EXPRESSION AND SECRETION OF OXA-2
BETA-LACTAMASE by STREPTOMYCES LIVIDANS

Thesis submitted to the University of Surrey
for the degree of Doctor of Philosophy
in the Department of Microbiology

By

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SUMMARY

The OXA-2 beta-lactamase gene was first found on a conjugative plasmid R46 from a clinical isolate of Salmonella typhimurium. To test the expression and secretion of OXA-2 beta-lactamase in Streptomyces lividans a shuttle plasmid (pSU101) was created by fusing an Escherichia coli plasmid (pSU8) carrying the OXA-2 beta-lactamase gene with the S. lividans vector pIJ61. The OXA-2 beta-lactamase gene specified by the hybrid plasmid pSU101 was expressed in S. lividans, although at a lower level than in E. coli. Almost all the beta-lactamase activity was found in the culture supernatant of S. lividans, whereas in E. coli the enzyme was almost wholly cell associated. The identity of the enzyme was established by substrate specificity and isoelectric focusing.

The stability and integrity of the plasmid pSU101 in both E. coli and S. lividans was determined, in comparison with that of pSU8 and pIJ61 plasmids.

The promoter regions of the OXA-2 beta-lactamase gene were identified by using promoter-probe plasmid vectors; and by S1 mapping of the transcriptional start-sites coupled to the DNA sequencing of the OXA-2 beta-lactamase gene. Multiple transcriptional start sites were found in both hosts, with the origin of transcription apparently different in the two organisms.

Part of this work has been published as a scientific paper which is appended.
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Section 1. Actinomycetes

The actinomycetes existence has been recognized for over a hundred years (Goodfellow et al., 1984) and they represent a very large and widely distributed group of organisms that form rounded cells from rods (Parish, 1979). Most members of this group have a complex colony structure based on multinucleate, branching mycelia, with differentiation of the colony into regions playing vegetative and reproductive roles. This morphological diversity led the microbiologists to consider them as fungi or as a group of microorganisms occupying a position between the true fungi and true bacteria (Chater and Hopwood, 1973).

The morphological and physiological studies such as the determination of the actinomycetes fine structure (Hopwood and Glauert, 1960), cell wall structure (Cummins and Harris, 1958; Pollock et al., 1972), the possession of phages (Welsch, 1969) and the features of their genetic exchange system (Hopwood, 1967; Sermonti, 1969; Hopwood and Wright, 1972), confirmed their prokaryotic nature. They now constitute the order Actinomycetales, and can be described as bacteria which have the ability to form branching hyphae at some stage of their development (Goodfellow et al., 1984).

1.1 The General Features of Actinomycetes

1.1.1 Substrate mycelium

When a spore of an actinomycete is grown on a solid substrate, it usually gives rise to hyphae, which branch at intervals and spread radially. The resulting mycelium, consisting of hyphae that either penetrate the substrate or grow along its surface, has been described as primary, substrate or vegetative (Locci and Sharples, 1984). It has the ability to break down insoluble organic materials by extracellular enzymes (Chater and
The substrate mycelium is non-fragmented in most spore-forming actinomycetes, including the genus *Streptomyces*. Locci (1976) described the growth of substrate mycelium as monocentric because it develops from the growth extension of a single propagule and all portions of the growth remain in filamentous continuity with the original element. Transverse septa may be present, mainly in the older portions of the mycelium (Williams et al., 1973).

### 1.1.2 Aerial Mycelium

When a substrate mycelium is allowed to grow on the surface of solid media for 2-3 days, vertically developing filaments may be formed (aerial mycelium). With further development, a network of aerial hyphae may cover the colony surface, giving it a typical hairy or powdery appearance (Locci and Sharples, 1984). Many factors such as the composition of the growth medium, the incubation temperature and the presence of specific stimulating compounds affect the production of aerial mycelium (Kalakoutskii and Agre, 1976; Williams et al., 1976; Chater and Merrick, 1979; Pogell, 1979). In general, aerial growth appears to be less branched than the substrate mycelium and it is hydrophobic (Higgins and Silvey, 1966) and it is obligately aerobic in the genus *Streptomyces* (Francisco and Silvey, 1971). As soon as the sporulation process proceeds the aerial mycelium development ceases. Lytic processes also take place in ageing colonies and may play a role in spore liberation of some actinomycetes (Locci, 1971, 1976). Due to the parasitic nature of aerial growth on the substrate mycelium, the latter undergoes extensive breakdown (Wildermuth, 1970). It has been suggested that the production of antibiotics by actinomycetes may prevent the invasion of lysing substrate mycelia by motile bacteria, thus promoting aerial growth in the absence of strong competition (Chater and Merrick, 1979).
1.2 Actinomycetes in Nature

Actinomycetes occur in a wide variety of natural and man-made habitats, growing on a vast range of substrates, such as soil and plant material, fresh and salt water, manure and composts (Williams et al., 1984).

Soil has the greatest population density, while deep seas do not offer a very favourable habitat (Okami and Okazaki, 1978; Lechevalier, 1981).

The population of actinomycetes varies in different soils. This is related to many factors such as the amount of organic matter, relative moisture content, temperature, aeration, soil vegetation and soil pH. The viable count of actinomycetes in the fertile soils was over four millions per gram as obtained by Taber (1960). They occur in high numbers in the top few inches of the soil and decrease with depth (Keast et al., 1984).

The actinomycetes are less favoured by a high moisture content than are other bacteria and are able to grow well at relatively low moisture levels, even at 15 to 20% of the moisture holding capacity of the soil (Waksman, 1967; Williams et al., 1972; Williams et al., 1984). They tend to favour alkaline or neutral soils and are sensitive to acid at pH 5.0 or less, therefore their viable counts are generally lowest in acidic and anaerobic soils (Waksman, 1959). Actinomycetes in soil occur in the spore stage as well as in the mycelial stage. Mycelia develop more abundantly at a temperature range of 28-37°C. At lower and higher temperatures, the mycelium undergoes fragmentation, giving rise to abundant formation of spores. Sporulation is also favoured by a dry atmosphere (Waksman, 1967; Orchard, 1981; Williams et al., 1984).

Although most actinomycetes are strict saprophytes, some form parasitic or symbiotic associations with plants and animals. They may cause disease in man and lower animals as a result of exogenous infection due to the consumption of grasses and foodstuffs containing actinomycetes spores and mycelium, or of endogenous infection due to the presence of spores and mycelium as
regular inhabitants of the healthy mouth. In man, *Actinomyces israelii* is found in the mouth, pharynx and intestine, while *A. bovis* and some other actinomycetes are found in the gastrointestinal parts of many animals (Waksman, 1967). *A. bovis* is believed to be an obligate inhabitant of the intestinal system of animals. Actinomycetes have also been found in some insects (Bignell et al., 1981).
Section 2. The Genus Streptomyces

Among all the genera of actinomycetes, more Streptomyces species have been described than any other genus. The Streptomyces species exhibit great variety in their morphology, physiology and biochemical activities. The fact that the majority of antibiotic producing actinomycetes are found among the Streptomyces has made them a very important group of organisms (Waksman, 1967).

Although the name Streptomyces means "Chain fungus" (Chater, 1984), Streptomyces are aerobic, Gram-positive sporoactinomycetes which form well-developed and extensively branching substrate and aerial mycelium (Goodfellow and Cross, 1984). The diameter of the hyphae is usually between 0.7 and 0.8μ. The hyphae vary greatly in length, some are long with limited branching, others are short but much branched. The substrate mycelium is non-septate, particularly in young cultures. Propagation occurs by means of spores or fragments of mycelium. Spores or conidia are produced in special spore-bearing hyphae (sporophores) which arise from the aerial mycelium. Spores are formed by compartmentalisation of the cell protoplasm within the cell wall. Sporulation begins at the top of the aerial spore-bearing hyphae and proceeds towards the base. The spores are usually spherical and between 0.5 and 2.0μ in diameter (Kurylowicz et al., 1975). The surfaces of the spores may be smooth, warty or hairy (Waksman, 1967, 1961).

On artificial solid media, the substrate mycelium of most Streptomyces colonies is smooth or lichenoid, hard and densely textured, may be raised and adheres tightly to the medium. The colonies are usually covered wholly or partially by aerial mycelium, which may be pigmented. The nature and intensity of the pigment depend on the species and on the medium composition (Waksman, 1967).

The streptomycetes have the ability to break down and utilize insoluble organic material such as plant and fungal remains. The utilization of such substrates requires the production
of extracellular hydrolytic enzymes. In order both to produce sufficient quantities of such enzymes and to benefit from their action it would seem advantageous for the growing colony to be coherent and firmly attached to its substrate (Chater and Merrick, 1979).

2.1 Variations and Mutation in Streptomyces species

Streptomyces are highly variable. This variability is related to many factors, including the colonial morphology, nutritional conditions, genetic constitution and environmental factors. Different species, or even strains, differ in this respect, some resisting variation and others undergoing ready changes. The most variable properties of streptomycetes include degree of sporulation and colour of spores, surface and margin of colony, colony sectoring, amount and colour of exudate on colony surface, and also amount and colour of soluble pigment released in substrate. Others are susceptibility of the culture to phage and lysis, and production of antibiotics.

Some strains may retain the capacity to give high antibiotic yields while others may lose it as a result of variation (Waksman, 1967).

2.2 Streptomyces Genetics

The Streptomyces genome size has been estimated as $10.5 \times 10^3$ kb (Benigni et al., 1975) which is about three times that of Escherichia coli or Bacillus subtilis. This may be reflected by the long optical density doubling time, which has been recorded as 84 minutes (Hodgson, 1982).

The morphological complexity of the streptomycetes, as well as their medical and industrial importance as the producers of the majority of the known antibiotics (Berdy, 1980, 1974) makes the study of the genetics of this genus of considerable interest. It has been suggested that there are many reasons which make Streptomyces genetics interesting, for example:

1). As a central part of a complete description of the group.
2). As a tool to analyse, and thus to describe more completely, their other aspects, such as their structure, physiology, and ecology.

3). As the means by which to optimize their exploitation for human welfare (Chater and Hopwood, 1984).

4). The unusually high content (about 73%) of guanine and cytosine (G+C) (Enquist and Bradly, 1971) of Streptomyces DNA could have interesting consequences (Thompson and Gray, 1983), for example, most regulatory sequences examined in bacteria are typically A+T (Adenine and Thymine) rich (Rosenberg and Court, 1979; Adhya and Gottesman, 1978).

Most of the genetical studies since 1955 until the early 1970s were dealing in the description of the architecture and behaviour of the chromosome (Sermonti and Spada-Sermonti, 1955; Hopwood, 1957; Hopwood, 1967; Sermonti, 1969; Friend and Hopwood, 1971; Baumann et al., 1974). Then the interest shifted towards sexual biology and the roles of plasmids (Hopwood et al., 1973; Hopwood and Wright, 1976; Bibb et al., 1977; Friend et al., 1978) and the use of the genetic system to analyse primary metabolism such as carbon catabolite repression (Hodgson, 1980, 1982), antibiotic biosynthesis (Wright and Hopwood, 1976a,b; Kirby and Hopwood, 1977; Rudd and Hopwood, 1979, 1980; Rhodes et al., 1981), differentiation (Chater and Merrick, 1979) and Streptomyces phages (Lomovskaya et al., 1980).

2.3 Gene Cloning in Streptomyces

Gene cloning is very important in industrial genetic engineering because it offers the most direct route to assorting parts of antibiotics into new combinations, thus potentiating the development of new products; it should also permit the introduction of genes allowing growth on cheap growth media, and the application of increased gene dosage and/or gene expression to yield increases for existing products (Chater and Hopwood, 1984; Hopwood and Chater, 1980).
Techniques for the cloning of *Streptomyces* genes have been made possible by the availability of several *Streptomyces* plasmids (Bibb et al., 1980; Thompson et al., 1980; Hopwood et al., 1981), and temperate bacteriophages (Suarez and Chater, 1980; Chater et al., 1981) as DNA cloning vectors, and by the discovery that plasmid or phage DNA is taken up quite efficiently by *Streptomyces* protoplasts treated with polyethylene glycol (PEG) (Bibb et al., 1978; Krugel et al., 1980).

2.3.1 Host Strains

The hosts for all the cloning experiments described so far have been *Streptomyces lividans* 66 (Lomovskaya et al., 1972) and the genetically well-characterized *S. coelicolor* A3(2) (Hopwood et al., 1973; Chater and Hopwood, 1984). But *S. lividans* 66 has several advantages for general cloning purposes over *S. coelicolor* A3(2), in that *S. lividans* 66 is experimentally a particularly easy strain to handle as it grows and sporulates vigorously and reproducibly, protoplasts are obtained in high yield and DNA isolation is without complication. Most plasmid or phage DNA grown in *Escherichia coli* can be introduced into *S. lividans* 66, but not directly into *S. coelicolor* A3(2), presumably because of restriction (Hopwood et al., 1983). However, *S. coelicolor* A3(2) only slightly restricts DNA from *S. lividans* 66 while *S. lividans* is not known to restrict DNA from any other streptomycetes, for example the DNA cloning vectors derived from the plasmid SLP1 (Thompson et al., 1982a) cannot be used in *S. coelicolor* A3(2). An additional advantage of *S. lividans* 66 is its performance as a more reliable indicator for the plasmid related lethal zygosis (Ltz+) phenotype than *S. coelicolor* A3(2) (Hopwood et al., 1983; Hopwood and Chater, 1982).

Lethal zygosis (Ltz+) is a narrow zone of delayed sporulation around the donor strain (plasmid-bearing culture) when it is inoculated as a patch or as an isolated spore on a plate spread with recipient spores (plasmid-free strain). The colony-centred zone of inhibition is termed a "pock" (Bibb et al., 1977, 1978).
2.3.2 Plasmid Vectors

Plasmids are extrachromosomal genetic elements found in a variety of bacterial species. Most plasmids are found as covalently closed circular DNAs. Two linear DNA plasmids were first isolated from Streptomyces rochei producing the lankacidin group of antibiotics (Hayakawa et al., 1979), and designated pSLA1 and pSLA2 (Hirochika and Sakaguchi, 1982). Linear plasmids have also been found in a yeast, Kluyveromyces lactis (Gunge et al., 1981), and in Borrelia hermsii (Plasterk et al., 1985).

Plasmids of Streptomyces are known to exist in a wide range of sizes (<4kb to about 170kb) and copy numbers per chromosome (from one copy to several hundreds). These extrachromosomal genetic elements have been shown to play a role in determining many of the properties expressed by these organisms, including antibiotic production and resistance (Hopwood, 1978; Schrempf and Goebel, 1979), fertility (Bibb et al., 1977), and differentiation and melanin production (Schrempf and Goebel, 1979).

The ideal plasmid cloning vector should possess several properties. It should be relatively small and should replicate in a relaxed fashion. It should carry one or more selectable markers to allow identification of transformants and to maintain the plasmid in the bacterial population. Finally, it should contain a single recognition site for one or more restriction enzymes in regions of the plasmid that are not essential for replication. Preferably, these restriction sites, into which foreign DNA can be inserted, should be located within the genes coding for selectable markers so that insertion of a foreign DNA fragment inactivates the gene.

The small plasmid vectors are very useful, because the plasmid is easy to handle in that it is more resistant to damage by shearing, and is readily isolated from the host cells. The small plasmids are usually present as multiple copies, and this not only facilitates their isolation but leads to gene dosage effects for all cloned genes. Finally, the small size plasmid vectors may have
single substrate sites for most restriction endonucleases (Maniatis et al., 1982; Old and Primrose, 1982).

SCP2 and SCP2*

SCP2 is a covalently closed circular (CCC) DNA of a uniform monomer size (c.18 x 10^6 daltons), it was the first *Streptomyces* plasmid to be studied physically (Schrempf et al., 1975), and it has been identified as a sex factor within *Streptomyces coelicolor* A3(2) (Bibb et al., 1977). The ability to promote chromosomal recombination, and to show the "Ltz" reaction, is greatly enhanced in a variant form of the plasmid called SCP2*, which has not been physically distinguished from SCP2 by restriction analysis (Bibb and Hopwood, 1981).

SCP2* is 31kb in length, is present within mycelium at one copy per genome, and has been well characterized by restriction endonuclease cleavage analysis (Fig.1). Several restriction sites occur within SCP2* which are amenable to DNA insertion without damaging functions essential for plasmid replication and maintenance (Bibb et al., 1981).

![Figure 1](image-url)  
*Figure 1  Restriction enzyme cleavage map of plasmid SCP2*, from Bibb et al. (1977).*
SLP1 Family

It consists of a series of plasmids, SLP1.1 to SLP1.9, which exist as autonomously replicating molecules within *S. lividans*. Genetic and molecular characterization of the plasmids reveals that they are derived from the strA region of the chromosome of *S. coelicolor*. It is proposed that, before or during mating with *S. lividans*, the SLP1 sequences are excised from the chromosome, bringing varying regions of the surrounding chromosome with them, and can circularize to yield the SLP1 family of plasmids.

The SLP1 plasmids range in length from 9.4 to 12.2 kilobases and possess a mycelial copy number of approximately 4 to 5 per chromosome. They provide suitable vectors for DNA cloning since the segments of chromosomal DNA carried by the larger members of the family are dispensable, in which a foreign DNA can be inserted without damaging functions essential for plasmid replication and maintenance. The most useful member as a potential cloning vector was SLP1.2, which contains a single BamHI and two PstI restriction sites, all within the nonessential region (Fig.2) (Bibb et al., 1981; Bibb, 1981). Cloning of antibiotic resistance genes, such as neomycin phosphotransferase and thiostrepton, into the single BamHI site resulted in the formation of pIJ61 plasmid (Thompson et al., 1982a).

![Restriction enzyme cleavage map of plasmid SLP1.2 from Bibb (1981).](image)

Figure 2 Restriction enzyme cleavage map of plasmid SLP1.2 from Bibb (1981).
Series of plasmids (pIJ101-pIJ104) were first discovered in *Streptomyces lividans* ISP5434 (Hopwood *et al.*, 1981). Their sizes were (8.9, 4.2, 3.9, and 4.7kb) respectively; with high copy number (40-300) per chromosome. pIJ101 was found to be self-transmissible by conjugation, and has the lethal zygosis (Ltz+) phenotype. It promotes chromosomal recombination at high frequency in both *S. lividans* 66 and *S. coelicolor* A3(2). pIJ101 has been marked by the cloning onto it of a DNA fragment carrying thiostrepton resistance gene, resulting in the formation of pIJ326, pIJ330, pIJ333 and pIJ302 (Kieser *et al.*, 1982); and by cloning a DNA fragment coding for the enzyme tyrosinase, responsible for melanin synthesis, in addition to the thiostrepton resistance gene, into pIJ102, the plasmid pIJ702 was generated (Katz *et al.*, 1983). This has facilitated testing of the host range of the plasmid by transformation of protoplasts of a variety of *Streptomyces* spp., with selection for thiostrepton resistance. It has been found that pIJ101 can replicate in members of several diverse *Streptomyces* species; these are *S. albus*, *S. azureus*, *S. acrimycinj*, *S. griseus*, *S. glaucescens*, and *S. rimosus* (Hopwood *et al.*, 1981).

The multicopy and a wide host range properties, are very valuable in the construction of the new vectors, particularly in the studies concerning the empirical discovery of hybrid antibiotics by shotgun cloning of chromosomal segments between the members of a collection of diverse strains (Hopwood and Chater, 1982; Hopwood *et al.*, 1981).

### 2.3.3 Phage Vectors

Possible uses of cloning systems in applied (Hopwood and Chater, 1980) and basic research in *Streptomyces* are numerous, and some of those uses may be most easily achieved by the availability of phage cloning vectors. *Streptomyces* phages can easily be isolated from most soils of pH higher than 5.0, by plating of soil extracts. This can be done either directly or after a short period
of enrichment by incubation with freshly germinated *Streptomyces* spores (Dowding, 1973) in a conventional soft-agar overlay containing spores of an indicator strain. Plaques are usually seen after overnight incubation at 28 to 30°C.

Nearly all the *Streptomyces* phages examined, consist of polyhedral heads and long non-contractile tails, which is similar to Bradley's group-B (Bradley, 1967), and contain double-stranded DNA (Lomovskaya et al., 1980).

*Streptomyces* phage vectors possess potential advantages (Chater, 1980) such as:

1. Their wide host range and the ease with which it can be determined.

2. The introduction of DNA into prophages where it should be stable and potentially inducible to a multicopy form by using heat-inducible prophage.

3. The possibility of taking advantages of DNA packaging constraints in selection of cloned DNA.

4. The ease with which hybridisation to radioactive probe DNA can be used to detect plaques containing desired DNA sequences (Benton and Davis, 1977).

5. The use of efficient phage promoters to obtain high level of transcription of cloned DNA.

The best phage cloning vectors would be temperate and possess wide host range and small numbers of appropriate restriction enzyme target sites in their DNA. Deletion mutants should be obtainable to allow packaging into virions of phage DNA containing inserted DNA. Three phages appear to meet most of these criteria: ΦC31, R4 (Chater, 1980) and SH10 (Klaus et al., 1981). In present attempts to clone DNA, the favourable vector was ΦC31, mainly because of the availability of relatively large deletions, but also because of its extensive genetic characterization.
The temperate phage \(\phi\)C31 is so far the best studied Streptomyces phage (Suarez and Chater, 1980). It has a wide host range within the streptomycetes and lysogenizes many strains, but did not form plaques on Streptomyces coelicolor A3(2). Phage-sensitive variants were obtained after ultra-violet light (UV) treatment of \(S\). coelicolor A3(2) and its multiauxotrophic derivatives (Lomovskaya et al., 1971; Lomovskaya et al., 1970). These variants and \(S\). lividans 66 were then used as indicator strains. In most work strain 66 was used because the large plaques obtained with it allowed the detection of plaque morphology mutants.

Electron microscopy showed that \(\phi\)C31 phage particles have a head with a hexagonal outline (57 by 54nm) and a tail (non-contractile), (123 by 10nm) with a basal plate (Smirnova and Novikova, 1976). The length of \(\phi\)C31 DNA as it is estimated by restriction enzyme analysis was 41.31kb (63% G+C), but only 32kb (or less) of the DNA contains genetic information that is essential for plaque formation (Chater et al., 1981a,b).

Although other phages such as R4 and SH10 have great potential as wide host range Streptomyces DNA cloning vectors, the most highly evolved series of vectors has so far been obtained from \(\phi\)C31. Suarez and Chater (1980) constructed a chimaeric phage by insertion of the small multi-copy \(E\). coli plasmid pBR322 into one of the six \(EcoR1\) sites of a \(\phi\)C31 deletion mutant. The resulting hybrid could easily be transformed into \(E\). coli, where it replicated as a plasmid which could, in turn, be used to transfect \(S\). lividans protoplasts. This chimaeric phage has been used in the isolation of a new series of deletion mutants, one of them (\(\phi\)C31 \(\Delta\)M17::pBR322) has been useful in the development of cloning vectors such as the \textit{in vitro} sub-cloning of the \textit{Streptomyces azureus} thiostrepton resistance gene (\textit{tsr}) (Chater et al., 1982a). Further \textit{in vivo} and \textit{in vitro} manipulations of one of the phages so obtained gave vectors such as \(\phi\)C31KC400 (Harris et al., 1983), in which some non-essential \(\phi\)C31 DNA has been removed and replaced by the viomycin
phosphotransferase (vph) gene (which gives viomycin resistance) from Streptomyces vinaceus NCIB8852 (Thompson et al., 1982b) and the whole of the E. coli plasmid pBR322. The vector can therefore be propagated either as a phage in Streptomyces or as plasmid (pIJ505) in E. coli. When \( \Phi C31KC400 \) is used as a vector the PstI fragment can be replaced by fragments of foreign DNA of up to 6kb while retaining an intact viomycin phosphotransferase gene (Figure 3).

**Figure 3** Simplified restriction map of \( \Phi C31KC400 \) vector. Only sites for BamHI (B), PstI (P) and PvuII (Pv) are shown, (from, Harris et al., 1983).
Section 3. Mechanisms of Resistance to Beta-lactam Antibiotics

Resistance of bacteria in general to beta-lactam antibiotics can be achieved through a variety of mechanisms. These include:

1. Alteration of the outer membrane permeability.
2. Modification of the target enzymes to prevent interaction with the antibiotic.
3. Production of beta-lactamases.

The cell envelope provides a barrier between the bacterial cell and its environment, with the capsule or exopolysaccharide (if present) providing the primary barrier and therefore the initial obstacle in the penetration of beta-lactam antibiotics to their targets, the penicillin-binding proteins (PBPs). This can be done in three different ways (Slack and Nichols, 1982), first: the capsule or exopolysaccharide could form a static layer through which the antibiotic must diffuse to enter the bacterial cell envelope, the deeper the layer the longer the time the antibiotic will take to diffuse. The second mechanism would be the frictional resistance to diffusion afforded by the structural matrix of the polysaccharides. The third hypothetical mechanism would be a reduction in permeation of a charged antibiotic through an oppositely charged matrix (Helfferich, 1962). It has been shown that the capsular materials and exopolysaccharides of clinically important bacteria are commonly anionic or less frequently neutral, therefore a positively charged antibiotic would have to saturate any free binding sites in the matrix before penetrating to the bacterial surface (Stanier et al., 1977). Slack and Nichols (1981) showed (in vitro) that the penetration of a positively charged aminoglycoside has been reduced with hydrated exopolysaccharide from Pseudomonas aeruginosa, this effect was not seen with neutral or negatively charged beta-lactam.

The cell envelope of Gram-negative bacteria consists of an outer and inner membrane, separated by the periplasmic region and rigid peptidoglycan layer, which is the target of the antibiotic penicillin (Figure 4). Gram-positive bacteria have no outer
**Figure 4** Schematic representation of section through the cell envelope of a typical Gram-negative and a typical Gram-positive bacterium, (from DiRienzo et al., 1978).
membrane and they lack lipopolysaccharide, but have an enlarged external peptidoglycan layer that contains the cytosolic osmotic pressure. The outer and the inner membrane have a lipid bilayer structure (a bimolecular sheet of phospholipids) interdispersed with protein molecules. The outer membrane carries, in addition, Lipopolysaccharide; a complex molecule consisting of a lipid moiety covalently linked to a heteropolysaccharide (Glass, 1982; DiRienzo et al., 1978). It is known that the outer membranes from Gram-negative bacteria act as a barrier to hydrophobic antibiotics such as fusidic acid and rifamycin, but vary in their permeability to hydrophilic compounds (Chopra and Howe, 1978). It has been suggested that the penetration of the beta-lactam occurs through aqueous pores, formed by outer membrane proteins, known as porin channels (Nikaido et al., 1983), therefore modifications to the proteins which form these channels can cause a decrease in the penetration of beta-lactam antibiotic. It is thought that hydrophobicity and size of the antibiotic are important in deciding the ease with which the compound can pass through the porin channels. Therefore the hydrophobic compounds and the other compounds which are larger than 600 daltons do not pass through these pores easily.

It has been proved that the porins are not the only route of entry for beta-lactams. This has been shown by Curtis et al. (1985), where E. coli mutants losing the porin-forming proteins showed no change in susceptibility to some cephalosporins, but become resistant to first and second generation cephalosporins. Harder et al. (1981) showed that ampicillin can permeate the outer membrane easily in porin defective mutants. Sawai et al. (1982), suggested that there is a significant passage of ampicillin through the phospholipid.

The Lipo-polysaccharide (LPS) component of the outer membrane has an effective role in causing resistance in Gram-negative bacteria. Godfrey et al. (1984), showed that there was a correlation between LPS structure and resistance of four beta-lactam resistant strains of Pseudomonas aeruginosa. It has
been demonstrated that the LPS in *Pseudomonas aeruginosa* was usually associated with the OmpF porin and the state of the LPS directly influenced the number of open functional porin channels (Legakis and Shearer, 1984).

The alteration in the penicillin-binding proteins (PBPs) as a mechanism of resistance has to date been found primarily in Gram-positive clinical isolates, with the major mechanism being the decreased binding of the PBP to the beta-lactam antibiotic. Much work has been done examining the PBPs of methicillin-resistant *Staphylococcus aureus*, and several workers have attributed this resistance with PBP change (Brown and Reynolds, 1980, 1983; Hayes *et al.*, 1981; Georgopopadakou *et al.*, 1982; Utsui *et al.*, 1983; Hartman and Tomasz, 1984). The PBPs of cloxacillin resistant *Bacillus subtilis* strains have been shown to have an altered PBP2a (Kleppe *et al.*, 1982).

Resistance in *E. coli* due to an alteration in the cell PBPs has been recorded. Mecillinam binds preferentially to PBP2 resulting in the production of large spherical osmotically-stable cells. Spratt (1977, 1978) isolated a temperature-sensitive mecillinam-resistant mutant in which resistance was due to the modification of PBP2. The PBP2 was temperature-sensitive, so at certain temperatures (>42°C) this PBP was altered and could not be detected in PBP assay. The mutants were also very slow growing at 30°C compared to the parent.

There are many other reports which attribute the resistance in bacteria to PBP alterations; (e.g.) Dougherty *et al.*, (1980) showed that some strains of *Neisseria gonorrhoeae* (5 out of 20 isolates) were resistant to penicillin G due to reduction in the affinity of two of the PBPs. The most recent report of PBP alterations causing resistance was in *Haemophilus influenzae*, where different PBP profiles and PBP affinities, for beta-lactamase-negative ampicillin-resistant strains were obtained when compared to sensitive strains (Parr and Bryan, 1984; Mendelman *et al.*, 1984).
The most important mechanism of resistance to beta-lactam antibiotics to date is that afforded by beta-lactamase.

The relationship between resistance and beta-lactamase production in Gram-negative organisms is more complicated than in Gram-positive bacteria. In Gram-positive bacteria, since they have simple cell wall structure with no outer membrane, there seems to be only a minimal, if any, permeability barrier to penicillins, whereas, in the Gram-negative bacteria, presumably because of the lipid-containing, more complex, outer membrane, permeability to the beta-lactams may play a role in sensitivity or resistance. A further difference between Gram-positive and Gram-negative bacteria is that in the Gram-positive bacteria, usually relatively large amounts of extracellular beta-lactamase are found in the culture supernatant. Therefore resistance in Gram-positive bacteria is based on the destruction of the antibiotic outside the cell by the externally secreted beta-lactamases. In the case of Gram-negative bacteria, the enzyme tends to be cell-bound or enclosed (Saz and Lowery, 1979).

Beta-lactamases have a significant role in the rapid development of resistance to many beta-lactams among a wide range of bacterial species. For example, Simpson et al., (1980), showed that of 208 isolates from urinary tract infections, the majority owed their resistance to beta-lactamase. Bergan and Lernestedt (1983), have shown that clinical isolates of staphylococci were more resistant to antibiotics due to a higher incidence of beta-lactamase production. Ross (1984) showed that of the 900 organisms studied in his laboratory, all produced beta-lactamase. It has been shown that the resistance in Pseudomonas aeruginosa was related to the capability of chromosomal beta-lactamase to hydrolyze a beta-lactam substrate, e.g. piperacillin (Bryan et al., 1984; Bell et al., 1985). Seeberg et al. (1983), showed by genetic transfer experiments that chromosomal beta-lactamase of Enterobacter cloacae is responsible for resistance to expanded-spectrum cephalosporins. Nies et al. (1985), suggested that the R factor R1767 provides an excellent example for the
evolution of resistance plasmids, since this plasmid harbours two transposable elements - Tn2410 and Tn2411 - and mediates resistance towards ampicillin by the production of an OXA-2 beta-lactamase and to streptomycin, spectinomycin, tetracycline, chloramphenicol, sulfonamides, mercuric chloride, arsenate and carries genes for the production of colicin I.

