Development of plasmid vectors for regulated expression of cloned genes in *Streptomyces lividans* and *Escherichia coli*.

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by

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The research reported in this Thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

CONTENTS

ABBREVIATIONS

APS - ammonium persulphate

ATP - adenosine 5'-triphosphate

BDH - British Drug Houses

bp - base pairs

BRL - Bethesda Research Laboratories Ltd

BSA - bovine serum albumin

DEPC - diethyl pyrocarbonate

DMSO - dimethylsulphoxide

DMF - dimethylformamide

DTT - dithiotreitol

dNTP - deoxynucleoside 5'-triphosphate

dATP - deoxyadenosine 5'-triphosphate

dCTP - deoxycytidine 5'-triphosphate

dGTP - deoxyguanosine 5'-triphosphate

dTTP - deoxythymidine 5'-triphosphate

DNA - deoxyribonucleic acid

ssDNA - single stranded deoxyribonucleic acid

dH_2O - distilled water

EDTA - ethylene diaminetetra-acetic acid (disodium salt)

EtBr - ethidium bromide

IPTG - isopropylthio-B-D-galactoside

Kb - Kilo bases

NEN - New England Nuclear

PIPES - Piperazine-NN'-bis-2-ethane sulphonic acid

RBS - ribosome binding site

RNA - ribonucleic acid

mRNA - messenger ribonucleic acid

rRNA - ribosomal ribonucleic acid

SDS - sodium dodecylsulphate

TEMED - NNN'N'-Tetramethylethylenediamine

Tris - tris (hydroxymethyl) methylamine

XGal - 5-bromo-4-chloro-3-indolyl-B-galactoside

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SUMMARY

Using pIJ486 (Ward *et al.*, 1986) two bifunctional (*E. coli*/*Streptomyces*) expression vectors were constructed. They contained the promoters *lac* (pGLW49) and *tac* (pGLW50) just upstream of a promoter-less kanamycin phosphotransferase gene (*aph*II).

Both vectors conferred resistance to kanamycin in *E. coli*, but only the vector carrying the *tac* promoter conferred resistance to kanamycin in *S. lividans*.

A third bifunctional vector, pGLW51, was constructed. It contained not only the *tac* promoter but also a gene coding for the *lac* repressor (*lac*I).

Recombinants of *E. coli* and *S. lividans* containing pGLW51 showed elevated levels of resistance to kanamycin in the presence of IPTG. This indicated that regulation of the *tac* promoter by the *lac* repressor was occurring in both genera.

S_1 nuclease mapping of the *tac* promoter showed that the transcriptional start in *S. lividans* coincided with this transcriptional start in *E. coli*.

Since repression of p*tac* in *S. lividans* was very poor the level of transcription of the *lac*I gene was investigated and found to be very low.

Consequently, a vector carrying a *Streptomyces* promoter (the actinophage ØC31 repressor promoter) just upstream from the *lacI* gene was constructed (pGLW61).

This vector showed better repression of *ptac* in *E.coli* but not in *S. lividans*. Attempts were made to explain this fact which is possibly due to poor translation of the *lacI* mRNA in *S. lividans* coupled to "readthrough" of message from this new promoter to the *tac* promoter.

Using site directed mutagenesis, attempts were also made to improve expression from *ptac* in pGLW51 by shortening the distance between this promoter and the translational start of the *aphII* gene.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Streptomyces are common, highly oxidative, Gram-positive soil bacteria.

They have proved to be a very rich source of antibiotics, antitumour and antihypertensive agents, enzymes, herbicides and pesticides (Demain, 1984).

Since the first purified antibiotic, actinomycin, was obtained (Waksman and Woodruff, 1940) from an actinomycete, many medically-useful antibiotics followed. About 60% of the known antibiotics are produced by Streptomyces species (Berdy, 1980), including many commercially-important ones.

Much of the current interest for Streptomyces is not only due to their ability to produce antibiotics (and other "secondary" metabolites, see following pages) but is also due to the ability of these organisms to undergo a complex life cycle showing multiple morphological stages (Chater, 1984).

As the majority of spore-forming actinomycetes, Streptomyces are characterised by their branching vegetative mycelia, on which aerial, sporogenous hyphae develop.

The biosynthesis of many antibiotics occurs late in their life cycle, implying that the enzymes specific to their metabolism are only produced or activated then. Often this biosynthesis has been linked

to the development of aerial mycelium which eventually septates to form chains of spores.

Therefore, both the developmental cycle and the production of "secondary" metabolites anticipate a highly controllable gene expression in Streptomyces.

In recent years great advances were obtained in the development of recombinant DNA technology in Streptomcyes.

Polyethylene-glycol induced plasmid transformation of protoplasts has provided the basis for gene cloning in Streptomyces species (Bibb et al., 1978; Matsushima and Baltz, 1985; Acebal et al., 1986; Lampel and Strohl, 1986).

A gene from Streptomyces coelicolor conferring resistance to methylenomycin was the first gene cloned from a streptomycete (Bibb et al., 1980). Since then, many other genes involved in antibiotic resistance (e.g. Thompson et al., 1980; Thompson et al., 1982; Bibb et al., 1985) and biosynthesis (Feitelson and Hopwood, 1983; Malpartida and Hopwood, 1984; Chater and Bruton, 1985; Distler et al., 1985; Murakami et al., 1986; Stanzak et al., 1986) were cloned and characterised. Genes involved in carbon catabolism (Seno et al., 1984; Fornwald et al., 1987) and morphological differentiation (Piret and Chater, 1985) have also been isolated.

Many vectors are now available for cloning in Streptomyces. Most of the high-copy number vectors available, such as pIJ702 (Katz et al.,

1983) are derived from the high-copy number plasmid pIJ101, isolated from S. violaceoruber (Kieser et al., 1982).

SLP1.2 and SCP2 are low copy-number plasmids and have been used to construct other low copy number vectors, for example: pIJ61 (Thompson et al., 1982b) and pIJ922 (Lydiate et al., 1985) respectively.

Phage-derived cloning vectors have also been developed for Streptomyces. The most widely used actinophage has been the broad host range phage, ØC31 (Chater et al., 1981), that has a lysogeny pattern similar to phage lambda in E. coli. These phage derived vectors are useful in mutational cloning (Chater and Bruton, 1983) and in insertional inactivation of transcription units (Seno et al., 1983b).

Despite the advances made in the molecular genetics of streptomycetes, knowledge about primary and secondary metabolism and their regulatory mechanisms in this genera is very poor and insufficient.

The classification of secondary metabolites is often associated with a phase subsequent to active growth. This is not necessarily that simple and antibiotics can be produced during growth, or after growth, depending on fermentation conditions (Doull and Vining, 1988). Demain et al. (1983) emphasised that the timing of product formation should not be used to define a secondary metabolite.

Primary metabolism consists of the interrelated series of enzymic reactions which provide living cells with energy, with synthetic intermediates and with macromolecules such as proteins, nucleic acids

and lipids. In well regulated wild-type microorganisms, intermediates and end products of these reactions do not accumulate.

In organisms that produce secondary metabolites, certain steps of primary metabolism lack regulation. This results in overproduction of intermediates and end products of primary pathways which can subsequently induce pathways to form secondary metabolites (Malik, 1979; Drew, 1977).

Since secondary metabolism and growth "compete" for key intermediates and cofactors, understanding of the regulatory mechanisms that control their synthesis can be very useful to improve rationally the production of secondary metabolites.

Different approaches can be taken towards the comprehension of metabolic regulation "in vivo". Great progress has been made in the application of recombinant DNA technology to studies of regulation of metabolic pathways (reviewed by Nimmo and Cohen, 1987).

Biological pathways are controlled by enzymatic steps whose individual properties determine the overall rate of the process.

The concept of "rate limiting step" has been widely used to describe the potential control points in metabolic pathways. However, Kacser and Burns (1973) and Heinrich and Rapaport (1974) have emphasised that rarely is a single enzyme rate-controlling in a pathway. They have developed a theoretical method to analyse fluxes through pathways (called Metabolic Control Theory by Kell et al., 1989).

This theory defines the 'flux control coefficient' of a step in a pathway as the fractional change in flux through the pathway induced by a fractional change in the catalytic pathway capacity of the enzyme under study. In other words, the flux control coefficient is defined as the fractional change in pathway flux divided by the fractional change in enzyme concentration (or activity) when changes are infinitesimally small. Therefore, this coefficient expresses the degree to which an individual step influences the overall rate through a metabolic pathway. The sum of the flux-control coefficients of the enzymes in a metabolic pathway equals unity (this is known as the flux-control summation theorem, Kell et al., 1989).

The knowledge of flux-control coefficients of the enzymes in a particular pathway allows the choice of which enzyme(s) to clone, to modify or to deregulate in order to increase flux through that pathway. This will be the enzyme with the highest flux control coefficient.

The determination of these coefficients is not a simple task. Different approaches have been taken (Flint et al., 1980 and 1981; Middleton and Kacser, 1983; Groen et al., 1982) and a very elegant one was taken by Walsh and Koshland (1985). Their method was based on the ability in haploid organisms to modulate the level of an enzyme by placing its expression under the control of a promoter whose strength could be varied.

They put the citrate synthase gene of E. coli (whose product catalyses the reaction between oxaloacetate and the acetyl group of acetyl CoenzymeA that yields citrate in the citric acid cycle) under the

control of the tac promoter. The expression from this promoter was controlled by the level of IPTG in the medium, and the effects of varying citrate synthase levels on growth and pathway flux were studied in cultures using different carbon sources. They found that the flux-control coefficient of citrate synthase was low on glucose- and acetate-containing media, whilst on a solely acetate medium the flux control coefficient approached the maximum value of 1.0. When acetate was the sole carbon source, the carbon flow through the citric acid cycle was almost totally-dependent on the level of citrate synthase.

Therefore, this method is a powerful tool that allows the identification of reactions involved in the control of the rates of metabolic processes. Its application to Streptomyces would be very useful in the comprehension of metabolic regulation in this genera.

During the course of the work described here, attempts were made to develop plasmid vectors for regulated expression of cloned genes in Streptomyces lividans. Such vectors did not exist when this work was started, and they are essential for flux analysis in Streptomyces. The vectors which were constructed were based on the lac operator-repressor system which allows for modulation of gene expression.

1.2 Gene expression in Streptomyces

In a bacterial cell the rate of synthesis of any given protein is determined by the level of the corresponding mRNA and also by the efficiency with which that RNA is translated.

1.2.1 Transcription

It has been found that bacteria such as E. coli, B. subtilis and S. coelicolor contain multiple sigma factors and therefore multiple forms of RNA polymerase holoenzyme (Grossman and Losick, 1986). Each sigma factor (σ) confers on RNA polymerase the specificity to initiate transcription at a specific class of promoters (these have characteristic sequences). Thus, this pattern of multiple holoenzyme forms suggests that the expression of complex sets of genes may be partially coordinated by species of sigma factors that determine the utilization of particular promoters. See 1.2.1(a) (page 22a) for a discussion of promoter/polymerase kinetics.

In E. coli the major sigma factors is approximately 70,000 dalton (σ^{70}). One example of a minor sigma factor comes from studies on the heat-shock response upon a regulatory gene called htpR which codes for a sigma factor, σ^{32} (Grossman et al., 1984; Cowing et al., 1985). Other examples of minor sigma factors in E. coli come from studies of genes controlled by nitrogen availability. The ntrA gene product is a sigma factor that enables RNA polymerase to bind to the glnA (glutamine synthetase gene) promoter (Hirschmann et al., 1985). Also from studies of gene expression in coliphage T4, sigma factor σ^{gp55} enables RNA polymerase to initiate transcription from T4 late promoters (Kassavatis and Geiduschek, 1984).

Westpheling et al. (1985) demonstrated the existence of two distinct forms of RNA polymerase holoenzyme in Streptomyces coelicolor. These differed in their promoter recognition specificities. The holoenzyme containing sigma factor σ^{35} appeared to be analogous to holoenzymes from E. coli and B. subtilis containing respectively σ^{70} and σ^{43}.

These two sigma factors direct initiation from promoters that conform to the sequence of the consensus prokaryotic promoters (Hawley and McClure, 1983). On the other hand, the holoenzyme containing σ^{49} was capable of transcribing from the B. subtilis ctc promoter. This promoter is recognised by B. subtilis σ^{37} which directs transcription initiation of genes that are switched on in post-exponential phase cells (Ollington et al., 1981; Wang and Doi, 1984).

More recently, Buttner et al. (1988) showed that in S. coelicolor at least three forms of RNA polymerase, each associated with a different sigma factor (σ^{28}, σ^{49}, σ^{35}) were involved in the transcription of four different promoters of the agarase gene (dagA).

Westpheling and Brawner (1989) also reported the existence of two distinct transcribing activities involved in the expression of the Streptomcyes galactose operon. One initiates transcription in vitro specifically from $galP_1$, a glucose-repressed and galactose-induced promoter located at the 5' end of the galactose operon. The other initiates transcription specifically from $galP_2$, a low-level constitutive promoter internal to the operon.

It has been shown that a gene family of sigma factors, related to σ^{43} from B. subtilis and σ^{70} from E. coli (the "veg-like" sigma factors) is present in S. coelicolor (Takahashi et al., 1988).

Streptomyces show considerable promoter sequence heterogeneity. It seems possible that, at least partially, this promoter heterogeneity reflects the variability in RNA polymerase holoenzymes in the genera.

Despite the high G + C content of Streptomyces DNA, several promoters identified in the genera are relatively A + T rich and appear to be similar to the prokaryotic consensus promoters. Many of these were reviewed by Hopwood et al. (1986) and more recently other promoters that fall into this category have been identified in Streptomyces. Examples of these promoters are: pIJ101C (Deng et al., 1986) the aml promoters of S. limosus and S. venezuelae (Long et al., 1987; Virolle et al., 1988), the promoters P_1 and P_2 of the 16S rRNA gene of S. coelicolor (Baylis and Bibb, 1988), the tipA promoter (Murakami et al., 1989) and the gal P_1 promoter (Fornwald et al., 1987).

Two promoters of the aph gene of S. fradiae and two promoters of the tsr gene of S. azureus show some similarity to the prokaryotic consensus in the -10 region, but no resemblance in the -35 region (Bibb et al., 1985). The same has been found for promoters P_3 and P_4 of the 16S rRNA from S. coelicolor (Baylis and Bibb, 1988) and for the sph promoter from S. glaucescens (Vogtli and Hutter, 1987). These promoters that lack the conserved -35 region might be transcribed by a different form of RNA polymerase holoenzyme or may require some regulatory factors for initiation of transcription (Cowing et al., 1985).

Finally, other promoters, such as the endoH promoter from S. plicatus (Westpheling et al., 1985) and the vph promoter from S. vinaceus (Bibb et al., 1985), are markedly different from the prokaryotic consensus both in the -10 and -35 regions. These two promoters exhibit homology to the sequence recognised by the B. subtilis sigma factor 37, the sigma factor that recognises the B. subtilis ctc promoter. Westpheling et al. (1985) have shown that recognition of the endoH

promoter is mediated by a minor species of sigma factor, 49. Also reported to be significantly different from the prokaryotic consensus promoters are the sta promoter from S. lavendulae (Horinouchi et al., 1987) which shows homology to the endoH promoter, the hyg promoter from S. hygroscopicus (Zalacain et al., 1986), the gal P_2 promoter from S. lividans (Fornwald et al., 1987) and the dagA P_3 from S. coelicolor, reported to show some homology with the ctc promoter (Buttner et al., 1988.

Tandem promoters occur frequently in Streptomyces genes. These appear not only in antibiotic resistance genes (e.g. the ermE gene of S. erythraeus - Bibb et al., 1985 - and the aph gene of S. fradiae), but also in the dagA and the 16S rRNA gene of S. coelicolor. Multiple forms of RNA polymerase and multiple promoters can provide flexibility in the modulation of gene expression.

Very little is known about termination of transcription in Streptomyces, however it must have features that resemble this process in E. coli since the fd terminator efficiently terminates transcription in Streptomyces (Ward et al., 1986).

Some inverted repeats that can potentially form a hairpin loop structure in the mRNA have been identified downstream from coding regions (Wray and Fisher, 1988) and similar sequences have been shown to have transcriptional termination activity in S. lividans (Deng et al., 1987; Pulido and Jimenez, 1987).

1.2.2 Initiation of translation

In *E. coli* the Shine-Dalgarno sequence and its spacing from the translational initiation codon are among the many factors that play a role in initiation of translation (Gold *et al.*, 1981). In gram-positive bacteria (*Bacillus* and *Staphylococcus*) the free energies of formation of the mRNA-rRNA base pairing seem to be higher than those encountered for *E. coli* ribosome binding sites (McLaughlin *et al.*, 1981; Stormo *et al.*, 1982), i.e. the mRNA sequences show a higher degree of sequence complementarity to 3' and of 16S rRNA.

The nucleotide sequences preceding the translation start codons of many *Streptomyces* genes studied so far show variable complementarity to the 3' end of 16S rRNA (for examples see: Bibb *et al.*, 1985; Deng *et al.*, 1986; Adams *et al*, 1988; Hallam *et al.*, 1988; Virolle *et al.*, 1988) which has been sequenced for *S. lividans* (Bibb and Cohen, 1982) and *S. coelicolor* (Baylis and Bibb, 1988).

These sequences do not have the high level of complementarity found for the two Gram-positive genera studied previously. *Streptomyces* have an S1 like protein in their ribosomes (Roberts and Rabinowitz, 1989). It has been suggested that the inability of *B. subtilis* (and of other Gram-positive species) ribosomes to translate mRNAs from other species may be due to the absence of the largest 30S ribosomal protein found in *E. coli*, protein S1 (Higo *et al.*, 1982).

Roberts and Rabinowitz (1989) have shown that removal of the S1 protein from *E. coli* ribosomes results in translational behaviour that shows preferential activity on mRNA from Gram-positive sources. They

concluded that the S1 protein makes ribosomes able to translate mRNAs with weaker Shine-Dalgarno sequences.

Some Streptomyces genes present a striking contrast to the conventional interaction found in prokaryotes between ribosomes and Shine-Dalgarno sequences in initiation of translation. Horinouchi et al. (1989) showed that the transcriptional start point of the afsA mRNA of S. coelicolor corresponded to the first nucleotide of the ATG translational start codon This is not uncommon in Streptomyces: other genes have been reported to have overlapping transcriptional and translational starts such as:

- the 23S rRNA methylase gene from S. erythraeus (Bibb et al., 1985).
- the streptothricin acetyl transferase gene from S. lavendulae (Horinouchi et al., 1987).
- the aminoglycoside phosphotransferase gene from S. fradiae (Janssen et al., 1989).

Interestingly all these genes encode antibiotic resistance determinants, the exception being the afsA gene which product is a diffusable bioregulator involved in secondary metabolism (Hara and Beppu, 1982).

Therefore, expression of these genes must be enhanced at the onset of antibiotic production. Horinouchi (1989) suggested that their unusual translation mode might be related to an unknown mechanism of differential expression.

1.3 The lactose operon: a brief introduction (Dickson et al., 1975; Lewin, 1985)

When cells of E. coli are grown in the absence of a B-galactoside (e.g. lactose) they contain very few molecules of the enzyme B-galactosidase (which can cleave the lactose molecule into glucose and galactose, which can then be metabolised).

However, when an appropriate substrate is added to the media, new enzyme molecules are quickly synthesised.

Three genes map in the lactose operon:

- lacZ, which codes for the enzyme B-galactosidase (a 500,000 dalton tetramer).
- lacY, which codes for the enzyme B-galactosidase permease (a 30,000 dalton membrane-bound protein).
- lacA, which codes for the B-galactosidase transacetylase.

This cluster, lacZYA, is transcribed into a single mRNA from a promoter (the lac promoter) just upstream from the lacZ gene (Fig. 1.1).

A regulator gene, lacI, lies upstream from lacZ and forms an independent transcriptional unit. This gene codes for a repressor protein (a tetramer of four identical subunits of 38,000 dalton each) which binds a DNA sequence(s) (see following Section) downstream from the lac promoter, the lac operator(s) (lacO).

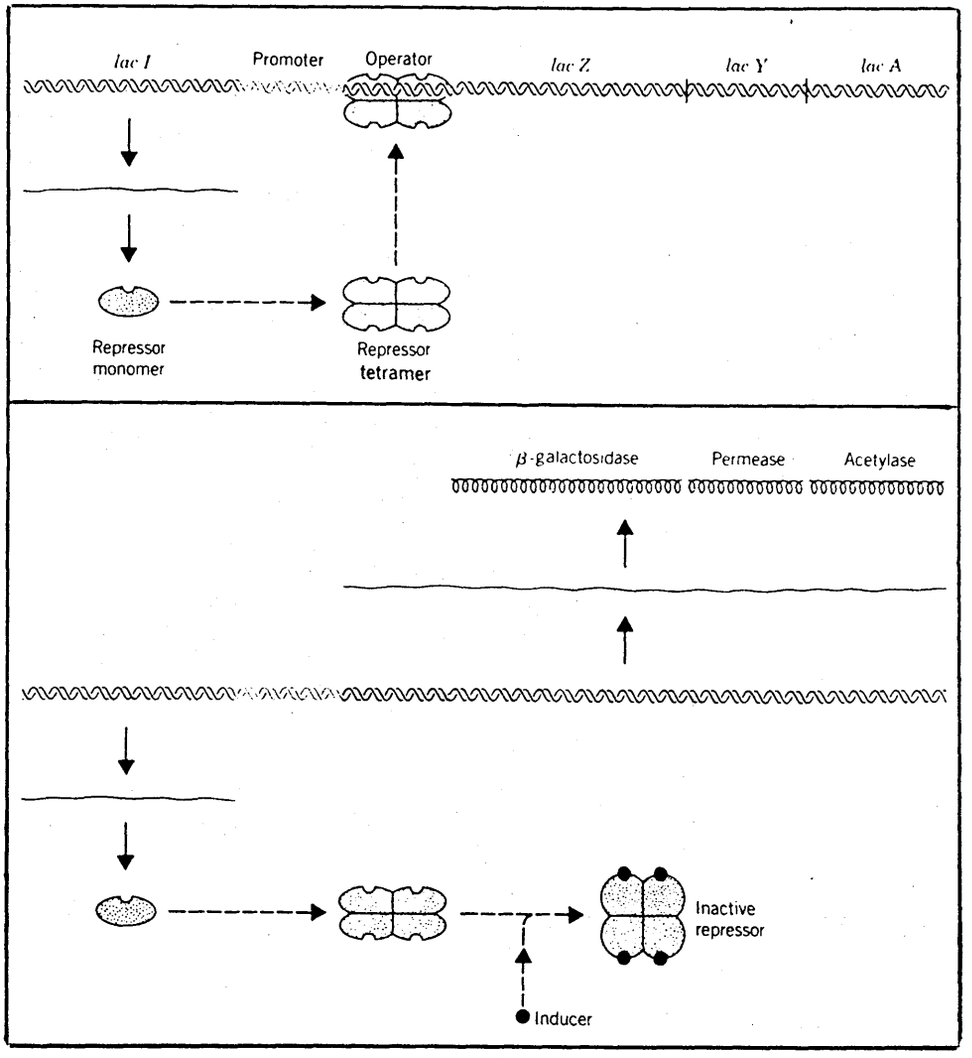


FIG.1.1 The original Jacob-Monod model of negative regulation of the lac operon.

The lacI gene encodes the repressor protein that binds to the operator sequence. This prevents transcription of the Z, Y and A genes which encode the lactose-metabolising enzymes.

However, when allolactose is present in the cell it binds to the repressor protein preventing it from binding to the operator. (taken from Lewin, 1983)

When the repressor binds at this site, it prevents transcription initiation by RNA polymerase at the lac promoter. This repression can be lifted by the action of an inducer molecule. This is a substrate or a substrate related molecule that is able to bind the repressor molecule changing its conformation, decreasing its affinity for DNA.

Isopropylthiogalactoside (IPTG) is an inducer of the lac operon. It resembles the natural inducers for B-galactosidase, but cannot be metabolised by the enzyme. The natural inducer of the operon is allolactose, a product of a molecular rearrangement which results in the transfer of a galactose molecule (one product from lactose breakdown by B-galactosidase) to glucose (the other product of lactose breakdown).

1.3.1 The lac repressor and the lac operator

The understanding of the recognition of specific DNA sequences by proteins constitutes a major problem in molecular biology. The determination of the crystal structures of DNA binding proteins such as the trp repressor (Scheritz et al., 1985) and the lambda proteins cro (Anderson et al., 1981) and cI (Pabo and Lewis, 1982) has led to comprehension of the interaction of these proteins with their respective DNA binding sites. A common feature of all these proteins is a helix-turn-helix structure on the protein. The first helix sits above the groove near the DNA backbone while a second helix fits partly or completely into the major groove (for a review: Pabo and Sauer, 1984).

The sequence from the lac repressor has been aligned with the protein of other DNA-binding proteins and it shares homologies with these in a region that spans through the helix-turn-helix domain (Sauer et al., 1982).

Recently, Boelens et al. (1987) using two-dimensional nuclear magnetic resonance showed that the second helix of the lac repressor makes contacts with the major groove of the lac operator.

In E. coli operators are generally located close to the promoters:

1. Downstream of the start transcription, as in the lac system (Gilbert et al., 1976).

2. Upstream of the promoter, as in the gal system (Adhya and Miller, 1979).

3. Overlapping with the promoters, as in the trp operon (Bennett and Yanofsky, 1978).

Irani et al. (1983) have proposed a novel repressor binding scheme for the galactose operon of E. coli. In their model, the tetrameric molecule of gal repressor binds to the operator upstream the promoter and to another operator that is within the first structural gene of the gal operon, and thus downstream of the promoter. Interactions between the repressor molecule and the two operators form a loop in the DNA which is important in the efficient blockage of transcription.

Similarly, Dunn *et al.* (1984) have reported the requirement for a second binding site in the *ara* system.

In the *lac* operon of *E. coli*, a second *lac* operator was detected 401 base pairs downstream from the first operator (Reznikoff *et al.*, 1974).

Since then several reports (Herrin and Bennett, 1984; Berse *et al.*, 1986; Mossing and Record, 1986) have illustrated the possibility of enhancing repression by introducing an additional *lac* operator sequence upstream from the *lac* promoter.

Mossing and Record (1986) replaced the *lacZ* gene with the *galK* gene on a plasmid and studied the effect of additional *lac*-operators placed at three different distances (118, 185 and 283bp) upstream from the *lac* promoter region. They reported a five-fold increase in the repression of *galK* when the second operator was placed 185bp away.

Eismann *et al.* (1987) showed that in *E. coli* the introduction of mutations into the DNA sequence of the second *lac* operator "*in vivo*" decreased repression of the *lac* operon five-fold. It has also been demonstrated (Kramer *et al.*, 1987) that the *lac* repressor tetramer binds to two *lac* operators on a linear DNA fragment, causing the intervening DNA fragment to form a loop. However, it remains to be determined how supercoiling and salt concentration may influence loop formation under physiological conditions.

How the second *lac* operator exerts its function is a question still to be answered. A bit more is known about the *lac* operator situated just

downstream from the promoter. In the lac operon the centre of symmetry of the lac operator is located 9 base pairs downstream from the start of transcription (Gilbert et al., 1976). Various methods have been used to determine the area of the DNA occupied by the lac repressor. DNase I protection experiments, for example, showed the protection of an area which extended several base pairs upstream the transcriptional start (Galas and Schmitz, 1978).

Identical experiments with RNA polymerase indicated that the area protected by RNA polymerase overlapped the area protected by the lac repressor (Reznikoff and Abelson, 1978).

Therefore, it was believed that the repressor, by binding to the operator was shielding the promoter sequence from being recognised by RNA polymerase.

However, Straney and Crothers (1987) have reported that the repressor inhibits RNA synthesis by a variety of mechanisms, not necessarily the exclusion of polymerase binding in the presence of repressor. They showed that, not only did the lac repressor increase, by more than 100-fold, the initial binding of RNA polymerase to E. coli lac UV5 promoter DNA, but also that the lac repressor can bind to the lac UV5 promoter at the same time as RNA polymerase. Their kinetic data suggests that the repressor acts by blocking the isomerization step in transcription initiation, not by blocking close complex formation. They suggested that the lac repressor "traps" the polymerase at the promoter, holding it in readiness for transcription when needed.

1.3.1(a) Positive regulation of lac promoter. (See page 22b)

1.3.2 The use of the lac promoter in expression vectors

The earliest use of the lac promoter system for the overproduction of protein was that made by Backman et al. (1976) and Backman and Ptashne (1978) on lambda repressor, the product of the cI gene of phage lamdba.

Then it became clear how useful these systems would be for the overexpression of genes in E. coli, and also the variety of parameters that affect expression of a gene. Inserting a restriction fragment bearing the lac promoter 200 base pairs upstream from the cI gene did result in higher levels of repressor, but not as expected. This was due to post-transcriptional problems. Backman's solution to this problem was to try different placements of the promoter relative to the cI gene and the best result was obtained when the promoter was closest to the gene.

Since then a variety of different vectors have been described for achieving the regulated, high-level expression of cloned genes in E. coli (Powels et al., 1985). These vectors can be used to identify the products of cloned genes, produce large amounts of a particular protein, study gene function "in vivo" etc. The ability to regulate expression of a cloned gene can be very important if the gene product is detrimental to the host cell, or for biochemical studies.

Amongst these vectors, many carry the lac promoter or the strong synthetic tac promoter.

The tac promoter (ptac) (de Boer et al., 1983; Amman et al., 1983) or trp-lac hybrid promoter is a strong consensus E. coli promoter. It consists of the -35 region of the trp promoter (a consensus -35 region) separated by 16 base pairs from the -10 region of the lac UV-5 promoter (a consensus -10 region) (Gilbert, 1976), and of a synthetic ribosome binding site. This promoter can be repressed by the lac repressor and be derepressed with IPTG.

The lac operator-repressor control system has been transferred into other genera and the lac repressor and operator functioned as transcriptional regulatory elements.

In B. subtilis the lac operator was placed downstream from the promoter for a penicillinase gene, creating a hybrid promoter controllable by the E.coli lac repressor, while the E. coli lac repressor gene was under the control of a promoter and ribosome binding site that allowed expression in B. subtilis (Yansura and Hemmer, 1984). The expression of the penicillinase gene was modulated by IPTG.

Tsuchiya and Morinaga showed that the lac operator repressor system was functional in the gram-positive Brevibacterium lactofermentum. lac gene expression was inducible by the addition of IPTG and functional repressor was produced from a lacI gene containing its original promoter.

More surprising are the reports that lac repressor can regulate expression from hybrid promoters containing a lac operator in

mammalian cells (Brown *et al.*, 1987; Hu and Davidson, 1987) and that IPTG can lift the repression.

The aim of this project was to build a plasmid vector(s) for regulated expression of cloned genes in *S. lividans*. As stated before, this vector would be a very useful instrument in flux studies in *Streptomyces*.

1.2.1(a) Kinetics of promoter/RNA polymerase interactions

The frequency of RNA chain initiation has been measured "in vivo" for some promoters of E. coli (Hawley et al., 1982).

The steps that precede initiation of RNA synthesis are:

$$R + P \underset{K_B}{\rightleftharpoons} RPc \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RPo$$

where R and P are free enzyme and promoter, respectively. These two species are at equilibrium with an intermediate (RPc, the "closed" complex) that undergoes a relatively slow isomerization to the transcriptionally active open complex.

The key intermediate in promoter function is RPc, the closed complex. This species undergoes the essentially irreversible isomerization to productive open complexes. It is also at equilibrium with free enzyme and free promoter. Therefore the overall frequency with which open complexes are formed depends on the fractional saturation of RPc and on the rate of RPc conversion to RPo.

An "in vitro" estimation of promoter strength requires an estimate of the two functional paramaters that characterize open-complex formation, K_B and k_2.

Hawley et al. (1982) have classified promoters into four functional classes:

- Class 1 - strong promoters are characterized by high K_B values and high isomerization rates.

- Class II - this class is limited in initiation frequency by relatively low binding constants. At high enzyme concentrations ($>10^{-7}$M) they can initiate as frequently as Class I.

- Class III - the weak promoters have poor binding constants and slow isomerization constants.

- Class IV - the fourth class is limited primarily by the isomerization rate.

1.3.1(a) Positive regulation of the lac promoter

Several loci in E. coli (and also in other bacteria) are subject to a positive control in which a high level of cyclic AMP is required for activity.

A large number of carbohydrates, including glucose, are taken up in Enterobacteriaceae via a phosphoenol pyruvate-dependent sugar phosphotransferase system. This system also regulates the level of intracellular cAMP in the cell (Postma, 1986).

The initial observations implicating cyclic AMP in regulation came from studies of the suppression of synthesis of catabolic enzymes when glucose was offered as an alternative carbon source (Postma, 1986).

The addition of extracellular cyclic AMP overcame this suppression at least partially.

The nucleotide functions by activating CRP (cyclic AMP receptor protein).

The crp gene codes for a protein of 210 amino acids. The purified protein behaves as a dimer and binds both to cAMP and to DNA (Riggs et al., 1971).

The N-terminal domain of CRP carries the binding site for cyclic AMP whereas the C-terminal domain carries the DNA binding site (McKay et al., 1982). Cyclic AMP causes conformational changes that alter the binding of the protein to DNA allowing it to recognize specific sequences (Schmitz, 1981).

The best evidence for its operation is the fact that initiation of lac transcription "in vivo" in the absence of cAMP-CRP is very low. The -10 and -35 regions of the lac promoter seem to be defective in some way and it is interesting that a simple change of -10 region from 5'TATGTT3' (plac) to 5'TATAAT3' (plac UV5) makes the promoter CRP independent (hence ptac is also CRP independent) (Reznikoff and Abelson, 1978). There is evidence to suggest that CRP binds to bases -57 and -66 upstream from the transcription start point (Dickson et al., 1977; Erbright, 1984) and kinetics of the initiation of transcription of plac show that CRP accelerates the initial binding of RNA polymerase (Malan et al., 1984).

Studies "in vitro" suggest that cAMP-CRP stimulates transcription from the lac promoter by a dual mechanism (Malan et al., 1984):

- it displaces RNA polymerase from an abortive alternative configuration;

- it stimulates transcription initiation by speeding up the rate of the initial binding of RNA polymerase to the promoter.

In Streptomyces, several cloned genes (e.g. the gyl operon) are sensitive to glucose repression. This mechanism does not appear to be similar to the one operating in E. coli. Streptomyces do not possess a phosphoenol pyruvate-dependent sugar phosphotransferase system (Sabater, Sebastian and Asensio, 1972) of the type which interfaces between available soluble glucose and the intracellular genetic regulatory system involving cAMP.

In S. coelicolor, glucose is utilised via glucose kinase, and glucose kinase mutants are not subject to glucose repression (Seno and Chater, 1983).

CHAPTER 2

MATERIALS AND METHODS

2. Materials and methods

2.1 Bacterial strains

All strains used are listed below:

Name	Genotype	Reference/Source
E. coli strains		
JM101	*supE*, *thi*, Δ(*lacpro* A, B)/F' *traD36*, *proA*, B, *lacI*^{q} ZΔM15	Yanisch-Perron *et al.* (1985)
DS941	*recF143*, *proA7*, *str31*, *thr1* *leu6*, *tsx33*, *mtl2*, *his4*, *argE3* *lacY*$^+$, *lacZ* ΔM15, *lacI*^{q}, *galK2* *ara14*, *supE44*, *xyl5*	Dave Sherratt
CB51	*dam*$^-$, *ara*, *thi*, Δ(*lacpro*)	Chris Boyd
1400	*supE*, *supF*, *hsdS*$^-$, *recA56* *met*$^-$, *thi*$^-$, λL512	Cami and Kourilsky (1978)
EMG9	*lacI*$^-$, *thi*$^-$, *str*^{R}	NCIMB
JM107 MA	*endA1*, *gyrA96*, *thi* *hsdR17*, *supE44*, *relA1* λ$^-$ Δ(*lacpro*)/F' *traD36*, *proAB*$^+$, *lacI*^{q}ZM15 *mcrAB*	Blumenthal *et al.* (1985)
S.lividans strains		
TK24	*str6*	Hopwood *et al.* (1983)

2.2 Plasmids and bacteriophages

The plasmids and bacteriophages used in this study, including those whose construction are described in this Thesis, are listed in Table 2.1.

2.3 Chemicals

Chemicals	Source
General Chemicals	BDH, Hopkins and Williams, Koch-light Laboratories, May and Baker, Formachem.
Media	Difco, Oxoid.
General Biochemicals	Sigma, Pharmacia, BRL.
Sodium-triisopropyl-napthalene sulphonate.	KODAK
Agarose	BRL
Acrylamide	BDH 'Electran'
Radiochemicals	NEN
Antibiotics	Sigma, Squibb and Sons

2.4 Culture media

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thymine, made up to 1 litre in distilled water and adjusted to pH 7.0 with NaOH.

L-Agar: As L-broth without glucose and the addition of 15g/l agar.

TABLE 2.1

PLASMID/ BACTERIOPHAGE	DESCRIPTION	SOURCE/ REFERENCE
pUC18	-	Yanisch-Perron *et al.*, (1985)
M13mp18/19	-	Yanisch-Perron *et al.*, (1985)
pIJ486	-	Ward *et al.*, (1986)
pIBI24	-	IBI Catalog No. 33860
pIJ2808	pUC19 derivative containing 0.9Kb insert carrying the 3' end of a 16S rRNA from *S. coelicolor*	H Baylis
ptacH ? ≡ GLW II	-	Chris Boyd
pGLW8	-	Fiona Stuart
pGLW49	pUC18 digested with EcoRI and HindIII ligated to pIJ486 digested with the same enzymes.	Chapter 3
pGLW50	pGLW8 digested with EcoRI and HindIII ligated to pIJ486 digested with the same enzymes.	Chapter 3
pGLW51	ptacH digested with EcoRI and HindIII ligated to pIJ486 digested with the same enzymes.	Chapter 4
pGLW53	1.1Kb SspI-SmaI fragment containing *ptac* and most of the *aphII* gene of pGLW51 cloned into the HincII site of pUC18.	Chapter 4
mGLW54	0.3Kb SstI-PstI fragment containing *ptac* and a small portion of the *aphII* gene from pGLW53 cloned into M13mp18.	Chapter 4
pGLW55A	0.5Kb AvaII-BclI fragment containing *placI* and part of the *lacI* gene from ptacH cloned into HincII site of pUC18.	Chapter 5
pGLW56	ExoIII deletion derivative of pGLW55A	Chapter 5
mGLW57	0.1Kb HindIII-EcoRI fragment from pGLW56, containing *placI*, cloned into M13mp19.	Chapter 5

PLASMID/ BACTERIOPHAGE	DESCRIPTION	SOURCE/ REFERENCE
pGLW58	0.1Kb HindIII-EcoRI fragment from pGLW56, containing placI, cloned into pIJ486.	Chapter 5
pX199 4.4	0.4Kb HindIII-SmaI fragment containing the promoter of the ØC31 repressor gene (Sinclair and Bibb, 1988) cloned into the HindIII site of pXE3 13.	M.C.M. Smith
pXE3 13	SCP2 based transcriptional fusion vector containing a promoter-less xylE gene.	T. Clayton (John Innes Institute)
ptac ØC31	0.4Kb HindIII fragment, containing the promoter of the ØC31 repressor from pX199 4.4 cloned into the DraII site (439) of ptacH.	Chapter 6
pGLW61	ptac ØC31 digested with EcoRI-HindIII ligated to pIJ486 digested with the same enzymes.	Chapter 6
pIJ583	pIJ486 carrying bldA as a BglII-PstI fragment of 870bp	Lawlor et al. (1987)
mGLW62	mGLW54 after site directed mutagenesis	Chapter 7
pGLW63	0.3Kb BamHI-HindIII fragment of mGLW62 containing mutated ptac-aphII cloned into ptacH.	Chapter 7
pGLW64	pGLW63 self-ligated after removal of the SmaI-SspI fragment containing the original ptac.	Chapter 7
pGLW65	pGLW64 self-ligated after removal of the 37 base pair fragment flanked by two EcoRI sites.	Chapter 7
pGLW51*	pGLW65 digested with HindIII and PstI (partially digested) ligated to pIJ486 digested with HindIII and PstI (partially digested).	Chapter 7
mGLW51*	0.3Kb PstI-XbaI fragment containing ptac from pGLW65 cloned into M13mp18.	Chapter 7

2YT-Broth: 10g bacto-tryptone, 10g yeast extract, 5g NaCl made up to 1 litre in distilled water.

