PACTAMYCIN RESISTANCE

IN *STREPTOMYCES*

by

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Summary.

The mechanism whereby *Streptomyces pactum* avoids auto-toxicity by its product, pactamycin, has been studied. Pactamycin is a potent inhibitor of translation, but does not inhibit either polyphenylalanine synthesis in response to polyuridylate or other partial reactions of ribosome function. Consequently, the coupled transcription-translation reaction, originally developed for *Streptomyces lividans* (Thompson *et al.*, 1984), was modified such that it functioned with ribosomal components from *S. pactum*. Using this system, ribosomes from *S. pactum* were shown to be highly resistant to pactamycin, due to a property of the 30S ribosomal subunit. Further characterisation of the resistance mechanism was hindered by an inability to reconstitute 30S ribosomal subunits from *S. pactum* that were functional in the coupled transcription-translation reaction. However, similar particles reconstituted from *S. lividans* fractions were active in this system and so the pactamycin resistance gene was cloned in *S. lividans*, as reconstitution analysis could then be performed on two strains of this organism.

The original pactamycin resistance clone contained an unstable plasmid such that in the absence of pactamycin, the resistance gene (*pct*) was deleted from the cloning vector and the majority of ribosomes were sensitive to the drug. Since there was insufficient pactamycin available for consumption in the culture medium, several subclones were generated, which contained *pct* in alternative plasmids. Pactamycin resistant ribosomes were prepared from all the subclones, without demand on the limited pactamycin resources. However, reactions containing subclone ribosomes were only 60-70% resistant to pactamycin, whereas those containing *S. pactum* ribosomes exhibited total resistance. The

incomplete resistance was not due to each ribosome being partially resistant but to the presence of a mixed population of resistant and sensitive particles. Nevertheless, analysis of ribosomes from one of the subclones revealed that the 30S ribosomal subunit caused resistance, and preliminary reconstitution studies indicated that pactamycin resistance is a consequence of 16S rRNA alteration. Since hybridisation analysis demonstrated that the resistance gene did not encode an rRNA operon, post-transcriptional modification of 16S rRNA is probably the mechanism of pactamycin resistance in *S. pactum*.

Resistance to celesticetin in the producing organism, *Streptomyces caelestis*, was also studied using the coupled transcription-translation system. Ribosomes from the producer were resistant to celesticetin and the structurally related antibiotic, lincomycin, due to a property of the 50S ribosomal subunit. When this resistance mechanism was compared with that previously described for the 50S ribosomal subunit of *Escherichia coli*, the results obtained were dependent upon the assay system employed. Polyphenylalanine synthesis in response to polyuridylate by ribosomes from *E. coli* and *S. caelestis* was highly resistant to lincomycin, whereas that performed by *S. lividans* ribosomes was sensitive. However when resistance was tested in a coupled transcription-translation reaction, ribosomes containing 50S subunits from *E. coli* were only slightly more resistant to lincomycin than those particles which contained 50S subunits from *S. lividans*, whereas ribosomes containing *S. caelestis* 50S subunits were highly resistant. Although the reason for this discrepancy has not been established, these findings illustrate the value of an alternative reaction, in which 'natural' protein is synthesised, to the study of antibiotic resistance.

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ABBREVIATIONS.

ATA	aurin tricarboxylic acid
A_x	absorbance at a wavelength of x nm in a 1 cm light path
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3':5'-cyclic monophosphate
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minute
CTP	cytosine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDTA	diaminoethane tetra-acetic acid
EF-G	elongation factor G
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
IF-2	initiation factor 2
kb	kilobase pairs
kD	kilodalton
N.C.I.B.	National Collection of Industrial Bacteria
PEG	polyethylene glycol
PEP	phospho(enol) pyruvate

RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
RNAse	ribonuclease
S	Svedberg unit (10^{-13} seconds)
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TP30	total protein derived from 30S ribosomal subunits
Tris	tris(hydroxymethyl)aminomethane
TTP	thymidine 5'-triphosphate
UTP	uridine 5'-triphosphate

CHAPTER 1

INTRODUCTION

Introduction.

Since the advent of chemotherapy, with the pioneering work of Ehrlich at the turn of the century, several thousand antibiotics have been discovered. Of these, a limited number have proved to be of great clinical value in the treatment of infections, either in their natural form or as semi-synthetic derivatives. However, the majority of antibiotics used in such treatments have been administered on a purely empirical basis, without a detailed insight into their selectivity or mode of action.

It is only in recent years that detailed studies on the molecular basis of antibiotic action have been carried out and this has subsequently proved invaluable to our understanding of some of the complex biosynthetic pathways in both prokaryotic and eukaryotic cells. Many antibiotics exert their toxicity upon the flow of genetic information from DNA to protein. This can occur at the level of DNA replication, for example novobiocin (Smith and Davis, 1965), RNA synthesis, in the case of rifamycin (Hartmann *et al.*, 1967) or protein synthesis, such as tetracycline (Gale and Paine, 1950). A variety of other functions are inhibited by antibiotics, including the biosynthesis of peptidoglycan (the complex polymer found in the cell wall of most bacteria) and the structural or functional integrity of an organism's membrane system. Consequently a cell may be susceptible to antibiotic attack on any one of a number of functional fronts. Furthermore, any individual cellular process is complex and consists of a sequence of reactions, each of which may be a potential antibiotic target site. Thus although rifamycin and streptolydigin both inhibit RNA synthesis and bind to RNA polymerase (Wehrli *et al.*, 1968; Cassani *et al.*, 1971), the former is considered to act when RNA polymerase initiates the synthesis

of an RNA molecule (Sippel and Hartmann, 1968; Umezawa *et al.*, 1968) whereas the latter inhibits the subsequent elongation of the RNA chain (Schlief, 1969; Cassani *et al.*, 1971). This degree of specificity has enabled the elucidation of steps in some complex reactions and mechanisms of antibiotic action to progress concurrently. Furthermore, a position has now been reached whereby many systems can be dissected into more simple steps, thus facilitating the study of structural and functional domains associated with individual steps. This is especially true with our understanding of the mechanism of protein synthesis and the structure of the ribosome.

The ribosome is a very abundant multimacromolecular aggregate which carries out essentially similar reactions in all cells. However, prokaryotic and eukaryotic ribosomes are distinguishable by their sedimentation coefficients. Ribosomes from bacterial origin, of which those from *Escherichia coli* are the best studied, have an 'S' value of 70, whereas those from eukaryotes have a sedimentation coefficient of 80S, although in each case the notional 'S' value represents a range of coefficients.

The *E. coli* ribosome has a mass of 2.3 megadaltons and can be readily dissociated into two unequal subunits when dialysed against a buffer containing 10^{-3} M magnesium ions. The smaller ribosomal subunit (sedimentation coefficient 30S) contains one RNA molecule of 1542 nucleotides (16S rRNA) and twenty one different proteins (numbered S1-S21 in decreasing order of size), whereas the larger '50S' subunit comprises two species of RNA (23S and 5S rRNA) and thirty one different proteins (numbered L1-L31).

The dissociation of eukaryotic ribosomes is less readily achieved than with prokaryotic ribosomes. The smaller subunit (40S) from

rat liver ribosomes contains 18S rRNA and about thirty proteins, whilst the 60S subunit contains 45-50 proteins and three species of RNA: 28S, 5.8S and 5S (for a review of ribosome structure see Liljas, 1982).

Although ribosomes from all sources carry out essentially the same process, *i.e.* the sequential and ordered polymerisation of amino acids, there are key differences in the process between prokaryotic and eukaryotic cells and to a lesser extent between members of each type. Since functional differences and obvious structural variations do exist between types of ribosome, it may be possible for an antibiotic to bind to a site only present on one class of ribosome, whereas another drug may recognise a target site which is equivalent in all ribosomes. In this way some antibiotics discriminate between ribosomes from different sources and their selective toxic effects are often a direct consequence of this. Furthermore, since antibiotics can bind to different sites on ribosomes and impair specific functions, characterisation of a binding site is effectively the localisation of a functional site, provided the antibiotic does act where it binds.

A number of different approaches have been taken to define antibiotic binding sites on the ribosome in an effort to pinpoint functional domains. These have included direct biochemical studies on sensitive ribosomes, comparisons between ribosomes from drug resistant mutants and those from wild-type cells and the study of resistance mechanisms in antibiotic producing organisms. Examples of such studies are presented below.

1.1 The binding site for chloramphenicol.

To indicate the value of alternative direct biochemical approaches to the definition of antibiotic binding sites, some of the

various studies on chloramphenicol binding to ribosomes are discussed below.

Historically, chloramphenicol has been an important antibiotic, since it was the first broad spectrum drug to be used clinically and was the first protein synthesis inhibitor to be shown to bind to the ribosome. The finding that [^{14}C] chloramphenicol could bind to bacterial ribosomes but not to those from several eukaryotes (Vazquez, 1964a) explained the earlier observation that the drug inhibited protein synthesis in intact bacteria (Gale and Paine, 1951) and in prokaryotic cell-free protein-synthesising systems (Lamborg and Zamecnik, 1960) but not in cell-free systems from eukaryotic sources (Rendi, 1959; Raacke, 1959; Bretthauer *et al.*, 1963).

Subsequently, chloramphenicol was shown to inhibit the elongation phase of protein synthesis. In the original model for this process (Traut and Monro, 1964), a peptide bond is formed between the peptidyl moiety of a tRNA molecule bound to one of the two tRNA binding sites, known as the ribosomal 'P' (peptidyl or donor) site and an aminoacyl-tRNA located in the other ribosomal binding site known as the aminoacyl, acceptor or 'A' site. After peptide bond formation, the deacylated tRNA is released from the ribosome and the growing peptidyl-tRNA is translocated to the P site. When the next aminoacyl-tRNA species enters the A site, the cycle is repeated. Recently, a '3 site' model for tRNA binding has been proposed, whereby the spent tRNA is transferred to an 'E' or exit site, prior to release from the ribosome (for review, see Nierhaus *et al.*, 1986). As yet, no results from research into modes of antibiotic action have been re-interpreted using a '3 site' model and therefore all the studies described below are based upon the original '2 site' model. The actual peptide bond forming step

in the elongation process is believed to be inhibited by chloramphenicol both *in vitro* (Traut and Monro, 1964) and in bacterial protoplasts (Cundliffe and McQuillen, 1967). In order to locate the ribosomal binding site for chloramphenicol and thus the functional domain(s) involved in peptide bond formation, studies with [^{14}C] chloramphenicol were carried out. These demonstrated the presence of a high affinity binding site on the 50S ribosomal subunit and a low affinity site on the 30S ribosomal counterpart (Vazquez, 1964b; Lessard and Pestka, 1972; Grant *et al.*, 1979).

A technique which has been instrumental to the localisation of drug target sites, including that of chloramphenicol, is the splitting and reconstitution of bacterial ribosomes. When ribosomes are incubated in lithium chloride solutions, or banded in caesium chloride gradients, specific proteins are removed, leaving core particles. Furthermore, alteration of the salt concentration during the dissociation step enables a number of different but defined particles to be produced, from which ribosomes can be reconstituted when incubated with the split proteins. This type of analysis has been taken to its logical conclusion such that ribosomal subunits can be reconstituted using purified components, thus allowing the functional contribution of individual ribosomal proteins to be assessed (Traub and Nomura, 1968; Nomura and Erdmann, 1970). The involvement of ribosomal protein L16 in chloramphenicol binding to ribosomes was established using a reconstitution approach. Ribosomal core particles lacking this protein were incapable of binding chloramphenicol until the protein was reconstituted back (Nierhaus and Nierhaus, 1973).

Protein L16 was also implicated in the chloramphenicol binding site by studies with the affinity analogue monoiodamphenicol. This

molecule reacts specifically with 70S ribosomes or 50S ribosomal subunits. Although L16 was the primary site for labelling, proteins L6, L24 and S3 were also targets, albeit to a lesser extent (Pongs *et al.*, 1973). However, when monobromamphenicol was used as an affinity probe, L2 and L27 were labelled (Sonnenberg *et al.*, 1973). Finally, when intact *E. coli* cells were administered monoiodamphenicol, ribosomal proteins S6, L16 and L24 were labelled to the greatest extent (Pongs and Messer, 1976).

Complementary studies have to some extent provided a spatial relationship between the ribosomal proteins labelled by chloramphenicol affinity analogues. When antibodies raised to purified ribosomal proteins are bound to ribosomes and observed by electron microscopy, models for the location of proteins on the ribosomal surface can be constructed. Early models suggested that ribosomal proteins L2, L6, L16 and L27 are all clustered on the ribosome (Tischendorf *et al.*, 1974), however the most recent model of Stöffler no longer has proteins L27 and L6 in close proximity (Stöffler and Stöffler-Meilicke, 1986). Hopefully the discrepancies in the locations of ribosomal proteins which are evident when models from different workers are compared, will be resolved in the near future. A consensus model would facilitate the interpretation of results obtained from affinity labelling experiments, since this type of analysis is often complicated by non-specific interactions between the probe and various ribosomal components. In this way, information derived from the use of radiolabelled antibiotic, affinity analogues, antigenic determinants, electron microscopy and reconstitution may be collated to try to pinpoint the chloramphenicol binding domain on the ribosome and consequently components of the peptidyl transferase centre.

Since chloramphenicol inhibits the peptidyl transferase reaction on prokaryotic but not eukaryotic ribosomes, the domain(s) involved in this function must differ in some way between the two types. This is further emphasised by the observation that the protein synthesis inhibitor, anisomycin (Grollman, 1967) only antagonises the peptidyl transfer reaction on eukaryotic ribosomes (Neth *et al.*, 1970; Battaner and Vazquez, 1971). Thus although chloramphenicol and anisomycin both inhibit peptide bond formation, the site of this fundamental step on prokaryotic and eukaryotic ribosomes must be sufficiently different to allow selectivity, despite the close similarities between the substrates and products of the reaction.

1.2 Resistance in mutants due to altered ribosomal proteins.

Comparisons between antibiotic sensitive and resistant ribosomes have enabled a number of antibiotic binding sites to be delineated. There are too many differences between rat liver ribosomes and those from *E. coli* to enable the determination of features which confer chloramphenicol resistance to the former. However, there are examples where ribosomes from more closely related organisms have differing antibiotic sensitivities. For example, *E. coli* ribosomes are more resistant to the unrelated antibiotics amicetin and lincomycin than those from *Bacillus stearothermophilus* (Chang *et al.*, 1966; 1969). When hybrid ribosomes (prepared by isolating ribosomal subunits from both sources and recombining them in each of the four possible combinations) were tested for their sensitivity to the drugs, it was shown that some properties of the 50S ribosomal subunit from *E. coli* conferred resistance *in vitro*. Further analysis has again been complicated by the many differences between the two types of ribosome, although it should

be possible to characterise the resistance further, by reconstitution of 50S ribosomal subunits using components prepared from the two sources.

Ideally, direct comparisons should be made between ribosomes from an antibiotic resistant mutant and a wild-type strain, since these may only differ in one or two components. Furthermore, if the organism involved in such studies has a well established genetic map, then the components involved can be implicated, although not proven, by classical genetic means.

1.21 Characterisation of streptomycin and spectinomycin resistant mutants.

E. coli mutants which are highly resistant to streptomycin have been isolated and the alteration involved has been fully characterised. When protein synthesis was carried out by extracts from the mutant, it was found to be streptomycin resistant, whereas that performed by extracts from wild-type cells was sensitive to the drug (Erdös and Ullmann, 1959; 1960). Furthermore, recombination studies between supernatant and ribosome fractions from resistant and sensitive extracts, showed that the mutant possessed antibiotic resistant ribosomes (Flaks *et al.*, 1962). Subsequently, streptomycin resistance was shown to result from a property of the small ribosomal subunit (Cox *et al.*, 1964; Davies *et al.*, 1964) and in the ultimate reconstitution experiments, the mutant ribosome was shown to be resistant by virtue of an altered ribosomal protein, namely S12 (Ozaki *et al.*, 1969), with an amino acid change at one of two key sites within the protein (Breckenridge and Gorini, 1970; Funatsu and Wittmann, 1972). However, although protein S12 is involved in streptomycin resistance and presumably therefore in drug binding, it cannot bind the drug. In

addition, core particles lacking S12 are substrates for streptomycin binding (Schreiner and Nierhaus, 1973). Other studies have indicated that proteins S3, S5, S9 and S14 are either directly involved in streptomycin binding to ribosomes, or enable the ribosome to attain a conformation conducive to drug binding (Chang and Flaks, 1970).

Just as alterations in ribosomal protein S12 can confer high level streptomycin resistance in *E. coli*, certain changes in protein S5 cause resistance to spectinomycin (Bollen *et al.*, 1969; Funatsu *et al.*, 1972). It is unfortunate that although the precise amino acid replacements involved in spectinomycin and streptomycin resistance are known, the modes of action of the two drugs are not as well defined.

Streptomycin has a number of effects on partial reactions of protein synthesis, including the release of initiator tRNA from preformed 70S initiation complexes (Modollel and Davis, 1970), as well as inhibition of aspects of elongation (Igarashi *et al.*, 1969) and termination reactions (Scolnick *et al.*, 1968). These multiple effects have made it difficult to ascribe a single mode of action to streptomycin.

Perhaps the most striking effect of streptomycin is its ability to cause mistranslation of the genetic code (Davies *et al.*, 1964). Although the incorporation of an incorrect amino acid into peptide linkage normally occurs at very low frequencies (but can be demonstrated *in vitro*), 'misreading' is greatly amplified by the presence of some aminoglycosides, including streptomycin. This drug-induced misreading was greatly reduced on streptomycin resistant ribosomes in cell-free systems synthesising polyphenylalanine (Anderson *et al.*, 1965). However, when natural mRNA was used as a template, there was no difference in

the level of misreading between extracts from sensitive and resistant strains in the presence of the drug (Tai *et al.*, 1978).

Further experiments, examining the effect of protein S12 alteration *in vivo* have indicated that this ribosomal protein controls the overall efficiency of tRNA binding to the ribosome, but has little effect on the codon-anticodon recognition process (Strigini and Gorini, 1970). Finally, the most recent interpretation of the action of streptomycin is that it totally abolishes the proofreading of incorrect tRNA species, but has only a small effect on the accuracy of the initial tRNA selection process (Ruusala and Kurland, 1984) Therefore protein S12 may be part of the functional domain involved in this process.

Spectinomycin does not cause misreading of mRNA codons (Davies *et al.*, 1965), nor does it inhibit the binding of acylated-tRNA to ribosomes *in vitro*, or affect the puromycin-dependent release of nascent peptides (Anderson *et al.*, 1967). The 'puromycin reaction' is a model assay for peptidyl transfer activity (Traut and Monro, 1964) since the antibiotic is an analogue of the 3' terminus of aminoacyl-tRNA (Yarmolinsky and de la Haba, 1959) and will participate (as an acceptor substrate) in a peptide bond-forming reaction with peptidyl-tRNA bound to the 'P' site of the ribosome, with the formation of peptidyl-puromycin (Allen and Zamecnik, 1962; Nathans, 1964; Smith *et al.*, 1965). The latter molecule interacts only weakly with the ribosome and usually dissociates. During normal protein synthesis, the newly formed peptidyl-tRNA would have been translocated to the donor or 'P' site to await the binding of another codon-determined acceptor acyl-tRNA species into the 'A' site, prior to peptide bond formation. The failure of spectinomycin to inhibit substrate binding or peptidyl transfer reactions led to the proposal that the antibiotic inhibited the

translocation reaction (Anderson *et al.*, 1967), a notion that was further supported by studies with intact bacterial protoplasts (Burns and Cundliffe, 1973). Since translocation involves the movement of ribosomes and mRNA with respect to each other and since the mRNA is believed to be associated primarily with the 30S ribosomal subunit, the S5 region of the ribosome may be involved in this interaction.

1.22 Characterisation of thiostrepton resistant mutants.

The problems encountered in trying to assign a functional significance to the alterations in ribosomal protein S12 arose from the multiplicity of apparently different effects exerted by streptomycin. However the model for the binding of thiostrepton to ribosomes and its mode of action, is much better defined. Thiostrepton is a potent inhibitor of protein synthesis in extracts of most bacteria, but not of eukaryotic cells. However, *E. coli* and most Gram-negative bacteria are insensitive to the drug *in vivo* whereas Gram-positive organisms are highly susceptible (Tanaka *et al.*, 1970; Weisblum and Demohn, 1970a). This drug (and several closely related compounds) inhibits the interaction of elongation factor G (EF-G) and GTP with the ribosome during the elongation phase of protein synthesis (Bodley *et al.*, 1970; Weisblum and Demohn, 1970b) and the binding of the elongation factor Tu-GTP-aminoacyl-tRNA complex into the ribosomal A site (Kinoshita *et al.*, 1971; Modollel *et al.*, 1971). Since both events involve the hydrolysis of GTP and these factors cannot bind simultaneously to the ribosome (Carbrer *et al.*, 1972; Miller, 1972; Richman and Bodley, 1972; Richter, 1972; Cundliffe, 1972b), a single GTPase site has been proposed (Cundliffe, 1971; Kinoshita *et al.*, 1971; Modollel *et al.*, 1971), which is believed to be the target domain for thiostrepton.

The localisation of the thiostrepton binding site was facilitated by the isolation of resistant mutants of *Bacillus subtilis* (Smith *et al.*, 1978) and *Bacillus megaterium* (Cundliffe *et al.*, 1979). The mutants possessed thiostrepton resistant ribosomes which lacked a ribosomal protein functionally and serologically equivalent to L11 from *E. coli* (Cundliffe *et al.*, 1979; Stark *et al.*, 1980; Wienen *et al.*, 1979). Full sensitivity to thiostrepton was restored when the mutant ribosomes were supplemented with *E. coli* protein L11 or the *B. megaterium* homologue, BM-L11 (Stark *et al.*, 1980). Protein L11 does not represent the thiostrepton binding site *per se* since binding of radiolabelled antibiotic to the protein could not be detected, whereas a weak but significant association with 23S rRNA was observed (M. Stark and E. Cundliffe, unpublished results). However the binding of thiostrepton to 23S rRNA was greatly enhanced, to a stoichiometry close to unity, by the presence of protein L11 (Thompson *et al.*, 1979). This binding ratio was scarcely reduced when thiostrepton was bound to a complex of 23S rRNA and protein L11 treated with ribonuclease T_1. The resultant complex contained an oligonucleotide of 61 bases (Schmidt *et al.*, 1981) and represents the smallest antibiotic binding site to be isolated from a ribosome.

If the approach of attributing functional domains to antibiotic binding sites is to succeed, evidence that the 61 base oligonucleotide and protein L11 are somehow involved in GTP hydrolysis and elongation factor interactions must be obtained. There is a partial reaction of protein synthesis in which ribosomes and EF-G alone catalyse the hydrolysis of GTP (Nishizuka and Lipmann, 1966) and thiostrepton strongly inhibits this 'uncoupled GTP hydrolysis' reaction. Ribosomes lacking protein L11 possessed a GTP hydrolytic activity of only 50%

compared with wild-type ribosomes, but this residual activity was more resistant to thiostrepton (Stark and Cundliffe, 1979). Moreover, when protein BM-L11 was reconstituted back onto the ribosomes, wild-type levels of activity and drug sensitivity were restored (Stark *et al.*, 1980). Furthermore, it was hydrolysis rather than EF-G-dependent binding of GTP, which was impaired in the mutants.

Evidence that protein L11 was involved in GTP related functions of the ribosome also came from studies in which mutants and wild-type strains were challenged to produce a 'stringent response'. Stringent strains of bacteria are capable of shutting down the synthesis of RNA from rRNA genes (Lazzarini and Dahlberg, 1971), tRNA genes (Primakoff and Berg, 1970; Ikemura and Dahlberg, 1973) and genes encoding ribosomal proteins (Dennis and Nomura, 1974) during conditions of amino acid starvation. This response is coupled to the appearance of guanosine tetra- and penta-phosphates (Cashel, 1969) which are believed to interact with RNA polymerase (Travers, 1976; van Ooyen and Gruber, 1976). These regulatory nucleotides are synthesised from ATP and either GDP or GTP, by a complex of mRNA, deacylated tRNA, the ribosome and stringency factor (Haseltine *et al.*, 1972; Sy and Lipmann, 1973; Pedersen *et al.*, 1973; Block and Haseltine, 1973). However, mutants lacking the ribosomal protein equivalent to L11 were unable to make the guanosine polyphosphates unless reconstituted with the missing protein (Smith *et al.*, 1978; Stark and Cundliffe, 1979; Stark *et al.*, 1980) and thus did not reduce their RNA synthesis under appropriate conditions. Three other 'relaxed' mutants have been described for *E. coli*, although only two have been characterised. Thus *rel* C strains contain an altered form of protein L11 (Parker *et al.*, 1976), whereas *rel* A mutants are unable to

produce a functional stringency factor (Block and Haseltine, 1973). However, these mutations did not result in thiostrepton resistance.

Finally, when EF-G was crosslinked to ribosomes, protein L11 was one of the primary targets (Maassen and Müller, 1981) as was part of the 61 residue oligonucleotide of 23S RNA protected by L11 from ribonuclease digestion (Sköld, 1983). These data taken together present a compelling illustration of thiostrepton binding to a single site within a region of 23S rRNA which interacts with protein L11 and is intimately associated with the EF-G dependent hydrolysis of GTP and the production of guanosine polyphosphates; two reactions that the antibiotic potently inhibits.

In addition to the examples of streptomycin, spectinomycin and thiostrepton resistant ribosomes, alterations in protein S2 have been linked with resistance to kasugamycin (Okuyama *et al.*, 1974); L6 to gentamicin (Buckel *et al.*, 1977); S5, S12 and S17 with neomycin (Cannon *et al.*, 1974; De Wilde *et al.*, 1975) and L4 with erythromycin (Wittmann *et al.*, 1973), although reconstitution analysis has not been carried out in any of the latter cases. However, despite the involvement of various protein alterations with resistance, there are no examples of a strong binding existing between a drug and a purified ribosomal protein. Moreover, the only example of any interaction is a weak association between erythromycin and protein L15, which was detected by equilibrium dialysis (Teraoaka and Nierhaus, 1978). In other cases, either the ribosomal protein involved in drug binding only adopts the necessary conformation as part of a larger structure, or more than one protein may form the binding site. There is one alternative explanation which for a long time was not seriously considered; perhaps the component of key

ribosomal domains with which antibiotics interact is the rRNA rather than the ribosomal proteins.

1.3 Resistance in mutants due to altered ribosomal RNA.

Recent studies have shown that some RNA molecules have catalytic roles in a number of processes. Thus the rRNA precursor in *Tetrahymena*, as well as mitochondrial mRNA and rRNA in fungi are capable of self-splicing (Cech *et al.*, 1981; Gorriga and Lambowitz, 1984). In addition, it is the RNA subunit of ribonuclease P which contains the active site for this enzyme, since tRNA maturation can be catalysed by the RNA in the absence of its protein counterpart under certain conditions (Guerrier-Takada and Altman, 1984; Guerrier-Takada *et al.*, 1983). Therefore, it may be that the RNA component of ribosomes has a direct functional role and is not merely a 'scaffold' on which ribosomal proteins are held in a suitable conformation. It has even been suggested that the first ribosome was an RNA molecule (Crick, 1968), at a time before proteins were employed as catalysts. If rRNA is functionally important in present day ribosomes, one might expect antibiotics to interact with it. Consequently it should be possible to obtain antibiotic resistant mutants with altered patterns of transcriptional modification or primary sequence changes in the RNA. In practice however, the latter type of alteration is unlikely to occur because of the redundancy of rRNA genes in bacteria (Yanofsky and Spiegelman, 1962) and eukaryotes (Darnell, 1968). Thus, a mutation in one *E. coli* rRNA operon would almost certainly be recessive to the other six wild-type loci present.

Ribosomes lacking a specific post-transcriptional modification have been isolated and characterised from a kasugamycin resistant

mutant of *E. coli* (Helser *et al.*, 1971; 1972). When 16S rRNA was isolated from the resistant ribosomes, it was found to lack the two adjacent N^6-dimethyladenosine residues at positions 1518 and 1519 in wild-type *E. coli* 16S RNA. That undermethylation was the cause of resistance was established when 16S rRNA from resistant ribosomes was methylated *in vitro* by extracts from sensitive cells and then reconstituted into active 30S subunits. The resulting ribosomal particles were found to be kasugamycin sensitive. The two dimethylated residues are close to a sequence in the 3' terminus of 16S rRNA which is complementary to a sequence 5' to the translational start codon in most bacterial mRNA (Shine and Dalgarno, 1975; Steitz, 1980) and which is thought to be important in the initiation process. It has been postulated that the interaction between these sequences allows the correct alignment of the start codon with the ribosomal P site, with a possible involvement of ribosomal protein S1 in *E. coli* (Draper and Von Hippel, 1978; Dahlberg and Dahlberg, 1975). Thus the site of kasugamycin resistance is at, or very close to, a key region of the ribosome for the initiation of protein synthesis. This is consistent with the finding that kasugamycin specifically inhibits this phase of translation, by destabilising the complex formed between the 30S ribosomal subunit, initiator tRNA and mRNA (Poldermans *et al.*, 1979). Therefore the drug appears to act at or close to its binding site on the ribosome.

RNA alterations have also been described for viomycin resistant mutants of *Mycobacterium smegmatis* (Yamada *et al.*, 1972). This antibiotic is unusual in that it binds to a single site on each ribosomal subunit. Interestingly the mycobacterial mutants possessed either an altered 30S or 50S ribosomal subunit and resistance in either case was shown to be an unspecified property of the RNA (Yamada *et al.*,

1978). If the viomycin resistances were characterised further they might help to define regions in the ribosome that are important in subunit interactions and the translocation reaction, since the drug has been reported to promote the association of ribosomal subunits, stabilise 70S couples (Yamada and Nierhaus, 1978) and inhibit translocation (Liou and Tanaka, 1976; Modollel and Vazquez, 1977).

Ribosomal RNA sequence alterations are unlikely to cause antibiotic resistance in *E. coli*, because the organism has seven chromosomal rRNA operons. However, the development of gene cloning systems has enabled one rRNA operon to be maintained at multiple copies within the cell, by virtue of its presence on a plasmid. This has led to the isolation of three antibiotic resistant mutants of *E. coli*, with altered rRNA sequences. Sequence analysis showed that a C to U transition at residue 1192 in 16S RNA resulted in spectinomycin resistance (Mark *et al.*, 1983; Sigmund *et al.*, 1984) whereas an A to U substitution at 2058 in 23S RNA conferred resistance to the macrolide erythromycin (Sigmund and Morgan, 1982; Sigmund *et al.*, 1984). A transition from G to A at the adjacent position 2057 resulted not only in resistance to erythromycin, but also to chloramphenicol (Ettayebi *et al.*, 1985). The pattern of antibiotic resistance is further complicated by the finding that the mutation at 2058 also conferred resistance to all macrolide antibiotics, the structurally unrelated lincosamides and streptogramin B type drugs, whereas the 2057 transition only conferred resistance to macrolides with 14 atom lactone rings. The modes of action of the MLS (macrolide, lincosamide, streptogramin B) antibiotics are not all the same. Whereas erythromycin has been labelled a translocation inhibitor because of its effects on

bacterial protoplasts (Cundliffe and McQuillen, 1967) and partial reactions *in vitro* (Igarashi *et al.*, 1969; Corcoran and Oleinick, 1969), lincomycin and some macrolides have been shown to inhibit peptidyl transferase assays (Monro and Vazquez, 1967; Cerna *et al.*, 1971; Mao and Robishaw, 1971). Since chloramphenicol also inhibits this reaction, it is highly probable that residues 2057 and 2058 lie within the peptidyl transferase domain of the ribosome.

The loop of RNA secondary structure in which G2057 and A2058 reside has also been implicated in chloramphenicol and erythromycin binding, from studies with yeast, mouse and human mitochondria (Figure 1.1). Although eukaryotic cells are resistant to these antibiotics, chloroplast and mitochondrial ribosomes are typically sensitive to these and other inhibitors of prokaryotic ribosomes (Wintersberger, 1965; Wheeldon and Lehninger, 1966; Ellis, 1969). Furthermore, mitochondria usually possess a single copy gene for each rRNA species, and therefore a mutation which confers antibiotic resistance will not be competing with wild-type rRNA from other copies in the genome.

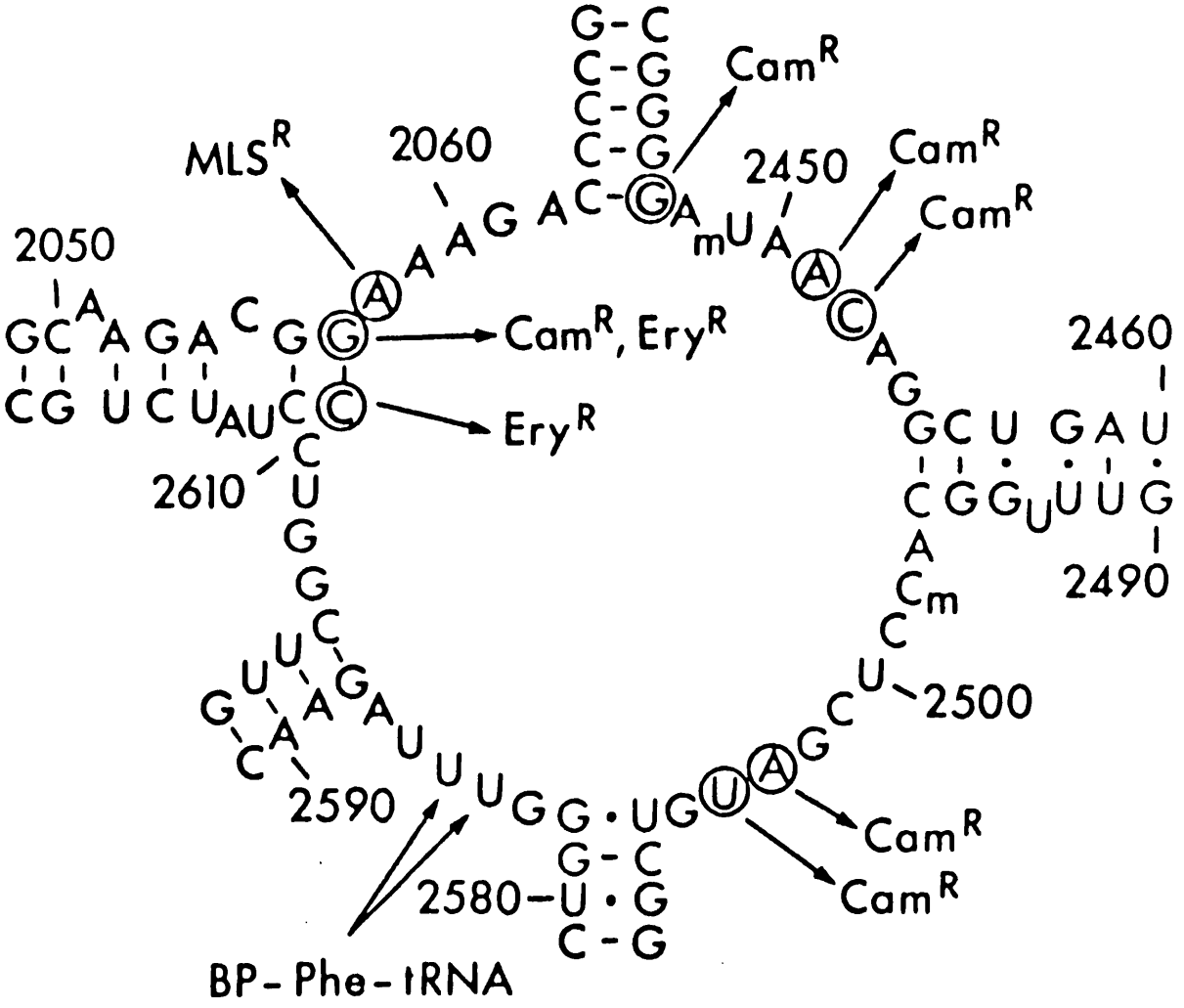
A number of yeast mitochondrial mutants have been isolated which have sequence changes in their large subunit rRNA. Thus an **A** to G change at a position equivalent to 2058 (in *E. coli* 23S RNA) led to erythromycin resistance (Sor and Fukuhara, 1982) as did a C to G transversion at position 2761, although this also conferred resistance to the macrolide spiramycin. Moreover, when position 2611 was a U, the mitochondria were only resistant to spiramycin (Sor and Fukuhara, 1984). Since erythromycin is a 14 atom lactone ring macrolide, whereas the corresponding ring of spiramycin contained 16 atoms, it may be that other members of each class of macrolide share cross-resistance in these examples.

Legend to Figure 1.1.

Secondary structure model for domain V of *E. coli* 23S rRNA.

This figure, modified from Moazed and Noller (1987), shows the sites at which RNA alterations result in resistance to chloramphenicol (Cam), erythromycin (Ery), all MLS antibiotics (MLS) or combinations of these drugs. Some sites have been transposed onto the *E. coli* 23S rRNA secondary structure from equivalent eukaryotic rRNA domains The sites which were affinity labelled by benzophenone-derivatised tRNA (BP-phe-tRNA) are also shown.

Fig 1.1


MLS^R
2060
Cam^R
2450
Cam^R
Cam^R
2050
Cam^R, Ery^R
2460
Ery^R
2610
2490
2500
Cam^R
Cam^R
2590
2580
BP-Phe-tRNA

Studies with chloramphenicol resistant ribosomes from human, mouse and yeast origins have identified four sites which can be altered to give resistance (Dujon, 1980; Blanc *et al.*, 1981; Kearney and Craig, 1981; Slott *et al.*, 1983). These did not include the site at 2057 previously described. Thus a total of seven sites can be mutated which alter the interaction of certain peptidyl transfer inhibitors with the ribosome. Although these sites are somewhat scattered in the primary sequence, they are tightly clustered in the secondary structure models for rRNA (Woese *et al.*, 1983) and probably form a loop with functional significance in peptidyl transfer.

Clustering of antibiotic resistance mutations in rRNA is not confined to the large subunit. Three mutations in the small subunit rRNA from yeast mitochondria and the protozoan *Tetrahymena thermophila* have been located, which result in resistance to paromomycin or hygromycin B. (Li *et al.*, 1982; Bruns *et al.*, 1985; Spangler and Blackburn, 1985). Both antibiotics are aminoglycosides and cause misreading in eukaryotic cells (Palmer and Wilhelm, 1978; Palmer *et al.*, 1979), therefore investigations into their binding sites may locate a functional domain involved in codon-anticodon recognition. The first mutation characterised was a C to G transversion resulting in paromomycin resistance in yeast mitochondria. The base change responsible was at a position equivalent to 1409 in *E. coli* 16S RNA and would disrupt the postulated base-pair formed with G1491 at the end of a highly conserved helical region of RNA secondary structure. The importance of this base-pair was confirmed when a paromomycin resistance mutant of *Tetrahymena* was shown to have a G to A transition at residue 1491. Thus paromomycin resistant ribosomes can be obtained by disrupting either base at the terminus of a helix which begins with base-pair C1409-G1491. That this

region is involved in the interaction of some aminoglycosides was emphasised when a hygromycin B resistant *Tetrahymena* was found to have a U to C change at position 1495, *i.e.* only four nucleotides away from the base-pair involved in paromomycin resistance. Unfortunately, this protozoan is naturally resistant to other aminoglycosides, otherwise the effect of these base changes on sensitivity to members of the kanamycin and gentamicin families of antibiotics could have been examined. *Tetrahymena* has proved a useful organism for the study or rRNA alterations, because the multiple copies of the rDNA present in the somatic macronucleus are derived by amplification of a single copy in the germline micronucleus (Wilhelm *et al.*, 1978).

The first antibiotic resistant ribosomes to have the nature of the mutations precisely located were the streptomycin resistant mutants with alterations in S12. Recently, a second ribosomal alteration which confers streptomycin resistance has been defined. A transition from C to U at a position equivalent to 912 in *E. coli* 16S RNA probably results in resistance in *Euglena gracilis* chloroplasts (Montandon *et al.*, 1985). The events which caused the mutation also caused the deletion of two of the three rRNA operons in the chloroplast genome. Furthermore, each of two streptomycin resistant strains obtained had wild-type S12 sequences and only a single base change at 912. This nucleotide is highly conserved in eubacterial and chloroplast RNA and is therefore likely to be of functional significance. It will be interesting to discover whether this region of RNA secondary structure is close to positions 1409 and 1495 in the tertiary folding of the ribosome and whether it interacts with ribosomal protein S12.

The antibiotic resistant ribosomes described above have all been isolated from mutant strains and possess either an altered protein (or

absent protein) or RNA molecule. In some examples, the organisms were clearly slower growing than the wild-type strains, indicating that the mutation was disadvantageous to the cell. This poses a problem, since it may not be possible to select for mutants resistant to every antibiotic, as the necessary RNA base change or amino acid substitution may be too deleterious to the function of the ribosome. Although this may only happen in rare cases, it prompts an inquiry into the mechanism(s) of self-defence employed by those organisms which synthesise antibiotics where the previously studied resistance mechanisms result in ribosomes with impaired function.

1.4 Antibiotic resistance mechanisms in antibiotic producing organisms.

Investigations into the measures that antibiotic producers take to defend themselves against their potentially lethal metabolites have proved fruitful in the study of antibiotic target sites. There are three broad mechanisms which could be effective against autotoxicity. Firstly, the production of antibiotic may be spatially or temporally compartmentalised, or efficiently excluded, with a permeability barrier to exogenous drug. Alternatively, the antibiotic may be inactivated by substitution or hydrolysis. Finally and of particular interest here, the antibiotic target site may be modified and thus rendered refractory to drug binding and action (see Demain, 1974; Vining, 1979; Cundliffe, 1984).

The vast majority of antibiotics are produced by actinomycetes and fungi, although examples from other eubacteria including *Bacillus*, *Clostridia*, *Corynebacteria*, *Pseudomonas* and *Staphylococcus* have been described. The examples described below all concern antibiotics which inhibit protein synthesis and are produced by actinomycetes.

1.41 Resistance due to exclusion systems.

Of the three approaches that an organism might consider using as a defence mechanism, the first is the most difficult to study. Antibiotic biosynthesis has often been termed part of secondary metabolism; biochemical activities which take place after active growth has stopped. If antibiotics which inhibit ribosomes are produced after protein synthesis has ceased, the amount of antibiotic produced will depend on the stability of the enzymes in the biosynthetic pathway. Furthermore, if the cell is to recover, the ribosome must not be irreversibly inactivated and all the drug must be exported from the cytoplasm to allow protein synthesis to resume. Spatial compartmentalisation may occur as part of the resistance to streptomycin evident in *Streptomyces griseus* (see below), where active drug is produced by removal of a phosphate group during exclusion from the cytoplasm. Two examples of permeability and exclusion as a mechanism of resistance are described below.

Streptomyces sp. 3022a produces chloramphenicol, but has ribosomes which are as sensitive to the antibiotic as those from non-producing bacteria, both in their ability to bind the drug and synthesise polyphenylalanine in response to a polyuridylate template *in vitro* (Vining *et al.*, 1968; Malik and Vining, 1972). Similar results were obtained irrespective of growth phase at the time of harvesting the culture or whether the medium supported antibiotic production.

Although *S. sp. 3022a* grown under non-producing conditions is sensitive to chloramphenicol, the cells do recover after an elapse of time which is proportional to the exogenous drug concentration (as is the level of resistance acquired). Furthermore, resistance only persists whilst exogenous drug is present and is presumed to be due to a loss of

permeability to the molecule. The relationship between drug concentration and recovery time is believed to be a consequence of a constitutively produced intracellular chloramphenicol inactivating enzyme (Malik and Vining, 1971). Most of the chloramphenicol inactivation systems that have been described involve acetylation (reviewed in Shaw, 1983), however that present in *S. sp. 3022a* involves hydrolysis and subsequent acetylation. The enzyme is thought to deal with the slow uptake of chloramphenicol which persists as a result of a leaky permeability barrier which operates when the cells are grown under non-producing conditions.

An alternative resistance mechanism however, is employed when the culture is grown under conditions which support chloramphenicol biosynthesis. The uptake and degradation noted in non-producing conditions end as soon as production starts and the culture becomes fully insensitive to the drug (Malik, 1972). This mechanism is clearly more efficient than the one which is not coordinated to antibiotic biosynthesis.

A similar situation is observed when *S. griseus*, a streptomycin producer, is grown under conditions conducive to drug production. The culture becomes drug resistant with the onset of biosynthesis (Woodruff, 1966) and uptake studies indicate that the drug influx is markedly reduced in cells at this growth phase (Cella and Vining, 1975). Although the exclusion is not complete, no bioactive material was detected in the cytoplasm, presumably due to the action of a streptomycin-6-phosphotransferase present within the cell (Miller and Walker, 1969; Nimi *et al.*, 1971). Thus in at least two antibiotic producing *Streptomyces*, a permeability barrier plays an important role in drug tolerance.

1.42 Resistance due to antibiotic inactivation.

Paradoxically, a cell which inactivates endogenous antibiotic to protect its ribosomes, will not be producing active drug and will therefore not be a producer. Studies of *S. griseus* have shown that although a permeability barrier exists, a streptomycin phosphorylating activity is also present. However, a phosphatase has also been detected, which is associated with streptomycin export (Walker and Walker, 1971). Therefore, since streptomycin phosphate is the penultimate compound in the biosynthetic pathway (Nomi *et al.*, 1967), the cell may operate a system whereby streptomycin is initially inactivated, but is then converted to a toxic product during export. In this way a cell could use inactivation to protect itself and yet still produce antibiotic.

Aminoglycoside inactivating enzymes have also been demonstrated in other aminoglycoside producers, although this type of defence strategy is not confined to this group of antibiotics. Several examples of antibiotic inactivation systems present in producers are shown in Table 1.1.

Clearly, a wide range of antibiotics can be enzymically inactivated. However, since few uptake studies have been performed, it is difficult to assess whether these mechanisms are the sole means by which organisms protect themselves, or whether they play a minor role, dealing with leaky exclusion systems. The situation is further complicated by the finding that an increase in the dosage of the kanamycin acetyltransferase gene in *Streptomyces kanamyceticus* resulted in increased antibiotic production and a greater resistance to kanamycin during early growth phases (Crameri and Davies, 1986). This is probably not a reflection of resistance limiting antibiotic production, since an inducible target site modification system, rendering the ribosome totally

Table 1.1.

Examples of antibiotic inactivation that have been implicated in the prevention of autotoxicity in antibiotic producing *Streptomyces*.

Antibiotic	Producer	Inactivation	Reference
Blasticidin S	*S. morookiensis*	Acetylation	Sugiyama *et al.*, 1986
Capreomycin	*S. capreolus*	Acetylation and phosphorylation	Skinner and Cundliffe, 1980
Hygromycin B	*S. hygroscopicus*	Phosphorylation	Leboul and Davies, 1982
Kanamycin	*S. kanamyceticus*	Acetylation	Hotta *et al.*, 1981
Neomycin	*S. fradiae*	Acetylation and phosphorylation	Davies *et al.*, 1979
Paromomycin	*S. rimosus*	Phosphorylation	White and Davies, 1978
Puromycin	*S. alboniger*	Acetylation	Pérez-González *et al.*, 1983
Streptomycin	*S. griseus*	Phosphorylation	Sugiyama *et al.*, 1981
Streptothricin	*S. lavendulae*	Acetylation	Keeratibipul *et al.*, 1985
Viomycin	*S. vinaceus*	Phosphorylation	Skinner and Cundliffe, 1980

insensitive to kanamycin, is turned on when the organism is grown in antibiotic production medium (Nakano *et al.*, 1984).

That inactivation systems alone can confer antibiotic resistance has been demonstrated for a number of enzymes, by cloning the genes encoding them into an antibiotic sensitive host. When DNA fragments from *Streptomyces vinaceus* and *Streptomyces fradiae* were ligated into plasmids and introduced into *Streptomyces lividans*, viomycin and neomycin resistant transformants were obtained (Thompson, C. J. *et al.*, 1982a). Analysis of crude extracts from the viomycin resistant clones demonstrated the presence of a viomycin phosphotransferase activity and studies with cell-free extracts from the neomycin resistant transformants showed that either a phosphotransferase or acetyltransferase was present (Thompson, C. J. *et al.* 1982b). Similar results have been obtained with enzymes which inactivate hygromycin B (Malpartida *et al.*, 1983), paromomycin (Pérez-González and Jiménez, 1984), puromycin (Vara *et al.*, 1985), streptomycin (Ohnuki *et al.*, 1985; Vallins and Baumberg, 1985) and streptothricin (Kobayashi *et al.*, 1986). Although these results demonstrate that the enzymes can confer resistance to exogenous antibiotic when the genes encoding them have been transferred into *S. lividans*, their true role in antibiotic producers has yet to be fully established.

1.43 Resistance due to target site modification.

Modification of a producer's ribosomes, so that they are resistant to the action of an endogenous protein synthesis inhibitor, is clearly a valuable defence strategy. However, target site modification is not restricted to producers of ribosome inhibitors, since this type of resistance mechanism has also been described in organisms which

synthesise antagonists of RNA polymerase (Watanabe and Tanaka, 1976; Blanco *et al.*, 1984; Roza *et al.*, 1986), isoleucyl-tRNA synthetase (Hughes *et al.*, 1980) and elongation factor Tu (Glockner *et al.*, 1982).

The first ribosome modification system to be characterised was that present in *Streptomyces azureus*, a thiostrepton producer. Whereas the previously described mutants of *B. megaterium* (lacking ribosomal protein BM-L11) were moderately resistant to thiostrepton, *S. azureus in vivo*, and its ribosomes *in vitro* were totally resistant to the antibiotic (Dixon *et al.*, 1975). Subsequently, resistance was shown to be due to the action of a specific RNA methylase (Cundliffe, 1978), which introduces a single methyl group at a position equivalent to A1067 in *E. coli* 23S rRNA (Thompson, J., *et al.*, 1982a). The 2-O-methyl-adenosine produced by this reaction (Cundliffe and Thompson, 1979) resides in the 61 base oligonucleotide protected by ribosomal protein L11 from ribonuclease T_1 digestion (Schmidt *et al.*, 1981). These findings provide further support for thiostrepton interacting within a region previously shown to be involved in GTP hydrolysis. Furthermore, the site of methylation may pinpoint the precise site of thiostrepton binding to 23S rRNA.

The results from *in vitro* experiments were supported when DNA fragments from *S. azureus* were ligated into plasmids and introduced into *S. lividans* (Thompson, C. J. *et al.*, 1982a). The thiostrepton resistant transformants generated in these studies were shown to possess the resistance methylase and contain 23S rRNA which could not be methylated by the enzyme purified from *S. azureus* (Thompson, C. J. *et al.*, 1982b).

It was shown subsequently that other actinomycetes which produce antibiotics related to thiostrepton in structure or function also contain an RNA pentose methylase specific for A1067 (Thompson and

Cundliffe, 1980; Cundliffe and Thompson, 1981; Thompson, J. *et al.*, 1982b).

The second example of ribosome modification to be characterised followed up a previous observation that *Streptomyces erythraeus*, an erythromycin producer, possessed ribosomes that were unable to bind the drug, whereas those from *S. griseus* were fully susceptible (Teraoaka and Tanaka, 1974). The *S. erythraeus* ribosomes were also cross-resistant to all MLS antibiotics tested. Reconstitution analysis proved that dimethylation of 23S rRNA at an adenosine, equivalent to residue 2058 in *E. coli* 23S RNA, resulted in resistance to macrolides (Skinner and Cundliffe, 1982; Skinner *et al.*, 1983). This nucleotide is also dimethylated in 23S rRNA from clinical isolates of staphylococci and streptococci, which are resistant to MLS antibiotics (Lai and Weisblum, 1971; Lai *et al.*, 1973). In addition, the presence of N^6-monomethyl- and N^6-dimethyl-adenosine in 23S rRNA has been noted in a number of *Streptomyces* which produce MLS antibiotics (Graham and Weisblum, 1978; Fujisawa and Weisblum, 1981), however a causal relationship between methylation and resistance remains to be established.

Residue A2058 in 23S rRNA has been implicated in the interaction of erythromycin with the ribosome from studies of mutations in mitochondrial rDNA, rDNA present on a multicopy plasmid in *E. coli* and by RNA modification in antibiotic producers and clinical isolates. Taken with the location of other macrolide and chloramphenicol resistance mutations, there is a growing body of support for the involvement of the loop of RNA secondary structure shown in Figure 1.1, in peptidyl transferase activity.

More recently, target site modifications involving the small ribosomal subunit have been described. *Micromonospora purpurea*, a

gentamicin producer, possesses ribosomes resistant to gentamicin and kanamycin (Piendl and Böck, 1982), whereas an istamycin producer, *Streptomyces tenjimariensis* has kanamycin and apramycin resistant ribosomes (Yamamoto *et al.*, 1981; Skeggs *et al.*, 1985). However, a third pattern of cross-resistance occurs in the nebramycin producer, *Streptomyces tenebrarius*. Ribosomes from this organism are resistant to apramycin and tobramycin (both of which are components of the nebramycin complex) together with other antibiotics of the kanamycin and gentamicin families (Yamamoto *et al.*, 1982; Skeggs *et al.*, 1987). Reconstitution experiments have shown that all three resistance patterns can be attributed to properties of the 16S rRNA, either in the producer, or in aminoglycoside resistant clones of *S. lividans* harbouring genes from the producers (Piendl *et al.*, 1984; Thompson *et al.*, 1985; Skeggs *et al.*, 1985, 1987).

S. lividans JR14 contains the kanamycin-gentamicin resistance determinant from *M. purpurea* cloned into the multicopy plasmid pIJ702 (Thompson *et al.*, 1985). The DNA encodes an RNA methylase which acts on a guanosine at a position equivalent to 1405 in *E. coli* 16S rRNA, although in this case, the substrate for the enzyme was native 30S ribosomal subunits, rather than free RNA (Thompson *et al.*, 1985; Beauclerk and Cundliffe, 1987). A similar substrate was required by a methylase present in *S. lividans* TSK41, encoded by a DNA fragment from *S. tenjimariensis*. Aminoglycoside resistance in this strain was due to methylation of A1408 (Skeggs *et al.*, 1985; Beauclerk and Cundliffe, 1987). Although both enzymes confer kanamycin resistance, the patterns of cross-resistance are dissimilar. Thus A1408 methylation causes apramycin resistance, whereas methylation at G1405 results in gentamicin resistance. *S. tenebrarius* was resistant to all three antibiotics. This

could have been due to a novel methylase acting at both of the above sites, or at a different site, or by the presence of two methylases. The question was resolved when DNA from *S. tenebrarius* was cloned into *S. lividans* and kanamycin resistant clones selected. These could be subdivided into two groups; those that were also gentamicin resistant, e.g. *S. lividans* TSK31 and those that were resistant to apramycin (*S. lividans* TSK51). Methylation was the cause of resistance in each case and a comparison of the restriction maps coupled with Southern analysis has demonstrated strong homology between the cloned DNA in *S. lividans* JR14 and TSK31 and between that in *S. lividans* TSK41 and TSK51 (Skeggs *et al.*, 1987). Therefore *S. tenebrarius* contains two methylases which confer aminoglycoside resistance.

By a detailed characterisation of the antibiotic resistance mechanisms in aminoglycoside producers, the probable binding sites for the aminoglycosides apramycin, gentamicin and kanamycin have been delineated. Furthermore, subtle differences in their binding domains have been established, since kanamycin resistance results from modification of either of two sites, whereas only single and distinct sites have been mapped for apramycin and gentamicin so far. It remains to be seen whether other aminoglycoside producing strains have target site modification systems and if they do, whether the sites are different from those already described. An inducible ribosomal resistance to kanamycin and gentamicin has been demonstrated in *S. kanamyceticus* (Nakano *et al.*, 1984), however the gene responsible shows no obvious similarity to those isolated from *M. purpurea* and *S. tenebrarius*, which confer a similar phenotype (D. Holmes and P. Skeggs, personal communication). It would therefore be interesting to

discover how and where any target site modification system might operate.

The methylation events which confer aminoglycoside resistance in the producers occur at residues which are very close in the RNA secondary structure to sites in *E. coli* 16S rRNA, equivalent to the paromomycin and hygromycin B resistance alterations in yeast mitochondria and *Tetrahymena*. The likely involvement of this region of rRNA in interactions with mRNA and tRNA was discussed in section 1.3. These studies demonstrate again the value of studying antibiotic resistance mechanisms in producers, towards defining functional sites in the ribosome.

1.5 Gene cloning in *Streptomyces*.

The identification of the sites of action of aminoglycoside resistance methylases was greatly facilitated by recombinant DNA methodology. The shotgun cloning of the two ribosomal resistance genes from *S. tenebrarius* into *S. lividans* made the extensive purification of the enzymes unnecessary for studies of their separate effects. Furthermore, if crude extracts from *S. tenebrarius* had been used to study aminoglycoside resistance methylases, *S. lividans* rRNA might have also become methylated at sites which had no bearing on ribosomal resistance. Thus by comparing an antibiotic resistant clone with the host strain containing the vector, the biochemical characterisation is simplified since the difference between the two cells may be a consequence of the action of one gene product.

Gene cloning experiments have also led to the discovery of an inducible modification strategy in *S. kanamyceticus*. Kanamycin resistance in the producer had been previously attributed to the

kanamycin acetyltransferase present, since sensitive ribosomes are present in cells grown under non-inducing conditions. As a result of genetic manipulation, the ribosomal resistance gene was removed from an environment in which its expression was controlled, to one in which it was constitutively expressed. In addition, since the gene was cloned into a high copy number vector, there may be increased levels of the gene product in *S. lividans*, which might aid future biochemical analysis.

Increasing the amount of gene product may be important for the study of some of the resistance methylases. By simple analogy with other enzymes, just a few copies of the thiostrepton resistance methylase, for example, would probably be capable of modifying all the new 23S rRNA synthesised in a cell generation. In this context, it is interesting to note that attempts to detect mRNA in *S. azureus*, complementary to the cloned *tsr* gene have failed, whereas they have been successful with thiostrepton resistant clones of *S. lividans* (G. Janssen, personal communication). This suggests that transcription of the *tsr* gene in *S. azureus* is either very weak or tightly controlled. Furthermore, when promoter sequences from a number of *Streptomyces* genes were compared, those preceding the *tsr* gene were found to be the weakest when tested for their ability to direct transcription of a promoterless indicator gene (Ward *et al.*, 1986). Thus in *S. azureus*, the amount of methylase within the cell is probably very low.

In addition to aiding the biochemical characterisation of antibiotic target sites, resistance determinants have proved useful in the development of *Streptomyces* molecular biology. The first actinomycete gene to be isolated conferred resistance to methylenomycin (Bibb *et al.*, 1980). Previously, the genes for both the biosynthesis of, and resistance to, this antibiotic had been shown genetically to reside

on a large plasmid SCP1 of *Streptomyces coelicolor* (Kirby and Hopwood, 1977), although this particular plasmid has never been isolated, probably due to its large size (>200kb) and low copy number (Chater and Bruton, 1983; Hopwood *et al.*, 1979). The vectors employed in the original cloning experiments were SCP2* and SLP1.2. SCP2* is a derivative of SCP2, a plasmid which naturally resides in *S. coelicolor* at 1-2 copies per chromosome (Bibb *et al.*, 1977).

