CHAPTER ONE
INTRODUCTION

1.1 *Salmonella typhi*

Salmonella typhi is a flagellated, non-encapsulated, non-sporulating and facultative anaerobic Gram-negative bacillus. It ferments glucose but not lactose or sucrose, and reduces nitrate to nitrite. It is motile and has the capability to synthesize peritrichous flagella. It has a somatic (O) antigen (oligosaccharide), a flagellar (H) antigen (protein), and an envelope (K) antigen (polysaccharide), and a lipopolysaccharide macromolecule complex that forms the outer portion of the cell wall (Hoffman, 1984).

S. typhi ranges in size from 2 to 3 x 0.6 μm, and is a member of the Enterobacteriaceae (Parker, 1983). *S. typhi*, usually present in relatively small number in the environment, is also fastidious, easily inhibited by coliforms, and capable of acquiring R plasmid transmitted antibiotic resistance (Hoffman, 1984). *S. typhi* is specifically a human pathogen that causes the acute systemic febrile illness typhoid fever. Ingression of *S. typhi* is mostly through the gastrointestinal tract, especially the upper small bowel (Hornick, 1992, 1994).

1.2 Typhoid fever

In 1829, Pierre Louis first called it typhoid, meaning "typhus-like", but did not differentiate the typhoid of Paris and typhus that was then common in Great Britain. In 1837, William Wood Gerhard, a former student of Louis working in Philadelphia, clearly distinguished typhoid from typhus fever, both clinically and pathogenically. In 1880, Eberth described *Bacillus typhosus* in histological sections of mesenteric lymph nodes and the spleen (Hoffman, 1984). Four years later in 1884, Gaffky successfully cultured and isolated *S. typhi* in pure culture from the spleens of infected patients. He also reported that the infection was usually waterborne and not airborne (Edelman and Levine, 1986).

Typhoid fever is characterized by prolonged fever and sustained bacteremia, without endothelial or endocardial involvement. Typhoid fever is usually associated

with bacterial invasion of and multiplication within the mononuclear phagocytic cells of the liver, spleen, lymph nodes, and Peyer's patches (Hoffman, 1984; Hornick, 1992, 1994). The clinical features of typhoid fever in Malaysia, as seen during the 1987 outbreaks in Penang, were persistent fever (>1 week), headache, diarrhoea, malaise, anorexia, rigors, and cough. Increased incidences of vomiting and splenomegaly were reported among children infected with this disease (Lim, 1992; Pang *et al.*, 1992a, b).

In many developing countries, typhoid fever is still among the most important infectious diseases. Amid urban and rural populations, it affects all age groups and has very high mortality and morbidity rates. This disease is usually endemic, and epidemics frequently occur owing to poor sanitation and lack of control measures (Mirza and Hart, 1993). There are several attributes shared by countries that report increased typhoid severity: (1) rapidly increasing populations; (2) rapidly increasing urbanisation; (3) inadequate facilities for processing human wastes; (4) decreasing water supply; (5) close contact between humans, food, and heavily contaminated water supplies; and (6) overburdened health care delivery systems (Edelman and Levine, 1986). The method of infection of this fever is most commonly acquired by ingesting contaminated food or water, but rarely transmitted by direct finger to mouth contact with the faeces, urine, respiratory secretions, vomits, or pus from an infected individual (Hoffman, 1984).

The real impact of typhoid fever is difficult to estimate because the clinical picture may be confused with many other febrile infections like rickettsial fever. Nevertheless, according to the literature and the extensive field trials conducted for the assessment of candidate vaccines, about 16 million cases of typhoid fever were estimated to occur annually in the world, causing about 600,000 deaths (Ivanoff, 1994). Ivanoff (1994) also estimated that the mean population-based annual incidence of typhoid fever in endemic developing countries to be between $150/10^5$/year in South America to more than $1,000/10^5$/year in some Asian countries.

1.3 Diagnosis and treatment of typhoid fever

In 1896, Pfeiffer and Kalle successfully made the first vaccine for human use against typhoid. They used heat-killed organisms to make the vaccines and verified that protective antibodies, which passively protected guinea pigs against experimental infection, were developed (Edelman and Levine, 1986).

In the same year, Gruber, Durham and Widal each independently reported that convalescent-phase serum mixed with *S. typhi* caused the organisms to "stick together in large balls and lose their motility". Thus, the term agglutinins and the classic serologic test for infection *S. typhi* were born (Hoffman, 1984; Edelman and Levine, 1986).

Case fatality continued to increase to 30%, even with successful diagnosis and vaccination. However in 1948, Theodore Woodward and his colleagues announced that the antibiotic chloramphenicol (Cm) sterilized the blood cultures of 10 Malaysian typhoid patients (Woodward *et al.*, 1948). This ushered in the modern era of antibiotic as a treatment for typhoid fever. Ever since then, Cm has been the antibiotic of choice for treating both typhoid and paratyphoid fevers (Edelman and Levine, 1986). In institutions that have been equipped to provide appropriate supportive health care, it was noted that the fatality rate since the introduction of Cm was only 1 to 2% (Hornick, 1992).

In cases where typhoid fever was suspected, absolute diagnosis should be sought by attempting to isolate the culpable microorganism from the blood, faeces, urine, or occasionally the sputum or purulent exudates. An increment of the agglutinin titre by fourfold, especially against the O antigen, by the Widal reaction without recent immunization confirms the diagnosis (Hornick *et al.*, 1970; Hornick, 1992).

There is a vaccine that confers some protection against typhoid fever, but it is not very effective and has been shown to have side effects (Hoeprich, 1994). Currently, three vaccines against typhoid fever are used. Two are injected and one is orally administered (Salyers and Whitt, 1994b). The first successful injected vaccine uses killed whole bacteria. However, this vaccine has numerous side effects owing to the presence of lipopolysaccharide (LPS). In addition, this vaccine must be readministered periodically. The second type of vaccine is safer and much more effective as it consists of capsular material (Vi antigen) (Hoeprich, 1994). The oral vaccine, on the other hand, uses an attenuated strain, *S. typhi* Ty21a. This strain is used here as it was originally thought to be avirulent owing to a mutation in *galE* (Hornick *et al.*, 1970; Hoeprich, 1994). *galE* is involved in the galactose utilization pathway that consequently affects the production of the O antigen. Of late, Ty21a strain was found to have more than one mutation. This was encountered when a mutant was found to be still virulent albeit having a disruption in its *galE* gene. This ascertained that the avirulence of Ty21a is feasibly due to some other mutation. In

spite of the fact that this vaccine was proved to be safe and fairly effective in human field trials, its efficacy has differed drastically in different trials (96% protection in Egypt; 60 to 80% protection in Chile) (Hoeprich, 1994). The differences in the protection rate as seen in the conducted different field trials are still unknown. Even though Ty21a is still currently used in most trials to date, there is still concern for its unknown nature of mutation in the strain and whether this strain can evoke long-term immunity (Levine *et al.*, 1987). Developing a safe, effective vaccine against typhoid fever is currently the focus of intensive research and remains an important item on the world health agenda.

The bacteria in carriers are localized in the liver and billiary tract. Therefore, the antibiotics used to clear carriers of *S. typhi* must be bactericidal and attain high concentrations in liver and the billiary tract. Antibiotics are a fundamental part of the treatment of *S. typhi*, not only to save patients with active typhoid fever but also to clear bacteria from carriers, a major factor in the spread of typhoid fever (Salyers and Whitt, 1994b).

1.4 Antibiotics

1.4.1 Characteristics of antibiotics

Antibiotics are low-molecular-weight compounds that inhibit the growth of or kill bacteria. Most of the antibiotics used are produced by microorganisms, especially *Streptomyces* species and fungi (Lerner *et al.*, 1992; Salyers and Whitt, 1994a).

To be useful for therapy of human infections, an antibiotic must satisfy several criteria. For most applications in empirical treatment, the antibiotic should have a broad spectrum of activity. It should inhibit the growth of or kill various types of bacteria. As it is not always possible to wait for the isolation and identification of the causative organism before therapy to begin, broad-spectrum antibiotics are useful because the same symptoms can be caused by different bacterial species. Anyway, these antibiotics have an important drawback. They do not only attack pathogens but also reduce the numbers of resident microflora. This will then allow other pathogens that normally are outcompeted by the microflora to overgrow and cause other adverse infections (Silver and Bostian, 1993; Salyers and Whitt, 1994a).

Some antibiotics kill bacteria (bactericidal), whereas others only inhibit the growth of the bacteria (bacteriostatic). Patients with an intact immune system, both types of antibiotics are effective because these antibiotics allow the immune system to

catch up with and eliminate the invading bacteria. Unfortunately, bacteriostatic antibiotics can be ineffective in immunocompromised patients. Nearly all antibiotics are directed against targets that are specific to bacteria, therefore, minimising the toxicity of the antibiotics to humans. Examples of such targets are bacterial peptidoglycan, bacterial ribosomes, enzymes involved in folate metabolism, and bacterial enzymes involved in DNA supercoiling (Levy, 1992; Salyers and Whitt, 1994a).

