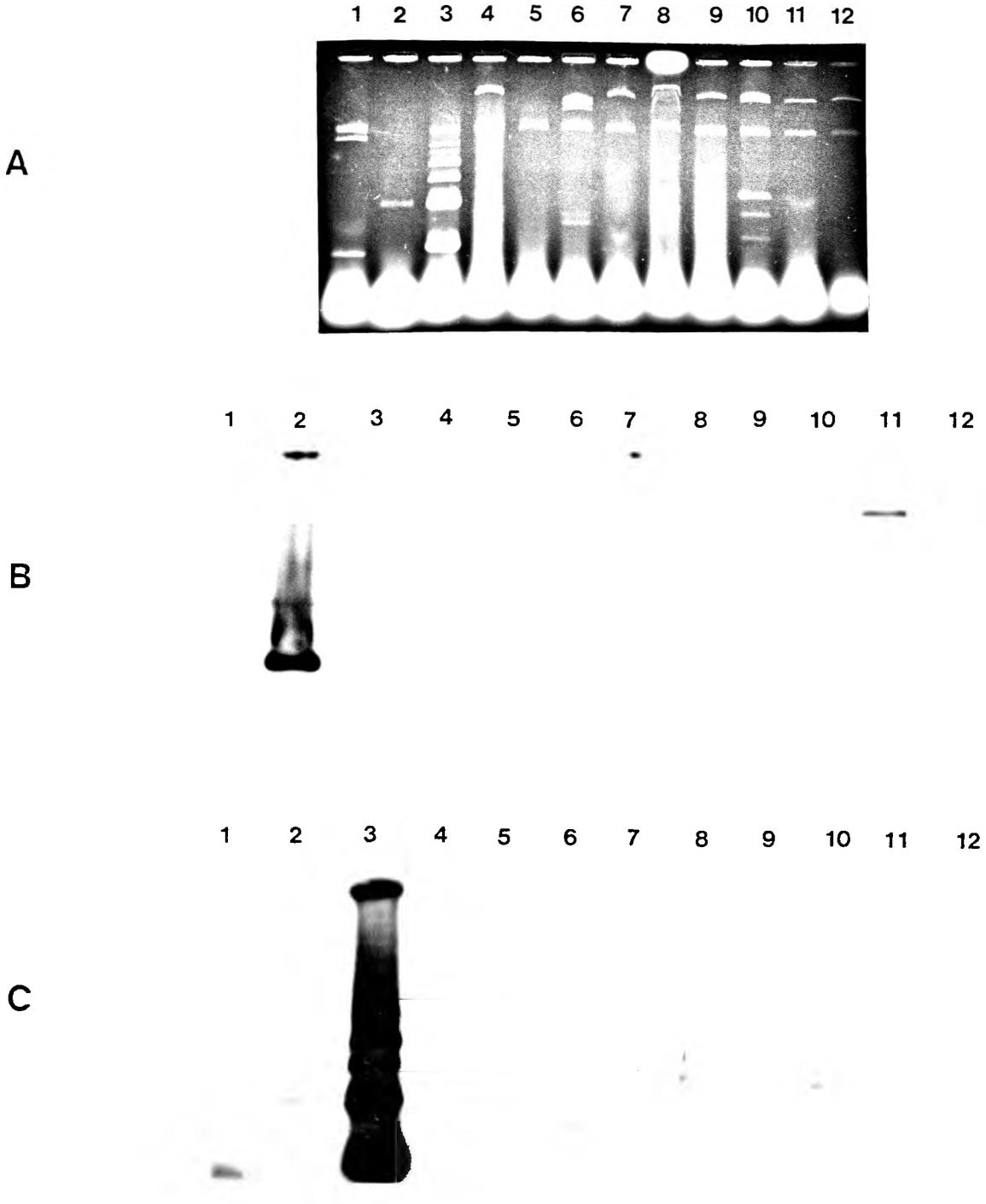


Figure 2.1. A: Gel electrophoresis of plasmid and chromosomal DNA extracted from E.coli isolates. Lane 1, Tp^S E.coli; 2, pFE506 (type I DHFR); 3, pUC4-12 (type II DHFR); 4 - 12, E.coli isolates (MIC >1 024 mg/l). B: autoradiogram of the gel in A hybridized with a type I DHFR gene probe. C: autoradiogram of the gel in A hybridized with a type II DHFR gene probe. Tp = trimethoprim.

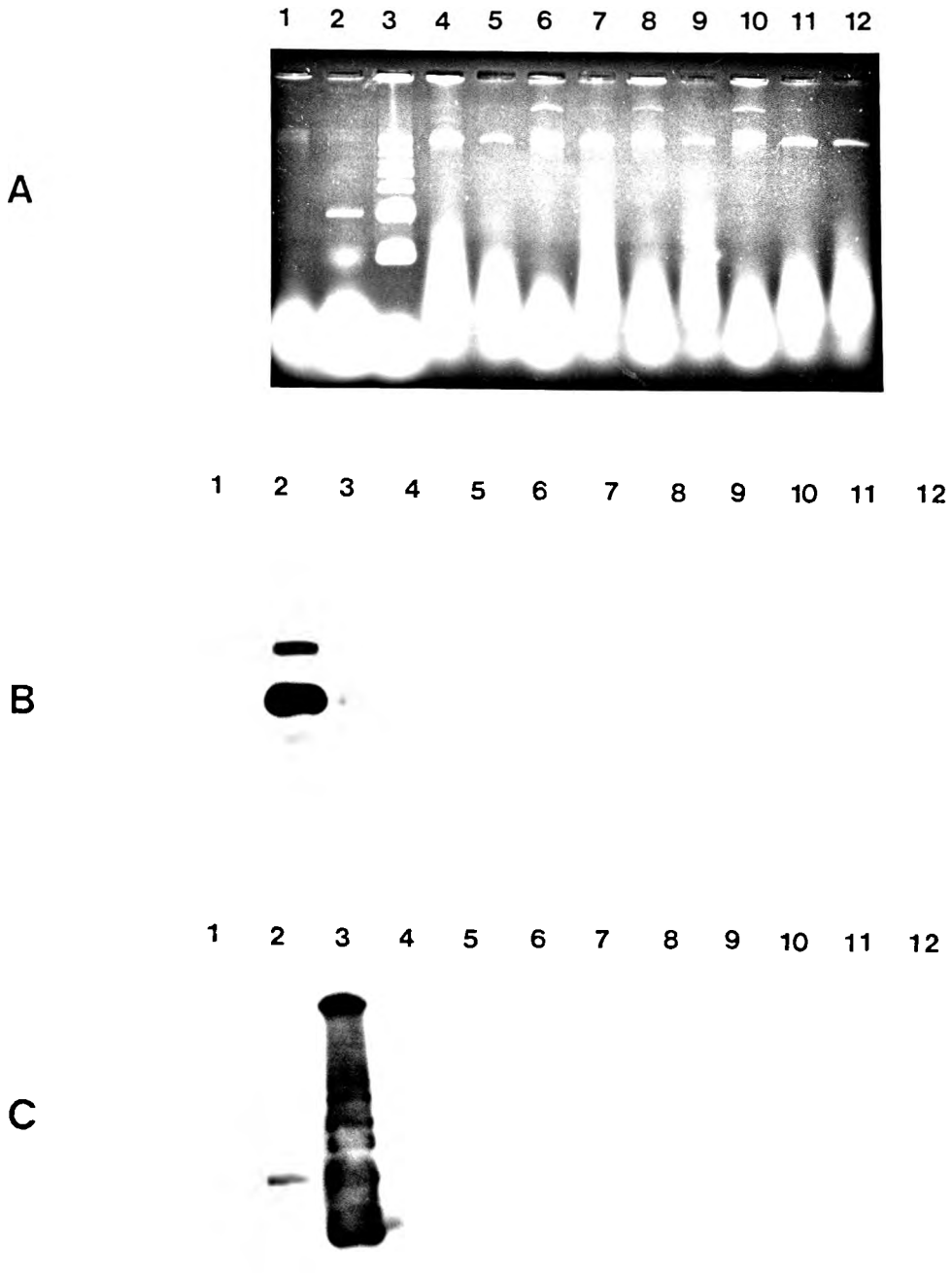


Figure 2.2. A: Gel electrophoresis of plasmid and chromosomal DNA extracted from various Proteus species. Lane 1, Tp^S Proteus; 2, pFE506 (type I DHFR); 3, pUC4-12 (type II DHFR); 4-12, Proteus isolates (MIC >1 024 mg/l). (Lane 6 = P.mirabilis (J120)). B: autoradiogram of the gel in A hybridized with a type I DHFR gene probe. C: autoradiogram of the gel in A hybridized with a type II gene probe. Tp = trimethoprim

2.4 DISCUSSION

Table 2.4. compares trimethoprim resistance reported from several European centres with that from developing countries. In these studies resistance to trimethoprim alone was determined and the bacteria were isolated from hospitalized patients.

The incidence of resistance in urinary isolates from London, Edinburgh and Turku was similar, falling within the range 8.6% to 12.3% (Huovinen, Mantyjarvi & Toivanen, 1982; Brumfitt, Hamilton-Miller & Wood, 1983; Amyes, 1986). Of these resistant strains the percentage which were highly resistant varied somewhat: 29.5% to 78%. However, of all the urinary isolates tested in these surveys high-level resistance only occurred in 2.7% to 9.5%.

In comparing the values obtained in the Indian study with those of Western Europe a marked difference is evident. A total of 64.1% of the Indian strains (as compared to 8% to 12% of European strains) was resistant to trimethoprim, 89.6% of these being highly resistant. Therefore, a massive 57.3% of all strains isolated were highly resistant to trimethoprim (Amyes, Doherty & Young, 1986). The values obtained for the three South African hospitals fall between those of Western Europe and India,

Table 2.4. Comparison of trimethoprim resistance in various centres.

	DATE	PLACE	TOTAL Tp RESISTANCE (%)	%HLR/RES ISOLATES	% HLR/TOTAL No.ISOLATES	TOTAL No.OF ISOLATES	REFERENCE
	1980/1	Turku (Finland)					
		Turku University	12.2	(29.5)	3.6	436	Huovinen et al. (1982)
		Kuopio University	8.6	(31.4)	2.7	430	Huovinen et al. (1982)
	1981	London (UK)	12.2	78	(9.5)	1 444	Brumfitt et al. (1983)
	1984	Edinburgh (UK)	12.3	(65.0)	8	323	Amyes (1986)
URINARY	1984	Vellore (India)	64.1	89.6	57.3	284	Young et al. (1986)
ISOLATES	1985	Dar es Salaam					
		(Tanzania)	-	-	27	-	Amyes (1986)
	1986/7	Johannesburg					
		(South Africa)					
		Baragwanath	53.5	78.4	42.0	398	This study
		Johannesburg	41.1	58.2	23.9	606	This study
		Hillbrow	63.6	63.2	40.2	214	This study
	1984	Paris (France)	24.3	95.4	(23.2)	5 494	Goldstein et al. (1986)
	1985	Dar es Salaam					
		(Tanzania)	36.0	-	22.0	-	Amyes (1986)
ENTERO-	1986/7	Johannesburg					
BACTERIA		(South Africa)					
		Baragwanath	50.7	65.4	33.1	872	This study
		Johannesburg	37.7	56.1	21.1	871	This study
		Hillbrow	59.1	34.3	20.2	721	This study

Note: The figures in parenthesis were not quoted in the referenced paper.

although they resemble the Indian values more closely. Resistance to trimethoprim ranged from 41.1% to 63.6% while high-level resistance occurred in 66.3 to 79.3% of all resistant isolates. Therefore, between 27.2% and 43% of all urinary isolates were resistant to >1 024 mg/l trimethoprim. In this, therefore, South Africa can be classed with other developing countries.

The reason (or reasons) for the high frequency of resistance is a matter for speculation. Antibiotics are not available over the counter as in other developing countries and thus this form of abuse cannot contribute to the high incidence of resistance. However, the amount of trimethoprim dispensed per annum to both in-patients and out-patients at these hospitals is large. Baragwanath Hospital uses 47.5 kg per annum as cotrimoxazole, Hillbrow Hospital 16 kg as cotrimoxazole and 2.3 kg as trimethoprim and Johannesburg Hospital 1.2 kg as cotrimoxazole and 20 kg as trimethoprim. These amounts are of the same order as those used in the Muhimbili Hospital, Dar es Salaam, and in the Christian Medical College Hospital, Vellore, but significantly more than that used in the Royal Infirmary, Edinburgh, (Amyes & Young, 1987). Because the greater proportion of trimethoprim dispensed at Baragwanath Hospital is prescribed to out-patients (43 kg per annum), patient

non-compliance in completing the course of antibiotics may be an important factor in the prevalence of resistance. With such large amounts of trimethoprim consistently present in the community the selective pressure created may also promote the spread of R-plasmids carrying trimethoprim-resistant DHFR genes. Comparative figures for the Hillbrow and Johannesburg Hospitals were not available.

In enterobacteria as a whole isolates from Western countries were generally less resistant than those from developing countries. Taking the values reported from Paris (Goldstein, Papadopoulou & Acar, 1986) as an example, resistance in enterobacteria was 24.3% as compared to 36% in Dar es Salaam and 37.7% to 59.1% in Johannesburg.

Comparison of high-level resistance from the same centres shows great consistency, however, with only Baragwanath Hospital having a higher proportion of enterobacteria resistant to >1 024 mg/l trimethoprim. Intrinsic mechanisms of resistance and/or plasmid-mediated intermediate resistance must, therefore, account for the higher resistance percentage in Tanzania and South Africa.

Comparison of resistance in urinary and non-urinary isolates shows that although there are more resistant

non-urinary isolates, high-level resistance is much more prevalent in urinary isolates. Since trimethoprim is one of the drugs of choice in the treatment of urinary tract infections its use (which results in high urinary concentrations) appears to be creating selective pressure for high-level resistance determinants in these bacteria. In the preliminary hybridization study only eight out of 36 isolates (22%) hybridized with the type I probe and none with the type II probe. These results were unexpected as high-level resistance mediated by the type I DHFR has been shown to be common in many countries. Resistance in the remaining isolates may, therefore, be due to one or more unidentified DHFRs.

CHAPTER 3 IDENTIFICATION OF A NOVEL PLASMID-ENCODED DIHYDROFOLATE REDUCTASE MEDIATING HIGH-LEVEL RESISTANCE TO TRIMETHOPRIM

3.1 INTRODUCTION

Enzymes are proteins specialized to catalyze biological reactions. They are remarkably specific and have great catalytic power, some depending on co-factors and/or co-enzymes for activity. Enzymes are classified according to the type of reaction they effect e.g. oxido-reductases are involved in oxidation-reduction reactions, transferases effect the transfer of functional groups and ligases are required in reactions where bonds are created.

Enzymes have many different properties which are used in their characterization. The most fundamental of these are the kinetics of the reaction they catalyze. The general theory of enzyme action and kinetics, developed in 1913 by L. Michaelis and M.L. Menton and extended in 1925 by G.E. Briggs and J.B.S. Haldane, gave rise to the well-known Michaelis-Menton equation

$$v_o = \frac{Vmax[S]}{Km + [S]}$$

where v_o is the initial rate of the reaction and [S] is the substrate concentration. The Michaelis constant (Km)

reflects the rate at which the enzyme-substrate complex is formed while the maximum velocity of the reaction (Vmax) reflects the rate at which the enzyme-product complex dissociates. The values of Km and Vmax are determined experimentally and are characteristic for each enzyme. In order to obtain accurate values for Km and Vmax single or double reciprocal plots of the Michaelis equation can be used. The equation for the Lineweaver-Burk plot is derived by taking the reciprocal of both sides of the Michaelis-Menton equation and rearranging to yield

$$\frac{1}{v_o} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax}$$

When $1/v_o$ is plotted against $1/[S]$, a straight line is obtained with a slope of Km/Vmax, an intercept of 1/Vmax on the $1/v_o$ axis and an intercept of -1/Km on the 1/[S] axis.

A second useful transformation of the Michaelis-Menton equation is obtained by multiplying the above equation by Vmax and rearranging to yield

$$v_o = -Km\,\frac{v_o}{[S]} + Vmax$$

A plot of v_o vs $v_o/[S]$ (Eadie-Hofstee plot) yields a straight line from which values for Km and Vmax can be obtained.