Beta-lactamases may have a common evolutionary origin and good candidates for their common ancestors are the cell wall synthetic enzyme. It is thought that beta-lactamases may have evolved from penicillin-sensitive enzymes, that is the PBPs, involved in peptidoglycan biosynthesis (Tipper and Strominger, 1965; Pollock, 1967). If this is so, beta-lactamase and cell wall synthetic enzymes would share some common regions of amino acid sequence. This proposal has been supported by Pratt and Govardhan (1984) in that beta-lactamases and D-alanyl-D-alanine transpeptidases are evolutionarily related, and they found that the extended amine acceptor binding site of transpeptidases may be to some extent present on serine beta-lactamases. The amino acid sequence from the 4 group A beta-lactamase (Ambler, 1980) and the two D-alanine carboxypeptidase (Yocum et al., 1979; Waxman and Strominger, 1980) from Bacillus subtilis and Bacillus stereothermophilus show significant homology in their NH2-terminal and active site regions. The two enzyme types share the same acyl-enzyme catalytic mechanism and can both be classed as serine enzymes. Sequence homology has been demonstrated between E. coli PBP5 (D-alanine carboxypeptidase) and the TEM beta-lactamases in its NH2-terminal region (Waxman et al., 1982). Broom-Smith et al. (1985), found two regions of similarity amongst the sequences of PBP1A, PBP1B, PBP3, PBP5, PBP6, and TEM beta-lactamase. One of these regions included in the active-site serine residue which was characterised by the sequence Gly-Ser-Xaa-Xaa-Lys-Pro, and has been found at the active site of all PBP5 and class A and class C beta-lactamases, except the D-alanine carboxypeptidase from Streptomyces strain R39. The second region occurs at a variable distance at 17-38 residues to the amino-terminal side of the active
serine and this latter region may also constitute part of the binding site for penicillin.

The three dimensional structural data obtained by X-ray crystallographic analysis should resolve the similarities and differences between the PBPs and beta-lactamases. Significant similarity between D-alanyl-D-alanine-peptidase from *Streptomyces* R61 (penicillin target enzyme) and the beta-lactamase from *Bacillus licheniformis* 749/c (penicillin hydrolyzing enzyme), was found by X-ray crystallography in the spatial arrangement of the elements of secondary structure, although the two enzymes have distinct catalytic properties and lack relatedness in their overall amino acid sequences except the near active-site serine (Kelly et al., 1986). Samraoui et al. (1986), found that the tertiary structure of beta-lactamase I from *Bacillus cereus* is similar to that of the penicillin-sensitive D-alanyl-D-alanine carboxypeptidase from *Streptomyces* R61. These studies provide strong support for the hypotheses that beta-lactamases arose from penicillin-sensitive enzymes involved in bacterial wall peptidoglycan metabolism.

The penicillin-sensitive D-alanyl-D-alanine carboxypeptidase-transpeptidase from *Streptomyces* R61 shares some characteristics with the class C beta-lactamases; for example, in their relative rates of acylation and deacylation at the active serine residue (Knott-Hunziker et al., 1982), their relative molecular mass (38,000 and 39,000 dalton), and there is some similarity between the sequences immediately adjacent to the active-site serines of the both types (Kelly et al., 1985). From these results we may deduce that the penicillin-sensitive enzyme from *Streptomyces* R61 and class C beta-lactamase have a common evolutionary origin. Since this enzyme shares an extensive region of common tertiary structure with class A beta-lactamase, then the penicillin-sensitive enzyme, the class A and the class C beta-lactamases must all have a common evolutionary origin, although no sequence similarity has been detected between the class A and class C beta-lactamases.
In summary, resistance in Gram-negative bacteria results from the interplay between the various mechanisms of resistance. This may be reflected by the emergence of resistance to a new antibiotic. An ideal beta-lactam antibiotic would penetrate external polysaccharides and outer membrane efficiently, not be destroyed by beta-lactamases, not induce beta-lactamase production and bind PBPs with high affinity.
Section 4. Beta-Lactamases

4.1 General Properties

Beta-lactamases (Penicillin/Cephalosporin beta-lactam amidohydrolase, EC3.5.2.6) are enzymes which hydrolyze the amide bond in the beta-lactam ring of penicillins, cephalosporins and related beta-lactam compounds, rendering them antibacterially inactive (Figure 5). It has been reported that this enzyme was first discovered by Abraham and Chain (1940) in an extract of Escherichia coli. Later it has been found in many other species of bacteria; Gram-negative species include: Klebsiella aerogenes (Bondi and Dietz, 1944), Proteus vulgaris and Pseudomonas aeruginosa (Perault, 1945), Proteus morganii (Lahelle, 1948), Shigella sp. (Bondi and Dietz, 1946) and Serratia marcescens (Czekalowski, 1950). Whereas Gram-positive species include: Nocardia sp. (Drake, 1946), Streptomyces spp. (Woodruffe and Foster, 1945), Microbacterium spp. (Iland, 1946), Bacillus spp. (Duthie, 1944), Staphylococcus aureus (Kirby, 1944).

Beta-lactamases constitute a heterogenous group of enzymes with molecular weights ranging from 12,000 - 65,000. They are produced in both Gram-positive and Gram-negative bacteria and contribute significantly to resistance in each case. The beta-lactamase gene can be either chromosomally or plasmid-mediated; the TEM group beta-lactamases may be able to utilise both locations (Curtis et al., 1972; Richmond and Sykes, 1972).

The majority of beta-lactamases produced by Gram-positive organisms are extracellular and inducible enzymes which appear in quantity only in the presence of an inducer (beta-lactam compound) (Citri and Pollock, 1966; Hennessey, 1967). Gram-negative organisms on the other hand produce both inducible and constitutive enzymes which are cell bound (Richmond and Sykes, 1973). The extracellular nature of the beta-lactamases produced by Gram-positive organisms
makes detection relatively easy, whereas the detection of enzymes produced by most of the Gram-negative organisms is difficult. This is due to the outer membrane of the bacterial cell wall which acts as a permeability barrier (Costerton and Cheng, 1975). Then the detection can be done by releasing the enzyme out of the cell by disruption techniques such as osmotic shock (Neu, 1968) or sonication.

**Figure 5** Site of action of beta-lactamases on penicillin (a) and cephalosporin (b).
4.2 Classification of Beta-Lactamases

The production of different types of beta-lactamases that can hydrolyze every known beta-lactam prompted the development of several classification systems, designed mainly to accommodate Gram-negative enzymes. The beta-lactamases produced by Gram-positive bacteria represent a relatively homogenous enzyme group, varying little in biochemical and physical parameters (Pollock, 1971). Richmond and Sykes (1973), have devised the most commonly quoted system. This system is composed of five main classes, based on substrate profile and the interaction of the enzymes with various inhibitors.

Class I, included all the enzymes that hydrolyze cephaloridine at a higher rate than that of benzyl-penicillin and ampicillin, and they are generally chromosomally determined, e.g., the enzymes synthesized by *Aerobacter cloacae* strain P99 (Goldner et al., 1968), and by *Enterobacter cloacae* 214 (Hennessey, 1967; Hennessey and Richmond, 1968), and the inducible enzyme from *Pseudomonas aeruginosa* (Sabath et al., 1965).

Class II, included the chromosomally mediated penicillinases, e.g., those produced by *Proteus morgani* and *Proteus mirabilis* (Jack and Richmond, 1970; Sawai et al., 1968).

Class III, only one enzyme type has been included in this class which represents the R factor-mediated beta-lactamases such as TEM beta-lactamase (Datta and Richmond, 1966; Lindqvist and Nordstrom, 1970; Sawai et al., 1970).

Class IV, included three types of chromosomally mediated penicillinase; e.g., that produced by *Aerobacter cloacae* strain 53 (Jack, 1971; Jack and Richmond, 1970).

Class V, heterogenous group that hydrolyzes oxacillin and carbenicillin, e.g. OXA-1 and OXA-2 (Dale and Smith, 1972, 1974; Matthew et al., 1975, 1979).

Data collected by Matthew and Harris (1976), suggested that beta-lactamases were genus, species and subspecies specific and therefore constituted a group of enzymes probably as diverse as the
bacteria themselves. Then Sykes and Matthew (1976), have devised a system based principally on the location of the beta-lactamase gene. Subdivisions within the system were then based on parameters such as substrate profile, molecular weight, isoelectric point and immunological cross reactivity.

Using the isoelectric focusing technique (Matthew et al., 1975) enabled beta-lactamases to be classified not just on substrate profile but also on their pI values.

As we know, for every novel beta-lactam that has been produced, a beta-lactamase has been found to inactivate it. Therefore the above classification systems were insufficient to accommodate this great diversity of proteins, since they were based on data collected from different laboratories, and methods for the determination of each parameter vary from place to place and are not always comparable. Systems based on sequencing data are the most reliable, since amino acid sequences are unique in that they are digital in nature, whereas most other biological informations are analogue. Ambler (1980), has devised a classification system based mainly on homologies of amino acid sequences. This system encompasses both Gram-positive and Gram-negative enzymes and is composed of three classes A, B and C.

Class A enzymes, including those from Staphylococcus aureus PCI, Bacillus licheniformis 749/C, Bacillus cereus 569/H (type 1) and the plasmid mediated TEM beta-lactamase show about 20% homology in their amino acid sequences (Ambler, 1979)

Class B, contains only the Bacillus cereus (type 2) beta-lactamase which differs in its hydrolytic mechanism from class A enzymes by its requirement for zinc metal for its activity.

Class C, comprising the chromosomally determined cephalosporinases such as the AmpC enzyme, whose amino acid sequence has been studied by Jaurin and Grundstrom (1981). Subsequent amino acid sequencing of the active sites of the Pseudomonas aeruginosa beta-lactamase and that of Enterobacter cloacae P99 has shown these two enzymes have extensive homology with AmpC such that they too are classed as members of class C (Knott-Hunziker et al., 1982; Joris et al., 1984). The comparison of the OXA-2 beta-lactamase DNA sequence
(Dale et al., 1985) with the published sequence of class A and class C beta-lactamase showed no significant matching, this leads to a suggestion that the OXA-2 enzyme may be the first member of class D. Bicknell et al., (1985) showed that the zinc requiring beta-lactamase from Pseudomonas maltophilia shares only a few properties with that of the Bacillus cereus type 2 enzyme and therefore will need to be grouped separately from B. cereus. This system therefore enables classification of similar enzymes encompassing broader activities than the earlier classification systems. However with the availability of more DNA and amino acid sequences it is possible that this system will also prove to be insufficient and require sub-groups within each class.

4.3 The Oxacillin-Hydrolyzing Beta-Lactamases

Oxacillin is a semisynthetic penicillin that is usually resistant to hydrolysis by beta-lactamase (penicillinase). However, the beta-lactamase mediated by the R-factor R46 (also known as R1818 or R-Brighton) hydrolyzes oxacillin more rapidly than benzylpenicillin (Smith, 1969). Enzymes that do this are classified as oxacillin-hydrolyzing beta-lactamases (Hedges et al., 1974). Interest in this group of beta-lactamases obviously derives from its unusual substrate profile. Two such enzymes have been purified: those specified by R-GN238 (Yamagishi et al., 1969) and by R46 (Dale and Smith, 1971). Dale and Smith (1972) were able to distinguish between these two beta-lactamases, particularly on the basis of molecular weight, and showed that these two types of enzymes were sufficiently different as to constitute two subgroups. The enzymatic and molecular properties of oxacillin-hydrolyzing enzymes specified by 14 different R-factors have been studied. The results revealed that this enzyme class could be separated into 2 major and 1 minor subgroup (Dale and Smith, 1974). The three groups were designated OXA-1, 2 and 3 by Matthew and Hedges (1976), OXA-1 and OXA-2 corresponding to groups Va and Vb in the Richmond and Sykes classification system (Richmond and Sykes, 1973). No comparable group to OXA-3 is found in that system.
The OXA-2 type of beta-lactamase is a plasmid mediated enzyme which is found in a small proportion of ampicillin-resistant clinical isolates of Gram-negative bacteria. This beta-lactamase was originally found in a strain of *Salmonella typhimurium* and is specified by the resistance plasmid R46 (Anderson and Datta, 1965). This enzyme possesses a number of features in which it is different from nearly all other known beta-lactamases; its ability to hydrolyze oxacillin six times more rapidly than benzylpenicillin and its interaction with anthraquinone dyes such as Cibacron blue (Dale and Smith, 1971, 1974; Monaghan et al., 1982). Further studies showed that this beta-lactamase was not inhibited by p-chloromercuribenzoate (pCMB) or cloxacillin but was inhibited by clavulanic acid and olivanic acid, had a pI of 8.65 and from amino acid analysis had a high arginine content (Holland, 1983; Holland and Dale, 1984; Dale et al., 1985).

The molecular weight of the OXA-2 beta-lactamase was 44,600 as determined by Sephadex-gel filtration (Dale and Smith, 1971), whereas that determined from SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was 28,400 (Dale and Smith, 1976). This anomaly in the molecular weight suggested that this enzyme may be dimeric. OXA-2/OXA-3 hybrid studies support the hypothesis that both OXA-2 and OXA-3 beta-lactamases are dimeric in their native state. Remeasurement of the monomer molecular weight gave a value of 32,000 daltons (Holland and Dale, 1984) and that of the native enzyme using sedimentation analysis, was 65,000 daltons (Mossakowska, D. unpublished data). The latter value was close to the expected value for the dimeric protein.

Transposition of an OXA gene was first reported by Yamamoto et al. (1981). That 20kb transposon, Tn2603, mediates resistance to ampicillin, streptomycin, sulphonamide, and mercury. The ampicillin resistance is due to the production of an OXA-1 beta-lactamase. It has been shown that OXA-2 beta-lactamase gene was carried by transposon Tn2410 which is derived from plasmid R1767 originally isolated from *Salmonella typhimurium* (Schmidt et al., 1982). In this case resistance is part of transposon, the beta-lactamase
gene is associated with genes for resistance to sulphonamide and mercury (Kratz et al., 1983; Nies et al., 1985). Despite the different ampicillin resistance gene, Tn2603 and Tn2410 transposons are similar in size, physical map and in their end sequence, suggesting that these transposons could have been generated from a common ancestor (Meyer et al., 1985).

The location of the OXA-2 beta-lactamase on a transposon means that this gene has a capability of transferring itself rapidly. Although OXA type beta-lactamases are not as common as TEM, the nature of the gene location can mean that they could become a future problem in drug resistance.

4.4 Location of Beta-Lactamases in the Bacterial Cell

There is a clear difference between the beta-lactamases from Gram-positive species and their counterparts from Gram-negative bacteria. Whereas the former are all synthesized in relatively large amounts and liberated into the surrounding medium (extracellular enzymes; Pollock, 1962), the latter are all cell-bound (Richmond and Sykes, 1973). It is thought that the Gram-negative beta-lactamases are located in the periplasmic region (Mitchell, 1961) that is contained between the inner or cytoplasmic membrane and the outer membrane (see figure 4) and can be released by osmotic shock, a technique which disrupts the outer membrane but leaves the cytoplasmic membrane intact (Neu and Heppel, 1964; Neu and Chou, 1967). Studies concerning the release of plasmid and chromosomally-mediated beta-lactamases prompted the hypothesis that the location of beta-lactamases within the cell was dependent on the genetic location of the enzyme's structural gene (Neu, 1968). It therefore became generally accepted that plasmid mediated beta-lactamases which were released by osmotic shock, were periplasmic, whereas chromosomal enzymes which were not released, were therefore more tightly bound. Exceptions to this generalization have been recorded, for example, R-factor-specified beta-lactamases of Proteus mirabilis could not be liberated by osmotic shock (Neu and Winshell, 1970), and it is thought that the
location of the enzyme's structural gene has no bearing on the location of that enzyme in the cell (Curtis et al., 1972). Smith and Wyatt (1974), have shown that the release of beta-lactamase by osmotic shock is apparently dependent on the molecular weight of the enzyme itself. Enzymes less than or equal to molecular weight of 30,000 are released whereas those above that size are retained. The equivalence of a molecular sieve and the outer membrane of Gram-negative bacteria has been established. It seems likely that although the outer membrane is damaged by osmotic shock treatment, it retains some of its sieving properties. Presumably, enzymes below a particular exclusion limit are released by the treatment depending upon the level of membrane damage, and enzymes above that size are retained. The nature of the component to which a periplasmic enzyme may be bound, would influence its release. The classification of an enzyme as non-periplasmic if it is not released by osmotic shock is probably invalid.
Section 5. Secretion of Protein across Membranes

The secretion of protein from Gram-positive bacteria is easier than that of Gram-negative bacteria. This may be related to their simple cell wall structure, which is represented by an enlarged external peptidoglycan layer and lacks lipopolysaccharide. Whereas the cell envelope of Gram-negative bacteria consists of two distinct membrane systems, the outer membrane and the inner or cytoplasmic membrane, separated by the periplasmic region and rigid peptidoglycan layer. Both the inner and the outer membrane have a lipid bilayer structure. The outer membrane carries in addition, Lipopolysaccharide - Figure 4 (Glass, 1982; DiRienzo et al., 1978).

The process of protein secretion involves both insertion of protein into membranes and passage of hydrophilic protein through hydrophobic membrane barriers. Gram-negative bacteria, such as Escherichia coli have soluble proteins localized to a periplasmic space that is contained between the cytoplasmic and outer membranes. Thus, proteins synthesized in the cytoplasm are exported to final destinations in both the outer membrane and the periplasm (Michaelis and Beckwith, 1982; Sabatini et al., 1982). The translocation of protein into the periplasmic space involves synthesis of protein in a precursor form which has a higher molecular weight than the mature protein due to the presence of a short peptide extension. This extension, usually between 15 and 30 amino acids in length (referred to as the signal sequence or leader sequence), comprises a hydrophobic sequence of at least 11 amino acid residues between the basic section (a short stretch of residues of the amino terminus that normally includes several positive charges) and the cleavage site (the site at which the signal region is removed from the mature protein). The hydrophobic region is subdivided into three sections by proline or glycine residues, (Figure 6), (Inouye et al., 1979; Inouye and Haledoua, 1980).

From the amino acid sequence, the length of the signal peptide was determined as 21 amino acid residues for OXA-2
beta-lactamase gene (Dale et al., 1985), 23 for TEM beta-lactamase, 24 for beta-lactamase of Staphylococcus aureus, and 26 for penicillinase of Bacillus licheniformis (Oliver, 1985).

Although the mechanism of protein secretion is yet unknown, there are various hypotheses describing the protein secretion across the cell's membranes as:

Signal Hypothesis:

a). Linear Model (Blobel and Dobberstein, 1975).

In this model, it is proposed that the signal peptide at the amino terminus of the precursor is first recognized by receptor proteins in the membrane, which then form a trans-membrane tunnel. Through this tunnel, the signal peptide as well as the remainder of the protein are linearly translocated from the inside to the outside of the cell, then the signal sequence is hydrolyzed into pieces outside of the cell (Figure 7a).

b). Loop Model (Inouye et al., 1979)

In this model, the positively charged first section of the leader sequence allows the initial attachment of the precursor protein to the negatively charged inner surface of the cytoplasmic membrane. Insertion of protein into the membrane proceeds, via hydrophobic interactions, between the appropriate sections in the signal region and the lipid bilayer. The protein extends through the membrane in loop configuration with the NH2-terminus of the signal region still attached to the inner surface of the cytoplasmic membrane. The role of the proline or glycine residues within section II of the signal region, may be important in the bending of the peptide at these positions to form the loop. At the end of the process, the cleavage site is exposed to proteases within the periplasm which cleave off the signal region. The mature protein is thereby released outside the cytoplasmic membrane (Figure 7b).
Figure 6  The general structure of a signal sequence composed of 25 amino acids in length.
1 = Basic region, 2 = Hydrophobic region, 3 = Recognition site for the cleavage of the signal sequence. Section 2 is divided into 3 subsections a, b and c by proline or glycine at positions 6 and 11.

Figure 7  Signal hypothesis; a - linear model; b - loop model for the translocation of secretary proteins across membranes.
The Membrane-triggered folding hypothesis (Wickner, 1980)

This was originally proposed to explain data concerning the secretion of the coat protein of bacteriophage M13. The coat protein was shown to be synthesized as a precursor, free in the cytoplasm, termed procoat, containing an amino-terminal signal sequence of 23 amino acid residue. The role of the amino-terminal leader sequence is not to mediate binding of nascent polypeptides to the membrane, but rather to confer a conformation on the precursor that renders it soluble. Interaction of this precursor within the membrane triggers a change in conformation, resulting in association with the cytoplasmic side of the membrane. Proton motive force effects insertion across the lipid bilayer (Figure 8).

Randall and Hardy (1984), have developed a model which describes exporting the periplasmic proteins in E. coli: This model combines features of both the signal hypothesis and the membrane-triggered folding hypothesis, and can be summarized as follows: As soon as the signal sequence emerges from the ribosome it interacts with a signal recognition particle; the binding of this particle blocks further elongation until the entire complex interacts with a membrane-associated docking protein. Then the amino-terminal leader sequence acts as in the membrane-triggered folding hypothesis, in mediating the binding of the nascent polypeptide to the membrane; this causes a conformational change in the precursor, resulting in association with the cytoplasmic side of the membrane. The mature protein, is then transported across the membrane (Figure 9).
Figure 8 Membrane-triggered folding hypothesis: insertion of coat protein of phage M13 into the cytoplasmic membrane of *E. coli*. LP, leader peptidase. The flower and the star represent unspecified conformations.
Figure 9 Export of a soluble protein through the cytoplasmic membrane of *E. coli* into the periplasmic space according to Randall and Hardy model (Randall and Hardy, 1984). The flower and star represent unspecified conformation. LP, leader peptidase.
The precise role of the signal sequence and its removal is still unclear. It appears to be necessary for transport in most cases since mutational changes in its amino acid sequence eliminate translocation (Bedouelle et al., 1980). Kadonaga et al. (1984), have shown that the signal peptide of the beta-lactamase derived from pBR322 is essential for secretion of the protein across the membrane. However, fusion of the DNA segment coding for the signal sequence with a non secretory protein results in a precursor that is not secreted. This indicates that some features of the mature beta-lactamase are necessary for proper processing. Pollitt and Zalkin (1983), have shown that all the information for correct processing is contained in the first 183 amino acids at the N-terminal end of the beta-lactamase from pBR322. The DNA sequence of the promoter, ribosome binding site and signal peptide from Bacillus subtilis alpha-amylase gene was attached to the beta-lactamase gene from E. coli which is devoid of this DNA region. The fusion protein, when introduced back into E. coli, results in correctly processed beta-lactamase. However, an incomplete signal peptide missing only the last six amino acids results in a cytoplasmic location for the protein (Palva et al., 1982). This not only emphasizes the need of signal peptide for the translocation of a protein, but also that a signal peptide from a Gram-positive organism can be recognised by a Gram-negative cell.

The role of carboxy-terminal-sequence in the export of several periplasmic proteins has been studied. This sequence is necessary for the transport of the TEM-1 beta-lactamase across the cytoplasmic membrane (Koshland and Botstein, 1980). Experiments utilising mutants which cause premature termination of the polypeptide chain indicate that deletion of even a small section of the carboxy-terminal region from the TEM-1 enzyme resulted in the failure of enzyme secretion into the periplasm. Abortive transportation resulted in spite of the normal processing of the signal sequence. Furthermore, experiments utilising fused proteins constructed from the signal region of the E. coli maltose-binding protein joined to the beta-galactosidase protein, have shown that
transport of this enzyme does not occur in the E. coli system (Bassford et al., 1979). These results suggest that the presence of the signal region is not sufficient to ensure secretion.

Beta-lactamases have provided a useful model for the study of processing and secretion in both eukaryotic and prokaryotic systems. The signal peptide of pBR322 beta-lactamase has been used to export insulin to the periplasm (Chan et al., 1981), and can transfer the COOH-terminal region of alpha-globin across dog pancreas vesicle membrane (Lingappa et al., 1984). Muller et al. (1982), showed that pre-beta-lactamase is correctly translated and processed in an in vitro eukaryotic system, when the DNA of pBR322 beta-lactamase is translated in vitro by wheat germ cells extract and is then added to dog pancreas microsomes. The TEM-1 beta-lactamase gene has been cloned into a suitable vector and transformed into Saccharomyces cerevisiae. The enzyme is expressed in a precursor form which is correctly processed to the mature enzyme in the eukaryotic cell (Roggenkamp et al., 1981). Conversely, eukaryotic, prokaryotic or hybrid signal sequence can direct the secretion of rat insulin in E. coli (Talmadge et al., 1980a). This indicates that some aspects of protein secretion are common to both the eukaryotic and prokaryotic system.
Section 6. Aim of the Project

Beta-lactamase is an enzyme which has attracted workers from a very wide range of the physical and biological sciences. Those scientists have worked and published on this enzyme over the past 40 years. The beta-lactamase has received so much attention, since it is bacterial, and may be obtained in large amount from non-pathogenic organisms which are extremely easy to grow (e.g. Bacillus sp.); it is easy, safe and cheap to produce in large amounts; it has a large number of readily available substrates; it has an extremely wide distribution in the bacterial kingdom, and is readily accessible to genetic analysis. Finally, it is extremely important in the clinical field, as its presence in certain pathogenic bacteria, determine their resistance to beta-lactam antibiotics, and to some extent dictate the choice of antimicrobial chemotherapy (Hamilton-Miller and Smith, 1979).

Our interest is to study the possibility of the expression of the OXA-2 beta-lactamase gene in Streptomyces lividans TK64, and its secretion.
EXPERIMENTAL WORK

Section 7. Materials

7.1 Strains

All the strains and their characters which have been utilized in this project are listed in Table 1.

7.2 Antibiotics

All the antibiotics were obtained from Sigma. Nitrocefin was obtained from Glaxo Research Limited. Thiostrepton was kindly provided by Mr. S.J. Lucania of E.J. Squibb and Sons, New Brunswick, N.J., USA.

7.3 Media

L.broth and L.agar were prepared according to the following recipe:

L.broth (1 litre volume)

- 10g Tryptone (oxoid)
- 5g Yeast extract (oxoid)
- 5g Sodium Chloride.

The volume was made up to 1 litre with distilled water and the pH adjusted to 7 with sodium hydroxide. The sodium chloride was substituted with 10mM sodium acetate when used for beta-lactamase preparation from Streptomyces strains, because chloride ions inhibit OXA-2 enzyme. To make L.agar, 15g/litre of Agar Technical No. 3 (Oxoid) was added.
### Table 1. Bacterial Strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Designation</th>
<th>Plasmid Content</th>
<th>Relevant Characters</th>
<th>Source/Reference</th>
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<tr>
<td><em>Escherichia coli</em> K12</td>
<td>HB101</td>
<td>None</td>
<td>F&lt;sup&gt;-&lt;/sup&gt;, proA&lt;sub&gt;2&lt;/sub&gt;, recA&lt;sup&gt;-&lt;/sup&gt;, Sm&lt;sup&gt;r&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>pKK232-8</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Boliver and Backman (1979)</td>
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<td>ED8767</td>
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<td></td>
<td>Provided by Dr. T. Kieser</td>
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<td>Δlacpro, thi, supE,</td>
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<td></td>
<td></td>
<td>F&lt;sup&gt;′&lt;/sup&gt;traD36, proAB</td>
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<tr>
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<td>JD322</td>
<td>pSU5</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pSU8</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;, Tc&lt;sup&gt;s&lt;/sup&gt;</td>
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<td>NOA1</td>
<td>pSU101</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;, Thr&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pIJ61</td>
<td>Nm&lt;sup&gt;r&lt;/sup&gt;, Thr&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>3131</td>
<td>pIJ702</td>
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<td>pIJ424</td>
<td>Th&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>Mel&lt;sup&gt;+&lt;/sup&gt;, pro&lt;sup&gt;-&lt;/sup&gt;</td>
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</table>

Cm = Chloramphenicol, Ap = Ampicillin, Tc = Tetracyclin, Sm = Streptomycin, Nm = Neomycin, Th = Thioestrepton, pro = Proline, Mel = Melanin
R2YE medium (Thompson et al., 1980)

103.00g Sucrose  
0.25g K2SO4  
10.12g MgCl2.6H2O  
10.00g Glucose  
0.10g Difco casaminoacids.

The volume was made up to 800ml with distilled water. 80ml aliquots were dispensed in 250ml Duran bottles containing 2.2g oxoid agar and autoclaved.
At time of use, the medium was remelted and to each bottle the following constituents were added after they had been prepared and autoclaved separately.

1.00ml KH2PO4(0.5%)  
8.00ml CaCl2.2H2O (3.68%)  
1.50ml L.proline (20%)  
10.00ml TES buffer (5.73% pH 7.2)  
0.20ml Trace element solution  
0.50ml NaOH  
5.00ml Oxoid yeast extract (10%)

Trace element solution (per litre)

40.00mg ZnCl2  
200.00mg FeCl3.6H2O  
10.00mg CuCl2.2H2O  
10.00mg MnCl2.4H2O  
10.00mg Na2B4O7.10H2O  
10.00mg (NH4)6Mo7O24.4H2O
It consists of minimal medium MM (Hopwood, 1967) supplemented with amino acids and L.Tyrosine.

**MM (per litre)**
- 0.50g Asparagine
- 0.50g K2HPO4
- 0.30g KOH
- 0.20g MgSO4.7H2O
- 0.01g FeSO4.7H2O
- 15.00g Oxoid Agar

The volume was made up to 980ml with distilled water, autoclaved, then 20ml Glucose (50%) was added.

To make MMT: 200ml MM was supplemented with the following sterile solutions:
- 4.00ml Difco casaminoacids (30% w/v)
- 1.50ml Amino acids solution.
- 10.00ml L.tyrosine

**Amino Acids solution (per 100ml)**
- 1000mg Larginine
- 750mg Lcystine
- 750mg Lhistidine
- 750mg DLhomoserine
- 750mg Lleucine
- 750mg Lphenylalanine
- 750mg Lproline
- 150mg adenine
- 150mg uracil
- 10mg nicotinamide

Sterilised by autoclaving at 115°C, 15 minutes.
2xYT Medium (per litre)

16g Tryptone (oxoid)
10g Yeast extract (oxoid)
5g Sodium Chloride

The volume was made up to 1 litre with distilled water, dispensed into the required volumes and autoclaved.

To make 2xYT agar, 1.5g Oxoid agar to each 100ml broth was added before autoclaving.

H top agar (per litre)

10g Tryptone (oxoid)
8g Sodium Chloride
8g Agar (oxoid)

The volume was made up to 1 litre with distilled water, boiled with stirring to melt the agar. Then dispensed into 3ml aliquots using small glass bottles (Bijous) and autoclaved.

Tryptone Soya Broth (TSB)

30g/litre Oxoid Tryptone Soya Broth powder (CM129).

Brain Heart Infusion Broth

37g/litre Oxoid Brain Heart Infusion Broth powder (CM225).

Yeast Extract - Malt Extract Medium (YEME) (Chater et al., 1982b)

3g Oxoid Yeast Extract
5g Oxoid Peptone (Bacteriological, L34)
3g Oxoid Malt Extract
10g Glucose
340g Sucrose
The volume was made up to 1 litre with distilled water, and stirred to dissolve the whole constituent. Then dispensed into Erlenmeyer flask containing stainless steel spring, as required, and autoclaved. Following autoclaving 2ml/litre MgCl2.6H2O (2.5M) was added.

For preparing protoplasts, 25ml/litre Glycine (20%) was added to the above ingredients.

**Spore pre-germination medium (2X)**

1% Oxoid Yeast extract
1% Difco casamino acids
0.01M CaCl2 (prepared and autoclaved separately as 5M solution)

**Peptone medium (Leyh-Bouille, et al., 1971)**

10.0g Oxoid peptone (Bacteriological, L34)
1.0g K2HPO4
1.0g MgSO4.7H2O
2.0g NaN03
0.5g KCl

The volume was made up to 1 litre with distilled water, and agitated to dissolve the whole ingredients. The medium was dispensed into 75ml aliquots in 250ml flasks and autoclaved.