Minimal Agar: 7g K_2HPO_4, 2g KH_2PO_4, 4g $(NH_4)_2SO_4$, 0.25 trisodium citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar made up to 1 litre in distilled water.

Davis and Mingoli (D&M) Salts (X4): 28g K_2HPO_4, 8g KH_2PO_4, 16g $(NH_4)_2SO_4$, 1g trisodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1 litre with distilled water.

Supplements: When required, supplements were added to the above minimal medium at the following concentrations:

glucose 2mg/ml thiamine vitamin B1 20ug/ml

amino acids 40ug/ml

Yeast extract-malt extract (YEME): 3g Difco yeast extract, 5g Difco bacto peptone, 3g Oxoid malt extract, 10g glucose, 340g sucrose made up to 1 litre in distilled water. After autoclaving add 1/20 volume of 100mM $MgCl_2$. For preparing protoplasts also add 1/20 volume of 10% (w/v) glycine.

R2 Medium: R2A- 44g agar, 0.5g K_2SO_4, 20.2g $MgCl_2.6H_2O$, 5.9g $CaCl_2.2H_2O$, 20g glucose, 6g proline, 0.2g casamino acids, 4ml trace elements solution (Hopwood et al., 1985), made up to 1 litre in distilled water.

R2B- 11.5g MOPS, 10g yeast extract, 203g sucrose, adjusted to pH 7.4 with NaOH, made up to 1 litre in distilled water.

Combine equal volumes of R2A (melted and cooled to 55°C) and R2B plus 1ml of 1% (w/v) KH_2PO_4 prior to use.

2.5 Sterilization

All growth media were sterilised by autoclaving at 120°C for 15 mins; supplements and buffer solutions at 108°C and $CaCl_2$ at 114°C for 10 mins. Amino acid and other heat sensitive solutions were sterilized by filtration.

2.6 Buffers and Solutions

Electrophoresis

10X TBE Buffer pH 8.3: 109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1 litre in distilled water, pH to 8.3.

10X TAE Buffer pH8.2: 48.4g Tris, 16.4g Na acetate, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH adjusted to 8.2 with glacial acetic acid.

Agarose gel loading buffer: 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 25% (w/v) ficoll, 0.5% (w/v) S.D.S., 50mM EDTA.

Sequencing gel loading buffer: 0.1% bromophenol blue, 0.1% xylene cyanol, 10mM Na_2EDTA, 95% (v/v) formamide (de-ionized with a mixed-bed resin).

Northern blotting loading buffer: 50% (w/v) glycerol, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol, 1mM EDTA.

DNA Manipulations

10X Core Buffer: 500mM Tris-HCl (pH 8.0), 100mM $MgCl_2$, 500mM NaCl. Stored at 4°C.

10X Ligation Buffer: 660mM Tris-HCl (pH 7.6), 66mM MgCl, 100mM DTT. Stored at -20°C.

ATP Stock Solution (10mM): 6mg of ATP were dissolved in 0.8ml of dH_2O. The pH was adjusted to 7.0 with NaOH. The volume was adjusted to 1ml with dH_2O. The solution was dispensed into 10ul aliquots and stored at -70°C.

TE Buffer: 10mM Tris-HCl, 1mM EDTA, pH 8.0.

TES Buffer: 20mM Tris-HCl (pH 7.5), 70mM $MgCl_2$, 500mM NaCl. Stored at -20°C.

10X Kinase Buffer: 500mM Tris-HCl (pH7.6), 100mM $MgCl_2$, 50m DTT, 1mM spermidine, 1mM EDTA. Stored at -20°C.

1X Exonuclease III Digestion Buffer: 66mM Tris-HCl (pH 8.0), 0.66mM $MgCl_2$. Stored at 4°C.

1X Exonuclease III Stop Buffer: 0.2M NaCl, 5mM EDTA (pH 8.0). Stored at 4°C.

Phenol: All phenol used in the purification of DNA or RNA contained 0.1% (w/v) 8-hydroxyquinoline and was buffered against 0.5M Tris-HCl (pH 8.0).

Phenol/Chloroform: 50 parts phenol, 49 parts chloroform, 1 part isoamyl alcohol.

Birnboim Doly I (BDI): 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA. Add lysozyme to 1mg/ml immediately before use, if necessary.

Birnboim Doly II (BDII): 0.2M NaOH, 1% (w/v) SDS stored in a plastic container.

Birnboim Doly III (BDIII): 5M KOAc (pH 4.8); mix equal volumes of 3M CH_3COOK and 2M CH_3COOH, pH should be 4.8.

STET Buffer: 8% (w/v) sucrose, 5% (w/v) Triton X-100, 50mM EDTA, 50mM Tris-HCl (pH 8.0).

PEG Solution: 20% (w/v) PEG 8000. 2.5M NaCl.

Column Buffer: 100mM NaCl, 10mM Tris HCl, pH 7.5, 1mM EDTA.

S_1 Nuclease Mapping Solutions

probe denaturing solution: 3.75M NaOH, 0.125M EDTA.

probe elution buffer: 1.5M NaCl, 0.2M NaOH, 5ug/ml single-stranded calf thymus DNA.

hybridization buffer: 40mM Pipes adjusted to pH 7.4 with NaOH, 400mM NaCl, 1mM EDTA, 80% (v/v) formamide (de-ionized with mixed-bed resin). Stored at -70°C.

(5X) S_1 nuclease digestion buffer: 1.4M NaCl, 150mM $NaCH_3COO$, pH 4.4, 22.5mM $Zn(CH_3COO)_2$. Stored at -70°C.

S_1 nuclease stop buffer: 2.5M NH_4CH_3COO, 50mM EDTA. Stored at 4°C.

RNA Manipulations and Northern Hybridization

All solutions were prepared in DEPC-treated dH_2O.

Kirby mixture: 1g tri-isopropylnapthalene sulphonate, 6g 4-amino salycilate (Na salt), 50mM Tris-HCl (pH 8.3), 6ml phenol, made up to 100ml in distilled water.

MFF: 500ul formamide, 162ul formaldehyde (37% v/v), 100ul (10X)MOPS, 283ul H_2O. Made fresh.

100X Denhardt's solution: 10g Ficoll, 10g polyvinylpyrolidone, 10g bovine serum albumin. Made up to 500ml with dH_2O.

20X SSC: 3M NaCl, 0.3M Na_3 Citrate.

10X MOPS: 0.2M Morpholinopropanesulphonic acid, 0.05M sodium acetate, 0.01M EDTA.

10X DNase RQ buffer: 400mM Tris-HCl, pH 7.9, 100mM NaCl, 60mM $MgCl_2$.

Bradford Assay Solutions

Bradford reagent: 100mg of Coomassie Brilliant Blue G-250 was dissolved in 50ml 95% (v/v) ethanol. 100ml of 85% (v/v) orthophosphoric acid was added to the above mixture which was then made up to 1000ml with demineralized water. This was then filtered through Whatman No. 1 paper and stored in a dark bottle.

Standard: 1mg/ml BSA in 150mM NaCl. This was diluted to appropriate concentrations.

Aph II Solutions

Buffer for French pressing: 20mM Tris-HCl, 10mM $Mg(CH_3COO)_2$, 25mM NH_4Cl, 10mM KCl, 2mM DTT, pH 7.5 at 4^o with HCl. The buffer was made 10X concentrated, without DTT and kept at 4^oC. When needed it was diluted and DTT added to the correct concentration.

AphII assay buffer: 66.7mM Tris-maleic acid, 41.7mM $MgCl_2$, 400mM NH_4Cl, 1.7mM DTT, pH adjusted to 7.1 with maleic acid at 4^oC. The buffer was made 5X concentrated and kept at 4^oC. When needed it was diluted and DTT added to the correct concentration.

[γ-^{32}P]ATP solution: 15ul of 50mM ATP, pH 7.2 with NaOH, 1ul of [γ-^{32}P] ATP, made up to 1ml with dH_2O. The specific activity of the [γ-^{32}P] ATP used was 6000 Ci/mmol.

Streptomyces Transformation Solutions

Transformation mix (T-mix): 2.5% (w/v) sucrose, 100mM $CaCl_2$, 2.5mM K_2SO_4, 1 ml trace elements, 50mM maleic acid, adjust to pH 8.0 with 1M Tris. Add 25% (w/v) PEG 1000 before use.

Medium P: 5.73g TES, 103g sucrose, 2.93g $MgCl_2.7H_2O$, 0.5g K_2SO_4, 3.68g $CaCl_2.2H_2O$, 2ml trace element solution. Adjust to pH 7.4 with NaOH and make up to 1 litre in distilled water.

Lysozyme solution: 10% (w/v) sucrose, 25mM TES buffer (pH 7.2), 2.5mM K_2SO_4, 2ml trace elements, 2.5mM $MgCl_2$, 2.5mM $CaCl_2$. Add 0.3mg/ml lysozyme and 0.5ml of 1% (w/v) KH_2PO_4/100ml solution immediately before use.

2.7 Antibiotics and Indicators

The antibiotic concentrations used throughout for both liquid and plate selection were as follows:

NAME	SELECTIVE CONCENTRATION	STOCK SOLUTION	STORAGE TEMPERATURE
Ampicillin	50ug/ml	20mg/ml (water)	-20°C
Kanamycin	5-250ug/ml	20mg/ml (water)	-20°C
Thiostrepton	25ug/ml	10mg/ml (DMSO)	4°C

Stock solutions were added to molten agar, cooled to 55°C.

X-gal (5-bromo-4-chloro-3-indolyl-B-galactosidase) was used in conjunction with the host strains JM101 and DS941 and the pUC and M13mp vectors, providing a screen for plasmids with inserts in the polylinker region. Recombinants containing inserts are generally white while those lacking inserts are blue. X-gal was stored at a concentration of 20mg/ml in DMF at -20°C and added to L-agar plates to a final concentration of 20ug/ml.

2.8 E. coli Growth Conditions

Liquid cultures for transformation and DNA preparations were grown routinely in L-broth at 37°C with vigorous shaking. JM101 liquid cultures for ssDNA preparations were grown in 2YT at 37°C in a roller drum for 5-6 hours.

Growth on plates was on L-agar or minimal media plus supplements; antibiotics were added as required. Plates contained 25mls of agar solution and were incubated overnight. All dilutions were carried out in L-broth. Strains were stored in 20% (v/v) glycerol and 1% (w/v) peptone at -70°C. Inocula from these stocks were streaked out on L-agar plates, incubated and single colonies were re-streaked on selective plates.

Gradient Plates: Levels of kanamycin resistance were assayed on L-agar gradient plates (containing 50ug/ml of ampicillin), according to the method of Bryson and Szybalski (1952).

Square petri dishes with 10cm sides were used and each layer contained 50ml of agar. The bottom layer contained the kanamycin and was allowed to set forming a slant. The top layer contained no kanamycin and was poured over the solidified bottom layer.

When IPTG was added, both layers contained 0.5mM IPTG.

The gradients were allowed to form at 37°C for two hours prior to inoculation. Five or ten ul samples of E. coli cultures grown for 16h on L-broth were applied to the plates and streaked four times with a toothpick across the agar surface from high to low kanamycin levels. The plates were incubated for 16-18 hours at 37°C.

2.9 Streptomyces growth conditions

Liquid cultures for protoplasting, DNA and RNA preparations were grown in YEME for S. lividans strains. Cultures were incubated at 30°C with vigorous shaking for approximately 2 days. The cultures were inoculated from slopes stored at -20°C or spore suspensions also stored at -20°C.

Strains were stored on R2 agar slopes (15mls) which were inoculated and incubated for 4-5 days until sporulation occurred, and then frozen at -20°C. Spore suspensions in 20% (v/v) glycerol were also stored at -20°C.

Growth on protoplast regeneration plates was at 30°C and antibiotics were overlayed on plates after 16-24 hours of incubation. Plates were

further incubated for 3-4 days. S. lividans protoplasts were regenerated on R2 agar plates.

Growth on plates was also on Emerson agar to which antibiotics and IPTG were added as required.

2.10 E. coli in vivo techniques

Transformation with plasmid DNA: Plasmids were introduced to different strains by genetic transformation. An overnight culture of the recipient was diluted 1 in 100 into 20ml L-broth and was grown to a density of approximately 10^8 cells/ml (about 90 min - 2 hours). The cells were harvested (12000g, 5 min, 4°C) and resuspended in 10ml of cold 50mM $CaCl_2$. The cells were pelleted again, resuspended in 1ml of cold 50mM $CaCl_2$ and kept on ice for at least 15 min before use. 200ul aliquots of the competent cells were added to the plasmid DNA, mixed gently and left on ice for up to 1 hour. The cells were heat shocked (2 min, 42°C) and returned to the ice for a further 15 min. An equal volume of L-broth was added and the cells incubated at 37°C for 1 hour to allow expression of the plasmid resistance genes. The cells were plated out on the appropriate selections. For transformation of ampicillin resistant plasmids no expression time was necessary. Unused cells were frozen at -70°C in 20% (v/v) glycerol.

Transfection of JM101 with M13: This followed exactly that procedure described above, except that after heat-shock the cells were incubated on ice for 15 mins, after which 200ul of fresh log phase JM101 culture was added. To this mixture was then added 30ul of IPTG (15mg/ml) and

30ul X-gal (40mg/ml). The cells were then mixed, added to 3ml of molten soft-agar (pre cooled to 47°C), and then plated onto thoroughly dried plates containing D & M minimal agar (containing 5ug/ml vitamin B_1 and 2mg/ml glucose).

2.11 Streptomyces in vivo techniques:

Preparation of protoplasts: 25ml cultures were grown in the appropriate medium for 30-36 hours. Mycelia were pelleted at 12000g for 10 minutes and washed twice in 10.3% (w/v) sucrose. The mycelia were resuspended in 4ml of lysozyme solution and incubated at 37°C for 15-30 minutes. The mycelia were examined microscopically to monitor protoplast formation. Then 5ml of P medium was added, triturated a couple of times and the protoplast were filtered through cotton wool. The protoplasts were pelleted at 12000g for 10 minutes and washed twice in P medium. Protoplasts were resuspended in 4ml of medium P, dispensed into 200ul aliquots and frozen at -70°C.

Transformation of protoplasts: DNA was added to protoplast and within 30 seconds 0.5ml of T mix was added. The protoplasts were diluted in medium P to 2ml after 10 seconds. They were plated out on regeneration plates which had been dried overnight.

2.12 In vitro techniques

Plasmid DNA preparation: three methods were used to obtain DNA from cells.

Birnboim and Doly (1979) DNA preparation: 200ml cultures of stationary phase cells were harvested by centrifugation (12000g, 5min at 4°C). The pellet was resuspended in 4ml of Birnboim-Doly I solution and incubated on ice for 5min for E. coli cultures and incubated at 37°C for 15 min with 100ug/ml lysozyme for Streptomyces cultures. 8ml of Birnboim-Doly II solution were added and the solution left on ice for 5 min before 6ml of cold Birnboim-Doly III solution was added, gently mixed and left on ice for a further 5 min. The cell debris and most chromosomal DNA was removed by centrifugation (32000g, 5 min at 4°C) and the plasmid DNA precipitated by addition of an equal volume of isopropanol followed by centrifugation at 39200g for 15 min. This DNA was further purified by banding on a CsCl/EtBr gradient. The DNA was resuspended in 1 ml of TE and 4.5g of CsCl dissolved in 3.5ml of TE. The DNA and CsCl solutions were added together with 250ul of EtBr (10mg/ml). The gradients were centrifuged in a Beckman VTi65 vertical rotor at 289,000g for 4 hours at 20°C. Two bands were visible, a lower supercoiled plasmid band and an upper chromosomal and relaxed plasmid DNA band. The lower band was removed using a 1ml syringe and the EtBr removed by repeyted butanol extractions (using water saturated butanol). The salts were removed by dialysis in 2 X 500ml 1 X TE. The DNA was then ready for use.

Mini DNA preparation using the method of Holmes and Quigley (1981): 1.5ml of an overnight E. coli culture containing the plasmid of interest was harvested by centrifugation in a 1.5ml eppendorf tube and resuspended in 350ul of STET buffer. 25ul of STET buffer containing lysozyme at a concentration of 10mg/ml was added and the tube vortexed briefly. This solution was boiled for 40 sec and centrifuged in an eppendorf microfuge for 15 min at 4°C. The pellet was discarded using

a toothpick and 40ul of 3M $NaCH_3COO$ and 400ul of cold isopropanol was added, followed by microcentrifugation for 7 min which precipitated the nucleic acid. The pellet was washed twice in 70% (v/v) ethanol and dried briefly in a vacuum drier before being resuspended in 20ul - 50ul 1 X TE. This DNA was suitable for digestion and other *in vitro* manipulations.

Mini DNA preparation using patches of Streptomyces from solid media (a modified Birnboim and Doly, 1979, method): Approximately $2cm^2$ of mycelium grown on solid medium were used. The mycelium was suspended in 250ul of Birnboim-Doly I solution and incubated at 37°C for 1 hour with 1mg/ml lysozyme. 200ul of Birnboim-Doly II were added and the solution left at room temperature for 2-3 minutes and then put on ice for 5 minutes.

150ul of ice-cold Birnboim-Doly III solution was added and gently mixed. The whole solution was put at -20°C for 15 minutes. The cell debris was removed by centrifugation in a microfuge in the cold for 10 min. The supernatant was removed into a clean tube and extracted once with equal volume of saturated phenol and once with equal volume of chloroform.

The clean supernatant was precipitated with ethanol and the dry DNA dissolved in 20-40ul of 1 X TE. This DNA was suitable for digestion with restriction enzymes.

Ethanol precipitation of DNA: The DNA solution was made 0.3M with respect to $NaCH_3COO$ and 2 volumes of cold ethanol added. After mixing, the DNA was precipitated by cooling on ice for up to 1 hour

and pelleted by centrifugation (27000g, 15 min, 4oC for large volumes or 12000g, 15 min, 4°C for small volumes in eppendorf centrifuge tubes). The pellet was usually washed in 70% (v/v) ethanol and dried briefly in a vacuum drier.

Restriction of DNA: Restrictions were usually performed in a total volume of 20ul containing between 0.25ug and 1ug of DNA, 2ul of 10X restriction buffer (provided by enzyme manufacturer) and 1-10 unit/ug DNA of enzyme, the volume being made up with distilled water. For larger scale restrictions the volumes were scaled up accordingly. The reactions were allowed to proceed for 1 to 2 hours at the appropriate temperature. Reactions were stopped either by the addition of gel loading buffer or by rapid heating to 70°C for 5 min followed by rapid cooling on ice.

Ligation of DNA fragments: The restriction fragments to be ligated were mixed such that the insert was in 3 times molar excess over the vector (10 times excess for blunt and ligations) and made up to 20ul by the addition of 2ul 10 X ligation buffer, 2ul 4mM ATP and distilled water. T4 DNA ligase was added (0.01 units/ug DNA for 'sticky' end ligation and 1 unit/ug for 'Blunt' end ligation) and the solution ligated for 4 hours at room temperature (or overnight at 16°C). Aliquots of the ligation mix were used to transform competent cells.

Filling recessed 3' ends of double-stranded DNA: Protruding 5' ends were filled in using the DNA polymerase activity of the Klenow

fragment of E. coli DNA polymerase I. The reaction was set up as follows:

restriction fragment (up to 1ug)	10ul
2.5mM solution of all dNTPs	1ul
10 X sequencing buffer	2.5ul
Klenow fragment of DNA polymerase	1U
dH_2O	up to 25ul

The reaction was incubated at 37°C for 20 minutes and then extracted once with phenol and once with chloroform, prior to precipitation with ethanol.

Total RNA preparation (Hopwood et al., 1985a): RNase is a very persistent enzyme and precautions were taken against contamination of equipment and solutions. All glassware was incubated overnight at 300°C. Distilled water was treated with DEPC (0.1% [v/v] of total volume) overnight and autoclaved. All solutions were made up from DEPC-treated water and with previously unused chemicals.

100ml cultures of Streptomyces mycelia were harvested by filtration. The mycelia were resuspended in 12g of 0.45mm glass beads, 5mls of Kirby mix, vortexed for 2 mins and centrifuged at 6000g for 5 mins. The supernatant was poured off into an equal volume of phenol/chloroform, vortexed and centrifuged at 6000g for 5 mins. The aqueous phase was removed and again added to an equal volume of phenol/chloroform, vortexed and centrifuged at 6000g for 5 mins. The aqueous phase was removed and precipitated in an equal volume of isopropanol and 1/10 volume of 3M $NaCH_3COO$. The isopropanol solution

was centrifuged at 12100g for 5 mins and the pellet resuspended in 1ml of DEPC-treated dH_2O.

To the total nucleic acid preparation 1/10 volume of (10X) DNase buffer was added, followed by RNase-free DNase [DNase RQ (Promega)] to 50 U/ml. The reaction mixture was incubated at 37°C for 20-30 minutes then extracted with an equal volume of phenol/chloroform solution. The aqueous phase was precipitated with 1/10 volume of sodium acetate and equal volume of isopropanol. The dryed pellet was redissolved in DEPC-treated dH_2O.

The concentration and purity of the RNA preparations were checked by taking O.D. absorbance readings at 260nm and 280nm.

$1A_{260}$ = 40ug/ml

A_{260}/A_{280} = 2.1 indicates a preparation almost free of protein.

Preparation of labelled probes:

(a) "Random primed" DNA labelling method.

Labelling of fragments of DNA with ^{32}P followed the procedure of Feinberg and Vogelstein (1983 and 1984) and a Boehringer Mannheim kit was used. The labelling reaction was set up in the following way:

25-50ng of denatured DNA fragment (denatured by heating for 10 min at 95°C and subsequent cooling on ice).

1ul of each unlabelled dNTP, from 0.5mM stocks.

2ul of reaction mixture (contained the hexanucleotide mixture and the 10X concentrated reaction buffer).

5ul (50uCi) of [α^{32}P] dCTP (800 Ci/mmol).

1ul (2U) of Klenow enzyme

made up to 20ul total volume with dH_2O.

The reaction mixture was incubated at 37°C for 30 minutes, and stopped by heating to 65° for 10 minutes.

(b) <u>End-labelling of oligonucleotide probes</u>

Synthetic oligonucleotides were ethanol-precipitated, vacuum-dessicated and resuspended in dH_2O. Their concentrations were determined using spectrophotometry (an A_{260} of 1 is equivalent to 40ug/ml of single-stranded DNA).

The following reaction mix was used:

10X Kinase buffer		4ul
oligonucleotide		300ng (30pmoles)
gamma-^{32}P-ATP (6000 Ci/mmol)		5ul (50uCi)
T_4 polynucleotide Kinase		1ul (10U)
dH_2O	to	40ul

The reaction was incubated at 37°C for 1 hour.

Sephadex-G50 column chromatography:

Random-primed and oligonucleotide probes were separated from unincorporated radioisotopes by gel filtration on Sephadex-G50 columns (20cm in length, 0.5cm in diameter). 5ul of Dextran Blue (50mg/ml) and 5ul of Phenol Red (50mg/ml) were added to the reaction mixtures after the reactions had been completed. The samples were then loaded onto Sephadex-G50 columns previously equilibrated with column buffer (100mM NaCl, 10mM Tris HCl, pH 7.5, 1mM EDTA). Fractions of approximately 500ul were collected. Those containing the Dextran Blue contained label incorporated into DNA by the "random primed" method. For oligonucleotide probes, fractions eluted with and just after the Dextran Blue were pooled. 1ul of the pooled fractions was added to 1ml of dH_2O and subjected to Cherenkov scintillation counting. Specific activities of the probes were usually around 10^6-10^7 cpm/ug. Both types of probe were boiled prior to their use, for 5 minutes.

2.13 Gel electrophoresis

Both DNA and RNA were visualized on horizontal neutral agarose gels. 0.8% (w/v) gels were most commonly used and 1-2% (w/v) gels were used for fragments of <1.5Kb.

Mini gels were used for rapid analysis of DNA digested with restriction enzymes or after precipitation steps. A BRL model H6 gel kit was used. 0.16g agarose was added to 20ml of TBE, boiled and cooled to 60°C. EtBr was added to 200ng/ml and the molten agarose poured into a 7.6cm X 5.1cm gel caster and an 8 well slot former (4.1 X 0.8mm wells) was placed in position. After the gel had set the slot

former was removed and the gel placed in the tank with 500ml of TBE. The gel was electrophoresed for 30-45 mins at 100V. The separated DNA molecules were visualised on a 302nm UV transilluminator.

Two types of full-size gels were used to isolate and to estimate the size of DNA molecules:

1. A Yale gel tank (23cm X 12.5cm) was used. 150-200 mls of agarose made up in TBE or TAE, was poured into the gel apparatus with the required slot former in place. When the gel was set, the slot former was removed and overlayed with the appropriate buffer on the gel to prevent it drying out during electrophoresis. The gel was loaded with samples and electrophoresed at 70V overnight or at 200V for 3-4hrs. The separated DNA molecules to be isolated from the gel were visualised with a 302nm hand held UV lamp or on the 302nm UV transilluminator.

2. 200ml gels - These gels were made by pouring 200ml of molten agar containing 200mg/ml EtBr into a 16.5 X 23cm gel former with a 20 space well former. The gels were run in TAE or TBE buffer in a gel tank with a capacity of 3 litres overnight at 20V. The DNA molecules were visualized on a 302nm UV transilluminator.

Photographing of gels. Ethidium bromide stained gels were viewed on a 302nm UV transilluminator and photographed using Polaroid type 67 land film or using a Pentax 35mm SLR loaded with Ilford HP5 film. Both cameras were fitted with Kodak Wratten filters (No. 23A).

Extraction of DNA from agarose gels using Geneclean. A Geneclean kit from Bio 101 was used. After staining, the gel was placed on a 302nm UV transilluminator and the band of interest excised. The agarose chip was added to 2-3 volumes of "NaI" solution, then heated at 55°C for a few minutes until the agarose had completely dissolved. 5ul of "glassmilk" suspension was then added, rapidly mixed, and placed on ice for 5 minutes. The mixture was spun (5 seconds in a microfuge) and the supernatant was carefully discarded. The pellet was washed three times in ice-cold "NEW" solution by adding 500ul, resuspending the pellet, respinning and discarding the supernatant. After the final wash, care was taken to remove all traces of "NEW" solution. DNA was eluted from glass beads by adding 20ul TE (pH 8.0) and incubating at 55°C for 5 minutes. The glass beads were pelleted by spinning for 30 seconds then the supernatant was removed carefully. The supernatant was respun to ensure the complete removal of all glass beads. The DNA was suitable for restriction, ligation, end-labelling etc. The compositions of the "NaI", "Glassmilk" and "NEW" solutions are undisclosed in the manufacturers instructions.

Sequencing gels. 6% (w/v) denaturing polyacrylamide gels were most commonly used and made up from the following stocks - 40% (w/v) acrylamide stock (acrylamide; bisacrylamide; 19:1), urea, 10X TBE.

6% acrylamide gel

15ml 40% (w/v) acrylamide

54g urea

10ml 10X TBE

Made up to a final volume of 100mls in dH_2O. The urea was dissolved by heating at 37°C and the sequencing gel solution stored at 4°C. Before pouring the gel, 300ul of 10% (w/v) APS and 50ul of TEMED were added to 50ml of the stock solution.

Preparation of glass plates and pouring the gel: The plates (40cm X 20cm) were cleaned thoroughly with alcohol and water and assembled using two spacers and taped together. The gel solution was poured from a beaker down one edge of the plates while tilting the plates at an angle of about 30°. The gel was laid at an angle of 5° and the comb inserted to the top of the well teeth. The gel polymerized at room temperature usually within 30mins.

Electrophoresis of sequencing gels: The gel was pre-electrophoresed for 30min at a constant power of 40W. Prior to loading, the samples were heated to 95°C for 5 mins, placed on ice and loaded on to the gel. 6% (w/v) gels were electrophoresed for 1.75-2hrs to read the first 100 nucleotides and for 4.5-5hrs to read up to 400 nucleotides.

2.14 Northern hybridisation

1.5% (w/v) agarose gels containing formaldehyde were prepared and run according to the methods of Maniatis et al. (1982). For each 100ml of agarose solution required, 1.5g of RNase-free agarose was melted in 73ml of DEPC-treated water. The solution was allowed to cool to about 60°C, whereupon 10ml of 10X MOPS buffer (Section 2.6) and 16.2ml of 37% (w/v) formaldehyde were added and the gel cast immediately. RNA solutions were denatured by the addition of 8 volumes of MFF (Section

2.6), followed by heating for 15min at 60°C. One volume of loading buffer was added before the gel was loaded. The gels were run at about $10Vcm^{-1}$ in 1X MOPS (which was recirculated) until the bromophenol blue dye front approached the end of the gel.

The formaldehyde gel was placed face down on 3 sheets of Whatman 3MM paper soaked, and in contact with 20 X SSC. A piece of Hybond-N nylon membrane (Amersham) cut to the size of the gel was pre-soaked in 20 X SSC and placed on top of the gel ensuring that no air bubbles were trapped. This was covered by further layers of Whatman 3MM paper and 3-4 layers of absorbent nappy pads cut to size. The stack was topped with a glass plate weighted down with two 500ml bottles of water. Transfer was allowed to proceed overnight. After blotting the nylon membrane was briefly washed in 2 X SSC and allowed to dry in air. The membrane was then wrapped in Saran Wrap and irradiated (RNA side down) on a 302nm UV transilluminator for 2-5 minutes.

Pre-hybridisation fluid (composition shown on Table 2.2) was added to nylon filters sealed into plastic bags and these were prehybridised for at least 2 hours (usually 4 hours) at the appropriate temperature with gentle agitation. Approximately 1ml of prehybridisation fluid was added per $5cm^2$ of membrane, and prehybridisation was performed at the same temperature at which hybridisation was to be carried out.

Hybridisation was carried out in the same solution, except that the probe was added to the membrane by opening up the bag and resealing. The time of hybridisation was usually 16-18 hours and it was performed with gentle agitation.

COMPONENT (ml)	for "random-primed" probes	for oligonucleotide probes
20 X SSC	10	10
0.4M $NaPO_4$ (pH7.0)	2	2
50% (w/v) Dextran sulphate	4	4
100 X Denhardt's solution	2	2
0.25M EDTA (pH8.0)	1.6	1.6
10% (w/v) SDS	1	2
Formamide	14	8
dH_2O	5.4	10.4
TOTAL VOLUME	40 ml	40 ml

TABLE 2.2: Composition of the prehybridisation/hybridisation mixture.

The membranes were washed at the appropriate temperature at the appropriate stringency. The washed filters were sealed in plastic bags and exposed at -70°C Kodak X-Omat film in an autoradiography cassette using an intensifying screen. Film was developed using a Kodak X-Omat automatic processor.

2.15 DNA sequencing

DNA sequencing reactions were performed on single-stranded M13 templates using the di-deoxy chain termination technique (Sanger et al., 1977). All template preparations, solutions and reaction conditions were as described in the "M13 Cloning/Dideoxy Sequencing Instruction Manual" published by Bethesda Research Laboratories.

DNA sequencing reactions were also performed on plasmids. The same technique was used but, in this case, "Guidelines for quick and simple Plasmid Sequencing" published by Boehringer Mannheim was followed.

2.16 S_1 nuclease mapping

2.16.1 High resolution S_1 nuclease mapping using single-stranded DNA probes

Preparation of the single-stranded probe

Single-stranded probes were prepared by synthesising a radio-labelled complementary strand using single-stranded M13 templates (Fig. 2.1).

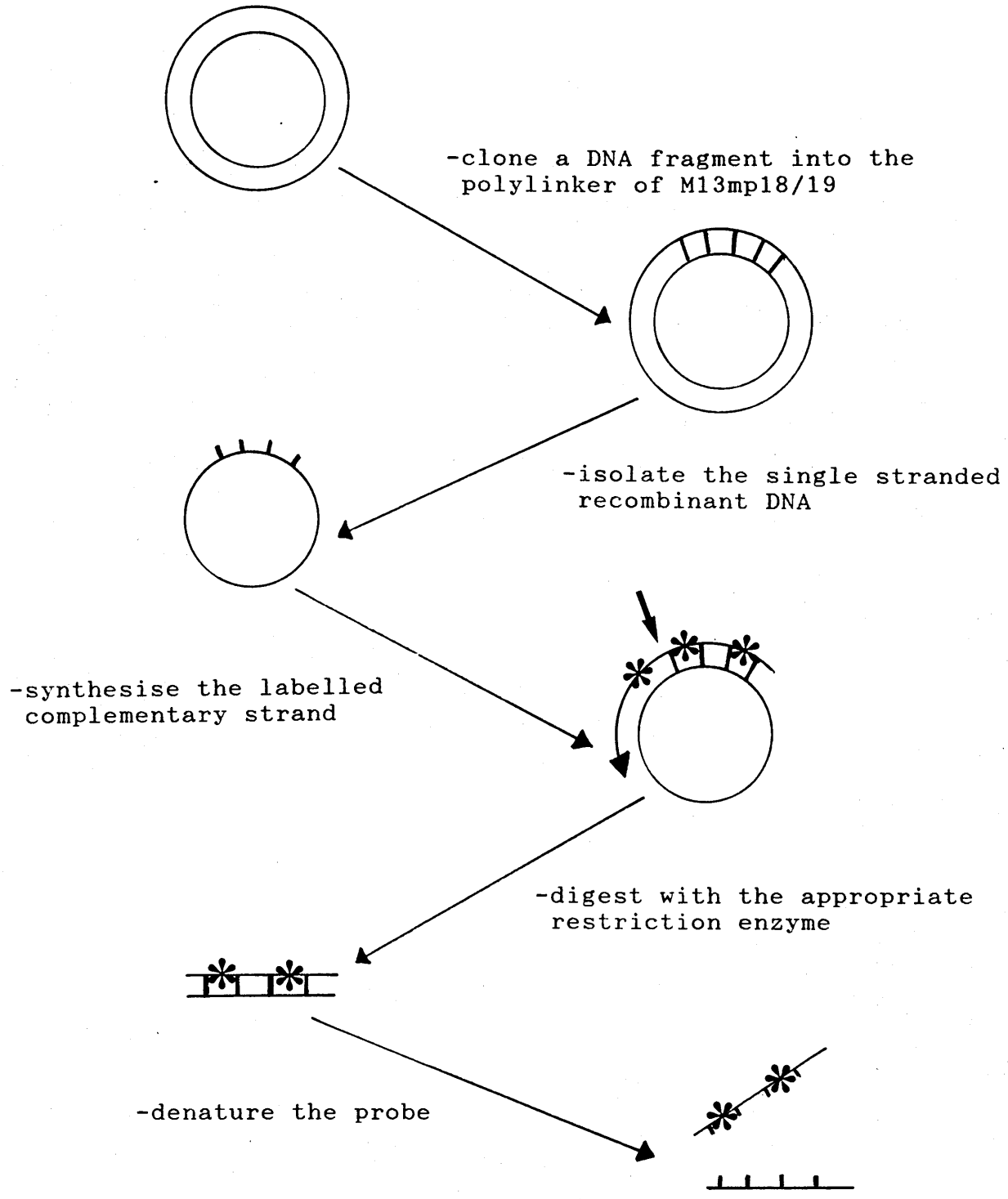


FIG.2.1 Flow chart of single stranded probe preparation for high resolution S1 nuclease mapping.

The primer annealing reaction was performed at 50°C for 5 minutes and then placed at 37°C for 10 minutes. The composition of the annealing reaction mix was:

1 - 2ul	SS template (1ug/ul)
2ul	M13 17-base sequencing primer (BRL) (5ng/ul)
21 -22ul	dH_2O
4ul	10X sequencing buffer (or core buffer)

After annealing 4ul of 0.5mM solutions of the nucleotides dGTP, dCTP and dTTP together with 50uCi (5ul) of labelled dATP (800Ci/mmol) and 1U of Klenow were added to the reaction mix. The reaction mixture was incubated at 37°C for 30 minutes, 4ul of 0.5mM cold dATP and 1ul (1U) of Klenow were then added and the incubation continued at 37°C for a further 30 minutes.

14ul of the appropriate restriction buffer and 10 U of the appropriate restriction enzyme were then added to the reaction mixture and incubated at the correct temperature for two hours.

The reaction mixture was denatured by the addition of 4ul of probe denaturing solution and run overnight on a 2% (w/v) TAE gel, at 50V.

The position of the probe band was monitored by placing Kodak X-omat film, wrapped in foil, directly on Saran Wrap that was in contact with the gel.

Isolation and purification of the probe from the gel

When the probe band position was identified a cut was made on the gel just in front of it. A strip of DE81 Whatman paper was placed in this cut and the gel was then run for 30 minutes at 150V.

The DE81 paper with the bound probe was placed in a 1.5ml Eppendorf tube with 300ul of probe elution buffer, was mashed with a syringe needle and centrifuged for 1 minute. The supernatant was transferred into a clean Eppendorf tube.

200ul of fresh elution buffer was added to the DE81 paper and the paper was remashed with the buffer. After centrifugation for 1 minute the clean supernatant was pooled with the one obtained from the first wash. Finally the pooled supernatant was centrifuged for 2-3 minutes to exclude any DE81 paper.

Enough 3M sodium acetate, pH 5.5, was added to the supernatant to bring the pH of the supernatant to 7. The probe was precipitated with ethanol and dissolved after drying in 50-100ul of DEPC-treated water.

Hybridisation

10ul of probe were precipitated with 10-200ug of total RNA, using ethanol. The dried pellet was resuspended carefully in 25ul of hybridisation buffer. The sample was incubated at 85°C for 5 minutes in a dri-block and left at 37°C overnight.

S_1 nuclease digestion

300ul of S_1 nuclease digestion buffer containing 100-200 units of S_1 nuclease, 5ug native calf thymus DNA and 5ug of single-stranded calf thymus DNA were added to each sample. The reactions were run at 37°C for 30min. 75ul of S_1 nuclease stop buffer and 10ug of tRNA were added to each sample before extracting once with phenol.