Subsequently a number of antibiotic resistance genes were isolated using plasmid SLP1.2 (Figure 1.2), one of several episomes obtained after genetic crosses between *S. coelicolor* and *S. lividans*. In the former strain, the SLP1.2 DNA forms an integral part of the chromosome, whereas in *S. lividans* it replicates autonomously at 4-5 copies per chromosome (Bibb *et al.*, 1981). The presence of this plasmid in an organism is made manifest when grown on a lawn of plasmid-free cells. The plasmid is self-transmissible and after conjugal transfer to a recipient, there is a circle of restricted growth (a pock) around the donor mycelium. Such growth inhibition is a characteristic feature of many transmissible plasmids in *Streptomyces*.

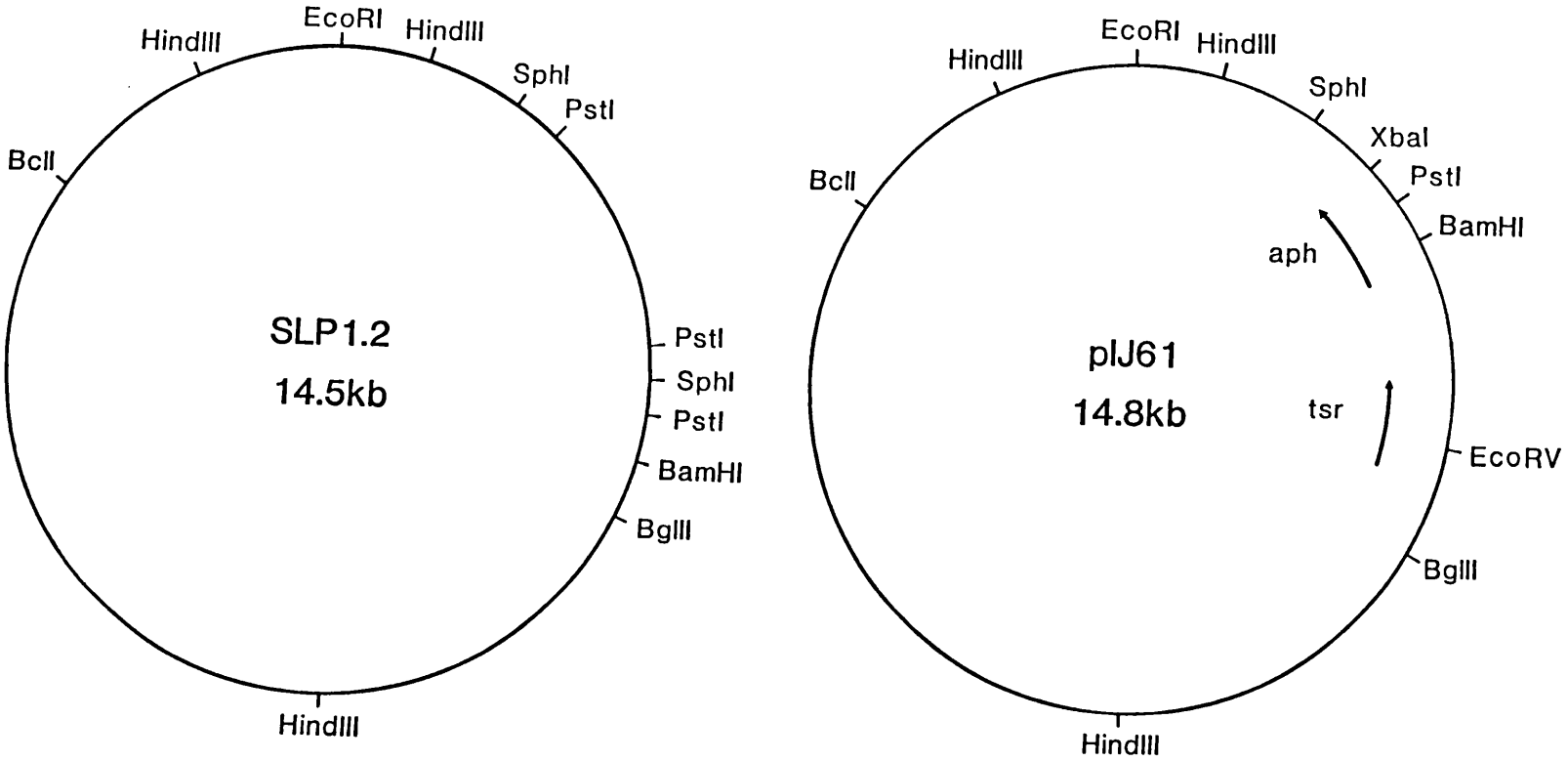
Plasmid SLP1.2 was useful as a cloning vector since it contained a unique cleavage site for BamHI and a cluster of three sites for PstI. When DNA was ligated into these sites, the maintenance, transfer and replication of the plasmid were unimpaired. Therefore these sites were used for cloning the thiostrepton resistance gene (*tsr*) from *S. azureus*, the viomycin phosphotransferase encoding gene (*vph*) from *S. vinaceus* and DNA fragments coding for the neomycin phosphotransferase (*aph*) and acetyltransferase (*aac*) from *S. fradiae* (Thompson, C. J. *et al.*, 1980; 1982a; 1982b). Unlike methylenomycin, the antibiotics thiostrepton, neomycin and viomycin, are readily available and thus suitable for use

Legend to Figure 1.2.

Restriction maps of plasmids SLP1.2 and pIJ61.

SLP1.2 was isolated from *S. lividans* after a genetic cross with *S. coelicolor*. A more versatile derivative of this plasmid, pIJ61, contains the genes for thiostrepton resistance (*tsr*) and neomycin resistance (*aph*) from *S. azureus* and *S. fradiae* respectively.

Fig 1.2


EcoRI
HindIII
HindIII
SphI
PstI
BclI
SLP1.2
14.5kb
PstI
SphI
PstI
BamHI
BglII
HindIII
EcoRI
HindIII
HindIII
SphI
XbaI
PstI
BclI
BamHI
aph
pIJ61
14.8kb
tsr
EcoRV
BglII
HindIII

as dominant selectable markers in improved vectors. One such plasmid, pIJ61 (Figure 1.2), is a derivative of SPL1.2 containing the *tsr* and *aph* genes. Since there are unique sites for BamHI and PstI within *aph*, insertion of DNA fragments into these sites results in the loss of neomycin resistance, allowing the enumeration of thiostrepton resistant transformants containing plasmids with inserts (Thompson, C. J. *et al.*, 1982c). One possible drawback of pIJ61 as a cloning vector is its restricted host range, since only *S. lividans* and *Streptomyces reticuli* have so far been established as hosts.

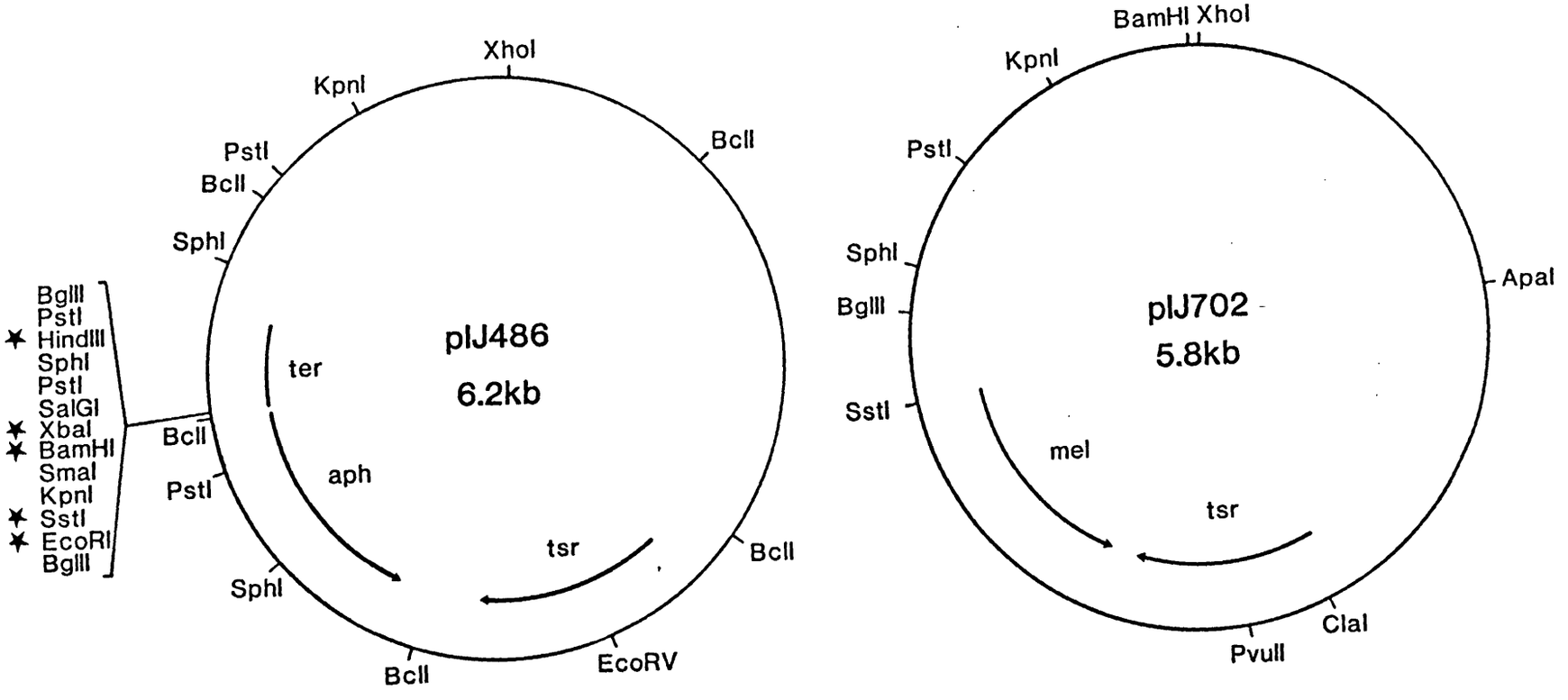
A series of vectors with a larger host range and a much higher copy number (between 40-300 per chromosome) have been developed from a self-transmissible plasmid, pIJ101 (Kieser *et al.*, 1982). This was one of several closely related plasmids isolated from various members of the *Streptomyces violaceoruber* group. A non-transmissible derivative, pIJ702 (Figure 1.3), is one of the most frequently used *Streptomyces* cloning vectors, since it contains *tsr* for the selection of primary transformants and unique BglII, SphI and SstI sites within a region of DNA involved in the synthesis of melanin from tyrosine (Katz *et al.*, 1983). *S. lividans* mycelium containing this plasmid produces a black pigment when grown on a medium supplemented with tyrosine, however, when DNA is inserted at any of the three unique sites within the melanin gene, pigment production is abolished. This provides a rapid means for detecting the number of recombinant molecules after primary selection of transformants with thiostrepton. This plasmid has been used to clone antibiotic production genes (Jones and Hopwood, 1984; Feitelson and Hopwood, 1983; Murakami *et al.*, 1986), genes for antibiotic inactivating enzymes (Malpartida *et al.*, 1983; Pérez-González and Jiménez, 1984; Vallins and Baumberg, 1985) and aminoglycoside ribosomal resistance

Legend to Figure 1.3.

Restriction maps of plasmids pIJ486 and pIJ702.

The cloning vectors pIJ486 and pIJ702 are derivatives of the high copy number plasmid pIJ101 and contain the thiostrepton resistance gene (*tsr*) from *S. azureus* as a selectable marker. Plasmid pIJ486 contains a polylinker of convenient restriction sites upstream from a promoterless neomycin phosphotransferase gene (*aph*) from transposon Tn5. The insertion of correctly orientated promoter sequences into the polylinker region and introduction into *Streptomyces* results in transformants which are resistant to neomycin. The presence of a terminator sequence from coliphage fd prevents transcription of the *aph* gene from any promoter sequences in the vector. Plasmid pIJ487 contains the polylinker sequence in the opposite orientation, but is otherwise identical to pIJ486 in structure. Restriction sites which are unique to the polylinker are indicated *. Plasmid pIJ702 contains DNA (*mel*) from *S. antibioticus* which directs the synthesis of the black pigment, melanin. Insertion of DNA into the SphI, BglII or SstI restriction sites within *mel* usually prevents pigment production.

Fig 1.3


XhoI
KpnI
PstI
BclI
SphI
BglII
PstI
HindIII
SphI
PstI
SalGI
XbaI
BamHI
SmaI
KpnI
SstI
EcoRI
BglII
BclI
PstI
SphI
BclI
EcoRV
BclI
BclI
pIJ486
6.2kb
ter
aph
tsr
BamHI XhoI
KpnI
PstI
SphI
BglII
SstI
ApaI
pIJ702
5.8kb
mel
tsr
ClaI
PvuII

genes (Nakano *et al.*, 1984; Thompson *et al.*, 1985; Skeggs *et al.*, 1985; 1987).

Two other non-transmissible derivatives of pIJ101 have been developed as promoter probe vectors (Ward *et al.*, 1986). In addition to *tsr*, plasmids pIJ486 and pIJ487 possess a promoterless structural gene for an aminoglycoside phosphotransferase from transposon Tn5 (Figure 1.3). The expression of this gene is only achieved when DNA fragments with promoter activity are cloned in the correct orientation into an adjacent polylinker of useful restriction sites.

For the cloning of some genes, low copy number vectors may be desirable. Plasmids such as pIJ940 (Figure 1.4) have been developed from SCP2*. They possess a fairly broad host range and are present at about 1-2 copies per chromosome. However, the major advantage is the great stability that these vectors possess, even when containing DNA inserts 35kb in size (Lydiate *et al.*, 1985). Vectors in this series have been used to clone entire antibiotic biosynthetic pathways, for example that for the biosynthesis of actinorhodin in *S. coelicolor* (Malpartida and Hopwood, 1984). Smaller derivatives of SCP2* were found to have an increased copy number of approximately 30. The shuttle plasmid pOJ160 (Figure 1.4) is a useful variant of the smaller replicon, since it can replicate in *E. coli* and *Streptomyces* (R. Baltz, personal communication). Transformants in the enteric host are selected by resistance to apramycin , whereas in *Streptomyces*, transformants can be selected using either apramycin or thiostrepton. Another useful property of this plasmid is the presence of DNA coding for the α-peptide of β-galactosidase, cloned from pUC19 (Yanisch-Perron *et al.*, 1985). This enables recombinant molecules in *E. coli* to be detected by a simple

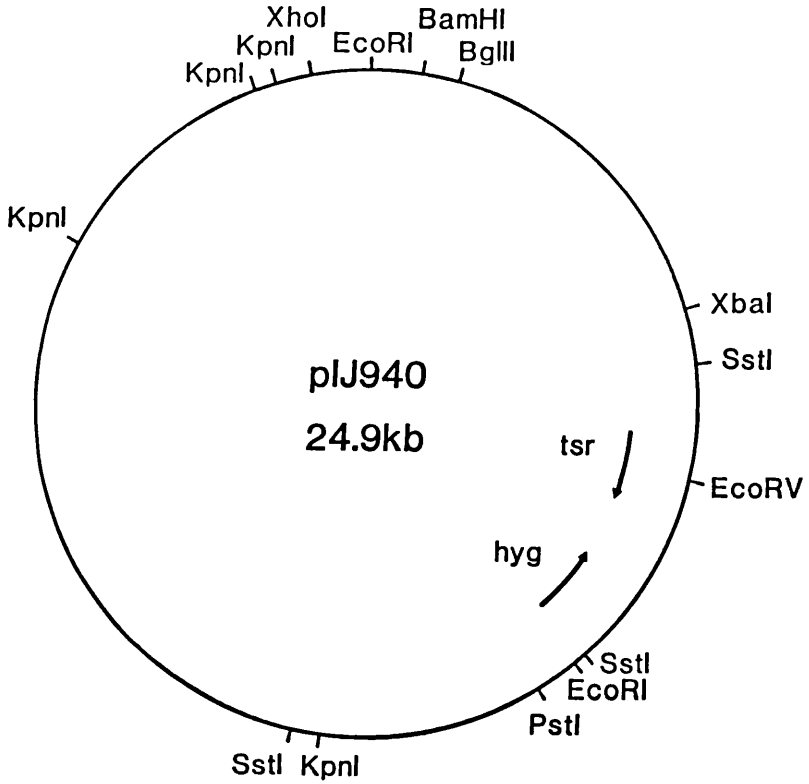
Legend to Figure 1.4.

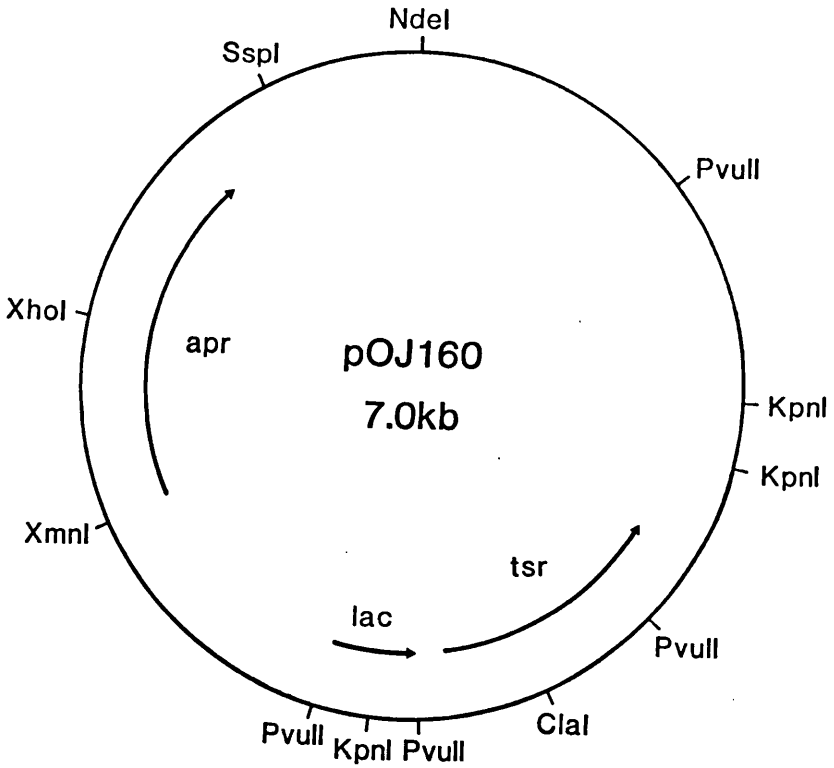
Restriction maps of plasmids pIJ940 and pOJ160.

Plasmids pIJ940 and pOJ160 are both derivatives of the *S. coelicolor* plasmid SCP2 and contain the thiostrepton resistance gene (*tsr*) from *S. azureus*. Plasmid pIJ940 is a conjugative low copy number derivative containing the hygromycin B resistance determinant (*hyg*) from *S. hygroscopicus*.

Plasmid pOJ160 is a non-transmissible derivative which can replicate in *Streptomyces* (plasmid copy number of 30) and in *E. coli* (high copy number). The apramycin resistance gene (*apr*) on pOJ160 is expressed in both hosts. Plasmid pOJ160 also contains DNA (*lac*) which encodes the inducible expression of the β-galactosidase α-peptide, from pUC19.

Fig 1.4


XhoI
KpnI
KpnI
EcoRI
BamHI
BglII
KpnI
XbaI
SstI
EcoRV
pIJ940
24.9kb
tsr
hyg
SstI
EcoRI
PstI
SstI
KpnI


NdeI
SspI
PvuII
XhoI
apr
pOJ160
7.0kb
KpnI
KpnI
XmnI
tsr
lac
PvuII
PvuII
KpnI
PvuII
ClaI

blue-white colour test, using the chromogenic substrate X-gal (see Methods for further details).

In addition to the range of plasmids available for DNA manipulation in *Streptomyces*, a number of bacteriophage vectors have been developed, based on the temperate actinophage ϕC31 (Harris *et al.*, 1983; Rodicio *et al.*, 1985). Several derivatives contain the *tsr* gene and/or *vph* gene to enable the selection of stable lysogens. There are two types of vector in use, differing principally in the presence or absence of an *att* site. This DNA sequence enables the phage genome to become integrated into the chromosome at a complementary sequence, to form a lysogen. Such phages can be used to study the expression of cloned genes at a more 'physiological' gene dosage. Bacteriophages lacking the *att* site can still lysogenise a host, but only if they contain inserted DNA which is homologous to a sequence in the chromosome. However, if the insert does not contain a complete trancriptional unit, a mutation will arise after integration. This method of 'mutational cloning' allows genes to be cloned without the prior isolation of specific mutants and enables the transcriptional patterns within cloned DNA fragments to be analysed. In this way, the operons involved in the biosynthesis of methylenomycin (Chater and Bruton, 1983; 1985) and actinorhodin (Malpartida and Hopwood, 1986) have been characterised.

One of the major goals in *Streptomyces* molecular biology is to clone and study genes involved in antibiotic biosynthesis. The elevation of gene dosage using multicopy plasmids may lead to increased drug production. Another aim is to produce novel antibiotics by combining pathways with common intermediates. That novel compounds can be produced was demonstrated when genes from the actinorhodin

biosynthetic pathway were introduced into an organism which produced a similar antibiotic (Hopwood *et al.*, 1985). Although the novel secondary metabolites produced in this study lacked antibiotic activity, their synthesis holds optimism for the future of novel drug creation.

The cloning of antibiotic biosynthetic pathways has been facilitated by the observation that genes for production and resistance are often closely linked (Kirby and Hopwood, 1977; Rhodes *et al.*, 1981; Murakami *et al.*, 1986). In principle therefore, it should be possible to isolate antibiotic biosynthetic genes by cloning large DNA fragments which contain resistance genes. This approach has been successfully used to isolate genes involved in the biosynthesis of oxytetracycline (Rhodes *et al.*, 1984), streptomycin (Ohnuki *et al.*, 1985) and erythromycin (Stanzak *et al.*, 1986). Analysis of these gene clusters should shed important light on the regulatory signals involved in throwing the switch from primary to secondary metabolism.

One further and potentially very important application of research into antibiotic producing organisms, is that it may enable the mechanisms of antibiotic resistance in clinical isolates to be presaged. Resistance in clinical isolates of bacteria has been due to antibiotic inactivation, target site modification and drug impermeability. Furthermore, the resistance determinants are often encoded on extrachromosomal elements and so may not be of an organism's parental genetic blueprint. Thus, Walker and Walker (1970) suggested that resistance genes originated in antibiotic producing organisms. Support for this idea came when it was shown that aminoglycoside inactivating enzymes from producers had similar properties to those present in resistant clinical isolates (Beneviste and Davies, 1973). Furthermore, the *aph* gene isolated from *S. fradiae* has been sequenced (Thompson and

Gray., 1983) and has significant homology with *aph* genes found on transposons Tn5 and Tn903. Finally, similarities also exist between the mechanism of MLS resistance in staphylococci and streptococci and that functioning in *S. erythraeus*, since the 23S rRNA methylase present in each case dimethylates a position equivalent to A2058 in *E. coli* (Weisblum, 1975; Ranzini and Dubin, 1983; Thakker-Varia *et al.*, 1985; Skinner *et al.*, 1983).

Thus, the study of antibiotic resistance mechanisms in antibiotic producers has not only yielded information on the precise location of some important antibiotic binding sites, but has also contributed greatly to the advances in *Streptomyces* molecular biology. Furthermore, it promises much for the pharmaceutical industry and the treatment of resistant bacteria in hospitals.

1.6 The study of protein synthesis *in vitro*.

The major requirements for the study of antibiotic resistance mechanisms are a sensitive assay system and an appropriate control organism to allow relevant comparisons. In *Streptomyces*, the general cloning host *S. lividans* is a suitable organism, since in many cases resistance is studied in clones carrying resistance determinants from antibiotic producers. In addition there are no constitutively expressed resistance mechanisms in this strain.

Previous studies on ribosome modification systems have employed functional assays based on partial reactions of protein synthesis or synthetic messenger-directed polypeptide production. Since thiostrepton interferes with the interaction of elongation factors with the ribosome and GTP hydrolysis, the 'uncoupled' GTP hydrolysis reaction, catalysed by ribosomes and EF-G, is a highly appropriate assay system. Resistance to

MLS antibiotics in *S. erythraeus* was studied in a polyphenylalanine-synthesising system programmed by a polyuridylate template. Although erythromycin does not inhibit this reaction very strongly, spiramycin (another macrolide) is a potent inhibitor. Furthermore, since the MLS resistance phenotype in other organisms is associated with a single gene, it was assumed that the spectrum of antibiotic resistance observed in *S. erythraeus in vivo* was due to a single ribosomal alteration. Therefore by studying a pattern of cross-resistance to spiramycin, the mechanism of MLS resistance in *S. erythraeus* was elucidated. The polyuridylate-directed assay was also used to study the ribosomal modifications in aminoglycoside producers, since the system was susceptible to the action of most aminoglycosides and was therefore convenient for studying the biochemical basis of the patterns of cross-resistance observed in these organisms.

Although the artificial mRNA-directed system has been valuable in the study of many antibiotic resistance mechanisms, there are some antibiotics of interest which do not inhibit this assay or other partial reactions and for which there are no known cross-resistances. Pactamycin is a good example of such an antibiotic. This drug potently inhibits protein synthesis in prokaryotes and eukaryotes (Young, 1966; Colombo *et al.*, 1966) and may therefore be acting at a ribosomal domain of such importance that it has been conserved during evolution. Since the antibiotic is synthesised by *Streptomyces pactum* (Bhuyan *et al.*, 1961), an *in vitro* assay system applicable to the study of resistance in a *Streptomyces* producer was deemed desirable. Polyuridylate-directed systems are only poorly inhibited by the drug, and since pactamycin's mode of action is far from clear, a simplified assay has not been developed. Furthermore, some of the effects that have been demonstrated

with pactamycin may not be attributable to its primary effect on an organism's metabolism. Thus, a general complex system in which a ribosome passes through many, if not all the modulations of protein synthesis, would be more appropriate for the study of the action of this antibiotic.

A protein-synthesising system directed by natural mRNA would be a suitable assay for studying resistance to ribosome inhibitors. Such systems have already been developed from extracts of *E. coli* (Nathans *et al.*, 1962) and *B. subtilis* (Legault-Demare and Chambliss, 1974). The natural mRNA used in these reactions was either an RNA bacteriophage genome or the major transcripts synthesised from a DNA bacteriophage after infection. However, no RNA phages have been isolated in *Streptomyces* and research directed towards the patterns of transcription observed after phage infection of mycelium, is in its infancy. Therefore the only natural mRNA available would be total endogenous mRNA. Alternatively, a coupled transcription-translation system could be employed. This system would have similar antibiotic sensitivities to those of a natural mRNA system, but would have greater flexibility of template. A coupled transcription-translation system from *Streptomyces* could also be developed for studying actinomycete gene expression *in vitro*, since many important aspects of control mechanism in *E. coli* have been elucidated in this way. Some examples of the applications of *E. coli* coupled transcription-translation systems are presented below.

1.7 *E. coli* gene expression *in vitro*.

The study of protein synthesis *in vitro* started in the laboratory of Zamecnik, where it was demonstrated that ribosomes were the site of peptide synthesis and that the process required ATP, GTP and

tRNA (Hoagland *et al.*, 1958). Following this, it was shown that an artificial RNA template, polyuridylic acid, stimulated the synthesis of polyphenylalanine (Nirenberg and Matthaei, 1961) and subsequent studies on this basis led to the elucidation of the genetic code.

The coat protein of the *E. coli* bacteriophage f2 was the first protein to be synthesised in a cell-free extract (Nathans *et al.*, 1962) and since then a number of viral RNA molecules have been used as templates. Bacterial mRNA has often proved very difficult to isolate, since many molecules have short half-lives, but this problem was overcome by DeVries and Zubay (1967) who prepared a DNA-dependent protein-synthesising system programmed by transducing phage DNA. Subsequently, a large number of different proteins have been produced *in vitro*, in extracts programmed by supercoiled plasmid molecules, relaxed covalently closed plasmids and even DNA fragments. The protein-synthesising systems have ranged from simple cell extracts to highly defined systems, involving thirty purified proteins. Some systems allow completion of polypeptide chains, whereas others restrict protein synthesis to di- or tri-peptide formation. The latter are equally suited for studying effectors of transcription and translation, since most regulatory systems operate before the synthesis of short peptides.

Using combinations of these systems, a number of problems concerning gene expression have been addressed. The classic example is the stimulation of synthesis of enzymes in the *lac* operon by the regulatory nucleotide cAMP (Chambers and Zubay, 1969). By uncoupling transcription from translation, the former activity was shown to be the target for stimulation by cAMP and the inducer analogue, isopropyl-thiogalactopyranoside (reviewed in Chen and Zubay, 1983). Similar systems have enabled the purification of the cAMP binding protein (CAP),

The activity of the regulatory nucleotide, guanosine tetraphosphate, has also been studied in crude cell extracts. This nucleotide stimulates the expression of the *lac* operon *in vitro* (Yang *et al.*, 1974) whereas the converse effect is observed with ribosomal protein operons, genes for elongation factors and rDNA (reviewed by Cozzone, 1980; Chen and Zubay, 1983). Although the precise mechanism has yet to be established, an interaction with RNA polymerase is suspected (Travers and Baralle, 1976; Kingston *et al.*, 1981).

Partially purified systems have been extensively used to support some features of the translational feedback model for regulation of ribosomal protein synthesis (reviewed by Nomura *et al.*, 1984). The model proposes that certain key ribosomal proteins inhibit the translation of their own polycistronic mRNA, when present in excess over rRNA. This was demonstrated directly with the L11 operon present on a transducing phage and purified ribosomal protein L11 (Yates *et al.*, 1980; Baughman and Nomura, 1983) and indirectly, by adding excess rRNA (Yates and Nomura, 1981) to bind the regulatory protein and thereby prevent the inhibition of translation.

Highly defined systems have been used to study the *in vitro* synthesis of elongation factor Tu, the RNA polymerase subunits α,β,β' and the effects of L-factor, a protein which modulates the activity of RNA polymerase at transcription termination or pausing sites (Zarucki-Schulz *et al.*, 1979; Kung *et al.*, 1975).

The dipeptide-synthesising system has certain advantages over the other systems. Fewer proteins are required to synthesise a dipeptide compared with a full polypeptide and there is no recourse to electrophoresis to analyse the results. However, prior knowledge of the N-terminal amino acid sequence or the nucleotide sequence of the gene is

essential. In addition to the study of regulatory mechanisms, dipeptide systems have proved useful in the identification of translational start points within a gene. When such a system was programmed by plasmids carrying the gene encoding initiation factor 2 (IF-2), two dipeptides were produced. These corresponded to the N-terminal dipeptides of the two forms of IF-2 found *in vivo* (Plumbridge *et al.*, 1985). This suggests that the two proteins arise from alternative translational start points, rather than by proteolytic cleavage of the larger form, which is believed to account for the two forms of initiation factor 3 (Lestienne *et al.*, 1982).

Although *E. coli*-derived systems have been used successfully to study the expression of many genes, they have limited value in the study of genes from Gram-positive organisms. Although some genes from *B. subtilis* are expressed in *E. coli*, for example *leu*, *rib*, *citG* (Mahler and Halvorson, 1977; Rabinovich *et al.*, 1978; Moir, 1983), others involved in the cellular differentiation process are not. For the most part, this may be due to the presence in *Bacillus* of multiple forms of RNA polymerase holoenzyme, produced at different stages in the developmental cycle, each differing in their sigma subunit and consequently their specificity of promoter sequence (Losick and Pero, 1981; Johnson *et al.*, 1983). Similarly, there are few examples of the expression of *E. coli* genes in *B. subtilis*; since for example , several antibiotic resistance genes from enteric bacteria are inactive in the latter strain (Ehrlich and Sgaramella, 1978).

In addition to transcriptional barriers to heterospecific gene expression, there are also differences in the translational process. In one study, *E. coli* ribosomes supported protein synthesis with five out of five mRNA species derived from Gram-negative sources and six out of

six mRNAs from Gram-positive cells. However ribosomes from *B. subtilis* were inactive with mRNA of Gram-negative origin, but functioned with four transcripts from Gram-positive sources (Stallcup *et al.*, 1974). Similar results were obtained when ribosomes from *E. coli* and *Clostridium pasteurianum* were compared (Stallcup and Rabinowitz, 1983).

When promoter sequences from *E. coli* and *B. subtilis* genes were compared, a consensus was determined for conserved sequences approximately 10 and 35 base-pairs upstream from the transcriptional start point (Hawley and McClure, 1983). However, when *Streptomyces* promoters are considered, some show few similarities with the eubacterial consensus and even in those cases where the correspondence is stronger, the promoters have usually been non-functional in *E. coli* (Hopwood *et al.*, 1986). Nevertheless there are some promoter sequences in the *Streptomyces* genome which do operate in *E. coli*, but these constitute a minority (Bibb and Cohen, 1982; Jaurin and Cohen, 1985). Conversely, although there may be barriers to the heterospecific expression of *Streptomyces* genes, *Streptomyces* RNA polymerase is capable of utilising at least some promoter sequences from *E. coli*, *Serratia marcescens* and *Bacillus licheniformis* (Bibb and Cohen, 1982).

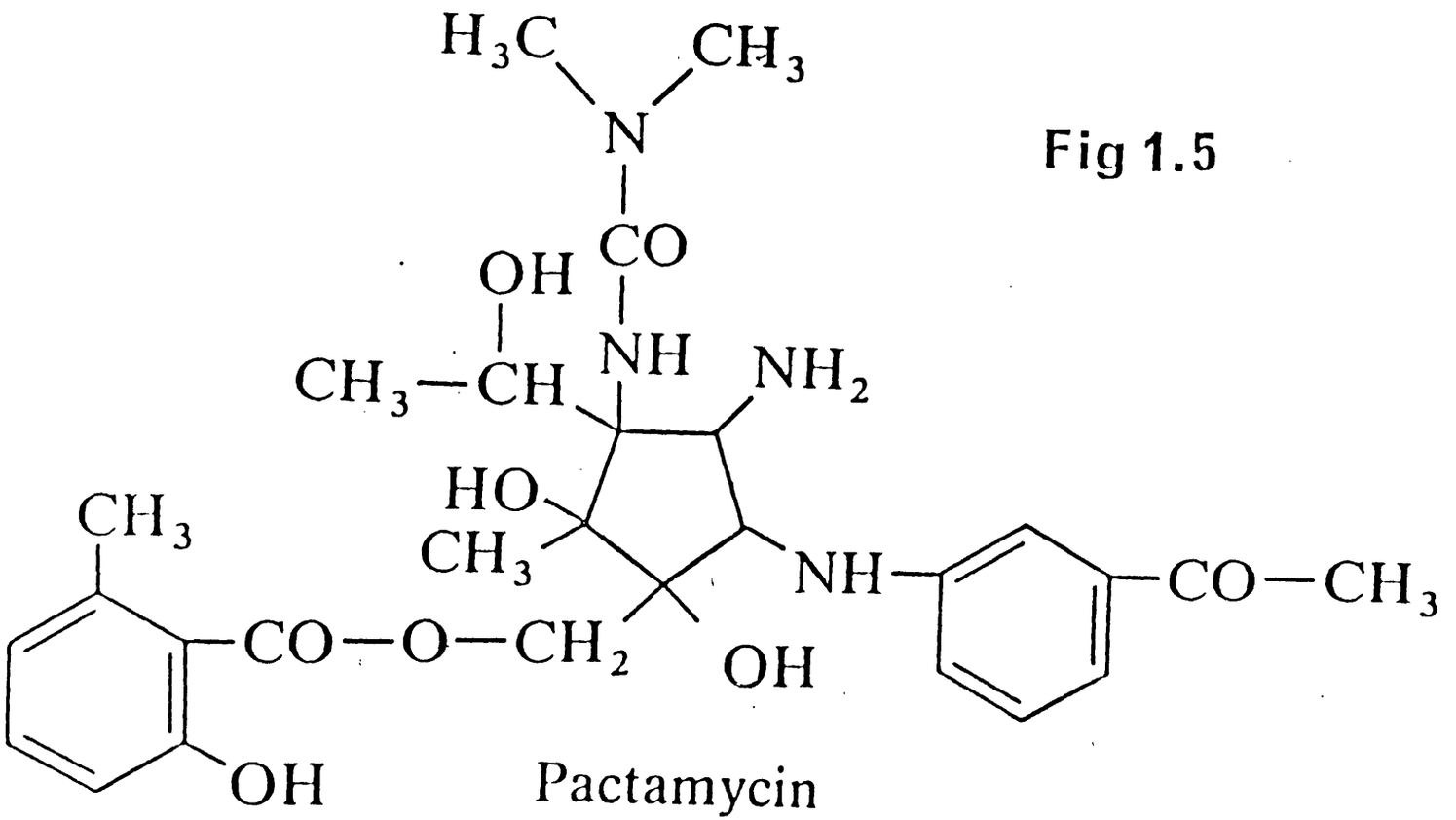
Another important aspect of gene expression in *Streptomyces* is the presence of more than one form of RNA polymerase holoenzyme, as shown in *S. coelicolor* (Westpheling *et al.*, 1985). Thus some of the more atypical promoters isolated from *Streptomyces* may be recognised by RNA polymerases which have no functional homologue in *E. coli*. Alternatively, the inactivity of some *Streptomyces* promoters may be due to the inability of *E. coli* RNA polymerase to separate the GC-rich DNA strands within the promoter, rather than a failure to recognise a specific sequence, since *Streptomyces* DNA has a relatively high G+C

content (Bibb *et al.*, 1983; Westpheling *et al.*, 1985). This is consistent with the relatively high A+T content of the *Streptomyces* promoters which do function in *E. coli* (Jaurin and Cohen, 1985).

In view of the various results, it seemed that *in vitro* expression of *Streptomyces* genes would best be conducted in a coupled transcription-translation system derived from *S. lividans*. This would enable the number, size and location of gene products to be determined for given fragments of DNA. Such a system could also be developed to show the effects of regulatory nucleotides and proteins on gene expression *in vitro*. Furthermore, it would provide a suitable assay for investigation of the modes of action of antibiotics and the mechanisms of resistance encountered among actinomycetes, especially if the system could be fractionated into subcellular components. This would hopefully lead to the discovery of novel target site modification systems and the localisation of antibiotic binding sites.

1.8 Pactamycin.

Pactamycin, a hydrophobic molecule of molecular weight 558 (Wiley *et al.*, 1970 and Figure 1.5) is produced by *Streptomyces pactum* (Bhuyan *et al.*, 1961) and is well suited for study in a coupled transcription-translation system. The drug is active against bacteria of both Gram types and eukaryotes (White, 1962) where protein synthesis is the primary site of inhibition (Young, 1966; Colombo *et al.*, 1966; Bhuyan, 1967; Cundliffe and McQuillen, 1967). Pactamycin has no effect on the charging of tRNA molecules (Bhuyan, 1965; Colombo *et al.*, 1966) but primarily inhibits the activity of ribosomes. This was shown by combining ribosomes and supernatant fractions from pactamycin-treated and untreated reticulocytes, in the four possible pairings and testing



Pactamycin

Fig 1.5

them for activity (Felicetti *et al.*, 1966). Although protein synthesis is the primary focal point, RNA synthesis is stimulated by pactamycin in *E. coli* starved of amino acids (Kersten *et al.*, 1967; Ezekiel and Elkins, 1968) and certain stable RNA species from *B. subtilis* were undermethylated when the culture was grown in subinhibitory pactamycin concentrations (Kersten *et al.*, 1968).

Studies with tritiated pactamycin have shown that at 0°C there is a rapid binding of the drug to a single site on the 30S or 40S ribosomal subunit (Cohen *et al.*, 1969a; MacDonald and Goldberg, 1970). A single site was also demonstrated for the intact 70S or 80S particle, presumably *via* a site on the smaller subunit, since the larger counterpart did not possess a site. However polysomes or monosomes bearing mRNA did not bind the drug (Stewart and Goldberg, 1973), although complexes of initiator tRNA, mRNA and 30S subunits did possess a binding site.

Treatment of polysomes with ribonuclease results in monosomes bearing a small fragment of mRNA. These particles remained refractory to drug binding, whereas ribosomes released from mRNA by 'run-off' (induced by NaF) regained their ability to bind the antibiotic. This was also true of ribosomes released from mRNA by puromycin. However when in the presence of puromycin, the release of ribosomes was blocked by the elongation inhibitor fusidic acid, pactamycin failed to bind even though the nascent peptide had been removed (MacDonald and Goldberg, 1970; Stewart and Goldberg, 1973). Accordingly, it was proposed that either tRNA or a protein factor blocked the pactamycin binding site.

Different results however, were obtained when pactamycin binding to polysomes was investigated at 35°C. When the antibiotic was present

during polysome formation, a slow and accumulative drug binding to the polysomes was observed. Furthermore, inhibitors of the elongation cycle inhibited the binding, suggesting that the slow binding at 35°C was a result of competition between pactamycin and some component of the translational apparatus during cycles of elongation.

To try to establish what specific effects might be a consequence of pactamycin action, the binding of various molecules to the *E. coli* ribosome was studied. Although the drug had no effect on the binding of mRNA to ribosomes, it could inhibit the binding of both polylysyl-tRNA and lysyl-tRNA to ribosomes programmed by polyadenylate (Cohen and Goldberg, 1967). At 7mM Mg^{++} concentration, polylysyl-tRNA binding could be inhibited by approximately 60% in the presence of pactamycin and the binding of lysyl-tRNA was inhibited by about 30%. However when the Mg^{++} concentration was increased to 14mM, pactamycin concentrations which totally inhibited polylysine synthesis had little effect on the binding of either tRNA species, thus questioning the relevance of the results obtained at lower ionic conditions. A destabilisation of the initiation complex was also proposed, since pactamycin was capable of releasing 60% of the pre-bound polylysyl-tRNA, although this effect was also reduced at higher Mg^{++} concentrations.

Similar experiments using N-acetylphenylalanyl-tRNA as a tRNA specific for P site binding in response to polyuridylic acid, showed that the drug could inhibit tRNA binding to the ribosome when added prior to complex formation and could release pre-bound tRNA when added subsequently (Cohen *et al.*, 1969a). This effect was also confined to conditions of low Mg^{++} and high NH_4^+ concentrations. The 70S quasi-initiation complex formed under these conditions was found to have a less compact structure in the presence of pactamycin, especially in an

ionic environment which favoured ribosome dissociation (Cohen and Goldberg, 1969b).

The results with synthetic messengers and bacterial ribosomes suggested that pactamycin might specifically inhibit the initiation of protein synthesis. This view received further support in various experiments with eukaryotic ribosomes. Reticulocyte ribosomes completing the synthesis of pre-existing polypeptides were more resistant to pactamycin than ribosomes obtained from reticulocytes pre-incubated with NaF and therefore carrying out initiation-dependent protein synthesis (MacDonald and Goldberg, 1970). Furthermore when the incorporation of radiolabelled formylmethionine and methionine into protein was followed, to assay initiation and elongation respectively, the former was found to be considerably more sensitive to pactamycin (Lodish *et al.*, 1971). Although a preferential inhibition of initiation has been noted in these and other eukaryotic systems (Stewart-Blair *et al.*, 1971; Ayuso and Goldberg, 1973), there was little difference between the sensitivity of initiation and elongation in bacterial extracts programmed by bacteriophage f2 RNA or with preformed polysomes (Tai *et al.*, 1973; Goldberg *et al.*, 1973).

In an attempt to define the mode of action further, the structure and function of the initiation complex has been studied in eukaryotic systems. In one report (Seal and Marcus, 1972) pactamycin did not prevent methionyl-tRNA (met-tRNA) binding to wheat-embryo ribosomes programmed by tobacco mosaic virus RNA, but caused the tRNA to be unreactive with puromycin. The authors proposed that met-tRNA binding proceeds *via* two steps; in the first step, initiation factors catalyse met-tRNA binding to the ribosome and in the second, another factor converts the met-tRNA to a puromycin reactive form. Pactamycin

was accused of inhibiting the second step. In addition, the drug was said to act after the formation of the 40S-60S couple, since when reactions were analysed on sucrose gradients there was no radiolabelled met-tRNA in the 40S region in the presence of pactamycin.

This result however, was not obtained in a system using reticulocyte ribosomes. In essentially similar experiments, pactamycin caused an accumulation of smaller ribosomal initiation complexes, either by inhibiting the joining of the larger subunit, or by promoting the formation of an inactive 80S complex which readily dissociated (Kappen *et al.*, 1973). The non-functional 40S complex had an altered conformation, since it was resistant to mild nuclease treatment, unlike the normal intermediate. Pactamycin also caused the appearance of an oligomer which sedimented at a rate between that of monosomes and disomes. The '1.5-mer' was proposed to be a ribosome couple and a pactamycin-inactivated 40S subunit on the mRNA at the initiation site. When the reaction mixture was treated with puromycin, a proportion of the radiolabelled met-tRNA associated with the 1.5-mer disappeared and a similar amount appeared at the 40S region of the gradient. This was the expected result, since the ribosome couple was released by puromycin, leaving the 40S initiation complex in a puromycin unreactive form.

A more detailed analysis showed that under certain conditions, pactamycin could exert two effects on eukaryotic translational initiation. Firstly, the drug could block the formation of a stable 80S initiation complex and secondly, it could prevent protein synthesis after the initial dipeptide had been formed (Kappen and Goldberg, 1973; 1976; Kappen *et al.*, 1973). The first blockade was shown to be an impairment of the joining reaction, although the extent of pactamycin inhibition varied between lysates. These workers proposed that pactamycin

sequestered 'joining factor' on the ribosome, so that the greatest inhibition was observed when this factor was most limiting. In the presence of pactamycin, 50% of the radiolabelled methionine associated with ribosomes both *in vitro* and in reticulocytes, was in the form of the first dipeptide of globin, methionine-valine. This dipeptide was shown to be located in the ribosomal P site, since it was reactive towards puromycin. If pactamycin was added after the formation of the dipeptide, there was no effect on subsequent peptide synthesis. This was consistent with the previous observation that pactamycin failed to bind to ribosomes bearing mRNA. The inhibition of tripeptide synthesis when pactamycin was present throughout, may have been due to a block of either peptidyl transfer or the binding of aminoacyl-tRNA into the A site. Since the puromycin reaction had not been inhibited by pactamycin in a number of systems (Felicetti *et al.*, 1966; Cohen and Goldberg, 1967; Cundliffe and McQuillen, 1967), the latter inhibition is the more likely.

An inhibition of aminoacyl-tRNA binding to the ribosomal A site had previously been proposed for the action of pactamycin from studies with bacterial protoplasts (Cundliffe and McQuillen, 1967; Cundliffe, 1972a). Puromycin addition to protoplasts resulted in the release of nascent peptide. This reaction was blocked by erythromycin, presumably by blocking elongation and thus keeping peptidyl-tRNA in the A site. However, when protoplasts were pre-incubated with chloretetracycline, aminoacyl-tRNA binding to the A site was inhibited, thus preventing the erythromycin inhibition of peptide release induced by puromycin. This was because all the peptidyl-tRNA was confined to the P site, *i.e.* the correct site for puromycin. Since pre-incubation of protoplasts with pactamycin produced a similar result, this drug may also prevent aminoacyl-tRNA binding to the A site.

Although the precise effect of pactamycin on eukaryotic ribosomes is still subject to some disagreement, a specific inhibition of initiation can be achieved under certain conditions. This feature has been used by various groups to determine the gene order on eukaryotic polycistronic viral mRNAs (Summers and Maizel, 1971; Taber *et al.*, 1971; Butterworth and Rueckert, 1972). The mapping strategy depends on a single translation initiation site on the mRNA and upon normal production of the proteins in equal amounts. If pactamycin is added to a translation reaction after polysome formation, new rounds of initiation will be prevented and consequently the amount of any one protein produced (relative to that in the absence of pactamycin) will depend on the number of ribosomes that translate that part of the mRNA. Therefore, regions of the template closest to the initiation site will be depleted of ribosomes first and will therefore give rise to less protein than will cistrons at the 3' end of the mRNA.

Studies to define the pactamycin binding site have involved an [^{125}I] derivative of the antibiotic and its intrinsic photoreactivity (Tejedor *et al.*, 1985). The pactamycin analogue possessed similar biological reactivity to the native drug *in vivo* and in cell-free extracts. When the probe was photoincorporated into *E. coli* ribosomes, a specific interaction was observed. When the 70S ribosomal particle was used, similar amounts of radioactivity were associated with the ribosomal proteins and rRNA. However when 30S ribosomal subunits were the target, 70% of the radiolabel was associated with the rRNA. In addition to the changes in distribution between the two types of ribosomal particle, the identity of the most radiolabelled proteins changed. Proteins S2 and S21, which were the second and third most labelled protein in the 30S ribosomal subunit, were not labelled when

ribosomes were employed for photoincorporation. Also, the amount of radiolabel associated with protein S4 increased when 70S ribosomes were used. Clearly then, there are structural changes in the pactamycin binding domain during subunit association. Protein S18 was the most labelled protein in the small subunit experiments, but since this protein contains a highly exposed cysteine residue (Kang *et al.*, 1974) and is often a target for affinity probes, specific labelling of this protein is often questioned. The authors however, expressed confidence in the specificity of radiolabelling, since it was reduced when ribosomes were used, whereas subunit association had little effect on non-specific probes (Michalski and Sells, 1975).

The large subunit proteins L13, L4, L6 and L2 accounted for 34% of the radiolabel associated with ribosomal proteins when 70S particles were employed. These proteins probably therefore, reside on the 50S subunit at a site opposite the cluster of small subunit proteins which are targets. The authors suggested that the small subunit proteins hit by the probe supported the view of pactamycin as a translational initiation inhibitor, since proteins S2, S13, S18 and S21 had all been cross-linked to one or more initiation factor (Cooperman *et al.*, 1981; Pon *et al.*, 1982; Bioleau *et al.*, 1982) and proteins S4, S13, S18 and S21 had been implicated in the mRNA binding site on the ribosome (Ofengand, 1980).

Studies with affinity probes could in principle be complemented by the analysis of pactamycin resistance in mutants. However, although a resistant mutant of *B. subtilis* has been obtained and the mutation mapped close to the origin of replication, there has been no further genetic definition of the locus, or biochemical analysis of the resistance mechanism (Harford and Sueoka, 1970). Cultures of *Bacillus*

amylofaciens have the ability to take up and degrade pactamycin, but they are not resistant to the drug. They are initially inhibited by the drug, but soon recover from the antibiotic with a recovery time which is dependent upon the concentration of drug in the medium (Both *et al.*, 1971).

The study of resistance to pactamycin in the antibiotic producing organism will be of particular interest, especially if a ribosomal modification system is in operation. Localisation of the pactamycin binding site by characterisation of the modification site, would probably locate a key functional domain and might help resolve the mode of action of the drug. Since the binding site has been highly conserved during evolution, it probably resides in a crucial region of the ribosome.

1.9 Aim of the present research work.

The main aim of the present work was to fractionate the coupled transcription-translation system from *S. lividans* developed by S. Rae and J. Thompson and use it to characterise antibiotic resistance mechanisms in the producers of celesticetin and pactamycin. The antibiotic celesticetin is discussed in the introduction to Chapter 4.

CHAPTER 2

METHODS

Methods.

1 Origin, maintenance and growth of organisms.

1.1 Bacterial strains.

The bacteria used in this work were obtained from the sources indicated in Table 2.1.

1.2 Growth and preservation of *Streptomyces*.

Spores from *Streptomyces caelestis*, *Streptomyces lividans* and *Streptomyces pactum* were obtained by growth on NE agar, which contained 1% (w/v) glucose, 0.2% (w/v) yeast extract, 0.1% (w/v) beef extract, 0.2% (w/v) casamino acids and 2% (w/v) agar, adjusted to pH 7.0 with KOH.

All *Streptomyces* strains were preserved as spore suspensions in glycerol. These were prepared by removing spores from the surface of an NE agar plate using 5-10 ml of sterile water and filtering them through a cotton wool plug to remove mycelium. Spores were then concentrated by centrifugation in a Hereaus Christ centrifuge at 3,000 rev min^{-1} for 10 min at room temperature, resuspended in 1 ml 20% (v/v) glycerol and stored at -20°C.

Confluent plates of *Streptomyces* spores were prepared by spreading a loop of spore suspension over the surface of an NE agar plate, followed by incubation at 30°C for 4-5 days. For the preparation of subcellular components for *in vitro* protein synthesis, spores and aerial mycelium were removed from a confluent NE agar plate by agitation in 10 ml 0.1% (v/v) Triton X100, and used to inoculate 2 × 1 l YEME medium (see below) supplemented with 0.5% (w/v) polyethylene glycol

Table 2.1.

Sources of bacteria.

Organism	Strain	Source
Escherichia coli	NM522	Dr. M. J. R. Stark, Leicester Biocentre.
Streptomyces caelestis	NCIB 9751	N.C.I.B.
Streptomyces lividans	M387	Prof. D. A. Hopwood, John Innes Institute, Norwich. Strain TK64 containing plasmid pIJ487.
Streptomyces lividans	TC73	Prof. D. A. Hopwood, John Innes Institute, Norwich. Strain 1326 containing plasmid pIJ61.
Streptomyces lividans	TK21	Prof. D. A. Hopwood, John Innes Institute, Norwich. Plasmid-free derivative of strain 66.
Streptomyces lividans	TSK1	Dr. P. Skeggs, this laboratory. Strain TK21 containing plasmid pIJ702.
Streptomyces pactum	NCIB 9445	N.C.I.B.

6000, in a 2 l baffled flask. Cultures were grown at 30°C for 14-20 hr in a New Brunswick orbital shaker at 220-250 rev min^{-1}.

Cultures for the preparation of DNA or protoplasts were obtained by inoculating 0.1 ml of spore suspension into 25 ml YEME medium supplemented with 0.5% (w/v) glycine and 34% (w/v) sucrose in a 250 ml flask containing a stainless steel coiled spring to aid aeration. Incubation was for 36-42 hr at 30°C in a New Brunswick orbital shaker at 250-300 rev min^{-1}.

YEME medium contained 1% (w/v) glucose, 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, 0.3% (w/v) malt extract and 0.1% (w/v) $MgCl_2.6H_2O$.

1.3 Growth and preservation of *E. coli*.

All *E. coli* strains were grown on LB agar at 37°C for 16-24 hr. LB agar contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride and 2% (w/v) agar. Cells from an LB agar plate which contained 1,000-5,000 colonies were removed in 5 ml minimal salts medium [0.2% (w/v) glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 90 mM $Na_2HPO_4.12H_2O$, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl] and centrifuged in a Hereaus Christ centrifuge at 3,000 rev min^{-1} for 10 min at room temperature. The cells were then resuspended in 0.5 ml minimal salts medium containing 20% (v/v) glycerol, and stored at -20°C.

E. coli cultures were grown in LB medium at 37°C on an orbital shaker at 200-300 rev min^{-1}.

2 Cell-free protein synthesis: conditions for assay and preparation of components.

The following methods were used to prepare subcellular fractions for *in vitro* protein synthesis. Except where stated, all manipulations were carried out at 0-4°C and the final products were divided into small portions, rapidly frozen in an industrial methylated spirits-CO_2 bath and stored at -70°C.

2.1 Preparation of coupled transcription-translation systems from *S. lividans* and *S. pactum*.

This method is essentially as published (Thompson *et al.*, 1984). Mycelium from 6 × 1 l cultures, incubated for 14-16 hr at 30°C, were harvested by centrifugation at 9,000 rev min^{-1} for 10 min in a Beckman JA10 rotor. The mycelium was resuspended in 400 ml buffer I [10 mM HEPES-KOH pH 7.6 at 20°C, 10 mM $MgCl_2$, 1 M KCl, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol] and washed by centrifugation as above. The washing procedure was repeated twice with buffer I and twice with buffer II [50 mM HEPES-KOH pH 7.6 at 20°C, 10 mM $MgCl_2$, 60 mM NH_4Cl, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol]. After the final wash, the mycelium was resuspended in 150 ml buffer II, collected by filtration onto Whatman No. 1 paper, weighed and resuspended in 2.5 ml buffer II per gramme wet weight. Typically, the yield of culture was about 2-3 grammes per litre of culture. The suspension was then passed through a chilled French pressure cell at 10,000-12,000 psi. Unbroken mycelium and cell-debris were cleared from the preparation by centrifugation at 15,000 rev min^{-1} for 30 min in a Beckman SW27 rotor. The supernatant was removed and recentrifuged under identical conditions. The resulting 30,000×g supernatant was designated "S30" and

was typically 200-300 A_{260} units ml^{-1}. Then, while the bulk of the preparation was held at 0°C, a small portion of S30 was treated with micrococcal nuclease for increasing incubation times to determine the time necessary to remove endogenous DNA and RNA, that would otherwise contribute to the plasmid-independent activity of the system. S30 (30 A_{260} units) was incubated with 1 µl nuclease (Stock solution; 150 U μl^{-1} in 50 mM glycine-KOH pH 9.2 at 20°C, containing 5 mM $CaCl_2$) in 1 mM $CaCl_2$ at 30°C. Samples containing 5 A_{260} units were removed at 10 min intervals and EGTA-KOH (pH 7.0 at 20°C) was added to 2 mM final concentration, in order to chelate calcium ions and render the calcium-dependent nuclease inactive. A portion (2 A_{260} units) of each nuclease-treated sample was then assayed for coupled transcription-translation activity in the presence and absence of exogenous plasmid, to determine the minimum incubation time needed to remove plasmid-independent activity. The remainder of the S30 was then appropriately treated with nuclease prior to storage at -70°C. Extracts prepared in this way retained activity for more than twelve months.

2.2 Preparation of S100 and crude ribosome fractions.

A high speed supernatant fraction was prepared from a nuclease-treated S30 by centrifugation at 50,000 rev min^{-1} for 2.5 hr in a Beckman Ti75 rotor. The 100,000×g supernatant (S100) was stored at -70°C. The crude ribosome pellet was resuspended in buffer III [10 mM HEPES-KOH pH 7.6 at 20°C, 10 mM $MgCl_2$, 60 mM NH_4Cl and 5 mM 2-mercaptoethanol] and centrifuged for 5-16 hr at 40,000 rev min^{-1} in a Beckman Ti75 rotor. The pellet was resuspended in buffer III and stored at -70°C.

2.3 Preparation of crude initiation factors for protein synthesis.

This method is based on that developed for the preparation of crude initiation factors from *Bacillus subtilis* (Legault-Demare and Chambliss, 1974). *S. lividans* mycelium from cultures grown for 16-20 hr at 30°C was harvested at 9,000 rev min^{-1} for 10 min in a Beckman JA10 rotor, washed twice in buffer IV [buffer I minus glycerol] and once in buffer III. The pellet from the final wash was resuspended in a small volume of buffer III and passed through a precooled French pressure cell at 10,000-12,000 psi. The resulting suspension was cleared by centrifugation at 15,000 rev min^{-1} for 30 min in a Beckman SW27 rotor. The 30,000×g supernatant was then recentrifuged at 45,000 rev min^{-1} for 4 hr in a Beckman Ti70 rotor. The crude ribosome pellet was resuspended in buffer III, stirred slowly at 0°C for 16 hr and recentrifuged at 45,000 rev min^{-1} as above. The pellet was then resuspended in buffer V [10 mM HEPES-KOH pH 7.6 at 20°C, 10 mM $MgCl_2$, 1 M NH_4Cl and 5 mM 2-mercaptoethanol] and slowly stirred for 16 hr at 0°C. Ribosomes were removed from the preparation by centrifugation as above and the upper four-fifths of the supernatant was collected. Seven volumes of saturated ammonium sulphate solution (pH 7.0 with ammonia) was slowly added to three volumes of supernatant and kept at 0°C for 1.5 hr with continuous stirring. The resultant precipitate was collected by centrifugation at 15,000 rev min^{-1} for 15 min in a Beckman JA21 rotor and the pellet was resuspended in buffer III (1.5 ml per 6 l of original culture). This "factor" preparation was extensively dialysed against buffer III and stored at -70°C.