Another criterion is that an antibiotic must be able to reach the part of the human body where the infection is occurring. Some antibiotics are not absorbed from the gastrointestinal tract, some enter the bloodstream but do not cross the blood-brain barrier into spinal fluid, and some do not penetrate abscesses very well or enter phagocytic cells. Thus, the distribution of the antibiotic relative to the likely distribution of infecting bacteria must be taken into account (Salyers and Whitt, 1994a).

The targets of clinically used antibiotics are peptidoglycan synthesis (β-lactams and glycopeptides), protein synthesis (aminoglycosides, tetracyclines, macrolides, and lincosamides), DNA gyrase (quinolones), RNA polymerase (rifampin), and production of tetrahydrofolic acid (trimethoprim and sulfonamides) (Salyers and Whitt, 1994a).

In the treatment of typhoid fever, Cm is still the antibiotic of choice as it remains effective in patients infected with susceptible strains, and also inexpensive. Although effective *in vitro* against *S. typhi*, sulfonamides (Su), tetracyclines (Tc), and aminoglycosides are still ineffective *in vivo*. Only Ac, amoxicillin, trimethoprim-sulfamethaxazole (Tp-Su), and thiamphenicol have been used successfully in large series of patients. Cephalosporin and trimethoprim (Tp) alone have been reported to be effective in small groups of patients (Hoffman, 1984).

1.4.2 Aminoglycosides

Aminoglycoside antibiotics have been used for years to treat Gram-negative bacilliary infections. Their rapid bactericidal activity has made them as primary players, especially in blood stream infections. Their potential for synergistic activity with β-lactams and the relative infrequency of stable aminoglycoside resistance in bacteria during therapy have made these agents ideal to combat multiresistant

organisms, such as *Pseudomonas* and *Enterobacter*, that are usually found in nosocomial infections (Rubens *et al.*, 1981). The clinical utility of aminoglycosides depends on the pathogen, the site of infection, and the nature of the infected host.

Although aminoglycosides have the potential for nephro- and ototoxicity, dosage management based on monitoring of serum level and renal functions has enhanced their utility. Moreover, allergic reactions and other adverse effects are rare. In spite of the advent of new β-lactams and fluoroquinolones, the aminoglycosides continue to serve as important antibacterial agents for serious infections (Salyers and Whitt, 1994a).

All aminoglycosides consist of a central six-membered aminocyclitol ring linked to two or more aminosugar residues by glycosidic bonds, display high polarity, and are water-soluble polycations, which generally stabilize to heat and pH change, within the range of 5 to 8, (Gottlieb and Shaw, 1967a).

Bacterial ribosomes are usually targeted by the aminoglycosides (Spotts and Stanier, 1961; Weisblum and Davis, 1968). These ribosomes are good targets for antibiotics because they differ appreciably from ribosomes of mammalian cells. Aminoglycosides act by binding to the 30S subunit of the bacterial ribosome. The bound 30S subunit binds to the mRNA and places an fMet-tRNA in the P site, but the 50S subunit does not join the 30S to form the active ribosome, thus, no protein synthesis occurs (Gottlieb and Shaw, 1967b; Salyers and Whitt, 1994a).

Aminoglycosides are bactericidal, probably because accumulation of the aberrant 30S subunits causes toxicity to the cell. Access of aminoglycosides to their ribosomal target(s) requires diffusion through aqueous pores in the outer membrane (in Gram-negative bacteria) formed by porin proteins. This is then followed by active accumulation across the plasma membrane (in all bacteria) to intracellular concentrations far above those outside the cell (Anand and Davies, 1960a; Bryan and Kwan, 1983). The initial ionic binding of cationic amines of aminoglycosides to negatively charged residues of the outer membrane and lipopolysaccharide is rapid and nonsaturable (Jackson *et al.*, 1990). This was demonstrated by the increased rate and extent of bacterial killing as that to the increased concentration of the aminoglycoside in the growth medium (Vogelman and Craig, 1985, 1986). This ionic binding also seems to explain the observed post-antibiotic effect of aminoglycosides, whereas bactericidal effect continues even after removal or depletion of

aminoglycoside from the growth medium (Bundtzen *et al.*, 1981).

Aminoglycosides are effective against a number of pathogenic bacteria, but their side effects limit their use. Prolonged use of aminoglycosides can lead to loss of hearing and to impairment of kidney function. Streptomycin (Sm) is one of the aminoglycosides used to treat typhoid infections (Salyers and Whitt, 1994a).

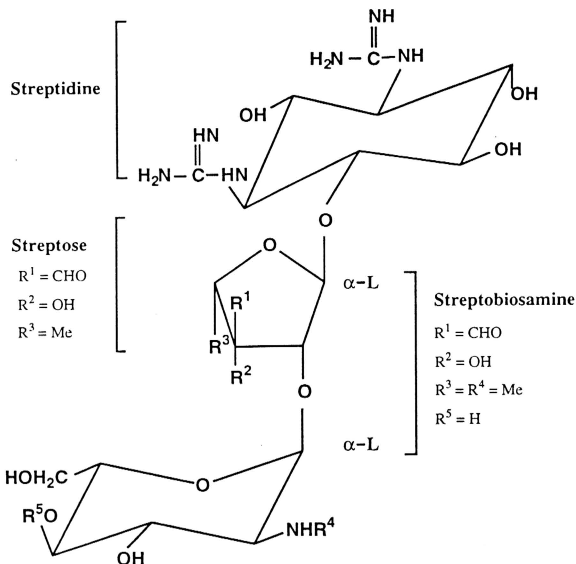
1.4.3 Streptomycin (Sm)

Sm was first isolated from *Streptomyces griseus*, after systematic examination of soil microorganisms for antibacterial activity (Schatz *et al.*, 1944). The aminocyclitol of Sm is streptidine (Figure 1), whereas that of all other available aminoglycosides is 2-deoxystreptamine (Figure 2) (Gottlieb and Shaw, 1967a).

Though the exact mechanism of the bactericidal effect of aminoglycosides is still not completely elucidated, the mode of action of Sm is the best studied of all the aminoglycosides. As related earlier, the primary target of Sm is the bacterial ribosome (Anand and Davis, 1960a, b; Spotts and Stanier, 1961; Cox *et al.*, 1964; Weisblum and Davis, 1968). In addition to that, it also inhibits protein synthesis, impairment of cellular respiration, inhibition of RNA and DNA synthesis, breakdown of RNA, and damage to the plasma membrane (Arnad *et al.*, 1960; Dubin *et al.*, 1963).

Sm binds to the 30S ribosomal subunit at its interface with the 50S subunit. The binding of Sm is dependent on protein S12. This is because reduced binding of Sm occurs when S12 is mutationally altered (Ozaki *et al.*, 1969). However, proteins S3, S5, and L6 may also have some effects on the binding (Schreiner and Nierhaus, 1973; Hummel *et al.*, 1980). The binding of Sm to ribosomes disrupts peptide chain elongation by destabilizing polysomes to break down to monosomes. This "streptomycin monosomes" can neither initiate formation of peptide chain nor dissociate at normal rates to free, active ribosomal subunits (Modollel and Davis, 1968, 1969, 1970; Wallace and Davies, 1973; Wallace *et al.*, 1973a, b).

Sm can also cause misreading of the genetic code owing to incorrect codon-anticodon interaction on the ribosome. This then results in formation of "missense" proteins and inadequate production of proteins needed for vital bacterial processes (Davies *et al.*, 1965).



	R^1	R^2	R^3	R^4	R^5
Streptomycin	CHO	OH	Me	Me	H
Dihydrostreptomycin	CH_2OH	OH	Me	Me	H
Dihidrodeoxystreptomycin	CH_2OH	H	Me	Me	H
Hydroxystreptomycin	CHO	OH	CH_2OH	Me	H
N-demethylstreptomycin	CHO	OH	Me	H	H
Mannosidostreptomycin	CHO	OH	Me	Me	Man*

* Mannosyl

Figure 1: Structure of streptomycins. (From Gottlieb and Shaw, 1967a.)