The aqueous phase was precipitated with ethanol and the dried pellets resuspended in 4ul of sequencing gel loading dye. After heating at 95°C for 3-5 minutes, the samples were placed on ice and loaded onto a denaturing polyacrylamide gel.

2.16.2 Low resolution S_1 mapping

Preparation of double-stranded probes

Probes were prepared by end-labelling the DNA strand complementary to the mRNA at its 3' end using [α-^{32}P]dNTP and the Klenow fragment of *E. coli* DNA polymerase. 2-4ug of DNA were digested (in a total volume of 20ul) with a suitable enzyme that produced protruding 5' termini.

This was labelled by addition of 2 0uCi of the appropriate [α-^{32}P]dNTPs (800Ci/mmol) and of 1 Unit of Klenow fragment, and incubated at room temperature for 15 minutes (this method is illustrated in Fig. 6.5).

After denaturation of the enzyme at 70°C for 15 minutes, 25ul of the appropriate restriction buffer and 10U of the appropriate second

restriction enzyme were added to the reaction mixture and incubated at the temperature advised by the manufacturer for 1 hour.

The reaction mixture was denatured by the addition of 8ul of probe-denaturing solution and run overnight on a 1.5% (w/v) TAE gel at 50V.

From this step onwards, the technique followed was exactly the same as for high resolution S_1 mapping with two exceptions:

1. After precipitation of the probe together with the RNA, the pellet was dissolved in 15ul of 3M trichloroacetate, 50mM Pipes pH 7.0, 5mM EDTA (at 45°C in concentrated chaotropic salt solutions, RNA:DNA hybridization is very efficient but DNA-DNA duplexes remain completely denatured - Murray, 1986).

   After heating at 65°C for 5 min, the samples were incubated at 45°C overnight.

2. The final pellets were dissolved in 10ul of water and loading buffer was added to each. The samples were run on a 1% (w/v)agarose gel in TBE. The gel was dried and autoradiographed.

2.17 Unidirectional digestion with exonuclease III (Henikoff, 1984)

20ug of plasmid were digested with two restriction enzymes:

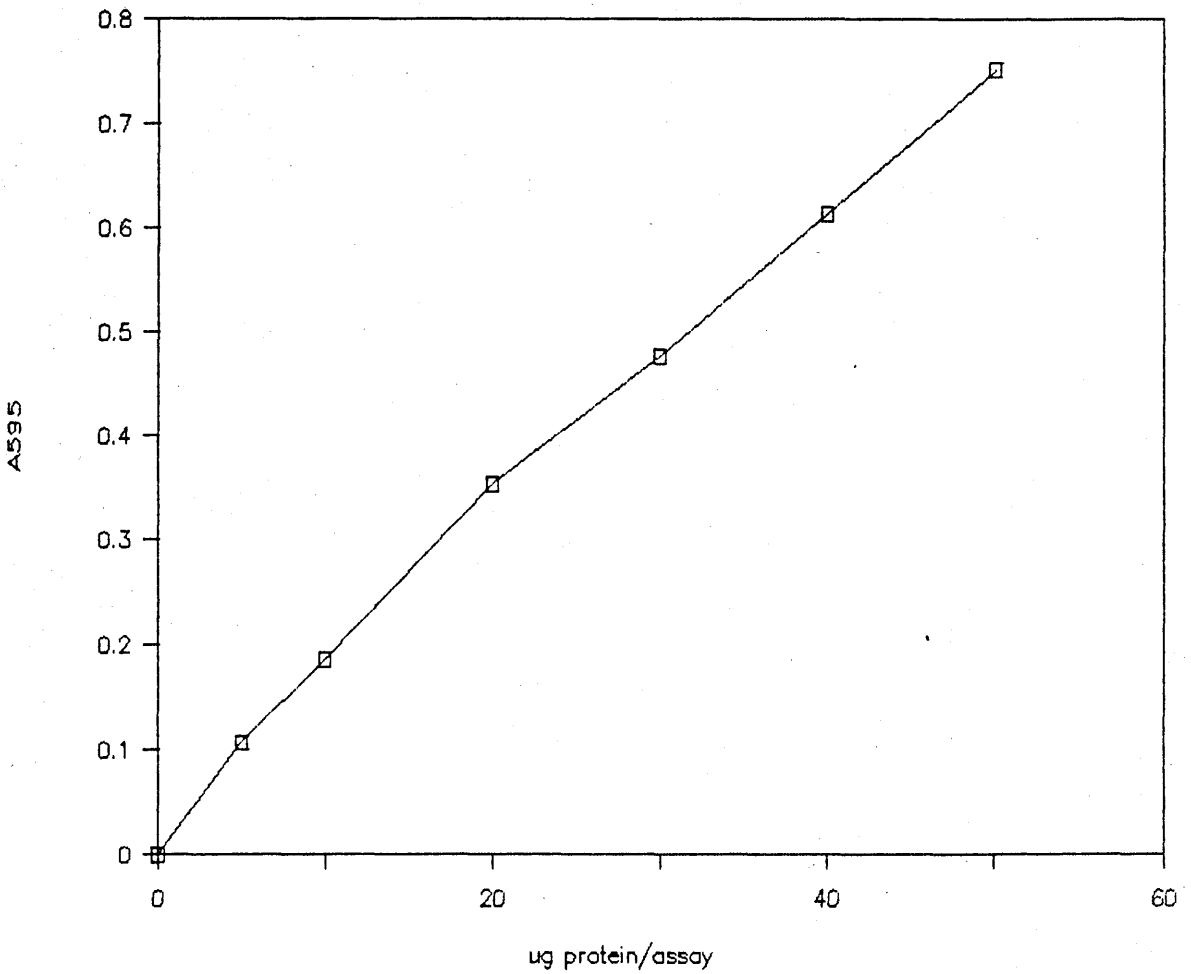
(a) one generating 5' overhanging ends.

(b) one generating 3' overhanging ends.

The sample was precipitated and resuspended in 100ul of ExoIII digestion buffer. 650 units of Exo III were added and digestion proceeded at 30°C. 7.5ul aliquots were removed at 40 second intervals and mixed with 22.5ul of ExoIII stop buffer and incubated at 70°C for 10 minutes. The samples were precipitated, dried and resuspended in 40ul of S_1 digestion buffer containing 4 Units of S_1 nuclease and incubated at 37°C for 20 minutes. 6ul of S_1 stop buffer was added to each sample and the aliquots extracted with 25ul of phenol followed by 25ul of chloroform. The samples were precipitated using ethanol and resuspended in 10ul of a 20mM Tris-HCl, pH 8.0, 7mM $MgCl_2$ solution. 0.1 unit of Klenow was added and the samples incubated for 2 minutes at 37°C. 1ul of a 0.1mM mix of the four deoxyribonucleotides was added and the incubation continued for a further 2 minutes. 40ul of 1 X ligation buffer with 1 unit of T_4 DNA ligase was added and incubated at 16°C. 20ul of each time point sample was transformed into competent cells of E. coli DS941.

2.18 Bradford protein assay for total protein Estimation

50ul of cell extract or 50ul of an appropriate dilution of the cell extract in 150mM NaCl were added to 2.5ml of Bradford reagent (Bradford, 1976), mixed well, and incubated at room temperature for 10 minutes. The A_{595} was measured. A standard curve was also constructed using a range of BSA concentrations (0-50ug/assay). One of these curves is shown on Fig. 2.2. Only determinations falling within the range of 5-50ug protein/assay were used. Otherwise samples were diluted before assaying.



ug of protein per assay	A595
5	0.106
10	0.185
20	0.354
30	0.477
40	0.613
50	0.751

FIG. 2.2: Example of a standard curve constructed for a Bradford protein assay using a range of BSA concentrations.

2.19 Aminoglycoside phosphotransferase (AphII) assay

The method used was described by Haas and Dowding (1975). Enzyme activity is assayed by means of a phosphocellulose paper binding assay which measures transfer of radio-label from a suitable co-factor ([γ-^{32}P]ATP) to the antibiotic, in this case kanamycin.

Preparation of crude cell extracts:

E. coli and S. lividans cells were harvested during active growth (growth curves of S. lividans are shown in Fig. 4.3) and were washed twice with 25ml of Davies and Mingoli salts. The washed cells were resuspended in 2-3ml of French pressing buffer and French pressed (in an SLP Aminco motorized press) at 16,000 psi (the Streptomyces cells were pressed twice). 1.5ml of the pressed extract was then centrifuged in a microfuge for 10 minutes to remove the cell debris. 1ml of clear crude extract (kept on ice) was put into a clean tube and used for AphII and Bradford assays.

Each assay contained:

10ul of AphII assay buffer (Section 2.6)
10ul of [γ-^{32}P]ATP solution (Section 2.6)
2ul of Kanamycin (1mg/ml)
10ul of extract

The mixture was incubated at 35°C for 20 minutes, and 10ul samples of each reaction mixture was pipetted onto a premarked piece (approximately 1cm^2) of Whatman P-81 phosphocellulose paper.

After 30 seconds at room temperature, the papers were placed in 400ml of hot (70-80°C) distilled water for 4 minutes. The papers were then dried, placed in scintillation vials with 1ml of scintillation fluid and counted.

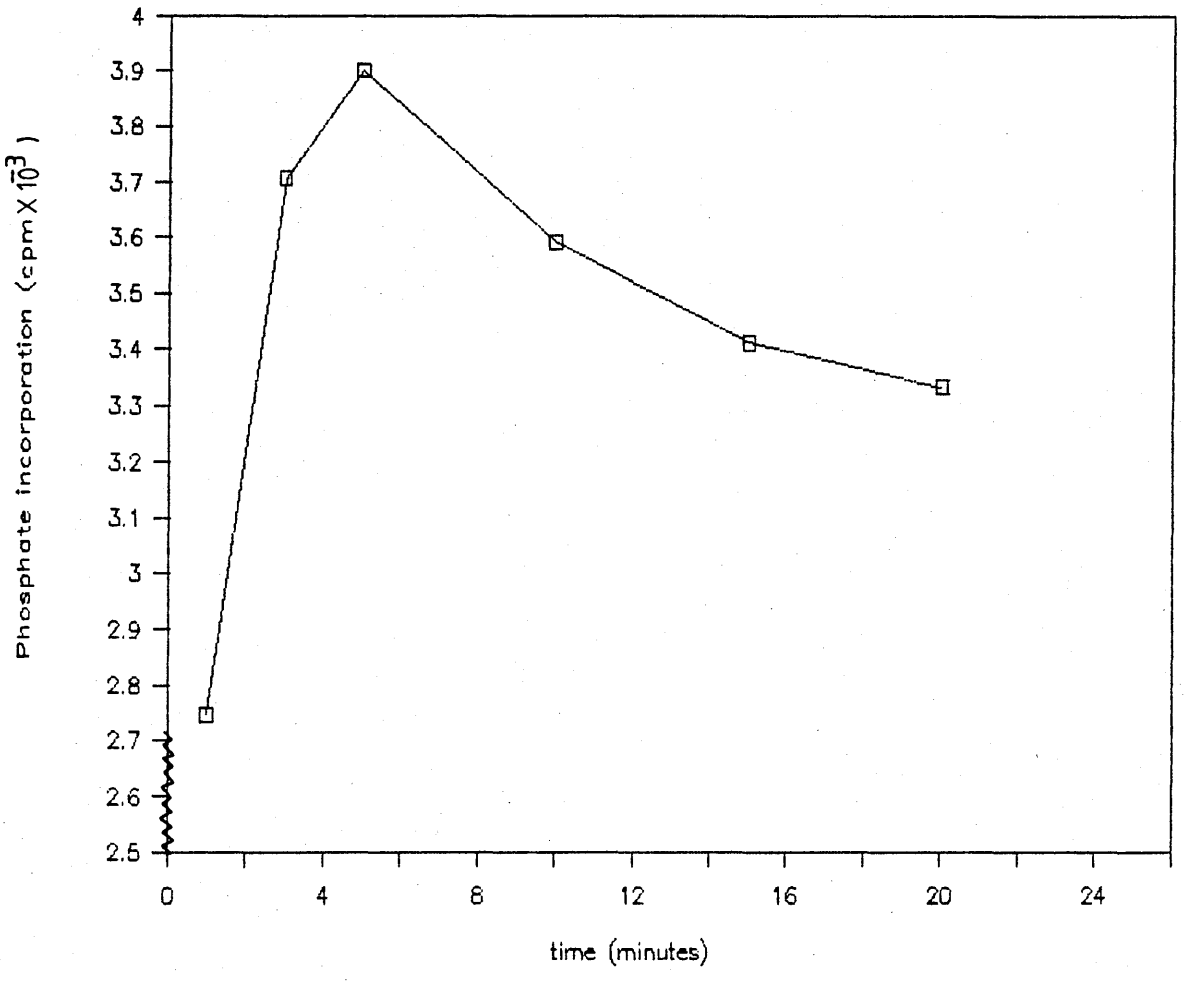
Validation of the techniques for E. coli extracts

Using cell extracts from E. coli 1400 containing pGLW50, the best assay conditions for E. coli transformants were determined.

Initially the assay was performed using the above conditions. Triplicate reactions were set up and 3ul samples of the reaction mixture were taken at various time points. Neither at 35°C nor at 20°C was the incorporation of γ-^{32}P into kanamycin linear with time (Fig. 2.3).

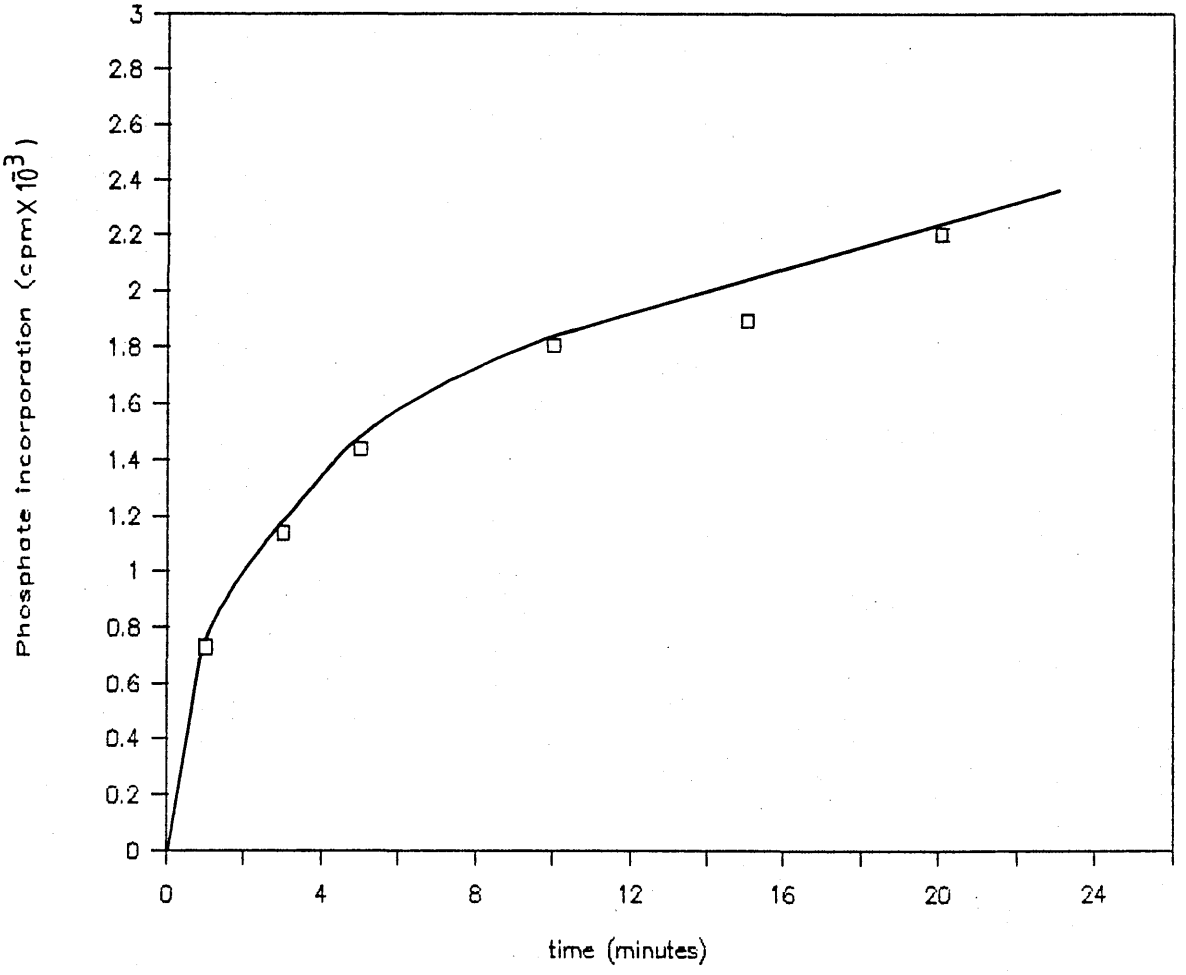
At 35°C the incorporation, after reaching a maximum value (around 5 minutes) decreased. This could be due to phosphatases present in the extract which were also active at 35°C.

To try to slow down the reaction, the assay was then performed at 0°C. In this way the incorporation of ^{32}P into kanamycin proved to be linear for the first 5-6 minutes. Fig. 2.4 shows that by doubling the amount of extract (enzyme), the velocity of incorporation doubled, and that by doubling the amount of kanamycin (substrate) no significant change in the velocity of the reaction occurred.



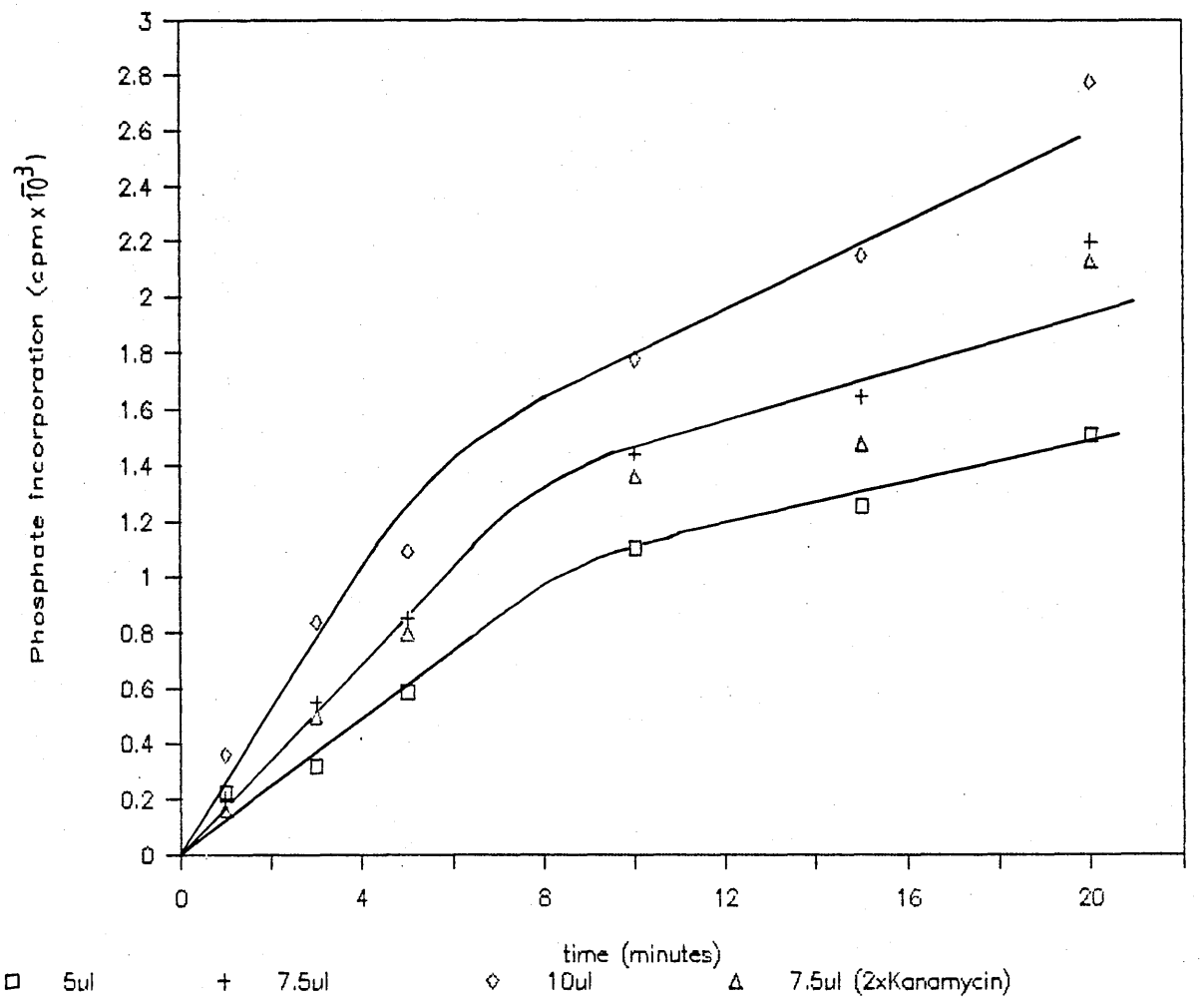
time(min)	Average incorporation (cpm)
1	2747
3	3706
5	3901
10	3593
15	3411
20	3333

FIG. 2.3A: Incorporation of ^{32}P into kanamycin, at 35°C, using 10ul of crude extract of E. coli 1400 containing pGL50.



time(min)	Average incorporation (cpm)
1	728
3	1136
5	1439
10	1807
15	1892
20	2205

FIG. 2.3B: Incorporation of ^{32}P into kanamycin, at 20°C, using 10ul of crude extract of E. coli 1400 containing pGL50.



time(min)	Average icorporation(cpm)		
	5ul extract	7.5ul extract	10ul extract
1	220	204	360
3	321	545	838
5	587	849	1090
10	1103	1440	1776
15	1250	1644	2148
20	1508	2200	2775

FIG. 2.4: Incorporation of ^{32}P into kanamycin (at 0°C) using different amounts (5, 7.5 and 10ul) of crude extracts of E.coli 1400 containing pGLW50. The assay was also performed with 7.5ul of extract but twice the amount of kanamycin (data shown only on the graph).

Thus, the rate of the reaction was proportional to the concentration of enzyme

Thus, for E. coli extracts the assay was performed on ice for 5 minutes. The assay mixture was composed of:

20ul of AphII assay buffer (Section 2.6)
20ul of [γ-^{32}P]ATP solution (Section 2.6)
4ul of kanamycin (1mg /ml)
5ul of crude extract

Validation of the technique for S. lividans extracts

Using cell extracts from S. lividans TK24 containing pGLW50, the best assay conditions for S. lividans transformants were determined

The assay was performed in triplicate at three different temperatures: 35°C, 20°C and 0°C. 5ul samples were taken at various time points and spotted onto the phosphocellulose paper. The assay mixture contained:

20ul AphII assay buffer (Section 2.6)
20ul of [γ-^{32}P]ATP solution (Section 2.6)
4ul of kanamycin (1mg/ml)
10ul of crude extract

(Note: Before adding the extract, the other reagents were incubated at the appropriate temperature for at least 2 minutes.)

At 35°C, the assay with S. lividans extracts (Fig. 2.5) was linear in the first 5 minutes of incubation, whereas at 20°C and 0°C activity was too low and therefore not detected initially.

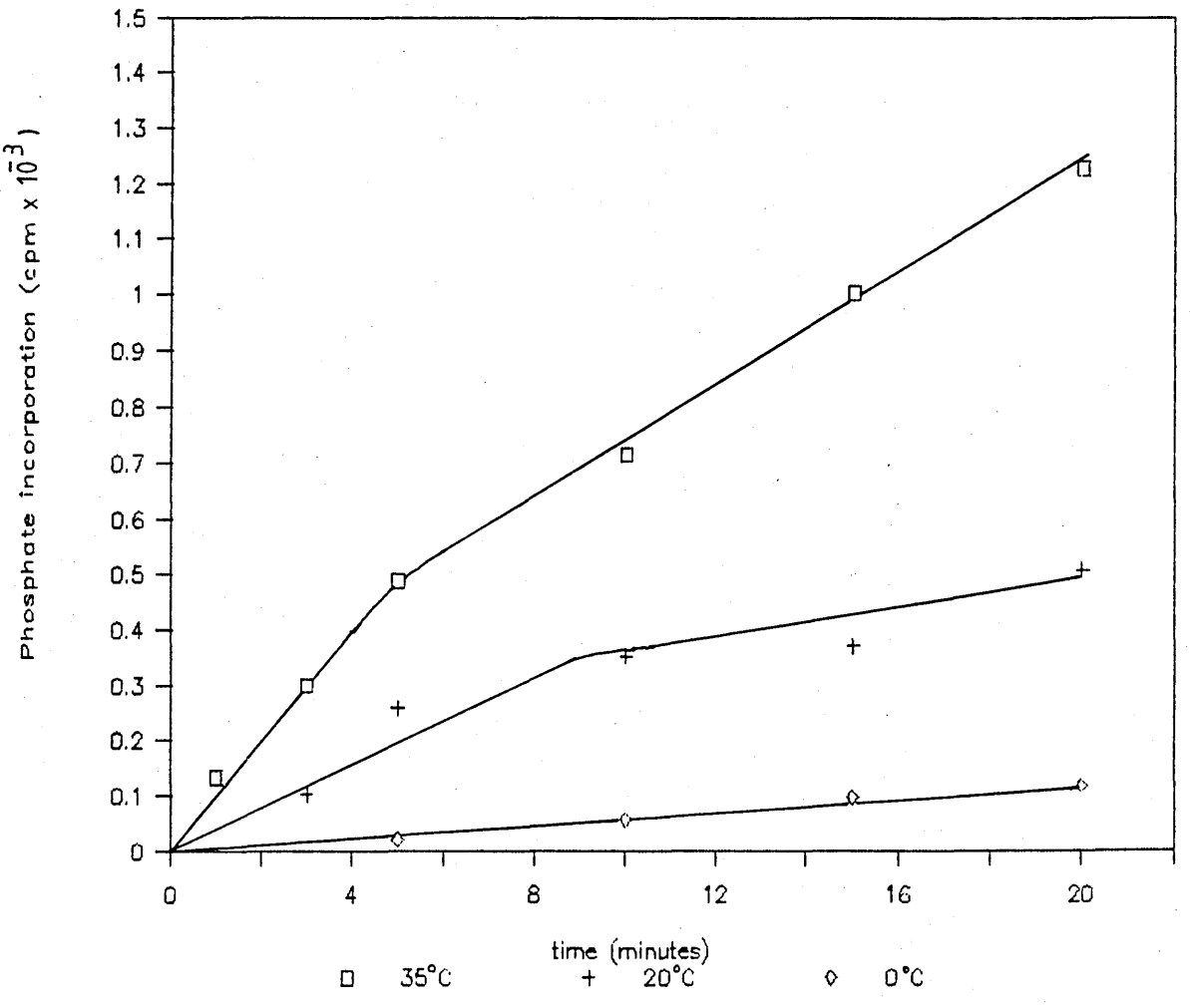
Fig. 2.6 illustrates the proportionality between enzyme concentration and reaction rate. As found for E. coli extracts, the reaction rate doubled when twice the volume of extract (enzyme) was added. This experiment was performed as described above, at 35°C, with 5 and 10ul of crude extracts of S. lividans TK24 containing pGLW50 (the final volume of the reaction was the same, when only 5ul of extract was used, extra 5ul of AphII assay buffer was added to the reaction).

Thus, the assay of S. lividans crude extracts was performed at 35°C for 4 minutes. The assay mixture was composed of:

20ul AphII assay buffer (Section 2.6)
20ul of [γ-^{32}P]ATP solution (Section 2.6)
4ul of kanamycin (1mg/ml)
10ul of crude extract

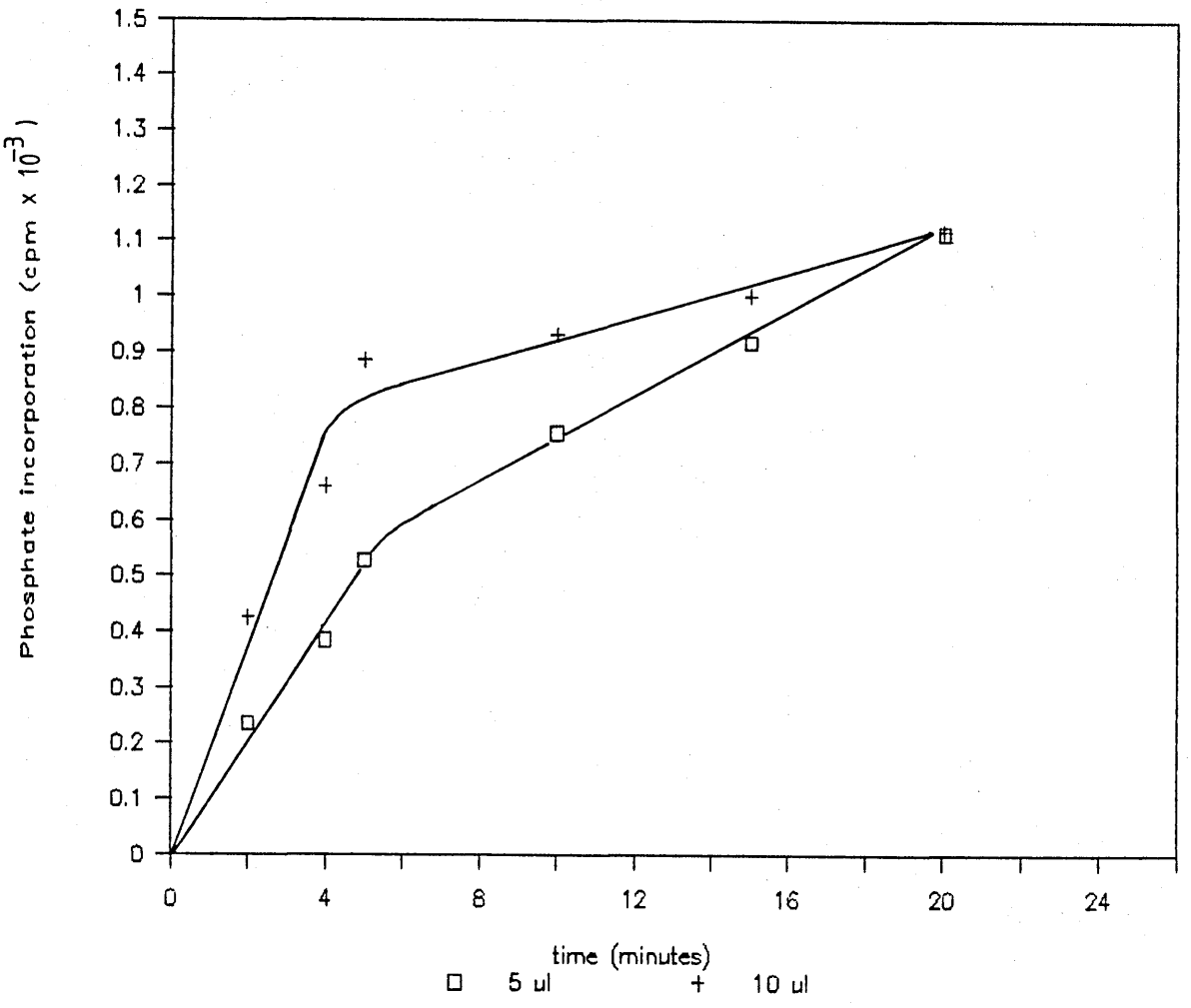
Calculation of AphII specific activities for E. coli and S. lividans extracts.

Each assay was performed in triplicate and an average of counts incorporated per minute was found. Each time the assay was performed triplicate controls containing either no extract or no kanamycin were set up. After incubation these were spotted onto phosphocellulose paper, washed and counted alongside with the other samples. The average counts of the background controls were then subtracted from the average counts obtained for each sample.



time(min)	Average incorporation (cpm) 35°C	20°C	0°C
1	135		
3	299	103	
5	487	258	21
10	714	352	57
15	1002	371	97
20	1227	507	118

FIG. 2.5: Incorporation of ^{32}P into kanamycin (at 35, 20 and 0°C) using 10ul of extract of S. lividans containing pGLW50.



time(min)	Average incorporation (cpm) (5ul)	(10ul)
2	236	425
4	384	660
5	527	885
10	755	932
15	918	1002
20	1115	1119

FIG. 2.6: Incorporation of ^{32}P into kanamycin at 35°C using 5 and 10ul of crude extract of S. lividans containing pGLH50.

To determine the specific activity of the label used each time the assay was performed, 5ul of [γ-^{32}P]ATP solution were spotted onto phosphocellulose and counted without being washed.

AphII activity (A) was calculated using the following equation:

$$\underset{(U/ml)}{A} = \frac{(\text{average sample counts} - \text{average background counts}) \times \text{total reaction volume (ul)}}{\text{volume spotted onto paper (ul)}} \times \frac{1}{\text{number of incubation minutes}} \times \frac{1}{L} \times 100$$

1 Unit = 1nmol of ^{32}P incorporated per minute.

L = specific activity of label used (5ul of [γ-^{32}P]ATP solution were counted and 20ul were used in each reaction. Each reaction contained 0.015 nmole of ATP, therefore

$$L = \left(\frac{0.015}{\text{counts} \times 4}\right).$$

and the specific activity of the crude extract (SA) was calculated using the following equation:

$$\underset{(U/mg)}{SA} = \frac{A}{P}$$

P = amount of protein in assayed sample (mg/ml).

2.20 Site directed mutagenises

This method is described in Section 7.2.

CHAPTER 3

CHOOSING A PROMOTER TO DEVELOP CONTROLLABLE STREPTOMYCETE EXPRESSION VECTORS

3.1 Introduction

Several inducible promoters have been reported in Streptomyces recently.

Fornwald et al. (1987) showed that transcription of the galactose operon in Streptomyces lividans was controlled by two independently-regulated promoters: galP1, which was responsible for galactose-dependent transcription of the operon and galP2, a constitutive promoter.

Smith and Chater (1988) cloned the entire operon for glycerol utilization (glyABX) of Streptomyces coelicolor A3(2) and showed that transcription of the operon was glycerol-inducible and glucose-repressible. It has also been shown that the expresion of the agarase gene (dagA) of S. coelicolor A3(2) is subject to catabolite repression (Kendall and Cullum, 1984; Hodgson and Chater, 1981).

Further examples are a thiostrepton-induced promoter which was found in S. lividans by T. Murakami et al. (1988) and the gene encoding glutamine synthetase (gln A) which has a nitrogen-regulated promoter (Wray and Fisher, 1988).

Control of these promoters (with the exception of the thiostrepton regulated promoter) is necessarily achieved by a change in the nutritional state of the medium because the "inducer" molecule is also a metabolic source for growth.

For flux studies in the pathways that lead to antibiotic synthesis, for *Streptomyces* differentiation studies etc., it would be much simpler if a promoter could be found to be induced by a molecule not involved in catabolism.

In *E. coli*, such a system is well described: repression by binding of the *lac* repressor to the *lac* operator and derepression by action of metabolically inert inducers such as isopropyl-B-D-thiogalatoside (IPTG) (Beckwith and Zipser, 1970; Miller and Reznikoff, 1980). This approach was taken by Walsh and Koshland (1985) who placed a citrate synthase from *E. coli* under the control of the *tac* promoter.

Horinouchi and Beppu (1985) demonstrated indirectly that the *tac* promoter was expressed in *S. lividans*, a finding that was in agreement with the work by Jaurin and Cohen (1984), who showed that *Streptomyces* have an RNA polymerase able to recognize and use various components of *E. coli* transcriptional signals.

In this Chapter attempts to use the *E. coli* promoters *tac* and *lac* (*plac*) in *Streptomyces* will be described. pIJ486 (Ward *et al.*, 1986), a promoter-probe vector, was chosen to analyse the potential promoter activity of these promoters in *S. lividans* TK24.

Promoter-probe vectors have proved excellent tools in the isolation and characterization of transcriptional signals. Several have been developed for studies of gene expression in *Streptomyces*.

Bibb and Cohen (1982) constructed promoter-probe plasmid vectors for *S. lividans*, using expression of *E. coli* chloramphenicol

acetyltransferase gene (CAT) as an indicator of promoter activity. Using these vectors they showed that promoters from both gram-positive and gram-negative bacteria were able to promote expression of the CAT gene in S. lividans.

Horinouchi and Beppu (1985) used a low copy number promoter-probe vector (pARC 1) that allowed the chromogenic identification of transcriptional control signals in S. lividans based on the expression of pigment gene(s). Since the brown pigment is a secondary metabolite (shunt product of actinorhodin biosynthetic pathway), its biosynthesis is possibly affected by physiological conditions.

pIJ486 and pIJ487 have useful characteristics that the above vectors lack. They rely on the expression of a promoter-less aminoglycoside 3'-phosphotransferase II gene (aphII), derived from the transposon Tn5 (Beck et al., 1982), to detect transcriptional activity. Expression of the aphII gene confers resistance to kanamycin and neomycin in both E. coli (Beck et al., 1982) and S. lividans (Foster, 1983). These vectors also have other very useful features:

- a polylinker 5' to the promoter-less aphII gene.

- a terminator, from E. coli phage fd, 5' to the polylinker, preventing transcriptional read-through from vector promoters.

- translational stop codons 3' to the polylinker in each of the three reading frames and 5' to the aphII gene. This ensures that only transcriptional fusions are detected.

More recently other low copy promoter probe vectors have been built for Streptomyces. Forsman and Jaurin (1987) built a vector containing a unique BamHI site just upstream, the ampC B lactamase gene of E. coli. Thus this vector allows for chromogenic identification of promoters in S. lividans by the use of nitrocefin. It also has a transcriptional terminator signal upstream, the cloning site (BamHI) that prevents readthrough transcription from plasmid-borne promoter.

Feitelson (1988) has built a promoter-probe vector that comprises pARC1 as well as the transcriptional terminator and the polylinker of pIJ486. These were inserted just upstream of the reporter gene of pARC1.

