AN INVESTIGATION OF THE REGION OF DNA REQUIRED FOR *STREPTOMYCES PENEMAFACIENS* PLASMID pSPN1 REPLICATION

ANTHONY SMITH

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ABBREVIATIONS

A	adenine
Ap^r	ampicillin resistance
ATP	adenosine triphosphate
bp	base pairs
C	cytosine
DNA	deoxyribonucleic acid
dNTP's	deoxyribonucleotide triphosphates
ds	double stranded
EDTA	ethylene diamine tetra-acetic acid
G	guanine
IHF	integration host factor
IPTG	isopropyl-beta-D-thiogalacto-pyranoside
kb	kilobase
LA	Luria agar
LB	Luria broth
MES	2(N-morpholino)ethanesulphonic acid
MIC	minimum inhibitory concentration
*ori*C	chromosomal origin of replication
*ori*V	vegetative origin of replication
Pol I	DNA polymerase I
Pol II	DNA polymerase II
Pol III	DNA polymerase III
RNA	Ribonucleic acid
RNA-Pol	RNA polymerase
RNase	Ribonuclease
SDS	Sodium Dodecyl Sulphate

Str^r	Streptomycin resistance
ss	single stranded
T	thymine
TES	N-tris(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid
Tsr	thiostrepton resistance
Tris	tris(hydroxymethyl)aminoethane
X-Gal	5-bromo-4-chloro-3-indolyl-b-galactoside
#	number

ABSTRACT

Plasmid pSPN1 is a 26.5kb cryptic plasmid, originally isolated from *Streptomyces penemafaciens* ATCC 31599. A 12.5kb *Bgl*II fragment of pSPN1 was cloned into the vector pLR2, and this confefrred on pLR2 which lacks a *Streptomyces* origin of replication, the ability to replicate in a number of *Streptomyces* species.

A vector pBlue was constructed by inserting a streptomycin resistance gene from plasmid pIJ4642 into the ampicillin resistance gene of the vector Bluescript. The resistance gene was able to function in both *E.coli* and *Streptomyces* species and thus pBlue could serve as a vector for shortening and sequencing in *E.coli* as well as a origin-probe vector in *Streptomyces*. The origin-containing *Bgl*II fragment of pSPN1 was cloned into pBlue to create pFull, which was able to be selected for and replicate in *Streptomyces*. The conditions affecting selection of pFull in *Streptomyces* were investigated and optimized. The copy number of pFull was found to be 0.2 per chromosome.

Attempts were made to clone origin-containing fragments smaller than the 12.5kb *Bgl*II fragment. Initially a *Sau*3A partial library was made of the origin-containing fragment, this however did not produce any replicating plasmids. As an alternative approach, pFull was extensively mapped and a series of deletion derivatives were constructed. The derivatives were tested for the ability to replicate in *Streptomyces*. Judging from the deletions that were and were not able to replicate it is apparent that at least 5.5kb of DNA is required for pFull and hence for pSPN1 to replicate.

CHAPTER 1

GENERAL INTRODUCTION

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Plasmids are non-essential autonomously replicating extrachromosomal double stranded (ds) deoxyribonucleic acid (DNA) elements that are distinct from viruses in that they have no extracellular stage. Plasmids are usually covalently closed circular (ccc) DNA, however linear plasmids have been identified in a number of organisms including *Streptomyces rochei* (Hirochicka *et al.*, 1984) and *Thiobacillus versutus* (Woldarczyk and Nowicka, 1988). Plasmids range in size from a few hundred base pairs (bp) to several hundred thousand base pairs and copy numbers can vary from less than 1 to greater than 800 per chromosome (Hopwood *et al.*, 1986). The majority of plasmids are cryptic. Those whose function is known confer useful characteristics such as drug resistance, increased substrate range and production of antibiotics to the host bacterium (Hopwood *et al.*, 1986). These advantages and the ability to spread between bacteria allow plasmids to confer rapid short term selective adaptation on their hosts, however under nonselective conditions plasmids are an energy burden on the host and in some cases can be lost rapidly.

A common property of plasmids is their stable inheritance in a growing population of bacteria. This can be achieved by strategies such as overreplication (Cambell, 1981), partitioning mechanisms (Austin, 1988), killing of plasmid free segregants (Gerdes *et al.*, 1986) and infectious conjugal transfer (Levin, 1986) as well as intramolecular resolution of plasmid multimers to monomers which ensures more discrete molecules for partitioning to each daughter cell (Austin *et al.*, 1981). These different strategies require that: i) Plasmid genes for replication control must be expressed adequately by the host at the proper time in the cell cycle. ii) The plasmid's initiation complex must interact

1.1.1 The minimal requirements for initiation of replication

The minimal replicon of ColE1 is a 600bp region from which replication takes place unidirectionally in the theta-shaped Cairns type manner (Inselburg, 1974; Tomizawa *et al.*, 1974; Tomizawa *et al.* 1975). This process uses only host encoded proteins (Donoghue and Sharp, 1987). For initiation, DNA dependant ribonucleic acid (RNA) polymerase, ribonuclease H, DNA polymerase I (Pol I), DNA gyrase and topoisomerase I are required (Hillenbrand and Staudenbauer, 1982; Itoh and Tomizawa, 1978; Itoh and Tomizawa, 1980; Minden and Marians 1985). The gyrase is thought to open out the DNA and to provide the topological driving force for the replication fork movement (Orr and Staudenbauer, 1981). Topoisomerase I modulates the superhelicity which may aid transcription of the primer promoter and hence assist in the production of the primer (Minden and Marians, 1985). A primer precursor called RNA II is transcribed from 555bp upstream of the origin (*ori*) and terminates heterogeneously downstream of the *ori* giving an approximately 700bp transcript (Itoh and Tomizawa, 1980; Tomizawa *et al.*, 1981; Tomizawa and Masukata 1987). If the 5' end of the nascent RNA II forms the correct secondary structure a process called coupling takes place (Tomizawa and Itoh, 1982), whereby the 3' end of RNA II forms a reversible hybrid with its DNA template near the *ori* (Fig. 1.1). There are three mechanisms by which initiation may continue. Type I appears to be the main type (Kingsbury and Helinsky, 1970; Ohmori *et al.*, 1987) and is RNase H and Pol I dependant (Dasgupta *et al.*,1987). RNase H cleaves the hybridat a sequence of five Adenosines and generates mature RNA II, Pol I elongates the primer from the 3' OH as well as digesting the primer with it's 5'-3' exonuclease activity, further cleavage by RNase H also takes place (Fig. 1.2)(Selzer and Tomizawa, 1982). In the absence of RNase H and Pol I, type II mechanism is used. Due to RNA II coupling to the leading strand, the lagging strand forms a displacement loop where replication starts, provided that at least 40 nucleotides are available for the replisome replication complex to bind (Masukata *et al.* 1987). Host Dna G primase and Dna B helicase form a replisome

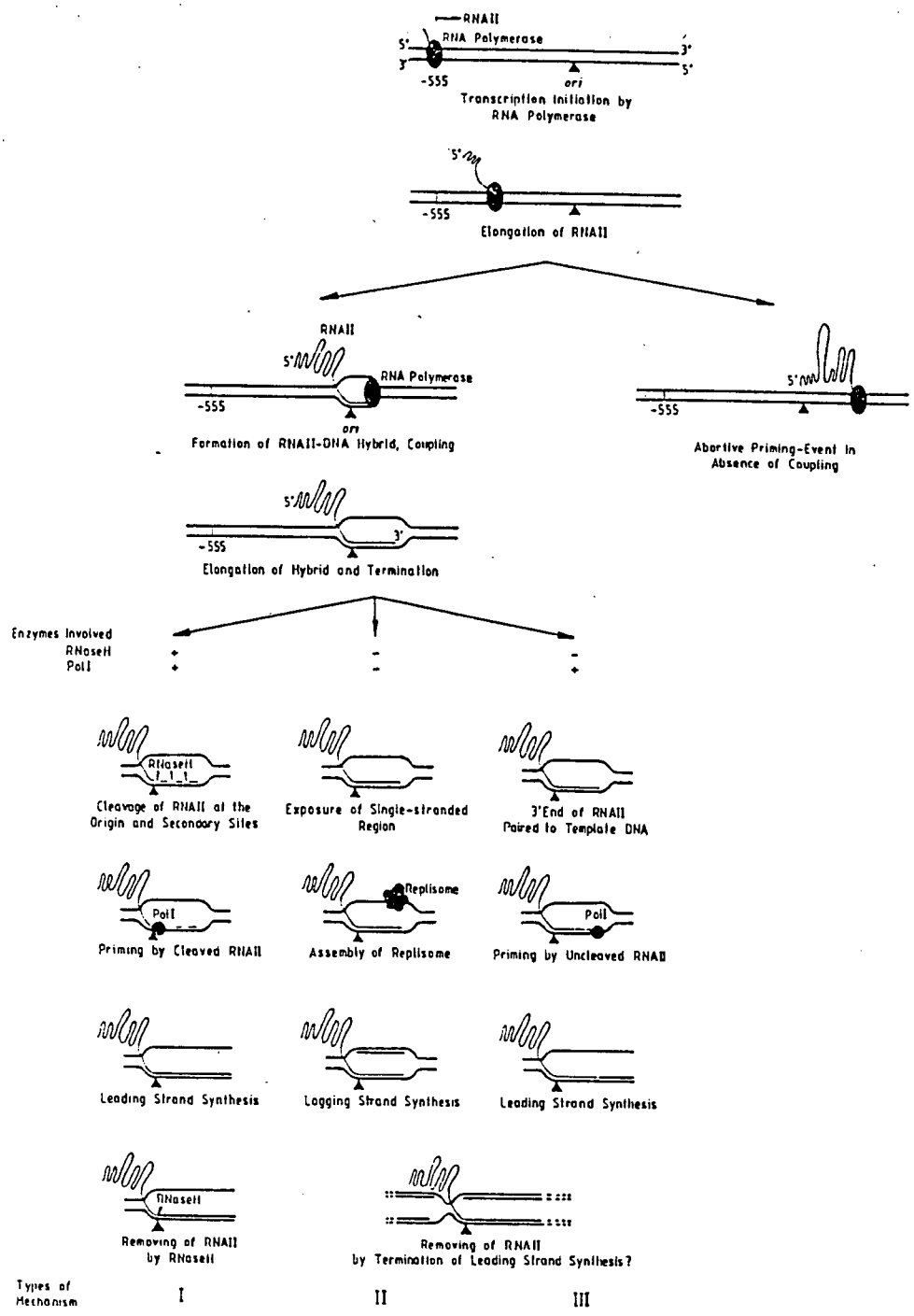

Figure 1.1 Diagrammatic representation of the three mechanisms of ColE1 replication initiation. Transcription starts at position -555 with respect to the *ori* which is indicated by a black triangle. DNA is marked in thick lines while thin lines indicate RNA, folds in the different RNAs represent secondary structure. The direction of replication is shown by arrows (from Keus and Stahl, 1989).

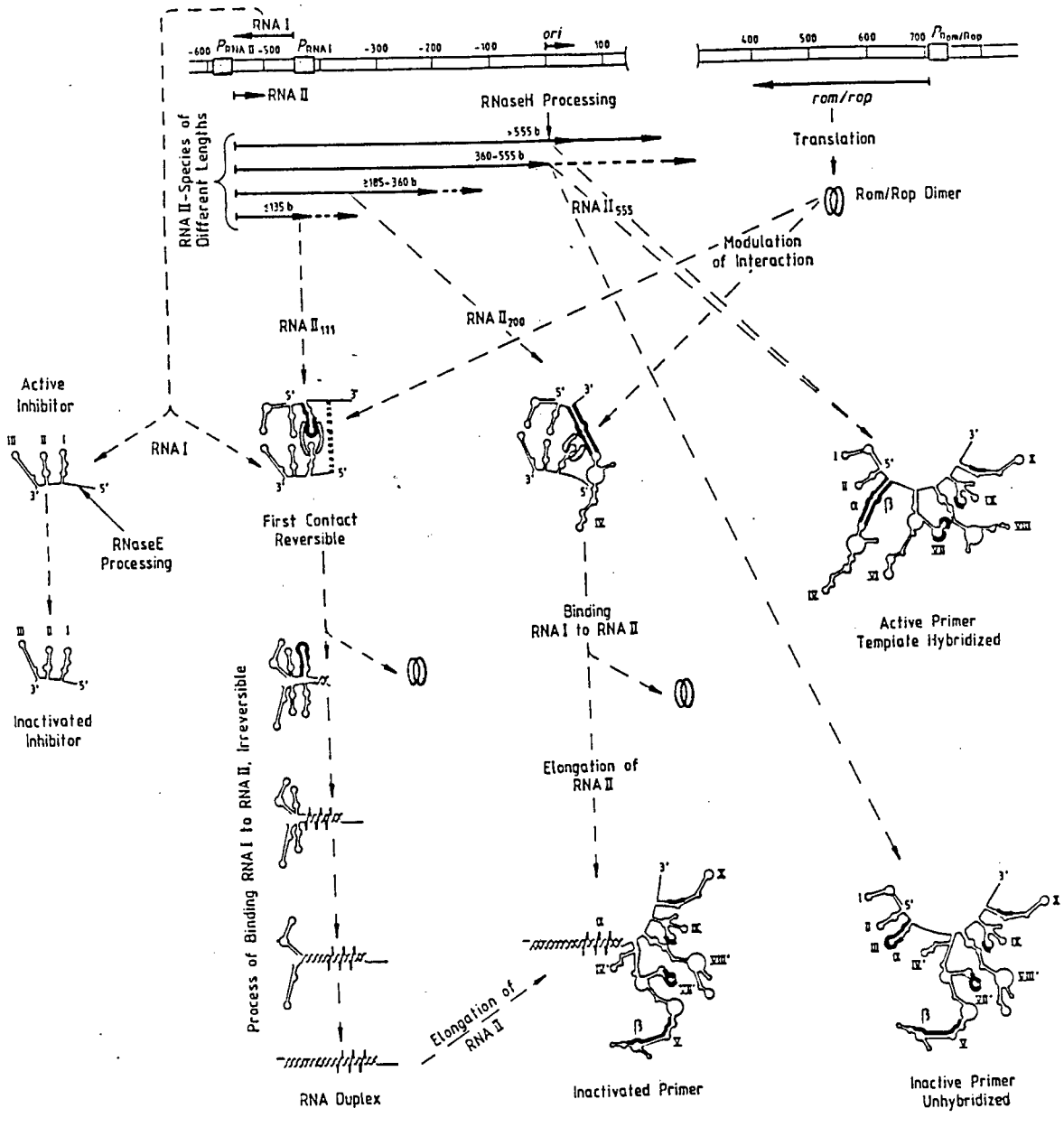

Figure 1.2 An illustration of the genetic regulation of the ColE1 plasmid origin of replication. DNA comprising the origin is indicated at the top, the thick arrows indicate direction and length of RNA transcripts. Thin arrows indicate possible paths of interaction between Rom/Rop, RNA I and RNA II, Roman numerals indicate stem-loop structures. Dark boxes in the primer transcripts, indicate regions involved in tertiary pairing during coupling of RNA II to its template (from Keus and Stahl, 1989).

on the displacement loop and replication can then proceed from various sites in a region that extends approximately 500bp downstream of the *ori* (Kornberg, 1982). In some cases only RNase H is lacking and type III mechanism is used. Pol I recognizes coupled but unprocessed RNA II molecules that have a region of less than 50 nucleotides hybridized to the template DNA and uses these as a primer, however full length RNA II transcripts are not used as primers (Dasgupta *et al.*, 1987). In mechanisms II and III where RNase H is not available for primer removal, termination of leading strand synthesis is thought to remove the unprocessed RNA II.

1.1.2 ColE1 regulation

In addition to the strength of the RNA II promoter, there are two other factors that modulate replication. The main regulatory circuit is an inhibitor-target system which regulates primer functioning as follows. An inhibitor RNA molecule of 108 nucleotides called RNA I is transcribed in the opposite direction and from the opposite strand to RNA II, from a start 445bp upstream of the *ori* to a few bases from the RNA II start (see Fig 1.1) (Tomizawa, 1986; Itoh and Tomizawa, 1978; Morita and Oka, 1979). This inhibitor hybridizes with RNA II rendering it unable to bind to the DNA and hence to function as a primer. Synthesis of RNA II takes 12 seconds to reach completion and generate the full length of 700bp. However only when the RNA II has a length of between 110 and 360bp is RNA I able to hybridize to and inhibit RNA II (Tomizawa, 1986). A high ratio of inhibitor to target is required to ensure that RNA I binds to RNA II in an effective manner given the short period in which inhibiting hybridization can occur. The RNA I promoter is five times stronger than the RNA II promoter, and this, coupled to the requirement for correct folding of pre-RNA II, means that 1 in 20 pre-primer transcripts generates a replication event under stable copy number conditions (Lin-Chao and Bremer, 1987).

The availability of RNA I is also effected by such factors as charged tRNA levels (Yavachev and Ivanov, 1988) and growth conditions (Ivanov *et al.*, 1988) hence these factors also effect copy number. RNA I forms a tRNA like structure with a single stranded tail of 9 nucleotides at the 5' end (Morita and Oka, 1979; Tamm and Polisky, 1985; Tamm and Polisky, 1983; Yavachev and Ivanov, 1988). The initial reversible contact termed "kissing", between RNA I and RNA II takes place in the single stranded loops of RNA I stem-loop I and RNA II stem-loop III (Lacatena and Cesareni, 1983), this aligns the 5' end of RNA I with an unfolded complementary region near the 3' end of RNA II. A more recent proposal by Tomizawa is that the 5' end of RNA I interacts with a complementary loop near the 3' end of RNA II. The strands hybridize weakly at first but become progressively more tightly bound, passing through 2 major intermediates till zipping together has occurred, disrupting the secondary structure of the RNAs and hence rendering RNA II useless as a primer (Tomizawa, 1984; Tomizawa, 1985; Tomizawa, 1990).

The third regulatory component is a 63 amino acid protein called Rom (RNA I inhibition modulator) or Rop (repressor of primer). Rop doubles the RNA I - RNA II rate of interaction (Tomizawa and Som, 1984; Dooley and Polisky, 1987), although the basal rate is dependant on the length of the RNA II transcript (Tomizawa, 1986). The simplest model of Rop action envisages Rop binding to the stems of RNA I and II and thus stabilizing the interaction of complementary loops in the initial "kissing" reaction by forming a Rop dimer and the correctly aligning the RNA I - RNA II complex (Cesareni *et al.* 1984; Cesareni and Banner, 1985; Helmer-Critterich *et al.*, 1988). Recently it has been shown by Tomizawa (1990), that Rop acts once RNA I and RNA II have already bound. Rop binds to the first major intermediate in a rapid and reversible reaction, it then speeds up the rate of conversion of the first major intermediate to the second major intermediate. The Rop - second major intermediate complex is stable and Rop does not readily dissociate from the complex, conversion of the second major intermediate to the

inactive RNA I - RNA II hybrid finally occurs and primer formation is inhibited. The ColE1 displays a direct inhibitor-target system and the replication of this plasmid is probably the best understood to date.

1.2 RK2

RK2 is a 60kb broad host range plasmid of Gram negative bacteria (Lanka *et al.*, 1983; Pansegrau and Lanka, 1987) with a copy number of 4-7 in *E.coli* and 3 in *Pseudomonas aeruginosa* (Figurski *et al.*, 1979; Grinter, 1984; Itoh *et al.*, 1984). The plasmid which belongs to incompatability group IncN codes for tellurite and tetracycline resistance and contains a copy of transposon Tn1 (Bradley and Taylor, 1987; Pansegrau and Lanka, 1987). The genes for replication cover a region of 20kb in three clusters, designated *oriV*, *trfA* and *trfB* (Stalker *et al.*, 1981; Figurski and Helinski, 1979). From the above and other evidence it has been shown RK2,RP4, RP1, R18 and R68 are different isolates of the same plasmid.