2.4 Preparation of salt-washed ribosomes.

Salt-washed ribosomes were obtained from 30,000×g supernatants prepared as described in section 2.3 above, with one additional step: DNAase (5 μg ml^{-1} final concentration) was added to the suspension after passage through the French pressure cell. The S30 was then layered over an equal volume of 20% (w/v) sucrose in buffer VI [buffer V with the $MgCl_2$ concentration adjusted to 30 mM] and centrifuged for 5-16 hr at 45,000 rev min^{-1} in a Beckman Ti70 rotor. The supernatant (designated S100*) was then dialysed against buffer III and stored at -70°C. This fraction was used as a source of soluble factors for protein-synthesising systems directed by polyuridylate. Brown membranous material was removed from the ribosome pellet in a small volume of buffer III by gentle agitation with a glass rod, before the ribosomal pellet was resuspended in the same buffer and stored at -70°C.

Salt-washed ribosomes from *E. coli* MRE 600 were prepared by a similar method and were a kind gift from Dr. A. Beauclerk.

2.5 Preparation of ribosomal subunits.

Salt-washed ribosomes (450 A_{260} units) were dialysed against 3 × 1l buffer VII [10 mM HEPES-KOH pH 7.6 at 20°C, 1 mM $MgCl_2$, 150 mM NH_4Cl and 5 mM 2-mercaptoethanol] in order to dissociate the particles into their component subunits. The dissociated ribosomes were then layered onto linear 10-30% (w/v) sucrose gradients in buffer VII and centrifuged for either 1.5 hr at 40,000 rev min^{-1} in a Sorvall TV 850 rotor or for 16 hr at 18,000 rev min^{-1} in a Beckman SW27 rotor. The gradients were then pumped through an Isco UA5 density gradient fractionator with 60% (w/v) glycerol and the A_{254} was monitored continuously. Fractions containing 50S and 30S ribosomal subunits were

separately pooled, the magnesium chloride concentrations raised to 10 mM and the particles harvested by centrifugation at 40,000 rev min^{-1} for 16 hr in a Beckman Ti70 rotor. The ribosomal subunits were finally resuspended in buffer III at 150-300 A_{260} units ml^{-1} and activated by incubation at 30°C for 30 min (Zamir *et al.*, 1974) prior to storage at -70°C. Ribosomal subunits from *E. coli* MRE 600 were a kind gift from Dr. A. Beauclerk.

2.6 Preparation of 16S rRNA.

RNA was prepared from 30S ribosomal subunits by deproteinisation with phenol. Ribosomal subunits in buffer III plus 0.1% (w/v) SDS were shaken with an equal volume of phenol saturated with Tris-HCl (pH 7.5 at 20°C). The phases were separated by low speed centrifugation and the aqueous phase re-extracted with phenol before 16S rRNA was precipitated from the aqueous phase with 3 volumes of ethanol in the presence of 300 mM sodium acetate at -70°C for 1 hr. The RNA was then collected by centrifugation at 8,000 rev min^{-1} for 10 min in a Sorvall HB4 rotor. The pellet was washed with 80% (v/v) ethanol, dried *in vacuo* for 10 min and dissolved in water. After re-precipitation with ethanol and salt as above, the RNA was finally resuspended in buffer VIII [10 mM HEPES-KOH pH 7.6 at 20°C, 0.2 mM $MgCl_2$] at 50 A_{260} units ml^{-1} and stored at -70°C. The integrity of each 16S rRNA preparation was checked by electrophoresis in a denaturing gel system (Lehrach *et al.*, 1977). RNA samples (0.5-1 μg) in formaldehyde-phosphate buffer [6% (w/v) formaldehyde, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4] containing 50% (v/v) formamide, 20% (v/v) glycerol and 0.01% (w/v) BPB, were denatured by incubation at 65°C for 3 min prior to loading onto a 1% agarose gel cast in formaldehyde-phosphate buffer. After electrophoresis for 40 min at

8 V cm^{-1} in the same buffer, the gel was incubated in 10% (w/v) trichloroacetic acid for 20 min and then extensively washed in distilled water to remove formaldehyde. The RNA was stained for 20 min using ethidium bromide (1 $\mu g\ ml^{-1}$ in 0.5 M ammonium acetate) and photographed on a uv transilluminator.

2.7 Preparation of total protein (TP30) from 30S ribosomal subunits.

A solution containing 8 M urea and 4 M LiCl was treated with Bentonite to remove any trace amounts of ribonuclease present and centrifuged briefly in an MSE Microcentaur. The supernatant was passed through a Swinnex filration unit (0.2 μm, Millipore) and mixed in equal volumes with 4 A_{260} units of 30S ribosomal subunits (150-300 A_{260} units ml^{-1} in buffer III) and incubated at 0°C for 72 hr. The 16S rRNA was removed by centrifugation at 48,500 rev min^{-1} for 10 min in a Beckman 30° airfuge rotor and the supernatant was dialysed extensively against buffer IX [30 mM HEPES-KOH pH 7.6 at 20°C, 20 mM $MgCl_2$, 1M KCl and 6 mM 2-mercaptoethanol]. The TP30 preparation was used immediately for the reconstitution of 30S ribosomal particles.

2.8 Reconstitution of 30S ribosomal subunits.

The procedure adopted was that of Traub *et al.*, 1971. 2 A_{260} units of 16S rRNA in buffer VIII was diluted to 0.75 ml in buffer X [30 mM HEPES-KOH pH 7.6 at 20°C, 20 mM $MgCl_2$, 59 mM KCl, 1 mM DTT, 1 mM spermidine trihydrochloride and 5 mM 2-mercaptoethanol] and incubated at 40°C for 5 min. TP30 from 4 A_{260} units 30S ribosomal subunits in 0.25 ml buffer IX plus 1 mM DTT, 1 mM spermidine trihydrochloride and 100 units placental ribonuclease inhibitor, was added and the incubation at 40°C continued for 20 min. The final ionic

strength of the reaction was 0.37 which has been reported as optimal for the reconstitution of *E. coli* 30S ribosomal subunits (Traub *et al.*, 1971). The reconstituted particles were harvested by centrifugation at 40,000 rev min^{-1} in a Beckman Ti75 rotor for 16 hr and resuspended in 30 µl buffer III.

2.9 Conditions for coupled transcription-translation assays.

Assays were performed in 30-50 µl volumes and contained 26% (v/v) synthesis mix (see below), 2-3.3 µg plasmid DNA, [^{35}S] methionine (27 µCi ml^{-1}, 10 Ci $mmol^{-1}$), magnesium acetate to give 12 mM final Mg^{2+} concentration and the components of cell-free extracts to be tested. When S30 was used, 2 A_{260} units were included. Otherwise 25 pmol unwashed ribosomes, 12-24 pmol salt-washed ribosomes or ribosomal subunits were employed, together with crude initiation factor preparation and S100. The inputs of the latter two components were optimised for each preparation. When the activity of reconstituted 30S ribosomal subunits was assayed, 40 pmol subunits were included.

Synthesis mix contained 200 mM HEPES-KOH pH 7.6 at 20°C; 140 mM ammonium acetate; 280 mM potassium acetate; 7 mM DTT; 5 mM ATP (sodium salt; pH 7.0 with Tris); 3.4 mM CTP, GTP, and UTP (all sodium salts; pH 7.0 with Tris); 100 mM PEP (trisodium salt; pH 7.0 with Tris); 19 amino acids (minus methionine) each at 1.4 mM; 7.5% (w/v) PEG 6000; 260µM calcium folinate and 100 units pyruvate kinase in 20% (v/v) glycerol.

Incubations were at 30°C. 5-10 µl samples were removed into 0.1 M KOH at various time intervals and heated at 95°C for 7 min to hydrolyse methionyl-tRNA. Following the addition of excess 10% (w/v) TCA, acid-precipitable material was collected on Whatman GF/C filters,

extensively washed with 5% (w/v) TCA and dried under infra-red light. The radioactivity retained on the filters was estimated by liquid scintillation spectrometry using a toluene-based scintillation fluid (Fisofluor No. 3).

2.10 Conditions for protein synthesis directed by polyuridylic acid.

Cell-free synthesis of polyphenylalanine was carried out in 50 µl reaction mixtures. Reactions were started by the addition of 25 µl "poly-U cocktail" to ribosomes (4 pmol) or ribosomal subunits (4 pmol of each) and S100 or S100* in a volume of 25 µl. The "poly-U cocktail" contained 40 mM HEPES-KOH (pH 7.6 at 20°C); 20 mM $MgCl_2$; 200 mM KCl; 5 mM ATP (sodium salt); 0.75 mM GTP (sodium salt); 10 mM PEP (trisodium salt); 19 amino acids (minus phenylalanine) each at 75 µM; L-[u-^{14}C] phenylalanine (10 µCi ml^{-1}, 518 mCi $mmol^{-1}$); 2 mg ml^{-1} *E. coli* tRNA (phenylalanine specific); 1 mg ml^{-1} polyuridylate (potassium salt) and 200 units ml^{-1} pyruvate kinase in 20% (v/v) glycerol. The final concentrations of magnesium and monovalent cations were 15 mM and 150 mM respectively.

The reactions were performed at 30°C. 10 µl samples were removed into approximately 1 ml 0.1 M KOH at 10 min time intervals and processed as described in 2.9 above.

2.11 Polyacrylamide gel analysis of the products of protein synthesis *in vitro*.

Samples for gel analysis were produced as above (section 2.9) except that [^{35}S] methionine (14 Ci ml^{-1}; 800 Ci $mmol^{-1}$) was used. After 20 min incubation at 30°C, 2 µl unlabelled methionine (44 mg ml^{-1}) was added to the reaction and the incubation continued for 10 min, to allow

completion of all radiolabelled peptides. Then a 2 µl sample was removed to estimate the radioactive content of the hot TCA-precipitable material. The samples were then mixed with one third volume of loading buffer [375 mM Tris-HCl pH 8.8 at 20°C; 4% (w/v) SDS; 35% (v/v) glycerol; 2.7 M 2-mercaptoethanol and 0.01% (w/v) BPB], heated for 10 min at 100°C prior to loading onto a 10% (w/v) polyacrylamide gel (14.5 × 10.5 × 1.5 cm) containing 0.1% (w/v) SDS, prepared according to the standard procedure of Laemmli (1970). Electrophoresis was typically for 5 hr at 20 mA constant current. The gels were then fixed in 7% (v/v) acetic acid, treated with AMPLIFY and dried onto Whatman No.1 paper, prior to fluorography using Fuji RX film at -70°C.

3 Isolation and manipulation of DNA.

3.1 Preparation of total genomic DNA.

The isolation of total DNA from *S. lividans* and *S. pactum* was carried out using the lytic procedure of Smith (in Hopwood *et al.*, 1985).

A 25 ml *Streptomyces* culture was incubated at 30°C for 40-42 hr and then harvested by centrifugation at 3,000 rev min^{-1} for 10 min at room temperature in a Hereaus-Christ centrifuge. The mycelium was washed by centrifugation through 10.3% (w/v) sucrose, as above. The pellet was then resuspended in 4 ml "lysozyme solution" [2 mg ml^{-1}, 25 mM Tris-HCl pH 8.0 at 20°C, 25 mM EDTA-KOH pH 8.0, 10.3% (w/v) sucrose] and incubated for 10 min at 37°C. After the addition of 4 ml Kirby mix [2% (w/v) sodium triisopropylnapthalene sulphonate, 12% (w/v) sodium 4-aminosalicylate, 0.1 M Tris-HCl pH 8.0 at 20°C, 6% (v/v) phenol equilibrated with 0.1 M Tris-HCl pH 8.0 at room temperature], the preparation was agitated on a vortex mixer for 1 min. 8 ml phenol

(saturated as above): chloroform: isoamyl alcohol (50:50:1) was added, the mixture agitated for a further 15 s and then centrifuged at 3,000 rev min^{-1} for 10 min. The supernatant was removed, re-extracted with an equal volume of phenol: chloroform: isoamyl alcohol and centrifuged as above. Following the precipitation of DNA and RNA from the aqueous phase with an equal volume of isopropanol in the presence of 300 mM sodium acetate, nucleic acid was spooled out using a glass hook. The precipitate was then washed in 80% (v/v) ethanol and dissolved in 5 ml TE buffer [10 mM Tris-HCl pH 8.0 at 20°C, 1 mM EDTA-KOH pH 8.0 at 20°C] containing 40 $\mu g\ ml^{-1}$ RNAse (pre-heated to 90°C for 10 min to inactivate any contaminating deoxyribonuclease present). The preparation was then incubated at 37°C for 30 min, extracted with phenol: chloroform: isoamyl alcohol and reprecipitated with isopropanol and salt as above. The DNA was finally resuspended in 1 ml TE buffer and stored at 4°C.

3.2 Preparation of plasmid DNA from *Streptomyces*.

Since all the *Streptomyces* plasmids used in this study contained the thiostrepton resistance gene as a primary selectable marker, cultures were routinely grown in media supplemented with thiostrepton (20 $\mu g\ ml^{-1}$). All plasmids were prepared by the alkaline lysis method of Kieser (1984), from 25 ml cultures grown for 40-48 hr at 30°C.

Mycelium was harvested by centrifugation at 3,000 rev min^{-1} for 10 min at room temperature, washed by centrifugation through 10.3% (w/v) sucrose and resuspended in 5 ml final volume of "lysozyme solution" (section 3.1 above). Following incubation at 37°C for 20 min, 2.5 ml of freshly prepared alkaline SDS [0.3 M sodium hydroxide, 2% (w/v) SDS] was added and the mixture agitated immediately on a vortex mixer. The cell

lysate was incubated at 70°C for 20 min and then slowly cooled to room temperature. The preparation was then extracted with 2 ml unbuffered phenol: chloroform (50:50) and centrifuged for 15 min at 3,000 rev min^{-1}. The aqueous phase was removed, extracted once with neutral phenol: chloroform: isoamyl alcohol and once with chloroform. Nucleic acid was precipitated by addition of an equal volume of isopropanol in the presence of 300 mM sodium acetate and incubated at room temperature for 20 min. The precipitate was then collected by centrifugation at 8,000 rev min^{-1} for 10 min in a Sorvall HB4 rotor, washed with 80% (v/v) ethanol, dried *in vacuo* for 10 min and resuspended in 1 ml TE buffer containing 40 $\mu g\ ml^{-1}$ RNAse. After incubation at 37°C for 30 min, the preparation was extracted with phenol: chloroform: isoamyl alcohol and reprecipitated as above. The DNA was dried and finally resuspended in 1 ml TE buffer.

If the plasmid DNA was required for cloning experiments, or was contaminated with large amounts of chromosomal DNA, supercoiled DNA was isolated by centrifugation in caesium chloride gradients. Plasmid DNA in less than 1 ml was diluted to 4 ml with TEN buffer [30 mM Tris-HCl pH 8.0 at 20°C, 5 mM EDTA-KOH pH 8.0, 50 mM sodium chloride]. Caesium chloride (4.2 g) was dissolved in the DNA solution and 0.2 ml ethidium bromide (10 mg ml^{-1}) added. This solution was then centrifuged at 50,000 rev min^{-1} for 16 hr at 20°C in a Beckman VTi65.2 rotor. When visualised in ultraviolet light, two bands could be seen. The DNA in the lower band was collected, extracted with isopropanol (saturated with caesium chloride) until all the ethidium bromide was removed and then precipitated with sodium acetate and isopropanol at -20°C. The DNA was collected by centrifugation for 10 min in an MSE Microcentaur

microcentrifuge, washed with 80% (v/v) ethanol, dried *in vacuo* and resuspended in 0.1 ml TE buffer.

3.3 Large scale preparation of *E. coli* plasmids.

The neutral lysis method of Godson and Vapnek (1973) was used to isolate *E. coli* plasmids in large quantities.

Cultures (100-200 ml) were grown to stationary phase at 37°C, in LB medium supplemented with either 200 μg ml^{-1} ampicillin or apramycin, depending on the selectable marker present on the plasmid. Cells were harvested by centrifugation at 6,000 rev min^{-1} for 10 min in a Beckman JA10 rotor and the bacterial pellet resuspended in 10 ml ice-cold 10% (w/v) sucrose in 50 mM Tris-HCl (pH 8.0 at 20°C). After the addition of 2 ml of fresh lysozyme solution [10 mg ml^{-1} in 0.25 M Tris-HCl pH 8.0 at 20°C] and 8 ml of 0.25 M EDTA-KOH (pH 8.0), the solution was mixed by inversion and kept at 0°C for 10 min. Cell lysis was achieved by the addition of 4 ml of 10% (w/v) SDS, followed immediately by 6 ml of 5 M NaCl. The lysis mixture was gently agitated and incubated at 0°C for 60-90 min. The preparation was then centrifuged for 30 min at 30,000 rev min^{-1} in a Beckman Ti70 rotor at 4°C and the supernatant removed and extracted with neutral phenol: chloroform: isoamyl alcohol and then with chloroform. The nucleic acid in the aqueous phase was precipitated by the addition of 2 volumes ethanol and incubation at -20°C for 1 hr. Recovery of DNA, removal of RNA and subsequent caesium chloride density gradient centrifugation were as described in section 3.2. When the plasmids were destined for use in coupled transcription-translation reactions, RNAse was not used.

3.4 Small scale preparation of *E. coli* plasmids.

When a large number of plasmids were required for brief analysis, a modification of the procedure described by Holmes and Quigley (1981) was employed. Small patches of strains to be tested were prepared on LB plates supplemented with 200 μg ampicillin or apramycin and incubated overnight at 37°C. The cells were removed from the plate and resuspended in 0.35 ml STET buffer [8% (w/v) sucrose, 5% (v/v) Triton X100, 50 mM Na_2EDTA and 50 mM Tris-HCl pH 8.0 at 20°C]. After the addition of 25 μl lysozyme [10 mg ml^{-1} in 50 mM Tris-HCl pH 8.0 at 20°C], the cells were briefly agitated on a vortex shaker, heated at 100°C for 40 s and then centrifuged for 10 min in an MSE microcentrifuge at room temperature. Nucleic acid was precipitated from the supernatant by the addition of 40 μl of 3 M sodium acetate and 0.42 ml isopropanol and incubation at -20°C for 15 min. The precipitate was collected by centrifugation for 10 min in a microcentrifuge, resuspended in 0.15 ml TE buffer, extracted with neutral phenol: chloroform: isoamyl alcohol and reprecipitated. The nucleic acid was harvested as above, washed with 80% (v/v) ethanol, dried *in vacuo* and finally resuspended in 0.1 ml TE buffer containing 40 μg ml^{-1} RNAse.

3.5 Agarose gel electrophoresis of DNA samples.

DNA preparations were routinely analysed in horizontal 0.7% (w/v) agarose gels cast in TEA buffer [40 mM Tris-acetate pH 8.0 at 20°C, 2 mM Na_2EDTA]. DNA samples containing 10% (v/v) sample buffer [TEA buffer supplemented with 50% (v/v) glycerol, 0.01% (w/v) xylene cyanol FF and 0.01% (w/v) BPB] were loaded onto gels (8 cm × 8 cm × 3 mm) which were submerged in TEA and electrophoresed at 80 V for 30-40 min. DNA was visualised by staining in 1 μg ml^{-1} ethidium bromide

for 15 min and observation on a uv transilluminator. Lambda phage DNA fragments from digestion with the restriction endonuclease HindIII were used as size markers for linear molecules (Daniels *et al.*, 1980).

3.6 Restriction, phosphatase treatment and ligation of DNA.

DNA was cleaved with various restriction endonucleases under the conditions specified by the manufacturers. Reactions were typically in volumes less than 50 µl. When the DNA was required for further manipulation, it was subjected to further treatment. After extraction with an equal volume of neutral phenol: chloroform: isoamyl alcohol and then chloroform, the DNA was precipitated from the aqueous phase with 3 volumes of ethanol in the presence of 300 mM sodium acetate, using an industrial methylated spirits- CO_2 bath for 20 min. The precipitate was collected by centrifugation for 10 min in an MSE Microcentaur microcentrifuge, washed in 80% (v/v) ethanol, centrifuged for 5 min, dried under vacuum for 10 min and finally resuspended in TE buffer.

In many experiments, vector DNA was also treated with calf intestinal alkaline phosphatase (CIAP) during restriction. This enzyme removes the terminal phosphates after cleavage and therefore abolishes the recircularisation of vector molecules in ligation mixtures. Typically, 0.5-1 unit of enzyme were used to treat up to several microgrammes of DNA. Before phenol-chloroform extraction and subsequent precipitation, the CIAP was inactivated by heating the sample at 75°C for 15 min in the presence of 0.1% (w/v) SDS, 10 mM Tris-HCl pH 8.0 at 20°C, 100 mM sodium chloride and 1 mM Na_2EDTA.

For shotgun cloning experiments, 1 µg linear vector (CIAP treated) was ligated with 5-6 µg genomic DNA fragments at 40 µg ml^{-1} final DNA concentration in 66 mM Tris-HCl (pH 7.5 at 20°C), 6.6 mM

$MgCl_2$, 1 mM DTT and 0.4 mM ATP. 1 unit T4 DNA ligase was added and incubation was carried out at room temperature for 16-20 hr. The DNA was precipitated with salt and ethanol as above and resuspended in 20 µl TE buffer.

In subcloning experiments, vector and donor DNA fragments were ligated at approximately 1:2 molar stoichiometry, in 25-50 µl under ionic conditions as described above. The ligation mixture was then used directly for transformation experiments without recourse to precipitation.

3.7 Isolation of specific DNA molecules from agarose gels.

In some experiments it was necessary to purify a particular DNA fragment prior to ligation. The DNA species of interest was separated by electrophoresis in a 1% (w/v) low melting point agarose gel in TEA buffer under conditions described previously (section 3.4). The DNA was stained in ethidium bromide (1 µg ml^{-1}) and visualised on a uv transilluminator.

The method employed was the "freeze-squeeze" procedure described by Traut and Renz (1982). Gel slices containing the required DNA were removed from the gel with the minimum amount of agarose and incubated in the dark in a solution containing 0.3 M sodium acetate and 1 mM Na_2EDTA for 45 min at 30°C. The gel slice was then transferred to a small 0.5 ml Eppendorf tube punctured at the bottom and plugged with a small amount of siliconised glass wool. The tube was placed in a large 1.5 ml eppendorf tube and then the two tubes were incubated in an industrial methylated spirits-CO_2 bath for 15 min prior to centrifugation in an MSE Microcentaur microcentrifuge for 10 min. The solution in the large tube was removed and DNA precipitated from it with

sodium acetate and ethanol as described previously. The DNA was then resuspended in 10-30 µl TE buffer.

3.8 Southern hybridisation of DNA.

a) Transfer of DNA.

DNA samples to be transferred to GeneScreen*plus* membranes (New England Nuclear) were separated by electrophoresis in a 0.7% agarose (LITEX) gel in TEA buffer at 6 V cm^{-1} for 3 hr. To allow more efficient transfer of large DNA molecules, the DNA in the gel was "acid-nicked" by incubation in 0.25 M HCl for 15 min at room temperature. The gel was then incubated in a solution containing 0.4 M NaOH and 0.6 M NaCl for 30 min and then in 1.5 M NaCl plus 0.5 M Tris-HCl pH 7.5 at 20°C for 30 min.

To prepare the membrane for DNA transfer, it was cut to the size of the gel, washed in distilled water and then incubated in a solution containing 3 M sodium chloride and 0.3 M sodium citrate for 15 min.

The blotting apparatus was set up with a wick of Whatman 3 MM paper supported on a glass plate. The ends of the wick were placed in a solution containing 3 M sodium chloride and 0.3 M sodium citrate and the gel placed on this, with the top surface uppermost. The membrane was placed onto the gel and spacers composed of double layered Saran wrap were fitted to prevent the buffer from bypassing the gel and membrane. Five pieces of dry filter paper were placed above the membrane and a 10 cm stack of absorbent paper towels were held in position above these, under a 1 kg weight. Transfer was allowed to continue for 16-24 hr at room temperature, after which the membrane was removed from

the gel, incubated in 0.4 M NaOH for 1 min to denature the transferred DNA and then briefly in an excess of a solution containing 0.2 M Tris-HCl (pH 7.5 at 20°C), 0.3 M sodium chloride and 0.03 M sodium citrate. The membrane was dried at room temperature and then incubated at 42°C for 6 hr in 10 ml prehybridisation buffer in a sealable plastic bag. This contained 50% (v/v) deionised formamide, 1% (w/v) SDS, 1 M NaCl and 10% (w/v) dextran sulphate.

b) Preparation of radiolabelled DNA fragments.

Radiolabelled probes for Southern hybridisation were prepared by a method using random hexadeoxynucleotide primers (Feinberg and Vogelstein, 1983).

The DNA fragment to be labelled was excised from a 1% (w/v) low melting point agarose gel after electrophoresis at 10 V cm^{-1} for 40 min and visualisation in ethidium bromide on a uv transilluminator. The weight of the gel slice was determined and 1.5 ml distilled water added per gramme of agarose. From this an approximate value for the DNA concentration was calculated. The gel slice was incubated at 100°C for 7 min and then kept at 37°C for 60 min. 25 ng DNA fragment was radiolabelled in a 25 µl reaction mixture at room temperature for 16 hr. The reaction contained 50 mM Tris-HCl (pH 8.0 at 20°C), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 µM each of dATP, dTTP, dGTP, 200 mM HEPES-NaOH pH 6.6 at 20°C, 1.35 A_{260} units hexadeoxynucleotides, 0.4 mg ml^{-1} enzyme grade BSA, 25 µCi [α-^{32}P] dCTP (3,000-4,000 Ci $mmol^{-1}$) and 2 units large fragment of DNA polymerase I.

The reaction was stopped by the addition of 0.1 ml buffer [20 mM Tris-HCl pH 7.5 at 20°C, 20 mM NaCl, 2 mM EDTA-KOH pH 8.0, 0.25% (w/v) SDS and 1 µM dCTP] and unincorporated nucleotides were removed by

gel filtration through a Sephadex G50 column. The column was prepared in a siliconised 1 ml Gilson tip plugged with siliconised glass wool, with G50 that had been pre-incubated in 3 mM Tris-HCl (pH 7.0 at 20°C) plus 0.2 mM Na_2EDTA. The column was rinsed with this buffer and then allowed to drain. After the sample had been loaded and allowed to run into the column, more buffer was added. Ten 0.2 ml fractions were then collected and 1 µl of each was placed in a xylene-based scintillation fluid (Fisofluor No.1 from Fisons) and the radioactivity estimated. Two peaks of radioactivity were obtained. Fractions from the first peak were pooled and denatured by incubation at 100°C for 10 min together with 1 mg salmon sperm DNA, immediately prior to hybridisation.

c) Hybridisation of DNA.

After 6 hr prehybridisation at 42°C, the denatured probe and salmon sperm DNA were introduced into the plastic bag containing the membrane. This was then resealed and incubated for a further 16 hr at 42°C, with constant agitation. After this period of hybridisation, the membrane was removed from the bag, washed twice in 100 ml of 0.3 M NaCl plus 0.03 M sodium citrate for 5 min at room temperature, twice in 200 ml of a solution containing 0.3 M NaCl, 0.03 M sodium citrate and 1% (w/v) SDS at 65°C for 30 min and then twice in 100 ml of 15 mM NaCl, 1.5 mM sodium citrate at room temperature for 30 min. This washing procedure removed from the membrane, probe DNA which had less than 85% homology with the bound DNA. The filter was then dried and radioactive bands visualised by autoradiography with Fuji RX film.

4 Preparation of bacteria for transformation with plasmid DNA.

4.1 Preparation, transformation and regeneration of *S. lividans* protoplasts.

This method is that of Bibb *et al.*, 1978, modified by Thompson, C. J. *et al.*, 1982a. Since transformation is *via* protoplasts and these are highly susceptible to traces of detergent, all glassware was acid-washed and sterile plastic items were used.

Mycelium from a 25 ml culture incubated at 30°C for 38-40 hr, was harvested by centrifugation at 3,000 rev min^{-1} for 10 min in a Hereaus Christ centrifuge and washed twice by centrifugation through 10.3% (w/v) sucrose at room temperature. Protoplasts were generated by incubation of the washed mycelium in 4 ml lysozyme solution (1 mg ml^{-1} lysozyme in L buffer) at 30°C for 30 min. L buffer contained 10.3% (w/v) sucrose, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.4 mM K_2SO_4, 0.4 mM KH_2PO_4, 25 mM TES-NaOH (pH 7.2 at 20°C) and 0.2% (v/v) trace element solution. The trace element solution contained (per litre) 40 mg $ZnCl_2$; 200 mg $FeCl_3.6H_2O$; 10 mg $CuCl_2.2H_2O$; 10 mg $MnCl_2.4H_2O$; 10 mg $Na_2B_4O_7$ and 10 mg $(NH_4)_6Mo_7O_{24}.4H_2O$. The suspension was triturated three times, incubated for a further 15 min at 30°C and then diluted with 5 ml P buffer (as L buffer, but with 10 mM $MgCl_2$ and 25 mM $CaCl_2$). Protoplasts were separated from residual mycelium by passage through a cotton wool plug, and collected by centrifugation at 3,000 rev min^{-1} for 10 min in an MSE bench top centrifuge. The protoplast pellet was resuspended in 0.5 ml P buffer and the concentration of protoplasts determined by measuremnt of the optical density at 600 nm (J. Thompson and P. Skeggs, personal communication, have established that 1 OD unit is equivalent to 1.5×10^9 protoplasts).

Protoplasts (4×10^9) were diluted with 5 ml P buffer and centrifuged as above, immediately prior to transformation. The pellet was resuspended in a minimal volume of P buffer (approximately 0.1 ml), to which was added 25 ng supercoiled plasmid DNA or 20-30 µl ligation mix. Then 0.5 ml T buffer [25% (w/v) PEG 1000, 2% (w/v) sucrose, 1 mM K_2SO_4, 75 mM $CaCl_2$, 35 mM Tris-maleic acid pH 8.0 at 20°C and 0.2% (v/v) trace element solution] was added, followed by 5 ml P buffer, not later than 30 s after T buffer. The protoplasts were harvested by centrifugation, resuspended in a minimal volume of P buffer and then diluted to 1 ml with the same buffer.

Protoplasts (4×10^8) were spread onto each of up to 10 plates of regeneration medium (R2YE), since this number has been reported to be optimal for regeneration (Thompson, C. J. *et al.*, 1982a). R2YE agar contained 10.3% sucrose, 1.4 mM K_2SO_4, 50 mM $MgCl_2$, 1% (w/v) glucose, 0.4 mM KH_2PO_4, 20 mM $CaCl_2$, 0.3% (w/v) L-proline, 0.5% (w/v) yeast extract, 0.01% (w/v) casamino acids (Difco), 25 mM TES-NaOH pH 7.2 at 20°C, 5 mM NaOH, 0.2% (v/v) trace element solution and 2.2% (w/v) Difco agar. The plates were incubated in a laminar flow hood for 3-4 hr prior to use. In this time, they had lost approximately 15% weight.

After 18-22 hr incubation at 30°C, primary transformants were selected by flooding the plates with 2 ml thiostrepton suspension (0.5 mg ml^{-1} in water). After further incubation for 4-5 days, the number of transformants was estimated. In all the *Streptomyces* transformation experiments in this work, thiostrepton resistance was used as the selectable marker.

4.2 Preparation and transformation of competent cells from *E. coli*.

Strain NM522 was used as the cloning host for all the manipulations in *E. coli*. This strain does not possess a functional β-galactosidase gene since the α-peptide portion is absent. However, when plasmid vectors containing the α-peptide region are introduced into this strain, β-galactosidase function is restored by intragenic complementation. Expression of the gene is greatest when an inducer is present, e.g. isopropyl thiogalactopyranoside (IPTG). This system is particularly useful when organisms are grown in a medium containing IPTG and Xgal. The latter is a chromogenic substrate for β-galactosidase, which produces a blue colour, therefore cells containing an intact plasmid-borne α-peptide region are blue. However, if DNA is inserted into a polylinker of useful restriction sites within the DNA for the α-peptide, then no functional β-galactosidase activity is produced and the colonies are white. Thus there is a convenient colour test to determine whether plasmid vectors contain inserts.

The method for obtaining competent cells is that of Mandel and Higa (1970). 5 µl of *E. coli* NM522 stored in glycerol at -20°C was used to inoculate 1 ml LB liquid medium, which was incubated overnight at 37°C. The overnight culture was added to 100 ml LB medium and shaken vigorously at 37°C until an optical density at 600 nm equal to 0.2 units was reached. Cultures were kept on ice for 10 min and then centrifuged in a Hereaeus Christ centrifuge at 3,000 rev min^{-1} for 10 min at 4°C. The cell pellet was resuspended in 40 ml ice-cold 0.1 M $CaCl_2$ and kept at 0°C for 30 min. The cells were harvested again at 4°C and resuspended in 0.1 M $CaCl_2$ to 1 ml final volume. The cells were incubated for 1-24 hr at 0°C, prior to transformation.

In order to determine the transformation efficiency of a preparation of competent cells, 10 ng supercoiled pUC18 DNA (Yanisch-Perron *et al.*, 1985) in a volume less than 30 µl was added to 0.2 ml competent cells and incubated at 0°C for 30 min. The transformation mixture was transferred to 42°C for 2 min, diluted with 1 ml LB medium and incubated at 37°C for 1 hr. A portion of the transformation mixture (0.2 ml) was spread over the surface of an LB plate supplemented with ampicillin (50 µg ml^{-1}), to select for transformants. After 16 hr incubation at 37°C, the transformation efficiency was determined.

When DNA had been ligated into the α-peptide encoding DNA of pUC18 or pOJ160 and the blue-white colour test required to detect plasmid molecules containing inserts, the 1.2 ml transformation mixture was added to 17 ml soft LB agar, supplemented with ampicillin or apramycin (50 µg ml^{-1}), 70 µg ml^{-1} IPTG and 0.4 mg ml^{-1} Xgal at 42-45°C. 3 ml was then spread onto each of six LB agar plates supplemented with 50 µg ml^{-1} of the appropriate antibiotic and incubated at 37°C for 16 hr.

White colonies were removed from the plates of ampicillin resistant primary transformants, spread onto LB agar plates containing 200 µg ml^{-1} of this drug and incubated at 37°C. When vectors containing the gene for β-lactamase were employed, this was essential since the enzyme is secreted into the medium, where it destroys the selection pressure, enabling plasmid-free cells to grow and contaminate genuine transformants. This is not a problem with pOJ160, because apramycin acetyltransferase is not secreted into the medium.

5 Quantitation of nucleic acid and ribosomal components.

The concentration of nucleic acid or ribosomal components was determined by measuring their absorbance at 260 nm. The following conversion factors were used: 1 A_{260} unit is equivalent to 50 $\mu g\ ml^{-1}$ DNA, 45 $\mu g\ ml^{-1}$ RNA, 29.4 pmol ml^{-1} ribosomes, 46 pmol ml^{-1} 50S ribosomal subunits or 87 pmol ml^{-1} 30S ribosomal subunits.

6 Materials.

6.1 Enzymes.

The following enzymes were obtained from the Sigma Chemical Co.: lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas) and pyruvate kinase (type III from rabbit muscle). Calf intestinal alkaline phosphatase (molecular biology grade), deoxyribonuclease I (from bovine pancreas) and micrococcal nuclease (from *Staphylococcus aureus*) were purchased from Boehringer Mannheim. Restriction endonucleases, T4 DNA ligase and the Klenow fragment of DNA polymerase I were all from Bethesda Research Laboratories.

6.2 Biochemicals.

The following biochemicals were purchased from Sigma Chemical Co.: ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, dTTP, PEP (all sodium salts); DTT, L-amino acids, calcium folinate, spermidine trihydrochloride, IPTG, HEPES, TES, Tris, 2-mercaptoethanol, polyuridylate (potassium salt), *E. coli* unfractionated tRNA and *E. coli* tRNA (phenylalanine specific). Urea, low melting point agarose and BSA were obtained from Bethesda Research Laboratories. SDS and acrylamide were from Serva Ltd., and Sephadex G50 plus hexadeoxynucleotides (cat. no. 2166) were

from Pharmacia Fine Chemicals. PEG 1000 (Koch-Light), sodium triisopropylnapthalene sulphonate (Kodak), sodium 4-aminosalicylate (Aldrich), Xgal (Anglian Biotechnology) and Triton X100 (ICI) were obtained from the sources indicated.

6.3 Reagents for radiochemical analysis.

L-[u-^{14}C] phenylalanine, [^{35}S] methionine, [α-^{32}P] dCTP, [^{14}C] methylated protein mixture and AMPLIFY were obtained from the Radiochemical Centre, Amersham. Polaroid Type 57 4×5" land film was used to photograph agarose gels and Fuji RX film was used for autoradiography and fluorography. Fisofluor No. 1 and No. 3 (Fisons) were used for liquid scintillation spectrometry.

6.4 Antibiotics.

The antibiotics used in this work were obtained from the sources indicated:- ampicillin, aurin tricarboxylic acid, erythromycin, rifampicin and tetracycline (Sigma Chemical Co.); celesticetin, lincomycin and pactamycin (Upjohn Company, Kalamazoo, Michigan, U.S.A.); apramycin (Eli Lilly, Indianapolis, U.S.A.); carbomycin (Pfizer, Sandwich, Kent); chloramphenicol (Parke Davis and Company, Hounslow, London); spiramycin (May and Baker, Dagenham, Essex); thiostrepton (Squibb, Princeton, New Jersey, U.S.A.).

Lipiamycin and streptolydigin were a kind gift from Dr. J. Salas (Universidad de Oviedo, Spain).

CHAPTER 3

CHARACTERISATION AND FRACTIONATION OF A COUPLED TRANSCRIPTION-TRANSLATION SYSTEM FROM *STREPTOMYCES LIVIDANS*

1 Introduction.

Cell-free protein-synthesising systems programmed by DNA templates have been prepared from *Escherichia coli* and *Bacillus subtilis*. However, such extracts are not ideally suited for the study of *Streptomyces* gene expression *in vitro*, since many of these genes are not functional in *E. coli* (Bibb and Cohen, 1982; Hopwood *et al.*, 1986). In addition, *Streptomyces* possess some promoter sequences which are dissimilar to those typical of *E. coli* and those that are vegetatively expressed in *B. subtilis* (Hawley and McClure, 1983; Bibb *et al.*, 1985a; Janssen *et al.*, 1985). Therefore, to enable the numbers and sizes of polypeptides encoded on fragments of the *Streptomyces* genome to be determined, a coupled transcription-translation system from *Streptomyces lividans* was developed in this laboratory by Stewart Rae.

Streptomyces lividans possesses several features which make it well suited for the preparation of a coupled transcription-translation system. Firstly, the organism grows and sporulates in a reproducible and vigorous manner and is therefore easy to manipulate in the laboratory. Also, *S. lividans* has been successfully used for cloning supernumary genes from *Streptomyces*, *Micromonospora* and *E. coli* and therefore lacks strong barriers to the expression of at least some heterospecific genes. Furthermore, since cloned DNA from various bacteria could survive in *S. lividans* after transformation, a powerful restriction system is absent. Clearly the presence of a restriction endonuclease in a cell-free extract is undesirable, since it could degrade exogenous DNA templates, resulting in decreased coupled transcription-translation activity. Finally, *S. lividans* is not constitutively resistant to many antibiotics and therefore cell-free protein synthesis should be sensitive to most, if not all, ribosome

inhibitors. Consequently, comparisons between components of cell-free extracts from antibiotic resistant *Streptomyces* and *S. lividans* should enable the coupled transcription-translation system to be used for characterising resistance mechanisms in antibiotic producing organisms.

In addition to my own work on the fractionation of the *S. lividans* coupled transcription-translation system, this chapter also describes the initial development of the system by Stewart Rae and Dr. Jill Thompson, since their work forms an important background to the present work. I am very grateful to them both, for kindly giving me permission to present their results.

2 Results.

2.1 Preparation of a coupled transcription-translation system from *S. lividans*.

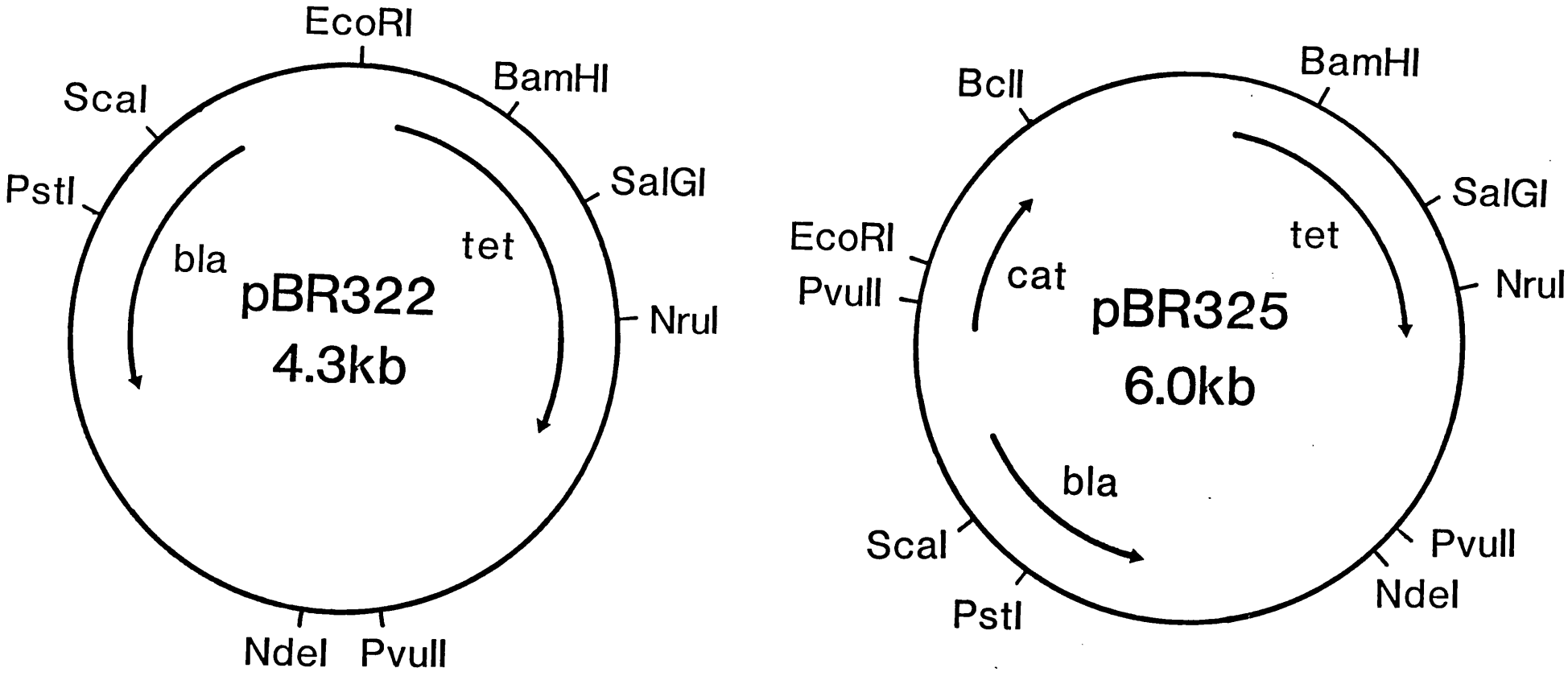
Cell-free extracts were prepared from *S. lividans* cultures as described in Methods (Section 2.1), using HEPES buffers throughout, since previous studies had shown that *Streptomyces* protein-synthesising systems directed by polyuridylic acid, were strongly inhibited by Tris buffers (E. Cundliffe, unpublished data). The S30 extract was then assayed for DNA-dependent protein synthesis under conditions similar to those described for *E. coli* coupled transcription-translation reactions. Plasmids pBR322 and pBR325 (Figure 3.1) were chosen as templates, since their nucleotide sequences were known (Sutcliffe, 1979; Prentki *et al.*, 1981) and therefore the sizes of the polypeptides encoded by plasmid-borne genes could be predicted. *Streptomyces* vectors were not used for the initial characterisation since no DNA sequence data was available and the sizes of the plasmid encoded gene products were not known.

Legend to Figure 3.1.

Restriction map of plasmids pBR322 and pBR325.

Plasmids pBR322 and pBR325 are derivatives of plasmid pMB1 Both vectors contain the ampicillin resistance determinant (*bla*) from transposon TnA and the tetracycline resistance gene (*tet*) from plasmid pSC101. In addition pBR325 contains the chloramphenicol resistance determinant (*cat*) from phage P1Cm.

Fig3.1


EcoRI
BamHI
ScaI
SalGI
PstI
bla
tet
pBR322
4.3kb
NruI
NdeI
PvuII
BclI
BamHI
SalGI
EcoRI
PvuII
cat
tet
pBR325
6.0kb
NruI
bla
ScaI
PvuII
PstI
NdeI

The coupled transcription-translation assay included a cocktail of small molecules (LMM), which only differed from that used for *E. coli* extracts (Pratt *et al.*, 1981) in the choice of buffering agent. With this modified LMM, a significant amount of radiolabelled methionine was incorporated into acid-precipitable material. However, the *E. coli* systems produced locally were considerably more active. Therefore to determine whether any components of the LMM were inhibitory to the *Streptomyces* system, components of the LMM (Table 3.1) were considered for omission. The reaction mixture formed a white precipitate when the LMM was added, which was presumably due to the calcium ions in the LMM. When calcium ions were omitted from the LMM, the reaction mixture did not contain a precipitate and the activity of the coupled transcription-translation reaction was increased 10-fold (Figure 3.2). Subsequent reactions were therefore performed in the absence of added Ca^{2+}. This result (of S. Rae) was the single largest stimulation of the activity of the system during its development.

Two other components of the LMM, cAMP and *E. coli* unfractionated tRNA, may not have been essential in the reaction and so their effects on the *S. lividans* cell-free system were examined. When cAMP was omitted from the LMM, there was no discernible effect on the activity of the reaction (S. Rae, unpublished data) and so subsequent batches of LMM were prepared without cAMP, allowing the effect of cAMP on gene expression to be examined independently. When *E. coli* tRNA was omitted from the LMM, the activity of the *S. lividans* coupled transcription-translation reaction was increased 2-fold (S. Rae, unpublished data). It was not established whether the deleterious effect of tRNA was representative of tRNA obtained from any supplier or whether it was peculiar to one batch tested. However, since the

Table 3.1.

Composition of the low molecular weight mix (LMM) for coupled transcription-translation by *E. coli* extracts.

Component	Final concentration in reaction
Tris-acetate, pH 8.2	58 mM
Dithiothreitol	1.8 mM
ATP, pH 7.0	1.3 mM
CTP, GTP, UTP, pH 7.0	0.9 mM each
PEP, pH 7.0	28 mM
19 amino acids (minus methionine)	0.36 mM
PEG 6000	2% (w/v)
Calcium folinate	36 $\mu g\ ml^{-1}$
cAMP, pH 7.0	0.66 mM
tRNA, *E. coli* unfractionated	0.17 $mg\ ml^{-1}$
Ammonium acetate	37 mM
Calcium acetate	10 mM
Potassium acetate	74 mM

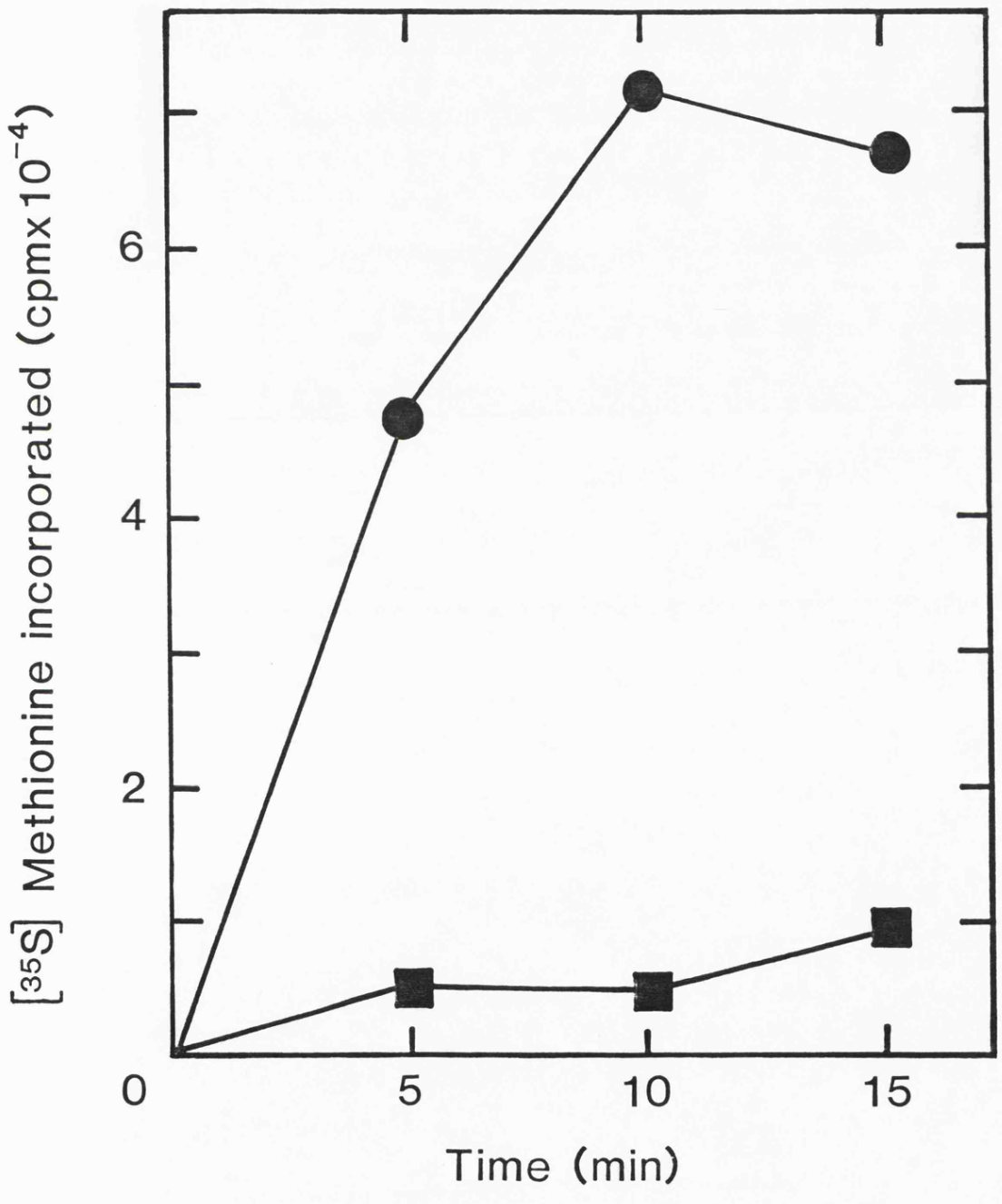
Legend to Figure 3.2.

Effect of calcium ions on the coupled transcription-translation activity of an *S. lividans* S30.

The reactions (30 µl) contained 2 A_{260} units of extract, 11 µCi [^{35}S] methionine (1190 Ci mmol^{-1}), 2.3 µg pBR325 DNA, 10 mM Mg^{2+}, 30% (v/v) synthesis mix and were performed in the presence (■) and absence (●) of 10 mM calcium acetate.

This experiment was performed by S. Rae.

Fig 3.2



exogenous tRNA appeared to be dispensable for activity in the reaction, it was omitted from subsequent LMM preparations. Pyruvate kinase was also added to the LMM, since it stimulates polyphenylalanine synthesis by *Streptomyces* extracts programmed with polyuridylate. Consequently the cocktail of small molecules used throughout the development of the *Streptomyces* coupled transcription-translation system differed from the *E. coli* LMM by the inclusion of HEPES plus pyruvate kinase and by the omission of Tris, cAMP, tRNA and Ca^{2+}. The *Streptomyces* cocktail will subsequently be referred to as "synthesis mix".

One of the most important aspects of any translation system is the amount of protein synthesis that an extract can support in the absence of added template. The 'background' activity observed in the *S. lividans* S30 varied between extracts, but was typically 30-60% of that observed in the presence of plasmid. Previous studies with *B. subtilis* cell-free systems showed that the majority of endogenous template was mRNA, with the remainder coming from transcription and translation of DNA fragments in the extract. In order to reduce this plasmid-independent synthesis, extracts were pre-incubated with a cocktail of components which allowed the mRNA to be translated and subsequently degraded. The extracts were then dialysed to remove the amino acids which had been supplied in the cocktail, so that the specific activity of the radiolabelled amino acid used to monitor protein synthesis was not reduced. This approach enabled the endogenous protein synthesis by *B. subtilis* extracts to be reduced to about 25% of the DNA-dependent activity (Legault-Demare and Chambliss, 1974).

A very different approach however, was adopted to remove templates from some eukaryotic cell-free translation systems. In these extracts, endogenous templates are degraded by a Ca^{2+}-dependent nuclease

from *Staphylococcus aureus*, which is subsequently inactivated by the addition of EGTA (Pelham and Jackson, 1976). This chelating agent has a high affinity for divalent cations and the removal of Ca^{2+} silences the enzyme. Although this approach completely removes background activity, it is limited in its application, as it can only be used for assays which do not depend on calcium. Fortunately, the *S. lividans* coupled transcription-translation reaction does not require these ions and so the nuclease method was used to eliminate plasmid-independent protein synthesis.

The time of nuclease treatment necessary to remove endogenous templates was determined by performing a pilot reaction for each extract. Samples of S30 were incubated with a fixed amount of nuclease for various periods of time and then assayed for their ability to support protein synthesis in the presence and absence of plasmid. The results shown in Figure 3.3 are typical of such an experiment. After 30 minutes nuclease treatment, protein synthesis was totally dependent on added template.

Once the preparation and nuclease treatment of *S. lividans* coupled transcription-translation systems had been established by S. Rae, a number of experiments were carried out to determine optimal concentrations for various components of the assay system. Previously, 10 mM Mg^{2+} concentration had been shown to be optimal for activity in *S. lividans* extracts (S. Rae, unpublished data). However, this result was obtained with extracts which had not been treated with nuclease. Since this treatment would have increased the nucleotide pool in the extract and since nucleotides are capable of binding cations, the free Mg^{2+} concentration may no longer have been optimal. To investigate the effect of this parameter on coupled transcription-translation activity, a

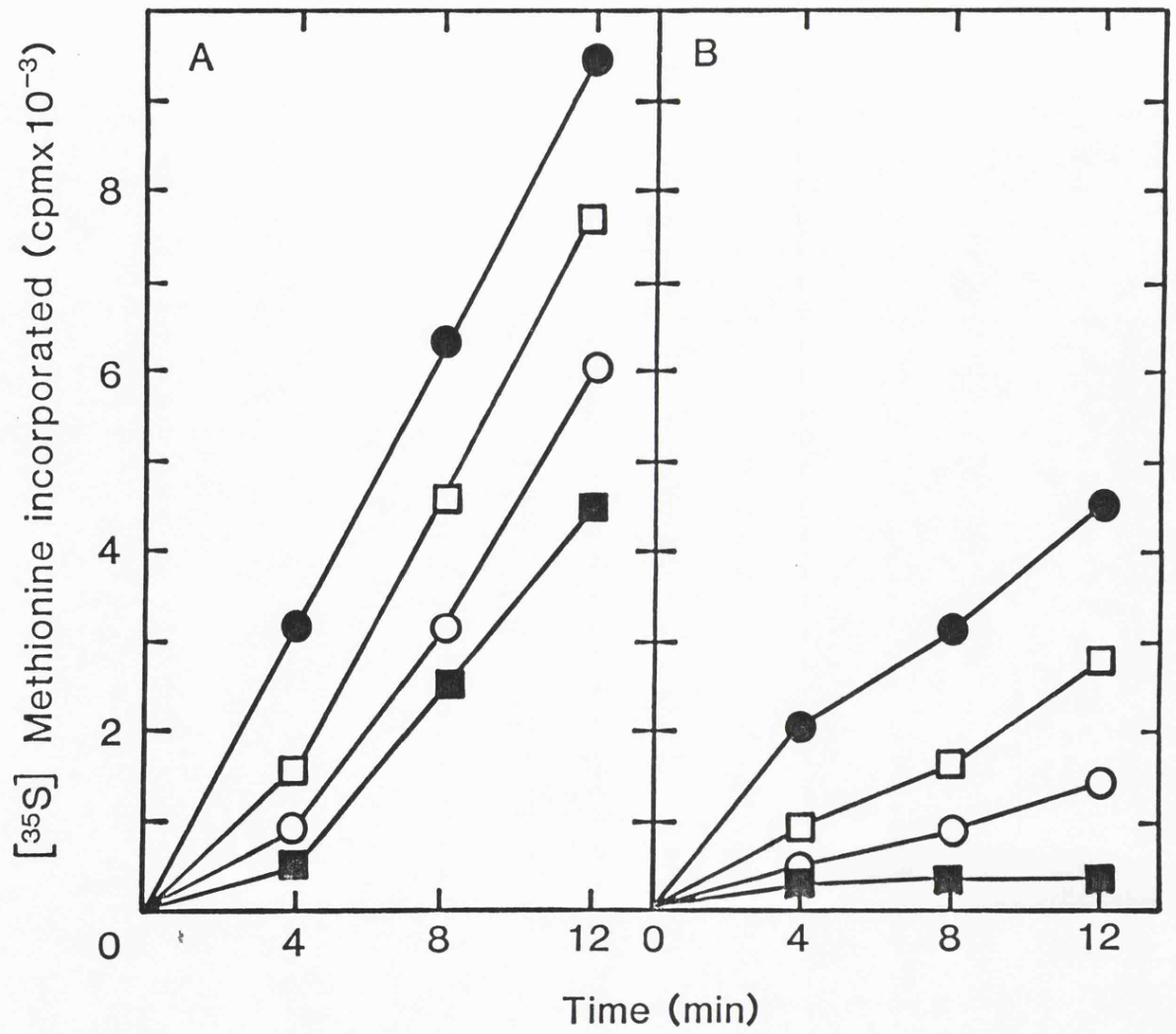
Legend to Figure 3.3.

Removal of endogenous protein synthesis in *S. lividans* extracts with micrococcal nuclease.

A portion of *S. lividans* TK21 extract was incubated with micrococcal nuclease in the presence of Ca^{2+}. Small samples were removed after various incubation times and the nuclease activity was stopped by the addition of EGTA. Each sample (2 A_{260} units) was then assayed for coupled transcription-translation activity in the presence (panel A) and absence (panel B) of plasmid.

Duration of nuclease treatment: 0 min (●), 10 min (□), 20 min (○) and 30 min (■).

Fig 3.3


A
B
[35S] Methionine incorporated (cpmx10-3)
0
2
4
6
8
0
4
8
12
0
4
8
12
Time (min)

series of reactions were carried out at different Mg^{2+} concentations. The results from these experiments are presented in Figure 3.4A, and show that the optimal Mg^{2+} concentration was between 12 and 14 mM. Since synthetic mRNA-directed systems can bypass the normal translational initiation process at 15 mM Mg^{2+}, it was decided to use 12 mM Mg^{2+} for coupled transcription-translation reactions, in order to ensure that a proper initiation process was in operation.