**Glucose-minimal medium**

6.0g Na2HPO4
3.0g KH2PO4
1.0g NH4Cl
0.5g NaCl
15.0g Oxoid Agar.
The volume was made up to 1 litre with distilled water, autoclaved and cooled to 50°C, then the following sterile reagent was added and mixed:

- 1ml 1M MgSO4
- 1ml 0.1M CaCl2
- 1ml 1M thiamine HCl
- 10ml 20% Glucose
7.4 **Buffers**

**P (Protoplast) buffer** (Okanishi et al., 1974; Hopwood and Wright, 1978).

- 10.300g Sucrose
- 0.025g K2SO4
- 0.202g MgCl2.6H2O
- 0.20ml Trace element solution (as prepared previously)

The volume was made up to 80ml with distilled water and autoclaved. After autoclaving the following sterile solutions were added:

- 1 ml KH2PO4 (0.5%)
- 10 ml CaCl2.2H2O (3.68%)
- 10 ml TES buffer (5.73%, pH 7.2)

**L (lysis) buffer** (Thompson et al., 1982b)

The following sterile solutions were mixed and kept at 4°C until required:

- 100.00ml Sucrose (10.3%)
- 10.00ml TES (5.73%, pH 7.2)
- 1.00ml K2SO4 (2.5%)
- 0.20ml Trace element solution
- 1.00ml KH2PO4 (0.5%)
- 0.10ml MgCl2.6H2O (2.5 M)
- 1.00ml CaCl2 (0.25M)

Just before use, a sample of the above stock lysis solution was taken out and the lysozyme was dissolved in a concentration of 1 mg/ml, followed by filter sterilisation.
T (Transformation) buffer (Thompson et al., 1982b)

The following sterile solutions were mixed:

- 25.00ml Sucrose (10.3%)
- 75.00ml Distilled water
- 0.20ml Trace element solution
- 1.00ml K2SO4 (2.5%)

To 9.3ml of the above solution the following sterile solutions were added:

- 0.20ml CaCl2 (5M)
- 0.50ml *Tris-Maleic acid buffer

For use, 2.5g PEG 1000, previously sterilized by autoclaving was added to 7.5ml of the above solution.

* 1M solution of Tris-base and the pH was adjusted to 8.0 by adding maleic acid.

10X Ligation buffer:

- 0.66M Tris - HCl (pH 7.6)
- 50.00mM MgCl2
- 50.00mM dithiothreitol (prepared as 1M, filter sterilized)

10X Nick-translation buffer:

- 0.50M Tris - HCl (pH 7.2)
- 0.10M MgSO4
- 1.00mM dithiothreitol (filter sterilized)
- 500.00ug/ml bovine serum albumin (BSA)

Stored at -20°C as small aliquots.
Prehybridisation Solution

- 6X SSC
- 0.5% SDS
- 5X Denhardt's Solution
- 100.0 ug/ml denatured Salmon sperm DNA (sigma).

Denhardt's Solution (50X)

- 0.5g Ficoll (molecular weight = 400,000)
- 0.5g Polyvinylpyrrolidone (molecular weight = 40,000)
- 0.5g Bovine serum albumin
  Water up to 50ml

SSC Buffer (20X)

175.3g NaCl and 88.2g Sodium Citrate, dissolved in 800ml distilled water. The pH was adjusted to 7.0 with a few drops of a 10N solution of NaOH. Then the volume was adjusted to 1 litre and autoclaved.

Buffers for Restriction Endonuclease Digestion

Low-salt Buffer (10X LOW):
- 10mM Tris-HCl (pH7.5)
- 10mM MgCl2
- 1mM Dithiothreitol

Medium-salt Buffer (10X MED):
- 50mM NaCl
- 10mM Tris-HCl (pH7.5)
- 10mM MgCl2
- 1mM Dithiothreitol
High-salt Buffer (10X Hi):
100mM NaCl
50mM Tris-HCl (pH7.5)
10mM MgCl2
1mM Dithiothreitol

Buffer of Smal (10X SMA):
20mM KCl
10mM Tris-HCl (pH8.0)
10mM MgCl2
1mM Dithiothreitol (DTT)

Sodium-phosphate buffers:

These buffers were prepared according to the directions given in "Data for Biochemical Research", 2nd Edition, (Dawson et al., 1969).

7.5 Chemicals:

BDH:

Acrylamide (Electran), Ammonium persulphate (Analar), Ammonium sulphate (Analar and enzyme grade), Boric acid, Dimethyl dichlorosilane (Repelcote), Dithiothreitol (DTT), D-glucose (Analar), Ethylene diamine tetra-acetic acid - EDTA (disodium salt, Analar), Glycine (Analar), 8-Hydroxyquinoline (Analar), PAGE-blue-83 (Electran), Phenol (Analar), Sodium acetate, Sucrose (Analar), Tetramethylethylenediamine (TEMED).
BCL (Boehringer Mannheim);

Caesium chloride (Analar), Deoxynucleoside triphosphates (dNTPs), Dideoxynucleoside triphosphates (ddNTPs).

BRL (Bethesda Research Laboratories Inc.,);

Agarose (Ultra-pure and Electrophoresis grade), Phenol (Ultra-pure redistilled), Urea (Ultra-pure and enzyme grade), Vanadyl Ribonucleoside complexes.

Sigma;

Bovine Serum Albumin, Coomassie Brilliant Blue G-250, Diethylpyrocarbonate (DEPC), Ethidium bromide, IPTG (Isopropyl-Beta-D-thio-galactopyranoside), Lysozyme (grade 1), NN'-methylene-bis-Acrylamide, Gamma-Methacryloyxpropyl-trimethoxy silane, MOPS (3-[N-Morpholino] propane sulfonic acid), PIPES (Piperazine-N,N'-bis [2-ethane-sulfonic acid]), Polyethylene Glycol (PEG), Ribonuclease Type 1-A, tRNA, Sodium dodecyl sulphate (SDS), TES (N-tris [Hydroxymethyl] methyl-2-amino ethane sulfonic acid), Trizma Base (Reagent Grade), X-gal (5-bromo-4-Chloro-3-indolyl-beta-galactoside).

M and B Limited; Sodium Chloride.

Restriction enzymes were obtained from BRL, BCL or New England Biolabs.

Calf Intestinal Alkaline Phosphatase (CIP), T4-ligase and Bacteriophage Lambda DNA were purchased from BCL.

DNA polymerase I and Klenow fragment were obtained from BRL or Amersham International plc.
S-35 and P-32 (alpha and gamma) Deoxyadenosine Triphosphate, Triethyl-ammonium salt, were obtained from Amersham International plc.

M13mp9 DNA (RF) was obtained from New England Biolabs.

The Ampholine Polyacrylamide gel (PAG) plates, pH 3.5 - 9.5, were obtained from LKB.

The Broad pI calibration kit (pH 3 - 10) for pI determination using isoelectric focusing, was obtained from Pharmacia Fine Chemical AB.

**7.6 Films:**

- **Kodak:** Colour Film CP100
  - X-Ray Film X-Omat
- **Fuji:** X-Ray Film RX
- **Polaroid:** Positive/negative 4x5 land film type 665
Section 8. Methods

8.1 Maintenance of strains

8.1.1 Escherichia coli strains

The Escherichia coli strains were maintained by streaking out on plates of L-agar for single colonies and stored at 4°C, for not longer than one month. This method is used for the working strains. These strains were stored at -70°C for a long time (2-3 years) as glycerol cultures. Glycerol cultures can be prepared by adding an equal volume of 40% glycerol to an overnight culture in L.broth of the strain to be stored. The culture was frozen rapidly, and thawed rapidly at 37 °C prior to use. A freezing mixture containing 6.3g K2HP04, 0.45g Sodium citrate, 0.09g MgSO4.7H2O, 0.9g (NH4)2SO4, 1.8g KH2PO4, 44g glycerol in 500ml H2O was, sometimes, used instead of 40% glycerol to dilute the overnight cultures.

8.1.2 Streptomyces lividans strains

The Streptomyces lividans strains were maintained by inoculating over the entire surface of the R2YE agar plates, plus antibiotic, where appropriate, and incubated at 30°C until they yielded well sporulating growth, then kept at 4°C.

The strains were stored for a long time at -20°C as a spore suspension, which was prepared by adding 10ml of sterile Ringer solution containing 0.01% Tween 80 as wetting agent to a plate of well sporulated culture; the surface of the culture was then scraped with a sterile glass spreader, first with gentle pressure and then gradually more vigorously, so as to suspend the spores. Then the crude suspension was poured into a sterile universal and agitated vigorously using a vortex mixer for a few minutes. The suspension was filtered through non-absorbent cotton wool, then the spores were sedimented by centrifugation for 5-10 minutes at c.3000 rpm, and resuspended in a suitable volume of sterile Ringer solution containing 0.01% Tween 80 and stored at -20°C.
8.2 Pregermation of Streptomyces spores (Hopwood et al., 1985a)

Dense spore suspension was pelleted by centrifugation (MSE-Chilspin) at 4500 rpm for 10 minutes and resuspended in 5ml TES (0.05M pH 8) buffer. This was heat shocked at 50°C for 10 minutes and cooled under cold tap water. An equal volume of double strength pre-germination medium was added and the spores were incubated at 37°C on a shaker for 2-3 hours. The pre-germinated spores were pelleted and resuspended in water or TES buffer with vigorous agitation on a vortex mixer to disperse the clumps. The resulting germinated spores were used to inoculate the required growth medium.

8.3 Preparation of plasmid DNA

8.3.1 Large-scale isolation of plasmid DNA from E. coli

Many methods have been used to isolate plasmid DNA. All of them involve three basic steps: growth of bacteria and amplification of the plasmid; harvesting and lysis of the bacteria; and purification of the plasmid DNA. The following procedure was used routinely to isolate the plasmid from E. coli strains which harbour small plasmids.

Reagents:

TES: 10mM Tris, 1mM Na2EDTA, 0.17M NaCl, pH 8.0
TE: 10mM Tris-HCl, pH 7.5, 1mM Na2EDTA.
Brij/Doc solution: 1% Brij 58, 0.4% SDS in 0.01M Tris-HCl pH 8.0, 0.001 Na2EDTA.

Procedure

The strain under study was grown overnight at 37°C with shaking in 5ml L-broth containing 20ug/ml of the appropriate
antibiotic. Then 4ml of the starter culture was inoculated into 150ml of pre-warmed L-broth (plus the appropriate antibiotic as 20ug/ml final concentration) and incubated at 37°C with shaking for 3-4 hours until the optical density of the culture at 650nm was at 0.9. At this stage the chloramphenicol to 170ug/ml (or spectinomycin to 150ug/ml where the cells are chloramphenicol resistant) was added. The culture was left shaking vigorously for a further 4-18 hours. The cells were then harvested by centrifugation (MSE-High speed 18) at 6000 rpm, 4°C for 15 minutes and resuspended in 2.6ml of 25% sucrose in 0.05M Tris pH 8.0. The cell suspension was kept on ice and the cells were lysed by the addition of 0.4ml of Lysozyme solution (20mg lysozyme/ml in 0.25M Na2EDTA, pH 8.0) with intermittent swirling on ice for five minutes. Then 2.6ml of 0.25M Na2EDTA (pH 8.0) was added with intermittent swirling on ice for 5 minutes. The cells were completely lysed by the addition of 4ml of Brij/Doc solution rapidly from a 10ml pipette, and mixed well by drawing up and expelling three times. The mixture was left on ice for 20-30 minutes or until lysis had occurred. After that the cell debris and the bulk of the chromosomal DNA were pelleted by centrifugation at 15,000 rpm, 4°C for 45 minutes. The supernatant (cleared lysate) was pooled into a fresh tube to which was added solid caesium chloride at the rate of 0.95g/ml cleared lysate and ethidium bromide (20mg/ml in water, stock) at 500ug/ml as a final concentration. The cleared lysate was distributed into quick sealed polyallomer tubes which were spun in a 75 Ti rotor of a Beckman L8-M ultracentrifuge at 40k rpm for 60 hours at 18°C. The DNA bands were viewed via U.V. Longwave illumination and the plasmid bands were removed by puncturing the side of the tube with a 21G syringe needle, and pooled. The ethidium bromide was removed from the plasmid DNA by three extractions with equal volume of isopropanol saturated with caesium chloride solution. The plasmid DNA was dialysed against 4 x 500ml of TE buffer or TES buffer, and ethanol precipitated.
8.3.2 Large-scale isolation of plasmid DNA from Streptomyces strains

The alkaline lysis procedure (Kieser, 1984; Kendall and Cullum, 1984) was routinely used, in which most of the chromosomal DNA was removed by alkaline denaturation.

Procedure

The strains of Streptomyces lividans were grown at 30 °C for 3-4 days with shaking in 400ml L.broth containing thiostrepton as 5ug/ml. The mycelium was harvested by centrifugation (MSE-High speed 18) at 10,000 rpm 4 °C for 30 minutes, washed with 10.3% sucrose and resuspended in 40ml of 25mM Tris-HCl, pH 8.0, 25mM Na2EDTA and 10.3% sucrose containing 2mg/ml lysozyme. Following incubation at 37 °C for 30-60 minutes, the cells were lysed with 100ml of 0.15M NaOH, 1% SDS and placed on ice for 10 minutes. 80ml of 4M sodium acetate pH 5.5 were added and the mixture was left to stand on ice for 30 minutes. Denatured chromosomal DNA and protein were removed by centrifugation at 12,000 rpm for 30 minutes at 4°C. The supernatant was removed, mixed with 100ml of 50% w/v PEG 6000, left on ice for one hour, and the precipitated DNA recovered by centrifugation for 10 minutes at 5000 rpm. The resulting pellet was resuspended in 10ml of 10mM Tris-HCl pH 8.0, 1mM Na2EDTA. The plasmid DNA was further purified by caesium chloride-ethidium bromide gradient centrifugation (Chater et al., 1982) by adding 1.05g CsCl per millilitre DNA and ethidium bromide solution (10mg/ml) to 500ug/ml as final concentration. Centrifugation was carried out by using the 75Ti rotor of a Beckman - L8M ultracentrifuge at 36000 rpm for 60 hours. The CCC DNA band was collected from the gradient using U.V. long wave illumination to visualise DNA. The ethidium bromide was removed from the plasmid DNA by three extractions with equal volume of isopropanol saturated with Caesium Chloride. The plasmid DNA was dialysed against 4 x 500ml of TE buffer (10mM Tris, 1mM Na2EDTA pH 8.0), and ethanol precipitated.
8.3.3 Small scale plasmid preparation

The alkaline lysis procedure, (Kieser et al., 1984) was used for small scale isolation of the plasmids from both Streptomyces and E. coli strains. S. lividans strains were grown for 24 hours at 30°C in 10ml Trypton Soya Broth (TSB) supplemented with 5ug/ml thiostrepton, when appropriate. E. coli strains were grown overnight at 37 °C in 5ml L.broth supplemented with 50ug/ml ampicillin, or 20ug/ml chloramphenicol when appropriate. Harvesting was by centrifugation at 4,500 rpm, 4°C for 10 minutes without additional washes.

Reagents

Lysozyme solution: Lysozyme (2mg/ml) in 0.3M sucrose, 25mM Tris (pH8.0), 25mM Na2EDTA.
Alkaline/SDS: NaOH (0.3M), 2% SDS
Acid phenol/chloroform: Phenol (Analar) 5g, 5ml chloroform (Analar), 1ml H2O, 5mg 8-hydroxyquinoline (Analar).
Neutral phenol/chloroform: Acid phenol/chloroform equilibrated first with 0.5 volume 1M Tris (pH 8.8) and then with 0.5 volume 0.1M Tris (pH 8.0)
Unbuffered sodium acetate: 3M Sodium acetate.
Spermine-HCl (Sigma): 100mM Spermine-HCl filter sterilized.
TE Buffer: 10mM Tris-HCl (pH 8.0), 1mM Na2EDTA

Procedure

The pelleted cells were resuspended in a total volume of 500ml lysozyme solution containing 50ug/ml RNase in a 1.5 ml eppendorf tube, and incubated for 30 minutes at 37 °C for Streptomyces or 0°C for E. coli. Following incubation the tubes were mixed gently and 250ul of alkaline SDS solution was added to each tube, followed by vortex mixing. The mixes were incubated at 70°C
for 15 minutes, then cooled to room temperature. 80ul acid phenol/chloroform was added with immediate vortex mixing until the phases were thoroughly mixed. The two phases were then separated by spinning for 2 minutes in Eppendorf centrifuge, and the aqueous phase (upper) was transferred into a fresh tube containing 70ul 3M unbuffered sodium acetate. 700ul isopropanol was added, and mixed by inverting the tubes several times. The mixtures were kept at room temperature for 5 minutes, then the plasmid DNA was pelleted by centrifugation. The pellet was redissolved in 50ul TE buffer, then 5ul 3M unbuffered sodium acetate and 25ul neutral phenol/chloroform were added and mixed by vortexing. The aqueous phase was separated by centrifugation and transferred to a fresh tube. The plasmid DNA was precipitated by adding 50ul isopropanol, and redissolved in 500ul TE buffer. 25ul 100mM spermine-HCl was added, mixed, and kept at room temperature for five minutes. The DNA was pelleted by centrifugation and dispersed by vortexing in 300ul 0.3M sodium acetate, 10mM MgCl2, then 700ul ethanol was added, mixed and kept at room temperature for 60 minutes. After that the plasmid DNA was precipitated by centrifugation, redissolved in 50ul TE buffer, and stored at 4°C.
8.4 In vitro manipulation of DNA

8.4.1 Ethanol precipitation

DNA precipitation was carried out by dispensing the DNA solution into aliquots of 300μl in 1.5ml polypropylene microcentrifuge tubes. Aliquots of 30μl of sodium acetate 3M, 30μl of magnesium acetate 0.3M and 750μl of absolute ethanol were added. The mixture was inverted several times and left at -20°C overnight. The DNA was then precipitated by centrifugation for 5 minutes in an Eppendorf centrifuge. The DNA was washed 3 times in cold absolute ethanol, dried under vacuum and resuspended in 50ul of TE buffer. DNA suspensions were stored at 4°C.

8.4.2 Quantitation of DNA

To determine the DNA concentration and protein contamination, the spectrophotometric method was routinely used by which the amount of UV irradiation absorbed by the bases was measured (Maniatis et al., 1982).

Procedure

DNA preparations were diluted 10 or 20-fold in the final dialysing buffer. The optical density at 260 and 280nm was measured using a Beckman model 24 U.V. spectrophotometer and a 0.5ml quartz cuvette. The ratio between the readings at 260nm and 280nm (OD260/OD280) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD260/OD280 of 1.8 or greater. An optical density of 1.0 at 260nm is equivalent to a DNA concentration of 50ug/ml, and to an RNA concentration of 40ug/ml. DNA concentration (ug/ml) = OD260 x 50 x Dilution.
8.4.3 Dephosphorylation of DNA: (Maniatis et al., 1982; Kendall and Cullum, 1984).

The terminal 5' phosphates can be removed from DNA by treatment either with bacterial alkaline phosphatase (BAP), or with calf intestinal alkaline phosphatase (CIP), (Chaconas and Van de Sande, 1980). The latter enzyme has the considerable advantage that it can be completely inactivated by heating to 68°C, and it was used to dephosphorylate the digested vector DNA when ever it is required in the present study to prevent the intra-vector ligation and hence to increase the proportion of cloned fragments among subsequent transformants.

Procedure

The linearised DNA vector after it had been digested with the appropriate restriction enzyme was extracted once with phenol/chloroform and ethanol precipitated. The DNA was redissolved in 50mM Tris-HCl pH 8.0, 0.1mM EDTA. 0.1 unit of CIP per each microgram DNA was added and incubated at 37°C for 30 minutes. The reaction was terminated by heating to 70°C for one hour. Followed by two phenol extractions and ethanol precipitation.

8.4.4 Recovery of DNA from low-melting-temperature Agarose

The low-melting-temperature agarose was dissolved in electrophoresis buffer (TBE) by heating to 70°C. The gel was cooled to 37 °C and ethidium bromide to a final concentration of 0.5ug/ml was added. The gel was poured at 4 °C to ensure that it set properly. The DNA samples were then loaded and the electrophoresis was carried out at 4°C to ensure that the gel did not melt during the run. The desired fragment of the gel was cut out and transferred to an appropriate tube to which about 5 volumes of 20mM Tris-HCl (pH 8.0) and 1mM EDTA was added. The tube was heated to 65°C for five minutes to melt the gel. The melted gel slice was extracted at room temperature with an equal volume of phenol. The
phases were separated by centrifugation at 20°C and the aqueous phase was recovered and re-extracted with phenol/chloroform and then with chloroform. Followed by ethanol precipitation.

8.4.5 Endonuclease Digestion of DNA

Single restriction digests

A 2ul aliquot of pSU101 DNA at a concentration of approximately 2.0ug/ul, was mixed with 16ul of sterile deionised water, 2ul of ten times the concentration of the appropriate restriction buffer (10X) and 0.5ul of the restriction enzyme in question. Each enzyme has a particular buffer composition which optimises the restriction digest. The digest buffers were prepared according to the manufacturers instructions or as recommended by Maniatis et al. (1982). The digests were incubated at 37°C for one hour.

Double restriction digests

Single digests were prepared as described above. Following incubation at 37°C the digests were heated at 65°C for 5-10 minutes in order to denature the restriction enzyme. A 0.5ul aliquot of the second enzyme was added directly if the restriction buffer of the first enzyme was suitable for the second enzyme, otherwise, correction of the salt concentration was done. The digest was incubated for a further one hour at 37°C, followed by heating at 65°C as above. The digests were run on a 1% or 0.8% agarose gels.

8.4.6 Agarose Gel Electrophoresis

Buffer

Tris-borate (TBE, 0.089M Tris, 0.089M boric acid, 0.002M Na2EDTA) was used as electrophoretic gel running buffer. It has a good resolution of DNA fragments and has high buffering capacity. The borate gels can be run at high voltage.
Procedure

The gel apparatus used was a horizontal slab gel, mainly the mini-gel tank was used. The spacers and the comb were fixed in the appropriate positions. The agarose gel concentration generally used was a 1% solution, prepared in borate buffer. The gel solution was boiled gently until dissolved, cooled and poured into the electrophoresis chamber. The gel was left to set for 30-45 minutes at room temperature. The comb and spacers were carefully removed and the electrophoresis buffer (TBE) was added in amount just enough to cover the gel to a depth of about 1mm. The gel was loaded with the DNA samples containing 10ul of the DNA preparation, and 3ul of borate loading buffer (50% glycerol, 5% SDS, 0.025% bromophenol blue). The gel was run at the appropriate voltage until the marker dye had reached the bottom end of the gel. The gel was then stained inside the same gel tank, with ethidium bromide at 0.5ug/ml for 30 minutes and destained with fresh buffer for 30 minutes. The DNA bands within the gel were viewed via longwave U.V.illumination using a transilluminator (Ultra-Violet Products Inc.). The gel was photographed when necessary.
8.5 Transformation Methods

8.5.1 Transformation of Escherichia coli.

The recipient strain commonly used was HB101, which is E. coli K-12 derivative.

Preparation of Competent Cells (Kushner, 1978).

Reagents

Solution A: 10mM MOPS, 10mM RbCl pH 7.0.
Solution B: 100mM MOPS, 50mM CaCl2 10mM RbCl pH 6.5

Procedure

The transformation strain was grown overnight at 37°C in 10ml L.broth. A 0.1ml aliquot of the overnight broth was sub-cultured into 20ml of prewarmed L.broth and grown at 37°C with shaking until the optical density at 650nm was approximately 0.15. The culture was dispensed in 1.5ml aliquots and pelleted by Eppendorf centrifuge. The cells were resuspended at 0°C in 0.5ml of solution A, and recovered by centrifugation at 4°C. Then the cells were resuspended in 0.5ml of solution B, stored at 0°C for 60 minutes, pelleted (as above) and resuspended in 0.2ml of solution B. The cells were then competent.

Transformation procedure

A volume of DNA in question (10-20ul) was added to each tube of competent cells and incubated at 0°C for 30-45 minutes with occasional shaking. Then diluted ten times with L.broth and incubated at 37°C for two hours. Aliquots (0.1ml) were plated out onto the appropriate selection plate. All the inoculated plates were incubated at 37°C for 24 hours.
8.5.2 Transformation of *Streptomyces lividans*

The transformation was routinely done using protoplasts of *S. lividans* TK64 (Hopwood et al., 1983), in the presence of polyethylene glycol (PEG).

**Preparation of protoplasts:**

Using the procedure described by Chater et al. (1982b), spores of *S. lividans* TK64 were grown in 25ml YEME (plus 34% sucrose, 0.005M MgCl2, 0.5% glycine) in a 250ml flask containing a coiled stainless steel spring at the bottom, for 30-36 hours at 30 °C with shaking. The mycelium was harvested by centrifugation at 3000 rpm for 15 minutes and washed twice with 10.3% (w/v) sucrose. Then the mycelium was resuspended in 4ml lysozyme solution (1mg/ml in L.buffer, Thompson et al., 1982) and incubated at 30°C for 15-20 minutes. It was then mixed by pipetting three times in a 5ml pipette and incubated for a further 15 minutes. 5ml of P-buffer was added and mixed as above. The protoplasts were passed through Millipore SCWP025 filters-8.0um pore size (Jaurin and Cohen, 1984) and sedimented gently at 2500 rpm for 7 minutes in a bench centrifuge (MSE-Chilspin). The protoplasts were washed twice with a 4ml P-buffer. In this stage the protoplasts were ready for transformation.

**Transformation procedure** (Thompson et al., 1982b; Bibb et al., 1978)

The above protoplast pellet (4x10⁹ protoplasts) was suspended in the drop of P-buffer left after pouring off the supernatant. The DNA to be transformed in 10-20ul was added and 0.5ml of PEG1000 solution (2.5g PEG dissolved in 7.5ml T-buffer) was immediately added and mixed by pipetting. After 60 seconds, 5ml P-buffer was added. The protoplasts were sedimented by gentle centrifugation and resuspended in 1ml P-buffer. 0.1ml aliquots were plated out onto R2YE plates which had been dried in a laminar flow cabinet for 2-3
hours, and incubated at 30°C. The transformants were selected for thiostrepton resistance by overlaying the regeneration plates with soft agar (0.6%) containing 50μg/ml thiostrepton at a suitable time (20 hours) after inoculation to allow for phenotypic expression.
8.6 Techniques using Radio labelled DNA

8.6.1 DNA labelling by nick-translation

The DNA probe used in colony hybridisation was labelled by nick-translation process (Maniatis et al., 1975; Rigby et al., 1977). In this process the *Escherichia coli* DNA polymerase I adds nucleotide residues to the 3'-hydroxyl terminus that is created when one strand of a double-stranded DNA molecule is nicked. In addition, the enzyme by its 5' to 3' exonucleolytic activity, can remove nucleotides from the 5' side of the nick. The elimination of nucleotides from the 5' side and the sequential addition of the nucleotides to the 3' side results in movement of the nick (nick translation) along the DNA.

**Procedure**

The nick translation reaction was set up by mixing, in a microcentrifuge tube, 1ug DNA (pSU8 or pSU5), 5ul 10x nick-translation buffer, 1ul of a 1mM dNTPs solution (dCTP, dGTP, dTTP), 10uCi (1ul) of [alpha-32P]dATP and H2O up to 44ul. This mixture was chilled to 0°C, then 2ul of diluted DNase (0.01ug/ml) was added and mixed by vortexing, followed by the addition of 5 units of *E.coli* DNA polymerase I. The contents of the tube were mixed and incubated at 16 °C for two hours. After incubation the reaction was stopped by adding 2ul of 0.5M EDTA. The nick-translated DNA was separated from unincorporated dNTPs by chromatography on a Sephadex G50 column (15cm x 0.7cm diameter) following the addition of 10ul Orange G (1mg/ml). The nick-translated DNA was eluted with 10mM Tris-HCl pH 8.0, 100mM NaCl, 1mM EDTA well ahead of Orange G, whereas the unincorporated dNTPs were eluted with the dye.

8.6.2 Colony hybridisation

*Streptomyces lividans* transformants containing a recombinant
plasmid can be identified by hybridisation with DNA homologous to the cloned DNA. Using a modified procedure of that described by Hanahan and Meselson (1980) and Schrempf (1982), the colonies of transformants to be tested were grown on nitrocellulose filters (BA85 Schleicher and Schull) placed on selective media (R2YE + 50μg/ml thiostrepton). A reference set of colonies was prepared. Colonies on the filter were lysed to release their DNA by placing the filter, (colony side up), onto three sheets of Whatman 3MM paper, previously soaked with the lysozyme solution (4mg/ml in 10mM Tris-HCl, 1mM EDTA pH 8.0), for 30-45 minutes. The DNA was denatured by placing the filter onto three sheets of Whatman 3MM paper soaked with 1% SDS and 1M NaOH for 20 minutes. The excess liquid was then removed by placing the filter on a dry sheet of Whatman 3MM paper, followed by transferring the filter onto 3 sheets of Whatman 3MM paper, soaked in neutralising solution (1.5M NaCl, 0.5M Tris-HCl, pH 7.5) for five minutes, this step was repeated three times. The filter was then rinsed briefly with ethanol and dried at room temperature, between two sheets of Whatman 3MM paper. Following drying, the filter was baked in a vacuum oven at 80°C for two hours. The DNA at this stage becomes fixed to the part of the filter originally occupied by the colony. The pre-hybridisation was carried out by placing the baked filter into a plastic bag, together with 5ml of the pre-hybridisation solution (Material section 7.4). The bag was sealed after removing air bubbles, and submerged in a 70°C water bath for 5-12 hours. The filters were then hybridised with nick-translated radio-labelled DNA prepared with either pSU8 or pSU5 (nick-translation was carried out as described in section 8.6.1). pSU8 probe was used to hybridise the DNA obtained from the transformants resulting from cloning experiment I, whereas pSU5 probe was used to hybridise those transformants resulting from cloning experiment II. The appropriate probe was denatured by boiling for 10 minutes and added to the filter together with a fresh pre-hybridisation solution (5ml), the bag was sealed as above and incubated submerged in a 70°C water bath overnight. Following incubation the filters were recovered and washed three times with 2xSSC and 0.5% SDS for five minutes each, at room temperature with gentle agitation, and two
times with 0.1xSSC and 0.1% SDS for 30 minutes each at 70°C. The filters were then left to dry and mounted for autoradiography, which was carried out by placing a sheet of X-ray film next to the samples, and intensification screen over the film. Then the mounted filters were inserted into a light-proof cassette and stored at -70°C for 2-3 days.

8.6.3 End-labelling of DNA with T4 Polynucleotide Kinase

Polynucleotide Kinase is used to transfer the Gamma-phosphate of ATP to a free 5'OH group in either DNA or RNA. The enzyme also has a phosphatase activity. Two reactions are, therefore, possible (Richardson, 1965; Maxam and Gilbert, 1980).

In the forward reaction, the enzyme catalyses phosphorylation following removal of 5'-terminal phosphates with alkaline phosphatase.

In the exchange reaction, the kinase catalyses the exchange of an existing 5'phosphate with the Gamma phosphate of ATP. The latter reaction has to be carried out in the presence of excess ATP and ADP. The enzyme requires magnesium ion as co-factor and dithiothreitol (DTT) or 2-mercapto ethanol as reducing agent. The enzyme is strongly inhibited by ammonium ions and the substrates should not be dissolved in or precipitated from buffer containing ammonium salts prior to treating with kinase.

Following the exchange reaction procedure, described by Arrand (1985), the HaeIII digested φX174 DNA was end-labelled.