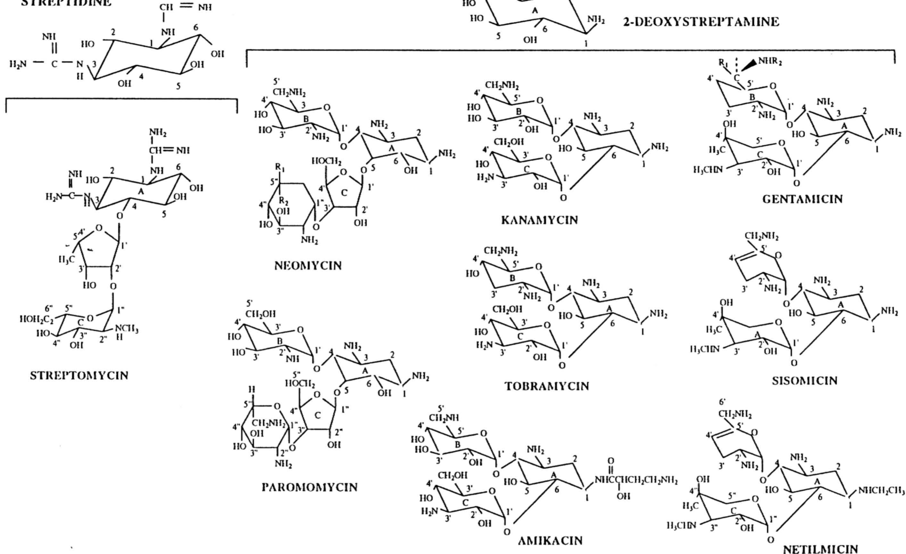


Figure 2: Chemical structures of the aminoglycosides. Neomycin contains approximately equal amounts of neomycin B (R_1=H; R_2=CH_2NH_2) and C (R_1=CH_2NH_2; R_2=H). Kanamycin is principally kanamycin A (structure shown). Gentamicin is gentamicin C complex, with approximately equal amounts of gentamicin C_1 (R_1=R_2=CH_3), C_{1a} (R_1=CH_3; R_2=H). (From Lerner *et al*., 1992.)

Nevertheless, the lethal effect of Sm is not due to general inhibition of protein synthesis and an ability to cause codon mistranslation (Bryan and Kwan, 1983). A model, proposed for the lethal effect of Sm, suggests that the lethality probably results from the accumulation of effects on the cytoplasmic membrane or from a gradual disruption of membrane integrity (Bryan and Kwan, 1983). The mechanism of Sm action still remains to be speculated (Hornick, 1992).

A model was proposed by Davis *et al.* (1986) incorporating much of the available information on the mechanism of streptomycin action. This proposed model involves five essential steps: (i) slight entry of Sm into the cell; (ii) interaction with chain-elongating ribosomes, resulting in misreading; (iii) incorporation of false proteins into the cell membrane, creating abnormal channels; (iv) increased and irreversible entry of Sm through these channels, creating more misreading and formation of channels; and (v) blockage of initiating ribosomes.

1.4.4 Mechanisms of Sm resistance

Three basic mechanisms may be involved in Sm resistance (Sm^R) in bacteria (Benveniste and Davies, 1973; Schmidt and Klopfer-Kaul, 1984; Salyers and Whitt, 1994a):

(i) Sm-modifying enzymes,

(ii) Mutational alterations of ribosomes, and

(iii) Mutations interfering with Sm uptake.

1.4.4.1 Sm-modifying enzymes

Enzymatic modification of a specific substituent group on the aminoglycoside, e.g., acetylation of an amino group, adenylation of a hydroxyl group, or phosphorylation of a hydoxyl group, is the most common mechanism of resistance to Sm in clinical isolates of aerobic Gram-negative bacteria (Figure 3) (Hornick, 1992). The level of resistance to Sm conferred by modifying enzymes is relatively high owing to the little amount of unmodified Sm that reaches the ribosome (Bryan, 1984). The location of these enzymes in the cell has been investigated and found to be loosely associated with the outer layer of the cytoplasmic membrane (Dickie *et al.*, 1978). Other evidence suggests that at least one aminoglycoside-modifying enzyme resides in the cytosol or is loosely associated with the inner leaflet of the plasma membrane (Perlin and Lerner, 1981).

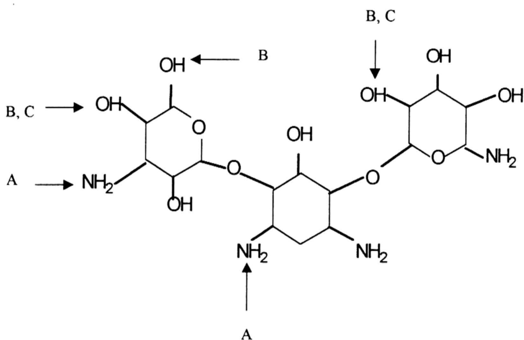


Figure 3: Action of aminoglycoside-inactivating enzymes. Note: A, acetyl; B, adenyl; C, phosphoryl. (From Salyers and Whitt, 1994a.)

1.4.4.2 Alterations in ribosomal Sm binding site

Although mutational changes in ribosomal binding proteins may account for resistance to Sm, this mechanism has been relatively unimportant for resistance to 2-deoxystreptamine aminoglycosides in clinical settings (Moellering, 1983; Lerner *et al.*, 1992). An investigation in a neonatal intensive care unit disclosed infection and colonisation with a strain of *Escherichia coli* that was multiply resistant to aminoglycosides, apparently from mutational alteration of ribosomes (Galas and Branscomb, 1976; Gaynes *et al.*, 1984). Nevertheless, this type of resistance is rare.

1.4.4.3 Mutations interfering with Sm uptake

Advent of Sm^R mutants with decreased uptake of Sm has been observed in Gram-negative bacteria (Anand and Davies, 1960a). The type of resistance is less common than that caused by Sm-modifying enzymes, and there is a complete cross-resistance to all aminoglycosides.

In most cases, the resistant isolates are small colony variants produced by mutations affecting the membrane electrical potential and various components of the electron transport chain (Anand and Davies, 1960b; Bryan and Kwan, 1983). In some cases, especially with *P. aeruginosa*, decreased aminoglycoside uptake is not linked to altered membrane electrical potential or electron transport or with abnormal aerobic growth. An alteration in the outer membrane or the lipopolysaccharide has been found in mutants, and in some cases the responsible alterations have not been verified (Lerner *et al.*, 1992). Nevertheless, higher level of resistance to Sm may follow from mutations in cells containing a modifying enzyme that is not sufficient to confer Sm resistance on its own (Perlin and Lerner, 1986). A transient resistance to Sm develops following the early concentration-dependent phase of rapid killing after exposure of bacteria to an aminoglycoside (Daikos *et al.*, 1990). This period of refractoriness to the bactericidal effect of aminoglycosides lasts for several hours and is maximal during recovery of growth after the postantibiotic effect. It also depends on the temporary cessation of energy-dependent uptake of aminoglycosides into the bacteria, and it seems to be an adaptive response rather than a result of selection of unstable mutants (Lerner *et al.*, 1992).

1.5 Epidemiology of antibiotic resistance

Treatment of typhoid fever patients is now conducted through effective antibiotics. Commonly, Cm, Ac, and Ctm make up the first line antibiotics against *S. typhi*. Until 1950, these antibiotics were effective in patients with acute infection, reducing complications and mortality. However, since 1950 progressive resistance to one or two of these first line antibiotics has been reported (Calquhoun and Weetch, 1950; Njoku-Obi and Njoku-Obi, 1965; Anderson and Smith, 1972; Paniker and Vimala, 1972; Anderson, 1975; Brown *et al.*, 1975; Chun *et al.*, 1977; Agarwal *et al.*, 1981; Datta *et al.*, 1981; Acar and Goldstein, 1982; Goldstein *et al.*, 1986; Rowe *et al.*, 1987; Gupta *et al.*, 1990; Karmaker *et al.*, 1991; Rowe *et al.*, 1991). Resistance to all three first line antibiotics were noticed in Pakistan, India, China, and the Arabian gulf in 1989 (Threlfall *et al.*, 1992; Mirza and Hart, 1993). The first line therapeutic regiments are thus rendered ineffective. Thus, the less effective agents, such as fluoroquinolones (Akhtar *et al.*, 1989; Asperilla *et al.*, 1990; Butler *et al.*, 1997), must be administered.

Until 1990, there were only 0.25% Cm resistant *S. typhi* strains in the UK (Ivanoff, 1994). Eventually, the situation changed dramatically and in that year, 20% of the strains were found to be resistant not only to Cm but also to Tp and Ac. Tp and Ac have been used as alternatives to Cm for treating typhoid fever (Rowe *et al.*, 1987, 1991; Threlfall *et al.*, 1991).

In the 1980s, among the *S. typhi* isolates from India (156 isolates), Singapore (520 isolates), and Indonesia (>5,000 isolates), less than 3% were resistant to Cm, but in a study of isolates from 100 patients in Bangkok, 45% were found to be resistant (Hoffman, 1984). In Malaysia, transferable antibiotic resistance in *S. typhi* was first reported by Jegathesan and Khor (1980), followed by Koh *et al.* (1983), Phipps *et al.* (1991), and Ansary *et al.* (1995).