The study of enzyme inhibitors has provided important information on the mechanism and pathway of enzyme catalysis. There are three types of reversible enzyme inhibition viz. competitive, uncompetitive and noncompetitive. These can be experimentally distinguished by the effects of the inhibitor on the reaction kinetics of the enzyme and these may be analyzed in terms of the basic Michaelis-Menton rate equation.

i) A competitive inhibitor reacts with the enzyme in such a way that it competes with the normal substrate for binding at the active site. The inhibitor is not changed in any way during this reaction. The inhibitor constant (Ki) is the dissociation constant of the enzyme-inhibitor complex.

ii) In uncompetitive inhibition the inhibitor binds to the enzyme-substrate complex to give an enzyme-substrate-inhibitor complex which cannot undergo any further reaction. Uncompetitive inhibition is rare in single substrate reactions but is more common where two substrates are involved.

iii) Noncompetitive inhibitors bind to either the enzyme

or the enzyme-substrate complex at a site other than the active site. This causes the formation of abnormal enzyme-substrate complexes which cannot undergo further reaction.

The three types of inhibitors can be distinguished by plotting $1/v_o$ vs $1/[S]$ for different, fixed concentrations of inhibitor. The plots obtained are characteristic for each type of inhibition.

Other distinguishing features used to characterize enzymes include determinations of molecular weight, sub-unit structure, isoelectric point (pI), substrate and inhibitor profiles, thermolability or stability and amino acid and DNA sequences. Sophisticated techniques such as X-ray crystallography have also provided valuable information on the three-dimensional structure of enzymes alone and in combination with their substrate and/or co-enzyme. Substrate, inhibitor and co-enzyme binding sites have been identified in this way.

Dihydrofolate reductases (DHFRs) catalyze the conversion of dihydrofolate to tetrahydrofolate in a reaction which requires the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). Although all DHFRs catalyze the same reaction they are not identical molecules and have been assigned to different groups using the approaches to characterization listed above.

Prior to 1987 the molecular basis of high-level trimethoprim resistance in South African isolates had not been investigated. The pilot hybridization study, described in Chapter 2, suggested that the type I and type II DHFR genes were not as common in local isolates as expected. It was possible, therefore, that another resistant DHFR was mediating high-level resistance in these organisms. Several isolates whose DNA did not hybridize with either of the probes were investigated for DHFR activity. One organism, Proteus mirabilis (J120), was selected for further investigation.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria

P.mirabilis (J120) which was resistant to ampicillin, tetracycline and trimethoprim was isolated from wound irrigation fluid. *Escherichia coli* K12 strain J62-2 (*pro his trp* rif) (Bachmann, 1972) was used as the recipient in conjugation experiments.

3.2.2 Minimum inhibitory concentration (MIC) determination

The MICs of trimethoprim for *P.mirabilis* (J120) and the *E.coli* transconjugants were determined as previously described in Chapter 2. Davis Mingioli minimal medium supplemented with 2.8% glucose was used.

3.2.3 Plasmid demonstration and sizing

DNA was extracted from the bacteria by the method of Kado & Liu (1981). Samples from each culture were electrophoresed in a flatbed, 0.7% agarose gel

at 3.75 V/cm for 5 h. The gel was stained with ethidium bromide (1 mg/l final concentration) for 2 to 3 h. Plasmid sizes were determined from the distance they had migrated in comparison with standard plasmids as previously described (Amyes & Gould, 1984).

3.2.4 Conjugation studies

Bacterial conjugation studies were performed by the method of Amyes & Gould (1984). The donor (*P.mirabilis* (J120)) and recipient (*E.coli* J62-2) strains were inoculated into nutrient broth and grown for approximately 5 h at 37°C in an environmental shaker (Gallenkamp). Fresh nutrient broth (4.5 ml) was warmed to 37°C and inoculated with 0.1 ml of the donor and 1.0 ml of the recipient. The mixture was incubated overnight at 37°C. After vortexing the broth 0.1 ml aliquots were plated onto DM minimal medium plates supplemented with 2.8% glucose and containing histidine (50 mg/l), tryptophan (50 mg/l), proline (50 mg/l), trimethoprim lactate (10 mg/l) and rifampicin (25 mg/l). The plates were incubated at 37°C for 16 h.

3.2.5 DHFR assay

DHFR activity was assayed by the method of Osborn & Heunnekens (1958). Reactions were performed in a Pye Unicam spectrophotometer (Phillips) at 37°C. The reaction mixture consisted of 40 mM sodium phosphate buffer pH 6.0, 0.1 mM NADPH, 10 mM β-mercaptoethanol, enzyme, trimethoprim or methotrexate where appropriate and distilled water to 0.95 ml. This was allowed to equilibrate for one minute before the reaction was started by the addition of dihydrofolate to 0.05 mM. The reaction was followed by noting the decrease in absorbance at 340 nm over a period of 3 minutes. The activity of the enzyme was defined as:

Δ OD/min x 82.5 x dilution factor (nmol DHF reduced /min/ml).

3.2.6 DHFR preparation and purification

Three litres of bacterial culture were grown overnight in nutrient broth (Oxoid) on a shaking platform. The bacteria were harvested by centrifugation (3 800 g) at 4°C for 10 min.

All further manipulations were performed at 4°C. The bacterial pellet was washed with 50 ml DM minimal medium, pelleted again and finally resuspended in 20 ml ice-cold buffer A (50 mM sodium phosphate buffer pH 7.4 containing 10 mM β-mercaptoethanol and 1 mM EDTA). The suspension was sonicated for 3 x 45 sec using a MSE Soniprep 150 then clarified by centrifugation at 40 000 g for 1 h. The resultant supernatant was the crude enzyme preparation. Nucleic acids were removed by precipitation with 1% streptomycin sulphate and the enzyme was precipitated with ammonium sulphate as detailed in the results section. The final ammonium sulphate precipitate was pelleted and resuspended in 2 ml Buffer A. The enzyme activity and protein concentration (Waddell, 1956) were determined at each stage.

3.2.7 Molecular weight determination

The molecular weight of the enzyme was determined by Sephadex exclusion chromatography as previously described (Amyes & Smith, 1974). Partially purified DHFR preparation (2 ml) was applied to a Sephadex G-75 column (2 cm^2 cross sectional area x 90 cm)

which had been previously calibrated using ovalbumin (Mr = 45 000), chymotrypsinogen (Mr = 25 500) and cytochrome c (Mr = 12 384). The enzyme was eluted from the column with Buffer A containing 1 mg/l bovine serum albumin at a flow rate of 10 ml/h. Fractions (2 ml) were collected using a Model 2214 fraction collector (LKB) and analyzed for DHFR activity. Finer determination was obtained with a Sephadex G-50 column (2 cm^2 x 90 cm) when chymotrypsinogen, cytochrome c and the β subunit of insulin (Mr = 3 878) were employed as molecular weight standards.

3.2.8 Heat sensitivity

Aliquots of enzyme preparation were maintained at 45 °C in a preheated waterbath from 15 seconds to two minutes. A sample was removed every 15 seconds, rapidly cooled on ice and the enzyme activity assayed.

3.3 RESULTS

3.3.1 MIC, conjugation and DHFR purification

The clinical strain P.mirabilis (J120) was highly resistant to trimethoprim (MIC >1 024 mg/l). The trimethoprim resistance determinant was transferred into E.coli J62-2. A single, large plasmid of approximately 79 kb, designated pUK672, was demonstrated in both the wild type and its transconjugant. This plasmid was presumed to carry the trimethoprim resistance determinant.

Cell-free extracts from both the original clinical strain (J120) and its E.coli J62-2 transconjugant (strain J62-2(pUK672)) were assayed for DHFR activity. Both strains had similar levels of activity (Table 3.1.) which were 20- to 40-fold higher than those in plasmid-free cells. The presence of trimethoprim in the growth medium did not significantly elevate the enzyme levels, suggesting that expression was not inducible.

DHFR activity was purified from both J120 and J62-2(pUK672). After removal of the nucleic acids with streptomycin sulphate the protein was selectively precipitated with ammonium sulphate.

Table 3.1. The specific activities of dihydrofolate reductase in P.mirabilis (J120) and its transconjugant.

Bacterial Strain	Designation	Plasmid	SA of DHFR (nmol DHF reduced /min/mg protein)
P.mirabilis	J120	(pUK672)	42.2
E.coli	J62-2	(pUK672)	20.8
E.coli	J62-2	R	1.1

Previous plasmid-encoded DHFR enzymes have all precipitated when the ammonium sulphate concentration was raised from 50 to 80% saturation. However, in this case 69.2% of the DHFR activity had precipitated at 50% saturation (Table 3.2.). When precipitate arising from this step was applied to a Sephadex G-75 column a single peak of dihydrofolate reductase activity was found at fraction 58. This trimethoprim-sensitive DHFR peak had a molecular weight of 23 600 and was assumed to be the chromosomal enzyme. However, when gel filtration was repeated with bovine serum albumin

Table 3.2. Purification of dihydrofolate reducatase from P.mirabilis (J120).

Stage	Volume (ml)	Protein conc. (mg/ml)	DHFR Activity (nmol/min/ml)	SA (nmol/min/mg protein)	Purification (-fold)	Recovery (%)
Bulk	17	28.17	1 188	42.17	1	100
50% $(NH_4)_2SO_4$ supernatant	17.5	5.08	49.5	9.74	0.23	4.3
50% $(NH_4)_2SO_4$ pellet	2	84.5	6 987.75	82.7	1.96	69.2
80% $(NH_4)_2SO_4$ pellet	2	67.73	759	11.21	0.27	7.5

(10 mg/l) added to the elution Buffer A, a larger DHFR peak emerged at fraction 75. The enzyme in this peak was resistant to trimethoprim.

3.3.2 Molecular weight of the plasmid DHFR

The elution behavior of the trimethoprim-resistant DHFR activity on Sephadex G-75 suggested an enzyme with a low molecular weight. Therefore, the precipitate arising from increasing ammonium sulphate saturation from 35% to 60% was applied to a Sephadex G-50 column (2 cm^2 x 90 cm), which had been calibrated with proteins of known molecular weight. After elution with Buffer A containing bovine serum albumin (10 mg/l) extracts from both J120 and J62-2(pUK672) gave a single peak corresponding to a molecular weight of 10 000 (Figure 3.1.).

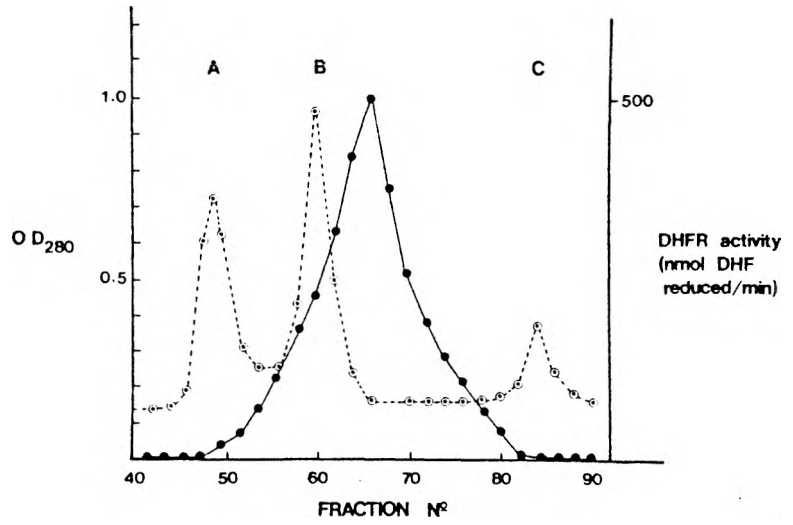


Figure 3.1. Sephadex G-50 gel filtration of the type VI dihydrofolate reductase. •——• , Dihydrofolate reductase activity; ⊙----⊙ , elution of standard marker protein (OD_{280}). A, chymotrypsinogen (Mr = 25 500); B, cytochrome c (Mr = 12 384); C, β chain of insulin (Mr = 3 878).

3.3.3 Sensitivity of the plasmid DHFR to antifolate compounds

The enzymes eluted from the Sephadex G-50 peaks were assayed in the presence of increasing concentrations of trimethoprim. Both the DHFR from the original strain and its E.coli transconjugant lost 50% of activity in the presence of around

200 μM trimethoprim (Figure 3.2.). The concentration of methotrexate required for 50% inhibition was 7.25 μM. In both cases the enzyme activity was inversely proportional to the logarithm of the inhibitor concentration over the concentration range tested.

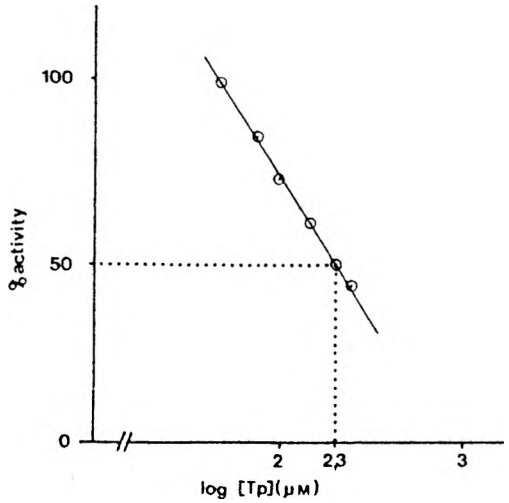


Figure 3.2. Determination of the ID50 for trimethoprim of the type VI DHFR from P.mirabilis (J120).

3.3.4 DHFR activity under limiting substrate concentrations

The activities of the enzymes were examined under partial saturation with dihydrofolate and full saturation with NADPH (50 μM). Control studies

showed that increasing the concentration of NADPH above 20 μM did not affect the rate of the reaction. The results analyzed by Lineweaver-Burk plots (Figure 3.3.) showed that the Km of dihydrofolate for the enzyme was 66 μM. The assays were repeated in the presence of 100 μM trimethoprim and it was found that the maximum velocity of the reaction was unchanged (Figure 3.3.), indicating that although this enzyme was relatively resistant to trimethoprim it was eventually competitively inhibited by the drug. The Ki of trimethoprim for the novel plasmid DHFR was about 50 μM.

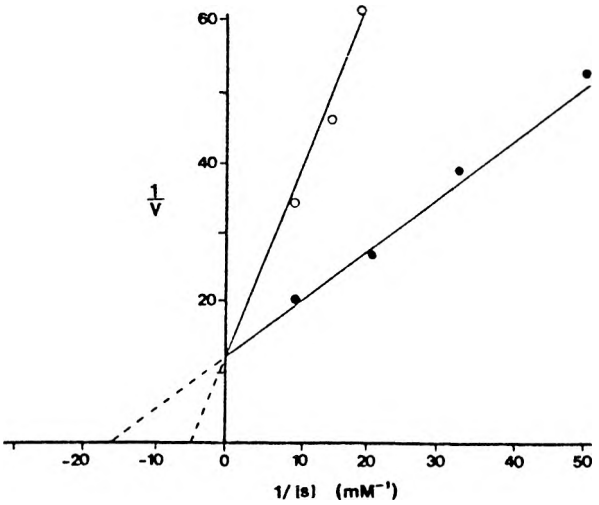


Figure 3.3. Lineweaver-Burk plots of partially purified type VI plasmid dihydrofolate reductase from the transconjugant strain J62-2 (pUK672).
•——• ,No trimethoprim; o——o , 100 μM trimethoprim.

3.3.5 Heat sensitivity of the DHFR

The novel enzyme was extremely heat labile, losing 59% of its activity in 24 seconds when heated to 45 °C.

These results are summarized in Table 3.3.

Table 3.3. Properties of the type VI DHFR encoded by plasmid pUK672.

Host strain	Mr	I50 Tp (µM)	I50 Mtx (µM)	TD50 (min)	Km DHF (µM)	Ki Tp (µM)
P.mirabilis (J120)	10 000	200	7.25	0.4	31.25	75
E.coli J62-2	10 000	150	10	0.35	66	50

Tp: trimethoprim; Mtx: methotrexate; SA: specific activity

I50: inhibitor concentration required to reduce enzyme activity by 50%.

TD50: time required for 50% inactivation of the enzyme when heated to 45 °C.

3.4 DISCUSSION

Plasmid pUK672 encoded a DHFR enzyme that was insensitive to inhibition by trimethoprim and conferred a high-level of resistance to trimethoprim on its host strain. The properties of this enzyme were different to those of all other types of plasmid-encoded DHFR enzymes occurring in gram-negative rods and staphylococci (Amyes & Towner, 1990). This new enzyme was designated DHFR type VI. The type VI DHFR differed from the others in most of its biochemical properties. It seemed to be an unstable enzyme. This was demonstrated in the rapid loss of activity found on separation of the enzyme by gel filtration. The enzyme activity was preserved, however, if the background protein concentration was kept high. No other plasmid dihydrofolate reductase loses so much activity on the removal of extraneous protein. A further indicator of the enzyme's instability was its rapid denaturation at 45 ^{o}C. Only the type I plasmid DHFR from gram-negative bacteria denatures so rapidly. However, the type VI enzyme shares few other properties with the type I dihydrofolate reductases.