Procedure

1.2ug DNA of φX174 (HaeIII digested) was dissolved in 25ul of exchange Kinase buffer (12mM MgCl2, 1mM DTT, 0.3mM ADP, 0.5μM ATP, 50mM imidazole-HCl, pH 6.4). 50uCi [gamma-P.32] ATP (Amersham 3000Ci/mmol) and 5 units of T4-polynucleotide Kinase were added. The reaction mixture was incubated at 37°C for 60 minutes. 3ul of the end-labelled material was taken off and treated in the same way used with S1 hybrid samples before loading onto the gel. The rest of the end-labelled material was kept in -70°C.
8.7 Measurement of Protein concentrations

The protein concentration was routinely measured by the Bradford method (Bradford, 1976), which involves the binding of Coomassie Brilliant Blue G-250 to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, so the protein concentration can be measured by the increase in the absorption at 595 nm. Bovine serum albumin (BSA) was used as the protein standard.

Preparation of protein reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of one litre, followed by filtration and kept in the dark.

Protein assay

Protein solution (BSA) containing 25, 50, 75, 100, 125 μg in a volume of up to 0.1 ml was pipetted into 12 x 100 mm test tube. The volume in the tube was adjusted to 0.1 ml with appropriate buffer. Five millilitres of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 minutes and before one hour in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The concentration of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.
8.8 Staining of PAG plates with protein stain

According to the manufacturer's instructions (LKB), the following solutions were prepared.

Fixing solution: 57.5g trichloroacetic acid and 17.25g sulphosalicylic acid, were added to 500ml distilled water.

Destaining solution: 500ml ethanol and 160ml acetic acid were mixed together. The resulting solution was diluted to 2 litres volume with distilled water.

Staining solution: 0.460g PAGE blue-83 (Electran) was dissolved in 400ml destaining solution

Preserving solution: 40ml glycerol was added to 400ml of the destaining solution.

Procedure

The PAG plates were placed into a tray containing fixing solution and left for 30-60 minutes. This solution precipitates the proteins and allows the Ampholines to diffuse out. Following fixation, the PAG plates were washed by placing them in destaining solution for five minutes. The destaining solution was removed and the PAG plates were stained for ten minutes at 60°C in staining solution, using a covered dish. The PAG plates were then destained with several changes of the destaining solution until the background was clear. The preservation was performed by placing the destained PAG plates in the glycerol preserving solution for one hour. The PAG plate was then mounted on a piece of Whatman 3MM filter paper, previously soaked with the glycerol preserving solution. The distance of each pI marker from the cathode side of the gel plate was measured, while the gel was still wet to avoid the errors caused by drying. The gel plate was then dried using the Gel Slab Dryer (Bio-Rad, model 224), and photographed.
8.9 Spectrophotometric assay of OXA-2 beta-lactamase

Enzyme activity was measured routinely using a spectrophotometric assay described by Samuni (1975). Beta-lactamase mediated hydrolysis was followed by measuring the rate of change in optical density at a wavelength which is characteristic of the substrate in question. This optimal wavelength was determined by comparing the relative absorption spectra from hydrolyzed and unhydrolyzed substrate (Holland, 1983). The wavelengths 240 and 263nm for benzylpenicillin and oxacillin respectively, represent the differential peaks between the two spectra.

Procedure

The assay was carried out using a Beckman model 24 U.V. spectrophotometer linked to a Beckman strip chart recorder and a Haake circulator for temperature regulation. For a typical assay, 2ml aliquots of 0.05M sodium phosphate buffer at pH 7.0 were dispensed into test and standard spectrophotometric cells. The buffer and substrates were kept at 30°C while the enzyme was kept in ice. A 0.5ml aliquot of the substrate, usually at a 2.5mg/ml concentration, was added to both cells and the contents of each cell were thoroughly mixed. The reaction was started when an appropriate volume of the beta-lactamase preparation was added to the test cell and thoroughly mixed into the solution by inverting the cell. Hydrolysis of the substrate in question was followed at the appropriate wavelength. The reaction temperature was maintained at 30°C.

8.10 Preparation of crude beta-lactamase

8.10.1 From Escherichia coli strains

The strain in question was inoculated into 10ml of prewarmed L-broth and incubated at 37 °C for approximately four hours. The starter culture was sub-cultured into 75ml of prewarmed L.broth in
a 250ml conical flask and grown overnight at 37°C on an orbital
shaker (MKV model, set at amplitude 5). The cells were harvested
and washed with an equal volume of 0.05M sodium phosphate buffer at
pH 7.0. The cells were then resuspended in 5ml of the same buffer
and sonicated (MSE sonicator) on ice for 1-2 minutes at
amplitude 3. The disrupted cell suspension was centrifuged
(MSE-High Speed 18) at 10,000 rpm for 20 minutes and the
supernatant was stored at 4°C.

8.10.2 From Streptomyces lividans strains

A spore suspension of the strain in question was prepared
and pregerminated as described previously (section 8.1.2 and
section 8.2). This was used to inoculate 75ml of prewarmed L.broth
in a 250ml conical flask containing a stainless steel spring and
grown overnight at 30 °C on an orbital shaker. The mycelium was
harvested by centrifugation and the supernatant was collected in
sterile container.

The crude exocellular beta-lactamase was precipitated from
the culture supernatant by adding solid ammonium sulphate at 60%
saturation level. The quantity of salt to be added was obtained
from the standard table listed in "Data for Biochemical Research",
2nd Ed., (Dawson et al., 1969). The salt was added slowly and in
portions to the culture supernatant which was maintained at 4°C.
The mixture was stirred gently until all the salt had dissolved and
was left at 4 °C overnight. Following storage at 4°C, the mixture
was centrifuged at 12,000 rpm for 30 minutes. The supernatant was
decanted and the precipitate was redissolved with 3ml 0.05M sodium
phosphate buffer.

The endogenous enzyme was extracted by disrupting the
mycelium, after it had been washed twice with 0.05M sodium
phosphate buffer and resuspended in 3ml of the same buffer, by
sonication on ice for 3-10 minutes. Followed by centrifugation at
10,000 rpm for 20 minutes and the supernatant was stored at 4°C.
8.11 Purification of OXA-2 beta-lactamase

The crude preparation of OXA-2 beta-lactamase was purified by affinity chromatography on boronic acid gel according to the method reported by Cartwright and Waley (1984), except that 0.5M sodium acetate was substituted for 0.5M sodium chloride in both the loading and the elution buffers since chloride-ions inhibit OXA-2 beta-lactamase.

The L-boronic acid affinity gel was made as follows: 20ml of Affigel 10 (Bio Rad) was washed with propan-2-ol and then water at 4 °C and transferred to 20ml of 1M KHCO3 containing 2g of m-aminophenyl-boronic acid hemisulfate (Aldrich Chemical Co.) and 2g of sorbitol. The suspension was agitated at room temperature for one hour, the pH being maintained at 8.0 with solid KHCO3.

Using a mini column containing 5ml of the L-boronic acid affinity gel previously washed with the loading buffer (20mM triethanolamine hydrochloride buffer, 0.5M sodium acetate pH 7.0), the crude preparation of OXA-2 enzyme diluted in 5ml of the loading buffer, was loaded into the column. Followed by extensive washing with loading buffer until the absorption at 280nm of washings was zero. The beta-lactamase was then eluted with 0.5M borate/0.5M sodium acetate pH 7.0. The column was regenerated by washing with this borate buffer, and stored at 4 °C to be used for the next sample.

8.12 Photography

All photographs, except the polaroids, were taken with a OM-10 Olympus camera fitted with an 50mm Macrolens. The polaroid photographs were taken with an MP4 Land camera using a polaroid positive/negative 4x5 Land film type 665.
8.13 DNA sequencing

8.13.1 Transformation of recombinant M13RF molecules into E. coli JM101 strain

JM101 is a derivative of E. coli K12 and is a host for growth of the single-stranded phage M13 and its recombinants (Messing et al., 1981). In doing transformation, it is necessary to ensure that these cells have not lost the plasmid necessary for F-Pilus synthesis, as this structure is necessary for M13 infection. Since this plasmid also carries a gene coding for proline synthesis (while the host cell has a proline gene deletion), selection by growth on glucose/minimal medium plates can be used. This will allow the growth only of those cells bearing the plasmid.

To make the competent cells, a single colony from a glucose minimal medium plate was picked and grown overnight in 10ml 2xYT medium with shaking at 37°C. 2ml of this overnight culture was used to inoculate prewarmed 40ml 2xYT broth, followed by incubation with shaking at 37°C for three hours. At the same time a prewarmed 20ml of 2xYT broth was inoculated with one drop of overnight culture and incubated at 37°C, to provide fresh cells which will be ready for use at the plating out stage.

At the end of the incubation period the cells were gently spun down from the 40ml culture using the MSE-Chilspin bench centrifuge at 3000 rpm for 5 minutes. The cells were resuspended in 20ml of sterilised 50mM CaCl2, pre-chilled on ice, and left on ice for 20 minutes. Then the cells were spun down as above and resuspended in 4ml cold 50mM CaCl2. These competent cells were kept on ice until used for transformation, but not more than four hours.

Aliquots of 0.3ml of competent cells were transferred to sterile 1.5ml microcentrifuge tubes and kept on ice, two tubes for each ligation. Then 5ul of the ligated DNA and controls was added per tube of competent cells and kept on ice for 40 minutes. The cells were heat shocked at 42°C for 3 minutes and then all the
tubes were returned to ice bath.

To each tube 10μl of 100mM IPTG (Isopropyl-Beta-D-thio-galactopyranoside), 30μl of 2% X-gal (5-bromo-4-Chloro-3-indolyl-Beta-galactoside) in dimethyl formamide and 100μl of fresh E. coli JM101 cells was added and mixed by pipetting and quickly the contents of each tube were transferred to a 3ml of molten H top agar, mixed by rolling and poured immediately onto a prewarmed (37°C) 2xYT agar plate. All the plates were left at room temperature to set, then the plates were inverted and incubated at 37°C overnight.

8.13.2 Preparation of single-stranded DNA template

Infected cells from recombinant colourless plaques were grown up to produce single-stranded template for the sequencing reaction. Each single colourless plaque was picked up, using a cocktail stick, and used to inoculate 1.5ml of diluted (1:100 warm 2xYT) E. coli JM101 overnight culture. The cultures were grown with vigorous shaking at 37°C for 6-8 hours. Followed by separating the supernatants out of the cells using the Eppendorf centrifuge for 5 minutes. The supernatants were transferred to fresh microcentrifuge tubes and recentrifuged as above, to ensure that all cells were removed. 250μl of 27% polyethylene glycol 6000 (PEG) in 3.3M NaCl was added to each supernatant, (1.3ml), obtained after the second centrifugation step, and mixed by inverting the tubes. The mixtures were left to stand at room temperature for 15 minutes, then phage particles collected following two centrifugation steps to remove all PEG traces. The pellets were resuspended in 100μl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 50μl of phenol mix (100g Analar-phenol + 100ml Chloroform + 4ml Isoamylalchohol + 0.1g 8 hydroxy-quinoline - equilibrated with TE) was added and mixed by vortexing for 15 seconds. The tubes were left to stand for 15 minutes at room temperature, then vortexed for a further 15 seconds, and the two layers were separated by centrifugation for 3 minutes. The aqueous (upper) layer was transferred to a fresh
microcentrifuge tube containing 10ul of 3M sodium acetate pH 6.0, then 250ul ethanol was added. All tubes were left overnight at -20 °C to precipitate the DNA. After ethanol precipitation, the pellet was redissolved in 100ul of TE buffer. To make the resulting viral DNA pure enough for sequencing reaction, 1ul of proteinase K (5ug/ml) was added to the DNA solution and left for 20 minutes at room temperature, followed by a phenol extraction and ethanol precipitation as above. Finally the DNA pellet was redissolved in 25ul TE buffer and kept at -20°C. The above procedure was adapted from the method described by Messing and Vieira (1982).

To check the quality and quantity of the prepared template DNA a 2ul aliquot was run on a 1% agarose minigel. One main band was clearly visible, with another minor band slightly ahead of it.

The single-stranded DNA template is ready to prepare the sequencing reaction.

8.13.3 Sequencing reaction

1 - Annealing

Using a microcentrifuge tube for each sample, mixed by spinning, 5ul single-stranded DNA template, 1ul M13 primer (17 nucleotide, from Celltech) 1ul 10X annealing buffer (100mM Tris-HCl, pH 8.5, 50mM MgCl2) and 3ul of sterile double-distilled water. The annealing reactions were incubated in a laboratory oven at 60 °C for 60-90 minutes, in tightly capped tubes. Followed by a brief spin to collect the samples to the bottom of the tubes.

2 - Sequencing reaction

Materials

(i) Stock dNTPs and ddNTP nucleotides (purchased from Boehringer Mannheim - BCL) were made in water at 10mM, and kept at -20°C.
(ii) dNTP buffer: 10mM Tris-HCl, 1mM EDTA pH 8.0.

(iii) Working solutions, kept at -20°C:
   a). 0.5mM dNTPs prepared by diluting the 10mM dNTPs stock with water.

   b). dNTP* Mixes are made volume in ul.

<table>
<thead>
<tr>
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<th>T*</th>
<th>C*</th>
<th>G*</th>
<th>A*</th>
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<tbody>
<tr>
<td>0.5mM dTTP</td>
<td>1</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>0.5mM dCTP</td>
<td>20</td>
<td>1</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>0.5mM dGTP</td>
<td>20</td>
<td>20</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>dNTP buffer</td>
<td>20</td>
<td>20</td>
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<td>20</td>
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</table>

   c). Chase = Mixture of the four dNTPs in 0.25mM as final concentration.

   d). ddNTP: These can be altered according to requirement, but for example the concentrations used for running a short gel (90 minutes) were:

   ddATP  0.1mM
   ddCTP  0.1mM
   ddGTP  0.4mM
   ddTTP  1.0mM

   and those for a longer gel up to 4 hours running were:

   ddATP  0.05mM
   ddCTP  0.05mM
   ddGTP  0.2mM
   ddTTP  0.5mM

   and those for very long gels up to 6 hours running were

   ddATP  0.05mM
   ddCTP  0.03mM
   ddGTP  0.15mM
   ddTTP  0.5mM
e) dNTP/ddNTP mix: to each dNTP* mix, an equal volume of the corresponding ddNTP working solution was added, for example:
50ul A* + 50ul ddATP
50ul C* + 50ul ddCTP
50ul G* + 50ul ddGTP
50ul T* + 50ul ddTTP

f) Formamide dye mix: This was made with 100ml formamide that has been deionised by stirring gently with 5gm of Amberlite MB1 (BDH) ion exchange resin for 30 minutes. The resin was removed by filtration. Then 30mg Xylene cyanol FF, 30mg bromophenol blue and 0.75g EDTA were added. This can be kept for one month.

Procedure

Before removing the annealed primer/template from the oven, a set of four microcentrifuge tubes for each template, were labelled using a colour code, T = green, C = blue, G = black, A = red. The dNTP/ddNTP mixes were dispensed into the appropriate tubes in 2ul aliquots, for example, 2ul of dTTP/ddTTP mix was added to the T tube, ...etc. The annealed primer/template was then removed out of the oven, allowed to cool, and spun briefly to bring any condensation to the bottom of the tube, 1 unit of Klenow (Amersham or BRL) was added which is 0.2-0.3ul depending on the enzyme batch.
10uCi of S35-dATP (Amersham, SJ264, 410 Ci/mmol, approximately 1.3ul) was added to each template, followed by briefly spinning to mix the component and as soon as possible, 2.7ul of each mix was dispensed into each T, C, G and A tubes. This was left for 20 minutes at room temperature. At the end of the incubation time, 2ul of Chase solution was added to each tube; the tubes were spun and left for a further 15 minutes. At this stage, if the reactions were not to be electrophoresed immediately, the contents of each tube were divided into half and stored at -20°C. If only one gel was to be run, then half of the tube contents were stored at -20°C for up to a period of 14 days. To the second half of the samples, 2ul of
formamide dye mix was added. Tubes containing the dye were spun and boiled for 3 minutes (ensuring that they were tightly capped) in batches of 8 tubes at a time. These were taken out and loaded onto the gel with a drawn out plastic tip (3.2ul) as the next lot of tubes were boiled. Rapid loading onto the gel was advisable to prevent the formation of secondary structure in the DNA. Also prior to loading, it was important to flush out each well since urea diffuses out of the gel into the wells making the loading difficult.

3 - Preparation of the sequencing gel and running of the samples

The stock acrylamide was prepared as 38% acrylamide and 2% bis-acrylamide (NN'-methylene-bis-acrylamide) in water. The solution, 100ml, was stirred gently for 30 minutes with 5g mixed bed resin (Amberlite MB1-BDH), followed by filtration to remove the resin. This was stored in the dark at 4°C.

The 40 x 20cm glass plates were scrubbed with detergent and rinsed. They were rinsed with distilled water and allowed to dry. The notched plate, was wiped in the fume cupboard with Repelcote (2% dimethyl-dichlorosilane in 1,1,1-trichloroethane-BDH) and allowed to dry. The bottom plate (i.e. the unnotched plate) was wiped with a mixture consisting of 10ml ethanol, 150ul of 10% acetic acid, 30ul of gamma-methacryloxypropyl-trimethoxy-silane (sigma) in the fume cupboard and allowed to dry. Both plates were then thoroughly rinsed with ethanol and after drying were assembled together with Vinyl tape using Raven "Plasticard" as spacers.

7.5ml of stock acrylamide was taken and allowed to warm up to room temperature. This was added to 21g of Urea (ultrapure, although analar can be used) and 5ml of 10X TBE buffer (162g Tris-base, 27.5g boric acid, 9.5g EDTA in 1 litre distilled water, this gives a stock solution of pH 8.8). The final volume of the gel was made up to 50ml with sterile distilled water and the solution warmed at 37°C for the urea to dissolve. To this was added 300ul of freshly prepared 10% ammonium persulphate and 50ul of TEMED (NNN'N'-tetramethylethylenediamine). The gel mixture was poured
carefully using a 50ml syringe so that air bubbles were not introduced into the gel. The comb was inserted and the plates were clamped together using fold-back spring clips. The gel was then left to set for at least one hour. After that the tape was removed from the bottom of the gel which was clamped onto the gel apparatus (Raven scientific); the tanks were immediately filled with running buffer (1 x TBE). The gel was prerun for 10 minutes at 40 Watt with constant voltage (1.4KV). The samples were loaded as previously described and the gel run at the same voltage as above. For a short gel the running time was approximately 90 minutes, and for a long gel 270 minutes.

After the gel had been stopped, the top plate was removed and the gel fixed for 10 minutes in 10% methanol, 10% acetic acid. The gel was removed and fixed in a fresh solution as above. The gel was then rinsed under running water for about 5 minutes and dried in an oven at 80°C for one hour. The dried gel was left to cool and exposed to an X-ray film such as Kodak X-Omat or Fuji RX. The length of exposure for an S-35 gel could be estimated by passing a Beta-Geiger counter over the dried gel prior to exposure. An exposure of 2-3 days at room temperature for a reasonably hot gel was normal.
8.14  S1 nuclease mapping of transcribed DNA sequences

A modified procedure adapted from the methods described by Berk and Sharp (1977), Sharp et al. (1980), Maniatis et al. (1982), Burke (1984), Hopwood et al. (1985a).

Reagents

1. Hybridisation buffer (5X)
   0.2M PIPES (Piperazine-N,N’-bis [2-ethane-sulfonic acid])
   pH 6.4
   2.0M NaCl
   5 mM EDTA
   The solution was DEPC treated, autoclaved and dispensed into aliquots of 200ul in sterile, DEPC treated eppendorf tubes. To each tube 800ul Formamide, previously deionized with a mixed-bed resin (Amberlite MB1, BDH) was added, The tubes stored at -70°C. Each tube was thawed once only.

2. S1 digestion buffer
   0.28M NaCl, 0.05M sodium acetate pH 4.6, 4.5mM ZnSO4. This was made up, DEPC treated, autoclaved then 20ug/ml thermally denaturated Salmon sperm DNA was added. Stored in small aliquots (1ml) at -70°C and thawed once only.

3. S1 termination solution (stored at 4°C)
   2.5M Ammonium acetate
   0.05M EDTA

Procedure

1. The single strand labelled DNA probe, which was prepared on the day of the experiment (section 17.2), was dispensed into sterile, DEPC treated 1.5ml eppendorf tubes at 0.5ug DNA in each tube. The probe was denatured at 70°C and ethanol precipitated with 1ug of yeast tRNA (sigma) as carrier, then redissolved in 30ul hybridisation buffer.
2. RNA samples: *E. coli* HB101 0.5mg, *E. coli* JD348 0.8mg, *E. coli* NOA1 0.8mg, *S. lividans* NOA2 1mg, *S. lividans* TC73 0.6mg and *S. lividans* TK64 0.5mg, were ethanol precipitated and thoroughly dried. The pellet was dissolved by repeated pipetting in the solution derived from step 1.

3. The tubes were tightly capped and submerged up to their necks in 72°C water bath for 10 minutes.

4. The setting on the water bath was turned down allowing temperature to equilibrate slowly (over c. 30-60 minutes) to 57°C which is above the Tm of the DNA-DNA duplex. The tubes were incubated at this hybridisation temperature for 3-4 hours, keeping the lid on the water bath.

5. Each sample was treated sequentially, 300ul chilled S1 digestion buffer containing 280 units S1 nuclease forcibly dispensed, while the bulk of the sample tube was still submerged in the water bath, followed by immediate mixing on a vortex mixer and placed in ice.

6. The sample tubes were incubated at 45°C for 45 minutes. The digestion was terminated by adding 75ul of S1 termination solution with vortex mixing. 10ug carrier tRNA was added and mixed by vortexing. 400ul isopropanol was added and the tubes were left for 5 minutes at room temperature. The tubes were spun for 5 minutes and the pellets were rinsed in 80% ethanol and dried.

7. The pellets were redissolved in 5ul formamide dye mix. S1 hybrids were denatured by boiling for three minutes, and quickly loaded onto polyacrylamide-Urea thin sequencing gel (Hentschel et al., 1980) using the end labelled φX174 (Hae III fragments) as marker. The end labelling was carried out as described in section (8.6.3). The glass plates and the gel were prepared exactly as described in sequencing, section (8.13.3), except using smaller plates (20 x 20cm) and the slab gel apparatus which is suitable to this size.
8. The gel was run at 40 Watts, with constant voltage (0.9KV), for 40 minutes. Then the gel was fixed, dried and exposed to an X-ray film, as it had been done to the normal sequencing gel. The exposing time was 18 hours only.
Section 9 Cloning strategies of OXA-2 beta-lactamase gene (bla)

The OXA-2 bla gene was obtained from pSU5 and pSU8 plasmids, which are different recombinant plasmids containing a fragment derived from the R. factor R46, whose restriction map was published by Brown and Willetts (1981).

The plasmid pSU5, was constructed by inserting the Bgl II fragment of R46 which carries the ampicillin resistance gene into the BamHI site of pED815 vector, a derivative of pBR325 in which the ampicillin resistance (TEM beta-lactamase) gene has been inactivated by a deletion around the PstI site (N. Willetts, unpublished). The insert in pSU5 was found to have suffered a deletion which did not appear to affect the bla-gene.

In other work, in order to get a smaller recombinant plasmid, a BamHI-HindIII fragment of pSU5 was cloned by ligating with BamHI-HindIII digested pACYC184 to yield the plasmid pSU8 (Dale et al., 1985). Figure 10 illustrates the construction of pSU5 and pSU8 plasmids.

To test the expression and secretion of OXA-2 beta-lactamase in Streptomyces lividans, three different strategies were used, involving two different Streptomyces vectors, pIJ61 and pIJ702.

pIJ61 (Thompson et al., 1982a): It was constructed from SLP1.2 plasmid by the addition of a neomycin-resistance gene (aph, for aminoglycoside phosphotransferase) from Streptomyces fradiae ATCC 10745 and a gene (tsr) for thioestrepton resistance from Streptomyces azureus ATCC 14921, and deletion of two segments of non-essential SLP1.2 DNA. This plasmid has several sites suitable for DNA insertion; the BamHI and PstI sites are particularly useful because they lie within the coding sequence of the aph gene (Figure 11). Insertion of DNA at either of these sites, therefore, abolishes neomycin resistance.

pIJ702 (Katz et al., 1983): It is derived from the pIJ101
plasmid, so it has a high copy number (40-300 per chromosome), and can replicate in members of several diverse Streptomyces species. pIJ702 carries the tsr gene for vector selection and the mel gene coding for the enzyme tyrosinase, responsible for melanin synthesis. The cloning sites normally used are those for SphI, SstI or BglII which are all within the mel gene (Figure 12). Insertion of DNA at any of these sites destroys the function of the mel gene, so transformed colonies can easily be identified by their colour. Transformants harbouring the unaltered vector plasmid form black colonies when grown on tyrosine-containing regeneration media because they can convert tyrosine into melanin, whereas recombinant plasmids produce white colonies.

The plasmid DNAs, pSU5 and pSU8 were isolated from E. coli strains JD322 and JD348 respectively; those of pIJ61 and pIJ702 were isolated from Streptomyces lividans strains, TC73 and 3131 respectively. The isolation of plasmid DNA was carried out in accordance with the methods described in section (8.3.1) and (8.3.2). Aliquots of each DNA sample were run on a 1% Agarose gel to check for nuclease and chromosomal DNA contamination. Neither nuclease or chromosomal DNA contamination was detected; the DNAs, therefore, were ready to be used in the cloning experiments.
**Figure 10** Structure of R46 and derived plasmids. The top line represents the structure of R46 (51.7Kb), linearised at the *SmaI* site adjacent to the replication region of the plasmid. The broken line indicates the portion deleted in pKM101. The region containing the OXA-2 beta-lactamase gene is shown expanded in the lower part of the figure, together with the corresponding regions of pKM101, pSU5 and pSU8.

*uvp*, ultraviolet protection; *tra*, conjugal transfer functions; *As*, *Tc*, *Su*, *Sm*, *Ap*, resistance to arsenate, tetracycline, sulphonamide, streptomycin and ampicillin, respectively; *rep*, replication.

Restriction enzyme sites indicated: *Bgl*, *BglII*; *P*, *PstI*; *Hin*, *HindIII*; *E*, *EcoRI*; *Hpa*, *HpaI*; *B*, *BamHI*. 
Figure 11  Restriction map of the plasmid pIJ61 from Thompson et al. (1982).

Figure 12  Restriction map of the plasmid pIJ702 from Katz et al. (1983).
9.1 Cloning of OXA-2 bla gene into pIJ61 plasmid vector

An attempt was made to clone a segment of DNA containing the OXA-2 bla gene from pSU8 into the pIJ61 plasmid, previously cleaved with suitable restriction enzymes. The restriction digests of both pSU8 and pIJ61 DNA samples were set up as follows:

A: Restriction digest of pSU8 plasmid

100ul pSU8 DNA (11ug) + 35ul H2O + 15ul 10X MED buffer
+ 18 units BamHI

B: Restriction digest of pIJ61

30ul pIJ61 DNA (3ug) + 24 ul H2O + 6ul 10X MED buffer
+ 6 units BamHI

Digests A and B were incubated at 37°C for 90 minutes, after that 5ul of each digest was taken out and heat shocked at 65°C for 5-10 minutes to denature the BamHI enzyme, and run on a 1% agarose gel to check the completion of the digestion. Whereas the remaining samples were kept in ice. Both digest A and B were successful. The remaining parts of both digests, A and B, were then heat shocked at 65°C for 5-10 minutes, followed by phenol extraction and ethanol precipitation (section 8.4.1). The DNA of each sample was resuspended with 1x of High salt restriction buffer and 18 units of XbaI restriction enzyme was added, and incubated at 37°C for 90 minutes. Each digest was checked for completion as above. All digest A sample was then run on a 1% low-melting-temperature agarose gel, in order to separate the two resulting bands. The required band (c. 3.0Kb), containing the OXA-2 bla gene, was cut out of the gel and the DNA was recovered from the agarose in accordance with the method described in section (8.4.4).

Digest B was ethanol precipitated as in section (8.4.1) in order to get rid of the smaller BamHI-XbaI fragment. The DNA was resuspended in TE buffer (10mM Tris-HCl, 1mM Na2EDTA, pH 7.5).

The recovered BamHI-XbaI fragment of pSU8, was ligated with the digested pIJ61 DNA (digest B), in a 1:3 ratio of the insert.
to the vector. A set of controls was done to ensure the efficiency of the ligation. The ligation mixture and controls are listed in Table 2. All the ligation mixture and controls were incubated at 12°C overnight.

The transformation results will be represented together with that of the next cloning experiment.
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<tr>
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<th>Ligation Mixture (ul)</th>
<th>Controls (ul)</th>
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<tr>
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<tr>
<td><strong>BamHI-XbaI pSU8</strong> (smaller fragment)</td>
<td>20 (60 ng)</td>
<td>-</td>
</tr>
<tr>
<td><strong>BamHI-XbaI pIJ61</strong></td>
<td>6 (180 ng)</td>
<td>6</td>
</tr>
<tr>
<td>uncut pIJ61</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IOX Ligation Buffer</td>
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<tr>
<td>5 mM ATP</td>
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<td>T4-ligase</td>
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</table>

ATP = Adenosine triphosphate.
9.2 Cloning of OXA-2 bla gene into pIJ702 plasmid vector

In this attempt the BamHI-PstI fragment (c. 3.5Kb) of pSU5 which carries the OXA-2 bla gene, was ligated with the BglII-PstI digested pIJ702.

The restriction digests of both pSU5 and pIJ702 DNA samples were set up as follows:

A: Restriction digest of pSU5.
100ul pSU5 DNA (12ug) + 35ul H2O + 15ul 10X MED buffer
+ 18 units BamHI

B: Restriction digest of pIJ702.
50ul pIJ702 (5ug) + 13ul H2O + 7ul 10X MED buffer
+ 10 units BglII

Digests A and B were incubated at 37°C for 90 minutes. Following incubation, both digests were checked for the completion of the digestion by running a sample of each on a 1% agarose gel, as in the previous experiment. Both digests showed a single band, and no undigested material appeared in the gel. Then the remaining parts of both digests were heat shocked to inactivate the BamHI and BglII enzymes. 18 units of PstI restriction enzyme was added to both digests (A and B) without any previous ethanol precipitation, because the second enzyme can work in the same digestion buffer as the first enzyme. Both digests A and B were incubated at 37°C for a further 90 minutes and as described above, each digest was checked for the completion. Then both digests were heat shocked, digest A was run on a 1% LMT agarose gel to separate the two resulting bands. Then the required band (c. 3.5Kb) which carries the OXA-2 bla gene, was cut out of the gel, and the DNA was recovered from the agarose in accordance with the method described in section (8.4.4). Digest B was ethanol precipitated and resuspended in TE buffer.

The recovered BamHI-PstI fragment of pSU5, was ligated with the BglII-PstI digested pIJ702, in a 1:3 ratio of insert to the
vector. A set of controls was done to ensure the efficiency of the ligation. Ligation mixture and controls were set up as in Table 3. All ligation mixture and controls were incubated at 12°C overnight.
### Table 3 Ligation mixture and controls: Cloning of OXA-2 bla gene into pIJ702 vector

<table>
<thead>
<tr>
<th>Ligation Mixture (ul)</th>
<th>Controls (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

- **BamHI-PstI pSU5 (smaller fragment)**
  - Ligation Mixture (12 (1.2 ug))
  - Controls (ul): 1, 2, 3

- **BglII-PstI pIJ702**
  - Ligation Mixture (20 (0.4 ug))
  - Controls (ul): 20, 20, -

- **uncut pIJ702**
  - Ligation Mixture (-)
  - Controls (ul): -

- **IOX Ligation Buffer**
  - Ligation Mixture (5)
  - Controls (ul): 5, 5, 5, 5

- **5mM ATP**
  - Ligation Mixture (5)
  - Controls (ul): 5, 5, 5, 5

- **H2O**
  - Ligation Mixture (8)
  - Controls (ul): 20, 20, 35

- **T4-ligase**
  - Ligation Mixture (2)
  - Controls (ul): 2, -
The ligated DNA resulting from experiment I (section 9.1) and experiment II (section 9.2), and those of the controls, were introduced into c. $3 \times 10^9$ *Streptomyces lividans* TK64 protoplasts using the polyethylene glycol transformation procedure. The preparation of protoplasts and the transformation were carried out in accordance with the methods described in section (8.5.2).

