Horizontal Gene Transfer and Evolution of Antibiotic Gene Clusters

By

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I dedicate this work to my mother Lina, my brother Peter and especially to my father George who always taught me the importance of being educated.

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Chapter 3 Phylogeny of *Streptomyces* spp.

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SUMMARY

Genome sequencing projects from diverse bacteria and the release of genetically engineered microorganisms make the topic of horizontal gene transfer (HGT) fashionable. Sequence analysis indicates the high frequency and the importance of horizontal gene transfer to the microbial evolution. Much knowledge about horizontal transfer has been derived from studies using soil microcosms, fluorescent-marked donors and recipients and from study of the increase of the antibiotic resistance as a result of use and often abuse of antibiotics. However, little is known about the transfer of chromosomally-located antibiotic biosynthesis genes in natural populations. The streptomycin pathway-specific regulator, *strR*, has been found in a set of diverse streptomycetes both phenotypically and genetically that previously have already been described to carry the resistance (*strA*) and one biosynthetic (*strB1*) gene from the same cluster. Phylogenetic analysis of both 16S rRNA and *trpB* gene fragments showed that two isolates were closely related to *S. coelicolor* which are known not to produce streptomycin or to contain any biosynthesis or resistance genes. The remaining pair did appear to be closely related to each other, particularly from the *trpB* analysis. *trpB* gene proved to be useful for resolving phylogenetic relationships between strains with highly conserved gamma region of the 16S rRNA gene. However, one of these strains is the only one from the *S. griseus* distant isolates that produced streptomycin and possessed many other genes from the cluster. The phylogenetic incongruency between 'species' tree and 'gene' tree can be attributed to horizontal gene transfer. The sequence identity of the detected genes is extremely high (99%) indicating a recent transfer event. Besides the physical proximity of *strRAB1* genes, there is also a well-characterised functional correlation of these genes in the streptomycin producer, *S. griseus*. However, *strRA* genes are silent in *coelicolor*-like isolates ASSF15 and ASB37. Therefore, this transfer may have other ecological role than a simple resistance phenotype such as the evolution of antibiotic cluster. It was not possible to prove the integration site of *strRAB1* genes within ASB37 genome but a possible site was identified.

Pathway-specific regulator StrR activates the transcription of *strB1* and other genes from the streptomycin cluster by binding to the upstream promoter regions. This protein binds as a tetramer. N-terminal deleted StrR derivatives were still able to bind as pseudodimers (two monomers). Two putative domains in the N'-terminus of the protein responsible for the protein dimerisation during the binding process have been identified.

ABBREVIATIONS

aa	Amino acid
aph	Aminoglycoside phosphotransferase
ATCC	American Type Culture Collection
°C	Degrees Celsius
CCC	covalently closed, circular
DMSO	Di-methyl sulphoxide
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
E.	*Escherichia*
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
EtOH	Ethanol
g	1 x force of gravity
G + C	Guanine and cytosine
g / l	gram / litre
HGT	Horizontal gene transfer
H-T-H	Helix-turn-helix
hur	hydroxyurea phosphotransferase gene
kb	kilo-base pairs
LB	Luria broth
Log	Logarithm (Base 10)

µg	Micrograms
µm	Micrometres
µg/g	Micrograms per gram
µg/ml	Micrograms per millilitre
mg	Milligram
mg/g	Milligram per gram
mg/ml	Milligram per millilitre
min	Minutes
ml	Millilitre
mol	Mole
ng	Nanogram
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
SARC	Streptomycin and related compounds
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
Sm	Streptomycin
SMA	Soya mannitol agar
spp.	Species

strA	Streptomycin-6-phosphotransferase gene
strB1	Amidinotransferase gene
strR	Streptomycin pathway-specific regulator
stsC	Pyridoxal phosphate dependent aminotransferase
strS	Pyridoxal phosphate dependent aminotransferase
TAE	TRIS-acetate EDTA
TBE	TRIS-borate EDTA
TE	TRIS-EDTA
TRIS	Tris (hydroxymethyl) aminomethane
TSB	Tryptone soya broth
UPGMA	Unweighted pair-group arithmetic average clustering
UV	Ultraviolet
V	Volts
v / v	Volume / volume
w / v	weight / volume

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DECLARATION

This thesis has been completed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself. Phylogenetic tree construction presented in Chapter 3 was undertaken in collaboration with Dr. Pam Wiener and Dr. Sharon Egan. All sources of information have been acknowledged by means of reference.

Dimitris Kallifidas

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PUBLICATIONS AND PRESENTATIONS

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Chapter 1

Introduction

1.1 *Streptomyces*

1.1.1 Morphology and Phylogeny

Streptomycetes are a group of aerobic Gram-positive bacteria belonging to the class Actinobacteria with complex colony morphology. The DNA composition of this family falls within a relatively narrow range of 63 to 78 percent GC. Species of *Streptomyces* produce filaments which are usually 0.5 to 1.0 μm in diameter and of indefinite length, often lacking cross walls in the vegetative phase. The group also develop a true mycelium but their mycelium does not fragment into bacillary or cocoid forms. Instead growth occurs at the tips of filaments and is often accompanied by branching so that the vegetative phase consists of a complex tightly woven matrix, resulting in a compact convoluted colony. As the colony ages, characteristic aerial filaments called sporophores are formed, which project above the surface of the colony and give rise to spores (conidia) (Wildermuth and Hopwood, 1970). Since the latter are more resistant to desiccation than hyphal fragments streptomycete spores seem to play a role as dispersal agents rather than resting agents (Hodgson, 1992). The spores and sporophores are often pigmented and contribute a characteristic colour to the mature colony; in addition pigments sometimes are produced by the substrate mycelium and contribute to the final colour of the colony. The dusty appearance of the mature colony, its compact nature and its colour make detection of streptomycete colonies on agar plates relatively easy.

Although a few streptomycetes can be found in aquatic habitats, they are primarily soil organisms. In fact, the characteristic earthy odour of soil is caused by the production of series of streptomycete metabolites called geosmins. Alkaline and neutral soils are more favourable for the development of *Streptomyces* species than are acid soils. Higher number of *Streptomyces* are usually found in well-drained soils such as sandy loams or soils covering limestone.

Streptomycetes are usually saprophytic bacteria. Their saprophytic lifestyle is made possible by their ability to degrade many kinds of organic molecules due to production of extremely large and diverse range of hydrolytic enzymes, including

proteases, nucleases, lipases and enzymes hydrolysing polysaccharides (Williams *et al.*, 1983a). Other streptomycetes are able to produce parasitic or symbiotic relationships with plants and animals. Plant pathogenic streptomycetes have been known for a very long time.

The *Streptomyces* genus is represented by a large number of species and subspecies. The ability of *Streptomyces* to produce many valuable bioactive compounds led to taxonomic confusion. Producers of novel natural products were described as new species and patented. However, many of these strains were synonyms resulting in the over-classification of members of this genus. In 1964 the International Streptomyces Project (ISP) was initiated to solve this problem. Shirling and Gottlieb (1968a, 1968b) described standard tests that were used for the identification and classification studies. Such tests involve spore chain morphology, spore surface ornamentation, spore colour, substrate mycelium and soluble pigments, production of melanin pigment and the utilisation of a range of carbon sources. This led to the re-analysis of many isolates and designation of type strains which were subsequently deposited into culture collections. Williams *et al.* (1983b) incorporated these tests in a numerical taxonomic study. A probablistic data matrix for the identification of *Streptomyces* was constructed using information from the numerical taxonomic database (Williams *et al.*, 1983a).

Williams *et al.* (1983b) subdivided the genus *Streptomyces* into species groups. The larger one is *S. albidoflavus* with 76 strains. This group can be subdivided into three other clusters namely *S.albidoflavus* subsp. *albidoflavus, S. albidoflavus* subsp. *anulatus* and *S. albidoflavus* subsp. *halstedlii*. Although the number of described streptomycetes was significantly reduced, the numerical taxonomy approach could not resolve the overspeciation problem completely. Additional chemotaxonomic and molecular methods are now used in order to refine the species relatedness within the *Streptomyces* genus.

One approach is the analysis of the patterns derived from metabolic products of the organisms such as fatty acids and proteins. Fatty acid analysis has been used to distinguish between bacterial genera (Goodfellow, 1989). Although fatty acid profiles were used to assign unknown streptomycetes to established taxa (Saddler *et al.*, 1987), it is rather unreliable method for species identification within the genus *Streptomyces* and requires standardised conditions. In contrast, protein pattern profiles can be used for

genus to species taxonomic refinements. In a study for the elucidation of taxonomy of plant pathogenic streptomycete isolates, DNA-DNA hybridisation showed similar results with protein profiles (Paradis *et al.*, 1994). Whole cell analysis that involves non-oxidative thermal degradation of cells resulting in a fingerprint for the organism requires stringent standardisation for reproducibility. While biochemical tests showed some doubtful results (Goodfellow *et al.*, 1987), the use of antisera showed genus to species specificity (Kirby and Rybick, 1986). In fact, monoclonal antibodies show more reproducible results although the poor nutrient status of soil may inhibit the expression of their binding substrates (antigens) in the studied streptomycetes. Actinophages can be used for host-identification at the genus and the species level (Wellington and Williams, 1981). In general, streptomycete phages are genus specific (Korn-Wendisch and Schneider, 1992), although some cross-reactivity has also been detected with other genera, including *Nocardia, Streptosporangium* and *Mycobacterium* (Bradley *et al.*, 1961).

New advances in DNA technology have contributed considerably to bacterial taxonomy (Anderson and Wellington, 2001). DNA-DNA hybridisations of total chromosomal DNA have been used to determine species identity within streptomycetes. DNA fingerprints can be provided by restriction of the genome with infrequently cutting endonucleases, a technique called low-frequency restriction fragment analysis (LFRFA). The combination of these whole genome-oriented methods can determine relatedness from genus to strain. However, genome instability (amplifications, deletions) is an obstacle to the effectiveness of this approach.

The comparison of rRNA sequences is a particularly powerful tool in streptomycete taxonomy. rRNA sequence comparisons have also been useful for answering questions concerning the horizontal transfer of genes within the genus (Wiener *et al.*, 1998). These genes are highly conserved within bacteria but have enough sequence variations to allow genus (α and β regions) and species (γ regions) differentation. Genes that are conserved between species, such as housekeeping genes, are additional targets for taxonomy studies. In two studies (Wiener *et al.*, 1998; Egan *et al.*, 2001), tryptophan synthase genes were used in addition to 16S rRNA comparisons to refine interspecific relationships between streptomycete soil isolates and to provide

evidence for the gene flow of antibiotic resistance and biosynthetic genes in natural populations.

1.1.2 Antibiotic production

Perhaps the most striking property of the streptomycetes is the ability to produce a large number and wide variety of secondary metabolites, many of which have important applications in human medicines and in agriculture as antibiotics or as compounds with other useful biological properties. It has been reported that *Streptomyces* species produced almost half the total number of antibiotics synthesised by all the other organisms (Goodfellow and O'Donnell, 1993). Some organisms produce more than one antibiotic and often the several kinds produced by one organism are not even chemically related. Similar antibiotics may also be formed by different species found in widely scattered parts of the world.

Antibiotics can be classified into major chemical families including aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines. Antibiotics are not essential for growth and tend to be strain specific (Hotta *et al.*, 1996). They have a wide range of chemical structures and biological activities and are derived by unique biosynthetic pathways from primary intermediates and metabolites. These pathways are often long and complex; the reactions are catalysed by enzymes with substrate specificities different from those of primary metabolism. The formation of secondary metabolites is directed by optimised sets of genes associated with genes for resistance mechanisms, regulators, transport and extracellular processing functions (Piepersberg, 1994). This molecular network seems to control both the timing and the level of gene expression. Finally, the control mechanisms are well integrated with the physiology of the producing organisms (Vining, 1992a).

Much of the information about the molecular analysis of antibiotic production is drawn from two model species. *S. griseus*, the producer of streptomycin, is a successful soil coloniser and can be isolated from a wide range of soils. *S. coelicolor* A3(2), is the most well studied genetically (Hopwood *et al.*, 1995) and produced at least four

antibiotics namely actinorhodin, undecylprodigiosin, calcium-dependent antibiotic, and methylenomycin A. The latter is plasmid specified.

1.2 Global regulation of antibiotic production

Streptomyces produce antibiotics in a growth phase-dependent manner. Production occurs at the end of exponential phase or at low growth rates and is coordinately regulated with morphological differentiation. There also seems to be a more general and innate 'clock-like' mechanism for the induction of antibiotic production and sporulation (Neumann *et al.*, 1996). Thus, *S. griseus* shows biphasic kinetics during exponential phase. Early in growth there is a 'decision phase' under the influence of effector molecules, during which the decision is made whether or not to produce streptomycin. If the decision is 'yes' the pathway specific activator gene is expressed and commitment to streptomycin production is no longer easily reversible by external signals. The differentiation cycle of *S. griseus* is completed on entering stationary phase, during which streptomycin and spores are produced (Neumann *et al.*, 1996). In this phase, *S. griseus* also regains the decision-making state by recovering sensitivity to effector molecules. The extraordinary phenomenon of decision-phase regulation is probably widespread among streptomycetes (Holt *et al.*, 1992; Blanco *et al.*, 1994), and not only limited to secondary metabolism.

In effect, nutrient depletion can cause antibiotic production. While there are many examples of metabolite interference with antibiotic synthesis, especially glucose, NH^+_4 and PO^-_4, the underlying mechanisms are generally unknown (Gräfe *et al.*, 1982; Demain, 1992). Even within one species, the production of different secondary metabolites appears to be triggered by the depletion of different nutrients.

Genetic regulation of antibiotic production can be found at the level of transcription, translation and post-translation employing diverse molecular mechanisms. Protein phosphorylation, and potentially phosphorylation cascades play a role in triggering antibiotic production; presumably AfsK, $AfsQ_2$, AbsA sense signals that cause

phosphorylation of their regulatory counterparts (AfsR, $AfsQ_1$ and the product of the gene cluster located downstream of *absA*) which can then stimulate transcription of the antibiotic biosynthetic pathways, perhaps via the pathway specific regulators (Horinouchi, 1993; Ishizuka *et al.*, 1992; Adamidis *et al.*, 1990). These sensors and regulators compose a two-component regulatory system.

bldA gene has been shown to specify the tRNA that translates the rare codon UUA as a leucine affecting in that way antibiotic synthesis at translational level. Such codons are absent from the essential vegetative growth genes but are distributed among antibiotic genes. This codon presence in phylogenetically distant organisms such as *S. griseus* and *S. coelicolor* indicates that the role of *bldA* in secondary metabolism and differentiation is widespread among streptomycetes (Distler *et al.*, 1987). *bldA* may regulate antibiotic production by allowing the translation of UUA codon-containing RNA late in growth only.

Antibiotic production is associated with reduced growth rate following amino acid limitation. One of the signals implicated is the increased ppGpp level. Many studies showed a positive correlation between ppGpp and the onset of secondary metabolism (Chakraburtty and Bibb, 1997; Ochi *et al.*, 1997) although this could be an indirect effect of impaired protein synthesis. However, it has been shown recently that an increased level of ppGpp under conditions of nutrient sufficiency could act as an activator of at least the pathway-specific regulator of actinorhodin in *S. coelicolor* A3(2) (Hesketh *et al.*, 2001) indicating a direct link between stringent response and antibiotic production. The final target of ppGpp is RNA polymerase (Chatterji *et al.*, 1998) allowing an effective interaction between RNA polymerase and the appropriate σ-factor for the selective transcription of antibiotic promoters.

The genome sequencing project of *S. coelicolor* A3(2) revealed an unexpectedly large number of σ-factors. In such a metabolically active organism, the presence of different σ-factors may be correlated with the recognition of a wide range of promoters following the appropriate environmental and physiological signals (Buttner, 1989). The use of a multiplicity of promoter structures and concomitant promoter-recognising sigma factors of RNA polymerase is therefore very important for the onset of antibiotic production (Aigle *et al.*, 2000).

Streptomycetes, like eukaryotes utilise cyclic AMP (cAMP) as an intracellular signal and secondary messenger to regulate growth and differentiation processes. Characterisation of a *S. coelicolor* M145 adenylate cyclase-deficient mutant has revealed that cAMP facilitates germination, aerial mycelium and antibiotic biosynthesis (Süsstrunk *et al.*, 1998). The fact that synthesis of polyketide antibiotic actinorhodin was induced by the concentration of cAMP (<20 μM) found in the medium suggested it may serve as a diffusible signalling molecule to co-ordinate antibiotic biosynthesis (Süsstrunk *et al.*, 1998). In contrast, high levels of cAMP in ArpA-deficient *S. griseus* mutant suppress the streptomycin production and aerial mycelium formation while it had no obvious phenotypic effect in the wild type strain (Kang *et al.*, 1999). These results may suggest a possible negative role of cAMP in the physiological and morphological differentiation in *S. griseus*.

Other diverse diffusible signalling molecules in prokaryotes are the γ-butyrolactones. The so-called 'autoregulators' are found in a wide variety of bacteria (Gräfe *et al.*, 1982). Even though in some cases they are considered as secondary metabolites themselves, they can trigger antibiotic production in several streptomycetes (Ohnishi *et al.*, 1999); in some cases they also play a role in morphological differentiation (Ueda *et al.*, 1998; Yamazaki *et al.*, 2000). The best-known example of a butyrolactone autoregulator is A-factor from *Streptomyces griseus* (Khokhlov *et al.*, 1967). A-factor homologues can be found *in S. virginiae* (virginiae butanolide; Yamada *et al.*, 1987), in *S. lavendulae* (IM-2; Kitani *et al.*, 1999), in *S. viridochromogenes* (I-factor; Gräfe *et al.*, 1982), *S. coelicolor* A3(2) (SCB1; Takano *et al.*, 2000) (Fig. 1.1). The extremely low effective concentration and remarkable pleiotropic effects of A-factor and A-factor homologues led to their recognition as microbial hormones. The extremely low concentrations of these molecules suggest involvement of specific binding proteins in the regulatory process (Miyake *et al.*, 1990; Kim *et al.*, 1989). The genetic organisation of the regulatory cascade is different depending on the diffusible molecule concerned. In *S. griseus* the key gene for A-factor biosynthesis, *afsA*, is not clustered with the rest of cascade (Horinouchi and Beppu, 1994) while in *S. virginiae* the VB and virginiamycin (VM) pathways are tightly linked. In *S. coelicolor* A3(2), the genes for the regulation and biosynthesis of at least one butyrolactone compound (SCB1) are found to be also

clustered (Takano *et al.*, 2001) and responsible for a γ-butyrolactone-mediated mechanism of antibiotic biosynthesis. This may reflect the divergent evolution of the physiological role of these autoregulators. Indeed, A-factor that is produced in a growth-dependent manner (Ando *et al.*, 1997a) controls at a very early stage common for both secondary metabolism and sporulation. In contrast, VB that is produced just before the antibiotic production (Kinoshita *et al.*, 1997) controls a downstream stage in the regulatory cascade only for secondary metabolism.

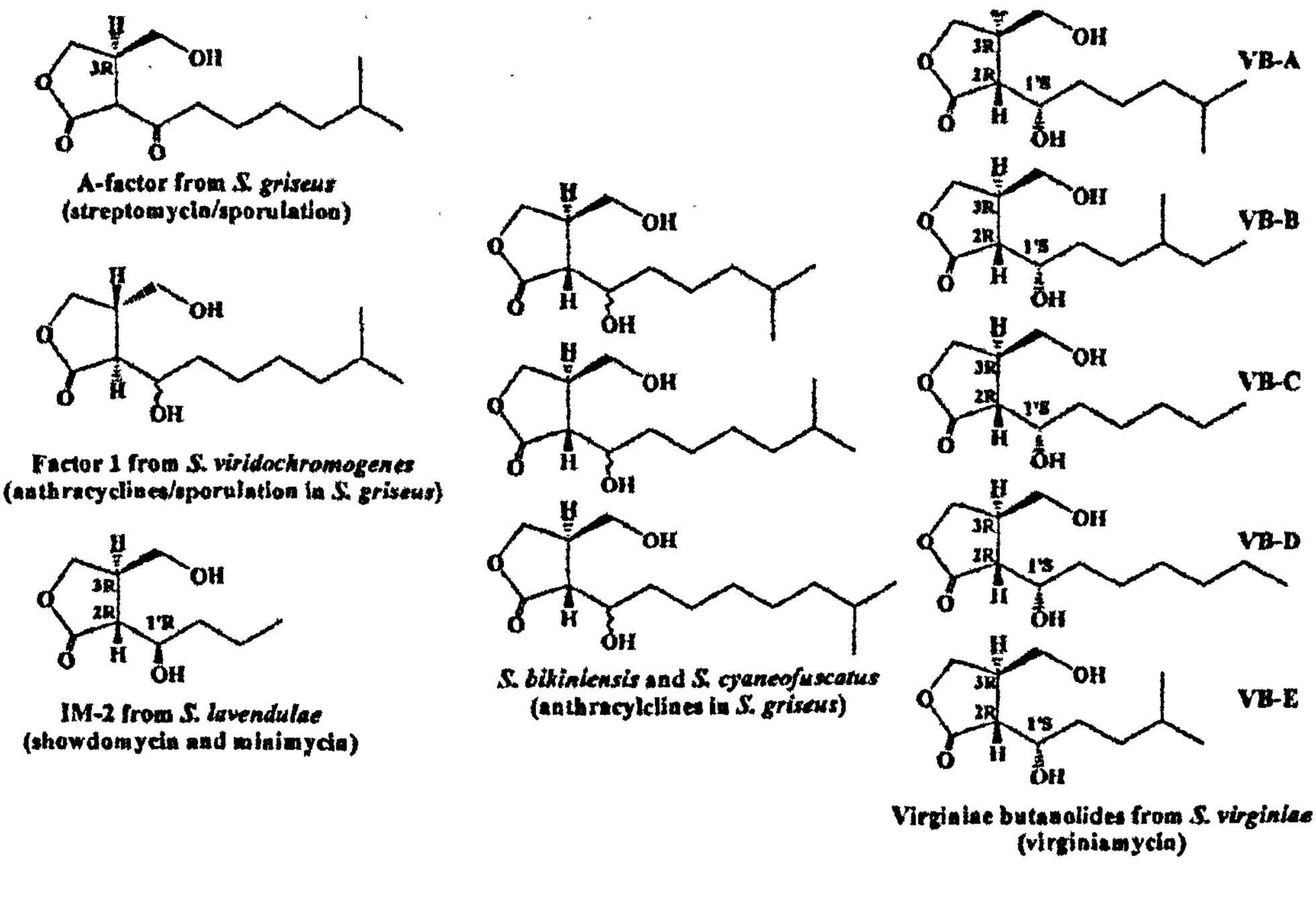


Figure 1.1: Autoregulators isolated from streptomycetes with γ-butyrolactone ring, and the antibiotics and the developmental stages induced by them. (Reproduced from Takano *et al.*, 2000).

Furthermore, A-factor receptor protein cannot bind to virginiae butanolides (Miyake *et al.*, 1989) implying strict ligand specificity and showing two distinct families of butyrolactones. This specificity is determined by the –hydroxyl (VB) or –keto (A-factor) group at the 6-position of the butyrolactone ring and the lengths of the acyl side chain (Fig. 1.2). The specific pair of an autoregulator and its binding protein in a given strain should function as an intraspecific potent system for discriminating different chemical signals from other species to prevent miscommunication among various actinomycetes species in the ecosystem (Horinouchi and Beppu, 1992).

On the other hand, an interspecific stimulation is also apparent as communication between different *Streptomyces* spp. by sharing a common autoregulator occurs. The extremely low effective concentrations of this group of autoregulators satisfy one of the prerequisites for this function, i.e. communication through the species barrier for symbiotic differentiation of various actinomycetes species in the ecosystem (Beppu, 1992). Further studies showed wide distribution of inter-species cross-talk mechanisms distinct from the hitherto known autoregulator systems in *Streptomyces* (Ueda *et al.*, 2000).

Biró *et al.* (1980) reported factor C from *S. griseus* which restored sporulation of a bald mutant of *S. griseus* and the corresponding gene (*farC*) in high copy number increased streptomycin production 20-40 fold. Southern hybridisation revealed the presence of *farC* in several other *Streptomyces* species (Birkó *et al.*, 1999).

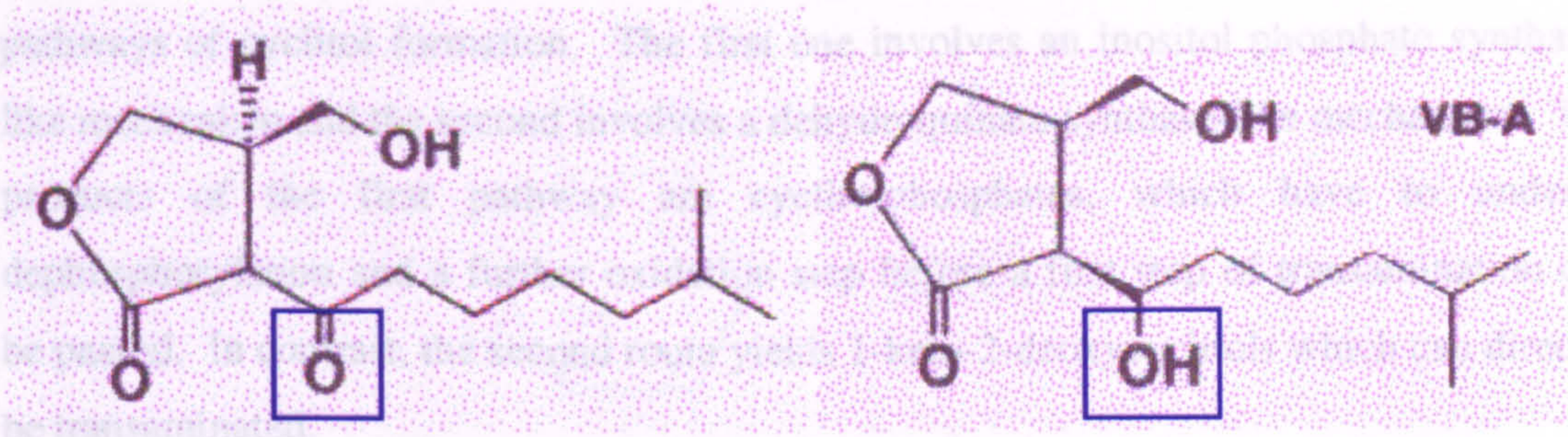


Figure 1.2: Two distinct butyrolactone families with different ligand specificity. A-factor family *(S. griseus*) has a ketone at the 6-position and the virginiae butanolide family (*S. virginiae*) has a hydroxyl group at the 6-position.

1.3 Streptomycin, an aminoglycoside antibiotic

Streptomycin (Sm) was discovered in 1944 by Selman Waksman and is produced by *Streptomyces griseus*. It was important because it inhibited many organisms resistant to sulphonamides and penicillin. Its antibacterial spectrum includes many Gram-negative species including some organisms in the salmonella group. It is inhibitory for several species of *Mycobacterium*, and was used to treat tuberculosis. Because of its irreversible side effects, mainly oto- and nephro-toxicity (Pratt and Fekety, 1986), it is now only used in agriculture to control plant pathogens of fruit trees (Sundin *et al*., 1995).

Streptomycin is soluble in water but is insoluble in ether, acetone and chloroform. It is stable in crystalline form when stored at 50°C and even in aqueous solution has excellent stability.

Streptomycin is a member of the aminoglycosides. These compounds are mainly based on amino-N-containing sugars and/or cyclitol derivatives, which are either oligo-or monosaccharides in nature. The predominant representatives of this group are the (amino)cyclitol-aminoglycosides (Rinehart and Suami, 1980). However, other amino-sugar based microbial secondary metabolites, such as monomers and disaccharidic compounds lacking a cyclitol moiety are also included.

Aminoglycosides can be classified into two groups according to chemical pathways of cyclitol formation. The first one involves an inositol phosphate synthase-like mechanism and the second involves a dehydroquinate synthase-like mechanism. The products of the first pathway are cyclitolphosphates, which have to undergo dephosphorylation and a further oxidation step before a first step of transamination can be passed. In contrast, the second route yields 1-keto-2-deoxycyclitols which can directly be transaminated.

Streptomycin that is synthesised by the first pathway is composed of scyllo-inositol derived aminocyclitol (streptidine), a 6-deoxyhexose component (streptose) and an aminohexose derivative (N-methyl-L-glucosamine) (Fig. 1.3). Structurally similar compounds with different side groups are hydroxystreptomycin, dihydrostreptomycin, bluensomycin (Table 1.1) and the similar form of pseudodisaccharide, spectinomycin. Most of the 2-deoxystreptamine (2DOS)-containing aminoglycosides use the second cyclitol chemical pathway and share the common pseudodisaccharidic intermediate paromamine (Fig. 1.4). Examples of this group are gentamicin, butirosin, neomycin, kanamycin.

The above groupings of the compounds can be changed if their function is to be considered. In this case, streptomycin groups together with all (2DOS)-aminoglycosides having a bactericidal effect. On the other hand, examples of compounds with bacteriostatic effect are spectinomycin and kasugamycin.

1.3.1 Biosynthesis of streptomycin

The biochemical pathway for streptomycin production is branched, resulting in the formation of each of the three moieties that comprise streptomycin; (i) streptidine (ii) L-dihydrostreptose (iii) N-methyl-L-glucosamine (NMLGA). The enzymatic steps in streptomycin biosynthesis as postulated from genetic (Piepersberg, 1995; Distler *et al.*, 1992) and enzyme studies (Walker, 1990; Walker, 1995; Walker, 1975) are: (a) the formation of the activated precursors, streptidine-6-phosphate, dTDP dihydrostreptose

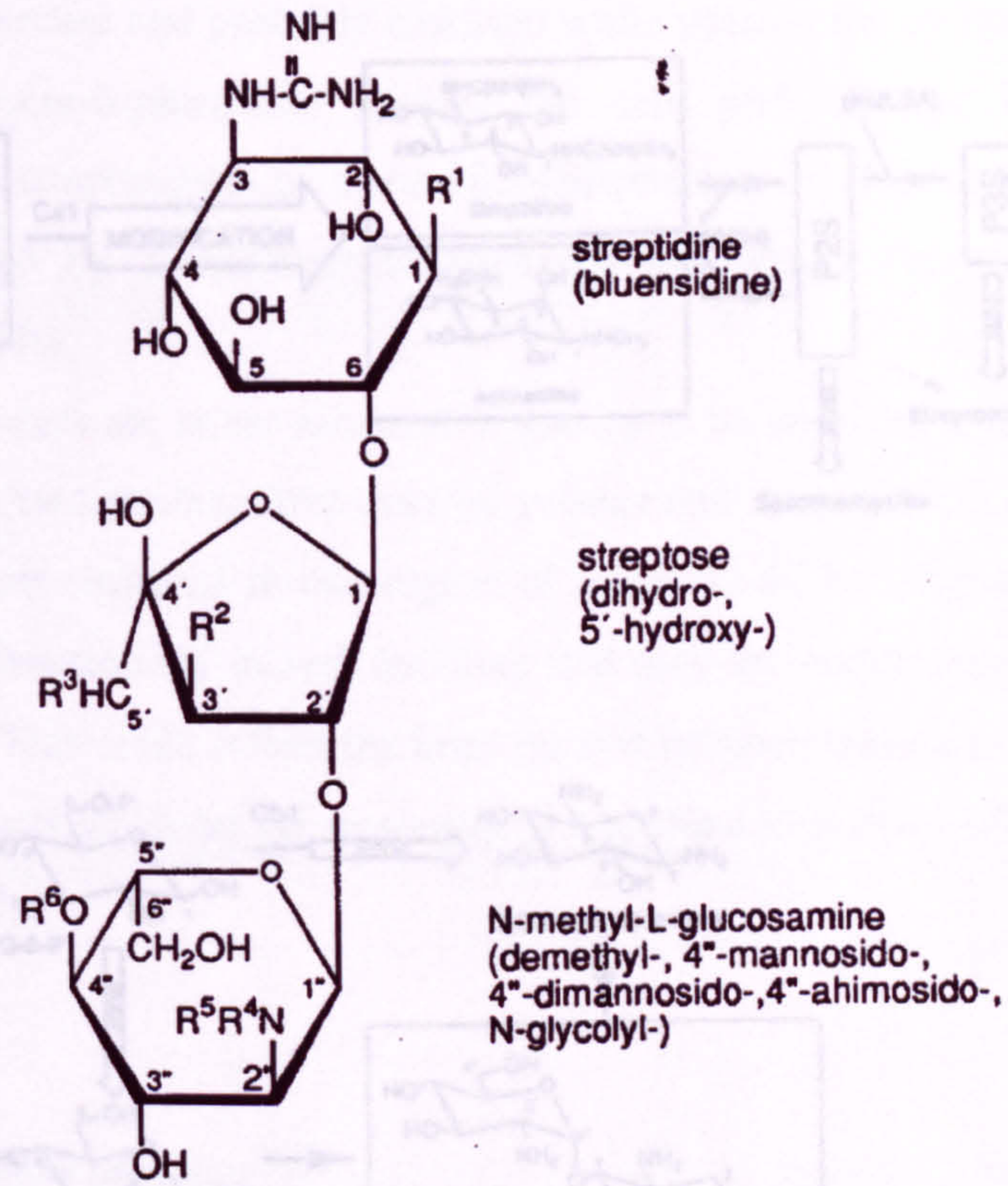


Figure 1.3: Chemical structure of streptomycin and related compounds. (Reproduced from Piepersberg, 1997).

Derivative	R1	R2	R3	R4	R5	R6
Streptomycin	HN-CNH-NH_2	-CHO	$-CH_3$	$-CH_3$	H	H
Dihydro-streptomycin	HN-CNH-NH_2	CH_2OH	$-CH_3$	$-CH_3$	H	H
5'-Hydroxy-streptomycin	HN-CNH-NH_2	-CHO	CH_2OH	$-CH_3$	H	H
Bluensomycin	O-CO-NH_2	CH_2OH	$-CH_3$	$-CH_3$	H	H

Table 1.1: Different side groups for streptomycin and other derivative compounds.

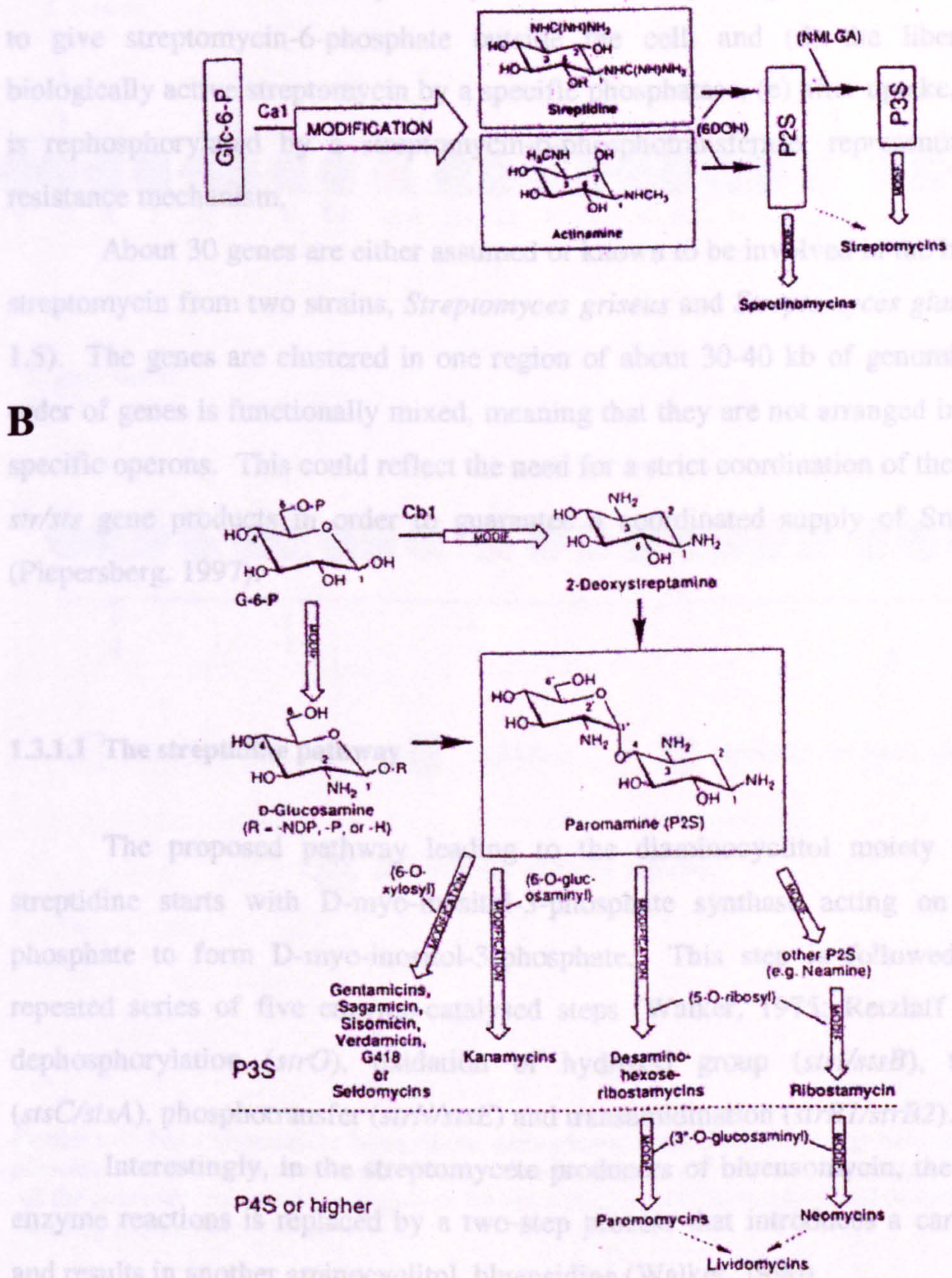


Figure 1.4: An overview of pathways for synthesis of aminocyclitol-containing aminoglycosides. (A) *Scyllo*-inositol derived aminonocyclitol ring. Full modification of activated monomers is preceded by the final condensation step that leads to pseudodisaccharide (spectinomycin) or pseudotrisaccharide (streptomycin). (B) 2-deoxystreptamine (2 DOS)-containing aminoglycosides. Formation of pseudodisaccharide intermediate, paromamine, is a common feature of this group. This step is followed by the core modifications of sugar moieties that leads to pseudotrsaccharides (e.g. gentamicins/kanamycins) or pseudotetrasaccharides (neomycins). (Reproduced from Piepersberg, 1997).

and NMLGA, (b) condensation of the precursors to dihydrostreptomycin-6-phosphate, (c) which becomes secreted and probably oxidised while passing the cytoplasmic membrane to give streptomycin-6-phosphate outside the cell; and (d) the liberation of the biologically active streptomycin by a specific phosphatase, (e) after uptake, streptomycin is rephosphorylated by a streptomycin-6-phosphotransferase, representing the major resistance mechanism.

About 30 genes are either assumed or known to be involved in the biosynthesis of streptomycin from two strains, *Streptomyces griseus* and *Streptomyces glaucescens* (Fig. 1.5). The genes are clustered in one region of about 30-40 kb of genomic DNA. The order of genes is functionally mixed, meaning that they are not arranged in subpathway-specific operons. This could reflect the need for a strict coordination of the expression of *str/sts* gene products in order to guarantee a coordinated supply of Sm biosynthesis (Piepersberg, 1997).

1.3.1.1 The streptidine pathway

The proposed pathway leading to the diaminocyclitol moiety of Sm called streptidine starts with D-myo-inositol-3-phosphate synthase acting on D-glucose-6-phosphate to form D-myo-inositol-3-phosphate. This step is followed by a twice-repeated series of five enzyme-catalysed steps (Walker, 1975; Retzlaff *et al.*, 1993): dephosphorylation (*strO*), oxidation of hydroxyl group (*strI/stsB*), transamination (*stsC/stsA*), phosphotransfer (*strN/stsE*) and transamidination (*strB1/strB2*).

Interestingly, in the streptomycete producers of bluensomycin, the second set of enzyme reactions is replaced by a two-step process that introduces a carbomoyl group and results in another aminocyclitol, bluensidine (Walker, 1990).

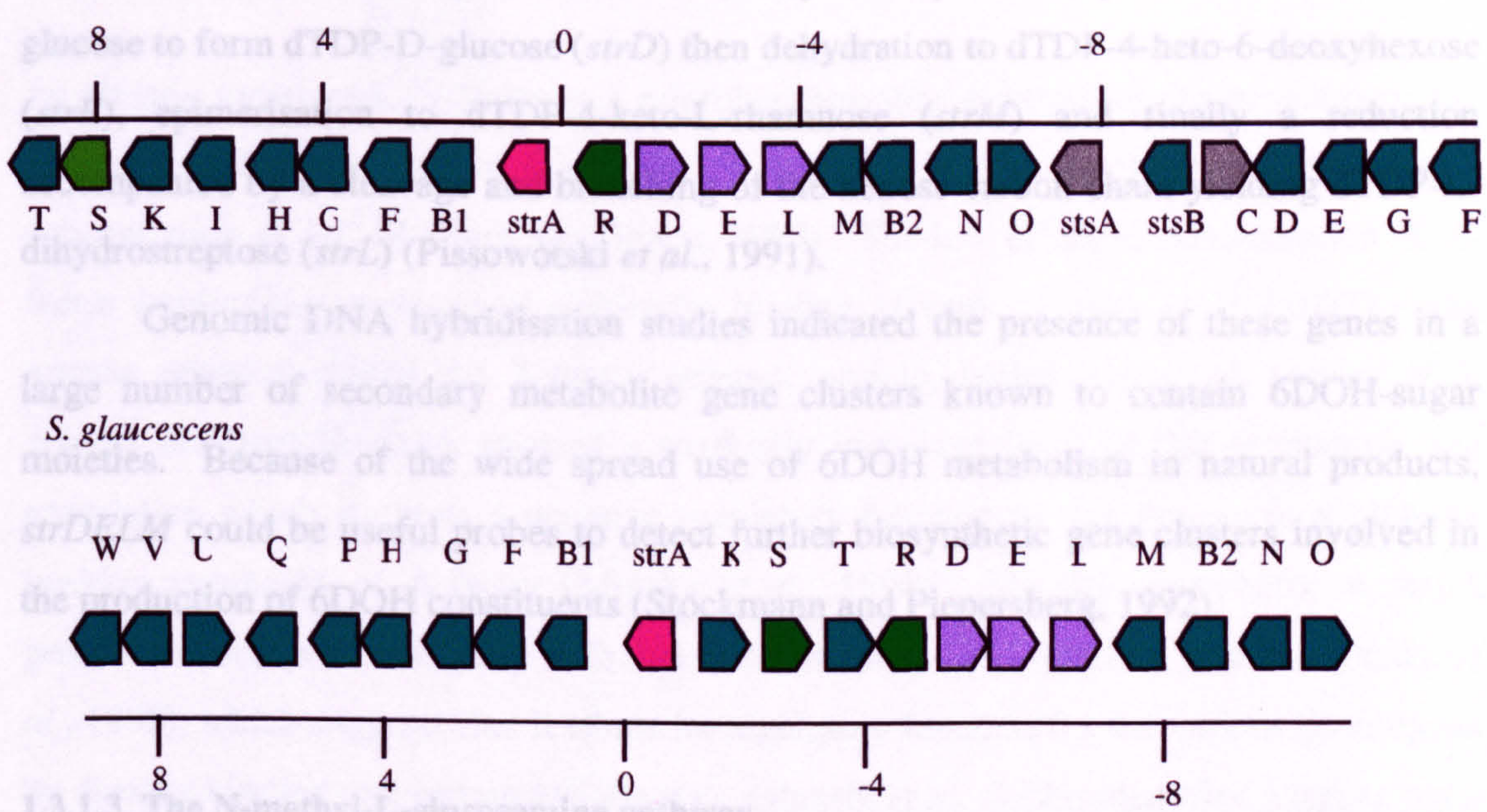


Figure 1.5: The streptomycin biosynthetic gene cluster from *Streptomyces griseus* and *Streptomyces glaucescens*. About 30 genes are assumed or known to be involved in the biosynthesis of streptomycin. All the genes are clustered including those for resistance, regulation, transport and extracellular processing functions (Piepersberg, 1995).