The most striking feature of the type VI enzyme was its low molecular weight. The size of the type VI enzyme was very similar to the sub-unit size of the type II plasmid

dihydrofolate reductases (Smith et al., 1979). However, the type II enzyme sub-units are not functional in a disaggregated form. Interestingly, the levels of resistance to trimethoprim and methotrexate conferred by the type VI enzyme were higher than those for any of the other plasmid-encoded DHFRs except for the type II. It is possible that the type VI enzyme is related to the type II, though a lack of direct hybridization of the type VI gene with the type II gene probe (Wylie & Koornhof, 1989) suggests that any such relationship is not close.

This is the first new plasmid-encoded dihydrofolate reductase to be found in Africa and, indeed, is the only one identified on this continent apart from the type Ia (Young & Amyes, 1986b).

CHAPTER 4 NUCLEOTIDE SEQUENCE OF THE DIHYDROFOLATE REDUCTASE TYPE VI GENE

4.1 INTRODUCTION

The development of techniques for rapid sequencing of DNA has led to many advances in molecular biology. The better known techniques are those of Maxam & Gilbert (1980) and Sanger et al. (1977). Maxam & Gilbert developed a sequencing technique which employed base-specific, chemical cleavage of end-labelled DNA. This method involves modification of a base, removal of the modified base from its sugar and DNA strand scission at that sugar. While this method produces adequate results the procedure developed by Sanger is more commonly used as it offers significant advantages in terms of rapidity and simplicity of protocol. It is based on the use of deoxynucleotide analogues which are randomly incorporated into a growing strand of DNA to give specific chain termination. One enzyme commonly used for this purpose is the Klenow fragment of E.coli DNA polymerase I. It has several properties which make it suitable for DNA sequencing:

i) it can only extend the DNA strand in the 5' to 3' direction from the primer as it does not possess the 5' to 3' exonuclease activity of the intact enzyme

ii) it faithfully synthesizes a complementary strand

from a single-stranded template

iii) it is able to incorporate dideoxynucleotides in place of deoxynucleotides

iv) it has an absolute requirement for a primer with a 3'-hydroxyl group.

Dideoxynucleoside triphosphates (ddNTPs) lack a 3'-hydroxyl group and, therefore, when incorporated into a growing strand of DNA cause chain termination. By controlling the ratio of ddNTP to the appropriate dNTP, incorporation of the dideoxynucleotide, and hence chain termination, occurs randomly.

Another enzyme frequently used in DNA sequencing is a DNA polymerase described by Tabor & Richardson (1987). It is a modified DNA polymerase from bacteriophage T7 with additional properties which make it eminently suitable for use in "dideoxy" sequencing. Its processivity i.e. the average number of nucleotides polymerized on a particular primer-template prior to dissociating is high, being in the region of 2 000. In addition to its high rate of polymerization it has a wide tolerance for and utilization of nucleotide analogues.

Sequencing is performed using four separate reactions. Each reaction contains all four dNTPs (one or more of which is radioactively labelled), single-stranded DNA template (which has been primed), enzyme and one of the

four ddNTPs. Four sets of fragments are generated, each set specifically terminating with either ddA, ddC, ddG or ddT. The fragments are separated using thin polyacrylamide gels and the sequence is read from the position of the bands across the four tracks. Between 200 and 400 bases can be determined from an average gel but the use of gradient gels, wedge gels and metre-long gels can increase the number of readable bases significantly. The quality of DNA used in sequencing has an important effect on the readibility of the autoradiograms. Contamination with either extraneous DNA or RNA results in random priming which, in turn, causes multiple banding and/or high background "noise". This makes interpretation of the sequence difficult. The development of the M13 bacteriophage cloning vectors (Messing et al., 1977) has largely overcome these limitations. M13 is a single-stranded, filamentous phage, the life cycle of which is suitable for cloning of "foreign" DNA and the preparation of single-stranded template. The bacteriophage enters the host cell (E.coli F') where it is stripped of its protein coat. The single-stranded viral DNA is then converted to a double-stranded replicative form (RF). This is followed by replication to give 100+ progeny RF molecules from which new single-stranded viral DNA is synthesized. RF DNA which is

required for cloning purposes can be purified from the cells at this point using standard methods for plasmid purification. The viral genome is then packaged into viral coat protein and extruded from the host without lysis. Some 200 phage progeny are produced per cell per generation and can be harvested free of contaminating material from the host cells by polyethylene glycol (PEG) precipitation. The single-stranded DNA can then be purified and used for DNA sequencing. Cloning of "foreign" DNA into the replicative form of M13 has been facilitated by the insertion of a multiple cloning site (MCS) at a point in the genome where essential viral functions are not disrupted. The MCS contains several unique restriction enzyme sites which can be utilized for insertion of "foreign" DNA.

The DNA sequences of dihydrofolate reductase genes have been analyzed in terms of the structure of the genes, the predicted amino acid sequences and the regions controlling their expression.

Comparison of the nucleotide sequences of the types I, II and V DHFRs (Fling and Richards, 1983; Swift, McCarthy & Heffron, 1981; Sundström et al., 1988) has revealed considerable homology between enzymes of the same group e.g. >78% between types IIa, b and c (Brisson and Hohn, 1984; Flensburg and Steen, 1986). There is no sequence

homology between the type I and type II and between the type II and type V genes (Fling and Richards, 1983; Sundström et al., 1988). The type I and type V DHFR genes do have a number of similar features. Both genes code for a polypeptide of 157 amino acids and there is an overall amino acid homology of 75% (Sundström et al., 1988). However, these genes do not hybridize with each other (Thomson and Amyes, 1988). The type I and type V DHFR genes have regions which are homologous to the chromosomal DHFR gene of E.coli K12 (Sundström et al., 1988).

The nucleotide sequence of the type VI DHFR was determined in order to establish its relationship with other DHFRs mediating high-level resistance to trimethoprim.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria

The gene encoding the type VI DHFR was isolated from *Proteus mirabilis* J120 (pUK672).

The cloning vectors used were pUC18 and bacteriophages M13mp18 and mp19 which were propagated in *E.coli* K12 JM103 and JM101 respectively (Yanisch-Perron, Vieira & Messing, 1985).

4.2.2 DNA isolation and purification

Plasmid DNA was purified using a Circleprep Kit (Bio 101 Inc., La Jolla, CA, USA) according to the manufacturer's instructions (Appendix C).

DNA fragments were purified by separation in and extraction from low melting point agarose as previously described in Chapter 2.

4.2.3 Cloning procedure

All cloning procedures using the vectors pUC18 and bacteriophages M13mp18 and mp19 were performed

according to the methods recommended by the supplier (Boehringer Mannheim). These are detailed in Appendices D and E.

Purified plasmid DNA (pUK672) was partially or fully digested with EcoRI and ligated with suitably restricted pUC18 using T4 DNA ligase. The hybrid plasmids were transformed into competent E.coli K12 JM103 and clones containing the DHFR gene were selected on a medium containing trimethoprim lactate (100 mg/l, Wellcome) in addition to ampicillin (100 mg/l), isopropyl-β-D-galactoside (IPTG) and X-gal (5-bromo-5-chloro-3-indolyl-β-D-galactopyranoside). Plasmid DNA from each clone was screened by the method of Maniatis et al. (1982). Similarly DNA fragments from pUK672 were ligated with suitably digested replicative form bacteriophage DNA and transformed into E.coli K12 JM101.

4.2.4 Sequence determination

Sequencing was performed by the chain termination method of Sanger, Nicklen & Coulson (1977) using an M13 sequencing kit (Boehringer) according to the manufacturer's instructions (Appendix F). The

sequence was determined from both strands with the exception of ç. 200 base pairs at the 5' end, the sequence of which was determined from one strand only. The sequence around the HindIII restriction site was confirmed using a 20-bp oligonucleotide primer which was complementary to the negative strand of the insert from position 266 to 285.

The sequence was analyzed using the Mount sequence analysis package (Genetics Software Centre, Department of Molecular and Cellular Biology, Biosciences West, University of Arizona, Tuscon, AZ85721, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation of the type VI DHFR gene

After partial digestion with EcoRI of the plasmid pUK672 the fragments were ligated with suitably restricted pUC18 and transformed into E.coli K12 JM103. A clone expressing trimethoprim resistance was found to contain a vector plasmid with an insert of c. 9.0 Kb. The insert DNA was excised from the vector, purified and digested a second time with EcoRI. The fragments were recloned into pUC18. Clones expressing trimethoprim resistance were screened and the vector with the smallest insert - c. 1.5 Kb - was identified. Attempts to remove the c. 1.5 Kb fragment from the plasmid revealed that the internal EcoRI site and the adjacent XbaI site of the vector had been damaged. However, the PstI site was functional and was used together with the second EcoRI site to remove the insert.

Digestion of the c. 1.5 Kb insert with a number of restriction enzymes showed that single sites for both XbaI and HindIII were present and that the fragments generated by each enzyme were

large enough to contain the DHFR gene. These fragments were cloned into pUC18 but only the PstI/XbaI insert expressed trimethoprim resistance. This insert, which was c. 1.0 Kb in size, also contained the HindIII site.

The biochemical properties of the enzyme expressed by this gene were identical to those expressed by the gene in P.mirabilis (J120).

For sequencing three fragments were cloned into M13mp18 and mp19. These were the c. 1.0 Kb PstI/XbaI fragment, the c. 600-bp PstI/HindIII fragment and the c. 400-bp HindIII/XbaI fragment (Figure 4.1.).

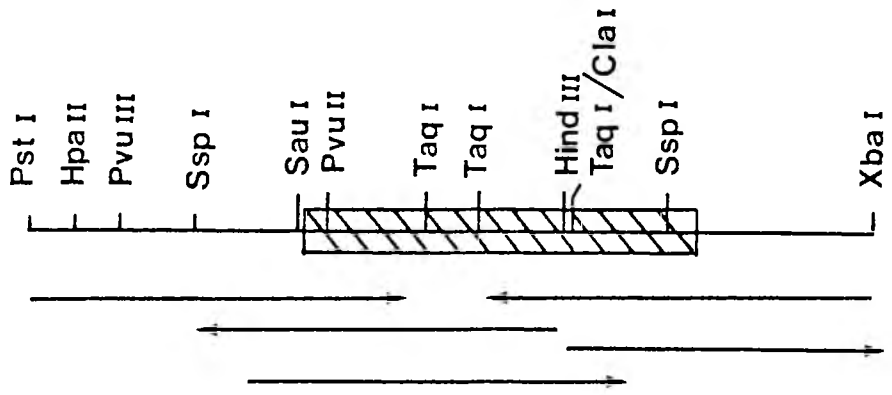


Figure 4.1. Restriction map of the 1 026 base pair insert containing the type VI DHFR gene (cross-hatched area). Arrows indicate the direction and extent of sequencing.

4.3.2 Nucleotide sequence of the type VI DHFR gene

Since the type VI enzyme had been previously assigned a molecular weight of 10 Kda a gene of about 240 to 300 base pairs was expected. Analysis of the sequence of the 1 026-bp insert yielded only one open reading frame (ORF) larger than 240 bases. This was 471-bp long and encoded an amino acid of 157 residues with a calculated molecular weight of 17 424 daltons. The nucleotide and predicted amino acid sequences are shown in Figure 4.2.

The proposed type VI structural gene was found to be bounded by an initiation codon (ATG) at position 336 and a termination codon (TAA) at position 807. At position 646 there was a HindIII restriction site. Other restriction sites are shown in Figure 4.1.

4.3.3 Analysis of the 5' and 3' nucleotide sequence

Analysis of the DNA sequence upstream of the ORF showed a set of possible -35 and -10 promotor sequences. The most highly conserved base pairs of the -35 consensus sequence in E.coli promotors are -TTGACA- (Rosenburg and Court, 1979). The hexamer

-TAGACA- found at position 260 of this sequence was identical in five of the six base pairs and could, therefore, have fulfilled this function. Harley and Reynolds (1987), in an analysis of 253 E.coli promotors, found that the optimum distance between the -35 and -10 regions was 17+1 base pairs. Taking this into consideration the hexamer -AGAAAT-, which was 18 base pairs downstream from the proposed -35 sequence, could have formed the -10 region. However, this sequence differed in three of the six base pairs found most commonly in E.coli -10 sequences viz. -TATAAT-. If these regions did function as the RNA polymerase binding site, they were further upstream than expected. However, longer leader sequences have been noted previously although their function is not known (Andrews et al., 1985; Swift, McCarthy & Heffron, 1981). There was a ribosomal binding site (-AGGA-) at an appropriate distance from the proposed initiation codon (Shine and Dalgarno, 1975).

A possible transcription termination site was present downstream of the coding region. An inverted repeat sequence centred at position 845/6 might be involved in the formation of a stem and loop structure in which the stem contains eight

Figure 4.2. Nucleotide sequence of the 1 026-bp insert. The DHFR coding region extends from base 336 to base 806. The deduced amino acid sequence is written below the DNA sequence. The -35 and -10 regions and the ribosomal binding site are underlined. The inverted repeat sequence is overlined.

```
   PstI
CCTGCAGTTGCCATGTTTTACGGCAGTGAAGAGATAGCGAGCGCTGATGTCCGGCGGTGC    60

TTTTGCCGTTACGCACCACCCCGTCAGTAGATGAACAGGAGGGACAGCTGATAGAAACAG   120

AAGCCACTGGAGCACCTCAAAAACACCATCATACACTAAATCAGTAAGTTGGCAGCATCA   180

CCCTATTTAACTTAATTCAAATATTTTAGAAAACTTAGTGATAGTAATACCTTAGGATAT   240

TTGTTAAAACTATATAGAGTAGACAAATGAGCGTTTAGTCGGCAGAAATATGCGCGATGA   300
                    -35                       -10

GCGCACTATTAAATTGTTAGCCCTCAGGAGGAAAA ATG AAA ATA TCT CTT ATG    353
                          SD        Met Lys Ile Ser Leu Met

GCA GCT GTT TCC GAG AAT GGA GTA ATT GGC TCT GGA TTG GAT ATA    398
Ala Ala Val Ser Glu Asn Gly Val Ile Gly Ser Gly Leu Asp Ile

CCT TGG CAT GTA CAA GGC GAG CAG CTC CTA TTC AAA GCC ATG ACT    443
Pro Trp His Val Gln Gly Glu Gln Leu Leu Phe Lys Ala Met Thr

TAC AAT CAA TGG CTT CTA GTT GGT CGT AAA ACC TTC GAC TCA ATG    488
Tyr Asn Gln Trp Leu Leu Val Gly Arg Lys Thr Phe Asp Ser Met

GGT AAA CTT CCG AAT AGA AAA TAT GCA GTG GTT ACT CGT TCT AAA    533
Gly Lys Leu Pro Asn Arg Lys Tyr Ala Val Val Thr Arg Ser Lys

ATT ATC TCG AAT GAC CCT GAT GTT GTG TAT TTC GCA AGT GTT GAA    578
Ile Ile Ser Asn Asp Pro Asp Val Val Tyr Phe Ala Ser Val Glu

TCG GCA TTA GCT TAC CTA AAC AAT GCG ACA GCA CAT ATC TTT GTT    623
Ser Ala Leu Ala Tyr Leu Asn Asn Ala Thr Ala His Ile Phe Val

TCT GGT GGT GGT GAA ATA TAT AAA GCT TTA ATC GAT CAA GCA GAT    668
Ser Gly Gly Gly Glu Ile Tyr Lys Ala Leu Ile Asp Gln Ala Asp

GTT ATC CAT CTT TCA GTG ATT CAC AAG CAT ATC TCT GGC GAT GTG    713
Val Ile His Leu Ser Val Ile His Lys His Ile Ser Gly Asp Val

TTT TTT CCT CCA GTT CCA CAG GGC TTC AAG CAA ACA TTT GAG CAA    758
Phe Phe Pro Pro Val Pro Gln Gly Phe Lys Gln Thr Phe Glu Gln

AGT TTC AGT TCA AAT ATT GAT TAC ACG TAC CAA ATT TGG GCA AAG    803
Ser Phe Ser Ser Asn Ile Asp Tyr Thr Tyr Gln Ile Trp Ala Lys

GGC TAA CAATCTGTTTAAGAGTGATTCGCAACGCGTGGAATTTTTACTATGCGTTGCG   860
Gly TER

TTTAGTGTTTAAGGTGGTATGCGGAGGCTTCGGTATTGCGTTGCTCACACCTTAACAGGG  920

CGTTATGTGTCTCTTGGATTTAGGTGAAATAAACTATGTTGAATATCATTAAATTTATTC  980

                                    XbaI
TTATTTCCTTAATGCTTTCAGTGATTTTTATCTATATTGCTCTAGA                1026
```

base pairs and the loop 16 bases. Other features consistent with rho-independent termination were not present.