0.1ml aliquots of the transformation mixes, were then plated out onto R2YE regeneration plates, which had been dried in a laminar flow cabinet for 2-3 hours. The plates were incubated at 30 °C. The transformants were detected by their acquisition of drug resistance (thiostrepton in this case). The drug resistance was detected by overlaying the regeneration plates with soft agar (0.6%) containing 50ug/ml thiostrepton at a suitable time after inoculation to allow for phenotypic expression (c. 20 hours).

Table 4 shows the number of thiostrepton resistant transformants obtained from cloning experiments, I and II. The results indicate that the cut, not ligated vectors (pIJ61 and pIJ702) gave no transformants; this means that the preparation of the vectors was very good, but some thiostrepton resistant colonies arose from the cut and religated vectors in a percentage of 15 and 2 of that resulted from the recombinant plasmids using pIJ61 or pIJ702 respectively. Therefore, most of the thiostrepton resistant transformants obtained on the non control plates are recombinants and might carry insert DNA.

These transformants were identified by different ways.
Table 4  Number of thiostrepton resistant colonies (tsr): Cloning of OXA-2 bla gene in pIJ61 and pIJ702 vectors.

<table>
<thead>
<tr>
<th>Cloning Experiments</th>
<th>Number of tsr colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>I  BamHI-Xbal pIJ61 + smaller</td>
<td>262</td>
</tr>
<tr>
<td>BamHI-Xbal pSU8 fragment</td>
<td></td>
</tr>
<tr>
<td>Controls:</td>
<td></td>
</tr>
<tr>
<td>1. pIJ61 cut, ligated</td>
<td>40</td>
</tr>
<tr>
<td>2. pIJ61 cut, not ligated</td>
<td>0</td>
</tr>
<tr>
<td>3. pIJ61 uncut</td>
<td>170</td>
</tr>
<tr>
<td>II BglII-PstI pIJ702 + smaller</td>
<td>3193</td>
</tr>
<tr>
<td>BamHI-PstI pSU5 fragment</td>
<td></td>
</tr>
<tr>
<td>Controls:</td>
<td></td>
</tr>
<tr>
<td>1. pIJ702 cut, ligated</td>
<td>76</td>
</tr>
<tr>
<td>2. pIJ702 cut, not ligated</td>
<td>0</td>
</tr>
<tr>
<td>3. pIJ702 uncut</td>
<td>contaminated</td>
</tr>
</tbody>
</table>
1. By Insertional inactivation:

The resulting transformants from using pIJ61 vector were difficult to identify by the insertional inactivation of the neomycin-resistance gene, because of the previous resistance to neomycin by the host organism. However, the insertion of the OXA-2 bla gene into the BglII site of pIJ702 causes insertional inactivation of melanin production. This can be detected by growing thiostrepton resistant transformants on tyrosine-containing media (see material, section 7.3). The transformants which carry the unmodified pIJ702 form black colonies, because they can convert tyrosine into melalin, while the recombinant plasmids produce white colonies, (Plate 1).

Table 5  Results of Insertional Inactivation of tyrosine gene

<table>
<thead>
<tr>
<th>Number of tested colonies</th>
<th>Mel-</th>
<th>Mel+</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>111</td>
<td>109</td>
</tr>
</tbody>
</table>

Table 5 shows that approximately half the number of the tested colonies are non-melanin producer. This indicates that the tyrosine gene has been inactivated, but this does not mean that all the Mel- colonies are carrying the required recombinant plasmid. They might carry a deleted and religated pIJ702 vector, and the deletion causes the inactivation of the tyrosinase gene.

The results also show a high number of non-recombinant (Mel+) colonies, higher than expected. This is probably due to
religation of the two BglII-PstI fragments of the vector pIJ702 which thus restored the Mel+ phenotype; or it may be possible that the insert has suffered a deletion and became too small to inactivate the tyrosine gene and therefore, the gene has expressed by reading through the insert.

Plate 1; Insertional inactivation of the Mel+ phenotype in Streptomyces lividans grown on MMT + thiostrepton. Black colonies (which are able to produce melanin from tyrosine) carry unmodified pIJ702; white colonies (melanin nonproducing) carry the recombinant pIJ702.
2. **By colony hybridisation:**

Due to the difficulties in screening of the thiostrepton resistant transformants obtained from cloning experiment I by insertional inactivation of neomycin resistance gene, and in order to find out whether the Mel- colonies (resulted from cloning experiment II) were carried the required recombinant plasmid; 220 thiostrepton resistant transformants from cloning experiment I and all of the Mel-(111) colonies were grown on nitrocellulose filters and lysed to release their DNA which was denatured and fixed to the part of the filter originally occupied by the colony.

pSU8 and pSU5 DNA was radiolabelled by nick-translation process and used as a probe to hybridise the homologous DNA. pSU8 probe was used to hybridise the DNA obtained from the transformants resulted from cloning experiment I, whereas pSU5 probe was used to hybridise the transformants resulted from cloning experiment II.

The colony hybridisation and DNA nick-translation was carried out in accordance with the methods described in sections (8.6.2) and (8.6.1).

![Table 6](image)
The results of experiment I (Table 6) showed a low percentage of positive colony hybridisation which may be because the clones were derived from the low copy number plasmid pIJ61, and also the transformants could not be screened by insertional inactivation of neomycin resistance gene. Therefore a random selection of the thiostrepton resistant colonies were tested by colony hybridisation. On the other hand, clones derived from pIJ702 showed a high percentage of positive spots; this is due to the screening of the thiostrepton resistant transformants by insertional inactivation of melanin production; in this way I could get rid of approximately half the number of thiostrepton resistant transformants, in addition, clones derived from the high copy number pIJ702 vector are more likely to be detected by colony hybridisation.

3. By Nitrocefin test:

Nitrocefin is a chromogenic substrate containing a beta-lactam ring; and allows rapid detection of beta-lactamase. Penicillinases and cephalosporinases hydrolyze the amide bond in the beta-lactam ring of nitrocefin. This reaction results in a distinctive colour change from yellow to red (Figure 13) (O'Callaghan et al., 1972).

Nitrocefin working solution was prepared, according to the manufacturer's instruction, by adding 0.5ml of dimethyl-sulphoxide (DMSO) to 5mg of solid nitrocefin. The compound dissolved immediately, 9.5ml of 0.05M sodium phosphate buffer pH 7.0 was then added, and shaken well to mix.

The test was carried out by growing the colonies to be tested on solid media (R2YE), then the nitrocefin solution, as 500ug/ml, dropped onto these colonies. The beta-lactamase producing colonies should turn red as well as the surrounding area within 10 minutes.

Unfortunately all the tested colonies showed no significant change in the colour of the nitrocefin, even after 2 hours,
compared with the parental strains (S. lividans TK64, S. lividans TC73, and S. lividans 3131). This indicates that either the OXA-2 beta-lactamase gene has not been expressed in the new host (S. lividans), or it has been expressed but inhibited by Chloride ions which are present as 50mM MgCl2.6H2O in R2YE media. It has been suggested that 15mM NaCl could inhibit 50% of the enzyme activity (Dale and Smith, 1974).

Figure 13 Reaction of the chromogenic cephalosporin-nitrocefin with beta-lactamase.
4. By restriction enzyme analysis:

The restriction enzyme analysis of the recombinant plasmid is the best way in which the inserted DNA can be identified. Six clones from the cloning experiment I, which showed positive spots with colony hybridisation, and 6 clones, Mel- and colony hybridisation positive, obtained from the cloning experiment II, were used for restriction enzyme analysis. The plasmids were isolated using the mini-lysis procedure as described in section (8.3.3).

The recombinant plasmids derived from pIJ61 vector were all the same size and showed the same pattern of restriction sites, when digested with HindIII, BamHI or EcoRI, as single digests; and when double digests were done e.g. BamHI/HindIII, BamHI/EcoRI, and EcoRI/XbaI; these plasmids were designated as pSU100. The restriction enzyme map of these plasmids is illustrated in figure 14, which shows the absence of the XbaI site and a deletion between BamHI and beyond the XbaI site compared with the restriction map of pIJ61 vector as illustrated by dotted line.

The recombinant plasmids derived from pIJ702, were in two different sizes, 4 clones were 4.9Kb in size designated pSU201, while the other two clones were 3.88Kb designated pSU202. These plasmids showed different patterns when digested with SalGI as single digest, this indicates that a deletion happened and caused the absence of two SalGI sites from the plasmid pSU202. The double digests such as BamHI/BglII and BamHI/PstI indicate that both BglII and PstI sites had disappeared and the deleted area was around these two sites as illustrated in figure 15 by a dotted line.

From overall restriction enzyme analysis we can deduce that either the colony hybridisation results were due to non-specific interactions, for example, detection of vector sequences or any other contaminated DNA; or the recombinant plasmids were highly unstable, so that insert was lost by the time restriction digests were done. Further more, the contamination with exonuclease could cut the vector back particularly in the case of pIJ702 and followed by religation. This deletion resulted in the inactivation of the tyrosinase gene and loss of melanin production.
Figure 14  Restriction enzyme analysis of the recombinant plasmid (pSU100) compared to the parental plasmid (pIJ61).
The positions of the following restriction enzyme recognition sites are indicated; H, HindIII; R, EcoRI; B, BamHI; X, XbaI.
Figure 15  Restriction enzyme analysis of the recombinant plasmid (pSU201, pSU202) compared to the parental plasmid (pIJ702).

The positions of the following restriction enzyme recognition sites are indicated; S, SalI; Bg, BglII; P, PstI; B, BamHI.
9.3 Construction of a shuttle vector by fusing pSU8 with pIJ61

One way of overcoming these problems is to create a shuttle vector, which is able to replicate in *Escherichia coli* as well as in *Streptomyces lividans*, because this has the advantage that manipulations can be carried out and characterised in *E. coli* before transferring the final product to *S. lividans*

A shuttle vector was therefore created by fusing an *E. coli* plasmid (pSU8), with the *S. lividans* vector pIJ61.

Both pSU8 and pIJ61 were digested with XbaI restriction enzyme as follows:

**A: Restriction digest of pSU8.**

100ul of pSU8 DNA (11ug) + 8ul H2O + 12ul 10XHi buffer
+ 15 units XbaI

**B: Restriction digest of pIJ61.**

100ul of pIJ61 DNA (10ug) + 8ul H2O + 12ul 10XHi buffer
+ 15 units XbaI

Digests A and B were incubated at 37°C for 60 minutes. Following incubation both digestes were checked for the completion of the digestion, by running a 5ul aliquot of each on a 0.8% agarose gel. Both digestes A and B were successful, and produced a single band indicating that they had been cut at their single XbaI site. The remaining parts of both digestes were heat shocked at 65°C for 5-10 minutes to inactivate the XbaI enzyme. Digest A, was further treated with calf intestinal alkaline phosphatase (CIP), to prevent intravector ligation, as described in section (8.4.3). Both digestes were, therefore ligated together using the ligation mixtures listed in Table 7. Two different ligation mixtures were utilised, and these differ in the relative concentrations of the donor and vector DNA. A set of controls has been made, and listed in Table 7 to ensure the efficiency of the ligation. All the ligation mixtures and controls were incubated at 12°C overnight.
<table>
<thead>
<tr>
<th></th>
<th>Ligation mixes (ul)</th>
<th>Controls (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pSU8 digest-CIP treated</td>
<td>10 (1ug)</td>
<td>10 (1ug)</td>
</tr>
<tr>
<td>pSU8 digest-NotCIP treated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pIJ61 - digest</td>
<td>10 (1ug)</td>
<td>30 (3ug)</td>
</tr>
<tr>
<td>pSU8 - uncut</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5mM ATP</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>10 x Ligation Buffer</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>H2O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T4-Ligase</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
The ligated DNA and controls were used to transform the CaCl$_2$-treated \textit{E. coli} HB101 cells, which are called competent cells. The preparation of competent cells and the transformation procedure was carried out in accordance with the methods described in section (8.5.1). The construction of the shuttle vector is illustrated in Figure 16. The transformation mixtures that were used are listed below.

**Transformation mixes:**

A = Ligation mix 1 + 200ul competent cells (\textit{E. coli} HB101)
B = Ligation mix 2 + 200ul competent cells
C = Control No. 1 + 200ul competent cells
D = Control No. 2 + 200ul competent cells
E = Control No. 3 + 200ul competent cells
F = Control No. 4 + 200ul competent cells
G = Control No. 5 + 200ul competent cells
H = 50ul TE buffer + 200ul competent cells

The transformants were selected for ampicillin resistance by plating out 0.1ml aliquots of the transformation mixes onto well dried L-agar plates containing 50ug/ml ampicillin, five replicates were made. The plates were incubated at 37°C for 24 hours.
Figure 16 Construction of pSU101 from the parental plasmids: pSU8, consists of a BamHI-HindIII fragment from R46 (□) carrying the OXA-2 beta-lactamase gene, inserted into pACYC184; the Streptomyces vector plasmid pIJ61 contains genes for thioestrepton resistance (ter, □□) and aminoglycoside phosphotransferase (aph, □□). The arrows indicate the direction of transcription of these genes. The positions of the following restriction enzyme recognition sites are indicated; R,EcoRI; X,XbaI; B,BamHI

108
### Table 8  Number of ampicillin resistant colonies:
Transformation of the shuttle vector in E. coli

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Transformation Mixes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A  B  C  D  E  F  G  H</td>
</tr>
<tr>
<td>1</td>
<td>25  7  3  1  &gt;300  17  UC  0</td>
</tr>
<tr>
<td>2</td>
<td>24  10 1  0  &gt;300  18  UC  0</td>
</tr>
<tr>
<td>3</td>
<td>15  17 2  1  &gt;300  15  UC  0</td>
</tr>
<tr>
<td>4</td>
<td>25  8  0  0  &gt;300  16  UC  0</td>
</tr>
<tr>
<td>5</td>
<td>20  12 1  0  &gt;300  18  UC  0</td>
</tr>
<tr>
<td>Total</td>
<td>109 54 7 2 &gt;1500 84 - 0</td>
</tr>
</tbody>
</table>

UC = Uncountable
Table 8 lists the colony counts obtained after plating out the transformation mixes A to H. The results indicate that the cut, CIP treated pSU8 plasmid vector (column D) gave only 2 colonies; this means that the vector has been completely linearised. The cut, CIP treated and religated vector (column C) gave very few colonies compared with that of the recombinant clones (columns A and B).

Of 109 ampicillin-resistant recombinant clones, 12 were tested for plasmid content and structure. Using the alkaline lysis procedure (section 8.3.3), the plasmids from these 12 clones were isolated. A 5μl aliquot of each sample was run on a 1% agarose gel to check the purity of the plasmid preparations, but unfortunately, two samples were contaminated with nuclease so the DNA was lost. Six other samples were not of the desired size because, two of them were very big as if more than the two DNA pieces have been ligated together, while the other four samples were very small; about the same size of pSU8 plasmid (data not shown). The remaining four samples were the expected size and were selected for restriction enzyme analysis. The four DNA samples were digested with XbaI restriction enzyme, at which site the fusion was performed. Following electrophoresis (data not shown) all the samples were shown to consist of two bands; one corresponded to pIJ61 band (XbaI digested), and the other corresponded to XbaI digested pSU8 band.

In order to determine the orientation of the fusion, the four samples were digested with HindIII restriction enzyme. Following electrophoresis, the four samples showed the same pattern of the bands, as illustrated in plate 2, this indicates that the orientation of the fusion was the same in all these four samples. One of these was selected for further study; designated pSU101 and the recombinant strain was called E. coli NOA1.
Plate 2  Restriction Enzyme analysis of four E. coli ampicillin resistant clones.

Track Number

1 = HindIII digest of lambda DNA

2, 3, 4, 5 = HindIII digest of the ampicillin resistant clones No. 1, 2, 3 and 4.

See figure 17 for a restriction map of pSU101. The sizes of the HindIII fragments have been estimated as 12, 5.4, 1.7, 1.4Kb.
Section 10  Construction of a restriction map of pSU101

A detailed restriction map of pSU101 was prepared in order to determine the orientation of the fusion of pSU8 with pIJ61. pSU101 DNA was isolated from strain *E. coli* NOA1 in accordance with the method described in section 8.3.1. This experiment was aided considerably by the availability of detailed restriction maps of both pSU8 and pIJ61 (figures 10 and 14). The restriction map was constructed from the information obtained by a series of single and double digests (as described in section 8.4.5), the digests were run on either 0.8% or 1% agarose gels. The size of the DNA fragments produced by restriction analysis were determined from a semilog plot of molecular size against mobility (mm), with reference to a series of standard size markers. The standards were usually HindIII and HaeIII digests of the bacteriophages, Lambda DNA and φX174 respectively.

Table 9 indicates the nature of the digests which were performed and the number and size of the fragments which were produced following restriction analysis. A series of preliminary single digests were performed including those which had known a single restriction site in the vector plasmid pIJ61.

A preliminary restriction map was constructed using the information obtained from these single digests. Further single and double digests were then selected with reference to the preliminary map in order to elucidate the complete restriction map. For example, a BclI digest indicated a single restriction site within pSU101. By selecting a double BamHI/BclI digest, the position of the BclI site was located within the larger of the two BamHI fragments.

Photographs of the two agarose gels featuring various restriction digests of pSU101 are shown in plates 3 and 4. Figure 17 illustrates the complete restriction map of pSU101, and the orientation of the fusion of pSU8 with pIJ61.
<table>
<thead>
<tr>
<th>Restriction digest</th>
<th>Fragments</th>
<th>Length in Kb</th>
<th>Restriction digest</th>
<th>No. of fragments</th>
<th>Length in Kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>XbaI</td>
<td>A</td>
<td>13.50</td>
<td>HindIII/XbaI</td>
<td>A</td>
<td>6.60</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoRI</td>
<td>A</td>
<td>13.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>A</td>
<td>16.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BglI</td>
<td>A</td>
<td>20.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BglII</td>
<td>A</td>
<td>20.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HindIII</td>
<td>A</td>
<td>12.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SalGI</td>
<td>A</td>
<td>9.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.70</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>2.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>1.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.95</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoRI/HindIII</td>
<td>A</td>
<td>7.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.40</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.80</td>
<td></td>
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<tr>
<td></td>
<td>D</td>
<td>3.00</td>
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</tr>
<tr>
<td></td>
<td>E</td>
<td>1.45</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>F</td>
<td>1.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BamHI/HindIII</td>
<td>A</td>
<td>5.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Kb = Kilobases

* This fragment was too small to be identified by restriction analysis on agarose gels. This value has been calculated according to the total sum of the fragment sizes subtracted from the 20.5Kb value for the composite pSU101 plasmid.
List of the DNA samples run on the Agarose gels illustrated in plates 3 and 4

Plate 3:

Track number

1 = \text{HindIII digest of Lambda DNA}.
2 = \text{XbaI digest of pLI61}.
3 = \text{XbaI digest of pSU8}.
4 = \text{HindIII digest of pLI61}.
5 = \text{HindIII digest of pSU8}.
6 = \text{XbaI digest of pSU101}.
7 = \text{HindIII digest of pSU101}.
8 = \text{EcoRI digest of pSU101}.

Plate 4:

Track number

1 = \text{HindIII digest of Lambda DNA}.
2 = \text{XbaI digest of pLI61}.
3 = \text{XbaI digest of pSU8}.
4 = \text{XbaI digest of pSU101}.
5 = \text{HindIII digest of pLI61}.
6 = \text{HindIII digest of pSU101}.
7 = \text{HindIII/XbaI digest of pSU101}.
8 = \text{EcoRI digest of pSU101}.
9 = \text{EcoRI/XbaI digest of pSU101}.
10 = \text{EcoRI/HindIII digest of pSU101}.
11 = \text{BamHI digest of pSU101}.
12 = \text{BamHI/XbaI digest of pSU101}.
13 = \text{EcoRI digest of pSU8}.
Plate 3.

Plate 4.
Figure 17 Restriction map of pSU101.
To test the expression and secretion of OXA-2 beta-lactamase in *Streptomyces lividans*, pSU101 DNA was isolated from *E. coli NA01* in accordance with the method described in section (8.3.1), and 1ug of this DNA was transformed into c. 4 x 10^9 *S. lividans* TK64 protoplasts. The preparation of protoplasts and transformation procedure were carried out in accordance with the methods described in section (8.5.2). 0.1ml aliquots of the transformation mixture were plated out onto well dried R2YE agar plates, five replicates were made, and incubated at 30°C. The transformants were selected for thiostrepton resistance by overlaying the regeneration plates with soft agar (0.6%) containing 50ug/ml thiostrepton at suitable time (20 hours) after inoculation. The plates were then incubated for a further 2 days.

The number of thiostrepton resistant transformants was uncountable; six of them were tested for the plasmid content and structure. The plasmids were prepared from these clones using the alkaline mini-lysis procedure as described in section (8.3.3). Digestion of the resulting DNA samples with the endonuclease *XbaI*, indicated that all of them were at the same size as the original pSU101 plasmid (data not shown). This means no deletion happened during the transformation process or inside the new host, which has been designated as *S. lividans* NOA2.
11.1 Testing the beta-lactamase production from *S. lividans* NOA2 in different liquid media

Three different liquid media were tested for the beta-lactamase production from *S. lividans* NOA2, these are L-broth, Brain-Heart-Infusion broth and peptone broth (Material section 7.3). Crude beta-lactamase from each culture was prepared in accordance with the method described in section (8.10.2). Each preparation was then assayed for enzyme activity using the spectrophotometric technique, as described in section (8.9), and the chromogenic beta-lactam nitrocefin as substrate.

As shown from the data in Table 10, the expression of OXA-2 beta-lactamase gene in *S. lividans* NOA2 grown in L-broth was very high compared to that when grown in Brain-Heart and peptone broth. The enzyme was almost entirely secreted into the supernatant, with only 4% intracellular activity, shown by disrupting the mycelium by ultrasonication.

The expression of the OXA-2 beta-lactamase gene was much less when the *S. lividans* NOA2 was grown in Brain-Heart-Infusion broth, and it was still predominantly secreted into the supernatant, with approximately 22% intracellular activity; but this level of expression was too low to be determined accurately. The level of expression of the OXA-2 beta-lactamase gene in peptone broth was almost undetectable.

The low level of beta-lactamase excretion in brain-heart and peptone broth may be due to the inhibitory effect of the chloride ions, as these media contain 8.6mM NaCl and 6.0mM KCl respectively.

According to these results, L-broth was used as the best medium for beta-lactamase production from *S. lividans* NOA2.
Table 10  OXA-2 beta-lactamase activity from S. lividans NDA2 grown in different liquid medium.

<table>
<thead>
<tr>
<th>Media</th>
<th>Enzyme activity (Arbitrary Units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture filtrate</td>
</tr>
<tr>
<td>L-broth</td>
<td>7.50</td>
</tr>
<tr>
<td>Brain-Heart-Infusion broth</td>
<td>0.34</td>
</tr>
<tr>
<td>Peptone broth</td>
<td>0.03</td>
</tr>
</tbody>
</table>
11.2 Analysis of the level of beta-lactamase production by strains NQA1 and NQA2 as compared with the control JD348 strain

A more detailed analysis of the level of enzyme production by the cloned strains, \textit{E. coli} NQA1 and \textit{S. lividans} NQA2 (pSU101) in comparison with the control strain \textit{E. coli} JD348 (pSU8), was carried out. Crude enzyme preparations were made from these strains in accordance with the methods described in sections (8.10.1) and (8.10.2). The measurement of protein concentration was performed as described in section (8.7). Enzyme activity was determined by spectrophotometric assay with the chromogenic substrate, nitrocefin, as described in section (8.9).

The results listed in Table 11 shows that OXA-2 expression from pSU101 in \textit{E. coli} was unexpectedly more than double that of pSU8 in \textit{E. coli}. This may be due to pSU101 having a higher copy number than pSU8. A direct measurement of the copy number of both the control pSU8 plasmid and pSU101 was not made, but the high concentration of pSU101 plasmid DNA compared to that of pSU8 obtained from a similar culture volume might reflect a higher copy number for pSU101 (data not shown).

Expression in \textit{S. lividans} was much lower, indicating probably some inefficiency in promoter recognition or to a difference in plasmid copy number. However, whereas in \textit{E. coli} the enzyme was almost wholly cell-associated, with \textit{S. lividans} it was located almost entirely in the culture supernatant. Virtually no significant nitrocefin-hydrolyzing activity was detected with either cell extracts or culture supernatants of the parental \textit{S. lividans} TK64. The beta-lactamase activity detected was, therefore, most likely to be mediated by the recombinant plasmid, but the possibility remained that it was due to depression of an endogenous enzyme.
<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Host</th>
<th>Plasmid</th>
<th>Enzyme level unit/ml</th>
<th>Relative activity</th>
<th>Protein mg/ml</th>
<th>Specific activity U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD348</td>
<td>E. coli HB101 culture filtrate cell extract</td>
<td>pSU8</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>NOA1</td>
<td>E. coli HB101 culture filtrate cell extract</td>
<td>pSU101</td>
<td>92.0</td>
<td>234</td>
<td>38</td>
<td>2.4</td>
</tr>
<tr>
<td>NOA2</td>
<td>S. lividans TK64 culture filtrate cell extract</td>
<td>pSU101</td>
<td>7.5</td>
<td>19</td>
<td>3.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>S. lividans TK64 culture filtrate cell extract</td>
<td>None</td>
<td>0.15</td>
<td>0.4</td>
<td>0.4</td>
<td>0.375</td>
</tr>
<tr>
<td></td>
<td>S. lividans TK64 culture filtrate cell extract</td>
<td></td>
<td>0.02</td>
<td>0.05</td>
<td>4.5</td>
<td>0.0044</td>
</tr>
</tbody>
</table>
Section 12 Characterization of the enzyme obtained from the cloned strains

In order to demonstrate that the secreted enzyme from *S. lividans* NOA2 was indeed the OXA-2 beta-lactamase I was able to take advantage of several unusual features of the OXA-2 enzyme, in which it is different from nearly all other known beta-lactamases; namely its ability to hydrolyze oxacillin at a higher rate than benzylpenicillin and its high pi value.

12.1 Substrate profile study

Crude enzyme preparations from each of the cloned strains carrying the hybrid plasmid pSU101 and that from the control, carrying pSU8 plasmid, were used in this experiment. Benzylpenicillin and oxacillin were chosen as differentiable substrates.

Using a spectrophotometric assay (section 8.9) I have verified that the enzyme from the *S. lividans* (pSU101) culture filtrate was indeed able to hydrolyze oxacillin, at a rate approximately three times that of benzylpenicillin, as shown in Table 12.

The enzyme specified by the plasmid pSU101 in *E. coli* showed a lower oxacillin/benzylicillin hydrolyzing ratio than that resulted from pSU8 in *E. coli*. This leads to a suggestion that pSU101 produced a second beta-lactamase which has the ability to hydrolyze benzylpenicillin rather than oxacillin.

Therefore the determination of the pi value of this enzyme was necessary.
<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Host</th>
<th>Plasmid</th>
<th>Molar substrate specificity profile</th>
<th>Oxacillin/Benzyl penicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD348</td>
<td>E. coli HB101</td>
<td>pSU8</td>
<td>0.0154 0.085</td>
<td>5.92</td>
</tr>
<tr>
<td>NOA1</td>
<td>E. coli HB101</td>
<td>pSU101</td>
<td>0.11 0.217</td>
<td>1.97</td>
</tr>
<tr>
<td>NOA2</td>
<td>S. lividans TK64</td>
<td>pSU101</td>
<td>0.012 0.033</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>culture filtrate cell</td>
<td></td>
<td>0.00016 0.000236</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>S. lividans TK64</td>
<td>None</td>
<td>0 0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>culture filtrate cell</td>
<td></td>
<td>0 0</td>
<td>0</td>
</tr>
</tbody>
</table>

Activity of each enzyme (micromoles of substrate hydrolyzed per minute per microlitre) was measured against the different substrates. All values were obtained with crude enzyme preparations.
12.2 Isoelectric Focusing Study (IEF)

The technique of isoelectric focusing is an electrophoretic one, but instead of the separation being carried out at a constant pH, the separation is carried out in a pH gradient which is established between two electrodes and is stabilized by carrier ampholytes. In this technique proteins migrate until they align themselves at their isoelectric point (pI) at which a protein possesses no net overall charge and will therefore concentrate at this point as migration ceases. This technique was used to determine the pI value of the enzyme produced by the cloned strains with comparison to both commercial standards and crude and pure OXA-2 beta-lactamase preparations from E. coli.

The purification protocol utilised was as described in section 13.

The following enzyme samples were examined by isoelectric chromatography:

1. Pure and crude OXA-2 enzyme extracted from strain JD348 carrying the pSU8 plasmid (control).
2. Pure and crude enzyme extracted from strain NOA1 (E. coli) carrying the hybrid plasmid pSU101.
3. Crude enzyme preparation from both the culture filtrate and cell extracts of Streptomyces lividans NOA2, carrying the hybrid plasmid pSU101.
4. Crude enzyme preparation from culture filtrate of the parental strain S. lividans TC73 carrying the pIJ61 plasmid.
5. Crude enzyme preparation from culture filtrate of the host strain S. lividans TK64 plasmid free.

The crude beta-lactamase extracts were prepared in accordance with the methods described in section (8.10.1) and section (8.10.2).
**Isoelectric chromatograph procedure:**

Isoelectric chromatography was performed on LKB pre-prepared Ampholine PAG plates pH 3.5 to 9.5, mounted on an LKB multiphor fitted with a cooling system or connected to cool running tap water. The apparatus and PAG plates were constructed according to the manufacturer's instructions. The sample applicators were applied at positions approximately in the centre of the samples template. Standard pI markers (broad pI calibration kit pH3 to 10, Pharmacia) were spread onto the surface of the Ampholine PAG plate at the side of the samples. The chromatograms were run for one hour and thirty minutes at 1.4 Kilovolts, 30 Watts. The sample applicators were removed after 30 minutes to avoid the tailing of the samples, then the samples were refocused. At the end of focusing the samples were developed for enzyme activity using nitrocefin. A piece of Whatman 3MM paper was cut to the size of the gel and soaked in a nitrocefin solution (500ug/ml nitrocefin dissolved in 0.05M sodium phosphate buffer, pH 7.2). The plates were developed by laying the filter paper over the gel. The development time was dependent on the enzyme activity of the samples. Prior to development, the gels were mounted for photography.

Plate 5 illustrates the results of the isoelectric focusing gel after the samples have been developed for enzyme activity, the development time was six minutes. Each enzyme sample produces an isoelectric spectrum consisting of one main band (the band with the greatest intensity in the isoelectric spectrum) representing the pI of the sample and several minor or satellite bands which are typically found with the OXA-2 enzyme (Matthew et al., 1975). These are less apparent in some tracks due to lower loading levels. Tracks b and c are clearly identical; tracks d, e, f and g showing main bands at similar pI to the main band in tracks b and c.
Plate 5  Isoelectric focusing gel developed for enzyme activity with nitrocefin

Track number

a - *S. lividans* TK64 culture filtrate (plasmid free).
b - *E. coli* (pSU8), crude cell extract.
c - *E. coli* (pSU101), crude cell extract.
d - *S. lividans* (pSU101), crude culture filtrate.
e - *S. lividans* (pSU101), crude cell extract.
f - *E. coli* (pSU8), pure cell extract.
g - *E. coli* (pSU101), pure cell extract.
h - *S. lividans* TC73 (pIJ61) crude culture filtrate.