The next component of the assay system to be investigated was methionine. Since radiolabelled methionine was used to monitor protein synthesis, it was important to determine the non-radioactive methionine concentration optimal for both the overall activity of the coupled transcription-translation system and for the incorporation of radiolabelled amino acid into protein by the extract. Clearly if the concentration of non-radioactive amino acid was too great, the incorporation of [^{35}S] methionine into protein would be a rare event. However, the system would be severely limited if no non-radioactive methionine was added. Therefore a number of assays were carried out, with a fixed amount of [^{35}S] methionine present in each, but with the exogenous methionine concentration varied between 1 and 6 μM. The results (Figure 3.4B) indicate that 3 μM methionine gives optimal incorporation of radiolabelled methionine into TCA-precipitable material.

The template input was another important parameter for examination, since the optimal amount was likely to vary with the size and genetic content of the DNA. The results in Figure 3.4C were obtained from experiments in which the amount of pBR322 was increased from 0 to 5 μg per reaction. The figure shows that when more than 2 μg pBR322 was added to the assay, only small increases in activity were observed. When the cost of preparing plasmid was taken into account, an

Legend to Figure 3.4.

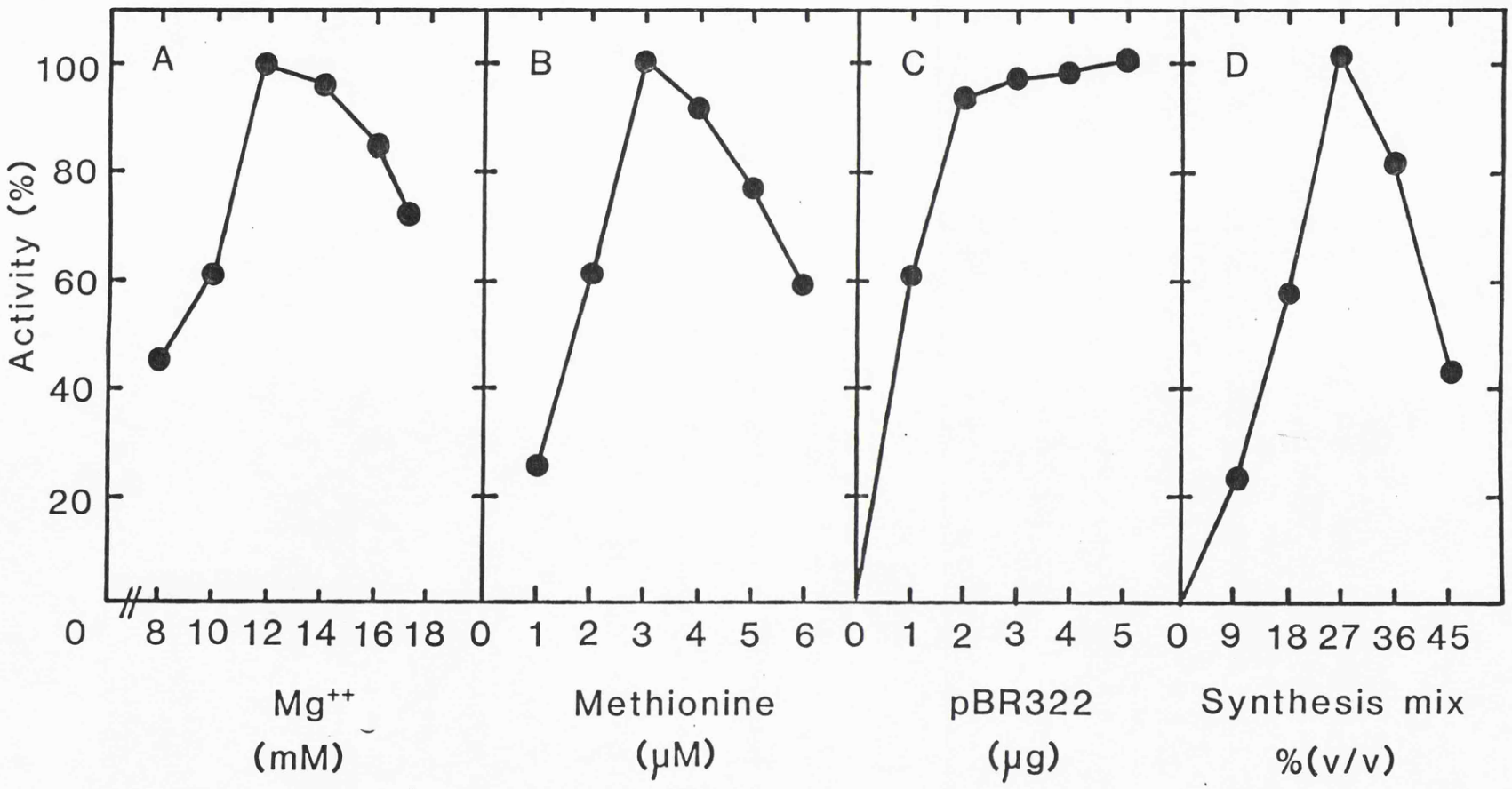
Optimisation of Mg^{2+}, methionine, pBR322 and synthesis mix inputs for coupled transcription-translation by *S. lividans* extracts.

All reactions (30 µl) contained nuclease-treated extract from *S. lividans* TK21 (2 A_{260} units), 8 µCi [^{35}S] methionine, 27% (v/v) synthesis mix, 3 µM methionine, 12 mM Mg^{2+} and 2 µg pBR322 DNA except where indicated below.

Component varied: (A) Mg^{2+}, 8-18 mM; (B) methionine, 1-6 µM; (C) pBR322 DNA, 0-5 µg; (D) synthesis mix, 0-45% (v/v).

100% activity is the incorporation of [^{35}S] methionine into TCA-precipitable material observed with the optimal input of the varied component.

Fig 3.4


A
B
C
D
Activity (%)
100
80
60
40
20
0
8 10 12 14 16 18
0 1 2 3 4 5 6
0 1 2 3 4 5
0 9 18 27 36 45
Mg++
(mM)
Methionine
(µM)
pBR322
(µg)
Synthesis mix
%(v/v)

input of 2 μg pBR322 was chosen for coupled transcription-translation reactions. The plasmid dependence of the *S. lividans* extract is also demonstrated in Figure 3.4C.

The final panel of Figure 3.4 shows the effect of altering the amount of synthesis mix present in the protein synthesis reaction. The results show that 27% by volume was the optimal amount and that the activity decreased when the synthesis mix input was increased to 45% of the reaction volume. Although the reason for the inhibition of the coupled transcription-translation system at high synthesis mix inputs was unclear, it was not a consequence of raising the combined K^+ and NH_4^+ concentration from 130 to 210 mM, because when this was increased independently, there was no discernible effect on activity (data not shown).

Although after the optimisation of one component, the remaining components were not re-optimised, the experiments described above show how a set of conditions were arrived at which routinely produced sufficient incorporation of radiolabel into protein.

Thus a typical reaction contained:

2 A_{260} units S30 extract

27% (v/v) synthesis mix

3μM methionine plus 0.8 μCi [^{35}S] methionine

12 mM Mg^{2+}

2 μg pBR322

To demonstrate that all the low molecular weight components necessary for coupled transcription-translation were supplied in the synthesis mix, nuclease-treated extracts were extensively dialysed and then assayed for protein synthesis directed by pBR322. Dialysis was

found to have no discernible effect on the activity of the system (data not shown). Although this result may support the notion that all the necessary components were supplied in excess, it could be interpreted to suggest that possible adverse and beneficial effects of dialysis had fortuitously counterbalanced. In either case, the dialysis of nuclease-treated extracts would be a logical progression for the coupled transcription-translation system, since it would make the assay amenable to investigations of the action of small effector molecules, such as cAMP and guanosine polyphosphates.

2.2 Sensitivity of extracts from *S. lividans* to inhibitors of RNA and protein synthesis.

Extracts of *S. lividans* are totally dependent upon plasmid DNA for protein synthesis. Consequently, antibiotics which inhibit the transcription of the DNA template should abolish the incorporation of [^{35}S] methionine into protein. S30 extracts were therefore pre-incubated with rifampicin (a semi-synthetic derivative of rifamycin), streptolydigin and lipiamycin prior to assay, in order to demonstrate the sensitivity of the coupled transcription-translation system to RNA polymerase inhibitors.

Rifamycin is one of the most potent inhibitors of prokaryotic RNA polymerases known. The drug binds tightly to the enzyme (Wehrli *et al.*, 1968) and selectively inhibits the initiation of transcription (Sippel and Hartmann, 1968), although it does not prevent RNA polymerase from binding to the template (Umezawa *et al.*, 1968). Streptolydigin also binds to RNA polymerase, albeit a weaker interaction than that between rifamycin and the enzyme, but the elongation of RNA chains is inhibited, rather than their initiation (Schlief, 1969; Siddikhol *et al.*, 1969:

Cassani *et al.*, 1971). Lipiamycin is also an inhibitor of the transcriptional elongation reaction.

Figure 3.5 shows that although lipiamycin and streptolydigin both strongly inhibited the coupled transcription-translation reaction, rifampicin was relatively ineffective. Thus, although 100 $\mu g\ ml^{-1}$ rifampicin inhibited the system by 60%, no further reduction in activity could be gained by increasing the drug concentration (data not shown). This result was very surprising since rifampicin potently inhibits the activity of RNA polymerases from a number of bacteria. It remains to be seen whether the insensitivity of the *S. lividans* cell-free system was a reflection of the reaction being optimised for translation or perhaps was due to the nuclease treatment. It is possible that free nucleotides in the extract might interfere with the rifampicin-RNA polymerase interaction, an explanation which could be tested using dialysed extracts. Nevertheless, streptolydigin and lipiamycin both inhibited the coupled transcription-translation system strongly and therefore either one could be used to block transcription and so fractionate the system at a functional level.

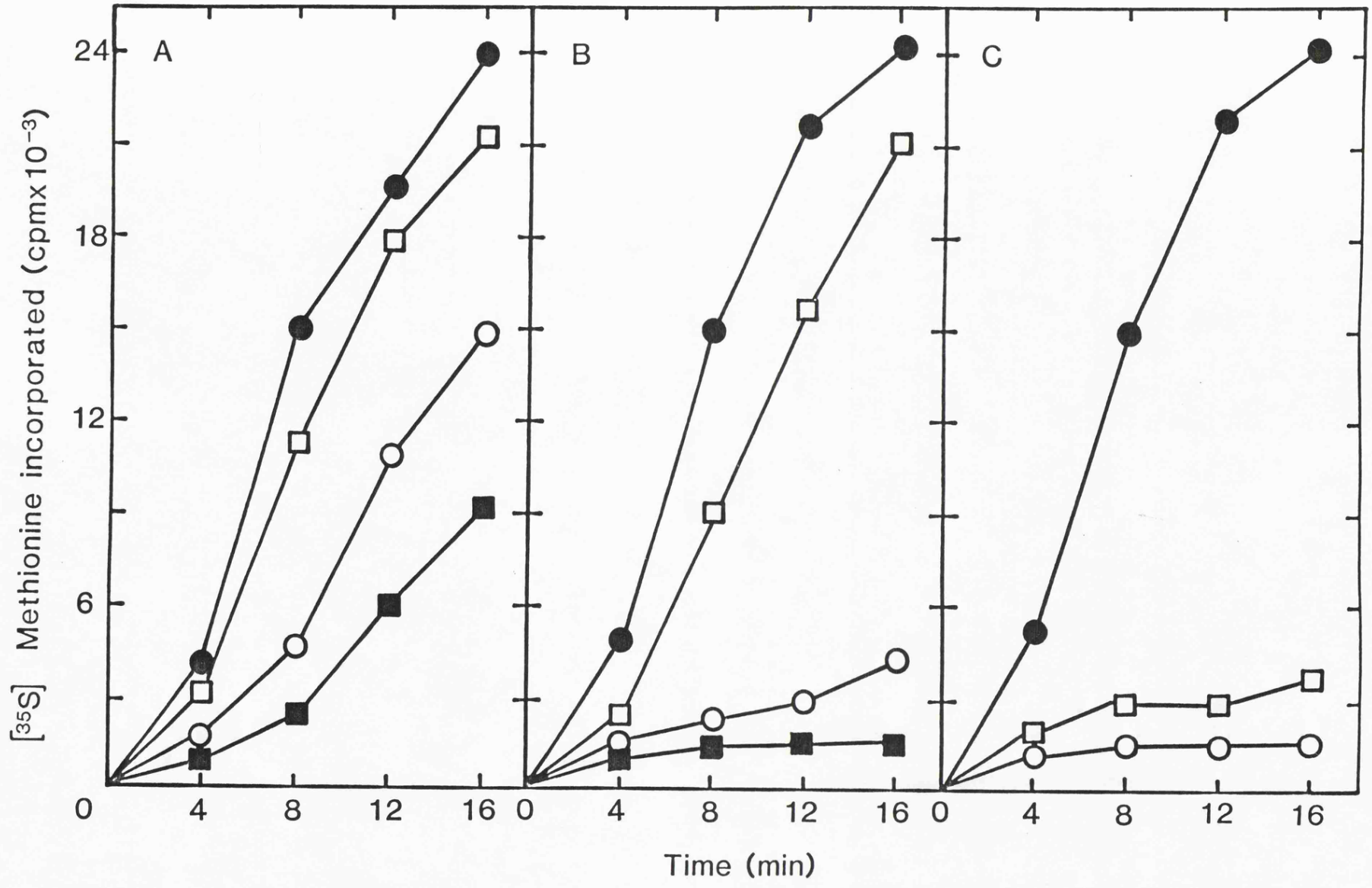
The protein-synthesising machinery in the *S. lividans* extract should be sensitive to most ribosome inhibitors, since the translational process in the extracts should be similar to the sequence of reactions which occurs *in vivo*. Previously, polyuridylate-directed systems had been used to study resistance to antibiotics, for example streptomycin (Ozaki *et al.*, 1969), viomycin (Skinner and Cundliffe, 1980), spiramycin (Skinner and Cundliffe, 1982) and kanamycin (Piendl *et al.*, 1984). In such 'artificial' mRNA systems however, ribosomes do not undertake factor-dependent initiation. Rather, translation of mRNA commences by ribosomes joining the template as 70S particles. Consequently some

Legend to Figure 3.5.

Sensitivity of *S. lividans* coupled transcription-translation reactions to inhibitors of RNA polymerase.

All reactions (30 µl) contained nuclease-treated extract from *S. lividans* TK21 (2 A_{260} units). Prior to assay for coupled transcription-translation activity, the S30 was incubated with solvent (●), or antibiotic at the following final concentrations: 1 µg ml^{-1} (□), 10 µg ml^{-1} (○) or 100 µg ml^{-1} (■). The antibiotics tested were rifampicin (panel A), streptolydigin (panel B) and lipiamycin (panel C).

Fig 3.5


A
B
C
24
18
12
6
0
4
8
12
16
[35S] Methionine incorporated (cpmx10⁻³)
Time (min)

antibiotics, for example kasugamycin, which specifically inhibit an aspect of the initiation factor-dependent reaction, have little effect on the synthesis of polyphenylalanine in response to polyuridylate. Surprisingly, even some antibiotics which inhibit the elongation cycle are also poor inhibitors of protein synthesis with polyuridylic acid as a template.

In order to determine whether the coupled transcription-translation system from *S. lividans* was more sensitive to some protein synthesis inhibitors than polyuridylate-directed reactions, the following experiments were carried out; 1 A_{260} unit S30 (nuclease-treated) was assayed in a 30 µl reaction for coupled transcription-translation activity or polyphenylalanine synthesis directed by polyuridylate, in the presence of aurin tricarboxylic acid, tetracycline, chloramphenicol or erythromycin.

Aurin tricarboxylic acid (ATA) has been shown to selectively inhibit the initiation of protein synthesis, but only when used at low drug concentrations (10 µM), since at greater inputs it also inhibits the elongation reaction (Grollman and Stewart, 1968; Tai *et al.*, 1973). The block of initiation by ATA is at the interaction of mRNA with the ribosome, as the drug prevented the binding of mRNA to the small ribosomal subunit, or to the ribosome, in a number of different assays (Grollman and Stewart, 1968; Siegelman and Apirion, 1971; Grollman and Huang, 1970; Lebleu *et al.*, 1970).

In the current study, ATA strongly inhibited both the coupled transcription-translation assay and the synthetic mRNA translation system (Figure 3.6A) at drug concentrations within the range for which specific effects on initiation have been described. Since similar inhibition was observed in each system, both the initiation factor-

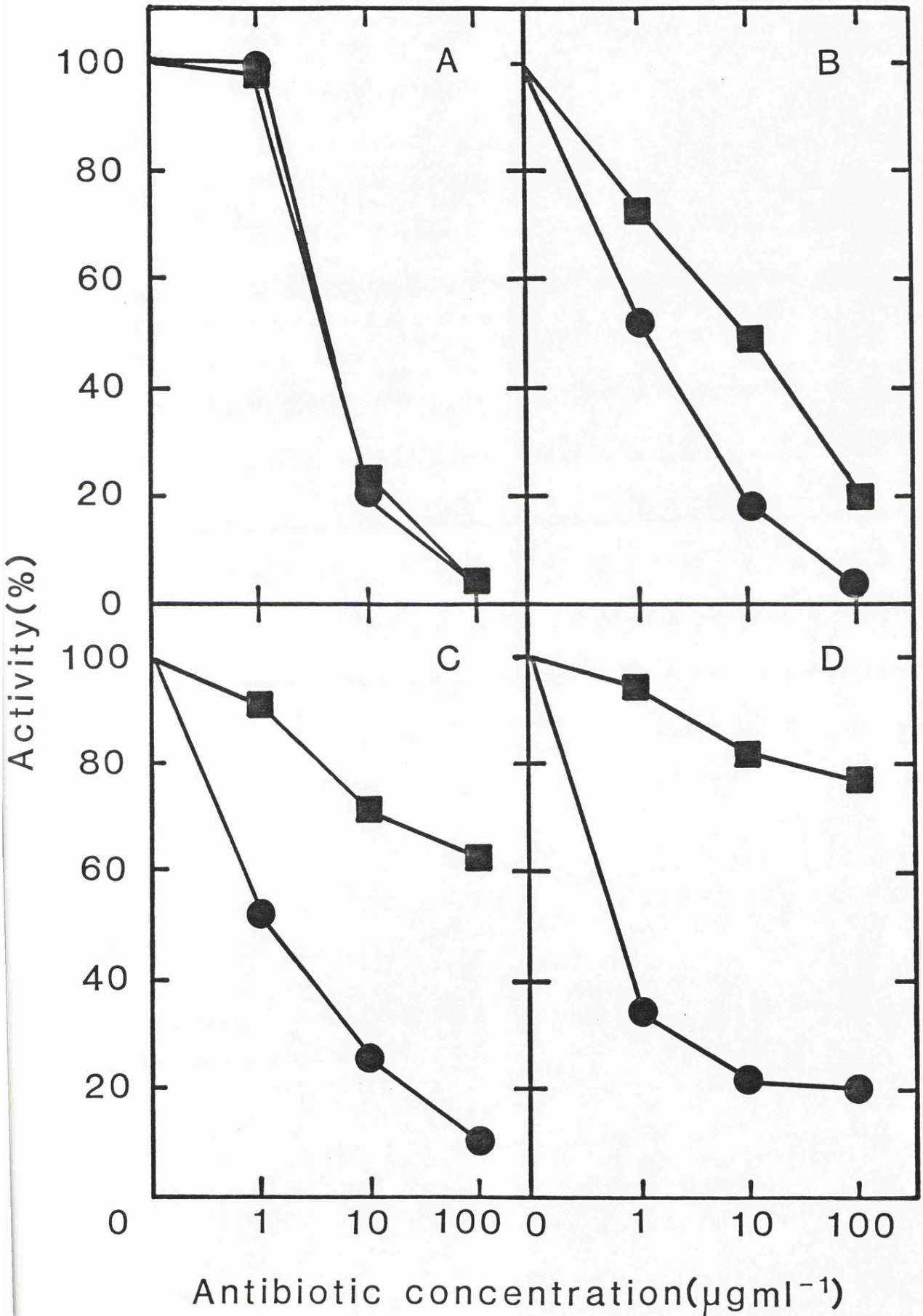
Legend to Figure 3.6.

Sensitivity of coupled transcription-translation and polyphenylalanine synthesis by *S. lividans* extracts to ribosome inhibitors.

All reactions (30 µl) contained nuclease-treated S30 from *S. lividans* TK21 (1 A_{260} unit). The extract was incubated with solvent or antibiotic prior to assay for polyphenylalanine synthesis (■) or coupled transcription-translation (●).

The antibiotics tested were: aurin tricarboxylic acid (panel A), tetracycline (panel B), chloramphenicol (panel C) and erythromycin (panel D).

Fig 3.6


A
B
C
D
100
80
60
40
20
0
Activity(%)
0
1
10
100
Antibiotic concentration(μgml⁻¹)

dependent binding of mRNA to the 30S ribosomal subunit presumed to occur in the coupled transcription-translation system and the quasi-initiation complexes formed in the system synthesising polyphenylalanine, appear to be equally sensitive to ATA.

Tetracycline is also believed to inhibit translation by preventing a function of the smaller ribosomal subunit, although there is no good data to suggest that it binds to this subunit exclusively. The currently accepted mode of action for tetracycline is that it specifically inhibits the binding of aminoacyl-tRNA into the ribosomal acceptor site, prior to peptide bond formation (Lucas-Lenard and Haenni, 1968; Gordon, 1969; Hill, 1969; Cundliffe and McQuillen, 1967). The response of the *S. lividans* extract to tetracycline in the two protein-synthesising systems is shown in Figure 3.6B. Although both systems were inhibited by tetracycline, the coupled transcription-translation reaction was more sensitive. The reason for the different sensitivities is not clear. There has been a report that tetraycline inhibits the binding of the initiator tRNA to ribosomes in response to the trinucleotide AUG (Sarkar and Thach, 1968). This may account for the greater sensitivity of the coupled transcription-translation system over the polyuridylate-directed assay, since the former initiates with formylmethionyl-tRNA.

The step in protein synthesis which follows the aminoacyl-tRNA binding reaction involves the formation of a peptide bond. Chloramphenicol is the best documented antibiotic which inhibits this step, as has been demonstrated *in vitro* (Traut and Monro, 1964) and in bacterial protoplasts (Cundliffe and McQuillen, 1967). Although chloramphenicol inhibited the peptidyl transfer between a number of substrates, its potency varied markedly with different templates. Thus

although bacterial extracts programmed with phage mRNA were usually sensitive to chloramphenicol, systems dependent on polyuridylic acid were fairly resistant, whilst polyadenylic and polycytidylic acid-programmed assays showed an intermediate sensitivity (Speyer *et al.*, 1963; Kucan and Lipmann, 1964). Figure 3.6C shows the relative sensitivities of the polyuridylic acid and pBR322-programmed systems from *S. lividans*, to chloramphenicol. Clearly the coupled transcription-translation system is considerably more sensitive to the drug than the polyuridylate-directed system, even though the antibiotic is believed to inhibit the same step in both assays.

Chloramphenicol is not the only antibiotic that acts at the elongation step of protein synthesis, but which only poorly inhibits polyphenylalanine synthesis. Many of the effects of erythromycin are consistent with the drug inhibiting the translocation of peptidyl-tRNA from the A site to the P site of the ribosome (Cundliffe and McQuillen, 1967; Igarashi *et al.*, 1969). The erythromycin sensitivities of the two protein-synthesising systems from *S. lividans* are shown in Figure 3.6D. Whereas a low level of inhibition was noted when 100 $\mu g\ ml^{-1}$ erythromycin was present in the polyuridylate-directed assay, the coupled transcription-translation system was sensitive to 1 $\mu g\ ml^{-1}$. Curiously though, complete inhibition was not observed in the pBR322-directed system, even when the drug concentration was increased to 100 $\mu g\ ml^{-1}$. Although the reasons for the relative insensitivity of the polyuridylate-dependent system to chloramphenicol and erythromycin was not clear, the results presented in Figure 3.6 show that the DNA-dependent assay was more sensitive to some inhibitors than the previously used synthetic mRNA translation systems. Consequently the

coupled transcription-translation system is a very appropriate assay for investigating antibiotic resistance mechanisms.

2.3 Analysis of the products of coupled transcription-translation by *S. lividans* extracts.

The experiments described in this section were performed in this laboratory by Dr. Jill Thompson.

The *E. coli* cloning vector pBR322 was selected for use as a template for coupled transcription-translation during the initial preparation and characterisation of the *S. lividans* extract, since the plasmid-borne resistance genes were known to be functional in *S. lividans* (Schottel *et al.*, 1981) and the nucleotide sequence of the replicon was known (Sutcliffe, 1979; Peden, 1983). In addition, the sizes of the plasmid-encoded polypeptides synthesised by *Streptomyces* extracts could be compared with those previously observed in *E. coli* protein-synthesising systems (for example, Pratt *et al.*, 1981). Unfortunately, very little data was available on the gene products encoded by *Streptomyces* plasmids at the time of the development of the coupled transcription-translation system from *S. lividans*. Consequently the high copy number plasmid pIJ350 (Kieser *et al.*, 1982) was included for analysis, since it could be prepared in large quantity.

When an *S. lividans* coupled transcription-translation system was programmed with supercoiled pBR322 DNA and the radiolabelled protein products were analysed by electrophoresis and fluorography, seven polypeptides were observed which were not synthesised in the absence of exogenous DNA (Figure 3.7 lanes b and d). Amongst these polypeptides were two species of approximately 34 and 31 kD, which were consistent with the previously observed sizes of the tetracycline

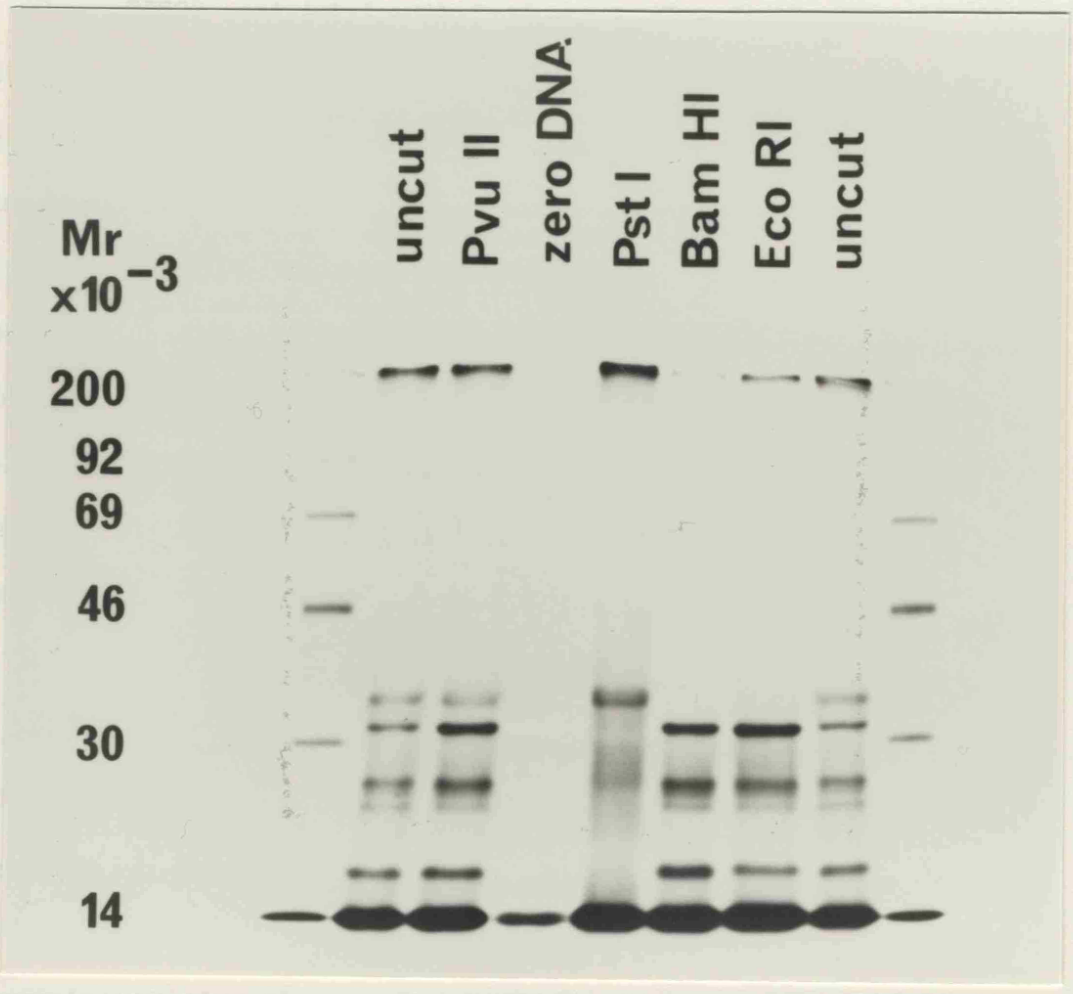
Legend to Figure 3.7.

Electrophoretic analysis of the products of coupled transcription-translation by *S. lividans* extracts programmed with pBR322.

The products of coupled transcription-translation were separated by electrophoresis on SDS-polyacrylamide gels and visualised by fluorography. The pBR322 template was either in a supercoiled form or in linear form following restriction with an endonuclease as indicated. [^{14}C] proteins were included as molecular weight markers.

This fluorogram was produced by Dr. J. Thompson.

Fig 3.7


Mr
x10⁻³
200
92
69
46
30
14
uncut
Pvu II
zero DNA
Pst I
Bam HI
Eco RI
uncut
a b c d e f g h i

resistance gene (*tet*) product and the β-lactamase preprotein respectively. Therefore in order to confirm the origin of these two polypeptides, linear pBR322 templates were prepared by restriction endonuclease cleavage at unique sites in the antibiotic resistance genes.

When pBR322 restricted with PstI was employed as a template for coupled transcription-translation, the 31 kD protein was not synthesised (Figure 3.7, lane e), thus supporting the identity of this polypeptide as the β-lactamase preprotein. However, four other protein bands were also absent after the electrophoresis and fluorography of the products of this reaction. Inspection of the nucleotide sequence of the β-lactamase gene (*bla*) did not reveal open reading frames which corresponded to the quadruplet of polypeptides synthesised *in vitro*. These four bands may have represented fragments of β-lactamase generated by proteases which may have been present in the *S. lividans* extract.

In another coupled transcription-translation reaction, BamHI-cleaved pBR322 was found to be a template for the synthesis of the five polypeptides derived from *bla*, but not for the 34 kD protein nor one other protein (Figure 3.7, lane f). This suggested that the 34 kD protein was probably the product of the *tet* gene. Surprisingly, the 34 kD protein was also absent when EcoRI-treated pBR322 was used as a template for coupled transcription-translation (Figure 3.7, lane g) even though the EcoRI restriction site lies 40-50 bp away from the transcriptional initiation site (Rodriguez *et al.*, 1979). This lack of template activity for the *tet* gene product may have been a result of limited exonuclease digestion at the termini of the linear DNA molecules, such that part or all of the promoter sequence was deleted. Similar problems had previously been encountered in some *E. coli* coupled transcription-translation systems (Yang *et al.*, 1980), but these had

been overcome by using a mutant strain which lacked the exonuclease activity associated with the *rec* BC locus. However, it should be noted that several workers have successfully used linear DNA molecules to direct coupled transcription-translation (Konings *et al.*, 1975; Lindahl *et al.*, 1976; Pratt *et al.*, 1981).

The final linear pBR322 template used to programme the *S. lividans* cell-free system was prepared by restriction of the plasmid DNA with PvuII. The polypeptides synthesised from this linear DNA were identical in size to those obtained in reactions directed by supercoiled pBR322 DNA (Figure 3.7, lane c). This was the anticipated result since the PvuII site is well separated from the *bla* and *tet* genes.

The only pIJ350-borne gene which has been sequenced is that which encodes the thiostrepton resistance methylase (Bibb *et al.*, 1985a). The nucleotide sequence predicts a protein of approximately 29 kd. When supercoiled pIJ350 DNA was employed as a template for coupled transcription-translation, two polypeptides of approximately 29 and 69 kd were observed following electrophoresis and fluorography of the reaction products (Figure 3.8). The smaller of these two polypeptides was shown to be derived from the thiostrepton resistance gene (*tsr*), as the protein was not synthesised when the *tsr* gene was inactivated by cleavage of pIJ350 DNA with the restriction endonuclease ClaI (Figure 3.8). The function and origin of the 69 kD protein are unknown, although it may be involved in plasmid maintenance or replication.

The results presented in this section indicate that the *S. lividans* S30 extract was indeed capable of specific coupled transcription-translation when programmed with DNA templates, since polypeptides with sizes consistent with those derived from the

Legend to Figure 3.8.

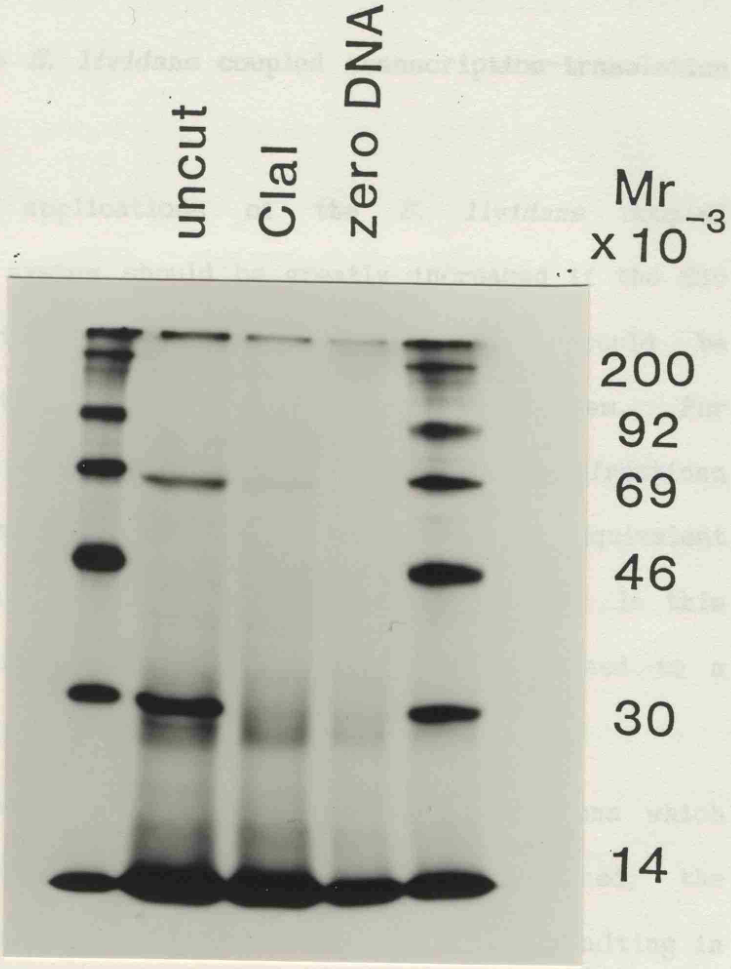
Electrophoretic analysis of the products of coupled transcription-translation by *S. lividans* extracts programmed with pIJ350.

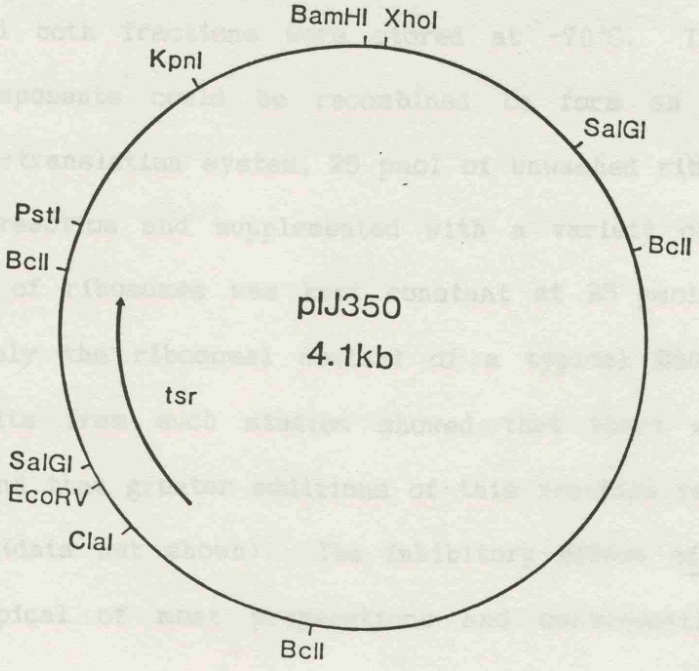
The products of coupled transcription-translation were separated by electrophoresis and visualised by fluorography. The pIJ350 template was either in a supercoiled form or in linear form following restriction with ClaI endonuclease. [^{14}C] proteins were included as molecular weight markers.

This fluorogram was produced by Dr. J. Thompson.

A restriction map of pIJ350 is also included in the Figure, to show the location of the thiostrepton resistance determinant (*tsr*) from *S. azureus*.

Fig 3.8


uncut
ClaI
zero DNA
Mr
x 10⁻³
200
92
69
46
30
14


BamHI
XhoI
KpnI
SalGI
PstI
BclI
BclI
pIJ350
4.1kb
tsr
SalGI
EcoRV
ClaI
BclI

nucleotide sequence and also those previously observed in other protein-synthesising systems, were synthesised.

2.4 Fractionation of the *S. lividans* coupled transcription-translation system.

The number of applications of the *S. lividans* coupled transcription-translation system should be greatly increased if the S30 extract could be fractionated into components which could be subsequently recombined to form a functional cell-free system. For example, a semi-purified assay could be used to interchange fractions from an extract which was sensitive to an antibiotic with equivalent fractions derived from an S30 which was antibiotic resistant. In this way, the mechanism of antibiotic resistance could be localised to a particular component.

Accordingly, in an attempt to prepare S30 subfractions which supported coupled transcription-translation when recombined, the *S. lividans* cell-free extract was centrifuged at high speed, resulting in a supernatant (S100) and a pellet of "unwashed" ribosomes. The latter were resuspended and both fractions were stored at -70°C. To test whether the two components could be recombined to form an active coupled transcription-translation system, 25 pmol of unwashed ribosomes were included in a reaction and supplemented with a variety of S100 inputs. The amount of ribosomes was kept constant at 25 pmol since this was approximately the ribosomal content of a typical S30-based reaction. The results from such studies showed that there was an optimal S100 input and that greater additions of this fraction resulted in reduced activity (data not shown). The inhibitory effect of large S100 inputs was typical of most preparations and consequently the

optimal amount of S100 for coupled transcription-translation was determined for each preparation using a fixed ribosomal input (25 pmol). Figure 3.9 shows that an assay containing 25 pmol unwashed ribosomes and an optimal amount of S100 had comparable activity to an unfractionated S30. This figure also shows that the S100 preparation was devoid of ribosomes since it had no coupled transcription-translation activity alone, but that the unwashed ribosome fraction had a low but significant activity in the absence of S100.

Once it had been established that active coupled transcription-translation systems could be prepared from unwashed ribosomes and a supernatant fraction, experiments were carried out to investigate whether purified ribosomes would support protein synthesis when supplemented with S100. Unwashed ribosomes from most bacterial sources are brown in appearance, due to contamination by membranous material. However, this contaminant can be removed by the centrifugation of unwashed ribosomes through a cushion of 20% (w/v) sucrose in a buffer containing 1 M K^+ or NH_4^+. The resulting "salt-washed" ribosomes are opalescent and those prepared from *E. coli* and *B. subtilis* usually lack their initiation factors and any associated elongation factors. A similar procedure has been used to obtain *Streptomyces* ribosomes which were highly active in assays in which polyphenylalanine was synthesised in response to polyuridylate (for example, Piendl *et al.*, 1984).

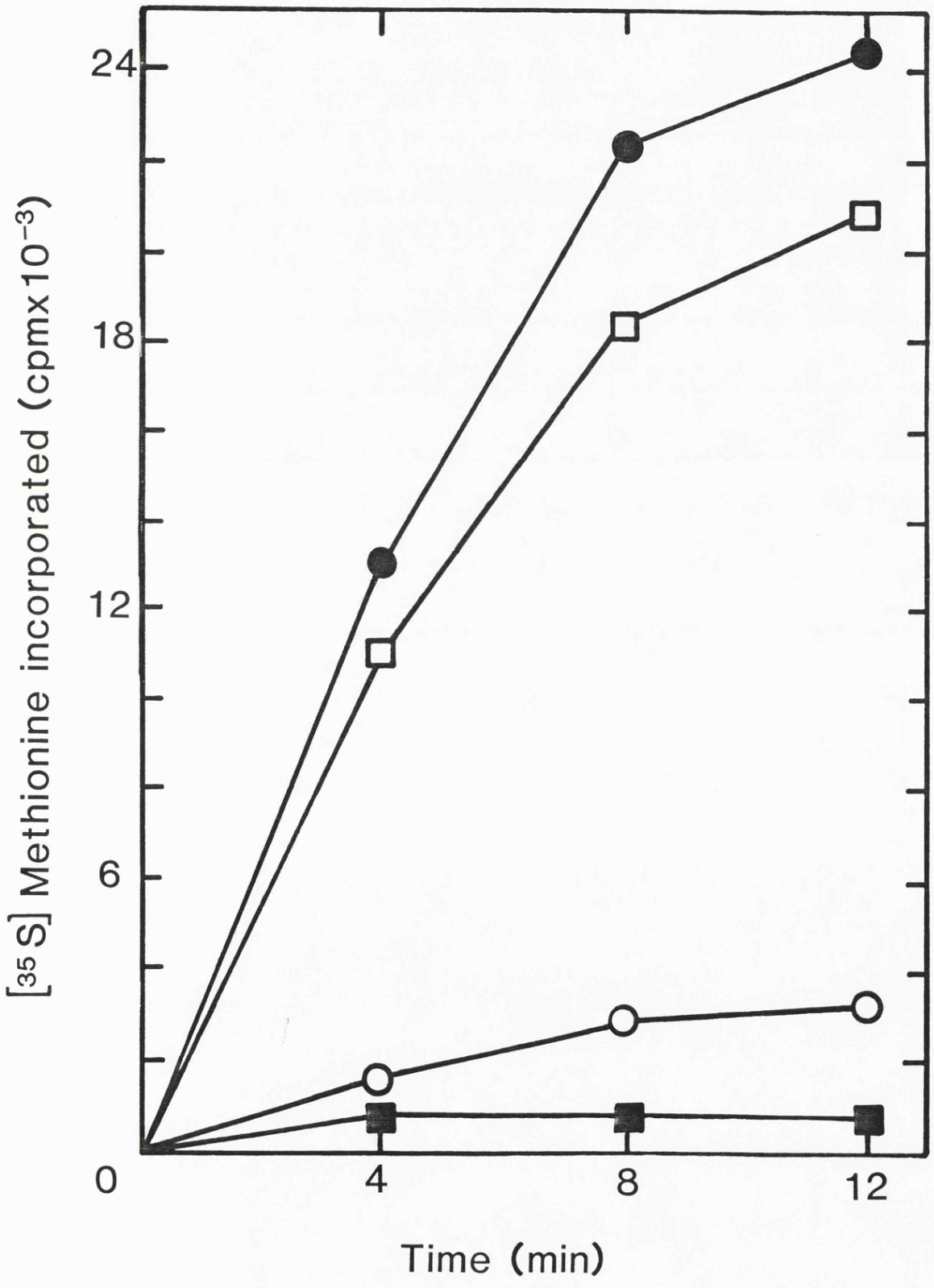
Salt-washed ribosomes from *S. lividans* were prepared as described in Methods (section 2.4) and tested for activity in a coupled transcription-translation reaction. When 25 pmol of these ribosomes were supplemented with an input of S100 optimal for 25 pmol unwashed ribosomes, the reaction possessed only 20% of the activity exhibited by

Legend to Figure 3.9.

Activity of a coupled transcription-translation reaction containing S100 and unwashed ribosome fractions.

Coupled transcription-translation reactions (30 µl) contained 2 A_{260} units of S30 (●), 4 µl S100 (■), 25 pmol unwashed ribosomes (○) or 4 µl S100 plus 25 pmol unwashed ribosomes (□). All fractions were prepared from *S. lividans* TK21.

Fig 3.9


24
18
12
6
0
4
8
12
[35 S] Methionine incorporated (cpmx10-3)
Time (min)

the unwashed ribosome-based system. The most probable explanation for the lower activity of the purified ribosomes was that the ribosomal complement of initiation factors had been removed during the washing procedure. Therefore to try to increase the activity of salt-washed ribosomes in the coupled transcription-translation reaction, a crude initiation factor fraction was prepared from *S. lividans*, following a protocol for the isolation of an equivalent fraction from *Bacillus subtilis* (Legault-Demare and Chambliss, 1974).

When the crude initiation factor preparation from *S. lividans* was tested for its ability to stimulate the activity of a reaction containing salt-washed ribosomes and S100, it was found to increase the amount of [^{35}S] methionine incorporation into protein three-fold (Figure 3.10A). This factor fraction was devoid of ribosomes since it would not support coupled transcription-translation in the absence of added ribosomes. These results support the notion that salt-washed ribosomes from *S. lividans* lose their quota of initiation factors during their preparation. Figure 3.10 also illustrates that the stimulatory effect of the *S. lividans* crude initiation factor preparation was not restricted to salt-washed ribosomes from *S. lividans*. Rather, the activities of salt-washed ribosomes from *Streptomyces caelestis* and *Streptomyces pactum* were also increased by approximately three-fold in the presence of the factor preparation.

Having established that the *S. lividans* crude initiation factor preparation could stimulate the activity of salt-washed ribosomes from a number of sources, this fraction was tested for its effect on a coupled transcription-translation reaction containing S30 or S100 and unwashed ribosomes. When the crude factor preparation was included in a coupled transcription-translation assay containing S100 and unwashed ribosomes

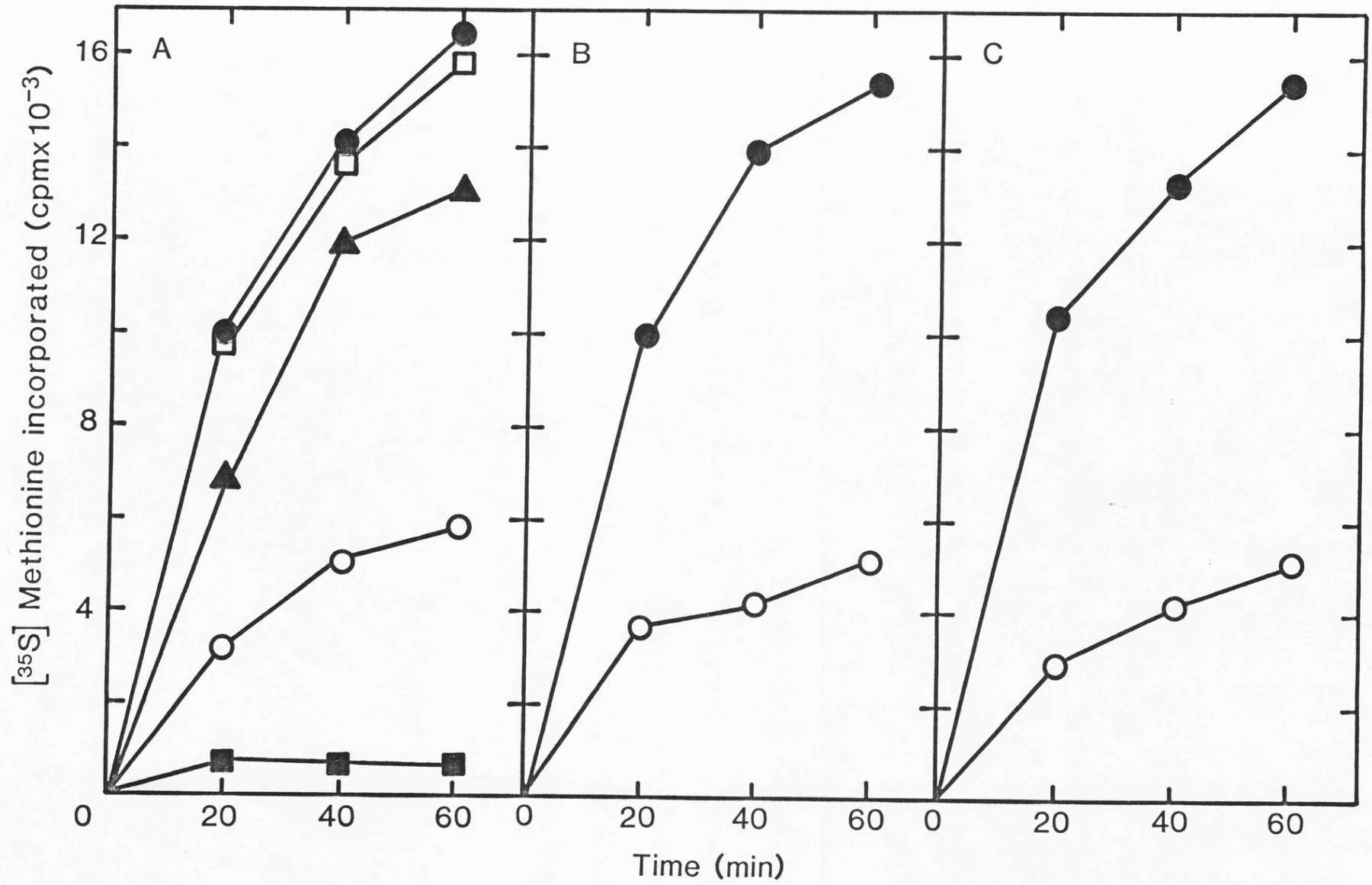
Legend to Figure 3.10.

Stimulation of the activity of salt-washed ribosomes in coupled transcription-translation reactions by a crude initiation factor preparation.

All coupled transcription-translation reactions (30 µl) contained S100 from *S. lividans* TK21. In addition, reactions contained either 5 µl crude initiation factor preparation (■), or 20 pmol salt-washed ribosomes together with zero (○), 1 µl (▲), 3 µl (□) or 5 µl (●) of crude initiation factor preparation from *S. lividans* TK21.

Sources of salt-washed ribosomes: *S. lividans* TK21 (panel A), *S. pactum* (panel B) and *S. caelestis* (panel C).

Fig 3.10


A
B
C
16
12
8
4
0
20
40
60
[35S] Methionine incorporated (cpmx10-3)
Time (min)

from *S. lividans*, there was no discernible effect on the reaction, even at high factor inputs (Table 3.2). A similar result was observed when crude factors were added to an unfractionated coupled transcription-translation reaction (Table 3.2). These findings indicate that unwashed ribosomes and ribosomes within S30 extracts are not limited by the availability of initiation factors for their activity, since a stimulation of protein synthesis would have been noted when reactions containing S30 were supplemented with crude factors.

After the preliminary characterisation of the *S. lividans* crude initiation factors in the *Streptomyces* coupled transcription-translation reaction, it was decided to investigate whether the factor fraction had any effect on polyphenylalanine synthesis by salt-washed ribosomes and S100 programmed by polyuridylate. When the crude factor preparation was added to S100 and unwashed ribosomes, a two-fold stimulation of polyphenylalanine synthesis was observed, with no further increase in activity when greater factor inputs were included (Figure 3.11). This result was unexpected since the factor-dependent initiation phase of protein synthesis is usually bypassed in synthetic mRNA-directed systems. Consequently, the stimulation of polyphenylalanine by the *S. lividans* factor preparation must have been due to either a different initiation process in the *S. lividans* assay, or to the presence of some other soluble protein(s) in the crude factor preparation which stimulated protein synthesis, for example, elongation factors. The latter explanation seems the most probable since elongation factors are essential for protein synthesis in all assay systems in which at least one peptide bond is synthesised. Although these factors are usually purified from an S100 fraction, some may be associated with unwashed ribosomes and therefore be retained in crude initiation factor

Table 3.2.

Effect of crude initiation factor preparation on the activity of S30 or unwashed ribosomes plus S100, in coupled transcription-translation reactions.

Subcellular fraction	Crude initiation factors	Activity(cpm)
S30	-	16,551
S30	1 µl	17,060
S30	2 µl	16,214
S30	3 µl	16,076
S100 + UR	-	12,947
S100 + UR	1 µl	11,981
S100 + UR	2 µl	12,195
S100 + UR	3 µl	11,833

Abbreviation: UR - unwashed ribosomes

Legend to Table 3.2.

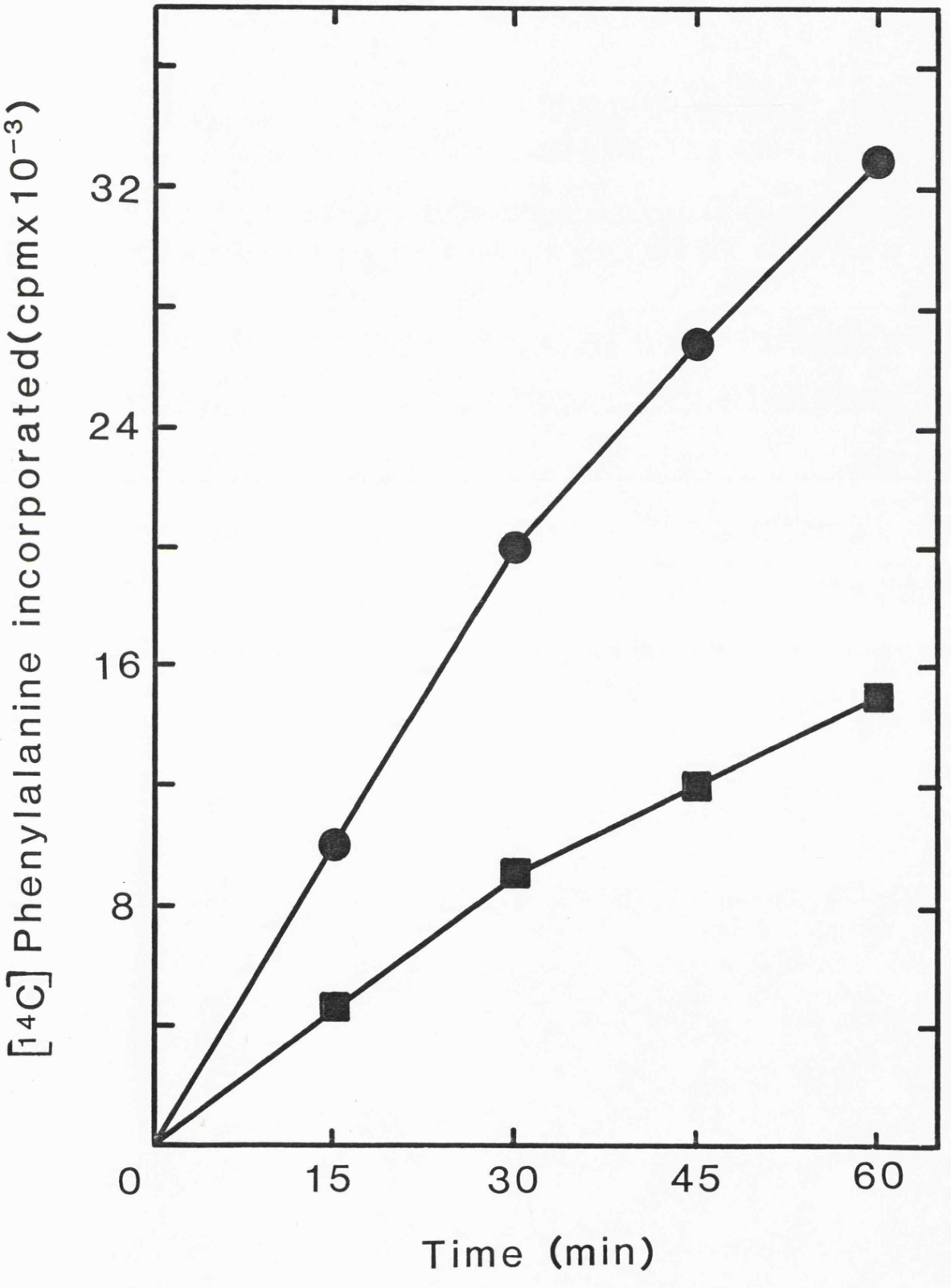
Reactions (30 µl) contained nuclease-treated S30 (2 A_{260} units) or unwashed ribosomes (25 pmol) and S100. All fractions were from *S. lividans* TK21. Samples (5 µl) were removed at 5 min intervals. 'Activity' is the incorporation of [^{35}S] methionine into TCA-precipitable material (cpm) per 5 µl sample, after incubation for 20 min.

Legend to Figure 3.11.

Effect of crude initiation factor preparation on polyphenylalanine synthesis by S100 and salt-washed ribosomes.

Reactions for the polyuridylate-directed synthesis of polyphenylalanine (50 µl) contained S100 and 4 pmol salt-washed ribosomes and were carried out in the presence (●) and absence (■) of 1 µl crude initiation factor preparation. All fractions were prepared from *S. lividans* TK21.

Fig 3.11


[14C] Phenylalanine incorporated(cpmx10-3)
32
24
16
8
0
15
30
45
60
Time (min)

preparations. No other experiments were performed to characterise the *S. lividans* factor preparation further.

2.5 Preparation of ribosomal subunits and reconstituted 30S particles for coupled transcription-translation reactions.

The previous section described the preparation of an active coupled transcription-translation system from a high speed supernatant fraction, crude initiation factors and salt-washed ribosomes. The next logical development was to produce a system which functioned using purified ribosomal subunits.

Ribosomes from *E. coli* readily dissociate into ribosomal subunits during centrifugation through sucrose gradients in a buffer containing 1 mM Mg^{2+} and 100-150 mM K^+, Na^+ or NH_4^+. Ribosomal subunits can even be prepared by the centrifugation of *E. coli* S30 extracts through similar gradients. However the separation of *Streptomyces* ribosomes into subunits is less readily achieved and several important points had to be learnt before subunit separation was routinely achieved. Firstly, unwashed ribosomes from *S. lividans* separated poorly and consequently salt-washed ribosomes were used as a starting material. Furthermore the ribosomes should appear opalescent and be devoid of contaminating membranous material. Finally, the ribosomes must be dialysed extensively against subunit dissociation buffer (see Methods) prior to centrifugation. Even when these procedures were followed, some preparations of ribosomes did not separate well. Nevertheless, the majority of salt-washed ribosome preparations separated satisfactorily so that ribosomal subunits could be isolated without sufficient cross-contamination to warrant repurification.

Once the purification procedure for *Streptomyces* ribosomal subunits had been established, 30S and 50S ribosomal particles from *S. lividans* were included in a coupled transcription-translation reaction with S100 and crude initiation factors. Such reactions typically displayed 60-90% of the activity exhibited by similar assays containing salt-washed ribosomes (for example, see Table 3.3). Furthermore, the ribosomal subunits were totally dependent on the crude initiation factor preparation for activity. This result implies that the S100 fraction from *S. lividans* does not contain a significant amount of one or more stimulatory factors. This in turn suggests that the activity displayed by salt-washed ribosomes and S100 was due to factors associated with the ribosomes rather than to components of the S100 fraction. Presumably, the residual ribosome-associated factors are removed during the preparation of ribosomal subunits.