4.3.4 Comparison of the amino acid sequences of the types I, V, VI and E.coli K12 chromosomal DHFRs

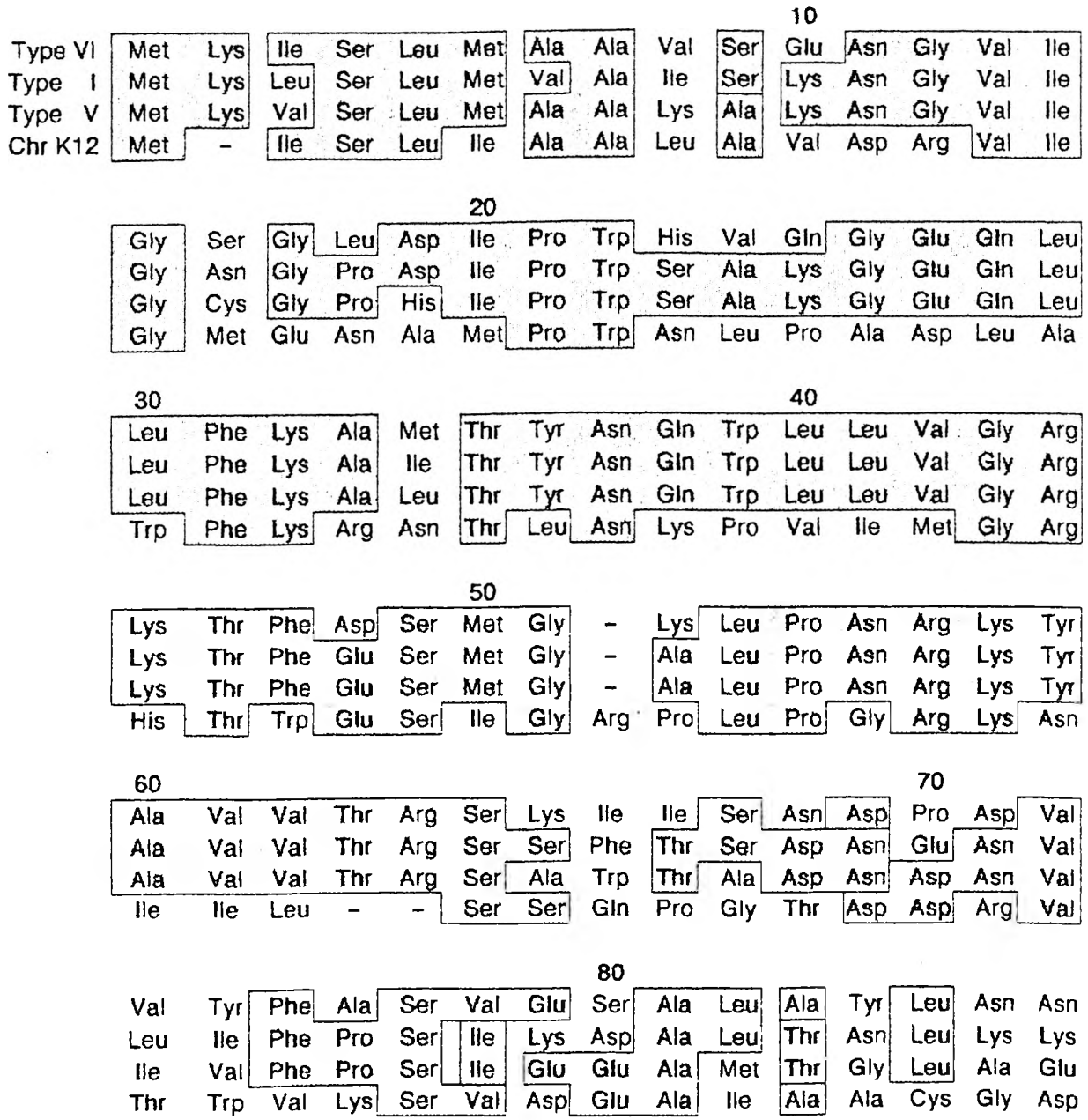
The types I, V and VI DHFR genes encode polypeptides of 157 amino acids while the gene for the E.coli chromosomal DHFR encodes a polypeptide of 159 residues (Smith and Calvo, 1980). These sequences are aligned in Figure 4.3. and numbered according to the E.coli DHFR.

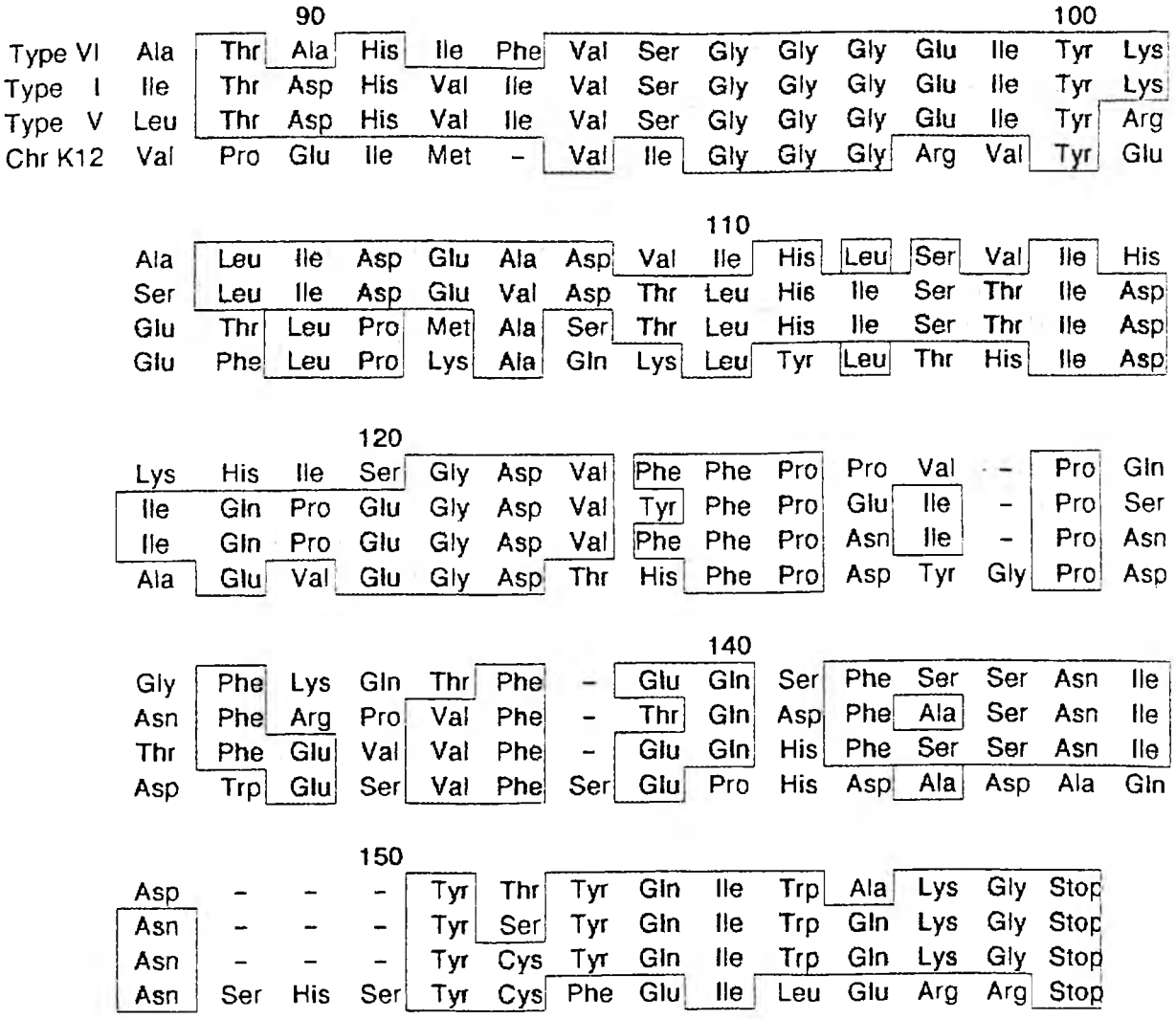
Overall amino acid homology between the type I and type VI enzymes was 63% but within the first 50 amino acids, where both co-enzyme and inhibitor binding take place (Matthews et al., 1978), there was 80% homology. There was even greater identity - 86.6% - in the residues 51 to 65.

Similarly comparison of the type V and type VI sequences showed 61% homology; 78% of the first 50 residues and 86.6% of residues 51 to 65 were identical.

The degree of homology between the chromosomal DHFR of E.coli K12 and the type VI was only 31%.

Figure 4.3. Comparison of the amino acid sequences of the types I, V, VI and E.coli K12 dihydrofolate reductases. The numbering is according to the E.coli DHFR. Conserved residues are shaded.




90
100
Type VI Ala Thr Ala His Ile Phe Val Ser Gly Gly Gly Glu Ile Tyr Lys
Type I Ile Thr Asp His Val Ile Val Ser Gly Gly Gly Glu Ile Tyr Lys
Type V Leu Thr Asp His Val Ile Val Ser Gly Gly Gly Glu Ile Tyr Arg
Chr K12 Val Pro Glu Ile Met - Val Ile Gly Gly Gly Arg Val Tyr Glu
110
Ala Leu Ile Asp Glu Ala Asp Val Ile His Leu Ser Val Ile His
Ser Leu Ile Asp Glu Val Asp Thr Leu His Ile Ser Thr Ile Asp
Glu Thr Leu Pro Met Ala Ser Thr Leu His Ile Ser Thr Ile Asp
Glu Phe Leu Pro Lys Ala Gln Lys Leu Tyr Leu Thr His Ile Asp
120
Lys His Ile Ser Gly Asp Val Phe Phe Pro Pro Val - - Pro Gln
Ile Gln Pro Glu Gly Asp Val Tyr Phe Pro Glu Ile - Pro Ser
Ile Gln Pro Glu Gly Asp Val Phe Phe Pro Asn Ile - Pro Asn
Ala Glu Val Glu Gly Asp Thr His Phe Pro Asp Tyr Gly Pro Asp
140
Gly Phe Lys Gln Thr Phe - Glu Gln Ser Phe Ser Ser Asn Ile
Asn Phe Arg Pro Val Phe - Thr Gln Asp Phe Ala Ser Asn Ile
Thr Phe Glu Val Val Phe - Glu Gln His Phe Ser Ser Asn Ile
Asp Trp Glu Ser Val Phe Ser Glu Pro His Asp Ala Asp Ala Gln
150
Asp - - - Tyr Thr Tyr Gln Ile Trp Ala Lys Gly Stop
Asn - - - Tyr Ser Tyr Gln Ile Trp Gln Lys Gly Stop
Asn - - - Tyr Cys Tyr Gln Ile Trp Gln Lys Gly Stop
Asn Ser His Ser Tyr Cys Phe Glu Ile Leu Glu Arg Arg Stop

The residues known to be involved in folate binding are 5, 6, 7, 15, 20, 22, 28, 30, 31 and 94 (Bystroff et al., 1990). The resistant DHFRs have identical residues to E.coli K12 DHFR at positions 5, 7, 22, 31 and 94. The remaining 5 amino acids differ from the K12 enzyme but the substituted residues (Met-6, Ile-20, Glu-27, Gln-28 and Leu-30) are identical in each of the resistant DHFRs. Similarly residues associated with methotrexate binding in E.coli are Ile-5, Ala-7, Asp-27, Leu-28, Phe-31, Ser-49, Ile-50, Leu-54, Arg-57, Ile-94 and Thr-113 (Matthews et al., 1977; Matthews et al., 1978). Only five of the 11 residues were conserved in the resistant DHFRs. These substitutions may explain why the binding affinities of the resistant DHFRs and the chromosomal enzyme differ.

Although the similarity in gene length, DNA and amino acid homology suggests common ancestry the biochemical properties of these enzymes were distinct. The type V and type VI DHFRs do, however, have certain physical properties in common. By Sephadex column chromatography the size of the type V DHFR was estimated to be 5 Kda (Thomson & Amyes, 1988), whereas the gene sequence data showed the size of the polypeptide to be 17 531 daltons

(Sundström et al., 1988). Similarly the molecular weight of the type VI DHFR was estimated to be 10 Kda (Wylie et al., 1989) whereas the gene determined a polypeptide of over 17 Kda. The reason for this behavior is unknown but it could be due to the hydrophobicity of the enzyme molecules (Towner et al., 1990). The molecular weight of the type V DHFR was subsequently reported to be 35 Kda by native polyacrylamide gel electrophoresis (PAGE) (Thomson, 1990) indicating that, like the type I DHFRs, the type V enzyme functions as a dimer. In preliminary investigations using SDS-PAGE the molecular weight of the type VI DHFR appears to be 17.5 Kda. It has not yet been determined whether it functions as a dimer but the similarities between these three enzymes support this possibility.

CONCLUSION

The investigations relating to trimethoprim resistance in South African isolates described here have merely served as an introduction into this field of study. Much work remains to be done, especially in molecular epidemiology, if trimethoprim resistance in South Africa is to be comprehensively understood and accurately assessed.

The incidence of trimethoprim resistance in gram-negative organisms isolated in South Africa is high. In order to establish trends in resistance patterns similar surveys should be performed at regular intervals. In addition the linkage of trimethoprim resistance genes with those of other antibiotics should be examined. This information would assist in formulating hospital antibiotic prescription policy and control of nosocomial spread of resistant organisms. In a country where cost-benefit considerations are of paramount importance the use of a drug as efficient and relatively inexpensive as trimethoprim should not be compromised by a high frequency of resistance.

The importance of the type VI DHFR in mediating resistance in bacterial populations in South Africa and elsewhere will be investigated. Local studies are being planned and a suitable probe will be developed from the cloned fragment and used in hybridization studies. Should the occurrence of this gene not

account for a major portion of the high-level trimethoprim resistance, further investigations into other mechanisms of resistance and their frequency will be necessary. It is possible that, because of the geographical isolation of South Africa from Europe and the USA, the genes mediating high-level trimethoprim resistance will have evolved differently and that other novel DHFRs will be identified.

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APPENDIX A.

NICK TRANSLATION

To avoid contamination by DNA-degrading nucleases found on the skin we recommend that protective gloves be worn whilst handling all reagents for the nick translation reaction.

a) Select DNA specific activity required.

b) Select the reaction conditions appropriate to the DNA specific activity required by referring to table 1.

c) Dissolve the DNA for labelling in either distilled water or 10mM Tris/HCl pH8·0, 1mM EDTA, to a concentration of 200µg/ml. Care should be taken to ensure that all impurities (such as agarose) which might interfere in the labelling reaction have been removed from the DNA.

d) To a conical polypropylene reaction tube (capacity up to 1·5ml) placed in an ice bath, add the appropriate volume of each reagent (see table 1) in the following order:

DNA solution
Nucleotide/buffer solution*
Radioactively labelled dNTP
Water
Enzyme solution (solution 2)

* When using kit N.5500 you will need to prepare this solution yourself as described on page 10

e) Cap the tube and mix gently by inverting several times. The tube may then be spun for a few seconds in a microcentrifuge to collect the contents at the bottom of the tube). Avoid vigorous agitation of the reaction mixture as this may cause severe loss of enzyme activity.

f) Place the tube in a constant temperature bath at 15°C. Careful control of temperature is necessary (see page 12).

g) Monitor the progress of the reaction, if required, as detailed on page 13.

h) When the desired DNA specific activity has been achieved, terminate the reaction using an appropriate method, such as:

— gel filtration
— addition of EDTA

Table 1. Volumes of components required for labelling DNA with phosphorus-32 using [α-^{32}P]dCTP, code PB.10165, in the standard protocol.