A study conducted on antibiotic resistant *S. typhi* strains isolated from Malaysia (Cheong, 1992) showed a lower frequency of such resistance over a period of ten years (1980 to 1989). The strains examined exhibited resistance to Sm (10%), Ctm (0.5%), Tc (0.4%), Ac (0.3%), kanamycin (Km) (0.2%), and Cm (0.1%).

Resistance patterns to aminoglycosides differ from region to region and among hospitals. Prevalence of various aminoglycoside-modifying enzymes, which generally confer high-level resistance, causes this difference to occur. Nevertheless, resistance patterns to aminoglycosides conferring low level resistance have been

noticed among isolates from Japan, Taiwan, Korea, Chile, and the United States (Shimizu *et al.*, 1985). The uptake of aminoglycosides in these isolates has been found to be reduced (Shimizu *et al.*, 1985). In East Asia, 88% of the aminoglycoside-resistant strains showed some type of 6'-acetylating activity, which confers resistance to the aminoglycosides most widely used there, e.g., dibekacin, kanamycin, and amikacin. Almost 94% of the isolates from Chile had 3-acetylating activity, but those isolates were from a single hospital outbreak. Meanwhile in the United States, 92% of the isolates that conferred resistance to at least one aminoglycoside were likely to have 2"-adenylating, 6'-acetylating, or 3-acetylating activity (Shimizu *et al.*, 1985).

1.6 Origin of antibiotic resistance

The root of antibiotic resistance genes has long been speculated (Figure 4). Initially, it was a tendency to assume that antibiotic resistance genes appeared after antibiotics were widely used in medicine. However, the genetic diversity within some classes of resistance makes it clear that these genes have been evolving for a much longer time (Hawkey, 1998).

There is a hypothesis that postulated that these resistance genes first evolved in antibiotic producing bacteria such as *Streptomyces* species. These resistance genes are a mechanism of protection from the antibiotics they produce (Salyers and Whitt, 1994a). It was the discovery of antibiotic modification as a form of self-protection in the streptomycetes that led to the proposal that antibiotic-producing microbes were the origins of the antibiotic resistance determinants found in other bacteria (Benveniste and Davies, 1973). In fact, genes for antibiotic production are frequently found in the same gene cluster with genes encoding resistance proteins (Hawkey, 1998). There is also another possibility that these antibiotic resistance genes evolved from housekeeping genes such as those encoding the sugar kinases and acetyltransferases to modify aminoglycoside antibiotics. In addition, a cryptic chromosomal gene or an aminoglycoside acetyltransferase gene appears to be present in most enterobacteria.

Mirza and Hart (1993) suggested that multiple antibiotic resistance does not represent dissemination of a single resistance plasmid. Instead uncontrolled use of antibiotics has probably encouraged the emergence of coliforms, in the normal enteric flora, which carry large self-transmissible multiple antibiotic resistance plasmids (Shears, 1993; Hawkey, 1998).

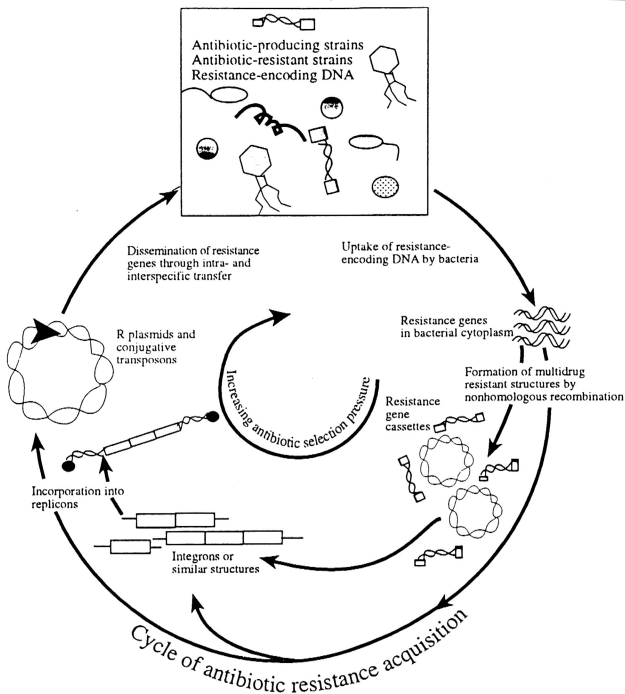


Figure 4: A schematic diagram displaying the possible route of acquisition of antibiotic resistance genes by bacteria under the selective pressure of antibiotics used. Following the single- or double-stranded DNA uptake by bacterial host, the resistance genes are incorporated into stable replicons by several different pathways. One of the pathways involves integron, as shown above, which has been exhibited by a large class of transposable elements in Enterobacteriaceae. The resistance plasmids could dwell as linear or circular form in the bacterial host and the dissemination of it is caused by one or more of the gene transfer mechanisms. (From Davies, 1994.)

These normal enteric bacteria serve as a reservoir for transmission of resistance plasmids to more pathogenic bacteria such as *S. typhimurium* (Hunter *et al.*, 1992) and *S. typhi* (Schwalbe *et al.*, 1990).

1.7 Transfer of antibiotic resistance genes

In many cases, resistance involves acquisition of a new gene, even though bacteria can become resistant to some antibiotics by mutating existing genes. These bacteria can acquire new genes by bacteriophage transduction or by transformation (uptake of DNA from the external environment), these types of transfers tend to occur mainly between members of the same species. Such narrow host range resistance transfer can be important clinically (Davies, 1994; Salyers and Whitt, 1994a).

A major clinical problem is the transfer of resistance genes across genus and species lines. Such broad host range transfer is most likely mediated by conjugation (transfer of DNA through a pore formed in the fused membrane of two bacteria) (Salyers and Whitt, 1994a).

Mycobacteria and streptomycetes (both soil microbes) participate in interspecific gene exchange with other bacterial genera by conjugation (Gormley and Davies, 1991). This may demonstrate the first step in the dissemination of antibiotic resistance genes to other bacterial species in nature.

Gene flux between bacterial replicons and their hosts is likely to be the rule than the exception, and appears to respond quickly to environmental changes (Levy and Novick, 1986). This gene pool is readily attainable to bacteria when they are exposed to the strong selective pressure of antibiotic usage, e.g., in hospitals, for veterinary and agricultural purposes, and as growth promoters in animal husbandry (Davies, 1994) (Figure 4).

A better knowledge of the components of this gene pool, particularly with respect to what might happen on the introduction of a new chemical entity as an antibiotic, might permit us to elude potential resistance mechanisms, and lead to more prudent use of antibiotics under circumstances when the presence of specific resistance determinants can be predicted (Hawkey, 1998).

1.8 R plasmids

R plasmids are autonomously replicating elements, which have been found in both Gram-negative and Gram-positive bacteria (Davies, 1994). The first report of R

plasmids in the West was made by Datta in 1962 (quoted by Falkow, 1975). Datta and Hughes (1983) showed that conjugative plasmids of the same incompatibility groups (Inc) existed in enterobacteria before and after the medical use of antibiotics. Thus, indicating that the evolution of R plasmids occurred by insertion of new resistance genes into a pre-existing enterobacterial plasmid gene pool (Jones and Stanley, 1992).

Conjugative R plasmids generally have at least two components. One component, the resistance transfer factor (RTF), has a molecular weight of 11×10^6 and carries a set of genes responsible for conjugative DNA transfer: genes regulating DNA replication and copy number, the transfer genes, and sometimes the gene of Tc^R. The other component, the resistance (r) determinant, is variable in size (from a few million to more than 100×10^6 molecular weight) and carries the antibiotic resistance genes (Watanabe, 1963; Mitsuhashi *et al.*, 1977). In many R plasmids, the r-determinant is usually flanked by homologous insertion sequence (IS) elements.

The R plasmids that transfer themselves by conjugation must carry a number of *tra* genes encoding proteins needed for the conjugation process. Some plasmids that cannot transfer themselves can still be transferred by conjugation because they are mobilized by self-transmissible integrated elements called conjugative transposons (Salyers and Shoemaker, 1994). Transfer of conjugative transposons takes place in three steps: i) excision to form a circular intermediate, ii) transfer of one strand of the circular intermediate to a recipient, and iii) integration into the recipient genome. Some conjugative transposons integrate almost randomly, whereas others integrate site-specifically.