In order to establish whether functions of the large or small ribosomal subunit were limiting protein synthesis in the fractionated cell-free system, experiments were performed to titrate the number of active subparticles. This was achieved by maintaining the crude initiation factor, S100 and one of the subunit inputs constant, and varying the supplement of the other ribosomal subparticle. The results from these studies are shown in Figure 3.12A. When the 30S ribosomal subunit content of the assay was fixed at 20 pmol, the input of the 50S moiety could be varied between 12 and 20 pmol without discernible effect on the coupled transcription-translation reaction. This suggests that functions of the small ribosomal subunit limit translation in the DNA-dependent reaction. This was consistent with results from the converse experiment, in which the input of smaller ribosomal subunit was varied. When the 30S to 50S subunit ratio was increased, protein synthesis was

Table 3.3.

Activity of ribosomal subunits in coupled transcription-translation reactions.

Subcellular components	Activity (cpm)
70S + CIF + S100	10,136
30S + 50S + CIF + S100	8,137
30S + 50S + S100	843
30S + CIF + S100	541
50S + CIF + S100	729

Abbreviations: 70S - salt-washed ribosomes
30S - small ribosomal subunits
50S - large ribosomal subunits
CIF - crude initiation factors

Legend to Table 3.3.

Reactions (30 µl) contained combinations of S100, crude initiation factor preparation, salt-washed ribosomes (20 pmol) and ribosomal subunits (20 pmol of each). All fractions were prepared from *S. lividans* TK21. Samples (5 µl) were removed at 10 min intervals. 'Activity' is the incorporation of [^{35}S] methionine into TCA-precipitable material (cpm) per 5 µl sample, after incubation for 40 min.

Legend to Figure 3.12.

Titration of active ribosomal subunits in pBR322 and polyuridylate directed protein synthesising systems.

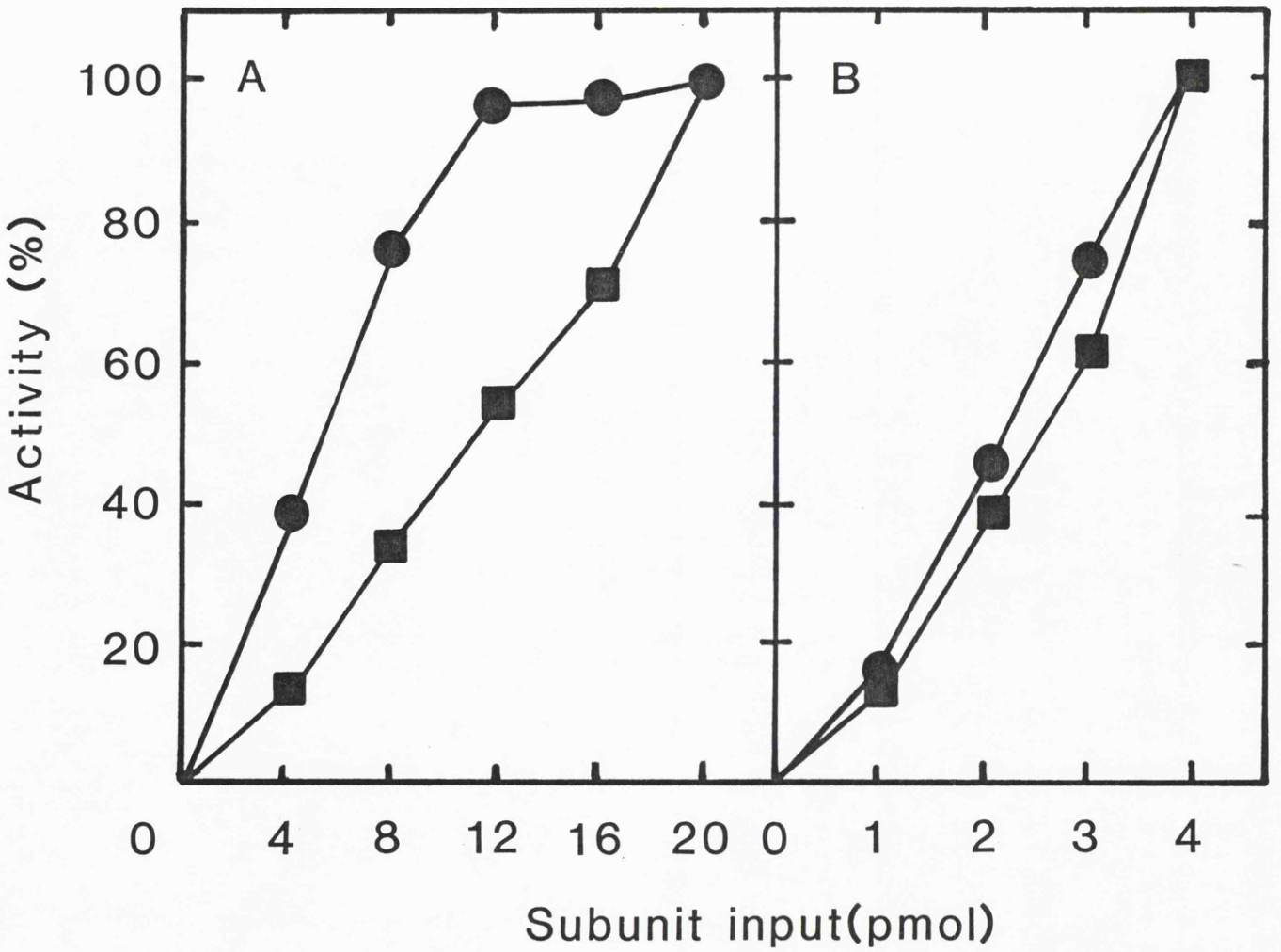
Panel A: Coupled transcription-translation reactions (30 µl) contained crude initiation factor preparation, S100 and either 20 pmol 50S ribosomal subunits with 0-20 pmol 30S counterparts (■) or 20 pmol 30S ribosomal subunits with 0-20 pmol 50S subunits (●).

100% activity is the incorporation of [^{35}S] methionine into TCA-precipitable material in assays containing 20 pmol of each subunit.

Panel B: Reactions for the synthesis of polyphenylalanine (50 µl) contained S100* and either 4 pmol 50S subunits and 0-4 pmol 30S counterparts (■) or 4 pmol 30S subunits with 0-4 pmol 50S particles (●). All fractions were prepared from *S. lividans* TK21.

100% activity is the incorporation of [^{14}C] phenylalanine into TCA-precipitable material in assays containing 4 pmol of each subunit.

Fig 3.12


A
B
100
80
60
40
20
Activity (%)
0
4
8
12
16
20
0
1
2
3
4
Subunit input(pmol)

stimulated. The relationship was directly proportional until the 30S ribosomal subunit input exceeded 20 pmol in the coupled transcription-translation assay.

A different result however, was obtained when similar experiments were performed on the polyuridylate-directed system. In this assay, the relative proportions of active particles in the ribosomal subunit preparations were similar (Figure 3.12B). Thus assays containing 4 pmol of 30S subunits and 2 pmol of 50S subunits possessed only 50% of the activity of a similar reaction containing 4 pmol of 50S subunits. The difference between the results obtained in the two assay systems was probably a consequence of the complex initiation presumed to be undertaken by 30S ribosomal subunits in the coupled transcription-translation reaction, compared with the pseudo-initiation event by which 70S ribosomes join polyuridylate in the polyphenylalanine-synthesising reaction.

The final step in the fractionation of the *S. lividans* coupled transcription-translation system undertaken during the course of this work, was to prepare functional 30S ribosomal subunits from purified 16S rRNA and 30S subunit protein (TP30). Previously workers in this laboratory had established the methodology for the reconstitution of 30S ribosomal particles from components of actinomycete ribosomes (Piendl *et al.*, 1984). These reconstituted particles were proficient in the translation of synthetic mRNA but were not tested for their ability to synthesise proteins from 'natural' mRNA templates. Therefore 30S ribosomal subunits were reconstituted from *S. lividans* 16S rRNA and TP30 using this methodology (described in Methods) and were assayed for activity in a coupled transcription-translation system supplemented with 50S subunits, crude initiation factors and S100. The reconstituted 30S

particles were active in this assay (Figure 3.13), although the level of activity was much less than that of native 30S subunits. In addition, there was considerable variation between the activities of different preparations.

The variability between reconstitution experiments could have been due to differences in the condition of the rRNA after incubation with the ribosomal protein preparation, since the TP30 may be contaminated with nucleases. To test this notion, rRNA was isolated from reconstituted particles and analysed by agarose gel electrophoresis under denaturing conditions. The analysis of rRNA isolated from various preparations of reconstituted 30S particles revealed a good correlation between the amount of intact 16S rRNA present and the activity of the reconstituted subunits (data not shown). Therefore TP30 preparations were incubated with placental ribonuclease inhibitor prior to their addition to the reconstitution reaction, in an attempt to inactivate any ribonucleases present. This inhibitor inactivates ribonucleases from a number of sources and has been used to increase the length of copy DNA (cDNA) transcription from mRNA templates by reverse transcriptase (deMartynoff *et al.*, 1980) and to improve the quality of polypeptide products in wheat-germ translation systems (Scheele and Blackburn, 1979). Spermidine was also added to the reconstitution reaction as the re-assembly of 30S ribosomal subunits from *B. subtilis in vitro* was stimulated by this polyamine, although no effect was noted with similar particles from *E. coli* (Kajegawa *et al.*, 1986).

The inclusion of ribonuclease inhibitor and spermidine in the reconstitution reaction increased both the number of successful experiments and the activity of the reconstituted particles. To determine whether this coincided with the presence of more intact 16S

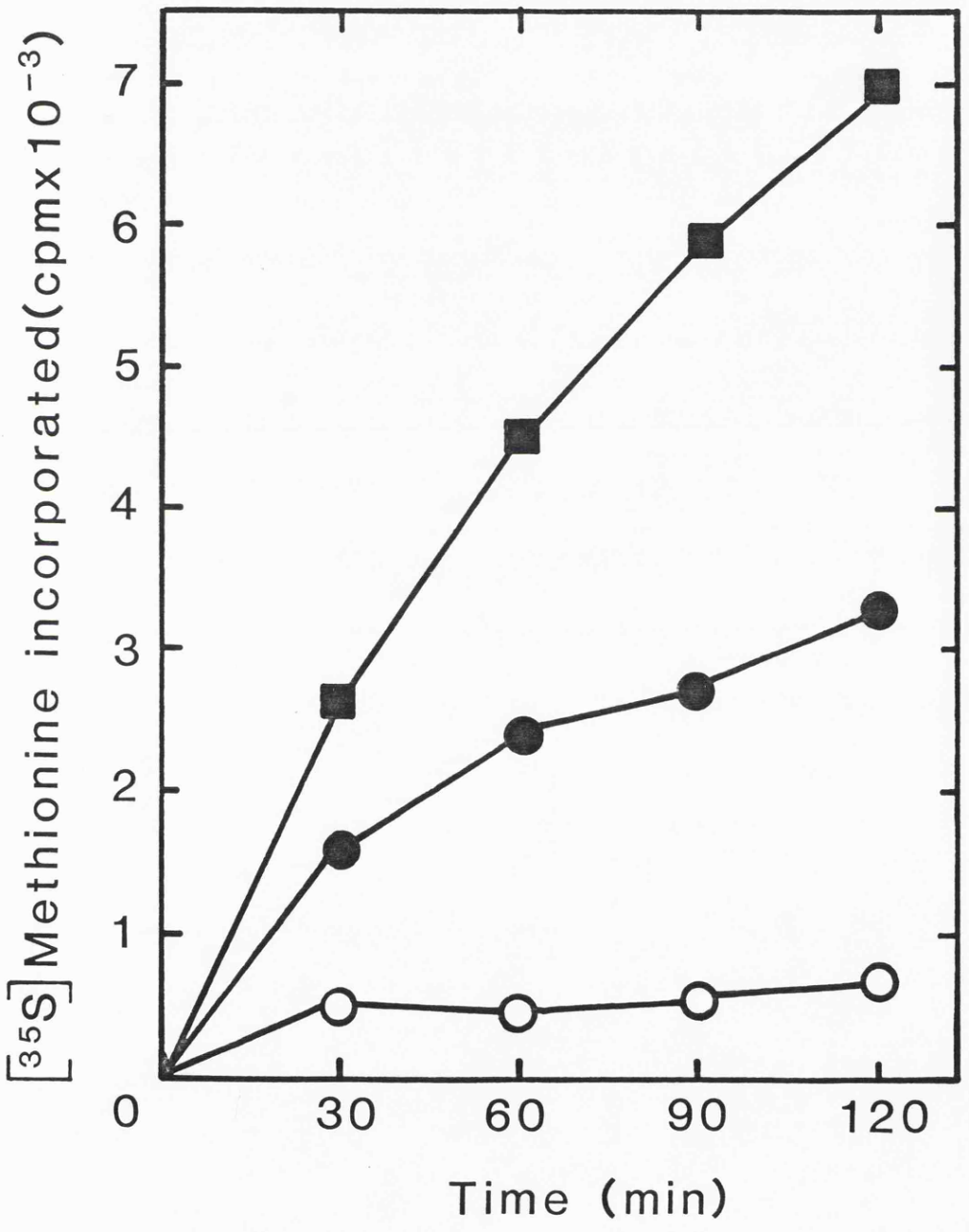
Legend to Figure 3.13.

Activity of reconstituted 30S ribosomal subunits in coupled transcription-translation reactions.

Coupled transcription-translation reactions (30 µl) contained S100, crude initiation factor preparation, 20 pmol 50S ribosomal subunits and 20 pmol native 30S ribosomal subunits (■), 40 pmol reconstituted 30S particles (●) or no 30S subunits (○).

All fractions were prepared from *S. lividans* TK21.

Fig 3.13


[35S]Methionine incorporated(cpmx10-3)
0
1
2
3
4
5
6
7
30
60
90
120
Time (min)

rRNA in the ribosomal subunits, RNA was isolated from the reconstituted particles and analysed. Ribosomal RNA extracted from 30S particles that had been reconstituted using the modified protocol was less degraded than that obtained from subunits prepared by the original procedure. One example of this observation is shown in Figure 3.14. Although the separate contributions of spermidine and the ribonuclease inhibitor to the success of the reconstitution experiments was not examined, their inclusion enabled more reproducible reconstitutions of *S. lividans* 30S ribosomal subunits to be performed.

3 Discussion.

This chapter has described the preparation and properties of S30 extracts from *S. lividans*, which were capable of coupled transcription and translation of genes encoded by plasmid DNA molecules. Furthermore, the activity of this system was totally dependent upon exogenous DNA, since prior treatment of the extract with micrococcal nuclease efficiently degraded exogenous templates.

When the *S. lividans* S30 was programmed with the *Streptomyces* plasmid pIJ350, considerably less protein was synthesised than in similar reactions containing the *E. coli* cloning vehicle, pBR322. Although this may have been due to differences in the purity of the two plasmid preparations, it was more likely to have been a consequnce of the genetic content of the plasmids. For instance, pBR322 encodes two antibiotic resistance determinants whereas pIJ350 contains only one resistance gene. In addition the promoter sequences of the pIJ350-borne resistance gene were found to be the weakest when several such sequences from *Streptomyces* antibiotic resistance genes were analysed *in vivo* (Ward *et al.*, 1986). Both of these features could have been

Legend to Figure 3.14.

Effect of ribonuclease inhibitor on the integrity of 16S rRNA during reconstitiution of 30S ribosomal subunits.

A TP30 preparation was divided into two portions and used to reconstitute two batches of 30S ribosomal particles using one 16S rRNA preparation. One TP30 portion was incubated on ice with ribonuclease inhibitor and spermidine prior to reconstitution.

The reconstituted 30S subunits were harvested, resuspended and then extracted with phenol. RNA was recovered from the aqueous phase by precipitation with ethanol plus sodium acetate and analysed by electrophoresis under denaturing conditions, in a 0.7% (w/v) agarose gel. 16S rRNA was extracted from native 30S ribosomal subunits by treatment with phenol

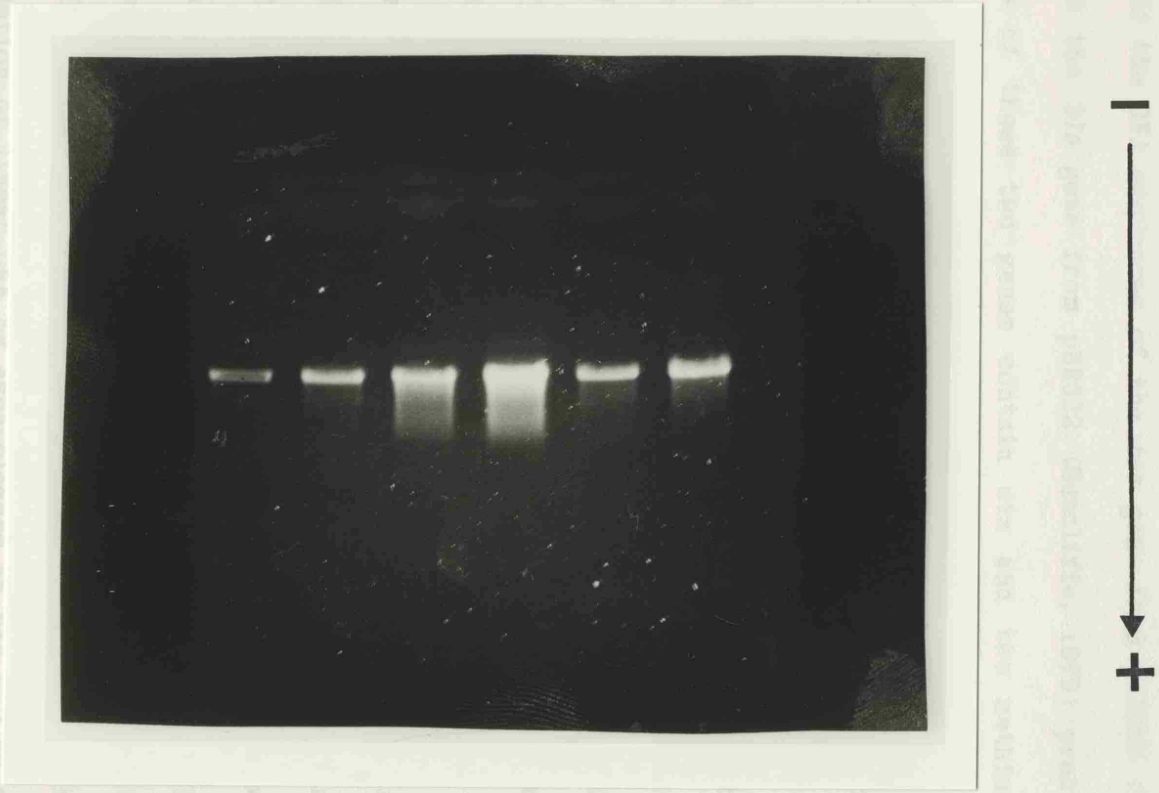
Lanes a and b: RNA (0.5 μg and 1 μg) from native 30S ribosomal subunits.

Lanes c and d: RNA (2 μg and 3 μg) from 30S particles reconstituted in the absence of ribonuclease inhibitor and spermidine.

Lanes e and f: RNA (1 μg and 2 μg) from 30S particles reconstituted in the presence of ribonuclease inhibitor and spermidine.

Fig 3.14

a b c d e f



responsible for the different template activities of the two plasmids. The greater incorporation of [^{35}S] methionine into protein in extracts programmed by pBR322 was not due to the relative abundance of methionine residues in the products of coupled transcription-translation, since inspection of the DNA sequences of the *tsr* gene from pIJ350 (Bibb *et al.*, 1985a) and the *bla* gene from pBR322 (Sutcliffe, 1979) predicts that the products of these two genes contain six and two methionine residues respectively.

The ability to synthesise specific protein products from plasmids with low template activity was facilitated by the extremely low background synthesis with the nuclease-treated S30. In the *B. subtilis* coupled transcription-translation system described previously (Leventhal and Chambliss, 1979), endogenous protein synthesis represented 25% of the total protein synthesis. This relatively high background would make it very difficult to observe products from poorly utilised templates.

The low endogenous synthesis by the *S. lividans* extract also enabled linear DNA molecules to be used as templates. Although linear pBR322 and pIJ350 DNAs, generated by incubation of plasmid DNA with restriction endonucleases, were capable of directing coupled transcription-translation, the activity was only 10-20% of that obtained with supercoiled DNA molecules. This lower template activity may have been due to degradation of linear DNA by exonucleases in the S30 or to a preference of the transcriptional machinery for supercoiled DNA, or to a combination of these factors. The inability of the S30 to synthesise the *tet* gene product from pBR322 molecules treated with EcoRI probably indicates that some nuclease activity was present in the extract.

There have been conflicting reports on the effect of DNA supercoiling and the action of DNA gyrase inhibitors on prokaryotic gene

expression *in vitro*. In one study, similar levels of colicin EI were synthesised by *E. coli* extracts programmed with supercoiled or relaxed Col EI plasmid DNA. However colicin EI synthesis from a relaxed template was inhibited seven-fold by the gyrase inhibitor novobiocin, whereas the expression of other Col EI-encoded genes was unaffected (Yang *et al.*, 1979). In contrast, a more recent series of *in vitro* studies have shown that a similar DNA gyrase inhibitor did not specifically inhibit the expression of any Col EI genes from a relaxed plasmid preparation (Pratt *et al.*, 1981). It would therefore be interesting to determine whether there are any differences in the proteins synthesised by *S. lividans* extracts programmed with relaxed and supercoiled plasmids in the presence and absence of novobiocin.

The initial characterisation of coupled transcription-translation by *S. lividans* extracts involved plasmids pBR322 and pIJ350. However, preliminary studies in this laboratory have shown that genes on *Staphylococcus aureus* plasmids and bacteriophage lambda are also expressed in this system (S. Rae, unpublished observations). These results are consistent with the results of other workers who have demonstrated that *S. lividans* can utilise promoter sequences from Gram-negative and Gram-positive bacteria *in vivo* (Bibb and Cohen, 1982; Horinouchi and Beppu, 1985). Therefore it appears that *S. lividans* lacks strong barriers to the expression of at least some heterospecific genes.

The activity of the *S. lividans* S30 in coupled transcription-translation reactions was increased by determining the optimal concentrations and inputs of various components of the assay. The most significant results from these studies were that calcium ions and unfractionated *E. coli* tRNA inhibited the reaction. The Ca^{2+}-independence of the assay enabled micrococcal nuclease to be used to abolish

'background' protein synthesis from endogenous templates in the extract. Fortunately this treatment did not inactivate the extract by degrading all the *S. lividans* tRNA, indicating that the tRNA was either present at an excess in the S30 so that it did not limit the overall reaction (even if a proportion was degraded by the nuclease) or that the tertiary structure of the tRNA made it a poor substrate for the enzyme.

Although the optimisation experiments have enabled the overall activity of the *S. lividans* coupled transcription-translation system to be increased, it has not been established whether the final activity was limited by transcription, translation or by the action of nucleases or proteases. The effect of including protease inhibitors in the buffers during the preparation and assay of extracts was not tested. However, the mycelium was centrifuged three times through 1 M KCl solutions, a treatment which removes surface-bound proteases from *B. subtilis* cells (Millet, 1970) and increases the activity of cell-free protein-synthesising systems from this organism (Legault-Demare and Chambliss, 1974). Since *Streptomyces* also produce extracellular proteases, it was hoped that the KCl washing procedure would be effective for *S. lividans* mycelium. The only precaution taken against mRNA degradation was to include placental ribonuclease inhibitor in the reaction mixture. However since this protein did not increase the incorporation of [^{35}S] methionine into TCA-precipitable material (data not shown), it was not included in coupled transcription-translation assays and its effect on the quality of the reaction products was not investigated.

Coupled transcription-translation in the *S. lividans* extract has been optimised for plasmid pBR322. With this template, the most active extracts were prepared from young mycelium (14-16 hour cultures) and older cultures yielded inactive S30s. However since *Streptomyces*

contain at least two different sigma factors (Westpheling *et al.*, 1985), it may be that only extracts of young mycelia contain an RNA polymerase with an appropriate sigma factor for the utilisation of promoters present on pBR322. Consequently extracts from older cultures may have been capable of coupled transcription-translation, but only if they had been programmed by genes which are expressed later in the life-cycle. Furthermore, extracts from older cultures may contain fewer active ribosomes, since ribosome preparations from such cultures were usually considerably less active than those from young mycelia. Presumably though, if the reasons for the inactivity of extracts from older mycelia are the requirement for an alternative template and more active ribosomes, it should be possible to construct a functional system by combining an S100 from an older mycelial extract with an active ribosome fraction whilst supplying a suitable template. Alternatively, the transcriptional capabilities of various extracts with different templates could be monitored directly by modifying the composition of the synthesis mix such that [^{3}H] UTP incorporation into nucleic acid could be measured.

Besides the study of coupled transcription-translation and transcription alone, the *S. lividans* S30 could be used to analyse the translation of different mRNA species by *Streptomyces* ribosomes. Such studies would be facilitated by the ability to synthesise large quantities of specific RNA molecules *in vitro*, using RNA polymerase from coliphage T7 and plasmid vectors which contain the specific promoter sequence recognised by this enzyme. A similar approach has enabled RNA molecules to be synthesised which function as mRNA templates in wheat-germ (Krieg and Melton, 1984) and *E. coli* translation systems (Watanabe *et al.*, 1986). This type of analysis would be particularly useful for

studying the translation of those *Streptomyces* mRNA which do not contain leader sequences between the transcription and translation start points (Hopwood *et al.*, 1986) and therefore lack a Shine-Dalgarno sequence. Since this sequence is believed to be important for the correct alignment of the mRNA with the 30S ribosomal subunit, there must be other features which enable these mRNA species to be translated.

Clearly, the coupled transcription-translation capabilities of *S. lividans* extracts should enable the study of *Streptomyces* gene expression *in vitro* to progress along similar lines to that in *E. coli* and *Bacillus subtilis*. In addition, the work in this chapter has indicated that the system could be exploited to study antibiotic action and resistance, since cell-free protein synthesis in the *S. lividans* extract was sensitive to all but one of the inhibitors of transcription and translation tested and the extract could be fractionated into components from which an active system could be reconstructed.

The key step in the fractionation of the *S. lividans* extract was the preparation of a crude initiation factor fraction which stimulated the activity of salt-washed ribosomes from various *Streptomyces*. Furthermore this fraction restored activity to ribosomal subunits, as these particles were totally inactive in coupled transcription-translation assays when supplemented with S100 alone. The crude initiation factor preparation was not purified further but its effect on coupled transcription-translation by S30 extracts and on polyphenylalanine synthesis by S100 and salt-washed ribosomes was investigated. The factor preparation had no discernible effect on the activity of the S30 extract or of S100 and unwashed ribosomes in the DNA-dependent reaction. This result is in marked contrast to those obtained from similar experiments using *E. coli* and *B. subtilis*

translation systems (Legault-Demare *et al.*, 1973; Legault-Demare and Chambliss, 1974), in which a two-fold stimulation of the activity of S100 and unwashed ribosomes was observed. Since in the *B. subtilis* systems, the unwashed ribosome-S100 combination and the S30 extract displayed similar activities, the authors proposed that *B. subtilis* ribosomes were not saturated with initiation factors *in vivo*, when the organisms were grown under conditions for preparing cell-free protein-synthesising systems. The results from the current work indicate that *Streptomyces* ribosomes are saturated with initiation factors, at least when grown under the conditions for the preparation of active cell-free extracts.

The crude initiation factor preparations from *B. subtilis* and *S. lividans* were also very different in their effects on polyuridylate-directed protein synthesis. Whereas the *Streptomyces* factors stimulated the activity of a salt-washed ribosome-S100 combination two-fold, the *B. subtilis* fraction strongly inhibited the equivalent *B. subtilis* system (Legault-Demare and Chambliss, 1974). No explanation was given for the inhibitory properties of the *B. subtilis* factors in this system, although the authors pointed out that it was unlikely to be due to nucleases in the factor preparation, since these would have inhibited the natural mRNA system also. The stimulatory effect of the *Streptomyces* factors on the polyuridylate-directed reaction was unexpected, but probably indicates that elongation factors were present in the preparation. The majority of elongation factors partition with the S100 during centrifugation, however a significant amount may associate with the unwashed ribosome fraction (the starting material for crude initiation factor preparation). At first sight, the two-fold stimulation of activity by the crude factors suggest that the elongation factors are

equally divided between the S100 and unwashed ribosome fractions. However, the S100 used in the polyuridylate system was not dialysed and consequently, although the S100 input in the reaction was optimal for the incorporation of [^{14}C] phenylalanine into polypeptide, it may have been a compromise between the input of elongation factors and the dilution of the radiolabelled amino acid by the amino acids in the S100. Hence, greater inputs of S100 could have supplied optimal amounts of elongation factors but reduced the specific activity of the [^{14}C] phenylalanine, so that a reduced incorporation of the radiolabel into TCA-precipitable material was observed.

The crude initiation factors and the S100 fractions have not been characterised further. However ribosomes have been fractionated such that 30S ribosomal particles, reconstituted from purified 16S rRNA and small ribosomal subunit protein (TP30), could be prepared and used in the coupled transcription-translation reaction in place of native 30S subunits. This type of fractionated system has not been attempted previously for any organism other than *E. coli* and *B. subtilis*. The only reconstitutions of actinomycete 30S ribosomal subunits which have resulted in particles capable of protein synthesis have involved this laboratory (Piendl *et al.*, 1984; Thompson *et al.*, 1985; Skeggs *et al.*, 1985), but these particles have never been tested for their ability to translate non-synthetic mRNA.

Although the activity of reconstituted 30S ribosomal subunits from *S. lividans* in the coupled transcription-translation reaction was often low, these subunits were as active in polyuridylate-directed reactions as those prepared by other workers in this laboratory. The synthetic activities of the ribosome preparations ranged from 35-60 phenylalanine residues polymerised per ribosome per hour

(phe ribosome^{-1} hr^{-1}) for salt-washed ribosomes, to 6-15 phe ribosome^{-1} hr^{-1} for reconstituted 30S particles (data not shown). However ribosomal particles with these activities were only obtained when isolated from cultures incubated for 14-20 hr, rather than for 40-48 hr. This was consistent with a previous observation that older *Streptomyces* cultures yielded less active subcomponents for protein synthesis than cultures incubated for 24 hr (Jones, 1976).

Finally, the fractionated coupled transcription-translation system should provide a particularly suitable system for studying antibiotic resistance mechanisms in *Streptomyces*. This system is a more realistic protein-synthesising reaction than the previously favoured assay based upon the polyuridylate-directed synthesis of polyphenylalanine. This is probably reflected in the greater antibiotic sensitivity of the reaction programmed by transcripts derived from pBR322. It should be possible to use the fractionated system to investigate ribosomal resistance directly, since ribosomes from at least twelve *Streptomyces* were active in the reaction when supplemented with S100 and crude factor preparations from *S. lividans* (Dr. A Beauclerk and M. Calcutt, unpublished data). Furthermore, the activity of reconstituted 30S particles in the reaction, although low, should be great enough to enable ribosomal resistance to 30S subunit inhibitors to be attributed to either rRNA or to the ribosomal proteins.

CHAPTER 4

CHARACTERISATION OF ANTIBIOTIC RESISTANCE MECHANISMS IN TWO ANTIBIOTIC PRODUCERS

1 Introduction.

The *Streptomyces* coupled transcription-translation system provides a convenient approach to the characterisation of antibiotic resistance mechanisms in those organisms which produce protein synthesis inhibitors. The application of this system should be facilitated by the ability to fractionate the extract into subribosomal components which can then be exchanged with similar fractions from antibiotic resistant and sensitive *Streptomyces*. This therefore should provide a relatively simple method for determining whether or not a producing organism possesses antibiotic resistant ribosomes.

This chapter will describe the analysis of antibiotic resistance mechanisms in two *Streptomyces* which synthesise inhibitors of protein synthesis, using the methodology developed for coupled transcription-translation in *S. lividans* extracts. *Streptomyces pactum* was chosen for study because its product, pactamycin, inhibits all ribosomes tested so far. This observation strongly suggests that the antibiotic inhibits an important step in translation, since its binding site is ubiquitous. However, attempts to identify the mode of action of pactamycin have been largely unsuccessful. A selective inhibition of translational initiation has been demonstrated in eukaryotic cell-free systems, but only under precisely defined conditions. This selectivity has not been observed in bacterial extracts programmed by natural mRNA or viral RNA. In addition, elevated pactamycin concentrations inhibited aspects of the elongation phase of protein synthesis in most assay systems. Therefore, the precise manner in which pactamycin affects translation remains unclear.

A perhaps surprising feature of research into pactamycin is that no resistance mechanisms have been characterised. It was hoped

therefore, that an investigation into the mechanism of resistance in the producer would lead to the isolation of pactamycin resistant ribosomes and the characterisation of a target site modification. These in turn would enable the drug binding site to be pinpointed, which might shed some light on the mode of action of the antibiotic.

The other organism included in this study was *Streptomyces caelestis*, which produces the lincosamide antibiotic, celesticetin (De Boer *et al.*, 1955). Unlike pactamycin, lincosamides are not active against eukaryotic ribosomes but inhibit bacterial protein synthesis (Josten and Allen, 1964; Vazquez, 1966a) *via* an interaction with the 50S ribosomal subunit (Vazquez, 1966b). Very little research has been carried out on the mode of action of celesticetin, presumably because it is closely related in structure to the better studied antibiotic, lincomycin (see Figure 4.0). The peptidyl transferase reaction has been shown to be sensitive to lincomycin (Monro and Vazquez, 1967), a finding which was consistent with the ability of the drug to compete with chloramphenicol for ribosomal binding (Fernández-Munoz *et al.*, 1971). However, effects on the binding of some tRNA analogues to the ribosome have also been observed (Celma *et al.*, 1970, 1971). Because of the obvious structural similarities between the two drugs, lincomycin and celesticetin may share a common ribosome binding site and thus resistance to both antibiotics may occur by the same mechanism.

Previously, lincomycin resistance had been noted in organisms which were resistant to all MLS type antibiotics, for example *Streptomyces erythraeus*. Resistance in this organism is due to dimethylation of an adenosine residue in 23S rRNA at a site equivalent to A2058 in the analogous rRNA of *E. coli*. However, there have been reports in which ribosomal resistance to lincomycin was not linked with

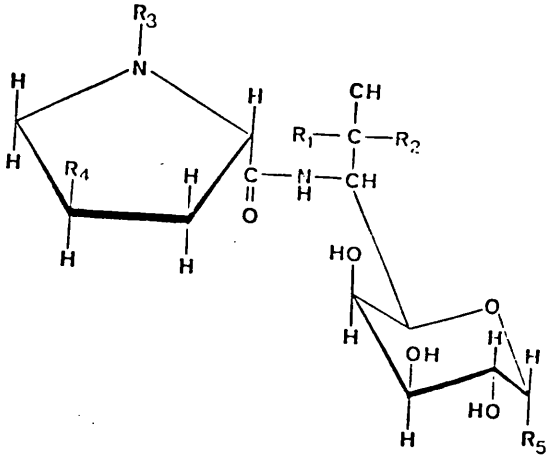
Legend to Figure 4.0.

Structures of celesticetin, erythromycin, lincomycin and spiramycin.

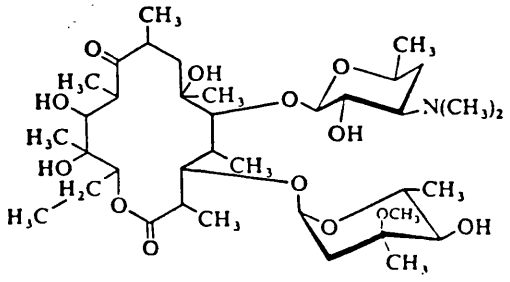
Celesticetin and lincomycin are modifications of the lincosamide structure shown.

	celesticetin (Mr 528.6)	lincomycin (Mr 406.5)
R_1	OCH_3	H
R_2	H	OH
R_3	CH_3	CH_3
R_4	H	$(CH_2)_2CH_3$
R_5	$SC_2H_4OCOC_6H_4$-2-OH	SCH_3

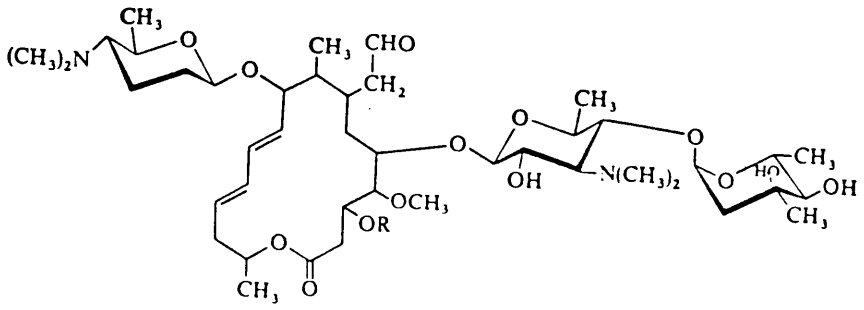
The structures of the macrolide antibiotics erythromycin (Mr 734) and spiramycin (Mr 843) are also shown.



LINCOSAMIDE



ERYTHROMYCIN



SPIRAMYCIN

Fig 4.0

MLS cross-resistance. In one instance, *S. lividans* was found to be resistant to lincomycin but not macrolides, when grown in the presence of subinhibitory erythromycin concentrations (R. Skinner, unpublished results). Also *Streptomyces cirratus*, a macrolide producer, was shown to be resistant to clindamycin (a semi-synthetic derivative of lincomycin), but sensitive to macrolides (Graham and Weisblum, 1979). There were indications in both cases that rRNA might be involved in resistance. Firstly, when rRNA was prepared from *S. lividans* which had been induced to lincomycin resistance, it was a poorer substrate for the MLS resistance methylase purified from *S. erythraeus*, than RNA extracted from ribosomes prepared from uninduced cultures. The presence of N^6-monomethyladenosine at residue A2058 in the 23S rRNA following induction in *S. lividans*, is one possible interpretation of this result. Secondly, 23S rRNA from *S. cirratus* contained monomethyladenosine whereas similar rRNA from other *Streptomyces* which were not lincomycin resistant, lacked this modification. However, no studies of resistance in functional assays were presented in the latter example, and a causal relationship between methylation and resistance was not established in either case.

Although lincomycin and celesticetin have often been considered to act similarly, there has been one important observation which demonstrated that the two lincosamides could act differently. *Staphylococcus aureus* 1206 was found to possess an inducible MLS resistance phenotype which could be induced by erythromycin and its derivatives (Allen, 1977). However celesticetin, but not lincomycin, could also induce this resistance mechanism and since one of the key elements in the translational attenuation model for induction (for review see Weisblum, 1984) is a sensitive ribosome with inducer bound to it,

there may be a subtle difference in the binding of celesticetin and lincomycin to the ribosome. It was hoped that an investigation into celesticetin resistance in the producing organism would lead to a full characterisation of a ribosomal resistance to lincosamide antibiotics.

This chapter describes the exploitation of the coupled transcription-translation system in the study of resistance to pactamycin and celesticetin in their respective producing organisms.

2 Results.

2.1 Pactamycin resistance in *Streptomyces pactum*.

The primary prerequisite for a study of pactamycin resistance in the producing organism was a sensitive assay. Since the antibiotic binds to ribosomes and inhibits their function (by a mechanism which is not understood), a generalised assay in which polypeptides are synthesised seemed more appropriate for such a study than a partial reaction of the translational process. Thus experiments were carried out to examine the pactamycin sensitivity of two protein-synthesising reactions, one in response to the synthetic mRNA, polyuridylic acid and the other employing plasmid pBR322 as a template for coupled transcription-translation. The reactions contained an equal amount of *S. lividans* S30 and were performed in a similar assay volume, in order to keep the drug to ribosome ratio and the antibiotic concentration constant. The results from this study (Figure 4.1) clearly show that the coupled transcription-translation system was the most suitable assay for demonstrating pactamycin sensitivity. Polyphenylalanine synthesis in response to polyuridylate was only poorly inhibited by pactamycin, even when the drug was present at a 500-fold molar excess over

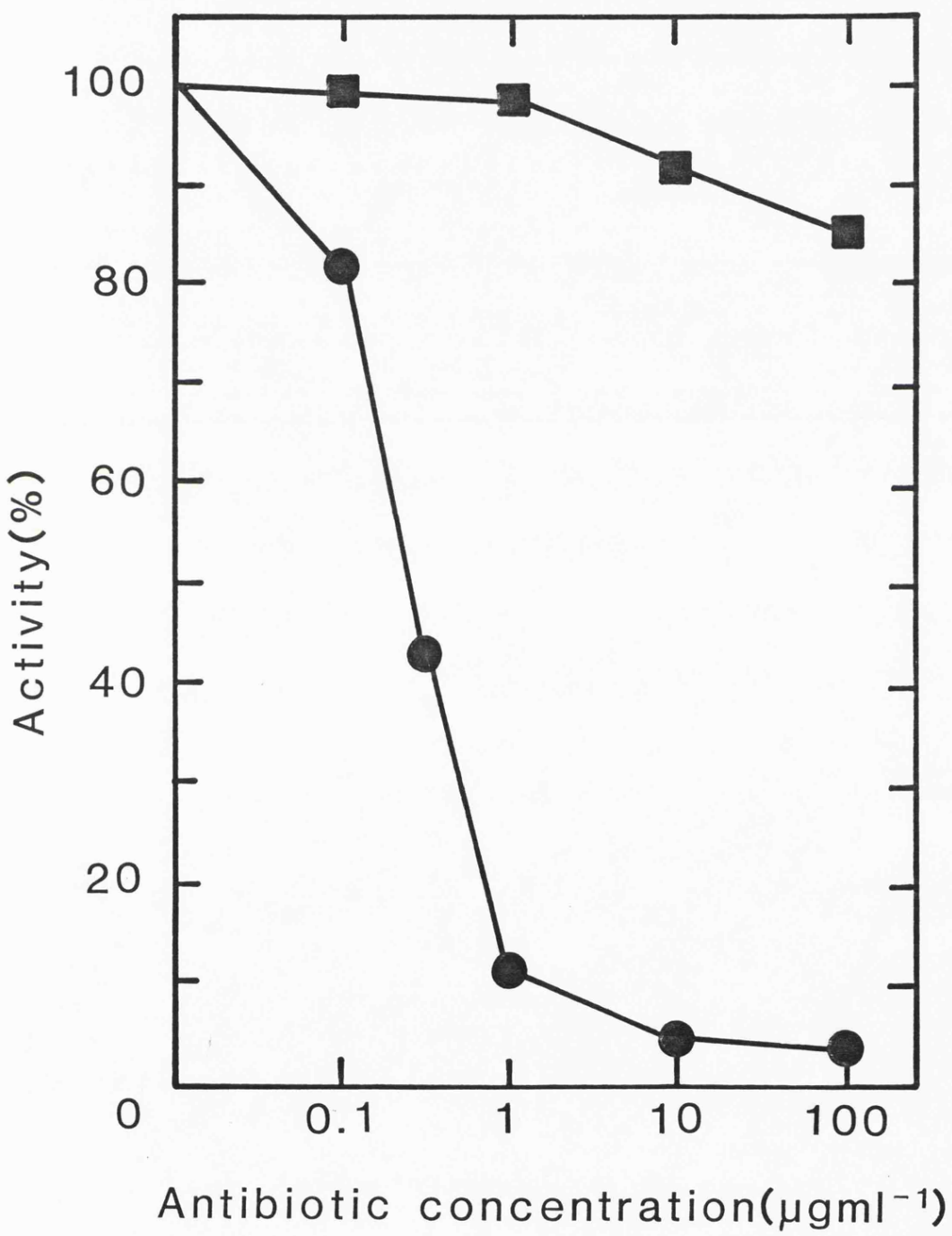
Legend to Figure 4.1.

Pactamycin sensitivity of coupled transcription-translation and polyphenylalanine synthesis by *S. lividans* extracts.

All reactions (30 µl) contained nuclease-treated S30 (1 A_{260} unit) from *S. lividans* TK21. The extract was incubated with DMSO or pactamycin prior to assay for polyphenylalanine synthesis (■) or coupled transcription-translation activity (●).

100% activity is the incorporation of radiolabelled amino acid into TCA-precipitable material in a drug-free control.

Fig 4.1


100
80
60
40
20
0
Activity(%)
0.1
1
10
100
Antibiotic concentration(μgml⁻¹)

ribosomes, whereas the DNA-dependent system possessed only 30% activity at equimolar pactamycin to ribosome inputs.

S. pactum is highly resistant to pactamycin *in vivo*. Therefore to determine whether protein synthesis *in vitro* was also resistant, the method for the preparation of coupled transcription-translation systems from *S. lividans* was carried out with cultures of *S pactum*. The resultant S30 extract was then tested for its ability to support protein synthesis in the presence and absence of plasmid DNA. The results in Figure 4.2 show that similar levels of activity were achieved whether pBR322 was present or not. A similar situation had been observed previously, in some *S. lividans* extracts, but this had not been a problem, as treatment with micrococcal nuclease had totally abolished plasmid-independent synthesis. Accordingly, the *S. pactum* S30 was treated with micrococcal nuclease, for increasing periods of time (as described in Methods) and then assayed for plasmid-dependent protein synthesis. The results obtained (Figure 4.2) were unexpected, since the activity of the extract in the presence and absence of pBR322 decreased to a similar extent. The reason for this was not established, but it was not due to the presence of Ca^{2+} in the S30, because the activity of extract which had been incubated with nuclease in the absence of added Ca^{2+} was virtually unaffected (data not shown). Nevertheless, the pactamycin sensitivity of an untreated *S. pactum* S30 was tested. The results presented in Figure 4.3 show that incorporation of [^{35}S] methionine into protein by the *S. pactum* extract was insensitive to pactamycin, at drug concentrations which totally inhibited a nuclease-treated *S. lividans* extract. At first sight, this result suggested that a resistance mechanism might be present *in vitro*. However, if the [^{35}S] methionine incorporation had been due to run-off translation by

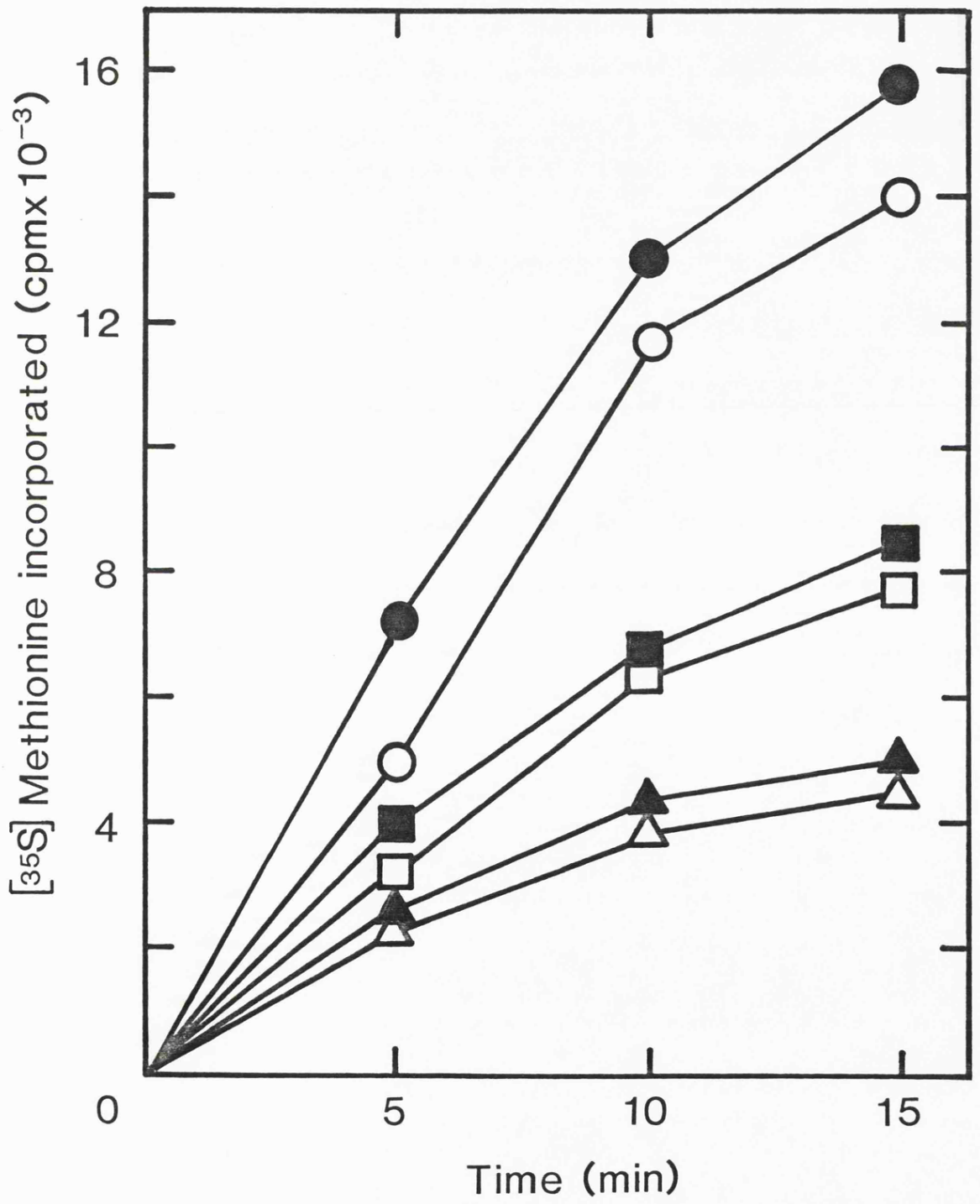
Legend to Figure 4.2.

Effect of micrococcal nuclease treatment on coupled transcription-translation activity by *S. pactum* extracts.

A portion of *S. pactum* extract was incubated with micrococcal nuclease in the presence of Ca^{2+}. Small samples were removed after various incubation times and the nuclease activity was stopped by the addition of EGTA. Each sample (2 A_{260} units) was then assayed for coupled transcription-translation in the presence (shaded symbols) and absence (open symbols) of plasmid.

Duration of nuclease treatment: 0 min (●), 15 min (■) and 30 min (▲).

Fig 4.2

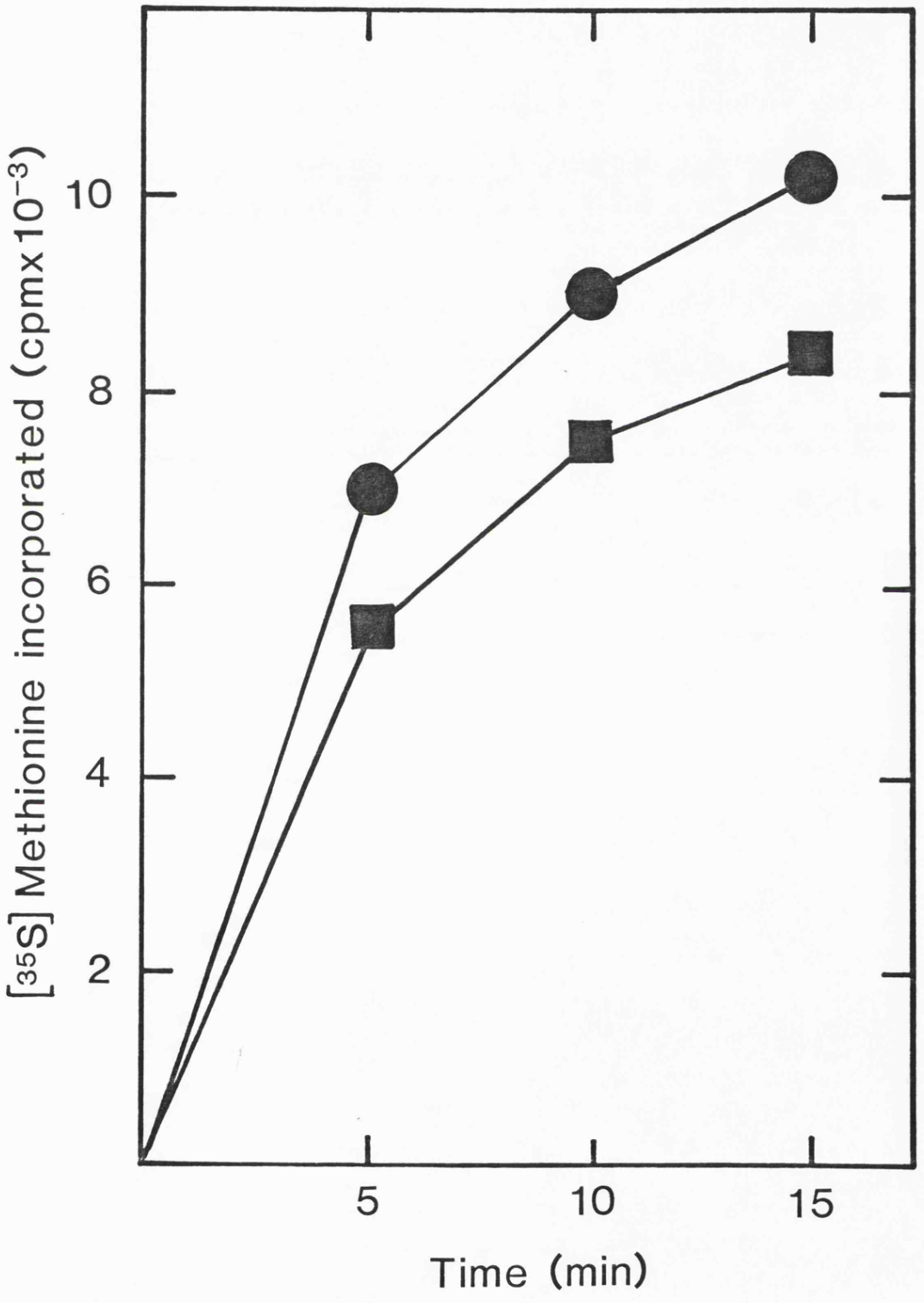

16
12
8
4
0
5
10
15
[35S] Methionine incorporated (cpmx10-3)
Time (min)

Legend to Figure 4.3.

Sensitivity of *S. pactum* coupled transcription-translation reactions to pactamycin.

All reactions (30 µl) contained *S. pactum* S30 (2 A_{260} units). The extract was incubated with DMSO (●) or 10 µg ml^{-1} final pactamycin concentration (■), prior to assay for coupled transcription-translation activity.

Fig 4.3


[35S] Methionine incorporated (cpmx10-3)
10
8
6
4
2
5
10
15
Time (min)

preformed polysomes, the insensitivity might have resulted from a reduced ability of pactamycin to bind to ribosomes in such complexes, compared with their binding to vacant ribosomal particles.

In order to establish whether *S. pactum* ribosomes were pactamycin resistant or not, nuclease-treated extracts from *S. lividans* and *S. pactum* and an untreated extract from the latter organism, were fractionated into S100 and unwashed ribosomes and then used to reconstruct four coupled transcription-translation systems. These homologous and heterologous systems were tested for their pactamycin sensitivity. As expected, a system composed of both *S. lividans* fractions was very sensitive to pactamycin (Figure 4.4A). However systems containing unwashed ribosomes from a nuclease-treated *S. pactum* extract were highly resistant to the antibiotic, irrespective of whether the S100 fraction was derived from *S. lividans* or from an untreated *S. pactum* S30 (Figure 4.4B,C). Since these ribosomes should not be complexed with mRNA after nuclease treatment, these data suggest that the *S. pactum* extract contained pactamycin resistant ribosomes. The final panel in Figure 4.4 shows that a system containing unwashed ribosomes from *S. lividans* and S100 from an untreated *S. pactum* S30 was also sensitive to pactamycin. Thus, if the *S. pactum* extract contained a pactamycin inactivating enzyme, in addition to the proposed ribosomal modification system, it was inactive under the conditions used for coupled transcription-translation reactions.

S. pactum ribosomes were probably the components of the unwashed ribosome fraction which conferred pactamycin resistance in the coupled transcription-translation reaction. However resistance may have been due to the presence of an inactivating enzyme in this fraction. In a previous study, a viomycin inactivating enzyme was found

Legend to Figure 4.4.

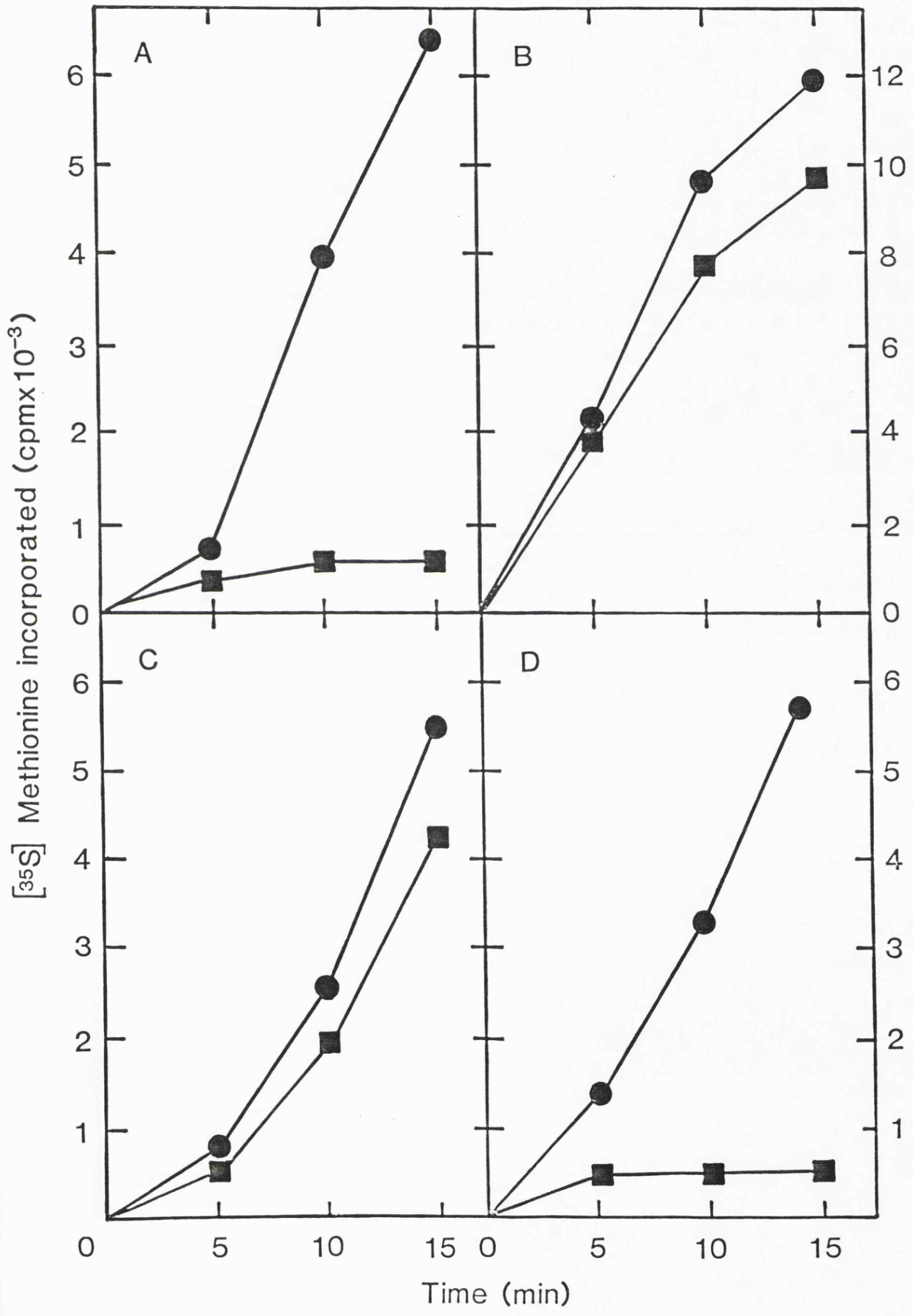
Localisation of pactamycin resistance to the unwashed ribosome fraction from *S. pactum*.