	Procedure to be used: A	B	C	D	notes (overleaf)
DNA specific activity required† (dpm/µg)	5 x 10^7	1 x 10^8	2 x 10^8	4 x 10^8	
Quantity of DNA to be labelled (µg)	2	1 to 2	1 to 2	1	(a)
Volume of nucleotide/buffer solution (solution 1 kit N.5000) (µl)	20	20	20	20	(b)
Volume of [α-^{32}P]dCTP solution, code PB.10165 (µl)	10	10 to 20	25 to 50	50	(c) (d)
Picomoles of [α-^{32}P]dCTP added	250	250–500	625 to 1250	1250	
Volume of enzyme solution (solution 2) (µl)	10	10	10	10	(e)
Volume of water	Sufficient to bring total incubation volume to 100µl				(f)

† at the activity reference date of the labelled nucleotide

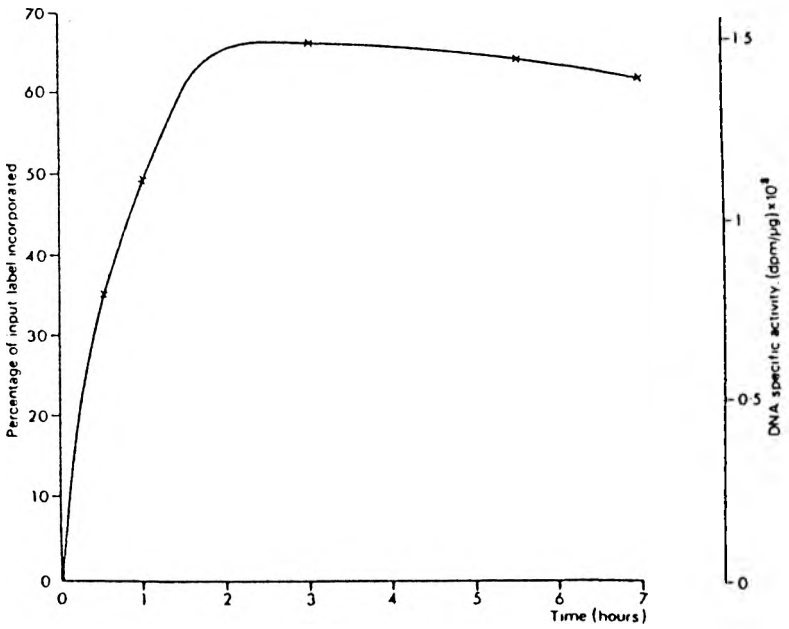

Figure 1. Time course of nick translation reaction using [α-^{32}P]dCTP and procedure B.

Monitoring the progress of the reaction

If required the progress of the labelling may be monitored as follows. Plastic or siliconized glass tubes must be used to avoid adsorption of DNA.

1) For DNA labelled with ^{32}P, ^{35}S or ^{125}I:

a) Transfer a 1 or 2μl aliquot of the reaction mixture to a tube containing 200μl water or 0·2M EDTA. Mix well.

b) Transfer 20μl of this 'stopped' solution to a tube containing 50μl carrier DNA solution (solution 3). Mix well. Use this mixture in step 4.

4) To the diluted sample from step 1 (b) or step 2, add 2ml ice-cold 10% trichloroacetic acid (TCA) solution, mix well, and allow to stand in an ice-bath for 10–15 minutes. The labelled and carrier DNA will co-precipitate (Note that TCA is corrosive and care should be taken in its handling).

5) Collect the precipitated DNA by vacuum filtration on a glass fibre or nitrocellulose filter disc.

6) Wash the filter disc with a further 2ml 10% TCA solution and dry the filter disc thoroughly, for example using an infra-red lamp.

7) Count the dried filter disc by liquid scintillation (all nuclides), Cerenkov (^{32}P) or gamma (^{125}I) counting, taking care that the count rate is not so high as to saturate the counter.

Termination of the reaction

The nick translation reaction may be stopped in various ways. The method chosen must, of course, be compatible with the proposed use of the labelled DNA product. For example:

a) The reaction mixture may be loaded directly on to a 10cm column of Sephadex G-10 or G-50 suspended in buffer containing 150mM NaCl, 10mM EDTA, 0·1% sodium dodecyl sulphate (SDS) and 50mM Tris/HCl, pH7·5. 10-drop or 1ml fractions are collected and monitored for radioactivity. The labelled DNA is eluted first and may be recovered from the pooled fraction by alcohol precipitation. The DNA isolated by this technique is suitable for use as a probe in hybridization experiments.

b) The reaction mixture may be extracted with phenol to separate the DNA from protein.

c) A general proteolytic enzyme (for example, trypsin) may be added which will digest the DNase I and DNA polymerase I.

d) 0·2M EDTA may be used to chelate the metal ions essential for enzyme activity.

APPENDIX B.

DNA TRANSFER AND HYBRIDIZATION

2. Membrane Handling

Membranes do not have to be prewetted and can be removed from gel or agar surfaces without risk of tearing. Biodyne A membranes may be handled in any convenient manner. Gloves or forceps should be used to prevent membrane contamination. Forceps will not damage the membranes. Either scissors or a sharp scalpel must be used to cut the membranes. A vacuum oven is not required for baking of nucleic acids onto Biodyne A membranes; a conventional air circulating oven is adequate.

3. Stock Solutions

3.1 Denaturing solution: 1.5M NaCl, 0.5M NaOH.

3.2 Neutralizing solution: 3M NaAcetate, pH 5.5.

3.3 20X SSC: 3M NaCl, 0.3M NaCitrate, pH 7.

3.4 Nonhomologous DNA: 2 mg/ml Herring Sperm DNA. Dissolve in water overnight, then sonicate for 1 hour. Store at 4°C.

3.5 100X Denhardt's (1966): 2% (w/v) Ficoll (400,000 m.wt.), 2% (w/v) Polyvinylpyrrolidone (360,000 m.wt.), 2% (w/v) Bovine Serum Albumin. Store at 4°C.

3.6 20X SSPE: 3.6M NaCl, 0.2M NaPhosphate, pH 8.3, 0.02M EDTA.

3.7 10% (w/v) Sodium dodecyl sulphate (SDS).

3.8 Wash Buffer: 5mM NaPhosphate pH 7.0, 1mM EDTA, 0.2% sodium dodecyl sulphate.

3.9 1M NaPhosphate, pH 6.5

3.10 Formamide: Deionised using a mixed-bed ion exchange resin such as Bio-Rad AG501-X8.

4. DNA Transfer Procedures

These protocols have been optimised for use with Biodyne A nylon membranes. They may differ significantly from published procedures.

4.2 Southern Transfer

Southern Transfers are performed by a modification of the procedure described by Southern (1979).

4.2.1 Place two reservoirs containing 20X SSC (3.3) side by side, and span with a glass plate.

4.2.2 Place a sheet of dry filter paper from one reservoir, over the glass plate, to the adjacent reservoir. Saturate the filter paper with 20X SSC (3.3).

4.2.3 After electrophoresis, remove gel from apparatus, and place in 150ml of denaturing solution (3.1) for 30 minutes with gentle agitation.

4.2.4 Replace buffer with neutralising solution (3.2). Agitate gently for 30 minutes.

4.2.5 Drain excess buffer from the gel.

4.2.6 Place gel on the paper/glass support and surround with strips of a waterproof material such as plastic film to ensure that the blotting buffer goes through the gel, not around it.

4.2.7 Carefully place a piece of dry or prewetted Biodyne A membrane on top of and completely covering the gel surface. Roll a clean pipette over the membrane to remove trapped air bubbles.

4.2.8 Place two sheets of filter paper over the membrane.

4.2.9 Cover with a 3" stack of paper towels, a glass plate and a 1kg weight.

4.2.10 Carefully inspect to ensure that the paper towels do not contact the gel.

4.2.11 Allow transfer of DNA from the gel to the membrane to proceed for at least 12 hours.

4.2.12 Remove membrane from gel surface. Do not rinse as this may result in loss of nucleic acid from the membrane.

4.2.13 Bake at 80°C for 1 hour.

7.1.1 Prehybridization

7.1.1.1 Denature nonhomologous DNA (3.4) by the addition of 1/10 volume 1N NaOH and heating at 65°C for 10 minutes.

7.1.1.2 Neutralise the DNA solution by the addition of 1/10 volume 1N HCl.

7.1.1.3 Hybridization solution contains (final concentration): 5X Denhardt's buffer (3.5), 5X SSPE (3.6), 0.2% (w/v) Sodium Dodecyl Sulphate (3.7), 500 µg/ml denatured nonhomologous DNA.

7.1.1.4 Seal the membrane in a plastic bag with 4ml of hybridization solution per 100 cm^2 of membrane.

7.1.1.5 Seal the bag inside the second bag with a damp paper towel.

7.1.1.6 Immerse the bag in a 65°C bath for 1 hour.

7.1.2 Hybridization

7.1.2.1 Open the bags containing the membrane and remove excess hybridization solution. Roll a pipette over the inner bag to remove as much of the solution as possible.

7.1.2.2 Add the labeled probe to the nonhomologous DNA and denature and neutralise as described in 7.1.1.1 and 7.1.1.2. Final probe concentrations in excess of 10^6 cpm per ml can result in some background.

7.1.2.3 To the membrane add 2ml of fresh hybridization solution per 100cm² of membrane.

7.1.2.4 Reseal the double bags and immerse at 65°C for duration of hybridization.

7.1.3. Washing

7.1.3.1 After hybridization, dip the membrane in wash buffer (3.8).

7.1.3.2 Place the membrane in a plastic bag.

7.1.3.3 Add 250ml of wash buffer per 100cm² of membrane.

7.1.3.4 Agitate the bags vigorously (200 rpm) for 30 minutes at room temperature, then discard the buffer.

7.1.3.5 Repeat steps 7.1.3.3 and 7.1.3.4 two times.

7.1.3.6 The membrane is now ready for autoradiography (or detection of biotinylated probe with an avidin conjugate). *Do not* allow membrane to dry if the probe is to be removed for subsequent rehybridization experiments (section 8).

3. Rehybridization

Biodyne A will sustain multiple cycles of hybridization and probe removal. Do not allow membrane to dry following the wash procedure. Removal of probe is carried out as follows:

8.1 Place the membrane in a plastic bag with 100ml of 10mM NaPhosphate, pH 6.5 (3.9), 50% (v/v) formamide (3/10) per 100cm² of membrane.

8.2 Incubate at 65°C for 1 hour.

8.3 Wash the membrane once in 250ml of 2X SSC (3.3), 0.1% (w/v) SDS (3.7) per 100cm² of membrane for 15 minutes at room temperature with vigorous agitation (100 rpm).

8.4 The membrane can now be prehybridized and hybridized with the desired probe.

9. References

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APPENDIX C.

PLASMID PURIFICATION

THE CIRCLEPREP™ KIT CONTAINS:

CIRCLEGROW capsules, container 0. 2 to 3 Capsules will make 50 ml of a special high-density growth medium for maintaining high copy numbers of plasmid in host cells without amplification

PRE-LYSIS BUFFER, Tris/EDTA/glucose solution, 120 ml Bottle 1

ALKALINE LYSIS REAGENT, 0.2 N NaOH and 1% SDS, 120 ml Bottle 2. Store at room temperature to avoid precipitation of SDS

NEUTRALIZING SOLUTION, 3 M potassium acetate, 120 ml Bottle 3

LITHIUM CHLORIDE SOLUTION, near saturated solution in 7ml vial 4
Store at room temperature to avoid precipitation of LiCl.

CIRCLEPREP GLASSMILK, 1 vial containing 1.5 ml, 5.

BINDING BUFFER, KBr/NaI/Tris mixture in 60 ml Bottle 6
Promotes binding of DNA, but not RNA or protein to CIRCLEPREP GLASSMILK

WASH SOLUTION, 30 ml of salt solution in 60 ml Bottle 7.
NOTE: Add 30 ml of 100% ethanol before use

Sieve material.

BIOFLOAT for floating microcentrifuge tubes in water bath during elution incubation.

Instructions.

Isopropanol is used during the CIRCLEPREP procedure to precipitate plasmid DNA but is not supplied in the kit. Also, as indicated above, 30 ml of 100% ethanol must be added to Bottle # 8, WASH SOLUTION, before use.

A. Plasmid Growth, Alkaline Lysis of Cells and Precipitation of Cellular DNA:

0 Grow cells in CIRCLEGROW medium overnight.

1 Add 4 ml of PRE-LYSIS BUFFER and resuspend cell pellet

2 Add 4 ml of ALKALINE LYSIS REAGENT to lyse cells.

3 Add 4 ml of NEUTRALIZING SOLUTION
Pellet precipitate in centrifuge and transfer supernatant through sieve to new tube. Add 1 volume of isopropyl alcohol, centrifuge precipitate and drain pellet.

B. Elimination of cellular DNA and RNA.

Second alkaline denaturation and precipitation of linear DNA

Add 0.5 ml of water and dissolve pellet. Transfer to a 2 ml microcentrifuge tube.

2 Add 250 ul of ALKALINE LYSIS REAGENT

3 Add 250 ul of NEUTRALIZING SOLUTION.

Spin for 2 minutes in microcentrifuge to pellet precipitate
Transfer supernatant to new 2 ml microcentrifuge tube.
Add 0.6 to 1 volume of isopropanol; centrifuge precipitate
Dissolve pellet in 500 ul of water.

(Recommended Option: Repeat steps B 2 and 3 one or more times.)

LiCl precipitation of RNA and ssDNA

4 Add 300 ul of LiCl solution; 5 minutes at room temperature; spin for 2 minutes and transfer supernatant to new tube

C. Purification of Plasmid DNA on silica:

5 Add 75 ul of CIRCLEPREP GLASSMILK. Let sit at room temperature for 5 minutes. Spin for 5 seconds and discard supernatant

6 Wash pellet 2 times with 1 ml BINDING BUFFER

Remove all liquid from pellet.

7 Wash pellet 2 times with 1 ml WASH SOLUTION

Elute plasmid DNA in 100 to 300 ul of water.

APPENDIX D.

pUC CLONING

Principle

pUC18 and pUC19 have been chosen from the pUC plasmid vector series for use here because they demonstrate the following advantages:

- They have a large number of unique cleavage sites in one polylinker region into which restriction fragments can be inserted with the help of T4 DNA ligase.
- Vectors with inserted DNA are easily detected after transformation of E. coli host strains. When plated on solid media containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), E. coli cells containing recombinant vectors form colorless colonies. Cells containing non-recombinant pUC vectors form blue colonies on X-gal plates (see fig. 13 on p. 19).
- The small pUC plasmids can easily be introduced into E. coli cells by transformation even when they contain large DNA inserts. They are replicated to a high copy number.
- Compared to earlier representatives of the pUC vector series, pUC18 and pUC19 are characterized by an increased number of cleavage sites in the polylinker region which allows insertion of a large variety of different restriction fragments (see table 3 and fig. 71) as well as by its entirely redetermined base sequence (1).

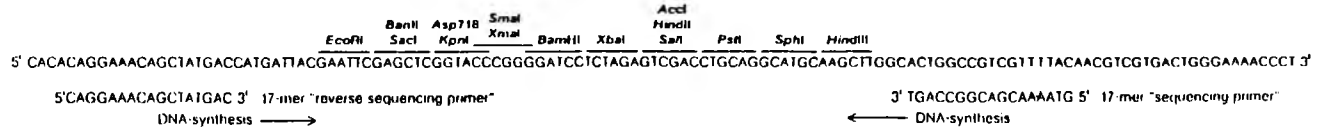
Application

Characterization of inserted fragments is facilitated by these characteristics of the pUC plasmids:

- Restriction mapping is facilitated by the small size of the vector.
- Plasmid preparation yields are high and allow easy isolation of the inserted fragments. One can readily obtain sufficient insert DNA for e.g. hybridization experiments or sequencing (2).
- The nucleotide sequence of inserted fragments can quickly and easily be determined by the double-strand sequencing method (3) with the help of the M13/pUC sequencing primer (see p. 137).
- Furthermore, it is possible to express genes in pUC plasmids under control of the *lac* promoter (4).