1.3.1.2 The L-dihydrostreptose pathway

A route for 6-deoxyhexose (6DOH) dihydrostreptose biosynthesis starts from D-glucose to form dTDP-D-glucose (*strD*) then dehydration to dTDP-4-heto-6-deoxyhexose (*strE*), epimerisation to dTDP-4-keto-L-rhamnose (*strM*) and finally a reduction accompanied by a cleavage and branching of the hexose carbon chain yielding dTDP-L-dihydrostreptose (*strL*) (Pissowotski *et al.*, 1991).

Genomic DNA hybridisation studies indicated the presence of these genes in a large number of secondary metabolite gene clusters known to contain 6DOH-sugar moieties. Because of the wide spread use of 6DOH metabolism in natural products, *strDELM* could be useful probes to detect further biosynthetic gene clusters involved in the production of 6DOH constituents (Stockmann and Piepersberg, 1992).

1.3.1.3 The N-methyl-L-glucosamine pathway

NMLGA synthesis has not yet been studied as intensively as the other branches of the pathway. Putative genes that can be involved in this pathway are *strFPQX* encoding for isomerase, oxidoreductase, glucose synthase and epimerase respectively (Mansouri and Piepersberg, 1991; Distler *et al.*, 1992).

The streptidine and dihydrostreptose subunits are then condensed by a glycosyltransferase step. The NMLGA moiety is then added by the same reaction to form dihydrostreptomycin-6-phosphate which is converted to streptomycin-6-phosphate as it passes through the cytoplasmic membrane. The export of the antibiotic in its inactive form is mediated by genes *strVW*, which encodes for proteins of an unusual type of ABC-transporter. In the streptomycin pathway, the final extracellular dephosphorylation step for the release of the biologically active antibiotic is catalysed by StrK.

Within the streptomycin cluster there are also located the pathway-specific regulator *strR*, and the resistance gene *strA*.

1.3.2 Regulation of streptomycin biosynthesis

1.3.2.1 A-factor

As already mentioned, A-factor is a microbial hormone controlling Sm production, Sm resistance and sporulation in *S. griseus*. It acts as a switch for these phenotypes at a concentration of 10^{-9} M (Hara and Beppu, 1982). The low effective concentration of A-factor implies the presence of a specific binding protein. Onaka *et al.* (1995) using reverse genetics cloned the A-factor receptor protein, called ArpA. A mutant *of S. griseus* deficient in ArpA showed a markedly increased level of Sm production and sporulation which suggests a negative regulatory function of this protein in a globally positive regulation of A-factor (Miyake *et al.*, 1990). The helix-turn-helix of ArpA protein showed great similarity with this motif of many DNA-binding proteins (Onaka *et al.*, 1995), which suggests that it exerts its regulatory function for the various phenotypes by directly binding to a certain key gene(s). Ohnishi *et al.* (1999) identified a target gene, *adpA*, of the A-factor receptor and proposed a model for A-factor regulatory cascade leading to the onset of streptomycin biosynthesis in *S. griseus* (Fig. 1.6). A-factor is synthesised by the action of AfsA in a growth-dependent manner (Ando *et al.*, 1997b). When A-factor reaches a critical level at 'decision phase' (Neumann *et al.*, 1996) it binds to ArpA which has bound the promoter region of AdpA (A-factor dependent protein) and dissociates ArpA from the promoter, thus leading to transcription and translation of *adpA*. AdpA then activates transcription of *strR* by binding upstream activation sequences (UAS) (Vujaklija *et al.*, 1991). Induced StrR then activates in turn the transcription of most of the streptomycin biosynthetic genes by binding multiple sites in the gene cluster (Retzlaff and Distler, 1995). The timing of the onset of Sm biosynthesis is therefore determined by the intracellular concentration of A-factor. In that way, there is a unifying model for regulation of Sm expression integrating both pleiotropic and streptomycin specific signalling elements. Recently, a σ factor essential for morphological development in *S. griseus* is found to be under the transcriptional control of AdpA

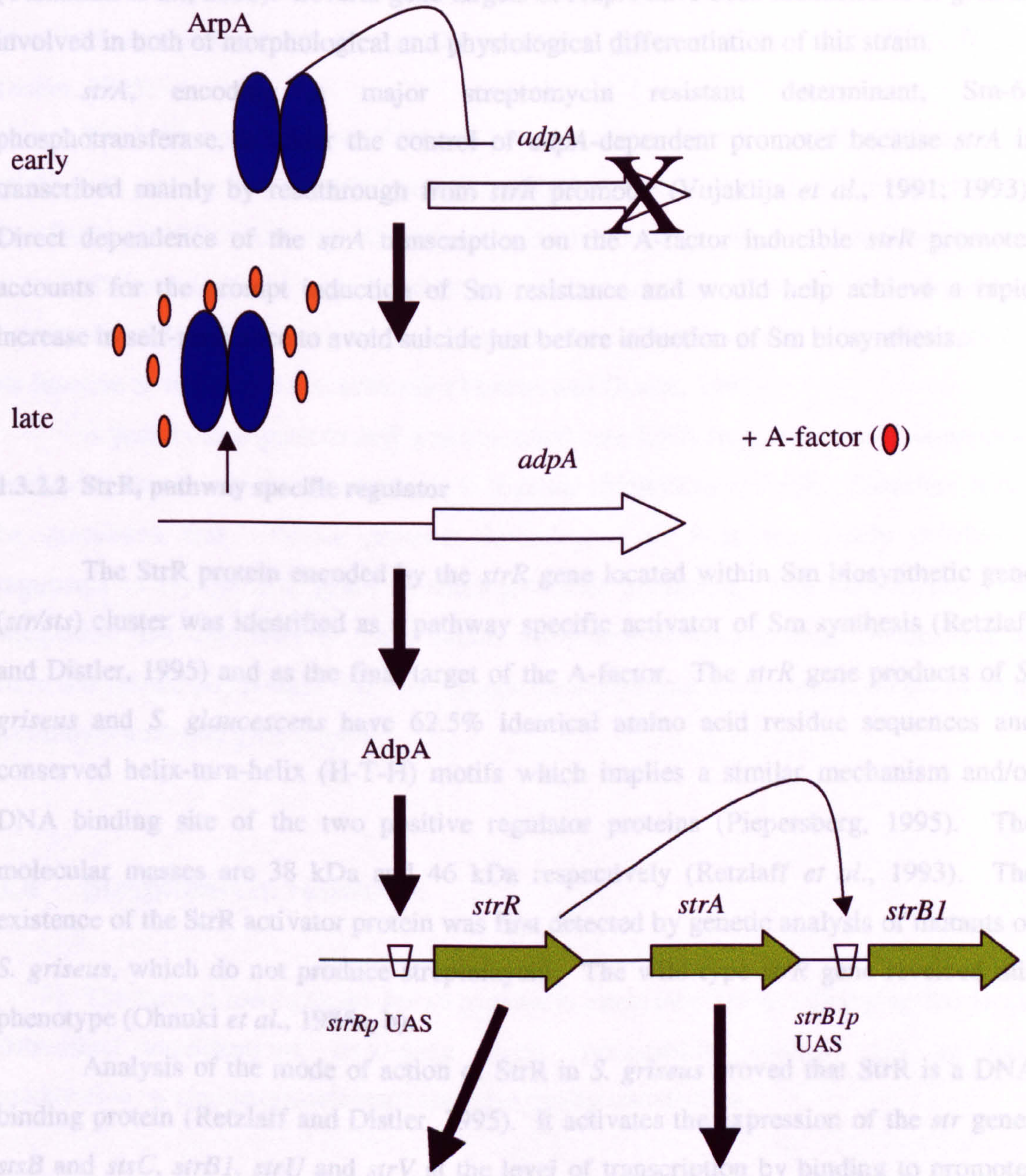


Figure 1.6: A-factor cascade for the regulation of streptomycin biosynthesis in *Streptomyces griseus*. Early in growth, when the concentration of A-factor is still low, an A-factor receptor (ArpA) binds and represses a transcriptional activator *adpA*. As the culture becomes denser, the concentration of the A-factor builds up to a critical level, at which it binds to ArpA, causing ArpA to dissociate from the DNA, and thus switching on the transcription of the *adpA*. Then AdpA protein switches on strR which is co-transcribed together with the streptomycin resistance gene *strA*. Then StrR can activate the transcription of other streptomycin biosynthesis genes.

(Yamazaki *et al.*, 2000). Several gene targets of AdpA have been identified in *S. griseus* involved in both of morphological and physiological differentiation of this strain.

strA, encoding a major streptomycin resistant determinant, Sm-6-phosphotransferase, is under the control of *adpA*-dependent promoter because *strA* is transcribed mainly by readthrough from *strR* promoter (Vujaklija *et al.*, 1991; 1993). Direct dependence of the *strA* transcription on the A-factor inducible *strR* promoter accounts for the prompt induction of Sm resistance and would help achieve a rapid increase in self-resistance to avoid suicide just before induction of Sm biosynthesis.

1.3.2.2 StrR, pathway specific regulator

The StrR protein encoded by the *strR* gene located within Sm biosynthetic gene (*str/sts*) cluster was identified as a pathway specific activator of Sm synthesis (Retzlaff and Distler, 1995) and as the final target of the A-factor. The *strR* gene products of *S. griseus* and *S. glaucescens* have 62.5% identical amino acid residue sequences and conserved helix-turn-helix (H-T-H) motifs which implies a similar mechanism and/or DNA binding site of the two positive regulator proteins (Piepersberg, 1995). The molecular masses are 38 kDa and 46 kDa respectively (Retzlaff *et al.*, 1993). The existence of the StrR activator protein was first detected by genetic analysis of mutants of *S. griseus*, which do not produce streptomycin. The wild type *strR* gene reversed this phenotype (Ohnuki *et al.*, 1985a, b).

Analysis of the mode of action of StrR in *S. griseus* proved that StrR is a DNA binding protein (Retzlaff and Distler, 1995). It activates the expression of the *str* genes *stsB* and *stsC*, *strB1*, *strU* and *strV* at the level of transcription by binding to promoter upstream sequences (Retzlaff and Distler, 1995). These sequences, together with a fourth StrR-binding site identified within the reading frame of the *strR* gene, are palindromes with the consensus sequence: GTTCGACTG$(N)_{11}$CAGTCGAAC (Retzlaff and Distler, 1995). The function of the StrR-binding site within the *strR* gene is still unclear. It could be either negative feedback regulation of StrR expression or activation of the *strA* promoter located approximately 400 bp downstream of this element. In the *str/sts* gene

cluster of *S. glaucescens*, an additional StrR-dependent activation site could also be identified upstream of the divergently orientated genes *strX* and *strV* (Retzlaff and Distler, 1995).

The helix-turn-helix motif located between the amino acid positions 207 and 227 of the StrR protein was identified as a prerequisite for its DNA binding activity (Thamm and Distler, 1997). Although C-terminal truncated StrR proteins were able to interact with StrR binding sites, they failed to activate transcription from the StrR-dependent promoter *strB1p*. Therefore, the C-terminus domain of StrR seemed to be necessary for its function as transcriptional activator (Thamm and Distler, 1997).

A gene homologous to *strR* was identified on a DNA fragment of *S. flavopersicus* conferring spectinomycin resistance in *S. lividans* (Piepersberg, 1995). Therefore it can be speculated that activator proteins, homologous to StrR are widely distributed regulators of biosynthetic genes in aminoglycoside producers. This also supports the suggestion that especially in those strains using myo-inositol synthase mechanism to form Sm-related compounds, the biosynthetic capacity could have evolved on the basis of a common ancestral gene cluster.

1.4 Streptomycin resistance

The known mechanisms for streptomycin resistance are by: changing the target (ribosomal modification), preventing access (permeability control) and antibiotic inactivation by phosphotransferases and adenylating transferases.

1.4.1 Ribosomal modification

As happens to all ribosome-binding antibiotics, mutation leading to resistance can result in an altered ribosomal protein S12 (streptomycin resistance) or methylated 16S rRNA (gentamicin resistance) such that the ribosome has reduced affinity for the antibiotic (Cundliffe, 1989).

1.4.2 Permeability control

Streptomycin resistance can be facilitated by preventing the antibiotic from entering the cell as it has been observed in *S. griseus*. Streptomycin uptake is reduced during the stationary phase of growth. Strains of *Bradyrhizobia japonicum* were developed that could fix nitrogen more efficiently to provide the plants with more nitrogen and increase productivity without increasing the economic input into the farming system of Cerrado region in Brazil. This microflora of this site is dominated by members of *Streptomyces* genus. Studies with streptomycin sensitive and resistant strains of bradyrhizobia isolates showed that differences in the exopolysaccharides from the cell walls of resistant strains were linked to an increase in impermeability to streptomycin.

1.4.3 Streptomycin modification

The genes encoding modification of aminoglycosides do not appear to undergo facile mutational changes that generate enzymes with altered substrate activity (Davies, 1994). Rather the response of bacteria to the introduction of a new antibiotic is to acquire a different gene. Shaw *et al.* (1993) compared the nucleotide and protein sequences of the known aminoglycoside acetyltransferases, phosphotransferases and adenyltransferases responsible for resistance in both pathogenic bacteria and antibiotic producing strains. Different proteins in the same functional class may show as little as 44% amino acid similarity.

Two resistant phosphotransferase enzymes have been detected in *S. griseus*, encoded by the genes *aphD* (aph(6')) and *aphE* (aph(3')). The latter does not seem to have a counterpart in *S. glaucescens*. AphE can phosphorylate the NMLGA moiety at the 3' position and is not located in the Sm biosynthetic cluster while AphD does so at streptidine 6' position.

AphE ia also more related to the neomycin resistance gene *aphA* of *S. fradiae* and it has been suggested that this gene had transferred into *S. griseus* which under selection of streptomycin production adapted to phosphorylating this molecule (Shaw *et al.*, 1993). Based on the above gene similarity, a constructed hybrid enzyme consisting of the AphE

N-terminus and AphA C-terminus showed the localisation of the substrate recognition sites on the C-terminal portion (Heinzel *et al.*, 1988).

Resistance conferring genes for exporter proteins actively transporting antibiotic molecules out of the producer cells have been identified only in the gene clusters for non-aminoglycoside antibiotics like macrolides, tetracyclines, anthracyclines, and lincosamides (Cundliffe, 1989). Two genes, conserved in the Sm production gene clusters of both *S. griseus* and *S. glaucescens*, *strV* and *strW*, encoding ABC transporters do not seem to confer resistance phenotypes. Therefore they could serve the purpose of transporting inactive final products from the cells only (Piepersberg, 1997).

In addition, a cryptic chromosomal gene for an aminoglycoside resistance appears to be present in many enterobacteria (Shaw *et al.*, 1993) as well as a cryptic kanamycin-resistant gene in *S. griseus* (Piepersberg, 1997).

In terms of contribution to antibiotic production, some resistance genes can also stimulate aminoglycoside production. For instance, the 6'-acetyltransferase gene (*aacA*) from *S. kanamyceticus* acts to stimulate antibiotic production when cloned on multicopy plasmids into producers of kanamycin and neomycin (Crameri and Davies, 1986).

Modifying enzymes are also found in a wide range of other bacteria, generally located on transposons and plasmids (Shaw *et al.*, 1993). Tn*5* and RSF1010 encode *strB*, a phosphotransferase which also phosphorylates at the 6'-position of streptidine but has less than 30% identity at the sequence level with the *S. griseus* resistance gene. A second streptomycin modifying gene is also located on this plasmid and transposon. In Tn*5*, there is an aph(6') gene which phylogenetically is clustered together with the *strA* gene from the streptomycin-producing strain *S. griseus*. In RSF1010, *strA* gene modifies the 3'-hydroxy group of streptomycin and resembles to aph(3') gene from *S. griseus*. The plasmids and transposons, which encode these genes, have been isolated from many bacteria including *Methylbacterium* (Mazodier *et al.*, 1982), *E. coli* (Genilloud *et al.*, 1988), *Rhizobium* (Putnozky *et al.*, 1983) and a clinical isolate *Shigella flexneri* (Shaw *et al.*, 1996).

The current hypothesis that most resistant determinants could have originated from the producing organisms (Benveniste and Davies, 1973) can be regarded as proven by nucleic acid and protein sequence comparisons of aminoglycoside resistance

determinants from producer organisms and clinical isolates (Davies, 1994; Marshall *et al.*, 1998).

Both the bacterial antibiotic phosphotransferases and the eukaryotic protein kinases recognise similar substrates (Piepersberg *et al.*, 1988) preferably cationic substances with free hydroxyl groups. The antibiotic kinases do share conserved domains with eukaryotic protein kinases, essential for nucleotide binding (Shi *et al.*,1998). Consistent with that, Shaw *et al.* (1993) pointed out that sugar kinases and acyltransferases may have evolved to modify aminoglycoside antibiotics and Piepersberg *et al.* (1988) suggested that the genes for protein kinases were ancestral sources for some class of aminoglycoside-modifying enzyme genes. These protein kinases may have been incorporated into the rest of the biosynthetic cluster of aminoglycosides during the time of biochemical evolution and being subjected to further dissemination.

1.5 Mode of action

Streptomycin impairs prokaryotic ribosome function by interacting with the 30S ribosome subunit and causing misreading of the messenger RNA. The fidelity of aminoacyl-tRNA selection involves an initial selection and proofreading by the ribosome. During decoding the anticodon triplet pairs with its cognate codon on the mRNA and protects bases on the 16S rRNA from chemical modification in the same region as the aminoglycosides (Moazed and Noller, 1986). The decoding site is composed of nucleotides 1400-1410 and 1490-1500, which form a stem-loop-stem structure that is known as the A-site of the *E. coli* 16S rRNA (Moazed and Noller, 1986). The asymmetric internal loop id the antibiotic binding site. Decoding is achieved by the interaction of the A-site with the backbone of the codon-anticodon helix, thereby protecting residues A1492 and A1493 from chemical modification (Moazed and Noller, 1986).

Binding of the antibiotics to the RNA induces a conformational change in the RNA, displacing the two universally conserved residues A1492 and A1493 towards the stem part of the decoding site, thereby probably switching the A-site into a high affinity

state for mRNA-tRNA recognition and reducing the rejection rate of near-cognate tRNAs (Karimi and Ehrenberg, 1994). This increased affinity of the A-site for tRNA in the presence of antibiotics might result in misreading of the genetic code.

Certain aminoglycosides like neomycin B, inhibit ribozymes by displacement of essential divalent metal ions. So far, there is no evidence that magnesium ions are involved in the decoding process, but this cannot be excluded (Schroeder *et al.*, 2000).

1.6 Evolution of antibiotic production

The great chemical diversity of antibiotics reflects the variety of functions and/or mode of actions of these compounds even though this issue has been debated for many years. Understanding the role of antibiotic structures and the importance of their production is essential, as it seems to be a major force that drives and shapes their evolutionary mechanisms.

1.6.1 Ecological role of antibiotic production

The predominant view on the role of antibiotics is that they possess a biological activity that endows the producer with increased fitness. This is achieved by either offering a growth advantage or protection from competition or predation (Vining, 1990). Siderophores are low molecular weight iron-chelating compounds that have strong selective value in iron-deficient environments (Crosa, 1989) improving the producer's ability to grow. The production of antibiotics targeted through a variety of biochemical mechanisms at other microorganisms is an important factor in the competition for resources. Mazzola *et al.*, (1992) tested such ecological competence of strains in soil by using phenazine producing and mutant non-producing *Pseudomonas fluorescens* strains. The reduced ability of the mutant non-producing strains to survive in soil was due to a diminished ability to compete with the resident microflora. In many cases, antibiotic production coincides with the onset of aerial mycelium formation and pleiotropic genes can regulate the expression of both phenotypes. One general potential adaptive benefit of

this interlinking of morphological and physiological differentiation could be the protection of the colony against invasion and outgrowth by other bacteria at the stage of colony development when aerial hyphae are growing parasitically on the lysing substrate mycelium (Chater, 1992).

Small diffusible molecules like γ-butyrolactones can be considered as secondary metabolites, so one metabolite controls the production of others (e.g. streptomycin) within the producing organism and acts externally on other organisms as well. In *S. coelicolor*, loss of the production of certain butyrolactones has no effect on morphology or antibiotic production. Therefore, these signalling molecules could also serve to fool any nearby *S. griseus*-like organism into preparing itself for sporulation at the earliest possible stage so that it no longer competes for growth (Chater, 1992).

Despite the above possible and proposed function of antibiotics, their ecological role is still obscure due to problems encountering the extraction of antibiotics from soil. This may be due to the charged nature of many compounds which meant that they are strongly bound to particles in the soil (Williams *et al.*, 1982). Moreover, soil type may also have an important effect on antibiotic detection and activity. Another factor is the nutrient-poor status of the soil which has meant that producing organisms were at a very low population level and if producing compounds, were doing so at very low levels. When soils are nutrient amended, antibiotics may often be detected (Wellington *et al.*, 1993).

The lack of sufficient antibiotic extraction from soil and the failure to attribute a biological activity to many secondary metabolites led many scientists to speculate that antibiotics play no role in increasing the fitness of the producer. Consequently, some other hypotheses about the biosynthesis of secondary metabolites have been put proposed (Nisbet, 1992) such as biochemical dead-ends, by-products of primary metabolism, detoxification mechanisms, the result of limited growth and the result of weak regulation of metabolism. However, these proposals do not address the evolutionary significance of secondary metabolites or the advantages that they confer on the producing microorganisms. The sophistication of their biosynthesis, its regulation and genetic organisation argue for its being an activity of benefit to an organism. Were it not maintained by selection, chance mutations in the genetic determinants of such a process,

even one that imposed a very low metabolic load, should eventually have led to its elimination (Vining, 1992a).

Moreover, in order to address more efficiently the antibiotic usefulness, it is important to consider how biological activity is defined. Biological activity studied at a molecular level *in vitro* can have a different meaning to biological activity studied at a whole organism level. At a molecular level, biological activity against a defined molecular target is a rare property for a molecule to possess. These observations led Firn and Jones (2000) to replace the term 'biological activity' with the term 'biomolecular activity', and it should be defined as the ability of a molecule to interact with a biologically functional molecule such that its biological function is changed significantly. However it is predictable that the frequency of molecules possessing 'biological activity' will be higher if activity is assessed by targeting an organism instead of a protein, as an organism contains thousands of potential protein targets.

However, even the absence of biological activity for the producer does not exclude the functionality of such antibiotic compounds within the ecosystem in a way to ensure a selective pressure for their production and retention. Consistent with that, symbiosis between different microorganisms may be another important aspect to understand the dynamics of microbial flora in various environments. Symbiosis between two microbial strains is achieved through feeding of an essential factor by one supporting strain for growth of the other strain. Ohno *et al.* (1999) showed that *Symbiobacterium thermophilum* essentially requires some ubiquitous metabolite(s) of low molecular weight produced by various bacterial species as growth factor(s). Different *Streptomyces* species may show symbiotic behaviour by sharing common signalling substances to adjust their activities for secondary metabolism and cell differentiation (Ueda *et al.*, 2000). We therefore need some new viewpoints to understand the diversity in the secondary metabolites which are functional not in the producing organisms but in the environment.

1.6.2 Streptomycin may have an old evolutionary history

Organic compounds like amino acids and nucleic acids could exist in the primitive earth atmosphere as indicated by the components of 'primordial soup' reactions

and chemical analyses of the constituents of meteorites (Lazcano and Miller, 1996). In addition to amino acids and nucleic acids, many other organic constituents of primitive earth are known to be components of biologically active secondary metabolites, some of which have antibiotic activity (Davies *et al.*, 1992). It has been proposed that molecules related to secondary metabolites played important roles in biochemical evolution as modulators or effectors, enhancing or controlling the biological activities of primitive macromolecules (Davies, 1990).

In this precellular world, RNA forms could function as ribozymes providing the catalytic property necessary for an effective peptide bond formation. Antibiotic-related structures could bind to RNA and by promoting conformational changes they could mediate the peptide synthesis process. As larger polypeptides were produced, their interaction with RNA improved system efficiency displacing the low molecular size effectors. The latter molecules by retaining the initial function could now act as competitive inhibitors of translation. Evidence of this prebiotic effector hypothesis comes from aminoglycosides. These antibiotics are known to inhibit translation by binding directly to specific RNA. Davies (1990) suggested that these molecules were once such a class of RNA-catalysis effectors involved in translation. Streptomycin and many analogues were shown to inhibit the first step of self-splicing of group I introns indeed. Gentamicin and other 2-deoxystreptamine aminoglycosides inhibited the second step of splicing, where quanine binding as a promoter of the reaction is not necessary. Therefore, 2-DOS aminoglycosides must bind to the intron DNA at a site different from the guanine site (von Ahsen *et al.*, 1992).

Interestingly, other translational inhibitor acting antibiotics like tuberactinomycin seem to promote the formation of intron oligomers, in a reaction analogous to RNA ligation (Davies *et al.*, 1992). These results may describe the modern antibiotics as 'molecular fossils' that could have played roles in biochemical evolution of nucleic acid structures by serving as effectors of ligation systems or splicing reactions.

After the formation of functional ribosomes had been completed, some of these low molecular mass molecules could have lost their original functions. However, natural selection for the development of compounds with different biological role (antibacterial action) but sharing pre-existing biochemical mode of action (interference with

ribosomes), could select for the recruitment of prebiotic effectors. Therefore, their biosynthetic potential has been evolved much later than their existence. In that way, a functional continuity between precellular and cellular physiology could be established.

Chemically, this rather unexpected evolutionary route can be explained on the basis of heterotrophic nature of producers. Vining (1992b) made the interesting observation that heterotrophic bacteria are more prone to develop secondary metabolites than autotrophs. The prebiotic synthesis of organic compounds by reduction of CO_2 to monomers used by the first organisms would also be considered a heterotrophic origin (Lazcano and Miller, 1999). The biosynthetic pathways of the earliest organisms arose after exhaustion of the prebiotic compounds used in the heterotrophic origin of life. From the biochemical point of view, secondary metabolites represent adaptive extentions of the pre-existing metabolic apparatus, presumed to be the essential primary pathways that supported normal life functions in ancestral organisms (Vining, 1992a).

This functional evolution has been coupled by a mechanistic evolution aiming to generate and further diversify of the corresponding biosynthetic pathways. Duplication of primary metabolic genes and mutation of the copies furnished altered proteins making them available for the secondary metabolism biosynthetic machinery. Serial recruitment of relatively inefficient primary enzymes endowed with broad substrate specificity (Jensen, 1976) could also have allowed for the initial diversification of enzymatically catalysed reactions in a selective process analogous to the recruitment of the prebiotic antibiotic forms.

Weakly selected genes from cellular biochemistry could also escape evolutionary loss (by genetic drift) by intraspecific DNA rearrangements incorporating them into partly developed gene clusters for a certain property or recruiting next to already modified genes for a certain function. The new gene organisation could be later subjected to horizontal gene transfer in a naïve genome allowing further optimisation of the selected property.

Genes organised into clusters can propagate both by vertical transmission and by horizontal gene transfer; unclustered genes can be inherited only by vertical transmission because mechanisms that promote gene transfer are limited by the size of DNA fragments they can mobilise (Lawrence, 1999).

All the biosynthetic genes for secondary metabolites investigated so far, have been found located in large clusters. The gathering of production genes to form clusters similarly as observed in plasmids or in viruses, seems to have been a means of conservation under strong selective pressures and for easy horizontal gene transfer of functional units (Piepersberg, 1991). The evidence to date that nucleotides and amino acid sequences of secondary metabolic genes show much weaker homology with genes of related primary pathways in the same organism than with those of related primary and secondary genes in other organisms suggests that gene transfer has been an important factor in the spread of secondary metabolism (Vining, 1992a). An example of such gene transfer is related to 6-deoxyhexose metabolism. This synthesis of 6-deoxyhexose constituents is widely used in natural products including streptomycin. Indeed, one of the genes involved in the biosynthesis of 6-deoxysugar side chains of avermectin in *Streptomyces avermetilis* is homologous to the *strD* gene of *S. griseus* which putatively encodes a dTDP-glucose synthase catalysing the first step in the biosynthesis of the streptose moiety (Distler *et al.*, 1987). In fact *strDELM* genes could be useful probes to detect further biosynthetic gene clusters involved in the production of 6DOH constituents (Stockman and Piepersberg, 1992). Therefore antibiotic production genes seem to be used at least in various combinations to form different pathways of increased chemical and functional diversity of the end-product (Mansouri and Piepersberg, 1991).

Streptomyces have been described as having a 'fluid' genome and the antibiotic clusters are frequently in unstable genomic segments being subjected to deletion, amplication and recombination (Schrempf, 1991). Recombination and subsequent horizontal gene transfer of the biochemical tools for the antibiotic production seems to be far more efficient and rapid diversification process than sequential modification of gene function by the accumulation of point mutations (Syvanen and Kado 1998).

Overall, the evolution of antibiotic production may be divided into two phases. In the first stage of biochemical evolution, the selective pressure for a large number of diverse antibiotic structures may be expected to be weak. Gene duplication and divergent evolution seems to have played a dominant role for an increase in genome size and for the early and relatively slow development of enzyme function and gene subclustering. As the complexity of cell structure increased, the cellular interactions posed a stronger

selection pressure for the diversification of these end-products. During this second phase of evolution, intra- and inter-genomic rearrangements in the form of horizontal gene transfer may be the major mechanism for the rapid multiplicity and diversification of biosynthetic pathways.

1.7 Horizontal gene transfer

The strategy of DNA acquisition allows microorganisms to share evolutionary success of others or to adjust it in the new host physiology and/or under different environmental conditions (Arber, 2000). In contrast to the evolution of new traits through the modification of existing sequences, the origin of new abilities through horizontal gene transfer has three requirements. First, there needs to be a means for donor DNA to be delivered into the recipient cell. Second, the foreign DNA must also be in or get into a format that allows long-term maintenance and replication (e.g. either as a self-replicating extrachromosomal element or by inserting into the recipient's genome). And third, the incorporated genes must be expressed in a manner that benefits the recipient microorganism.

Vehicles for such genetic exchange are known to be plasmids, transposons, congugative transposons, integrons and bacteriophage.

The mechanisms of transfer and maintenance influence the patterns of horizontal gene transfer. For example, mismatch repair systems in some species prevent homologous recombination based insertion of distantly related genes but have no effect on insertion via non-homologous recombination (Eisen, 2000a). The expression of the transferred genes can be influenced by the extent and type of adaptation of the newly acquired sequences in the recipient lineage and the phylogenetic distance between the donor and the recipient. In the process called amelioration (Lawrence and Ochman, 1998) the acquired sequence adjusted to the base composition and codon usage of the resident genome in order to reach an optimum expression level; unless all the necessary expression determinants are provided by the vehicle of transfer (Hall and Collis, 1995).

Therefore, both adaptation and vehicle of transfer can influence the frequency of a successful horizontal gene transfer.

1.7.1 Mechanisms of gene transfer

Three mechanisms of gene transfer in bacteria have been identified: transformation, conjugation and transduction.

1.7.1.1 Transformation

This process involves the uptake and incorporation of naked DNA. Transformability is a hereditary trait and bacterial cells that are transformable enter a physiologically regulated state of competence for uptake of exogenous DNA (Nielsen *et al.*, 1998). In the Gram-positive *Streptococcus pneumoniae* and *Bacillus subtilis* accumulation of a low molecular weight peptide in the growth medium stimulates the expression of genes involved in the development of competence, whereas in a Gram-negative like *Haemophilus influenzae* competence seems to be internally regulated and is usually expressed in late exponential or early stationary phase (Nielsen *et al.*, 1998). Although the presence of specific uptake sequences enhances the transformation efficiency between related species many of the naturally competent bacterial species, such as *B. subtilis* and *Streptococcus pneumoniae*, do not display sequence preference and are capable of high levels of transformation (Davison, 1999). Productive gene transfers mediated by natural transformation occur mainly between strains of the same species. Streptomycetes, in contrast, are not known to show transformability. Others like *Neisseria gonorrhoeae* are always in a competence state (Lorenz and Wackernagel, 1994). The latter study listed 44 species of naturally transformable prokaryotes although this number may be an underestimate due to failure of identifying conditions needed for the development of the competence.

Although natural transformation systems tend to take up linear DNA segments, plasmid DNA that has escaped from degradative enzymes can still transform competent

bacteria in oral and possibly other gastrointestinal habitats (Mercer *et al.*, 1999). Natural transformation requires that the freed DNA will remain stable and will be integrated into the recipient's genome by homologous recombination (Mercer *et al.*, 2001) unless the DNA segment contains a transposable element. Environmental DNA can be stabilised by adsorption to sand and clay particles thereby becoming 100- to 1000-fold more resistant to DNAase. Such adsorbed DNA may retain its transforming ability for weeks or even months (Lorenz and Wackernagel, 1994).

Transformation has been shown to occur in a variety of natural ecosystems such as soil. Nielsen *et al.* (1997a,b) found that competence disappeared rapidly upon introduction of bacteria into oligotrophic soil. Nutrient amendment permitted prolongation of competence and induced competence in cells that could no longer be transformed. Higher phosphate levels also increased the transformation efficiency. Soil moisture also affected the transformation frequency.

1.7.1.2 Conjugation

This process involves a bacterial cell-cell interaction which can mediate gene transfer in the environment. This is a broad host range mechanism and can mediate gene transfer between domains (for example between bacteria and plants and bacteria and yeast). In Gram-negative bacteria, conjugation begins when a donor attaches an appendage called a pilus to a recipient. Then the pilus retracts drawing together the donor and recipient. Generally many donors extend pili at about the same time. Consequently, extension of pili causes bacterial cells to aggregate into clusters. After aggregation occurs, bridges or pores are formed between donor and recipient cells and plasmids pass through the bridges from the donor to the recipients. Conjugation in Gram-positive bacteria does not involve pili. Instead, the putative recipient secretes substances that prompt potential donors to produce proteins, often called clumping factors, able to bring bacterial cells together. When the cells associate, they form the pores needed for DNA transfer.

Conjugation may involve transfer of a self-transmissible plasmid like F-plasmid and RP4 of *E. coli*. Mobilisation of a non self-transmissible plasmid can also occur by

the action of the other conjugative plasmid. In principle, it requires only the addition of a short DNA sequence, origin of conjugal transfer (*oriT*), to a replicative element to render it mobilisable by conjugation by cis-action of transfer functions of another plasmid (Davies, 1994).

Co-integration is the fusion of two different plasmids. Thus the non self-transmissible non-mobilisable plasmid may be transferred by its cointegrated self-transmissible counterpart. Conjugation can also mediate the transfer of chromosomal sequences by plasmids that integrate into the chromosome and by conjugative transposons which also facilitate plasmid mobilisation and cointegrate formation.

1.7.1.3 Transduction

New genetic material can also be introduced into a bacterium by a bacteriophage that has replicated within a donor microorganism and packaged random DNA fragments (generalised transduction) or the DNA adjacent to the phage attachment site (specialised transduction). As part of their life cycles, bacteriophages attach to bacteria and inject their DNA. This DNA then serves as a blueprint for making more copies of the bacteriophage, which burst from the infected bacterium and go on to infect other cells. At times, however, some of the new particles carry bacterial instead of viral DNA. The amount of DNA that can be transferred in a single event is limited by the size of the phage capsid, but may range upwards of 100 kb. Like transformation, transduction is generally regarded as a mechanism of narrow host range gene transfer. The reason for thinking that transduction mediates DNA transfers primarily between closely related bacteria is that most of the bacteriophage receptors so far described have been limited to a single bacterial species. However, this may be an artifact of the small number of phages and hosts that have been studied so far. Another factor that limits the range of productive transduction events is that generalised transduction transfers linear segments of DNA, which must be integrated into the recipient's genome by homologous recombination. Such DNA segments could be lost in hosts whose DNA failed to have sufficient homology with the incoming segments to allow them to be integrated.

On the other hand, phages have evolved ways to counteract the host restriction endonucleases protecting the transferred sequences from degradation. However, phages are very common in the environment (Jiang and Paul, 1998) and are relatively stable, being protected by the protein coat. Phages are also more compact and thus more diffusible than naked DNA. Finally, temperate phages may continue to coexist with the bacteria in the form of lysogens and be liberated in some distant future, in response to environmental factors like high nutrient level (Marsh *et al.*, 1993).

A phage isolated from a freshwater habitat, was able to transduce *Pseudomonas aeruginosa* and also members of the populations of natural lake-water environments (Ripp *et al.*, 1994). Phage-mediated transfer has been suggested to explain the distribution of the phage-encoded, pyrogenic exotoxin C among different phylogenetic lineages of *Streptococcus pyogenes* (Kapur *et al.*, 1992). Where the infection efficiency of the temperate actinophage of KC301 was sufficiently high to retard vegetative growth of Streptomyces lividans in sterile soil, sporulation compensated for the reduction in propagule numbers (Marsh *et al.*, 1993). Generalised transduction has been reported in *S. venezulae* and *S. coelicolor* (Burke *et al.*, 2001).

Since *Streptomyces* are not known to show transformability, the other two mechanisms (conjugation, transduction) are more important in mediating gene transfers in this genus. *Streptomyces* possess both covalently closed circular plamids (Hopwood and Kieser, 1993) and linear plasmids (Kinashi *et al.*, 1994). Plasmid transfer has been demonstrated *in situ* (Wellington *et al.*, 1990) and both mechanisms function in soil (Marsh and Wellington, 1994; Ravel *et al.*, 1998; 2000).

1.7.2 Methods for inferring horizontal gene transfer

One method that has been used to determine whether HGT has occurred involves inferring phylogenetically trees for many genes in many genomes. Smith *et al.* (1992) suggested several criteria for the evaluation of such possible horizontal gene transfer: (i) the tree should be constructed with regard to a specific protein or gene from a number of distantly related organisms with that of the known phylogenies for those species. If an

incongruency is seen between the 'gene tree' and 'species tree' then a case can be made for a horizontal gene transfer. (ii) the tree ought to be rooted by sequences from appropriate species or duplicated genes whose classification are generally accepted. (iii) the case is strengthened if more than one tree building yields the same topology. (iv) it is helpful when sequences of other molecules from the same organism yields trees with the conventional topology. (v) the life history of the potential gene donor and recipient organisms should involve contact between them, so transfer seems feasible. (vi) in addition, the genes being considered, must have phylogenetic information. (vii) the homology being compared must represent orthologous (diverged after a speciation event) and not paralogous (diverged after a duplication event) genes (Syvanen, 1994).

A common alternative method for inferring HGT involves using similarity search techniques, i.e. determine the 'best match' for each gene in a genome. HGT is frequently involved for those genes that have best matches to supposedly distant species (Eisen, 2000a). However, different evolutionary rates for related genes and gene loss can yield inaccurate results.

In theory, detecting genes with uneven distribution patterns could identify gene transfers. However, uneven distribution pattern can also be caused by gene loss or rapid sequence divergence (such that homologues are not detected in some lineages even when they are present) (Eisen, 2000a).

As it takes time for a transferred gene to ameliorate to the recipient's genome a foreign gene in a genome can be detected by identifying genes with unusual features like G+C anomalies or codon usage. However, it is important to note that selection and mutation can also cause unusual nucleotide composition. In addition, this method can neither detect transfers between species with similar compositions nor those that have occurred a long time ago such that amelioration has been completed.

1.8 Examples of horizontal gene transfer

There are now numerous examples of horizontal gene transfer between bacterial species, genera or families and even between bacteria and eukaryotes.

1.8.1 Antibiotic resistance

Resistance to an antimicrobial is a natural consequence of bacterial cell adaptation following exposure to antibiotics and is a direct result of competitive advantage conferred by the resistance phenotype.

Currently, plasmids encoding resistance to five or more antibiotics can be readily found in *E. coli, Salmonella* and other Gram-negative bacteria isolated from the general community as well as hospital sources. Multiresistance plasmids found in pathogens must have been created in the past five decades, and the use of antibiotics did not increase the spread of bacterial clones carrying such plasmids but encouraged the dissemination of antibiotic resistance genes (Hughes and Datta, 1983).

Direct examination of the nucleotide sequences of resistance genes in different bacteria has clearly confirmed horizontal gene transfer between bacteria from different habitats. An example is the tetracycline resistance gene *tetM*, which was found in soil *Streptomyces* spp. as well as colonic *Peptostreptococcus* species, suggesting that soil microbes may transfer genes to intestinal microflora (Salyers and Shoemaker, 1996). Similarly, almost identical *tetQ* genes are shared by *Bacteroides* spp. which are normal flora of the human gut, the distantly related genus *Prevotella ruminicola*, present in the rumens and intestines of farm animals and *Prevotella intermedia*, isolated from the human oral cavity (Nicolich *et al.*, 1994). These studies suggest that normal microflora of the human gut may act as reservoir of resistance genes which may subsequently be transferred to pathogens.

A striking characteristic of the *Bacteroides* conjugative transposons is that conjugation by these transposons is itself inducible by low levels of tetracycline. Tetracycline is used in animals feed as growth promoter and in human medicine as treatment for acne and this may have contributed to the spread of tetracycline resistance over the past 30 years (Salyers and Shoemaker, 1996).

1.8.2 Biodegradation pathways

Horizontal gene transfer has also led to the dissemination of gene clusters involved in the catabolism of xenobiotics in polluted environments. Bacteria have been isolated that are able to degrade most man-made pollutants and most of the degradative genes are part of operons of ten or more genes, or even regulons of several operons with accompanying control circuits. These operons are carried by wide host range conjugative or mobilisable plasmids (van der Meer, 1997).

1.8.3 Pathogenicity islands

Virulence factors of pathogenic bacteria (adhesins, toxins, invasins and others) may be encoded by particular regulons of the pathogenic genome termed 'pathogenicity islands'. Recent studies have discovered that horizontally acquired 'pathogenicity islands' are major contributors to the virulent nature of many pathogenic bacteria (Groisman and Ochman, 1997). These chromosomally encoded regions typically contain large clusters of virulence genes (10-200 kb in size) and can, upon incorporation, transform a benign organism into a pathogen.

The sequences flanking pathogenicity islands frequently contain short direct repeats reminiscent of those generated upon integration of mobile genetic elements and ORFs, within certain pathogenicity islands display sequence similarity to bacteriophage integrases. Many pathogenicity islands are situated at tRNA and tRNA-like loci, which appear to be common sites for the integration of foreign sequences.

Bukhalid and Loria (1997) analysing the plant pathogen, *S. scabies*, identified a pathogenic or virulence gene, *nec1*, which was sufficient to confer a necrogenic phenotype upon the non-pathogen *S. lividans 66 TK24*. The codon bias and GC content of *nec1* are atypical relative to high GC coding regions characteristic of *Streptomyces* strains suggesting that it was acquired from another taxon. Further genetic analysis of the immediate region bordering *nec1*, revealed the presence of a new insertion element,

IS*1629*, suggesting that *nec1* may have been spread among *Streptomyces* strains via transposition events (Healy *et al.*, 1999).

Examples of horizontal gene transfer are also proposed on the basis of genome analysis. Several between-genome comparisons show that all genomes contain some genes that are more similar to homologues in distant genomes than to homologues in closer relatives, or indeed, that are not found at all in genomes of closer relatives. Nelson *et al.* (1999) concluded by such reasoning that 24% of the genome of the bacteria hyperthermophile *Thermotoga maritima* has been obtained from archaeal hyperthermophiles. Lawrence and Ochman (1998) have concluded that 18% of the genes in the *Escherichia coli* K12 genome were introduced by lateral gene transfer in the past 100 million years.