Development time 6 minutes.
A pH calibration curve from one particular chromatogram is illustrated in figure 18. This was constructed by staining the gel plate with protein stain (PAGE blue-83), and plotting the pH value of each pH marker (plate 6) against its distance in centimetres from the cathode side.

Staining of the gel plate was carried out in accordance with the method described in section 8.8.

A protein band is seen in the crude extract of E. coli (pSU101) (plate 6, track a) which corresponds to that seen with purified OXA-2 beta-lactamase (track i) as well as to the main band seen with nitrocefin stained gels. Therefore the expression level of pSU101 in E. coli is sufficiently high to detect by protein staining, unlike that of pSU8. The main bands of the samples b, c, d, e and f did not appear due to the low protein concentration.

The pH of each sample was determined by measuring the distance of the main band from the cathode side of the gel plate and finding the corresponding pH value of that distance from the pH calibration curve. The results were identical: in each case a major band at pH=8.6 was obtained, which is equivalent to that obtained by Holland (1983) for OXA-2 enzyme. The 8.6 pH value for the OXA-2 beta-lactamase is consistent with previously recorded behaviour of this enzyme. Dale (1970) reported that the OXA-2 enzyme was positively charged at pH 8.3, others have reported different pH values for OXA-2 enzyme, such as 7.3 (Matthew and Hedges, 1976), and 8.1 (Matthew et al., 1975).

From the isoelectric focusing study, it can be concluded: Firstly; the enzyme produced by both E. coli and S. lividans carrying the pSU101 is indeed the OXA-2 beta-lactamase: secondly; there is no evidence that any of these strains produce a significant level of any other beta-lactamase.
Figure 18  pI calibration curve, used to determine the pI of the OXA-2 enzymes.
Plate 6  Isoelectric focusing gel stained by protein stain used in the construction of the pI calibration curve

Track number

a - E. coli (pSU101), crude cell extract.
b - E. coli (pSU8), crude cell extract.
c and d - S. lividans (pSU101), crude culture filtrate.
e and f - S. lividans (pSU101), crude cell extract.
g - S. lividans TK64 (plasmid free), crude culture filtrate.
h - S. lividans TC73 (pIJ61), crude culture filtrate.
i - purified OXA-2 beta-lactamase.
j - Protein Markers, consisting of the following proteins:

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tyrosinogen</td>
<td>9.30</td>
</tr>
<tr>
<td>2. Lentil lectin-basic band</td>
<td>8.65</td>
</tr>
<tr>
<td>3. Lentil lectin-middle band</td>
<td>8.45</td>
</tr>
<tr>
<td>4. Lentil lectin-acidic band</td>
<td>8.15</td>
</tr>
<tr>
<td>5. Myoglobin-basic band</td>
<td>7.35</td>
</tr>
<tr>
<td>6. Myoglobin-acidic band</td>
<td>6.85</td>
</tr>
<tr>
<td>7. Human carbonic anhydrase</td>
<td>6.55</td>
</tr>
<tr>
<td>8. Bovine carbonic anhydrase</td>
<td>5.85</td>
</tr>
<tr>
<td>9. Beta-lactoglobulin A</td>
<td>5.20</td>
</tr>
<tr>
<td>10. Soy bean trypsin inhibitor</td>
<td>4.55</td>
</tr>
<tr>
<td>11. Amyloglucosidase</td>
<td>3.50</td>
</tr>
</tbody>
</table>

The blue precipitate appearing toward the anode side is due to the presence of other proteins in the crude preparation of the samples.
Section 13 Purification of OXA-2 beta-lactamase by affinity chromatography

Affinity chromatography is a powerful technique for the purification of almost any biomolecule based on its biological function or individual chemical structure. The technique is a form of absorption chromatography in which the substance to be purified is specifically and reversibly absorbed by a complementary binding substance. Cartwright and Waley (1984), showed that beta-lactamases which are inhibited by both boronic and borate ions can be purified from crude cell extracts on boronic acid affinity columns. Non specifically bound proteins are eluted with high ionic strength buffers whereas beta-lactamase can be specifically eluted with borate ions.

Crude beta-lactamase from the cloned (NOA1 and NOA2) and control (JD348) strains was prepared as described in section (8.10). Following extraction the samples were precipitated with ammonium sulphate at 60% saturation level and resuspended in 3ml of 0.05M sodium phosphate buffer pH 7.0. Using a mini column containing 5ml of L-boronic acid affinity gel and loading the crude beta-lactamase preparation, showed that OXA-2 enzyme bound specifically. No detectable activity came through, during the loading, but a lot of protein eluted. Following washing of the column, the enzyme was eluted with borate buffer. Enzyme elution was measured using the microtitre nitrocefin assay. The enzyme containing fractions were pooled and precipitated with ammonium sulphate at 60% saturation levels.

The preparation of L. boronic acid affinity gel, running and elution of the samples from the column were carried out at 4°C as described in section (8.11).

Table 13 indicates the purification of the OXA-2 enzyme achieved by affinity chromatography on L. boronic acid affinity gel. The results listed in this table show that the recovery of enzyme activity is low, possibly due to incomplete elution of the
sample. There was not enough purified material to measure the protein concentration in order to estimate the recovered enzyme specific activity; however, the absence of detectable bands on a protein stained isoelectric focusing gel indicates that most of the contaminating proteins have been removed.
Table 13  Purification of OXA-2 beta-lactamase by affinity chromatography.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Host</th>
<th>Plasmid</th>
<th>Enzyme activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sample applied</td>
</tr>
<tr>
<td>JD348</td>
<td>E. coli HB101</td>
<td>pSU8</td>
<td>39.4</td>
</tr>
<tr>
<td>NOA1</td>
<td>E. coli HB101</td>
<td>pSU101</td>
<td>92.0</td>
</tr>
<tr>
<td>NOA2</td>
<td>S. lividans TK64</td>
<td>pSU101</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Culture filtrate</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Cell extract</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Section 14  Study of the stability of pSU101 plasmid

In view of the possibility of earlier problems being due to plasmid instability and loss of integrity, it was necessary to look at the plasmid pSU101 in both E. coli and S. lividans strains.

14.1  Stability of pSU101 plasmid in E. coli

Strains used are: NOA1 = E. coli carrying pSU101
JD348 = E. coli carrying pSU8

The two strains were grown on L-agar plates containing 50μg/ml ampicillin at 37 °C overnight. A single colony of each strain was used to inoculate 10ml L-broth supplemented with 20μg/ml ampicillin. This starter culture was incubated at 37°C for 18 hours. To prepare the first subculture, an aliquot of 50ul from the starter culture was used to inoculate 10ml L-broth without antibiotic; this was incubated at 37°C for 18 hours with shaking (rolling tube, Denley Spiramix). The second subculture was prepared by inoculating 10ml L-broth free of antibiotic with 50ul aliquot from the first subculture. The subculturing was repeated daily up to 5 days. A ten fold dilution series was prepared at the end of each subculture, using L-broth. Aliquots of 100ul out of the appropriate dilution were plated out onto L-agar plates either supplemented with ampicillin as 20μg/ml or free of antibiotic. Three replicates for each dilution were made.

The ratios of ampicillin resistant colonies to the total number of the colonies grown on L-agar plates free of antibiotic were plotted against the number of subcultures. This is illustrated in figure 19(a) and figure 19(b), for pSU101 and pSU8 respectively. The results indicate that both plasmids pSU101 and pSU8 were stable even after 40 generations. This is not as rigorous a test as using extended periods of continuous culture, which has been done in many cases, but is sufficient for practical purposes.
Figure 19  Plasmid stability in *Escherichia coli*,
a - pSU101;  b - pSU8.
The integrity of both pSU8 and pSU101 plasmids was determined by isolating the plasmids from 24 ampicillin resistant isolates, resulted from the 5th subculture. Twelve of them were carrying pSU8 plasmid and the other twelve were carrying the pSU101 plasmid. The plasmids were isolated in accordance with the method described in section (8.3.3).

The restriction enzyme analysis showed that the pSU8 plasmid was totally intact and was the same size in all twelve isolates as the control pSU8 plasmid DNA (data not shown). Whereas the plasmid pSU101 has suffered multiple deletions and rearrangement, comparing with the control pSU101 DNA. Plate 7 illustrates the difference in the sizes between the original pSU101 DNA and that resulted after the fifth subculture which designated as pSU101B. pSU101B is a representative of nine plasmids which showed the same pattern of restriction digests, the plasmids of the other three isolates had been lost, because they were nuclease contaminated.

A detailed restriction map of pSU101B was prepared, in order to define the deleted region. Figure 20 illustrates that the deletion has occurred within the plJ61 plasmid and shows that the OXA-2 beta-lactamase gene was not affected.
Plate 7 Restriction enzyme analysis comparing pSU101B with pSU101

Track number

1 = \textit{HindIII} digest of Lambda DNA
2 = \textit{BamHI}/\textit{BglII} digest of pSU101B
3 = \textit{XbaI}/\textit{BglII} digest of pSU101B
4 = \textit{BamHI}/\textit{HindIII} digest of pSU101B
5 = \textit{BamHI}/\textit{BclI} digest of pSU101B
6 = \textit{SalGI} digest of pSU101B
7 = \textit{BamHI}/\textit{BglII} digest of pSU101
8 = \textit{XbaI}/\textit{BglII} digest of pSU101
9 = \textit{BamHI}/\textit{HindIII} digest of pSU101
10 = \textit{BamHI}/\textit{BclI} digest of pSU101
11 = \textit{SalGI} digest of pSU101
12 = \textit{HaeIII} digest of \(\Phi X174\) DNA
Figure 20  Linear representation of restriction map of pSU101 and pSU101B.  
Black box, pLJ61; Hatched box, pACYC184; Open box, R46 insert; Dotted box, deleted region.  
The positions of the following restriction enzyme recognition sites are indicated; H, HindIII;  
R, EcoRI; Bc, BclI; S, SalGI; Bg, BglII; Ba, BamHI; X, XbaI.
14.2 Stability of pSU101 in *Streptomyces lividans*

Strains used are: NOA2 = *S. lividans* TK64 carrying pSU101  
TC73 = *S. lividans* 66 carrying pIJ61

To ensure that the original inoculum was 100% plasmid carrier, a selection pressure of 50μg/ml thiostrepton in agar plates was used to prepare the starter inoculum.

**Procedure**

To yield well sporulating growth, stock spore suspensions of both strains, NOA2 and TC73, were inoculated over the entire surface of well dried, R2YE agar plates supplemented with thiostrepton as 50μg/ml. The plates were incubated at 30°C until sporulation occurred. A spore suspension was prepared from each strain as described in section (8.1.2). This was used as the starter inoculum.

To determine the effect of sporulation on the plasmids stability within the generated spores, ten fold serial dilutions of the starter inoculum of both types were prepared. Aliquots of 0.1ml of the appropriate dilutions were plated out onto well dried R2YE plates with and without selective agent (thiostrepton as 50μg/ml). The plates were incubated at 30°C for 2-3 days. The results showed that the pIJ61 plasmid was absolutely stable, and the percentage of the plasmid loss did not exceed 1%. The NOA2 strain showed an apparent 6% plasmid loss (pSU101), which is not high enough to be sufficient in terms of this experiment.

To study the stability of the plasmids, pIJ61 and pSU101, during repeated subculturing in liquid medium, 3ml of the starter inoculum was pregerminated, as described in section (8.2), and used to inoculate 50ml YEME medium, free of selective agent, in 250ml flasks containing a stainless steel spring. The flasks were incubated at 30°C for 24 hours with shaking (MKV, orbital shaker).
Following incubation 2ml of the culture was used to inoculate prewarmed 50ml YEME medium to prepare the second subculture. This technique was repeated daily, for five days. At the end of each subculture, a ten fold serial dilution up to $10^{-6}$ was prepared from each culture. Aliquots of 0.1ml from the appropriate dilutions were plated out onto well dried R2YE plates with and without selective agent, in order to measure the plasmid loss. All the plates were incubated at 30°C for 2-3 days.

The ratios of thiostrepton resistant colonies which possess plasmids to the total number of the colonies grown on non-selective R2YE plates, were plotted against the number of subcultures. This is illustrated in figure 21. The results indicated that both plasmids pIJ61 and pSU101 were stable even after 25 generations. The percentage of the plasmid loss at the end of the experiment was 13.4 and 8.5 for pIJ61 and pSU101 respectively, comparing with that of the first subculture. However, since the loss is not progressive, these figures probably over-estimate the real degree of plasmid instability.

To check the integrity of pIJ61 and pSU101 in S. lividans, restriction enzyme analysis was performed on both plasmids. Twelve thiostrepton resistant isolates obtained from the fifth subculture of each type (either carrying pIJ61 or pSU101), were picked and the plasmids were isolated, using the alkaline mini-lysis procedure as described in section (8.3.3).

The restriction enzyme analysis showed that pIJ61 was still intact comparing to the size of the control pIJ61 DNA (data not shown), while pSU101 had suffered multiple deletions and rearrangement. The plasmids that have been obtained from all twelve isolates showed the same pattern of restriction digests; two of them were used for map construction and designated as pSU101C and pSU101D. Plate 8, is a photograph of an agarose gel featuring various restriction digests of pSU101C and pSU101D compared with that of the original pSU101 DNA. Figure 22, shows the restriction
maps of both the original pSU101 and that of pSU101C, from which the deleted region can be defined. These maps show that the region carrying the OXA-2 beta-lactamase gene was not included within the deleted region.
Figure 21  Plasmid stability in *Streptomyces lividans*.

a - pSU101;  b - pIJ61.
Plate 8  Restriction enzyme analysis of pSU101C and pSU101D

Track number

1  =  HindIII digest of Lambda DNA
2  =  SalGI digest of pIJ61
3  =  SalGI digest of pSU101
4,5 =  SalGI digest of pSU101C and pSU101D respectively
6,7 =  XbaI digest of pSU101C and pSU101D respectively
8,9 =  BamHI/BglII digest of pSU101C and pSU101D respectively
10,11 = PstI/BglII digest of pSU101C and pSU101D respectively
12 =  HaeIII digest of φX174 DNA
Figure 22  Linear representation of restriction map of pSU101 and pSU101C.
Black box, pLJ61; Hatched box, pACYC184; Open box, R46 insert; Dotted box, deleted region.
The positions of the following restriction enzyme recognition sites are indicated; H, HindIII;
R, EcoRI; Bc, BclI; S, SalGI; Bg, BglII; Ba, BamHI; X, XbaI.
Identification of regulatory regions controlling transcription can be done by using special promoter-probe plasmids, e.g. pIJ424 for S. lividans and pKK 232-8 for E. coli.

In the construction of the promoter-probe plasmid pIJ424, the promoter-less Kanamycin phosphotransferase (aphII) gene from Tn5 has been used as indicator for promoter activity. This gene, when expressed, confers resistance to Kanamycin. The promoter-probe plasmid pIJ424, is a derivative of a high copy number, wide host-range plasmid pIJ101 (Hopwood et al., 1985a).

Figure 23(a) illustrates the restriction map of the plasmid pIJ424. The ribosome binding site and the in-frame stop codon lie between the BclII cloning site and the start codon of the aphII gene. Insertion of a DNA fragment containing promoter activity in the BclII site can activate the gene. The resultant transformants show resistance to Kanamycin. The level of resistance varies between 2ug/ml and more than 200ug/ml, depending on promoter strength and plasmid copy number.

The plasmid pKK232-8 is a pBR322-derived plasmid vector (Brosius, 1984). This plasmid is useful for the selection of bacterial and phage transcription promoters, because it contains a promoter-less chloramphenicol acetyltransferase (CAT) gene. This gene can be activated by the insertion of a promoter-containing DNA fragment.

Figure 23(b) illustrates the restriction map of the promoter-probe plasmid vector pKK232-8. Multiple cloning sites are positioned 5'-proximally to the resistance gene (CAT) to facilitate insertion of DNA fragments and subsequent excision for subcloning. This plasmid contains transcription terminators distal to the CAT gene which allow the cloning of strong promoters. An additional transcription terminator has been placed upstream from the multiple cloning site to prevent transcription originating from pBR322 promoters.
Both pIJ424 and pKK232-8 were used to determine the location of the OXA-2 beta-lactamase gene promoter.
Figure 23 Restriction maps of promoter-probe plasmid vectors.  
(a) pIJ424;  
(b) pKK232.8
15.1 Promoter-probe experiment in E. coli

The DNA fragment containing the OXA-2 beta-lactamase gene was isolated from pSU8 plasmid previously cleaved with BamHI and HindIII restriction enzymes. This DNA fragment was recovered from agarose gel using low-melting-temperature agarose as described in section (8.4.4).

The promoter-probe plasmid vector pKK232-8 was either isolated from E. coli strain ED8767 (kindly provided by Dr. T. Kieser) in accordance with the method described in section (8.3.1), or obtained as a commercial product from Pharmacia.

The BamHI-HindIII pSU8 fragment, carrying the OXA-2 beta-lactamase gene was Sau-3A digested and randomly cloned into the single BamHI site of pKK232-8. The restriction enzyme digests for both DNA were set up as follows:

A: Sau 3A digest of BamHI-HindIII pSU8 fragment
80ul of pSU8 fragment DNA + 10ul H2O + 10ul 10x MED restriction buffer + 5 units Sau-3A enzyme.

B: BamHI digest of pKK232-8
10ul of pKK232-8 (1.7ug) + 80ug H2O + 10ul 10x MED buffer + 4 units BamHI enzyme.

Digests, A and B, were incubated at 37 °C for 1 hour. Following incubation, both digests were heat shocked at 65 °C for 5-10 minutes to inactivate the enzyme. Each digest was checked by running a 5ul aliquot of each on a 1.2% agarose gel. Both digests, A and B, were successful; digest A produced three visible bands following electrophoresis, whereas digest B produced a single band, indicating that pKK232-8 had been cut at this single BamHI site. Half of digest B was further treated with calf intestinal alkaline phosphatase (CIP). Both digests were ligated together using the ligation mixture listed in Table 14. A set of controls was set up as described in Table 14.
| Ligation mixture | 
|------------------|------------------|
| Ligation mix (ul) | Controls (ul) |
|                  | 1   | 2 | 3 | 4 | 5 |
| Digest A         | 15 (60ng) | - | - | - | - |
| Digest B - CIP treated | 15 (150ng) | 5 | 5 | - | - |
| Digest B - Not CIP treated | - | - | - | 5 | 5 | - |
| pKK232-8 uncut   | - | - | - | - | - | 2 |
| 5mM ATP          | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| 10 x Ligation Buffer | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| H2O              | 2.0 | 27 | 27 | 27 | 27 | 30 |
| T4-Ligase        | 2   | 1  | - | 1 | - | - |

Table 14  Ligation mixture and controls: Promoter-probe experiment in E. coli.
The ligation mixture and controls were incubated at 12°C overnight. 20μl of the ligated DNA was then used to transform *E. coli* HB101. The preparation of competent cells from strain HB101 and the transformation procedure was carried out in accordance with the method described in section (8.5.1). The transformation mixtures that were used are listed below.

**Transformation mixes:**

A = ligation mix + 200μl competent cells (*E. coli* HB101).
B = control No.1 + 200μl competent cells.
C = control No.2 + 200μl competent cells.
D = control No.3 + 200μl competent cells.
E = control No.4 + 200μl competent cells.
F = control No.5 + 200μl competent cells.
G = 20μl TE buffer + 200μl competent cells.

The transformants were selected for ampicillin resistance by plating out 0.1ml aliquots of the transformation mixes onto well dried L-agar plates containing 50μg/ml ampicillin; three replicates were made. The plates were incubated at 37°C for 24 hours.

Table 15 lists the colony counts obtained after plating out the transformation mixes A to G on ampicillin containing media. The results indicate that the controls, column B and C, were satisfactory compared to the numbers of ampicillin resistant transformants in column A. Therefore, most of the ampicillin resistant transformants obtained on the non-control plates were recombinants and might carry insert DNA.

To investigate the expression of the chloramphenicol gene, 270 ampicillin resistant clones obtained from the transformation mix A and all the clones resulting from both transformation mixes, B and C (as control), were inoculated on well dried L-agar plates containing 20μg/ml chloramphenicol. The plates were incubated at
Table 15  Number of ampicillin resistant colonies obtained from promoter-probe experiment in *E. coli*.

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Transformation Mixes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>106</td>
</tr>
</tbody>
</table>

UC = Uncountable
37 °C for 24 hours. As expected, all the control clones failed to grow on the chloromphenicol containing media, whereas 40 out of 270 clones (14.8%) showed strong resistance to chloramphenicol, and another 22 clones (8.15%), showed weak resistance. The expression of the chloramphenicol gene (CAT) indicates that the inserted fragment into the promoter-probe vector (pKK232-8) has a promoter activity.

To determine the size of the inserted fragments, the plasmids were isolated from twelve of the strong chloramphenicol resistant clones. The isolation of the plasmid was carried out in accordance with the method described in section (8.3.3). The restriction enzyme analysis listed in Table 16, showed that inserts of different sizes, ranging from 0.05 to 2.0Kb, had been ligated with the promoter-probe plasmid vector. This could be done by measuring the increase in the size of the smaller PstI fragment of the plasmid pKK232-8.

Plate 9 illustrates the PstI digests of some chloramphenicol resistant clones compared with that of the vector pKK232-8.
Table 16 Sizes of inserted fragments in the promoter-probe pKK232-8

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Insert size in Kb</th>
<th>Clone number</th>
<th>Insert size in Kb</th>
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<tbody>
<tr>
<td>3</td>
<td>0.60</td>
<td>16</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>0.68</td>
<td>17</td>
<td>nuclease digested</td>
</tr>
<tr>
<td>8</td>
<td>0.25, 1.9</td>
<td>20</td>
<td>0.58</td>
</tr>
<tr>
<td>10</td>
<td>0.05</td>
<td>21</td>
<td>0.73</td>
</tr>
<tr>
<td>13</td>
<td>0.50</td>
<td>22</td>
<td>0.60</td>
</tr>
<tr>
<td>14</td>
<td>0.50</td>
<td>44</td>
<td>0.1, 2.0</td>
</tr>
</tbody>
</table>
Plate 9 Restriction digests of chloramphenicol resistant clones

Track number

1 and 16 = HindIII digests of Lambda DNA
2 = HindIII digest of pKK232-8
3 = PstI digest of pKK232-8
4, 6, 8, 10, 12 and 14 = HindIII digests of chloramphenicol resistant clones no. 3, 7, 8, 10, 13, 14 respectively.
5, 7, 9, 11
13 and 15 = PstI digests of the same above clones.
Promoter-probe experiment in Streptomyces lividans

The DNA fragment containing the OXA-2 beta-lactamase gene was prepared and digested with Sau 3A endonuclease exactly as described in the previous experiment (section 15.1). This digest was called digest A.

The promoter-probe plasmid vector used in this experiment is pIL424 which has been isolated from S. lividans strain TK492 (kindly provided by Dr. T. Kieser). The isolation of the plasmid was carried out in accordance with the method described in section (8.3.2).

The cloning was performed using the single Bglll site of the vector pIL424. The restriction enzyme digest of this vector was set up as follows:

**B: Bglll digest of pIL424**

200ul of pIL424DNA + 25ul H2O + 25ul 10x LOW buffer + 10 units Bglll enzyme.

The digest (B) was incubated at 37°C for 1 hour. Following incubation, 10ul sample of the digest was taken out and heat shocked at 65 °C for 5-10 minutes to inactivate the Bglll enzyme, while the remaining part was kept on ice. To ensure that the vector had been completely linearised at its single Bglll site, 1ul of PstI enzyme was added to the sample. The sample was further incubated at 37 °C for 1 hour, followed by running the sample on a 1% agarose gel. The digest was successful, it showed the three expected bands, with the absence of the very small one (0.213Kb) which has run out of the gel. This indicates that the vector pIL424 had been cut at its single Bglll site. The remainder of digest B was heat shocked at 65°C for 5-10 minutes, half of it was further treated with calf intestinal alkaline phosphatase (CIP). Digest A and digest B were, therefore, ligated together using the ligation mixture listed in Table 17 together with a set of controls which was prepared to ensure the efficiency of the ligation. The ligation mixture and controls were incubated at 12°C overnight. 20ul of
<table>
<thead>
<tr>
<th>Controls (ul)</th>
<th>Digest A</th>
<th>Digest B - CIP treated</th>
<th>Digest B - Not CIP treated</th>
<th>piJ424 uncut</th>
<th>5mM ATP</th>
<th>10 x Ligation Buffer</th>
<th>H2O</th>
<th>T4-ligase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>4.0</td>
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<td>1</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
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<td>4.0</td>
<td>4.0</td>
<td>27</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 17: Ligation mixture and controls: Promoter-probe experiment in S. lividans.
each of the resulting ligated DNA was used to transform *S. lividans* TK64 protoplasts. The protoplast preparation and transformation was carried out in accordance with the method described in section (8.5.2).

After transformation, the regeneration of protoplasts was carried out by plating out aliquots of 0.1ml of each transformation mixture onto well dried R2YE media, five replicates were made. All the plates were incubated at 30°C. The transformants were selected for thiostrepton resistance by overlaying the regeneration plate with soft agar (0.6%) containing 50ug/ml thiostrepton at a suitable time after inoculation (c. 20 hours). The plates were then incubated for a further 2-3 days at 30°C. Numbers of thiostrepton resistant clones are listed in Table 18.

To test the expression of Kanamycin gene, 75 isolates of the recombinant clones and 25 isolates of the control No. 1 were inoculated on R2YE agar plates supplemented with kanamycin as 50ug/ml. As expected, all the control isolates failed to grow, whereas 18 isolates (24%) out of the tested recombinant clones were resistant to kanamycin. This indicates that the inserted fragment within the promoter-probe plasmid pIJ424 has a promoter activity and could activate the aphII gene.

Five isolates of the kanamycin resistant clones were used for plasmid isolation, in order to determine the size of the inserted fragment. The isolation of the plasmid was carried out in accordance with the method described in section (8.3.3). The restriction enzyme analysis revealed the presence of an insert of the same size (0.68Kb) in all the tested clones. This could be done by measuring the increase in the size of the medium PstI fragment of the plasmid pIJ424. Plate 10 illustrates the PstI digests of two of the kanamycin resistant clones compared with that of the plasmid pIJ424.
Table 18 Number of thiostrepton resistant clones obtained from promoter-probe experiment in *S. lividans*.

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Recombinant clones</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>121</td>
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<td>2</td>
<td>88</td>
<td>8</td>
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<td>3</td>
<td>106</td>
<td>10</td>
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<tr>
<td>4</td>
<td>122</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>118</td>
<td>4</td>
</tr>
</tbody>
</table>

UC = Uncountable
Plate 10  PstI digests of two kanamycin resistant clones

Track number

1 = HindIII digest of Lambda DNA
2 = PstI digest of pIJ424
3 = PstI digest of kanamycin resistant clone No. 2
4 = PstI digest of kanamycin resistant clone No. 5
Section 16 DNA sequencing

The results obtained from the promoter-probe experiments showed that the inserted fragments in both vectors, pKK232-8 and pLJ424, have promoter activity. The location of the inserted fragment was difficult to detect within the OXA-2 beta-lactamase gene by restriction enzyme analysis, since the cloning experiments utilised a shotgun technique, in which the whole OXA-2 beta-lactamase gene was Sau-3A digested and randomly cloned to the promoter-probe plasmid vectors without selection for a specific fragment which might contain the promoter region. The nucleotide sequence of these fragments comparing with the complete sequence of OXA-2 beta-lactamase gene (Dale et al., 1985), will define the location of these fragments.

Several methods have now been developed for determining the nucleotide sequence of a cloned fragment of DNA (Gilbert, 1981; Sanger, 1981), of these the Sanger method of chain termination sequencing (Sanger et al., 1977) offers significant advantages in terms of rapidity and simplicity of protocol. It is based upon the use of deoxynucleotide analogues which are randomly incorporated into a growing DNA strand to give specific chain termination. The enzyme E. coli DNA polymeraseI (particularly its large fragment, Klenow) is used in the sequencing reaction. It has the ability to build the complementary strand if given a single-stranded DNA template, a primer with a 3'-hydroxyl group and all the four deoxynucleoside triphosphate (dNTPs). In the case of chain termination sequencing reactions, the primer, which consists of a short fragment of DNA, is annealed to its complementary site on the template. The DNA polymeraseI will extend this primer 5' to 3' direction, adding a nucleotide at a time until a full complementary strand is synthesised giving a complete double stranded molecule. The enzyme also has the ability to incorporate a dideoxynucleotide (ddNTP), which lacks a 3'-hydroxyl group, instead of a deoxynucleotide. Therefore, if this happens into the growing strand of DNA, chain termination will result since there is no 3'-hydroxyl
group available for formation of the next phosphodiester bond.

During sequence analysis four separate reactions are carried out. Each reaction is supplied with all four dNTPs but only one of the four ddNTPs. Thus in the case of the reaction with ddATP, when the enzyme comes to incorporate deoxyadenosine monophosphate it has the choice between dATP and ddATP as substrate. If it incorporates the dideoxynucleotide then the reaction stops by chain termination. If it incorporates the deoxynucleotide then the reaction continues and the enzyme is again presented with a choice between dATP and ddATP when it comes to incorporate the next adenosine moiety.

By carefully controlling the ratio between dATP and ddATP, incorporation of the dideoxynucleotide, and hence chain termination, will be random. The end result of such a reaction is a family of DNA fragments of different lengths, each terminated at the 3'-end with a dideoxyadenosine monophosphate.

By carrying out four separate reactions, each with only one of four ddNTPs, four separate sets of fragments are formed, each set specifically terminated with either ddT, ddC, ddG or ddA. The 5'-ends share a common starting point, the primer. The development of high definition thin polyacrylamide gels (Sanger and Coulson, 1978) has made possible the separation of DNA fragments differing in size by only a single nucleotide; thus, these dideoxy terminated fragments can be accurately size-separated on such a gel. If one of the dNTPs in the reaction mixture is radioactively labelled at the alpha-position then the fragments can be detected by auto-radiography. Each band on the gel arises from termination at a specific point along the chain and from this the base sequence can be deduced.

The sequence is read from the autoradiograph by recording the positions of bands across the four tracks, each representing the positions of ddA, ddC, ddG or ddT terminated fragments. The smallest fragment, (that is the first band at the bottom of the
gel), is located and the track (A, C, G or T) in which this band appears is recorded. The next smallest fragment is then located, and its lane (A, C, G or T) is recorded. So, moving up the gel from track to track, the sequence of the DNA is recorded in a 5' to 3' direction from the primer.
16.1 M13 cloning system

M13 cloning techniques have been developed by Messing and his colleagues for the generation of single-stranded DNA templates suitable for sequencing by the Sanger "dideoxy method" (Gronenborn and Messing, 1978; Messing and Vieira, 1982; Messing et al., 1981).

M13 is a single-stranded filamentous phage. Its life cycle can be exploited for the preparation of pure single-stranded template (Schreir and Cortese, 1979). The phage enters a suitable host cell, (E. coli F' such as JM101 and JM103) by way of the F pilus. On entering the cell, the virus is stripped of its protein coat. The single-stranded viral DNA is then converted to a double-stranded replicative form (RF). This stage is followed by DNA replication to give 50-100 RF molecules per cell, from which new single-stranded viral DNA is synthesised. This is then packaged into viral coat protein and extruded from the host without cell lysis, thus completing the infectious cycle.

In this way, some 200 phage particles are produced per cell, per generation. The phage can be harvested, free of contaminating material from the host cells, by polyethylene glycol (PEG) precipitation of the culture supernatant. The single-stranded DNA can then be stripped of its viral protein coat by treatment with phenol. After ethanol precipitation this simple procedure will yield sufficiently pure single-stranded DNA for Sanger "dideoxy sequencing".