Unique cleavage sites		Insertable fragments	
Acc I	GT/CGAC	*Cla* I	AT/CGAT
		Nar I	GG/CGCC
		Asu II	TT/CGAA
		Aha II	G(A/G)/CG(T/C)C
		Mae II	A/CGT
		Hin P1I	G/CGC
		Hpa II/*Msp* I	C/CGG
		Taq I	T/CGA
Asp 718	G/GTACC	*Asp* 718	G/GTACC
Bam HI	G/GATCC	*Bam* HI	G/GATCC
		Bgl II	A/GATCT
		Bcl I	T/GATCA
		Xho II	(A/G)/GATC(T/C)
		Sau 3A	/GATC
Eco RI	G/AATTC	*Eco* RI	G/AATTC
		Eco RI*	/AATT
Hind II	GTC/GAC	every enzyme which produces blunt ends	
Hind III	A/AGCTT	*Hind* III	A/AGCTT
Kpn I	GGTAC/C	*Kpn* I	GGTAC/C
Pst I	CTGCA/G	*Pst* I	CTGCA/G
		Nsi I	ATGCA/T
Sac I, *Ban* I	GAGCT/C	*Sac* I	GAGCT/C
Sal I	G/TCGAC	*Sal* I	G/TCGAC
		Xho I	C/TCGAG
Sma I	CCC/GGG	every enzyme which produces blunt ends	
Sph I	GCATG/C	*Sph* I	GCATG/C
		Nsp HI	(A/G)CATG/(T/C)
		Nla III	CATG/
Xba I	T/CTAGA	*Xba* I	T/CTAGA
		Spe I	A/CTAGT
		Avr II	C/CTAGG
		Nhe I	G/CTAGC
Xma I, *Ava* I	C/CCGGG	*Xma* I	C/CCGGG
		Bsp MII	T/CCGGA
		Cfr 10I	(A/G)/CCGG(T/C)

Table 3: Unique cleavage sites in the pUC 18/19 polylinker and insertable fragments produced by restriction endonucleases



The kit contains

1. **E. coli JM83**
   One ampule 1 with lyophilized cells of the host strain E. coli K12 JM83 (*ara, rpsL, Δ(lac-proAB), Φ*80, *lacZ*ΔM15).
2. **E. coli JM109**
   One ampule 2 with lyophilized cells of the host strain E. coli K12 JM109 (*recA*1, *endA*1, *gyrA*96, *thi, hsdR*17, *supE*44, *relA*1, *Δ(lac-proAB)*, [F', *traD*36, *proA*⁺*B*⁺, *lacI*^{q}*Z*ΔM15]).
3. **pUC18 DNA**
   One vial 3 with 100 µl pUC18 DNA, 0.1 µg/µl; in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l, pH 8.0 (25 °C).
4. **pUC19 DNA**
   One vial 4 with 100 µl pUC19 DNA, 0.1 µg/µl, in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0 (25 °C).
5. **pUC18 DNA, *Eco* RI-cleaved**
   One vial 5 with 40 µl pUC18 DNA, cleaved with *Eco* RI, 25 ng/µl; in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0 (25 °C).
6. **T4 DNA ligase**
   One vial 6 with 25 µl T4 DNA ligase, 1 unit/µl.
7. **10 × Ligation buffer**
   One vial 7 with 250 µl 10-times-concentrated ligation buffer.
8. **X-gal solution**
   One vial 8 with 1 ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 2% (w/v), in dimethylformamide.
9. **IPTG solution**
   Three vials 9 with 850 µl isopropyl-β-D-thiogalactoside, 100 mmol/l, each; sterile filtered.

Stability

The kit can be stored at −20 °C until the date indicated. Repeated thawing and freezing should be avoided. After initial thawing, vials 3 and 4 containing pUC DNA, should not be frozen again but stored at +4 °C.
Immediately before use, mix gently and collect the solutions at the bottom of the vials by a short centrifuge spin.

Additionally required solutions and media

a. Appropriate restriction enzymes and restriction buffers.

b. Phenol, equilibrated, pH 7.0 (acc. to Maniatis, 5).

c. Phenol/chloroform/isoamyl alcohol: 25/24/1, equilibrated, pH 7.0 (acc. to Maniatis, 5).

d. Chloroform/isoamyl alcohol: 24/1 (acc. to Maniatis, 5)

e. LiCl solution, 4 mol/l; sterile filtered.

f. Ethanol A. R., 95% and 70% (v/v).

g. TE buffer: Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0.

h. $CaCl_2$ solution, 50 mmol/l; sterile.

i. Tris-HCl, 50 mmol/l, pH 7.2; sterile.

k. Ampicillin solution, 20 mg/ml; sterile filtered.

l. **LB liquid medium**
   Dissolve 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco) and 5 g NaCl with deionized water and fill up to 1 l.

m. **LB plates**
   Add 15 g Bacto agar (Difco) to 1 l LB liquid medium (l), autoclave and pour 25 ml each into sterile petri dishes and allow to solidify.

n. **LB/Amp/X-gal/IPTG plates**
   Autoclave 500 ml LB agar (m), cool down to approx. 50 °C, add under sterile conditions:
   2.5 ml ampicillin solution (k) ≙ 100 µg/ml,
   1 ml X-gal solution (solution 8) ≙ 0.004%. Wear gloves!
   2.5 ml IPTG solution (solution 9) ≙ 0.5 mmol/l
   Pour 25 ml each into sterile petri dishes and allow to solidify.

o. **M9 minimal-medium plates** (with vitamin B1 and glucose)
   10 × M9 salt solution:
   Dissolve 60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl and 5 g NaCl with deionized water, fill up to 1 l and autoclave.
   For 0.5 l medium sterilize the following components separately, cool down to approx. 50 °C and mix under sterile conditions:
   450 ml deionized water with addition of 7.5 g Bacto agar (Difco), autoclaved,
   50 ml 10 × M9 salt solution, autoclaved,
   0.5 ml $MgSO_4$ solution, 1 mol/l, autoclaved,
   0.05 ml $CaCl_2$ solution, 1 mol/l, autoclaved,
   2.5 ml vitamin B1 solution, 1 mg/ml, sterile filtered,
   5 ml glucose solution, 20% (w/v), autoclaved
   Pour 25 ml each into sterile petri dishes and allow to solidify.

Cloning experiment

I. Preparation of cleaved pUC vector DNA

1. Add the following to a sterile microfuge tube (20 µl final volume):
   10 µl ≙ 1 µg pUC18 or pUC19 DNA (solutions 3 or 4),
   2 µl 10 × restriction buffer (a),
   7 µl sterile redist. water,
   1 – 2 µl ≙ 2 – 5 units restriction enzyme (a).
2. Incubate for 1 h at 37 °C.
3. Add 205 µl TE buffer (g) and extract once with 200 µl phenol/chloroform/isoamyl alcohol (c), and once with 200 µl chloroform/isoamyl alcohol (d).
4. Add 25 µl sterile LiCl solution (e) and 750 µl ethanol, 95% (f).
5. Chill for 15 min at −70 °C (ethanol/dry ice mixture).
6. Centrifuge for 10 min, remove supernatant carefully and discard.
7. Add 1 ml ethanol, 70% (f), to the precipitate
8. Centrifuge for 5 min.
9. Remove supernatant. Dry precipitated DNA briefly under vacuum.
10. Dissolve in 10 µl sterile TE buffer (g) (≙ 0.1 µg DNA/µl).
11. Check 1 µl ≙ 0.1 µg on an 0.8% agarose gel.
    1 µl ≙ 0.1 µg uncleaved pUC DNA (solution 3 or 4) and 4 µl ≙ 0.1 µg pUC18 *Eco* RI fragments (solution 5) can be used for comparison.
    The DNA should be completely linearized and recovered without any loss.

II. Preparation of fragments for insertion

1. Add the following to a sterile microfuge tube (20 µl final volume):
   2 µg DNA to be cloned,
   2 µl 10 × restriction buffer (a),
   fill up to 19 µl with sterile redist. water,
   add 1–2 µl ≙ 2–10 units restriction enzyme (a).
2. Incubate for 1 h at 37 °C.

3–9. as described in section *I*.

10. Dissolve in 10 µl sterile TE buffer (g) (≙ 0.2 µg DNA/µl).

III. Ligation

1. Immediately before setting up a sticky-end ligation reaction, make dilutions of T4 DNA ligase and 10 × ligation buffer in following manner:
   - Mix 1 volume 10 × ligation buffer (solution 7) with 9 volumes sterile redist. water to make 1 × ligation buffer.
   - Add 1 µl T4 DNA ligase (solution 6) to 4 µl 1 × ligation buffer to give final enzyme concentration of 0.2 units/µl. The diluted enzyme solution is stable for only a few hours at +4 °C.
2. Add the following to a sterile microfuge tube on ice:
   1 µl ≙ 0.1 µg cleaved pUC DNA,
   variable µl ≙ 0.1–0.2 pmol cleaved insert DNA (e.g. 0.15–0.3 µg 2kb fragments),
   1 µl 10 × ligation buffer (solution 7)
   1 µl = 0.2 units T4 DNA ligase diluted as above for sticky-end ligations, or 1 µl = 1 unit T4 DNA ligase undiluted solution 6 for blunt-end ligations.
3. Incubate overnight (6 h minimum) at 15 °C for sticky-end ligations or at 22 °C for blunt-end ligations.

IV. Preparation of competent E. coli cells (see also VII)

1. Remove a single colony of E. coli JM83 from the LB plate, or JM109 from the M9 minimal-medium plate respectively, and inoculate into 5 ml LB liquid medium (l). Incubate the culture overnight at 37 °C with shaking.
2. Inoculate 40 ml LB liquid medium (l) with 0.4 ml fresh overnight culture, and incubate with shaking for approx. 2.5 h at 37 °C to A_{550} = 0.5 with JM83, or to A_{550} = 0.2 with JM109 respectively.
3. Centrifuge cells for 10 min at 3000 g in a pre-cooled rotor (e.g. Sorvall SS-34 rotor at 5000 rpm).
4. Resuspend pellet in 20 ml ice-cold sterile $CaCl_2$ solution (h). Allow to stand on ice for 30 min.
5. Centrifuge cells as described above (step 3).
6. Resuspend pellet in 4 ml ice-cold sterile $CaCl_2$ solution (h). These competent cells can be stored on ice up to 24 hours. The cell pellet may also be resuspended in ice-cold sterile glycerol/$CaCl_2$ solution (glycerol, 15% (v/v); $CaCl_2$, 50 mmol/l) and stored in 300 µl aliquots at −70 °C. Transformation efficiency may drop in the process.

V. Transformation

1. Mix the ligated DNA (section *III*) or control DNA (section *VI*) with sterile Tris-HCl solution (i) to give a final volume of 50 µl (e.g. 5 µl ligation mixture + 45 µl Tris-HCl solution).
2. Mix DNA in a sterile thin-walled test tube on ice with 300 µl competent E. coli cells and incubate on ice for 40 min.
3. Warm test tubes in a water bath to 42 °C (3 min) and let them stand at room temperature for 10 min.
4. Add 1 ml LB liquid medium (l) and roll or shake for 30–60 min at 37 °C (possibly transfer into sterile 10 ml culture tubes).
5. Distribute 0.2 ml each on LB/Amp/X-gal/IPTG plates (n).
6. Incubate plates *upside down* at 37 °C.

VI. Recommended controls

1. Cell competence check:
   Dilute 1 µl pUC18 DNA (solution 3) in 1000 µl sterile Tris-HCl solution (i).
   Use 50 µl (≙ 5 ng pUC18 DNA) of this solution for transformation (section *V*).
   After incubation, more than 10^5 blue colonies/µg pUC18 DNA (typically $1-2 \times 10^6$ blue colonies/µg pUC18 DNA) should be obtained.
   Lower colony count indicates that the quality of the competent cells is inadequate.
2. Checks for ligation with enclosed control DNA:
   Add the following to a sterile microfuge tube (100 µl final volume):
   4 µl ≙ 100 ng control DNA pUC18, *Eco* RI-cleaved (solution 5),
   85 µl sterile redist. water,
   10 µl 10 × ligation buffer (solution 7),
   1 µl ≙ 0.2 units T4 DNA ligase (dilute solution 6 with 1 × ligation buffer 1 + 4; see section *III*).

   Incubate overnight (6 h minimum) at 15 °C. Use 5 µl of the mixture (≙ 5 ng pUC18 DNA) for transformation (section *V*).
   The number of blue colonies per µg vector DNA should be at least 50% of the number obtained in control *VI*, 1 (typically > 70%).
   Alternatively, the increase in the molecular weight of the control DNA after separation by electrophoresis on an 0.8% agarose gel can be monitored.
3. Control for vector DNA cleavage:
   Use 1 µl ≙ 100 ng cleaved vector DNA (from section *I*) or as comparison 4 µl ≙ 100 ng control DNA (solution 5) for transformation (section *V*).
   The colony yield should be no more than 1% of that found in section *VI*, 1. Otherwise the vector DNA was not entirely cleaved and the preparation according to section *I* must be repeated.
4. Check for sterility:
   Use 50 µl sterile Tris-HCl solution (i) without DNA addition for transformation (section *V*). This serves to check whether competent cells, buffers and media were produced without bacterial contamination. No colonies should be observed.

VII. Note on the use of the E. coli strains

1. Revitalization of the E. coli strains from the ampules containing lyophilized bacteria:
   - Scratch ampule approx. 1 cm from the end with an ampule file (wear safety goggles!)
   - Immerse in ethanol for 2 min to sterilize ampule.
   - Roll into sterile cotton wool and break open.
   - Tilt glass rod in the ampule into a test tube with 5 ml LB liquid medium (l) under sterile conditions and mix briefly (Vortex).
   - Streak bacteria onto an LB plate (m) using a sterile loop. Incubate overnight at 37 °C. (We recommend also incubating a test tube with liquid medium so that the bacteria may be restreaked if necessary.)

2. Storage:

   For short-term storage of the E. coli strains (approx. 1–2 months), a streak on LB plates (m) should be made with JM83, for JM109 on M9 minimal-medium plates (o), and stored at +4 °C.

   E. coli JM109 carries the *lacZ*Δ*M15* mutation, which is necessary for blue/colorless discrimination with X-gal, on an F-episome. Growth on M9 minimal-medium indicates presence of the F-episome. For cloning experiments, therefore, a single colony of JM109 should always be picked from an M9 minimal-medium plate.

   For longer storage periods of the strains the usual procedures may be applied (e. g. glycerol culture).

3. Selection of the appropriate E. coli strain:

   E. coli JM83 is characterized by good transformability and high yields of plasmid DNA. Plasmid DNA which is isolated from this strain using rapid preparation methods, is suitable for sequencing of double-stranded DNA (3).

   However, this strain is both restriction- and modification-positive$^+$ (r_k, $m_k$$^+$) (1), i. e. when cloning DNA fragments which are prepared from a modification-negative E. coli strain or from organisms other than E. coli K, transformation efficiency is markedly reduced. This problem can be avoided by using the restriction-negative E. coli strain JM109 (1). No limitations are observed when cloning non-modified DNA in this strain. However, E. coli JM109 has a somewhat lower transformation efficiency than JM83.

Quality control

Ligation of the control DNA (pUC18, *Eco* RI-cleaved), as described in control *VI*, 2, and subsequent transformation yields at least 50% of the number of colonies per µg vector obtained with uncleaved pUC18 DNA (typically > 70%)

References

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APPENDIX E.

M13 CLONING

Principle

From the filamentous M13 phage, J. Messing et al. constructed a number of cloning vehicles, e.g. M13mp18 and M13mp19 (1). The double-stranded DNA of these phages can be cleaved with a number of restriction endonucleases at unique cleavage sites in a polylinker (see table 2 and fig. 64).

Vectors with inserted DNA are easily detected after transfection of appropriate E. coli host strains. When plated on solid media containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), recombinant phage vectors give rise to colorless plaques. Non-recombinant phage vectors give rise to blue plaques on X-gal plates (see fig. 13).

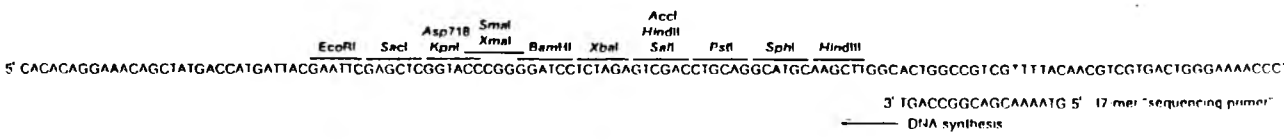
M13 phages multiply in infected E. coli cells first by forming double-stranded circular DNA ("replicative form" = RF DNA). When the RF DNA concentration reaches approx. 200 copies per cell, single-stranded (ss) DNA, comprising only of the (+) strand continues to be synthesized from the RF DNA. The newly synthesized DNA is packaged into phage particles, which are then secreted from the cell into the medium (2).