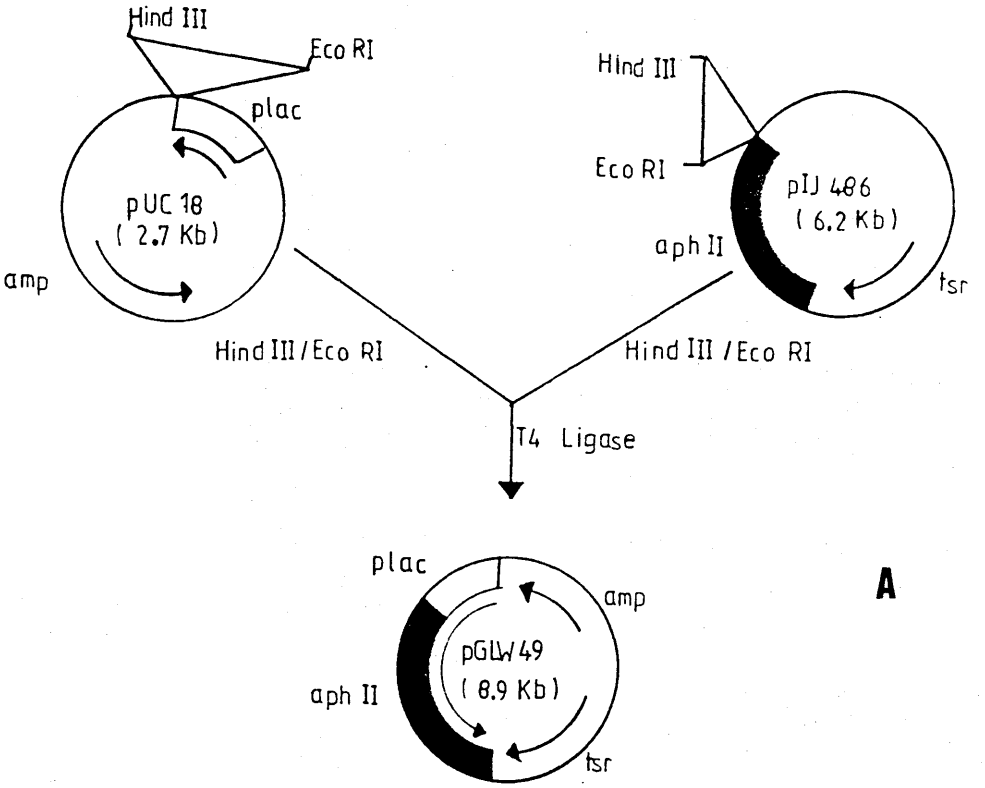
3.2 Results

3.2.1 Construction of pGLW49 and pGLW50

Using pIJ486, two bifunctional vectors for E. coli and S. lividans were constructed.

To obtain pGLW49 (Fig. 3.1A), pUC18 and pIJ486 were digested with EcoRI and HindIII and then ligated. In this way, the lac promoter of pUC18 was placed just upstream of the promoterless aphII gene.

Similarly, pGLW50 (Fig. 3.1B), was obtained by ligating pIJ486 and pGLW8 that had previously been digested with EcoRI and HindIII. In this construct the tac promoter of pGLW8 was placed just upstream of the promoterless aphII gene.



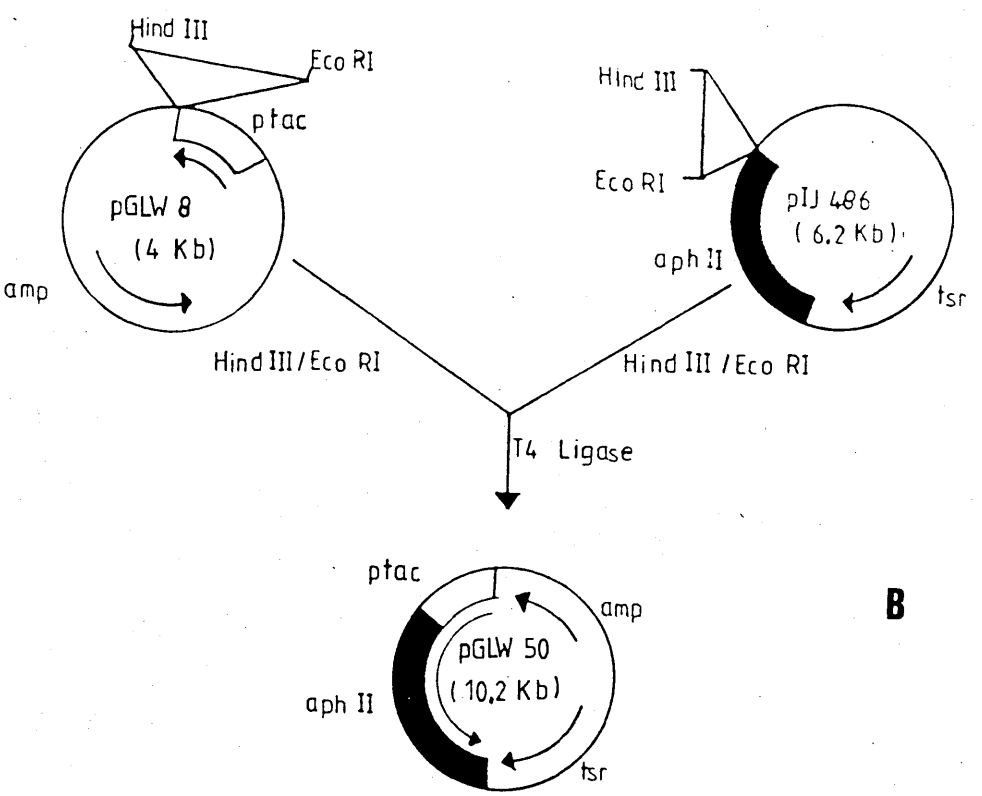


FIG. 3.1: Construction of pGLW49(A) and pGLW50(B).

E. coli 1400 was transformed with the ligation mixtures and transformants were selected on L-agar containing ampicillin. These plates were then replica plated onto L-agar containing ampicillin and kanamycin, and transformants containing both constructs conferred resistance to kanamycin on *E. coli*.

Therefore, mini DNA preparations of pGLW49 and pGLW50 from *E. coli* were used to transform protoplasts of *S. lividans* TK24. These transformants were selected with thiostrepton.

3.2.2 Assay of promoter activity from ptac and plac in Streptomyces

Spores from *S. lividans* TK24 containing pGLW49, pGLW50 and pIJ486 were plated in a dilution series on Emerson agar with thiostrepton, to assess the spore count.

Approximately equal numbers of spores of each recombinant were plated on Emerson plates with kanamycin concentrations, ranging from 0-130ug/ml, and thiostrepton at a concentration of 25ug/ml. Several independent clones of each construct were tested for *ptac* and *plac* activity.

TK24 recombinants containing pIJ486 and pGLW49 did not grow in the presence of kanamycin above 10ug/ml. However, TK24 carrying pGLW50 showed resistance to kanamycin up to 130ug/ml (highest concentration tested).

To ensure that no gross structural changes had occurred in the recombinants, plasmid DNAs were isolated from S. lividans containing pGLW49 and pGLW50 and were used to transform E. coli 1400. In this way it was confirmed that pGLW49 and pGLW50 still conferred resistance to kanamycin in E. coli.

Mini DNA preparations were made from several of these E. coli transformants and they were all shown to contain the expected plasmid: pGLW49 or pGLW50.

3.3 Discussion

The above results led to the conclusion that ptac in pGLW50 was functional in both E. coli and S. lividans. However, plac in pGLW49 failed to confer resistance to kanamycin on S. lividans, while it was functional in E. coli.

What was wanted was a functional promoter that could possibly be controllable in Streptomyces. This was achieved by finding that the tac promoter in pGLW50 was active in S. lividans.

It is not surprising that ptac was recognized as a promoter in S. lividans. Not only do several Streptomyces promoters appear to be similar to the prokaryotic consensus, as for example the two ermE promoters and the associated orfP_1 promoter of S. erythraeus (Bibb, Jannsen and Ward, 1985), but also two Streptomyces promoters from plasmid pIJ101 (Kieser et al., 1982), pIJ101C (Deng et al., 1986) and

pIJ101A (Buttner and Brown, 1987) have been found to promote transcription from the same start point in *E. coli* and *Streptomyces*.

Jaurin and Cohen (1985) as well as Forsnan and Jaurin (1987) have isolated from *S. lividans* various *Streptomyces-E. coli* type promoters (SEP) that show transcriptional activities in both genera. These promoters resemble typical *E. coli*-type promoters in base composition and structural organization. In addition it has been known that at least one type of *Streptomyces* RNA polymerase holoenzyme can recognize prokaryotic "consensus" promoters from other prokaryotic species (Bibb and Cohen, 1982). Moreover, an RNA polymerase species from *S coelicolor* was isolated by Westpheling *et al.* (1985). It initiates transcription from the *veg* promoter of *Bacillus subtilis*, a promoter which conforms to the *E. coli* consensus sequence (Moran *et al.*, 1982).

Is *plac* not recognized by RNA polymerase in *S. lividans*? Or is it just a very weak promoter?

The sequences of the -10 and -35 regions of *ptac* and *plac* are compared to the -10 and -35 regions of some *Streptomyces* promoters in Fig. 3.2.

From this comparison it is not obvious why *plac* would not be active in *Streptomyces*, but then, at least in *E. coli*, promoter recognition is only one of several parameters that determine promoter strength and activity (Brummer and Bujard, 1987). Even though signals for promoter recognition are within the -10 and -35 sequences, sequence elements outside this region can contribute to promoter strength (Kammerer *et al.*, 1986; Bujard *et al.*, 1987; see also Discussion Chapter 5). Thus, promoters with similar structures can exhibit very different strengths

E. coli consensus	TTGACa - 17 bp - TAtAaT	(1)
Streptomyces consensus	TTGaca - 18 bp - tAGgaT	(2)
pIJ 101A	TTGCGC - 18 bp - CAGACT	(3)
pIJ 101B	TTGACA - 17 bp - CAGTAT	(4)
ptac	TTGACA - 16 bp - TATAAT	(5)
Plac	TTTACA - 18 bp - TATGTT	(6)

(capital letters correspond to highly conserved nucleotides, small letters correspond to significantly conserved nucleotides)

FIG.3.2 The sequences of *plac* and *ptac* are compared to the sequences of the *E. coli* and *Streptomyces* consensus promoters and to the sequences of the *Streptomyces* promoters pIJ 101A and B.

(1) Hawley and McClure (1983)

(2) Hopwood *et al* (1986)

(3) Buttner and Brown (1985)

(4) Buttner and Brown (1987)

(5) de Boer *et al* (1983)

(6) Dickson *et al* (1975)

due to alternate optimization. Therefore other features of plac could be affecting its expression in Streptomyces.

S_1 mapping of the lac promoter, using RNA of S. lividans containing pGLW49 could be used to distinguish between lack of transcription from plac and lack of translation of the plac-aphII message in S. lividans.

Again, comparing the spacing between plac and the aphII message in pGLW49 and ptac and the aphII message in pGLW50 (Fig. 3.3), there seems to be no obvious reason for a translational problem in pGLW49.

Calcutt and Cundliffe (1989) constructed a plasmid (pLST801) that has the aminoglycoside phosphotransferase gene from Tn5 under the transcriptional control of the lac promoter.

pLST801 was used as template for coupled transcription-translation by fractionated cell extracts of different Streptomyces species. This suggested that "in vitro" a form of Streptomyces RNA polymerase initiated transcription from plac (or from some other sequence in pLST801 which might act as a promoter in Streptomyces) and that the lac/aphII mRNA was translated by extracts containing Streptomyces ribosomes.

It is possible that the promoter driving aphII transcription in pLST801 is plac. If this is the case it is probable that the aphII gene is not expressed in S. lividans containing pGLW49 due to some restriction to transcription or translation in this plasmid.

Streptomycetes do not possess a phosphoenol pyruvate-dependent sugar phosphotransferase system (Sabater, Sebastian and Asensio, 1972) of the kind which, in *E. coli*, interfaces between available soluble glucose and the intracellular genetic regulatory system involving cAMP.

Since the *lac* promoter, in *E. coli*, requires cAMP-CRP for transcription and this is not present in *S. lividans*, it might be possible that *plac* cannot be transcribed in this microorganism.

On the other hand, it is also conceivable that *S. lividans* RNA polymerase can start transcription from *plac* independently of cAMP-CRP.

It must also be pointed out that the spacing between the -10 and the -35 regions of *plac* and *ptac* is different (18 and 16 base pairs) (Stefano and Gralla, 1982). They suggested that during the course of binding to RNA polymerase the regions of DNA containing the -10 and -35 sequences must be twisted to allow simultaneous binding to the polymerase. Stefano and Gralla (1982) have shown that the length of the spacer that separates these sequences affects the rate of open-complex formation.

Although *Streptomyces* RNA polymerase is able to express from promoters that have spacers varying from 14-20 base pairs (Hopwood *et al.*, 1986) it is possible that the different spacing of *plac* and *ptac* will affect their expression in *S. lividans*.

(1) dag P1	TTGTCA-18bp-TAGCAT-7bp-A-218bp-GAAGGAG-7bp-GUG
(2) orf P1	CTGCGA-18bp-TAGCAT-7bp-A-26bp -GAACGGA-7bp-AUG
(3) ptac	TTGACA-16bp-TATAAT-6bp-A-26bp - AGGA -11bp-AUG
(4) plac	TTTACA-18bp-TATGTT-6bp-A-27bp - AGGA -7bp-AUG
ptac (pGLW50)	TTGACA-16bp-TATAAT-6bp-A-65bp - AGGA -9bp-AUG
plac (pGLW49)	TTAACA-18bp-TATGTT-6bp-A-67bp - AGGA -9bp-AUG

FIG.3.3 The expression signals of plac in pGLW49 and of ptac in pGLW50 are compared to the expression signals of the Streptomyces genes dagP1,orfP1 and of plac and ptac.

A much more extense list of streptomycete expression signals is given by Hopwood et al (1986).

(1) Buttner,cited by Hopwood et al (1986)

(2) Bibb et al (1985)

(3) de Boer et al (1983)

(4) Dickson et al (1975)

CHAPTER 4

CONTROL OF TRANSCRIPTIONAL ACTIVITY FROM *ptac* IN *STREPTOMYCES*

4.1 Introduction

The tac promoter includes the lac operator region and can, in E. coli, be repressed by the lac repressor (coded by the lacI gene) and induced by IPTG (de Boer et al., 1983).

In Chapter 3 it was shown that the tac promoter was functional in S. lividans.

It was not known whether this promoter could be repressed by the lac repressor in this strain, and if repressed whether it could be induced with IPTG.

In this Chapter it will be described how a bifunctional expression vector was constructed containing not only ptac but also the E. coli lacI gene.

S. lividans and E. coli recombinants containing this plasmid were used to study the induction levels of ptac, using aminoglycoside phosphotransferase II assays.

Attempts to use dot-blot assays to assess levels of aphII mRNA were preceeded by Northern blotting to check if the mRNA was intact.

S_1 mapping of the tac promoter was employed to determine the precise location of the transcriptional start of this promoter both in E. coli and in S. lividans, and to ensure that ptac was the promoter driving the transcription of the aphII gene.

4.2 Results

4.2.1 Construction of pGLW51. Transformation of E. coli 1400 and E. coli EMG9 lacI⁻ with this plasmid.

pIJ486 and ptacH were digested with EcoRI and HindIII and ligated together to make pGLW51 (Fig. 4.1).

E. coli 1400 was transformed with this ligation mixture and recombinants were selected on L-agar containing ampicillin. Replica plates were made onto L-agar containing ampicillin, kanamycin and IPTG. From six recombinants growing on these plates plasmid mini preparations were made and the presence of pGLW51 was confirmed in all of them.

Since 1400 is a lacI$^+$ strain, as a control for possible repression of the tac promoter by repressor coded by the lacI chromosomal copy, E. coli EMG9 lacI$^-$ competent cells were transformed with pGLW50 and pGLW51.

Both recombinant strains of E. coli containing pGLW50 and pGLW51 were grown, with and without 0.5mM IPTG, on plates containing increasing concentrations of kanamycin and ampicillin at a concentration of 100ug/ml. Results from these experiments are given on Table 4.1.

These results showed that:

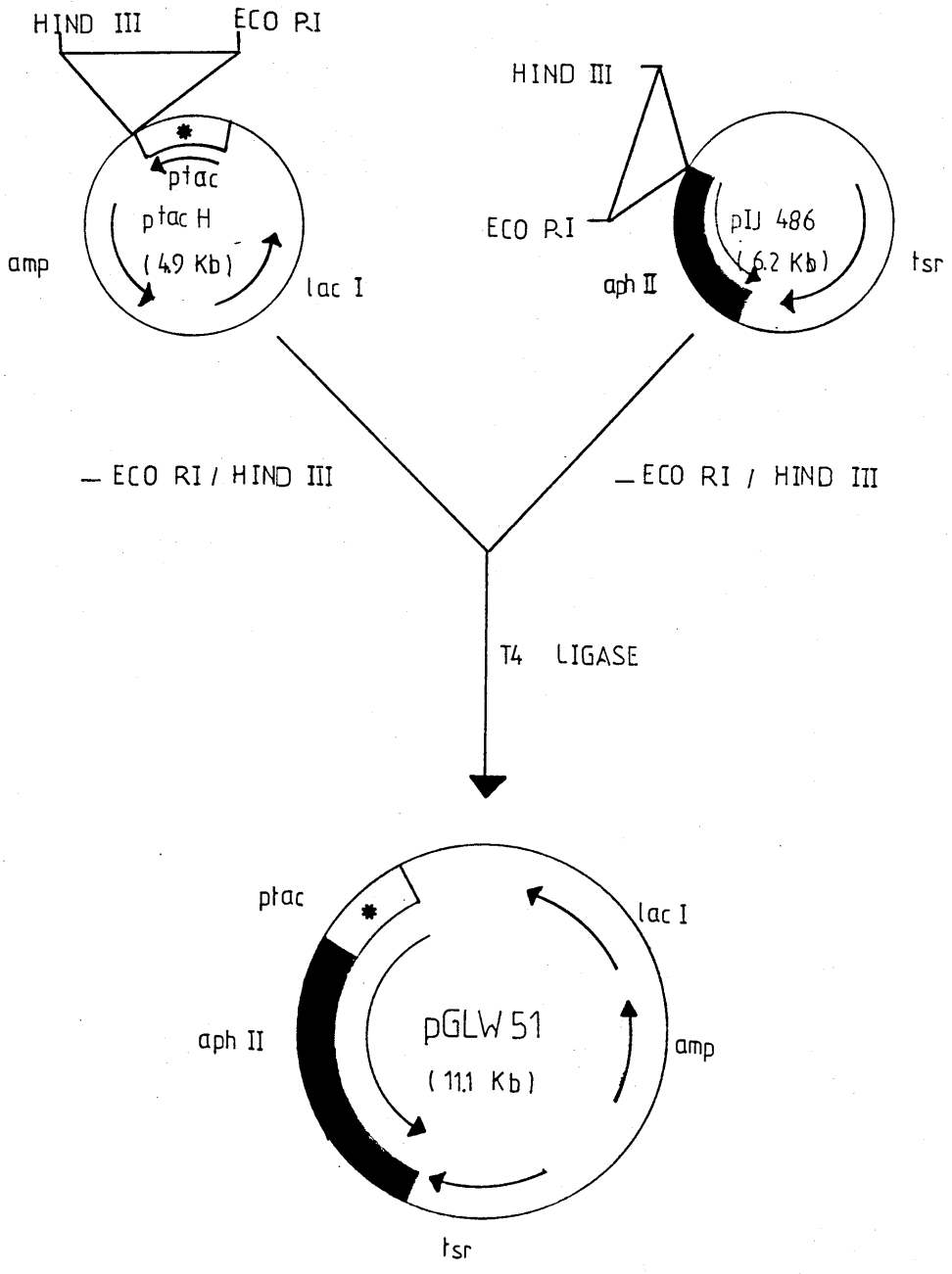


FIG. 4.1: Construction of pGLW51.

	L-agar containing Kanamycin	L-agar containing Kanamycin and IPTG (0.5mM)
1400 pGLW50	growth above 300ug/ml	growth above 300ug/ml
1400 pGLW51	growth stopped at 50-75 ug/ml	growth above 300ug/ml
EMG9 lacI⁻ pGLW50	growth above 300ug/ml	growth above 300ug/ml
EMG9 lacI⁻ pGLW51	growth stopped at 50-75 ug/ml	growth above 300ug/ml

TABLE 4.1: Levels of growth of *E. coli* 1400 and EMG lacI⁻ containing pGLW5(and pGLW51 in the presence of Kanamycin (with and without IPTG).

- repression of ptac was the same in both strains containing pGLW51 and the effect of the expression of the lacI chromosomal copy in E. coli 1400 was negligible.
- this repression was lifted when IPTG was present in the media.
- the repression was not complete, since both strains containing pGLW51 grew in the presence of kanamycin up to a concentration of 50ug/ml, when no IPTG was present.

4.2.2 Transformation of Streptomyces lividans TK24 with pGLW51

A mini DNA preparation of pGLW51 from E. coli 1400 was used to transform S. lividans TK24 protoplasts. Transformants were selected with thiostrepton.

Spores from S. lividans containing pGLW50 and pGLW51 were plated on Emerson agar with thiostrepton, to assess the spore count. Approximately equal numbers of spores of each recombinant strain were plated on three series of plates (all containing 25ug/ml thiostrepton).

1. Containing increasing kanamycin concentrations (0-120ug/ml).
2. As 1, but containing 5mM IPTG.
3. As 1, but containing 10mM IPTG.

These plates (Fig. 4.2) showed that the tac promoter was repressed but not fully, in S. lividans containing pGLW51 and that this promoter could be induced to different levels of expression by adding different concentrations of IPTG to the medium (S. lividans containing pGLW51 showed more growth in the presence of 10mM IPTG than with 5mM IPTG).

In S. lividans containing pGLW51, aphII specific activity was 1.5 fold lower than in S. lividans containing pGLW50 (Table 4.2). On plates (Fig. 4.2) S. lividans containing pGLW50 grew up to 100ug/ml of kanamycin (when no IPTG was present).

This suggests that a very small difference in aphII activity is enough to confer higher resistance to the antibiotic in this strain on solid media. Of course, the levels of aphII activity during spore germination and outgrowth may complicate the interpretation of this experiment.

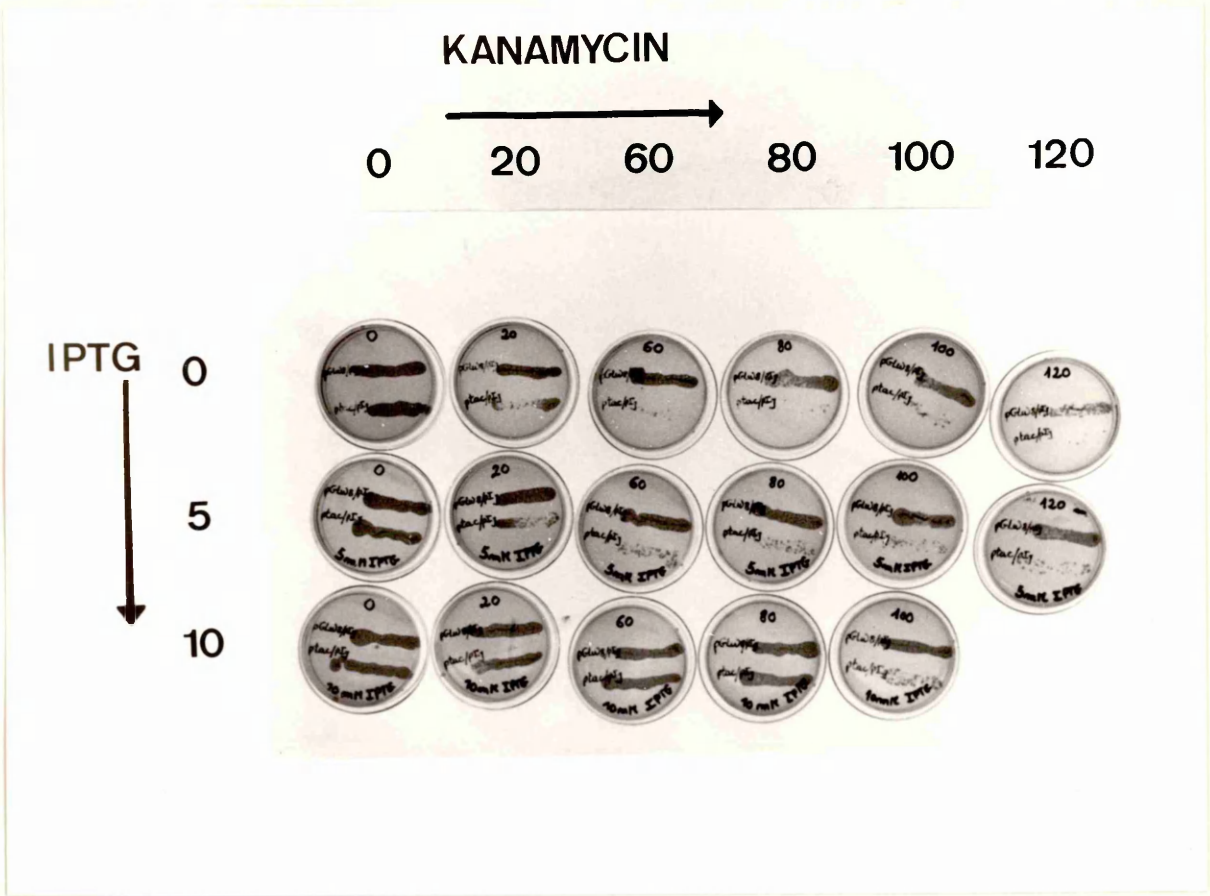


FIG. 4.2 Plates containing increasing kanamycin concentrations (0-100ug/ml) and containing 0,5 and 10 mM IPTG,were streaked with spore suspensions of S. lividans TK 24/pGLW50 (top of each plate) and S. lividans TK 24/pGLW51 (bottom of each plate).

Recombinants of S. lividans containing pGLW51 showed elevated levels of resistance to kanamycin in the presence of IPTG.

4.2.3 Aminoglycoside phosphotransferase II (aphII) assay

The aphII assay was performed as described in Chapter 2. As stated then, the assay had to be performed using different conditions for recombinants of *E. coli* 1400 and for recombinants of *S. lividans* TK24. Therefore the results obtained in both strains are not comparable directly.

In *E. coli* expression of *aph*II was 2.5-fold lower (Table 4.2) when the *lac* repressor was present (pGLW51), whereas in *S. lividans*, expression of *aph*II was 1.6-fold lower when the *lac* repressor was present (pGLW51).

4.2.3.1 Induction of aphII expression in cultures of S. lividans containing pGLW51

Growth of *S. lividans* containing pGLW50 and pGLW51 was followed at 30°C (Fig. 4.3). Accordingly, and at the beginning of the log phase for each strain, 1 litre cultures were induced with 15mM of IPTG. 100ml samples were taken regularly and assayed for aphII activity.

4.2.4 Northern blot analysis

Total RNA of *S. lividans* containing pGLW50, pGLW51 and pIJ486 was analysed by Northern blots.

E. coli 1400 (Assay performed at 0°C)

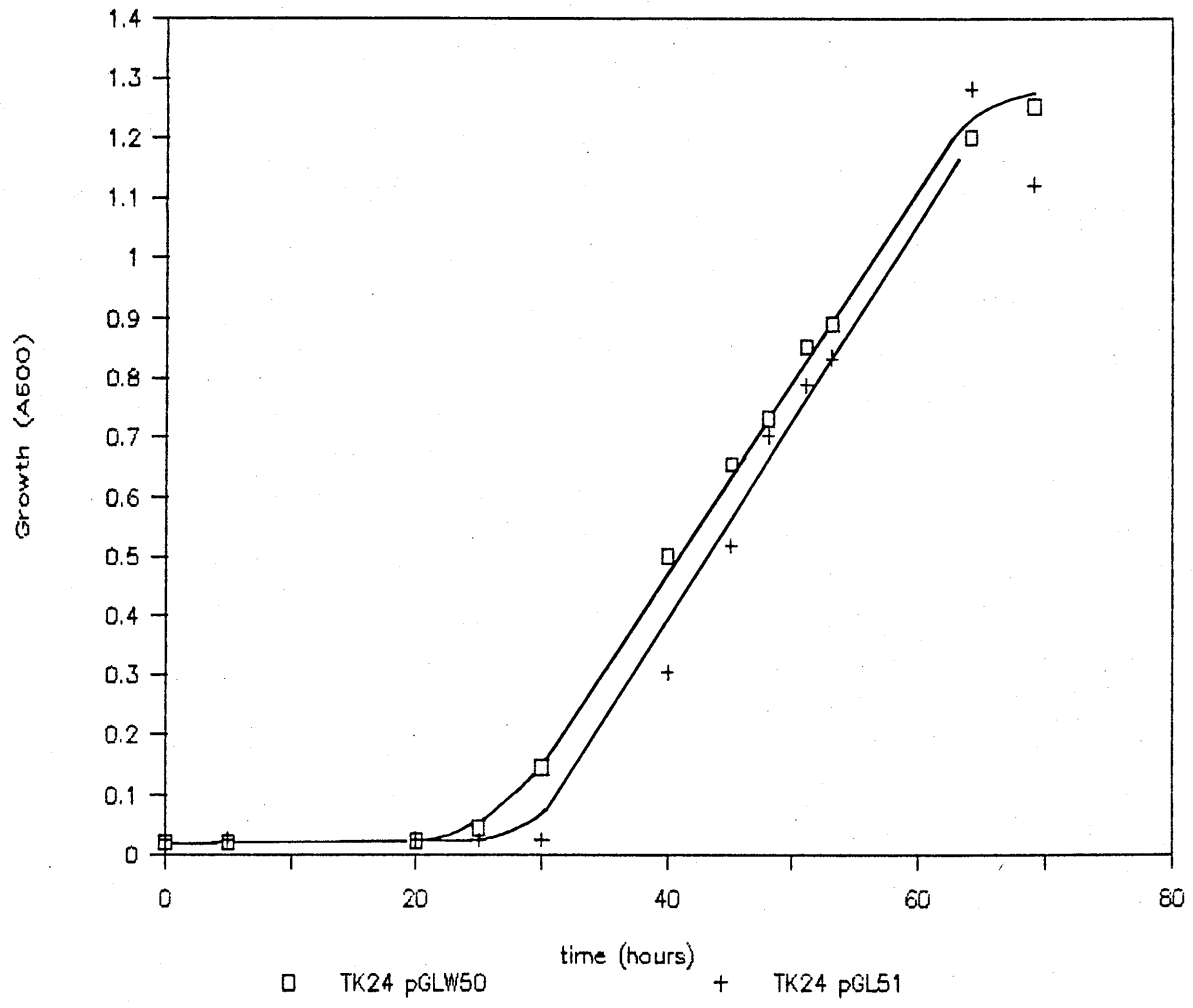
NO PLASMID	pGLW50	pGLW51
No activity	$6.02 \times 10^{-3} \pm 0.106 \times 10^{-3}$ U/mg	$2.4 \times 10^{-3} \pm 0.100 \times 10^{-3}$ U/mg

S. lividans TK24 (assay performed at 35°C)

NO PLASMID	pGLW50	pGLW51
No activity	$2.42 \times 10^{-4} \pm 0.107 \times 10^{-4}$ U/mg	$1.55 \times 10^{-4} \pm 0.098 \times 10^{-4}$ U/mg

1U = 1nmol of ^{32}P incorporated per minute

TABLE 4.2: AphII specific activities of *E. coli* and *S. lividans* containing pGLW50, pGLW51 and no plasmid. (Each assay was performed 5 times.)



time (hour)	A600 TK24 pGLW50	A600 TK24 pGLW51
0	0.021	0.024
5	0.021	0.025
20	0.023	0.025
25	0.045	0.024
30	0.146	0.024
40	0.502	0.305
45	0.655	0.518
48	0.732	0.702
51	0.852	0.789
53	0.891	0.834
64	1.202	1.282
69	1.252	1.123

FIG. 4.3: Growth curves of S. lividans TK24 containing pGLW50 and pGLW51.

The 100ml cultures (in YEME containing Thiostrepton) were inoculated with approximately 3 X 10^7 spores and grown at 30°C.

AphII specific activities

pGLW51		pGLW50	
time (h)	U/mg ($\times 10^4$)	time (h)	U/mg ($\times 10^4$)
34	1.5	34	2.7
36	1.4	36	2.1
38	1.3	38	2.4
39	1.3	40	2.0
40	1.5	42	2.2
41	1.8	44	2.0
42	2.0		

1U = 1 nmol of ^{32}P incorporated/min/35°C

TABLE 4.3: Induction of *S. lividans* Tk24 containing pGLW50 and pGLW51 with 15mM of IPTG.
IPTG was added to the culture at 34 hours after inoculation.

Initially the blots were:

1. Probed with a radio-labelled (labelled using random priming) 1.1Kb fragment cut with HindIII and XbaI from pGLW53. This fragment included the tac promoter and the aphII gene (see Fig. 4.5 for construction of pGLW53).

2. Hybridised at 48°C.

3. Washed twice with 2X SSC containing 0.5% (w/v) SDS at 65°C, for 15 minutes and twice with 1X SSC containing 0.5% (w/v) SDS at 65°C, for 15 minutes.

Fig. 4.6 shows one of these blots. The gel was divided in half and the RNA samples were loaded in such a way that one half of the gel was a replica of the other half.

After blotting, half of the blot was hybridised with the above probe (from pGLW53), the other half was hybridised with a 0.9Kb radio-labelled fragment, digested with EcoRI from pIJ2808. pIJ2808 is a derivative of pUC19 that contains approximately 900bp of the 3' end of 16S rRNA from S. coelicolor.

These blots suggested that the bands of RNA that were hybridising to the 1.1Kb probe (containing ptac and the aphII gene) were the same that were hybridising to the 16S rRNA probe.

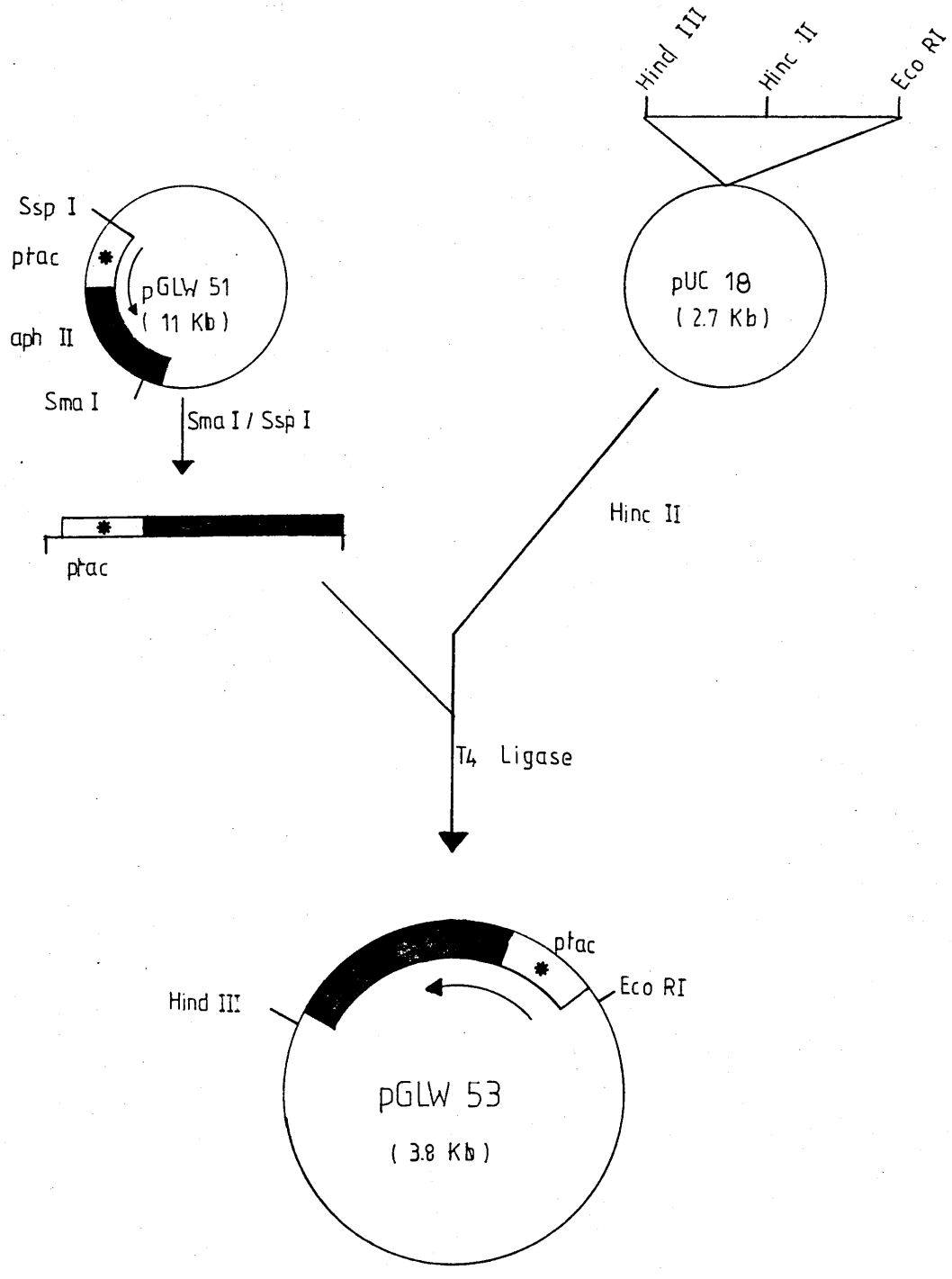


FIG. 4.5: Construction of pGLW53.

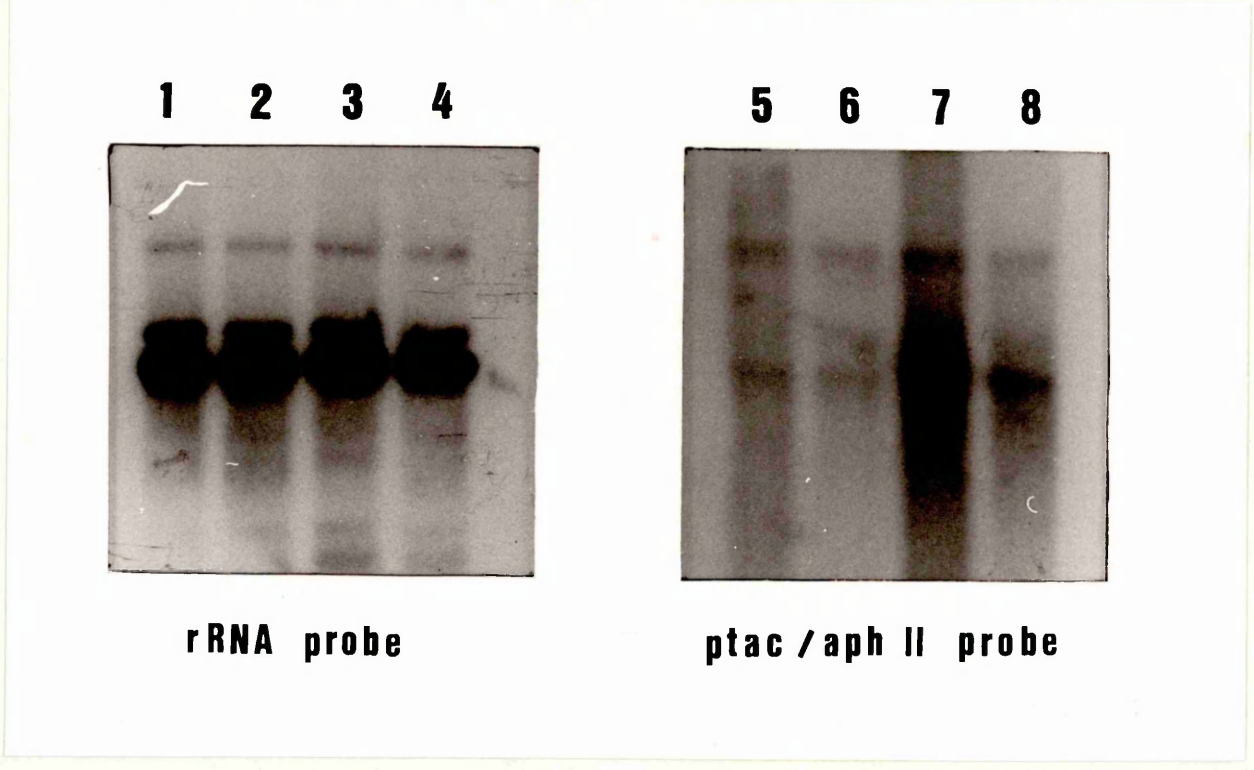


FIG. 4.6: Northern blot probed with rRNA and ptac/aphII probes. The filter probed with the rRNA probe was exposed for four hours while the filter probed with the ptac/aphII probe was exposed for sixty hours. Both filters were washed at 65°C for fifteen minutes:

i) twice with 2 X SSC + 0.5% (w/v) SDS

ii) twice with 1 X SSC + 0.5% (w/v) SDS

1=5-total RNA from S. lividans TK24/pGLW51 (not DNAse treated)

2=6-20ug total RNA from S. lividans TK24/pGLW51 (DNAse treated)

3=7-total RNA from S. lividans TK24/pGLW50 (not DNAse treated)

4=8-20ug total RNA from S. lividans TK24/pGLW50 (DNAse treated)

Control experiments were done using not only total RNA from S. lividans containing pIJ486 (has no ptac and therefore no aphII message is produced), pGLW50 and pGLW51, but also from S. lividans containing no plasmid. Blots were hybridised to both probes and as Fig. 4.7 shows, they also suggested that the 1.1Kb probe and the 16S rRNA probe were hybridising to the same RNA bands. Since neither S. lividans containing pIJ486 nor S. lividans without a plasmid produced the ptac/aphII message, and RNA from these strains was also hybridising to the two probes, it was concluded that the bands observed in the blots corresponded to rRNA bands.