The M13 life cycle can be exploited for the preparation of pure single-stranded recombinant DNA by using the double-stranded RF DNA as a cloning vector. Fragments of foreign DNA can be inserted into a suitable restriction enzyme site. M13RF DNA carrying such a double-stranded insert can be introduced into a suitable competent host cell by a transformation step. The resultant phage growth will lead to production of the hybrid molecule in both double-stranded (RF) and single-stranded (mature virus) forms, thus offering both an amplification step and a means of producing the insert DNA in single-stranded form.
16.2 Preparation of the DNA fragments to be sequenced from the promoter-probe plasmid vectors

1. From pKK232-8:

Four chloramphenicol resistant E. coli clones were chosen for the isolation of the DNA fragments that showed promoter activity; these are clone numbers 3, 13, 21 and 22. The Smal-HindIII fragments of 0.68, 0.58, 0.8 and 0.68Kb in size respectively, were isolated from the recombinant promoter-probe plasmid vector pKK232-8 after it had been cleaved by Smal and HindIII restriction enzymes considering the digestion optimum conditions for each enzyme as recommended by Maniatis et al. (1982). Following electrophoresis the interesting fragment was recovered from the agarose gel in accordance with the method described in section (8.4.4). The recovered Smal-HindIII fragments were designated as fragment E1, E2, E3 and E4 respectively.

2. From pIJ424:

Two kanamycin resistant Streptomyces lividans clones were selected for the isolation of the promoter-probe plasmid vector from which the inserted fragment which showed promoter activity was cleaved off. These are clone number 2 and number 5. The plasmid vector first cleaved with PstI restriction enzyme, resulted in producing three bands following electrophoresis. The medium band carrying the inserted fragment was first recovered from the agarose gel. Following purification, the obtained DNA was double digested with Smal and BclI restriction enzymes to get the Smal-BclI (c. 1Kb) fragment. This fragment was recovered from the agarose gel and designated as fragment S5 and S6 respectively. The preparation of the restriction digests was as recommended by Maniatis et al. (1982), and the extraction of DNA from agarose gel was carried out in accordance with the method described in section (8.4.4).
16.3 Preparation of the M13mp9 vector:

M13mp9 (RF) is double stranded DNA, of 7235 nucleotides long. It carries unique cloning sites (SmaI, XmaI, AccI, HincII, EcoRI, BamHI, SalGI, PstI and HindIII). The vector must be prepared so as to match the fragments to be cloned. The restriction digest was set up as follows:

\[
5\mu l \text{M13mp9 DNA (0.5ug)} + 4\mu l \text{H2O} + 1\mu l \text{IOXSMA buffer} + 3 \text{ units SmaI}
\]

The digest was incubated at 30°C for 60 minutes. Following the incubation the digest was kept on ice. To check that RF DNA digestion was complete, a small portion (1\mu l) of the digest was compared with a sample of undigested vector by gel electrophoresis on a 1% agarose gel. RF DNA has an increased mobility when cut. The digest was successful and produced a single band. The remaining part of the digest was heat shocked at 65°C for 5-10 minutes, followed by phenol extraction and ethanol precipitation. The DNA was resuspended with the suitable digestion buffer (MED) for the second enzyme. To half of the resulted DNA, 0.5\mu l BamHI enzyme was added, whereas to the other half 0.5\mu l of HindIII enzyme was added. Both digests were incubated for further 60 minutes at 37°C. Then both digests were further treated with calf intestinal alkaline phosphatase (CIP) in accordance with the method described in section (8.4.3).

The isolated DNA fragments that have promoter activity from both, pKK232-8 and pIJ424, were cloned with the resulting double cut vector (M13mp9) using the ligation mixtures listed in Table 19 together with a set of controls which was prepared in order to check that the vector has been prepared correctly. All the ligation mixes and controls were incubated at 10°C overnight, followed by heating at 65°C for 10 minutes to stop the reaction. 10\mu l of each was used to transform 0.3ml competent cells of E. coli JM101 strain. The preparation of JM101 competent cells and transformation was carried out as described in section (8.13.1).
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Ligation Mixes (ul)</th>
<th>Controls</th>
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<td></td>
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<th>Inserts:</th>
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<tbody>
<tr>
<td>E.1</td>
<td>10 - - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td>E.2</td>
<td>- 10 - - - -</td>
<td>- - - - -</td>
</tr>
<tr>
<td>E.3</td>
<td>- - 10 - - -</td>
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<tr>
<td>E.4</td>
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</tr>
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<td>S.5</td>
<td>- - - - 10 -</td>
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</tr>
<tr>
<td>S.6</td>
<td>- - - - - 10</td>
<td>- - - - -</td>
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</table>

<table>
<thead>
<tr>
<th>Vector M13mp9</th>
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<tbody>
<tr>
<td>Uncut</td>
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<td>2 - - - -</td>
</tr>
<tr>
<td>SmaI-digested</td>
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<td>- 2 2 - -</td>
</tr>
<tr>
<td>SmaI-HindIII CIP</td>
<td>2 2 2 2 - -</td>
<td>- - - 2 -</td>
</tr>
<tr>
<td>SmaI-BamHI CIP</td>
<td>- - - - 2 2</td>
<td>- - - - 2</td>
</tr>
</tbody>
</table>

| 10X Ligation Buffer  | 1.5 1.5 1.5 1.5 1.5 1.5 | 1.5 1.5 1.5 1.5 1.5 |
| 10mM ATP             | 1.5 1.5 1.5 1.5 1.5 1.5 | 1.5 1.5 1.5 1.5 1.5 |
| H2O                  | - - - - - -        | 10 10 10 10 10 |
| T4 Ligase            | 1 1 1 1 1 1        | - 1 - 1 1 |

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16.4 Identification of recombinants

Although M13 does not lyse its host cell, the growth of the cell is retarded as a consequence of supporting phage growth. Thus, when plated out, those cells infected with M13 will show up as areas of slower growth, which look like turbid plaques on a lawn of uninfected cells. Transformants can therefore be easily distinguished from non-transformed cells. In addition, the Messing M13 strains have been modified to allow the visual discrimination of recombinants from non-recombinants. Under appropriate conditions the M13 vector will grow up to form a blue plaque, but this process is disrupted by the introduction of a fragment of foreign DNA into the M13 insertion site. As a result recombinant phages (those carrying passenger DNA) will give colourless or "white" plaques.

This discrimination is based on the presence or absence of the enzyme Beta-galactosidase. *E. coli* host cells infected with any of Messing's M13 vectors (such as M13 mp9) will, in the presence of the lac operon inducer IPTG, produce a functional beta-galactosidase. Such cells will hydrolyze the substrate X-gal to give a blue dye (bromo-chloroindole). Insertion of foreign DNA into an appropriate site in the DNA vector interferes with the production of beta-galactosidase in infected cells. Thus recombinant M13 gives colourless plaques on an *E. coli* lawn, in contrast to the blue plaques given by the intact vector.

Table 20 lists the plaque counts obtained after plating out transformation mixes 1 - 6 and controls 1 - 5. The results indicate that the cut vector gave no plaques (control No. 3) this means that the vector has been completely linearised. The cut and religated vector control gave only blue plaques. Therefore, the colourless plaques obtained on the non-control plates are recombinants carrying insert DNA.
Table 20 Plaque counts from transformation M13mp9 recombinants

<table>
<thead>
<tr>
<th>Transformation Mixes</th>
<th>Plaque Counts</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White</td>
<td>Blue</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2, 2</td>
<td>0, 0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2, 0</td>
<td>0, 0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0, 0</td>
<td>0, 0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2, 1</td>
<td>0, 0</td>
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<td>5</td>
<td>0, 0</td>
<td>0, 0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7, 13</td>
<td>0, 0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0, 0</td>
<td>&gt;500, &gt;500</td>
</tr>
<tr>
<td>2</td>
<td>0, 0</td>
<td>250, 270</td>
</tr>
<tr>
<td>3</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>4</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>5</td>
<td>0, 0</td>
<td>6, 5</td>
</tr>
</tbody>
</table>

Each single colourless plaque was picked up, using a cocktail stick, and propagated in 1.5ml of diluted (1:100 warm 2 x YT) E. coli JM101 overnight culture, to produce single stranded template for the sequencing reaction.

The preparation of single stranded template and the sequencing reaction were carried out in accordance with the methods described in section (8.13.1) and section (8.13.2).
Sequencing data were collected and analysed using the computer programs developed by Staden (1982) of the MRC laboratory, Cambridge; a version of these programs adapted to run on a Prime computer was supplied by K.J. Indge. The implementation was carried out by Dr. T.N. Bryant at Microbiology Department - University of Surrey, July 1984.

The published sequence of the OXA-2 beta-lactamase gene (Dale et al. 1985) has been extended. The DNA sequencing of the extended part was carried out (in part) by myself, using the AvaI-EcoRI clones which had been previously prepared by Dr. J.W. Dale. The extended sequence consists of 582 nucleotide bases starting from base number 1 (Figure 24).

From the nucleotide sequencing data, it has been determined that the fragments E1 and E4 are similar because they showed the same nucleotide sequencing pattern and are designated as clone A. The fragments E2 and S6 showed different nucleotide sequences and are designated as clone B and clone C respectively. Plate 11, is a photograph of an autoradiogram, illustrates the resolution efficiency of a sequencing gel that has been run for 4 hours.
Figure 24 Nucleotide sequence of OXA-2 beta-lactamase gene
Plate 11: DNA sequencing autoradiogram.
The origin of clone A and clone B is illustrated in Figure 25, where the BamHI - HindIII fragment (from pSU8) containing the OXA-2 beta-lactamase gene, was Sau 3A digested and randomly cloned into the BamHI digested promoter-probe plasmid vector pKK232-8. Then the SmaI - HindIII fragment was isolated from the recombinants which showed promoter activity and cloned into SmaI - HindIII digested M13mp9 vector to be sequenced.

The nucleotide sequence of clone A, as illustrated in Figure 26a, starts with a SmaI site, but there is not a BamHI/Sau 3A (GGATCX) hybrid site which was expected to be found in addition to some sequence of the vector pKK232-8. This indicates that a deletion may have happened. However, the nucleotide sequence showed that the inserted fragment (E1 and E4) position 13-129 (underlined sequence) matches the OXA-2 nucleotide sequence (Figure 24) at position 1447-1564, but clone A deviates from OXA-2 before the Sau 3A site, (position 130 in clone A), and the rest of the sequence determined does not match any other part of the OXA-2 gene sequence or that of the vector M13mp9. Comparison between the nucleotide sequence of clone A and that of an Alu1 clone, which is obtained from the BamHI - HindIII fragment of pSU5 (J.W. Dale unpublished data) showed extensive homology in this region.

The published OXA-2 sequence was constructed from clones derived from the pKM101 plasmid (a deletion derivative of R46) which has a different deletion from that in pSU5 or pSU8 (Figure 10), whereas the Alu1 fragment and clone A have the same origin. This reflects the divergence effect between the pKM101, and pSU8 and pSU5 plasmids, i.e., the position at which these sequences diverge represents the end point of one or both of the deletions in pKM101 and pSU5.

Since this fragment showed homology to the part of the OXA-2 sequence which is located approximately at the end of the gene, the promoter activity shown by this fragment when cloned into the promoter-probe vector pKK232-8 was not specified by the promoter of the OXA-2 gene, but may be related to the promoter of another gene adjacent to the OXA-2 gene in the R-factor R46, that is the
spectinomycin resistance gene, or most probably the sulphonamide resistance gene. Clone A has therefore been disregarded in the search for the promoter of the OXA-2 gene.

The nucleotide sequence of clone B (Figure 26b), starts with 124 base pairs between the SmaI site and the start of the insert (presumably from the pKK232-8 vector), followed by the sequence (GGATC) which represents the ligation position of the inserted fragment with the BamHI site of the promoter-probe vector pKK232-8. The nucleotide sequence showed that the inserted fragment (E2) position 126-291 (underlined sequence), matches the OXA-2 nucleotide sequence (Figure 24) at position 884-1049, but in the opposite direction to the transcription of the OXA-2 gene. The sequence of this fragment ends with the Sau 3A/BamHI hybrid site (GATC). The following 28 base pairs could be of the vector pKK232-8 which ends with the HindIII site (AAGCTT). The rest of the sequence is of the M13mp9 vector.

Although this clone has the expected structure, its orientation and position rule it out as a possible OXA-2 promoter. It is likely that it is not actually used as a promoter in its normal surroundings.
Figure 25  Origin of clone A and clone B.
Figure 26  Nucleotide sequence (a) clone A, (b) clone B.
Figure 27 represents the origin of clone C. The Sau 3A fragments, generated from the BamHI - HindIII fragment (of pSU8) containing the OXA-2 beta lactamase gene, were randomly cloned into the BglII digested Streptomyces promoter-probe plasmid vector pIJ424. Then the SmaI-BclI fragment which showed promoter activity was isolated from the recombinant and cloned into SmaI - BamHI digested M13mp9 vector. The nucleotide sequence of this clone was expected to start with pIJ424 sequence; in fact, the sequence from the SmaI site was identical with the sequence in pSU8 starting with the proximal AvaI site. This could be explained by the presence of a SmaI site in the cloned fragment, although according to previous reports (Brown and Willetts, 1981; Langer and Walker, 1981) there is no SmaI site in this region. This may indicate that in pSU8 there has been a point mutation which establishes an additional SmaI site at this AvaI site. The nucleotide sequence of clone C as illustrated in figure 28, showed that the sequenced part of the cloned fragment (S6) is completely identical to the OXA-2 nucleotide sequence at position 1-590. Since the homology between these nucleotide sequences occurred upstream from the beginning of the OXA-2 gene, the promoter activity of the fragment S6, which activated the kanamycin (aph) gene in pIJ424, was probably related to that of the OXA-2 gene. This can be confirmed and a more accurate position defined by the use of S1 mapping (next section).
Figure 27  Origin of clone C.
Figure 28  Nucleotide sequence of clone C. The gaps shown in this sequence are due to some ambiguity in the gel readings, and have not been resolved.
Nuclease-S1 mapping is used to map the location of the ends of RNA molecules and of any splice points within them in relation to specific sites (e.g. position of restriction endonuclease cleavage) within the template DNA.

This technique was used to localise the promoters of several genes for example, \textit{ermE}P1, \textit{ermE}P2 and \textit{orf}P1 (Bibb et al., 1985b); p\textit{L}J101\textit{A} (Buttner and Brown, 1985), and \textit{aphP}1, P2 and \textit{tsrP}1, P2 (Janssen et al., 1985). In this study an attempt was made to localise the promoter of the OXA-2 beta-lactamase gene in both \textit{Escherichia coli} and \textit{Streptomyces lividans}.

17.1 RNA preparation

The RNA from both \textit{E. coli} and \textit{S. lividans} was isolated by using a modified method adapted from those of Scherrer and Darnell (1962), Glisin et al. (1974), Gabain et al. (1983), Jaurin and Cohen (1984), Hopwood et al. (1985a). To obtain a good preparation of RNA, the vanadyl-ribonucleoside complexes (ERL), were used during the initial stages of extraction at a concentration of 10mM to inhibit ribonuclease activity. All solutions were treated with 0.1\% diethylpyrocarbonate (DEPC) for at least 12 hours prior to autoclaving. Pipette tips and microcentrifuge tubes were submerged in a beaker containing a mixture of 0.1\% DEPC in 50\% ethanol and left overnight, then autoclaved after the solution was drained out. Similarly the Pasteur pipettes were treated with DEPC, autoclaved and dried at 100\°C.

Procedure

Strains used:

\textit{E. coli} JD348 (pSU8)
\textit{E. coli} NQA1 (pSU101)
\textit{E. coli} HB101
The cultures of both *E. coli* and *S. lividans* strains were prepared as described in section (8.10.1) and (8.10.2). When the culture had reached the appropriate stage of growth, it was immediately chilled by placing the flasks in an ethanol/solid CO2 bath for 2-3 minutes. The cells and mycelium were collected by centrifugation using a bench-top centrifuge (MSE-Chilspin) at 4500rpm, 4 °C for 10 minutes, followed by washing, twice, with ice cold DEPC-treated 100mM NaCl. The pellets were resuspended in 0.3M sucrose, 0.01M sodium acetate (pH4.5) containing 2mg/ml Lysozyme and 10mM vanadyl-ribonucleoside complexes and incubated at 37 °C for 5 minutes. Cells were lysed at 65 °C by addition of an equal volume of 1% SDS (Sodium dodecyl sulfate), with vigorous agitation from time to time to help the lysis. Total cellular RNA was isolated by hot phenol extractions; an equal volume of preheated phenol (ultra-pure, BRL) to 60 °C was mixed with the cells and shaken for 3 minutes in 60 °C water bath. The temperature in the mixture was approximately 50 °C at the end of the extraction, and was reduced to 4 °C by rapid chilling on ice. The homogenate was transferred to sterile disposable polypropylene tubes (Falcon 2006) and centrifuged to separate the phases at 2000rpm for 10 minutes at 4 °C. The aqueous (upper) layer was removed to a fresh polypropylene tube and its volume was measured. 0.4g caesium chloride per millilitre RNA solution was added and dissolved. This homogenate was layered onto 1.25ml of a 5.7M Caesium chloride, 0.1M EDTA cushion in an ultracentrifuge tube (SW 40 Polyallomer tubes). The tubes were centrifuged in a Beckman SW 40 rotor at 35000rpm for about 15 hours at 25 °C. This procedure takes advantage of the fact that the buoyant density of RNA in caesium chloride is much greater than that of other cellular macromolecules. During centrifugation, the RNA forms a pellet on the bottom of the tube while most of the DNA and protein floats upward in the caesium chloride solution. The supernatant was discarded and the walls of the centrifuge tubes
were thoroughly dried. The RNA pellet was dissolved with DEPC treated double-distilled, sterile water. The RNA preparations were stored at -70°C in 70% ethanol containing 0.3M sodium acetate.

Spectrophotometric measurements for each preparation of RNA were carried out at 280nm and 260nm in a double beam UV spectrophotometer (Philips Model PU8820). The concentration of RNA samples were calculated from the absorbance at 260nm, and levels of purification were estimated by their relative absorbance ratios. The results in Table 21 indicate that all RNA preparations are essentially free of protein, because their relative absorbance ratios range from 1.95 - 2.
### Table 21 Qualitative and quantitative assessment of RNA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Absorption at 260nm</th>
<th>Absorption at 280nm</th>
<th>260nm/280nm ratio</th>
<th>RNA mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> HE101</td>
<td>2.8</td>
<td>1.4</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td><em>E. coli</em> JD348</td>
<td>0.8</td>
<td>0.41</td>
<td>1.95</td>
<td>3.2</td>
</tr>
<tr>
<td><em>E. coli</em> NOA1</td>
<td>0.387</td>
<td>0.194</td>
<td>1.99</td>
<td>3.1</td>
</tr>
<tr>
<td><em>S. lividans</em> NOA2</td>
<td>0.947</td>
<td>0.480</td>
<td>1.973</td>
<td>3.8</td>
</tr>
<tr>
<td><em>S. lividans</em> TC73</td>
<td>1.061</td>
<td>0.534</td>
<td>1.98</td>
<td>4.2</td>
</tr>
<tr>
<td><em>S. lividans</em> TK64</td>
<td>0.605</td>
<td>0.31</td>
<td>1.95</td>
<td>2.4</td>
</tr>
</tbody>
</table>
17.2 Probe preparation

The mapping of RNA transcripts requires the use of single-stranded DNA probes of high specific activity. The availability of single-stranded phage cloning vehicles (Messing et al., 1981) provides an opportunity to synthesize such probes. One of the M13 clones (HC2) which had been previously prepared by Dr. J.W. Dale was used as the probe. The HpaI - BamHI fragment of the OXA-2 beta-lactamase gene (1.4Kb) was cloned in M13mp9 in an orientation so that the resulting single-stranded DNA probe generated by primer extension will complement the mRNA. The nucleotide sequence of the OXA-2 beta-lactamase gene indicated that the initiation start codon of the gene is within this fragment.

The single-stranded DNA template of HC2 clone was prepared as described in section (8.13.1), and annealed with a universal sequencing primer (Celltech-primer) as described in section (8.13.2). Then the primer was extended with DNA polymerase I Klenow fragment by adding 2ul each of dTTP, dCTP and dGTP at 0.5mM and 2ul (20uCi) alpha-P-32 dATP (Amersham PB.10204, 3000 Ci/mmol). The mixture was incubated at room temperature for 20 minutes followed by adding 2ul Chase (as used in the sequencing reaction). The reaction was then left at room temperature for a further 15 minutes. The reaction tube was heated at 70°C for 10 minutes to inactivate the enzyme and to denature the DNA duplex. The resulting complementary radioactive strand was used as a probe to hybridise to the RNA.

The single-stranded probe synthesized from M13mp9 clone (HC2), which includes the initial part of the transcribed sequence, was used for S1 mapping of the transcripts from the E. coli and S. lividans recombinant strains containing pSU101 (NOA1 and NOA2). Additional strains used as controls were E. coli JD348 carrying pSU8, E. coli HB101, S. lividans TC73 (pIJ61) and S. lividans TK64. A sample of total RNA (0.5 - 1mg) isolated from each strain was hybridised with 0.5ug of the single-stranded probe. The resulting hybrids were digested with S1 nuclease and the protected
fragments analysed on Polyacrylamide-Urea thin sequencing gel using the end labelled φX174 (HaeIII fragments) as markers.

The S1 mapping of the transcripts and the end-labelling of the DNA marker were carried out in accordance with the methods described in section (8.14) and section (8.6.3).

As can be seen in plate 12, RNA from both E. coli strains, either carrying pSU8 (lane c) or pSU101 (lane d) showed six discrete protected bands which represent different transcriptional start sites of the OXA-2 gene. This may be due to RNA polymerase heterogeneity; it has been suggested that bacteria such as E. coli, Bacillus subtilis and Streptomyces coelicolor contain multiple sigma factors and thus multiple forms of RNA polymerase holoenzyme. Each sigma confers on RNA polymerase the ability to initiate transcription at a specific class of promoters (Grossman and Losick, 1986). But it is also possible for a single RNA polymerase to initiate transcription at different sites.

The higher level of OXA-2 specific mRNA from E. coli NOA1 (lane d) may be due to a higher copy number of the pSU101 plasmid; also the higher level of expression of pSU101 compared to that of pSU8 can be explained by increased transcription. The first band represents the main transcriptional start point of the OXA-2 gene; the most efficient promoter, therefore, is located within this area of the OXA-2 nucleotide sequence, while the other bands (2-6) indicate the presence of less efficient promoters.

Plate 13 (lane e) illustrates that the RNA isolated from S. lividans NOA2 carrying the hybrid pSU101, showed two main S1-resistant fragments different from that obtained from E. coli strains, and another two bands of less intensity (barely visible in the photograph) corresponding to the first and third bands of the E. coli strains.

No S1-resistant fragment appeared from the hybridisation of the RNA obtained from both E. coli (lane b) and S. lividans (lane f.
and g) strains which do not have the OXA-2 gene. This indicates that the S1-resistant fragments were not artefacts due to protection from endonucleolytic cleavage by S1 by regions of secondary structure, and show the absence of S1-protected hybrids involving transcripts from chromosomal or pLJ61 genes. The bands that appear at the top of the photograph may be due to the reannealing of the complementary strands of the probe itself.

The size of each S1-resistant fragment was measured with reference to the ϕX174 (HaeIII fragments) standard marker. Since the position of one end is known with reference to the OXA-2 sequence (HpaI site at position 1006), the position of the other end, the transcriptional start point, can be determined by subtraction. More precise identification would require:

a) comparison with a sequencing gel, and;

b) RNA polymerase binding assay.

The conserved DNA sequences, located about 10 and 35 base pairs upstream of the transcriptional start site, of each promoter have been tentatively identified and listed in Table 22. The results indicate that each promoter of the OXA-2 gene in E. coli contains, in front of the transcriptional start point, two regions that show considerable similarity to the "-10" and "-35" sequences of the consensus prokaryotic promoter (Hawley and McClure, 1983), while these regions of the most efficient OXA-2 promoter, P(7), in S. lividans were similar to the consensus sequences of Streptomyces promoters (Hopwood et al., 1986). The "-10" and "-35" regions of the promoter P(8) in S. lividans could not be identified because their location is outside the sequenced part of the OXA-2 beta-lactamase gene.

The low expression level of OXA-2 beta-lactamase in S. lividans may be related to the long distance (28bp) between the "-10" and the "-35" consensus sequences of the promoter P(7). The appearance of the two other promoters in S. lividans corresponding to P(1) and P(3) of OXA-2 in E. coli may indicate that the RNA
polymerase of *S. lividans* can recognize these promoter sequences but at a very low efficiency.

It has been reported that the *S. lividans* RNA polymerase can recognize *E. coli* promoters, for example, that promote initiation of the *ampC* beta-lactamase in *E. coli* (Jaurin and Cohen, 1984) and others (Bibb and Cohen, 1982). The low efficiency of recognition of the *E. coli* OXA-2 promoter by *S. lividans* RNA polymerase may reflect genus or species-specific factors that interfere with gene expression in certain heterologous combinations, or that the *S. lividans* RNA polymerase requires accessory factors to transcribe these promoters.
Plate 12  Autoradiogram of S1 mapping of the transcripts from E. coli HB101, negative control (b); E. coli JD348 carrying pSU8, positive control (c); E. coli NOA1 carrying pSU101 (d); S. lividans NOA2 carrying pSU101 (e); S. lividans TC73 carrying pIJ61, negative control (f); S. lividans TK64 plasmid free, negative control (g); end labelled φX174 HaeIII fragments, standard markers (a and h). Numbers along the left margin are marker sizes in bases.

The protected DNA fragments are pointed by arrows. Enlarged version of upper part is illustrated in plate 13.
Plate 13  Same as plate 12. The photograph was enlarged and lane h was not included.
Table 22  Comparison of the E. coli consensus promoter sequence (Hawley and McClure, 1983) with the potential -10 and -35 sequences of the OXA2 promoter regions

<table>
<thead>
<tr>
<th>Promoter</th>
<th>S1 Resistant fragment size (bp)</th>
<th>-35 region</th>
<th>Distance (bp)</th>
<th>-10 region</th>
<th>Distance (bp)</th>
<th>mRNA start</th>
<th>Position of mRNA start on OXA-2 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli in E. coli</td>
<td></td>
<td>TTGACA</td>
<td>17</td>
<td>TATAAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* P(1) 550</td>
<td></td>
<td>TTGACA</td>
<td>22</td>
<td>TGTAAT</td>
<td>6</td>
<td>A</td>
<td>472</td>
</tr>
<tr>
<td>P(2) 480</td>
<td></td>
<td>TTGACC</td>
<td>14</td>
<td>TAACCG</td>
<td>6</td>
<td>G</td>
<td>537</td>
</tr>
<tr>
<td>* P(3) 445</td>
<td></td>
<td>GCGCAT</td>
<td>17</td>
<td>TGTTAT</td>
<td>4</td>
<td>G</td>
<td>563</td>
</tr>
<tr>
<td>P(4) 255</td>
<td></td>
<td>TTGGCG</td>
<td>17</td>
<td>TICTCT</td>
<td>4</td>
<td>A</td>
<td>755</td>
</tr>
<tr>
<td>P(5) 66</td>
<td></td>
<td>TGACAC</td>
<td>14</td>
<td>TACACT</td>
<td>3</td>
<td>T</td>
<td>949</td>
</tr>
<tr>
<td>P(6) 57</td>
<td></td>
<td>TGACAC</td>
<td>14</td>
<td>TACACT</td>
<td>6</td>
<td>A</td>
<td>952</td>
</tr>
<tr>
<td>OXA2 in S. lividans</td>
<td></td>
<td>TTGGCG</td>
<td>28</td>
<td>GAGCAG</td>
<td>6</td>
<td>G</td>
<td>317</td>
</tr>
<tr>
<td>P(7) 740</td>
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<td>NI</td>
<td>NI</td>
<td>NI</td>
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<td>NI</td>
<td></td>
</tr>
<tr>
<td>P(8) 1030</td>
<td></td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td></td>
</tr>
</tbody>
</table>

bp = Base Pairs  
NI = Not Identified  
* has been recognized by S. lividans
Figure 29 illustrates the sequenced part of OXA-2 gene and adjacent regions, and shows the position of the most efficient promoters; P(1) in *E. coli* and P(7) in *S. lividans*. The location of the homologous part of the promoter probe fragments with the nucleotide sequence of the OXA-2 gene are also shown. From this figure it can be seen that the most efficient promoters P(1) and P(7) are included within the fragment S6 (clone C) also P(2) and P(3), whereas the less efficient promoters P(5) and P(6) are included within the promoter probe fragment E2, but in the wrong orientation. However, these assignments of promoters and transcriptional start sites are tentative. Further experiments, including the use of probes with different end points, would be needed to confirm the nature and origin of these transcripts.
Figure 29 OXA-2 structural gene and its promoter regions. The location of the cleavage sites for *AvaI*, *Sau3A* (S), *HpaI*, *EcoRI*, were deduced from the sequence. Transcriptional start points are indicated by circles. The lower line shows size, position, and orientation of the promoter probe fragments.
18.1 Cloning strategies of OXA-2 beta-lactamase gene

The main objective of this study is to determine whether the OXA-2 beta-lactamase is secreted by *Streptomyces lividans*. To test the expression and secretion of the Gram-negative OXA-2 beta-lactamase in *S. lividans*, three different strategies were used involving two different *Streptomyces* vectors, pHJ61 and pHJ702. In the first and second attempt the OXA-2 gene was isolated from two different *Escherichia coli* plasmids, pSU8 and pSU5, and cloned into pHJ61 and pHJ702 (respectively), followed by transformation into *S. lividans* TK64. The clones obtained initially were colony hybridisation positive, showed insertional inactivation (pHJ702 clones) and were positive for beta-lactamase production, on first test but later negative. The restriction enzyme analysis of some plasmids obtained from these clones showed no indication of the presence of the insert. Therefore the insertional inactivation may be due to a deletion, for example contamination with exonuclease would cut the vector back; following religation, this would show as a deletion causing, in the case of pHJ702 the inactivation of the tyrosinase gene and inhibition of melanin production. The positive colony hybridisation results could be due to non-specific interaction for example, detection of vector sequences (e.g. transposon or any other related sequences since the probe used contained sequences outside the structural gene). The failure in getting recombinant plasmids carrying the OXA-2 beta-lactamase gene, is most likely to be due to instability of the recombinant plasmids, so that inserts were lost by the time screening of the recombinant transformants were done. This phenomenon was also observed, when Shareck *et al.* (1984) tried to construct the plasmid pFSH100, by joining the *Streptomyces* plasmid pHJ101 with an *E. coli* plasmid, pSAS1206. The restriction enzyme analysis of the presumed hybrid plasmid, following transformation in *S. lividans*, revealed that most of the *E. coli* plasmid pSAS1206 had been deleted, as well as part of *S. lividans* host plasmid, pHJ101. As one way of
overcoming these problems, a shuttle plasmid (pSU101) was created by fusing an *E. coli* plasmid (pSU8) carrying the OXA-2 beta-lactamase gene with the *S. lividans* vector pIJ61. This has the advantage that manipulations were carried out and characterised in *E. coli* before transferring the final product to *S. lividans*. 
The expression of OXA-2 beta-lactamase specified by the hybrid plasmid pSU101 in Escherichia coli

The results listed in Table 11 showed that OXA-2 expression from pSU101 in E. coli was unexpectedly more than double that of pSU8 in E. coli. This high expression level may have resulted from an alteration in the plasmid copy number or the level of transcription/translation of the gene.

Although the copy number of pSU101 and pSU8 has not been determined, the plasmid pSU101 appeared to have a very high copy number compared to that of pSU8, on the basis of the amount of plasmid DNA obtained from similar cultures, in the absence of chloramphenicol amplification.