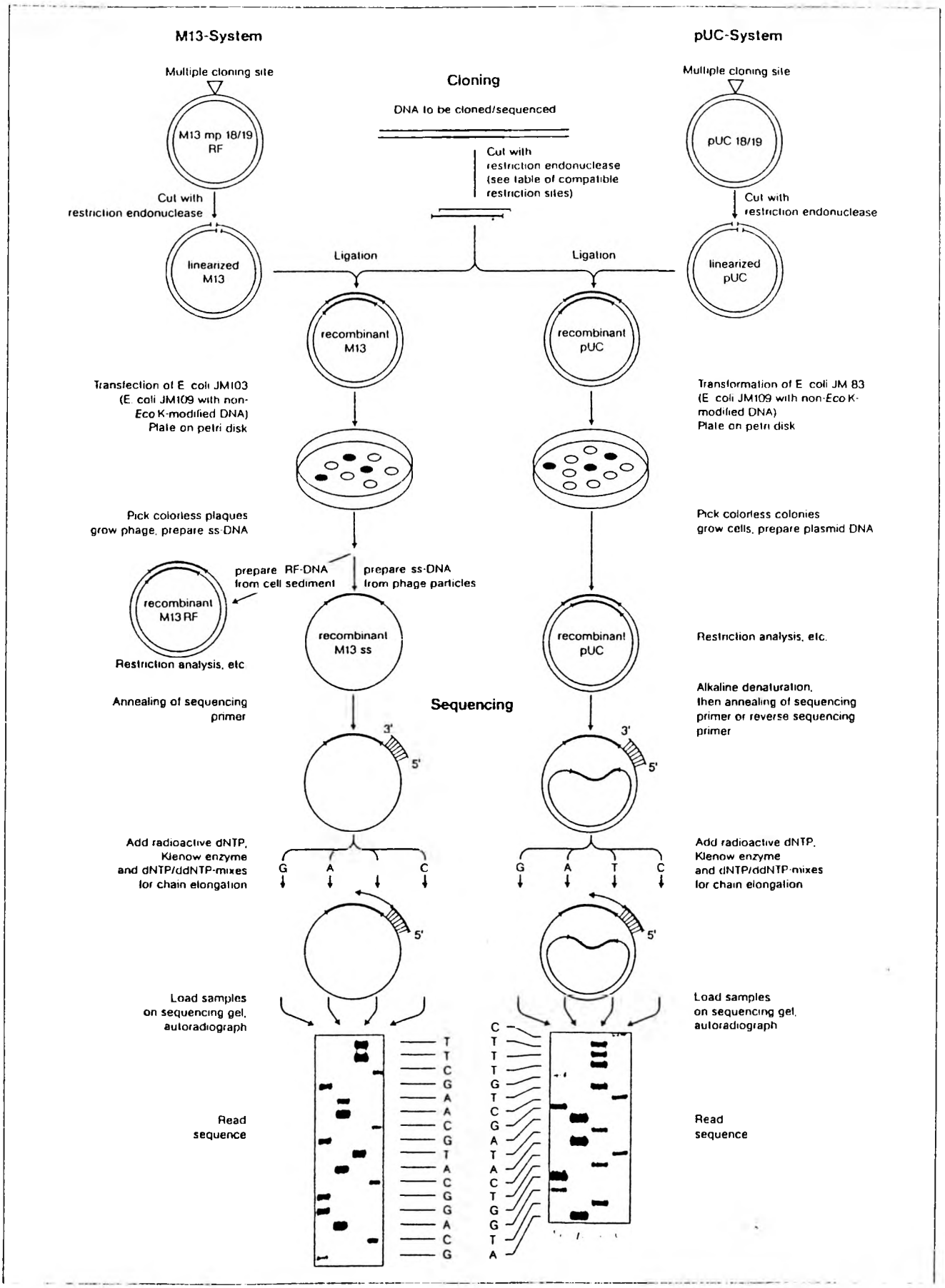
This means that by using standard plasmid preparation techniques, double-stranded RF DNA can be isolated from infected cells and used for restriction analyses or for further cloning experiments.

On the other hand, after the E. coli cells have been removed by centrifugation, phage particles can be isolated from the medium and ss (+) DNA prepared from the phage. ssDNA can be used for sequencing [dideoxy method (3, 4)], for *in vitro* mutagenesis experiments with synthetic oligonucleotides (5) or for preparing radioactively-labeled strand-specific DNA probes (6, 7).

Unique cleavage sites		Insertable fragments	
Acc I	GT/CGAC	*Cla* I	AT/CGAT
		Nar I	GG/CGCC
		Asu II	TT/CGAA
		Aha II	G(A/G)/CG(T/C)C
		Mae II	A/CGT
		Hin P1I	G/CGC
		Hpa II/*Msp* I	C/CGG
		Taq I	T/CGA
Asp 718	G/GTACC	*Asp* 718	G/GTACC
Bam HI	G/GATCC	*Bam* HI	G/GATCC
		Bgl II	A/GATCT
		Bcl I	T/GATCA
		Xho II	(A/G)/GATC(T/C)
		Sau 3A	/GATC
Eco RI	G/AATTC	*Eco* RI	G/AATTC
		Eco RI*	/AATT
Hind II	GTC/GAC	every enzyme which produces blunt ends	
Hind III	A/AGCTT	*Hind* III	A/AGCTT
Kpn I	GGTAC/C	*Kpn* I	GGTAC/C
Pst I	CTGCA/G	*Pst* I	CTGCA/G
		Nsi I	ATGCA/T
Sac I	GAGCT/C	*Sac* I	GAGCT/C
Sal I	G/TCGAC	*Sal* I	G/TCGAC
		Xho I	C/TCGAG
Sma I	CCC/GGG	every enzyme which produces blunt ends	
Sph I	GCATG/C	*Sph* I	GCATG/C
		Nsp HI	(A/G)CATG/(T/C)
		Nla III	CATG/
Xba I	T/CTAGA	*Xba* I	T/CTAGA
		Spe I	A/CTAGT
		Avr II	C/CTAGG
		Nhe I	G/CTAGC
Xma I	C/CCGGG	*Xma* I	C/CCGGG
		Bsp MII	T/CCGGA
		Cfr 10I	(A/G)/CCGG(T/C)

Table 2: Unique cleavage sites in the M13mp18/19 polylinker and insertable fragments produced by restriction endonucleases





Principle of M13/pUC cloning and sequencing

The kit contains

1. E. coli JM103
   One ampule 1 with lyophilized cells of the host strain E. coli K12 JM103 (*thi, rpsL, endA, sbcB*15, *supE, Δ(lac-proAB)*, [F', *traD*36, *proA*⁺*B*⁺, *lacI*^{q}*ZΔ*M15]).
2. E. coli JM109
   One ampule 2 with lyophilized cells of the host strain E. coli K12 JM109 (*recA1, endA1, gyrA*96, *thi, hsdR*17, *supE*44, *relA*1, *Δ(lac-proAB)*, [F', *traD*36, *proA*⁺*B*⁺, *lacI*^{q}*ZΔ*M15]).
3. M13 mp18 RF DNA
   One vial 3 with 100 µl M13mp18 RF DNA, 0.1 µg/µl; in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0 (25 °C).
4. M13 mp19 RF DNA
   One vial 4 with 100 µl M13mp19 RF DNA, 0.1 µg/µl; in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0 (25 °C).
5. M13 mp18 RF DNA, *Eco* RI-cleaved
   One vial 5 with 40 µl M13mp18 RF DNA, cleaved with *Eco* RI, 25 ng/µl; in Tris-HCl, 10 mmol/l, EDTA, 1 mmol/l; pH 8.0 (25 °C).
6. T4 DNA ligase
   One vial 6 with 25 µl T4 DNA ligase, 1 unit/µl.
7. 10 × Ligation buffer
   One vial 7 with 250 µl 10-times-concentrated ligation buffer.
8. X-gal solution
   One vial 8 with 1 ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 2% (w/v), in dimethylformamide.
9. IPTG solution
   One vial 9 with 1 ml isopropyl-β-D-thiogalactoside, 100 mmol/l; sterile filtered.

Stability

The kit can be stored at − 20 °C until the date indicated. Repeated thawing and freezing should be avoided. After initial thawing, vials 3 and 4 containing the M13 RF DNA, should not be frozen again but stored at + 4 °C.

Immediately before use, mix gently and collect the solutions at the bottom of the vials by a short centrifuge spin.

Additionally required solutions and media

a. Appropriate restriction enzymes and restriction buffers.

b. Phenol, equilibrated, pH 7.0 (acc. to Maniatis, 8).

c. Phenol/chloroform/isoamyl alcohol: 25/24/1, equilibrated, pH 7.0 (acc. to Maniatis, 8).

d. Chloroform/isoamyl alcohol: 24/1 (acc. to Maniatis, 8).

e. LiCl solution, 4 mol/l; sterile filtered.

f. Ethanol A. R., 95% and 70% (v/v).

g. TE buffer: Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0.

h. $CaCl_2$ solution, 50 mmol/l; sterile.

i. Tris-HCl, 50 mmol/l, pH 7.2; sterile.

k. **M9 minimal-medium plates** (with vitamin B1 and glucose)
   10 × M9 salt solution:
   Dissolve 60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl and 5 g NaCl with deionized water, fill up to 1 l and autoclave.
   For 0.5 l medium sterilize the following components separately, cool down to approx. 50 °C and mix under sterile conditions:
   450 ml deionized water with addition of 7.5 g Bacto agar (Difco), autoclaved,
   50 ml 10 × M9 salt solution, autoclaved,
   0.5 ml $MgSO_4$ solution, 1 mol/l, autoclaved,
   0.05 ml $CaCl_2$ solution, 1 mol/l, autoclaved,
   2.5 ml vitamin B1 solution, 1 mg/ml, sterile filtered,
   5 ml glucose solution, 20% (w/v), autoclaved
   Pour 25 ml each into sterile petri dishes and allow to solidify.

l. **2 × TY liquid medium**
   Dissolve 16 g Bacto tryptone (Difco), 10 g Bacto yeast extract (Difco) and 5 g NaCl with deionized water and fill up to 1 l and autoclave.

m. **2 × TY plates**
   Add 15 g Bacto agar (Difco) to 1 l 2 × TY liquid medium (l), autoclave and pour 25 ml each into sterile petri dishes and allow to solidify.

n. **H medium plates and H top agar**
   Dissolve 10 g Bacto tryptone (Difco) and 8 g NaCl with deionized water, fill up to 1 l and autoclave.
   For pouring plates add 12 g Bacto agar (for top agar: 8 g Bacto agar) and autoclave.
   Pour 25 ml each into sterile petri dishes and allow to solidify.

Cloning experiment

I. Preparation of cleaved M13 vector DNA

1. Add the following to a sterile microfuge tube (20 µl final volume):
   10 µl ≙ 1 µg M13 mp18 or M13 mp19 DNA (solution 3 or 4),
   2 µl 10 × restriction buffer (a),
   7 µl sterile redist. water,
   1 – 2 µl ≙ 2 – 5 units restriction enzyme (a)
2. Incubate for 1 h at 37 °C.
3. Add 205 µl TE buffer (g) and extract once with 200 µl phenol/chloroform/isoamyl alcohol (c), and once with 200 µl chloroform/isoamyl alcohol (d).
4. Add 25 µl sterile LiCl solution (e) and 750 µl ethanol, 95% (f).
5. Chill for 15 min at − 70 °C (ethanol/dry ice mixture).
6. Centrifuge for 10 min, remove supernatant carefully and discard.
7. Add 1 ml ethanol, 70% (f), to the precipitate.
8. Centrifuge for 5 min.
9. Remove supernatant. Dry precipitated DNA briefly under vacuum.
10. Dissolve in 10 µl sterile TE buffer (g) (≙ 0.1 µg DNA/µl).
11. Check 1 µl ≙ 0.1 µg cleaved M13 DNA on a 0.8% agarose gel electrophoresis. 1 µl ≙ 0.1 µg uncleaved M13 RF DNA (solution 3 or 4) and 4 µl ≙ 0.1 µg M13 mp18 *Eco* RI-fragments (solution 5) can be used for comparison.
    The DNA should be completely linearized and recovered without any loss.

II. Preparation of fragments for insertion

1. Add the following to a sterile microfuge tube (20 µl final volume):
   2 µg DNA to be cloned,
   2 µl 10 × restriction buffer (a),
   fill up to 19 µl with sterile redist. water,
   add 1–2 µl ≙ 2–10 units restriction enzyme (a).
2. Incubate for 1 h at 37 °C.

3–9. as described in section I.

10. Dissolve in 10 µl sterile TE buffer (g) (≙ 0.2 µg DNA/µl).

III. Ligation

1. Immediately before setting up a sticky-end ligation reaction, make dilutions of T4 DNA ligase and 10 × ligation buffer in following manner:
   - Mix 1 volume 10 × ligation buffer (solution 7) with 9 volumes sterile redist. water to make 1 × ligation buffer.
   - Add 1 µl T4 DNA ligase (solution 6) to 4 µl 1 × ligation buffer to give final enzyme concentration of 0.2 units/µl. The diluted enzyme solution is stable for only a few hours at + 4 °C.
2. Add the following to a sterile microfuge tube on ice:
   1 µl ≙ 0.1 µg cleaved M13 DNA,
   variable µl ≙ 0.1–0.2 pmol cleaved insert DNA (e.g. 0.15–0.3 µg 2kb fragments),
   1 µl 10 × ligation buffer (solution 7)
   1 µl = 0.2 units T4 DNA ligase diluted as above for sticky-end ligations, or 1 µl = 1 unit T4 DNA ligase undiluted solution 6 for blunt-end ligations.
3. Incubate overnight (6 h minimum) at 15 °C for sticky-end ligations or at 22 °C for blunt-end ligations.

IV. Preparation of competent E. coli cells (see also VII)

1. Remove a single colony of E. coli JM103 or JM109 from M9 minimal-medium plate and inoculate in 5 ml 2 × TY liquid medium (l). Incubate the culture overnight at 37 °C with shaking.
2. Inoculate 40 ml 2 × TY liquid medium (l) with 0.4 ml fresh overnight culture, and incubate for approx. 2 h at 37 °C to A_{550} = 0.2 with shaking.
3. Centrifuge cells for 10 min at 3000 g in a pre-cooled rotor (e.g. Sorvall SS-34 rotor at 5000 rpm).
4. Resuspend pellet in 20 ml ice-cold sterile $CaCl_2$ solution (h). Allow to stand on ice for 30 min.
5. Centrifuge cells as described above (step 3).
6. Resuspend pellet in 4 ml ice-cold sterile $CaCl_2$ solution (h).

   These competent cells can be stored on ice up to 24 hours.

   The cell sediment may also be resuspended in ice-cold sterile glycerol/$CaCl_2$ solution (glycerol, 15% (v/v); $CaCl_2$, 50 mmol/l) and stored in 300 µl aliquots at −70 °C. Transfection efficiency may drop in the process.

V. Transfection

1. Inoculate 20 ml 2 × TY liquid medium (l) with 1 drop E. coli JM103 or JM109 from a fresh overnight culture and shake at 37 °C.
2. Mix the ligated DNA (section *III*) or control DNA (section *VI*) with sterile Tris-HCl solution (i) to give a final volume of 50 µl (e.g. 5 µl ligation mixture + 45 µl Tris-HCl solution).
3. Mix DNA in a sterile thin-walled test tube on ice with 300 µl competent E. coli cells and incubate on ice for 40 min.
4. Warm test tubes in a water bath to 42 °C (3 min), and put on ice.
5. Melt H top agar (n), cool down to 42 °C. For each transfection experiment mix:
   200 µl E. coli, exponentially growing cells (culture from section *V*, 1., A_{550} approx. 0.3),
   40 µl IPTG solution (solution 9),
   40 µl X-gal solution (solution 8). Wear gloves!
6. To each test tube containing competent cells, add under sterile conditions:
   270 µl JM103/IPTG/X-gal mixture,
   3 ml H top agar (n).
   Mix carefully (roll) and pour immediately onto a warmed H medium plate (n).
7. After solidification of top agar, incubate plates *upside down* at 37 °C.

VI. Recommended controls

1. Cell competence check:

   Dilute 1 µl M13mp18 RF DNA (solution 3) in 1000 µl sterile Tris-HCl solution (i). Use 50 µl (≙ 5 ng M13mp18 RF DNA) of this solution for transfection (section *V*).

   After incubation, more than 250 blue plaques (≙ 5×10^4 plaques/µg M13mp18 DNA), typically 500 to 1000 blue plaques (≙ $1–2 \times 10^5$ plaques/µg M13mp18 DNA) should be obtained. Lower plaque count indicates that the quality of the competent cells is inadequate.
2. Checks for ligation with the enclosed control DNA:

   Add the following to a sterile microfuge tube (100 µl final volume):

   4 µl ≙ 100 ng control DNA, M13mp18, *Eco* RI-cleaved, (solution 5),
   85 µl sterile redist. water,
   10 µl 10 × ligation buffer (solution 7),
   1 µl ≙ 0.2 units T4 DNA ligase (solution 6 diluted 1 + 4 with 1 × ligation buffer, see section *III*, 1).