All reactions (30 µl) contained 25 pmol unwashed ribosomes and S100 from either *S. pactum* or *S. lividans* TK21. The various fractions were incubated with DMSO (●) or 10 µg ml^{-1} final pactamycin concentration (■), prior to assay for coupled transcription-translation activity.

Source of components: (A) ribosomes and S100 from *S. lividans*, (B) ribosomes and S100 from *S. pactum*, (C) ribosomes from *S. pactum* and S100 from *S. lividans* and (D) ribosomes from *S. lividans* and S100 from *S. pactum*.

S. pactum S100 was prepared from extracts which had not been treated with micrococcal nuclease. All other fractions were from nuclease-treated extracts.

Fig 4.4


A
B
C
D
[35S] Methionine incorporated (cpmx10-3)
Time (min)
0
5
10
15
1
2
3
4
6
8
12

to be associated with unwashed ribosome preparations and conferred resistance on this fraction (J. Thompson, unpublished data). Therefore to determine whether *S. pactum* ribosomes really were pactamycin resistant, salt-washed ribosomes were prepared from *S. lividans* and the pactamycin producer, since these particles were considerably more refined than their unwashed counterparts. The pactamycin sensitivity of these ribosomes was then assayed in a coupled transcription-translation reaction supplemented with crude initiation factors and S100 from *S. lividans*. The results from these experiments (Figure 4.5) clearly demonstrate that *S. pactum* ribosomes were resistant to high pactamycin concentrations, whereas those from *S. lividans* were very sensitive to the drug. Thus *S. pactum* employs a target site modification system which presumably prevents autotoxicity *in vivo*.

Previously, antibiotic binding studies had shown that pactamycin readily bound to the prokaryotic 30S ribosomal subunit, or the 40S equivalent from eukaryotes. It was therefore of interest to find out whether pactamycin resistance was also a property of the smaller ribosomal subunit. Consequently ribosomal 30S and 50S subunits were prepared from salt-washed ribosomes of *S. lividans* and *S. pactum* to enable pactamycin resistance to be further characterised.

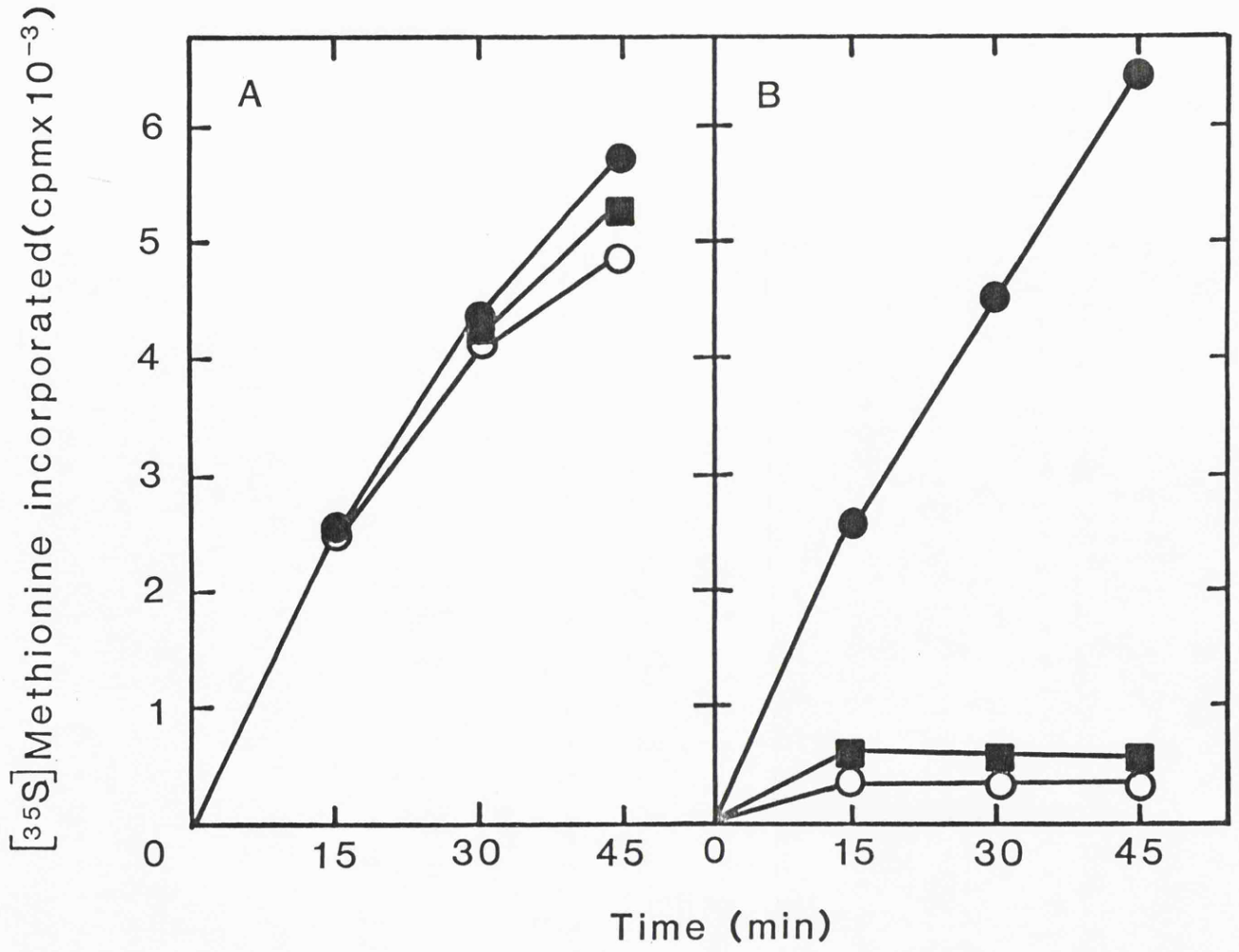
Once isolated, ribosomal subunits were recombined to form homologous and heterologous ribosomes and then assayed for pactamycin resistance in a coupled transcription-translation reaction, supplemented with appropriate fractions from *S. lividans*. As expected, protein synthesis was resistant to pactamycin when the ribosome contained a 30S subunit from *S. pactum* (Figure 4.6B,C). In addition, there was no contribution to resistance by the 50S ribosomal subunit from *S. pactum*, since hybrid ribosomes containing these particles were as sensitive as

Legend to Figure 4.5.

Sensitivity of salt-washed ribosomes from *S. lividans* and *S. pactum* to pactamycin.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Salt-washed ribosomes (20 pmol) from *S. pactum* (panel A) and *S. lividans* TK21 (panel B) were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} (■) or 100 µg ml^{-1} (○) final concentration, prior to assay for coupled transcription-translation activity.

Fig 4.5


[35S]Methionine incorporated(cpmx10-3)
A
B
0
1
2
3
4
5
6
0
15
30
45
0
15
30
45
Time (min)

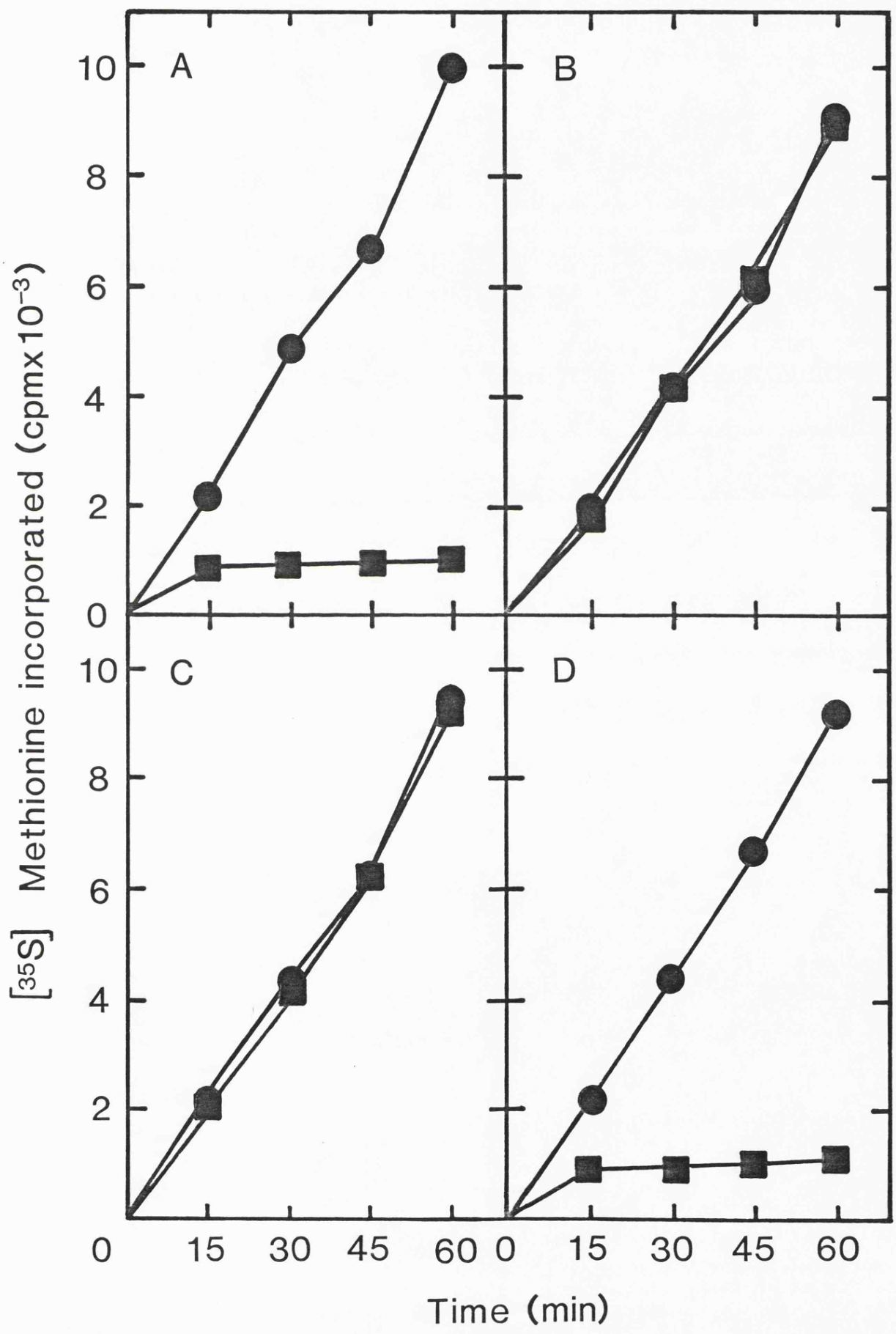
Legend to Figure 4.6.

Localisation of pactamycin resistance to the 30S subunit of the *S. pactum* ribosome.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Ribosomal subunits (20 pmol of each) were incubated with DMSO (●) or 10 µg ml^{-1} pactamycin (■) prior to assay for coupled transcription-translation activity.

Source of ribosomal subunits: (A) 30S and 50S subunits from *S. lividans* TK21, (B) 30S and 50S subuits from *S. pactum*, (C) 30S subunits from *S. pactum* and 50S subunits from *S. lividans* TK21 and (D) 30S subunits from *S. lividans* TK21 and 50S subunits from *S. pactum*.

Fig 4.6


A
B
C
D
10
8
6
4
2
0
0
15
30
45
60
[35S] Methionine incorporated (cpmx10-3)
Time (min)

homologous *S. lividans* ribosomes (Figure 4.6A,D). Thus the ribosomal alteration which results in pactamycin resistance and the antibiotic binding site reside on the same ribosomal subunit.

The next step in the localisation of the pactamycin resistance modification (and presumably the drug binding site) was the reconstitution of hybrid 30S ribosomal particles from 16S rRNA and TP30 isolated from *S. lividans* and *S. pactum* (for details, see Methods). Although 30S ribosomal subunits reconstituted from *S. lividans* fractions were functional in the coupled transcription-translation reaction, reconstituted 30S ribosomal particles containing rRNA or ribosomal proteins from *S. pactum* had no significant activity in this assay system (data not shown). The reason for the inactivity of these particles was not determined and consequently it has not been possible to characterise the resistance mechanism in *S. pactum* further. However, because active 30S ribosomal subunits could be reconstituted from *S. lividans*, it was decided to clone the gene(s) responsible for pactamycin resistance in *S. lividans* and characterise the resistance mechanism in this organism. The cloning and biochemical characterisation of pactamycin resistance determinant(s) will be described in Chapter 5.

2.2 Celesticetin resistance in *Streptomyces caelestis*.

The preceding section illustrated the value of a fractionated coupled transcription-translation reaction for investigating resistance to a protein synthesis inhibitor. A similar approach was therefore adopted to determine whether ribosomes from *S. caelestis* were resistant to the lincosamide antibiotic celesticetin.

Since salt-washed ribosomes from several *Streptomyces* had previously been shown to function in coupled transcription-translation

reactions (when supplemented with crude initiation factors and S100 from *S. lividans*), no attempt was made to prepare S30 extracts from *S. caelestis* that were active in this assay. Rather, ribosomes were prepared from the celesticetin producer and the question of ribosomal resistance was addressed directly. The results (Figure 4.7) clearly show that coupled transcription-translation reactions containing ribosomes from *S. caelestis* were highly resistant to celesticetin, whereas similar reactions containing *S. lividans* ribosomes were inhibited by low drug concentrations. Therefore it appeared that *S. caelestis* employed a ribosomal modification mechanism, presumably to prevent auto-inhibition.

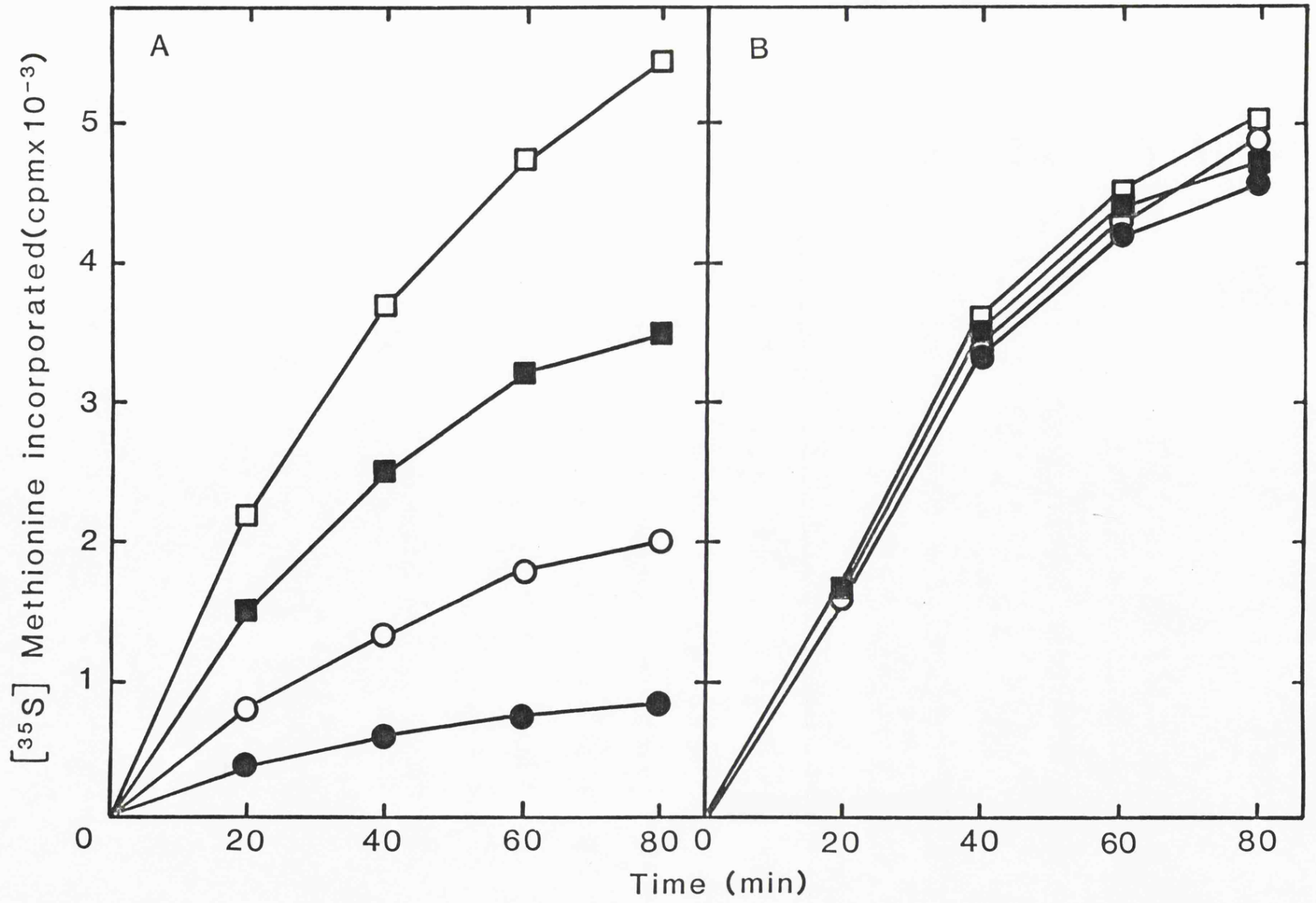
Ribosomal resistance to lincosamides had previously been linked with cross-resistance to other members of the MLS group of antibiotics. Accordingly, ribosomes from *S. caelestis* were tested for activity in the presence of the lincosamide, lincomycin and the macrolides; carbomycin, erythromycin and spiramycin. Ribosomes from *S. lividans* were used as a control, because even though this organism possesses an inducible lincomycin resistance mechanism, subcellular components were prepared from cultures grown under non-inducing conditions. The results from these experiments (Figure 4.8) demonstrate that ribosomes from *S. caelestis* and *S. lividans* were equally sensitive to the macrolide antibiotics involved in this study. However, ribosomes from the celesticetin producer were resistant to lincomycin, whereas those from the control organism were sensitive to this antibiotic. Thus the classical 'MLS' resistance mechanism was not evident in *S. caelestis*, although the ribosomes from this bacterium were resistant to both lincosamides tested, possibly by virtue of the same ribosomal alteration.

Legend to Figure 4.7.

Sensitivity of salt-washed ribosomes from *S. lividans* and *S. caelestis* to celesticetin in coupled transcription-translation reactions.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Salt-washed ribosomes (20 pmol) from *S. lividans* TK21 (panel A) and *S. caelestis* (panel B) were incubated with DMSO (□) or celesticetin at 1 µg ml^{-1} (■), 10 µg ml^{-1} (○) or 100 µg ml^{-1} (●) final concentration, prior to assay for coupled transcription-translation activity.

Fig 4.7

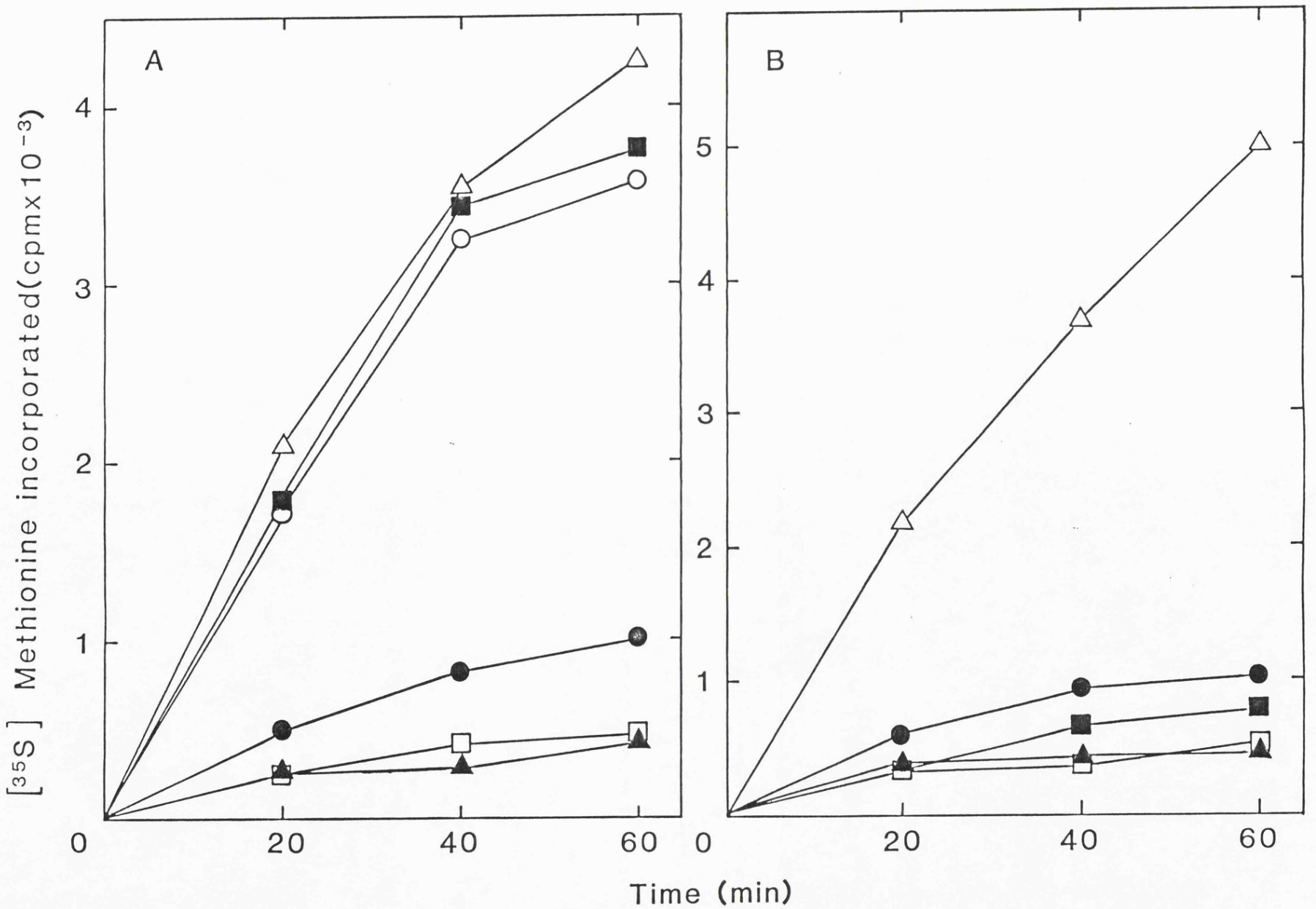

A
B
[35S] Methionine incorporated(cpmx10-3)
0
1
2
3
4
5
0
20
40
60
80
Time (min)

Legend to Figure 4.8.

Sensitivity of salt-washed ribosomes from *S. caelestis* and *S. lividans* to lincosamides and marolides in coupled transcription-translation reactions.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Salt-washed ribosomes from *S. caelestis* (panel A) and *S. lividans* TK21 (panel B) were incubated with solvent (△) or one of the following antibiotics at 100 µg ml^{-1} final concentration, prior to assay for coupled transcription-translation activity: celesticetin (■), carbomycin (▲), lincomycin (○), spiramycin (□) or erythromycin (●).

Fig 4.8


A
B
[35S] Methionine incorporated(cpmx10-3)
Time (min)
0
20
40
60
1
2
3
4
5

In order to characterise the *Streptomyces* lincosamide resistance further, 50S and 30S ribosomal subunits from *S. caelestis* and *S. lividans* were recombined to form homologous and heterologous ribosomes and then assayed in a coupled transcription-translation reaction for activity in the presence of lincomycin. The results (Figure 4.9) clearly show that ribosomes containing 50S subunits from *S. caelestis* were highly resistant to lincomycin, whereas those containing equivalent subcomponents from *S. lividans* were sensitive to the antibiotic.

Having established that *S. caelestis* ribosomes were lincomycin resistant *in vitro*, the ribosomal resistance mechanism was compared with that present in an organism which does not produce such antibiotics. It had previously been shown that *Escherichia coli* ribosomes were resistant to lincomycin *in vitro* (Chang *et al.*, 1966). This had been demonstrated by preparing hybrid ribosomes from ribosomal subunits derived from *Bacillus stearothermophilus* and *E. coli* and testing them for their ability to bind radiolabelled lincomycin. In order to determine whether ribosomes from *E. coli* and *S. caelestis* were resistant to the same concentrations of lincomycin, salt-washed ribosomes from these two organisms were assayed for resistance in a protein-synthesising system directed by polyuridylic acid. A coupled transcription-translation reaction would have been the assay of choice, but *E. coli* ribosomes were found to be inactive in the *Streptomyces*-derived system (data not shown) and an equivalent *E. coli* system was not readily available. However ribosomes from a number of sources including *E. coli*, had comparable activity to those from *S. lividans* in a polyuridylate-dependent system, when supplemented with a soluble protein fraction from *S. lividans*. The results from these studies (Figure 4.10)

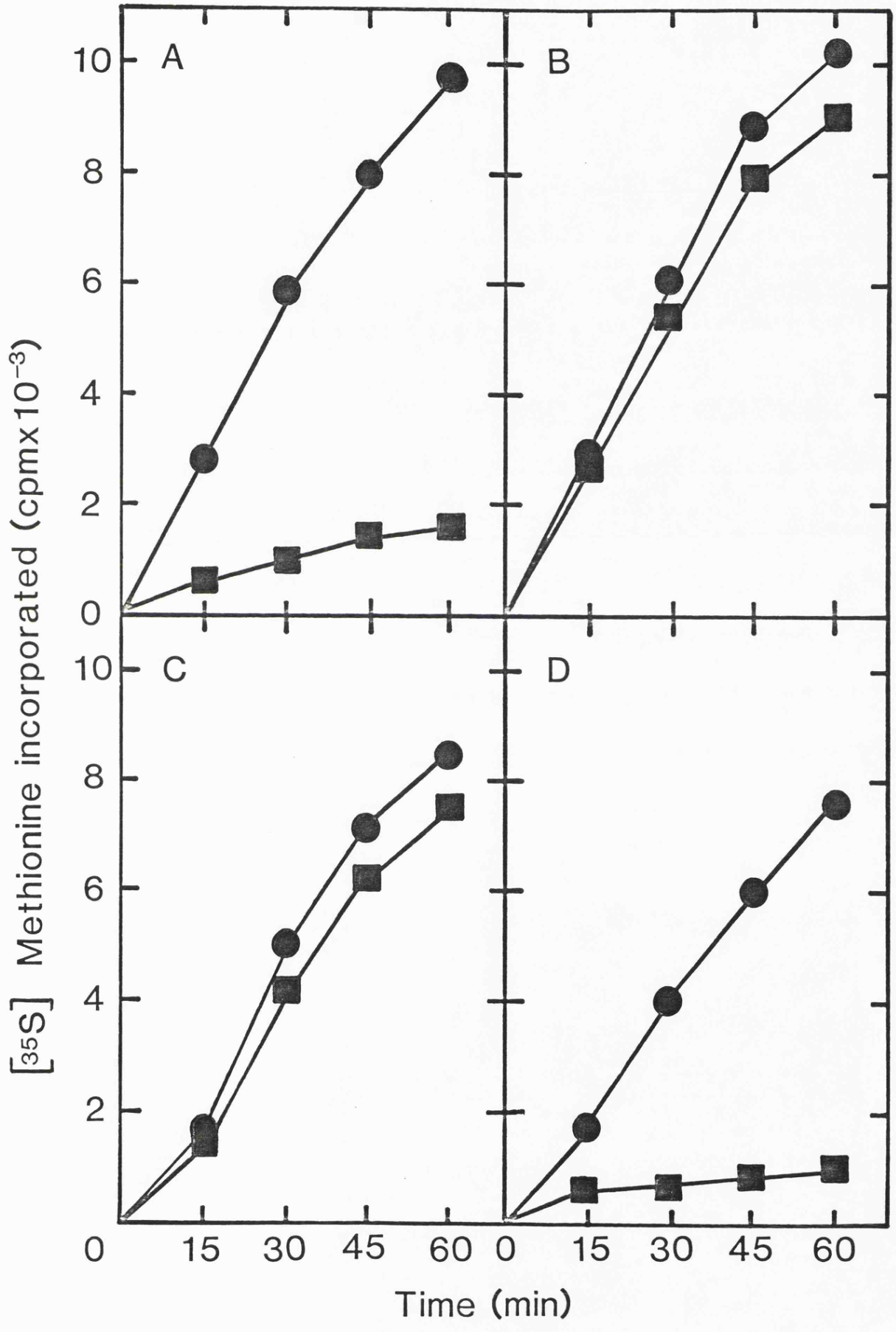
Legend to Figure 4.9.

Localisation of lincomycin resistance to the 50S subunit of the *S. caelestis* ribosome.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Ribosomal subunits (20 pmol of each) were incubated with H_2O (●) or lincomycin at 100 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Source of ribosomal subunits: (A) 30S abd 50S subunits from *S. lividans* TK21, (B) 30S and 50S subunits from *S. caelestis*, (C) 30S subunits from *S. lividans* TK21 and 50S subunits from *S. caelestis* and (D) 30S subunits from *S. caelestis* and 50S subunits from *S. lividans* TK21.

Fig 4.9

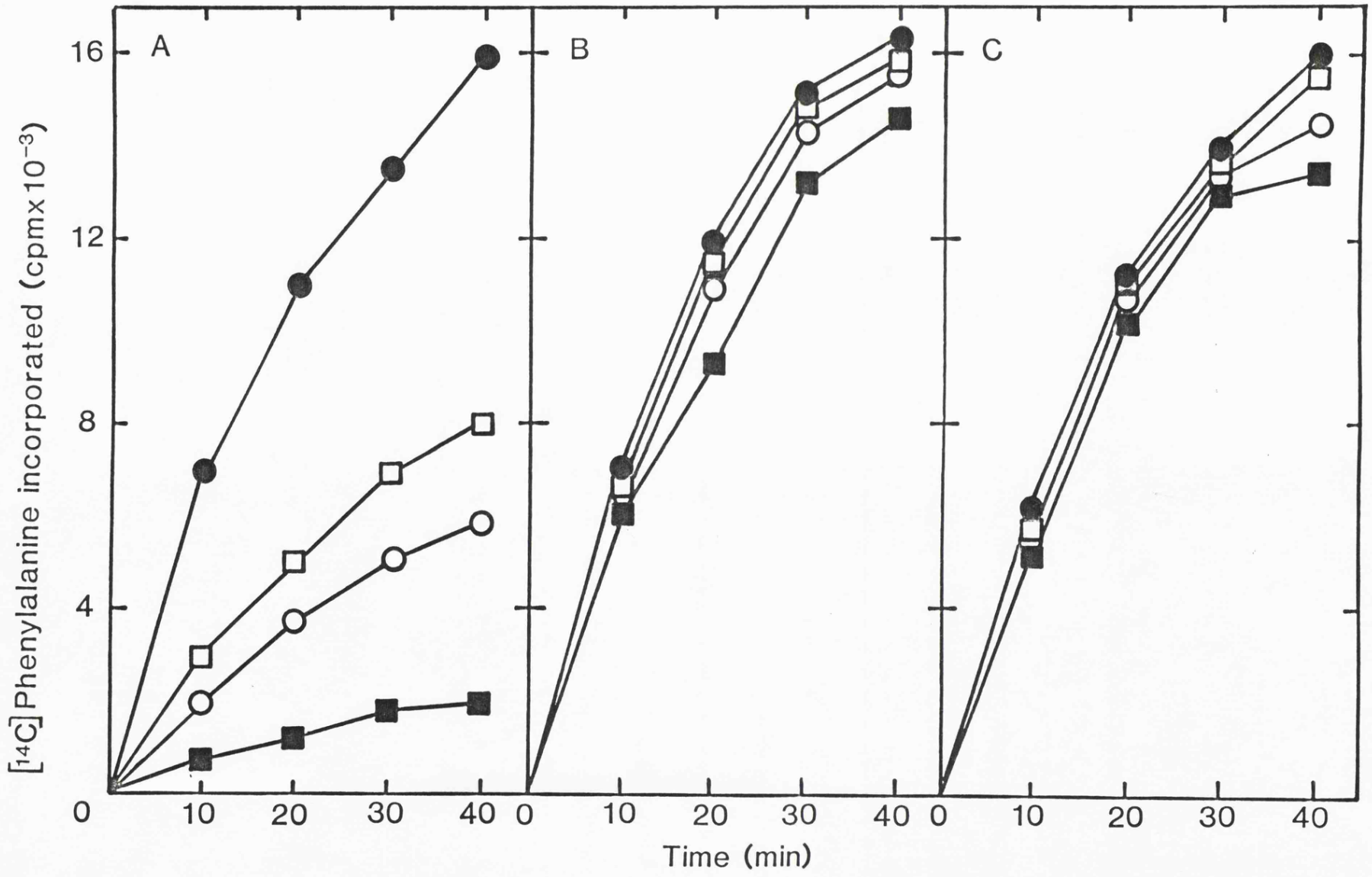

A
B
C
D
10
8
6
4
2
0
15
30
45
60
[35S] Methionine incorporated (cpmx10-3)
Time (min)

Legend to Figure 4.10.

Sensitivity of polyphenylalanine synthesis by ribosomes from *E. coli*, S. caelestis and *S. lividans* to lincomycin.

All reactions (50 µl) contained S100* from *S. lividans* TK21. Salt-washed ribosomes (4 pmol) from *S. lividans* TK21 (panel A), *E. coli* (panel B) and *S. caelestis* (panel C) were incubated with H_2O (●) or lincomycin at 4 µg ml^{-1} (□), 40 µg ml^{-1} (○) or 400 µg ml^{-1} (■) final concentrations, prior to assay for polyphenylalanine synthesis.

Fig 4.10


A
B
C
16
12
8
4
0
10
20
30
40
[14C] Phenylalanine incorporated (cpmx10-3)
Time (min)

demonstrate that ribosomes from *E. coli* and *S. caelestis* were indistinguishable in their ability to synthesise polyphenylalanine, even in the presence of 100 fold more lincomycin than was needed to inhibit the *S. lividans* ribosomes. Furthermore, ribosomes from *E. coli* and *S. caelestis* were found to be equally resistant to celesticetin in similar polyuridylate-directed assays (Figure 4.11). It seemed possible therefore that a similar resistance mechanism might be present in *E. coli* and *S. caelestis* which rendered their ribosomes resistant to lincosamides.

Previously, the lincomycin resistance of *E. coli* ribosomes had been shown to be a property of the 50S ribosomal subunit (Chang *et al.*, 1966). Consequently, it was decided to prepare homologous and heterologous 70S ribosomes from ribosomal subunits isolated from *E. coli* and *S. lividans* and test their ability to synthesise polyphenylalanine in the presence of lincomycin. The results of this experiment (Figure 4.12) confirm the previous finding that high level lincomycin resistance was a property of the 50S ribosomal subunit from *E. coli*, just as had been the case with the lincomycin resistance of ribosomes from *S. caelestis*.

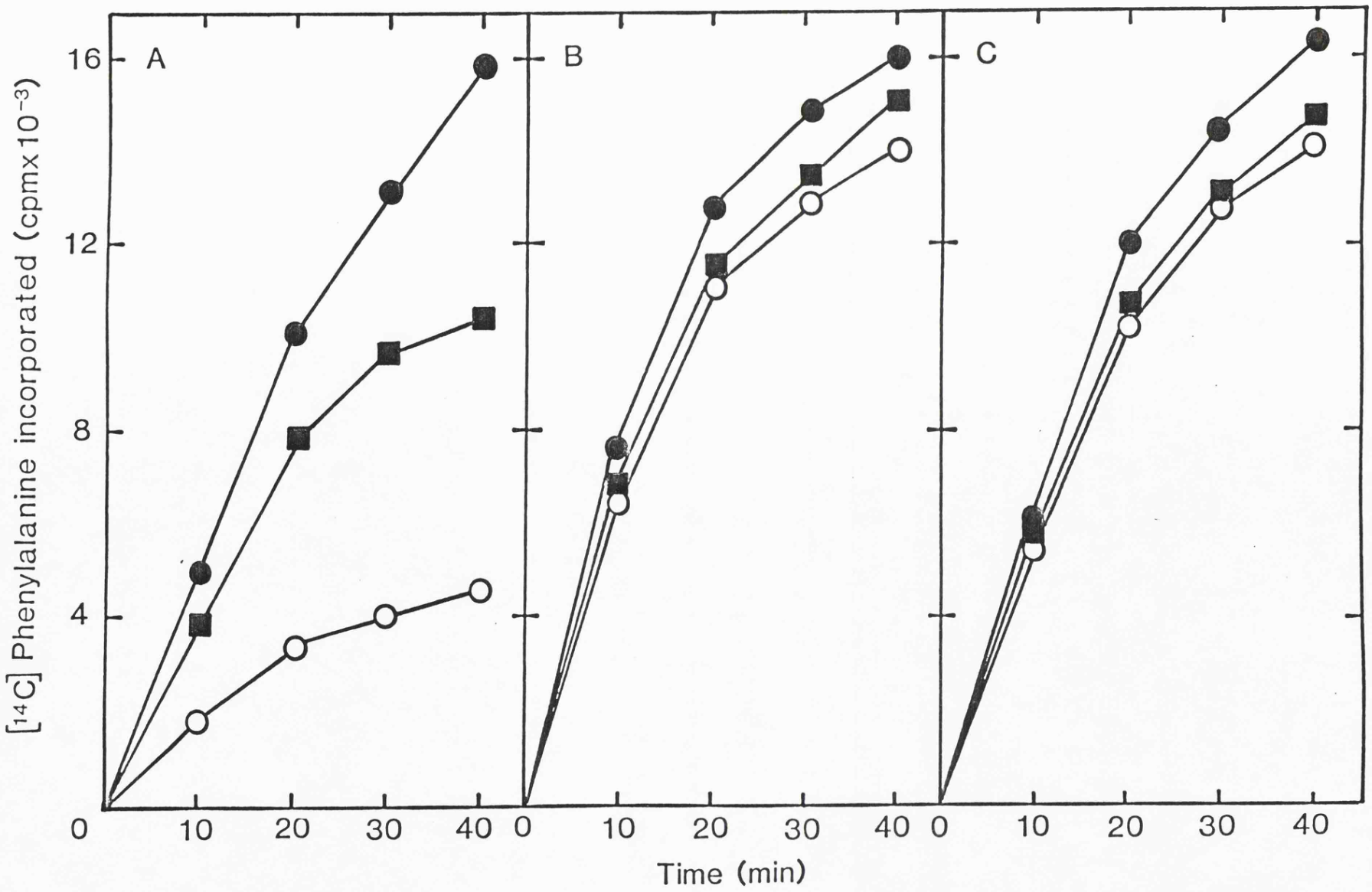
A translation system in which a natural protein rather than polyphenylalanine was synthesised would have been the ideal system for comparing the lincosamide resistances exhibited by ribosomes from *E. coli* and *S. caelestis*. The inactivity of *E. coli* ribosomes in a *Streptomyces* coupled transcription-translation reaction may have been due to incompatability between the 30S subunit of the *E. coli* ribosome and the crude initiation factor preparation from *S. lividans*. This notion is consistent with the finding that *E. coli* ribosomes were active in polyphenylalanine-synthesising systems in which factor-dependent initiation was not obligatory. Therefore 50S subunits were prepared

Legend to Figure 4.11.

Sensitivity of polyphenylalanine synthesis by ribosomes from *E. coli*, *S. caelestis* and *S. lividans* to celesticetin.

All reactions (50 µl) contained S100* from *S. lividans* TK21. Salt-washed ribosomes (4 pmol) from *S. lividans* TK21 (panel A), *E. coli* (panel B) and *S. caelestis* (panel C) were incubated with DMSO (●) or celesticetin at 20 µg ml^{-1} (■) or 200 µg ml^{-1} (○) final concentrations, prior to assay for polyphenylalanine synthesis.

Fig 4.11


A
B
C
16
12
8
4
0
10
20
30
40
[14C] Phenylalanine incorporated (cpmx10-3)
Time (min)

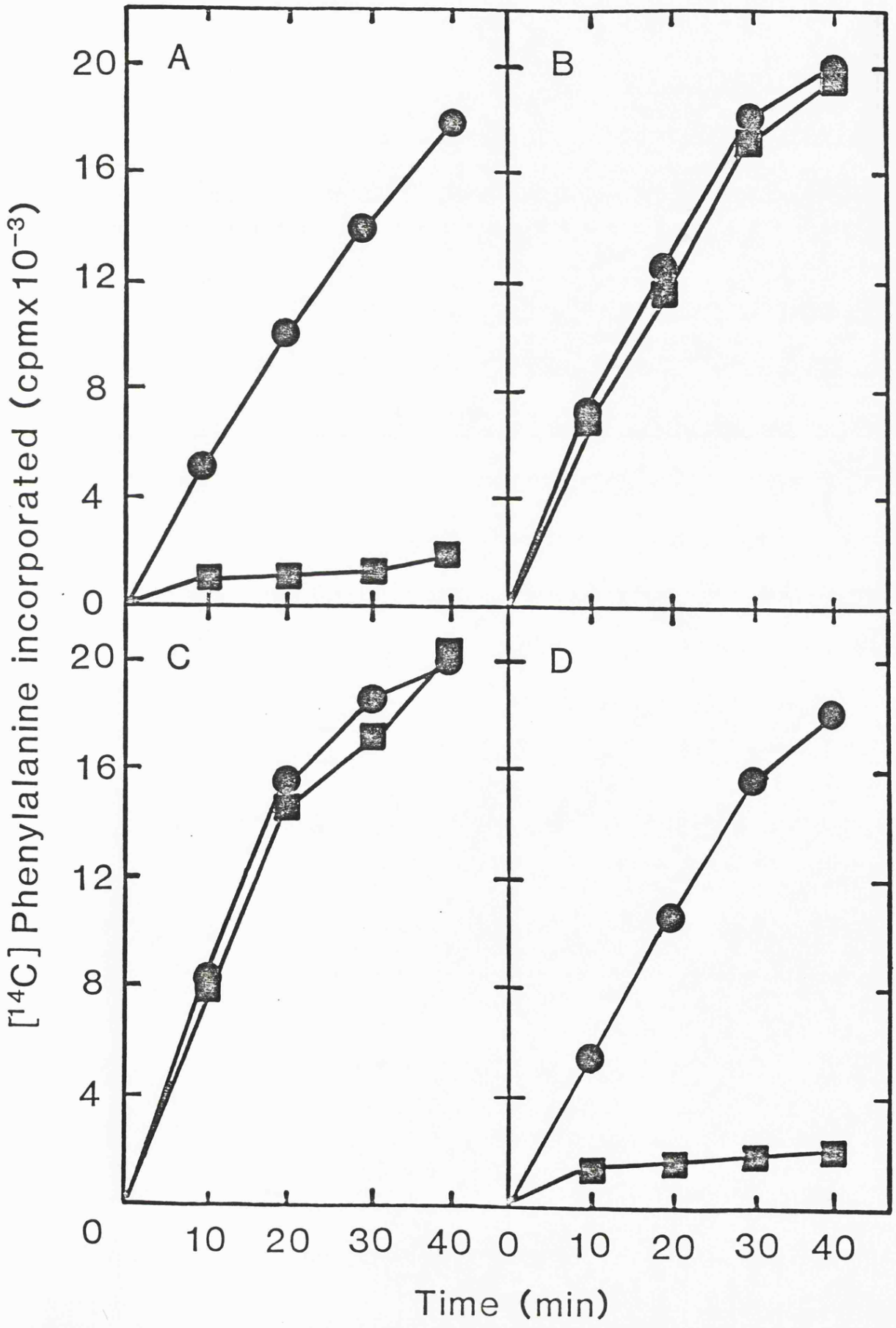
Legend to Figure 4.12.

Localisation of lincomycin resistance to the 50S subunit of the *E. coli* ribosome.

All reactions (50 µl) contained S100* from *S. lividans* TK21. Ribosomal subunits (5 pmol of each) were incubated with H_2O (●) or lincomycin at 400 µg ml^{-1} final concentration (■), prior to assay for polyphenylalanine synthesis.

Source of ribosomal subunits: (A) 30S and 50S subunits from *S. lividans* TK21, (B) 30S and 50S subunits from *E. coli*, (C) 30S subunits from *S. lividans* TK21 and 50S subunits from *E.coli* and (D) 30S subunits from *E.coli* and 50S subunits from *S. lividans* TK21.

Fig 4.12


A
B
C
D
20
16
12
8
4
0
10
20
30
40
[14C] Phenylalanine incorporated (cpmx10-3)
Time (min)

from *E. coli* ribosomes and tested for their ability to form ribosomal couples with 30S ribosomal subunits from *S. lividans*, that were active in the coupled transcription-translation reaction. The data presented in Table 4.1 show that hybrid ribosomes containing 50S ribosomal subunits from *E. coli* possessed equal activity to those 70S particles comprised of *S. lividans* ribosomal subunits.

The ability of the hybrid ribosomes containing 50S subunits derived from *E. coli* to function in the DNA-dependent assay system, made feasible the direct comparison of the lincosamide resistant properties of *E. coli* and *S. caelestis* 50S ribosomal subunits in this reaction. Accordingly, 30S ribosomal subunits derived from *S. lividans* were combined with 50S counterparts from *E. coli*, *S. caelestis* or *S. lividans* and tested for their lincomycin resistance in the coupled transcription-translation reaction. The results from this experiment (Figure 4.13) confirm that the 50S ribosomal subunit from *S. caelestis* confers ribosomal resistance to lincomycin. Surprisingly however, the results also show that hybrid ribosomes containing 50S subunits from *E. coli* were significantly more sensitive to lincomycin than the ribosomes containing the corresponding subunit from *S. caelestis*, although at low drug concentrations they were more resistant than homologous *S. lividans* ribosomes. This unexpected finding has yet to be rationalised, since it was anticipated that the drug concentrations chosen for the polyuridylate-directed assay should have discriminated between the different levels of resistance of the *E. coli* and *S. caelestis* ribosomes.

In conclusion then, it appears that ribosomes from *E. coli* and *S. caelestis* were resistant to lincomycin, possibly *via* different mechanisms. However, for an unknown reason, the different resistance levels of the two types of ribosome were only observed in the

Table 4.1.

Activity of *E. coli* ribosomes and ribosomal subunits in a *Streptomyces* coupled transcription-translation reaction.

Source of ribosomal components:		Activity
S. lividans	*E. coli*	(cpm)
70S	-	11,285
-	70S	1,064
50S, 30S	-	9,602
-	50S, 30S	952
30S	50S	9,213
50S	30S	994

Abbreviations: 70S - salt-washed ribosomes

30S, 50S - ribosomal subunits

Legend to Table 4.1.

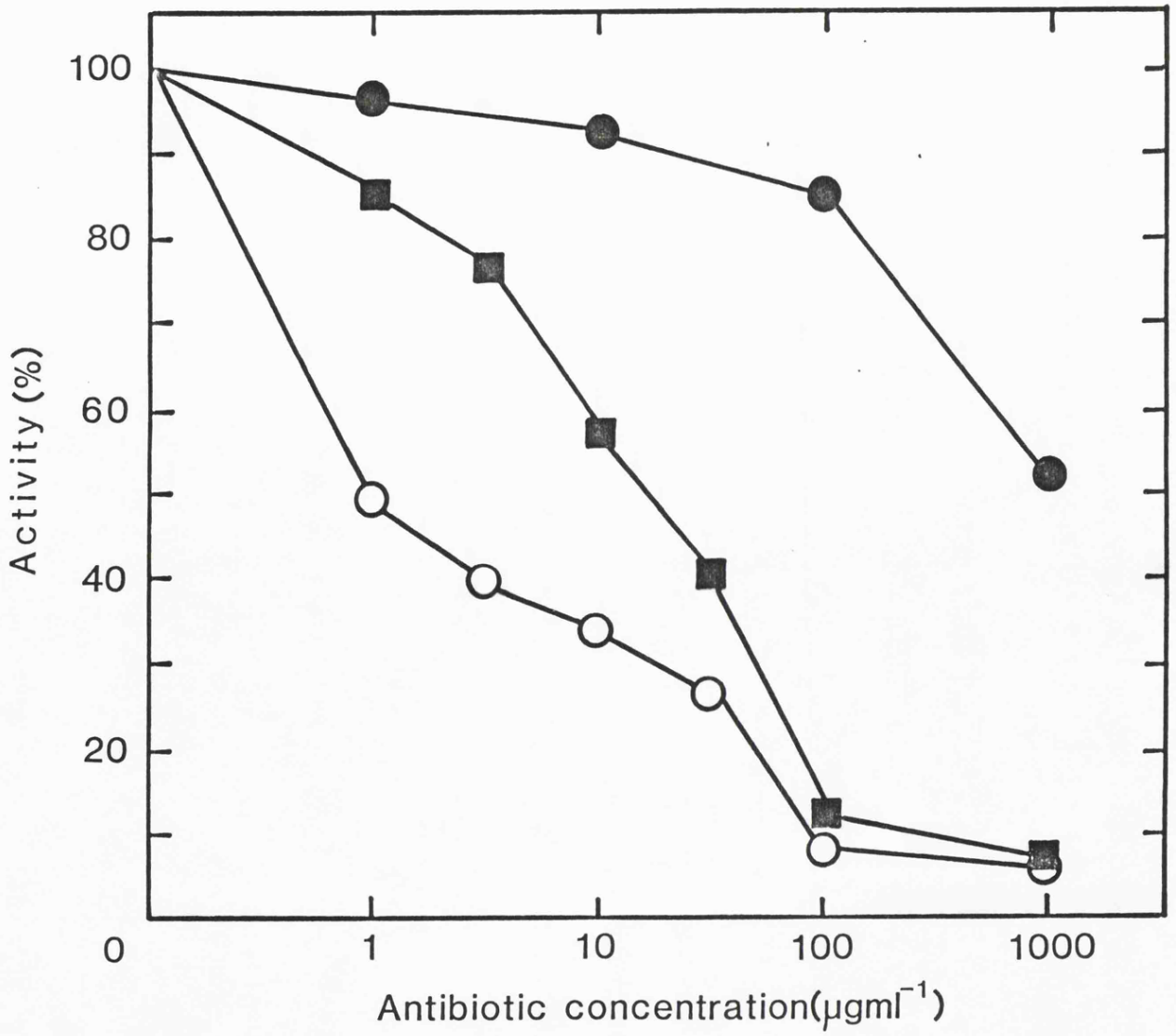
Reactions (30 µl) contained S100 plus crude initiation factor preparation from *S. lividans* TK21 and salt-washed ribosomes (20 pmol) or ribosomal subunits (20 pmol of each) from the sources indicated. Samples (5 µl) were removed at 10 min intervals. 'Activity' is the incorporation of [^{35}S] methionine into TCA-precipitable material (cpm) per 5 µl sample after incubation for 40 min.

Legend to Figure 4.13.

Sensitivity of ribosomes containing 50S Subunits from *E. coli*, *S. caelestis or S. lividans* to lincomycin in coupled transcription-translation reactions.

All reactions (30 µl) contained S100, crude initiation factor preparation and 30S ribosomal subunits (20 pmol) from *S. lividans* TK21. 50S ribosomal subunits (20 pmol) from *E. coli* (■), *S. caelestis* (●) or *S. lividans* TK21 (○), were incubated with H_2O or lincomycin, prior to assay for coupled transcription-translation activity.

Fig 4.13


100
80
60
40
20
0
Activity (%)
1
10
100
1000
Antibiotic concentration(μgml⁻¹)

Streptomyces coupled transcription-translation system. Unfortunately, neither resistance mechanism was further characterised, as efforts were concentrated on the elucidation of pactamycin resistance in *S. pactum*.

3 Discussion.

This chapter has described how a fractionated coupled transcription-translation system from *Streptomyces*, has been exploited to study the mechanism of resistance to celesticetin and pactamycin, in *S. caelestis* and *S. pactum* respectively. In order to characterise pactamycin resistance in the producer, cell-free extracts were prepared from 'young' cultures of *S. pactum*. However, these extracts exhibited a 'background' activity equal to the protein synthesis observed in the presence of exogenous DNA. Furthermore, the S30 was inactive after treatment with micrococcal nuclease. There were several possible explanations which could have accounted for this unexpected response to nuclease treatment, including the presence of a restriction endonuclease in the extract which degraded pBR322, or the presence of a high Ca^{2+} concentration in the cell, such that the addition of EGTA did not inactivate the nuclease. Of these explanations, only the latter was tested and this was subsequently eliminated. Further studies with *S. pactum* S30 extracts were not carried out because the successful fractionation of the coupled transcription-translation system enabled *S. pactum* ribosomes to be studied in a reaction supplemented with *S. lividans* S100.

Ribosomes from *S. pactum* were found to be highly resistant to pactamycin by virtue of a modified 30S ribosomal subunit. This finding was consistent with the previous observation that radiolabelled pactamycin bound to the 30S subunit of bacterial ribosomes and the 40S

ribosomal subunit from eukaryotes. Ideally, reconstitution analysis of the *S. pactum* 30S ribosomal subunit would have enabled the component(s) involved in pactamycin resistance to be localised further. However, attempts to reconstitute 30S ribosomal particles from *S. pactum* subcomponents , for activity in the coupled transcription-translation were unsuccessful, whereas similar particles derived from *S. lividans* fractions were functional in this assay. Consequently it was not possible to implicate 16S rRNA or one or more ribosomal proteins in pactamycin resistance.

Since *S. pactum* ribosomes were not cross-resistant to any antibiotics for which ribosome modification systems have been described previously, a novel target site modification strategy has been discovered. Ribosomal resistance to macrolides, the thiostrepton group of antibiotics and members of the gentamicin and kanamycin families, are all a result of rRNA methylation. Therefore future experiments should include attempts to identify an RNA methylase in *S. pactum* extracts which functions on an *S. lividans* ribosomal substrate. However reconstitution analysis would still be required to demonstrate a causal relationship between methylation and resistance.

The ribosomal resistance to lincosamide antibiotics evident in *Streptomyces caelestis* was established directly by comparing salt-washed ribosomes from this organism and *S. lividans* in a fractionated coupled transcription-translation reaction and was subsequently shown to be a property of the 50S ribosomal subunit. However ribosomes from *S. caelestis* were dissimilar to the lincosamide resistant ribosomes from *S. erythraeus* since they were sensitive to macrolide antibiotics. Consequently dimethylation of an adenosine residue equivalent to A2058

in *E. coli* 23S rRNA (the mechanism of MLS resistance in *S. erythraeus*) is unlikely to occur in the celesticetin producer.

The mechanism of lincosamide resistance in *S. caelestis* was not characterised further. However future work should include a search for a 23S rRNA methylase present in the producer which acts on ribosomal substrates from *S. lividans*. In addition, purified 23S rRNA from *S. caelestis* should be tested for its ability to act as a substrate for the purified erythromycin resistance methylase from *S. erythraeus*, to determine whether it is mono- or di-methylated by this enzyme. If the stoichiometry of methylation approached unity, this could represent circumstantial evidence that N^6-monomethylation of *S. caelestis* 23S rRNA at an adenosine equivalent to A2058 in *E. coli* 23S rRNA is the mechanism of lincosamide resistance in *S. caelestis*. Alternatively, if RNA methylation is the mechanism of resistance, but the *S. caelestis* equivalent of residue A2058 is not involved, it would be interesting to locate the site of modification and study its relationship to the other RNA alterations that give resistance to peptidyl transferase inhibitors and are located in the loop of RNA secondary structure shown in Figure 1.1.

The lincosamide resistance exhibited by *S. caelestis* ribosomes was compared with that of *E. coli* ribosomes, using a combination of translation systems. Although ribosomes from both sources were highly resistant to the action of lincomycin in a translation reaction directed by a synthetic polynucleotide, the responses of the ribosomes to lincomycin could be readily distinguished in the coupled transcription-translation system. *S. caelestis* ribosomes and hybrid 70S particles containing 50S ribosomal subunits from *S. caelestis* and 30S counterparts from *S. lividans* ribosomes were highly resistant to lincosamides in the

DNA-dependent system whereas hybrid ribosomes containing 50S ribosomal subunits from *E. coli* and 30S ribosomal particles from *S. lividans* were only slightly more resistant than homologous *S. lividans* ribosomes. The reason for the discrepancy between the lincomycin resistance of 50S ribosomal subunits from *E. coli* displayed in the two assay systems, has not been established. It would be interesting to test the action of lincomycin on partial reactions of protein synthesis, carried out by *E. coli* and *S. caelestis* ribosomes and in a fractionated *E. coli* coupled transcription-translation reaction or natural mRNA-directed system.

In conclusion, the coupled transcription-translation reaction has been used not only in the study of resistance to an antibiotic which poorly inhibits other protein-synthesising systems, but also to distinguish between two lincosamide resistance mechanisms which had appeared to be identical in an alternative assay.

CHAPTER 5

THE GENERATION AND BIOCHEMICAL CHARACTERISATION OF PACTAMYCIN RESISTANT CLONES OF *STREPTOMYCES LIVIDANS*

1 Introduction.

The ability to "shotgun clone" antibiotic resistance genes from antibiotic producers into sensitive organisms, has greatly facilitated the characterisation of some resistance mechanisms. For example, the neomycin producer *Streptomyces fradiae*, contains two enzymes, neomycin phosphotransferase and neomycin acetyltransferase (Davies *et al.*, 1979), each of which is capable of inactivating this drug *in vitro* (Thompson, C. J. *et al.*, 1982b). When the genes encoding the phosphotransferase (*aph*) and acetyltransferase (*aac*) were introduced separately into *Streptomyces lividans*, the sensitivity of the host organism was decreased 10-20 fold (Thompson C. J. *et al.*, 1982a,b). However neither of the *S. lividans* clones was as resistant as *S. fradiae*, even though in the *aph* containing clone, there was approximately four fold more gene product than in the producer. The situation was rationalised when the *aph* and *aac* genes were present together in *S. lividans*, since the resistance increased to a level 50-fold greater than that observed when either of the neomycin resitance determinants were present alone. This result clearly demonstrated a synergistic relationship between the activities of the two neomycin inactivating enzymes, although the reason for the effect was not apparent.

The cloning of antibiotic resistance determinants has also enabled genes of known function to be isolated. This has allowed the investigation of factors which control transcription and translation of *Streptomyces* genes to proceed (reviewed by Hopwood *et al.*, 1986).

It was hoped that just as the shotgun cloning of antibiotic resistance genes has aided other studies, the cloning of pactamycin resistance determinants from *Streptomyces pactum* would facilitate the characterisation of resistance in the producing organism. Previous

studies had been hampered by an inability to prepare functional 30S ribosomal subunits reconstituted with components derived from *S. pactum*, even though similar particles prepared from *S. lividans* had been active in coupled transcription-translation reactions. It was decided therefore, to clone the gene(s) responsible for the pactamycin resistance phenotype of *S. pactum* in *S. lividans*, so that subsequent reconstitution experiments could be carried out using ribosomal components isolated from pactamycin resistant and sensitive strains of *S. lividans*. This chapter describes the isolation and characterisation of pactamycin resistant clones of *S. lividans*.

2 Results.

2.1 Cloning of pactamycin resistance determinants into *S. lividans*.

The versatile plasmid pIJ702 (Katz *et al.*, 1983) was used as the vector for cloning genomic DNA fragments from *S. pactum* into *S. lividans*. A useful property of this vector is its copy number of between 40 and 300 molecules per chromosome. Besides making the plasmid easy to purify, the high gene dosage of any cloned pactamycin resistance genes may result in the synthesis of large amounts of gene product.