To try to overcome what seemed to be unspecific hybridisation of the 1.1Kb probe to rRNA, total RNA (treated and not treated with DNAse) from S. lividans containing no plasmid and pGLW50 was blotted and hybridised at higher temperatures: 50°C, 55°C and 60°C. The filters were washed at high stringency at 65°C. In this way unspecific hybridisation still occurred (data not shown).

4.2.4.1 Probing Northern blots with an oligonucleotide.

An oligonucleotide containing 32 nucleotides which matched an internal sequence of the aphII mRNA perfectly was synthesized (Fig. 4.8). The oligonucleotide 161 was end-labelled with ^{32}P and used to probe blots of total RNA of S. lividans containing pIJ486 and pGLW50. Hybridisation was performed at three different temperatures: 37°C, 41°C and 45°C. The blots were washed twice in 6X SSC at 40°C.

Very faint bands appeared in these blots (Fig. 4.9), and they corresponded to rRNA bands.

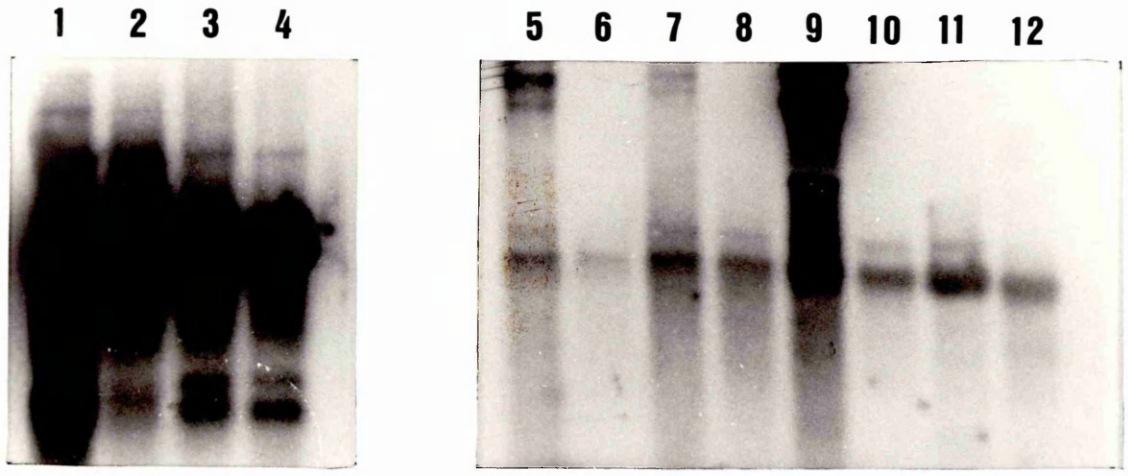


FIG. 4.7: Northern blot probed with rRNA and ptac/aphII probes. The filter probed with the rRNA probe was exposed for three hours while the filter probed with the ptac/aphII probe was exposed for one hundred and twenty hours. Both filters were washed at 65°C for 15 minutes.

i) twice with 2 X SSC + 0.5% (w/v) SDS

ii) twice with 0.1 X SSC + 0.5% (w/v) SDS

1-total RNA from S. lividans TK24/pGLW51

2-total RNA from S. lividans TK24/pGLW50

3-total RNA from S. lividans TK24/pIJ486

4-total RNA from S. lividans TK24

5-total RNA from S. lividans TK24/pGLW51

6-20ug of total RNA from S. lividans TK24/pGLW51 (DNAse treated)

7-total RNA from S. lividans TK24/pGLW50

8-20ug of total RNA from S. lividans TK24/pGLW50 (DNAse treated)

9-total RNA from S. lividans TK24/pIJ486

10-20ug of total RNA from S. lividans TK24/pIJ486 (DNAse treated)

11-total RNA from S. lividans TK24

12-20ug of total RNA from S. lividans TK24 (DNAse treated)

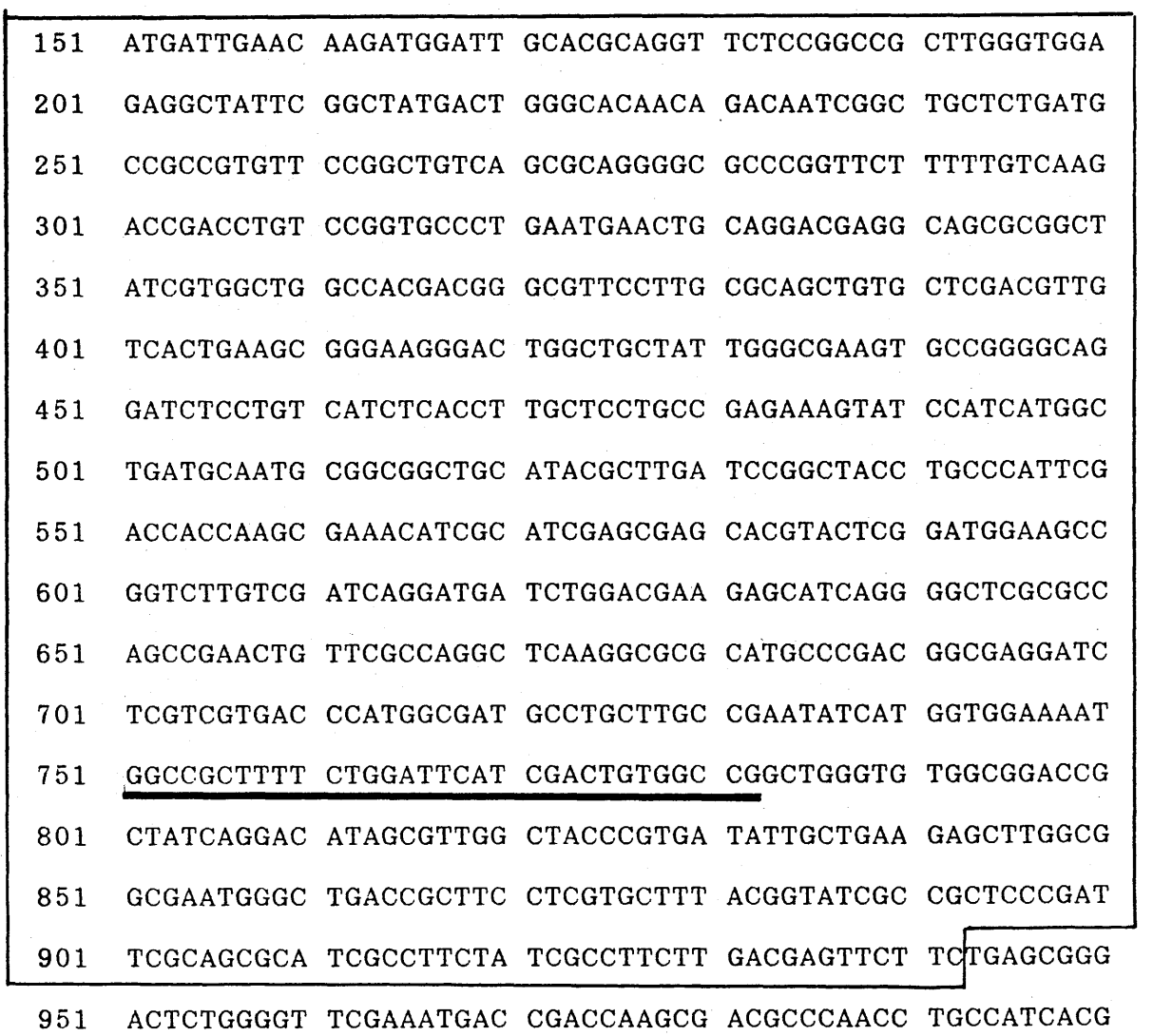


FIG.4.8 The coding region of the aphII gene is boxed by solid lines (Beck et al,1982).
The sequence complementary to the oligonucleotide 161 is underlined.

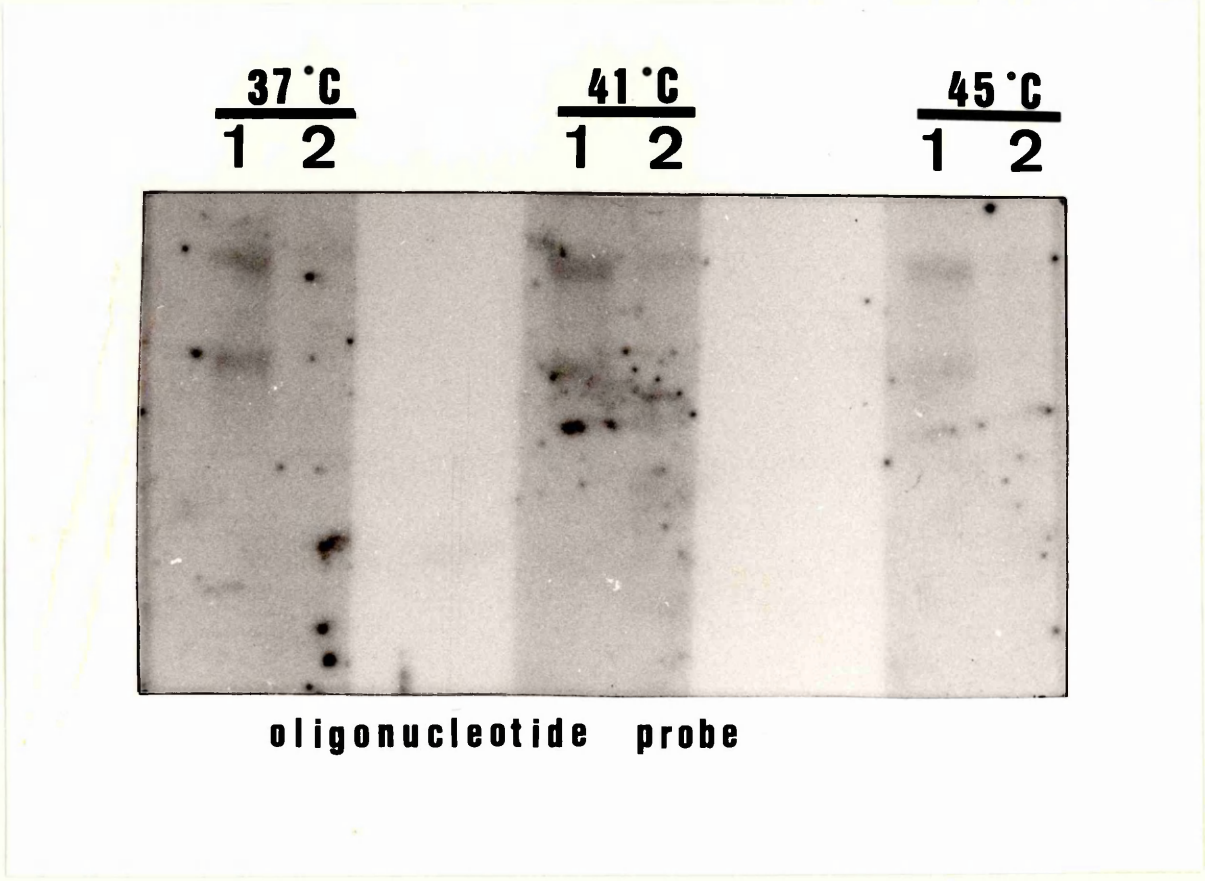


FIG.4.9 Northern blot probed with radiolabelled oligonucleotide 161.Each filter was hybridised at a different temperature: 37°,41° and 45°C.

1=25ug of total RNA from S. lividans TK 24/pGLW50

2=25ug of total RNA from S. lividans TK 24/pIJ 486

Since Northern blots failed to show the aphII transcript, dot-blot assays for this transcript could not be performed.

4.2.5 S_1 mapping of the tac promoter in E. coli and S. lividans

4.2.5.1 Constructing mGLW54 to make single stranded probes for S_1 mapping of ptac.

The 0.3Kb fragment obtained from the digestion of pGLW53 with SstI and PstI was purified from a 1.5% (w/v) TAE agarose gel. This fragment was subcloned into M13mp18 digested with the same enzymes (Fig. 4.10) to construct mGLW54.

The ligation mixture was used to transform competent cells of E. coli JM101. Single-stranded DNA prepared from recombinants containing mGLW54 was used to make the single stranded probe for S_1 mapping of ptac. This method was described in Section 2.16.1. For this promoter, XbaI was used to cut the extended probe.

4.2.5.2 S_1 mapping of ptac

The 0.3Kb single-stranded radio-labelled probe was hybridised to total RNAs from S. lividans TK24 recombinants containing pIJ486, pGLW50 and pGLW51 and to RNAs from E. coli 1400 recombinants containing pGLW50 and pGLW51.

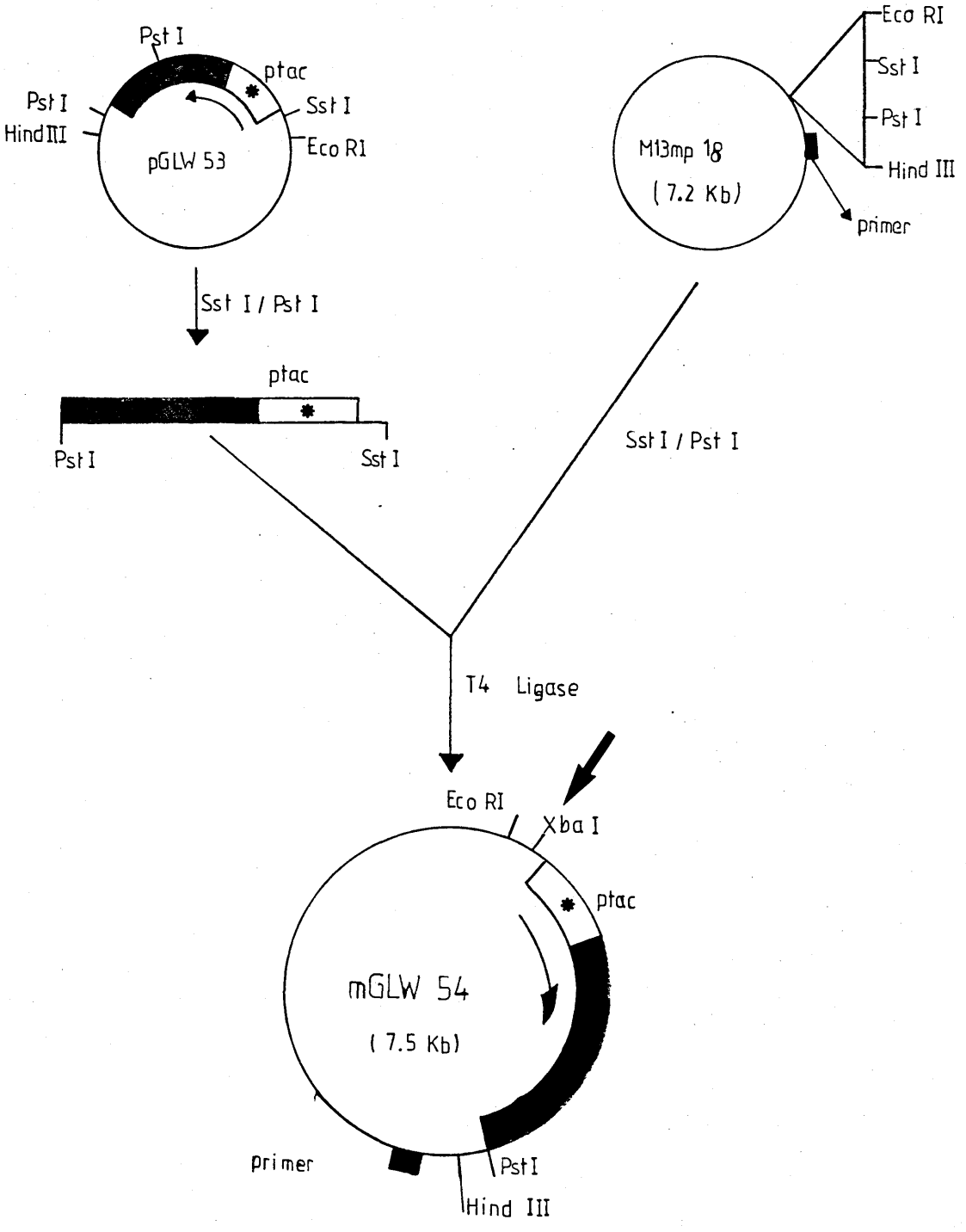


FIG. 4.10: Construction of mGLW54.

XbaI (indicated with an arrow) was the enzyme used to cut the extended probe.

The hybridisation was performed at 37°C overnight, and digested with 100 units of S_1 nuclease according to Section 2.16.1. The digested products were electrophoresed on a 6% (w/v) denaturing polyacrylamide gel.

No protected bands were identified with control RNA from S. lividans TK24 containing pIJ486 and tRNA (Fig. 4.11). A doublet appeared in these and all other samples, but this was due to incomplete digestion of the probe.

For the E. coli and the other S. lividans RNA samples, the most intense band indicating a transcriptional start site corresponded to a protected fragment of 267-268 base-pairs.

A dideoxy sequencing ladder was run alongside the samples digested with S_1 nuclease. Allowing for the additional DNA included in the primer (Fig. 4.12) the deduced transcriptional start site was identified for S. lividans and E. coli, and in agreement with that observed previously for E. coli (de Boer et al., 1983).

4.3 Discussion

The tac promoter can be regulated (poorly) in S. lividans. The lac repressor protein, encoded by the E. coli lacI gene, is expressed in S. lividans and represses expression from ptac.

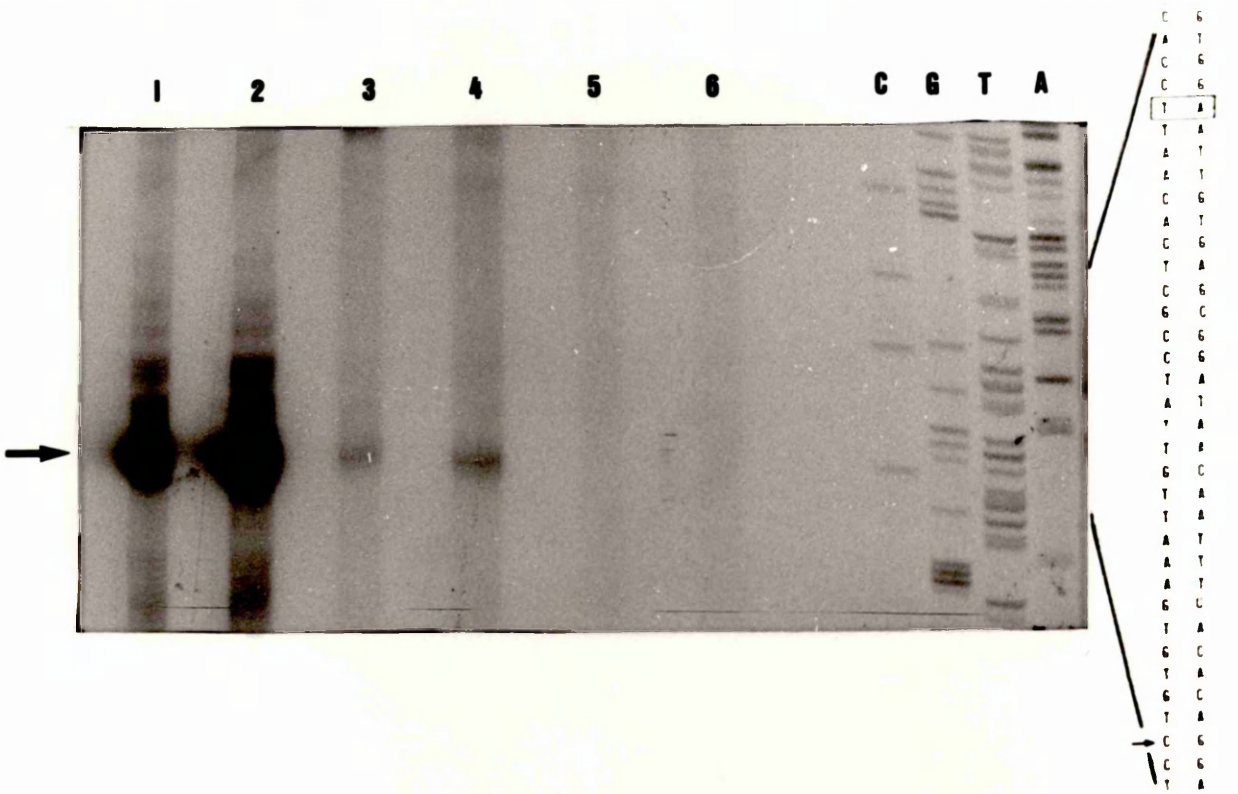


FIG. 4.11: High resolution S_1 nuclease mapping of ptac in S. lividans TK24 and E. coli 1400 containing pGLW51 and pGLW50.

1-20ug of total RNA from E. coli 1400/pGLW51

2-20ug of total RNA from E. coli 1400/pGLW50

3-30ug of total RNA from S. lividans TK24/pGLW51

4-20ug of total RNA from S. lividans TK24/pGLW50

5-20ug of total RNA from S. lividans TK24

6-20ug of tRNA

The arrow indicates the start point coincident with the major protected band.

The boxed base pair indicates the expected start point in E. coli (de Boer et al., 1983).

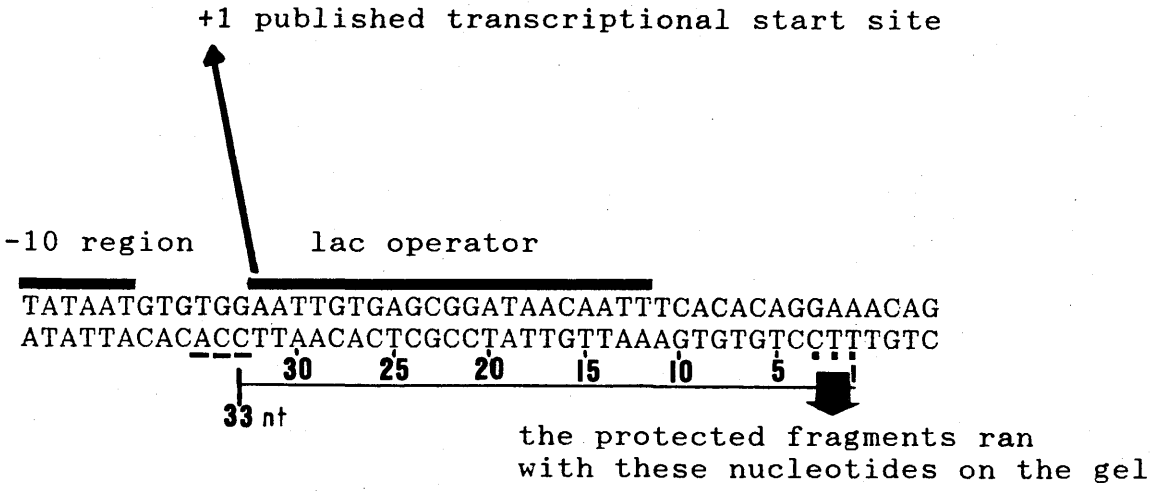


FIG.4.12 How the transcriptional start of ptac was deduced for E. coli and S. lividans.

The sequencing ladder ran 33 nucleotides behind the corresponding protected fragments . The protected fragments are marked with dots and the corresponding start sites are underlined.

Plate results, as well as aphII assay results, showed that derepression and modulation of expression could be achieved by exposing the S. lividans cells to different concentrations of IPTG. This indicated that IPTG was taken up by these cells. However, these results also showed that repression was far from complete either in E. coli or in S. lividans.

E. coli 1400 containing pGLW51 showed a specific activity of aphII which was 2.5 fold lower than the same strain containing pGLW50, but 2.4 U/mg of aphII specific activity were still detected. This was consistent with the observation that E. coli containing pGLW51 showed some resistance to kanamycin when no IPTG was present in the media (Section 4.2.1).

The result agrees with reports by Amann et al. (1988) and Stark (1987) that suggested that for tighter control of the tac promoter in E. coli, the wild type lacI gene might not be sufficient and the "up" promoter mutation, $lacI^q$ (Calos, 1978) was necessary.

Also, in the wild-type lac operon a second, lac operator-like sequence, 401bp downstream of the first lac operator has been identified (Reznikoff et al., 1974). Insertion of an additional lac operator upstream from the lac promoter-operator region can also increase repression by lac repressor "in vivo" (Herrin and Bennett, 1984; Berse et al., 1986; Mossing and Record, 1986). Recently, the participation of the "second lac operator" in "in vivo" repression has been demonstrated (Eismann et al., 1987) and it has been suggested that one lac repressor tetramer may bind to the two lac operators on one DNA fragment, thus causing the intervening DNA to form a loop

(Kramer et al., 1987). Therefore it is possible that the tac promoter, that lacks this second lac operator-like sequence, might be less repressible than the wild-type lac promoter.

For S. lividans containing pGLW51, the specific activity aphII was 1.55 U/mg which is rather high when compared to the same activity found for S. lividans containing pGLW50. The explanation given for poor repression in E. coli does not necessarily imply a weak promoter in the lacI gene. Although several E. coli genes are translated in S. lividans (Bibb and Cohen, 1982; Foster, 1983; Gil and Hopwood, 1983), the marked asymmetry of codon usage in Streptomyces could reduce the level of lacI translation, or there could even be some assembly difficulty of the lacI homotetramer.

It would be difficult to know theoretically whether the $lacI^q$ promoter would be a better Streptomyces promoter than the wild type lacI promoter. For example, Bagdasarian et al. (1983) showed that the tac and the $lacI^q$ promoters were active in Pseudomonas putida, but repression of the tac promoter by the $lacI^q$ encoded repressor was not complete in this gram-negative bacterium.

Attempts to quantify the aphII message were not successful. The profile of the Northern blot analysis was badly distorted by very large quantities of ribosomal RNA in the tracks. A similar problem was reported by Smith and Chater (1988). Thus dot-blot assays which have been shown to be very useful in the quantification of specific messages by others, could not be used. It is possible that, in addition to this problem, the half-life of the aphII mRNA could be rather short.

High resolution S_1 mapping identified the start of transcription by ptac in S. lividans and E. coli as being identical and in agreement with the start observed for E. coli by de Boer et al (1983). Two Streptomyces promoters, pIJ101A and pIJ101C, from the high-copy number plasmid pIJ101 were shown previously to promote transcription from the same start point in both genera (Deng et al., 1987; Buttner and Brown, 1987). This implies that the sigma factor 35 of RNA polymerase from Streptomyces (Buttner et al, 1988; Westpheling et al, 1985) recognises similar sequence determinants to the ones recognized by E. coli Sigma factor 70.

Abundance of mRNA as determined by S_1 nuclease in solution also agreed with the aphII assay and plate results in showing that repression was not complete in both genera. The start sites were found in E. coli and S. lividans containing pGLW50 and also pGLW51. Thus, despite the presence of the lac repressor in pGLW51 aphII mRNA was still made.

Nevertheless, pGLW51 is a useful vector for the characterization and analysis of pathway fluxes in Streptomyces. The aphII promoter-less gene could be replaced by a gene coding for an enzyme thought to be important in the control of the overall rate through a metabolic pathway. The expression of this gene could then be modulated by varying the level of IPTG in the medium. However, tighter control of the tac promoter would make this vector a much more useful tool.

It has not been mentioned that the variation in the copy number of these bifunctional vectors in either genera, but particularly in Streptomyces, could constitute a limitation to their use. However,

these expression vectors are based on pIJ486, a Streptomyces vector known to be stable (Ward et al., 1986). On the other hand, in the course of this work whenever DNA was prepared from Streptomyces, the ratio chromosomal DNA/plasmid DNA seemed to be constant.

CHAPTER 5

PROMOTER-PROBE AND S_1 MAPPING ANALYSIS OF *placI* IN *S. lividans*

5.1 Introduction

Repression of ptac in pGLW51 was shown to be incomplete both in E. coli and S. lividans (see Chapter 4).

In S. lividans, in particular, repression was very poor. A possible reason for this was that the promoter of the repressor gene, placI, was weak in S. lividans and therefore not enough repressor was made to prevent transcription from the tac promoter.

In this Chapter, results from high resolution S_1 mapping of placI in E. coli and S. lividans are presented together with promoter probe analysis results of this promoter in S. lividans using pIJ486. These experiments were designed to test if the lac repressor gene was being transcribed efficiently from its own promoter in S. lividans.

5.2 Results

5.2.1 Construction of pGLW55A

Since there were no available restriction sites close to the promoter of lacI, which could be used to subclone this promoter from ptacH into other vectors, a larger fragment was subcloned into pUC18 and then digested with exonuclease III.

As illustrated in Fig. 5.1, the 497 base pair BclI/AvaII fragment from ptacH, containing placI, was isolated from a gel and the protruding ends were made blunt using the Klenow fragment of DNA polymerase I

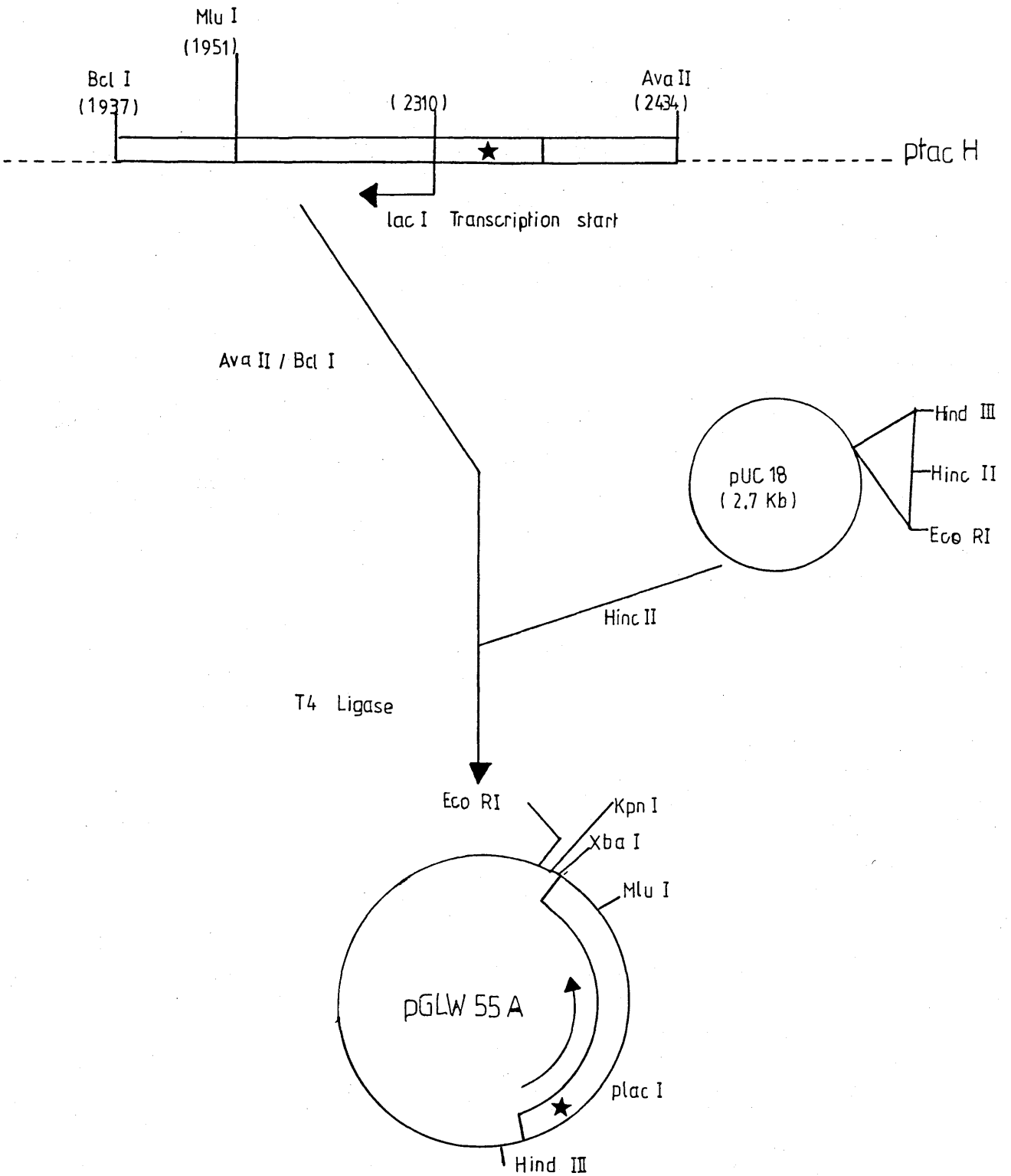


FIG. 5.1: Construction of pGLW55A.

(Section 2.12). The fragment was then ligated to pUC18 which had been digested with HincII.

Competent cells of E. coli DS941 were transformed with the ligation mixture and transformants were selected on L-agar plates containing ampicillin, IPTG and X-gal.

The unique MluI site in the cloned sequence was used to determine the orientations of the inserts in the plasmids recovered from overnight cultures of white colonies using mini DNA preparations. pGLW55A was identified as having the correct orientation and was used for digestions with exonuclease III.

5.2.2 Construction of exonuclease III deletions from pGLW55A

pGLW55A was digested with XbaI (5' protruding ends) and KpnI (3' protruding ends) and the exonuclease III digestion procedure described in Section 2.17 was followed.

Several recombinants of DS941 from time points 80, 120, 160 and 200 seconds were screened. Plasmid DNAs from the recombinants were digested with PvuII and examined by agarose gel electrophoresis to determine the extents of the deletions. The majority of the deletions were in the expected size range assuming a digestion rate of approximately 150 base pairs per minute.

Plasmid DNA was prepared by the Birnboim-Doly method from six recombinants containing deletions (29, 30, 32, 33, 36 and 39).

Plasmid DNA sequencing using only ddATP and ddTTP mixes (T/A tracking) of these plasmids was performed.

Fig. 5.2 shows that the plasmids 29, 30, 36 and 39 had all lost the EcoRI site as a result of bidirectional digestion. This was probably due to incomplete digestion with KpnI, the enzyme used to produce the 3' overhang.

Plasmid 33 was shown to have lost the -10 region of placI. Plasmid 32 (pGLW56), had the transcriptional start of placI 44 base pairs away from the EcoRI site, and was used for subsequent subcloning.

5.2.3 Sequencing and S_1 mapping of placI in E. coli and S. lividans

5.2.3.1 Construction of mGLW57

The EcoRI/HindII fragment of pGLW56 containing the lacI promoter was subcloned into the polylinker of M13 mp19 originating mGLW57 (Fig. 5.3).

Competent cells of E. coli JM101 were transformed with this ligation mixture. White plaques from plates containing X-gal and IPTG were screened for the presence of mGLW57.

Single stranded DNA from a recombinant containing mGLW57 was used to prepare the single stranded probe for S_1 mapping of placI.

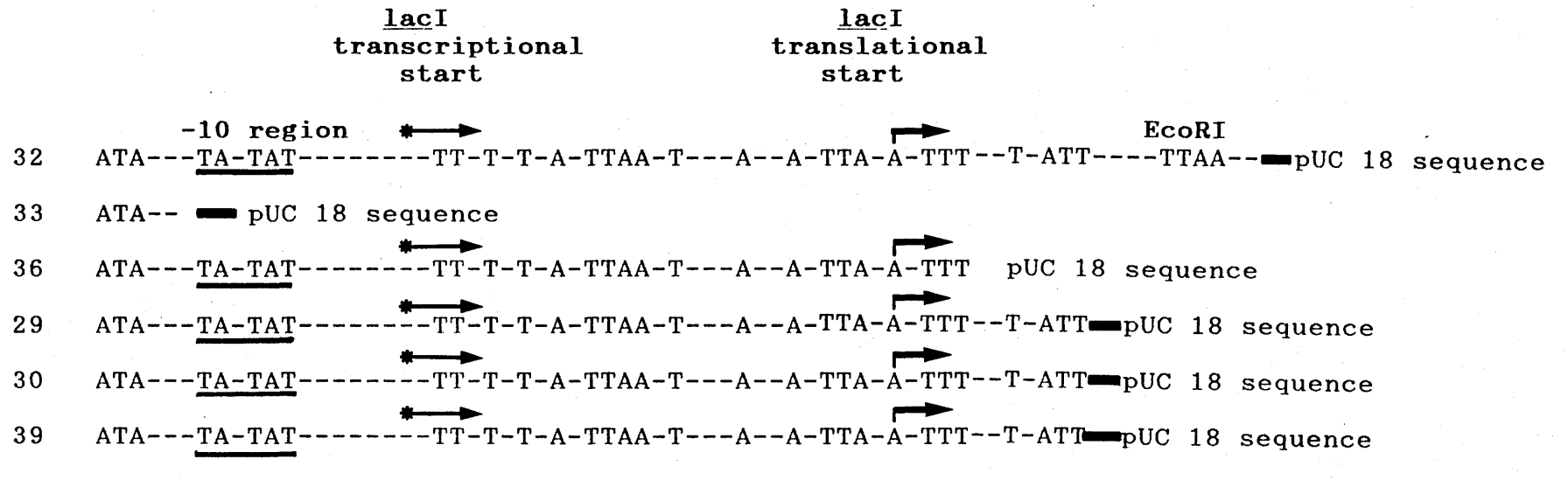


FIG 5.2 DNA sequences (only ddATP and ddTTP) of the regions surrounding the transcriptional start of lacI of six recombinants containing deletions of pGLW55A .