Of all the claims that have been made over the years, those reporting transfers between eukaryotes and prokaryotes are the most controversial. However, glyceraldehyde-3-phosphate dehydrogenase and glucose phosphate isomerase genes are good examples of horizontal gene transfer from eukaryotes to prokaryotes while Fe-superoxide dismutase seems to follow the opposite direction of transfer (Smith *et al.*, 1992).

Overall, comparative genomics suggest that horizontal gene transfer may be the rule rather than the exception (Eisen, 2000b) playing an important role in genome shaping and in molecular evolution of microorganisms leading eventually to speciation.

1.9 Aims

The general aim of this study was to investigate to what extent horizontal gene transfer can affect the distribution and diversity of aminoglycoside genes and gene clusters in nature using streptomycin as a model system. The more specific aims were:

- To use a housekeeping gene, *trpB*, that encodes for an enzyme involved in tryptophan biosynthesis in order to refine the phylogenetic relationships in a set of diverse streptomycetes.

- To develop and apply primers and probes of genes involved in aminoglycoside antibiotic production in order to test the presence of these genes in a sample of natural isolates.

- To clarify whether and how the isolates acquire aminoglycoside genes or part of their gene cluster as a result of a gene transfer.

- To assess whether the genes, found in isolates, are clustered.

- To investigate whether the transferred genes are expressed in the new hosts.

- To elucidate the binding properties of the streptomycin pathway-specific regulator StrR.

Chapter 2

Methods and Materials

2.1 Media

All media were made with distilled water unless otherwise stated and sterilised by autoclaving at 121°C for 15 min.

Media for *E. coli*

Luria Broth (LB)	Bacto-tryptone 10 g/l Yeast Extract 5 g/l NaCl 10 g/l Adjusted to pH 7.0
LB-Agar	LB-medium Agar 15 g/l

Media for *Streptomyces* spp.

SMA-Agar	Soya 20 g/l Mannitol 20 g/l Agar 22 g/l
TSB-Medium	Tryptone Soya Broth 30 g/l

Table 2.1: Media used in this study.

2.2 Antibiotics

Where antibiotic supplemented media required, the following antibiotic concentrations were used:

Table 2.2: Antibiotics used in this study.

Antibiotic	**Stock solution (mg/ml)**	**Final concentration (μg/ml)**
Ampicillin (in SDW)	50	50
Kanamycin (in SDW)	50	50
Chloramphenicol (in ethanol)	35	35
Streptomycin (in SDW)	50	1-50

2.3 Buffers, reagents and solutions

Buffers, reagents and solutions were made with distilled water unless otherwise stated and sterilised by autoclaving at 121°C for 15 min. Any additional buffers, reagents or solutions were prepared as described in Sambrook *et al.* (1989).

Table 2.3: Solutions and buffers used in this study.

Solution / Reagent	Constituents
TE	100 mM Tris-HCL 10 mM EDTA
50 x TAE	Tris base 242 g/l Glacial acetic acid 57.1 g/l 0.5 M EDTA (pH 8.0) 100 ml/l
5 x TBE	Tris base 54 g/l Boric acid 27.5 g 0.5 M EDTA (pH 8.0) 20 ml/l
Spermine HCl	100 mM
Lysozyme solution (in TE buffer)	Lysozyme 2 g RNAse 50 mg/l Sucrose 300 g/l
20 x SSC	NaCl 175.3 g/l Tri-sodium citrate 88.2 g/l
20 x SSPE	NaCl 175.3 g/l NaH_2PO_4 27.6 g/l EDTA 7.4 g/l
50 x Denhartds	Ficoll-400 10 g/l Polyvinylpyrolidone-360 10 g/l Bovine serum albumin 10 g/l
Prehybridisation solution (35 ml) Hybridisation solution (15 ml)	20 x SSC 12.5 ml/50 ml 10% SDS (w/v) 2.5 ml/50 ml 50 x Denhartds 5 ml/50 ml Herring DNA sperm (1 µg/ml) 500 µl/50 ml

Loading buffer	Sucrose 60% 100 mM EDTA Bromophenol blue 0.25% (w/v)
Denaturation solution	10 M NaOH 50 ml/l NaCl 87.66 g/l
Neutralisation solution	Tris base 121.14 g/l NaCl 87.66 g/l Adjust to pH 8.0
Binding buffer (4x) (Gel retardation assays)	50 mM Tris-HCl pH 7.5 250 mM KCl 20 mM $MgCl_2$ 3 mM DTT 20% glycerol
Solution P1	50 mM Tris-HCl 10 mM EDTA 40 µg/ml RNAse
Solution P2	0.2 M NaOH 1% SDS
Solution P3	3 M Potassium acetate pH 5.5
Protein running buffer (5x)	25 mM Tris-HCl 250 mM glycine 0.1% SDS
Protein loading buffer	50 mM Tris-HCl 100 mM dithiothreitol 2% SDS 0.1% bromophenol blue 10% glycerol
Sonication buffer	20 mM Tris-HCl base 0.1 mM DTT
TBS	Tris base 108 g/l NaCl 150 mM
Transfer buffer	Tris 10 g/l Glycine 48 g/l SDS 1%

TBST	50 mM Tris-HCl pH 7.5 150 mM NaCl Tween 80 0.2%

2.4 Bacterial strains

Streptomycete strains were stored as suspensions of spores and mycelial fragments in glycerol (10% v/v) at –20°C (Wellington and Williams, 1978). All strains acquired from DSM, Annette Mehling (A.M.), E.M.H. Wellington culture collection (*) unless otherwise stated.

Table 2.4: Strains used in this study.

Strain name	Strain origin	Additional information
S. griseus	DSM40644	SM production
S. griseus M881	(A.M)	mutant; non SM
S. bikiniensis	DSM40581	OHSM production
S. bluensis	DSM40564	BM production
S. glaucescens	DSM40716	OHSM production
S. hygroscopicus var *glebosus*	DSM40823	BM production
S. coelicolor A3(2) production	*	Actinorhodin
S. subrutilis	DSM40445	OHSM production
S. galbus	DSM40480	SM production
S. lividans TK24	*	-
S. lividans TK23	*	-
S. humidus	DSM40386	SM production
S. fradiae	(ISP4037)	Neomycin production
Micromonospora purpurea	*	Gentamicin

S. diastatochromogenes	(ISP5745)	-
ASSF13	*	Huddleston, 1996
ASSF15	*	Huddleston, 1996
ASSF22	*	Huddleston, 1996
ASB27	*	Huddleston, 1996
ASB33	*	Huddleston, 1996
ASB37	*	Huddleston, 1996
E. coli	(ATCC29839)	SM^S
E. coli	(ATCC29842)	SM^R
TOP10 (*E. coli*)	Invitrogen	
BL21(DE3)pLysS (*E. coli* B strain derivative)	Invitrogen	Protein overexpression
STBL2 (*E. coli* JM109 derivative)	Gibco BRL	Stabilises unstable DNA

SM: streptomycin; OHSM: hydroxystreptomycin; BM: bluensomycin,
SM^S: streptomycin sensitive, SM^R: streptomycin resistant.

2.5 Plasmids

Table 2.5: Plasmids used in this study.

Plasmid	Insert	Host	Reference
pET 11a	-	BL21(DE3)pLysS	Studier *et al.*, 1990
pRBD13 (pUC18 based)	290 bp fragment containing the full palindromic StrR-binding site	TOP10 cells	Retzlaff and Distler, 1995
pB1p/2 (pUC18 based)	156 bp fragment containing one half of the palindromic StrR-binding site	TOP10 cells	Retzlaff and Distler, 1995

pSTR11 (pET based)	Full length of *strR* gene	BL21(DE3)pLysS	Retzlaff and Distler, 1995
pNstrR1 (pET based)	N-truncated form of *strR* having a deletion of the first 105 bp	BL21(DE3)pLysS	This study
pNstrR2 (pBAD based)	N-truncated form of *strR* having a deletion of the first 276 bp	TOP10 cells	This study
pNstrR3 (pBAD based)	N-truncated form of *strR* having a deletion of the first 459 bp	TOP10 cells	This study
pNstrR4 (pET based)	N-truncated form of *strR* having a deletion of the first 597 bp	BL21(DE3)pLysS	This study

2.6 Routine growth of strains

Streptomyces spores were inoculated into sterile liquid broth in a conical flask containing a spring. Spring baffles were used to favour dispersed growth since most strains tend to grow as rather compact masses or pellets of mycelium. For each 250 ml flask a 30 cm length of spring, 1.3 cm diameter coil, 19 sw gauge wire (Alliance Spring Co.) was used. Flasks were then incubated in orbital shakers at 30°C and 200 rpm until the streptomycete had grown. Crude monitoring of growth was done by packed cell volume method. Cultures were then centrifuged at 13 000 g and pellets resuspended in 10% glycerol. The mycelia could then be stored at -20°C and DNA extracted later.

2.7 Streptomycin resistance assays

Nutrient agar including the following concentrations of streptomycin sulphate were prepared in multi-well petri plates: 50, 20, 10, 5, 2, 1.5, 1, 0.5, and 0 µg/ml. 5 µl of spore

suspension was placed in each well. Growth was scored after 2 and 4 days as best (not different from growth with no antibiotic), good, poor, or absent. Tests were duplicated.

2.8 Streptomycin production bioassays

Brazilian isolates, *S. griseus* N2-3-11 and *S. lividans* TK23 were streaked to single colonies on SPM agar plates and incubated for 5 days at 30°C. Duplicates were then overlaid with overnight cultures of *E. coli* ATCC29842 (streptomycin-resistant strain) and ATCC 29839 (isogenic sensitive strain) in soft agar and incubated overnight at 37°C. Plates were examined for clear zones. The bioassay was repeated at least three times for each strain.

2.9 Molecular biology methods

2.9.1 Isolation of chromosomal DNA from *Streptomyces* (Procedure A)

This method yields DNA of a very high quality, suitable for all manipulations. Mycelia were incubated in 3 ml lysozyme solution for 2 hours at 37°C. Then 4 ml Kirby mix was added and agitated for 1 min on a vortex mixer. 8 ml of Tris-buffered phenol/chloroform was then added and sample agitated then centrifuged at 3,000 g to separate phases. The aqueous phase was then removed and re-extracted twice more with phenol/chloroform and then once with chloroform. The DNA was then precipitated by 20 min incubation at room temperature after addition of 0.1 vol 3 M sodium acetate and an equal volume of isopropanol. DNA was then pelleted by centrifugation at 8,000 g for 30 min, washed with 70% ethanol and resuspended in TE buffer. Samples were then run on an 0.8% agarose gel to assess DNA quality and quantified by comparison with the known DNA size ladder (Hopwood *et al.*, 1985).

2.9.2 Isolation of chromosomal DNA from *Streptomyces* (Procedure B)

This method is very rapid but the DNA is of poorer quality than that acquired by the use of procedure A. 50 mg mycelia were washed twice with 0.5 M sucrose and once with 0.5 M EDTA then resuspended in 500 µl lysis buffer and 10 mg ml^{-1} lysozyme. The cells were then incubated at 37°C for at least 2 hours. 125 µl of 10% SDS was then added to the suspension and gently inverted until the mixture thickened and went clear. 216 µl of 5 M potassium acetate was then added and mixed by inversion. The tube was then incubated on ice for 30 min and centrifuged at 14,000 g for 10 min at 4°C. The supernatant was then added to an equal volume of isopropanol and inverted then incubated at room temperature for 10 min. The sample was then centrifuged at room temperature at 14,000 g for 20 min. The DNA pellet was then washed in 70% ethanol and resuspended in TE buffer and 10 µg ml^{-1} RNase.

2.9.3 Isolation of chromosomal DNA from *Streptomyces* (Procedure C)

DNA was extracted from a 4 day old 50 ml broth culture of *Streptomyces* spp. grown in TSB. Cells were harvested by centrifugation at 3,000 g for 20 min, resuspended in 5 ml lysozyme solution and incubated at 37°C for 1 h with occasional agitation. 0.5 M disodium EDTA was added to a final concentration of 100 mM. SDS was added to a final concentration of 0.5% and the samples were immediately mixed by vigorous shaking. The mixture was allowed to stand for a few minutes and then 1/3 volume of phenol:chloroform (1:1) was added and was mixed well to emulsion. The mixture was further allowed to stand for another 10 min followed by centrifugation at 12,000 g for 30 min at 4°C and the upper layer decanted into a glass beaker. 7.5 M NH_4 acetate (0.5 volumes) and 1.5 volume of isopropanol was added and the mixture left at RT for 30 min. Precipitated DNA was spooled onto a fine glass rod, immersed in 70% EtOH, air dried for 2 min and resuspended in 400 µl SDW. Samples were stored at 4°C (T. Hosted, pers. comm.).

2.9.4 Alkaline lysis plasmid preparations

Small scale preparation of plasmid DNA was made using the QIAprep Spin Plasmid kit (Qiagen) according to the instructions specified by the manufacturers. Large scale preparation of plasmid DNA was made using the Qiagen Midi Kit (Qiagen) as per manufacturers instructions.

2.9.5 Cosmid isolation by alkaline lysis

10 ml of overnight grown culture (37°C) of *E. coli* harbouring the appropriate cosmids was spun down for 5 min at 6000 rpm. The cell pellet was resuspended in 100 µl of solution I by vortexing. 200 µl was removed to 1.5 ml Eppendorf tube and 400 µl of solution II was added and mixed by rapid inversion. 300 µl of solution III was then added and mixed by rapid inversion. The final mixture was centrifuged for 5 min at 13,000 rpm (Biofuge pico, Heraeus). 800-900 µl of supernatant was removed and mixed with 600 µl isopropanol followed by centrifugation for 2 min at 13,000 rpm (Biofuge pico, Heraeus). The pellet was washed with 450 µl of 70% ethanol and air-dried for 5 min. Finally the pellet was resuspended in 50 µl of sterile distilled water.

2.9.6 Agarose gel electrophoresis of DNA

Chromosomal and plasmid DNA as well as PCR products were analysed by gel electrophoresis. Agarose gels were typically between 0.8 and 2.0 % (w/v) in 1 x TAE containing 0.5 µg/ml ethidium bromide. Gels were visualised on a short wavelength UV transilluminator (UVP Inc.) Photographs were taken with a polaroid land camera and hood with a UV filter attachment using Polaroid 665 positive / negative film with 15-20

sec exposures. Gel images were also recorded using a UVP Life Sciences Grab It 2.0 system (Synoptics Ltd.).

2.9.7 DNA extraction and purification from agarose gels

DNA fragments were extracted from agarose gels using Qiagen gel extraction kits (Qiagen) or Geneclean II Kits (Bio 101) as per manufacturers instructions.

2.9.8 Enzymatic treatments of DNA

DNA digestion, ligation and dephosphorylation were carried out with the restriction enzymes, T4 DNA ligase and calf intestinal phosphatase respectively using the buffers and conditions specified by the manufacturers (GibcoBRL and New England Biolabs). Depending on the concentration of the DNA solution (plasmid or PCR products) appropriate volume of DNA (1-10 µl) was mixed with the desired restriction enzymes or combination of those (double digestions). Chromosomal DNA digestions were carried out using 15-25 µl of genomic DNA (1 mg/ml) with 1.5 µl (10 units/µl) of the appropriate restriction enzyme and incubated for 90 min at 37°C. The chromosomal digests were analysed on 0.8% agarose gel running overnight with constant voltage at 28V. Ligations were carried out with a ratio vector:insert of about 1:5.

2.9.9 Transformation of *E. coli* cells

Transformations with ligated DNA and circular plasmids of TOP10 (Invitrogen), STBL2 (GibcoBRL) and BL21(DE3)pLysS (Invitrogen) were carried out according to manufacturers instructions.

2.9.10 DNA quantification

Spectrophotometer readings were taken at 260 and 289 nm. An OD_{260} reading of 1 was taken to be equal to 50 µg/ml DNA. Purity was measured by the ratio OD_{260} / OD_{280}, a ratio of 1.8 was taken to be pure DNA (Sambrook *et al.*, 1989).

2.9.11 Polymerase chain reaction

A standard PCR amplification protocol was followed for all PCR amplifications in this study (resistance phosphotransferase *strA*, biosynthetic amidinotransferase *strB1*, tryptophan synthase *trpB*, Sm regulator *strR*). Specific variations on reaction conditions (annealing temperatures) and primers are shown in tables. Amplification reactions were performed in 50 µl reaction mixtures in 0.5 ml microfuge tubes. Amplification reactions were performed with 5 µl reaction buffer (x 10), 3 µl MgCl2 (25 mM), 2.5 µl DMSO, 2.0 µl dNTPs (10 mM each), 100 ng template DNA, 100 ng of each primer, 0.7 µl Taq DNA polymerase (0.5 units/µl). Reactions were carried out in a Hybaid Omnigene thermal cycler.

Step	*Conditions*	
Hot start	95°C for 5 min	
Denaturation	95°C for 1 min	35 cycles
Annealing	Tm (°C) variable for 45 sec	
Extension	72°C for 45 sec	
Final extention	72°C for 5 min	

Table 2.6: PCR conditions used for the amplification of gene fragments. PCR amplifications using Genome Walker kit (Clontech) were performed following manufacturer's conditions.

Table 2.7: Primers used in this study.

Primers / sequences	Target gene	Tm (°C)	Reference
StrRF1 5'-ggatcgggagggacgggcgg-3'	*strR*	62	This study
StrRR1 5'-acagccccgggccagctcgg-3'			
StrRF2 5'-ccctccgasttccccgcgcag-3'	*strR*	60	This study
StrRR2 5'-ccgatggcccggtcggacca-3'			
GSPR22F 5'-gtcgaatcattgcttccctccgattc-3'	*strR*	68	This study
RFR 5'-gatcctcacgttcgactgcggaaccc-3'			
TrpFF 5'-cccsggcatcggcccsgagct-3'	Tryprophan synthase	62	Egan *et al.*, 1998
TrpRR 5'-gaagtagcgggcggcsgtgtc-3'			
NstrR1 5'-gtgaCATATGgagccggtcgaatc-3' *Nde*I site	Generation of N'-truncated form of StrR for cloning in pET	63	This study
NstrR3 5'-ggcgacCATATGtcggcgaag-3' *Nde*I site	Generation of N'-truncated form of StrR for cloning in pET	58	This study
NstrR4 5'-ctgctCATATGgagaacccctc-3' *Nde*I site	Generation of N'-truncated form of StrR for cloning in pET	58	This study

StrRWR1 5'-gcGGATCCcgccgtcatccgacatcgc-3' *Bam*HI site	Used in combination with each of the NstrR primers for the generation of N'terminus mutated StrRs		This study
D103 5'aagAGATCTggagccaccgagatcgcc-3' *Bgl*II	Generation of N-truncated form of StrR for cloning in pBAD	62	This study
D100 5'-gaaAGATCTtcggcgaagacggtggg-3' *Bgl*II	Generation of N-truncated form of StrR for cloning in pBAD	58	This study
D101 5'-aGAATTCtcatccgacatcgctcaa-3' *Eco*RI	Used in combination with each of the D103 and D100 primers for the generation of N'terminus mutated StrRs		This study
StrAF 5'-gtgcggctgctcgaccacgac-3'	*strA*	63	This study
StrAR 5'-ccgtcctcgatgtcccacaggg-3'			
StrB1F 5'-cgaatgggacccgctggagg-3'	*strB1*	59	This study
StrB1R 5'-agccgcggcttgggcgcgg-3'			
AfsAF 5'-cacgatccgctgctggtcgc-3'	*afsA*	59	This study
AfsAR 5'-gggcgctggaagagggtcgg-3'			
ArpAF 5'-ccaaggggccttgtacttccac-3'	*arpA*	63	This study
ArpAR 5'-cgccacatctccgccacctgc-3'			

AdpAF 5'-cggtgcacgtcgatccgcgc-3'	*adpA*	59	This study
AdpAR 5'-aggagccgagctggcggcg-3'			

2.9.12 Southern blotting

Agarose gels were washed twice in denaturation solution for 20 min and once in neutralisation solution for 40 min whilst gently rotating. A tray was filled with 10 x SSC. Whatmann 3MM filter paper was laid over a glass plate within the tray to allow it to absorb. The gel was placed onto the filter paper. Amersham Hybond N membrane (pre-soaked in SDW for 5 min and 10 x SSC for 5 min) was laid on the gel. Two pieces of 3 mm blotting paper were placed above the membrane. X-ray film was placed around the gel to ensure upward capillary transfer only and paper towels and a weight were placed above the blotting paper to facilitate transfer. Capillary transfer was performed for 12 h, the filter washed in 2 x SSC and the DNA fixed to the filter using a short wave transilluminator (UVP Inc.) for 4 min (2 min either side). Filters were stored at –20°C in plastic wrap.

2.9.13 Dot blots

DNA samples to be probed were heated to 95°C and snap-chilled on ice. An equal volume of 20 x SSPE was added to each tube. The samples were then placed onto the membrane which was held in a vacuum-line manifold. The membrane was then transferred to denaturation solution for 5 min followed by neutralisation solution for 1 min, before fixing with UV light.

2.9.14 Colony blotting

Nytran nitrocellulose filters (Amersham) were orientated and laid down for 1 min on the plates containing the colonies needed to be screened. The filters were left to dry for a few seconds on 3 mm Whatman filter paper with the colony side up. Then the filters were placed with the colony side up on filter papers pre-soaked in 10% SDS (w/v), in denaturation solution, in neutralisation solution and 2 x SCC for 5, 15, 15 and 10 min respectively. The filters were dried between 2 sheets of Whatman paper and DNA fixed with to the nitrocellulose with UV for 2 min. Then 0.5 ml of 2 mg/ml proteinase K solution was overlaid on the nitrocellulose filters and evenly distributed followed by 1 h incubation at 37°C. Pre-wet Whatman papers in distilled water were placed on the filters and pressure was applied with a ruler. The papers were gently removed from the nitrocellulose filters removing the cell debris from the colonies. The filters were ready for hybridisation.

2.9.15 Hybridisation and stringency washes

Membranes to be probed were incubated with hybridisation solution following manufacturers' instructions, except non-homologous DNA which was added to a final concentration of 0.5 mg ml^{-1}. Membranes were hybridised with probe DNA overnight. After hybridisation, the membrane was washed at 65°C in 2x, 1x or 0.1 x SSPE buffers (Amersham) according to stringency required. Membranes were then sealed in polythene and exposed to X-ray film.

2.9.16 [^{32}P] labelling of probe DNA

DNA was denatured and snap-chilled on ice. The probe was labelled with [α-^{32}P dGTP] using a random primed labelling kit (Boehringer). The reaction was performed for 30 min and then stopped by addition of TE. Unincorporated label was then removed

from the probe by use of a Sephadex G25 spin column. Labelled probe DNA was then denatured before being added to the hybridisation solution.

2.9.17 Preparation of 5'-labelled DNA fragments

Plasmids pRBD13 (having 290 bp insert) and pB1p/2 (having 156 bp insert) were digested with *Eco*RI and *Eco*RI / *Hind*III respectively. The digested plasmids were treated with 1 µl of calf intestinal phosphatase (Boehringer) and run on agarose gel. The inserts were gel-extracted and labelled with [γ-^{32}P ATP] using T4 polynucleotide kinase (GibcoBRL). Unincorporated label was then removed from the probe by use of a Sephadex G25 spin column.

2.9.18 Autoradiography

Radioactive filters were placed in a Harmer film cassette containing intensifying screens with Fuji X-ray film. The cassette was stored at –70°C for a sufficient time to obtain the best exposure. The exposed film was developed in an automatic developer (Agfa Curix 60).

2.9.19 Cloning of PCR products

Purified PCR products were quantified by electrophoresis with known standards and extracted from the gel as described above. Aliquots used for cloning following manufacturers instructions (TOPO-TA cloning kit, Invitrogen). White colonies were picked, screened for plasmids and screened for correct sized inserts by digestion with restriction enzymes. For the generation of N-truncated forms of StrR, primers were engineered to carry restriction sites at the 5' end. The obtained PCR products were

cleaved with the corresponding restriction enzymes and after purification (Geneclean II kit, BIO101) they were used for a sticky-end ligation with the appropriate vector.

2.9.20 Genome walking using Genome Walker kit (Clontech)

Genome library of ASB37 was constructed and amplified by using genome-walking methodologies (Genome Walket kit; Clontech) according to manufacturers instructions. Briefly, genomic DNA was digested with restriction endonucleases that leave blunt ends. Double-stranded 'adaptors' were then ligated to blunt-ended restriction digestion products. Genomic library amplification reactions were then done by using nested adaptor oligonucleotide primers (Genome Walket kit; Clontech) and nested species specific oligonucleotide primers. Prior to sequencing the PCR products were gel extracted and cloned in TA vector (Invitrogen).

2.9.21 Cosmid library construction and packaging

The cosmid library was constructed using a SuperCos1 vector (Stratagene) as per manufacturers instructions. Partial *Sau*3A1 digestion of chromosomal DNA was performed in a total volume of 300 µl containing: 100 µg DNA, 30 µl 10 x reaction buffer, 3 µl BSA (10mg/ml), 40 µl *Sau*3A1 (160 units), and 101 µl of distilled water. The partial digest was performed at 37°C for 5 min. The packaging was performed with a Gigapack II XL Packaging extract (Stratagene) as specified by the manufacturers. The library was amplified in Stratagene XL1 Blue MR cells. 1000 clones were isolated and stored in 10% glycerol at –80°C.

2.9.22 Cosmid library screening

Cosmids were isolated from the 1000 clones by pooling the clones into groups of 12 and perform alkaline lysis DNA extraction. Each miniprep was spotted on nitrocellulose membrane and screened by hybridisation using *strA* PCR product as a probe. One group gave a positive signal and the 12 corresponding cosmids were isolated individually (by alkaline lysis method) and rescreened with the same *strA* probe. A single positive clone was identified.

2.9.23 Plasmid library construction

Chromosomal digests of ASB37 were carried out using *Stu*I enzyme. This enzyme leaves blunt-ended fragments and does not cleave within the *strR* gene sequence. The chromosomal digests were run on agarose gel and subsequently transferred on nitrocellulose filter and were probed with *strR* PCR product. A single positive signal was identified around the size of 4-5 kb and the corresponding area from the gel was extracted and purified (Geneclean II kit, BIO 101). The eluted DNA was subjected to shotgun cloning using Zero-blunt PCR cloning kit (Invitrogen). The resulting ligation was used to transform TOP10 cells (Invitrogen) which were spread on kanamycin-containing LB agar plates.

2.9.24 Plasmid library screening

The size-selected plasmid library was screened by colony blotting and hybridisation. Several colonies gave positive signals when hybridised against *strR* PCR product. The positive colonies were grown on LB medium supplemented with kanamycin and stored in 10% glycerol at -80°C. Plasmid DNA from these colonies was

isolated using Qiagen miniprep kit and was further screened by PCR (using primers targeting *strR* gene) and by restriction analysis.

2.9.25 DNA sequencing and analysis

At least three independent plasmids or PCR reaction products were used for sequencing. Approximately, 300 ng of plasmid DNA and 60 ng of PCR products were used for the sequencing reactions. DNA sequencing reactions were carried out by cycle sequencing with the Dye Terminator Kit of PE Applied Biosystems (Warrington, Cheshire, UK). Nucleotide sequences were analysed in BLAST 1.0 or 2.0 (http://www.ncbi.nlm.nih. gov/cgi-bin/BLAST/) and aligned in ClustalW (http://www2.ebi.ac.uk./clustalW/).

2.9.26 RNA extraction

RNA was extracted in a time-course fashion covering the different stages of growth of streptomycetes using RNeasy kit (Qiagen) according to manufacturer instructions.

2.9.27 cDNA synthesis and RT-PCR

1 µl of total RNA, 1 µl of the appropriate reverse primer and 2.5 µl of water were mixed in a 0.5 ml eppendorf tube. RNA and primer were denatured for 10 min at 65°C in a thermocycler and immediately were placed on ice. RNA / primer mixture was added to a mixture consisting of: 5 µl of expand RT buffer, 2 µl DTT(100 mM), 2 µl dATP (10 mM), 2 µl dTTP (10 mM), 2 µl dCTP (10 mM), 2 µl of dGTP (10 mM) and 1 µl of expand reverse transcriptase (50 units/µl; Boehringer). The total volume was 20 µl. The

final mixture was incubated for 45-60 min at 42°C and the reaction was stopped by placing it on ice. PCR of cDNA was carried out using the same condition as with the genomic DNA template.

2.10 Biochemical methods

2.10.1 Heterologous gene expression in *E. coli* BL21(DE3)pLysS

This approach is a tightly controlled T7 RNA polymerase-based gene expression in *E. coli*. *E. coli* BL21(DE3)pLysS was grown overnight in 2 ml of LB medium containing 50 µg/ml ampicillin and 35 µg/ml chloraphenicol at 37°C. This culture was diluted 1:100 with the same medium and was incubated at 37°C until the optical density (560 nm) of the cultures reached a value of 0.5. The induction of expression was started by addition of IPTG at a final concentration of 1 mM. The incubation time was between 60 and 120 min after the IPTG addition. 1 ml of culture before the IPTG addition and after the IPTG-incubation time were removed, centrifuged at 13,000 rpm (Biofuge pico, Heraeus) for 5 min and the pellets were resuspended in 40 µl of SDS-PAGE loading buffer. After boiling the samples for 5 min, they were analysed by SDS-PAGE gel electrophoresis.

2.10.2 Gel retardation assays

DNA-protein complex was formed at 25°C in 20 µl of DNA binding buffer. In general, 0.05 OD_{260} poly((dI-dC)•poly(dI-dC)) (Roche), 25 µg of sheared herring-sperm DNA, approximately 1-5 ng of ^{32}P-end-labelled fragment and 1-50 µg of protein were used for each assay. After 5 min of incubation the reaction mixture was applied to a 6% polyacrylamide gel with the running buffer containing 0.5 x TBE. The gel was run at 130

V for approximately 3 h, equilibrated in 10% acetic acid, dried and subjected to autoradiography.

2.10.3 SDS-polyacrylamide gel electrophoresis and Coomassie staining

The protein concentration of each sample was measured using the Bio-Rad assay developed by Bradford (1976) following the manufacturers instructions. Separation of proteins by 0.1% sodium dodecyl sulphate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Leammli (1970) and the gels were subsequently stained using the Coomassie-Brilliant Blue G250 reagent.

2.10.4 Cell free extracts

The cells were harvested by centrifugation (12,000 g) and the cell pellet was washed twice with 5 ml of sonication buffer and finally resuspended in 1.5 ml of the same buffer. The cells were subjected to sonication at 20 microns for 3 times of 20 seconds each. The cell debris were removed by centrifugation at 13,000 rpm (Biofuge pico, Heraeus) for 30 min (4°C). The supernatant was used as the cell-free extract and stored at –70°C.

2.10.5 Western analysis

Proteins were transferred to a PVDF-membrane (Amersham-Buchler, Braunschweig) by electroblotting using the Fastblot B33 apparatus (Biometra, Gottingen), and 0.3 x Laemmli gel running buffer containing 0.1% SDS as blotting buffer. The membrane was blocked with TBST containing 3% bovine serum albumin for 16 h at 4°C, and then probed with 1:4000 diluted anti-StrR antiserum for 30 min at 20°C.

StrR ptoteins were detected with alkaline phosphatase-conjugated mouse anti-rabbit immunoglobin G antibodies by the BM chromogenic Western blotting kit (Boehringer, Mannheim).

2.11 Phylogenetic analysis

2.11.1 UPGMA cluster analysis

Binary data from the RFLP analysis was analysed in the NTSYS package (Exeter publishing, NY) by use of SIMQUAL (simple matching coefficient), and SAHN (hierarchical clustering). Phenograms were obtained by TREE.

2.11.2 Phylogenetic analysis

All phylogenetic analyses were performed in PHYLIP (Felsenstein, 1985). Pairwise alignments were performed by Smith and Waterman local alignment algorithm (GCG) and multiple alignments by eye, then checked in CLUSTALW. Phylogenetic methods in PHYLIP include neighbour joining (DNADIST, NEIGHBOR), parsimony (DNAPARS) and maximum likelihood (MLK). Different methods of analysis were used for each dataset and groupings were the same across the different methods unless stated otherwise. Confidence limits on the branches were achieved by bootstrapping (BOOTSTRAP).

Chapter 3

Phylogeny of *Streptomyces* spp.

3.1 Introduction

Traditional taxonomic tools such as analysis of chemical or morphological characteristics can be useful in determining subgeneric relationships, but they do not accurately or fully reflect the phylogeny of *Streptomyces* (Ludwig *et al.*, 1993). Certain techniques have been used to analyse the relatedness between *Streptomyces* species, including comparisons derived from ribosomal protein sequence analysis (Kawamoto and Ochi 1998) and DNA-DNA hybridisation (Labeda 1998). Woese and Fox (1977) used molecular systematic analysis of ribosomal RNA (rRNA) molecules to provide the first useful evolutionary classification of microbes as well as a universal tree of life. All 16S rRNAs have in common three-dimensional structural elements of similar function. The primary structures are well studied and are mainly conserved with hyper-variable regions identified (Woese, 1987; Kim *et al.*, 1993). Edwards *et al.* (1989) designed 'universal' primers from highly conserved terminal regions, which amplify nearly the entire 16S rRNA gene. This allowed the subsequent analysis of entire 16S rRNA genes, which led to the determination of specific signature regions that could be used as specific target sites for different groups at different taxonomic levels. Some regions are unique at the genus level (Salama *et al.*, 1991) and more variable regions can be used for assigning groups to lower taxonomic levels (Klijn *et al.*, 1991). Stackebrandt *et al.* (1991; 1992) identified three regions that vary within 16S rRNA genes of streptomycetes; α (nt 982-998, *S. ambofaciens* nomenclature) and β (nt 1102-1122) which are suitable for resolution at the genus level. The third region, γ (nt 150-200) is the most variable and species-specific regions are normally located here. Some sequence variation between species can also occur outside of these regions. Analysis of 16S rRNA genes from a collection of *Streptomyces* showed a common insertion of 5-6 bp within the β region (Mehling *et al.*, 1995). This study designed genus and strain-specific primers from varying regions within the gene.

Phylogenetic analysis of molecular sequence is based on several assumptions, including that gene transfer or recombination of these essential genes does not occur, the evolution of the molecule reflects the evolution of the strain and that rates of evolution

are constant between groups. Gene transfer of 16S genes has never been determined definitively. It is known however, that most bacteria have several 16S rRNA operons; *Bacillus* spp. has been shown to possess eight, *E. coli* seven (Cilia *et al.*, 1996) *Streptomyces griseus* six (Kim *et al.*, 1993) and it is possible to envisage that any sequence differences between operons could result in different placements of the same strain in a phylogenetic tree. Ninet *et al.* (1996) examined two copies of the 16S rRNA gene from a mycobacterium strain and demonstrated that the sequences differed at 18 positions. These differences resulted in the phylogenetic analysis placing the genes at quite separate branches. Wang *et al.* (1997) demonstrated the presence of two distinct functional expressed types of 16S rRNA in *Thermobispora bispora*, which contains four operons. Sequence of the entire genes showed that 98 nucleotides were different, with 6 deletion-insertion regions, none of which occurred in the invariable or rarely variable nucleotides.

The high level of similarities within 16S rRNA genes also appears to facilitate recombination, both within the strain (Liu and Sanderson, 1998) and, it has been postulated, between closely related species. Haukka *et al.* (1996) found two 16S rRNA sequences from one strain that differed in six nucleotides and suggested that this could be explained by horizontal gene transfer. Recombination of homologous DNA leading to chimeric molecules composed of parts of two different sequences has been illustrated with 16S rDNA sequences. Mylvaganam and Dennis (1992) sequenced 16S rRNA genes from two non-adjacent operons from the archaebacterium *Haloarcula marismortui*, and analysis showed that there were 74 nt differences.

In contrast, the phylogenetic utility of 16S rRNA gene sequences has been assessed by many studies. Eisen (1995) analysed all available *recA* genes from many different bacteria, representative of all major groups, including $\alpha, \beta, \gamma, \delta$ and ε proteobacteria, cyanobacteria and high-GC Gram-positives. He found that the *recA* derived tree was mostly congruent with that of 16S rRNA, but some differences were observed. Snel *et al.* (1999) showed that a distance tree based on number of genes shared between genomes is remarkably similar to the tree based on rRNA sequences for those same species. It is important to note that these gene-content trees represent averages of patterns produced by phylogeny, gene duplication and loss, and horizontal transfer and

are not real phylogenetic trees. The fact that these 'trees' are very similar to phylogenetic trees of single genes (such as those for rRNA or *recA*) suggests that there may be an average phylogenetic history to species that is accurately reflected in the rRNA of life.

But even if 16S rRNA genes provide an accurate picture of the history of species, the high degree of conservation within 16S rRNA genes may cause problems for resolving phylogenetic relationships between some closely related species. This was well demonstrated in the phylogenetic analysis of *Micromonospora* spp. by Koch *et al.* (1996), where many groupings could not be resolved, due to little variation within the genes; identity between *Micromonospora* spp in this study ranged from 96.95 to 100%, and most were over 98% identical.

The high similarities between 16S rRNA genes from different genera and particularly within a genus indicates that analysis of other essential 'housekeeping' genes may be useful in determining phylogenetic relationships of closely related strains. Many studies have analysed a second gene to further define phylogenetic relationships of subspecies of bacteria (Thampapillai *et al.*, 1994; Christensen and Olsen, 1998; Wang *et al.*, 1997). Comparison of different genes can help identify which genes are useful for phylogenetic analysis and identify genes that may have different evolutionary histories. A second gene phylogeny can also support one derived from 16S rRNA genes. A further study by Ludwig *et al.* (1993) analysed *tuf* (EF-tu protein chain) and *atpD* (ATP synthase B-subunit) genes from different bacteria and again showed similar phylogenetic relationships to those obtained with 16S rRNA. However, the EF-tu gene has also been shown to be subject to horizontal gene transfer (Ke *et al.*, 2000). It would also be advantageous if the second gene was functionally unrelated to the other marker genes, e.g. a gene coding for a protein involved in amino acid metabolism and a gene involved in DNA replication, transcription or translation. Some of these genes do differ in their usefulness for resolving phylogenies of very closely related bacteria. Ludwig *et al.* (1993) reported that *atpD* was superior to *tuf* at resolving the phylogenetic relationships of bacteria.

Tryptophan biosynthetic genes in streptomycetes are one of the best-studied examples for gene-enzyme relationships, gene structure and control of expression (Hu *et al.*, 1999). TrpB polypeptides are more homologous in all bacteria studied than any other

tryptophan biosynthetic enzymes (Hu *et al.*, 1999). Ahmad *et al.* (1990) first used genes involved in aromatic amino acid biosynthesis to examine phylogenetic relationships of bacteria. The authors examined the ordering of tryptophan synthase genes from the biosynthetic gene cluster of many enteric bacteria and showed that this could help to further refine the relationships of enteric bacteria.

This study aimed to analyse the phylogenetic relationships of a group of phenotypically diverse *Streptomyces* natural isolates. Huddleston *et al.* (1997) analysed a collection of *Streptomyces* isolates from a site in Brazil where antibiotic production was implicated in the poor performance of nodulating bradyrhizobia inoculants. The authors found high levels of streptomycin resistance in soil isolates, which were diverse both phenotypically and genetically. Six of the original set of isolates were chosen that represented the wide range of phenotypic groups isolated from the soils and phylogenetic relationships to each other and known streptomycin-producing type strains determined by 16S rRNA sequence analysis. The distribution of streptomycin production was examined in comparison to the clades formed by many streptomycete type strains by 16S sequence analysis. However, phylogenetic analysis of near-full 16S rRNA sequences (Fig. 3.1) yields a tree in which all of the isolates, with the exception of ASSF15 and ASB37 (*S. coelicolor*-like strains) group in an unresolved clade with *S. griseus* (Wiener *et al.*, 1998). Here, a second 'housekeeping' gene, *trpB* was also analysed from the isolates and most streptomycin producers, to compare with the trees obtained from 16S rRNA sequence analysis and allow further refinement and resolution of phylogenetic relationships of these strains.

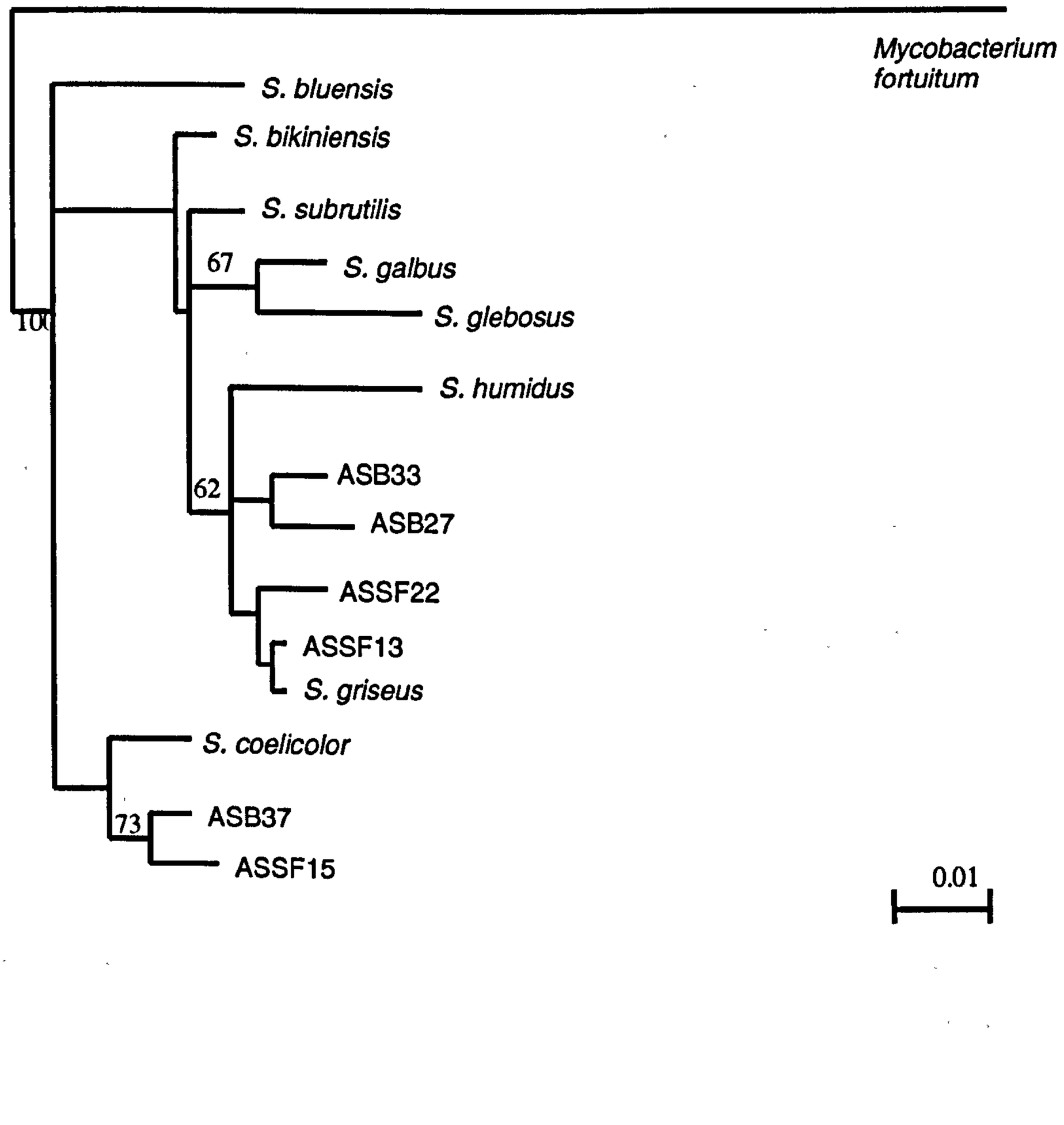


Figure 3.1: Phylogenetic relationships of isolates and type strains based on 16S rRNA sequences (1181 bp). *Mycobacterium fortuitum* was used as the outgroup. Strains included *Mycobacterium fortuitum, S. glebosus, S. bikiniensis, S. griseus, S. humidus, S. coelicolor, S. subrutilis* and natural isolates: ASSF13, ASSF15, ASSF22, ASB27, ASB33, ASB37. Bootstrap values over 60% (as derived from maximum likelihood analysis) are shown. The scale bar represents 1 % dissimilarity (Wiener *et al.*, 1998).