S. typhi strains that have acquired plasmid-encoded multiple antibiotic resistance were described in Mexico in 1973 (Olarte and Galindo, 1973). *S. typhi* strains resistant to the first line therapeutic drugs of Cm, Ac, or Ctm were also reported in Thailand and other parts of Southeast Asia in 1984 (Ling and Chau, 1984; Murray *et al.*, 1985). Since 1985, multiple antibiotic resistant *S. typhi* strains have been found to increase in rural Southern India (Anand *et al.*, 1990; Jesudason and John, 1992). The multiple antibiotic resistance in *S. typhi* strains responsible for the typhoid fever outbreaks in Mexico, India, Vietnam, and Thailand were R plasmid borne (Smith *et al.*, 1978). All these strains harboured IncHI R plasmids (Anderson, 1975), which are characterized by a thermosensitive mode of conjugal transfer (Taylor and Levine, 1980). In Malaysia, transferable antibiotic resistance strains of *S. typhi* were also found to harbour IncHI R plasmids (Wong, 1991; Chong, 1992;

Kadambeswaran, 1993; Narayanan, 1995; Wong *et al.*, 1996; Wong, 1999).

IncHI plasmids are distinguished by their thermosensitive mode of conjugation (transfer efficiency is optimal at 22-30°C) and their capacity to encode multiple antibiotic resistance (Taylor and Levine, 1980). These traits also implicated IncHI plasmids as potential vectors in the dissemination of antibiotic resistance among pathogenic and indigenous bacterial species in water and soil environments (Maher and Taylor, 1993). These strains have remained endemic and have been responsible for small outbreaks in many developing countries.

Self-transmissible or mobilizable plasmids, such as R plasmids, can acquire and transmit multiple antibiotic resistance genes. There are two ways that these plasmids can acquire multiple antibiotic resistance genes. One way is by acquiring sequential transposon insertions. However, most multiple antibiotic resistance plasmids apparently did not arise this way. A newly discovered type of integrating element, called an integron, is probably responsible for the evolution of many of these plasmids that carry multiple antibiotic resistance genes (Stokes and Hall, 1989).

1.9 Transposable genetic elements (TGE)

TGE are mobile genetic elements found in all genera of bacteria. Their presence in bacteria was first detected because of the rapid spread of antibiotic resistance among bacterial populations in nosocomial infections (Rubens *et al.*, 1981; Bennett and Hawkey, 1991).

TGE can transfer not only among species within the Gram-positive or Gram-negative group but also between Gram-positive and Gram-negative bacteria (Cohen, 1976; Salyers and Whitt, 1994b). Transfer of TGE takes place in three steps: i) excision to form a circular intermediate, ii) transfer of one strand of the circular intermediate to a recipient, and iii) integration into the recipient genome. Some TGE integrate, almost randomly, whereas others integrate site-specifically. TGE do not only transfer themselves but also mobilize co-resident plasmids, either providing transfer functions in *trans* or by inserting themselves into the plasmid (Cohen, 1976; Cohen and Kopecko *et al.*, 1976; Salyers and Whitt, 1994b).

TGE also can promote rearrangements of the genome, directly or indirectly (Schmitt, 1986), which could result in deletions, insertions, inversions, or translocations:

a) the transposition event itself may cause deletions or inversions or may lead to the movement of a host sequence to a new location; and

b) transposons could serve as substrates for cellular recombination systems functioning as "portable regions of homology"; two copies of a transposon at different locations (even on different chromosomes) may provide sites for reciprocal recombination.

In Gram-negative bacteria, TGE carrying antibiotic resistance genes have been divided into three groups based on their mechanistic differences, correlated relationships in genetic organization, and DNA sequence homology (Campbell *et al.*, 1979; Carlos and Miller, 1980; Kleckner, 1981; Schmitt, 1986).

Class I transposons mostly carry one or more antibiotic resistance gene(s) usually bounded by two direct or inverted copies of an IS element in the form of a compound transposon. IS are the simplest TGE and are normal constituents of bacterial chromosomes and plasmids. A standard strain of *E. coli* is likely to contain several (<10) copies of any one of the more common IS elements. The mechanism whereby compound transposons originated is easy to imagine. Two independent insertions of a given IS at both sides of an antibiotic resistance gene directly result in a mobile DNA segment containing that gene (Bukhari *et al.*, 1977). Examples of TGE in this class are Tn*5* (Berg, 1983) and Tn*10* (Lewin, 1995).

Class II transposons, on the other hand, contain short (~38 bp) inverted terminal repeats at its ends and encode the transposition functions in the central region (Martinez and de la Cruz, 1990). Class II transposons represent a group of related elements that are widely distributed in Gram-negative and Gram-positive bacteria (Heffron, 1983). This group can be divided into two families, exemplified by Tn*3* and Tn*21* (Schmidt and Klopfer-Kaul, 1984). These families show extensive structural and functional relatedness in terms of their integration sites and gene products (Schmidt *et al.*, 1984; Grindley and Reed, 1985). They also suggest that they may have evolved from an ancestral Tn by insertion of new antibiotic resistance genes at specific sites (Schmidt, 1984; Lafond *et al.*, 1989; Mercier *et al.*, 1990) (Figure 5).

There are two genes involved in the transposition process in class II elements; these are *tnpA* (encodes the transposase) and *tnpR* (encodes resolvase, a protein that recognises the *res* site and resolves cointegrate intermediates of the transposition process) (Schmitt, 1986).

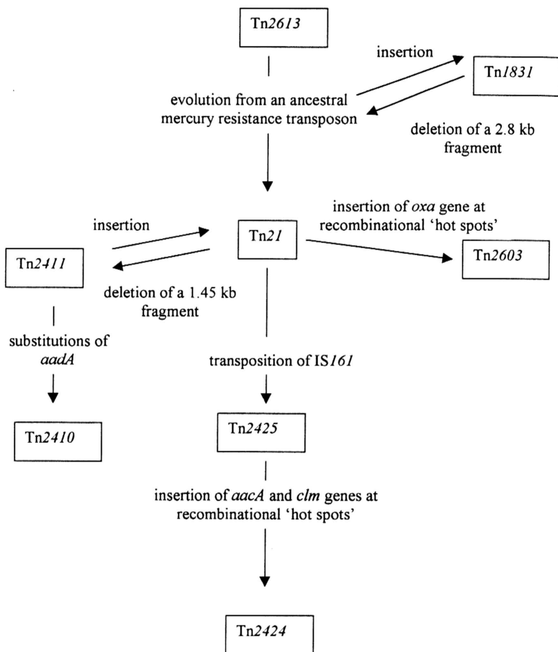


Figure 5: Evolutionary relationships of Tn*21* and Tn*21*-related transposons. (From Meyer *et al.*, 1983.)

Class III transposons are called episomes. This group is a complex, self-replicating element often containing IS and Tn elements (Cohen, 1976).

There are two modes of transposition, namely conservative (nonreplicative) and replicative transposition (Figure 6). Class I transposons transpose conservatively, whereas the class II transposons exhibit replicative transposition (Shapiro, 1979). During replicative transposition, the element is duplicated. Thus, the transposing entity is a copy of the original element (Lewin, 1995). Generally, this type of transposition also comprises cointegrate intermediates, i.e., fusions between the two copies of the (duplicated) element with the donor and target replicons.

Class II elements have an element encoded specific recombination system, which resolves the component replicons, each with a copy of the transposon, from these cointegrates. Nonetheless, "direct transposition" occurs in rare cases to circumvent the cointegrate formation. The introduction of staggered nicks and ensuing DNA synthesis generates the short duplication of target DNA flanking an inserted element (DR). This is a reflection of the transposition mechanism (Schmitt, 1986). In conservative (nonreplicative) transposition, the transposing element moves directly from one site to another as a physical entity, thus, the element is conserved (Lewin, 1995).

IS has the ability to move into genes or DNA sequences as unique units, indicating that the ends of these elements act as hot spots for recombination. Besides acting as hot spots sites for recombination, the ends of these elements also act as hot spots sites for deletions of large genetic elements. This was discovered through heteroduplex studies by electron microscopy on R plasmid DNA molecules (Nisen *et al.*, 1977; Ohtsubo and Ohtsubo, 1978; Ohtsubo *et al.*, 1981).

Most multiresistance TGE found today in natural isolates of Gram-negative bacteria are close relatives of Tn*21*. Recently acquired resistances to newly introduced antibiotics, more often than not, appear on plasmids or TGE carrying the Tn21 integration system.

1.10 Transposon Tn*21*

Tn*21* is the archetype of sub-group of the Class II and belongs to the Tn*21* subgroup of the Tn*3* family of TGE (Grinsted *et al.*, 1990). These elements have evolved by recruiting new resistance genes.

Conservative **Replicative**

tnpA

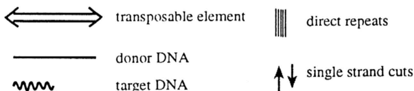
cointegrate

Direct *tnpA*

tnpR

res

Figure 6: Two modes of transposition operative in bacteria. In conservative (excisive) transposition the donor replicon is lost, whereas in replicative transposition both the donor and target replicons end up with a copy of the element. Note that the double-stranded donor and target DNAs are represented by single lines. Genes: *tnpA*, transposase; *tnpR*, resolvase, and *res*, resolution site. (From Schmitt, 1986.)