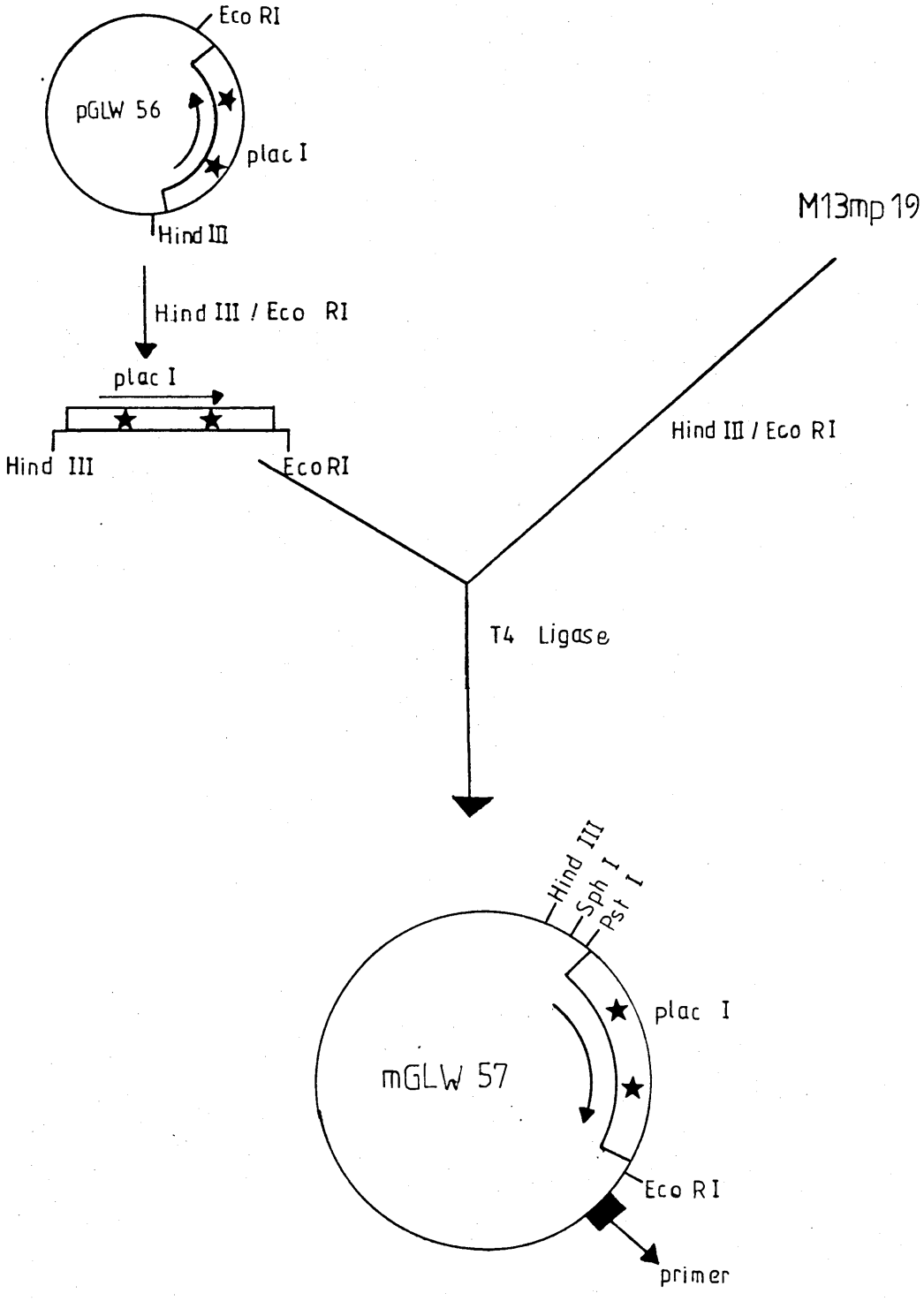


FIG. 5.3: Construction of mGLW57.

5.2.3.2 High resolution S_1 mapping of placI in S. lividans and E. coli

The 160 nucleotide single-stranded radio-labelled probe was made as described in Section 2.16.1. HindIII was the restriction enzyme used in this process.

The probe was hybridised to total RNA from E. coli 1400 and S. lividans TK24 containing pGLW50 (no lacI) and pGLW51 (lacI).

The hybridisation was performed at 37°C overnight (as described in Section 2) and digested with 200 U of S_1 nuclease.

The digested samples were electrophoresed on a 6% (w/v) denaturing polyacrylamide gel.

No protected bands were identified with control RNA (Fig. 5.4): tRNA and RNA from E. coli and S. lividans containing pGLW50 (carries no lacI gene). As was observed for the S_1 mapping of ptac (Section 4.2.5.2), some bands appeared in these control tracks but they were thought to be due to incomplete digestion of the probe.

For the RNA sample corresponding to E. coli containing pGLW51, an intense band indicated a protected fragment of 46- 48 nucleotides (Figs. 5.4 and 5.5).

A dideoxy sequencing ladder of mGLW57 was run alongside the S_1 digestion products allowing the identification of the main transcriptional start site (Fig. 5.6). This start site agreed with the published start site for placI in E. coli (Calos, 1978).

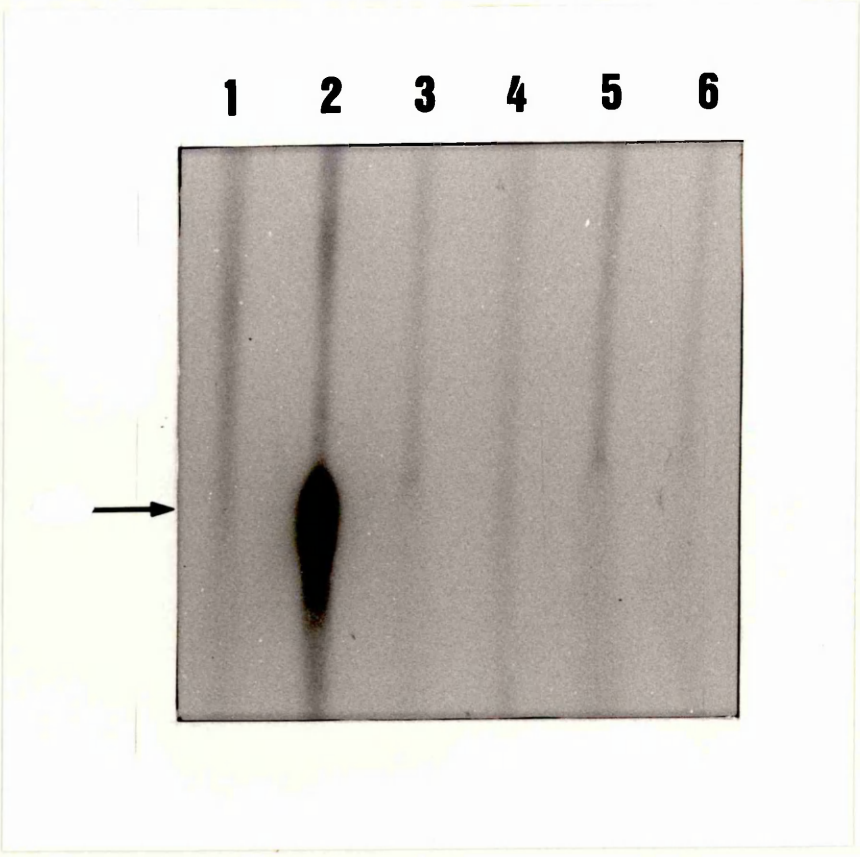


FIG. 5.4: High resolution S_1 nuclease mapping of the lacI promoter in E. coli 1400 and S. lividans containing pGLW50 and pGLW51.

1-20ug tRNA

2-40ug of total RNA from E. coli 1400/pGLW51

3-70ug of total RNA from S. lividans TK24/pGLW51

4-40ug of total RNA from E. coli 1400/pGLW50

5-70ug of total RNA from S. lividans TK24/pGLW50

6-30ug of total RNA from S. lividans TK24

The arrow indicates the start point coincident with the major protected band (46 nucleotides long).

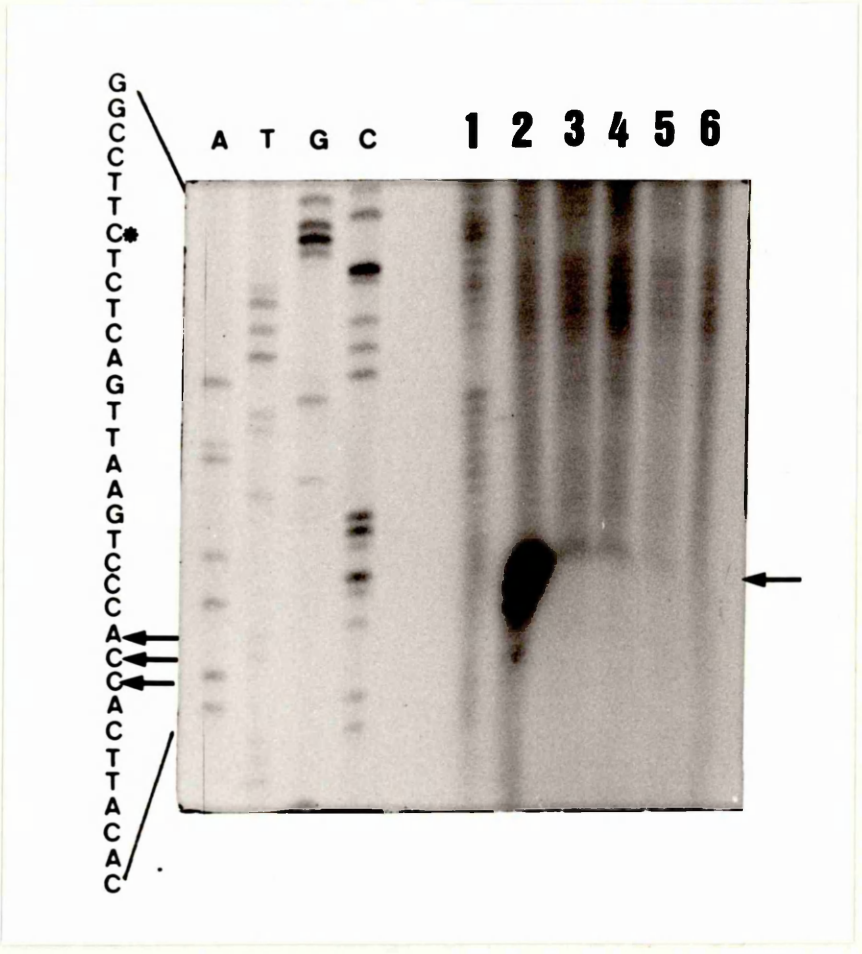


FIG. 5.5: High resolution S_1 nuclease mapping of the lacI promoter in E. coli 1400 and S. lividans TK24 containing pGLW51.

1-probe

2-40ug of total RNA from E. coli 1400/pGLW51

3-240ug of total RNA from S. lividans TK24/pGLW51

4-120ug of total RNA from S. lividans TK24/pGLW51

5-60ug of total RNA from S. lividans TK24/pGLW51

6-20ug of tRNA

The arrows indicate the start point coincident with the major protected bands. The starred nucleotide indicates the expected start in E. coli (Calos, 1978).

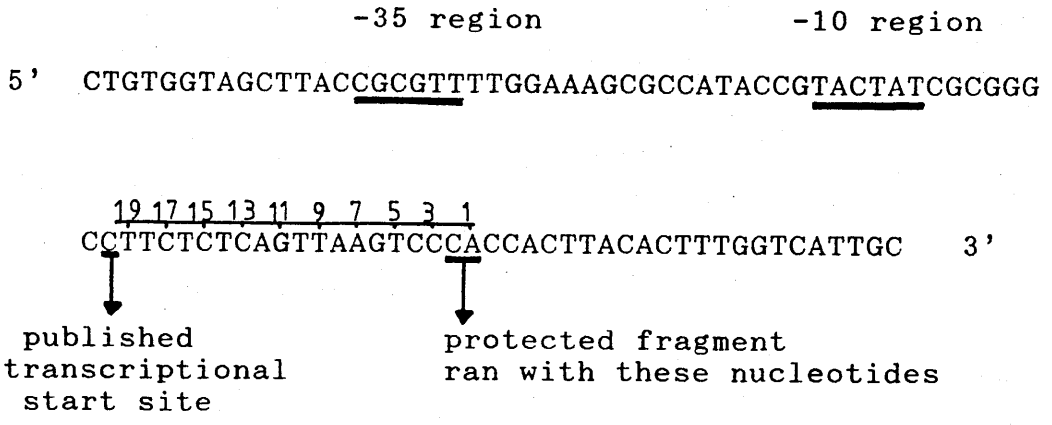


FIG.5.6 How the transcriptional start of placI was deduced for E. coli containing pGLW51.

The sequencing ladder ran 19 nucleotides behind the corresponding protected fragment , since the S1 nuclease digested samples lost the primer while the sequenced DNA did not.

No protected band was identified with RNA from S. lividans containing pGLW51 (Figs. 5.4 and 5.5) even when increasing concentrations of total RNA were used.

5.2.4 Promoter probe analysis of placI in S. lividans

pGLW58 (Fig. 5.7) was constructed by subcloning the EcoRI/HindIII fragment from pGLW56, containing the lacI promoter into the polylinker of the promoter-probe vector pIJ486.

S. lividans TK24 protoplasts were transformed with the above ligation mixture and transformants were selected with thiostrepton. Twenty independent recombinants were tested for their resistance to kanamycin. Spores of each were plated on Emerson plates containing increasing concentrations of kanamycin and thiostrepton at a concentration of 25ug/ml. All these clones failed to grow on plates containing 10ug/ml of kanamycin.

To ensure that these recombinants actually carried pIJ486 with the placI insert, plasmid preparations were made of four recombinants and all four were shown to contain the insert.

5.3 Discussion

In Chapter 4 it was inferred that the lacI gene was expressed to some extent in S. lividans, since the effect of the lac repressor on ptac was detectable by a decrease in resistance to kanamycin in S. lividans containing pGLW51, This repression was relieved by IPTG.

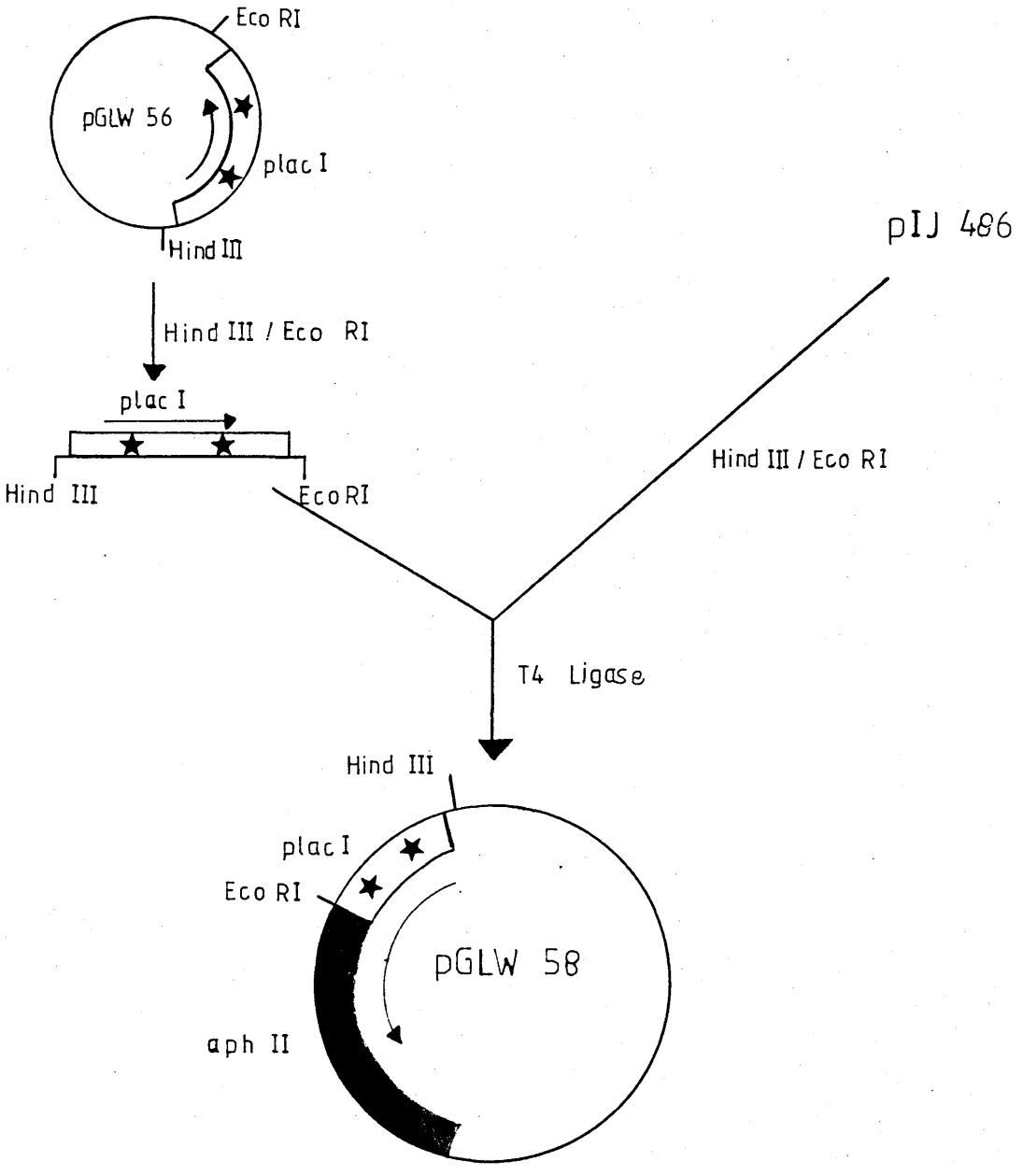


FIG. 5.7: Construction of pGLW58.

However, in S. lividans containing pGLW51 high resolution S_1 mapping of placI failed to detect the lacI mRNA, and therefore its start in the streptomycete whereas the mRNA of lacI was detected in E. coli and its start site was mapped.

This indicated that, in S. lividans either the lacI mRNA abundance was very low or/and that this mRNA had a very short half life and therefore was difficult to detect.

The EcoRI/HindIII fragment containing the lacI promoter did not show promoter activity when inserted into pIJ486, suggesting that the first of the above possibilities was more probable, without ruling out the second one.

However, the possibility of a different transcriptional start in S. lividans and E. coli cannot be eliminated. It is possible that the sequence recognized by the Streptomyces RNA polymerase is not the actual sequence of placI but another one. If this was the case it should still have been possible to detect the lacI mRNA (if this was not in low abundance and/or very labile) even though its start might have been upstream from the cloned region. This possibility would have resulted in full-length protection of the radiolabelled probe.

Thus from the results presented in this Chapter it was concluded that the promoter (placI or not), driving lacI transcription, is a very weak Streptomyces promoter.

This can explain, at least partially, the poor levels of repression of the *tac* promoter encountered in *S. lividans*, since not enough repressor was made to shut down transcription.

It must be added that the *lacI* promoter and the *Streptomyces fradiae aph*PI promoter have very similar -35 and -10 regions. While this *Streptomyces* promoter is strong in streptomycetes, it has not been observed to promote transcription in *E. coli* (Janssen, cited by Hopwood *et al.*, 1986). However, the majority of *Streptomyces* promoters do not function in *E. coli* while several promoters act as promoter sequences in *S. lividans* (Bibb and Cohen, 1982; Jaurin and Cohen, 1985).

This again raises the question of what makes a promoter functional in *E. coli* and *Streptomyces* and suggests that in the whole program of transcription these genera have common and different features.

Initiation of transcription involves a complex set of interactions between RNA polymerase and promoter (a segment of DNA that contains signals for the proper binding and subsequent activation of RNA polymerase holoenzyme to a form capable of initiating the synthesis of RNA). Comparison between the great number of prokaryotic promoter sequences revealed common features between many of these which had resulted in a so-called "consensus sequence" (Rosenberg and Court, 1979; Siebenlist *et al.*, 1980).

The "consensus sequence" consists of two highly conserved regions at -10 and -35 in relation to the transcriptional start. The importance

of these conserved regions in transcriptional initiation and of the distance between them (17 base pairs ± 1) is evident from:

1. The fact that they are so highly conserved and that many of the promoter mutations identified fall into these regions (Siebenlist et al., 1980; Rosenberg and Court, 1979).

2. Nuclease digestion experiments which show that the -35 and -10 sequences are included in the region that is protected from DNAse by RNA polymerase during "open" complex formation (Schmitz and Galas, 1979).

3. The fact that contacts between the functional groups of the promoter DNA (hydrogen-bonding groups in the grooves of the DNA double helix and backbone phosphates) and the protein side chains of the polymerase also occur in these regions (Simpson, 1982).

4. The fact that prokaryotic RNA polymerase holoenzymes having sigma factors other than the predominant bacterial form have -10 and -35 regions that do not conform to the consensus sequence (Pero et al., 1982; Gilman et al., 1982).

The steps in mRNA synthesis which involve the promoter are usually described as (McClure, 1985):

(i) promoter location.
(ii) "closed" complex formation between promoter and polymerase.
(iii) "open" complex formation (or isomerization).
(iv) mRNA initiation.

These steps are affected not only by the -10 and -35 sequences but also by other factors.

For example, there is some evidence suggesting that regulation of transcription initiation can also occur as a result of structural and conformational changes in the DNA template. Methylation at the -10 region of the IS10 transposase gene promoter, pIN, increases its activity 10 to 30 times (Roberts *et al.*, 1985). Methylation also influences *E. coli trp*R promoter activity (Marinus, 1985) and is required for expression of the *mom* gene of phage Mu (Plasterk *et al.*, 1983).

Template supercoiling can also be expected to influence both the RNA polymerase binding to the promoter and the opening of the double helix to form the open complex (Lilley, 1986). It has been shown that different promoters can be either binding-limited or isomerisation-limited. The *lac*UV5 promoter is an example of a promoter limited by weak binding in *E. coli* (Hawley and McClure, 1982).

In vitro and *in vivo* experiments have revealed that the response of promoters to changes in supercoiling levels can be quite complex: some promoters are unaffected by topology, some are stimulated, some are impaired (Lilley, 1986).

The current view on transcription initiation in *E. coli* and other prokaryotes is probably too simplistic, other unknown factors possibly affect this very complex process.

Thus it is difficult to understand why the *lacI* promoter is so poorly transcribed in *Streptomyces*. It might not just be a promoter sequence problem, it might be related to the supercoiling state of the plasmids used, or due to some unknown factor(s).

CHAPTER 6

CLONING OF A *STREPTOMYCES* PROMOTER UPSTREAM THE *lacI* GENE of pGLW61

6.1 Introduction

Having found that placI was a poor promoter in S. lividans (Chapter 5) and knowing that in E. coli by having a stronger promoter ($placI^q$) upstream the lac repressor gene, tighter repression of expression from the tac promoter was achieved (Walsh and Koshland, 1985; Amann et al., 1988), it seemed logical to insert a Streptomyces promoter upstream the lacI gene of pGLW51. In this way, transcription of the lac repressor would be expected to improve and (assuming that there was no significant limitation in the translation of lacI in Streptomyces) the repression of ptac in S. lividans would consequently be enhanced.

The choice of a Streptomyces promoter to clone upstream of the lacI gene fell upon the promoter of the phage ØC31 repressor gene. This promoter is a moderately strong promoter which is preferable as a very strong promoter might lead to the production of vast amounts of lac repressor (a DNA binding protein) which in excess could be deleterious to the cell.

6.1.2. The promoter of the phage ØC31 repressor gene

The repressor gene (c) of the Streptomyces temperate phage ØC31 was cloned and sequenced (Sinclair and Bibb, 1988; Sinclair and Bibb, personal communication).

A 397 base pair HindIII/SmaI fragment (Fig. 6.1) containing the 5' end of the coding region of this gene was shown to possess bi-directional promoter activity in vivo (i.e. when inserted into the promoter-probe vectors pIJ486 and pIJ487). This was consistent with the finding of

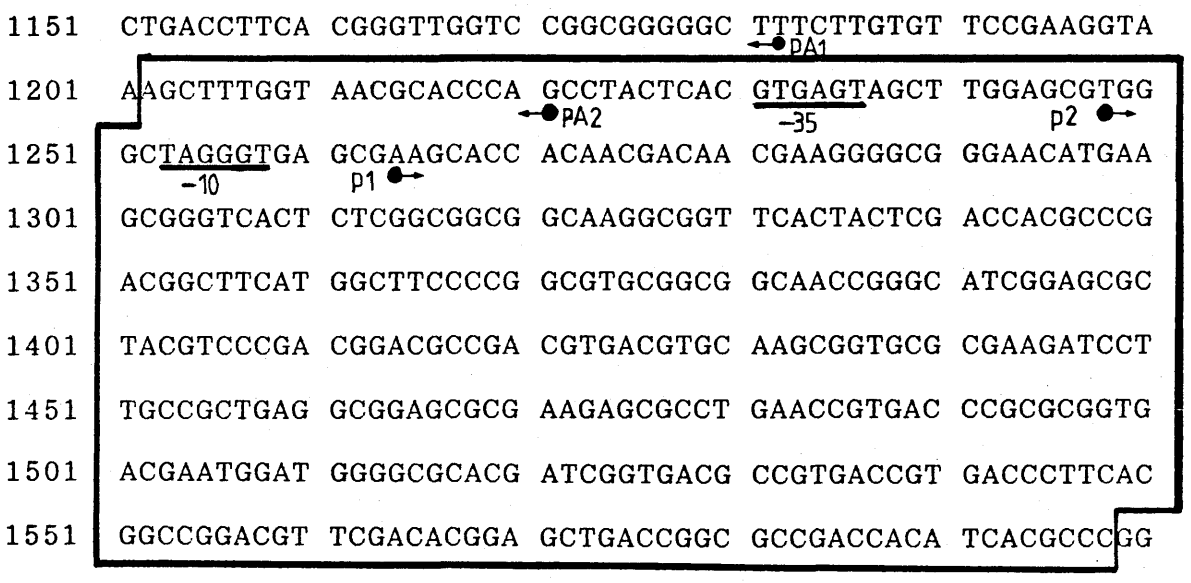


FIG.6.1 Nucleotide sequences surrounding the transcriptional initiation signals of the repressor gene of phage ØC31.

The nucleotide sequence of the 397bp Hind III/Sma I fragment that contains the 5' end of the repressor gene is contained in the box . The locations of p1,p2,pA1 and pA2 start sites are indicated by filled circles with arrows . The putative -10 and -35 regions of p1 are underlined.

two *in vitro* transcription start sites, identifying promoters p1 and p2 (*in vivo* these conferred resistance to kanamycin up to 50ug/ml), reading towards the repressor gene and two transcription start sites oriented in opposite direction, identifying promoters pA1 and pA2 (*in vivo* pA2 conferred resistance to kanamycin up to 25ug/ml).

High resolution nuclease S_1 mapping failed to reveal transcripts corresponding to pA1 and pA2 *in vivo* (probably because pA1 and pA2 were not active when the RNA preparations were made). The same technique did confirm the location of p1 in lysogenic cultures, and of p2 in RNA preparations from cultures containing the multi-copy promoter probe constructs.

While pA1 and pA2 and p2 show no similarities to any known consensus promoter sequences, p1 has a -10 region (TAGGGT) that resembles the consensus sequence (TATAAT) and a -35 region that is less consensus-like.

Thus, in this Chapter it will be described how the 0.4Kb fragment containing the ØC31 repressor was cloned upstream the *lacI* gene of pGLW51.

The new construct, pGLW61, was transformed into *E. coli* and *S. lividans* and compared to pGLW50 and pGLW51 both on plates containing kanamycin and by aphII assays.

The 0.4Kb fragment was obtained from pX1994.4, a gift from M.C.M. Smith. This plasmid contains the 0.4Kb fragment between two HindIII sites.

6.2 Results

6.2.1 Construction of ptac ØC31

ptacH contains two DraII restriction sites, at nucleotides 439 and 2433. The site at nucleotide 2433 is just upstream placI (nucleotide 2380; Fig. 6.2).

Using DraII, partial digests of ptacH were performed by diluting the enzyme to 0.067 or 0.033 U/ug of DNA. These digests were incubated at 37°C for 20 minutes and extracted once with phenol, once with chloroform and precipitated with ethanol. The precipitate, once dry, was dissolved in water and the 5' protruding ends were filled in using the Klenow fragment of DNA polymerase I (Section 2.12).

The 4.9Kb band corresponding to ptacH cut once with DraII was isolated by running the samples on a 1% (w/v) TAE gel. The DNA was purified using a "Geneclean" Kit and ligated to the 0.4Kb restriction fragment containing the promoter of the ØC31 repressor gene. This fragment was obtained by restriction of pX199 4.4 with HindIII followed by one phenol and one chloroform extraction. After precipitation the 5' ends were made blunt using Klenow fragment of DNA polymerase I and the sample was run on a 2% (w/v) TAE gel. The 0.4Kb fragment was isolated, purified from agarose fragments using a "Geneclean" Kit and ligated to ptacH which had been digested partially with DraII.

E. coli 1400 was transformed with this ligation mixture and transformants were selected on L-agar containing ampicillin.

Mini DNA preparations were made for 75 transformants and 10 were found to contain the 0.4Kb insert. Using PvuI (Fig. 6.2) it was possible to distinguish into which of the filled-in DraII sites the fragment containing the promoter of the ØC31 repressor gene had been subcloned and in which orientation.

All transformants containing the insert had it in position 439. Of the transformants containing no insert, 6 were used to check which DraII site had been filled in. This was performed by doing a double digest with EcoRI and DraII. All six transformants were found to have lost the DraII site at position 439.

Since there was a marked preference of the DraII enzyme to digest the plasmid at site 439, one of the above transformants which had lost this site (and now had a unique DraII site) was used to subclone the 0.4Kb fragment containing the ØC31 repressor promoter into the '2433' site.

So, DNA from a transformant containing ptacH with no DraII site at 439 was restricted with DraII. After being extracted with phenol and chloroform the DNA was precipitated and the 5' protruding ends were filled using Klenow fragments of DNA polymerase I. The sample was run on a 1% (w/v) TAE gel. The 4.9Kb band was purified using a "Geneclean" Kit and ligated to the 0.4Kb fragment obtained from pX199 4.4 as before.

The ligation mixture was used to transform E. coli 1400 and transformants were selected on L-agar containing ampicillin. The orientation of the 0.4Kb insert was found by restriction of DNA mini

preparations with PvuI. Several transformants were found to contain ptac ϕC31 (Fig. 6.2).

6.2.2 Construction of pGLW61

To obtain pGLW61 (Fig. 6.3), ptac ϕC31 and pIJ486 were digested with EcoRI and HindIII and then ligated to each other. Thus, the tac promoter was placed just upstream the promoter-less aphII gene. pGLW61 is identical to pGLW51, the only difference being the promoter of the ØC31 repressor gene which is inserted upstream the lacI gene.

E. coli 1400 was transformed with the ligation mixture and transformants selected on L-agar containing ampicillin. Using E. coli mini DNA preparations of transformants containing pGLW61, S. lividans TK24 was transformed with this plasmid and transformants were selected with thiostrepton.

6.2.3 Kanamycin resistance levels of S. lividans TK24 and E. coli 1400 containing pGLW61 on plates.

Four different recombinants of E. coli 1400 containing pGLW61 were streaked on gradient plates (Section 2.8) containing kanamycin between 0-600ug/ml, with and without IPTG. E. coli 1400 containing pGLW50 and pGLW51 were also streaked on the same plates. In all cases, E. coli was grown on L-broth containing ampicillin for 18 hours prior to inoculation of the plates and 10ul of each culture were used to streak the plates.

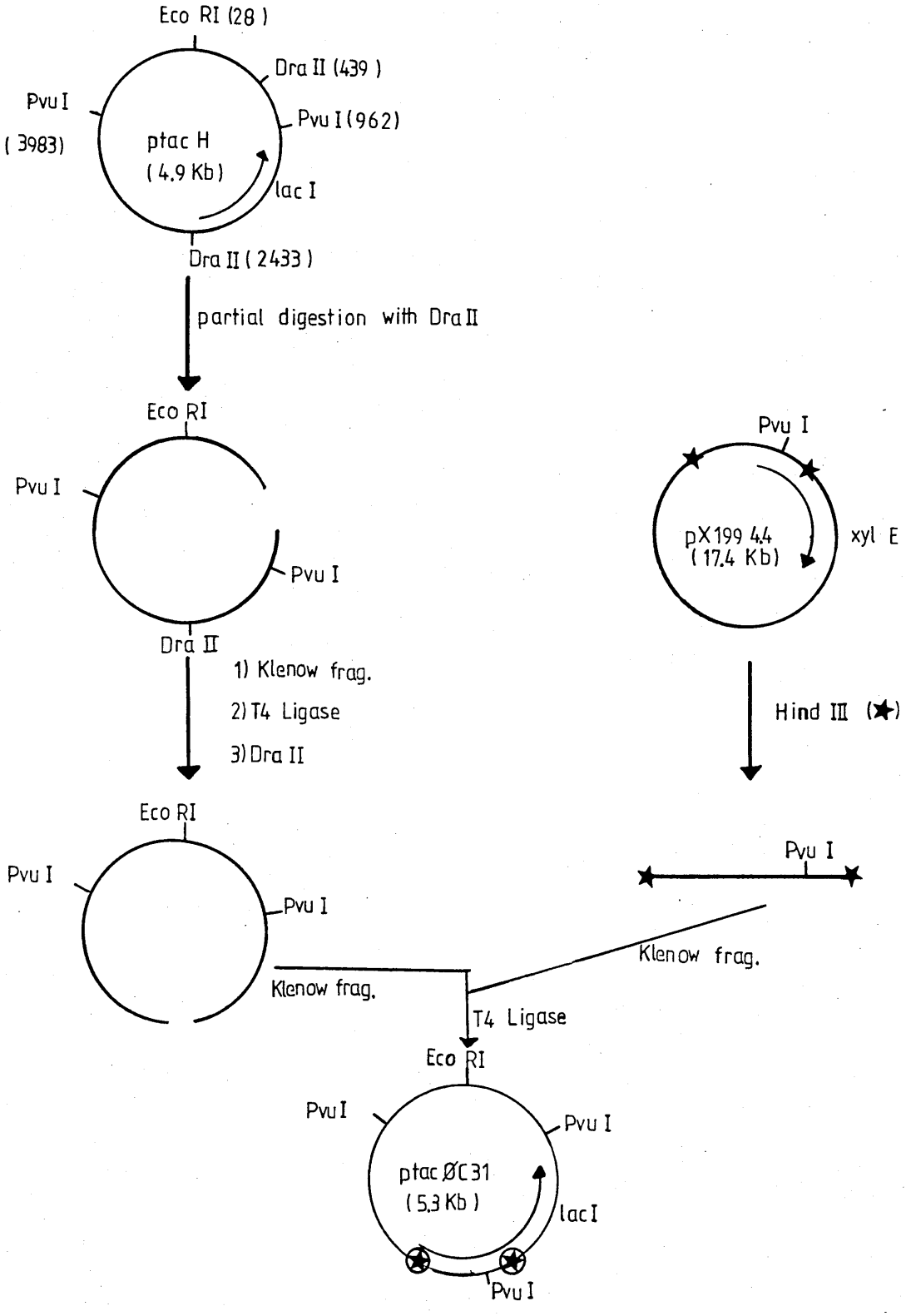


FIG. 6.2 Construction of ptac ØC31.

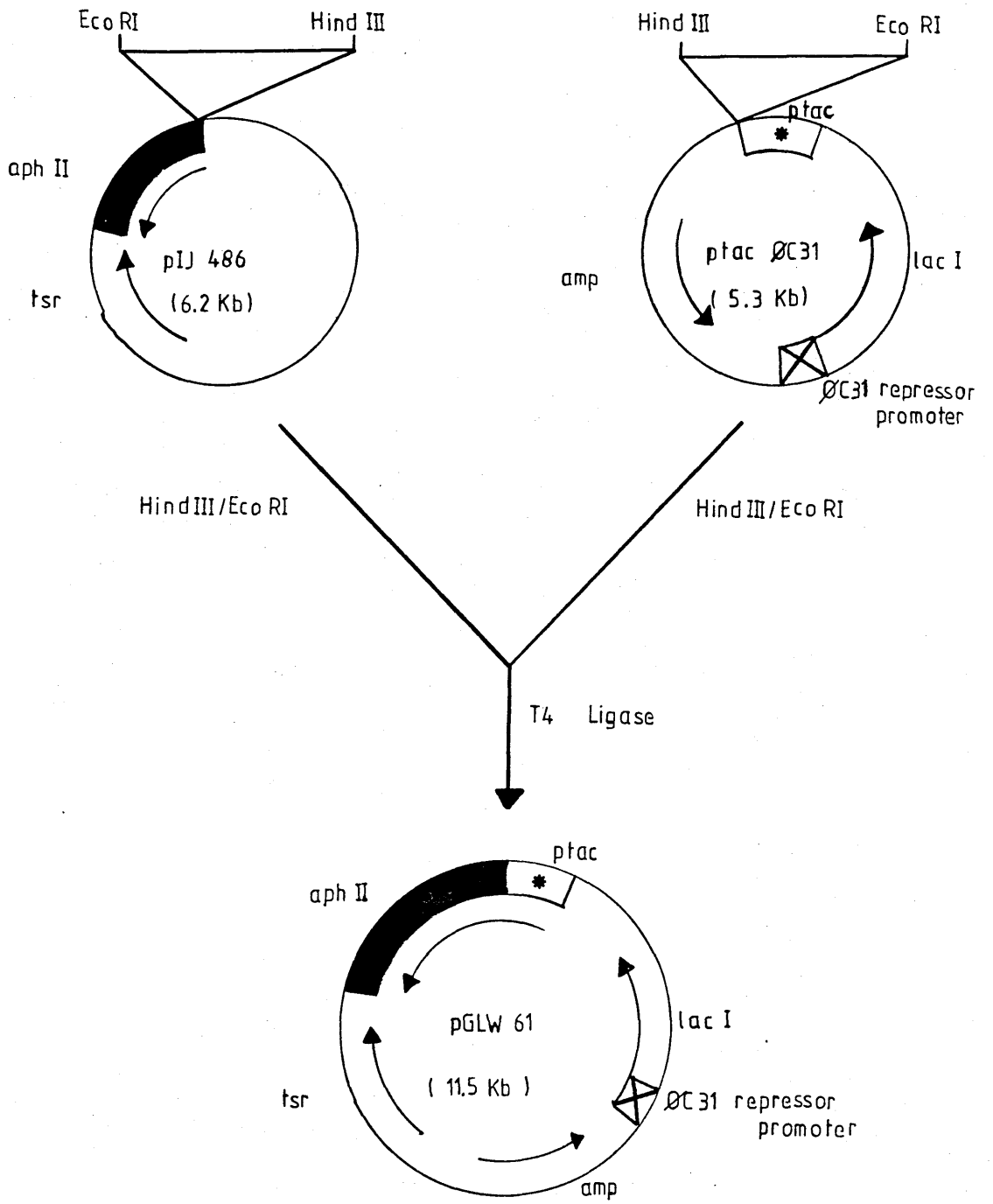


FIG. 6.3: Construction of pGLW61.

E. coli containing pGLW61 showed higher kanamycin resistance in the presence of IPTG (Fig. 6.4). Repression of *ptac* by the *lac* repressor was lifted in the presence of IPTG, in the same way as for *E. coli* containing pGLW51.