1.2.1 The structure of *oriV*

Replication from *oriV* is unidirectional by a Cairns type mechanism (Firshein and Caro, 1984). *oriV* consists of i) eight 17bp iterons; ii) a putative promoter surrounded by DnaA binding sites and a putative IHF (integration host factor) binding site; iii) a 49bp A+T rich sequence with a DnaA binding site; iv) a 67bp G+C rich sequence and v) three open reading frames, two of which are *copA/incA* and *copB/incB*, the copy number/incompatability determinants (Thomas *et al.*, 1984). The *oriV* requirements of different hosts vary, *E.coli* requires only a 393bp region and A2, a *trfA* protein (see Fig. 1.3) for replication (Stalker *et al.*, 1981; Thomas *et al.*, 1981; Kornacki *et al.*, 1984),

while *Pseudomonas aeruginosa* requires a 617bp region and proteins A1 and possibly A2 (Schmidthauser et al., 1983; Durland and Helinski, 1987). An A+T rich region and the DnaA binding site fall into the 393bp region necessary in *E.coli* (Gaylo *et al.*, 1987; Pinkney *et al.*, 1988), in *Pseudomonas aeruginosa* DnaA is unnecessary but there is the additional requirement for *copA/incA* and *copB/incB* incompatability loci(Schmidthauser and Helinski, 1985).

1.2.2 Replication initiation

The *trfA* operon produces three proteins, A1 and A2 are products of the same reading frame but A2 starts downstream of the A1 start (Shingler and Thomas, 1984). Both are thought to be double stranded DNA binding proteins which bind to the iterons in the *oriV* region (Smith and Thomas, 1984; Smith and Thomas, 1987; Krishnapillai, 1986). The third protein is called KilD and is a regulator (see section 1.2.3). Replication initiation in *E.coli* requires DnaA, DnaB helicase, DnaG primase, DNA gyrase and DNA polymerase III (Pol III) (Gaylo *et al.*, 1987; Pinkey *et al.*, 1988). The presence of an IHF binding site suggests that this is also required, Pol I is however not required. Replication proteins A1 and A2 bind DNA nonspecifically at low concentrations, at higher concentrations they bind specifically to the *oriV* where they are thought to associate with host proteins and hence initiate replication (Barth *et al.*, 1984). The *trfB* proteins B1 and B2 which are responsible for the *incC* determinant may play a role in *oriV* fine regulation, modulation of the *trfA* operon or effect the stability of A1 and A2 (Thomas, 1986). The actual course of events that results in the opening out of the *ori* and priming is as yet not understood.

1.2.3 The *kil/kor* system of regulation

The *kil/kor* system is a regulatory system located outside of the minimal replicon which allows RK2 to adapt to a range of hosts, the *kil* genes being lethal to the host while the *kor* gene products suppress the *kil* genes. There are five *kor* genes, namely *A*, *B*, *C*, *E*, and *F* and four *kil* genes *A*, *B*, *C* and *D*, these are located around the *ori* as shown in Figure 1.3. The 101 amino acid KorA protein inhibits *kilA* and *kilD* as well as autoregulating itself by inhibiting the *trfB* operon from which it is transcribed (Young *et al.*, 1985; Theophilius *et al.*, 1985; Bechhofer and Figurski, 1983). KorA stimulates KorC production and hence represses *kilC* indirectly (Figurski *et al.*, 1982; Young *et al.*, 1984). KorB represses the *trfA* operon and *trfB* operon as well as the *kil*B gene (Young *et al.*, 1987). *kor*F produces 2 polypeptides which inhibit *trf*A operon and the *trf*B operon (Jagura-Burdzy *et al.*, 1991), KilD on the other hand depresses the *trfA* operon, finally KorC inhibits *kilC* and KorE inhibits *kilA*. The promoters on which KorA and KorB act have operator like palindromes for these repressors to bind to (Theophilus *et al.*, 1985), these promoters are required to be broad host range promoters as is the case for *trfA* (Pinkney *et al.*, 1987).

In addition to the *kil/kor* system which regulates replication, RK2 is additionally stabilized by a 3.1kb non-essential region of DNA. The region contains a multimer resolution site and a resolvase, thus the plasmid is able to increase the number of discrete copies of RK2 available for segregation. The region also appears to have 2 other proteins which seem to act in a partitioning system thus giving rise to a very stable plasmid (Barth, 1979; Barth and Grinter, 1974).

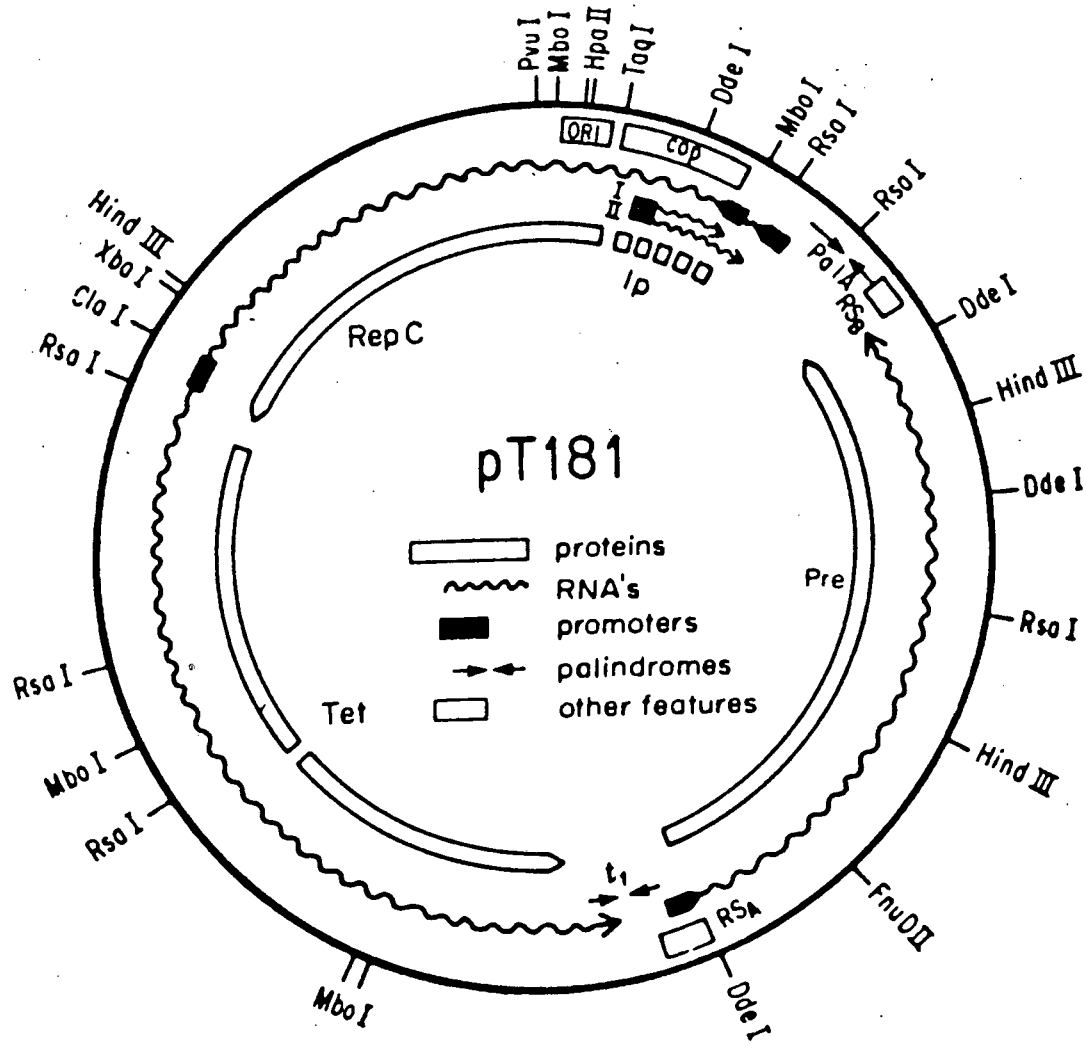

Figure 1.4 Genetic map of pT181. Wavy lines represent transcripts, solid blocks represent promoters and RSa and RSb represent recombination sites. Functional elements in clockwise order are: (*cop*) copy control, (*ori*) replication origin, (*repC*) initiator protein coding sequence, (tet) tetracycline resistance determinant (may comprise one or two genes), (t_1) terminator, (*pre*) coding sequence for a site specific recombinase. Palindrome A is marked as *palA* and Ip is a putative leader peptide for RepC (from Novick *et al.*, 1986).

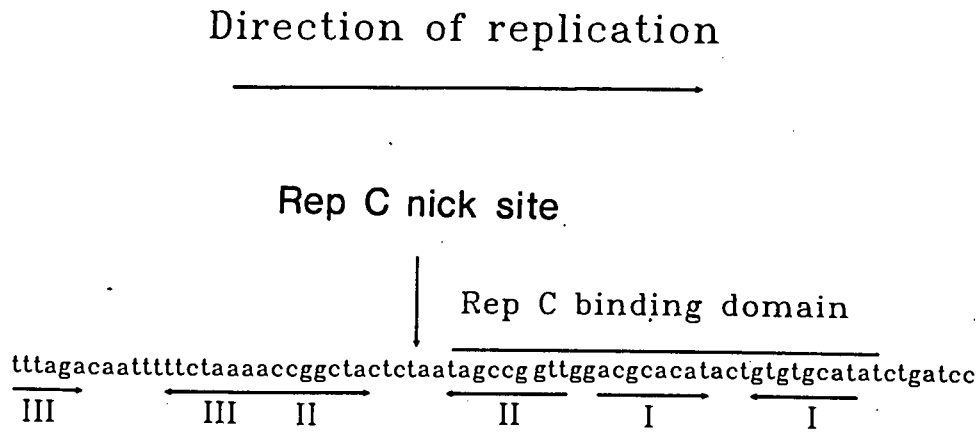

Figure 1.5 Detailed representation of the pT181 *ori*. Horizontal arrows numbered with Roman numerals indicate stem-loop forming sequences.(from Gennaro *et al.*, 1989)

The RepC protein binds to a specific 32bp region of the *ori* (Zock *et al.*, 1990), this binding increases the stability of a stem-loop structure IR II seen in Figure 1.5 (Noirot et al., 1990). The RepC introduces a single stranded nick into the loop of the stem-loop structure and becomes covalently bound to the 5' end of the nick (Majumder and Novick, 1988). RepC can nick other related plasmids such as pC221 with equal efficiency, but it has a vastly greater binding affinity for the pT181 *ori* and it is this binding which ensures efficient replication (Zock *et al.*, 1990). Repeat III is constant for all pT181 like plasmids while IR I has a variable sequence, the RepC binding site is IR I and thus it only binds pT181. The *cmp* gene appears to enhance replication by increasing the production of RepC, it may also increase the effectiveness of RepC utilization (Novick *et al.*, 1987).

The nicked strand is extended from the 3' end until it re-encounters the RepC protein and replication is terminated at the *ori*. The newly synthesized strand is circularized and the lagging strand displaced (Iordanescu and Projan, 1988). Replication of the displaced strand now proceeds from *palA*, the circular dsDNA is then hypersupercoiled by gyrase and relaxed by topoisomerase to produce a mature molecule (Majumder and Novick, 1988).

The replication of pT181 is regulated primarily at the level of RepC translation by two transcripts of 85 and 150bp which overlap the start of the RepC transcript but are transcribed in the opposite direction and from the opposite strand (Kumar and Novick, 1985). These transcripts control copy number by sequestering the RepC transcript Shine-Delgano sequence and attenuating RepC translation (Novick *et al.*, 1989). Under normal copy number conditions only 3% of RepC transcripts are translated, the rest are attenuated by RNA I (85bp) and RNA II (150bp) although the latter may only aid the efficiency of RNA I. The RepC transcript can form a number of hairpin loops using sequences I, II, III, and IV as shown in Figure 1.5. In the absence of RNA I, sequence I termed the preemptor hybridizes to sequence III and prevents the formation of a

terminator or sequestration of the Shine-Delgano sequence. In the presence of RNA I, the stem-loop structure formed by sequences I and II interacts with the loop of RNA I stem-loop causing a zippering type of hybridization analogous to that seen for ColE1 regulation by RNA I and II. This hybridization sequesters the Shine-Delgano sequence and causes the formation of a transcriptional terminator from sequences III and IV, thus ribosomes cannot bind and even if they did, they would terminate almost immediately (Novick *et al.*, 1989).

1.3.2 pUB110

The plasmid pUB110 as shown in figure 1.6 is 4548bp in size and has a copy number of 50 in *Bacillus subtilis* It codes for kanamycin and bleomycin resistance (Novick *et al.*, 1987; Viret and Alonso, 1988). As with other Gram positive plasmids, replication is by rolling circle. However it is unique in that the lagging strand origin (*oriL*) is functional in more that one host (Boe *et al.*, 1989). The leading strand origin requires a *cis*-acting 24bp *oriU* and the *trans* acting RepU protein translated from the 949bp *repU* gene (Alonso *et al.*, 1988). As for pT181, an *incA* incompatability determinant overlaps the *repU* gene and produces 2 counter transcripts of 80 and 250bp which are thought to act in a similar manner to RNA I and II of pT181 . The *oriU* nick site has been localized to 8bp but in this case there appears to be no secondary structure involvement (Maciag *et al.*, 1988). There are two stem-loop structures flanking *oriU* but no involvement in initiation has been proven for them (Alonso *et al.*, 1988). Once the *oriL* is nicked, RepU attaches covalently to the 5' end of the nick and replication is thought to proceed as for pT181.

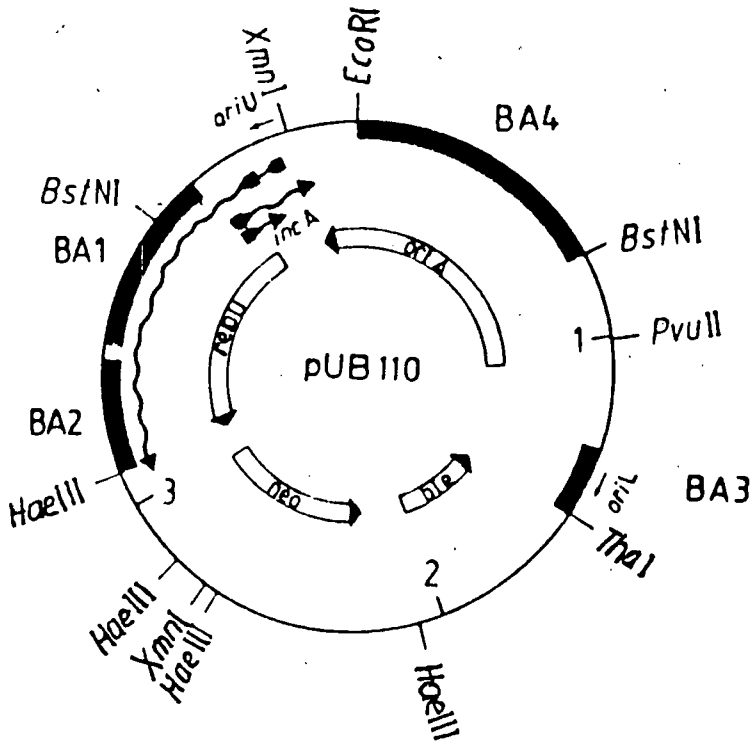

Figure 1.6 Structure of pUB110. Arrows in the inner circle indicate open reading frames, wavy lines indicate putative RNA transcripts and filled bars represent membrane binding sites. Arrows outside the circle indicate site and direction of the leading (*oriU*) and lagging (*oriL*) origins (from Alonso *et al.*, 1988).

The pUB110 lagging strand origin, *oriL* is different from that seen in other Gram positive plasmids, pUB110 accumulates very little ssDNA and *oriL* is functional in *Bacillus subtilis* and *Staphlycoccus aureus*. The lagging strand origin of rolling circle plasmids seems to effect host range or at least reduce replication efficiency in some species. The pUB110 *oriL* has no homology to other lagging strand origins and this may reflect its increased functional range (Boe *et al.*, 1989). *oriL* is a 140bp, *cis*-acting, orientation dependant region capable of forming secondary structures (Viret and Alonso, 1988). DnaG primase is required for *oriL* initiation, RepU involvement has been suggested but not conclusively proven (Alonso *et al.*, 1988). Whatever the exact mechanism, it is equally effective in both hosts. In the absence of correct primosome factors or *oriL*, nonspecific priming directed by DnaB takes place (Alonso *et al.*, 1988; Viret and Alonso, 1988), but at a lower efficiency than the normal mechanism.

1.4 Streptomyces plasmids

Streptomyces plasmids range in size from 1.8 to greater than 200kb. They have copy numbers from less than 1 to 800 and can either be cccDNA or linear DNA (Hopwood *et al.*, 1986). The plasmids are ubiquitous and while many confer no easily detectable phenotype, others have been shown to be involved in antibiotic resistance and production (Aguilar and Hopwood, 1982) and the formation of lethal zygosis pocks (Hopwood *et al.*, 1986). Many Streptomyces plasmids are refractory to physical isolation due to their large size or low copy number. They are in numerous cases episomes and can promote chromosomal recombination as well as showing the ability to self transmit (Hopwood *et al.*, 1986).

The best understood *Streptomyces* plasmid is pIJ101, which forms the basis of the cloning vectors pIJ702 and pIJ385 as well as many others (Hopwood *et al.*, 1986). pIJ101

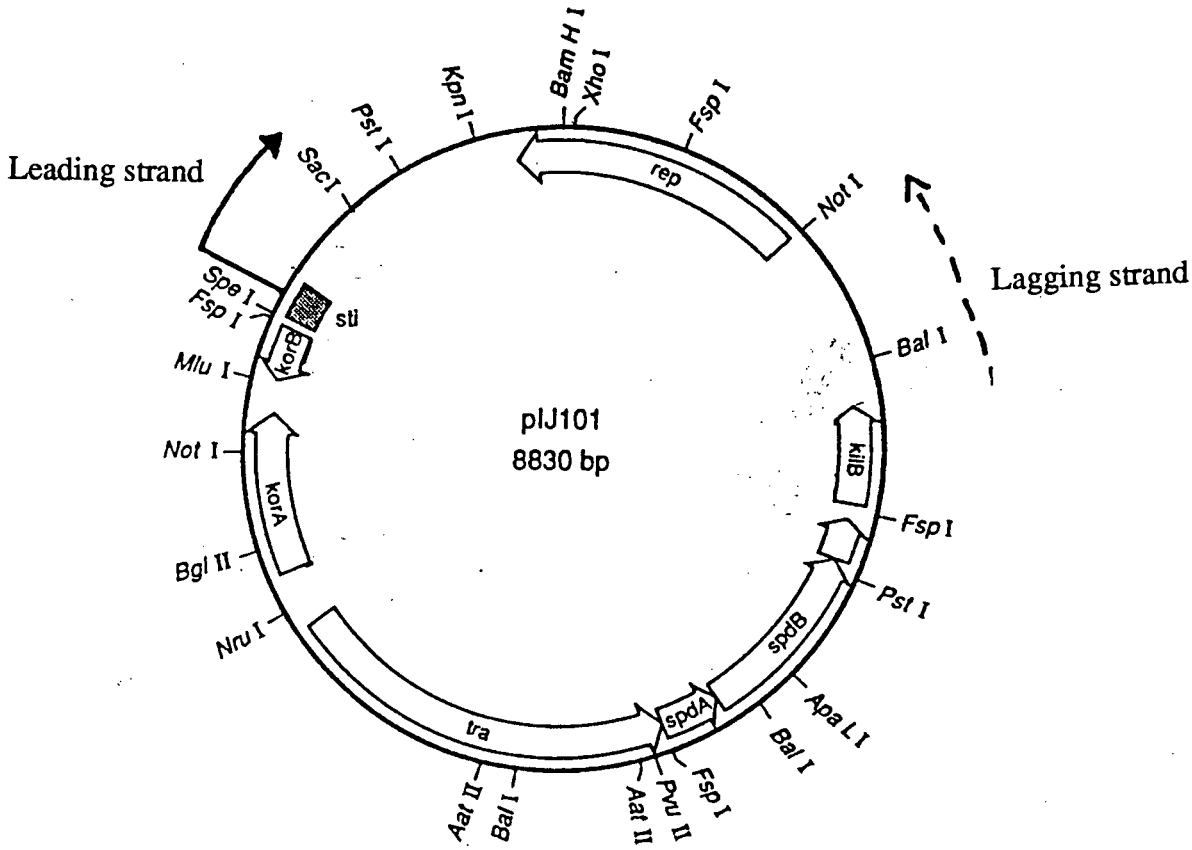

Figure 1.7 Genetic map of pIJ101. Sites and direction of leading and lagging strand origins are indicated with arrows outside the circle. Open reading frames are indicated by open boxes; arrow heads indicate direction of translation. The shaded box represents the region conferring strong incompatability (Sti) (from Stein *et al.*, 1989).

is a broad host range 8.9kb plasmid (Hopwood *et al.*, 1986) whose copy number varies between 40 and 300 depending on the culture age and nutrient conditions. Figure 1.7 shows a map of pIJ101. The minimal replicon is 2.1kb or less and contains an undetermined *ori* sequence and a 450 amino acid Rep protein. As with other Gram positive plasmids replication is by rolling circle from the leading strand origin. The Rep protein however bears no homology to other Rep proteins (Kendall and Cohen, 1988). An open reading frame of 56 amino acids adjacent to the *Rep* gene also exists on the 2.1kb fragment and may act as regulator of replication. Constructs using only the 2.1kb *ori* fragment accumulate ssDNA and occur at a reduced copy number, this however can be prevented by the cloning in *cis* of a 500bp fragment that is responsible for the strong incompatibly (Sti) determinant (Kieser *et al.*,1982). The Sti fragment has no open reading frames but does have extensive secondary structure, it is thought to be the origin of lagging strand synthesis. Two Sti^+ plasmids or two Sti^- plasmids are compatible, however a Sti^+ plasmid will soon dilute out a Sti^- plasmid, thus supporting the theory that it is the origin of lagging strand synthesis .