Different members of the Tn*21* subgroup harbour genes encoding resistance to mercuric chloride (*mer*), genes involved in the transposition processes, which consist of *tnpA* (transposase), *tnpR* (resolvase), and a modulator of the resolvase, *res*, (de la Cruz and Grinsted, 1982; Hyde and Tu, 1985; Ward and Grinsted, 1987; Grinsted *et al.*, 1990). They also harbour one or more additional antibiotic resistance genes (Kratz *et al.*, 1983; Meyer *et al.*, 1983; Tanaka *et al.*, 1983; Schmidt and Klopfer-Kaul, 1984).

The resistance gene "cassettes" containing one or more antibiotic resistance genes are inserted into one of two locations on Tn*2*1 or related elements. The mechanism of this insertion usually involves site-specific recombination by an integrase (Grinsted *et al.*, 1990).

In some cases, a single antibiotic resistance gene (e.g., the oxacillin resistance gene, *oxa2*, of Tn*2410*) replaces the *aadA* gene, whereas in other cases, two or three resistance genes are found at this site (Kratz *et al.*, 1983; Ouellette *et al.*, 1987). In the latter case, the *aadA* gene is frequently present and the additional genes are located on either side of *aadA* gene. In all cases, the resistance genes are inserted at unique sites, in the same orientation, and are transcribed from left to right (Figure 7) from a promoter in the 5' conserved region (left-hand end in Figure 7).

In Tn*21*-like transposon, other resistance genes were also found in the vicinity of the *aadA* gene (Schmidt, 1984; Schmidt and Klopfer-Kaul, 1984; Wiedemann *et al.*, 1986) (Figure 7) which led to the hypothesis of recombinational hot spots.

The hot spots, abbreviated hs1 or hs1* (3' to gene integration) and hs2 (5' to gene integration), provide signals for site-specific recombination and constitute a naturally occurring interspecific expression system (Schmidt *et al.*, 1989; Stokes and Hall, 1989; Wohlleben *et al.*, 1989). All of the genes found as insertions or substitutions next to *aadA* were flanked by some derivatives of these hot spots (Schmidt *et al.*, 1989). The hot spots act as recognition sites for a *trans*-acting-site-specific recombinase, which is not encoded by Tn*21* (Wiedemann *et al.*, 1986; Martinez and de la Cruz, 1990).

Analysis by heteroduplex and restriction enzyme, by comparing various structures of the Tn*21* backbone, has suggested that different recombinational events have occurred at the hot spots (Schmidt *et al.*, 1989). Thus, the major differences between the various members of the Tn*21* group of elements are simply a question of which accessory genes they have acquired (Grinsted *et al.*, 1990).

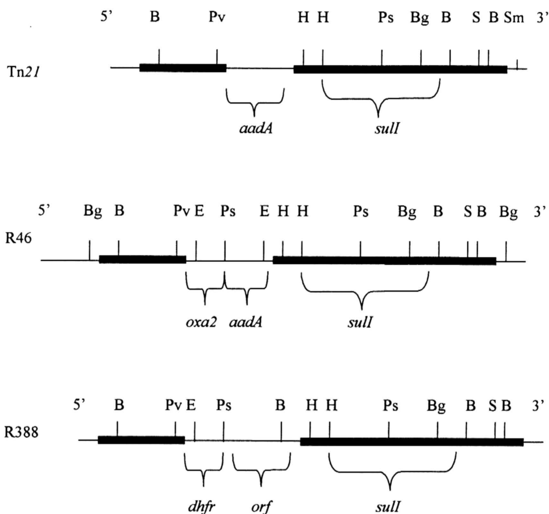


Figure 7: Physical maps showing the regions common to Tn*21*, R46, and R388. The thick black lines indicate the two conserved regions. Restriction sites are from published maps of Tn*21* (de la Cruz and Grinsted, 1982), R46 (Brown and Willetts, 1981), and R388 (Avila and de la Cruz, 1988) and sequence data (Zolg and Hänggi, 1981; Hall and Vockler, 1987; Sundström *et al.*, 1988; Stokes and Hall, 1989). Restriction sites shown are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II; Ps, *Pst*I; S, *Sal*I; Sm, *Sma*I. (From Hall *et al.*, 1991.)

Nücken (1991) also suggested that a site-specific recombination mechanism has been identified and characterized, directing integration of genes by way of discrete hot spots provided by Tn*21*-related elements. Tn*21*-like transposon could be significantly involved in the acquisition of new genes via hs1/hs2-mediated integrative recombination and horizontal gene flow among bacterial communities. The hot spot mediated site-specific integration mechanism is clearly of great importance in the evolution of Tn*21*-related elements or the dissemination of antibiotic resistance genes (Schmidt *et al.*, 1989). This recombination mechanism also represents an essential step in horizontal gene transfer in general.

Another mode of acquiring genetic determinants is through transposition, which genetically involves discrete blocks of DNA, some of which may be bracketed by copies of an insertion element. Some of these elements also display high deletogenic activity and may be responsible for the loss of plasmid determinants adjacent to the point of insertion. It can be suggested that the transposition of IS elements into the left part of Tn*21*-like elements is a likely cause of sequence variations in this group of related transposons (Timmis *et al.*, 1986).

1.11 IS elements

Insertion sequence (IS) elements are autonomous units, ranging in length from 800 to 1,800 bp. IS produce polar mutations containing Rho-dependent transcription stop signals (except for IS*1*) and also chain-termination mutations in all possible reading frames, which account for the polar effects. An IS element displays a characteristic structure in which its ends are identified by terminal inverted repeats (IR) consisting of 10-41 bp, the number depending on the element, while the adjacent ends of the flanking host DNA are identified by short direct repeats. The sequence of the direct repeat may vary among individual transposition events, but the length is constant for any particular IS element (a reflection of the mechanism of transposition). The most common lengths for the direct repeats are 5 and 9 bp (Mahillon and Chandler, 1998).

IS elements encode only determinants relevant to their own transposition and they are often found as terminal repeats of Tn (Figure 8). The termini of each IS element have been known to become integrated at multiple sites in bacterial and phage genomes, and found as terminal repeats of transposons (Carlos and Miller, 1980; Kleckner, 1981; Iida *et al.*, 1983).

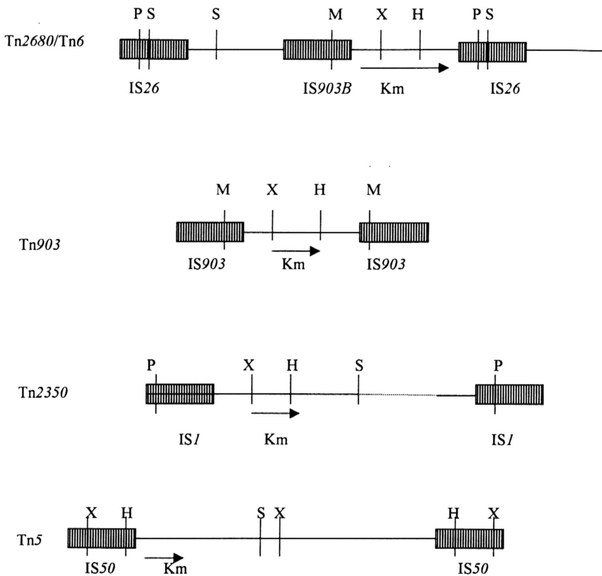


Figure 8: Structure of relevant Km^R transposons. Maps are redrawn from Iida *et al.* (1982), Labigne-Roussel *et al.* (1983), and Ohtsubo *et al.* (1980). The arrows with Km indicate the location and the orientation of the aminoglycoside phosphotransferase gene, *aph*. Restriction cleavage sites: S, *Sal*I; P, *Pst*I; H, *Hin*dIII; X, *Xho*I; M, *Mlu*I. (From Mollet *et al.*, 1985.)

Commonly, the IR are between 15 and 25 bp long, and the two copies are closely related rather than identical. IR sequences at the ends of the translocatable DNA elements may have an important role for translocation (Mahillon and Chandler, 1998). The presence of particular sequences at both ends of an element in an inverted order suggests that there is a protein or an enzyme which recognises the nucleotide sequences and somehow assembles the two ends of the element at the target site of translocation (Schmitt, 1986).

IS elements are compactly organized, containing one or more structural genes, regulatory information and transposition determinants, sometimes overlapping, all within a very limited coding capacity. These IS elements are frequently found to be associated with unstable genetic traits, and it has been suggested that they are sustained through enhancing mobility or through inducing beneficial regulatory mutations by direct selection acting on genetic versatility (Mahillon and Chandler, 1998).

Most IS elements insert at a variety of sites within the host DNA. Nevertheless, some also show with a varying degree of preference for particular hot spots. Insertion of an IS element abolishes the function of the gene into which the element is inserted. Additionally, it can also affect the functions of genes distal to the insertion site with respect to the promoter of the operon. Moreover, an IS element carrying a promoter region is capable of switching the activity of adjacent genes on or off according to the direction of the insertion of the IS segment (Cohen and Kopecko, 1976).