   Incubate overnight (6 h minimum) at 15 °C. Use 5 µl of the mixture (≙ 5 µg M13mp18 DNA) for transfection (section *V*).

   The number of blue plaques per µg vector DNA should be at least 50% of the number obtained in control *VI*, 1. (typically > 70%).

   Alternatively, the increase in the molecular weight of the control DNA after separation by electrophoresis on an 0.8% agarose gel can be monitored.
3. Control for vector DNA cleavage:

   Use 1 µl ≙ 100 ng cleaved vector DNA (from section *I*) or as comparison 4 µl ≙ 100 ng control DNA (solution 5) for transfection (section *V*).

   The plaque yield should be no more than 1% of that found in section *VI*, 1. Otherwise the vector DNA was not entirely cleaved and the preparation according to section *I* should be repeated.
4. Check for sterility:

   Use 50 µl sterile Tris-HCl solution (i) without DNA addition for transfection (section *V*). This serves to check whether competent cells, buffers and media were produced without phage contamination. No plaques should be observed.

VII. Notes on the use of the E. coli strains

1. Revitalization of the E. coli strains from the ampules containing lyophilized bacteria:
   - Scratch ampule approx. 1 cm from the end with an ampule file (wear safety goggles!).
   - Immerse in ethanol for 2 min to sterilize ampule.
   - Roll into sterile cotton wool and break open.
   - Tilt the glass rod in the ampule into a test tube with 5 ml 2 × TY liquid medium (l) under sterile conditions and mix briefly (Vortex).
   - Streak bacteria onto a 2 × TY plate (m) using a sterile loop. Incubate overnight at 37 °C. (We recommend also incubating a test tube with liquid medium so that the bacteria may be restreaked if necessary.)
2. Storage:

   For short-term storage of the strains (approx. 1–2 months) bacteria should be streaked on M9 minimal-medium plates (k). This is necessary for the maintenance of the F-episome of the strains which is essential both for the infectivity for M13 phages as well as for blue/colorless discrimination with X-gal.

   For cloning experiments we therefore recommend starting with a single colony picked from an M9 minimal-medium plate.

   For longer storage of the strains the usual procedures may be applied (e. g. glycerol culture).
3. Selection of the appropriate E. coli strain:

   The kit contains two E. coli strains. E. coli JM103 is an M13 host strain which is characterized by good transfectibility and high yields when preparing ssDNA and RF DNA.

   However, this strain is both restriction- and modification-positive (r_k^+, m_k^+) (1), i. e. when cloning DNA fragments prepared from a modification-negative E. coli strain or from organisms other than E. coli K, transfection efficiency is markedly reduced.

   This problem can be avoided by using the restriction-negative E. coli strain JM109. No limitations are observed when cloning non-modified DNA in this strain. However, E. coli JM109 has a somewhat lower transfection efficiency than JM103.

VIII. Preparation of recombinant M13 ssDNA

1. Make a 1 : 100 dilution of an E. coli JM103 overnight culture with 2 × TY liquid medium (l) and distribute 1.5 ml each into large sterile test tubes, one for each plaque to be picked.
2. Collect individual colorless plaques with sterile Pasteur pipettes or sterile toothpicks and inoculate into the test tubes.
3. Roll or shake for 5 h at 37 °C.
4. Transfer into 1.5 ml microfuge tubes and centrifuge for 5 min at 12000 g. Carefully remove supernatant without disturbing sedimented cells. (Supernatants may be stored at + 4 °C for several days but have to be centrifuged again afterwards. Do not add chloroform!)
5. Centrifuge supernatant again for 5 min in order to remove all cell debris.
6. Add 200 µl of a solution containing NaCl, 2.5 mol/l; polyethylene glycol 6000, 20% (w/v); allow to stand for 15 min at room temperature.
7. Centrifuge for 5 min at 12000 g. Remove supernatant and discard. Centrifuge again for 2 min and carefully remove all liquid remains of the phage pellet using a pulled-out Pasteur pipette.
8. Resuspend phage pellet in 100 µl TE buffer (g) by repeated suction in an automatic pipette, add 50 µl buffer-equilibrated phenol (b) and mix for 15 s (Vortex). Allow to stand for 15 min at room temperature and mix again for 15 s.
9. Centrifuge for 3 min at 12000 g; transfer aqueous top layer into new microfuge tube.
10. Add 10 µl LiCl solution (e) and 250 µl ethanol, 95% (f); allow to stand overnight at −20 °C.
11. Centrifuge for 10 min at 12000 g, remove supernatant. Add 1 ml ethanol, 70% (f), centrifuge again for 10 min. Remove supernatant and dry pellet briefly under vacuum.
12. Dissolve ssDNA in 50 µl TE buffer (g). The yield should be approx. 5 µg (check by agarose gel electrophoresis).

IX. Preparation of recombinant M13 RF DNA

1. Make a 1 : 100 dilution of an E. coli JM103 overnight culture with 2 × TY liquid medium (l) and distribute 1.5 ml each in large sterile test tubes, one for each plaque to be picked.
2. Collect individual colorless plaques with sterile Pasteur pipettes or sterile toothpicks and inoculate into the test tubes.
3. Roll or shake for 4–5 h at 37 °C.
4. Transfer into 1.5 ml microfuge tubes and centrifuge for 5 min at 12000 g. Remove supernatant and store at + 4 °C.
5. For small amounts of RF DNA:
   Using standard plasmid preparation methods, the bacterial pellet from No. 4 may be directly processed to M13 RF DNA. The bacterial pellet from the ssDNA preparation may also be used here.
6. For larger amounts of RF DNA:
   Inoculate 100 ml 2 × TY liquid medium (l) with 1 ml E. coli JM103 overnight culture and 1 ml phage supernatant from No. 4.
7. Shake for 4–5 h at 37 °C.
8. Centrifuge cells. RF DNA can be isolated according to standard plasmid preparation methods.

Quality control

Ligation of the control DNA (M13 mp18, *Eco* RI-cleaved), as described in control *VI*, 2, and subsequent transfection yields at least 50% of the number of plaques per µg vector obtained with uncleaved M13 mp18 DNA (typically > 70%).

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APPENDIX F.

M13 SEQUENCING

Principle

The nucleotide sequence of DNA fragments inserted into M13 vectors (1, 2) can quickly and easily be determined with the chain termination method of Sanger (3). Using this method a short sequencing primer (4), which is complementary to a region near the 3'-terminus of the M13 polylinker, is annealed to a single-stranded(ss) DNA isolated from M13 phage particles.

In the presence of all four deoxyribonucleoside triphosphates (dNTP) – one of which is radioactively labeled – Klenow enzyme (DNA polymerase I, large fragment) can be used to initiate DNA synthesis from the 3'-terminus of this sequencing primer toward the polylinker region. The reaction is carried out as four separate incubations, each of which is supplemented with a limiting amount of a different dideoxyribonucleoside triphosphate (ddNTP). In this way statistic base-specific chain terminations are effected at sites where a ddNTP is incorporated. Chasing with a large excess of unlabeled dNTP ensures that those chains where no ddNTP has been incorporated are polymerized to a high molecular weight. This prevents these unterminated chains from disturbing subsequent evaluation of the sequencing experiment.

After denaturation by formamide and heat the labeled samples are separated by electrophoresis on polyacrylamide gels containing urea. After autoradiography, the radioactively labeled fragments are visualized as bands on an X-ray film. As the distance of migration of the individual fragments is directly proportional to their length, the base sequence can be read directly from the order of the individual bands on the X-ray film (see fig. 13 on p. 19).

7-Deaza-dGTP, an analog of dGTP is also supplied with this kit. 7-Deaza-dGTP is used to replace dGTP in the chain elongation reaction to overcome the problem of band compression (5, 6) when sequencing G-C rich regions. Replacement of dGTP with 7-deaza-dGTP prevents the formation of undesired secondary structures which alter the electrophoretic mobility of single-stranded DNA fragments in polyacrylamide gels and lead to ambiguous or uninterpretable sequence data (see fig. 83 on p. 146). In addition, the use of 7-deaza-dGTP results in increased uniformity of band intensity, especially in the G-reaction.

[^{35}S]dATP-α-S has certain advantages in comparison to the more commonly used [α-^{32}P]dATP (7):

1. The longer half-life of [^{35}S] (87.2 days) compared to [^{32}P] (14.3 days) allows longer storage of [^{35}S]dATP-α-S. Sequencing samples that have already been prepared can be stored for longer periods before being run on gels.
2. Bands on autoradiograms are sharper with [^{35}S]-labeled samples than with [^{32}P]-labeled samples.
3. The lower energy of the β-particles emitted from [^{35}S] ensures safer handling.

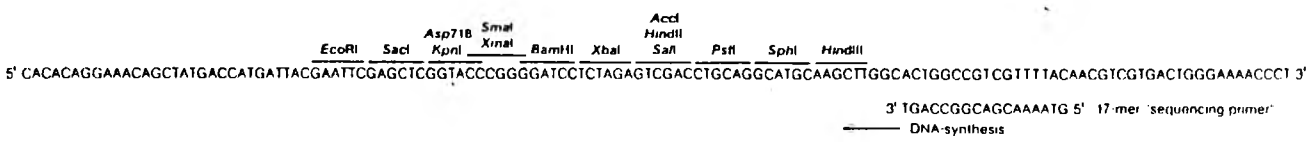
Any [α-^{32}P]dNTP or [^{35}S]dNTP-α-S can be used in the kit. For the reasons mentioned above we recommend the use of [^{35}S]dATP-α-S. Modifications necessitated by the use of [α-^{32}P]dNTPs are indicated at the corresponding steps

The kit contains

1. **dATP**
   One vial 1 with 250 µl dATP, 0.5 mmol/l, pH 7.5.
2. **dCTP**
   One vial 2 with 250 µl dCTP, 0.5 mmol/l, pH 7.5.
3. **dGTP**
   One vial 3 with 250 µl dGTP, 0.5 mmol/l, pH 7.5.
4. **dTTP**
   One vial 4 with 250 µl dTTP, 0.5 mmol/l, pH 7.5.
5. **ddGTP**
   One vial 5 with 25 µl ddGTP, 2 mmol/l, pH 7.5.
6. **ddATP**
   One vial 6 with 25 µl ddATP, 2 mmol/l, pH 7.5.
7. **ddTTP**
   One vial 7 with 120 µl ddTTP, 2 mmol/l, pH 7.5.
8. **ddCTP**
   One vial 8 with 25 µl ddCTP, 2 mmol/l, pH 7.5.
9. **Klenow enzyme**
   One vial 9 with 120 µl Klenow enzyme, 1 unit/µl.
10. **10 x Reaction buffer**
    One vial 10 with 350 µl 10-times-concentrated buffer for hybridization and chain elongation reaction.
11. **Deoxynucleotide mixture** (chase mixture)
    One vial 11 with 900 µl of a mixture of dATP, dCTP, dGTP, dTTP; 0.125 mmol/l each; pH 7.5.
12. **M13/pUC sequencing primer**
    One vial 12 with 220 µl M13/pUC sequencing primer 17-mer, 0.4 µmol/l, in water.
13. **Control DNA**
    One vial 13 with 50 µl single-stranded M13mp18 DNA, 0.1 µg/µl, in Tris/EDTA buffer (TE-buffer), pH 8.0.
14. **Formamide buffer** (stop buffer)
    Two vials 14 with 1 ml formamide buffer each.
15. **7-Deaza-dGTP**
    One vial 15 with 250 µl 7-deaza-dGTP, 0.5 mmol/l, pH 7.5.

Stability

The kit can be stored at –20 °C at least until the date indicated. Repeated thawing and freezing should be avoided. We recommend dividing the nucleotide solutions (solutions 1–8) into aliquots after thawing and storing them again at –20 °C afterwards. The control DNA (solution 13) should be stored at +4 °C after thawing.



Additionally required reagents

Radioactive label:

[^{35}S]dATP-α-S, 8 μCi/μl, > 600 Ci/mmol, or [α-^{32}P]dATP, 10 μCi/μl, 400–800 Ci/mmol, aqueous solution.

Preparation of the dNTP/ddNTP mixtures

When using radioactively labeled dATP, follow this pipetting scheme:

Nucleotide solutions	G mixture μl	G mixture μmol/l	A mixture μl	A mixture μmol/l	T mixture μl	T mixture μmol/l	C mixture μl	C mixture μmol/l
dGTP (3)*	2	5	40	100	40	100	40	100
dTTP (4)	40	100	40	100	2	5	40	100
dCTP (2)	40	100	40	100	40	100	4	10
ddGTP (5)	12	120	—	—	—	—	—	—
ddATP (6)	—	—	10	100	—	—	—	—
ddTTP (7)	—	—	—	—	50	500	—	—
ddCTP (8)	—	—	—	—	—	—	10	100
10× buffer (10)	20		20		20		20	
redist. water	86		50		48		86	
final volume	200		200		200		200	

* dGTP can be replaced by 7-deaza-dGTP (solution 15) in the dNTP/ddNTP mixtures to overcome problems in sequence evaluation caused by band compression.

If you use radioactively labeled dNTP other than dATP, leave the corresponding unlabeled dNTP out of all the mixtures. For such reactions add 4 μl dATP (solution 1) to the A mixture and 40 μl dATP to each of the G, T and C mixtures. Add redistilled water to a final volume of 200 μl.

Note: These dNTP/ddNTP mixtures have been optimized for sequencing DNA with a base composition (mol % G+C) of approx. 50%. When DNA with a very different base composition is sequenced, it may be necessary to modify the mixtures accordingly. The following rule should be applied in this case: If one of the base-specific sequence reaction mixtures gives bands on the autoradiogram which increase in intensity toward the high molecular weight region (500 bases), i. e. closer to the slot of the sequencing gel, the amount of ddNTP should be increased in that sequencing mixture. If the bands become distinctly weaker before a fragment size of approx. 400–500 bases has been reached, the amount of ddNTP should be reduced.

The final dNTP/ddNTP mixtures can be stored as aliquots at −20 °C for several months. It is not recommended to freeze again.

Standard assay

I. Annealing of the sequencing primer

Add to a sterile 1.5 ml microfuge tube:

5 μl control DNA (solution 13) or 0.5 μg self-prepared M13(ss)DNA,

2 μl ≙ 0.8 pmol M13/pUC sequencing primer (solution 12),

1.5 μl 10× reaction buffer (solution 10),

1 μl sterile redist. water.

Incubate mixture for 10 min at 55 °C and allow to cool slowly to room temperature afterwards.

II. Chain elongation

Add to hybridization mixture:

2 μl ≙ 16 μCi [^{35}S]dATP-α-S or 1 μl ≙ 10 μCi [α-^{32}P]dATP, 1 μl ≙ 1 unit Klenow enzyme (solution 9).

Mix by repeated suction with the pipette tip and add 2.5 μl of the reaction mixture into each of four labeled microfuge tubes (G, A, T, C).

Start reaction by adding 2 μl each of the appropriate dNTP/ddNTP mixture.

Incubate for 20 min at room temperature (or 15 min if [α-^{32}P]dATP is used).

Chase the reaction by adding 2 μl deoxynucleotide mixture (solution 11) to each microfuge tube and incubate for 15 min at room temperature.

Terminate the reaction by adding 4 μl formamide buffer (solution 14).

The assay may also be frozen and dried in a vacuum centrifuge before addition of the formamide buffer. For loading onto the polyacrylamide gel, smaller volumes are advantageous. In addition, it is possible to store the dried samples at −20 °C for several days.

III. Denaturation and gel electrophoresis

Immediately before loading the samples on the sequencing gel, denature the samples by incubation for 3 min at 95 °C in a water-bath, then quickly cool on ice and centrifuge briefly. Immediately load 1–2 μl of each sample into the appropriate slot of a pre-electrophorized and pre-heated (65 °C) sequencing gel. We recommend using 5% polyacrylamide gels with a 0.1–0.4 mm thickness gradient using the Macrophor® Sequencing System (LKB).

IV. Autoradiography and evaluation

After electrophoresis, agitate the sequencing gel in technical acetic acid, 10% (v/v), for 10 min in order to remove urea. Remove the gel from the bath, rinse briefly with deionized water, and dry for approx. 1 h at 80 °C. Lay an X-ray film on the dried gel, mark orientation and expose overnight (longer or shorter exposure may be necessary).

Quality control

After sequencing the control DNA and analysis on a 5% polyacrylamide gel (0.1–0.4 mm thickness gradient), a sequence of at least 250 nucleotides can be determined (typically 300–400 nucleotides) under the conditions described here.

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