The *cop* gene product is thought to inhibit Sti mediated lagging strand synthesis as it effects only Sti^+ plasmids, it presumably has no effect on Sti independent lagging strand synthesis (Deng *et al.*, 1988).

pIJ101 has four genes, *korA*, *korB*, *tra*(*kilA*) and *kilB* whose exact function is unclear. The *kor* genes are regulatory while *tra* is involved plasmid transfer and *kilB* in the formation of lethal zygosis pock formation. KorA represses the *korA* gene and *kilA* at a transcriptional level while it represses *kilB* at a translational level. KorB represses itself and *kilB* at a transcriptional level. The above four genes are implicated either directly or indirectly in plasmid transfer as are the *spdA* and *spdB* genes which appear to effect intramycelial plasmid transfer (Stein *et al.*, 1989; Kendall and Cohen, 1988).

CHAPTER 2

ISOLATION OF PLASMID pSPN1 FROM *STREPTOMYCES PENEMAFACIENS* AND CLONING OF A *BglII* FRAGMENT CONTAINING THE ORIGIN OF REPLICATION

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2.1 SUMMARY

The four *Bgl*II fragments of *Streptomyces penemafaciens* plasmid pSPN1, were cloned into the insertional inactivation vector pLR2. The 12.5kb fragment was found to contain an origin of replication that was functional in *Streptomyces lividans* 66 TK 24 and *Streptomyces coelicolor* A3 (2) M145. The possible involvement of pSPN1 in thienamycin production was investigated by testing for homology with other penem-antibiotic producers such as *Streptomyces fulvoviridis*, *Streptomyces cattleya* and *Streptomyces olivaceus*. On the basis of homology, pSPN1 does not appear to be involved in thienamycin production.

2.2 INTRODUCTION

Streptomyces are Gram-positive, heterotrophic bacteria which undergo a complex cycle of morphological differentiation. They produce a wide variety of commercially important antibiotics. Thienamycin is a very effective penem-antibiotic that at present is only produced commercially by chemical synthesis. *Streptomyces penemafaciens*, unlike *Streptomyces cattleya,* does not produce other antibiotics and thus offers a simpler system to studyThienamycin production. It was for this reason that *Streptomyces penemafaciens* was chosen for study.

While isolating chromosomal DNA from *Streptomyces penemafaciens*, a faint plasmid band was observed in the isopicnic gradient. The plasmid, when treated with restriction endonucleases gave discrete bands and was mapped for *Bgl*II, *Bam*HI, *Eco*RI and *Cla*I restriction sites. The 26.5kb plasmid was designated pSPN1 (Fig. 2.1). In addition to the bands produced by pSPN1, other much fainter bands were observed. A second plasmid designated pSPN2 was postulated to account for these bands. pSPN2 is about 1-2kb larger than pSPN1 and is substantially different from it (Smith, 1988).

Numerous Streptomycetes have been found to have plasmids and in many cases they have been shown to contain more than one compatible plasmid (Hopwood *et al.*, 1986). A number of plasmids have been implicated in antibiotic production (Hopwood *et al.*, 1986), however previous studies on *Streptomyces penemafaciens* have shown that although thienamycin production by this organism is very unstable, plasmid involvement is unlikely (Smith, 1988). Because of the unstable production of thienamycin, isolation by insertional inactivation, of the loci responsible for the production of thienamycin, is difficult. Although the vector may have inserted into and inactivated the pathway this would be hard to detect because of the high rate of spontaneous loss of production. As antibiotic resistance genes are almost always clustered with the production pathway, it is in some cases possible to use a cloned resistance gene to detect production pathways. *Streptomyces* are naturally resistant to penem-antibiotics hence this approach is not possible for thienamycin production. Polymerase chain reaction using primers to the isopenem synthase gene, a critical gene in penem-antibiotic production, has also failed to isolate any production genes (Dorrat, 1989).

No function has yet been linked to pSPN1, but because of its large size and low copy number, it may be suitable as the base for a *Streptomyces* cloning vector capable of holding large inserts or inserts where high gene dosage may be detrimental to the host

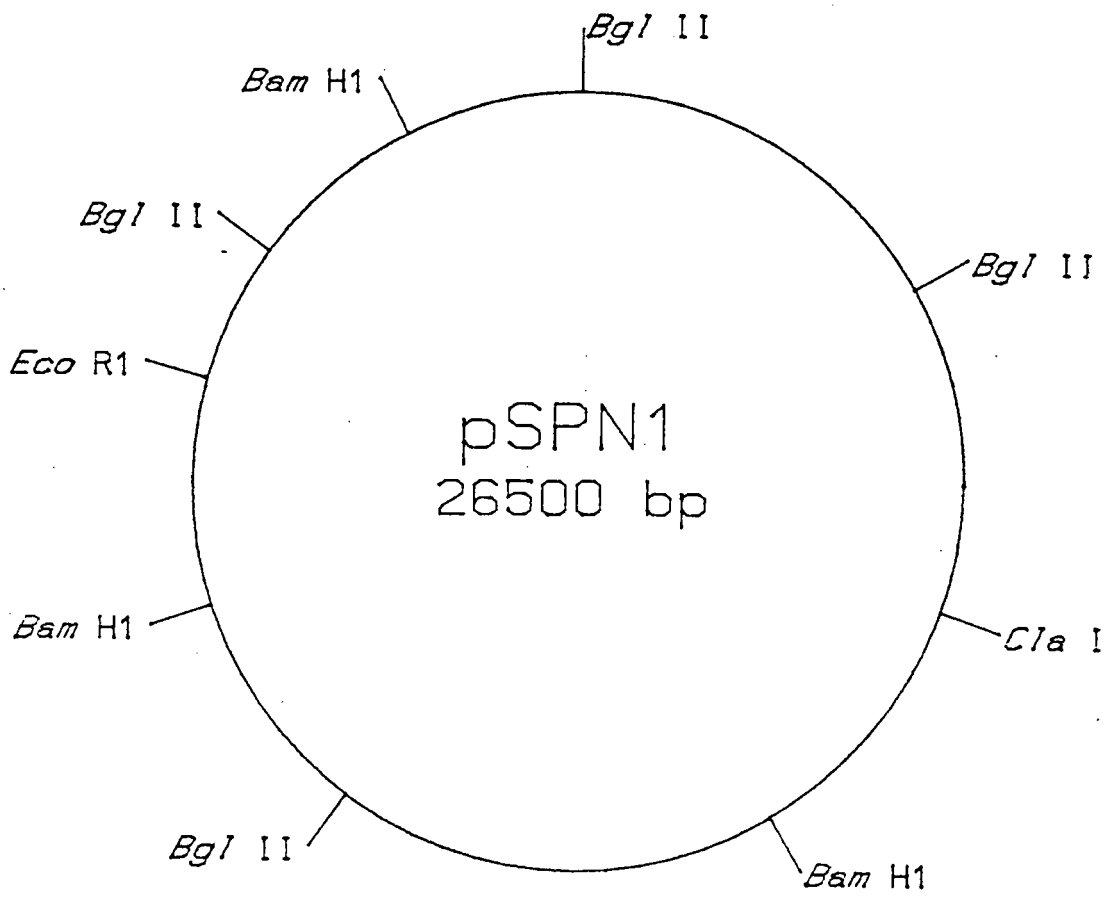

Figure 2.1 Restriction map of pSPN1.

(Smith, 1988). In order to study pSPN1 in greater detail, it was decided to clone the four *Bgl*II fragments that comprise the plasmid and to determine the location of the origin of replication.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and plasmids

Streptomyces penemafaciens ATCC 31599, *Streptomyces lividans* 66 TK24 str-6, *Streptomyces coelicolor* A3 (2) M145 SCP1$^-$ SCP2$^-$, *Streptomyces fulvoviridis* ATCC 15863, *Streptomyces cattleya* ATCC 35852 and *Streptomyces olivaceus* ATCC 3335 (Hopwood *et al.*, 1983; Hopwood *et al.*, 1985.), originated from the culture collection of Professor Ralph Kirby, Rhodes University. *E.coli* LK111 lac^q lacZΔM15 (Zabeau and Stanley, 1982.) was obtained from the culture collection of the Department of Microbiology, University of Cape Town. pLR591 (Hill *et al.*, 1989) was constructed by cloning pEcoR251 cut with *Bam*HI into the *Bgl*II site of pIJ702 and was obtained from Dr. Russel Hill. The plasmid pLR2 is a 1.3kb *Sma*I deletion of pLR591 (Figure 2.2) that is no longer able to replicate in *Streptomyces* as the *ori*V has been deleted. *E.coli* strains were stored in 50% glycerol at -20°C while *Streptomyces* spores were stored in 20% glycerol at -70°C.

2.3.2 Culture conditions

E.coli cells were grown in Luria broth or on Luria agar at 37°C. *Streptomyces penemafaciens* spores were germinated on ISP2 medium and protoplasts were regenerated on 1/2SRM medium, spores of other *Streptomyces* species were germinated

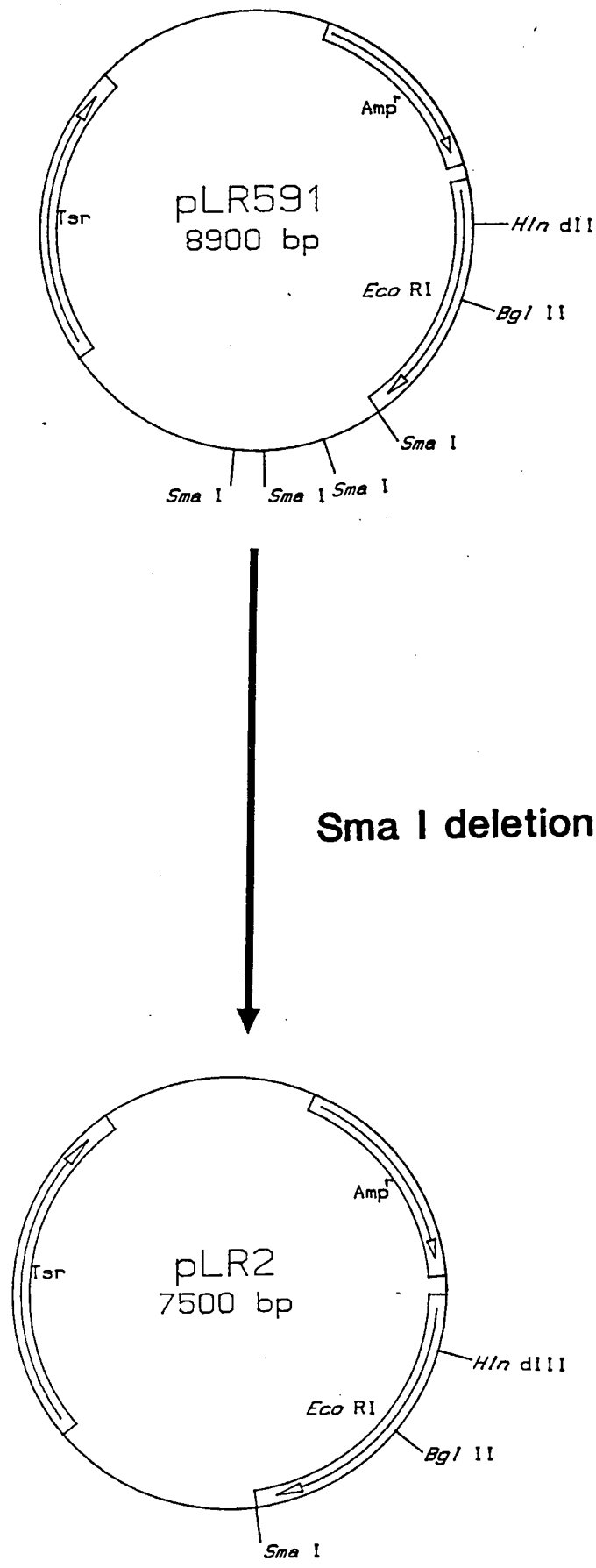

Figure 2.2 Diagrammatic representation of the construction of pLR2 by a *Sma* I deletion of pLR591.

on malt3 medium and protoplasts were regenerated on R2YE. All *Streptomyces* liquid cultures were grown in YEME from a 1ml innoculum of a spore suspension and all cultures of *Streptomyces* were grown at 30°C. Appendix A lists the composition of all media and the antibiotic concentrations used.

2.3.3 DNA extraction

The large scale preparation of *E.coli* plasmid DNA (maxiprep procedure), was by the alkaline lysis method of Maniatis *et al.* (1989). A 100ml culture was grown overnight at 37°C and then harvested by centrifugation at 5000g for 5 min, the pellet was resuspended in 5ml solution I (the composition of all solutions and buffers is described in appendix B). After 5 min at room temperature 10ml of solution II was added and the mixture was kept on ice for 5min, before the addition of 7.5ml of ice cold solution III. Cellular debris was removed by centrifugation at 15000g for 10 min, one volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 40000g for 15 min. The pellet was washed with 70% ethanol and resuspended in 4,8ml TE buffer, the DNA was then purified by isopicnic CsCl-EtBr ultracentrifugation as described by Maniatis *et al.* (1989).

Small scale preparation of *E.coli* plasmid DNA (miniprep procedure) was performed by a modified version of the method described by Maniatis *et al.* (1989). A 500µl aliquot of an overnight culture was harvested by centrifugation for 2-3 seconds on a bench top microfuge. The pellet was resuspended in 200µl of solution I and kept at room temperature for 5 min, 400µl of solution II was then added and the sample was kept on ice for 5 min. 300µl of ice cold solution III was added and after another 5min on ice, cell debris was precipitated by centrifugation for 15 min in a microfuge. One volume of isopropanol was added and after mixing, the DNA was precipitated by centrifuging for

15 min in a microfuge. The pellet was washed with 70% ethanol and dried under vacuum before being resuspended in 50µl sterile TE buffer.

Except where stated, *Streptomyces* plasmid DNA was isolated by procedure 2, for the isolation of total *Streptomyces* DNA as described by Hopwood *et al.* (1985). Only the plasmid band was removed after isopicnic ultracentrifugation.

All DNA concentrations were estimated on agarose gels using known concentrations of phage-Lambda as a reference standard. Accurate measurements were determined using a Beckman DU-40 spectrophotometer (Maniatis *et al.*, 1989). The best yields of *Streptomyces* DNA were generally from early log phase cultures.

2.3.4 DNA restriction digestions and agarose gel electrophoresis

All DNA restriction digests were performed using enzymes and buffers supplied by Boehringer-Manheim and according to their instructions. Digests for restriction analysis were stopped by the addition of stop buffer and 20µl aliquots were run on 0.8% agarose TBE buffer gels containing 400ng/ml ethidium bromide. The gels were run and photographed using a Polaroid camera as described by Maniatis *et al.* (1989).

2.3.5 Southern hybridization and colony blots

DNA from agarose gels was denatured, neutralized, and then either capillary blotted or vacuum blotted onto Hybond-N membrane (Amersham) and fixed by exposure to 254nm ultra-violet light for 3 min. All procedures were carried out as described by Amersham in their booklet, "Membrane transfer and detection methods".

Colony blots were performed by transferring colonies to Hybond-N circles and then placing the membrane, colony side up, on filter disks soaked in 2 times SSC containing 5% SDS. After 5 min the membrane and filter disks were transferred to a microwave oven and heated at 750 watts for 3 min. The DNA was then fixed to the membrane by exposure to 254nm ultra-violet light for 3 min (Buluwela *et al.*, 1989).

Radio-active probes were prepared using the Amersham nick translation kit (product number N.5000) as directed by the manufacturers. Hybridization was performed at 65°C by the method of Church and Gilbert (1984), and the membrane was then used to expose Agfa Curex X-ray film at -70°C in the presence of intensifying screens. The film was then developed according to the manufacturers instructions.

Non radio-active probe was prepared using the Digoxigenin-dUTP kit from Boehringer-Manheim (product number 1093 657) according to the manufacturer's instructions. The probe was used to hybridize to DNA immobilized on Hybond-N as described above. Bound probe was detected according to Digoxigenin-dUTP kit instructions and the results were then recorded by photocopying the membrane.

2.3.6 Protoplasting and transformation of Streptomycetes

A two stage culture was used to grow mycelium for protoplasting, 1ml of spore suspension was inoculated into 100ml of YEME and the culture was grown till early log phase (about 4 days), 10ml of this culture was then inoculated into 100ml YEME and grown for 24-36 hours. The culture was then protoplasted according to the method described by Hopwood *et al.* (1985), and the protoplasts were stored in 500µl aliquots at -70°C. The efficiency of protoplasting was tested by adding SDS to a concentration of

0.001% and plating the protoplasts onto regeneration medium, this killed all protoplasts and left only unprotoplasted mycelium unharmed and able to grow.

Protoplasts were transformed using the "Rapid small scale procedure for the transformation of *Streptomyces* protoplasts with plasmid DNA" described by Hopwood *et al.* (1985). Aliquots of 100µl of the transformation mix were inoculated onto R2YE plates containing bavastin and ampicillin and then spread using a 5ml glass pipette (Melton and Kieser, 1988). When growth was just visible (12-20 hours) antibiotic selection was applied in a SNA overlay as directed by Hopwood *et al.* (1985) Transformants were counted after another two days. The transformants were allowed to sporulate and then plated onto selective media (ISP2 or malt3 containing thiostrepton). After the colonies sporulated the plates were flooded with 10ml of 20% glycerol and the resultant spore suspension was stored as 1ml aliquots at -70°C.