In one shotgun cloning experiment, DNA fragments from the *S. pactum* genome, generated by incubation with KpnI, were ligated with pIJ702 that had been linearised by similar treatment and terminally dephosphorylated using CIAP. Ligated DNA was then used to transform protoplasts of *S. lividans* and, after regeneration, primary transformants were selected using thiostrepton (20 $\mu g\ ml^{-1}$), as described in Methods. After further incubation to allow sporulation, approximately 15,000

primary transformants (on 10 plates) were replica-plated onto two plates containing thiostrepton plus pactamycin and incubated for 60 hr. Throughout the cloning and subsequent manipulation of pactamycin resistance determinants, considerable effort was made to minimise the amount of pactamycin used, as only 100 mg of antibiotic was available. Consequently, the minimum inhibitory concentration of pactamycin which prevents the growth of *S. pactum* was not determined. However, pactamycin was employed at 40 $\mu g\ ml^{-1}$ as a selective concentration because the producer grew and sporulated vigorously on solid medium containing this concentration of antibiotic, whereas no growth was detected on plates inoculated with *S. lividans*.

In the cloning experiment described above, seven pactamycin resistant colonies were obtained. Spores and aerial mycelium from each colony were removed and inoculated into 20 ml liquid medium supplemented with thiostrepton, although one pactamycin resistant transformant (*S. lividans* TB5), chosen at random, was used to inoculate medium supplemented additionally with pactamycin. After incubation at 30°C for 60 hr, plasmid DNA was prepared from the seven cultures by an alkaline lysis procedure (see Methods).

The seven plasmid preparations were analysed by agarose gel electrophoresis, using supercoiled pIJ702 as a size marker. Each preparation contained a DNA species which was indistinguishable in mobility to pIJ702. However the DNA preparation from *S. lividans* TB5, grown in the presence of pactamycin, contained an additional DNA species, slower in mobility than pIJ702.

A number of subsequent studies enabled the identity of the two plasmids present in *S. lividans* TB5 to be established. The larger molecule (pTB5), was shown to consist of pIJ702 containing a 4.9 kb

insert at the KpnI site (Figure 5.1). This plasmid conferred pactamycin resistance, but deleted at high frequency to form pTB702, which was similar, but not identical to, pIJ702, since it contained a small amount of DNA from the 4.9 kb insert in pTB5 (see Figure 5.2). Furthermore pTB5 could only be prepared from cultures of *S. lividans* TB5 grown in the presence of pactamycin. The experimental evidence for these conclusions is presented below.

Initially, the plasmid DNA from *S. lividans* TB5 (grown in the presence of pactamycin) was tested for its ability to transform *S. lividans* TK21 to pactamycin resistance. Approximately 10-20 ng of pIJ702 and pTB5/pTB702 were introduced into protoplasts of *S. lividans*, spread over the surface of plates containing regeneration medium and incubated overnight at 30°C. One regeneration plate from each transformation was then flooded with a thiostrepton suspension whilst pactamycin selection pressure was applied to another. After 3-4 days further incubation, no growth was observed on the pactamycin plate onto which protoplasts transformed with pIJ702 had been spread, whereas protoplasts transformed with plasmid DNA from *S. lividans* TB5 grew as a confluent melanin-producing lawn, in the presence of this antibiotic. Therefore one component of the plasmid DNA from *S. lividans* TB5 contained pactamycin resistance determinant(s).

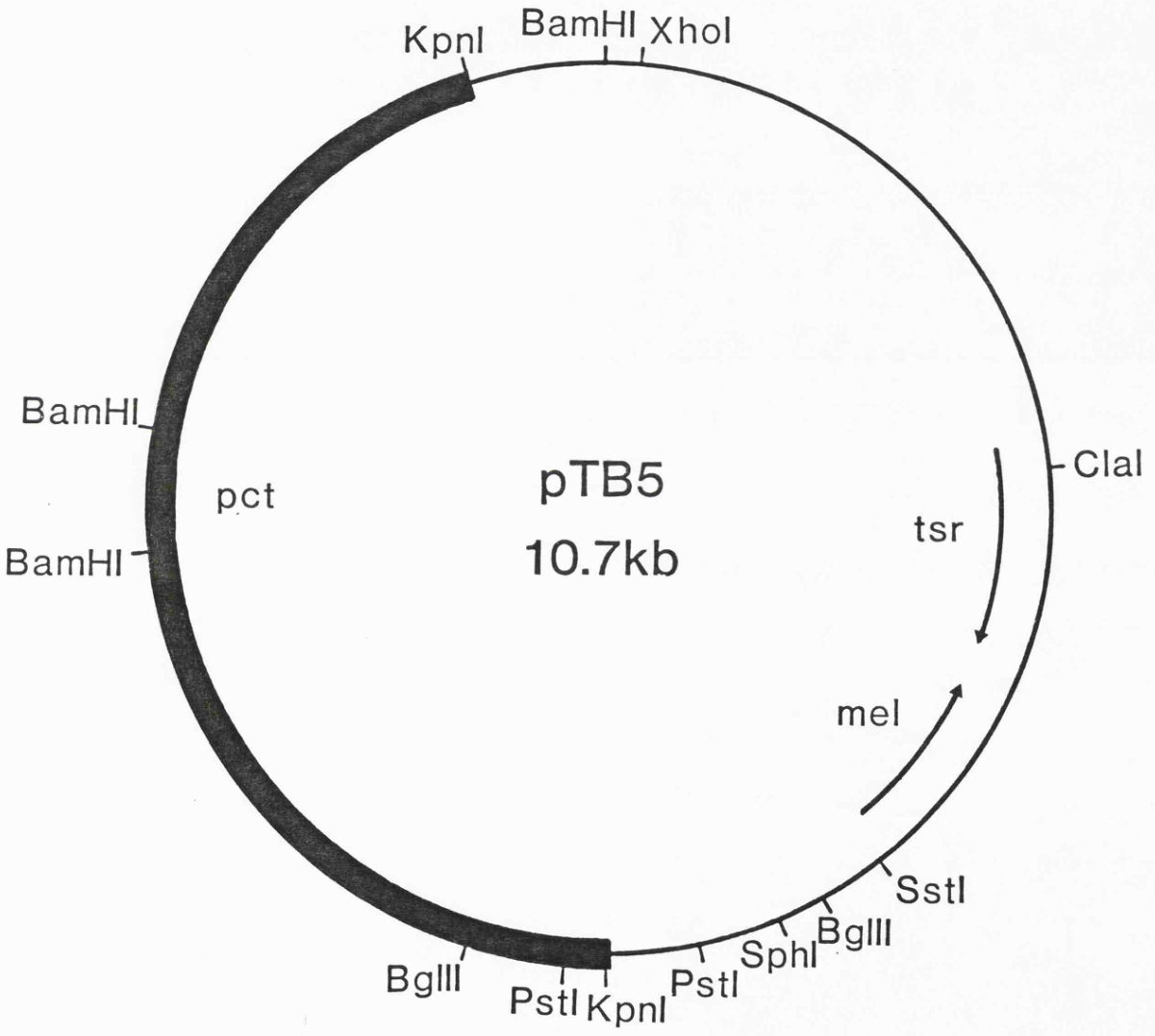
In order to establish the relationship (if any) between pTB5 and pTB702, further plasmid preparations were carried out. Although, in the experiment described above, most of the regeneration plate was covered with confluent growth of pactamycin resistant transformants, a few isolated colonies were present. Spores from one such colony were removed and used to inoculate liquid medium supplemented with thiostrepton, while spores from another colony were inoculated into

Legend to Figure 5.1.

Restriction map of plasmid pTB5.

Plasmid pTB5 is the larger of two plasmids isolated from a pactamycin resistant clone of *S. lividans*. The plasmid contains a 4.9 kb fragment of *S. pactum* DNA (*pct*) inserted into the KpnI site of pIJ702. The locations of the genes for melanin synthesis (*mel*) from *S. antibioticus* and thiostrepton resistance (*tsr*) from *S. azureus* are indicated.

Fig 5.1


KpnI
BamHI
XhoI
ClaI
SstI
BglII
SphI
PstI
KpnI
PstI
BglII
BamHI
BamHI
pct
pTB5
10.7kb
tsr
mel

Legend to Figure 5.2.

Structural relationship between plasmids pTB5, pTB702 and pIJ702.

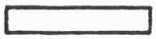
Plasmids pTB5, pTB702 and pIJ702 are shown in linear form with the unique BamHI site of pIJ702 at the termini.

Plasmid pTB5 was generated by insertion of a 4.9 kb fragment of *S. pactum* DNA (*pct*) into the KpnI site of pIJ702. This construct is unstable and a deleted derivative is always isolated from the pactamycin resistant clone of *S. lividans*.

Key.

pIJ101 DNA

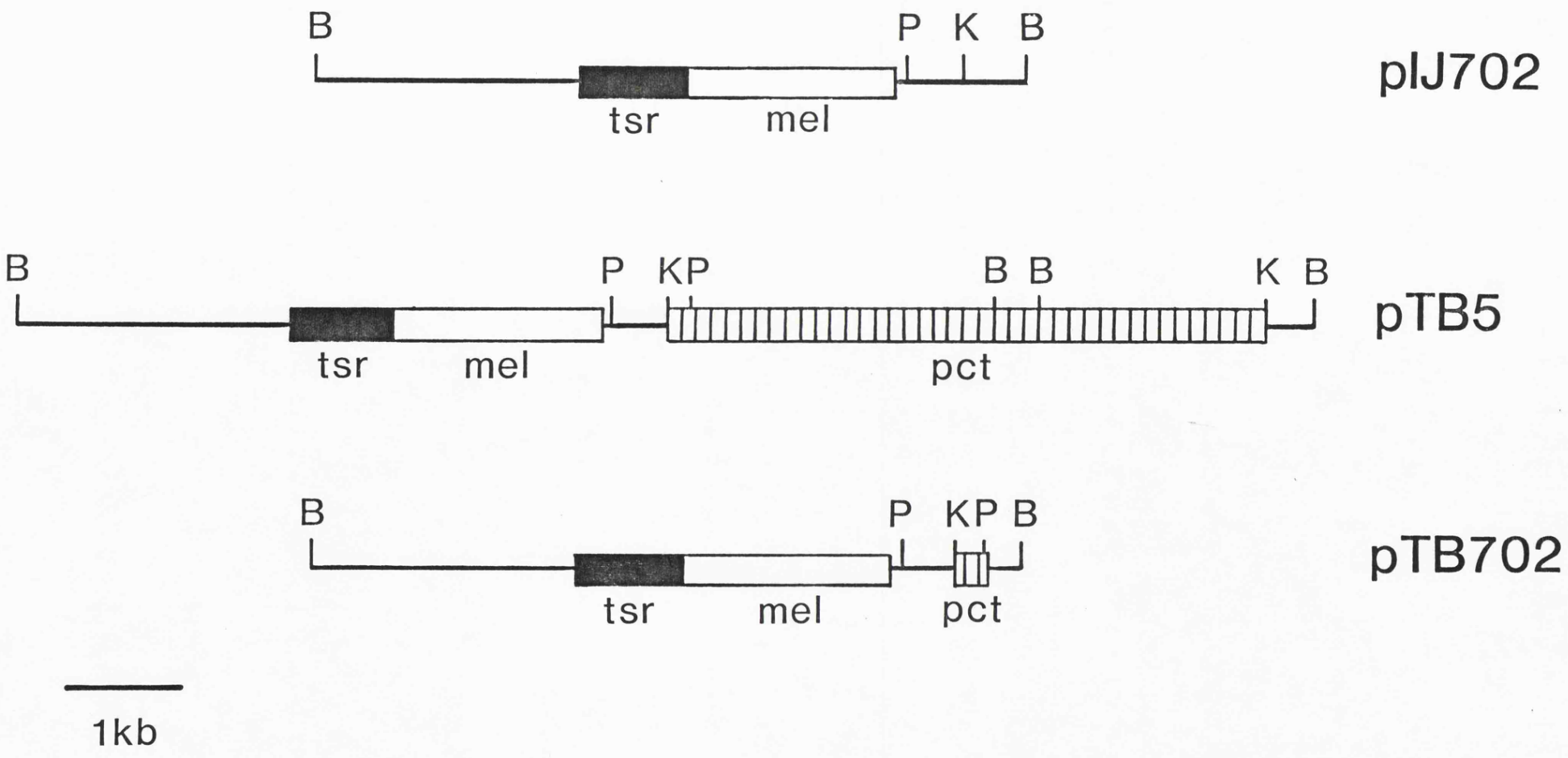
tsr gene from *S. azureus*.



mel gene from *S. antibioticus*.

pct DNA from *S. pactum*.

Fig 5.2


B
P K B
tsr
mel
pIJ702
B
P KP
B B
K B
tsr
mel
pct
pTB5
B
P KP B
tsr
mel
pct
pTB702
1kb

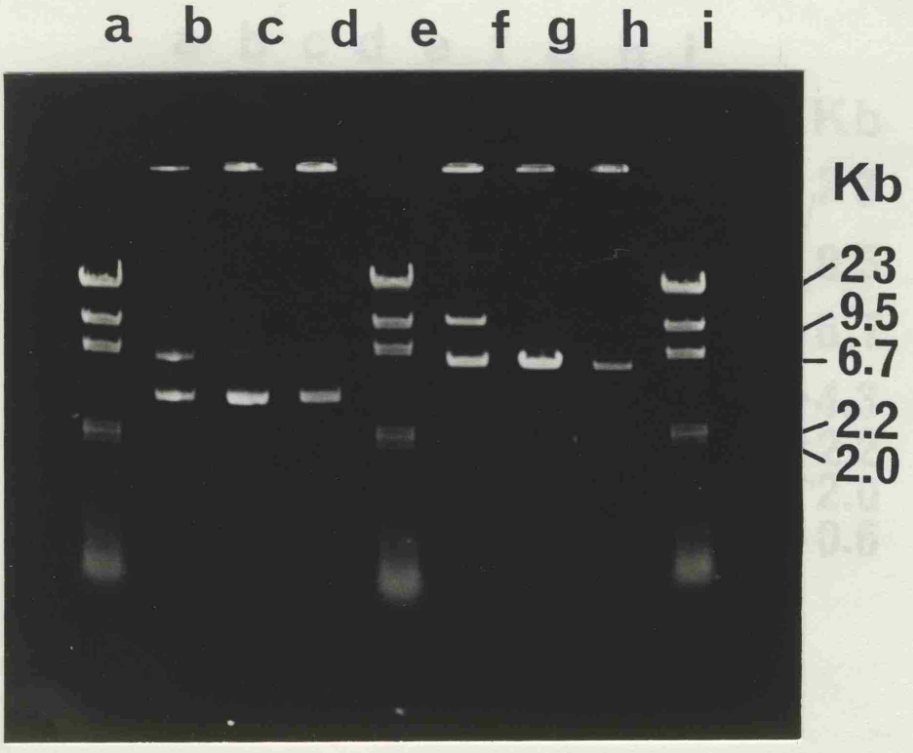
medium containing pactamycin. After 60 hr incubation at 30°C, plasmid DNA was isolated from the two cultures and analysed by agarose gel electrophoresis. The plasmid DNA prepared from the culture grown in the presence of pactamycin contained the two plasmids again, whereas the preparation from mycelium grown in medium supplemented with thiostrepton contained only pTB702. Since it was improbable that the pactamycin resistant transformant analysed here and the original *S. lividans* TB5 colony, had both been cotransformed by two plasmids, it seemed likely that pTB702 was a deleted product of pTB5 Furthermore when *S. lividans* TB5 was grown in medium supplemented with thiostrepton, only pTB702 could be isolated from it (Figure 5.3).

The notion that plasmid pTB702 was derived from pTB5 was supported by results from restriction analysis of the two molecules. The DNA fragments generated when pTB702, pTB5 and pIJ702 were cleaved by BglII, ClaI, KpnI or PstI and analysed by agarose gel electrophoresis, are shown in Figures 5.3 and 5.4. These results show that pTB5 contains a 4.9 kb KpnI insert and that both pTB5 and pTB702 possess a 0.6 kb PstI fragment, which is not present in pIJ702. Furthermore, these and other data from restriction analysis, indicate that the deletion event which led to the formation of pTB702, had one end-point located within the insert and the other at a site in pIJ702 DNA.

In conclusion, the presence of pactamycin appeared to enrich cultures of *S. lividans* TB5 for mycelia which contained plasmid pTB5, since in the absence of this selection, a deleted form of the plasmid predominated. It was assumed that the pIJ702-like molecules, isolated from the six other pactamycin resistant clones were also pTB702 molecules. Accordingly, all subsequent work focussed on the 4.9 kb KpnI fragment in pTB5.

Figure 5.3.

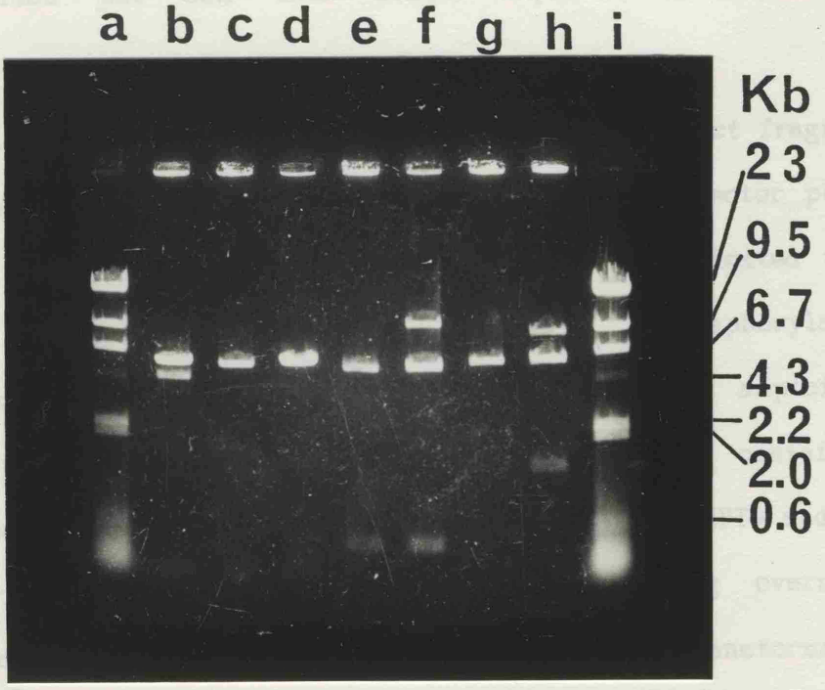
Restriction analysis of plasmids pTB5 and pTB702. I.



The following DNA samples were electrophoresed in a 0.7% (w/v) agarose gel: HindIII-cleaved DNA from bacteriophage lambda (a,e,i); uncut pTB5 (b), pIJ702 (c), pTB702 (d); ClaI-cleaved pTB5 (f), pIJ702 (g), pTB702 (h).

Figure 5.4.

Restriction analysis of plasmids pTB5 and pTB702. II.



The following DNA samples were electrophoresed in a 0.7% (w/v) agarose gel: HindIII-cleaved DNA from bacteriophage lambda (a,h);

KpnI-cleaved pTB5 (b), pTB702 (c);

PstI-cleaved pIJ702 (d), pTB702 (e), pTB5 (f);

BglII-cleaved pTB702 (g), pTB5 (h).

2.2 Subcloning of the pactamycin resistance determinants in *E. coli*.

The primary reason for cloning the pactamycin resistance gene(s) into *S. lividans* was to enable the preparation and characterisation of pactamycin resistant ribosomes from this organism. Unfortunately however, the resistant clone *S. lividans* pTB5, contained an unstable plasmid, so that even the preparation of the fragment presumed to confer pactamycin resistance (designated *pct*) necessitated the presence of pactamycin in the growth medium. Therefore efforts were made to subclone *pct* DNA into other, hopefully more stable, constructions.

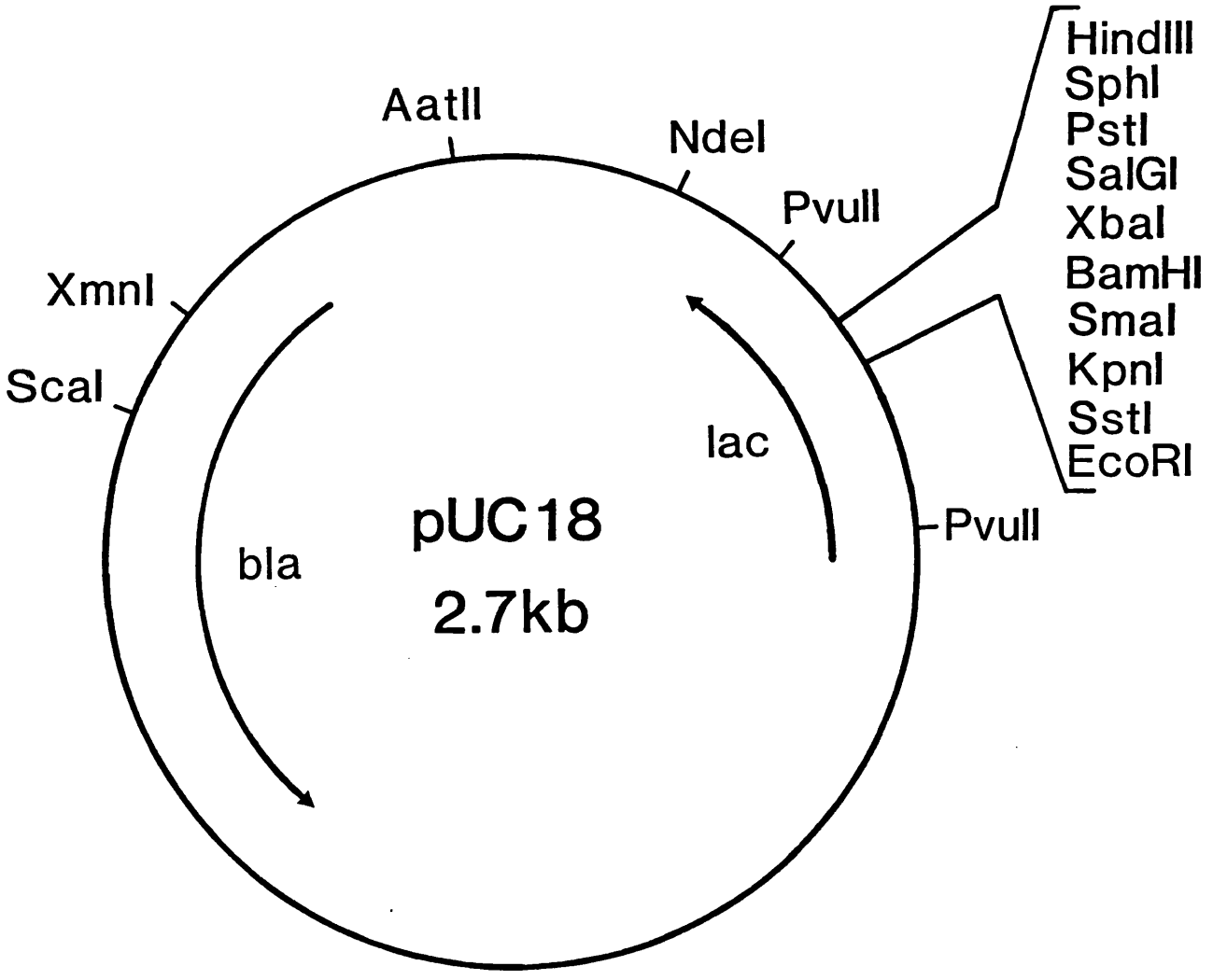
To facilitate subsequent manipulations, the 4.9 kb *pct* fragment was inserted into the KpnI site of the *E. coli* expression vector pUC18 (Figure 5.5). Briefly, 2 μg KpnI-digested pTB5 DNA was ligated with 0.5 μg pUC18 similarly treated and terminally dephosphorylated. Competent cells of *E. coli* NM522 were then transformed to ampicillin resistance by the ligation mixture and spread onto plates containing ampicillin as part of a soft agar overlay supplemented with IPTG and the chromogenic β-galactosidase substrate, X-gal. Following overnight incubation, blue and white colonies were observed on the transformation plates. Since colonies containing plasmids with DNA inserted at the KpnI site should be white, plasmid DNA was prepared from 20 white colonies, as described in Methods. The plasmids were then subjected to agarose gel electrophoresis and those molecules which were larger than pUC18, were restricted with KpnI. Subsequent analysis showed that some plasmids contained the 4.9 kb *pct* fragment, so these were then treated with PstI to determine the orientation of the inserted DNA in pUC18. Figure 5.6 shows the two possible constructs, designated pTB500 and pTB501. PstI cleavage of the former plasmid should generate 3.1 and

Legend to Figure 5.5.

Restriction map of plasmid pUC18.

The expression vector pUC18 contains the ampicillin resistance determinant (*bla*) from transposon TnA for selection of transformants and the DNA for the inducible expression of the α-peptide of β-galactosidase (*lac*). Insertion of DNA into the polylinker of convenient restriction sites within *lac* can be detected by a blue-white colour test when an appropriate host strain is grown in the presence of the chromogenic substrate X-gal and an inducer, e.g. IPTG.

Fig 5.5


HindIII
SphI
PstI
SalGI
XbaI
BamHI
SmaI
KpnI
SstI
EcoRI
AatII
NdeI
PvuII
XmnI
ScaI
lac
pUC18
2.7kb
bla
PvuII

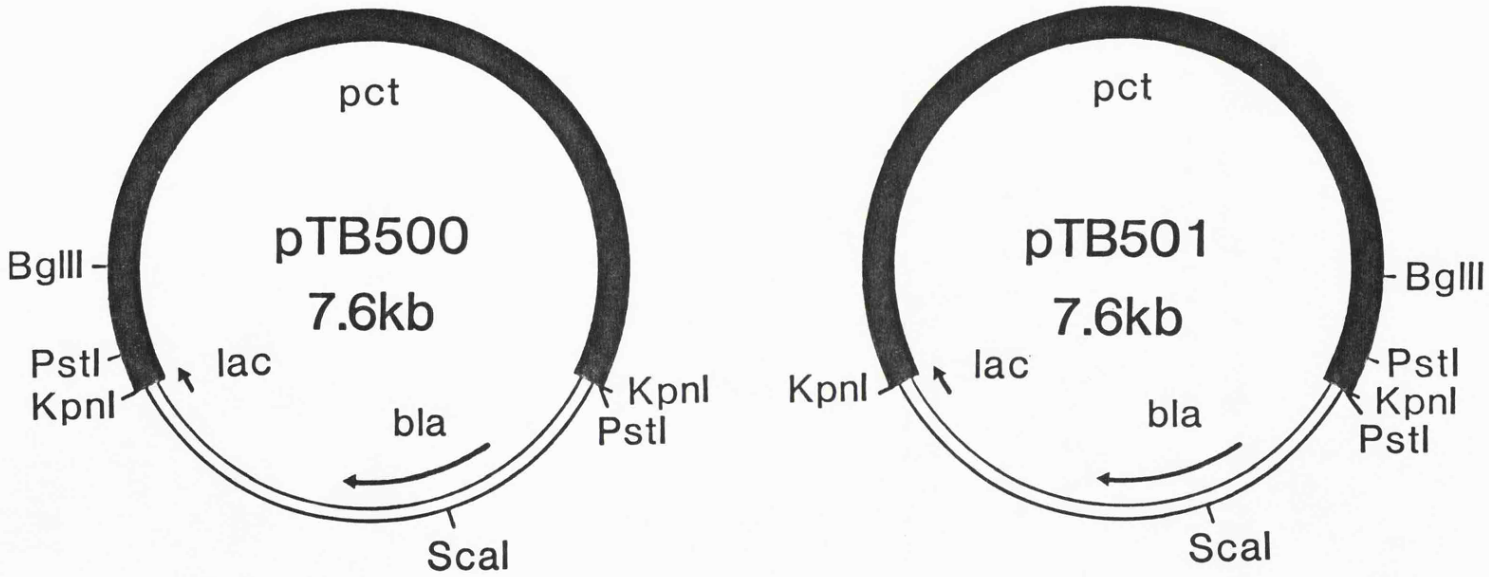
Legend to Figure 5.6.

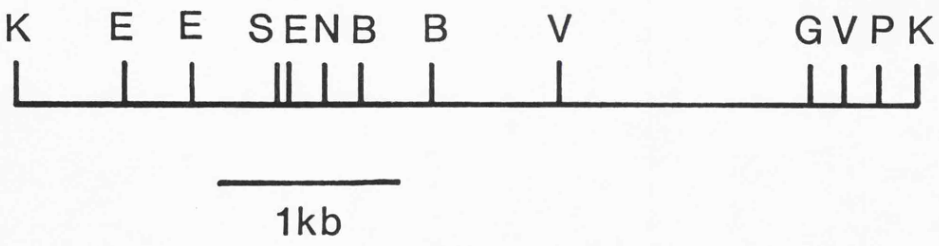
Restriction map of *pct* DNA, and plasmids pTB500 and pTB501.

Plasmids pTB500 and pTB501 were constructed by insertion of *pct* DNA from pTB5 into the unique KpnI site of pUC18. The two plasmids differ only with respect to the orientation of *pct* DNA in the vector.

The restriction map of *pct* DNA shows the locations of cleavage sites for the following restriction endonucleases: BamHI (B), BglII (G), EcoRI (E), KpnI (K), NruI (N), PstI (P), PvuII (V) and SalGI (S).

Fig 5.6


pct
pTB500
7.6kb
BglII
PstI
KpnI
lac
bla
KpnI
PstI
ScaI
pct
pTB501
7.6kb
BglII
KpnI
lac
bla
PstI
KpnI
PstI
ScaI


K E E S E N B B V G V P K
1kb

4.7 kb fragments, whereas PstI restriction of the latter should result in 7.6 kb and 0.2 kb DNA molecules. When the restriction digests were analysed by agarose gel electrophoresis, examples of both pTB500 and pTB501 were present (data not shown).

To determine whether *pct* DNA could confer pactamycin resistance on *E. coli*, either by expression from its *Streptomyces* promoter (if present) or from the *lac* promoter in pUC18, *E. coli* strains containing pTB500 and pTB501 were grown on solid medium supplemented with IPTG and ampicillin for one generation and then streaked onto similar plates that also contained pactamycin (40 $\mu g\ ml^{-1}$). After overnight incubation, no growth was detected on the pactamycin plates. This suggested that either the resistance gene was not expressed, or that the *pct* DNA had been deleted. Restriction analysis of plasmid DNA from both *E. coli* strains after two rounds of propagation on medium supplemented with ampicillin and IPTG, revealed that the 4.9 kb KpnI fragment was still present (data not shown). Therefore, the pactamycin sensitivity of *E. coli* strains containing pTB500 or pTB501 was probably due to lack of transcription and translation of the *Streptomyces* DNA, or due to a failure of the *pct* gene product to function in this host.

Although the *pct* DNA was not expressed in *E. coli*, it was stable in pTB500. Consequently large amounts of the 4.9 kb fragment could be prepared without any further consumption of pactamycin. However, before ligating the *pct* DNA into alternative *Streptomyces* vectors, a simple restriction map of the 4.9 kb fragment was constructed from the results of restriction analysis of pTB5, pTB500 and pTB501 (Figure 5.6). Unique sites for the enzymes BglII, NruI, PstI and SalG1 were found, together with two sites for BamHI and PvuII and three sites for EcoRI. Then, having established a map of the *pct* DNA, subcloning strategies were

devised to clone the pactamycin resistance determinant(s) back into *Streptomyces*.

2.3 Subcloning of the *pct* DNA into *Streptomyces*.

Several strategies were employed to reintroduce *pct* DNA into *S. lividans*, in an attempt to isolate a more stable pactamycin resistant strain, suitable for the preparation of ribosomal components and subsequent characterisation of the resistance mechanism.

In the first subcloning experiments, pTB500 DNA was ligated with a replicon derived from the low copy number vector, pIJ61 (Figure 1.2). Prior to this work, pIJ61 and pUC18 had been ligated together at their unique PstI sites, to create pTB611 and pTB612 (Figure 5.7). These plasmids enabled large quantities of pIJ61 DNA to be prepared in *E. coli* and were capable of replication in both *S. lividans* and *E. coli*. The bifunctional properties of pTB611 and pTB612 were demonstrated by transforming *S. lividans* protoplasts with 10-20 ng of each plasmid and selecting for thiostrepton resistant transformants. Six colonies from each transformation were then inoculated into medium supplemented with thiostrepton and incubated for 60 hr, after which plasmid DNA was prepared, analysed and used to transform *E. coli* competent cells to ampicillin resistance. Plasmid DNA was then isolated from several colonies from each transformation and restricted with either HindIII or SalGI which cleave both pTB611 and pTB612, three and six times respectively. When the digests were analysed by agarose gel electrophoresis, the patterns of DNA bands observed in the gel were indistinguishable from those derived from the original pTB611 and pTB612 preparations (data not shown). This result showed that plasmids

Legend to Figure 5.7.

Restriction maps of plasmids pTB611 and pTB612.

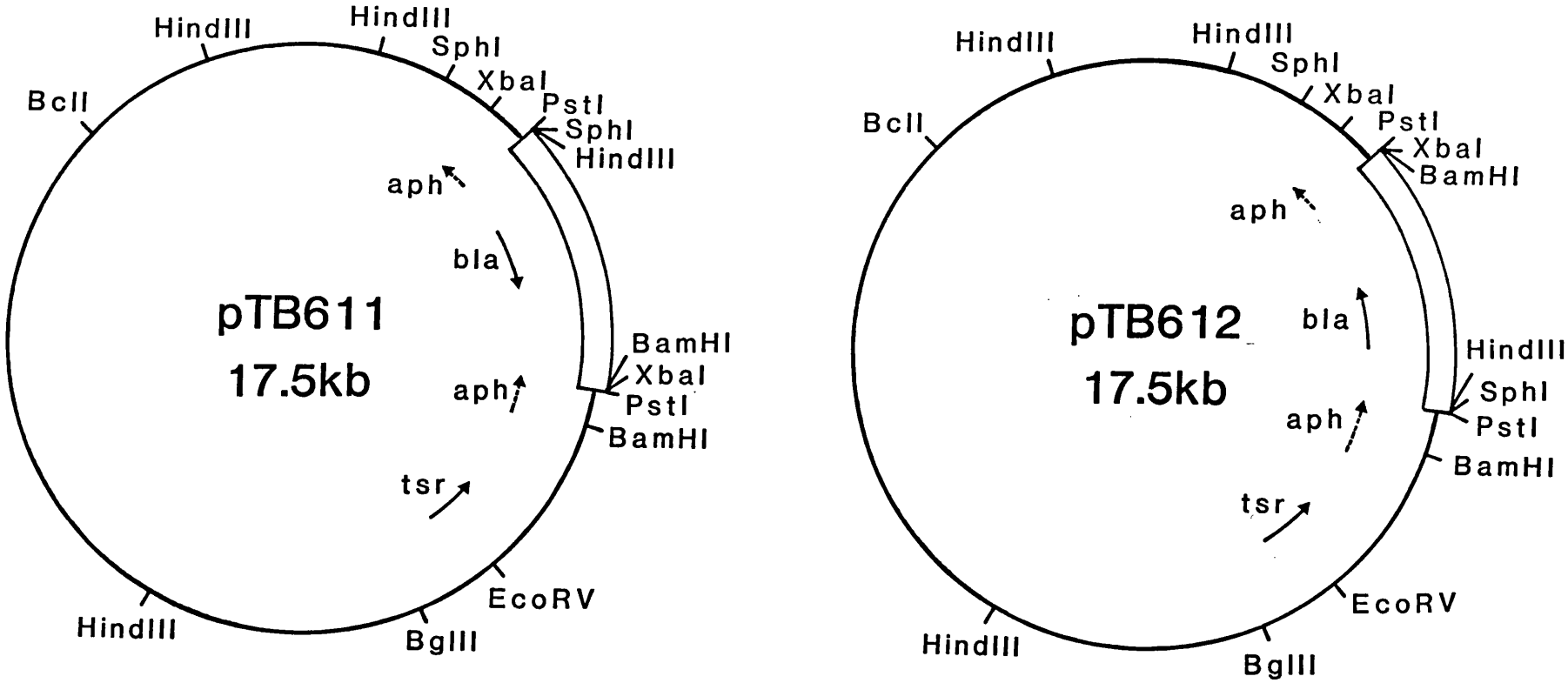
Plasmids pTB611 and pTB612 contain pIJ61 and pUC18 DNA ligated at their unique PstI sites and differ only in their relative orientations. Both plasmids contain the ampicillin resistance determinant (*bla*) from transposon TnA and the thiostrepton resistance gene (*tsr*) from *S. azureus*. The neomycin phosphotransferase gene (*aph*) from *S. fradiae* and the DNA encoding the α-peptide of β-galactosidase (*lac*) were inactivated by the construction of the shuttle vectors.

The large XbaI-SphI fragment of pTB611 was used to construct pTB613 (see Figure 5.8).

Key. ——— pIJ61 DNA.

═══ pUC18 DNA.

Fig 5.7


HindIII
HindIII
SphI
XbaI
PstI
SphI
HindIII
BclI
aph
bla
pTB611
17.5kb
BamHI
XbaI
PstI
BamHI
aph
tsr
EcoRV
HindIII
BglII
HindIII
HindIII
SphI
XbaI
PstI
XbaI
BamHI
BclI
aph
pTB612
17.5kb
bla
HindIII
SphI
PstI
BamHI
aph
tsr
EcoRV
HindIII
BglII

pTB611 and pTB612 were indeed capable of replication in both *S. lividans* and *E. coli* without suffering gross DNA rearrangements.

Plasmids pTB611 and pTB612 were not used as cloning vehicles because of the relative paucity of unique cloning sites in these vectors. Rather, pTB611 was used as a source of the pIJ61 replicon. Thus the large XbaI-SphI fragment of pTB611 was ligated to pTB500 which had been similarly restricted and terminally dephosphorylated. The ligation mixture was then used to transform *E. coli* NM522 to ampicillin resistance and restriction analysis of plasmids isolated from a number of purified transformants showed that pTB613 had been constructed (Figure 5.8). When this plasmid was introduced into *S. lividans* protoplasts, the resultant thiostrepton resistant transformants were found to be pactamycin resistant. Thus, assuming that the low copy number of pIJ61 (4-5 plasmid molecules per chromosome) was maintained in *S. lividans* containing pTB613, *pct* DNA could confer pactamycin resistance when present at a low gene dosage in the cell.

The aim of the next series of experiments was to produce a general method for introducing *pct* DNA fragments present in pUC18 into vector pIJ487 (Figure 1.3), such that after ligation and introduction into *S. lividans* protoplasts, the vast majority of transformants contained the desired construction. The subcloning strategy, was as follows. Theoretically, 50% of *E. coli* transformants containing a pIJ487-pTB500 fusion, obtained by ligating pIJ487 cleaved with SstI and similarly treated pTB500, should have the promoterless aminoglycoside phosphotransferase gene (*aph*) from pIJ487, under the control of the *lac* promoter in pTB500. Thus selection for kanamycin resistant transformants should lead to the isolation of pIJ487-pTB500 chimaeras with the structure of plasmid pTB614 (Figure 5.9). Digestion of such

Legend to Figure 5.8.

Restriction map of plasmid pTB613.

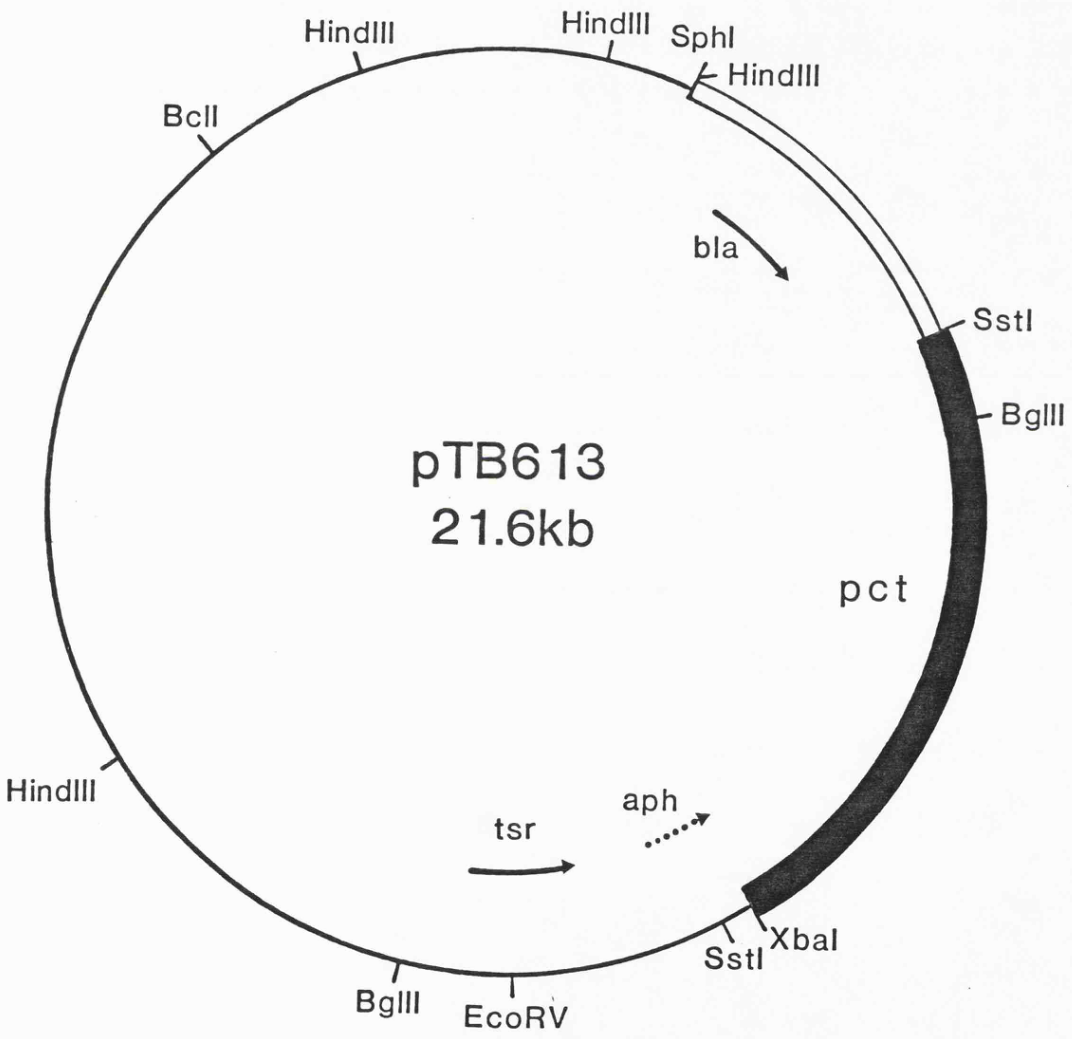
Plasmid pTB613 was constructed by inserting the large XbaI-SphI fragment of pTB611 into pTB500 restricted with the same enzymes. This plasmid can replicate in *E. coli* and *S. lividans* since it contains the replication functions from pUC18 and pIJ61. The locations of the thiostrepton resistance gene (*tsr*) from *S. azureus*, ampicillin resistance gene (*bla*) from transposon TnA, *pct* DNA from *S. pactum* and the truncated neomycin resistance gene (*aph*) from *S. fradiae* are indicated.

Key. ——— pIJ61 DNA.

═══ pUC18 DNA.

▬▬▬ *pct* DNA.

Fig 5.8


HindIII
HindIII
SphI
HindIII
BclI
bla
SstI
BglII
pTB613
21.6kb
pct
HindIII
aph
tsr
XbaI
SstI
BglII
EcoRV

Legend to Figure 5.9.

Restriction map of plasmids pTB614 and pTB615.

Plasmids pTB614 and pTB615 are the two possible constructs that can be obtained when pIJ487 and pTB500 are ligated at their unique SstI sites. Both plasmids contain the thiostrepton resistance gene (*tsr*) from *S. azureus*, the promoterless neomycin resistance gene (*aph*) from transposon Tn5, the ampicillin resistance determinant (*bla*) from transposon TnA, *lac* DNA from pUC18 and the pactamycin resistance determinant(s) from *S. pactum* (*pct*).

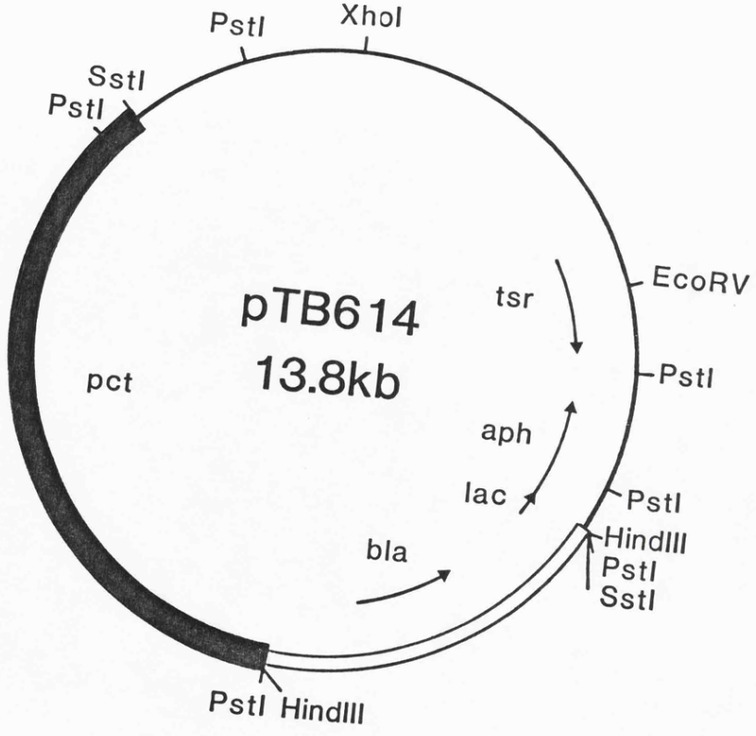
In practice, it was not possible to construct plasmid pTB614 in *E. coli*.

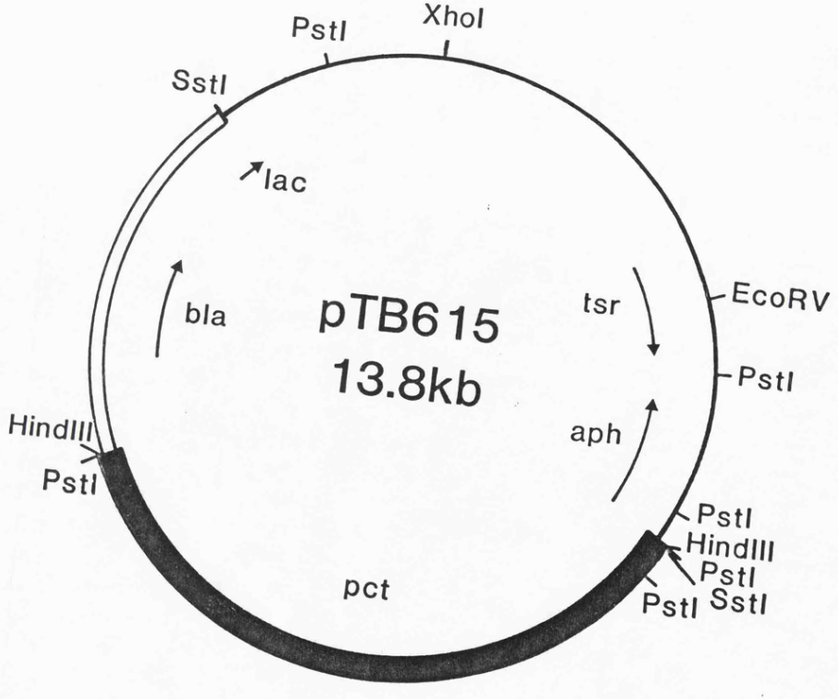
Key. pIJ487 DNA.

 pUC18 DNA.

 *pct* DNA.

Fig 5.9


PstI
XhoI
SstI
PstI
pTB614
13.8kb
tsr
EcoRV
pct
PstI
aph
lac
PstI
HindIII
bla
PstI
SstI
PstI
HindIII


PstI
XhoI
SstI
lac
bla
pTB615
13.8kb
tsr
EcoRV
PstI
aph
HindIII
PstI
PstI
HindIII
PstI
PstI
SstI
pct

plasmids with HindIII followed by ligation at low DNA concentrations should result in the formation of a *Streptomyces* replicon containing whatever DNA fragment had been cloned into the polylinker region of pUC18, between the HindIII and SstI sites. Therefore, after transformation of *S. lividans* protoplasts with the ligated DNA, the majority of thiostrepton resistant colonies should contain the desired construct. The application of this strategy however, depends upon an absence of constraint upon the orientation of pIJ487 DNA after ligation with pTB500 and a lack of HindIII and SstI sites in the cloned DNA.

When 3 μg SstI-treated pIJ487 was ligated with 1 μg of similarly digested pTB500 and introduced into *E. coli* competent cells, a number of transformants containing pIJ487-pTB500 fusions were isolated. However, all of those fusions that were analysed (12) had the configuration of plasmid pTB615 (Figure 5.9), *i.e.* the wrong orientation for the final step of the subcloning protocol. The reason for the limitation on pIJ487 DNA orientation in pTB500 was not established. The plasmid that was obtained, designated pTB615, was capable of transforming *S. lividans* protoplasts to thiostrepton resistance and furthermore, these transformants were resistant to pactamycin. Although subcloning strategy outlined above could have been modified by using *pct* DNA cloned in pUC19, this approach was not adopted because a convenient shuttle vector, pOJ160 (Figure 1.4) became available.

In a final series of experiments, various fragments of the 4.9 kb *pct* DNA were ligated into the bifunctional plasmid pOJ160. This vector was particularly useful because it contained the polylinker and β-galactosidase α-peptide DNA from pUC19, which enabled specific constructs to be rapidly screened and purified in *E. coli*. Protoplasts of *S. lividans* were then transformed with 10-30 ng of each plasmid and

spores from the thiostrepton resistant transformants were tested for their ability to grow in the presence of a disc containing 50 μg pactamycin. Although the majority of pOJ160-based constructs were prepared using *pct* DNA from pTB500, two plasmids (pTB625 and pTB626) contained *pct* DNA from pTB502, a derivative of pTB500 in which the 1.45 kb SalGI fragment had been deleted (see Figure 5.10).

The various *pct* fragments ligated into pOJ160 and their effect on the pactamycin sensitivity of *S. lividans* strains containing them are shown in Figure 5.11. These results indicate that the smallest fragment of *pct* DNA isolated so far, which confers pactamycin resistance, was only 0.6 kb smaller than the original 4.9 kb insert in pTB5. Nevertheless, the subcloning experiments have demonstrated the involvement of a 0.35 kb sequence in pactamycin resistance, since the absence of either flanking portion results in sensitivity to the drug. However, it should be noted that plasmid DNA has not yet been re-isolated from mycelia of pactamycin sensitive subclones, to check that the appropriate construction was still present after transformation. Previously though, even the highly unstable clone, *S. lividans* TB5, remained pactamycin resistant after one round of propagation on thiostrepton-containing medium. Thus the pactamycin sensitivity of certain subclones was probably due to absence of essential *pct* DNA prior to transformation, rather than as a result of subsequent DNA rearrangement(s).

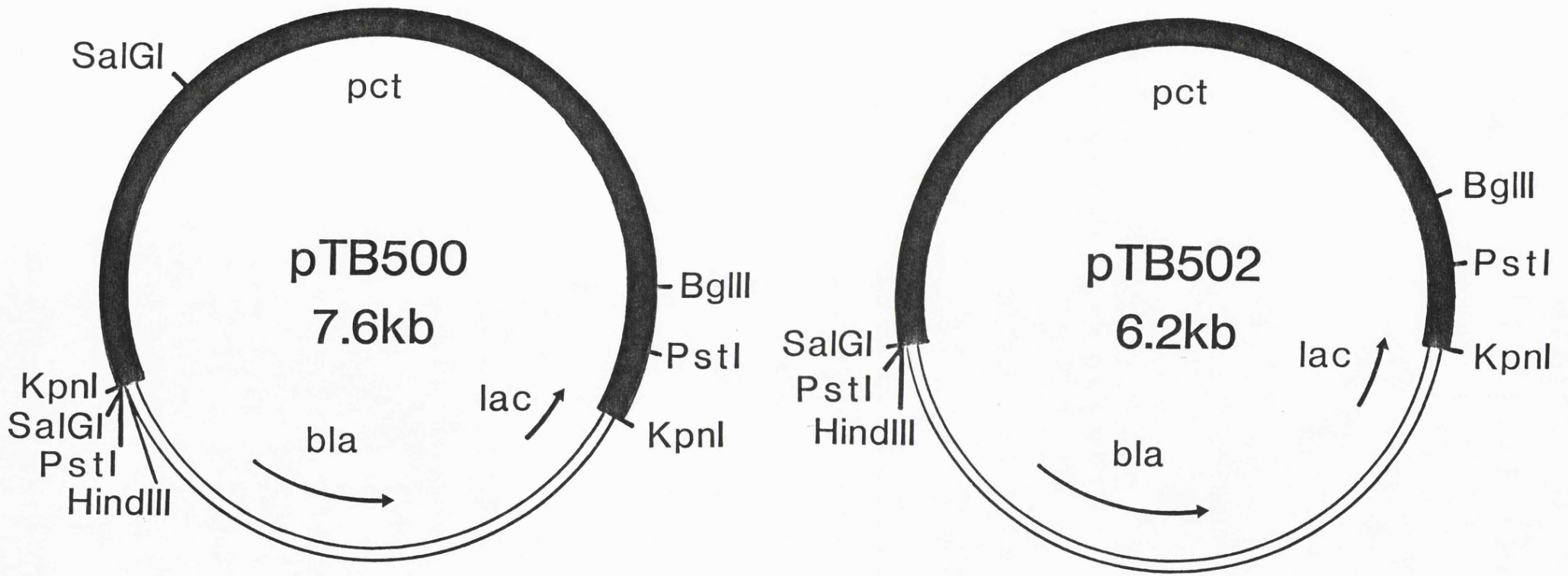
In conclusion, a variety of pactamycin resistant subclones of *S. lividans* have been produced, in an attempt to generate a stable clone suitable for the characterisation of the pactamycin resistance mechanism.

Legend to Figure 5.10.

Restriction map of plasmids pTB500 and pTB502.

Plasmid pTB500 and pTB502 contain *pct* DNA inserted in the polylinker region of pUC18. Plasmid pTB502 was generated by deletion of the small SalGI fragment from pTB500 *in vitro*. These two plasmids were used as a source of *pct* DNA for ligation into pOJ160 (see Figure 5.11).

Fig 5.10


SalGI
pct
pTB500
7.6kb
BglII
PstI
KpnI
lac
bla
KpnI
SalGI
PstI
HindIII
pct
BglII
pTB502
6.2kb
PstI
KpnI
lac
SalGI
PstI
HindIII
bla

Legend to Figure 5.11.

Localisation of pactamycin resistance determinant(s) within the *pct* DNA fragment.

Various portions of the *pct* DNA from pTB500 or pTB502 were ligated into the shuttle vector pOJ160 and tested for their ability to confer pactamycin resistance on *S. lividans*.

Plasmids pTB620 and pTB621 were generated by ligating the larger PstI fragment from pTB500 with pOJ160 treated with the same enzyme.

Plasmid pTB622 was generated by inserting the large HindIII-BglII fragment from pTB500 into pOJ160 treated with HindIII and BamHI.

Plasmids pTB623 and pTB624 were generated by ligating the 1.9 kb BamHI fragment from pTB500 with pOJ160 treated with BamHI.

Plasmids pTB625 and pTB626 were generated by inserting the large PstI fragment from pTB502 into PstI-treated pOJ160.

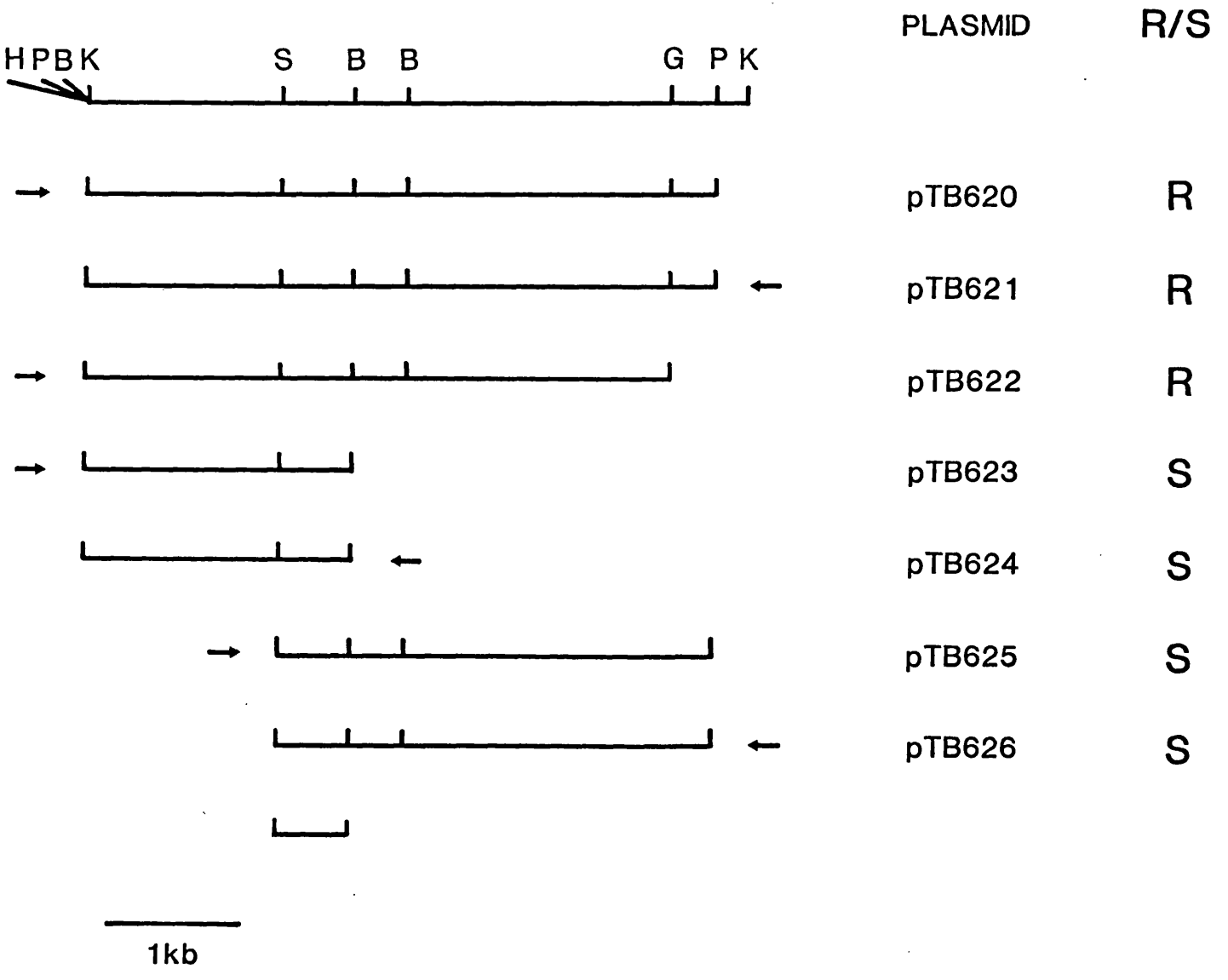
Abbreviations: BamHI (B), BglII (G), HindIII (H), KpnI (K), PstI (P), SalGI (S).

Key. ⟶ Direction of Transcription from *lac* promoter.

R Pactamycin resistant transformants.