3.2 Results

3.2 Phylogenetic relationships of *Streptomyces* spp. derived by analysis of a housekeeping gene *trpBA*

To further examine the phylogenetic relationships of the Brazilian isolates, in particular to try and further refine the *S. griseus* group and show supportive evidence for the groupings of ASSF15, and ASB37 with *S. coelicolor*, the tryptophan biosynthetic gene fragment *trpB* was chosen for sequence analysis. *Mycobacterium tuberculosis* was chosen as an outgroup, it being the most similar to all *trpB* sequences of *Streptomyces* and not in the genus. This gene fragment was shown to be more variable than the 16S rRNA gene (Table 3.1).

Examination of the *trpB* tree (Fig. 3.2) shows that ASSF15 and ASB37 once more group with *S. coelicolor*, and ASSF13 and ASSF22 are very closely related to *S. griseus*. In contrast to the tree derived from analysis of the 16S rRNA sequences, the remaining two isolates, ASB27 and ASB33, cluster together, significantly well away from the *S. griseus* cluster.

The differentiation of ASB27 and ASSF15 from ASSF13 and ASSF22 is not consistent with the 16S tree although the two pairs of isolates had many differing characteristics, including some key factors that were 'most diagnostic' in identification of Cluster 1 strains (Huddleston, 1996). Detailed analysis of the higher order secondary structure of 16S rRNA genes has shown that the hypervariable gamma region of the 16S rRNA gene forms a stem-loop structure (Kim *et al.*, 1993; Fig. 3.3). Detailed analysis of the stem structure of the hyper-variable gamma region of both ASB27 and ASB33 supported this, and suggested that these two isolates were distinct from the other two

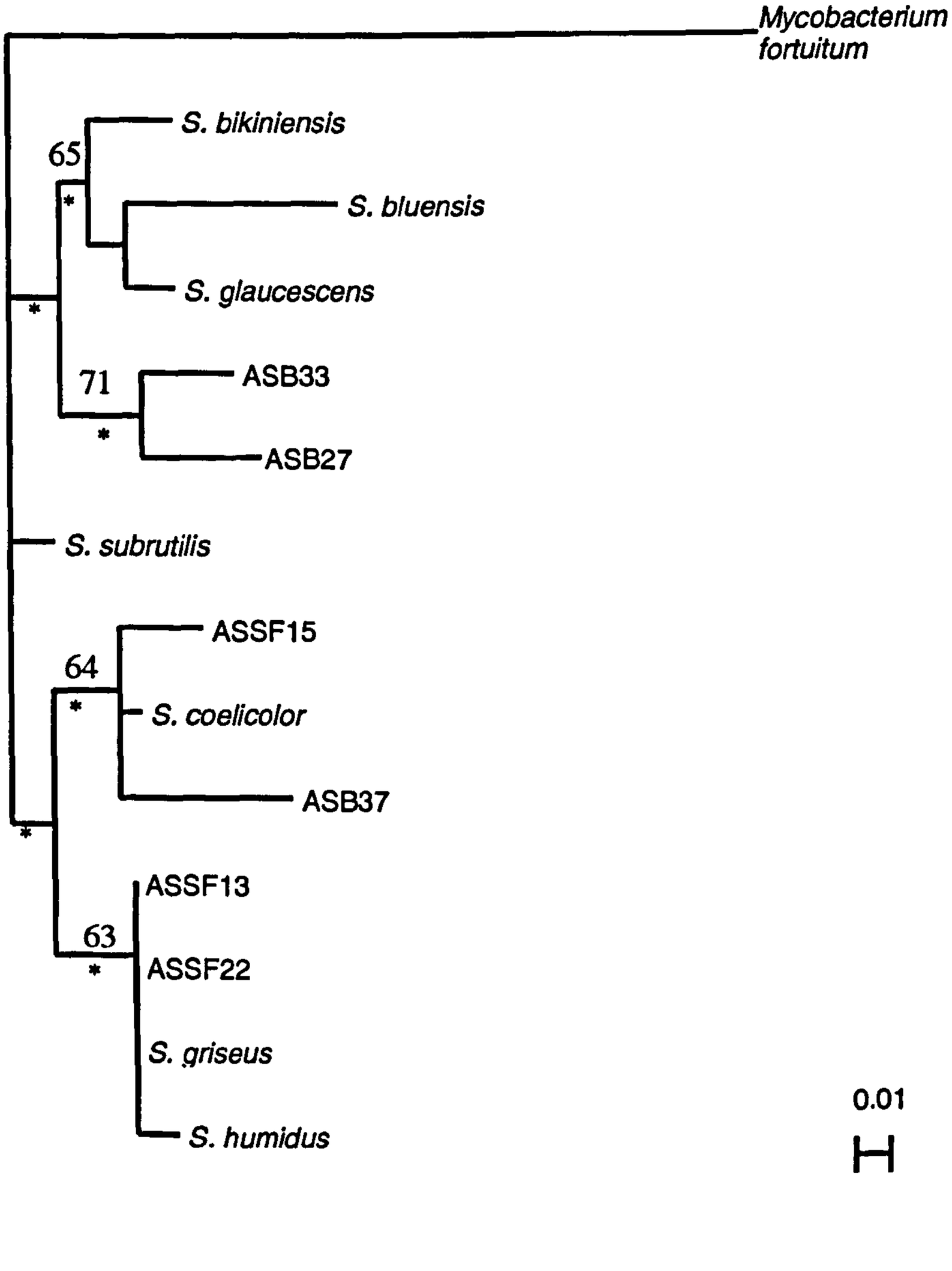


Figure 3.2: Phylogenetic relationships of isolates and representative type strains based on partial *trpB* sequence (185 bp). Third codon nucleotides were masked during the phylogenetic analyses to ensure that third position bias did not drive the tree topology. Bootstrap values over 60% (as derived from maximum likelihood analysis) are shown. Asterisks indicate branches which were also recovered in the parsimony and neighbor-joining analyses (Bootstrap values>75%). The scale bar represents 1 % dissimilarity.

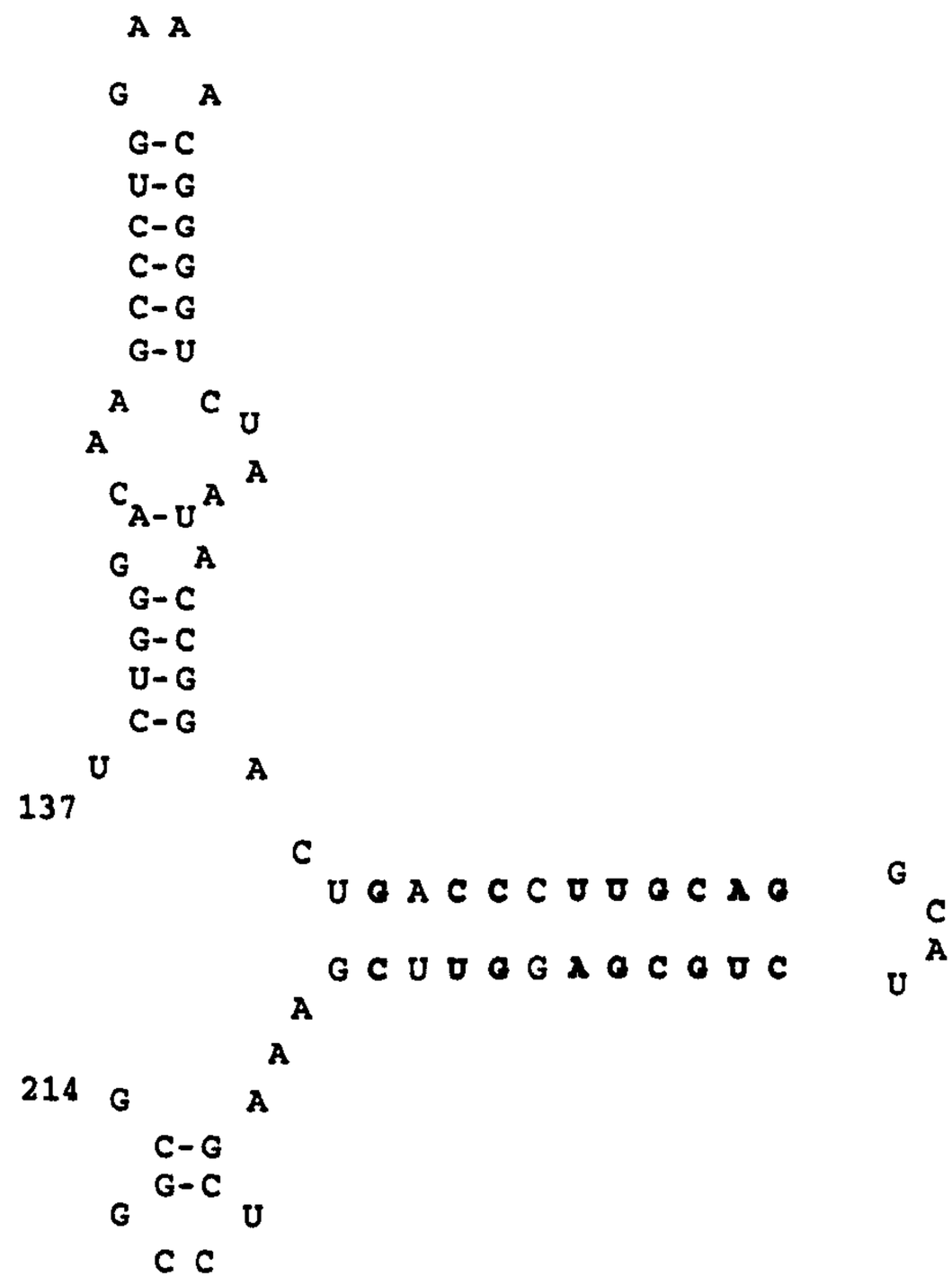


Figure 3.3: Putative secondary structure of part of the 16S rRNA molecule, including the gamma region (V2) for ASB37. Nucleotides in bold are analysed in Table 3.1.

Table 3.1: Distribution of gamma region 16S rRNA signature nucleotides in *Streptomyces* isolates and type strains.

Nucleotide position in 16S rRNA.*	Nucleotide pair in:			
	S. griseus DSM 40644 ASSF13, ASSF22	*S. coelicolor*[1] ASSF15, ASB37	ASB27	ASB33
176-201	C-G	G-C	C-U	C-U
177-200	C-G	C-U	C-G	C-G
178-199	U-G	C-G	U-G	U-G
179-198	U-G	U-A	U-G	U-G
180-197	G-C	C-G	C-G	C-G
181-196	U-A	G-C	C-G	U-A
182-195	C-G	C-G	A-U	C-G
183-194	C-G	A-U	A-U	G-C
184-193	C-G	G-C	A-U	C-G

* *S. ambofaciens* nomenclature
[1] E.M.H Wellington culture collection

groups containing *S. griseus* and *S. coelicolor.* ASB27 and ASB33 also show a common substitution of two amino acid residues, glycine and serine, which were not observed in any other streptomycetes or *M. tuberculosis* from the alignment (Fig 3.4; residue position 89 and 90). These two strains were also more similar to each other than to any other strains. The *trpB* gene and protein sequences of ASSF15 and ASB37 are very similar to *S. coelicolor,* as expected, even in the more variable gamma chain region (Fig 3.3). This was consistent with the phenotypic examination of these strains as they showed spiral spore morphology and production of blue and red pigments (Wiener *et al.*, 1998). The sequence of the genes from both ASSF13 and ASSF22 are identical to that of *S. griseus,* which supports the 16S rRNA tree.

Clearly, there is insufficient heterology in the 16S rRNA genes of these two isolates with *S. griseus* to show separation. Phylogenetic analysis of *trpB* provides strong evidence for supporting the separation of these isolates from *S. griseus.* Therefore, phylogenetic analysis of another housekeeping gene like *trpB*, can be useful for the determination of intra-generic relationships within the genus *Streptomyces*.

3.3 Discussion

As previously seen with other phylogenetic analyses of actinomycetes (Koch *et al.*, 1996) the 16S rRNA gene, although informative about some groups' relationships, may often not allow all areas of the phylogeny of a group of strains to be resolved. The 16S rRNA tree for the large collection of streptomycetes (Fig 3.5) showed that although some roots for primary clusters had low bootstrap values, other branches had high confidence limits and many of the groupings were similar to those observed from other methods, such as phenotypic analysis. The overall topology is consistent with previous analyses of 16S rRNA sequences (Kim *et al.*, 1996; Stackebrandt *et al.*, 1992) and similar to the phenogram (Fig 3.1).

Analysis of the 16S rRNA hypervariable gamma region in the *Streptomyces* isolates containing *strA* produced groupings (Table 3.1) which were also recovered in the phylogenies derived from the *trpB* analysis (Fig. 3.2). This suggests that in the

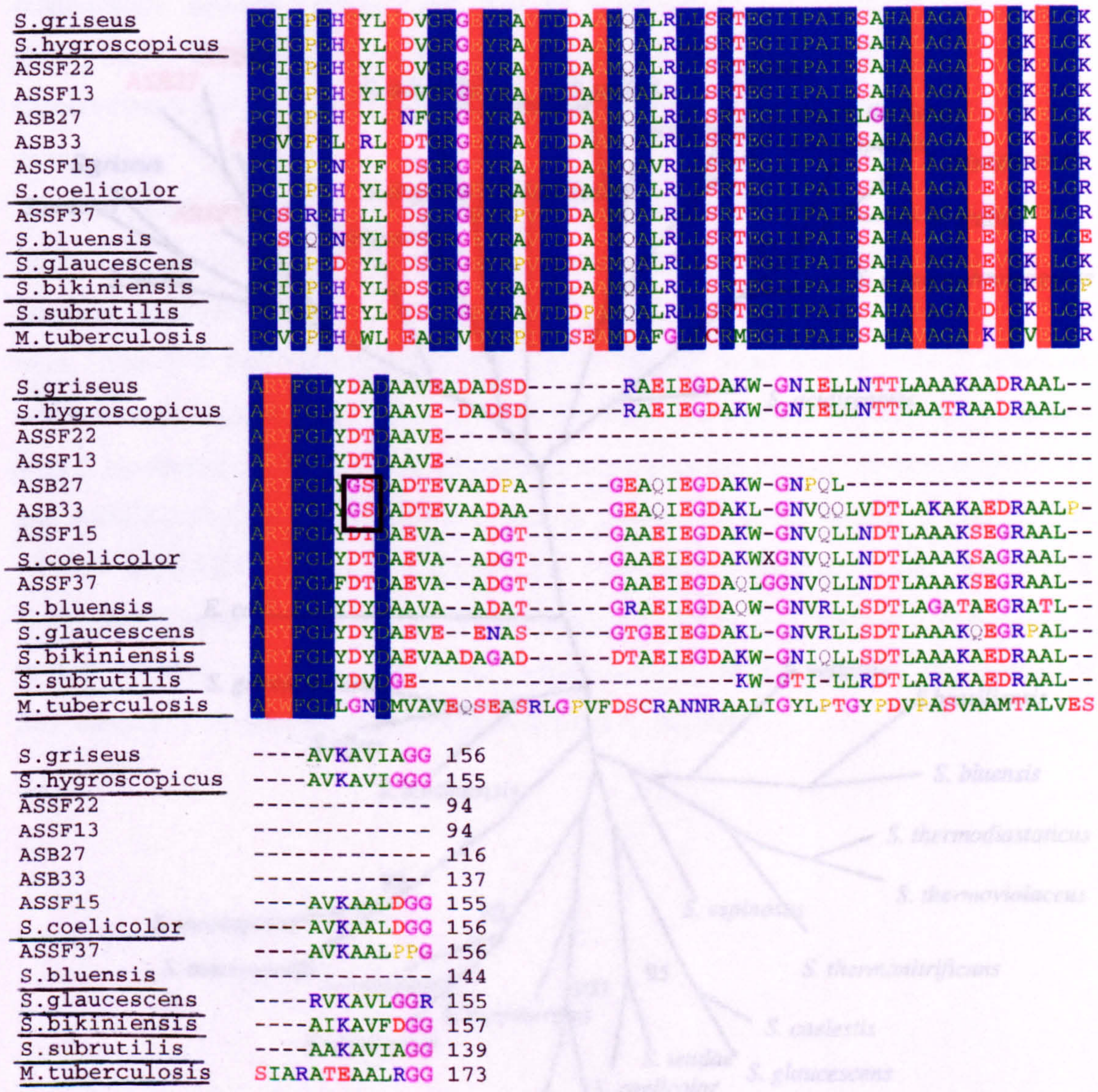


Figure 3.4: Alignment of the deduced amino acid sequence of tryptophan synthase gene from a diverse set of streptomycetes and *M. tuberculosis*. The unique substituted amino acids in the sequence of ASB27 and ASB33 are boxed. Identical residues are shown in blue background and similar in red background.

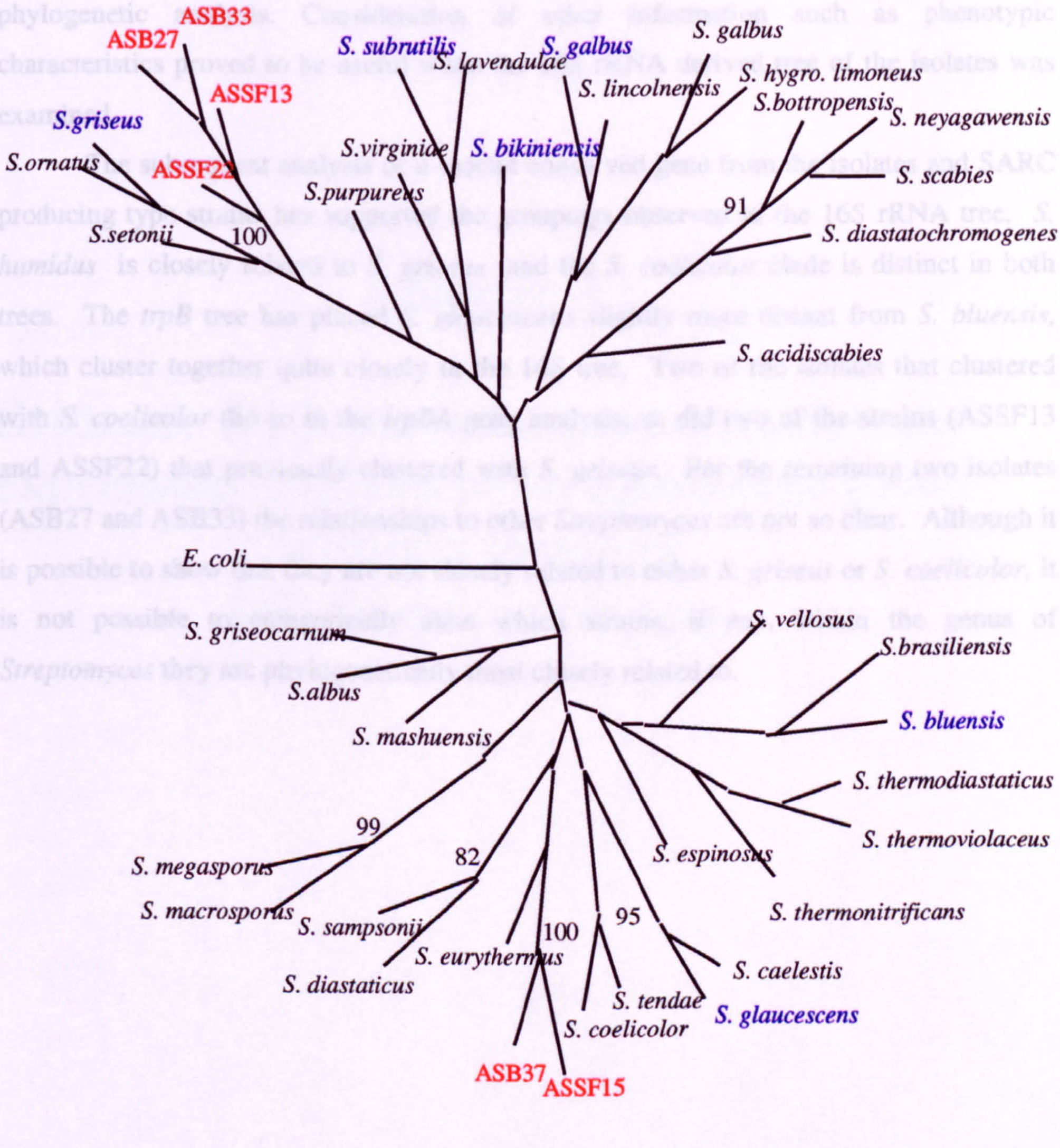


Figure 3.5: Phylogenetic analysis of 16S rRNA sequence of various streptomycetes based on partial sequence (1100 bp). SARC-producing type strains are highlighted in blue and Brazilian isolates in red. Tree is derived by parsimony analysis. Bootstrapped 100 times, values over 80% are shown. *E. coli* used as an outgroup. (Analysis performed by P. Wiener).

Streptomyces genus, the gamma region can be informative, particularly when examining relationships between strains that are not resolved by full-sequence 16S rRNA phylogenetic analysis. Consideration of other information such as phenotypic characteristics proved to be useful when the 16S rRNA derived tree of the isolates was examined.

The subsequent analysis of a second conserved gene from the isolates and SARC producing type strains has supported the groupings observed in the 16S rRNA tree. *S. humidus* is closely related to *S. griseus* and the *S. coelicolor* clade is distinct in both trees. The *trpB* tree has placed *S. glaucescens* slightly more distant from *S. bluensis,* which cluster together quite closely in the 16S tree. Two of the isolates that clustered with *S. coelicolor* did so in the *trpBA* gene analysis, as did two of the strains (ASSF13 and ASSF22) that previously clustered with *S. griseus.* For the remaining two isolates (ASB27 and ASB33) the relationships to other *Streptomyces* are not so clear. Although it is possible to show that they are not closely related to either *S. griseus* or *S. coelicolor,* it is not possible to categorically state which strains, if any, within the genus of *Streptomyces* they are phylogenetically most closely related to.

Chapter 4

Evidence for horizontal gene transfer and expression analysis of streptomycin genes within a group of soil *Streptomyces* spp.

4.1 Introduction

It has been proposed that resistance genes may have been originated from bacteria producing antibiotics (Davies, 1994). Confirmation of transfer of resistance genes from producers is illustrated from the finding that mycobacteria possess tetracycline-resistant determinants identical to those in the tetracycline-producing *Streptomyces rimosus* (Pang *et al.*, 1994). Based on high stringency hybridisation rather than sequence comparisons it has been shown the transfer of *tetKL* genes from *Streptomyces* spp. to *Staphylococcus* spp. (Salyers *et al.*, 1998). Two different clusters (*vanA* and *vanB*) can induce glycopeptide resistance to enterococci. The amino acid similarities and orientation of these genes resemble those found in glycopeptide-producing bacteria (Marshall *et al.*, 1998) indicating that the latter organisms may serve as a source of clinically relevant vancomycin resistance. However, the differences in amino acid compositions of *van* genes and G+C contents of flanking DNA regions do not indicate a direct transfer event (Marshall *et al.*, 1998). These inducible gene clusters are found on plasmids, transposons (Quintiliani and Courvalin, 1996) and even conjugative chromosomal elements (Quintiliani and Courvalin, 1994) indicating their possible dissemination into other bacterial species. Recently, *vanHBX* cluster with the same gene organisation as have been described in the above organisms was found in *S. coelicolor* A3(2) by the genome sequencing project (cosmid SC66T3).

Evidence of gene transfer in other actinomycetes is provided by analysis of 1,3-dichloropropane and 1,2-dibromoethane degradative pathways in *Rhodococcus* strain and two phylogenetically distinct bacteria. Site-specific integration as a mechanism of horizontal gene transfer may be responsible for the evolution of these degradative pathways (Poelarends *et al.*, 2000). Integrases were found associated with conserved segments of haloalkene dehalogenase gene in *Pseudomonas pavonaceae* 170 and *Mycobacterium* sp. strain GP1 when compared with the putative haloalkene-degradative donor *Rhodococcus rhodochrous*. Interestingly, in one organism these integrases were flanked by an IS. These integrases may recruit unrelated catabolic genes and disseminate them with the help of IS, mimicking in that way the features of integrons for the spread of antibiotic resistance (Poelarends *et al.*, 2000).

Linear plasmids ranging in size from 12 kb and 1 Mb have been found in actinomycete genera such as *Streptomyces* (Kinashi *et al.*, 1987), *Nocardia*, *Rhodococcus* and *Mycobacterium* (Kalkus *et al.*, 1990). The designation 'giant linear plasmid' is often used to describe the larger linear plasmids; however, there is no evidence for any essential difference between such large plasmids and smaller linear plasmids. Large linear plasmids confer advantageous phenotypes and have been shown to carry genes encoding antibiotic biosynthesis, resistance to heavy metals and ability to break down xenobiotics (Table 4.1). Members of the genus *Rhodococcus* have the ability to utilise isopropylbenzene as the sole carbon and energy source. *Rhodococcus erythropolis* was shown to contain a giant linear plasmid; loss of the plasmid was accompanied by the loss of isopropylbenzene and trichlorethene degragation. In addition, resistance to arsenite and also mercury was shown to be plasmid-encoded in this bacterium (Kesseler *et al.*, 1996).

Most of the genes directing the synthesis of antibiotics in streptomycetes appear to be chromosomal. There are several cases where the genetic evidence (high frequency transfer of biosynthetic determinants by protoplast fusion) suggests that a character is encoded by a plasmid but no plasmid can be physically detected by conventional methods (Stonesifer *et al.*, 1986). However, pulsed-field gel electrophoresis experiments have shown that a number of antibiotic-producing *Streptomyces* spp. harbour linear plasmids (Kinashi *et al.*, 1987). Moreover, plasmid SCP1 in *Streptomyces coelicolor* A3(2), which has been demonstrated by genetic methods to encode methylenomycin biosynthesis and resistance genes, is a 350-kb giant linear plasmid (Kinashi *et al.*, 1987). Evidence has been provided suggesting that a 520-kb linear plasmid (pKSL) may be required for the production of lasalocid acid and echinomycin (Kinashi *et al.*, 1987) and that a 17-kb linear plasmid may be involved in the production of the lankacidin group of antibiotics by *S. rochei* (Hayakawa et al., 1979). *S. fradiae* encodes a plasmid-like element that contains tylosin genes, amplifiable DNA and several antibiotic resistant genes which is similar to the SCP1 plasmid of *S. coelicolor* (Stonesifer *et al.*, 1986). Irrespective of whether actinomycete linear plasmids contribute to the formation of antibiotics or not, all linear plasmids detected so far are conjugative (Hopwood and Kieser, 1993).

Organism	*Plasmid*	*Size (kb)*	*Phenotype attributed*	*Reference*
Rhodococcus erythropolis	pBD2	210	Isopropylbenzene and trichlorethene catabolism, arsenite and mercury resistance	Kesseler *et al.*, 1996
***Rhodococcus* spp.**	pHG201	270	Autotrophy	Kalkus et al., 1990; 1993
	pHG204	180	Tallium resistance	
	pHG205	280	Autotrophy	
S. coelicolor	SCP1	350	Methylenomycin synthesis	Kinashi *et al.*, 1987 Stonesifer *et al.*, 1986
S. fradiae		420	Tylosin synthesis	
S. lasaliensis	pKSL	520	Lasalocid A synthesis	Kinashi *et al.*, 1987
S. parvulus		520	Actinomycin D synthesis	Kinashi and Shimaji, 1987
S. rimosus	pPZG101	387	Cryptic	Gravius *et al.*, 1994
S. venezuelae		130	Chloraphenicol synthesis	Kinashi and Shimaji, 1987
***Streptomyces* spp.**	pRJ3L	322	Mercury resistance	Ravel *et al.*, 1998; 2000
	pRJ28	330	Mercury resistance	

Table 4.1: Phenotypes attributed to linear plasmids.

Conjugation provides bacteria with the ability for horizontal gene transfer, which is the most cases limited to one species or at least to closely related organisms. Among actinomycetes, i.e. *Rhodococcus* and *Streptomyces*, conjugative transfer of DNA might

cross the species barrier. The catabolic genes of pBD2 plasmid in *Rhodococcus erythropolis* exhibit 55%-78% identity to analogous enzymes from Gram-negative bacteria (Kesseler *et al.*, 1996). Mating is possible between different *Streptomyces* species. Mercury resistant genes were located on giant linear plasmids in two Chesapeake Bay *Streptomyces* isolates (Ravel *et al.*, 1998) which can be transferred between *Streptomyces* species. In addition, the large *Streptomyces* plasmids encoding mercury resistance are widespread in mercury-contaminated sediments having an important role in gene transfer between streptomycetes in the environment (Ravel *et al.*, 2000).

4.1.1 Mechanisms of horizontal gene transfer in actinomycetes

4.1.1.1 Plasmids

Plasmids were originally thought to be the only elements to be transferred by conjugation, the mechanism that is capable of mediating very broad host range transfers. Conjugative R plasmids are responsible for most of the dissemination of antibiotic resistance genes. One of the best studied is R100 which contains transposons encoding tetracycline, mercury and chloramphenicol resistance. In a study using phyloplane ecosystem (Sundin *et al.*, 1994) streptomycin resistance transposon Tn*5393* was found on different plasmids associated with a wide range of *Pseudomonas syringae* chromosomal genotypes. These results suggest that plasmid transfer and transposition play an important role in the dissemination of antibiotic resistance.

Many plasmids have been characterised physically in streptomycetes and almost all those tested are conjugative (Hopwood *et al.*, 1983). Most are covalently closed circular DNA (CCC) ranging in size from less than 4 kb to more than 200 kb and with copy numbers between one and several hundreds. Interestingly, streptomycetes also contain double-stranded linear DNA plasmids of a wide range of sizes (Table 4.1). A typical example of broad host range conjugative plasmid in actinomycetes is pIJ101 (Fig. 5.1). The *tra* functions in this plasmid are regulated at the transcriptional level by

the4*korA* repressor and are responsible for intermycelial plasmid transfer. The *spd* functions mediate the intramycelial transfer of the plasmid. Plasmid-mediated gene transfers of this kind are restricted to closely related bacteria because homologous recombination is required to integrate the transferred DNA.

Another speciality of streptomycetes is the widespread occurrence of elements which occur integrated in the host chromosome but can excise to become autonomous plasmids. SLP1 of *S. coelicolor* and pSAM2 of *S. ambofaciens* can exist as autonomous plasmids with their own replication and transfer functions but they can also integrate by site-specific recombination into the host genome. The plasmid and host attachment sites *attP* and *attB* respectively, have at least 44 bp of DNA in common (Kieser and Hopwood, 1991). Integration and excision of the pSAM2 integrative plasmid is mediated by plasmid-encoded genes (*int/xis*). The attachment site in the host chromosome is part of a tRNA gene which is not disrupted by plasmid integration. The SLP1 and pSAM2 *att* sites would specify tyrosine and proline tRNAs, respectively. Since the tRNA sequence is highly conserved within actinomycetes, the host range of the plasmid seems to be broader when it is used for integration than when used as an autonomously replicating molecule (Hopwood and Kieser, 1993). Free CCC forms have never been detected in strains containing integrated SLP1. This has been attributed to a specific *imp* (inhibition of maintenance of plasmid) function that is deleted from the autonomously replicating SLP1 derivatives. In both pSAM2 and SLP1 plasmids, all the functions needed for integration are clustered (Hopwood and Kieser, 1993).

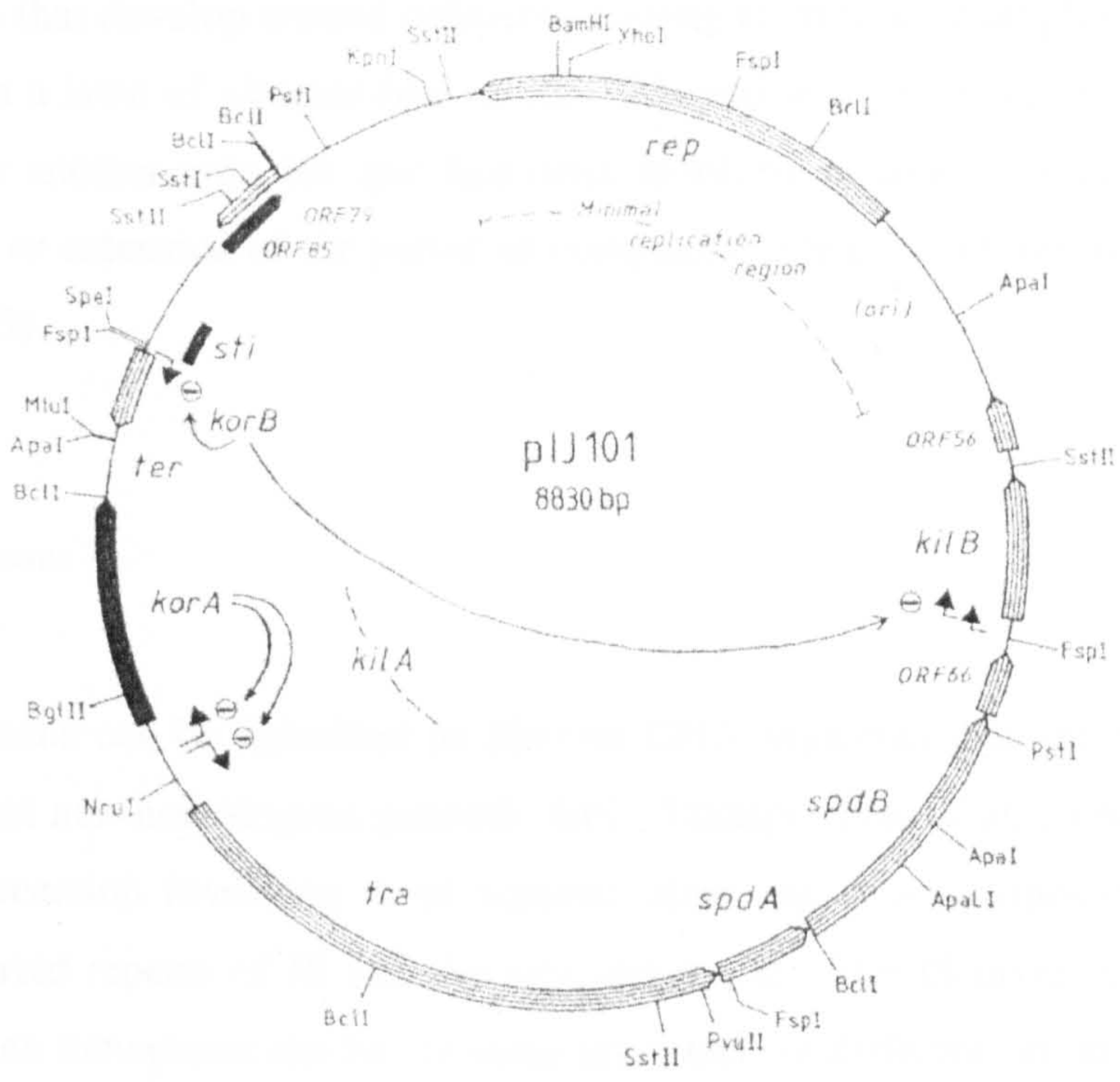


Figure 4.1: Functional map of pIJ101. Genes for intermycelial transfer (*tra*) and intramycelial (*spd*) are indicated. (Reproduced from Hopwood and Kieser, 1993).

4.1.1.2 Conjugation mechanism

The streptomycete plasmids do not encode genes involved in the establishment of a physical connection between donor and recipient. Since the non-motile streptomycetes grow as branched mycelium there might be no need for a specific aggregation system. The hyphea of donor and recipient might meet simply by growing together. Tra is the only essential protein for plasmid transfer and chromosome mobilisation. In most plasmids, *tra* is part of a Kor regulatory system. KorA is a DNA binding protein that represses tra and its own transcription. Unregulated expression of *tra* results in cell death. Downstream of *tra* follows the *spd* functions which may be involved in the intramycelial colonisation of the transferred plasmid. Streptomyces conjugative plasmids (linear or circular) are associated with pocks, macroscopically visible, circular areas of

retarded growth that develop around colonies growing from individual plasmid-carrying spores seeded in a lawn of plasmid-free spores. Disruption of *spd* reduces the pock size suggesting also another role for *spd* functions involved in quicker establishment of hyphal contacts or extension of the period of competence for plasmid transfer (Hopwood and Kieser, 1993).

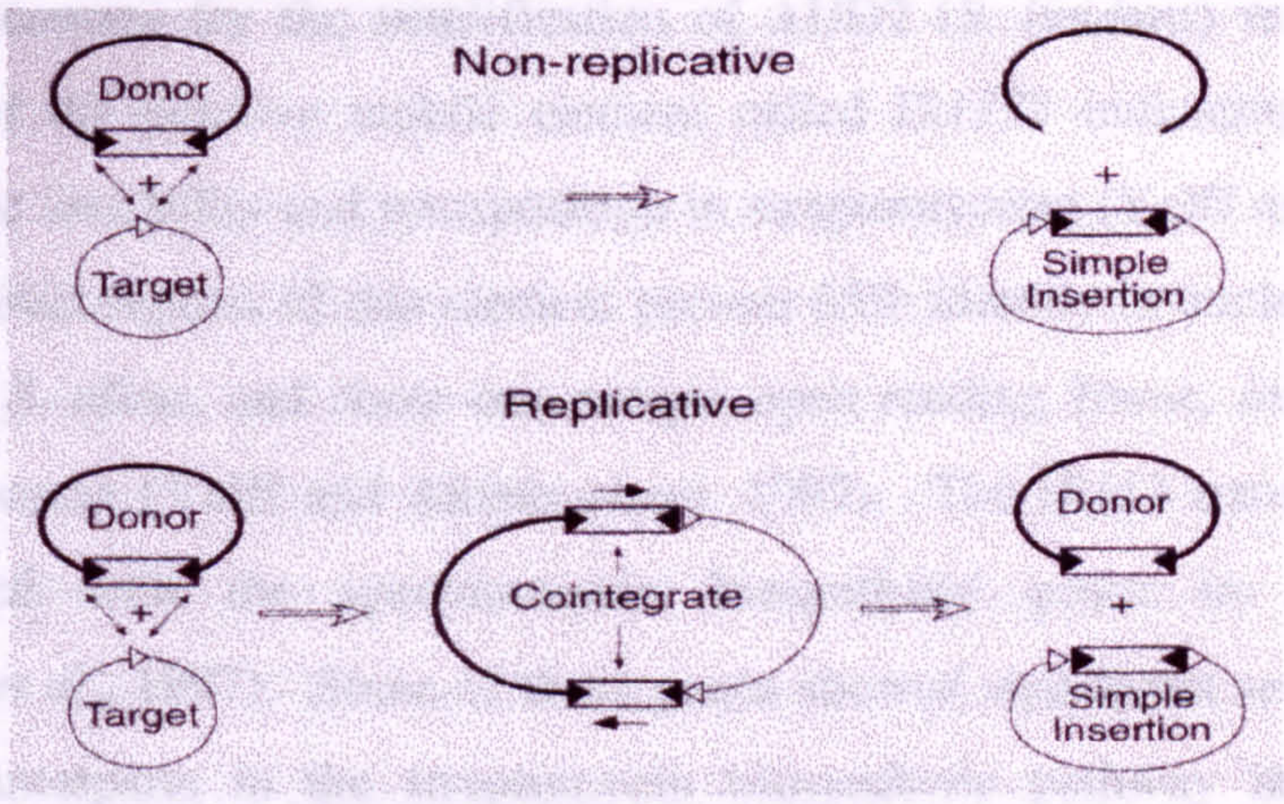
4.1.1.3 Transposons

Transposons can be described as discrete DNA segments that are able to move between different non-homologous genomic loci. Transposition of an element is thus a recombination reaction involving three separate sites: the two transposon ends often containing inverted repeats of IS and the new target site. The IS inverted repeats that flank the complex transposon can be the same (symetric) or different (asymetric). These transposons are probably formed by transposition of ISs to either side of a region of DNA. Indeed, any region of chromosomal DNA which becomes flanked by the same IS can potentially become a transposon. Transposase is encoded by a gene located within the element and binds specifically to inverted repeated sequences (Hallet and Sheratt, 1997). There are two modes of transposition, the replicative and the non-replicative one. Both modes of transposition usually generate short target duplication flanking the element in the new target locus (Fig 4.2). Several bacteriophages are in fact transposons or transposing bacteriophages. For example, bacteriophage Mu is a very large transposon that encodes not only the enzymes that regulate its transposition but also a large number of structural proteins necessary to construct the virion packaging (Graur and Li, 1999).

Transposons and insertion elements have been found in some members of the *Streptomyces* genus. IS*117* is a 2.5-kb transposable element of *S. coelicolor* A3(2) which has a strongly preferred integration site (Kieser and Hopwood, 1991). The CCC form, the so-called 'mini-circle', is a non-replicating transposition intermediate. The sequence of IS*117* shows one large open reading frame (ORF1), which may encode for a transposase/integrase function. Proteins with similar sequence have also been identified within insertion elements IS*110* of *S. coelicolor* A3(2), IS*116* of *S. clavuligerus* and

IS*900* of *Mycobacterium paratuberculosis*. There is no resemblance to tRNA gene at the integration point. Integration of the minicircle does not cause the target site duplication typical of most transposable elements (Kieser and Hopwood, 1991).

A



B

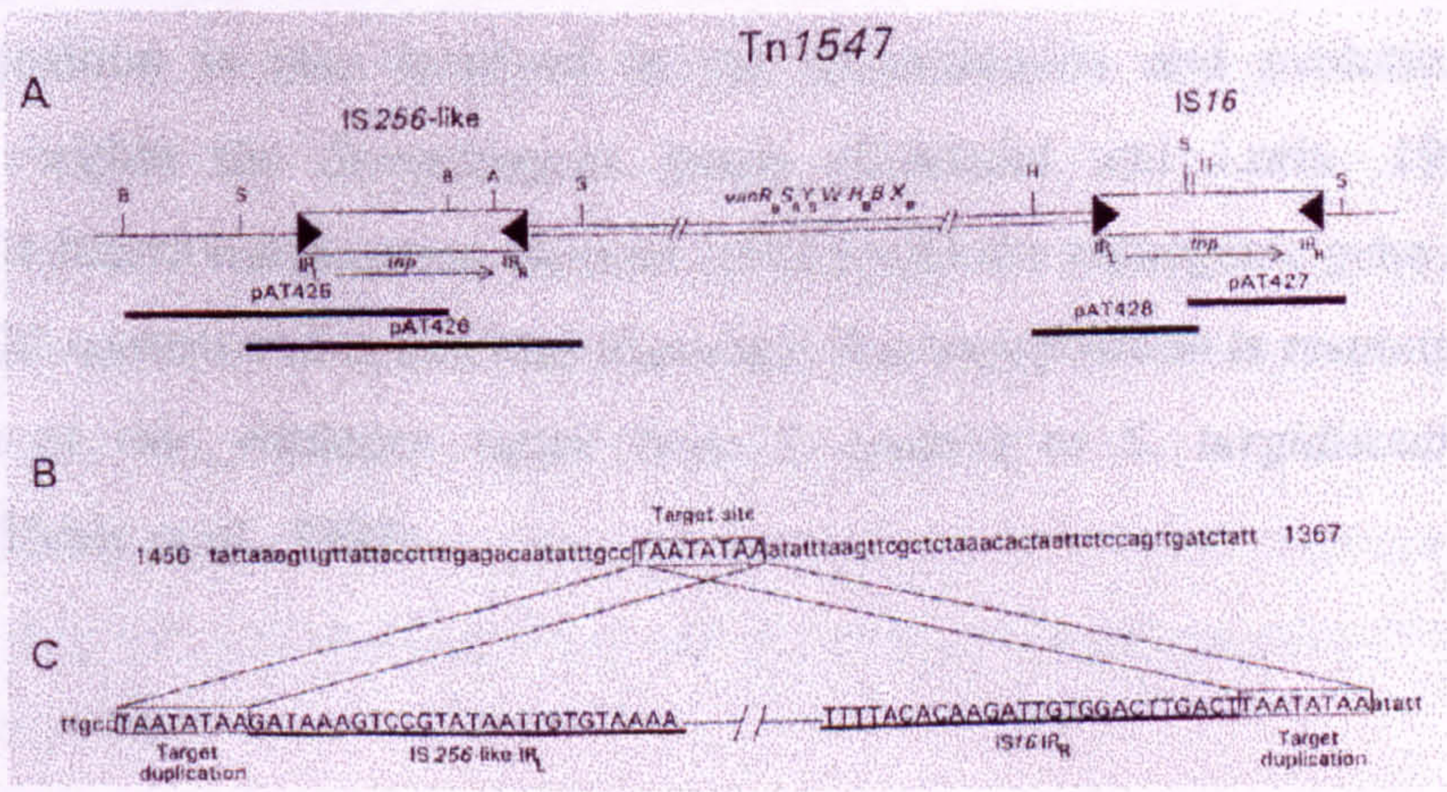


Figure 4.2: Transpositional recombination as a mediator of genetic and genomic rearrangements. (A) There are two modes of transposition. In non-replicated transposition, a fragment from the donor is excised and transferred to the target site. In replicative transposition, a cointegrate molecule is formed by the donor and the target fusion. The transferred fragment is duplicated and both donor and target acquire one copy of the fragment. Resolution of the cointegrate may occur by homologous recombination or site-specific recombination. (Reproduced from Hallet and Sherratt, 1997). (B) The new target locus is flanked by short target sequence duplications. An example of such post-transfer structure is the Tn*1547* that confers vancomycin resistance in *Enterococcus faecalis* (Reproduced from Quintiliani and Courvalin, 1996).