2.3.7 *E.coli* transformations

Competent *E.coli* cells were prepared by the calcium chloride method described by Maniatis *et al.* (1989) or when transformation efficiency was not critical, by the DMSO method of Chung and Miller (1988). In all cases 100µl of the transformation mix was plated onto Luria agar plates containing the appropriate antibiotic, these were then spread with a sterile spreader and incubated overnight at 37°C.

2.3.8 DNA purification and recombinant techniques

DNA requiring the removal of proteins was purified using phenol:chloroform extraction. One volume of phenol:chloroform was mixed with the sample, the phases were then

separated in a microfuge for 5 min and the aqueous phase was transferred to a new tube. The extraction was repeated a further two times. Then 1 volume of chloroform:isoamyl alcohol was added, the tube was mixed, and the phases were allowed to separate, the aqueous phase was then transferred to a new tube. The process was repeated another two times to remove the last traces of phenol. The aqueous phase was mixed with 0.05 volumes 5M CH_3COOK and 2 volumes of ethanol. After cooling at -70°C for 10 min the DNA was precipitated by centrifugation in a microfuge for 15 min. The pellet was dried under a vacuum and the DNA resuspended in sterile distilled water or TE buffer.

All ligations were performed using 5X ligation buffer (Appendix B) as described by Maniatis *et al.* (1989). All other routine methods not listed were performed according to the methods described by Maniatis *et al.* (1989).

2.4 Results

2.4.1 Cloning of the pSPN1 replication origin

The plasmid pSPN1 was digested with *Bgl*II and the fragments were shotgun cloned into the *Bgl*II site of the positive selection vector pLR2, using a 1:1 molar ratio of vector to insert. This ligation mix was used to transform *E.coli* LK111 to ampicillin resistance, DNA from the transformants was prepared by the miniprep method and then 5µl aliquots of the DNA were digested with *Bgl*II. The digests were run on a TBE agarose gel and then photographed as described in section 2.3.3. Four classes of clones were found and Southern blots confirmed that these corresponded to the 4 *Bgl*II fragments of pSPN1. The clones were designated pAS1.0 (10.6kb), pAS2.0 (11.6kb), pAS3.0 (14.1kb) and pAS4.0 (19.0kb) (Figure 2.3). DNA from these clones was prepared by the maxiprep procedure and was used to transform *Streptomyces lividans* 66 TK24 and *Streptomyces coelicolor*

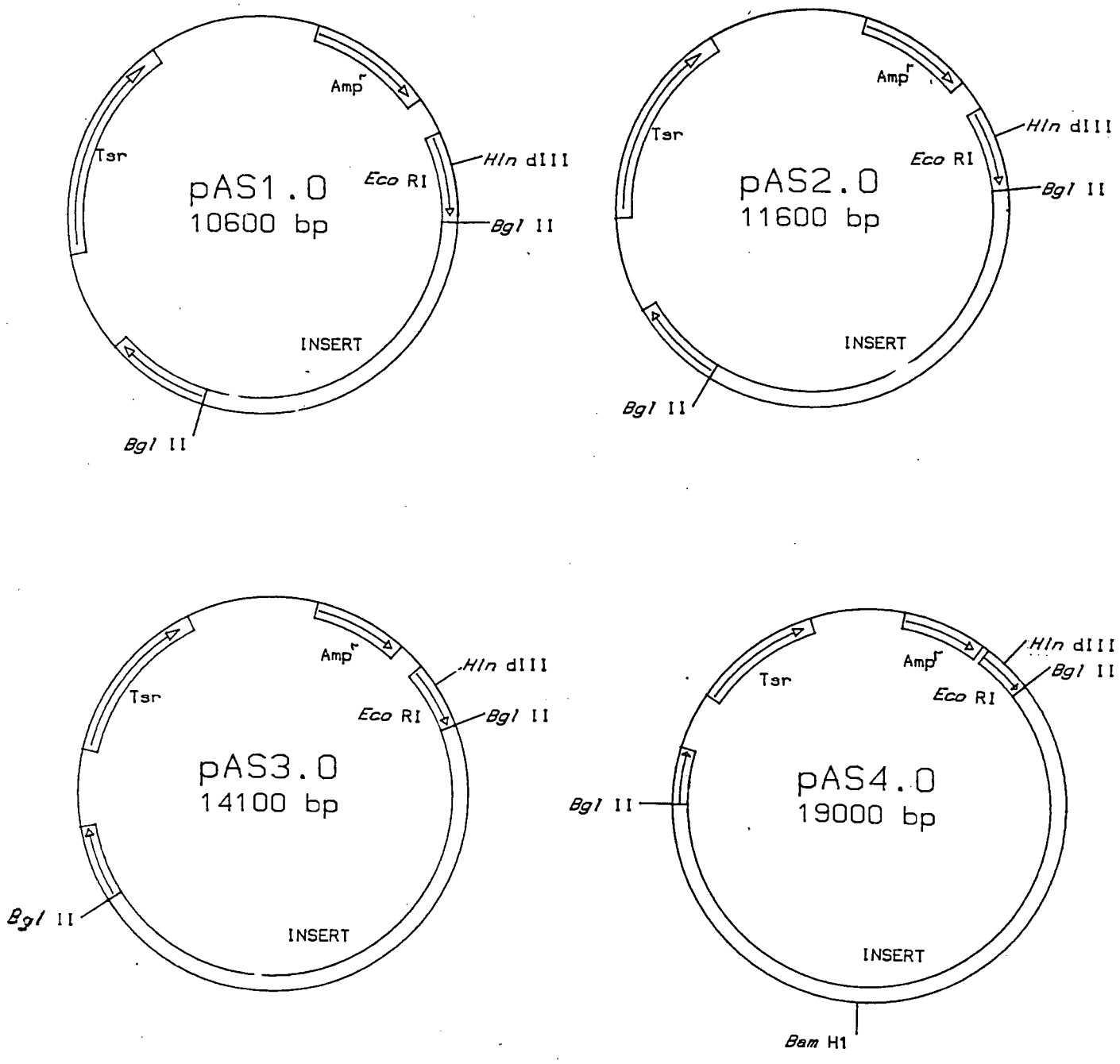

Figure 2.3 Genetic maps of pAS1.0, pAS2.0, pAS3.0 and pAS4.0.

M145 protoplasts. As pLR2 lacks a *Streptomyces* origin of replication, only pLR2 which contains the pSPN1 origin of replication will replicate and hence transform the protoplasts to thiostrepton resistance. Only pAS4.0, which contained the 12.5kb *Bgl*II fragment produced thiostrepton resistant colonies. Total DNA extraction and isopicnic ultracentrifugation yielded autonomously replicating pAS4.0. Restriction mapping of pAS4.0 isolated from *E.coli* showed that it was identical to pAS4.0 isolated from *Streptomyces lividans*, except that it was no longer cut by *Cla*I. The possible reason for this is that in certain cases the *Cla*I site becomes methylated in *E.coli* LK111 which is Dam$^+$, this inhibits restriction by *Cla*I at this particular site (Neesen and Volckaert, 1989). Constructs isolated from *Streptomyces* transformants were also cut with *Bgl*II, separated by TBE agarose gel electrophoresis, blotted to Hybond-N and probed with pAS4.0, all constructs isolated were unaltered pAS4.0.

2.4.2 Homology of pSPN1 to total DNA isolated from various *Streptomyces* species

In order to investigate the possible involvement of pSPN1 in thienamycin production, pSPN1 was used to probe total DNA isolated from other penem-antibiotic producing strains. DNA was prepared as in section 2.2.3, except that all DNA was removed from the isopicnic gradient. DNA samples of 5µg from *Streptomyces fulvoviridis*, *Streptomyces cattleya* and *Streptomyces olivaceus* were cut with *Bgl*II and then run on an agarose gel alongside 500ng of pSPN1 DNA cut with *Bgl*II. The gel was then blotted to Hybond-N and probed with pSPN1 DNA as described in section 2.3.5, faint bands were detected in lanes containing *Streptomyces fulvoviridis* and *Streptomyces cattleya* DNA, no bands of equal size were seen. As *Streptomyces fulvoviridis* showed the strongest homology to pSPN1, it was used for further study. Samples of *Streptomyces fulvoviridis* DNA were digested with *Bgl*II, *Apa*I, *Hha*I, *Kpn*I, *Pvu*II, *Sac*II, *Sma*I and *Bam*HI, each of these digests was then run on an agarose gel alongside pSPN1 DNA digested by the

same enzyme. The gel was then blotted to Hybond-N and a Southern blot was performed with pSPN1 as a probe, again there was faint homology but no equal sized homologous bands were seen although using 7 different enzymes were used to digest the DNA. If both organisms share a pathway they should both produce some identical sized fragments of DNA corresponding to DNA from the shared pathway. Due to the method of DNA preparation there was the possibility that the plasmid DNA was contaminated with *Streptomyces penemafaciens* chromosomal DNA and that this was responsible for the apparent homology, not the plasmid. As all *Streptomyces penemafaciens* chromosomal DNA preparations contain traces of pSPN1 DNA, this could not be used as a probe to determine whether chromosomal DNA was responsible for the homology. By using the cloned fragments obtained in section 2.4.1 as probes, *Streptomyces penemafaciens* chromosomal contamination of the probe could be eliminated. *Streptomyces penemafaciens* chromosomal DNA and each of the cloned fragments were used as probes to gels that had lanes containing, *Streptomyces penemafaciens* total DNA, *Streptomyces fulvoviridis* total DNA and pSPN1 DNA each digested with *Bgl*II. None of the cloned fragments had any homology to *Streptomyces fulvoviridis* DNA, however *Streptomyces penemafaciens* total DNA did. Based on the assumption that pathways for the production of an antibiotic show homology even when they occur in different organisms, this adds further weight to the argument that pSPN1 does not contain the genes encoding the biosynthetic pathway for thienamycin. Homology seen in previous blots was thus probably due to small amounts of *Streptomyces penemafaciens* chromosomal DNA in the pSPN1 probe.

2.4.3 Attempted transformation of species other than *Streptomyces lividans* and *Streptomyces coelicolor*

Streptomyces lividans and *Streptomyces coelicolor* were readily transformed with pAS4.0 using the method described by Hopwood *et al.* (1985). *Streptomyces cattleya* protoplasts either died or were insufficiently protoplasted despite filtering out the mycelium using mirror cloth, prior to their final centrifugation, a new adaptation for *Streptomyces cattleya* protoplast generation developed at Pan Labs in Seattle.

2.5 Discussion

Of the methods available to isolate *Streptomyces* DNA the procedure chosen was by far the quickest and easiest. This was the reason why it was initially chosen, however it also served as an easy method of determining if a plasmid was present. If the plasmid was autonomously replicating a plasmid band not found in the wild type was seen in the transformant. This would not occur if the plasmid was integrated into the chromosome or if the organism had mutated to antibiotic resistance. Once the plasmid was known to be autonomously replicating, attempts were made to isolate plasmid DNA using the plasmid DNA isolation procedures described by Hopwood *et al.* (1985). However these procedures were time consuming and yields were extremely low. Because of its large size only methods for the isolation of total DNA are sufficiently gentle enough to purify sufficient quantities of pSPN1 without shearing it.

It was fortunate to isolate the entire origin of pSPN1 on one of the *Bgl*II fragments as one of the sites may have been within the origin and thus cloning would have disrupted the origin. The cloned fragments were tested for replicative ability in strains known to be plasmid free as this allows easy detection of autonomously replicating plasmid. The

strains were also chosen as they are already known to be able to harbor a range of plasmids and thus would in all probability be able to harbor pSPN1. Since pSPN1 lacks a marker it was not feasible to test the whole plasmid for the ability to replicate. Once the origin was found to reside on the 12.5kb fragment, pAS4.0 became the focus of this study.

The apparent homology between *Streptomyces fulvoviridis* and *Streptomyces penemafaciens* is unlikely to be due to nonhomologous binding as other species such as *Streptomyces lividans* which have similar G+C ratios show no homology (results no shown). It is most probable that the homology seen between *Streptomyces fulvoviridis* and *Streptomyces penemafaciens* chromosomal DNA, reflects a close phylogenetic relatedness. Nothing more can be inferred from the data except that pSPN1 is not similar to any DNA elements in *Streptomyces fulvoviridis.*

When transforming *Streptomyces* with DNA from *E.coli*, a large proportion of DNA becomes restricted or is modified so that it is no longer functional. Thus when transforming *Streptomyces* with *E.coli* derived DNA, very high concentrations of DNA are required to overcome this problem or the DNA can be prepared in a Dam$^-$ *E.coli* strain (Neesen and Volckaert, 1989). Due to the slow growth rate of *Streptomyces* which can easily take 2 weeks between transformation of protoplasts and isolation of transformant DNA, constructs were manipulated in *E.coli* and only when they were fully complete were they transferred to *Streptomyces*. This strategy saves time and results in an increased number of transformants. It was thus decided to produce a vector capable of shuttling between *E.coli* and *Streptomyces* so that DNA manipulation could take place in *E.coli* and yet the construct could be tested in *Streptomyces* without subcloning.

CHAPTER 3

CONSTRUCTION OF A *STREPTOMYCES - E.COLI* SHUTTLE VECTOR FOR THE STUDY OF *STREPTOMYCES* ORIGINS OF REPLICATION

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CONSTRUCTION OF A *STREPTOMYCES - E.COLI* SHUTTLE VECTOR FOR THE STUDY OF *STREPTOMYCES* ORIGINS OF REPLICATION

3.1 Summary

A streptomycin resistance marker, functional in both *E.coli* and *Streptomyces* was cloned into the vector Bluescript. The 12.5kb replication origin-containing *Bgl*II fragment from pSPN1 was cloned into this newly constructed vector and it was found to confer the ability to replicate in *Streptomyces* on this vector. The copy number of this construct was found to be 0.2 in *Streptomyces* and conditions affecting its selection in *Streptomyces* species were investigated.

3.2 Introduction

Once the fragment containing the origin of pSPN1 had been isolated, it was decided to shorten this fragment to its minimal replicon in order to use it as the basis of a *Streptomyces* cloning vector. Bluescript is an ideal vector for performing deletions by Exonuclease III shortenings or using restriction sites to cut out large regions of DNA. Bluescript cannot replicate in *Streptomyces* and even with a cloned origin, a marker selectable in *Streptomyces* is necessary. It was thus decided to introduce a marker that can be selected for in *Streptomyces* into Bluescript, this would enable the cloned origin to be manipulated in *E.coli* and tested for replication in *Streptomyces* without subcloning. The existing vector pLR2 is unsuitable as it lacks a cloning cassette and thus would require deletions to be blunt ended and ligated into the *Bgl*II or *Hin*dIII sites which had been blunt ended, this would not allow for fragments to be excised for subcloning or

sizing. Exonuclease III digestion in order to shorten inserts is also impossible in pLR2 and the insert would have to be subcloned in order for it to be sequenced.

3.3 Materials and Methods

3.3.1 Bacterial strains and plasmids

Streptomyces lividans 66 TK21 (Hopwood *et al.*, 1983) was a kind gift from Mervyn Bibb of the John Innes Institute, *Streptomyces coelicolor* 2708 proA1 hisA1 argA1 cysD18 uraA1 SCP1$^-$ SCP2$^-$ (Hopwood *et al.*, 1985) was from the culture collection of Will Bourn, Department of Microbiology, University of Cape Town. Bluescript KS+ originates from Stratagene, pIJ4642 (Tobais Kieser, unpublished results) was obtained from Ralph Kirby, Department of Microbiology, Rhodes University. Plasmid pLR591 (Hill *et al.*, 1989) was obtained from Russel Hill (pLR591 is also known as pLR1).

3.3.2 Isolation of total Streptomyces DNA

Total *Streptomyces* DNA was isolated by a modified version of procedure 1 for the isolation of total *Streptomyces* DNA as described by Hopwood *et al.* (1985). One gram (wet weight) of mycelium from a 4-6 day old culture was resuspended in 5ml TE buffer containing 50mg Lysozyme (Boehringer-Manheim). The mixture was incubated at 30°C and titurated every 15 min until a drop of the suspension was cleared by the addition of a drop of 10% SDS. After the addition of 1.2ml 0.5M EDTA and 0.13ml Pronase solution, 0.7ml of 10% SDS was added and the mixture was incubated at 37°C for 2 hours. Six milliliters of equilibrated phenol (Appendix B) was then added and the mixture was shaken by hand for 10 min. A further 6ml of phenol was added and the mixture was

shaken for another 5 min. After centrifugation at 10000g for 15 min the aqueous phase was retained and 5ml TE was added to the organic phase which was then shaken for another 5 min. The phases were then separated by centrifugation at 10000g for 15 min and the supernatant was then added to that already collected, The phenol extraction was then repeated on the pooled supernatant. The remaining phenol was then extracted from the aqueous phase by adding 6ml of chloroform:isoamyl alcohol (24:1), shaking for 5 min and then centrifuging at 5000g for 15 min. The aqueous phase was again extracted with 6ml of chloroform:isoamyl alcohol and RNase A stock solution was added to a final concentration of 40μg/g. The mixture was then incubated for 1 hour at 37°C after which one volume of isopropanol was added and the DNA was precipitated by centrifugation 40000g for 15 min. The pellet was washed with ice cold 70% ethanol then resuspended in 5ml TE buffer and 500μl 5M potassium acetate. Two volumes of ethanol were then added and the DNA was reprecipitated by centrifugation at 40000g for 15 min. The pellet was resuspended in 1ml sterile TE buffer and stored at -20°C.

3.3.3 Determination of streptomycin minimum inhibitory concentrations for *Streptomyces* species

To determine the MIC of streptomycin for *Streptomyces* spores on solid malt3 medium, 100μl of spore suspension was plated on pates containing streptomycin in concentrations ranging from 0 to 1600μg/ml. Growth was assessed after 5 days and the MIC was taken to be the lowest concentration which resulted in absolutely no growth.

The MIC of streptomycin for protoplasts was determined by carrying out a *Streptomyces* transformation as previously described in section 2.3.6 and applying SNA overlays containing a range of streptomycin concentrations, after 20 hours of incubation at 30°C. Growth was assessed after a further 2 days incubation.

For the calculation of the MIC in YEME medium, 1ml of spore suspension was inoculated into 100ml of YEME containing streptomycin. This was performed for concentrations ranging from 0 to 100µg/ml, growth was assessed after 3 days and then again after 6 days to determine if the MIC was effected by the growth period.

3.3.4 Purification of DNA fragments out of an agarose gel

Fragments were initially purified using the Geneclean kit produced by Bio 101 or a homemade copy of it (Vogelstein and Gillespie, 1979), higher yields were obtained by electroelution of DNA from TAE agarose gels. For electroelution the DNA sample was run on a TAE gel which was then viewed under 302nm ultraviolet light. The relevant band was cut out and the gel block and 300µl of TAE buffer were placed in as small a piece of dialysis tubing as possible and returned to the electrophoresis apparatus and run at 100V for sufficient time for the band to run out of the gel and into the surrounding buffer. The buffer was then used to wash as much DNA off the sides of the tubing as possible, the TAE containing the DNA was mixed with 300µl phenol:chloroform. The mixture was separated in a microfuge for 5 min and the aqueous phase was extracted 3 times with chloroform:isoamyl alcohol. Two volumes of ethanol were added to the sample as was 0.05 volumes 5M CH_3COOK and after 10 min at -70°C the DNA was precipitated in a microfuge for 15 min. The DNA was resuspended in TE buffer or sterile distilled water if the sample was to be treated with calf intestinal alkaline phosphatase (CIP).