S Pactamycin sensitive transformants.

Fig 5.11


PLASMID
R/S
H P B K
S
B
B
G
P K
pTB620
R
pTB621
R
pTB622
R
pTB623
S
pTB624
S
pTB625
S
pTB626
S
1kb

2.4 Hybridisation analysis of *pct* DNA.

In order to confirm that the 4.9 kb KpnI fragment in pTB5 originated from the *S. pactum* genome, Southern analysis was carried out. A radioactive probe was synthesised using the entire 4.9 kb *pct* fragment as a template, whilst two *S. pactum* genomic DNA preparations, *S. lividans* total DNA and pTB500 (all cleaved with KpnI) were subjected to agarose gel electrophoresis and then transferred to a membrane. Having allowed the probe DNA to hybridise to membrane-bound DNA overnight, the membrane was subjected to a high stringency wash so that the probe only remained bound to DNA on the blot, with which it had greater than 85% homology. The autoradiogram in Figure 5.12 clearly demonstrates that the probe hybridised to a single KpnI fragment in the original *S. pactum* DNA used to generate pTB5, in a subsequent *S. pactum* genomic DNA preparation and in the pTB500 digest. Moreover, there was no hybridisation of the probe to *S. lividans* DNA even after a longer exposure (data not shown).

These data show that the *pct* DNA isolated from *S. lividans* TB5 originated in *S. pactum* and that there are no highly homologous sequences in the *S. lividans* genome. These data may suggest that *pct* DNA does not contain rDNA sequences or ribosomal protein genes, since although there is very little information available on such genes in *Streptomyces*, these sequences are probably highly conserved. If this notion was true and if the resistance mechanism in the clones proved to be ribosomal, the resistance could not be due to any peculiarity of the primary sequence of RNA or of any ribosomal protein. Rather, the conclusion would be that either post-transcriptional modification of rRNA or post-translational modification of ribosomal protein(s) was involved.

Legend to Figure 5.12.

Hybridisation of *pct* DNA with genomic DNA from *S. lividans* and *S. pactum*.

DNA fragments, generated by restriction with KpnI, were separated by electrophoresis in a 0.7% (w/v) agarose gel. The DNA fragments were then transferred to a nylon membrane by "Southern Blotting" (Southern, 1975) and then hybridised with [^{32}P] radiolabelled *pct* DNA from pTB500 (the 4.9 kb KpnI fragment). The membrane was washed at "high stringency" and subjected to autoradiography.

a. pTB500 (5 ng).

b. *S. pactum* genomic DNA II (2 μg).

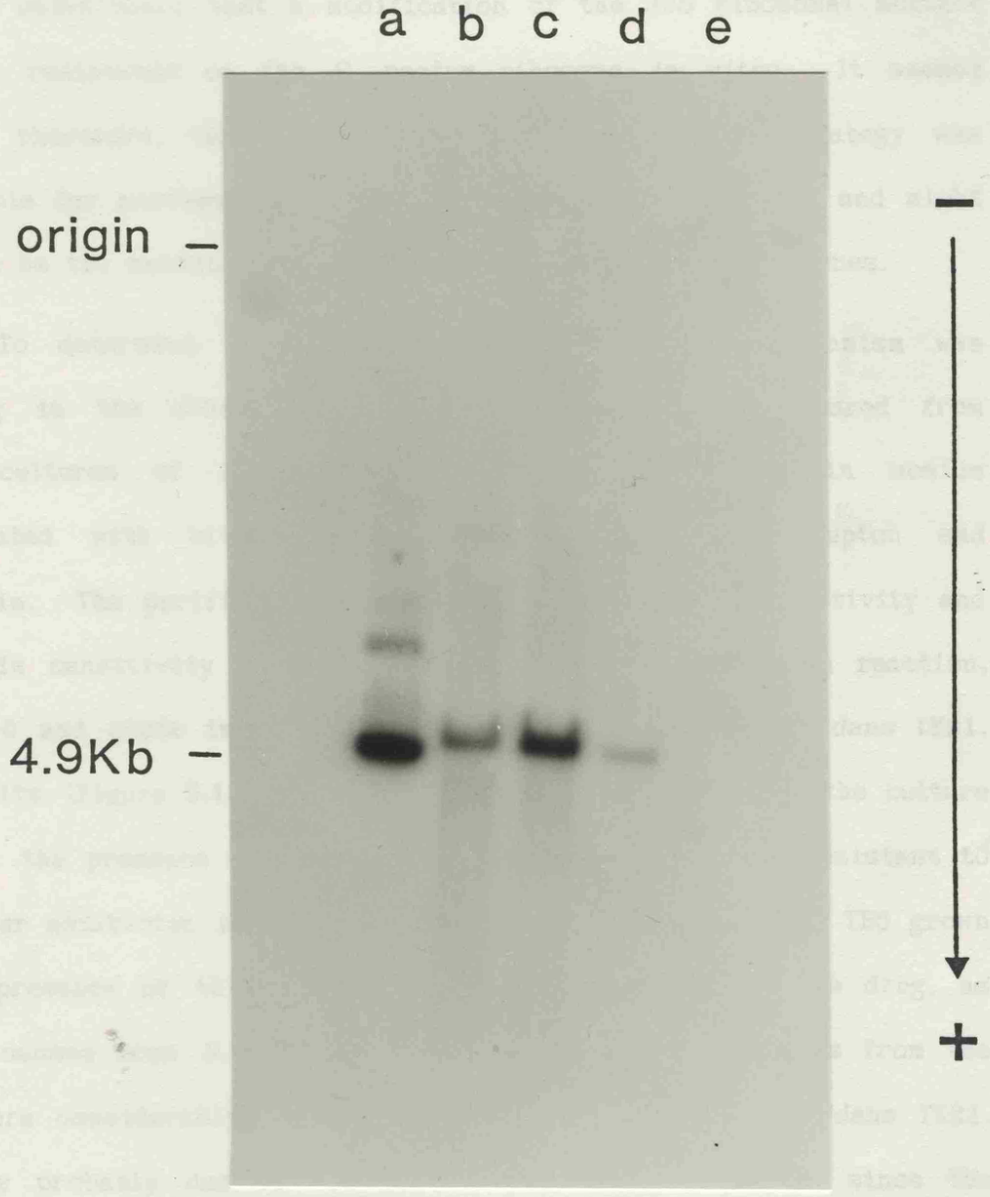
c. *S. pactum* genomic DNA II (5 μg).

d. *S. pactum* genomic DNA I (2 μg).

e. *S. lividans* genomic DNA) (5 μg).

S. pactum genomic DNA I was used in the generation of pactamycin resistant clones. *S. pactum* genomic DNA II was a subsequent preparation.

Fig 5.12



2.5 Biochemical characterisation of pactamycin resistant clones of *S. lividans*.

Prior to the cloning of pactamycin resistance determinants it had been established that a modification of the 30S ribosomal subunit conferred resistance on the *S. pactum* ribosome *in vitro*. It seemed probable therefore, that this target site modification strategy was responsible for pactamycin resistance in the producer *in vivo* and might therefore be the mechanism of resistance in the *S. lividans* clones.

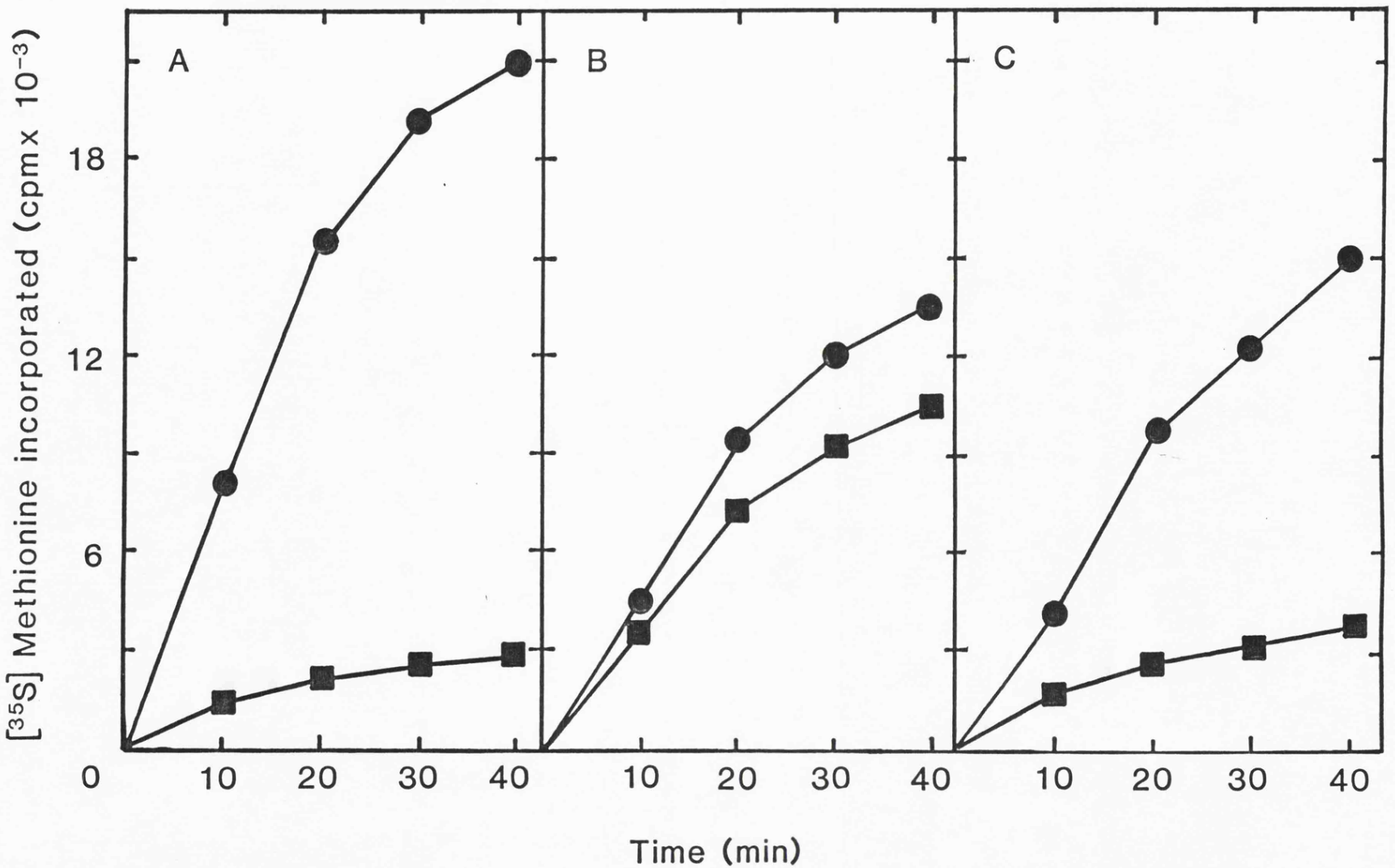
To determine whether a ribosomal resistance mechanism was operating in the clones, salt-washed ribosomes were prepared from 200 ml cultures of *S. lividans* TB5 grown for 48 hr in medium supplemented with either thiostrepton alone, or thiostrepton and pactamycin. The purified ribosomes were then assayed for activity and pactamycin sensitivity in a coupled transcription-translation reaction, with S100 and crude initiation factors prepared from *S. lividans* TK21. The results (Figure 5.13) show that ribosomes obtained from the culture grown in the presence of pactamycin and thiostrepton were resistant to the former antibiotic *in vitro*, whereas those from *S. lividans* TB5 grown in the presence of thiostrepton alone were sensitive to the drug, as were ribosomes from *S. lividans* TK21. However, the ribosomes from the clone were considerably less active than those from *S. lividans* TK21. This was probably due to the culture conditions employed, since the clone was grown in such a way as to minimise the amount of pactamycin used. Although pactamycin was required to obtain drug resistant ribosomes, there was no evidence to invoke the presence of an inducible resistance phenotype, since both *S. pactum* and *S. lividans* TB5 grew on medium containing pactamycin concentrations which fully inhibited *S. lividans* TK21, without prior exposure to subinhibitory concentrations

Legend to Figure 5.13.

Sensitivity of ribosomes from *S. lividans* TK21 and *S. lividans* TB5 to pactamycin.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Salt-washed ribosomes (20 pmol) from *S. lividans* TK21 (panel A), *S. lividans* TB5 grown in the presence of pactamycin (panel B) and *S. lividans* TB5 grown in the presence of thiostrepton (panel C) were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Fig 5.13


A
B
C
[35S] Methionine incorporated (cpm x 10-3)
0
6
12
18
10
20
30
40
Time (min)

of the antibiotic. Rather, pactamycin was most probably necessary to enrich the culture for mycelia which contained plasmid pTB5.

The finding that pactamycin resistant ribosomes could be prepared from *S. lividans* TB5, demonstrated that a ribosomal resistance mechanism had been cloned from *S. pactum*. However, the clone was unsuitable for the purification of such ribosomal particles, because the ribosomes were poorly active in coupled transcription-translation reactions, obtained in low yield and most importantly, their preparation required a pactamycin supplement in the growth media. In fact, all the available pactamycin would have been exhausted in the growth of only two litres of culture. Consequently, ribosomes were prepared from other *S. lividans* subclones containing pTB613, pTB615 and pTB620, to investigate whether they were pactamycin resistant when purified from cultures supplemented with thiostrepton alone. Ribosomes were not prepared from all the *S. lividans* control strains, since these organisms were as sensitive to pactamycin as *S. lividans* TK21 *in vivo*.

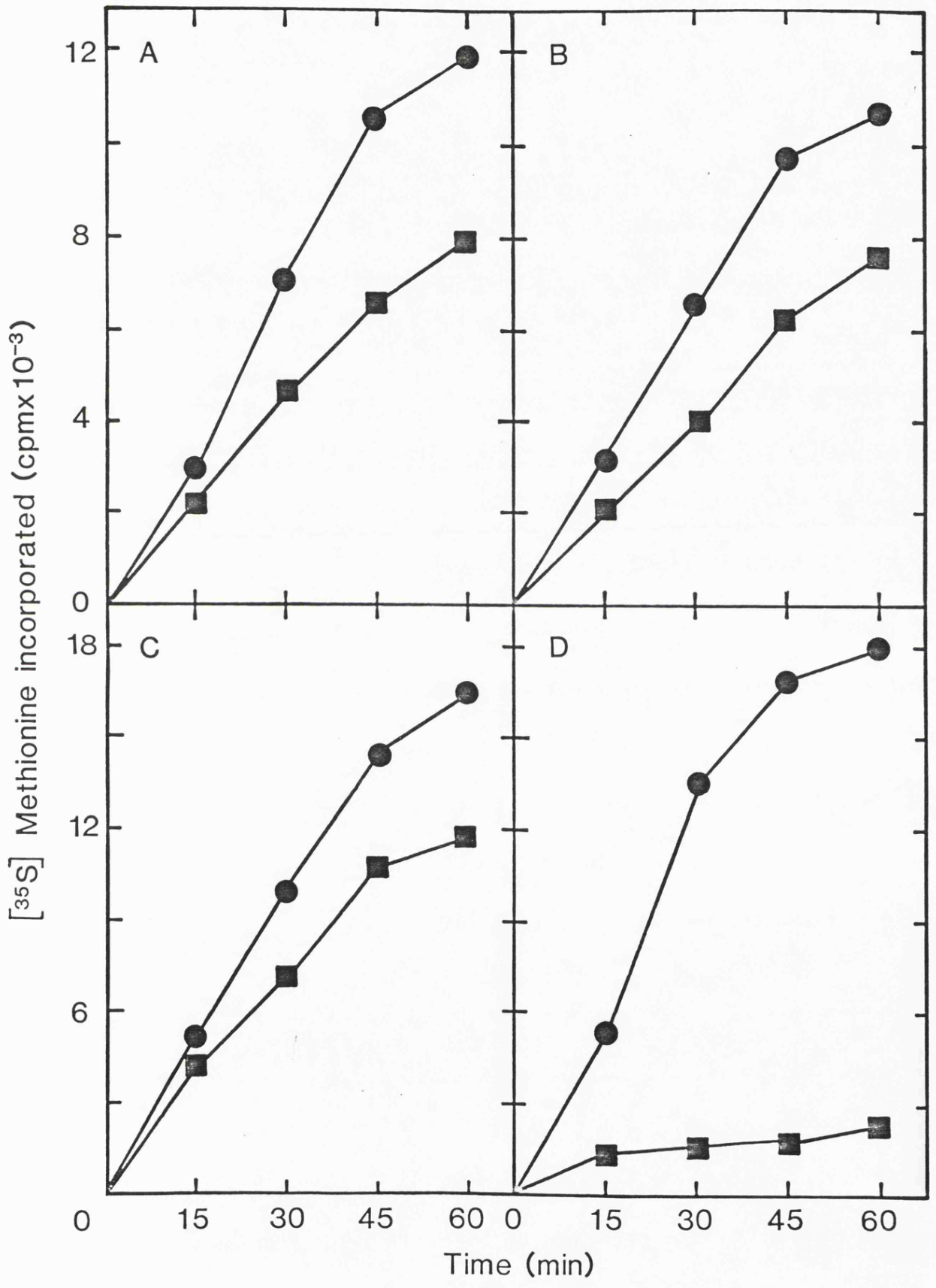
The graphs in Figure 5.14 show that ribosomes prepared from the three *S. lividans* subclones were resistant to pactamycin *in vitro*. However, none of the coupled transcription-translation reactions were totally resistant to pactamycin, unlike similar assays containing *S. pactum* ribosomes. Nevertheless, ribosomes prepared from all three subclones possessed 60-70% activity in the presence of pactamycin concentrations which totally inhibited ribosomes from the control strain. Therefore, since the subclone cultures were not grown in pactamycin containing medium, the resistance phenotypes of strains containing pTB613, pTB615 and pTB620 were considerably more stable than that of *S. lividans* TB5. However, at least one of the subclones was not totally stable. When ribosomes were prepared from cultures of

Legend to Figure 5.14.

Sensitivity of ribosomes from pactamycin resistant subclones of *S. lividans* to pactamycin.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Salt-washed ribosomes (20 pmol) from *S. lividans* containing pTB613 (panel A), pTB615 (panel B), pTB620 (panel C) and *S. lividans* TK21 (panel D) were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Fig 5.14


A
B
C
D
12
8
4
0
18
12
6
0
15
30
45
60
[35S] Methionine incorporated (cpmx10-3)
Time (min)

S. lividans containing pTB613, which had been inoculated with spores from a second propagation on thiostrepton-containing medium, the activity of the particles was inhibited by approximately 70% in the presence of pactamycin (Figure 5.15). Therefore, all subsequent preparations of ribosomal components from *S. lividans* subclones were purified from cultures which had been inoculated with spores and aerial mycelium from thiostrepton resistant primary transformants, to maximise the level of pactamycin resistance obtained *in vitro*. In addition, the cultures were incubated for the minimum time required to obtain sufficient mycelium for ribosome preparation (typically 16-20 hr at 30°C). Such ribosome preparations were reproducibly 60-70% resistant to pactamycin.

The incomplete pactamycin resistance of ribosomal preparations from the *S. lividans* subclones could have been due to the presence of sensitive ribosomes in a population of resistant particles, or alternatively it could have been a consequence of each ribosome being only partially modified. In order to establish which of these possible explanations was correct, salt-washed ribosomes from *S. lividans* TB620 (*S. lividans* TK21 containing pTB620) were assayed for protein-synthesising activity in the presence of various concentrations of pactamycin. Since this antibiotic is a potent inhibitor of coupled transcription-translation reactions, sensitive ribosomes should be fully inhibited by low pactamycin inputs whereas resistant particles should be unaffected, even at much greater drug concentrations. On the other hand, if each subclone ribosome was only partially resistant, the degree of inhibition should increase in response to greater pactamycin inputs. The results presented in Table 5.1 show that 1 $\mu g\ ml^{-1}$ pactamycin inhibited the activity of the subclone ribosome preparation by

Legend to Figure 5.15.

Sensitivity of ribosomes from *S. lividans* TB613 to pactamycin after two rounds of propagation in the absence of pactamycin.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Salt-washed ribosomes (20 pmol) from *S. lividans* TK21 (panel A) or *S. lividans* TB613 were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

The culture conditions for *S. lividans* TB613 were as follows: Plasmid pTB613 was introduced into *S. lividans* TK21 protoplasts and the resultant thiostrepton resistant transformants were plated onto NE agar supplemented with thiostrepton. When the organism had sporulated, spores were removed and used to inoculate similar plates. Once the colonies had sporulated, spores were removed and inoculated into liquid media supplemented with thiostrepton and incubated for 20 hr. Ribosomes were then prepared from these cultures.

Fig 5.15

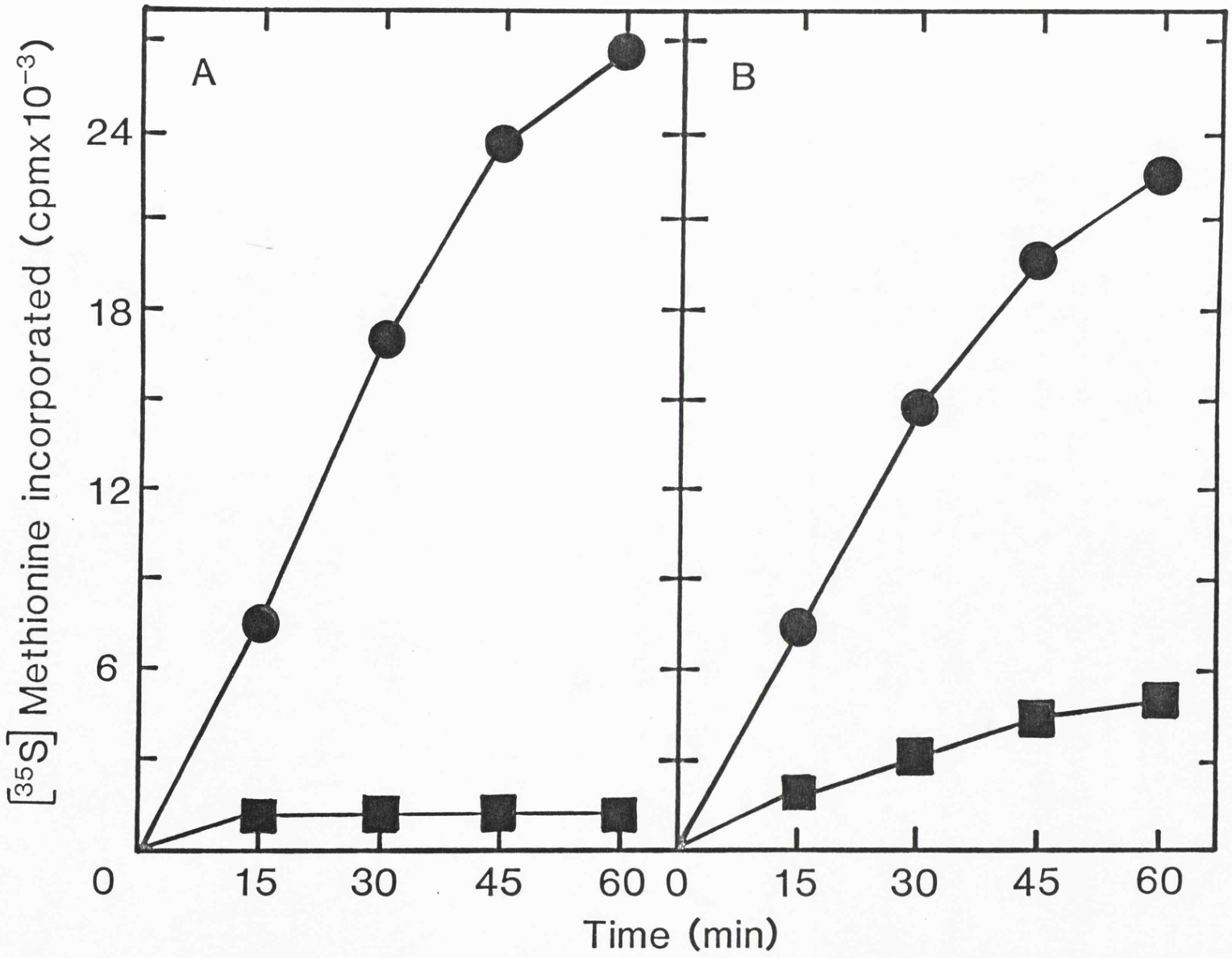

A
B
24
18
12
6
0
15
30
45
60
0
15
30
45
60
Time (min)
[35S] Methionine incorporated (cpmx10-3)

Table 5.1.

Effect of pactamycin on activity of ribosomes from *S. lividans* TB620 in coupled transcription-translation reactions.

Source of ribosomes	Final pactamycin concentration ($\mu g\ ml^{-1}$)	Activity (cpm)
S. lividans TK21	zero	19,970
S. lividans TK21	1	3,125
S. lividans TK21	10	1,673
S. lividans TK21	100	1,484
S. lividans TB620	zero	15,511
S. lividans TB620	1	11,907
S. lividans TB620	10	11,274
S. lividans TB620	100	10,931

Legend to Table 5.1.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Ribosomes were incubated in the presence of DMSO or pactamycin for 10 min prior to assay. Samples (5 µl) were removed at 10 min intervals. 'Activity' is the incorporation of [^{35}S] methionine into TCA-precipitable material (cpm) per 5 µl sample after incubation for 40 min.

approximately 30% and that greater drug concentrations did not increase the level of inhibition further. These data strongly suggest that the incomplete resistance observed *in vitro* was due to the pactamycin sensitivity of approximately 30% of the ribosomes in the preparation, rather than to partial resistance in all the particles.

Previously, ribosomal resistance to pactamycin in the producer had been shown to be a property of the 30S ribosomal subunit. Therefore to investigate whether small ribosomal subunits from *S. lividans* TB620 were similarly modified, salt-washed ribosomes from the subclone and a control strain were dissociated into their respective subunits. The purified 30S and 50S ribosomal subunits were then recombined to form homologous and heterologous ribosomes, which were then assayed for pactamycin sensitivity in coupled transcription-translation reactions. The results from one such experiment (Figure 5.16) clearly demonstrate that the level of pactamycin resistance observed in the homologous *S. lividans* TB620 ribosome could be attributed solely to a property of the smaller subunit. Furthermore, the level of pactamycin resistance exhibited by homologous and heterologous ribosomes, containing 30S subunits derived from *S. lividans* TB620, was similar to that found in native 70S particles. Thus, to a certain extent, the pactamycin resistance mechanism in the *S. lividans* subclone seemed analogous to that evident in *S. pactum*, although for some as yet undetermined reason, resistance was incomplete.

Having established the role of the 30S ribosomal subunit from *S. lividans* TB620 in pactamycin resistance, total reconstitution of the small ribosomal subunit was carried out in order to identify the ribosomal component responsible. Accordingly, 16S rRNA and total protein (TP30) were prepared from 30S ribosomal particles from the

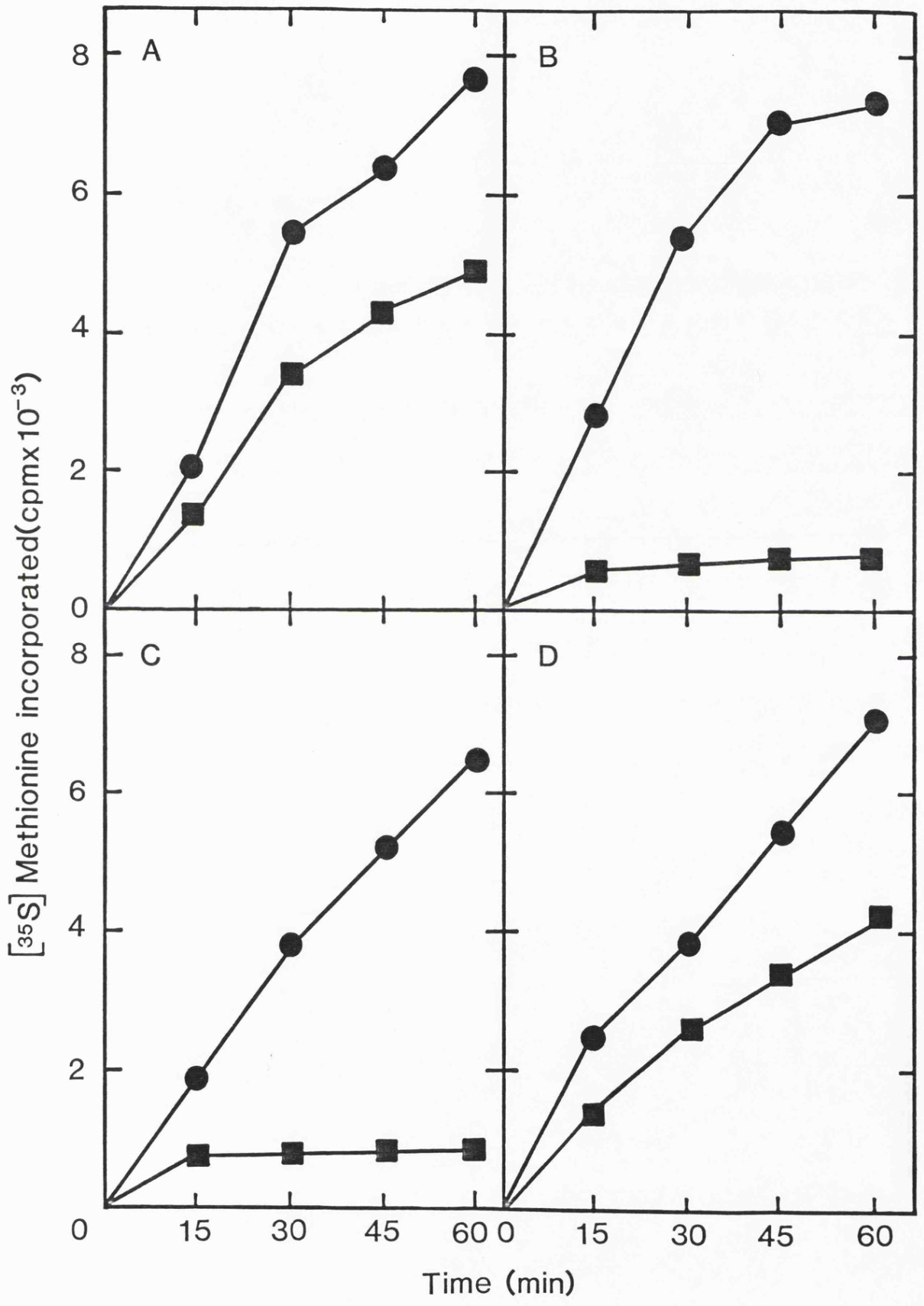
Legend to Figure 5.16.

Localisation of pactamycin resistance to the 30S subunit of the *S. lividans* TB620 ribosome.

All reactions (30 µl) contained S100 and crude initiation factor preparation from *S. lividans* TK21. Ribosomal subunits (20 pmol of each) were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Source of ribosomal subunits: (A) 30S and 50S subunits from *S. lividans* TB620, (B) 30S and 50S subunits from *S. lividans* TK21, (C) 30S subunits from *S. lividans* TK21 and 50S subunits from *S. lividans* TB620 and (D) 30S subunits from *S. lividans* TB620 and 50S subunits from *S. lividans* TK21.

Fig 5.16


A
B
C
D
8
6
4
2
0
15
30
45
60
[35S] Methionine incorporated(cpmx10-3)
Time (min)

subclone and a control strain. Homologous and heterologous 30S ribosomal subunits were then reconstituted from the purified components and tested for their pactamycin sensitivity in a coupled transcription-translation reaction, in which all other subcellular fractions were derived from *S. lividans* TK21.

Active 30S ribosomal subunits were frequently reconstituted from *S. lividans* TK21 components, but problems were often encountered in trying to reconstitute hybrid 30S particles containing ribosomal fractions from *S. lividans* TB620. The reason for the difficulties was not determined, although it may have been due to the lower activity of ribosomal components from the subclone. Nevertheless, in two experiments, activity and resistance to pactamycin in coupled transcription-translation reactions were observed. The results shown in Figure 5.17 were obtained from an experiment in which one TP30 preparation was divided into two portions and used to reconstitute 30S ribosomal subunits with 16S rRNA from either *S. lividans* TK21 or TB620. The results strongly suggest that rRNA was altered in the subclone, since 30S ribosomal particles containing 16S rRNA from *S. lividans* TB620 were significantly more resistant to pactamycin than those containing the equivalent rRNA from the control strain.

The pactamycin sensitivities of 30S ribosomal subunits reconstituted in the four possible combinations with 16S rRNA and TP30 from *S. lividans* strains TB620 and TK21 are shown in Figure 5.18. These data again support an involvement of 16S rRNA in pactamycin resistance *in vitro*. Although a low level of resistance was observed with 30S ribosomal subunits reconstituted with 16S rRNA from *S. lividans* TK21 and TP30 from *S. lividans* TB620, this was probably due to incomplete removal of 16S rRNA from the ribosomal proteins after LiCl-urea

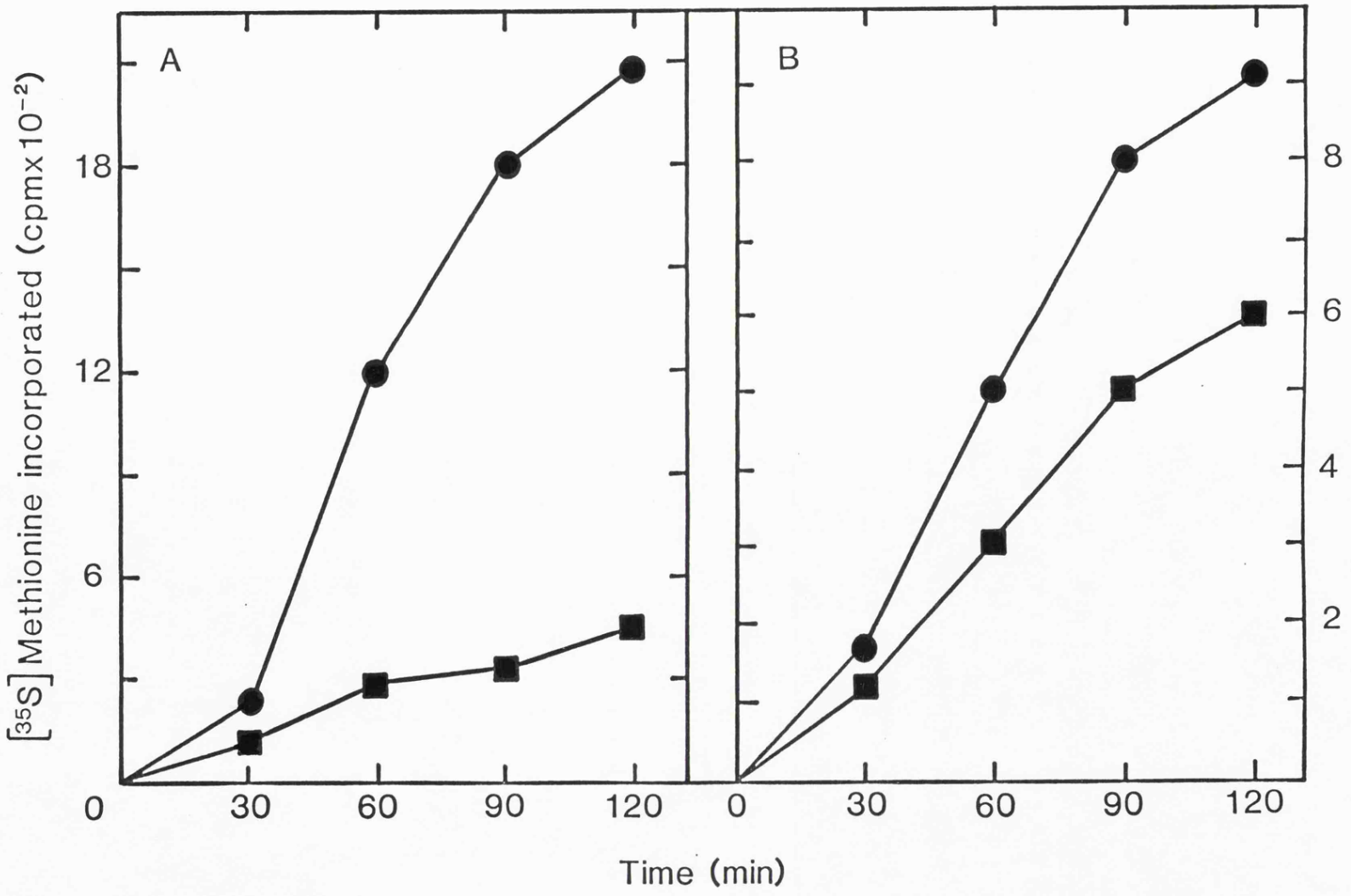
Legend to Figure 5.17.

Localisation of pactamycin resistance to 16S rRNA of ribosomes from *S. lividans* TB620.

All reactions (50 µl) contained S100, crude initiation factor preparation and 50S ribosomal subunits (20 pmol) from *S. lividans* TK21. Reconstituted 30S ribosomal subunits (40 pmol) were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Source of components: (A) 16S rRNA and TP30 from *S. lividans* TK21, (B) 16S rRNA from *S. lividans* TB620 and TP30 from *S. lividans* TK21.

Fig 5.17


A
B
[35S] Methionine incorporated (cpmx10-2)
18
12
6
0
8
6
4
2
0
30
60
90
120
Time (min)

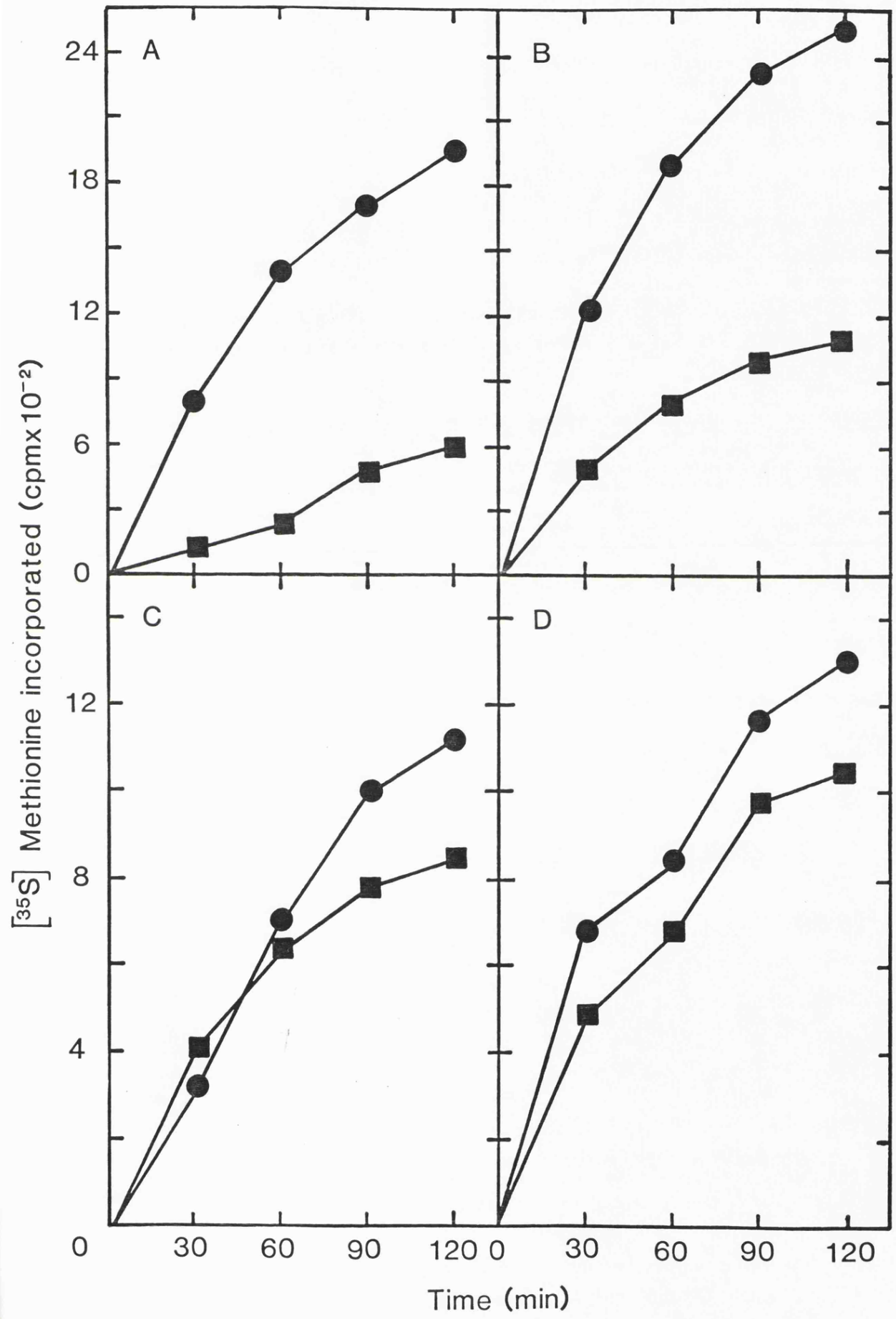
Legend to Figure 5.18.

Pactamycin sensitivity of homologous and heterologous 30S ribosomal subunits reconstituted from *S. lividans* TK21 and *S. lividans* TB620 components.

All reactions (50 µl) contained S100, crude initiation factor preparaton and 50S ribosomal subunits (20 pmol) from *S. lividans* TK21. Reconstituted 30S subunits (40 pmol) were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Source of components: (A) 16S rRNA and TP30 from *S. lividans* TK21, (B) 16S rRNA from *S. lividans* TK21 and TP30 from *S. lividans* TB620, (C) 16S rRNA from *S. lividans* TB620 and TP30 from *S. lividans* TK21 and (D) 16S rRNA and TP30 from *S. lividans* TB620.

Fig 5.18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12
8
4
0
30
60
90
120
[35S] Methionine incorporated (cpmx10⁻²)
Time (min)

extraction and precipitation. The results from two subsequent experiments support this explanation. When 30S ribosomal particles were reconstituted with TP30 prepared in a solution containing either lithium chloride from a fresh source, or a greater concentration of the salt (2.5 M compared with 2.0 M), they were more strongly inhibited by pactamycin in coupled transcription-translation reactions (Figure 5.19) than those described previously.

In conclusion, there is a body of data which strongly suggests 16S rRNA alteration as the mechanism of pactamycin resistance in the *S. lividans* subclones and presumably in *S. pactum* also. Unfortunately, time did not allow the resistance mechanism to be characterised further.

3 Discussion.

This chapter has described the isolation of a 4.9 kb KpnI fragment (*pct*) from the *S. pactum* genome, which conferred pactamycin resistance on *S. lividans*. This DNA fragment however, was highly unstable when inserted at the KpnI site of the multicopy plasmid pIJ702, since the recombinant molecule readily underwent DNA rearrangement(s) to produce a smaller plasmid (pTB702) which was related to the cloning vector. Nevertheless, it was possible to demonstrate that the *pct* DNA encoded a ribosomal modification system by analysing ribosomes prepared from cultures of the clone, *S. lividans* TB5, grown in the presence of pactamycin. The requirement for this antibiotic in the preparation of resistant ribosomes was not obviously due to an inducible resistance mechanism, since the clone grew on medium containing high pactamycin concentrations, without prior exposure to subinhibitory levels of the drug. Rather, pactamycin probably increased the proportion of mycelia which contained *pct* DNA, since in the presence of thiostrepton, plasmid

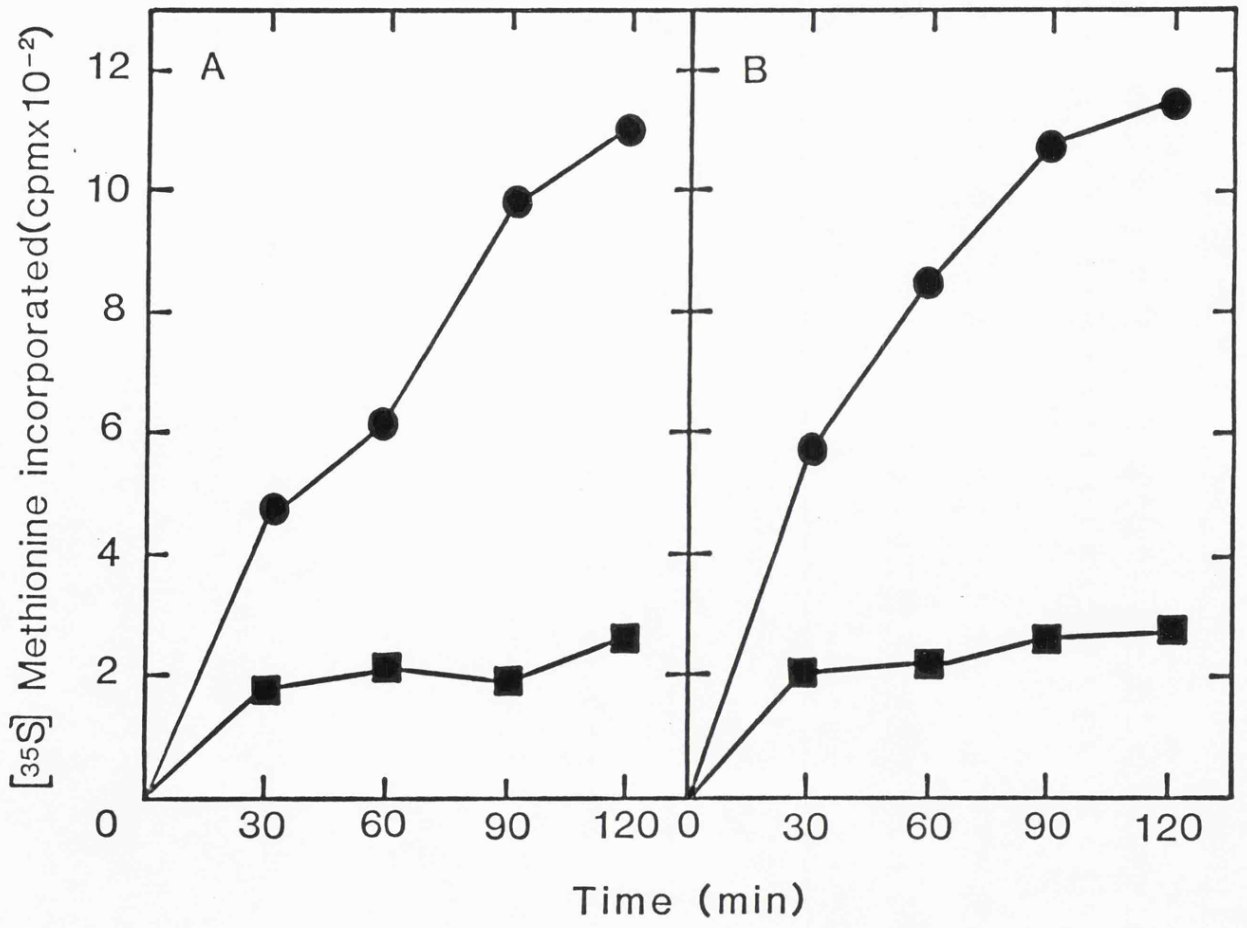
Legend to Figure 5.19.

Pactamycin sensitivity of 30S ribosomal subunits reconstituted from different preparations of TP30 from *S. lividans* TK21.

All reactions (50 µl) contained S100, crude initiation factor preparation, 50S ribosomal subunits (20 pmol) and 30S ribosomal subunits (40 pmol) reconstituted from 16S rRNA and TP30 from *S. lividans* TK21. The 30S subunits were incubated with DMSO (●) or pactamycin at 10 µg ml^{-1} final concentration (■), prior to assay for coupled transcription-translation activity.

Preparation of TP30: (panel A) LiCl from an unopened bottle was used for protein extraction from 30S ribosomal subunits, (panel B) the LiCl concentration for protein extraction was increased from 2 M to 2.5 M.

Fig 5.19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60
90
120
[35S] Methionine incorporated(cpmx10-2)
Time (min)

pTB702 would be maintained preferentially as it would be the smaller of the two incompatible plasmids.

The reason for the plasmid instability in *S. lividans* TB5 has not been determined. It appears though, that the deletion event which generated pTB702 occurred between specific sites, since two discrete bands were observed when the plasmid preparation from the clone was analysed by agarose gel electrophoresis. However, if larger deletions had occurred, they might have resulted in DNA molecules which could not replicate and so consequently were not isolated. Furthermore, the KpnI site in pIJ702 is "possibly in a function necessary for plasmid stability" (Kieser *et al.*, 1982), although it has been described as a cloning site (in Hopwood *et al.*, 1985) and successfully used in this laboratory to clone the *erm E* gene from *S. erythraeus* (A. Thiara, unpublished results). Instability problems in some *E. coli* cloning experiments have been due to the insertion of strong promoters into the cloning vehicle. RNA synthesis from these promoters caused instability by directing transcription into the replication regions of the plasmid and caused overproduction of proteins involved in the maintenance of plasmid copy number (Stueber and Bujard, 1982). Perhaps *pct* DNA sequences promote transcription through replication regions of pIJ702, resulting in plasmid instability.

In one of the few cases where instability of cloned DNA in *Streptomyces* has been reported, ligation of the culprit DNA into an alternative vector resulted in a more stable construction (Dehottay *et al.*, 1986). When *Streptomyces albus* DNA was ligated into the unique PstI site in pIJ61 and transformed into *S. lividans*, five of the six clones which expressed the β-lactamase from *S. albus* were lost after one subculture. Furthermore, when plasmid DNA was isolated from the

surviving clone and reintroduced into *S. lividans* protoplasts, 40% of the primary transformants failed to produce β-lactamase. However, when the 4.9 kb PstI fragment was transferred from pIJ61 to plasmid pIJ702, the resulting construct was totally stable.

It was hoped that subcloning the *pct* DNA into alternative vectors might lead to the generation of a clone from which pactamycin resistant ribosomes could be prepared without consuming more antibiotic. Fortunately, such ribosomes could be prepared from cultures of *S. lividans* containing pTB613, pTB615 and pTB620, grown in the presence of thiostrepton alone. Curiously however, only 60-70% of the ribosomes in the preparation were pactamycin resistant *in vitro*, whereas total resistance had previously been observed with *S. pactum* ribosomes. The level of resistance appeared to be independent of the presumed copy number of the cloning vector used, or the context of the *pct* DNA, since similar levels of resistance were obtained with ribosome preparations from subclones containing replicons based on pIJ61, pOJ160 and pIJ487, which have copy numbers of 4-5, 30 and 40-300 respectively. It may be relevant that plasmid copy number determinations have often been performed on cultures grown under dissimilar conditions to those employed for ribosome preparation, so that the actual copy number of the plasmids containing *pct* may not be as different in actively growing cultures as their nominal values suggest.

The subcloning of the pactamycin resistance determinants into various *Streptomyces* vectors was greatly facilitated by the insertion of *pct* DNA into the polylinker sequence of the expression vector pUC18. Unfortunately, *E. coli* strains containing *pct* DNA in this vector were not pactamycin resistant, even when grown in the presence of the *lac* inducer IPTG. It is perhaps not surprising that the resistance gene was

not expressed from a *Streptomyces* promoter (if present), since only a minority of such signals are used by *E. coli* RNA polymerase (Bibb and Cohen, 1982; Jaurin and Cohen, 1985) and none of the promoter sequences isolated so far from antibiotic resistance determinants from *Streptomyces* have functioned in *E. coli*. The absence of expression from the *lac* promoter may have been a reflection of the large size of the DNA insert and thus the possible occurrence of *Streptomyces* transcriptional terminators between the promoter and the start of the pactamycin resistance determinant Alternatively, the product of the pactamycin resistance determinant may not have been functional in *E. coli*. For example, if the resistance mechanism is 16S rRNA methylation, but an intermediate in ribosome assembly is the substrate, it may be that ribosome biogenesis is somehow different in *E. coli*, such that the putative methylase fails to recognise its target.

Attempts to isolate the pactamycin resistance gene(s) as a small DNA fragment have so far resulted in an insert only 0.6 kb shorter than the original 4.9 kb piece. Further experiments, including the ligation of *pct* DNA partially digested with Sau3A with pOJ160 cleaved with BamHI, should result in the isolation of a smaller DNA fragment which confers pactamycin resistance in *S. lividans* and perhaps even in *E. coli*.

It is not known whether the pactamycin resistance gene is expressed from its own promoter or from sequences in the cloning vectors. The absence of the 'natural' promoters might explain the incomplete resistance observed *in vitro*. In a previously reported cloning experiment, the *erm E* gene was isolated from *S. erythraeus* without its promoter sequences (Thompson, C. J. *et al.*, 1982b; Bibb *et al.*, 1985b). Although the gene was expressed in *S. lividans*, presumably

from promoter activities within the vector, the clone was less resistant to erythromycin than *S. erythraeus*. Furthermore, the ribosomes in the clone were not completely modified by the erythromycin resistance methylase *in vivo*, since 10% of the rRNA isolated from the clone ribosomes was a substrate for the purified enzyme *in vitro*. The *erm E* gene has subsequently been re-isolated together with its promoter sequences and the resultant *S. lividans* clones exhibited a similar level of erythromycin resistance to *S. erythraeus in vivo*. It remains to be established whether the increased resistance *in vivo* was primarily a consequence of the natural promoter signals being present or the higher copy number of the vector employed when *erm E* was re-isolated.

Further plasmid instability may have caused the incomplete pactamycin resistance observed *in vitro*. If more antibiotic had been available, ribosomes could have been prepared from subclones grown in the presence of pactamycin and then analysed to determine what proportion were resistant to the drug. Alternatively, a detailed analysis of the plasmids in the subclones could be undertaken to see whether any deleted or rearranged molecules were present under the growth conditions employed for ribosome preparation. A formal possibility that could account for the incomplete resistance *in vitro* is that there might be competition between the plasmid-borne resistance determinant(s) and a sensitive chromosomal counterpart, for example *pct* DNA could encode ribosomal protein or rRNA. Southern analysis demonstrated that there was no strong homology between *pct* DNA and the *S. lividans* genome, which should rule out the above explanation. If *pct* contained rDNA sequences, it might have been expected to hybridise to more than one KpnI fragment in the *S. pactum* chromosome (although this did not necessarily have to occur) as there is a redundancy of rDNA in

S. coelicolor (H. Baylis, personal communication) and presumably therefore in other *Streptomyces*. One final point relevant to the competition argument is that the context and copy number of *pct* DNA was probably different in the various subclones, yet there was no discernible effect on the level of pactamycin resistance *in vitro*. This result would not have been expected if *pct* DNA encoded genes for ribosomal components.

The precise mechanism of pactamycin resistance in *S. pactum* and *S. lividans* clones has not yet been determined. However, reconstitution analysis of 30S ribosomal subunits from clone ribosomes has suggested that a property of 16S rRNA is involved. Furthermore, since the *pct* fragment is unlikely to encode rDNA, the most plausible resistance mechanism is a post-transcriptional modification of the rRNA. A similar mechanism has been described for all the antibiotic producers with drug resistant ribosomes that have been characterised so far. If pactamycin resistance does result from specific methylation of 16S rRNA, purification of the methylase from clones which overproduce the enzyme should enable the site and nature of the methylation event to be determined. Identification of the methylation site should pinpoint a crucial region of the ribosome involved in pactamycin binding and the mode of action of the antibiotic.

Concluding remarks.

This thesis has described efforts to locate the ribosomal binding site for pactamycin by investigating the resistance mechanism adopted by the producing organism, *S. pactum*. Unfortunately, it has not been possible to fully characterise the target site modification present in this organism, although ribosomal reconstitution experiments have strongly suggested 16S rRNA modification as the mechanism of pactamycin resistance. It is hoped that future work on the site of this modification will identify a key region of 16S rRNA for ribosome function. Previous analyses of rRNA methylation in antibiotic producing organisms have enabled rRNA domains involved in GTP hydrolysis, peptidyl transfer and codon-anticodon recognition to be unveiled.

If 16S rRNA methylation proves to be the pactamycin resistance mechanism, it will not be easy to ascribe a function to the domain in which the methylated residue resides. This is because the mode of action of pactamycin is unclear, especially in bacterial systems. However, considerable data is being accumulated from studies of initiation factor, tRNA and antibiotic binding to ribosomes, so that a better understanding of the action of pactamycin may be gained from data derived from these other ligand binding studies. Whatever the precise mode of action of the drug, the function that it inhibits has probably been conserved during evolution, since the binding site appears to be ubiquitous. Consequently, when the site of RNA modification has been located, it would be interesting to mutagenise it, using a cloned RNA operon on a high copy number vector and then analyse ribosomes from the mutants in a number of partial reactions of protein synthesis. This approach may also shed light on the mode of action of pactamycin.

Besides its potential use in the characterisation of the pactamycin binding domain of ribosomes, the cloned pactamycin resistance determinant (*pct*) is of interest since it is another actinomycete gene of known function which can be studied. DNA sequence analysis of the *pct* gene may indicate sequences which promote transcription of the gene, and these could then be compared with sequences of other promoters from *Streptomyces* genes. It is unlikely that the *pct* gene will ever be used as a selectable marker on new cloning vectors, since the antibiotic is unavailable. However it would be interesting to determine whether the *pct* gene can be expressed in other eubacteria and even perhaps in eukaryotic cells, by using appropriate expression vectors. Whether other organisms can become pactamycin resistant will not only depend on the synthesis of the gene product but also whether similar structural features of the substrate for modification are present during ribosome biogenesis.

The second target site modification described here is that which confers celesticetin resistance on ribosomes from *S. caelestis*. It has not been established whether methylation of 23S rRNA is responsible for resistance, but if this proves to be the case, localisation of the site of methylation will be of particular interest because it probably resides in domain V of 23S rRNA (see Figure 1.1). The site may be at or very close to adenosine A2058, which is dimethylated by the MLS resistance enzyme from *S. erythraeus*.

Finally, studies of ribosome modification in antibiotic producers have given ribosomologists an alternative approach by which rRNA can be investigated. However, although the producers of celesticetin, erythromycin, pactamycin, thiostrepton and various aminoglycosides have assisted in this way by using rRNA modification

strategies which are, to all intents and purposes, constitutively expressed, there are as many examples of producers of translation inhibitors which have sensitive ribosomes. In some of these cases, it may be that the modification system is inducible under these conditions. However in others, rigorous examinations have revealed that target site modification is not the only mechanism by which producing organisms avoid suicide.

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Abstract.

The coupled transcription-translation system previously developed for *Streptomyces lividans* was modified such that it functioned using purified ribosomal subunits, a crude initiation factor preparation and a high speed supernatant fraction. This system was used to investigate antibiotic resistance mechanisms in two *Streptomyces* which synthesise inhibitors of translation. Resistance to either pactamycin in *Streptomyces pactum* or celesticetin in *Streptomyces caelestis* was due to ribosome modification. In each case, high level resistance was attributed solely to one ribosomal subunit, the 30S subunit of the *S. pactum* ribosome and the 50S subunit of the *S. caelestis* ribosome.

Shotgun cloning experiments have enabled a pactamycin resistance determinant from *S. pactum* to be isolated in *S. lividans*. However, in the original pactamycin resistant clone the plasmid was unstable and in the absence of pactamycin selection pressure, only a deleted form could be recovered. When ribosomes from resistant subclones were analysed, it appeared that a ribosome modification system from *S. pactum* had been cloned. Ribosome reconstitution studies indicated that a property of 16S rRNA was responsible for resistance. Since the cloned resistance determinant was not homologous to 16S rRNA (as judged by Southern analysis), pactamycin resistance in *S. pactum* is probably due to post-transcriptional modification of 16S rRNA.