IS*26* is a new IS element belonging to the IS*1* family, which upon integration, generates an 8 bp duplication of its target sequence. IS*26* is 820 bp long and carries 14 bp perfect terminal IR (Figure 9) (Mollet *et al.*, 1983). This sequence shows partial homology with the IR of other IS elements such as IS*1* and IS*102*/IS*903* (Mollet *et al.*, 1985).

A large ORF (frame I) within IS*26* encodes a peptide of 234 amino acids long. IS*26* also contains an ORF II (frame II) in the opposite direction starting inside frame I and using the same frame. This is not a small ORF, since it has no stop codon within IS*26*, which ends at a distance of 591 nucleotides from the start of frame II (Mollet *et al.*, 1983).

GGCACTGTTGCAAATAGTCGGTGGT...IS*26*...CATCGCTAAC**TTTGCAACAGTGCC**

GGTGATGCTGCCAACTTACTGATTT...IS*1*...CT**ATCAATAAGTTGGAGTCATTACC**

GGCTTTGTTGAATAAATCGAACTTT...IS*102*/...GAAATCT**GATTTATTCAACAAAGCC**

IS*903*

Figure 9: Sequence alignment of the termini of IS*26*R, IS*1*, and IS*102*/IS*903*. IS*102* and IS*903* are related and differ only by 60 nucleotides. All sequences are arbitrarily drawn with the largest open reading frame oriented from left to right. The terminal inverted repeats are shown in bold letters. The nucleotides homologous to those of IS*26* are underlined. (From Mollet *et al.*, 1985.)

1.12 Integrons

The first integron was detected in transposon Tn*21*, where it was found between the *mer* operon and the transposition genes (Brown *et al.*, 1986). Mercury compounds are used extensively in medicine as disinfectants and are also released in large quantities into the environment by industry. Almost certainly as a result of this, mercury resistance determinants are also present on many plasmids, particularly antibiotic resistance plasmids.

Integrons are genetic elements, which are widespread in Gram-negative bacteria and could be considered as natural vectors for the reshuffling and expression of antibiotic resistance genes (Stokes and Hall, 1989). Integron is also found either as part of transposons of the Tn*21* family, or independently on several groups of broad-host-range plasmids (Stokes and Hall, 1989).

Integrons contain determinants of a site-specific recombination system, integrase, which enables the integrons to capture genes (Stokes and Hall, 1989; Collis and Hall, 1992a, b; Hall and Collis, 1994). The most common integron comprises three separate parts, two conserved segments between which discrete units are integrated as cassettes (Stokes and Hall, 1989) (Figure 10). The 5' conserved segment contains a gene (*intI*) which encodes a putative site-specific recombinase, IntI (Martinez and de la Cruz, 1990; Mercier *et al.*, 1990) and includes the recombination site, *attI*. This segment also includes a common promoter region for expression of the integrated cassettes, P_{1-2} (Stokes and Hall, 1989).

The 3' conserved segment contains the type I sulfonamide resistance gene, *sulI* (Sundström *et al.*, 1988; Stokes and Hall, 1989), and two ORFs, ORF4 and ORF5 (Stokes and Hall, 1989). The *sulI* gene always seemed to be localized within an integron. The dissemination of *sulI* on R plasmids obviously relies on the stable location of *sulI* in an integron (Rådström *et al.*, 1991). The integrated cassettes found between these regions most often encode antibiotic resistance determinants, such as genes for aminoglycoside-modifying enzymes, β-lactamases, trimethoprim-resistant dihydrofolate reductases, and chloramphenicol resistance. The selective advantage obtained by the acquisition of other inserted resistance genes must have contributed to the spread of *sulI*. Most known trimethoprim resistance genes are found on *sulI* carrying integrons. This is due to the frequent use of the combination of trimethoprim

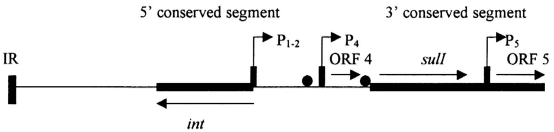


Figure 10: General structure of *sulI*-associated integrons. The positions and orientation of promoters ($P_{1\text{-}2}$, $P_{4\text{-}5}$) responsible for transcription of the inserted cassettes and ORF (ORFs 4 and 5) are indicated. The bold circle represents the 59 bp element. (Adapted from Hall and Collis, 1994.)

and various sulfonamides (Rådström *et al.*, 1991). The 3' sides of the resistance genes in the variable region are commonly associated with palindromic sequences that have been called the 59 bp elements or recombination hot spots (RHSs) and are sites of specific recombination (Martinez and de la Cruz, 1988, 1990; Hall *et al.*, 1991). In naturally occurring integrons, there appears to be no restriction on the number or order of inserted cassettes (Hall and Vockler, 1987). The cassettes are discrete units, which are independently mobile by means of integrase, and alteration in the arrangement of the cassettes in the insert region can occur by excision of individual cassettes or reassortment, or insertion of new cassettes (Collis and Hall, 1992a; b; Collis *et al.*, 1993). Thus, this contributes to integron diversity. Figure 11 shows the general structure of an integron.

Several factors potentially affect the expression of gene cassettes inserted into integrons: i) the relative strength of the different integron promoters encountered in the 5'-conserved segment, ii) the relative position of gene cassettes inserted in second, third, etc. position in the antibiotic-resistance operon may be less well expressed because of the 59 bp elements (Stokes and Hall, 1989), which are found at the downstream end of each inserted gene cassette and, which may act as inefficient terminators of transcription, iii) the presence of an internal promoter associated with an inserted gene cassette, and iv) copy numbers of the plasmid on which integrons are present. All of these factors may have an effect on the amount of mRNA produced for each antibiotic resistance gene integrated in the integron, and therefore on the susceptibility of the host strains to antibiotics (Lévesque *et al.*, 1994).

Besides insertion of a gene cassette into a primary site, insertion into a secondary site in a plasmid that does not contain an integron is also possible (Recchia and Hall, 1995a, b). Recchia *et al.* (1994) also speculated the occurrence of recombination events involving a circular gene cassette and a secondary site. The secondary site conforms to the consensus GNT (Gt/aT, Ga/tTNa/t) (Francia *et al.*, 1993; Recchia *et al.*, 1994), which has some similarity to the core-site consensus, and could lead to the insertion of cassette-associated antibiotic resistance genes at many different locations. The inserted gene would be expressed if the cassettes were correctly oriented with respect to a promoter in the recipient. Nevertheless, such insertions occur rarely and only if they are extremely stable. This is due to the cassette being flanked by only one specific recombination site making excision unlikely

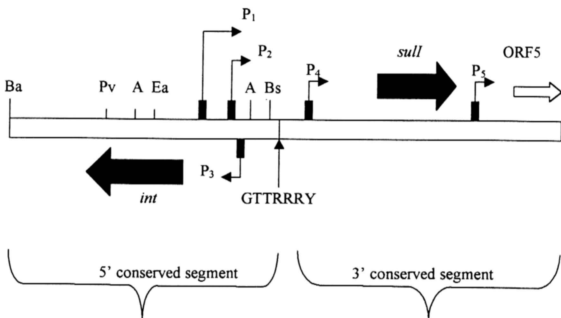


Figure 11: General structure of integrons. The location and orientation of different promoters ($P_{1\text{-}5}$) are shown. The existence of P_4 is controversial (Sundström *et al.*, 1988). The arrow marked *int* (arrow in the direction of transcription) denotes the gene for the integrase (Ouellette and Roy, 1987). The arrow marked *sulI* is the type I sulfonamide resistance gene common to most integrons. The putative product of ORF 5 shows some similarity with the puromycin acetyltansferase of *Streptomyces alboniger* (Bissonnette and Roy, 1992). A, *Ava*I; Ba, *Bam*HI, Bs, *Bst*I; Ea, *Eag*I; Pv, *Pvu*II. This structure was from Lévesque *et al.* (1994).

(Recchia *et al.*, 1994). These events are likely to be important in the spread of cassette-associated genes to many different locations.

The mechanism for the mobility of the integron is still unexplained but at least four different ways to distribute the integron are possible. The first is the mobility of the integron itself, as indicated by its different genetic location (Sundström *et al.*, 1988; Stokes and Hall, 1989). Second, the integron could be distributed by integration into transposons belonging to the Tn*21* family. The third possibility is the horizontal transfer of the integron via R plasmids, such as IncW plasmids, pSa and R388. The last mechanism of spread could be through the compound transposon (Tn*610*), found in *Mycobacterium fortuitum* and shown to carry part of an integron, including *sulI* and two flanking IS*6*-like insertion sequences (IS*6100*) (Martin *et al.*, 1990).