Two other transposable elements from streptomycetes, Tn*4556* and IS*493*, seem to insert into random sites and have been developed as tools for transposon mutagenesis (McHenney and Baltz, 1996). Some amplifiable units of DNA (AUDs) contain direct repeats which serve as substrates for recombination to promote amplification. Duplication necessary for the amplification of AUD2 (*S. lividans*) may arise through transposition of the putative mobile element called IS*1373* indicating the correlation between genetic instability and transposition in streptomycetes (Volff and Altenbuchner, 1997). Both direct repeats of this element present 65% identity in nucleic acid sequence with IS*112* of *S. albus*, and show one unique open reading frame, *insA*, encoding the putative transposase (Volff and Altenbuchner, 1997). The IS*112* transposase has been found clustered with the spectinomycin biosynthetic genes in *S. flavopersicus* (Lyutzkanova *et al.*, 1997). Some of these genes showed significant sequence similarity with their counterparts in the streptomycin biosynthetic pathway indicating that an ancestral production gene cluster for the streptomycin-like aminoglycosides could have developed into another variant by divergent evolution after degeneration and/or rearrangement (Piepersberg, 1997).

Transposition is also involved in the dissemination and evolution of plant pathogenicity within the *Streptomyces* genus (Bukhalid and Loria, 1997). The association of a functional insertion element IS*1629* with the pathogenic gene *nec1* which has atypical GC content and codon bias illustrates that transposition is responsible for the dissemination of this virulence factor from *S. scabies* to *S. turgidiscabies* and *S. acidiscabies* (Healy *et al.*, 1999).

4.1.1.4 Congugative transposons

Conjugative transposons move by site-specific recombination. In site-specific recombination, DNA strands are broken and exchanged at precise positions of two target DNA loci (Hallet and Sheratt, 1997). The first step in conjugative transposition is excision of the element from the donor DNA molecule to form a covalent circular closed (CCC) intermediate (Marra *et al.*, 1999). Excision is mediated by two transposon-

encoded proteins: integrases (Int) and excisinase (Xis). Coupling sequences of 6 bp that flank the transposon are formed at the site of excision (Marra *et al.*, 1999). Since the coupling sequences intervene between the *tra* genes and the corresponding promoter within the CCC intermediate, it was suggested that the nucleotide content of the coupling sequences could influence the frequency of conjugative transposition by affecting the *tra* gene expression (Marra *et al.*, 1999). They do so, however, by affecting transcript elongation due to RNA polymerase pausing or termination and not simply to differences in Int binding (Pethel and Churchward, 2000). Conjugative transposons carry their own integrase genes and can thus integrate into the genome of any host in which the integrase is expressed leaving the recipient fully transfer proficient. These integrases are members of λ integrase family and appear to have different mechanism of that of transposases (Salyers *et al.*, 1998). There is also no target duplication when the conjugative transposon integrates (Salyers *et al.*, 1998) and conjugative transposons differ considerably in their integration specificity. Conjugative transposons tend to have very broad host ranges. Tn*916* can transfer between Gram-positive and Gram-negative bacteria (Clewell *et al.*, 1995). Antibiotic resistance to sulfamethoxazole, trimethoprim and streptomycin in *Vibrio cholerae* 0139 are also linked to conjugative transposon (Waldor *et al.*, 1996). The tetracycline resistant gene *tetM* codes for a ribosomal protection protein and is always associated with conjugative transposons. The sequences of the *tetM* genes from a variety of Gram-positive and Gram-negative bacteria are virtually the same, suggesting a recent transfer event (Salyers *et al.*, 1998). The *tetM* gene was found in soil *Streptomyces* spp. as well as in colonic *Peptostreptococcus* species indicating that soil microbes may transfer genes to intestinal microflora (Salyers and Shoemaker, 1996).

4.1.1.5 *Streptomyces* phages and transduction

The temperate bacteriophage ϕC31 infects a large number of streptomycetes, including *Streptomyces lividans* and *Streptomyces ambofasciens* (Kuhstoss and Rao, 1991). The ϕC31 genome is about 41.5 kb and is linear with cohesive ends (Smith *et al.*,

1999). Analysis of the ϕC31 genome has revealed that ϕC31 has very similar head and tail genes to those of the *E. coli* phage HK97, two *Rhodobacter capsulatus* prophages and two *Mycobacterium tuberculosis* prophages (Smith *et al.*, 1999). These phages and prophages from evolutionary diverse hosts are likely to share a common head assembly mechanism. There is also evidence for shuffling of the individual genes between phages by horizontal exchange. The portal/capsid proteins from the two *Rhodobacter* prophages are more similar to the ones from *Streptomyces* phage ϕC31 than to each other (Smith *et al.*, 1999). The inability to discern which phage genome is most closely related to which is highly suggestive of horizontal transfer of genetic material between phages. ϕC31 DNA also serves as a specific integration target of *Streptomyces* transposable element IS*110* in a region of apparently non-coding DNA (Bruton and Chater, 1987). The attachment sites of phage and host share only three bases of homology in their core sequences (Kuhstoss and Rao, 1991). The ϕC31 integrase is a member of the resolvase/invertase family of site-specific recombinases (Thorpe and Smith, 1998) and the directionality in ϕC31 integrase is strictly controlled by the nonidentical recombination sites with no requirement to form the topologically defined structures that are more typical of the resolvases/invertases (Thorpe and Smith, 1998).

A generalised transducing phage has been reported in *S. venezuelae* and *S. coelicolor*. High-titer lysates can escape from the host restriction-modification systems and subsequent plaque formation indicates that that these phages appear to have a broad host range (Burke *et al.*, 2001). Isolation and characterisation of generalised transducing phages in streptomycetes may depend on the conditions expected to severely reduce superinfection killing during the selection of transductants (Burke *et al.*, 2001).

4.1.1.6 Chromosome instability and gene amplification of *Streptomyces* genome

On average, the chromosome of about 0.5% of the spores is affected by deletions removing up to 25% of the genome (Volff and Altenbuchner, 1998). For an average chromosomal size of about 8 Mb, this means that deletions can remove about 2 Mb of DNA, which exceeds by far the size of small bacterial genomes. Although internal

rearrangements have been described, most of the deletions have been detected at or near the chromosome ends and that very often involve one or both telomeres. The genetic and physical map of the *S. coelicolor* A3(2) chromosome show that there are no genes that are essential for growth under laboratory conditions in this region except *argG* involved in the arginine biosynthesis (Leblond and Decaris, 1994). Therefore, streptomycetes might be unique in tolerating large deletions that do not affect the viability of the cells under laboratory conditions. Genetic instability affects different phenotypical properties, often pleiotropically, including morphological differentiation, production of secondary metabolites such as pigments and antibiotics, antibiotic resistance, secretion of extracellular enzymes.

In *S. lividans*, chloramphenicol sensitive and Arg^- phenotypes result from deletion of a chloramphenicol resistance marker, *cmlR*, and of the arginosuccinate synthatase gene, *argG*, respectively (Schrempf, 1991). In *S. glaucescens*, the hydroxystreptomycin phosphotransferase and the tyrosinase structural genes are included in the deletions (Volff and Altenbuchner, 1998). In *S. griseus*, the *afsA* gene, which encodes a protein essential for A-factor production is deleted in some A-factor negative mutants (Horinouchi, 1999). Such terminal deletions may result from the collapse of replication forks at single-strand breaks in the chromosome which are not correctly repaired. Thus, mutations in *recA*, or the presence of mutagens increase this chromosomal instabilitty (Volff and Altenbuchner, 2000) and the unstable region appears to be the terminus of replication.

Large deletions are frequently associated with DNA amplifications. These amplified DNA sequences (ADS) consisted of tandem reiterations of a basic region of DNA called the amplifiable unit of DNA (AUD) and can account for up to 50% of the total DNA. The AUD1 element of *S. lividans* is found as highly reiterated 5.7 kb fragment in 90% of the Cml^s Arg^- mutants arising by genetic instability (Altenbuchner and Cullum, 1984). Another AUD element was found in a tylosin non-producing mutant of *S. fradiae*. The corresponding phenotype was caused by deletion accompanying the amplification, which removed tylosin biosynthesis genes (Baltz and Seno, 1988). In *Streptomyces achromogenes* amplification of AUD-Sar1 carrying a spectinomycin resistance gene resulted from selection for higher spectinomycin resistance (Volff and

Altenbuchner, 1998). Interestingly, the 90-kb AUD2 from *S. lividans*, which is delimited by two copies of the insertion elements IS*1373*, might be a giant mercury resistance transposon (Volff and Altenbuchner, 1997). However, with the exception of positively selected amplification, the selective advantage of the amplification remains unknown and the involvement of the genes present in the AUD in the amplification mechanism itself, is highly speculative. In mutants with amplifications, the deletions usually end near or within the amplified DNA. The presence of amplified DNA could buffer further deletions and thus prevent deletion of essential genes (Leblond and Decaris, 1994).

The discovery of the linearity of *Streptomyces* chromosomes was thought to provide the explanation for the observed high level of genetic instability, which could be due, for example, to degradation of non-protected telomeres (Leblond and Decaris, 1994). However, artificial and spontaneous circularised chromosomes were found to be at least as unstable as the corresponding linear chromosomes (Lin and Chen, 1997). Hence, linearity is clearly not the reason for the high level of genomic rearrangement observed in streptomycetes. However, chromosome linearity can facilitate homologous recombination events without generating multimers that they might be difficult to replicate and extremely unstable (Volff and Altenbuchner, 1998). Linear chromosomes may also allow for easier interspecies gene transfer. Homologous recombination between linear chromosome and linear plasmids can result in the acquisition of chromosomal genes by the linear plasmids which can then be transferred at high frequency into other strains. A study of mutant strains of the oxytetracycline-producer *S. rimosus* has shown that linear plasmids can acquire chromosomal genes via integration into the chromosome followed by imprecise excision (Gravius *et al.*, 1994). Similarly, the extreme ends of the *S. lividans* chromosome are similar or identical to one end of the linear plasmid SLP2 (Volff and Altenbuchner, 2000). This includes one copy of the transposable element Tn*4811*.

4.1.1.7 Transfer of antibiotic biosynthesis genes

There have been some hypotheses about the transfer of antibiotic biosynthesis genes. For example, Weigel *et al.* (1988) hypothesised that production of the β-lactam cephalosporin in fungi arose as a result of horizontal gene transfer from *Streptomyces*. This is however a contentious issue; Smith *et al.* (1992) concluded that the similarity of these genes was acceptable with no need to invoke a gene transfer event, because the genes' presence in both was due to a duplication event that occurred before prokaryote and eukaryote divergence (alternatively the cluster may also just have been lost in multiple extant fungal and streptomycete lines). Further analysis of the genes (Buades and Moya, 1996), suggested that the short branch lengths linking fungi and bacteria supported a gene transfer event. If antibiotic production plays an important role in the natural environment, then selection and transfer of these genes for production might be expected. Other studies have examined functional genes from different bacteria and found that the GC content of particular genes is quite different to the rest of the genome, (e.g. *rfb* genes for o-antigen synthesis in *Salmonella typhimurium*) which suggests that these genes may have been acquired by transfer events, although homologues in donor species have never been identified (Lan and Reeves, 1992; Syvanen, 1994).

Streptomycin and similar related compounds (SARCs) are produced by several streptomycete strains and it has been suggested that antibiotic production is strain-specific not species-specific (Hotta *et al.*, 1996), a phenomenon which has been observed in the well-characterised producing type strains. To address this hypothesis however, a thorough analysis of the phylogeny and antibiotic-producing ability of many natural streptomycete isolates needs to be performed. In addition, the delimitation of *Streptomyces* species using phenotypic data has led to many ill-defined species groups (Williams *et al.*, 1983a,b). It is probably the case that not rigorous analysis of sufficient data is available from natural isolates which are phylogenetically closely related enough to a producing type strain to show that all identified strains of a particular genotypic species do or do not possess the same antibiotic production ability.

Production of streptomycin is well characterised in *S. griseus* N2-3-11 and ATCC 12475 and is regulated by a plethora of factors affecting both the pleiotropic interlinking

of primary and secondary metabolism (nutrient availability, growth kinetics, phosphorylated guanine, protein phosphorylation, levels of cAMP) and the cellular physiology committed to antibiotic biosynthesis (codon usage, A-factor autoregulator, biosynthetic cluster of around 30 genes). The biochemical pathway for production is branched resulting in the formation of each of the three moieties that comprise streptomycin: a diaminocyclitol moiety (streptidine), 6-deoxyhexose sugar (streptose) and N-methyl-L-glucosamine (NMLGA). Modifications of this pathway are found in other streptomycetes and lead to the production of related compounds including hydroxy-streptomycin and bluensomycin.

At the genetic level, more than 25 biosynthetic genes that are involved in the biosynthesis of streptomycin have been described so far (Piepersberg, 1997). *strA* encodes a phosphotransferase which confers resistance to streptomycin avoiding auto-inhibition in the producer and has a central location in the cluster. Transcription of *strA* occurs first before any production genes and is initiated from the promoter of *strR*, the pathway–specific regulator which is located immediate upstream of *strA* leading to a unique transcript for these two genes. StrR functions as a transcriptional activator of a number of promoters within the *str* cluster including the one of *strB1* (Retzlaff and Distler, 1995; Thamm and Distler, 1997). The latter gene is the first biosynthetic gene located just downstream of *strA* and encodes for an amidinotransferase involved in the streptidine pathway. The streptomycin biosynthesis is under the control of A-factor, a microbial hormone which exerts its positive effect on Sm production by a well-defined regulatory cascade transferring the signal eventually to the *strR* promoter (Ohnishi *et al.*, 1999). Other genes within the cluster are involved in the biosynthesis and condensation of subunits and the export of the inactive compound. Extracellular phospahatase (*strK*) liberates the biologically active streptomycin molecule. Two additional genes analysed here are *strF*, a postulated hexosamine epimerase and *strS*, a pyridoxal phosphate dependent aminotransferase. Both of these genes are involved in NMLGA pathway Ahlert *et al.*, 1997; Piepersberg, 1995).

Huddleston *et al.* (1997) analysed a collection of *Streptomyces* isolates from a site in Brazil where antibiotic production was implicated in the poor performance of nodulating *Bradyrhizobia* inoculants. The authors found high levels of streptomycin

resistance in soil isolates which were phenotypically diverse. Southern blotting revealed that a homologue to the *str*A gene from *S. griseus* was found present in these strains. Hybridisation of the *strA* of these isolates to the *S. griseus* gene indicates high levels of similarity. Because the strains were phenotypically and genetically different, the authors suggested that gene transfer could be responsible for the distribution of these highly similar *strA* genes within these strains.

The best way to establish gene transfer events is to generate and then compare and contrast phylogenetic trees. Using this approach, Wiener *et al.* (1998) confirmed the original hypothesis of horizontal gene transfer of *strA* in the Brazilian isolates. Comparison of 'species' tree (16S rDNA) and 'gene' tree (*strA*) led to a phylogenetic incogruency. In addition the authors showed using PFGE analysis that *strA* had been integrated into the new host's chromosome. Egan (1998) showed that the Brazilian isolates also have the first streptomycin biosynthetic gene adjacent to *strA*, namely *strB1*, showing equally high sequence similarity with the *S. griseus* counterpart gene (>98%).

This study aimed to apply the hypothesis of gene transfer to other genes from the cluster (*strR, strS, strF*) in conjunction with a better examination of the phylogenetic history of Brazilian isolates by analysing another housekeeping gene, tryptophan synthase (Chapter 3). The overall distribution of SARC-production among known type strains was also considered and used to address the general hypothesis that gene transfer is an important factor in the evolution of antibiotic production genes in producing organisms. The example of the Brazilian isolates provides evidence that this is indeed the case.

4.2 Results

4.2.1 Analysis of *strR* sequences from *Streptomyces* species

Consensus primers were designed for *strR* gene based on *S. griseus* and *S. glaucescens* sequences. Four sets of primers were designed targeting at different positions within *strR* gene (Fig. 4.1). By using a range of different conditions relating to [Mg^{++}] and template concentration, optimised conditions gave the expected size of bands related to each primer pair (Fig. 4.2).

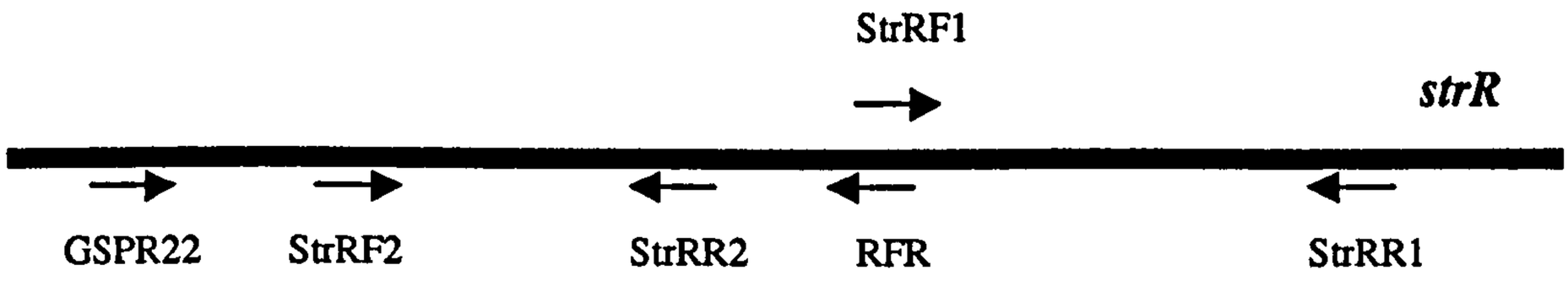


Figure 4.3: Schematic representation of *strR* gene showing the relative annealing sites of primers used.

The PCR products were transferred onto a nitrocellulose membrane and probed using *strR* PCR product generated from *S. griseus*. After hybridisation, the filter was treated with high stringency washings. All the samples from Brazilian isolates gave a strong positive signal except isolate ASB27 which gave a rather poor signal (Fig. 4.2). Further confirmation of the presence of *strR* gene in these isolates came from sequencing (Fig. 4.3). PCR products generated from the [GSP22F-RFR] set of primers were gel-excised and purified using Geneclean II kit (BIO101). The eluted DNA was used for cycle sequencing. The sequencing results from Brazilian isolates were in agreement with the probing data showing similarity with *S. griseus strR* gene in a range of 98% - 100% (Table 4.1). Sequence from ASB27 did not show any homology with *strR* sequence indicating the absence of that gene from this isolate.

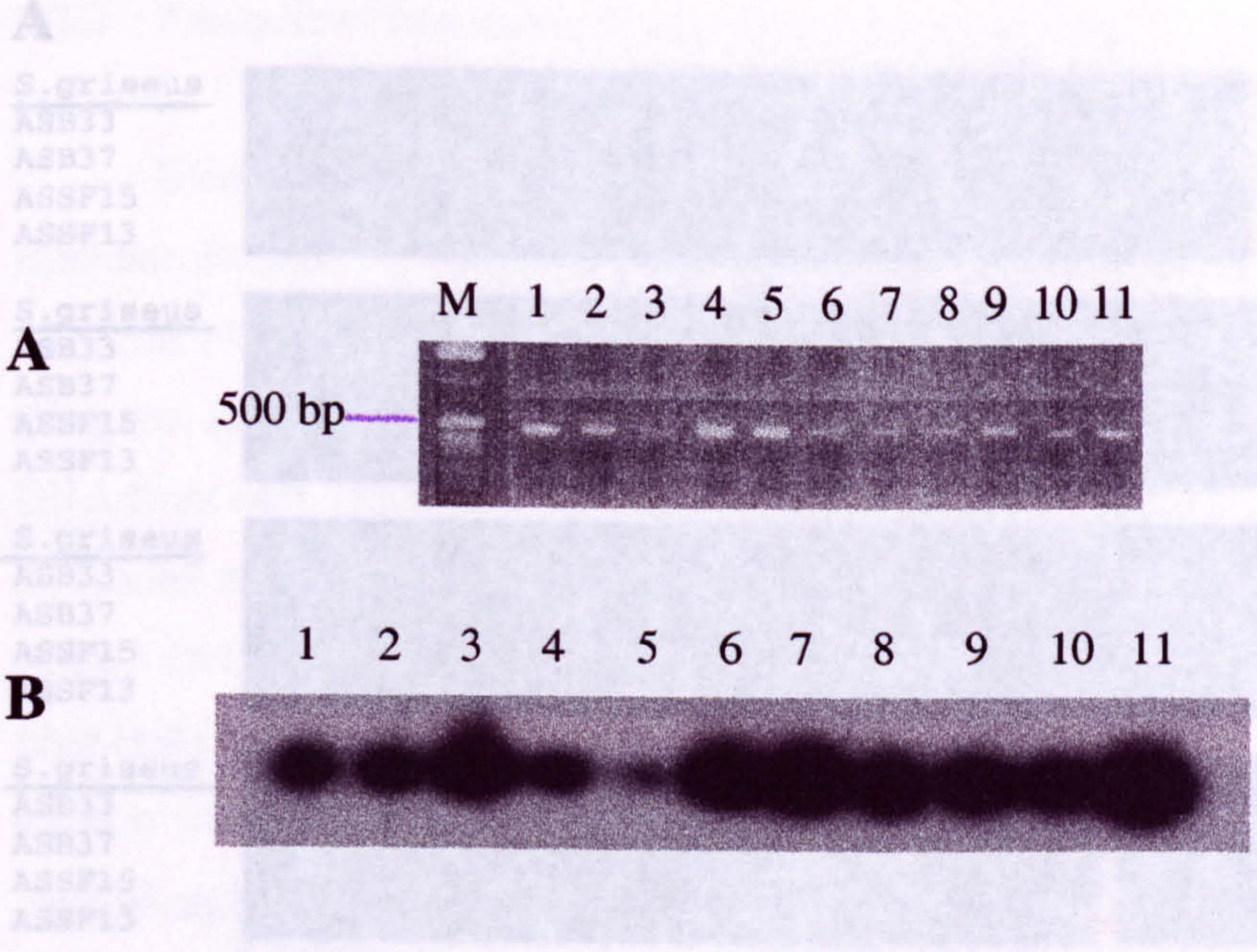
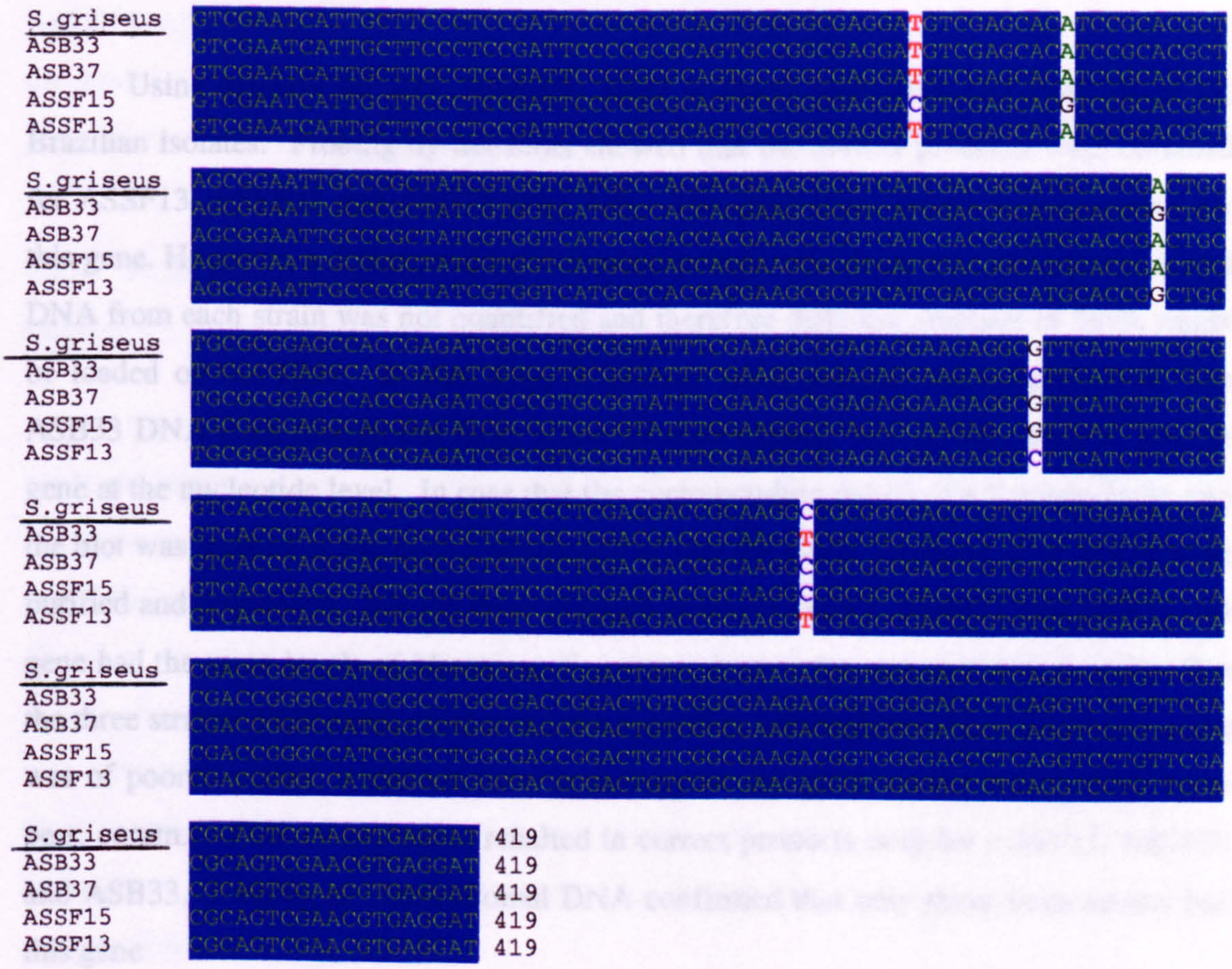


Figure 4.4: Detection of *strR* gene from diverse streptomycetes. (A) Purified PCR products from six Brazilian isolates generated using strRF1-strRR1 primers (400 bp). 1 kb ladder (M), ASSF13 (lanes 1,2), ASSF15 (lane 3), ASSF22 (lane 4-6), ASB27 (lane 7), ASB33 (lanes 8,9), ASB37 (lane 10), *S. griseus* (lane11). (B) Autoradiograph of PCR products hybridisation using *S. griseus* PCR fragment as a probe. *S. griseus* (lane 1), ASB37 (lane 2), ASB33 (lanes 3,4), ASB27 (lane 5), ASSF22 (lanes 6-8), ASSF15 (lane 9), ASSF13 (lanes 10,11).

A



B

Figure 4.5: Alignment of *strR* partial sequence from *S. griseus* and a set of Brazilian isolates. (A) Nucleotide sequence generated by PCR using GSPR22-RFR set of primers and analysed by cycle sequencing (ABI machine). (B) Translated amino acid sequence with reference to *S. griseus* StrR protein. Regions of conservation are highlighted in blue and similar residues are highlighted in red. All the alignments were performed using ClustalW and BioEdit programmes.

4.2.2 Analysis of two other genes from the streptomycin biosynthetic cluster

Using primers for *strF*, similarly sized products were observed from all of the Brazilian isolates. Probing by dot blots showed that the correct products were obtained for ASSF13, ASSF22 and ASB33 (Fig. 4.4). The other three strains were negative for this gene. However, a differential hybridisation pattern was observed. The chromosomal DNA from each strain was not quantified and therefore different amounts of DNA might be loaded on the filter. In that way, *S. griseus* DNA yielded less hybridisation than ASB33 DNA even though the latter strain showed slightly less identity than *S. geiseus* gene at the nucleotide level. In case that the corresponding gene had a low similarity and the blot was washed at too high a stringency so that the probe dissociated, all bands were purified and sequenced. For the three positive strains ASSF13, ASSF22 and ASB33, the gene had the same levels of identity to *S. griseus* as seen for *strA* and *strR* at >98%. For the three strains' PCR products that did not hybridise, although the sequence that resulted was of poorer quality, it did not have homology to any genes on the database (S. Egan pers. comm.). PCR of *strS* again resulted in correct products only for ASSF13, ASSF22 and ASB33. Probing of chromosomal DNA confirmed that only these three strains had this gene

Figure 4.6: Detection of *strF* gene in four streptomycetes. Dot blot analysis of chromosomal DNA from Brazilian isolates for the detection of *strF* gene. *S. griseus* (sample 1), ASB33 (sample 2), ASSF22 (sample 3), ASSF13 (sample 4).

4.2.3 Streptomycin production by the Brazilian isolates

Having the observation of partial detection of streptomycin cluster, it was interesting to test whether the isolates can produce the antibiotic. Using standard bioassay, the supernatants of the Brazilian isolates cultures taken at certain time points after inoculation (9, 17, 21, 24 and 36 h) were loaded on a paper disc on two nutrient agar plates covered with *E. coli* ATCC 29842 (streptomycin resistant) and ATCC 29839

(isogenic sensitive strain) respectively and incubated overnight. Supernatants from ASSF13, ASSF22 and ASB33 caused inhibition zones around the paper discs. Therefore the strains shown to have all the tested genes, they can also produce the actual antibiotic and perhaps they contain the complete set of biosynthetic genes (Table 4.1).

4.2.4 PCR amplification of *strA-strB1* intergenic region from selected Brazilian isolates

Because of the functional importance of the intergenic region *strA-B1* in the producer strain *S. griseus* (Retzlaff and Distler, 1995), it was attempted to amplify this fragment from the selected Brazilian isolates ASSF15 and ASB37. A forward primer (strA1F) was designed at the 3' end of the *strA* gene and a reverse primer (strB1R) at the 5' end of the *strB1* gene. Both primers bind to regions that were conserved in *S. griseus* and *S. glaucescens*. A band of the expected size (491 bp) related to *S. griseus* fragment was obtained from all the selected strains and cloned into a pZero blunt vector (Invitrogen) and the resulting plasmids were subjected to cycle sequencing. The sequencing results showed high similarity (99%) with the corresponding fragment of *S. griseus* (Table 4.1; Fig. 4.5).

isolates	*strR* presence/ %homology to *S. griseus* over 419 bp	*strF* presence	*strS* presence	*strA-strB1* intergenic region homology (%) to *S. griseus* (491 bp)	Sm production
ASSF13	+ / 99	+	+	N/A	yes
ASSF15	+ / 99	-	-	98	no
ASSF22	+ / 100	+	+	N/A	yes
ASB27	-	-	-	N/A	no
ASB33	+ / 99	+	+	N/A	yes
ASB37	+ / 100	-	-	98	no

Table 4.2: Detection of DNA sequences from *str* cluster in a set of six Brazilian isolates in correlation with streptomycin production. These Brazilian isolates have also *strR*, a regulatory gene found adjacent to previously detected *strAB1* genes (Wiener *et al.*, 1998).

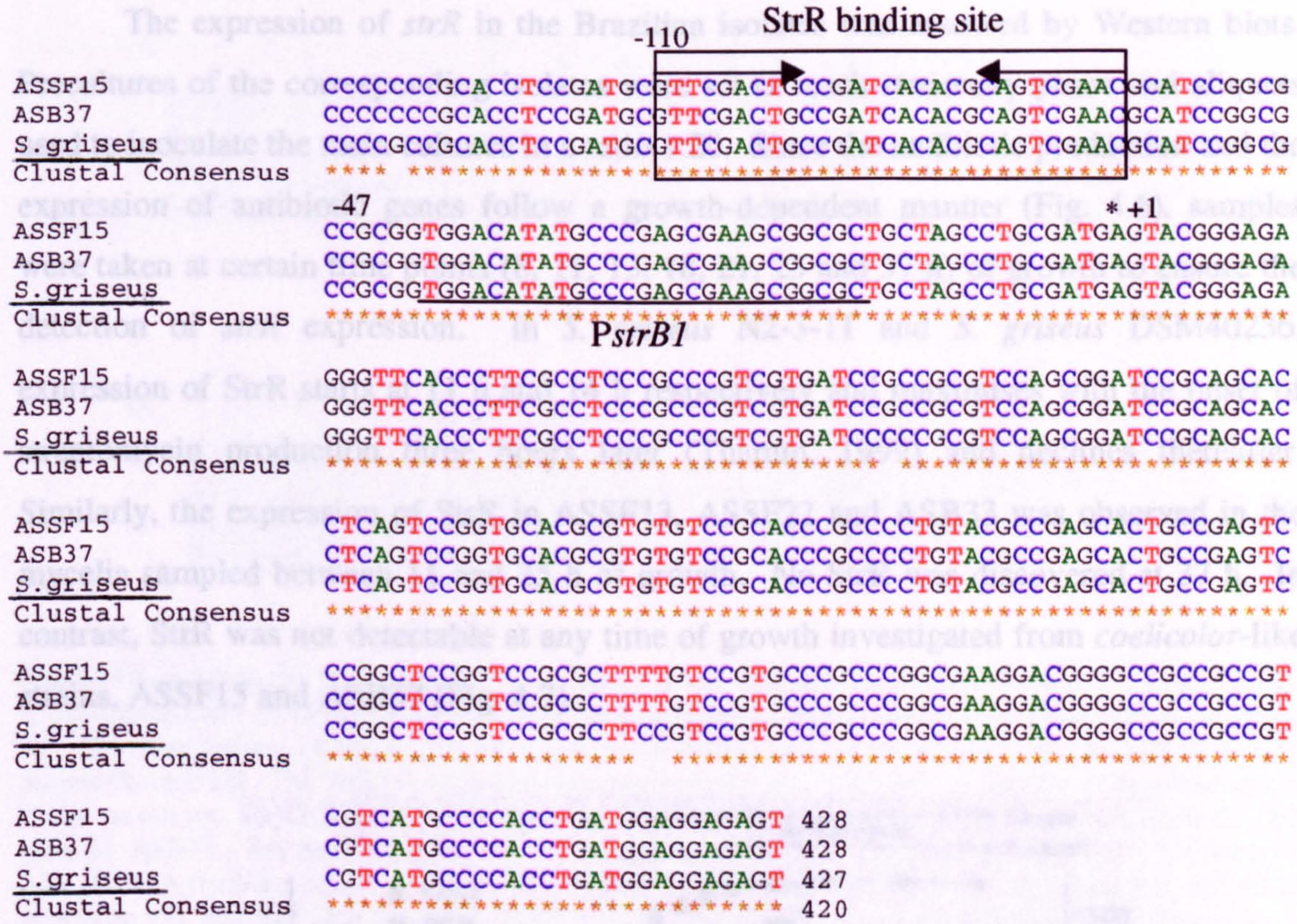


Figure 4.7: Alignment of the intergenic region *strA-B1* from *Streptomyces griseus*, ASSF15 and ASB37. The StrR binding site is boxed. Inverted repeats are indicated by arrows. The *strB1* promoter is underlined. The transcriptional start site of *strB1* is indicated by an asterisk.

4.2.5 Detection of *strR* expression by Western analysis

The expression of *strR* in the Brazilian isolates was assessed by Western blots. Precultures of the corresponding isolates were set to reach stationary phase and aliquots used to inoculate the main cultures in a ratio 1:20. Since the antibiotic production and the expression of antibiotic genes follow a growth-dependent manner (Fig. 4.6), samples were taken at certain time points (8, 11, 13, 18, 21, 25 and 37 h) of growth to ensure the detection of *strR* expression. In *S. griseus* N2-3-11 and *S. griseus* DSM40236, expression of StrR starts at 11 h and 14 h respectively and maximises with the onset of streptomycin production three hours later (Thamm, 1999) and declines thereafter. Similarly, the expression of StrR in ASSF13, ASSF22 and ASB33 was observed in the mycelia sampled between 11 and 25 h of growth. No StrR was discovered at 37 h. In contrast, StrR was not detectable at any time of growth investigated from *coelicolor*-like strains, ASSF15 and ASB37 (Fig. 4.7).

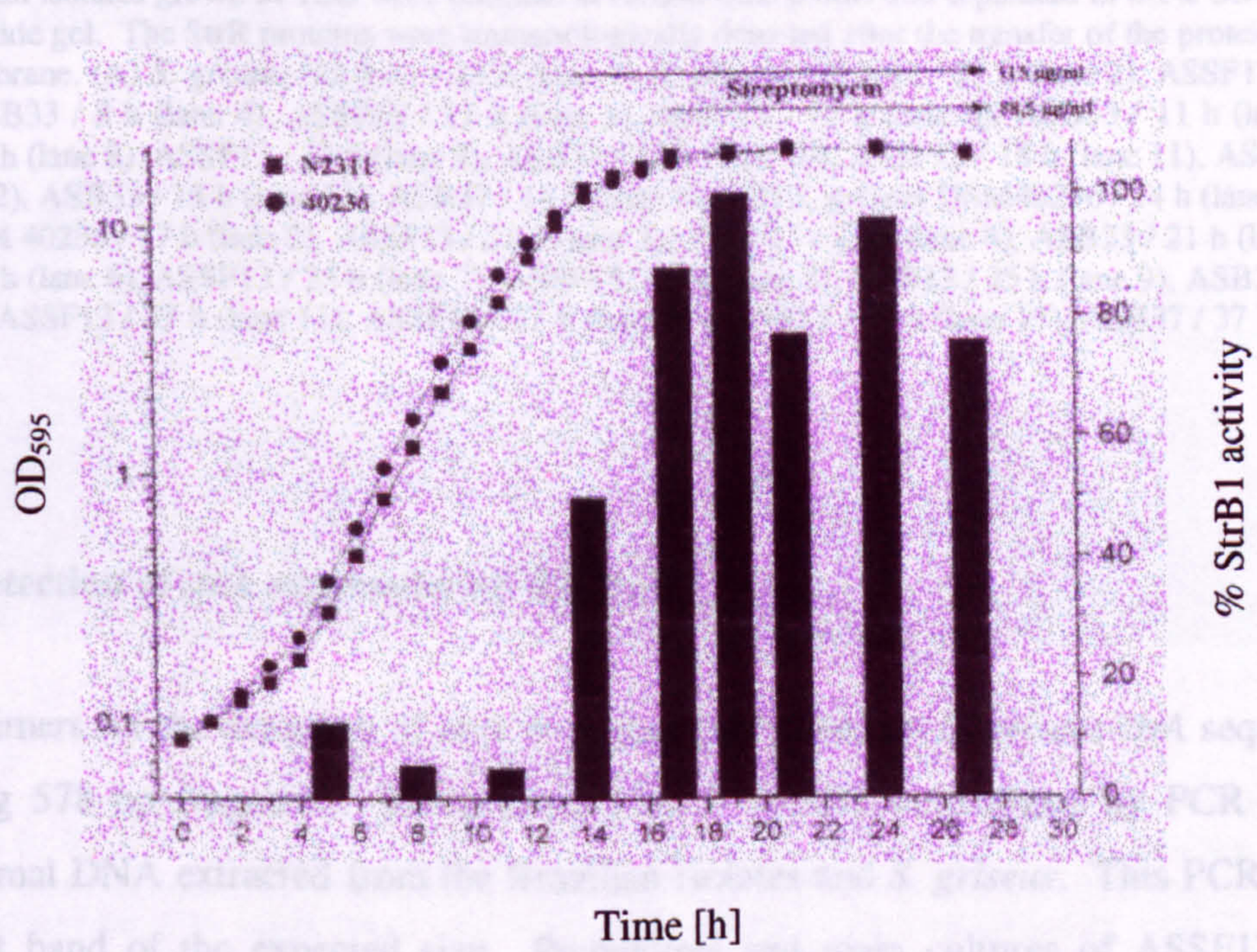


Figure 4.8: Growth curve, streptomycin production and StrB1 activity of *S. griseus* in TSB. The correlation between production of biomass (OD_{595}) for *S. griseus* DSM40236 and *S. griseus* N2-3-11 and StrB1 acitivity over time is shown. Streptomycin production starts at 14 h for *S. griseus* N2-3-11 and 17 h for *S. griseus* DSM40236 with maximum amounts produced at 27 h for both strains. The onset of streptomycin production coincides with the maximum activity of *strB1* gene which is achieved at 19 h of growth. (Reproduced from Thamm, 1999).

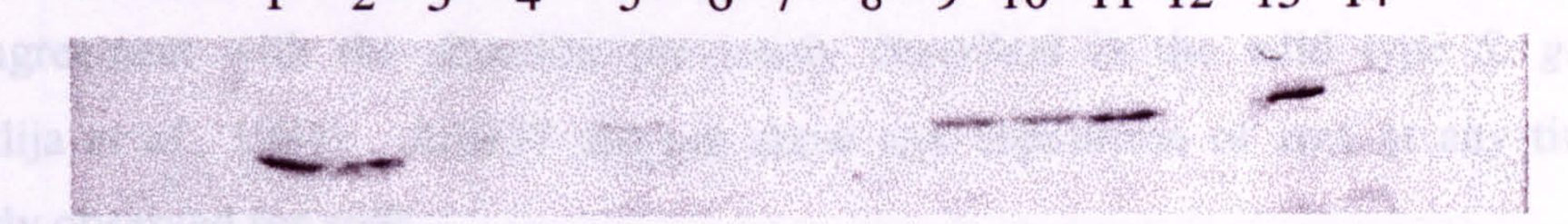


Figure 4.9: Analysis of StrR expression in Brazilian isolates. Immunological detection of StrR proteins in cell-free extracts of Brazilian isolates using StrR antiserum. 50 µg protein of cell-free extracts prepared from Brazilian isolates grown in TSB were sampled at certain time points and separated in 0.1% SDS-12% polyacrylamide gel. The StrR proteins were immunologically detected after the transfer of the proteins to a PVDF membrane. (A) *S. griseus* N2-3-11 / 13 h (lane 1), *S. griseus* N2-3-11 / 17 h (lane 2), ASSF13 / 8 h (lane 3), ASB33 / 8 h (lane 4), ASSF13 / 11 h (lane 5), ASSF15 / 11 h (lane 6), ASB33 / 11 h (lane 7), ASB37 / 11 h (lane 8), ASSF13 / 13 h (lane 9), ASB33 / 13 h (lane 10), ASSF13 / 18 h (lane 11), ASSF15 / 18 h (lane 12), ASB33 / 18 h (lane 13), ASB37 / 18 h (lane 14). (B) *S. griseus* DSM40236 / 14 h (lane 1), *S. griseus* DSM 40236 / 17 h (lane 2), ASSF13 / 21 h (lane 3), ASSF15 / 21 h (lane 4), ASB33 / 21 h (lane 5), ASB37 / 21 h (lane 6), ASSF13 / 25 h (lane 7), ASSF15 / 25 h (lane 8), ASB33 / 25 h (lane 9), ASB37 / 25 h (lane 10), ASSF13 / 37 h (lane 11), ASSF15 / 37 h (lane 12), ASB33 / 37 h (lane 13), ASB37 / 37 h (lane 14).