3.3.5 Dephosphorylation - Removal of 5' phosphate groups from DNA molecules

DNA to be Dephosphorylated was cut with the required restriction enzyme and then purified using phenol:chloroform before being precipitated. The DNA was then resuspended in sterile distilled water and 10µg of DNA was mixed with 10X CIP buffer (Boehringer-Manheim) and 10 units of molecular biology grade calf intestinal alkaline phosphatase (CIP) (Boehringer-Manheim). The mixture was incubated at 37°C for 1 hour then extracted with phenol:chloroform and resuspended in the required volume of TE buffer. The degree of Dephosphorlyation was tested by performing ligations with and without insert DNA. Levels of Dephosphorlyation greater than 50% were easily achieved, however when 100% Dephosphorlyation was required the Dephosphorylated DNA was resuspended in distilled water and the Dephosphorlyation procedure was repeated.

3.3.6 Blunt ending - the production of flush ends from stickey ends

The DNA was cut with the required restriction enzyme, purified using phenol:chloroform and then resuspended in TE buffer. To 5µg of cut DNA, 4µl of Amersham nick translation kit buffer and 4µl of a 2mM solution containing all four dNTP's was added, the volume was then made up to 19µl with sterile distilled water. One microlitre of blunting enzyme was then added, for 5' overhangs Klenow polymerase was used and for 3' overhangs T4 polymerase was used, the mixture was incubated at room temperature for 30 minutes. The DNA was then purified using a phenol:chloroform extraction and after precipitation it was resuspended in TE buffer or sterile distilled water if the DNA needed to be Dephosphorylated.

3.3.7 Plasmid copy number determination

The plasmid whose copy number needed to be determined was grown up under selection and two different DNA isolation procedures were performed, one procedure isolated only the plasmid DNA, while the other procedure isolated total DNA. The concentration of plasmid and total DNA was calculated spectrophotometrically and the DNA was then cut with a restriction enzyme which cuts the plasmid once. A dilution series of total DNA concentrations was run next to a dilution series of plasmid DNA. These concentrations ranges were roughly calculated using the formula below and a postulated value for the copy number. The gel was then blotted to Hybond-N and probed with purified plasmid, the autoradiograph showed only plasmid bands, the intensity of the band was proportional to the concentration plasmid DNA. If Xμg of total DNA produce a band of equal intensity to Yμg of plasmid it can be concluded that there is the same amount of plasmid DNA in both lanes. Since the size of the plasmid, the size of the chromosome and the amount of DNA in both lanes was known, the copy number of the plasmid was determined using the following formula.

$$\frac{\text{Plasmid size (kb) X Copy \#}}{\text{Chromosome size in (kb)}} = \frac{\text{μg of plasmid DNA in band}}{\text{μg of total DNA in Band}}$$

All recombinant techniques not described above are performed as described by Maniatis *et al.* (1989)

3.3.8 Assay to determine the effect of plate dryness on protoplast regeneration rate

R2YE plates were dried in a 60°C oven for varying lengths of time, 100µl of untransformed protoplasts was then spread on each plate using a 5ml pipette. The plates were then incubated at 30°C for 48 hours and the growth was graded from 0 (no growth) to +++ (confluent growth).

3.3.9 Assay to determine if time at which the streptomycin overlay is applied affects protoplast viability

Eleven R2YE plates were spread with 100µl of untransformed TK21 protoplasts each and another eleven plates were each spread with 100µl of TK21 protoplasts transformed with pC1 (see section 3.4.1). After 12 hours one plate of transformed and one of untransformed protoplasts were overlaid with 1000µg/ml of streptomycin in SNA. A further two plates were overlaid every subsequent hour until 22 hours had elapsed since the protoplasts had been plated. The protoplasts were grown for a further two days and the number of transformants was then counted.

3.3.10 Assay to determine if protoplast concentration affects streptomycin selection levels

A range of protoplast dilutions from 10^0 to 10^{-3} was prepared and eight R2YE plates were inoculated with each dilution. After 20 hours the eight plates of each dilution were overlaid with SNA containing streptomycin at concentrations that varied from zero to 1600 µg/ml. The number of transformants was counted after a further 2 days incubation at 30°C.

3.4. Results

3.4.1 Cloning of the streptomycin resistance gene from pIJ4642 into pLR591

In order to determine if the streptomycin resistance gene from pIJ4642 was functional in *E.coli* and *Streptomyces*, it was cloned into the *E.coli-Streptomyces* shuttle vector pLR591. A *Hin*dIII digestion of pIJ4642 released the streptomycin resistance gene and this was shotgun cloned into pLR591 that had been cut at a unique *Hin*dIII site. The fragments, were ligated in a 1:1 molar ratio and used to transform *E.coli*, transformants were selected on Luria agar plates containing ampicillin and streptomycin. DNA from the transformants was prepared by the miniprep method and cut with *Hin*dIII and *Sph*I. The digests were run on a TBE agarose gel whose restriction pattern confirmed that all the inserts were the streptomycin resistance gene from pIJ4642. A representative construct was chosen and designated pC1. *Streptomyces lividans* 66 TK21, the streptomycin sensitive strain of *Streptomyces lividans* 66 was then transformed with pC1. Transformed and untransformed TK21 were overlaid with SNA containing a range of streptomycin concentrations and it was determined that untransformed protoplasts were completely killed at concentrations above 500μg/ml while at concentrations of 1600μg/ml there were greater than 100 pC1 containing transformants per plate.

3.4.2 Construction of pBlue

In order to be able to select for Bluescript in *Streptomyces* species, the streptomycin resistance gene from pIJ4642 was cloned into Bluescript. The *Hin*dIII fragment containing the resistance gene was excised from pIJ4642, by *Hin*dIII and was

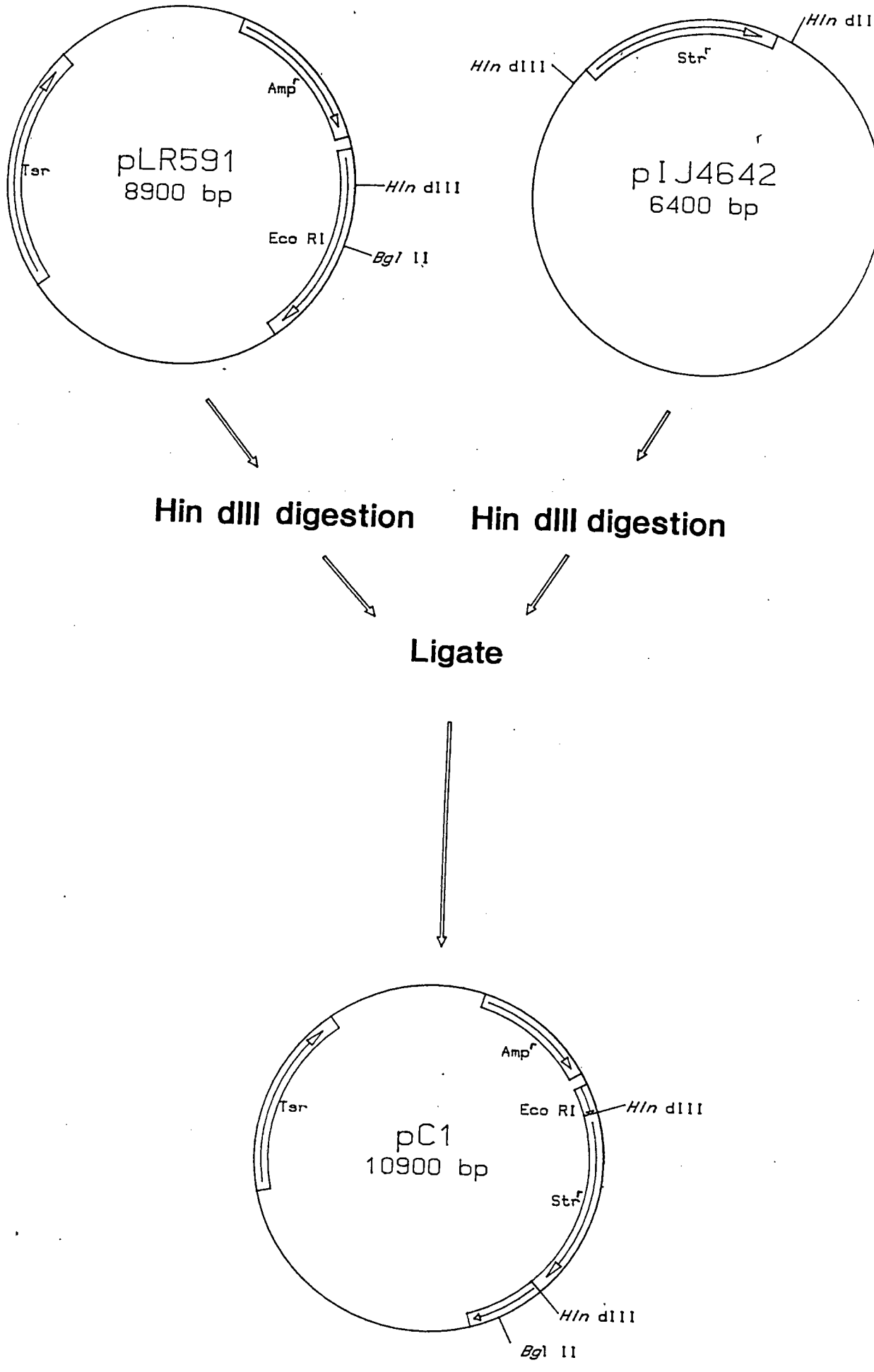

Figure 3.1 Diagrammatic representation of the construction of pC1 from pIJ4642 and pLR591.

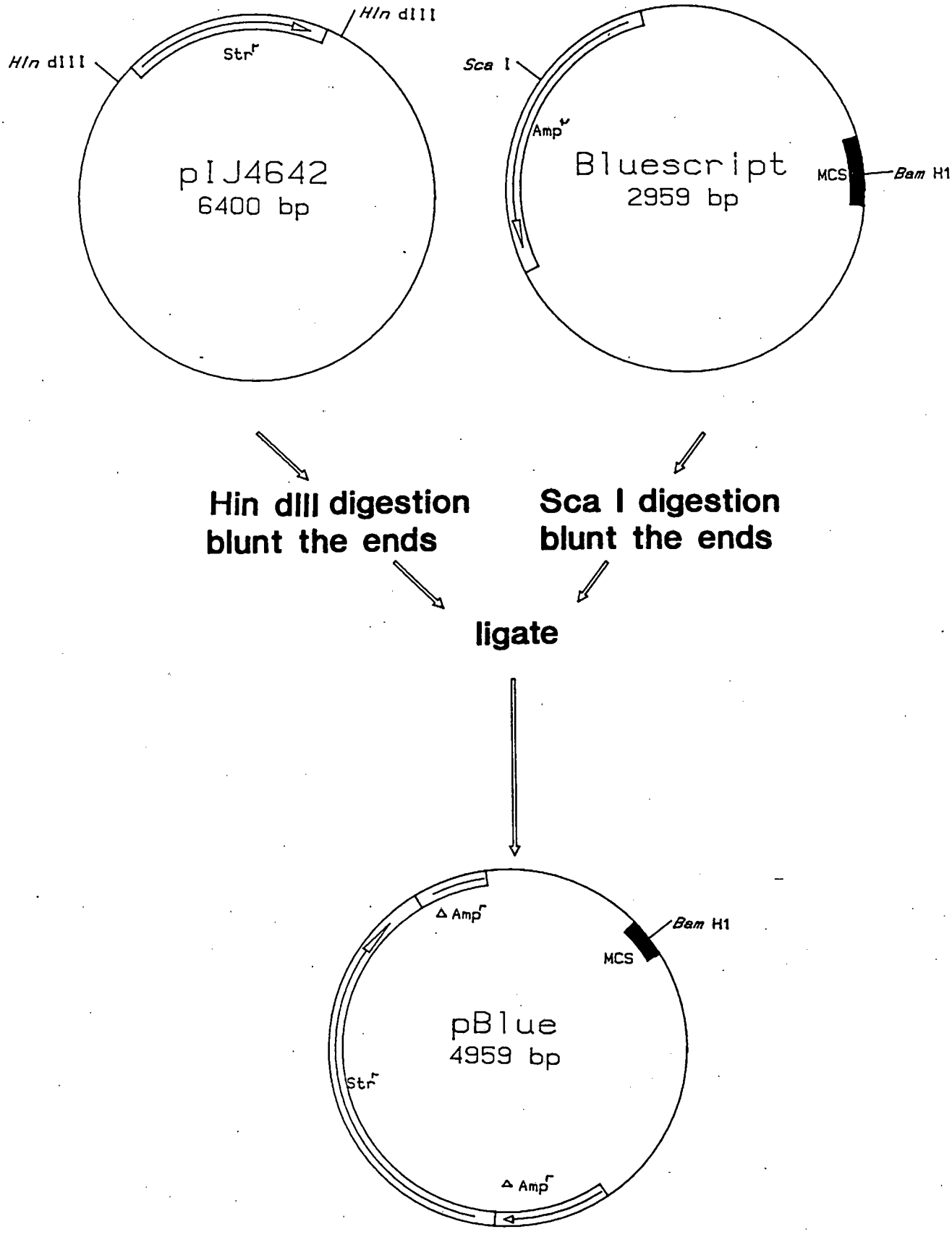

Figure 3.2 Diagrammatic representation of the construction of pBlue from pIJ4642 and Bluescript.

blunt ended (section 3.3.6). It was then ligated into the blunt-ended *Sca*I site of Bluescript. DNA from this ligation which had a 1:1 molar ratio of vector to insert was used to transform *E.coli*. *E.coli* transformants were selected on plates containing streptomycin, X-Gal and IPTG (Appendix A), white colonies were replica plated and blotted to Hybond-N. The membranes were then hybridized as previously described, Bluescript DNA was used as a probe. DNA from positive colonies was then prepared by the miniprep procedure and after digestion with *Sph*I, run on a TBE agarose gel. Two types of transformants were found, which corresponded to the insertion of the resistance gene in either orientation. Representative transformants which were determined by restriction digestion (see figure 3.3), these were selected and arbitrarily designated pBlue+ and pBlue-. The orientation of the insert did not appear to effect gene expression or plasmid viability, hence pBlue+ was used for all subsequent cloning. Although pBlue has a selectable marker that is effective in *Streptomyces* (see section 3.3.1), it cannot replicate in *Streptomyces* and thus it functions as an ideal vector to test the viability of a *Streptomyces* plasmid origin of replication or as a vector for insertional inactivation of *Streptomyces* genes.

3.4.3 Cloning of the origin containing *Bgl*II fragment from pSPN1 into pBlue

In order to test the viability of the pSPN1 origin in the vector pBlue and to create a positive control by which to judge the viability of deletions, the origin containing fragment of pSPN1 was cloned into pBlue. The 12.5kb *Bgl*II fragment was cut out of pAS4.0, gel purified by electroelution and ligated into Dephosphorylated pBlue cut with *Bam*HI (Figure 3.4). *E.coli* cells were transformed with this DNA and then plated onto plates containing streptomycin, X-Gal and IPTG (Appendix A). DNA from the streptomycin resistant *E.coli* transformants was prepared by the miniprep method and after digestion with *Xba*I and

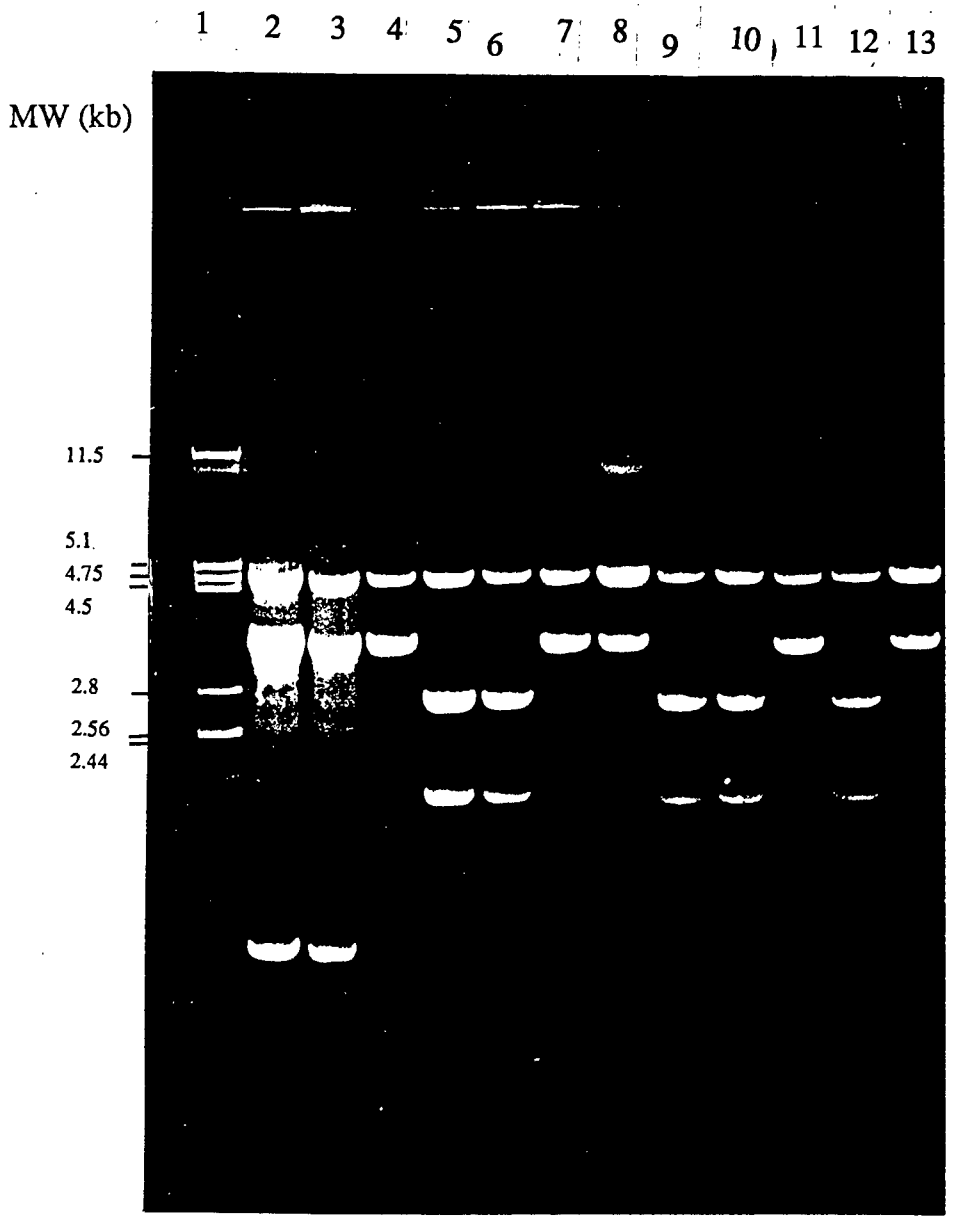

Figure 3.3 Bluescript containing the streptomycin resistance gene from pIJ4642. The leftmost lane contains a *Pst*I digest of phage Lambda, the remaining lanes contain streptomycin resistant Bluescript partially digested with *Sph*I and *Bam*HI to show the diffrent orientations of the resistance gene. One clone of each orientation was selected and arbitrarily designated pBlue+ (lanes 2,3,4,7,8,11 and 13) and pBlue- (lanes 5,6,9,10 and 12).