It is difficult to use these plates quantitatively but, taking into account that the inoculum for these plates was prepared and grown for the same length of time (and to stationary phase), it is possible to compare the results for the different strains. Repression of *ptac* in *E. coli* containing pGLW61 seemed to be slightly tighter than in *E. coli* containing pGLW51 - this last strain was able to grow at higher levels of kanamycin when no IPTG was present.

For *Streptomyces lividans* TK24, two series of plates with increasing kanamycin concentrations (0-120ug/ml), thiostrepton and one containing IPTG (10mM), and the other containing no IPTG were prepared. Each plate was streaked with approximately 6 X 10^3 spores of TK24 carrying pGLW50, pGLW51 and pGLW61.

S. lividans recombinants containing pGLW50 and pGLW51 behaved as expected (see Section 4.2), i.e. pGLW51 showed repression of *ptac* (of kanamycin resistance) which was lifted in the presence of IPTG. *S. lividans* TK24 containing pGLW61 did not show any effect and grew in the absence of IPTG with no sign of repression up to 120ug/ml of kanamycin.

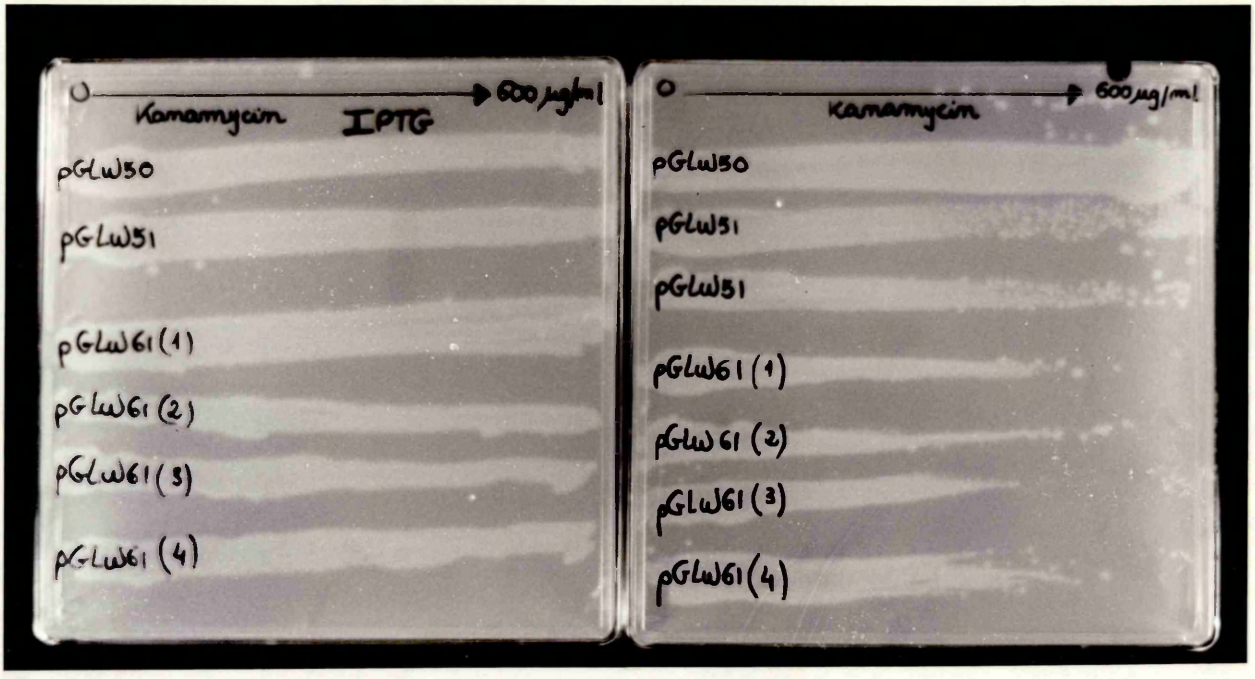


FIG.6.4 Gradient plates containing increasing kanamycin concentrations (0-600 ug/ml) , one containing no IPTG , the other containing 0.5 mM IPTG . These plates were streaked with overnight cultures of E. coli 1400 containing pGLW50,pGLW51 and pGLW61 (four different recombinants).

When no IPTG was present in the medium recombinants containing pGLW61 showed slightly lower levels of resistance to kanamycin than recombinants containing pGLW51.

6.2.4 AphII activity of E. coli 1400 and S. lividans TK24 containing pGLW61

The AphII assay was performed simultaneously as described in Chapter 2 for extracts of E. coli and S. lividans recombinants containing pGLW61, pGLW51 and pGLW50.

For E. coli (Table 6.1) the recombinant containing pGLW50 had a 3.1-fold higher AphII specific activity than that containing pGLW61. This suggests that in pGLW61 the tac promoter is better repressed than in pGLW51 (2.5-fold lower activity than E. coli containing pGLW50). This result is in good agreement with the results obtained from gradient plates that showed that E. coli containing pGLW61 was less resistant to kanamycin than the same strain carrying pGLW51.

For S. lividans (Table 6.2) the crude extracts containing pGLW50 had 1.6X higher AphII activity than that containing pGLW51. For the extract containing pGLW61 the AphII specific activity was two-fold higher than containing pGLW50.

These results are also in good agreement with those obtained from plates: pGLW61 containing S. lividans showed no signs of growth inhibition in the presence of kanamycin (at the levels tested).

STRAIN	SPECIFIC ACTIVITY (U/mg)
E. coli 1400/pGLW50	$6.08 \times 10^{-3} \pm 0.02 \times 10^{-3}$
E. coli 1400/pGLW51	$2.45 \times 10^{-3} \pm 0.05 \times 10^{-3}$
E. coli 1400/pGLW61	$1.99 \times 10^{-3} \pm 0.1 \times 10^{-3}$

1U = 1nmol of ^{32}P incorporated per minute at $0^{o}C$

TABLE 6.1: AphII specific activity of *E. coli* 1400 containing pGLW50, pGLW51 and pGLW61.

STRAIN	SPECIFIC ACTIVITY (U/mg)
S.lividans TK24/pGLW50	$2.4 \times 10^{-4} \pm 0.1 \times 10^{-4}$
S.lividans TK24/pGLW51	$1.5 \times 10^{-4} \pm 0 \times 10^{-4}$
S.lividans TK24/pGLW61	$4.7 \times 10^{-4} \pm 0.2 \times 10^{-4}$

1U = 1nmol of ^{32}P incorporated per minute at $35^{o}C$

TABLE 6.2: AphII specific activity of *S.lividans* TK24 containing pGLW50, pGLW51 and pGLW61.
(Each assay was performed three times.)

6.2.5 Low resolution S_1 mapping using a probe that included the lacI/lacZ region of pGLW61 and pGLW51.

A double-stranded probe corresponding to part of the lacI and of the lacZ gene of pGLW51 and pGLW61 was prepared according to Section 2.16.2 and as shown in Fig. 6.5.

The 1.66Kb probe was hybridised to RNA from S. lividans TK24 containing pGLW51 and pGLW61, to RNA from E. coli 1400 containing pGLW51 and pGLW61 and to tRNA. Each sample was digested with 200U of S_1 nuclease.

As Fig. 6.6 shows, no protected bands were identified with control tRNA. For the E. coli RNA samples, a single protected band, which corresponded to the fully-protected probe, appeared in the tracks. Thus, it was concluded that in E. coli containing either plasmid transcription through the lacI/lacZ region occurred.

A similar result was obtained with S. lividans RNA containing pGLW51 and pGLW61. However the signal present in the track corresponding to S. lividans carrying pGLW51 was much fainter than the signal for S. lividans carrying pGLW61, and both hybridisations were set up with 100ug of total RNA. This suggests that in S. lividans transcription through the lacI/lacZ region is much more efficient in pGLW61.

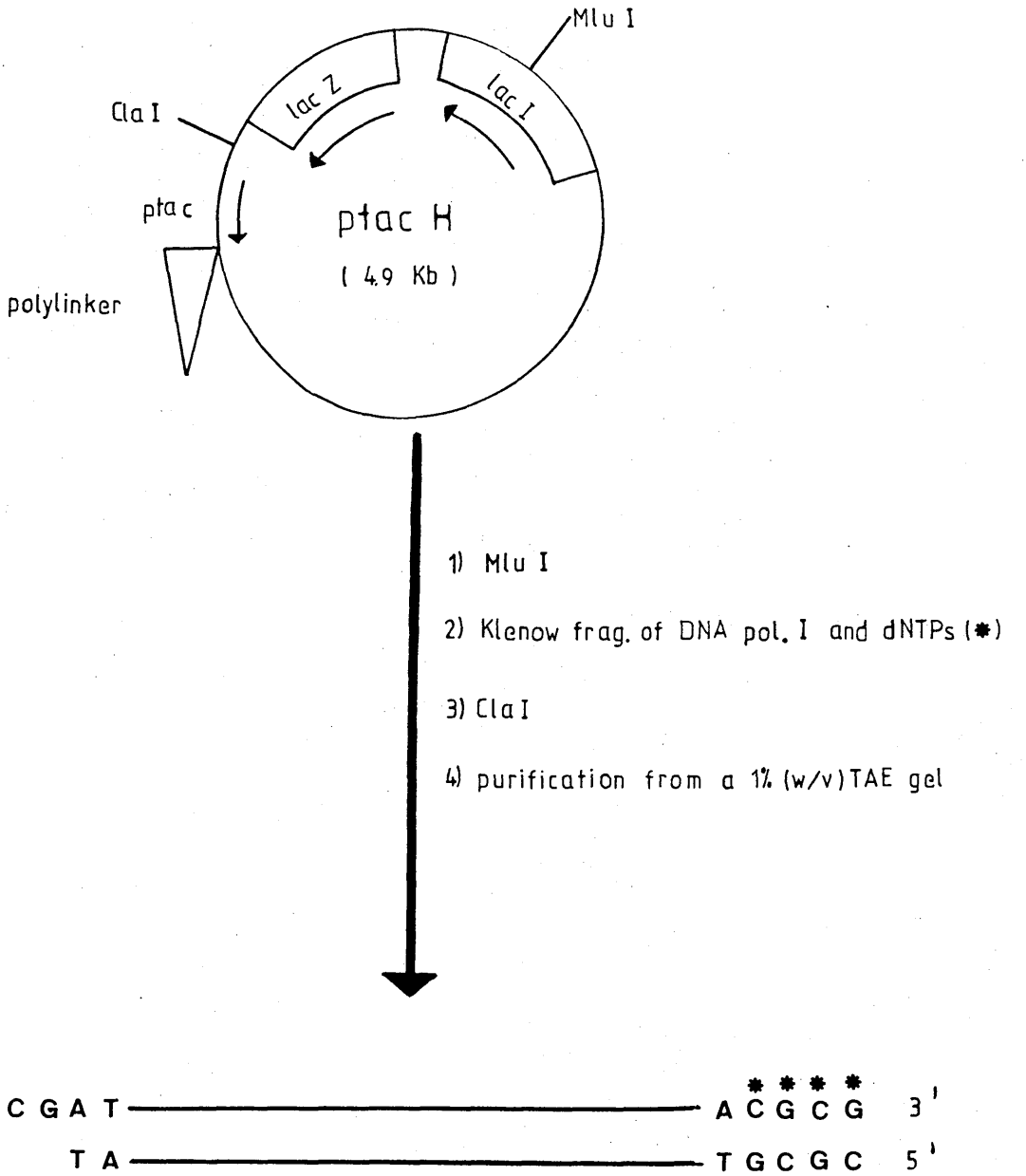


FIG. 6.5 Preparation of lac I/lac Z probe for low resolution S1 nuclease mapping . The 1.66 Kb DNA fragment was labelled using Klenow fragment of DNA polymerase I and radiolabelled dNTP's.

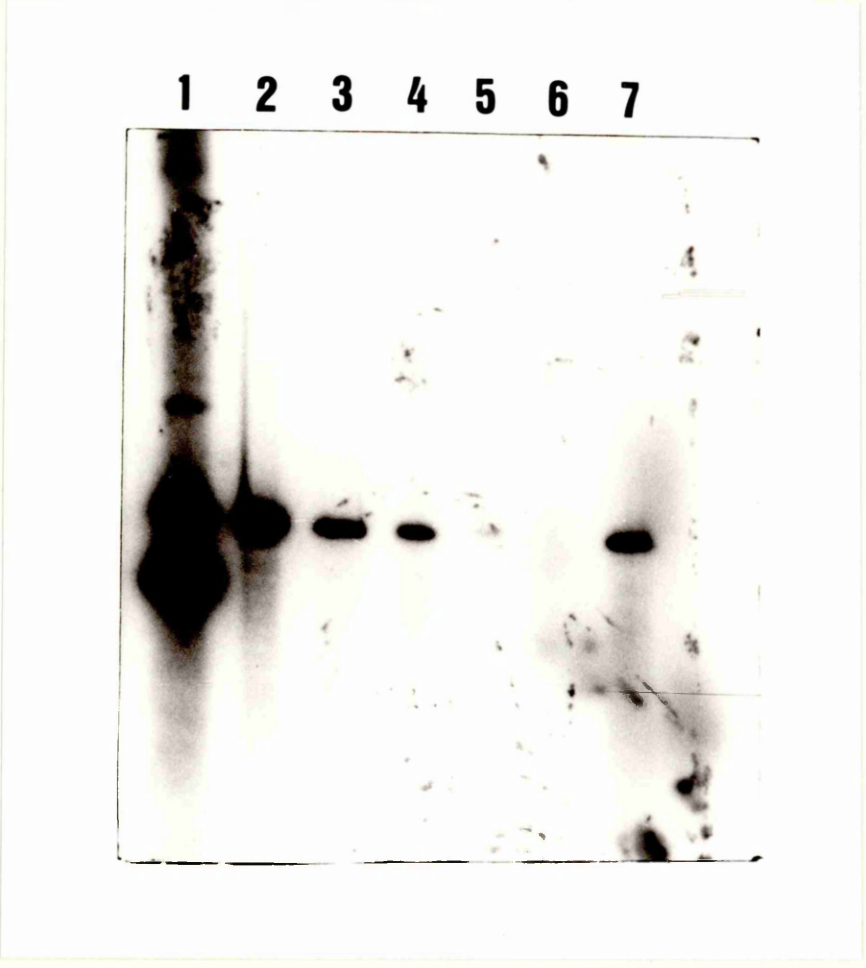


FIG 6.6: Low resolution S_1 nuclease mapping of the lacI/lacZ region in E. coli 1400 and S. lividans TK24 containining pGLW51 and pGLW61.

1-lacI/lacZ probe

2-20ug of total RNA from E. coli 1400/pGLW61

3-10ug of total RNA from E. coli 1400/pGLW61

4-100ug of total RNA from S. lividans TK24/pGLW61

5-100ug of total RNA from S. lividans TK24/pGLW51

6-10ug of tRNA

7-10ug of total RNA from E. coli 1400/pGLW51

6.2.6 Detection of the bldA gene product in S. lividans

In S. coelicolor the tRNA for leucine which recognises the codon UUA is encoded by the gene bldA. This tRNA accumulates late in the growth cycle of the microorganism and it has been suggested that the use of the UUA codon might be a mechanism for translational control in this species (Lawlor et al., 1987).

Since the lacI gene has five TTA codons, the absence of the tRNA recognising the UUA codon could possibly affect translation of the lacI gene in S. lividans. To test whether this tRNA was present in total RNA samples of S. lividans recombinants containing pGLW51 and pGLW61 a probe specific for this tRNA was used in S_1 nuclease protection experiments.

Probe 1 was prepared as described in Section 2.16.1, using the M13 phage derivative constructed by Lawlor et al. (1987). Probe 1 is specific for the tRNA-like transcript of the bldA gene of S. coelicolor and HindIII was the restriction enzyme used to digest the extended probe.

The approximately 230 nucleotide radio-labelled probe was hybridised to total RNA from S. lividans TK24 recombinants containing pGLW51 and pGLW61. As a positive control, total RNA from S. lividans TK24 containing pIJ583 (pIJ486 carrying bldA as a BglII-PstI fragment of 870 base pairs - Lawlor et al., 1987) was also hybridised to the probe. As a negative control, total RNA from an 8 hour culture of S. coelicolor J1501 cts (non-induced) was hybridised to the probe. It

was assumed that such a 'young' culture would not contain the bldA gene product.

The hybridisations were performed at 37°C, overnight and digested with 200 Units of S_1 nuclease according to Section 2.16.1.

The digested products were electrophoresed on a 6% (w/v) denaturing polyacrylamide gel.

As expected, no protected bands were identified with S. coelicolor RNA (this RNA was from an 8 hour old culture, and the bldA gene specifies a tRNA that accumulates late in growth) [Fig. 6.7].

For the other RNA samples a single protected band appeared and it ran approximately 140 nucleotides ahead of the full size probe. Therefore the size of the protected bands was close to 90 nucleotides and the expected protected band was of 87 nucleotides.

This suggested that in S. lividans TK24 grown in liquid culture for 38-42 hours, a tRNA homologous to the bldA gene product of S. coelicolor was produced. The bldA gene product was also detected in S. lividans TK24 containing no plasmid and containing pGLW50 (results not shown).

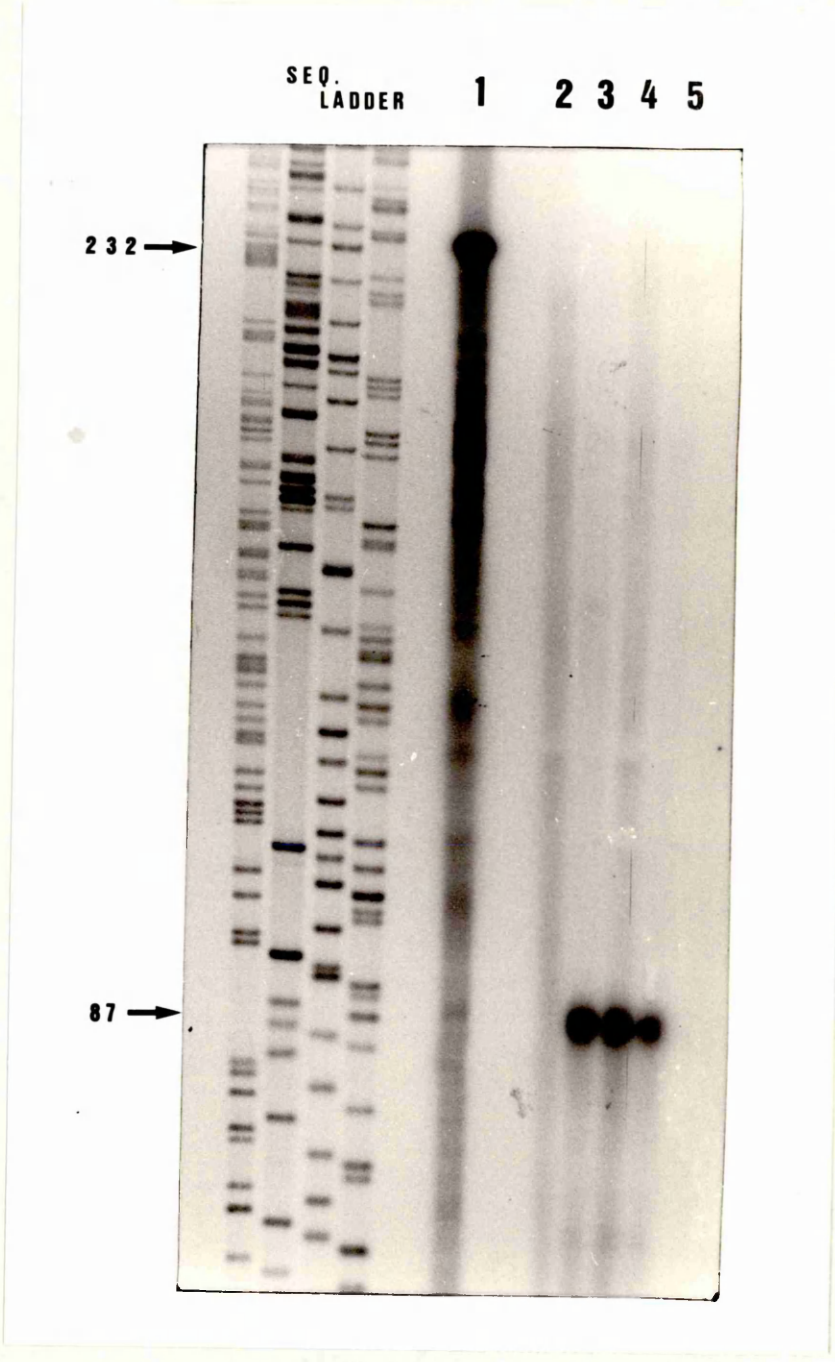


FIG. 6.7: S_1 nuclease protection of the bldA gene product.

1-probe

2-20ug of total RNA from S. coelicolor cts non-induced

3-10ug of total RNA from S. lividans TK24/pIJ583

4-20ug of total RNA from S. lividans TK24/pGLW51

5-20ug of total RNA from S. lividans TK24/pGLW61

Arrows indicate the full size probe (232 nucleotides) and the protected band (87 nucleotides).

6.3 Discussion

In E. coli, both the aphII assays and the gradient plates showed that insertion of the ØC31 repressor promoter upstream the lacI gene improved slightly the repression of the tac promoter.

This suggested that this promoter was recognized by E. coli RNA polymerase. As a result, there was an increment of lacI transcription which led to higher production of lac repressor.

It is not known whether the promoter p1 or p2 (or both) is being recognized in E. coli. It is more likely that p1 is recognized since it somewhat resembles the consensus bacterial promoter, but this would only be known with certainty by doing high resolution S_1 mapping of this region in E. coli.

M.C.M. Smith (personal communication) has also found that the ØC31 repressor promoter region cloned in pX199 4.4 [a low copy probe vector containing the catechol oxidase (xylE) as a reporter gene] could express low levels xylE activity in E. coli.

In S. lividans, it was also expected that by increasing the level of lacI mRNA this would result in higher concentrations of lac repressor in the cell, improving the repression of the tac promoter. However, this was not the case. The cloning of the ØC31 repressor promoter upstream the lac repressor gene resulted in no apparent repression, and S. lividans containing pGLW61 showed greater aphII activity than S. lividans containing pGLW50 (which carries no lac repressor gene).

A possible interpretation for the above is that the Streptomyces promoter cloned upstream the lacI gene is being used by RNA polymerase to start a transcript that includes the gene lacI, part of lacZ gene and also reading through to the ptac/neo gene of pGLW61.

However, this does not clarify why no further repression of ptac occurred when this construct was present in S. lividans. If the translation of the lacI gene was, for some reason, restricted in S. lividans even though there might be more lacI mRNA to be translated, this might not result in a higher level of lac repressor.

The above interpretations combined would explain why S. lividans containing pGLW61 shows no signs of repression of the tac promoter, and why it shows higher AphII activity than S. lividans carrying pGLW50.

Low resolution S_1 mapping was performed (Section 6.2.5) to compare the abundances of the lacI/lacZ mRNA in S. lividans containing pGLW61 and pGLW51. This experiment clearly showed that the abundance of the lacI/lacZ mRNA was much greater in S. lividans containing pGLW61. This result agrees with the fact that in pGLW61 there might be a message that reads through ptac (as opposed to starting in ptac) and if the lacI mRNA translation was not efficient then this might result in higher AphII activity and no apparent repression.

In E. coli (at least) the 1.6Kb probe used in the low resolution S_1 mapping experiments should hybridise to the mRNA that starts at the lacI promoter and terminates just before the lac promoter in pGLW51 and pGLW61 at the end of the lacI gene. The band corresponding to

this protection should be approximately 0.8Kb and was not seen on the 1% (w/v) agarose gels in which the S_1 digested samples were run, possibly because these were run too far. It is also possible that in the above constructs the terminator was not efficient. Selliti *et al.* (1987) have shown that, in *E. coli*, the *lacI* gene does not have a strong termination signal (and it is not known how effective this is in *Streptomyces*). However, if the *lac* repressor is present on the DNA template, a greatly increased fraction of the transcripts produced have 3' ends in the *lac* control region sequence.

Yet again, it is not known how well this system might work in *S. lividans* but if there is no sufficient *lac* repressor made and the above is true in this strain, then read through the *lacI* terminator is very likely to occur.

Therefore, from the low resolution S_1 mapping experiments it was concluded that in *E. coli* containing pGLW51 and pGLW61 and in *S. lividans* containing pGLW61 the *lacI*/*lacZ* region was efficiently transcribed. Thus, it is possible that some of this message read through the *tac* promoter and this could be one of the reasons why repression of the *tac* promoter was not complete in these constructs. The insertion of an efficient terminator at the 3' end of the *lacI* gene might improve repression of the *tac* promoter in these constructs.

It must be added, though, that the high resolution S_1 mapping experiments of the *tac* promoter (Section 4.2.5) showed poor signals for fully protected probe, and then it is not easy to differentiate between DNA/DNA hybridisation (the probe might not have been fully denatured, and so some probe could still be double stranded) and

DNA/RNA hybridisation. The method of hybridisation (in trichloroacetate) used for the low resolution experiments has been reported to be at least 50-fold more sensitive than the formamide method used for the high resolution S_1 mapping experiments (Murray, 1986). Thus, the full-length mRNA might not have been detected so easily in the earlier high-resolution S_1 experiments to map the tac promoter.

The other major question raised by the results presented in this Chapter is the efficiency of translation of the lacI mRNA.

Not a lot is known about translation and translation efficiency in Streptomyces. The codon usage in the species is markedly asymmetric, favouring codons with G or C in the third position (Hopwood et al., 1986). Despite this, several foreign genes have been expressed in Streptomyces, of these the Tn5 aphII gene (Ward et al., 1986), the lacZ gene of E. coli (King and Chater, 1986) and human interferon α-2 (Pulido et al., 1986) are examples. This indicates that the tRNA population cannot be too asymmetric since genes of lower G + C base composition are still expressed.

In S. coelicolor bldA mutants are not impaired in vegetative growth, but are unable to produce aerial hyphae or spores and unable to produce any of the four antibiotics produced by the wild-type strain.

Recent studies (Lawlor et al., 1987) revealed that the bldA gene encodes a tRNA that recognises the rare Streptomyces codon UUA (for leucine). This tRNA accumulates late in growth and it has been

suggested that the use of this codon may be a mechanism for translational control of differentiation.

Not only is this codon rare in Streptomyces, but also it seems that the presence of the UUA codon in a gene (TTA in the DNA) greatly impairs translation of this gene in S. coelicolor bldA mutants (K.F. Chater, personal communication).

However, using the S. coelicolor probe for bldA, a single signal was detected in all the S. lividans TK24 recombinant RNA samples tested using S_1 nuclease digestion. This suggested that S. lividans TK24 has a gene homologous to the S. coelicolor bldA gene and that in liquid culture, 38-42 hours (the time at which RNA was prepared and that cells were harvested for AphII assays) after inoculation this gene was being expressed.

Therefore, it was concluded that even though the lacI gene contained five TTA codons, this was probably not limiting translation of the gene, at least at the time at which experiments were performed, and that there must be another translational limitation for this gene in S. lividans. It is difficult to know what this translational limitation is. King and Chater (1986) for example have studied the expression of the E. coli lacZ gene in Streptomyces. They found that this gene was expressed at low levels in this genus and that activities were markedly higher in S. lividans than S. coelicolor. It was suggested that this was probably due to inefficient translation of lacZ mRNA and/or mRNA instability.

Why the *lacZ* gene is poorly expressed in *Streptomyces* and why it is better expressed in *S. lividans* than *S. coelicolor* is not clear, but then very little is known about translation in *Streptomyces*.

CHAPTER 7

AN ATTEMPT TO IMPROVE THE EFFICIENCY OF TRANSLATION OF THE aphII GENE

7.1 Introduction

Efficient expression of a gene requires the accurate positioning of powerful transcriptional and translational initiation signals relative to the coding sequence. Thus, even though transcription from the promoter of a gene might be very efficient (giving rise to abundant mRNA), translational initiation can limit the overall efficiency of the expression of that gene.

In E. coli, translational initiation is a multi-component process requiring interactions between RNA species (mRNA, 16S rRNA and fMet-tRNA fMet), initiation factors (IF-1, IF-2 and IF-3) and the ribosomal proteins (Lewin, 1985).

In E. coli, the initiation of protein synthesis begins at a start codon which in most cases is an AUG (Gold et al., 1981). This codon is at the centre of the RNA fragment which is not degraded by RNAse during initiation of translation. These regions, which are 30-40 bases in length, are called ribosome binding sites (RBS) (Gold et al., 1981; Kozak, 1983).

Shine & Dalgarno (1974) postulated that during translation initiation the purine-rich sequences five to nine bases (Shine-Dalgarno region) upstream from the start codon in the mRNA might pair with complementary sequences close to the 3' end of 16S rRNA. The Shine-Dalgarno region is thought to be involved in the positioning of the 30S ribosomal subunit with respect to the start codon on the mRNA.

Several hundred bacterial and phage mRNAs have been sequenced (Lodish, 1976; Iserentant and Fiers, 1980). Nearly all of the mRNAs include sequences with some complementarity to the 3' end of the 16S rRNA.

The sequences of the 3' ends of the 16S rRNA from S. lividans (Bibb and Cohen, 1982) and from S. griseus and other actinomycetes (Stackebrandt and Woese, 1981) have been determined. They all show identity of a 14 base pair sequence at the 3' end and to a sequence believed (from DNA sequence analysis) to be at the 3' end of the 16S rRNA from S. coelicolor A3(2) (Baylis and Bibb, 1987 and 1988).

Consideration of the currently available information on mRNA structure and translational initiation complicates the problem of constructing optimal translational initiation sequences. Many parameters influence the formation of the translational initiation complex including:

- the length of mRNA complementary to 16S rRNA (Lodish, 1976; Roberts et al., 1979; Iserentant and Fiers, 1980).

- the distance (spacer) between the Shine-Dalgarno sequence and the initiation codon (Goeddel et al., 1979; Siner et al.,1981).

- the nucleotide sequences on either side of the AUG initiation codon and of the spacer (Taniguchi and Weissmann, 1979; Ganoza et al., 1982; Matteucci and Heynekei, 1983; Hui et al., 1984).

- the nucleotide sequences upstream from the Shine-Dalgarno region (McCarthy et al, 1985).

In previous Chapters it was described how bifunctional expression vectors for E. coli and S. lividans were constructed. In these vectors (pGLW50, pGLW51 and pGLW61) the tac promoter was cloned upstream from the aphII gene of pIJ486.

The sequence of the DNA region that spans from the -10 region of the tac promoter to the translational start codon (ATG) of the aphII gene is shown in Fig. 7.1. Therefore these constructs have transcriptional starts which are 65 base pairs upstream from the aphII Shine-Dalgarno region and 79 base pairs upstream from the translational start codon, ATG.

As stated earlier, there is some evidence for the involvement of sequences upstream from the Shine-Dalgarno region in the initiation of translation. Roberts et al. (1979) constructed several plasmids carrying the lambda cro gene sequence downstream from the lac promoter. The fusion transcripts, under lac control, all shared the same 5' terminal lac sequence, but had different extents of the cro initiator region. Plasmids pTR213, pTR199 and pTR214 (Fig. 7.2) had a 20-fold difference in cro expression, even though the differences in nucleotide sequence were more than 15 nucleotides 5' to the Shine-Dalgarno region. Substantially different RNA secondary structures might account for the observed differences in cro gene expression.

Evidence supporting the role of messenger RNA secondary structure in the control of translation initiation is also provided by work done on the lamB gene (which encodes a major outer membrane protein) of E. coli (Hall et al., 1982). Two point mutations (one just upstream of the Shine-Dalgarno region, the other within the translated region)

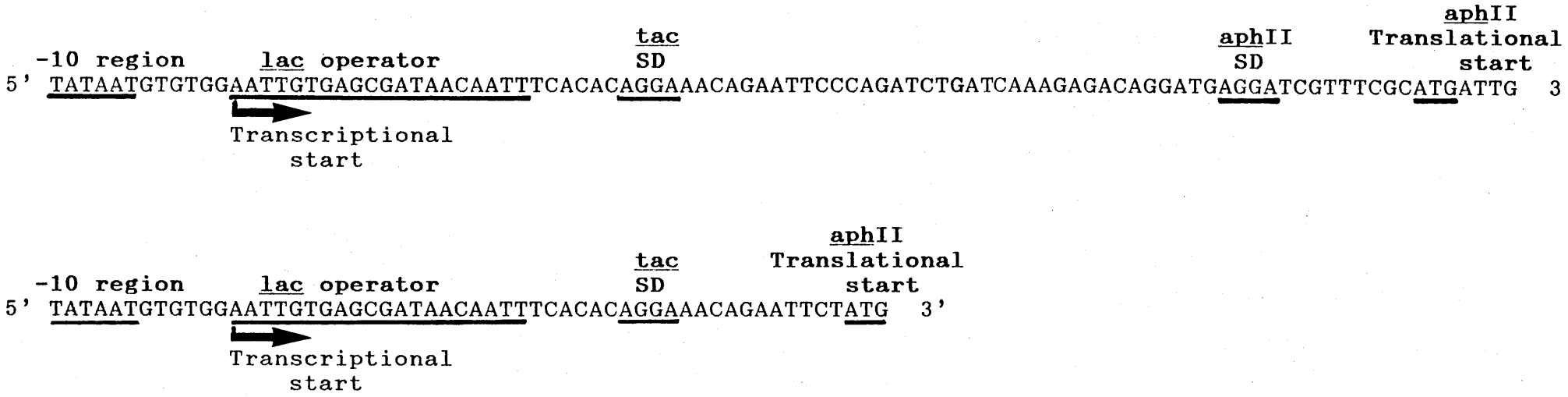


FIG.7.1 DNA sequence of pGLW50,pGLW51 and pGLW61 (top) that extends from the -10 region of the tac promoter to the translational start of the aphII gene as compared to the original ptac sequence (bottom) (de Boer et al,1983).

(SD= Shine-Dalgarno region)

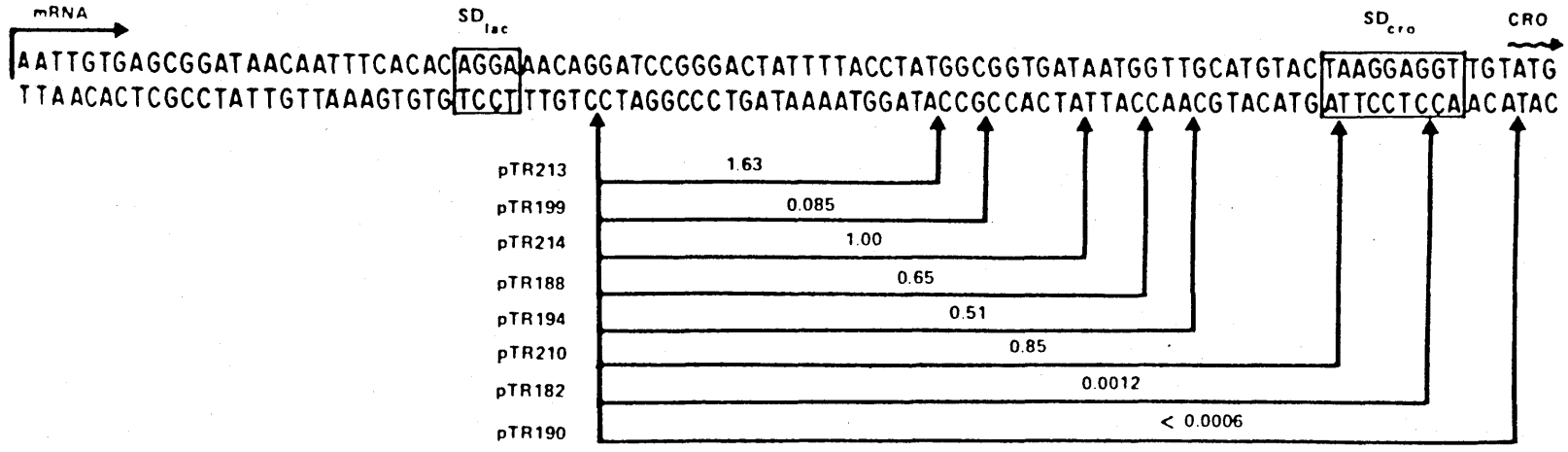


FIG.7.2 The DNA sequence of pTR 161 that extends from the transcriptional start of plac (straight arrow) to the translational start of the cro gene (wavy arrow) is shown above.

Using exonuclease III and S1 nuclease deletion plasmids of pTR 161 were constructed. For eight of these the extent of the deletions is indicated by brackets.

The numbers on the brackets represent the level of cro protein as a percentage of soluble protein in the cells transformed with each of the deletion plasmids. (taken from Roberts et al, 1979)

affected translation initiation as they favoured the formation of a hairpin structure in the lamB transcript which resulted in the Shine-Dalgarno sequence being inaccessible to ribosomes.

Individually, the two mutations reduced the amount of gene product, but together they suppressed each other by regenerating the wild-type instability of the hairpin structure.

In addition, it has also been known that certain sequences from natural RBS's can be used to promote highly-efficient translational initiation. For example, a region of the E. coli atpE RBS (approximately 40 base pairs upstream from the start codon) had been shown to promote efficient translational initiation of a number of genes (McCarthy et al., 1986).

Together with all this information about the possible influence of mRNA secondary structure and sequence in the process of initiation of translation, and possibly related to it, there is evidence for the improvement of gene expression by bringing the promoter closer to the translational start (Yarranton, 1989; Backman and Ptashne, 1978).

In this Chapter, an attempt to improve expression of the aphII gene of pGLW51, by bringing the tac promoter closer to the ATG codon of the aphII gene is described.