4.2.6 Detection of *strA* expression by RT-PCR

Primers for the detection of *strA* were designed based on *S. griseus strA* sequence amplifying 578 bp fragment. The primers (StrAF-StrAR) were tested by PCR using chromosomal DNA extracted from the Brazilian isolates and *S. griseus*. This PCR gave one bright band of the expected size. Precultures and main cultures of ASSF13 and ASB37 were prepared as described before (section 4.6) and samples for RNA extraction were taken at 16, 20, 24 and 43 h to allow comparisons with Western analysis of StrR expression (section 4.6). RNeasy kit (Qiagen) was used for the RNA extraction (Fig. 4.8). After running on a non-formaldehyde containing agarose gel, 2 bands

corresponding to 16S and 23S rRNA were obtained. Consistent with the results of the Western analysis, *strA* expression was shown only for ASSF13 (Fig. 4.9). The transcription of *strA* is coupled with that of *strR* as they occur at the same time points and is in agreement with the situation previously described in the wild type *S. griseus* (Vujaklija *et al.*, 1991). ASB37 did not show any expression of *strA* at any time as similarly observed for *strR*.

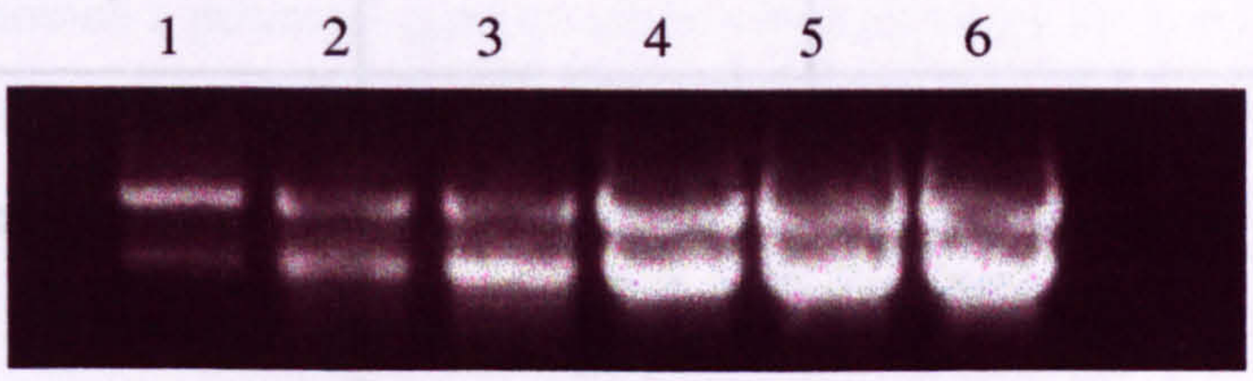


Figure 4.10: Total RNA extraction from cultures of ASSF13 and ASB37. Two bands were obtained corresponding to 16S and 23S rRNA respectively. Samples at selected time points of growth are shown. ASSF13 / 11 h (lane 1), ASB37 / 11 h (lane 2), ASSF13 / 18 h (lane 3), ASB37 / 18 h (lane 4), ASSF13 / 25 h (lane 5), ASB37 / 25 h (lane 6).

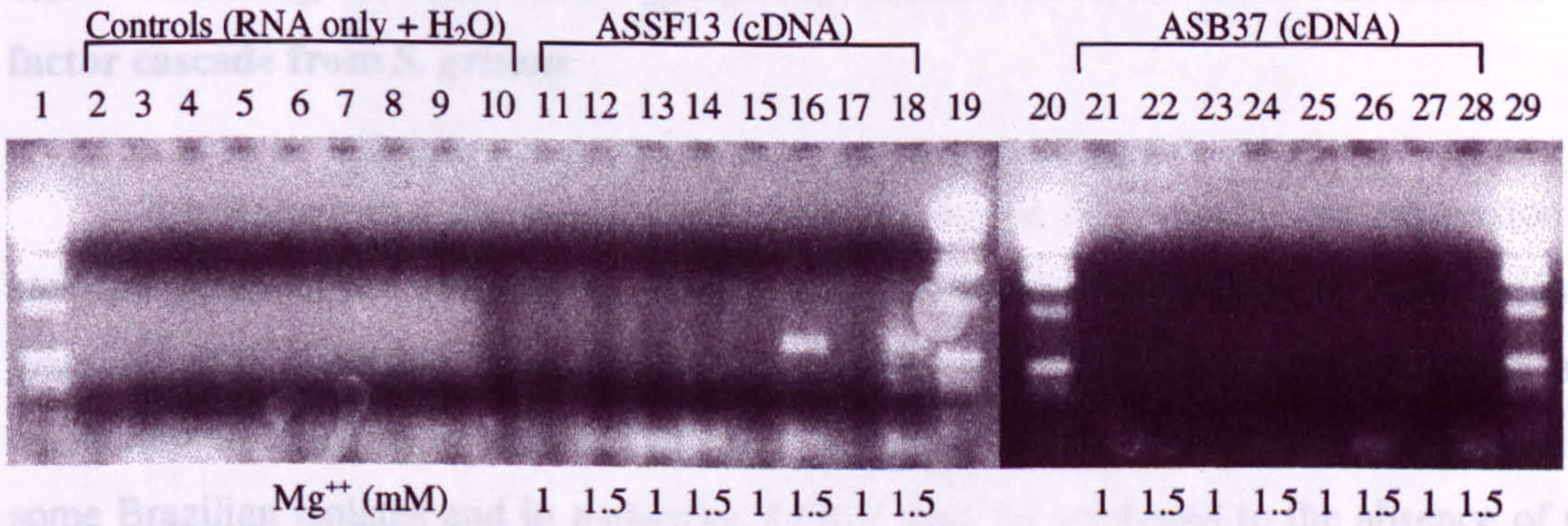


Figure 4.11: Analysis of *strA* transcription in ASSF13 and ASB37 isolates. Detection of *strA* transcripts over growth by RT-PCR using specific primers derived from *strA* sequence of *S. griseus* resulting in 578 bp fragment. Two different Mg^{++} concentrations (1.0 and 1.5 mM) were used for each PCR reaction of the experimental samples. 1 kb ladder (lanes 1,19,20,29), ASSF13 / 16 h / RNA only (lane 2), ASSF13 / 20 h / RNA only (lane 3), ASSF13 / 24 h / RNA only (lane 4), ASSF13 / 43 h (lane 5), ASB37 / 16 h / RNA only (lane 6), ASB37 / 20 h / RNA only (lane 7), ASB37 / 24 h / RNA only (lane 8), ASB37 / 43 h / RNA only (lane 9), H_2O (lane 10), ASSF13 / 43 h (lanes 11,12), ASSF13 / 24 h (lanes 13,14), ASSF13 / 20 h (lanes 15,16), ASSF13 / 16 h (lanes 17,18), ASB37 / 43 h (lanes 21,22), ASB37 / 24 h (lanes 23,24), ASB37 / 20 h (lanes 25,26), ASB37 / 16 h (lanes 27,28).

	strA	*strR*
RT-PCR	negative	
Western analysis		negative
Plate assays	No resistance after growth on glucose, maltose, mannitol, ISP-4, A-factor addition	

Table 4.3: Summary of expression analysis of *strA* (in ASB37) and *strR* (in all Brazilian isolates but ASSF22). In all cases there was Sm resistance as low as 5 μg / μl.

4.2.7 Assessing the lack of *str* genes expression in ASB37 with relevance to A-factor cascade from *S. griseus*

It is known that the streptomycin production and in particular the expression of *strRAB1* genes of the biosynthetic cluster is under the tight regulation of A-factor in *S. griseus*. The message from A-factor is transferred to the downstream genes and eventually to *strRp* by a well-defined cascade (Chapter 1). The lack of expression in some Brazilian isolates and in particular ASB37 may be attributed to the absence of the intermediate components of the cascade or the absence of the A-factor itself in this isolate.

Primers for the amplification of *afsA* gene were designed based specifically on the *afsA* sequence from *S. griseus*. The primers were designed to amplify a 477 bp fragment. The PCR gave the expected size product (Fig. 4.10). The band was excised from the gel, purified and sequenced. Surprisingly, despite the high specificity of primers, the sequence was almost identical with *scbA* gene found in *S. coelicolor* that involved in the

synthesis of another butyrolactone, SCB1. This result reconfirms the *S. coelicolor* background of ASB37 as described by phylogenetic analysis (Chapter 3). In the same way, primers were designed for *arpA* gene based on the sequence from *S. griseus*. The PCR was negative for ASB37 which is consistent with the observation from other labs that it was not possible to detect any A-factor-binding protein in cell extracts of *S. coelicolor*. Primers for *adpA*, the last step in A-factor cascade were designed based on the sequence from *S. griseus*. PCR gave the expected size product (481 bp) and sequencing showed a potential gene product with homology to AraC-type transcriptional activators found in *S. coelicolor* (Fig. 4.11). The best hit was 98% similarity with a gene from StC105 cosmid from genome sequencing project. The latter gene in *S. coelicolor* is 89% identical with *adpA* from *S. griseus* at the amino acid level.

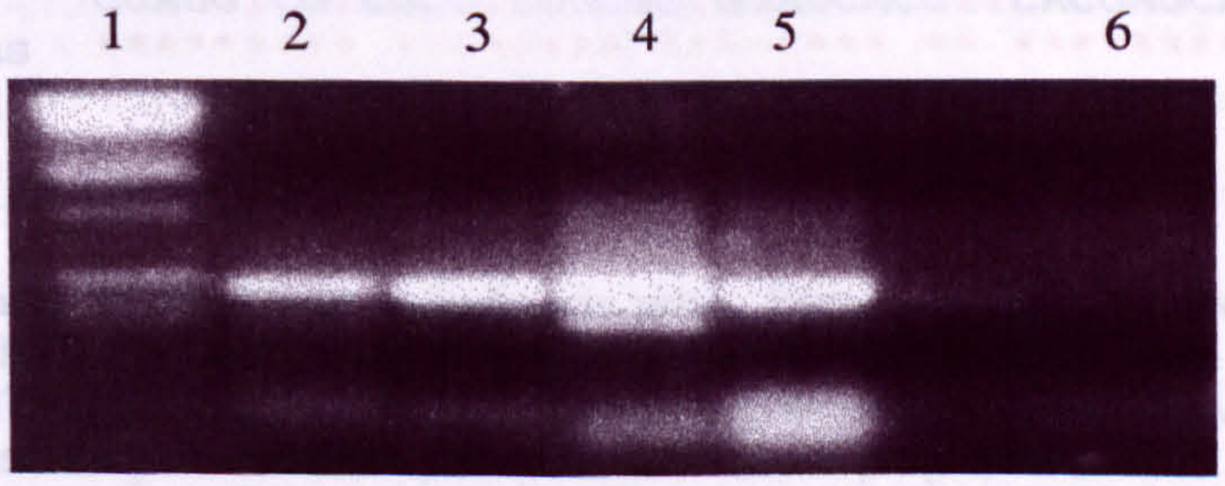


Figure 4.12: Detection of γ-butyrolactone biosynthetic gene (*scbA*) in *coelicolor*-like Brazilian isolates (ASB37 and ASSF15). PCR amplification of chromosomal DNA from *S. griseus* and selected Brazilian isolates using AfsAF-AfsAR pair of primers targeting 477 bp gene fragment. 1 kb ladder (lane 1), *S. griseus* (lane 2), ASSF13 (lane 3). ASSF15 (lane 4), ASB37 (lane 5), negative control (lane 6).

```
S. griseus        GTGGACGACGGGGATGTGCTCACCTCCGCCGGCACCGCCGCCGGGATCGA
S. coelicolor     GTGGACGACGGCGACGTGCTGACGTCCGCCGGCACCGCGGCCGGGATCGA
ASB37             GTGGACGACGGCGACGTGCTGACGTCCGCGG-CACTGTGGCCGGGATCGA
Clustal Consens   ********** ** ***** ** ***** * *** *  **********

S. griseus        TCTCTGCCTCCACATAGTCCGCACCGACCACGGCACGGAGGCCGCGGGGG
S. coelicolor     CCTGTGCCTGCACATCGTGCGCACGGACCACGGCAACGAGGCGGCCGGTG
ASB37             CCTGTGCCTGCACATCGTGCGCACGGACCACGGCAACGAGGCGGCCGGTG
Clustal Consens    ** ***** ***** ** ***** **********  ***** ** ** *

S. griseus        CACTCGCCCGCAGGCTCGTCGTGCCGCCACGGCGCAGTGGCGGGCAGGAG
S. coelicolor     CGCTGGCCCGCCGGCTGGTGGTCCCCCCGCGCCGCAGCGGCGGCCAGGAG
ASB37             CGCTGGCCCGCCGGCTGGTGGTCCCCCCGCGCCGCAGCGGCGGCCAGGAG
Clustal Consens   * ** ****** **** ** ** ** ** ** ***** ***** ******

S. griseus        CGCTACCTGGACAGGTCTTTACCGGAGGAGATCGGCTCCGACCCGCTCGC
S. coelicolor     CGCTACCTCGACAGGTCTTTACCGGAGGAGATCGGCGCCGACCCGCTCGC
ASB37             CGCTACCTCGACAGGTCTTTACCGGAGGAGATCGGCGCCGACCCGCTCGT
Clustal Consens   ******** ************************** ***********

S. griseus        CGAGGTCGTGGCCTGGGCCCTGGAGCATCTGCACGAGCAG
S. coelicolor     CGAGGTCGTCGCCTGGGCGCTGGAGCACCTCCACGAGCAG
ASB37             CGAGGTCGTCGCCTGGGCGCTGGAGCACCTTCACGAGCAG
Clustal Consens   ********* ******** ******** ** *********
```

Figure 4.13: Alignment of *adpA* and homologous genes from *S. griseus*, *S. coelicolor* A3(2) and Brazilian isolate ASB37. Sequence from *S. griseus* and *S. coelicolor* A3(2) come from the database. Sequence from ASB37 has been derived from PCR product generated using the [AdpAF-AdpAR] pair of primers. The alignment was performed using BioEdit and ClustaW programmes.

S. griseus	*afsA* → A-factor	*arpA*	*adpA*
ASB37	99% identity with *scbA* → SCB1	Not detected by PCR	98% identity with a *S. coelicolor* gene found in SCF105

Table 4.4: Relevance of A-factor cascade between *S. griseus* and ASB37.

4.3 Discussion

Previous studies (Huddleston *et al.*, 1997; Wiener *et al.*, 1998; S. Egan, 1998) showed that two genes from streptomycin biosynthetic cluster (*strA*, *strB1*) are identical in a set of phylogenetically diverse streptomycetes. This study proves that in addition to the above genes, another gene from the same cluster, *strR*, has been detected in the same set of isolates. The organisation of these three genes, *strRAB1*, in the producer strain *S. griseus*, indicates that these genes are both physically and functionally dependent. They are clustered together (Fig. 4.12) and their expression is co-regulated. Transcription of *strA* occurs mainly as a readthrough from *strR* promoter and StrR also initiates transcription of *strB1* by binding to *strB1* promoter.

The presence of nearly identical sequences of these genes in a set of streptomycetes that were diverse both phenotypically and genetically leads to phylogenetic incongruency. Similar phylogenetic discrepancies can be seen in the distribution of streptomycin production throughout the *Streptomyces* genus (Fig. 4.13). These results can be explained by a number of mechanisms like different rates of evolution, unreliable identification of orthology, gene loss, parallel evolution and gene transfer. Partial sequences of the *strA* genes from other SARC producing type strains indicate that their genes are quite different from *strA* as found in *S. griseus* (Egan, 1998). Therefore, since the rates of evolution in all the SARC producing strains are the same it is subsequently expected that the *strRAB1* genes in Brazilian isolates would be quite distinct from those in *S. griseus* if these genes were residing on isolates' genome for a long time.

A scenario involving genes which duplicated and diverged prior to speciation of the strains can be fairly confidently ruled out, which has posed problems for interpreting other putative cases of gene transfer (Smith *et al.*, 1992). The extremely high levels of similarity (>98%) between the *griseus*-like *str*A genes of the isolates indicates a very short divergence time which is inconsistent with an ancient duplication event.

Figure 4.14: The structural and functional dependence of *strRAB1* genes in ASSF15, ASB37 and *S. griseus*. (A) The transferred gene fragment from streptomycin cluster. (B) Co-transcription of *strR-strA* genes from *strRp* (Vujaklija *et al.*, 1993). Transcriptional activation of *strB1* by StrR binding to upstream activating sequences (UAS) within *strB1p* (Retzlaff and Distler, 1995).

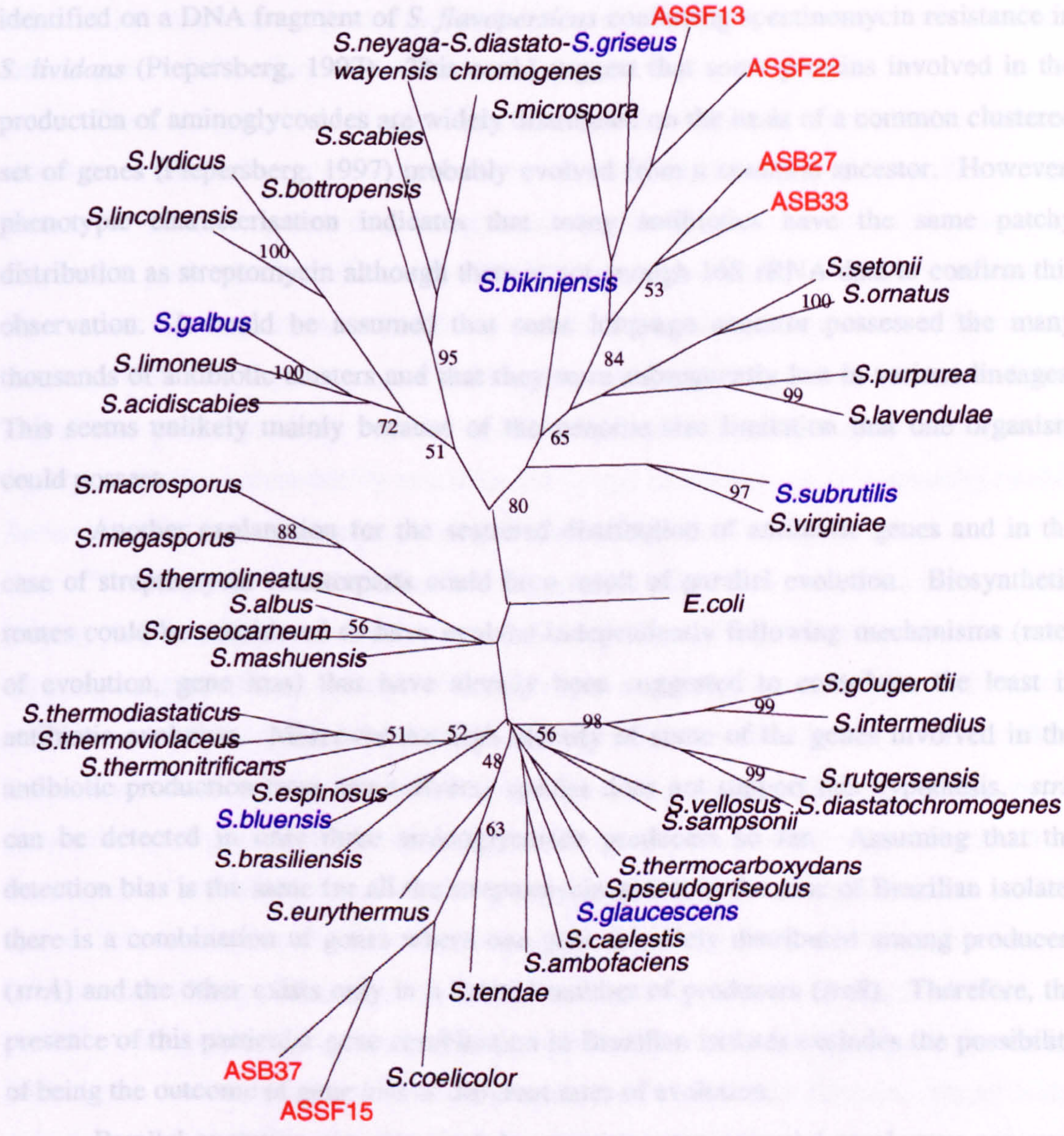


Figure 4.15: Distribution of streptomycin production within the genus *Streptomyces*. Phylogenetic analysis of *Streptomyces* based on parsimony analysis of 16S rRNA gene sequences. SARC-producing type strains are highlighted in blue and Brazilian isolates in red. Tree is derived by parsimony analysis. Bootstrapped 100 times. *E. coli* used as an outgroup. (Analysis performed by P. Wiener).

It could be possible that gene clusters have been completely lost multiple times in the history of streptomycetes (Piepersberg, 1995). A gene homologous to *strR* was identified on a DNA fragment of *S. flavopersicus* conferring spectinomycin resistance in *S. lividans* (Piepersberg, 1997). This could suggest that some proteins involved in the production of aminoglycosides are widely distributed on the basis of a common clustered set of genes (Piepersberg, 1997) probably evolved from a common ancestor. However, phenotypic characterisation indicates that many antibiotics have the same patchy distribution as streptomycin although there is not enough 16S rRNA data to confirm this observation. It could be assumed that some long-ago ancestor possessed the many thousands of antibiotic clusters and that they were subsequently lost in various lineages. This seems unlikely mainly because of the genome size limitation that one organism could possess.

Another explanation for the scattered distribution of antibiotic genes and in the case of streptomycin counterparts could be a result of parallel evolution. Biosynthetic routes could be considered to have evolved independently following mechanisms (rates of evolution, gene loss) that have already been suggested to contribute the least in antibiotic evolution. Moreover the high identity of some of the genes involved in the antibiotic production from some diverse species does not support this hypothesis. *strR* can be detected in only three aminoglycoside producers so far. Assuming that the detection bias is the same for all the streptomycin genes, in the case of Brazilian isolates there is a combination of genes where one gene is widely distributed among producers (*strA*) and the other exists only in a limited number of producers (*strR*). Therefore, the presence of this particular gene combination in Brazilian isolates excludes the possibility of being the outcome of gene loss or different rates of evolution.

Parallel evolution can also work by recruiting non-essential paralogous enzymes from the cellular metabolic network. In this way, the organisms could develop the biosynthetic capacity independently and in regard to certain environmental conditions showing the observed strain-specific and not species-specific behaviour of antibiotic production. Such approach could also support the high sequence similarity that biosynthetic genes are shown to share. However, in the absence of alternative option like horizontal gene transfer, such evolutionary strategy means that any organism should

possess a great deal of genetic information if it is to produce every necessary antibiotic in response to any possible environmental conditions. In contrast, since gene acquisition is balanced by simultaneous gene loss, horizontal gene transfer theoretically can introduce infinite genetic information without affecting genome size.

There is a general tendency in nature to use already tested genetic or biochemical material. In a sense, even the recruitment of enzymes with broad specificity, like protein kinases for the development of aminoglycoside resistance, can be considered as an intra-organismal gene transfer. The further distribution and evolution of antibiotic resistance also occurs by acquisition and not by evolutionary chance (mutation). Moreover, pathways for the degradation of recalcitrant aromatic compounds resemble lignin degradation pathways, from which they probably derive (Harwood and Parales, 1996). Finally, virulence occurs not by mutation but by the acquisition of pathogenicity islands determinants by horizontal gene transfer (de la Cruz and Davies, 2000).

The case of the Brazilian streptomycetes that are well characterised both phenotypically and genetically is a good example supporting horizontal gene transfer between distantly-related species. 16S rRNA and *trpB* analysis showed that ASSF15 and ASB37 were phylogenetically distinct from *S. griseus* and other isolates. In fact these strains were clustered together with *S. coelicolor.* Because *S. coelicolor* is known not to possess the *str*A gene, it can be inferred that at least one transfer event has occurred from the *S. griseus* group into the *S. coelicolor* group including strains ASSF15 and ASB37. Any transfer events must have occurred very recently since there are so few differences at the nucleotide level. What is also of interest is that these two recipients, ASSF15 and ASB37, were isolated from geographically distinct locations which suggests there may have been two independent transfer events (Santa Fé and Brasilia, respectively, Huddleston *et al.*, 1997).

16S rRNA data had been unable to resolve the phylogenetic positioning of ASB27 and ASB33. However, by examining the *trpB* housekeeping gene, both ASB27 and ASB33 were found to cluster away from the other four isolates and from *S. griseus.* These two strains cluster more closely with hydroxystreptomycin-producing *S. subrutilis*, although this probably reflects the fact that so few strains were included in this particular study. This suggests that at least one other separate transfer event has occurred.

Alternatively, because ASB27 and ASB33 are so similar as well as ASAF15 and ASB37 as shown by *trpB* analysis, the number of transfer events can be reduced by hypothesising two independent transfer events to the ancestors of these strains. However, this hypothesis suffers from the fact that ASB33 seems to have the entire cluster whereas ASB27 only has some of the genes. It could be envisaged that part of the cluster was lost after transfer even though this could be difficult to prove unless the precise selective pressure responsible for these transfers is known. Moreover, the high similarity of transferred genes between the isolates and the putative donor *S. griseus*, limits the divergence time between the possible initial transfer event and the subsequent speciation. What seems to be plausible is the fact that any donor can create the necessary selective pressure for gene transfer to occur but it is the recipient's genome that will determine the efficiency of such transfer event (e.g. integration). Therefore, any gene transfer vehicles like conjugative plasmids or transposons containing the corresponding *str* genes may be available in the ecosystem from which Brazilian strains had been isolated and can recognise an identical chromosomal region to which they can be integrated. From this aspect, some genes are known to be most prone to transfer (Eisen, 2000) but the same is also true for strains. Some of them are more vulnerable to gene transfer. For example, it has been observed that in some organisms, especially those with small population size like *Rickettsia*, horizontal gene transfer is absent as a means of evolution (Andersson and Andersson, 1999).

Two other strains, ASSF13 and ASSF22 were shown to be identical to *S. griseus* in terms of phenotypic tests, 16S rRNA and *trpB* analyses. These strains also possess the entire streptomycin cluster which suggests that it is possible to isolate *Streptomyces* strains from the environment that reflect the well characterised type strains held in culture collections.

Isolates ASSF15, ASB27 and ASB37 do not possess two other genes from the streptomycin biosynthetic cluster, *strF* and *strS*, and do not produce streptomycin. Previous studies showed the absence of other genes (*strN*) from the same cluster in these strains (Egan, 1998). The other strain ASB33 does produce streptomycin; it has all the tested genes and probably contains the entire cluster. In terms of expression, as expected *strR* is expressed in all the strains that produce the antibiotic and *strA* is found to be

transcribed in ASSF13, the only producing strain that was tested (Table 4.2). In *S. griseus*, previous studies (Piepersberg, 1997; Thamm, 1999) showed that streptomycin production follows a growth dependent manner, appeared initially around 14 h of growth with some biosynthetic enzymes -including StrR- having maximum expression at the same time. StrR is not detectable any more after 19 h of growth. In ASB33 the expression pattern resembles to that of the S. griseus-like isolates (ASSF13 and ASSF22). The peak expression of *strR* occurs a few hours (18 h) later and lasts correspondingly longer (25 h) than the type strain *S. griseus* (Fig 4.7). In *S. griseus*, *strR* and *strA* genes are co-transcribed through the A-factor responsive *strR* promoter (Vujaklija *et al.*, 1993). Consistent with that, *strA* transcripts were detected around the same time with the StrR expression in ASSF13. No further transcription seems to occur after 25 h of growth. These results suggest that the growth phase-dependent expression of *strRA* genes is controlled at the transcriptional level mainly caused by the availability of a specific σ-factor of RNA polymerase recognising the *strR* promoter. However, rapid degradation of transcripts and the corresponding proteins may also account for the observed mode of expression. In non-producing isolates there is no detectable expression of *strR* at any time and no transcription of *strA* in ASB37, the only non-producing isolate tested. From this study and others (Wiener *et al.*, 1998; Egan, 1998) it appears that the non-producing isolates showed streptomycin resistance as low as 5 µl/ml regardless of the growth conditions (C-source) or other stimulating effectors added to the media (A-factor). Therefore it seems that at least *strR* and *strA* are silent in these strains. In the natural environment, the strains could be found in niches where antibiotic production is at a low enough level for the strains to be resistant and thus have an advantage for carrying *strA*. They could also acquire the other two genes *strB1*, *strR* because they are adjacent to the resistant gene. However, besides the physical dependence of these three genes, the functional autonomy of this subcluster challenges the idea that this transfer may have another ecological role like the evolution of an antibiotic cluster.

It is known that expression of *strR* and subsequently *strA* and *strB1*, is under the control of A-factor in *S. griseus* (Horinouchi, 1999). In this type strain, A-factor receptor (ArpA) acts as a repressor-type regulator for an A-factor dependent gene (*adpA*) that serves as a transcriptional activator of *strR*, a pathway-specific regulator of Sm

biosynthetic gene cluster. A-factor, that is produced in growth-dependent manner (Ando *et al.*, 1997a,b), releases ArpA from the promoter of *adpA*, which then activates *strR*. It was obvious to attribute the lack of expression of transferred genes in the case of non-producing isolates to the absence of some parts or the whole cascade. This hypothesis was tested on ASB37, the *S. coelicolor*-like strain (Table 4.3). Using specific primers for A-factor biosynthetic gene (*afsA*) as found in *S. griseus*, PCR resulted a bright band corresponding to *scbA* gene that is involved in the formation of another butyrolactone SCB1 (Takano *et al.*, 2000). This result proved the common evolutionary origin of such butyrolactones. Although there is no identical A-factor in ASB37, *S. coelicolor* is known to restore Sm production and sporulation in A-factor deficient mutant of *S. griseus*, albeit at a 500-fold higher concentration than A-factor itself (Takano *et al.*, 2000). In any way, addition of crude A-factor in ASB37 did not increase the resistance levels. Detection of ArpA in ASB37 by PCR failed which is consistent with the results of other groups that they failed to detect any A-factor binding protein in cell extracts of *S. coelicolor*. If the rest of the A-factor cascade is intact in ASB37, the absence of ArpA should remove its negative effect switching constitutively on the *strR* expression. Consistent with this idea, primers designed from *S. griseus adpA* sequence were used to detect the presence of the A-factor dependent gene by PCR. This approach revealed the presence of highly homologous copy of *adpA* in ASB37, as also confirmed by the sequencing project of *S. coelicolor* (StC105 cosmid). The gene organisation upstream *adpA* in *S. griseus* and *S. coelicolor* is identical showing extremely high amino acid identities (Ohnishi *et al.*, 2000) that did not match to any other of many homologous regulators found in *S. coelicolor* (genome project). In the same sense, other parts of A-factor cascade showed much less homology with the corresponding genes in *S. coelicolor* (Onaka *et al.*, 1998). Although many butyrolactone receptors have been identified in *S. coelicolor* (Onaka *et al.*, 1998), none of them are able to bind *adpA* promoter site in the same strain (Onishi *et al.*, 2000). Trancriptional studies are necessary to elucidate the regulation of this gene. The presence of a highly similar copy of *adpA* gene in ASB37 and *S. coelicolor* A3(2) and the identical organisation of flanking regions at least in the case of *S. coelicolor* together with no established functions of these genes in these strains may also indicate a possible, quite

recent, and not optimised functionally, transfer of this DNA fragment from *S. griseus* to *coelicolor* population.

The presence of regulons of streptomycin biosynthetic route from *S. griseus* in the well-characterised phylogenetically distinct type strain *S. coelicolor* A3(2) and the continuous acquisition of genes of the streptomycin cluster by *S. coelicolor* natural isolates (ASB37) may have important implications on the evolution of the corresponding antibiotic. Although *S. coelicolor* A3(2) has a number of *adpA* homologues, the gene found in StC105 cosmid shows a significantly higher similarity (86%) with the counterpart copy in *S. griseus*. Accordingly, the lack of expression of transferred *str* genes in the Brazilian isolates must be looked at the *strR* upstream promoter regions comparing with those in *S. griseus* and testing their compatibility with *adpA* gene copy found in ASB37. For a better analysis of expression of *str* genes it is also important to detect the boundaries of these transfer events which could also indicate the vehicle of transfer (e.g. inverted repeats, phage remnants etc.).

The detection of genes like *scbA*, *adpA* in ASB37 and their possible presence in ASSF15 and sequencing of their flanking regions coupled with the data from *S. coelicolor* A3(2) genome sequencing project proved the correct identification of these isolates as *coelicolor* strains as indicated by 16S rRNA and *trpB* phylogenetic analysis.

The functional importance of intergenic region *strA-B1* consists of a palindrome sequence that comprises a StrR-binding site which serves for the transcriptional activation of *strB1*, an amidinotransferase enzyme involved in the streptidine biosynthesis. Amplification of this intergenic spacer from ASB37 showed virtually identical (~99%) sequence similarity relative to *S. griseus* corresponding sequence which indicates a recent transfer event. Egan (1998) found amidinotransferase activity in ASB37 and ASSF15, the level of which was indistinquisable from that of *S. griseus* and *S. bikiniensis*. Assuming the specificity of the assay used is not in question, the expression of *strB1* must then follow a StrR-independent mechanism. The streptidine subunit has been shown to have function in the cell wall of *S. griseus* (Barabas and Szabó, 1968), therefore the substrate could be available in the new hosts as an intermediate from another metabolic pathway which could account for the maintenance and expression of *strB1*.

The conservation of functional integrity of *strA-B1* intergenic region in terms of StrR-binding site could mean that post-transfer refinement of *strR* expression or other regulatory system can still affect the expression of *strB1* giving to the new host a selective advantage.

Whole genome sequencing projects revealed the major contribution of HGT in bacterial evolution (Doolittle, 1999a). It was also suggested that HGT has played an important role in the evolution of antibiotic clusters (Vining, 1992a). All biosynthetic genes for secondary metabolites investigated so far, have been found to be located in clusters including resistance mechanisms, regulators, transport and extracellular processing functions (Mansouri and Piepersberg, 1991; Piepersberg, 1994). Such clustering of genes could facilitate their spread by HGT (Piepersberg, 1991). The genetic material employed in the design of pathways seems to be used in modular fashion in order to acquire the maximum variation with the minimum of genetic and enzymatic reserves. The multiplicity of alternative end-products with related biosynthetic routes is more likely to happen by recombination mechanisms. Sequence analysis between homologous genes from streptomycin and hydroxy-streptomycin cluster of *S. griseus* and *S. glaucescens* respectively showed significantly higher similarity than within intergenic regions (Piepersberg, 1997). This indicates that the genetic information required for Sm production is very old and being frequently recombined in the actinomycetes. The principle of mixing gene clusters in order to increase the diversity of the end-product can be seen in other antibiotic biosynthetic pathways as well. A striking example is the genes involved in 6-deoxysugar metabolism. dTDP-glucose synthase from *S. fradiae* is similar to the respective *rfbA* from enterobacteria while the adjacent gene for glucose dehydratase in *S. fradiae* is more related to other counterparts in streptomycetes (e.g. *strE*) (Piepersberg, 1994). Genes involved in the biosynthesis of deoxyhexose subunit of avermectin in *S. avermetilis* is highly similar to *strD* of *S. griseus* (Mansouri and Piepersberg, 1991). The distribution of these constituents in natural products can be exploited in designing combinatorial biosynthetic pathways and protein engineering experiments for the production of novel antibiotics since specific attachment of one or more deoxysugars can be sufficient for the bioactivity of many antibiotics and antitumor polyketides (Doumith *et al.*, 2000).

The pyridoxal-phosphate dependent aminotransferases in streptomycin production (StsC, StsA) are thought to have evolved by gene duplication. However, the protein similarity between them is around 25%, that is too low to suggest gene duplication as being responsible for their occurrence during the evolution of the Sm pathway (Piepersberg, 1997). Perhaps, gene duplication and divergent evolution was followed by HGT and subsequent spread of these genes to other streptomycetes. The overall conclusion is that the gene pool containing the biochemical tools for the antibiotic production seems to be a highly fluid one, being frequently recombined and horizontally disseminated in prokaryotes. Although this recombination mechanism is random, it results however in a directed process aiming to end-product diversification independently of the biosynthetic route. The case of the Brazilian isolates, especially those with the partially detected *str* cluster (ASB27, ASSF15, ASB37), could be part of such an evolutionary process.

Chapter 5

Cloning of the flanking regions of the horizontally transferred *strR* gene in Brazilian isolate ASB37

5.1 Introduction

The topology of introgression of the horizontally transferred genes is important. Shared gene content phylogeny agrees with the one derived from rRNA sequences (Snet *et al.*, 1999) and *recA* produces trees that strongly resemble rRNA topology (Eisen, 1995). This suggests that those horizontal transfers that occur are either constrained by phylogenetic relationships indicated by rRNA tree or only occur for a moderate portion of the genome (Eisen, 2000b). Genome sequencing projects showed preferential target sites for insertion. Comparison of *E. coli* K-12 genome with genomes of *Klebsiella pneumoniae* and *Salmonella enterica* showed that integrations appear to be concentrated in areas of the genome where the rate of rearrangements is rapid, relative to the divergence times of these organisms, so that many sites of rearrangement have sustained changes in more than one lineage (McClelland *et al.*, 2000). Around the origin and terminus of replication is another "hot-spot" for genomic rearrangements and integration of foreign DNA (Lawrence and Ochman, 1998). Recombination and chromosomal inversions have been found tightly coupled with recombination (Eisen *et al.*, 2000; Valencia-Morales and Romero, 2000) due to the resolving of the knot-like structure necessary to the completion of replication (Corre *et al.*, 1997).

The catalytic role of the insertion sites in any intra- or inter-genomic rearrangement mechanism has been recognised so that transposon-mediated mutation has been divided in site-specific, transpositional recombination and random homologous recombination (Mahillon, 1998). The selection of the target site is based on both structural and functional characteristics of chromosomal regions. The insertion of many transposons is influenced by regional features like local DNA structure, the presence of protein binding sites or DNA supercoiling (Craig, 1997). Interestingly, DNA twists can control expression of certain genes in response to environmental changes (Wang and Syvanen, 1992). Conjugative transposon Tn*916* inserts at a region that includes several adenines followed by several thymines and often contains a static bend (Lu and Churchward, 1995). In general the mechanisms of transpositional shuffling and gene expression are coupled through DNA architecture (Dai and Rothman-Denes, 1999) that gives direction to the functional evolution.

In transpositional recombination, there is a duplication of a few bases of the target site on either side of the element as a result of the integration (Hallet and Sheratt, 1997). Normally in IS elements, such duplications immediately flank terminal sequences of the element that are inversely related to each other. Therefore the ends of the most IS elements are inverted repeats. In the case of IS*112* (*S. albus*) imperfect inverted repeats can be detected at its ends and was flanked by a 2 bp duplication at the target site (Rodicio et al., 1991). A related element, IS*493* caused 6 bp target site duplication upon insertion. A member of different family of insertion elements, IS*110* (*S. coelicolor*), contained imperfect inverted repeat albeit not absolutely terminal (Bruton and Chater, 1987). IS*110* is inserted in the right-hand end of ϕC31 phage DNA. There is extensive homology between the left and the right IS*110*-ϕC31 DNA junctions suggesting homologous recombination may be involved in the excision and transposition of IS*110* (Bruton and Chater, 1987). In contrast, the excision of conjugative transposons appear to be independent of homologous recombination as their integrases appear to have a different mechanism from that of transposases (Salyers *et al.*, 1998). In addition, there is no target site duplication when the conjugative transposon integrates (Salyers *et al.*, 1998).

A 2.1 kb segment of right end of streptomycete phage ϕC31 DNA was found to be sufficient to direct site-specific integration of plasmid vectors in *Streptomyces ambofaciens* and *Streptomyces fradiae* (Kuhstoss and Rao, 1991). Inspection of the sequence at the phage-host junction site revealed that recombination is conservative (Kuhstoss and Rao, 1991). An open reading frame coding for a 613-residue protein was identified in this region (Kuhstoss and Rao, 1991) belonging to the resolvase/invertase family (Thorpe and Smith, 1998) with some unique features. Its promoter is separated from the coding sequence upon integration (Kuhstoss and Rao, 1991). Integrase can act on both linear and supercoiled substrates (Thorpe and Smith, 1998). Comparison of phage and host attachment sites of ϕC31 reveals three bases of identity at the point of crossover (Kuhstoss and Rao, 1991). The direction of ϕC31 recombination is strictly controlled by these nonidentical recombination sites (Thorpe and Smith, 1998). Sequences around and in close vicinity of *attP* and *attB* sites contain imperfect inverted and direct repeats which may serve as potential binding sites for the phage integrase

(Kuhstoss and Rao, 1991). The ϕC31 attB site does not appear to be a tRNA. However, the DNA sequences around the attachment sites of *S. ambofaciens* and *S. fradiae* are almost 90% identical over at least 300 bp stretch. Coding regions of housekeeping genes in streptomycetes tend to be 85% to 90% conserved at DNA level.

Many PAIs are inserted at tRNA and tRNA-like loci, which appear to be common sites for the integration of foreign sequences (Hacker and Kaper, 2000). Unlike the ϕC31 integration site, the integrating actinomycete plasmids utilise *attB* attachment sites that resemble to tRNA genes (Hopwood and Kieser, 1993). Integration proceeds via recombination between the plasmid attachemt site (attP) and the host attachment site (attB). The *attP* and *attB* sites are identical over at least 43 bp. Because the *attB* sequence overlaps an end of the tRNA sequence, an intact tRNA gene remains after plasmid integration (Hopwood and Kieser, 1993). Excision of the plasmid regenerates the *att* site demonstrating that the transposition of the integrating element is conservative (Kieser and Hopwood , 1991). The integration and excision reactions for pSAM2 are catalysed by the products of plasmid-borne genes, which resemble the *int* and *xis* genes of phage λ which *attB* site does not resemble tRNA genes. This suggests that these elements might have been derived from temperate phages (Hopwood and Kieser, 1993).

Large deletions arising by genetic instability are frequently accompanied by high-copy-number tandem amplification of specific sequences called amplifiable units of DNA (AUDs). Two basic classes of amplifiable loci found in Streptomyces can be distinguished according to structural differences. Type I AUD are characterised by the absence of any special structure, are heterogeneous in size and are overlapping. In contrast type II AUD exhibits a repetitive structure stretching over several hundred base-pairs, flanking a central sequence. Amplified DNA sequences (ADS) associated with either the overexpression of antibiotic resistance or metabolite production are mostly from type II AUD (Volff and Altenbuchner, 1998). Amplification of an AUD element rarely occurs when it is present in one copy. The amplification itself is a two-step process. Single-copy amplifiable elements are converted by unequal crossing-over event to a duplicated structure (Schrempf, 1991). The presence of long direct repeats and certain DNA-binding proteins promote the amplification of specific chromosomal sequences. The type II AUD in S. fradiae is bound by 2.2 kb direct repeats within the

tylosin biosynthesis gene cluster (Baltz and Seno, 1988). The AUD1 element *in S. lividans* has a compound structure composed of three 1 kb and two 4.7 kb repeats alternating in the same orientation (Altenbuchner and Cullum, 1984). AUD2 carries mercury resistance genes in *S. lividans* and is flanked by the functional insertion element IS*1373* indicating that the duplication necessary for the amplification of AUD2 may arise through transposition of this insertion element (Volff and Altenbuchner, 1997). The current hypothesis about the amplification process involves the overreplication of the AUD by a rolling circle-like mechanism (Leblond and Decaris, 1994). Amplification of the best-studied AUD, the AUD1 (type II) from *S. lividans*, requires RecA-catalysed homologous recombination between two large direct repeats and a DNA binding protein encoded by the AUD itself (Volff and Altenbuchner, 2000). A minimal length of direct repeats is necessary for AUD1 amplification to occur. The mechanism of type I amplification remains unclear although RecA could play a role in this process as well (Volff and Altenbuchner, 1998).