1.13 M13mp18 and M13mp19 bacteriophages

The dideoxy sequencing method has been greatly facilitated by the development of the filamentous *E. coli* phage M13 as a cloning vector (Sanger *et al.*, 1980; Messing, 1983). Each M13 phage contains a single-stranded circular DNA molecule that replicates as a double-stranded circular replicative form (RF). The RF can be isolated as a plasmid, which can then be transformed into competent *E. coli* cells to yield either phage, which contains single-stranded DNA molecules, or additional double-stranded circular RF molecules. Therefore, a DNA fragment of interest can be cloned into the RF of M13 and then single stranded DNA for sequencing can be readily produced in large quantities.

All M13 vectors of the mp series are derivatives of recombinant M13 bacteriophage (M13mp1) that carries a short segment of *E. coli* DNA in its major intergenic region (Messing *et al.*, 1977). This intergenic region contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lacZ*), allowing the development of a simple color test to distinguish between vectors that carry a segment of foreign DNA and those that do not. The F' plasmid of the host cell carries a defective β-galactosidase gene that codes for an enzymatically inactive polypeptide lacking amino acids 11-41.

Currently, M13mp18 and M13mp19 (Figure 12), which contain cloning regions with 13 different cleavage sites that can accept DNA fragments produced by enzymatic digestion, are the cloning vectors of choice.

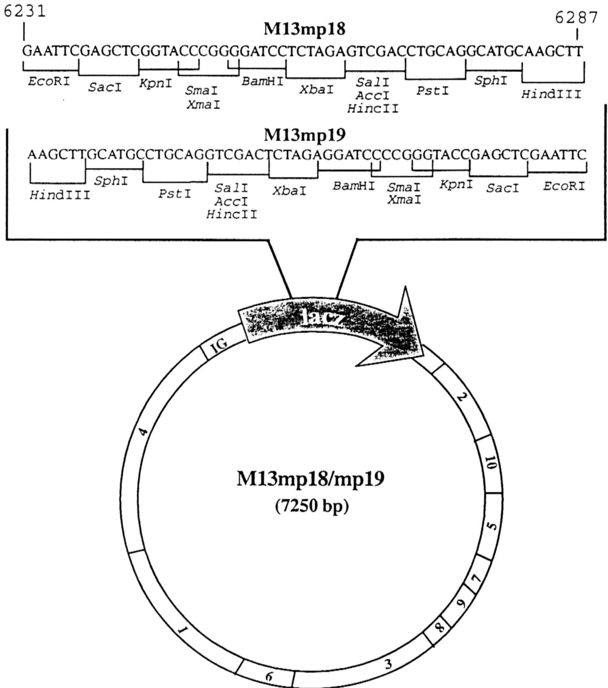


Figure 12: Cloning vectors M13mp18 and M13mp19. (From BioDirectory '98. Amersham Pharmacia Biotech., UK.)

The complete sequences of M13mp18 and M13mp19 have been determined (Yanisch-Perron *et al.*, 1985). These two vectors differ only in the orientation of the non-symmetrical polycloning region within the *lacZ* region (Figure 12).

By the use of these vectors, restriction fragments with different ends (generated by restriction endonucleases whose sites are present in the polylinker) can be readily cloned in either orientation with respect to the *lacZ* gene (i.e., forced cloning) (Figure 13). This procedure permits preparation of opposite strands of the insert from the two recombinant phages, a feature that is particularly important for dideoxy sequencing.

Therefore, the sequence of the nucleotides of the inserted DNA in either vector (M13mp18 or M13mp19) can be determined by using M13 universal primer. In addition, single-stranded probes complementary to each strand of the foreign DNA of interest can also be generated. In this study, M13mp18 is the sequencing vector of choice.

1.14 Previous studies on pST8

Previous studies on the multiple antibiotic resistant *S. typhi* S8 isolate (Kadambeswaran, 1993; Wong *et al.*, 1996; Lye, 1997; Wong, 1999) have shown that there is a large conjugative R plasmid designated pST8, which confers resistance to Ac, Cm, Ctm (Tp-Smz), Sm and Tc.

Wong (1999) has shown through genetic (transposition and transformation) and physical (plasmid and restriction digestion analyses) evidences that a multiple antibiotic resistance transposon (Tn*X8*) encoding $Ac^RCm^RCtm^RSm^RTc^R$ is present on pST8.

Wong (1999) transferred pST8 into *E. coli* UB5201 harbouring pUB307, a conjugative plasmid conferring resistance to Km and Tc, as the recipient replicon. Later, a conjugation between *E. coli* UB5201 harbouring pST8 and pUB307 and *E. coli* UB1637 was done. The resultant transconjugants were selected on plates containing appropriate antibiotics. Two transconjugants, $Ac^RCtm^RKm^RSm^RTc^R$ and $Ac^RCtm^RKm^RSm^R$, were obtained. The presence of $Ac^RCtm^RKm^RSm^R$ indicated that Tn*X8* was trapped in the Tc^R gene of pUB307 and has insertionally inactivated that particular gene.

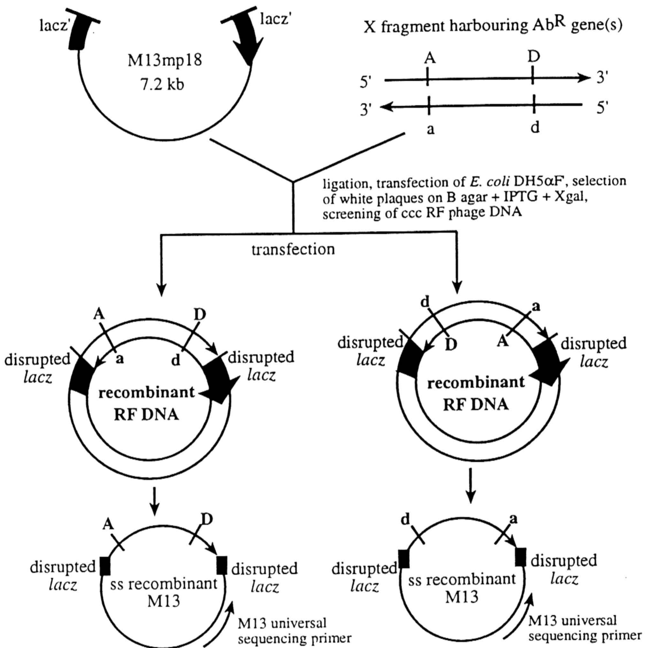


Figure 13: The orientations of inserted DNA fragments harbouring antibiotic resistance gene(s) in M13mp18. DNA insert harbouring antibiotic resistance gene can be inserted in M13mp18 in two opposite orientations. One orientation ensures the antibiotic resistance gene is expressed whilst the other does not.

A recombinant plasmid, designated pCL8, was extracted from $Ac^RCtm^RKm^RSm^R$ transconjugant. After digestion with *Sal*I, pCL8 generated five fragments ranging from 2.5 to >12 kb. The *Sal*I fragments of pCL8 were ligated with *Sal*I-linearized pKan, a cloning vector with Km^R gene (4.2 kb), and transformed into *E. coli* DH5α cells. Wong (1999) designated the recombinant pKan harbouring the 5.5 kb *Sal*I Sm^R fragment as pCLS55 (9.7 kb).

1.15 Objectives of this study

Multiple drug resistance is now common in typhoidal and non-typhoidal *Salmonellae* in both developing and developed countries throughout the world. In order to preserve the efficacy of drugs such as ciprofloxacin for the treatment of such diseases as typhoid fever and salmonella septicaemia, it is essential that the problems resulting from the overuse of antibiotics should be widely publicised. The use of antibiotics in both human and veterinary medicine should be strictly controlled whenever possible.

The wide variety of translocatable elements, their common occurrence on bacterial plasmids and chromosomes, and their ability to mediate recombination and rearrangement of non-homologous DNA suggest that they have a major role in bacterial evolution.

This project was undertaken with the specific aim to subclone and characterize the Sm^R genes derived from the multiple antibiotic resistance transposon present in *S. typhi* S8. The nucleotide sequences and deduced polypeptides of the Sm^R genes of pCLS55 and other transposons carrying these genes were then compared.

To achieve the objective, the following strategies were undertaken:

i) subcloning of the Sm^R from pCLS55 into plasmid vector pUC19 and *E. coli* DH5α by shot-gun subcloning,

ii) molecular characterization of the Sm^R genes (to determine their physical map for easier manipulations),

iii) determination of the nucleotide sequences of the subcloned Sm^R genes, after the fragment containing the genes was located and subcloned in M13mp18, and

iv) analyzing the nucleotide sequences and deducing the amino acid sequences of the subcloned Sm^R genes. This is to compare nucleotide sequences of the Sm^R genes with the sequences of other Sm resistance genes available in the Genbank.