It has been reported that the copy number of the plasmid ColEl and its derivatives is regulated by a plasmid specific repressor protein (Gelfand et al., 1978; Shepard et al., 1979; Twigg and Sherratt, 1980). The region coding for this repressor lies adjacent to the replication origin of the plasmid (Shephard et al., 1979).

Several features of plasmid replication have been observed which are consistent with the major tenets of the negative control model of replication put forward by Pritchard and his colleagues (Pritchard et al., 1969; Pritchard, 1978; Pritchard and Grover, 1981). It has been assumed that the replication control can be exerted at the level of initiation, rather than elongation, of replication of ColEl plasmid (Pritchard et al., 1969). This plasmid appears to encode two products which control the initiation of its replication, a small RNA molecule called RNA1 and a small polypeptide encoded by the rop (also called rop) gene. The latter is apparently not essential under most conditions. Thus ColEl appears to be entirely dependent on host enzymes for its replication.

Tomizawa and Som (1984), have emphasized the important role played by RNA and host's RNA polymerases in the initiation of ColEl replication and in the control of this process. The first event
appears to be the transcription of a region of the plasmid, at a point 555 base pairs upstream from the replication origin, to form an RNA molecule called RNAII. Some of the RNAII molecules hybridise to the complementary template DNA near the origin and are then cleaved at the origin by RNase H (a host-encoded enzyme specific for DNA:RNA hybrids). Cleavage by RNase H exposes an RNA end at the origin and this serves as a primer at which DNA synthesis can begin.

**ColE1** replication is controlled by a negative feedback mechanism. An RNA molecule (RNAI) inhibits the initiation of ColE1 replication. It does this by binding to RNAII and preventing this molecule from forming a secondary structure which it must have in order to hybridise to the template DNA near the replication origin. Furthermore, a region downstream from the ColE1 origin is also involved in the control of the initiation of plasmid replication and in the regulation of plasmid copy number.

Derivatives of ColE1 which have a deletion of a specific region, downstream from the origin have a higher copy number, so it was proposed that this DNA segment might encode another inhibitor of ColE1 replication. This is a small polypeptide of 63 amino acids encoded by the *rom* (or *rop*) gene, which enhances the binding of RNAI to RNAII so that RNAII functions less effectively as a primer. This reduces the copy number of ColE1 (Hardy, 1986).

Under steady state conditions there is an equilibrium between plasmid copy number and inhibitor concentration such that each plasmid replicates an average once per cell and cell cycle. The plasmid copy number and inhibitor concentration directly affect one another, such that if the copy number in a cell is too high, the concentration of inhibitor also becomes higher than normal, since the number of inhibitor genes is increased. This increased inhibitor concentration reduces the average number of replication per plasmid and cell cycle below one, while the cells continue to grow, until the concentration of inhibitor and plasmid copy number return to the normal level. Mutation in the inhibitor gene,
resulting in a less efficient inhibitor, causes an increase in copy number until the inhibitor concentration sets an average replication frequency of one per plasmid copy and cell cycle (Nordstrom et al., 1984).

Although there is no information about the replication control system of either pSU8 or the Streptomyces plasmid pIJ61, the multicopy (20 per chromosome) plasmid pACYC184 (Chang and Cohen, 1978), on which the plasmid pSU8 based, might have a replication control system similar to that of ColE1. Fusion of the plasmid pSU8 with the pIJ61 at the XbaI site (downstream of the pACYC184 replication origin) to form the plasmid pSU101, might disrupt the rom gene. Thus the binding of RNAI to RNAII will be less efficient, and the synthesis of RNAII continues, leading to increase the copy numbers of the plasmid pSU101 in E. coli.

The apparent increase in the copy number of the hybrid plasmid pSU101 relative to that of pSU8, created a gene dosage effect. Although the results of the enzyme activity (Table 11) showed that the level of OXA-2 beta-lactamase production by cells carrying the hybrid plasmid pSU101 has increased more than double relative to the control pSU8 strain, the isoelectric focusing results (Section 12.2) indicate a greater difference; the OXA-2 beta-lactamase production is sufficiently high (>2 fold) to be detected by protein staining. S1 mapping of the transcripts obtained from E. coli NOAI carrying the hybrid plasmid pSU101 and from the control pSU8 strain (JD348) as illustrated in plate 12, shows a higher level of transcription of OXA-2 beta-lactamase gene in pSU101 compared to that in pSU8, although the same transcriptional origin was used in both plasmids. Although this is probably due to plasmid copy number, an alternative explanation would be that a mutation has occurred in the promoter region in the plasmid pSU101 which therefore becomes more efficient than that in pSU8.

In some cases gene expression is limited by a transcription/translation control system. For example, a gene dosage effect was
not observed for the gene coding for the ribosomal protein structural genes (of *E. coli*) when cloned into a high copy number plasmid, due to a post transcriptional control system (Fiih et al., 1980).

Limitations in expression are more often at the transcriptional level. In most cases, the initiation of transcription has been shown to be the rate-limiting step in the expression of gene functions (Rose and Yanofsky, 1972). Regulation of the *ampC* expression could occur at the level of initiation or termination of transcription or both (Jaurin et al., 1981). Mutations in both the promoter and the attenuator for *ampC* causes increased *ampC* beta-lactamase production and the expression of *ampC* has become independent of the growth rate. Since the mutation at the attenuator, abolished termination of *ampC* transcription, Jaurin and his co-workers have suggested that the growth rate-dependent regulation of *amp* operon is exerted at the level of anti-termination, and they proposed that the ribosome binding site at the *amp* leader RNA has a regulatory role. Binding of ribosomes to the *ampC* leader region would protect about 12 bases downstream and this could prevent formation of the termination stem and loop structure.
The expression of OXA-2 beta-lactamase specified by the hybrid plasmid pSU101 in Streptomyces lividans

The results listed in section 11 show that the cloned OXA-2 beta-lactamase gene has been expressed in the new host S. lividans. Different levels of enzyme production were found, when the S. lividans strain, containing the hybrid plasmid pSU101, was grown in three different media (Table 10). The low level of beta-lactamase excretion in Brain-Heart infusion broth and Peptone broth may be due to the inhibitory effect of the chloride ions as these media contain 8.6mM NaCl and 6.0mM KCl respectively. In L. broth this problem has been overcome in that 10mM Sodium acetate was substituted for 8.6mM Sodium Chloride. It has been reported that all the oxacillin-hydrolyzing beta-lactamases are inhibited by chloride ions (Dale and Smith, 1974). Comparison of the enzyme production levels specified by the hybrid plasmid pSU101 in both E. coli and S. lividans (Table 11) indicate that the expression of OXA-2 beta-lactamase in S. lividans was much lower than that in E. coli; this may be related to promoter specificity or to a difference in plasmid copy number.

S1 mapping studies (Section 17) on the transcript obtained from S. lividans NOA2 carrying the plasmid pSU101, revealed that the most efficient transcription of the gene was carried out at a transcriptional start point different from that used in the initiation of the transcription in E. coli (OXA-2 P(1)), and therefore presumably a different promoter sequence was used. This does not necessarily mean that the S. lividans RNA polymerase is unable to recognize the E. coli promoter.

The low expression level of the OXA-2 beta-lactamase gene in S. lividans may be related to the long separation distance (28bp) between the "-35" and the "-10" consensus sequences, as shown in Table 22. It has been reported that the optimum distance to get very efficient promoter activity is 17bp (Hawley and McClure, 1983; Jaurin and Cohen, 1984). Alternatively, the mRNA produced may be less stable.
The expression of the OXA-2 beta-lactamase gene of a Gram-negative origin in the Gram-positive Streptomyces lividans, reflects the heterospecific gene expression, which was first demonstrated by Schottel et al. (1981) after construction of the shuttle vectors pSLP120 and pSLP125. The E. coli antibiotic-resistance genes of pACYC177 and pACYC184 coding for Kanamycin (Km) phosphotransferase and chloramphenicol acetyltransferase (CAT), respectively, were shown to be expressed in S. lividans, but they found that the promoter that normally accomplishes transcription of the chloramphenicol resistance gene in E. coli is not responsible for its expression in S. lividans; however the normal ATG start codon for the gene is utilised as the translational start signal in the new host. This was followed by other reports indicating the expression of some antibiotic resistance genes of E. coli origin in Streptomyces species. The expression of the tetracycline resistance gene of pBR322 in S. albus strain G and P was reported by Chater et al. (1982a); they have suggested that the promoter responsible for the tet gene expression in S. albus could be the same promoter used in E. coli. Bibb et al. (1983), have reported that the Tn5 Kanamycin phosphotransferase gene has been expressed in a detectable level in S. lividans. Jaurin and Cohen (1984) showed that the ampC gene of E. coli (chromosomally determined beta-lactamase) could be transcribed in S. lividans, and that the up-promoter mutation in the "-35" region, "-10" region, and the intervening "spacer" region creates a promoter that in E. coli is 16 times stronger than the wild type ampC promoter, and yields an approximately 30 fold increase in promoter strength in S. lividans. This led to a suggestion that S. lividans contains an RNA polymerase that recognizes and uses the various components of the E. coli transcriptional signals. Shareck et al. (1984), have reported that the sulfonamide resistance gene encoded by pSAS1206 is phenotypically expressed in S. lividans; and proposed that the transcription of this gene in S. lividans could be initiated from a promoter signal which is located in plasmid pIJ101.
The mRNA sequences study performed by Bibb and Cohen (1982) indicates that the ribosomes of *S. lividans* recognize sequences for the initiation of translation, that show little complementarity to the 3' end of their 16s rRNA. This differs from the observation made by McLaughlin *et al.* (1981), that led to the hypothesis, that the ribosomes of other Gram-positive bacterial species, such as *Bacillus* and *Staphylococcus*, in order to initiate translation, require a greater degree of such complementarity than do the ribosomes of Gram-negative species, such as *E. coli*. These conclusions were proffered as the reason for the observed lack of expression of Gram-negative genes in a Gram-positive host (Ehrlich, 1978; Ehrlich and Sgaramella, 1978).

The results in this study indicate that the *S. lividans* RNA polymerase can recognize two promoter sequences (P(1) and P(3)), that initiate transcription of OXA-2 beta-lactamase gene in *E. coli*, but with a very low efficiency.
RNA polymerase heterogeneity

Analysis of the sequences around the transcriptional start sites of the OXA-2 beta-lactamase gene, resulting from the S1 mapping (Plate 12) with RNA from E. coli containing the hybrid plasmid pSU101 and with RNA from the control pSU8 strain, suggests more than one promoter sequence as listed in Table 22. The S1 mapping (Plate 13) with RNA from S. lividans carrying the cloned OXA-2 gene (pSU101) also showed more than one promoter sequence. OXA-2 P(7), the most efficient promoter, showed considerable similarity (5 out of 6 base pairs) to the erm EP2 (Bibb et al., 1985b) at both -35 and -10 conserved sequences, but the separating distance between the two regions was longer than that of erm EP2. This may be a cause of the low level of OXA-2 transcription in S. lividans. The other two promoter sequences were apparently identical to OXA-2 P(1) and OXA-2 P(3). Since all the promoters are fundamentally similar, the presence of multiple transcriptional start sites is not evidence for RNA polymerase heterogeneity. Although it has been reported that S. coelicolor contains several RNA polymerase holoenzyme forms which are distinguishable by their ability to recognize different promoter classes (Westpheling et al., 1985a, b).

The discovery of more than one sigma factor for the RNA polymerase of S. coelicolor A3 (2) (Westpheling et al., 1985a, b) and the recent analysis of a variety of Streptomyces promoters (Bibb et al., 1985a, b; Buttner and Brown, 1985; Janssen et al., 1985) have demonstrated the occurrence of multiple classes of promoter sequences in Streptomyces. These investigations reflect the remarkable capacity of Streptomyces to support the transcription of heterologous genes from a diversity of prokaryotic sources.

The first demonstration that E. coli contains alternative forms of holoenzyme came from studies on the heat-shock response (Neidhardt et al., 1984; Grossman et al., 1985). This heat-shock
response is dependent upon a regulatory gene called htpR or rpoH; its product has extensive amino acid sequence homology to that of sigma-70 (Landick et al., 1984; Yura et al., 1984). Another example of an alternative sigma factor in E. coli comes from studies on gene expression in coliphage T4. The product of the phage T4 regulatory gene 55, is a sigma factor (Sigma-gP55) which enables RNA polymerase to initiate transcription from T4 late promoters (Kassavatis and Geiduschek, 1984).

In Bacillus subtilis cells, so far, five holoenzyme forms have been distinguished; of which three or four are involved in transcription of sporulation (spo) genes or genes under control of sporulation genes (Losick and Youngman, 1984). The principal holoenzyme form which presents in vegetatively growing cells of B. subtilis contains a sigma species of 43000 dalton (Gitt et al., 1985). This sigma factor is equivalent to E. coli sigma-70 in its capacity to direct initiation at promoters of prototypical structure (Moran et al., 1982).
Stability of the plasmid pSU101 in both Escherichia coli and Streptomyces lividans

The results of the stability experiment (section 14) showed that the shuttle vector, pSU101, is stably inherited in both E. coli (Figure 19a) and S. lividans (Figure 21a), even after 40 and 25 generations (respectively), in the absence of selection. This could be related to the high copy number of the plasmid pSU101.

The stable inheritance of a bacterial plasmid implies that there is an efficient mechanism to ensure that each daughter cell receives at least one copy of the plasmid at cell division. This is more applicable to the low copy number plasmids, since the need for an efficient segregation mechanism is less critical for multicopy plasmids. Some plasmids, including R1, pSC101, prophage P1 and F, have been shown to carry a region which enhances stability. This region has been designated par, indicating that it acts by ensuring accurate segregation (partitioning) of plasmids to daughter cells at cell division (Meacock and Cohen, 1980; Miki et al., 1980; Nordstrom et al., 1980). It has been reported that multimerization is a major cause of plasmid instability, because plasmid multimers are maintained at lower copy numbers than monomers (Summers and Sherratt, 1984). This phenomenon found in the multicopy plasmid ColE1, due to the homologous recombination between plasmids, causes a reduction in the plasmid copy number and thus instability. To overcome this problem, the plasmid ColE1 contains a determinant, cer, which is necessary for independent recombination events that efficiently convert any multimer to monomers (Summers and Sherratt, 1984; Sherratt, 1986).

Similar systems are encoded by the multicopy plasmid CloDF13 and the plasmid R46, to resolve the multimeric plasmids to monomers, by encoding the sit-specific resolution functions, parB and per, respectively (Hakkaart et al., 1984; Dodd and Bennett, 1986). Ogura and Higara (1983), have described another system that contributes to the stable inheritance of plasmid F. It temporarily inhibits cell division if it has not itself replicated. It seems
that if there is only one copy of F in a cell when cell division would normally occur, the plasmid inhibits this division until it has replicated, but it inhibits neither cell growth nor chromosome replication. Thus plasmidless daughter cells cannot be formed. Another F plasmid gene releases the inhibition of cell division once the F plasmid has replicated. A similar system causing a transient inhibition of cell division, exists in the R1 plasmid (Nordstrom et al., 1984).

The stable inheritance of the plasmid pSU101, appears to depend on a specific region of the plasmid. Due to the lack of the information about the partition mechanism of the plasmids, pSU8 and pIJ61 (pSU101 components), and how they maintain their stability at cell division, it is not easy to suggest which part of the plasmid is responsible for the maintenance of plasmid stability. Whether the stability is due to a partition function as that of the multicopy plasmid CoIE1 or it contains a special system that ensures duplication of the plasmid and an ordered partitioning at cell division remains to be studied.

Although the plasmid pSU101 was stably inherited, it suffered deletions. The restriction enzyme analysis of the plasmid pSU101B (Figure 20), which was obtained from E. coli at the end of the 5th subculture, revealed that most of that part originating from the Streptomyces plasmid (pIJ61) had undergone deletion. In contrast, figure 22 showed that most of the E. coli plasmid (pSU8) portion in the plasmid pSU101C had undergone deletion when the S. lividans strain, carrying pSU101, was subcultured up to 5 times without selection. This indicates that there is some selection for E. coli or S. lividans plasmid within the related host, and may be due to the known differences in the guanine-plus-cytosine content of their DNA (e.g. 73% for S. lividans and 48% for E. coli).
18.6 Enzyme secretion

The results listed in section 11 and section 12 indicated that the OXA-2 beta-lactamase has indeed been secreted by *S. lividans*, which is therefore able to recognize a typical Gram-negative signal sequence for both secretion and (presumably) cleavage to release soluble protein, although the latter point requires determination of the N-terminal amino acid sequence of the secreted protein.

Systems which allow proteins to be secreted into the medium are more advantageous where high yields of relatively pure protein are required. Systems like these may be used to great advantage, in commercially useful products.

The efficient secretion of the OXA-2 beta-lactamase may, therefore, provide a useful model for studying secretion in *S. lividans* and a way of obtaining secretion of other proteins. Studies on secretion will be facilitated by increasing the production of the enzyme. This can be achieved in two ways: firstly, by improving the existing system, using up-promoter mutations. As has been shown, the *S. lividans* RNA polymerase can recognize more than one class of promoter sequences, and the sequence analysis has shown that the most efficient promoter, that initiates transcription of the OXA-2 gene in *S. lividans*, has characteristic -35 and -10 regions separated by 28 bp (Table 22). The sequence of the -35 and -10 regions (TTGCGG and GAGGAT respectively) have five out of six base pairs homology with the "consensus" sequence for these regions (TTGACG and GAGGAT respectively) of the *Streptomyces* promoter (ermEP2, Bibb et al., 1985b). Up-promoter mutations containing base substitutions in the -35 and -10 regions of this promoter will create regions having precisely the consensus sequence of the ermEP2. Furthermore, the intervening "spacer" region between -35 and -10 regions is very important for efficient transcription (Jaurin and Cohen, 1984); this can be altered by deleting a region of 10 bp to get a separating distance equal to that of the ermEP2. This technique can
be used to change the conserved sequences around -35 and -10 regions of the other class of promoter, OXA2 P(1), which could be recognized by the S. lividans RNA polymerase and showed considerable homology (five out of six base pairs) to the typical prokaryotic consensus sequence of -35 (TTGACA) and -10 (TATAAT) (Hawley and McClure, 1983), since it has been reported that S. lividans contains an RNA polymerase that recognizes and uses the same "-35" and "-10" signals that promote initiation of the ampC in E. coli, and that up-promoter mutations in the -35 region, the -10 region, and the intervening "spacer" region had led to increase synthesis of ampC mRNA in S. lividans as well as in E. coli (Jaurin and Cohen, 1984). Alternatively, increased expression could be achieved by attaching a more efficient Streptomyces promoter such as that from the aph (aminoglycoside phosphotransferase) gene. The aph promoter on pSU101 can be brought closer to the OXA-2 gene by removing the region in between.

Secondly, an increased copy number could be obtained by creating similar fusions with a multicopy vector such as pIJ702. For example, fusion of pSU5 which is an E. coli plasmid carrying the OXA-2 beta-lactamase with the Streptomyces vector pIJ702 at a suitable site, in such a way as to permit the hybrid plasmid to replicate in both E. coli and S. lividans. An attempt was made to do this but the hybrid plasmid, resulting from the fusion of pSU5 cleaved at the BamHI site with the BamHI digested pIJ702, failed to replicate in S. lividans (Data not shown). This may be due to the fusion occurring with a region of pIJ702 which is essential for replication (Kieser et al., 1982).

This approach will provide a useful vector for the expression and secretion of foreign protein in S. lividans, that is by inserting the genes in question adjacent to the signal peptide cleavage site of OXA-2 in such a way that they are translated in phase.

There are at present relatively few reports of the successful application of the bacterial protein secretion system.
Natural extracellular proteins of Bacillus species have been produced commercially for some time, but the application of gene fusion technology is a relatively new development (Palva et al., 1981; Palva et al., 1983). It has been reported that the expression of foreign genes and secretion of the gene products in Bacilli is still limited. This is due to; first, a lack of suitable vectors to promote expression and secretion of the product of an inserted gene and the instability of the existing plasmids except for a few cases; even in those cases foreign DNA cloned into the plasmid is unstable (Gryczan et al., 1978; Clewell et al., 1979; Uhlen et al., 1981; Kreft et al., 1983; Ostroff and Pene, 1984a, b). Secondly, the inefficient transformation methods, either using competent cells (Canosi et al., 1978; de Vos et al., 1981) or protoplasts (Dubnau, 1983) of Bacillus cells.

Bacillus subtilis can use promoters from other Bacillus species and from closely-related Gram positive genera such as Streptococcus (Yagi et al., 1978) and Staphylococcus (Ehrlich, 1977; Kreft et al., 1978), but genes from E. coli (Lee et al., 1980; Mclaughlin et al., 1982; Moran et al., 1982) or from Streptomyces are generally not expressed in this host. This is owing to the requirement in B. subtilis for a strong complementarity between the mRNA ribosome-binding site and the 3' end of 16s rRNA. In addition, the recognition requirement for transcriptional initiation signals may be more stringent for the B. subtilis than for the E. coli RNA polymerase (Mclaughlin et al., 1981; Moran et al., 1982; Murray and Rabinowitz, 1982).

In E. coli, gene fusions have been used to promote the export of foreign proteins to the periplasm by coupling them to signal peptides (Talmadge et al., 1980b; Talmadge and Gilbert, 1982; Suzuki et al., 1982; Mosbach et al., 1983; Ohmura et al., 1984). There are hitherto no reports of the successful secretion of these hybrids to the supernatant, except in certain mutants. The Neisseria IgA protease is extracellular when it is produced by E. coli K12 (Koomey et al., 1982).
Many *Bacillus* genes are expressed readily in *E. coli* (Gray and Chang, 1981; McLaughlin et al., 1982), but most if not all *Streptomyces* genes failed to express in *E. coli* (Bibb and Cohen, 1982). This may be due to the high G+C content of *Streptomyces* DNA, including that in and around its promoter sequences, that presents a barrier to gene expression in heterologous hosts such as *E. coli* and *Bacillus* (Westpheling et al., 1985b).

Due to the complication in both *E. coli* and *Bacillus subtilis* secretion systems, Pugsley and Schwartz (1985) have suggested that *E. coli* nor *B. subtilis* are ideal microorganisms for the commercial application of protein secretion, and they may be superceded by other bacterial strains.

The ability of the *Streptomyces* species to excrete large proteins directly into the culture medium, and the production of a variety of secondary metabolites, particularly antibiotics, make these organisms a potentially interesting alternative host-vector system. The gene, specifying the production of the beta-galactosidase enzyme which is naturally excreted by *S. lividans* was cloned (Burnett et al., 1985) using the *Streptomyces* plasmid pIJ61 and transferred into *S. griseus* or *S. albus* which lack any beta-galactosidase activity. The results revealed the expression and excretion of the enzyme in both strains. They have suggested that this system could offer a potentially useful mechanism to express heterologous gene products. The gene is thereby fused to the beta-galactosidase gene at a point just downstream from the sequence where the proteolytic cleavage occurs, to produce a beta-galactosidase fusion protein. The beta-galactosidase sequence thereby provides the information necessary for synthesis and export of the fusion protein. The extracellular fusion protein will be cleaved at the cleavage site still present on the beta-galactosidase portion of the fusion and will release the heterologous protein in its native form.

There are several other advantages, that could make the
Streptomyces ideal industrial microorganisms, over both E. coli and Bacillus subtilis, such as; the availability of efficient systems for the direct cloning of genes into Streptomyces on plasmid and phage vectors developed from indigenous replicons (Chater et al., 1982b; Hopwood and Chater, 1982; Bibb et al., 1983; Hopwood et al., 1985a, b); the development of protoplast technology which is the key to the development of recombinant DNA technology in Streptomyces. In the presence of polyethylene glycol, protoplasts can take up DNA (Bibb et al., 1978) or fuse with each other (Hopwood et al., 1977; Baltz and Matsushima, 1983) or with liposomes (Makins and Holt, 1981); the great capacity of Streptomyces to support the transcription of heterologous genes from a diversity of prokaryotic sources such as E. coli, Bacillus and Serratia (Bibb and Cohen, 1982); and the discovery of more than one sigma factor for the RNA polymerase of S. coelicolor A3(2), which confer on the RNA polymerase the ability to recognize and respond to a characteristic class of promoters (Westpheling et al., 1985a, b).

The secretion of the cloned Gram-negative OXA-2 beta-lactamase by Streptomyces lividans demonstrates the potential of this system.
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Secretion by *Streptomyces lividans* of a cloned Gram-negative beta-lactamase

(Protein secretion; signal peptide recognition; extracellular enzymes; shuttle vectors)

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1. SUMMARY

The OXA-2 β-lactamase gene was first found on a conjugal plasmid R46 from a clinical isolate of *Salmonella typhimurium*. To transfer the gene to *Streptomyces lividans* a shuttle vector was created by fusing an *Escherichia coli* plasmid carrying the OXA-2 β-lactamase gene with the *S. lividans* vector pIJ61. The OXA-2 β-lactamase gene was expressed in *S. lividans*, although with a much reduced efficiency; virtually all of the β-lactamase activity was found in the culture supernatant. The identity of the enzyme was established by substrate specificity and isoelectric focusing. While previous reports have shown the expression of chloramphenicol-resistance [7] and tetracycline-resistance [8,9] genes from *E. coli* in

2. INTRODUCTION

Interest in gene cloning in *Streptomyces* has primarily been directed at the pathways for antibiotic synthesis. However, *S. lividans* has several advantages as an alternative to *E. coli* as a host for in vitro genetic manipulation, including broad promoter recognition and the ability to secrete some proteins [1]. The nature of the signals required in *S. lividans* for accurate secretion and processing of foreign proteins expressed from cloned genes has not been thoroughly investigated. In this paper, we show that *S. lividans* can efficiently secrete a β-lactamase originating from a plasmid in Gram-negative bacteria.

The OXA-2 type of β-lactamase is a plasmid-mediated enzyme of Gram-negative bacteria which is distinct from the more widely studied TEM β-lactamase [2]; its unusual properties and ease of detection make it a convenient marker for studies such as these, since it can be readily distinguished from the low level of endogenous β-lactamase found in many bacteria, e.g., by its ability to hydrolyse oxacillin and its high isoelectric point [3]. In common with other Gram-negative β-lactamases, it is thought to be secreted across the cytoplasmic membrane and released, by cleavage of the signal peptide, into the periplasm [4,5]. The gene has recently been sequenced in this laboratory and found to contain a putative 21 amino acid signal sequence at the N-terminus [6]. Since Streptomyces, as Gram-positive bacteria, have a simpler cell-wall structure with no outer membrane, transport of proteins across the cytoplasmic membrane will lead to secretion into the culture supernatant. The recognition and cleavage of the signal peptide can therefore be easily examined.
Streptomyces, the location of these proteins in *E. coli* indicates that they are less likely to be secreted in *Streptomyces*. Jaurin and Cohen [10] showed that the ampC gene of *E. coli* (chromosomally determined β-lactamase) could be transcribed in *S. lividans*, but did not report enzyme levels or location. The efficient secretion of the OXA-2 β-lactamase may therefore provide a useful model for studying secretion in *S. lividans* and a way of obtaining secretion of other proteins.

3. MATERIALS AND METHODS

The original source of the OXA-2 β-lactamase was a large conjugative plasmid (R46) found in a clinical isolate of *Salmonella typhimurium* [11]; a restriction map of this plasmid has been published [12]. From R46 was derived, by a series of sub-cloning steps, the plasmid pSU8 which consists of a fragment of R46 carrying the β-lactamase gene inserted between the BamHI and HindIII sites of pACYC184 [6]. The *Streptomyces* vector plasmid pIJ61 [13,14] contains genes for thiostrepton resistance (*tsr*) and aminoglycoside phosphotransferase (*aph*). The size of this plasmid in our hands (13.5 kb) differs slightly from that published elsewhere [13,14]. The bacterial strains used were *E. coli* HB101 and *S. lividans* TK64 [1]. Restriction enzymes, DNA ligase and calf intestinal phosphatase were obtained from commercial sources and used as described by Maniatis et al. [15]. Transformation of *E. coli* HB101 was carried out by the method of Kushner [16]; ampicillin-resistant clones were selected on L agar plus ampicillin (50 µg/ml). Protoplast transformation of *S. lividans* was performed as described by Chatet et al. [9], with selection for thiostrepton resistance. Thiostrepton was obtained from E.J. Squibb, New Brunswick, NJ, U.S.A.

For the measurement of β-lactamase activity, bacterial strains were grown in L broth and harvested by centrifugation. Cell extracts were prepared by ultrasonication. β-Lactamase activity was determined by spectrophotometric assay with the chromogenic substrate nitrocefin [17]. For the measurement of the relative rates of hydrolysis of different substrates, the spectrophotometric method of Samuni [18] was used. The pI value of the enzyme was determined by isoelectric focusing [19], calibrated with commercial standards (Pharmacia).

4. RESULTS AND DISCUSSION

To test the expression and secretion of the OXA-2 enzyme in *S. lividans*, a shuttle vector was constructed by fusing pSU8 with pIJ61 as shown in Fig. 1. Both pSU8 and pIJ61 were digested with XbaI, and the pSU8 digest was further treated...
Table 1  
<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Host</th>
<th>Plasmid</th>
<th>Enzyme level</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD348</td>
<td>E. coli HB101</td>
<td>pSU8</td>
<td>39.4</td>
<td>100</td>
</tr>
<tr>
<td>NOA1</td>
<td>E. coli HB101</td>
<td>pSU101</td>
<td>92.0</td>
<td>234</td>
</tr>
<tr>
<td>NOA2</td>
<td>S. lividans TK64</td>
<td>pSU101</td>
<td>cell filtrate</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>S. lividans TK64</td>
<td>none</td>
<td>cell filtrate</td>
<td>0.15</td>
</tr>
</tbody>
</table>

with calf intestinal phosphatase (CIP) before ligation and subsequent transformation of E. coli HB101. Of 109 ampicillin-resistant clones, 12 were tested for plasmid content and structure, and four were found to have the predicted structure as shown, all with the same orientation to the fusion. One of these was selected for further study (designated pSU101) and was introduced into S. lividans TK64 [1] by protoplast transformation with selection for thiostrepton resistance.

Table 1 shows that OXA-2 expression from pSU101 in E. coli was unexpectedly more than double that of pSU8 in E. coli. Expression in S. lividans was much lower, indicating probably some inefficiency in promoter recognition. However, whereas in E. coli the enzyme was almost wholly cell-associated, with S. lividans it was located almost entirely in the culture supernatant. Virtually no nitrocefin-hydrolyzing activity was detected with either cell extracts or culture supernatants of the parental S. lividans TK64. The β-lactamase activity detected was therefore most likely to be mediated by the recombinant plasmid, but the possibility remained that it was due to derepression of an endogenous enzyme. To demonstrate that it was indeed the OXA-2 β-lactamase we were able to take advantage of several unusual features of the OXA-2 enzyme, namely its ability to hydrolyse oxacillin and its high pI value. Using a spectrophotometric assay [18], we verified that the enzyme from the S. lividans culture filtrate was indeed able to hydrolyse oxacillin, at a rate approx. three times that of benzylpenicillin. The pI value was determined by isoelectric focusing [19] with comparison to both commercial standards and crude and pure OXA-2 β-lactamase preparations from E. coli. The results were identical: in each case a major band at pI = 8.6 was obtained, together with other minor bands which are typically found with the OXA-2 enzyme [19].

We conclude therefore that the OXA-2 β-lactamase can indeed be secreted by S. lividans, which is therefore able to recognise a typical Gram-negative signal sequence for both secretion and (presumably) cleavage to release soluble protein, although the latter point requires determination of the N-terminal amino acid of the secreted protein. Linking this signal sequence to a more efficient Streptomyces promoter will provide a useful vector for the expression and secretion of foreign proteins in S. lividans.

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REFERENCES