An integron is a genetic unit that includes the determinants of the components of a site-directed recombination system capable of capturing and mobilizing genes that are contained in mobile elements called gene cassettes (Hall and Collis, 1995). Although integrons are normally reported from Enterobacteriaceae and other Gram-negative bacteria, a truncated integron has been found in *Mycobacterium fortuitum* (Martin *et al.*, 1990) and a functional integron has been described in *Corynebacterium glutamicum*, a Gram-positive species (Nešvera *et al.*, 1998). The gene cassette in this integron showed even higher expression when compared to the expression in *Escherichia coli*. Genes specifying adaptation to xenobiotics can also spread as integrons (Poelarends *et al.*, 2000). The widespread use of antibiotics and the introduction of xenobiotics into the environment seem to lead to adaptation by similar molecular mechanisms.

The essential components of an integron are a recombinase, a member of the λ integrase family that catalyses both integration and excision of cassettes, an adjacent site, attI, that is recognised by the integrase and is the receptor site for the cassettes. In addition a promoter is suitably oriented for expression of the cassette-encoded genes (Ouellette and Roy, 1987; Fig. 5.3). Three different integrase genes *(intI)* have now been identified and these define three distinct integron families (class 1,2 and 3) (Arakawa *et*

al., 1995). Integrons although unable to move themselves, can be located in transposons which have the ability to copy themselves to other DNA molecules, or on plasmids (Fluit and Schimitz, 1999).

The cassettes are mobile elements that include a gene and an integrase-specific recombination site that is a member of a family of sites known as 59-base elements. Though the amino acid sequences of the integrases are only 40-60% identical, the same cassettes have been found in integrons from different classes, indicating that all integrases recognize the cassette-associated 59-base elements (Arakawa *et al.*, 1995). The latter together with the fact that no two of the 59-base elements sequenced to date are identical defines integron as a functionally flexible unit which can be used for engineering purposes.

Multiple cassette insertions can occur, and integrons containing several cassettes have been found in nature. Recently, a *Vibrio cholerae* super-integron was found harbouring hundreds of gene cassettes with functions extending beyond antibiotic resistance and pathogenicity (Rowe-Magnus and Mazel, 1999). Therefore, the role of integrons has been expanded in genome evolution.

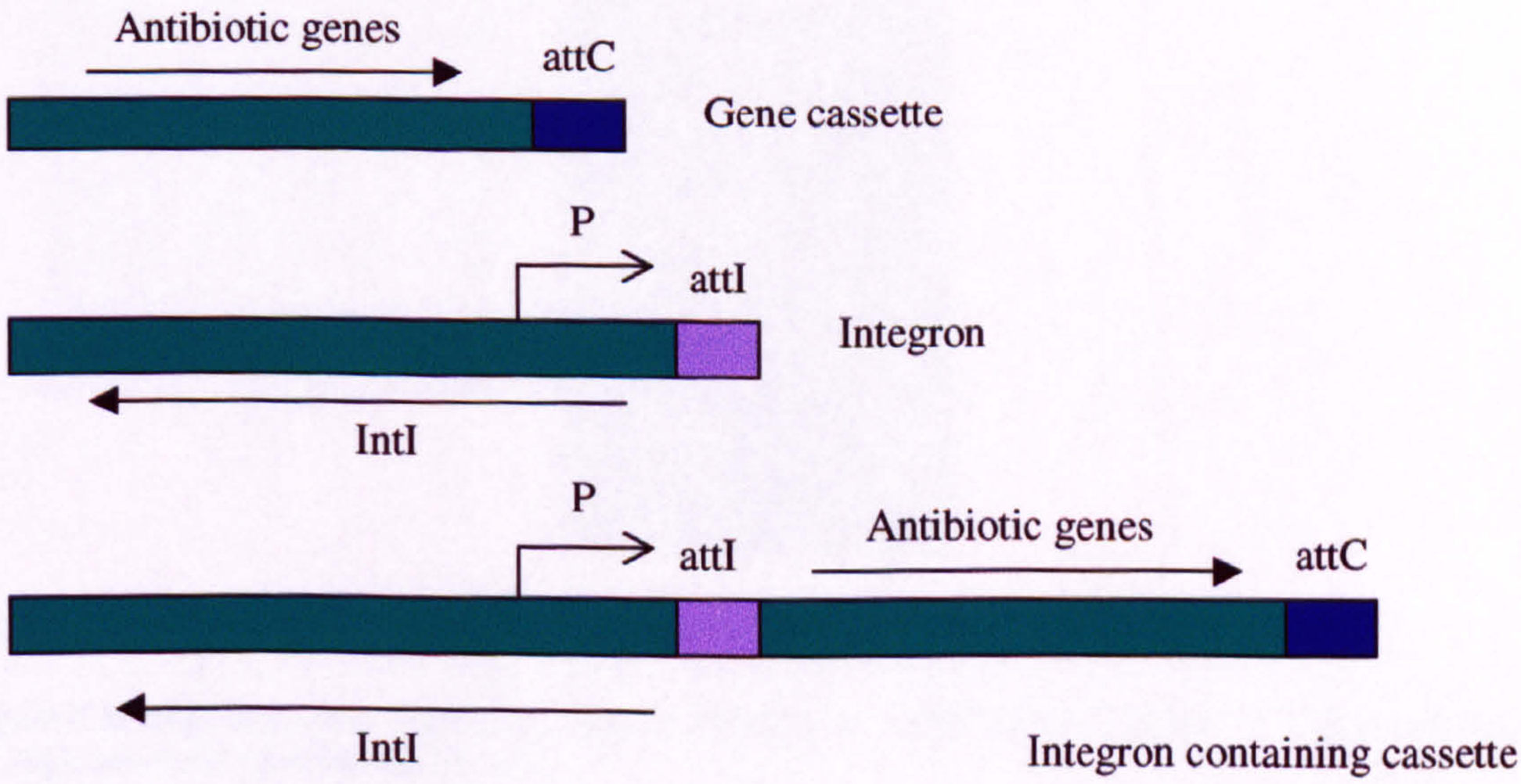


Figure 5.1: Integron structure.

This study aimed to identify the integration site of *str* subcluster gene transfer and to clone the upstream flanking of *strR* in ASB37 Brazilian isolate. This would facilitate the identification of the mechanism that was involved in the dissemination process and the detection of the presence or absence of other antibiotic-related genes and regulatory elements that may account for the lack of expression of the transferred genes.

5.2 Results

5.2.1 Plasmid library construction (size selection)

High molecular weight chromosomal DNA (Fig. 5.4) from ASB37 was isolated (section 2.9.3) and digested with a number of restriction enzymes. The chosen enzymes should fulfil certain criteria: (a) to cut within high %GC sequences, (b) not to cut the gene of interest (*strR*), (c) the gene of interest to be located on restriction fragment of suitable size for further analysis (cloning and sequencing). *Xho*I, *Pst*I, and *Stu*I and combinations of those were used to digest DNA from ASB37.

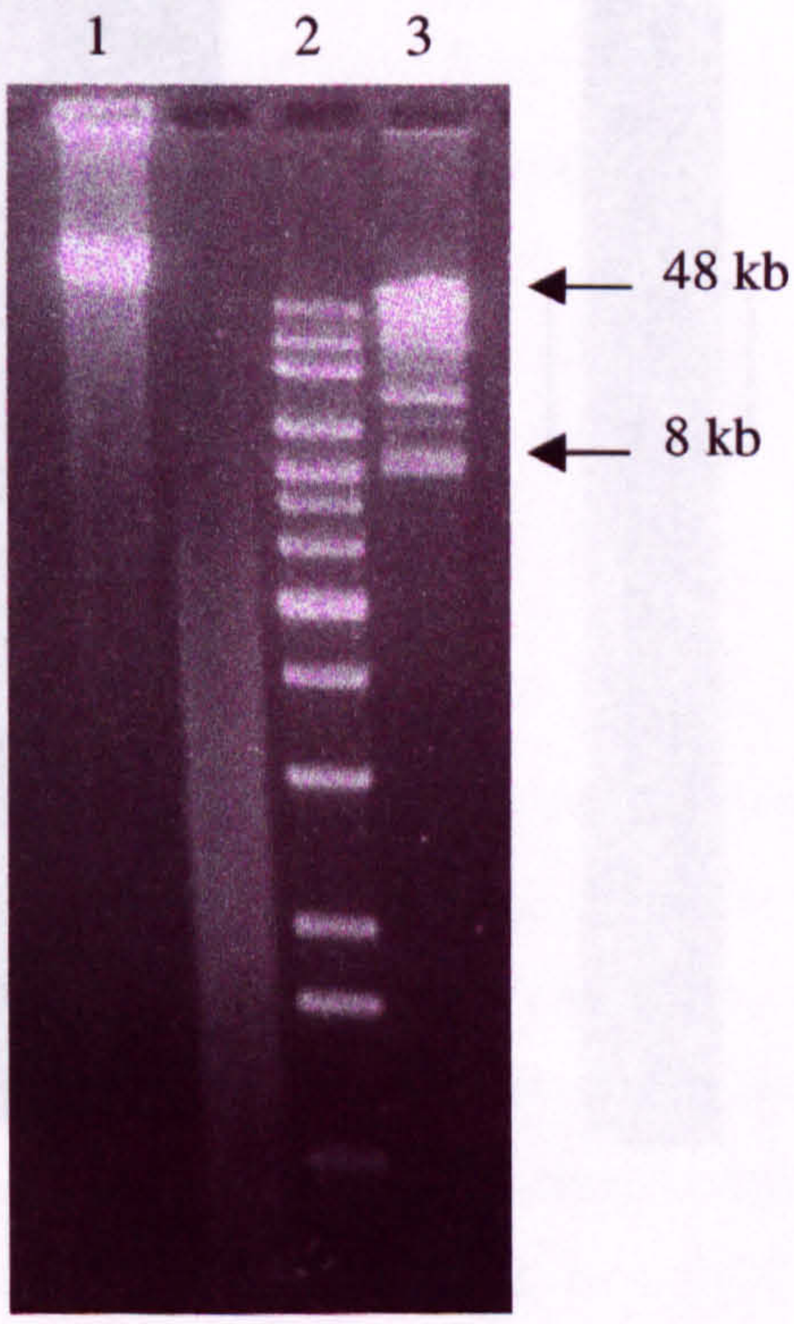


Figure 5.2: High molecular mass chromosomal DNA from ASB37 (1 µg / µl; lane 1). 1 kb ladder (lane 2), High molecular mass ladder (lane3).

The best restriction pattern was obtained with *Stu*I (Fig. 5.5A) that leaves blunt-ended fragments. Depending on the concentration of the chromosomal DNA each time, 15-25 µl of DNA (20 µg) was cut with 1.5 µl *Stu*I (15 units) in a total volume of 30 µl using the buffers specified by the manufacturers (Gibco BRL). The mixture was incubated at 37°C for 90 min. The digests were resolved on 0.8% agarose gel that used for Southern blotting. Total genomic DNA from ASB37 was analysed by Southern hybridisation using StrRF2-StrRR2 PCR product generated from *S. griseus*.

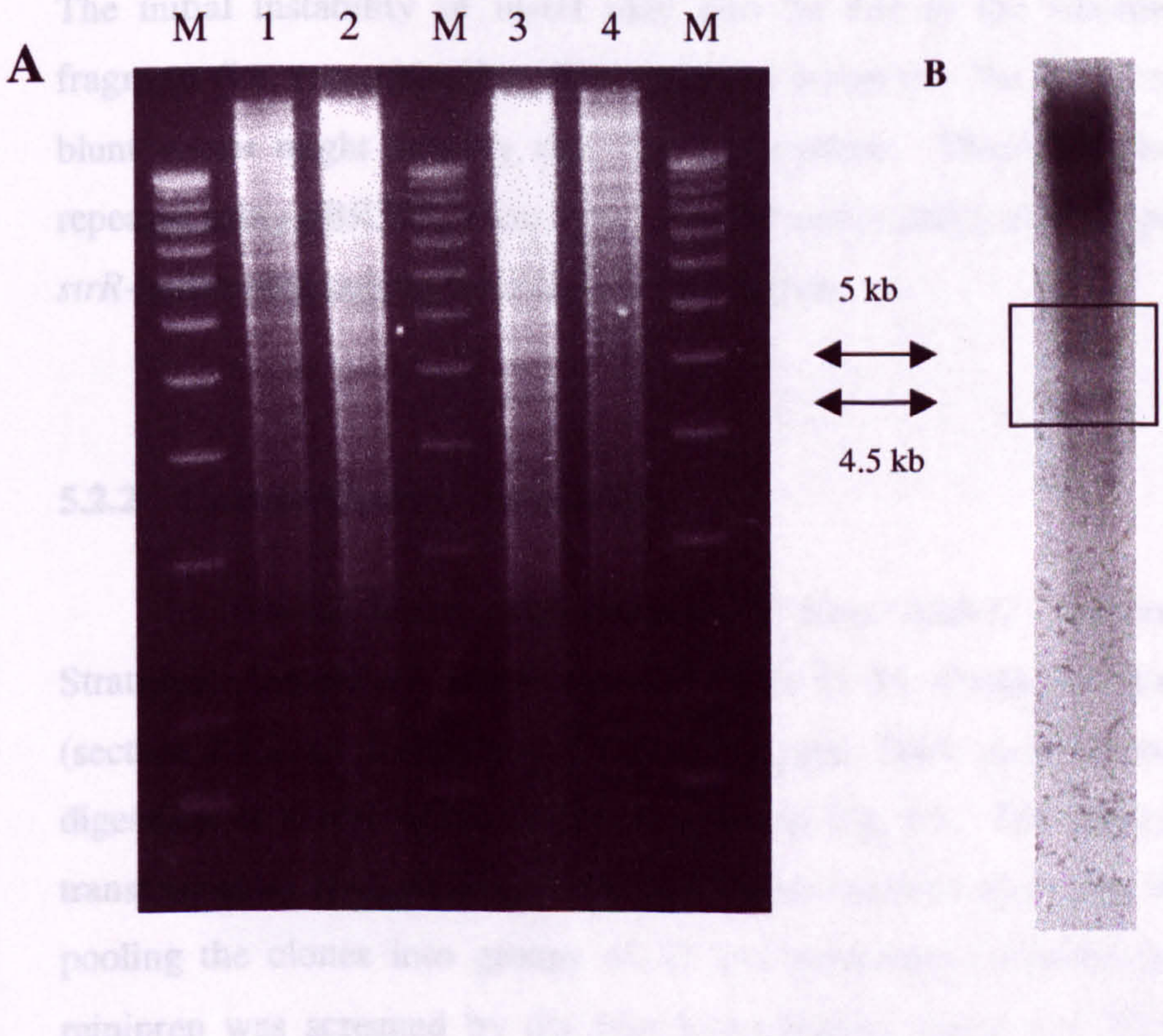


Figure 5.3: Detection of chromosomal fragment from ASB37 carrying *strR* gene. (A) Restriction digestion of chromosomal DNA from ASB37 using *Stu*I endonuclease. 1 kb ladder (lane M), 10 µl (lanes 1,4) and 25 µl (lanes 2,3) of restriction reaction was resolved on 0.8% agarose gel. (B) Southern hybridisation of total genomic DNA from ASB37 using 300 bp [α-^{32}P] dGTP-labelled *strR* PCR product. Two hybridised fragments of 4.5 and 5.0 kb were obtained.

Two hybridised fragments were obtained with approximate size 4.5 kb and 5.0 kb respectively (Fig. 5.5B). Chromosomal DNA corresponding to this size was gel-extracted, purified and shotgun cloned using pZero-blunt kit (Invitrogen). The transformants with the recombinant plasmids were screened by colony hybridisation using the same *strR* PCR product as before. Several colonies gave positive signals (Fig. 5.6). The colonies were grown and plasmid DNA was extracted and screened further by PCR using [StrRF2-StrRR2] set of *strR* primers. PCR products of the expected size (300 bp) were obtained (Fig. 5.7) and sequencing confirmed the presence of *strR* gene in one of these plasmids. However, restriction analysis of this plasmid revealed deletions associated with growth. Streptomycete DNA is known to be difficult structure to clone. The initial instability of insert may also be due to the structure of *strR* restriction fragment (i.e. recombination sites, inverted repeats). The high-copy number of pZero-blunt vector might amplify this cloning problem. Therefore, the whole strategy was repeated using pBR329, a low-copy number vector, but it was not possible to identify any *strR*-harbouring colony by colony hybridisation.

5.2.2 Cosmid library construction

A cosmid library was constructed from ASB37 chromosomal DNA using a Stratagene SuperCos1 vector and Gigapack II XL Packaging Extract as described in (section 2.9.21). Conditions for chromosomal DNA preparations and partial *Sau*3AI digestions of chromosomal DNA are given in Fig. 5.8. The library was amplified (1000 transformants) for screening. Cosmids were isolated from the 1000 transformants by pooling the clones into groups of 12 and performing alkaline lysis minipreps. Each miniprep was screened by dot blot hybridisation using *strA* PCR product as a probe generated using [StrAF-StrAR] set of primers (550 bp). One group gave a positive signal and each of the 12 cosmids were isolated individually (alkaline lysis miniprep) and re-screened for the *strA* by dot-blot hybridisation using the same PCR product as before. A single positive clone was identified (Fig. 5.9). However, similar deletions as found in the plasmid library were observed (Fig. 5.10). Although the unstable *strR*-containing

fragment may be responsible for such deletions, more than 80% of the rest of the cosmids showed deletions.

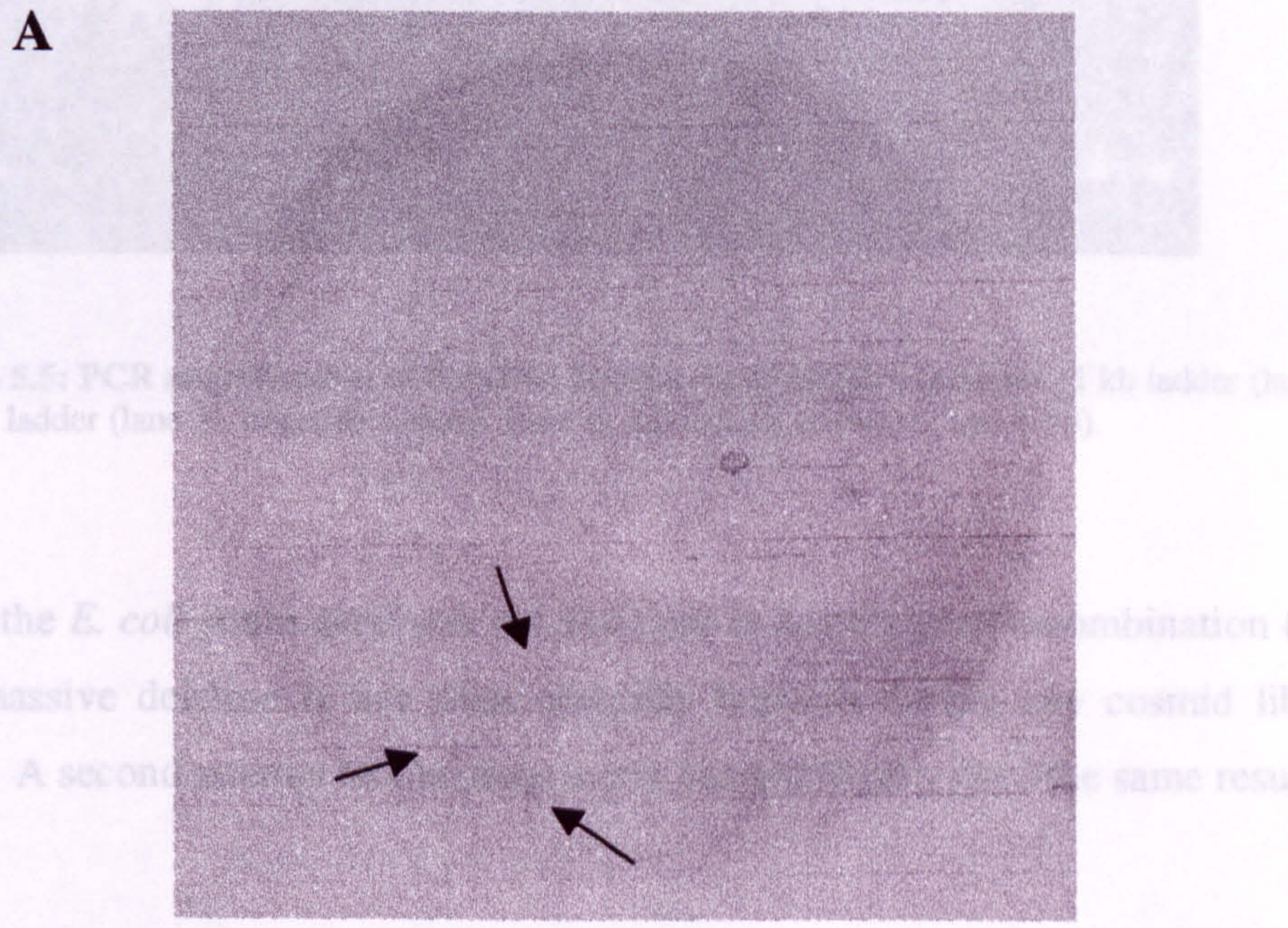


Figure 5.4: Screening of ASB37 plasmid library (size selection). (A) Colony hybridisation using (B) PCR product derived from *strR* gene as a probe.

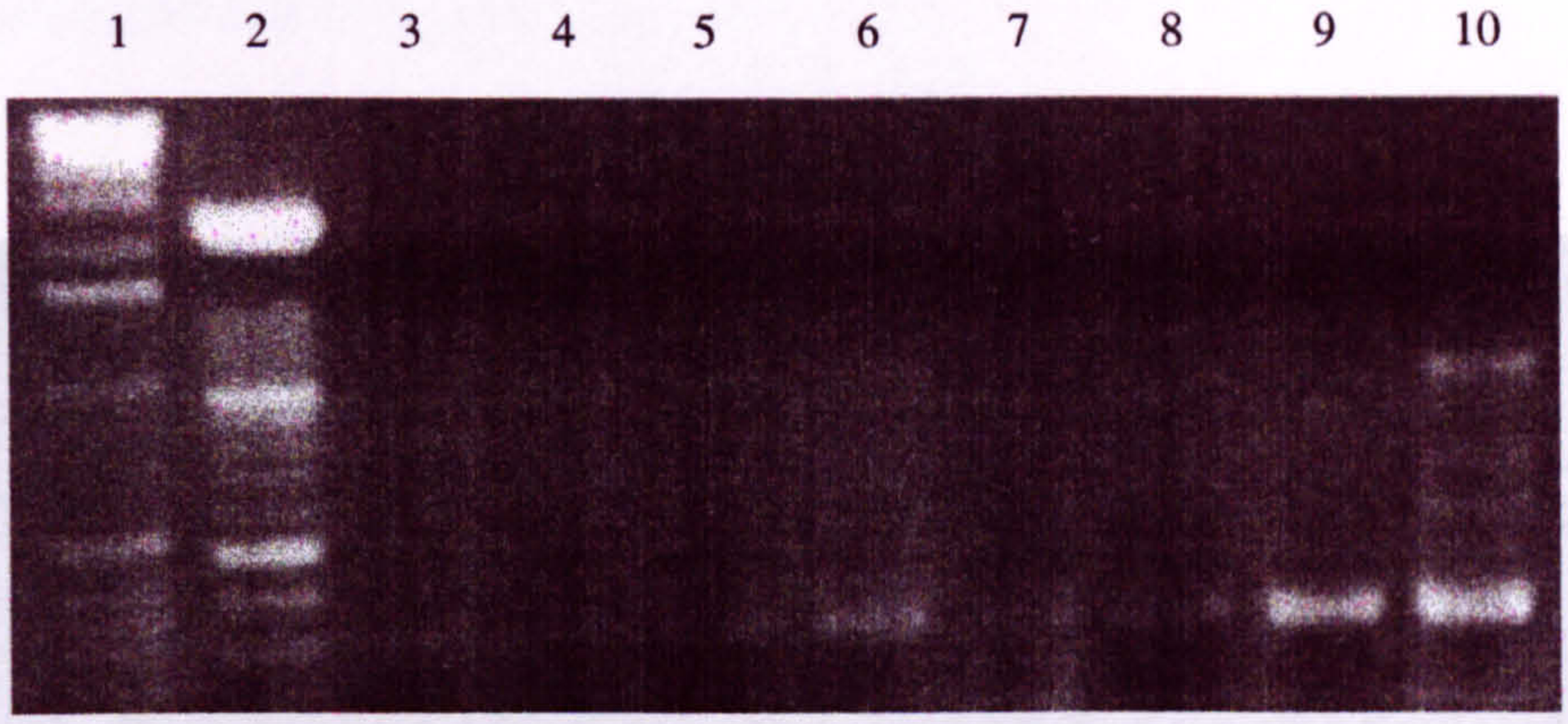


Figure 5.5: PCR amplification of the DNA from a set of positive colonies. 1 kb ladder (lane 1), 500 bp ladder (lane 2), negative control (lane 3), 8 different colonies (lane 4-10).

Even though the *E. coli* strain used was not deficient in homologous recombination (XRI blue) such massive deletion is not what normally happens during any cosmid library construction. A second attempt to construct a new cosmid library gave the same results.

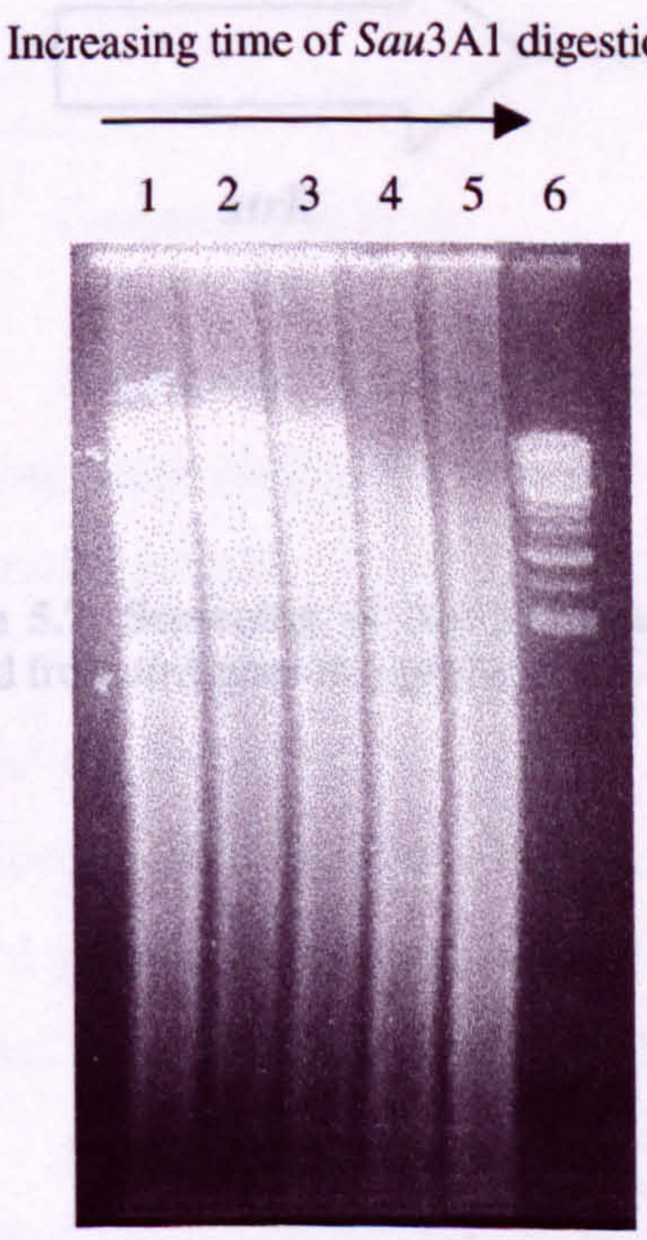


Figure 5.6: Chromosomal preparation for size selection and library construction. *Sau*3A1 endonuclease digestion with increasing reaction time from 1 to 5 min (lanes 1-5). 3 min reaction gave the appropriate restriction pattern for the construction of cosmid library.

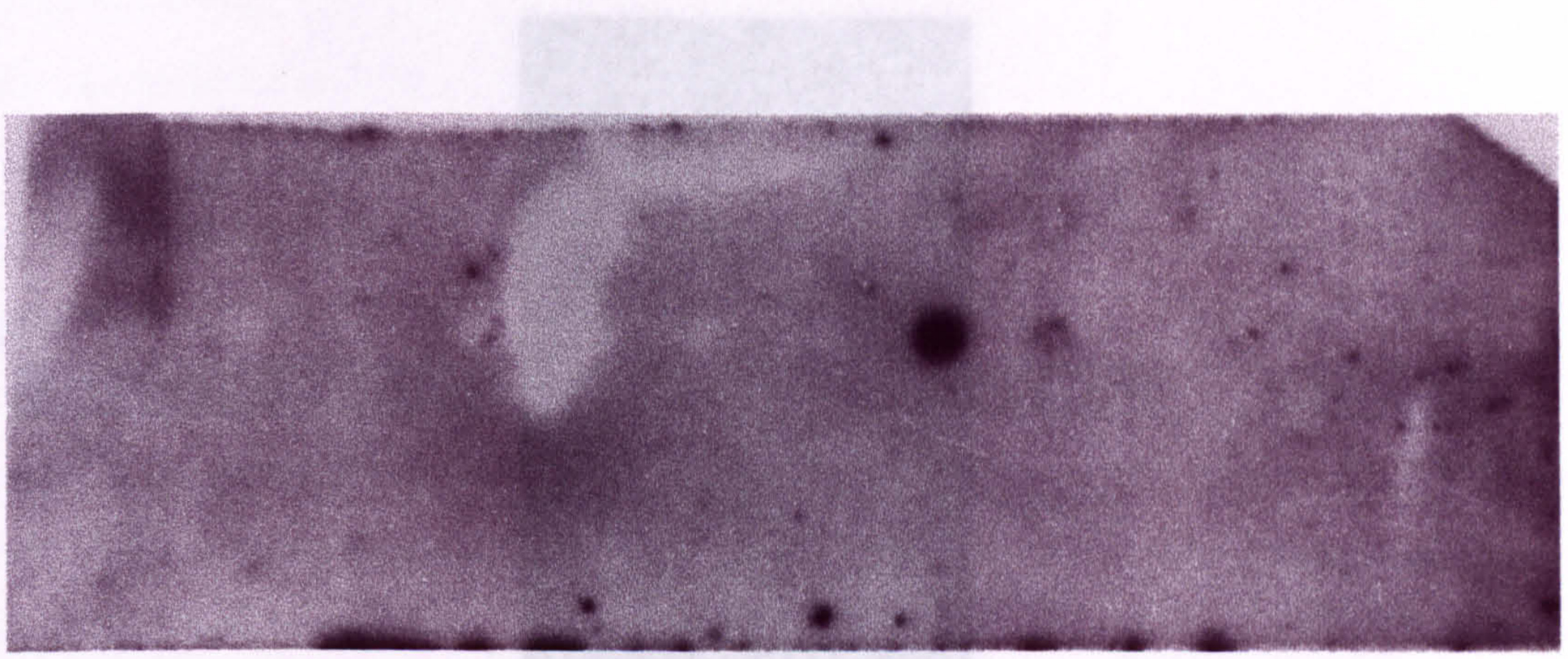


Figure 5.7: Screening of ASB37 cosmid library. (A) Colony hybridisation using (B) PCR product derived from *strA* gene as a probe.

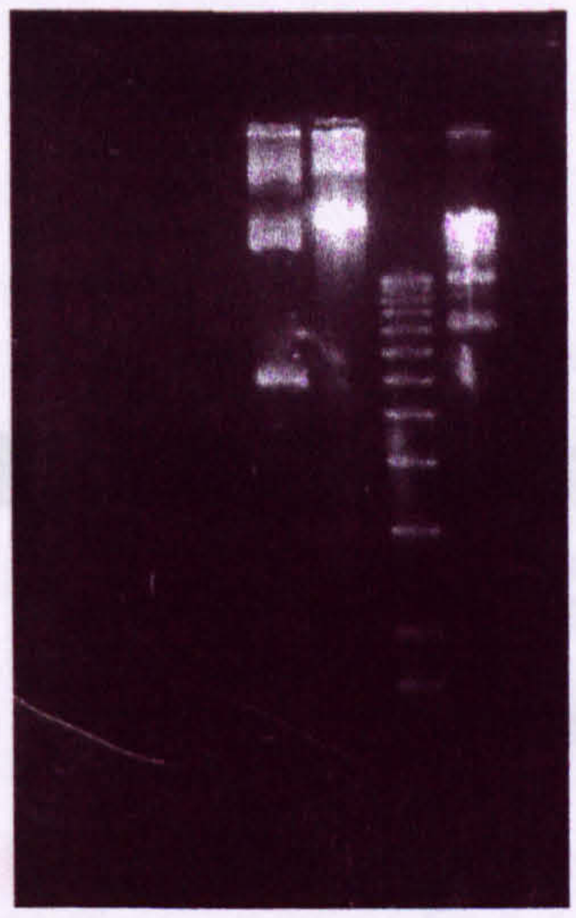


Figure 5.8: Deletions of ASB37 cosmid hybridised against *strA* probe. ASB37 uncut cosmid (lane 1), *Micromonospora carbonacea* uncut cosmid (lane 2), 1 kb ladder (lane 3), high molecular weight marker (lane 4). No intact ASB37 cosmid could be observed. By comparison with the also uncut cosmid, the ASB37 bands were not attributed to different conformation of the cosmid molecule (i.e. supercoiled). Multiple bands and DNA smearing had always been observed for ASB37 cosmid extracted at different times of growth of the host strain.

5.2.3 Genome walking

A genomic library of ASB37 was constructed and amplified by using genome-walking methodologies (Genome Walker kit; Clontech) according to the manufacturer's recommendations. Briefly, genomic DNA was digested with restriction endonucleases that leave blunt ends. Double-stranded "adaptors" were then ligated to blunt-ended restriction digestion products. Genomic library amplification reactions were then done by using nested adaptor oligonucleotide primers (Genome Walker kit; Clontech) and nested gene-specific (*strR*) primers. The nested gene-specific primers were designated as GSPR11 and GSPR22 (see Methods and Materials).

One of the blunt-end enzymes was *Stu*I, the same enzyme used for plasmid library construction. Using *Stu*I-derived genomic libraries, PCR yielded a unique 3.3 kb

fragment (Fig. 5.12) which is consistent with the observations based on the plasmid library approach that has been undertaken (Fig. 5.11).

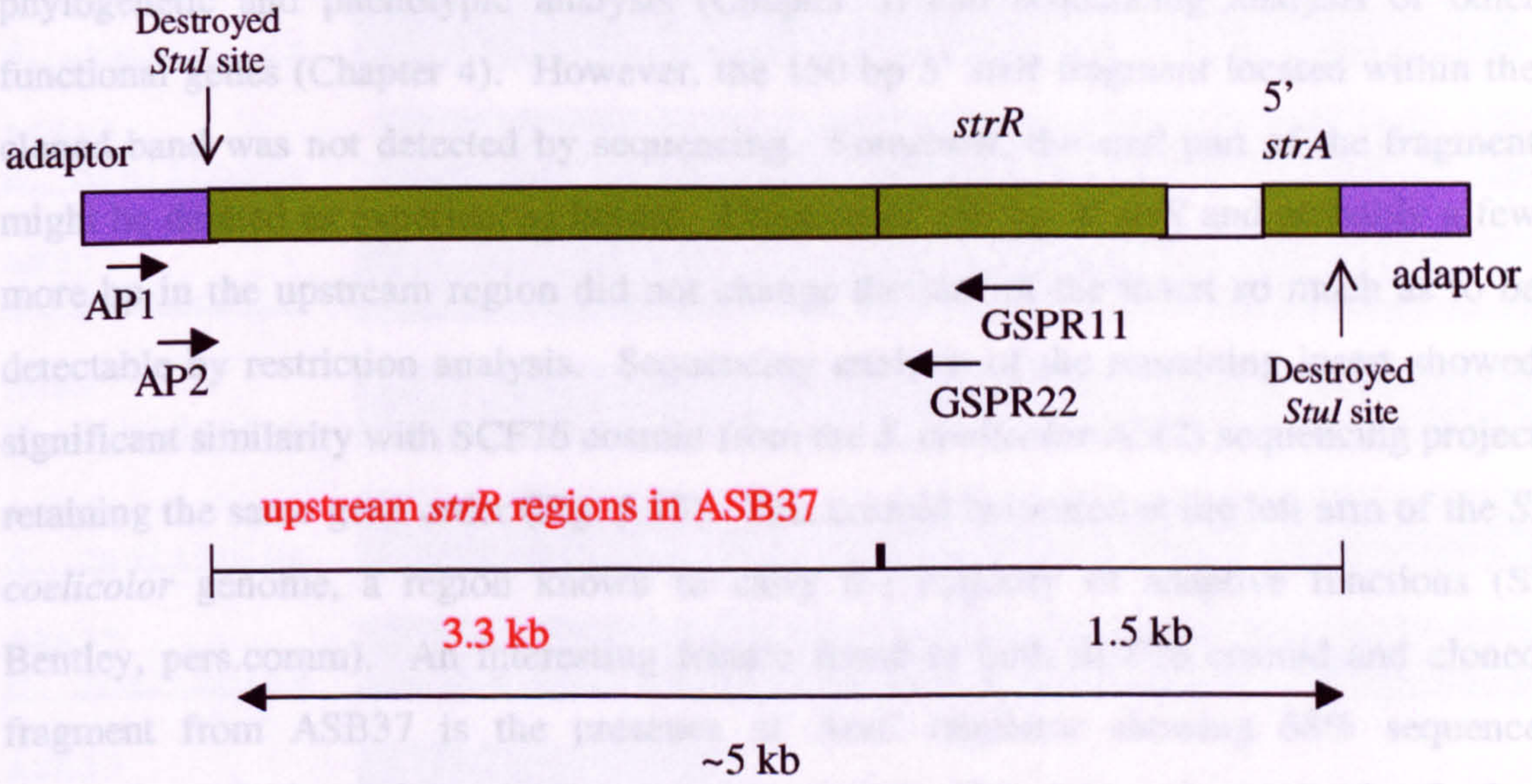


Figure 5.9: Schematic representation of genome walker approach for cloning of *strR* flanking regions from ASB37. PCR-based cloning of unknown regions using primers specific to the adaptor and *strR* sequences. Estimation of the size of the *strR* upstream flanking regions based on previous plasmid library hybridisations.

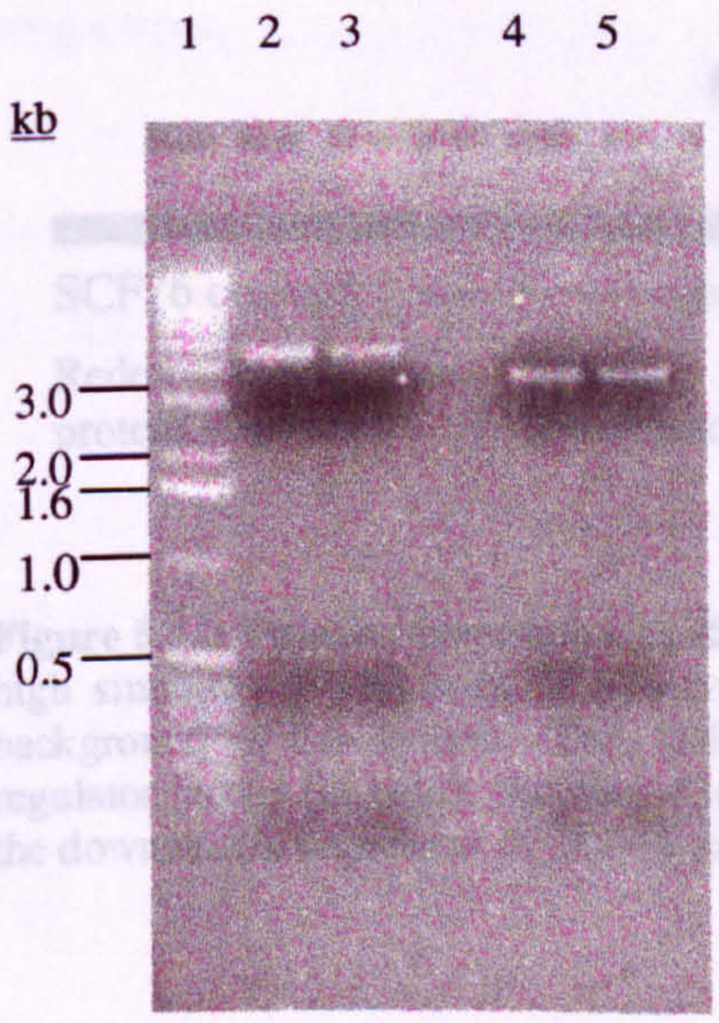


Figure 5.10: PCR amplification of *strR* upstream regions using Genome Walker Kit (Clontech). *StuI* genomic libraries yielded a unique band of the expected size (~ 3.3 kb) (lane 4,5). 1 kb ladder (lane 1), PCR amplification of *DraI* genomic libraries (lane 2,3).

The band was cloned into the TA-vector (Invitrogen) and the plasmid used to transform STBL2 cells (GibcoBRL) that can stabilise direct repeats within cloned sequences. Plasmid DNA was extracted using Qiagen miniprep and sequenced. The sequencing results confirmed the *S. coelicolor* background of ASB37 as has been suggested by phylogenetic and phenotypic analysis (Chapter 3) and sequencing analysis of other functional genes (Chapter 4). However, the 150 bp 5' *strR* fragment located within the cloned band was not detected by sequencing. Somehow, the *strR* part of the fragment might be deleted as experienced before. Deletion of 150 bp of *strR* and probably a few more bp in the upstream region did not change the size of the insert so much as to be detectable by restriction analysis. Sequencing analysis of the remaining insert showed significant similarity with SCF76 cosmid from the *S. coelicolor* A3(2) sequencing project retaining the same gene order (Fig. 5.13). This cosmid is located at the left arm of the *S. coelicolor* genome, a region known to carry the majority of adaptive functions (S. Bentley, pers.comm). An interesting feature found in both SCF76 cosmid and cloned fragment from ASB37 is the presence of AraC regulator showing 68% sequence similarity with the A-factor responsive gene (*adpA*) (Fig. 5.14). Interestingly, further upstream of this cosmid there is another region of *S. coelicolor* genome (SCF62 cosmid), which contains genes showing significant similarity with streptomycin genes from *S. griseus* (*strEM*).