For this an EcoRI restriction site was introduced (using site directed mutagenesis) between the Shine-Dalgarno region of the aphII gene and the ATG start codon of mGLW54 using site directed mutagenesis (Fig. 7.3). Following this, the 37bp fragment flanked by the two EcoRI

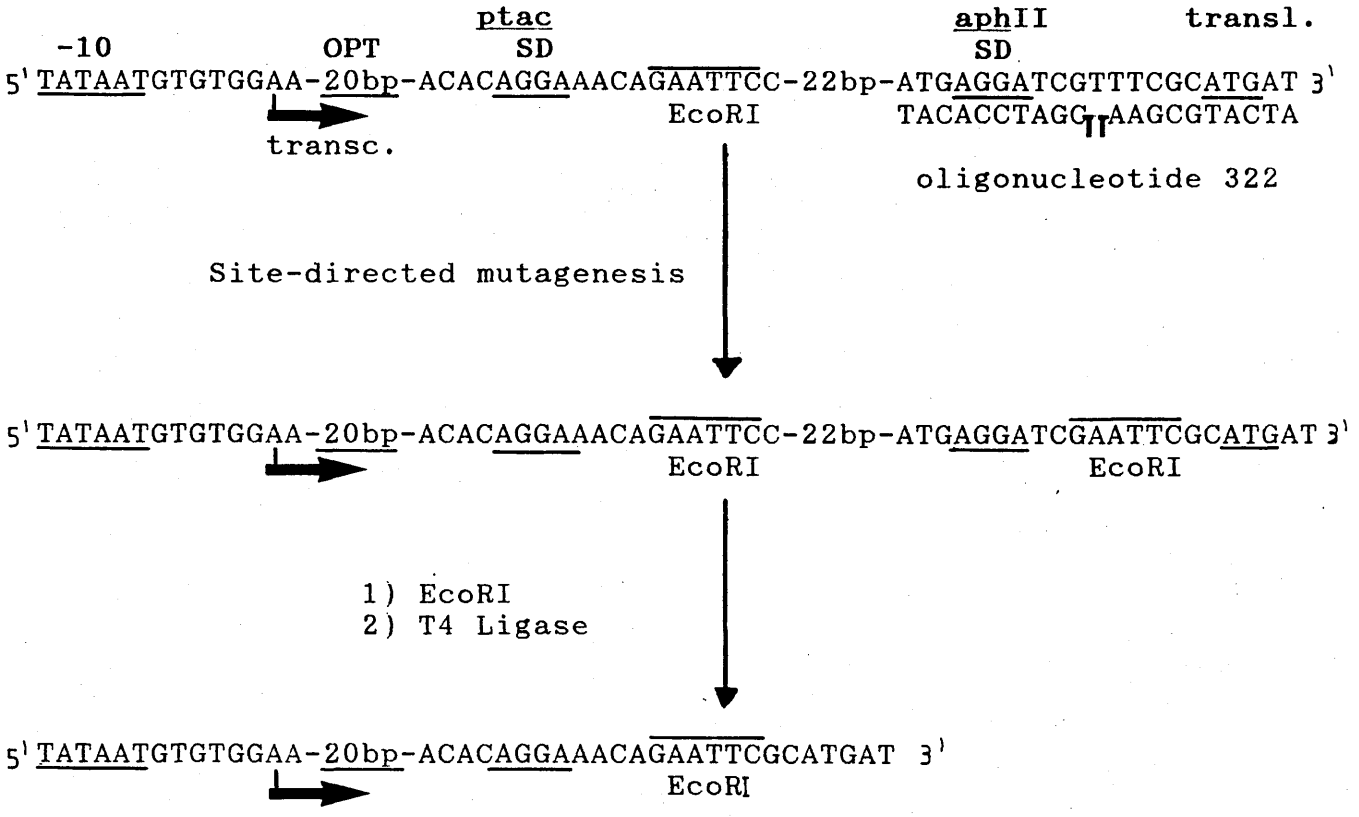


FIG.7.3 Shown is part of the sequence of mGLW54 extending from the -10 region of the tac promoter to the translational start of the aph gene.

Oligonucleotide 322, containing an EcoRI restriction site was used to mutagenise the sequence. The aph II transcriptional start was brought closer to the tac transcriptional start by digestion with Eco RI and religation.

(SD= Shine-Dalgarno region, OPT= lac operator, -10=-10 region, transl=translational start, transc=transcritional start)

restriction sites was removed, and the tac Shine-Dalgarno region left at 12bp from the ATG start of the aphII coding region. In this way a unique EcoRI site was left between the Shine-Dalgarno region and the ATG codon, allowing for introduction or removal of sequences in the area.

7.1.1 Brief introduction to the site directed mutagenesis method used

The method followed was the one described by Vandeyar et al. (1988). It is based on selective methylation of the mutant strand by the incorporation of 5- methyl dCTP.

A mutagenic oligonucleotide is annealed to a single-stranded template and extended by DNA polymerisation in the presence of 5-methyl-dCTP. Restriction of the hemimethylated DNA with MspI results in the nicking of the parental strand which is subsequently removed by treatment with exonuclease III. The mutants are recovered by transfection with the methylated strand of JM107MA (Blumenthal et al., 1985), a non-restricting E. coli host.

This method is very rapid; all reactions are done in the same tube, and it gives high mutation frequencies since it eliminates the non-mutant parental sequences in vitro.

7.2 Method

7.2.1 Phosphorylating the oligonucleotide using T4 kinase

The mutagenic oligonucleotide (100pmol) was 5' phosphorylated with 1 Unit of T4 polynucleotide kinase in a 40ul reaction containing 100mM Tris-HCl, pH 8.0, 10mM $MgCl_2$ and 1mM ATP. After 30 minutes of incubation at 37°C the reaction was stopped by heating at 70°C for 10 minutes.

7.2.2 Annealing the phosphorylated oligonucleotide to the single-stranded DNA

2ug of the single-stranded DNA to be mutated were annealed with 5pmol of the phosphorylated oligonucleotide at 70°C for 15 minutes, and then at room temperature for 10 minutes in 20ul of 20mM Tris-HCl pH 7.5, 10mM $MgCl_2$ and 50mM NaCl.

7.2.3 Synthesis of the mutated strand

The total reaction volume was increased to 100ul with 70mM Tris-HCl, pH 8.0 and 10mM $MgCl_2$ containing dATP, dGTP, dTTP and 5-methyl-dCTP to a concentration of 0.5mM each, ATP was also added to a concentration of 1mM.

To the above mixture 10 U of T4 DNA polymerase (Pharmacia) and 3 U of T4 DNA ligase were added. After 90 minutes at 37°C, the reaction was terminated by incubating at 70°C for 10 minutes.

A 2ul sample was taken (T_3) and used to transform competent cells of E. coli JM107MA.

7.2.4 Elimination of the parental strand

7.2.4.1 Digestion with MspI and HhaI

To the above reaction 10 U of MspI and 10 U of HhaI were added (the parental strand is nicked by MspI at C^5mCGG sequences, yielding sites for exonuclease III action and any uncopied single-stranded molecules are linearized by the HhaI digest). The reaction was incubated for 37°C for 45 minutes, a 2ul sample (T_4) was used to transform competent cells of E. coli JM107MA.

7.2.4.2 Digestion with exonuclease III

The parental strand was then degraded by the addition of 100 U of exonuclease III to the MspI/HhaI digest and incubated at 37°C for 45 minutes. This reaction was terminated by incubating at 70°C for 10 minutes and a 4ul sample (T_5) was used to transform competent cells of E. coli JM107MA.

7.2.5 Transformation of JM107MA

JM107MA cells were made competent and transformed with T_3, T_4 and T_5 as described in Section 2.10.

7.3 Results

7.3.1 Site directed mutagenesis of mGLW54 using oligonucleotide 322

mGLW54 single-stranded DNA was annealed to phosphorylated oligonucleotide 322 and the method followed was the one described in Section 7.2.

T_3, T_4 and T_5 were used to transform competent cells of *E. coli* JM107MA and 684, 128 and 9 transformants were obtained respectively.

Mini preparations of DNA were made for 20 transformants (1-9 from T_5 and 11-20 from T_4). DNA from these preparations was digested with EcoRI and run on 12% (w/v) polyacrylamide gels in TBE alongside pUC18 digested with EcoRI and PstI (which results in a 38bp fragment).

If any of the transformants was a mutant it should have an extra EcoRI site (Fig. 7.4). Therefore, the parental DNA (mGLW54) digested with EcoRI should result in two fragments of approximately 0.1 and 7.5Kb, while digestion of the mutant DNA (mGLW62) should result in three fragments of 37bp and approximately 0.1 and 7.5Kb.

Transformants 1 and 7 were shown to contain mGLW62. Single-stranded DNA from these two mutants was sequenced and the presence of the new EcoRI site was confirmed (Fig. 7.5).

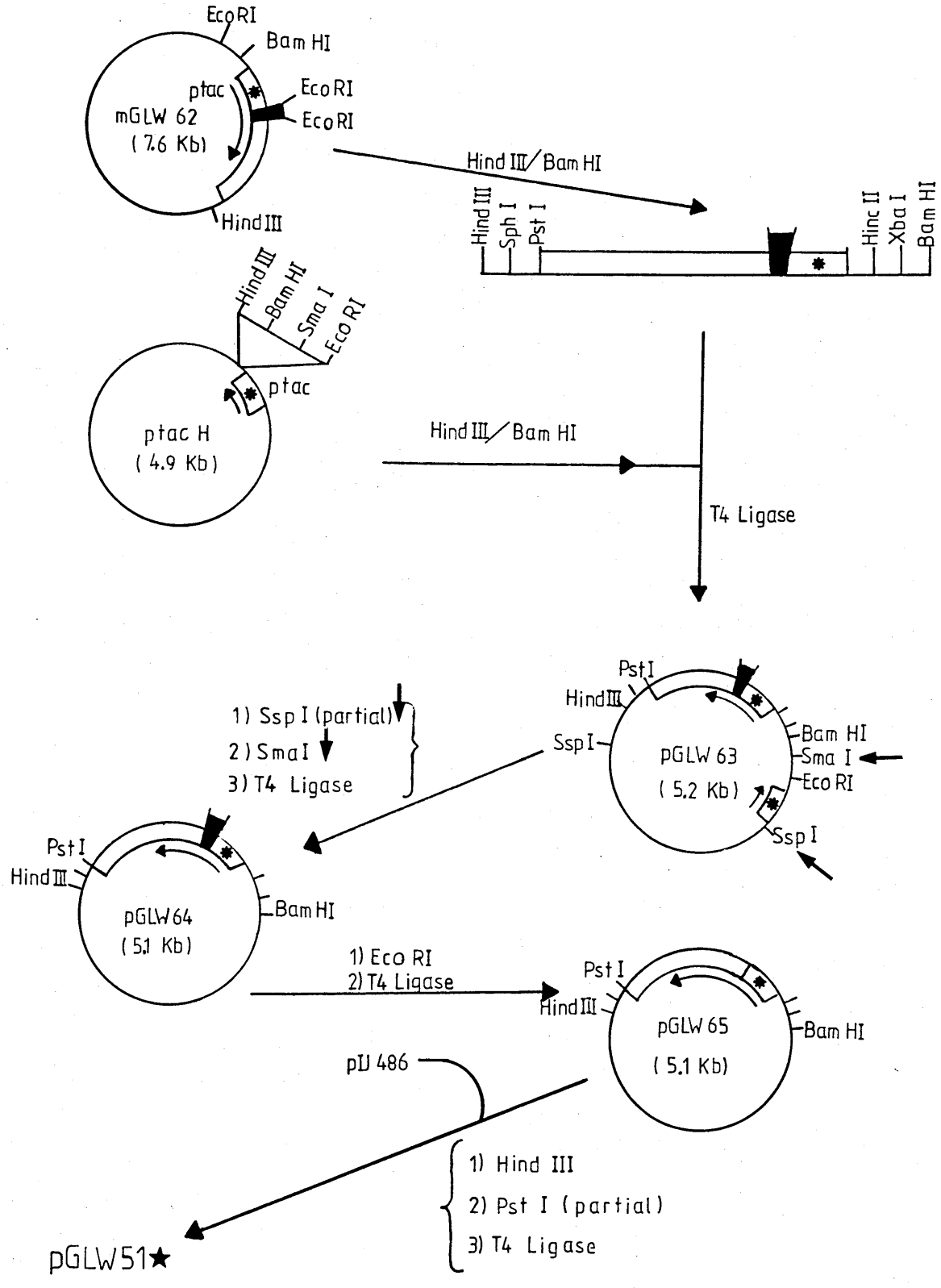


FIG.7.4 Construction of plasmids from mGLW62 to pGLW51*

FIG. 7.5: DNA sequence of mGLW54 (wild type) and DNA sequences of mGLW62 (mutants 1 and 7).

7.3.2 Construction of pGLW51*

The aim of the site directed mutagenesis was to introduce an EcoRI site closer to pre-existing EcoRI so that a construct similar to pGLW51 could be made in which the ribosomal binding site of the aphII gene would become much closer to the transcriptional start of ptac.

The EcoRI site was introduced; it was now a matter of removing the 37bp sequence and of introducing the new ptac/aphII sequence into a new construct. This process is explained in the following sections and illustrated in Fig. 7.4.

7.3.2.1 Construction of pGLW63

mGLW62 was digested with HindIII and BamHI and the 0.3Kb fragment was isolated from a 2.0% (w/v) TAE gel. After removing contaminating agarose with a "Geneclean" kit, this fragment was ligated to ptacH which had been digested with the same enzymes.

The ligation mixture was used to transform competent cells of E. coli DS941. Mini preparations of DNA were made for five transformants. These DNA samples were digested with HindIII and HincII and were all shown to contain the 0.3Kb insert. Thus, the five transformants carried pGLW63.

7.3.2.2 Construction of pGLW64

pGLW63 contained two tac promoters, therefore the next step was to remove the original tac promoter to originate pGLW64.

pGLW63 has two SspI sites (Fig. 7.4). Partial digestion of this plasmid was performed with this enzyme, under the usual conditions but in the presence of 25 and 50ug/ml of ethidium bromide. These two digests were run on a 2% (w/v) TAE gel and the 5.2Kb band (corresponding to a single cut with SspI) was purified using a "Geneclean" kit. The DNA recovered was then digested with SmaI and run on a 1% (w/v) TAE gel. The 5.1Kb band (a cut with SSPI[116] and a cut with SmaI should result in a 5.1Kb fragment) was purified and self ligated.

Competent cells of E. coli DS941 were transformed with the ligation mixture, the transformants were selected with ampicillin and mini preparations of DNA were made of five transformants.

By digesting this DNA with HindIII and XbaI simultaneously and running on a 2% (w/v) TBE gel, all transformants showed a 0.3Kb insert. By digestion with SspI, they all showed the presence of a single SspI site. Thus all transformants contained pGLW64.

7.3.2.3 Construction of pGLW65

pGLW65 had no other EcoRI sites apart from the ones contained in the ptac/aphII region (Fig. 7.4). Therefore the 37bp piece of DNA contained between these two sites could easily be removed.

pGLW64 was digested with EcoRI and run on a 1% (w/v) TAE gel. The 5.1Kb band was purified using a "Geneclean" kit and self-ligated. The ligation reaction was used to transform competent cells of DS941. Transformants were selected with ampicillin and two were shown to contain pGLW65.

7.3.2.4 Construction of pGLW51* (Fig. 7.6)

pGLW65 has two PstI sites. The small sequence of the aphII gene contained in this plasmid is flanked by a PstI site and the tac promoter.

pIJ486 has five PstI sites, two of these are within the aphII gene, one being the same one that flanks the aphII sequence contained in pGLW65.

Both pGLW65 and pIJ486 were digested totally with HindIII and then digested partially with PstI. For the partial digestions approximately 0.033 U of PstI were incubated with 1ug of DNA at 37°C for 30 minutes. These reactions were then run on a 1% (w/v) TAE gel.

From the pIJ486 digest a 6.0Kb band was isolated and purified using a "Geneclean" kit. From the pGLW65 digest a 5.1Kb band was isolated and purified using a "Geneclean" kit. The purified DNAs were then ligated together.

Competent cells of E. coli 1400 were transformed with the ligation mixture. Transformants were selected with ampicillin and mini

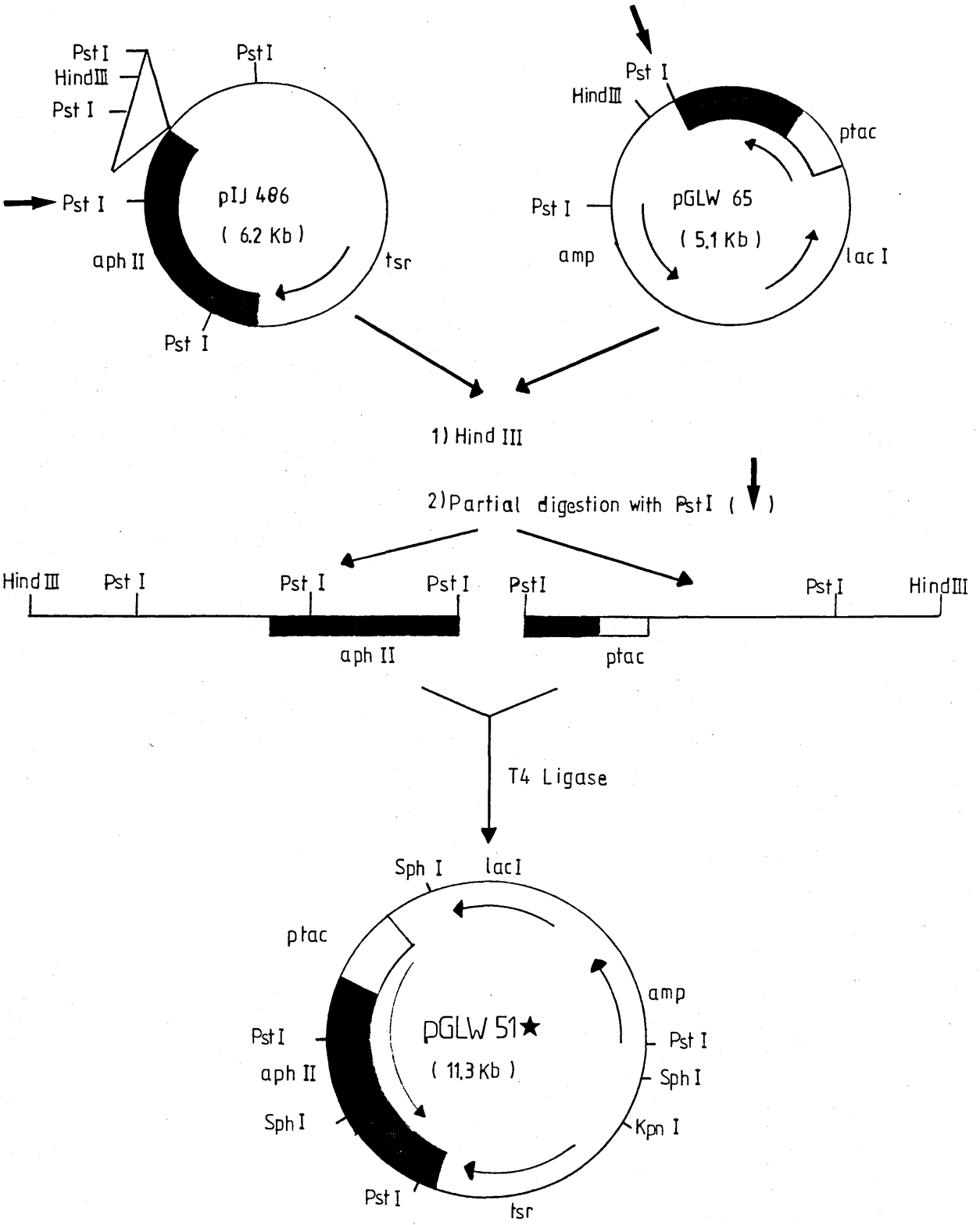


FIG.7.6 Construction of pGLW51*

preparations of DNA were made for sixteen (1-16). Transformant 8 was shown to contain pGLW51* by digestion with HindIII, PstI, KpnI, SphI.

7.3.2.5 Plasmid sequencing the tac/aphII region of pGLW51*

pGLW51* was digested with XbaI and PstI and run on a 1.5% (w/v) TAE gel. The 0.3Kb band was purified using a "Geneclean" kit and ligated to pIBI24 which had been digested with the same enzymes. The ligation mixture was used to transform competent cells of E. coli JM101.

The 0.3Kb insert was sequenced and no mutations were found apart from the 37bp deletion in the tac/aphII region.

7.3.3 Checking Kanamycin resistance levels of E. coli and S. lividans containing pGLW51*

Gradient plates (Section 2.8) containing 0-400ug/ml of kanamycin, with and without 0.5mM IPTG and ampicillin at a concentration of 100ug/ml were streaked out with E. coli 1400 containing pGLW50, pGLW51 and with ten transformants of E. coli 1400 containing pGLW51*.

While E. coli containing pGLW50 grew well up to 400ug/ml of kanamycin (with and without IPTG) and E. coli containing pGLW51 grew well up to that same level of kanamycin in the presence of IPTG, all the transformants containing pGLW51* failed to grow in the presence of Kanamycin (with and without IPTG). These same transformants grew well in the presence of ampicillin and the presence of pGLW51* was confirmed in every transformant by mini DNA preparations.

S. lividans TK24 protoplasts were transformed with pGLW51*. Transformants were selected with thiostrepton.

Approximately equal numbers of spores ($5x10^3$) of S. lividans containing pGLW50 and of six different S. lividans recombinants containing pGLW51* were streaked on plates containing kanamycin concentrations between 0-120ug/ml.

All the S. lividans recombinants containing pGLW51* failed to grow in the presence of 10ug/ml of kanamycin while S. lividans containing pGLW50 grew on 120ug/ml of kanamycin.

7.3.4 S_1 mapping of the tac promoter in E. coli and S. lividans containing pGLW51*

By bringing the tac promoter closer to the ribosomal binding site of the aphII gene it was expected that the expression of this gene would be increased at least in E. coli. The results presented in Section 7.3.3 showed that pGLW51* confers no kanamycin resistance to E. coli or S. lividans.

To be able to distinguish between lack of transcription from the tac promoter of pGLW51* and lack of translation of the ptac/aphII message, S_1 mapping of ptac was performed using RNA prepared from E. coli and S. lividans containing pGLW51*.

7.3.4.1 Construction of mGLW51* to make single-stranded probes

The 0.3Kb fragment obtained from the digestion of pGLW65 with PstI and XbaI was purified from a 1.5% (w/v) TAE agarose gel. This fragment was subcloned into M13mp18 digested with these same enzymes (Fig. 7.7) to construct mGLW51*.

The ligation mixture was used to transform competent cells of E. coli JM101. Single-stranded DNA prepared from recombinants containing mGLW51* was used to make the single-stranded probe for S_1 mapping of ptac. For this promoter, XbaI was used to cut the extended probe (made as described in Section 2.16.1).

7.3.4.2 S_1 mapping of ptac using total RNA from E. coli and S. lividans containing pGLW51*

The 313 nucleotide radio-labelled probe was hybridised to total RNA from S. lividans TK24 recombinants containing pGLW51* and to total RNAs from E. coli 1400 recombinants containing pGLW50 and pGLW51*.

The hybridisations were performed at 37°C overnight and digested with 200 Units of S_1 nuclease according to Section 2.16.1. The digested products were electrophoresed on a 6% (w/v) denaturing polyacrylamide gel.

No protected bands were identified with control tRNA (Fig. 7.8). For the three RNA samples a protected band appeared and it ran approximately 87 nucleotides ahead of the full size probe.

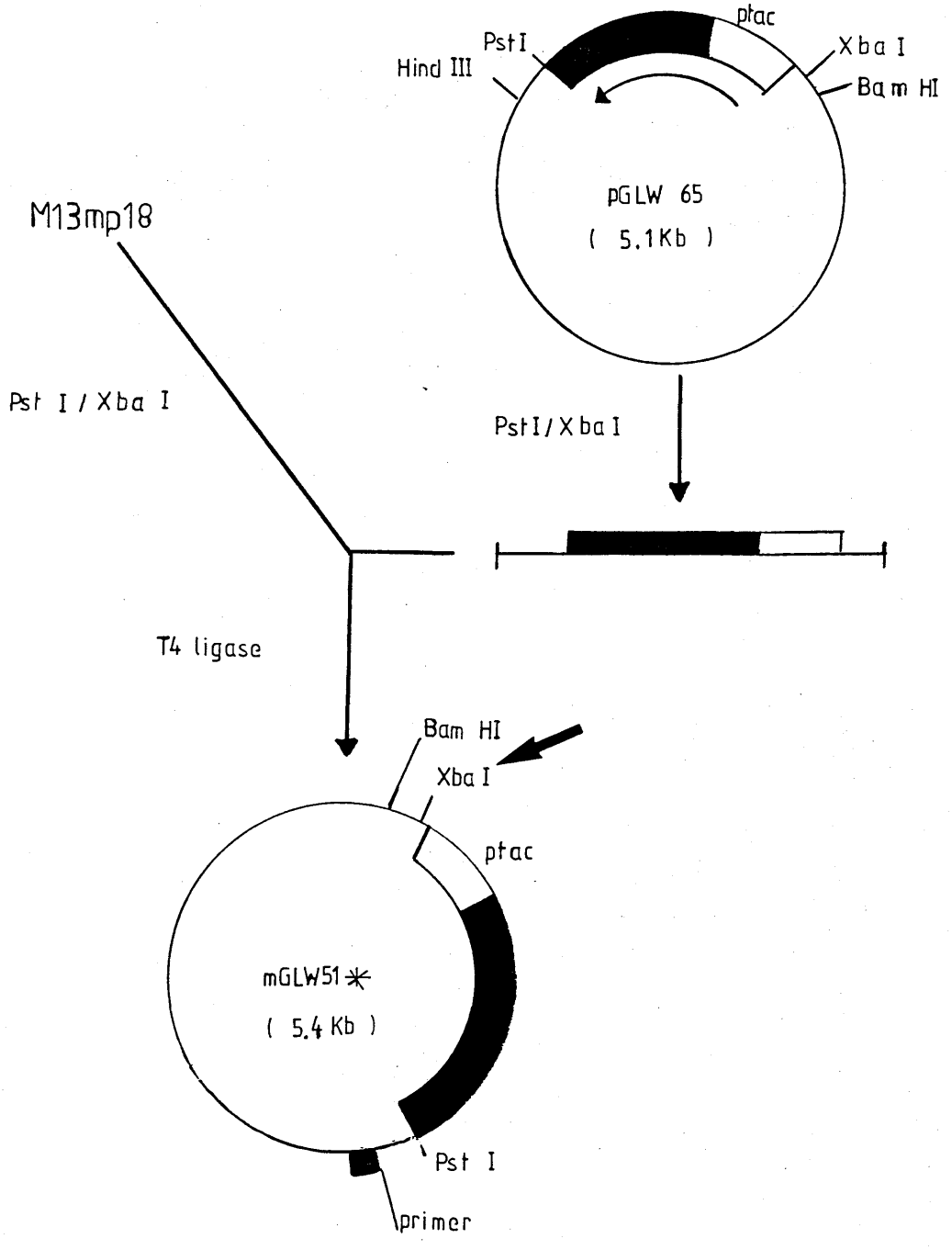


FIG. 7.7: Construction of mGLW51*.

XbaI (indicated with an arrow) was used to cut the extended probe.

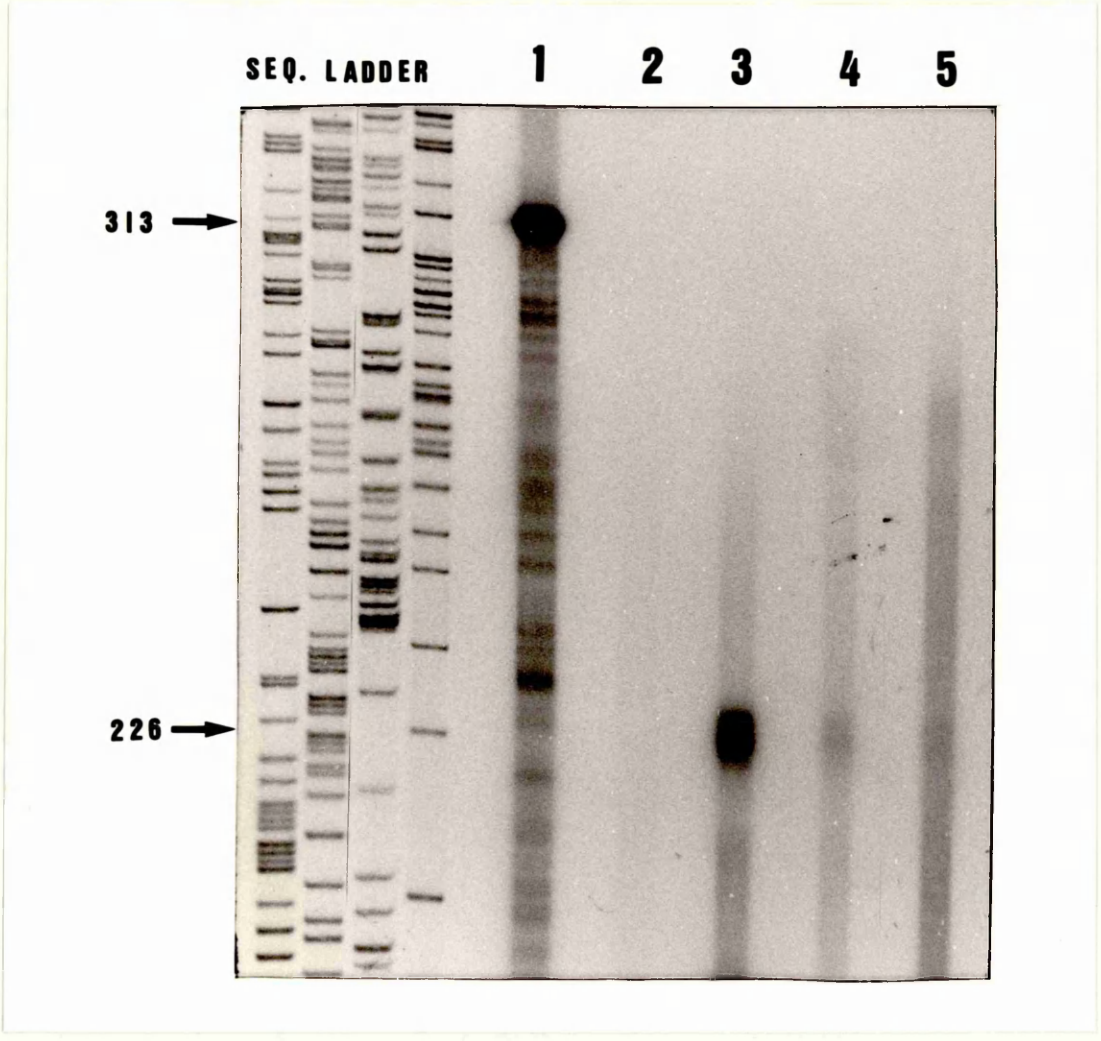


FIG.7.8 High resolution S1 nuclease mapping of ptac in S. lividans and E. coli containing pGLW51*.

1=probe

2=20ug of tRNA

3=100ug of total RNA from E. coli/pGLW50

4=10ug of total RNA from E. coli/pGLW51*

5=40ug of total RNA from S. lividans TK24/pGLW51*

Arrows indicate the full size probe (313 nucleotides long) and the major protected band (226 nucleotides long).

Allowing for the additional DNA included in the primer (34 nucleotides) and knowing that the transcriptional start of ptac was 52 nucleotides away from the XbaI site (which limited the probe), then the expected protected band should be 86 nucleotides shorter than the full size probe.

Thus, this experiment showed that initiation of transcription from ptac was occurring in E. coli and S. lividans containing pGLW51*.

7.4 Discussion

The results presented in this Chapter showed that pGLW51* failed to confer kanamycin resistance to E. coli and S. lividans. Nevertheless, transcription of the aphII gene carried by this plasmid occurred in both genera.

The possibility of a mutation somewhere in the aphII gene, even though unlikely, cannot be ruled out since only the ptac-aphII fusion area was sequenced. Restriction analysis of pGLW51* showed no differences from the expected pattern, but only by sequencing the whole gene would it be possible to know if any mutations did occur.

It is more likely that, by deleting the aphII Shine-Dalgarno region and by bringing the transcriptional start and the Shine-Dalgarno region of ptac closer to the aphII translational start, translation of the aphII gene was abolished in S. lividans and E. coli, while transcription from the tac promoter still took place. Since very little is known about the process of initiation of translation in

Streptomyces, it is interesting that in pGLW51* initiation of translation of the aphII gene was abolished both in E. coli and S. lividans.

As was stated in Section 7.1, many different factors seem to affect initiation of translation and this makes it difficult to determine the true cause(s) for the lack of expression of the aphII gene in recombinants containing pGLW51*.

The expression of the lac-cro fusion plasmids built by Roberts et al. (1979) illustrate this complexity. For example, in pTR199 (Fig. 7.2) the deletion is only three bases longer than that in pTR213 and five bases shorter than that in pTR214. However, recombinants carrying pTR199 produced ten times less cro protein than recombinants carrying the other two plasmids. Also surprising in the above work is the fact that recombinants carrying pTR182 and pTR190 produced extremely small amounts of cro protein. In these two plasmids, the cro Shine-Dalgarno region was deleted (partially in pTR182 and totally in pTR190), but the lac Shine-Dalgarno region was still present in both. Thus the lac Sine-Dalgarno sequence was brought close to the cro ATG codon, as in pGLW51* the tac Shine-Dalgarno sequence was brought close to the aphII ATG codon.

It is possible that the separation between the Shine-Dalgarno sequences and the ATG codon in pTR182 (10bp), pTR190 (5bp) and pGLW51* (12bp) is not ideal, but is this enough to prevent translation?

In the case of lacZ, the Shine-Dalgarno sequence is separated from its ATG by 7 base pairs, while in ptac the Shine-Dalgarno region is separated from its ATG by 11 base pairs.

On the other hand, Backman and Ptashne (1978) built a plasmid containing a "hybrid" ribosome binding site containing the lac Shine-Dalgarno region eight base pairs upstream from the ATG codon of the phage lambda CI (repressor) gene. Expression of the repressor in recombinants carrying this plasmid was extremely efficient.

Lim et al. (1987) have also reported the construction of different deletions of the arginine repressor gene argR which removed the control region from this region and placed the tac promoter upstream from the deleted argR region. The highest expression of arginine repressor was obtained from plasmids carrying deletions extending into the argR Shine-Dalgarno region.

However, each of the above (lac or tac) fusions created a different sequence between (and around) the Shine-Dalgarno region and the translational start codon, and it is possible that as a consequence some fusions are more efficiently translated.

The sequences of pGLW51 and pGLW51* that expand from the -10 region of ptac into the aphII coding sequence (40 base pairs downstream from the ATG codon) were analysed using FOLD. This computer program identifies stable secondary structures that might form in a particular mRNA. This analysis showed no significant change in the secondary structure of the ptac-aphII mRNA between pGLW51 and pGLW51* and eliminated the possibility that in pGLW51* the tac Shine-Dalgarno region or the ATG

codon might be involved in the formation of a hairpin loop, thus inaccessible to ribosomes.

In pGLW51* there is an EcoRI site between the Shine-Dalgarno region and the translational start. Therefore it will be possible to change the distance between these two sequences and investigate the effect this will have in the translation of the aphII gene.

CHAPTER 8

CONCLUSIONS AND FUTURE WORK

The main objective of this project was to construct a Streptomyces expression vector with a controllable promoter. The expression of genes cloned downstream from this promoter could then be modulated.

The bifunctional expression vectors constructed were based on the lac operator-repressor system; at the inception of the project it was not known if this system would be functional in Streptomyces.

Initially the activities of the lac and tac promoters were investigated in S. lividans using aphII as a reporter gene. The construct bearing the lac promoter (pGLW49) conferred kanamycin resistance to E. coli but not to S. lividans. The construct bearing the tac promoter (pGLW50) conferred resistance on both genera and the transcriptional start site for this promoter was shown to be the same in both genera using high resolution S_1 nuclease mapping.

A third plasmid (pGLW51) carrying not only the tac promoter but also the lac repressor gene, lacI, was then constructed. S. lividans and E. coli recombinants containing this vector showed resistance to higher concentrations of kanamycin in the presence of IPTG. This result was expected in E. coli. In S. lividans, it was consistent with the lacI gene being expressed at some level and with the repressor protein binding to the lac operator in this strain.

However, the repression of ptac as judged by aphII assays and S_1 nuclease mapping experiments was far from complete in pGLW51, both in E. coli and S. lividans. This vector is still useful since an induction level of 1.5-fold is sufficient for flux control studies.

From then on, attempts were made to improve the repression level in Streptomyces.

Certainly nothing was known about the transcription or translation of the lacI gene in S. lividans apart from the fact that the gene was being expressed at some level, since some repression of ptac (of kanamycin resistance) was detected.

High resolution S_1 mapping of the lacI promoter failed to detect the lacI transcript in S. lividans containing pGLW51, but detected this same transcript in E. coli carrying this plasmid.

From these and promoter-probe experiments using the lacI promoter in S. lividans it was concluded that the lacI promoter was very weak in S. lividans.

Based on these results, and on the knowledge that in E. coli stronger promoters driving lacI transcription can enhance repression of ptac and plac in this strain, a Streptomyces promoter was cloned upstream the lacI promoter. The promoter chosen was the promoter of the phage ØC31 repressor gene.

In E.coli the plasmid containing this promoter, pGLW61, showed more repression of the tac promoter than pGLW51, as judged by gradient plates containing increasing kanamycin concentrations and by aphII assays. Therefore, it was concluded that the promoter of the ØC31 repressor gene was active in E. coli. By increasing lacI mRNA levels, more lac repressor was produced, and consequently ptac was better

repressed than in previous constructs containing just the lacI promoter.

In S. lividans containing pGLW61, repression of ptac was not detected; in fact, aphII activity was higher in S. lividans containing pGLW61 than in S. lividans containing pGLW50 (the plasmid containing no lacI gene).

It seemed possible that the message starting at the promoter of the ØC31 repressor gene might be transcribed through the lacI/lacZ region of the plasmid. This mRNA could then conceivably continue transcription through the tac promoter and the aphII gene.

This possibility was investigated by performing low resolution S_1 mapping of the lacI/lacZ region using total RNA of S. lividans and E. coli containing pGLW51 and pGLW61. This experiment revealed that in all cases there was transcription through the lacI/lacZ region. However transcription through this region was much more efficient in S. lividans containing pGLW61 than in S. lividans containing pGLW51.

The apparent lack of repression of the tac promoter in S. lividans carrying pGLW61 could be explained by this message 'read-through' coupled with poor translation of the lacI gene.

The lacI gene contains five TTA codons which in S. coelicolor are known to require for translation a tRNA (the bldA gene product) which accumulates late in growth. Therefore the presence of this tRNA was analysed in different total RNA samples from S. lividans recombinants.

In all the S. lividans samples tested, the bldA gene product was detected. From this it was inferred that S. lividans probably had a bldA gene homologous to the S. coelicolor gene and that the presence of TTA codons in the lacI sequence was not limiting translation. Therefore lacI translation must be limited in S. lividans by some other factor(s).

It might be possible to restore repression of ptac which was lost when the ØC31 repressor promoter was cloned upstream from the lacI gene of pGLW51 by introducing an efficient Streptomyces terminator downstream from lacI and from the lac promoter (on the lacZ gene).

It is also possible that the introduction of a second lac operator upstream from ptac in pGLW51 and pGLW61 might improve repression in S. lividans, as it has been reported to do so in E. coli.

Removal of the lac operator that lies between the lacI and lacZ genes, in pGLW51 and pGLW61, might be another way of enhancing repression levels in both genera. This operator probably competes with the operator on ptac for binding of the repressor.

Purified antibodies for the lac repressor could help in the investigation of the efficiency of translation of the lacI mRNA in S. lividans. For example, in S. lividans is the enhancement of lacI transcription in pGLW61 relative to pGLW51 reflected in an enhancement of lac repressor production?

Preliminary Western blotting experiments (data not shown) failed to detect the *lac* repressor in crude protein extracts of *S. lividans* containing pGLW51 and pGLW61, while it detected the *lac* repressor in crude protein extracts of *E. coli* containing these two plasmids.

Finally, and most interesting, will be the cloning of *Streptomyces* genes of known biochemical interest downstream from the *tac* promoter (of pGLW51 or an improved version of it), modulation of their expression and study of the effect of this modulation in flux through a particular pathway.

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