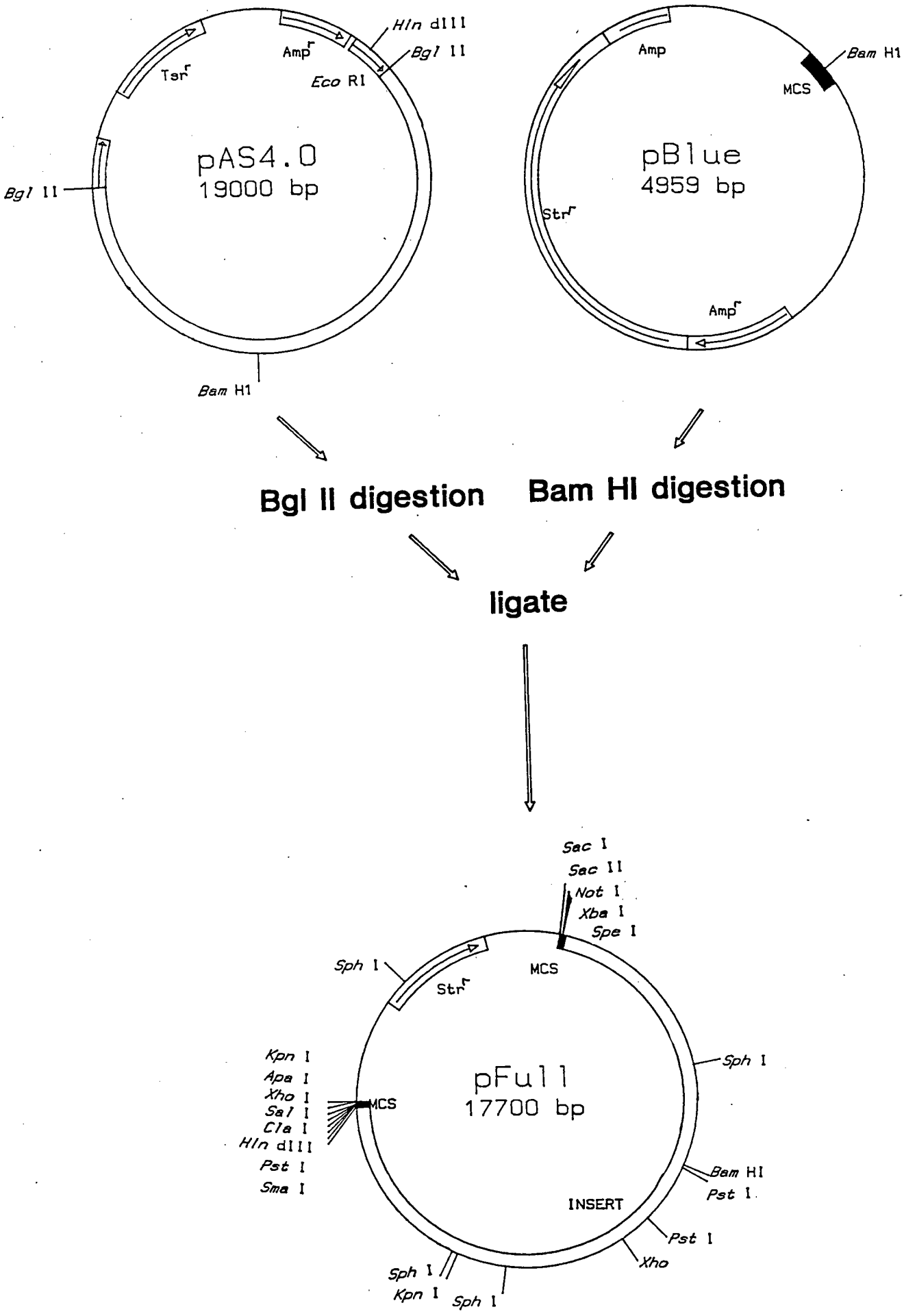

Figure 3.4 Diagrammatic representation of the construction of pFull from pAS4.0 and pBlue.

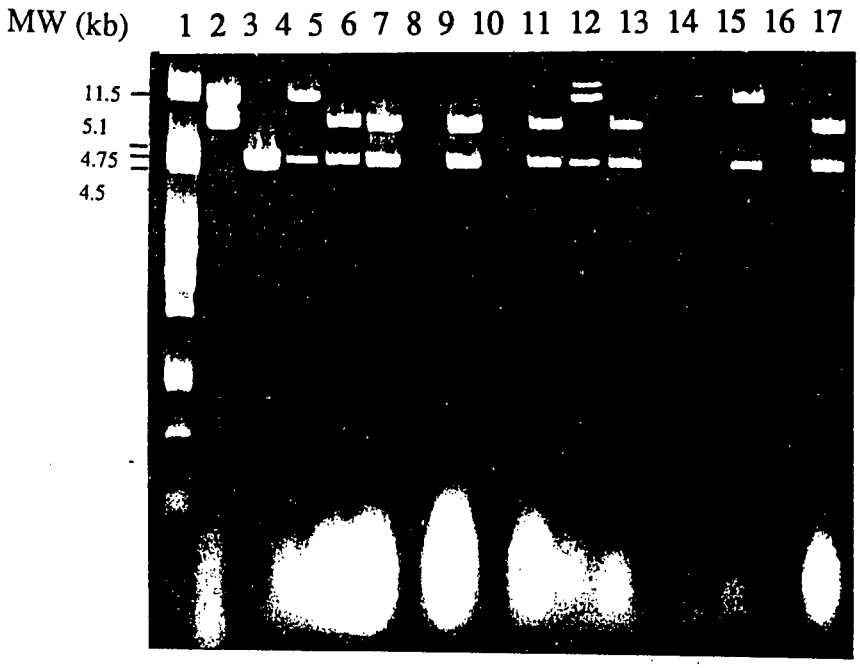

Figure 3.5 Digests of supposed pFull clones. Lane 1 contains a *Pst*I digest of phage Lambda; lane 2 pAS4.0 digested with *Bgl*II; lane 3 pBlue digested with *Bam*HI and lanes 4 to 17 are *Hin* dIII, *Xho*I digests of DNA isolated from transformants from the experiment discribed in section 3.4.3. The clone whose DNA was shown in lane 4 was designated pFull and contained the full 12.5kb *Bgl*II fragment from pAS4.0.

*Hin*dIII, the DNA was run on an agarose gel (see figure 3.5) before being blotted to Hybond-N and probed with pSPN1 DNA. A clone containing the full length insert was found and designated pFull. The construct was used to transform *Streptomyces lividans* 66 TK21 as described in section 2.3.6 pFull DNA was reisolated from streptomycin resistant colonies as an autonomously replicating entity. From numerous transformations it became apparent that the level of streptomycin required to kill untransformed protoplasts varied according to numerous factors discussed in section 3.4.5. At concentrations of 200-300μg/ml above the streptomycin concentration required to kill untransformed TK21 protoplasts, the transformed protoplasts were also killed. This gives a concentration window of 200-300μg/ml in which transformed protoplasts could be differentiated from untransformed protoplasts. A major problem was that this window fluctuated according to factors difficult to control. Transformants were thus always selected by plating onto at least 6 plates and selecting with overlays of 300, 400, 500, 600, 700 and 800μg/ml of streptomycin. Untransformed protoplasts were also selected with these concentrations as a control The benefits of this approach will be discussed in section 3.5.

3.4.4 Selection of *Streptomyces* using streptomycin

Initially *Streptomyces coelicolor* 2708 was selected as the recipient strain for streptomycin resistance conferring constructs. This strain unfortunately showed a very high rate of spontaneous mutation to streptomycin resistance and proved difficult to transform. *Streptomyces lividans* 66 TK21 was chosen as a recipient and found to produce occasional spontaneous revertants but in general showed a MIC of 50μg/ml streptomycin for spores. If however the plates were left for a few weeks, small pinprick colonies were often observed. These pinprick colonies were ignored as they represented a slight background growth which selection could not totally inhibit. Protoplasts showed a

MIC that fluctuated according to factors investigated in this section. Selection of *Streptomyces lividans* 66 TK21 in liquid culture showed that the MIC increased proportionately with amount of time for which the culture was allowed to grow. Cells containing constructs pC1 and pFull (sections 3.4.1 an 3.4.3) were shown after 3 days growth in YEME, to be able to confer resistance to greater than 100µg/ml of streptomycin, while untransformed spores showed no growth at concentrations greater than 40µg/ml. After 6 days of incubation the MIC of streptomycin for untransformed TK21 had risen slightly to 50µg/ml and thus 100µg/ml of streptomycin was used to select transformants. A flask was inoculated with untransformed TK21 as a control when growing up transformants.

3.4.5 Factors effecting pFull selection

When selecting pFull it became apparent that the MIC of streptomycin for untransformed TK21 varied considerably. Figure 3.6 shows that the degree of culture plate dryness effected the regeneration efficiency of protoplasts. Colony numbers increased with increasing dryness to a peak of regeneration efficiency and then declined as the plates became excessively dry. Figure 3.7 shows that after a certain number of hours the number of protoplasts expressing the resistance gene reached a maximum. The exact time taken to reach this maximum was however dependant on the regeneration efficiency of the protoplasts which was in turn dependant on incubation temperature and plate dryness. An assay was performed in order to determine whether the protoplast concentration affected streptomycin selection levels. The results are shown in Figure 3.8. It was apparent that selection was dose dependant and that the MIC varied between different batches of protoplasts as the concentration of protoplasts varies from batch to batch. All these factors had to be taken into account when performing transformations.

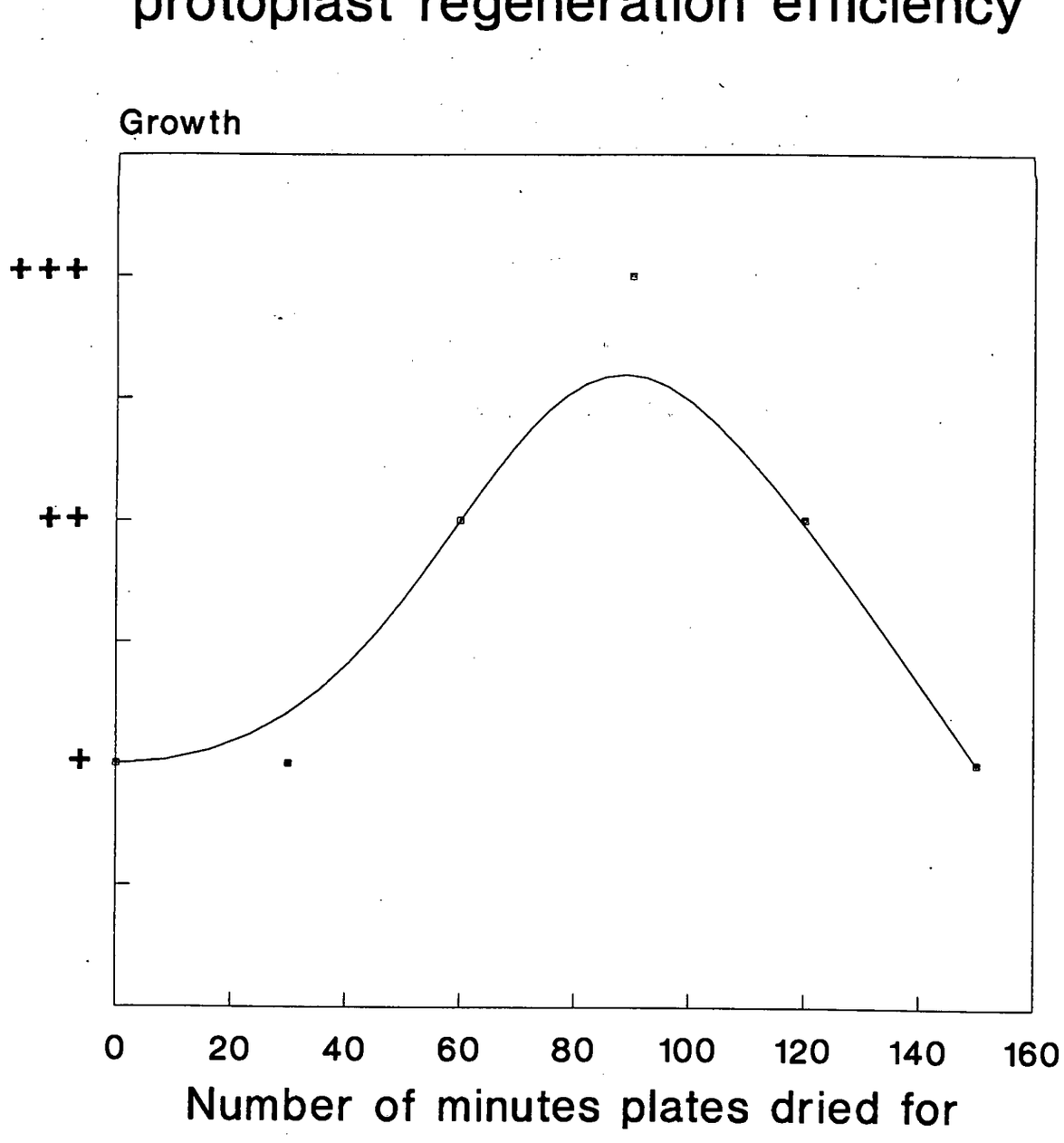

Figure 3.6 The effect of plate dryness on protoplast regeneration rate. This graph shows how regeneration is effected after R2YE plates are dried for varying lengths of time at 60 degrees centigrade.

Figure 3.7 Effect of varying incubation times before streptomycin overlay. The two curves correspond to protoplasts that are transformed with pC1 and protoplasts that are untransformed.

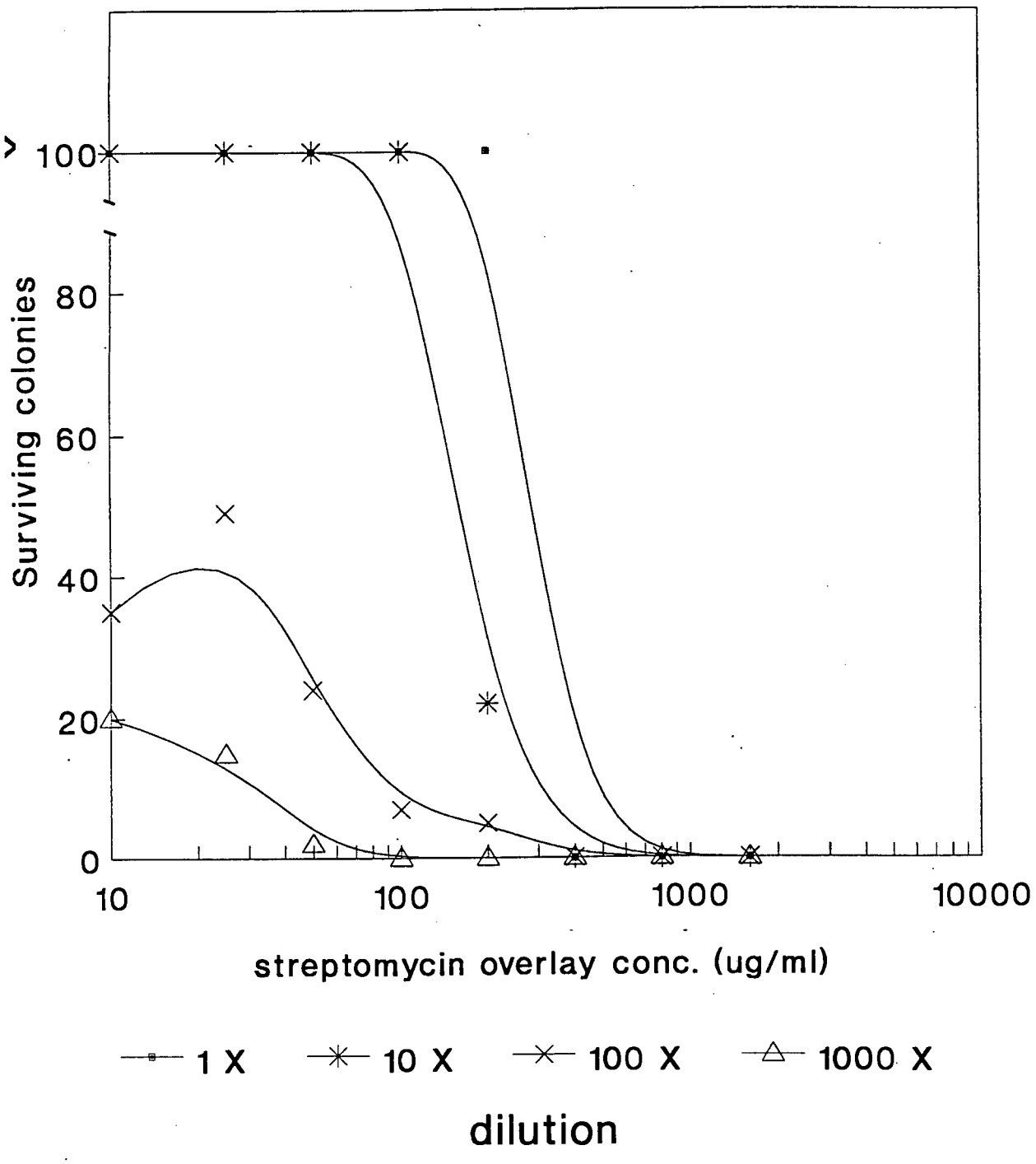

Figure 3.8 Effect of protoplast concentration on the selection ability of streptomycin. Dilutions of protoplasts are indicated below the graph.

3.4.6 Determination of the copy number of pFull in *Streptomyces lividans* 66 TK21

Total DNA was isolated from TK21 containing pFull (here the method described in section 3.3.2 was used). The DNA was cut with *Eco*RI and after being run on an agarose gel, was then blotted to Hybond-N and probed with pFull which, was isolated from *E.coli* so as to ensure that no *Streptomyces* chromosomal DNA contaminated the probe. As various nonspecific bands were observed in lanes containing total DNA, it was decided to excise the insert and probe with only the vector which contained a much lower G+C content than the rest of the DNA and thus avoid nonspecific hybridization. The DNA was then cut with the restriction enzymes *Xba*I and *Hin*dIII, thus excising the pSPN1 insert from pBlue+ as can be seen in Figure 3.4. A dilution series of pFull DNA concentrations and a dilution series of total DNA concentrations were run on an agarose gel. The DNA was then blotted to Hybond-N and probed with pBlue+ DNA as previously described. The bands seen in Figure 3.9 correspond to pBlue+. It can be seen that about 7ng of plasmid would produce a band of equal intensity to that produced by 10μg of total DNA. With the use of the formula described in the methods section, the copy number was calculated at 0.2 copies per chromosome, this assumes a genome size of 5000kb (Genthner *et al.*, 1985).

3.5 Discussion

It can be seen from the results presented in this chapter that selection was a problem in the system used here. The rate of mutation to streptomycin resistance was far greater than that observed for thiostrepton resistance, the most widely used selection for Streptomycetes. The rate of mutation seems to be accelerated by protoplasting, this resulted in a streptomycin resistant background in all *Streptomyces* transformations. When counting supposed transformants, the level of background streptomycin resistance

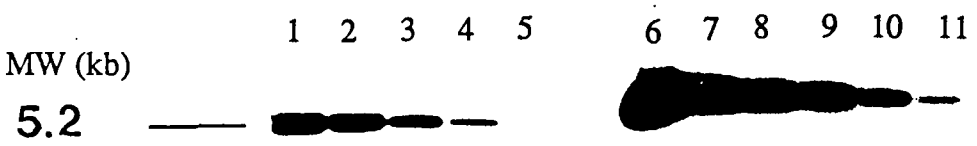

Figure 3.9 Southern blot for the calculation of pFull copy number. Lanes 1-5 contain 15µg,10µg,5µg,2.5µg and 1µg, respectively of total DNA isolated from *Streptomyces lividans* 66 TK21 containing pFull and digested with *Xba* I and *Hin* dIII. Lanes 6-11 contain 50ng,20ng,15ng,10ng,5ng and 2.5ng of pFull DNA isolated from *E.coli* and digested with *Xba* I and *Hin* dIII. This blot was probed with pBlue and bands correspond to homologous pBlue-pBlue hybridization.

was always be measured and it was the number of colonies above the background that was pertinent, not the absolute number. Mutation to streptomycin resistance was a minor problem which was overcome by screening larger numbers of supposed transformants.

The natural level of streptomycin resistance in TK21 protoplasts was constant. However if an increased number of protoplasts were plated the effective concentration of streptomycin dropped and this resulted in streptomycin sensitive colonies escaping selection. The amount of growth at selection is critical as it determines the effective streptomycin concentration. Selecting when there is a lot of growth will result in a higher MIC than would be seen if selection took place when there was only a small amount of growth. From section 3.4.4 it can be seen that by plating more protoplasts, allowing increased time before selection or increasing the growth rate, the MIC will increase. It is the growth rate that was the most difficult to control, this was dependant on the plate dryness which was in turn dependant on the age of the plate, even when they were stored in sealed packets. Since the dryness of plates changed between subsequent transformations using the same batch of plates and between batches of plates, this was a variable almost impossible to control. To ensure that the MIC remained constant, a constant number of protoplasts were plated onto plates of identical dryness and these were then allowed to grow for a fixed time before selection took place. It was thus sensible to keep easily controllable factors such as protoplast number and time before application of selection constant. Plate dryness was very difficult to control or asses accurately. Thus when selecting it was necessary to select over a range of concentrations and have controls for each of these concentrations.

Major problems arose when the maximum level of resistance was close to the MIC, as was the case for pFull. The streptomycin resistance gene could only confer resistance to a specific level of streptomycin. An increase in the number of gene copies would result in higher levels of resistance. Thus for low copy number plasmids such as pFull the

resistance level conferred was low. High copy number plasmids such pC1 had higher levels of resistance due to increased gene dosage. Selection of transformants was complicated by the fluctuating MIC. In high copy number plasmids it is not necessary to select near to MIC thus the fluctuations are not important. In low copy number plasmids, fluctuations can result in the concentration of streptomycin used for selection to fall below the MIC and thus prevent selection. When working with the low copy number origin of pSPN1, a range of selection concentrations was used. Thus only colonies selected at concentrations above the MIC observed for that transformation can be considered to be true transformants.

The copy number of pFull (0.2 per chromosome) was not unexpected as the plasmid yields have always been very poor. Copy numbers of less than one are possible in *Streptomyces* as some mycelial compartments may have numerous chromosomes (Chater, 1989). The copy number of pSPN1 was similar to that of a 2.6kb "mini-circle" isolated from *Streptomyces coelicolor* A3 (2) (Lydiate *et al.*, 1985), these plasmids do not however appear to have any other obvious similarities. It is possible that pFull can similar to the "mini-circle", exist as an integrated as well as an autonomous element.

The method of copy number determination used in these experiments is by far the best available as it limits the number of assumptions that must be made. This method merely requires the assumption that the genome of the test strain is similar in size to those of other Streptomycetes whose genomic size is known. If accuracy was vital the genome size could have been measured but this was unnecessary as in test calculations which used genome sizes of 1000kb more or less than that used in the final calculation, the end result was not significantly altered. Several methods of copy number determination assume copy number to be directly proportional to levels of antibiotic resistance. This is not always true, is difficult to verify and a known reference standard is required. Most *Streptomyces* plasmid copy numbers cover a range of values and thus cannot serve as an

accurate standard for the determination of other copy numbers. Therefore a well known standard such as genome size is far more suitable. The use of high pressure liquid chromatography to determine plasmid copy number is obviously the most accurate but this sort of accuracy was unnecessary. The main point to be made was that the copy number of pFull in *Streptomyces lividans* TK21 is less than one.

Despite the problems encountered in this study, pBlue offers a good system for the manipulation of *Streptomyces* origins of replication. Plasmids with copy numbers as low as pFull are uncommon and thus this system should work for most *Streptomyces* plasmids. The vector allows manipulation in *E.coli* an organism that is easy to handle and quick to grow, the *E.coli* origin of the vector also allows high yields of plasmid DNA to be prepared for *in vitro* work. The streptomycin marker allows for the testing of the altered construct in *Streptomyces* species without time-consuming subcloning of each altered construct. The streptomycin marker also allows for pBlue to be co-resident in *E.coli* with Bluescript and hence may help in studies which require *trans* complementation.

CHAPTER 4

DELIMITATION OF THE REGION OF pSPN1 NECESSARY FOR REPLICATION

CHAPTER 4

DELIMITATION OF THE REGION OF pSPN1 NECESSARY FOR REPLICATION

4.1 Summary

A *Sau*3A partial library of pAS4.0 was constructed in pBlue. The library was then used to transform *Streptomyces lividans* 66 TK21. None of the plasmids were able to replicate in this strain. A different approach to the problem was adopted. The origin-containing fragment of pSPN1 was extensively mapped and a series of deletion derivatives of pFull were constructed. These deletion derivatives were assayed for their ability to replicate in *Streptomyces*. From the data obtained it was possible to determine that a region greater than 5.5kb was required for pSPN1 replication. The location of this region was determined.

4.2 Introduction

Chapter 3 showed that the pSPN1 origin of replication is able to function in *Streptomyces lividans* 66 TK21 when transferred with the newly constructed vector pBlue. Also described were the conditions necessary to select transformants containing viable plasmids. This chapter is devoted to attempts to delineate the minimal replicon of pSPN1.

There are three feasible options available when attempting to shorten large fragments of DNA to sizes that are suitable for sequencing. The DNA can be partially digested with an enzyme that cuts frequently within the sequence and then ligated into a vector to form a library. The clones are then screened for activity and the smallest functional clone can

then be shortened by Exonuclease III or Bal31 to obtain a more precise determination of the minimal functional domain. The shortened fragments can be assayed for the exact point at which activity is lost and thus the minimal functional domain can be determined. The other approach involves mapping the fragment for rare restriction sites and then using these to delete large fragments of DNA. Deletion derivatives are then assayed for activity. In this manner the size of the DNA can be reduced till it is feasible to use Exonuclease III or Bal31. The use of Bal31 to produce deletions of pFull is not feasible as the insert is far larger than the vector and the selection marker or *E.coli* origin of replication would be deleted before a suitable amount of the insert was deleted. Initially Exonuclease III digestion was thought to be the best procedure for obtaining deletions of pFull, this however was unfortunately not possible. Bluescript is a vector designed for performing shortenings on *E.coli* derived DNA and as such, the restriction sites of the cloning cassette are chosen because they are rare in *E.coli*, the replication origin of pSPN1 like all *Streptomyces* DNA has a high G+C ratio and as a result contains numerous sites that are rare in *E.coli*. For Exonuclease III digestion it is necessary to have two unique sites, a 3' overhang to protect the vector from digestion and a 5' overhang for the enzyme to digest, on either side of the insert, in order to perform deletions from both sides of the insert. The construct pFull lacks the required sites and thus cannot be shortened from either direction (Fig 4.1). Attempts were thus made to shorten the insert by partial digestion and when this approach failed to be successful, shortening using restriction enzymes was used.

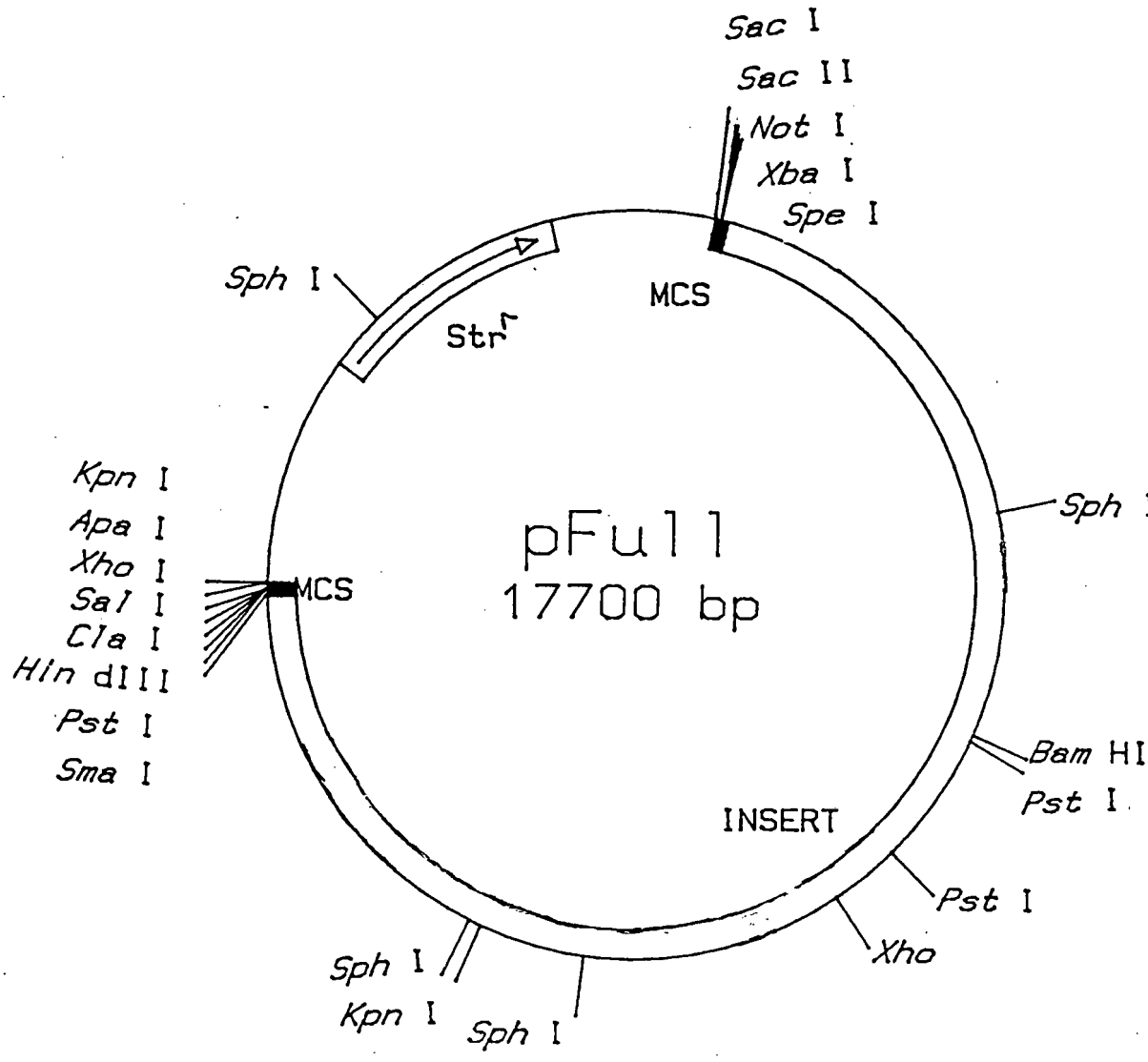

Figure 4.1 Genetic map of pFull. The region marked insert corresponds to pSPN1 derived DNA.

4.3 Materials and Methods

4.3.1 *Sau*3A partial digestion

Enough distilled water was added to 7 microfuge tubes containing 5µg of DNA and 2µl of 10X *Sau*3A restriction buffer so that the total volume of tubes 2-7 was 15µl. Tube 1 was filled to 19µl. One microlitre of 1u/µl *Sau*3A was added to tube 1, the contents mixed and 5µl transferred to tube number 2. The procedure was repeated until the last 5µl taken out of tube 7 was discarded. The tubes were incubated at 37°C for 30 min, 10µl of stop buffer was added, 2µl of the digest run on a slide gel in order to determine which tubes produce partial digestions. The contents of these tubes were pooled.

4.4 Results

4.4.1 Construction of *Sau*3A partial library of pSPN1 origin containing fragments from pAS4.0

Figure 4.2 gives a diagramatic representation of the construction of a *Sau* 3A partial library of pSPN1. The plasmid pAS4.0 was cut with *Bgl*II to excise the insert containing the pSPN1 origin of replication, phenol:chloroform was then used to purify the DNA which was resuspended in TE buffer. The DNA was then partially digested with *Sau*3A as described in section 4.3.1 and the pooled partial digests were run on a TAE gel. Insert and vector that were cut by *Bgl*II only were at the top of the lane while partials extended in a ladder below these bands. Agarose containing the ladder was cut out and cut into 5 equal sized blocks. The DNA was then electroeluted out of the agarose and resuspended in 20µl TE buffer. Fraction 1 contained the biggest fragments and fraction 5 the smallest. The reasons for fractionating the digests are discussed in section 4.5. The DNA from

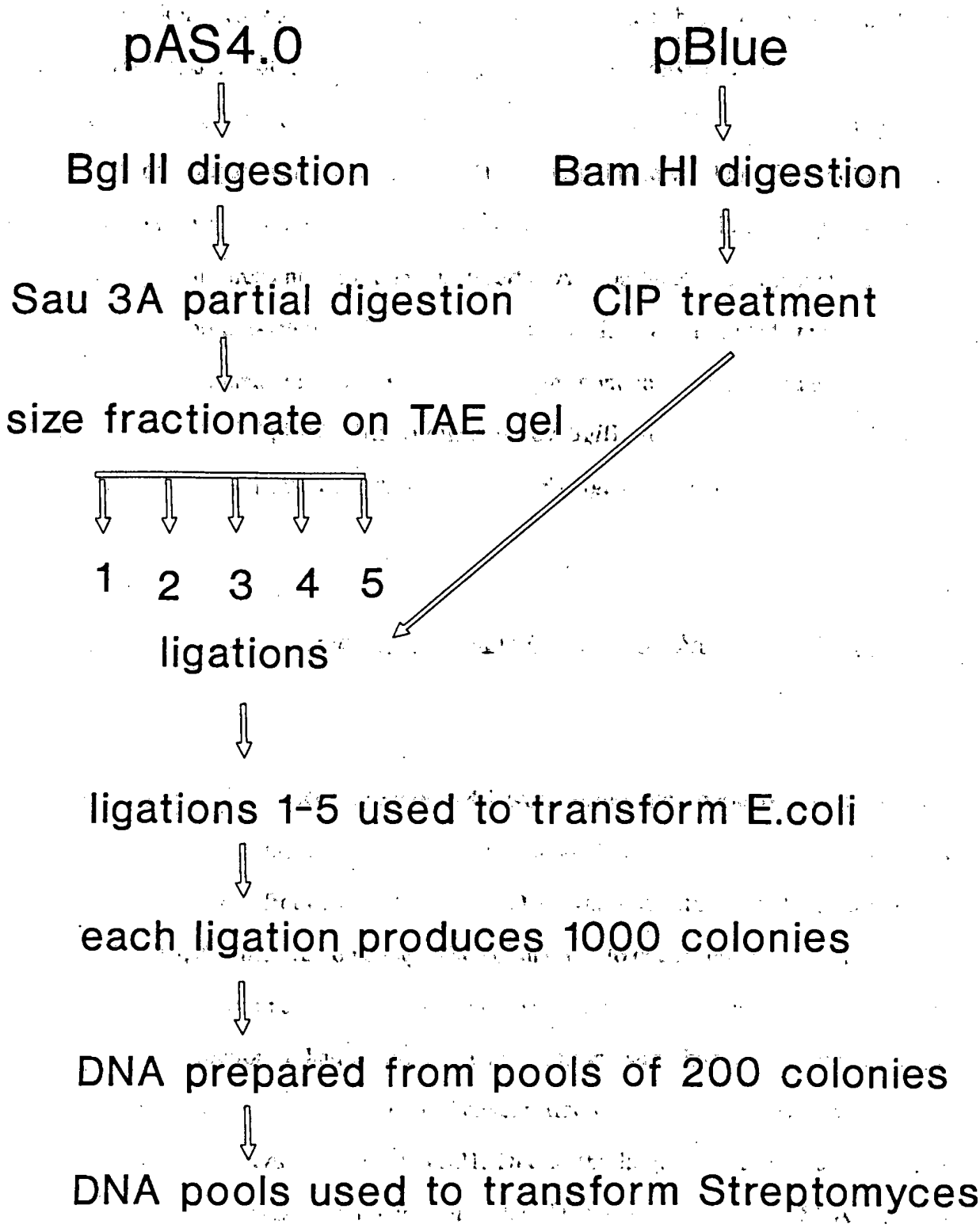

Figure 4.2 Diagrammatic representation of the construction of a *Sau* 3A partial library of pAS4.0 in pBlue and of the screening of this library in *Streptomyces lividans* 66 TK21.

each fraction was then ligated into pBlue which had been cut with *Bam*HI and Dephosphorylated, producing ligation mixes 1-5. The mixes which all contained an excess of vector DNA, were used to transform *E.coli* LK111. The transformants were selected on streptomycin, X-Gal and IPTG, approximately 1000 white colonies were isolated for each ligation mix. Since the vector had been completely Dephosphorylated these were all presumed to contain inserts. As the vector component of pAS4.0 constituted approximately 2/5 of the construct, it was assumed that 3/5 of the transformants contained partials of the origin containing *Bgl*II fragment and 2/5 contained partials of pLR2. Due to digestion by *Bgl*II all transformants contained only partial fragments of pLR2 or partials of the pSPN1 *Bgl*II fragment.

4.4.2 Transformation of *Streptomyces lividans* 66 TK21 with *Sau*3A partials of the pSPN1 origin

The transformants obtained in section 4.4.1 were formed into pools of 200 colonies and DNA prepared from them by the miniprep method. This gave five DNA pools for each size fraction isolated from the TAE gel. The DNA was cut with *Hin*dIII which linearized the constructs, and then run on an agarose gel in order to determine the size range of each pool. The pools for a particular fraction all gave a similar size range. As expected, pools formed from fraction 1 had a larger mean construct size than those from fraction 5 although there was some size overlap between fractions. The DNA pools were used to transform *Streptomyces lividans* 66 TK21. Due to the large number of transformations, each transformation was plated onto four plates. Two were selected with SNA containing 300µg/ml streptomycin and two with SNA containing 500µg/ml. Control transformations using pFull, pBlue and no DNA showed that selection with 300µg/ml gave approximately 50 colonies for pFull and two to three colonies when no DNA or pBlue was used. The DNA pools yielded between 0 and 19 colonies per plate, 16 colonies in

total were selected from pools that showed above average numbers of colonies. These were plated to malt3 containing 50µg/ml streptomycin. Only four grew, the others being nontransformants that had escaped selection . The four surviving colonies were grown up in YEME and the DNA was extracted as in section 2.3.3. No plasmid DNA was however seen in the DNA isolates and it was presumed that these were spontaneous streptomycin resistant mutants. The transformation experiment was repeated twice more, but no plasmid DNA was isolated in either case. Section 4.5 discusses why the probability of isolating a replicating construct is extremely low and the reasons why it was thus decided to abandon this approach.

4.4.3 Construction of restriction enzyme deletion derivatives of pFull

In order to identify the minimal replicon for sequencing or use in cloning vectors, various restriction enzyme deletions of pFull were constructed and their ability to replicate in *Streptomyces lividans* 66 TK21 was tested. The construct pFull was mapped with the restriction enzymes *Sph*I, *Pst*I, *Bam*HI, *Xho*I and *Kpn*I (Figure 4.1), the enzymes *Sma*I, *Sac*I and *Pvu*II were found to cut numerous times and it was not feasible to map all the sites for these restriction enzymes. As there is a unique *Bam*HI site in the center of the insert contained in pFull, it was decided to use this site to divide the 12.5 kb insert into two halves. The origin-containing pSPN1 insert of pFull was excised using *Xba*I and *Hin*dIII, and was cut in half using *Bam*HI. The fragments were then blunt-ended and using a 1:1 molar ratio of insert to vector, they were ligated into pBlue which had been cut with *Bam*HI and blunt-ended. The ligation mix was used to transform *E.coli* LK111. Transformants were selected on plates containing streptomycin and X-Gal and transformant DNA was prepared by the miniprep method. The DNA was digested with *Xba*I, *Hin*dIII and *Xho*I, allowing constructs containing the two fragments to be identified as they are of similar size. Only one orientation of each fragment was selected. After

confirming by a Southern blot that the insert was indeed part of pSPN1, these constructs were then isolated by the maxiprep procedure. The constructs were designated pAS4.1 and pAS4.2 (see figure 4.4), they were both in the same orientation with respect to pBlue as they are in pFull. The DNA was then used to transform *Streptomyces lividans* 66 TK21, but neither construct was able to replicate. It can thus be concluded that the minimal replicon overlaps the *Bam*HI site.

Construct pAS4.3 was made by digesting pFull with *Kpn*I, removing the enzyme using a phenol:chloroform extraction and religating the plasmid in a dilute ligation mix, so as to produce a *Kpn*I deletion of pFull. The same technique was used to produce pAS4.4 a *Xho*I deletion of pFull. Digestion of pFull with *Sph*I followed by TAE agarose gel electrophoresis and electroelution was used to purify the 5.3kb *Sph*I fragment that contains the unique *Bam*HI site which lies within the minimal replicon. This fragment was then blunt-ended and ligated into Dephosphorylated pBlue+ that had been cut then blunt-ended at its *Bam*HI site. This construct was designated pAS4.5. The same methodology was used in isolating the 3.3kb *Sph*I/*Xho*I fragment that was used to produce construct pAS4.6, again cloned into Dephosphorylated pBlue that had been blunt-ended at its *Bam*HI site. All these constructs were first isolated by the miniprep procedure, then they were analyzed by restriction analysis (Figure 4.3) and Southern blots were performed in order to determine that they did in fact contain the correct pSPN1 fragments.

4.4.4 Replication ability of pFull deletion derivatives

DNA from the deletion derivatives was isolated by the maxiprep method and it was used to transform *Streptomyces lividans* 66 TK21 as described in section 3.4.3. Presumed transformants were grown up under selection on malt3

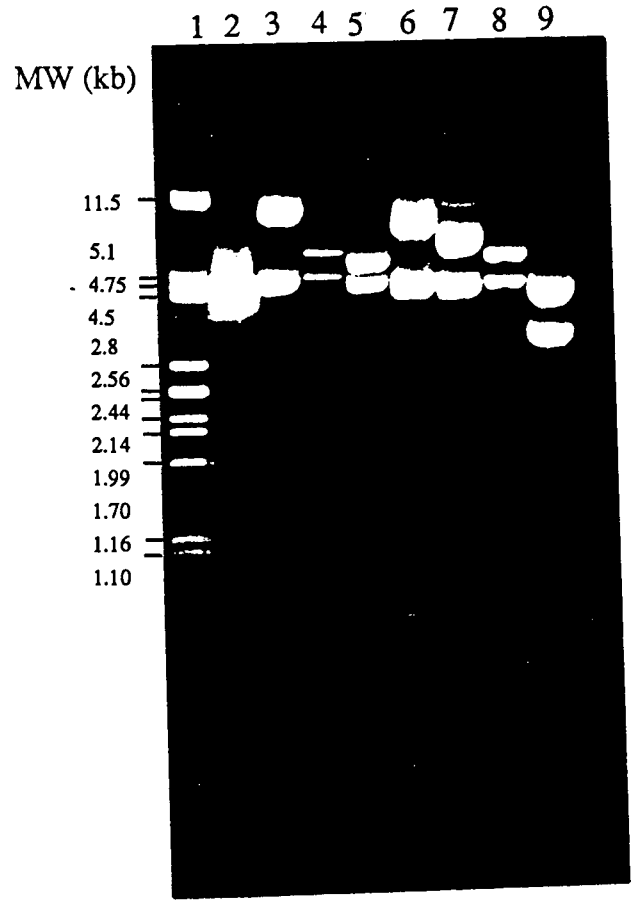

Figure 4.3 Gel showing deletions of pFull. Lane one contains a *Pst*I digest of phage Lambda. Lanes 2-9 contain various plasmids digested with *Xba*I and *Hin* dIII so that they are separated into insert and vector (pBlue). Lane 2 contains pBlue; lane 3, pFull; lane 4, pAS4.1; lane 5, pAS4.2; lane 6, pAS4.3; lane 7, pAS4.4; lane 8, pAS4.5 and lane 9, pAS4.6.

containing 50μg/ml streptomycin and the spores were used to inoculate flasks containing YEME. DNA was extracted from the cultures as described in section 2.3.3. Colonies containing plasmid were only isolated when pAS4.3 was used to transform the protoplasts, all other constructs were unable to replicate. Figure 4.4 shows that the minimal replicon of pSPN1 occupies a region greater than the 5.5kb of the *Sph*I fragment in pAS4.5 but less than the 9.3kb of the *KpnI/BglII* fragment in pAS4.3.

4.5 Discussion

The *Sau*3A partials of the pSPN1 origin were fractionated so that instead of screening all partials that transformed *Streptomyces lividans* 66 TK21 to streptomycin resistance, it was only necessary to screen those in the fraction containing the smallest partial size range. This enabled more colonies to be screened for plasmid content, a necessary procedure in view of the background level of non transformed colonies.

In retrospect the use of partials to isolate the smallest functional origin containing fragment was unwise. Due to the low rate of transformation obtained when *E.coli* DNA was used to transform *Streptomyces* species, DNA isolated by the miniprep procedure was only just concentrated enough to produce enough transformants to be visible against the background. In the pooled DNA, the concentration of a particular DNA species was 200 times less and thus was unlikely to survive the *Streptomyces* restriction system and be visible in the background. There are two approaches to overcome this problem, the partials can be individually transformed into *Streptomyces lividans* 66 TK21 or DNA from the pools can be prepared by the maxiprep procedure. Individual transformations using DNA prepared by the miniprep procedure, would require screening the 1000 colonies of each of the five fractions to isolate different partials and to ensure that the constructs do contain pSPN1

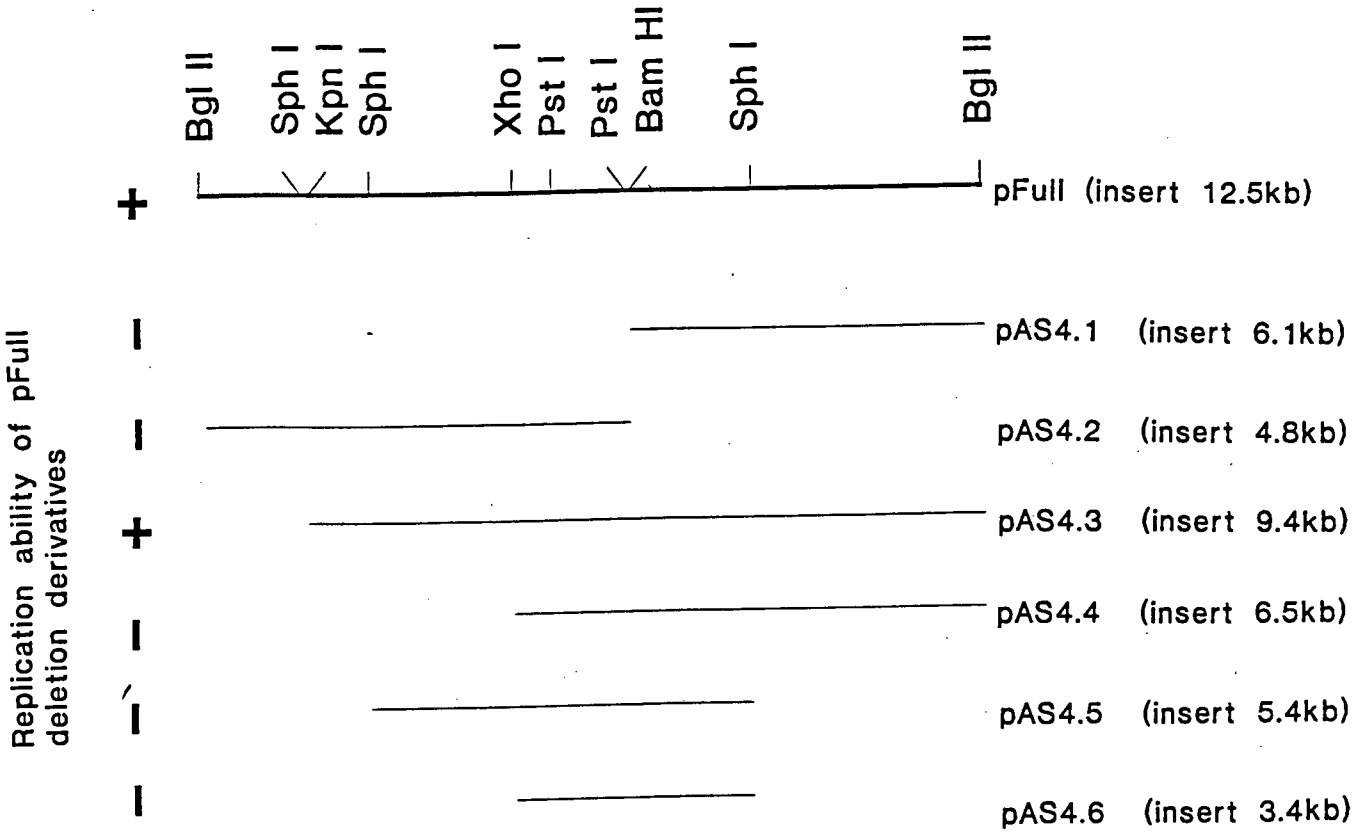

Figure 4.4 Deletion derivatives of pFull and their ability to replicate in *Streptomyces lividans* 66 TK21.

DNA. The number of different possible partials is enormous, this would then require hundreds of transformations with DNA of low concentration, this would be very time consuming. Preparing the pooled DNA by the maxiprep method is expensive as there are 25 different pools, five for each fraction, this approach also does not guaranty that the concentration of all DNA species will be sufficiently high for it to escape restriction in *Streptomyces*. Both methods rely on numerous transformations and as transformations were the weak point in the system used here, this did not assure a clearly discernable result.

After determining in section 4.4.4 that the minimal replicon of pSPN1 was greater than 5.5kb, it was apparent this method of partial library construction would never have isolated a functional origin of replication. The fractions selected from the gel were all less than 5.2kb and no fragment this small could contain the minimal replicon of pSPN1. The choice of upper cut off limit in selecting the partial sizes was based on the wrong assumption that the minimal replicon was at biggest 5kb. The limit of 5kb was based on the known minimal replicon sizes for *Streptomyces* plasmids, these are 2.2kb for pTA4001, 3.3kb for pVE1, 2.9kb for pSK2, 3.8 for pSK1, 4.0kb for pNM100, 2.9kb for pFJ103 and 5.9kb for SCP2* (Hopwood *et al.*, 1986). A point of interest is that both pSPN1 and SCP2* are similar in size and have similar sized minimal replicons, they however show no similarity of restriction pattern.

The delimitation of the region of pSPN1 necessary for replication is incomplete, in Figure 4.3 it can be seen that the right border of the minimal replicon lies between the *Kpn*I and the *Sph*I site to the left of it. The left border lies somewhere between the edge of the pBlue cloning cassette and the leftmost *Sph*I site. The left border cannot easily be defined more precisely as all the restriction sites that could be used to delete fragments from this side are also to be found in other parts of the DNA which have been shown to be essential for replication. Deletion of DNA by Exonuclease III digestion is still not

possible for the left border as no unique 3' site is available to protect the vector. With the deletion of the *Kpn*I fragment it is now possible to perform Exonuclease III digestion on the right border of pAS4.3 but this is pointless until it is possible to do the same to the left border.

CHAPTER 5

GENERAL CONCLUSIONS

With the exception of pIJ101, little is known about the replication of *Streptomyces* plasmids. The great commercial significance of *Streptomyces* makes it essential to understand the functioning of these elements as they are the basis of most genetic manipulation. In order to study the replication origin of pSPN1 in greater detail, it was decided to reduce the origin to it's minimal replicon. Once the minimal replicon was isolated, it could be used as the basis of a vector or sequenced for study in greater detail. Although the isolation of a minimal replicon may sound simple, it should be noted that *Streptomyces* genetic techniques are not as simple those for *E.coli*, this is due to the complexity of *Streptomyces* physiology and growth. This study has three main areas of interest, the plasmid pSPN1 as a whole, the construction of a vector for the study of *Streptomyces* origins and the delimitation of the origin of pSPN1.

From this study it was shown that pSPN1 is most probably not involved in thienamycin production, and as it shows no easily discernable phenotype, it can be said to be cryptic. The lack of homology to *Streptomyces fulvoviridis* shown by the plasmid is interesting when chromosomal DNA from *Streptomyces penemafaciens* is known to show homology. This difference in homology may indicate that *Streptomyces penemafaciens* gained pSPN1 by horizontal transfer after it diverged from *Streptomyces fulvoviridis*. Another interpretation of the data may be that the similarity between *Streptomyces penemafaciens* and *Streptomyces fulvoviridis* was only due to their penem-antibiotic pathways and that they are otherwise unrelated. Only phylogenetic studies based on sequence similarities will clarify this situation.

The use of shuttle vectors in the study of Streptomycetes is common as it allows genetic manipulation in easy to handle species such as *E.coli* and testing of the altered construct in *Streptomyces*. As no readily available vector existed for the study of the pSPN1 origin, a modified form of Bluescript was constructed and it proved ideal, except for two points. The streptomycin resistance gene functions adequately except when used in conjunction with a very low copy number origin. This compounded by a low but noticeable rate of mutation to streptomycin resistance made this study extremely difficult as the accurate selection of transformants was always doubtful. The lack of reliable selection made the routine job of transforming protoplasts a major step in each experiment. The second disadvantage of this system was the high G+C ratio of the restriction sites in the pBlue cloning cassette, as has been seen in this study these sites are often common in *Streptomyces* DNA. This system may still prove very useful for the exact delimitation of high copy number *Streptomyces* origins as it enables origins to be tested and shortened without subcloning. The system could also be used for insertional inactivation but, the low copy number of chromosomally inserted constructs may render then difficult to select for.

The pSPN1 origin occupies a large region of DNA and assuming that it has the same copy number as its derivative pFull, it has a very low copy number. The large amount of DNA required for replication has interesting implications. The region may contain *kil/kor* genes similar to those seen in pIJ101, in this case deletions made in this study may have either destroyed the minimal replicon or merely removed a controlling *kor* gene and thus made the construct lethal. Lethal constructs would appear nonreplicating although they could in fact replicate, thus the true minimal replicon may be smaller than proposed here. Most Gram positive plasmids replicate by the rolling circle mode of replication and thus have only a very small amount DNA that is essential. Plasmids that replicate by the theta mode of replication require more plasmid specific proteins and thus have a larger minimal replicon. This may be the case for pSPN1 as theta type replication is favoured

by large plasmids, if this is true for *Streptomyces* plasmids is still unknown. Plasmids that replicate by the theta mode are more stable as they do not go through a single stranded intermediate, this stability is manifested by the ability of the origin to carry a large amount of nonessential DNA. pSPN1 could serve as the basis of a vector capable of holding large amounts of DNA, its low copy number would render it suitable for genes that are lethal in high copy number.

This study has brought to light an novel *Streptomyces* plasmid that may help broaden the understanding of *Streptomyces* plasmids. It is hoped that further studies will reveal more about this interesting plasmid.

APPENDIX A

Antibiotic Stock concentrations

ampicillin	100 µg/ml in H_2O
streptomycin	100 µg/ml in H_2O
thiostrepton	50 µg/ml in DMSO
bavastin	500 g/l in H_2O

Antibiotic Working concentrations

ampicillin	100 mg/ml all solid and liquid media
streptomycin	30 mg/ml for all *E.coli* media
streptomycin	for *Streptomyces* see Chapter 3
thiostrepton	500 mg/ml in SNA overlays
thiostrepton	50 mg/ml in malt3
bavastin	100 mg/ml in malt3, R2YE and 1/2SRM

The Media should be cooled to below 55°C before adding the antibiotic stock solutions.

Luria broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
	H_2O to 1000 ml

Luria agar

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
agar	15 g
	H_2O to 1000 ml

ISP 2 Medium

Yeast extract	4 g
Bacto Malt extract	10 g
Dextrose	4 g
	H_2O to 1000 ml
	adjust pH to 7.3
	add 20g Agar

1/2 SRM Medium

Sucrose	102.50 g
K_2S0_4	0.25 g
$MgCl_2.6H_20$	10.12 g
Glucose	10.00 g
Casamino acids	0.10 g
Bacto Oatmeal Agar	3.00 g
Trace element solution	2.00 g
L-Proline	3.00 g
Yeast extract	2.00 g

MES Buffer	19.52 g
	H_2O to 1000 ml
	adjust pH to 6.1
	add 20g agar
post sterile additions:	10 ml 2M $CaCl_2.2H_2O$
	1 ml 5% KH_2PO_4

Trace Element solution

$ZnCl_2$	40 mg
$FeCl_3.6H_2O$	200 mg
$CuCl_2.2H_2O$	10 mg
$MnCl_2.4H_2O$	10 mg
$Na_2B_4O_7.10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg
	add H_2O to 1000 ml

malt3 medium

Difco Malt extract	21.6 g
Yeast extract	4.5 g
0.5M Na_2HPO_4	15.0 ml
4.0M NaH_2PO_4	3.3 ml
	H_2O to 1000 ml

adjust pH to 6.5 with 0.5M

Na_2HPO_4 and 4.0M NaH_2PO_4

R2YE medium

Sucrose	206.00 g
K_2SO_4	0.50 g
$MgCl_2.6H_2O$	20.24 g
Glucose	20.00 g
Casamino acids	0.20 g
	H_2O to 1600 ml

divide into two 800 ml aliquots

to each aliquot add 22g Oxoid number 1 agar

post sterile additions to each 800 ml aliquot:

0.50% KH_2PO_4	10 ml
3.68% $CaCl_2.2H_2O$	80 ml
20.00% L-Proline	15 ml
5.73% TES buffer pH 7.2	100 ml
Trace element solution	2 ml
1M NaOH	5 ml
10.00% Yeast extract	50 ml

SNA Medium

Difco nutrient broth powder	8 g

Oxoid number one agar	3 g
	H_2O to 1000 ml

YEME medium

Yeast extract	3 g
Bacto Peptone	5 g
Bacto Malt extract	3 g
Glucose	10 g
Sucrose	340 g
	H_2O to 1000 ml

post sterile additions:

$MgCl_2.6H_2O$	2ml/l
20% Glycine	25ml/l

X-Gal plates

50µl of 2% X-Gal in DMSO and 25µl of 100mM IPTG are spread on each plate, the plate is then allowed to dry for at least 2 hours before use.

APPENDIX B

Solution I

Glucose	50mM
Tris	25mM
EDTA	10mM

Solution II

NaOH	0.2M
SDS	1%

Solution III

5M CH_3COOK	60.0 ml
Glacial Acetic Acid	11.5 ml
H_2O	28.5 ml

Pronase solution

Dissolve 10mg/ml Pronase in H_2O and then incubate at 30°C for 10 min to predigest. Store as 1ml aliquots at -20°C.

Equilibrated Phenol

Melt fresh phenol at 60°C, then add 1/3 volume TE buffer and shake, allow phases to separate and remove the TE, repeat till TE has a pH of about 7.8 then add 0.1% 8-Hydroxyquinolene. Store at -20°C in a light proof bottle. To make Phenol:Chloroform:Isoamyl alcohol add 24 parts Chloroform and 1 part Isoamyl alcohol to 25 parts equilibrated Phenol, mix and store at -20°C.

RNase A solution

RNase A	10 mg
TE buffer	1 ml

The solution was heated to 100°C for 15min and then stored at -20°C.

Stop Buffer

Bromophenol blue	0.25%
Ficoll type 400	25%
EDTA	60mM

TBE buffer

Tris	89mM
Borate	89mM
EDTA	2mM

TE buffer pH8.0

Tris	10mM
EDTA	10mM
	adjust pH to 8.0

5X Ligation Buffer

1M Tris pH 7.6	2.5 ml
1M $MgCl_2$	0.5 ml
DTT	0.0077 g
ATP	0.0302 g
PEG 6000	2.5 g
	H_2O to 10ml

Filter sterilize and store at -70°C in 500µl aliquots.

50X TAE buffer

Tris-HCl	242.0 g
Glacial acetic acid	57.1 ml
0.5M EDTA pH 8.0	100.0 ml
	H_2O to 1000 ml

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