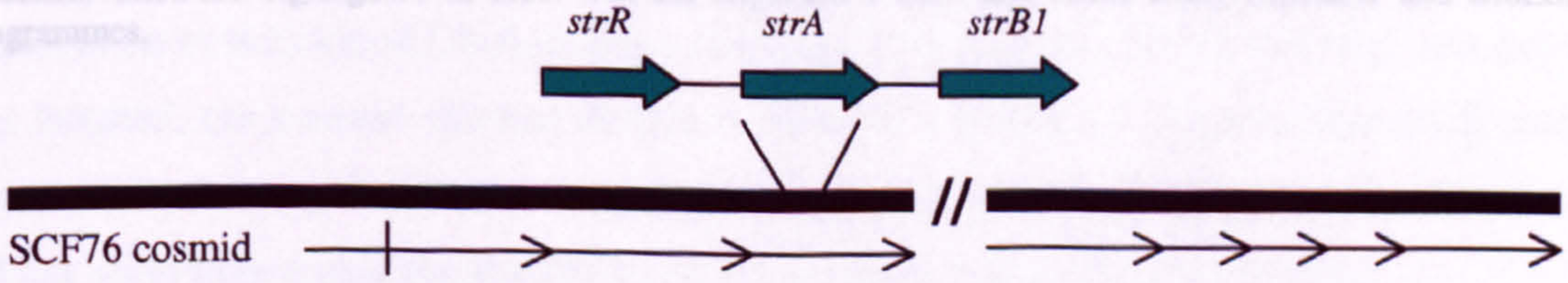


Figure 5.11: Putative insertion site of *str* subcluster within ASB37 genome. The sequence data showed high similarity (97%) with SCF76 cosmid from *S. coelicolor* A3(2) genome indicating the *coelicolor* background of this isolate. This DNA fragment contain a gene belonging to the *araC* transcriptional regulator family (in bold). Another AraC transcriptional activator and an ABC transporter are also found in the downstream sequences of SCF76 cosmid.

```
ASB37          CAGGTCGCCCGATCACTGCCGGTGTACATGCAGCGGGCCGGCGGCCAGTC
S. coelicolor  CAGGTCGCCCGCTCACTGGTGGTGTACATGCAGCGGGCCGGCGGCCAGTC

ASB37          GCAGTTCTCCGCCTCGCTCCAGGGCCCGGCTCCCCGCACGCCGGTGCTGT
S. coelicolor  GCAGTTCTCCGCCTCGCTCCAGGGCCCGGCTCCCCGCACGCCGGTGCTGC

ASB37          TGCTGGTCCAGGAGGCGGTGCAGGGCGATCCGACGGCGGACCACGGTCTG
S. coelicolor  GGCTGGTCCAGGAGGCGGTGCAGGGCGATCCGACGGCGGACCACGGCCTG

ASB37          GAGGCGCTGGCCGCCCGGGTGCGGGTCAGCCCGCGGCACCTGACGCGCAT
S. coelicolor  GAGGCGCTGGCCGCCCGGGTGCGGGTCAGCCCGCGGCACCTGACGCGCAT

ASB37          GTTCCGGGCCGAGCTGGACACGACGCCGATGAAGTACGTGGAGCTGATCC
S. coelicolor  GTTCCGGGCCGAGCTGGACACGACGCCGATGAAGTACGTGGAGCTGATCC

ASB37          GCTTCGACATGGCCAAGGCCCTGCTCGACGCCGGGCACAACGCCACCGAG
S. coelicolor  GCTTCGACATGGCCAAGGCCCTGCTCGACGCCGGGCACAACGCCACCGAG

ASB37          GCCGCCACCCGATCGGGCTTTTCGAGCTACGAGAGTCTGCGCCGGGCCTT
S. coelicolor  GCCGCCACCCGATCGGGCTTTTCGAGCTACGAGAGTCTGCGCCGGGCCTT

ASB37          CGCCCGGCACCTGGGA
S. coelicolor  CGCCCGGCACCTGGGA
```

Figure 5.12: Sequence analysis of putative insertion site of *strRAB1* genes in ASB37 shows high similarity with SCF76 cosmid and AraC transcriptional activator from *S. coelicolor* A3(2). Regions of conservation are highlighted in blue. All the alignments were performed using ClustalW and BioEdit programmes.

5.3 Discussion

The task of cloning the *strR* flanking regions proved to be very difficult. Despite the initial positive detection of clones, plasmid library construction yielded a series of deleted clones. Chromosomal DNA from ASB37 was digested with enzymes that could yield a well-resolved restriction pattern but result also in a restriction fragment containing *strR* and appropriate flanking sequence which would be sufficient for sequencing analysis. The best restriction patterns fulfilling these criteria were provided by *Stu*I enzyme using a restriction time of 90 min. The *strR* PCR product from *S. griseus* was gel-purified and used to probe the corresponding *Stu*I chromosomal digests of ASB37. Repetitive hybridisations showed a unique ~5 kb fragment hybridised against *strR*. Some radiation above this band must be due to non-specific hybridisation of the probe with the high amount of DNA left in this region. *Stu*I enzyme is a moderate GC cutter that leaves a lot of chromosomal DNA uncut. In addition some partially digested *strR* containing sequences could still be hybridised and give the observed high molecular mass signal in the southern blots. pZero blunt plasmid (Invitrogen) used for this cloning strategy is a high-copy number that can destabilise DNA sequences from high GC organisms like streptomycetes.

A cosmid library approach was used to maximise the size of the flanking region obtained from both ends of the *strR-strB1* transferred fragment in the ASB37 isolate. In addition the low-copy number of SuperCos1 vector might be proved useful for the stabilisation of the cloned DNA inserts. Although one positive cosmid carrying *strA* gene was isolated, the cosmid showed deletions related to growth of the transformed *E. coli* strain. *E. coli* hosts deficient in *rec* genes and therefore in homologous recombination did not seem to decrease the instability of the cloned *str*-containing sequences.

A genome walking approach (Clontech) was the third attempt undertaken to clone the *strR* flanking regions. Chromosomal digests of ASB37 DNA were ligated with adaptors (Clontech) of known sequence. PCR was employed using primers from the adaptors and *strR* gene. A PCR product of the same size as the *Stu*I restriction fragment from the plasmid library was obtained. However, the sequence did not confirm the presence of the expected part of *strR* gene. This could be due to the loss of this sequence

by deletion as had been experienced before. One way to verify this interpretation would be to design primers from the *strR* gene and the cloned flanking sequences and attempt to amplify this region using ASB37 chromosomal DNA. If these sequences are contiguous then a predictable PCR product must be obtained. Alternatively, the cloned flanking region can be used as a probe for hybridisation against *Stu*I digests of chromosomal DNA from ASB37 in order to prove the co-linearity with the corresponding *strR* hybridised fragment. In this way any PCR bias can be avoided.

The observed instability of *strR* containing sequences may be due to the presence of inverted and direct repeats or secondary structures. The existence of such structures renders the clonable region proficient for deletion by homologous recombination and genetic rearrangement. The *Streptomyces* genome frequently undergoes gross genomic rearrangement events which results in the deletion of extremely large seqments of chromosomal DNA. Spontaneous mutability is correlated with large scale DNA rearrangements, mainly giant deletions and amplifications in a limited genomic regions. The unstable region is present at the chromosomal extremeties. Replication of the *Streptomyces* linear chromosome proceedes bidirectionally from an origin of replication *oriC* located in the centre of the chromosome (Musialowski *et al.*, 1994). The unstable region appears to be at the terminus of replication, a 'hot spot' location for genomic rearrangements as described in other bacteria as well (Eisen *et al.*, 2000). Interestingly, SCF76 cosmid from *S. coelicolor* A3(2) genome is located at the left chromosomal end.

The DNA sequence of one end of the *Streptomyces* chromosome is inversely repeated to the other end. These terminal inverted repeats (TIRs) have sized between 24 kb and 559 kb (Volff and Altenbuchner, 2000). Many short palindromic repeats are present within the last few hundred basepairs of the TIRs (Lin *et al.*, 1993). Transposable elements are also found at the end of *S. lividans* chromosome (Tn*4811*) which can allows the exchange of the ends with another linear replicon (Volff and Altenbuchner, 1998). Amplification events in streptomycetes are usually accompanied by extensive deletions. The deletions usually end near or within the amplified DNA. An internal DNA segment bordered by direct repeated elements can undergo amplification. However, this internal sequence must encode for a DNA binding protein recognising the flanking repeats (book ref). A minimal size of the direct repeats is necessary for the amplification of the internal

segment plus one copy of the repeated element (Young and Cullum, 1987). A recombination event between the two direct repeats is mediated by RecA (Volff and Altenbuchner, 1998). DNA sequences on one side, but not the other, of the amplifiable region are especially prone to suffer deletion (Young and Cullum, 1987). The location of the amplifiable region is also important. Introduction of the AUD1 element in a region close to the replication origin did not result in any amplification (Volff and Altenbuchner, 1998).

Another mechanism of telomere rearrangement is the replacement of the chromosomal arm by interchromosomal homologous or illegitimate recombination between two non-allelic copies of duplicated genes located in opposite orientation on different chromosome arms. This may lead to gene duplication together with a deletion (Fischer *et al.*, 1997). Terminal inverted repeats can also be exchanged between the two chromosomal arms.

Genes located near the telomeres should be more frequently included in duplications and deletions generated by this mechanism than genes located far away from chromosomal ends. No housekeeping genes were located within 800 kb from one end and at least 300 kb from the other chromosomal end of *S. lividans*. In contrast, dispensable properties like antibiotic biosynthesis and resistance genes are found in the deletable region (Leblond and Decaris, 1994). Genes encoding for regulators of gene expression (repressors and σ-factors) are found in the amplifiable regions (Leblond and Decaris, 1994). DNA sequence from AUD90 (*S. ambofaciens*) encodes for proteins homologous with acyltransferases which are involved in the biosynthesis of the antibiotic erythromycin in *Saccharopolyspora erythraea*. The organisation of these genes and their expression is unique indicating the participation of these genes in a new biosynthetic pathway (Leblond and Decaris, 1994).

Chapter 6

Binding properties of N-terminal truncated streptomycin pathway-specific regulator StrR

6.1 Introduction

Antibiotic biosynthesis appears to be regulated by a complex network of pleiotropic as well as specific regulators composing a signal transduction cascade. Various environmental signals, such as changes in the availability of nutrients (phosphate, nitrogen and carbon source) or the synthesis of an extracellular autoregulator molecule (A- factor) participate in the regulation of Sm synthesis. Analysis of antibiotic clusters showed the presence of pathway-specific regulators which are the last sensors of the above signals and control the expression of biosynthetic genes mainly at the transcriptional level. The selectivity of particular promoters is determined by RNA polymerase holoenzyme that consists of the core enzyme with the subunit structure $\alpha_2\beta\beta'$ and one of the several species of σ subunit (Buttner, 1989). It is this interaction of σ subunits with the core enzyme that causes the functional differentiation of the various forms of holoenzymes. A second level of differentiation of RNA polymerase involves additional transcription factors for transcription initiation (Ishihama, 1988). Interactions between RNA polymerase and a regulatory protein that binds to neighboring DNA sites at the promoter results in cooperative binding of both components. Protein-protein contacts affect transcription in a number of ways which also reflect the different structural interactions occurring with either the α subunit or the σ subunit of holoenzyme (Ishihama, 1993). Transcriptional proteins can induce DNA unwinding affecting transcription by either realigning the –10 and –35 hexamers when their spacing is suboptimal at the promoter, that allows RNA polymerase binding (*merT* operon; Ansari *et al.*, 1995) or facilitating open complex formations (*ilv* regulon; Opel *et al.*, 2001). Alternatively, binding of proteins may cause DNA looping mediating both the repression by DNA contacts shifting (*ara* operon; Schleif, 1988) or activation by enhancing contacts between transcription protein and polymerase (*glnA*; Wedel *et al.*, 1990). Transcriptional activation, mediated by DNA binding proteins, is a central regulatory mechanism of antibiotic gene expression (Table 6.1). Biosynthetic gene clusters responsible for daunorubicin, actinorhodin, undecylprodiogiocin and β-lactams (cephamycin, and clavulanic acid) production in different *Streptomyces* spp. are regulated by *dnrI, actII-*

ORF4, *redD* and *ccaR* respectively (Tang *et al.*, 1996; Chater, 1992; Takano *et al.*, 1992; Alexander and Jensen, 1998; Pérez-Llarena, *et al.*, 1997)). These proteins are homologous and comprise a common family of streptomycetes pathway specific regulator proteins (SARPs) (Wietzorrek and Bibb, 1997). In addition, *dnrI* can complement *actII*-ORF4 mutations and *actII*-ORF4 can stimulate daunorubicin production in *S. peucetius*, whereas the other family regulators cannot show a similar cross-complementation effect (Chater and Bibb, 1997). Probably, not all the members of the family can recognise strict consensus binding sites, a situation that resembles with members of another regulatory family (LysR type) (Henikoff *et al.*, 1988). Despite the sequence similarity with OmpR-like proteins, DnrI binding sites overlap the promoter sequences they activate (Tang *et al.*, 1996) in contrast with the OmpR binding sites which are found upstream the promoter sequences. The binding sites of most transcription regulators (activators or repressors) either overlap the RNA polymerase binding site or are located upstream of this sequence as in the case of OmpR (Ishihama, 1993), correlating with the physical interactions occurring with the structural subunits of holoenzyme. Therefore, unlike the OmpR interaction with the α subunit of RNA polymerase (Ishihama, 1993), the location of the DnrI binding site may instead be indicative of interaction of this protein with the σ subunit of holoenzyme during the transcription process. However, such speculation is untested.

Computer analysis failed to reveal likely H-T-H DNA binding motifs in SARP proteins that may indicate the need for additional interaction with other proteins for efficient activation of biosynthetic structural gene promoters (Chater and Bibb, 1997).

In contrast, transcriptional regulators of aminoglycoside production share different structural characteristics. Helix-turn-helix motifs have been identified near the middle of *strR* and *spcR* genes involved in streptomycin and spectinomycin biosynthesis respectively (Retzlaff and Distler, 1995; Lyntzkahova *et al.*, 1997). StrR is a conventional transcriptional activator of multiple *str* genes by interacting with multiple binding sites with consensus sequences found upstream of the corresponding RNA polymerase recognition element (Retzlaff and Distler, 1995). Analysis of the binding sites reveals 9 bp inverted repeats separated by 11 bp corresponding to one complete turn

of DNA helix; consequently the repeats within any one promoter region are expected to occur on the same face of the DNA (Retzlaff and Distler, 1995).

BrpA is a pathway-specific activator for bialaphos production showing similarity to response regulator proteins of two-component systems (Raibaud *et al.*, 1991). The N-terminus contains the site for phosphorylation upon a particular signal which is transmitted to the C-terminus containing a DNA-binding motif responsible for the transcription activation of specific promoters.

Transcriptional regulator	Antibiotic biosynthesis	Protein family	H-T-H location	Consensus binding sequences	Binding site location	Organism
ActII-ORFIV	Actinorhodin	SARP	No H-T-H motif	No	Promoter overlap	*S. coelicolor*
RedD	Undecyl-prodigiosin	SARP	No H-T-H motif	No	Promoter overlap	*S. coelicolor*
DnrI	Daunorubicin	SARP	No H-T-H motif	No	Promoter overlap	*S. peuceticus*
CcaR	Clavulanic acid Cephamycin	SARP	No H-T-H motif	No	Promoter overlap	*S. clavigulerus*
BrpA	Bialaphos	LysR	C-terminus	No	Not Known	*S. hygroscopicus*
StrR	Streptomycin	StrR	Central	Yes	-110 upstream promoter region	*S. griseus*
HstrR	5'-OH-Streptomycin	StrR	Central	Yes	-110 upstream promoter region	*S. glaucescens*
SpcR	Spectinomycin	StrR	Central	Yes	Not Known	*S. flavopersicus*

Table 6.1: Sequence and structural characteristics of transcriptional regulators involved in antibiotic biosynthesis in streptomycetes.

As already discussed, the mechanistic coupling of gene transfer and gene transcription is determined by the regional architecture of the DNA helix. On the other hand, the function of newly acquired genes may reveal the nature of selective pressure of a particular combinatorial gene transfer events. By connecting transcriptional regulatory circuits to the action of natural genetic engineering systems, there is a plausible molecular

basis for coordinated changes in the genome subject to biologically meaningful feedback. Therefore, further understanding of the regulation of antibiotic gene expression- especially for streptomycin genes as being targets of horizontal gene transfer, in the case of the Brazilian isolates, will help to describe the forces that shape antibiotic evolution.

Retzlaff and Distler (1995) showed that StrR is a DNA binding protein that promotes transcription by recognising conserved inverted repeats within *str/sts* gene promoters. Binding of StrR is accompanied by oligomerisation which seems to be a requirement for StrR transcriptional activity (Thamm, 1999). C-terminal truncated StrR derivatives have lost their tetramerisation upon binding and the transcriptional activation of *strB1* promoter (Thamm, 1999; Thamm and Distler, 1997). Therefore, it may be assumed that several domains span the entire length of *strR* gene. Those responsible for tetramerisation and transcription activation seem to be found at the C-terminus with the actual binding domain (helix-turn-helix motif) being located at the center of the gene. This study aimed to test this model by examining the functionality of the N-terminal region of StrR. Deletion analysis of this region was used to identify a dimerisation domain required for DNA binding and/or regulating activity.

6.2 Results

6.2.1 Construction of N-truncated forms of *strR* gene

Certain methionines within the N-terminus of *strR* gene were used as the new start codons of truncated versions of *strR*. These sites were at nucleotide 105, 276, 459, 597 respectively (Fig. 6.1). Genomic DNA from *S. griseus* N2-3-11 was used as template for generation of N-truncated StrRs using PCR. Primers were designed to introduce *NdeI* sites incorporating methionine codons and to engineer the adjacent downstream amino acid, whenever required, into glutamate (Table 6.2) as this facilitates overexpression of the truncated genes (J. Distler, pers. comm.). Reverse primer was designed to introduce a *BamH1* site downstream the natural stop codon of *strR*. PCR was performed with 2.5 units of Pfu-Turbo polymerase (Stratagene) using buffers provided by the supplier and conditions as described in Chapter 2. PCR products of the expected size were obtained for all deleted *strRs* except for *NstrR2* (Fig. 6.2) despite several attempts.

Forward primer sequence	**Amino acid position**	**Nucleotide position**	**Amino acid change**
NstrR1: 5'gtgaCATATGGAGccggtcgaatc3' Nde I site	35	105	M(V to E)
NstrR2: 5'gccacCATATGGAGggagcc3' Nde I site	92	276	M(R to E)
NstrR3: 5' ggcgacCATATGtcggcgaag3' Nde I site	153	459	(L to M)S
NstrR4: 5'ctgctCATATGgagaacccctc3' Nde I site	199	597	(Q to M)E

Table 6.2: Engineered primers for the generation of N-terminus truncated StrRs. Restriction site *NdeI* is shown. Nucleotides in bold are the new start codons of the deleted StrR derivatives. Mutated codons are underlined.

```
atggagcatatttcagggaacagccccgagcaagtccgtgagaggtcggcggcggtgacgggtgcggtcg
 M  E  H  I  S  G  N  S  P  E  Q  V  R  E  R  S  A  A  V  T  G  A  V

aggaaagtgaactcaaattatccgccgtgacaatggtcccggtcgaatcattgcttccctccgattcccc
E  E  S  E  L  K  L  S  A  V  T  M  V  P  V  E  S  L  L  P  S  D  S  P
                                  └──────────► NstrR1

gcgcagtgccggcgaggatgtcgagcacatccgcacgctcgccgcatccggagcggaattgcccgctatc
 R  S  A  G  E  D  V  E  H  I  R  T  L  A  A  S  G  A  E  L  P  A  I

gtggtcatgcccaccacgaagcgcgtcatcgacggcatgcaccgactgcgcgccaccaaaatgcgcggag
 V  V  M  P  T  T  K  R  V  I  D  G  M  H  R  L  R  A  T  K  M  R  G
                                                              └──► NstrR2

ccaccgagatcgccgtgcggtatttcgaaggcggagaggaagaggcgttcatcttcgcggtgaagtccaa
A  T  E  I  A  V  R  Y  F  E  G  G  E  E  E  A  F  I  F  A  V  K  S  N

cgtcacccacggactgccgctctccctcgacgaccgcaaggccgcggcgacccgtgtcctggagacccat
 V  T  H  G  L  P  L  S  L  D  D  R  K  A  A  A  T  R  V  L  E  T  H

ccgtcctggtccgaccgggccatcggcctggcgaccggactgtcggcgaagacggtggggaccctcaggt
 P  S  W  S  D  R  A  I  G  L  A  T  G  L  S  A  K  T  V  G  T  L  R
                                     └──────────► NstrR3

cctgttcgactgccggggttccgcagtcgaacgtgaggatcgggagggacgggcgggcccggccgctgga
S  C  S  T  A  G  V  P  Q  S  N  V  R  I  G  R  D  G  R  A  R  P  L  D
                                                                    H
ccccaccgaggggcggaagctggcgagccggctgctccaggagaaccccctcggcgtcgctgcgtcagatc
 P  T  E  G  R  K  L  A  S  R  L  L  Q  E  N  P  S  A  S  L  R  Q  I
                                     └──────────► NstrR4
                T                   H
gccgcacaggccggcgtctccccgagcaccgcctccgacgtccgcaagcggctgagccgtggtgagagcc
 A  A  Q  A  G  V  S  P  S  T  A  S  D  V  R  K  R  L  S  R  G  E  S

cgctgcccgaacgcgatcgtcaacaggaagtgccggccgtcgcccggactccggcccgtgtctcccgggc
P  L  P  E  R  D  R  Q  Q  E  V  P  A  V  A  R  T  P  A  R  V  S  R  A

cgacgggagttgggcgccgcacaccgtggcgctgcgccacctcagccgggacccgtccgtgcggctcacc
 D  G  S  W  A  P  H  T  V  A  L  R  H  L  S  R  D  P  S  V  R  L  T

gaggacggtcgggcgctgctacgttggctgaacgtggtggccgtgcgcaaccaggactgggaccgcctcc
 E  D  G  R  A  L  L  R  W  L  N  V  V  A  V  R  N  Q  D  W  D  R  L

tgggcaacgtccctccgcactgcgtcaaggtcatagccgagctggcccggggctgtgccgacatctggca
L  G  N  V  P  P  H  C  V  K  V  I  A  E  L  A  R  G  C  A  D  I  W  H

tcgggtggcggaggaactggaccaggccggcatcgacgaggcggcgggccggtccttgagcgatgtcgga
 R  V  A  E  E  L  D  Q  A  G  I  D  E  A  A  G  R  S  L  S  D  V  G

tga
 *
```

Figure 6.1: New start sites of N-terminus truncated StrRs and their position in relation to helix-turn-helix (HTH) motif of the StrR protein.

6.2.2 Cloning and overexpression of N-truncated *strR* derivatives using pET expression system

The obtained PCR products were cloned into pET expression vector using the engineered *NdeI* and *BamH1* sites (Fig. 6.2). The target *strR* fragments were under the control of strong bacteriophage T7 transcription and translation signals and expression is induced by providing a source of T7 RNA polymerase in the host cell. BL21(DE3)pLysS was used as a host cell. The pLys plasmid encodes T7 lysozyme, a natural inhibitor of T7 RNA polymerase and thus reduces the basal level of expression of target genes. Overexpression was carried out as described in Chapter 2. Additional unique protein bands were found in *E. coli* induced with IPTG harbouring plasmids pNstrR1 and pNstrR4 although not so intense as the wild type (Fig. 6.3). The size of NstrR1 and NstrR4 overexpressed proteins corresponded well with the calculated molecular masses (33 kDa and 17 kDa respectively). The NstrR3 fragment was not overexpressed possibly due to its sequence alterations despite the usage of high fidelity polymerase.

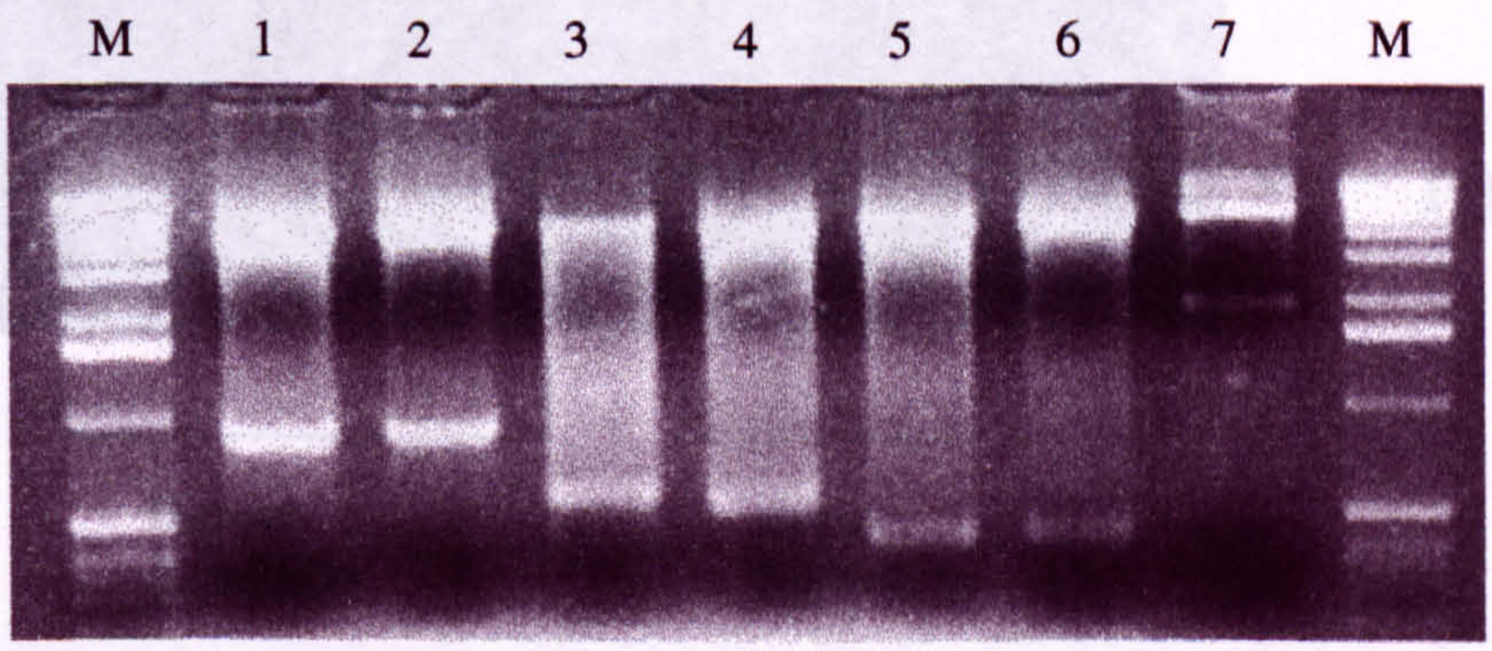


Figure 6.2: Cloning of N-terminal truncated StrR PCR products in pET11a vector. Recombinant plasmids were restricted with *NdeI/BamHI* endonucleases.1 kb ladder (lanes M), NstrR1 (lanes 1,2), NstrR3 (lanes 3,4), NstrR4 (lanes 5,6), pLRW11 plasmid containing the full length StrR (lane 7).

Three C-terminal truncated strRs (namely StrR299, StrR231 and StrR199) were kindly provided by Dr. Sven Thamm. Str199 protein lacks the putative H-T-H motif. In contrast, StrR299 and StrR231 lacking the C-terminal 51 and 119 amino acids respectively still comprised the H-T-H motif. These mutated StrRs were overexpressed and the determined molecular masses of the expressed proteins were in good agreement with the calculated molecular masses (32, 24.5 and 22.5 kDa) (Fig.6.4).

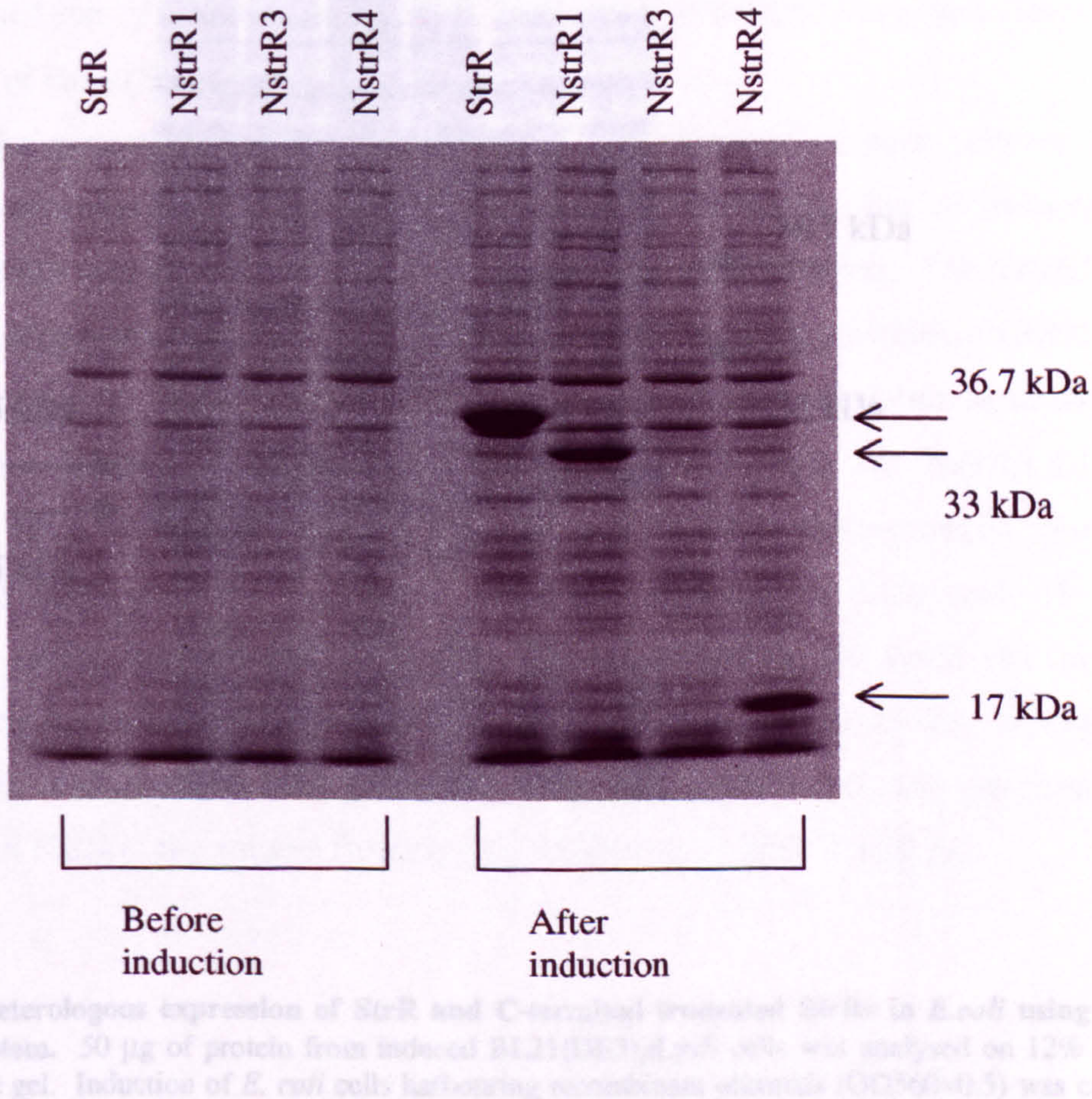


Figure 6.3: Heterologous expression of StrR and N-terminal truncated StrRs, NstrR1 and NstrR4 in *E.coli* using pET expression system. 50 µg of protein from induced BL21(DE3)pLysS cells was analysed on 12% SDS-polyacrylamide gel. Induction of *E. coli* cells harbouring pNstrR1 and pNstrR4 plasmids (OD560=0.5) was carried out by addition of IPTG at final concentration of 1 mM. Induction time 120 min.

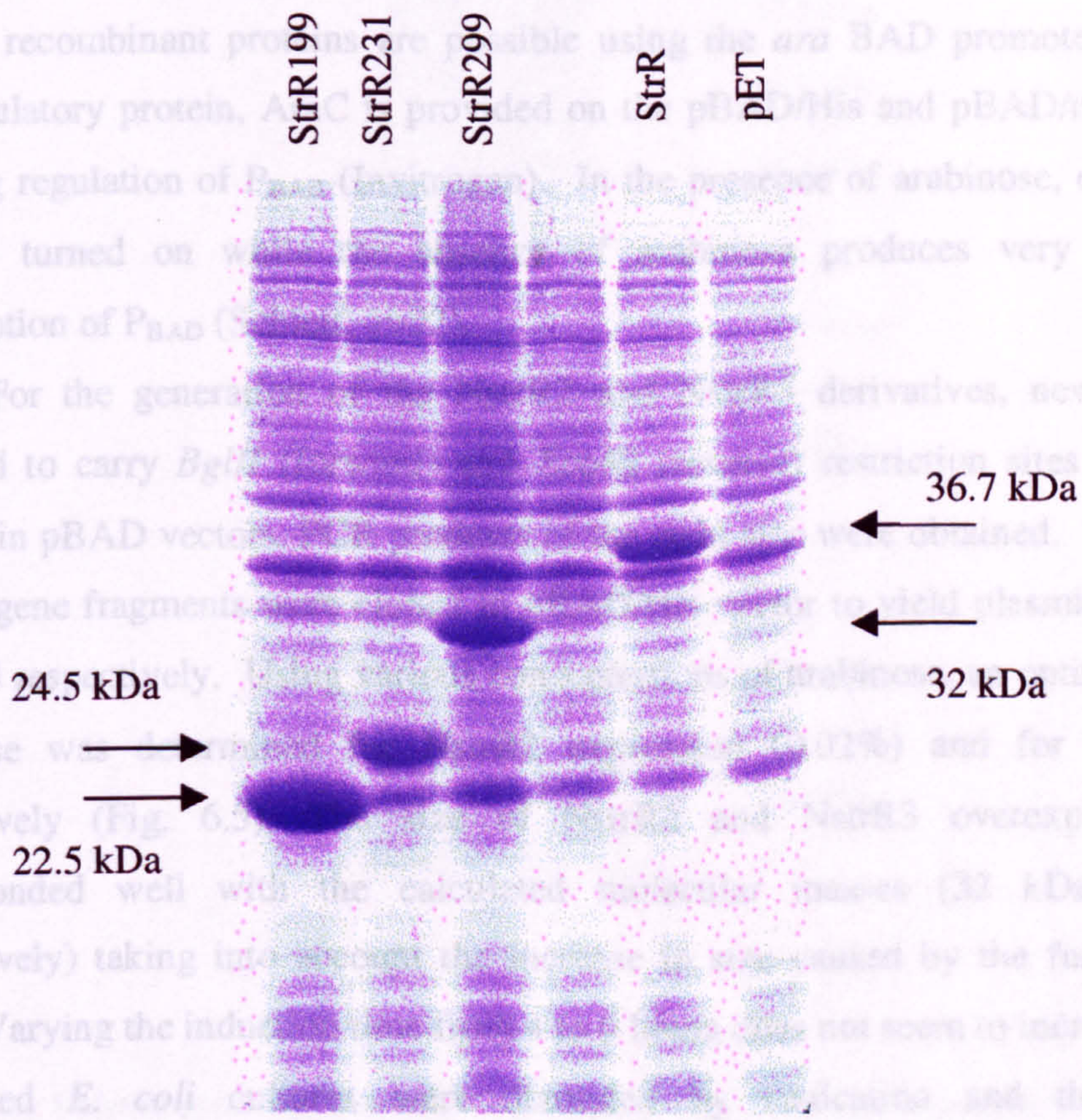


Figure 6.4: Heterologous expression of StrR and C-terminal truncated StrRs in *E.coli* using pET expression system. 50 μg of protein from induced BL21(DE3)pLysS cells was analysed on 12% SDS-polyacrylamide gel. Induction of *E. coli* cells harbouring recombinant plasmids (OD560=0.5) was carried out by addition of IPTG at final concentration of 1 mM. Induction time 120 min.

6.2.3 Cloning and overexpression of N-terminal truncated *strR* derivatives using pBAD expression system

The failure to generate the NstrR2 fragment or to overexpress the NstrR3 protein, led to the use of an alternative cloning and expression system. Optimum levels of soluble, recombinant proteins are possible using the *ara* BAD promoter from *E. coli*. The regulatory protein, AraC is provided on the pBAD/His and pBAD/myc-His vectors allowing regulation of P_{BAD} (Invitrogen). In the presence of arabinose, expression from P_{BAD} is turned on while the absence of arabinose produces very low levels of transcription of P_{BAD} (Schleif, 1992).

For the generation of the NstrR2 and NstrR3 derivatives, new primers were designed to carry *BglII* (forward) and *EcoRI* (reverse) restriction sites for subsequent cloning in pBAD vectors. PCR products of the right size were obtained. The *NstrR2* and *NstrR3* gene fragments were cloned in pBAD/His vector to yield plasmids pNstrR2 and pNstrR3 respectively. Using various concentrations of arabinose, an optimum amount of arabinose was determined for NstrR2 expression (0.02%) and for NstrR3 (0.2%) respectively (Fig. 6.5). The size of NstrR2 and NstrR3 overexpressed proteins corresponded well with the calculated molecular masses (32 kDa and 25 kDa respectively) taking into account the increase in size caused by the fused His tag (~3 kDa). Varying the induction time from 4 to 6 hours does not seem to increase expression. Expressed *E. coli* cultures were disrupted by sonication and the presence of overexpressed Nstrs in the soluble fraction was confirmed by SDS-PAGE gel.

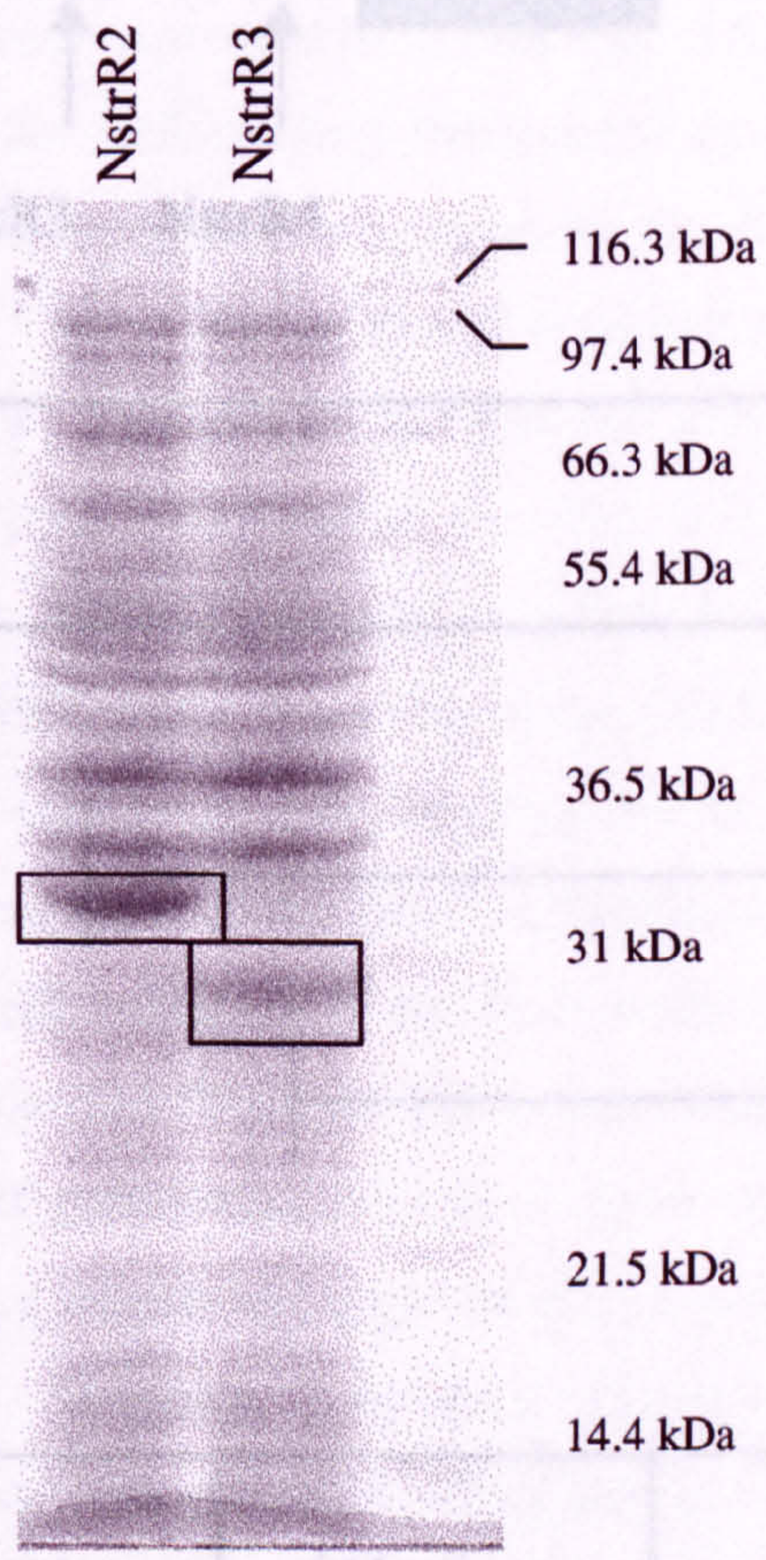
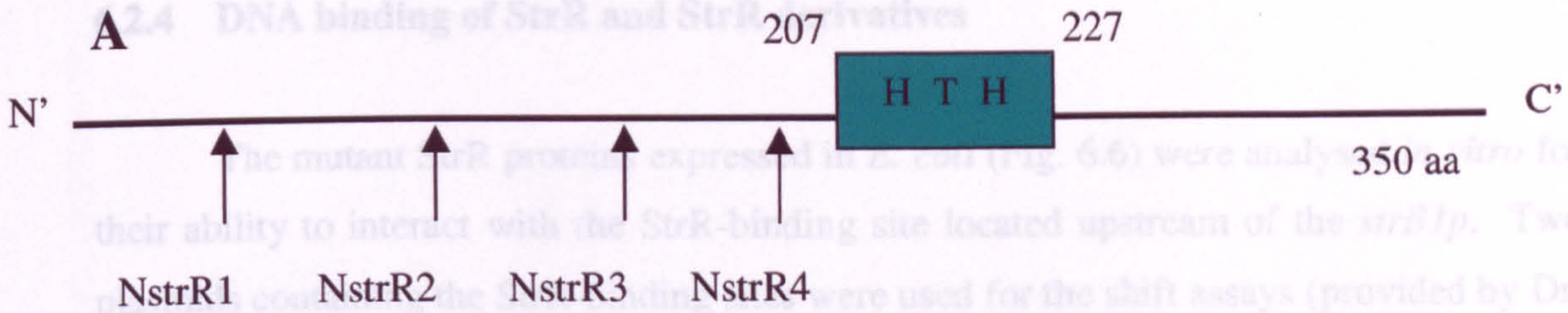


Figure 6.5: Heterologous expression of N-terminal truncated NstrR2 and NstrR3 in *E.coli* using pBAD expression system. 50 µg of protein from induced TOP10 cells was analysed on 12% SDS-polyacrylamide gel. Induction of *E. coli* cells harbouring pNstrR2 and pNstrR3 plasmids (OD560=0.5) was carried out by addition of L-arabinose at final concentration of 0.02% and 0.2% respectively. Induction time 5 h. The proteins carried a N-terminus His-tag.

A



B

gene	vector	induction	host
NstrR1	pET11a	IPTG	*E.coli*
NstrR2	pBAD	L-arabinose	*E.coli*
NstrR3	pBAD	L-arabinose	*E.coli*
NstrR4	pET11a	IPTG	*E.coli*

Figure 6.6: Schematic representation of the StrR protein and mutated N-terminal derivatives. (A) The *strR* gene (black bar) with the relative positions of new start codons are shown. The putative H-T-H motif of StrR protein is shown is boxed and its position within the *S. griseus* wild type StrR protein is indicated. (B) Summary of the expression and inductions systems used for the generation of N-terminal truncated StrRs.

6.2.4 DNA binding of StrR and StrR derivatives

The mutant StrR proteins expressed in *E. coli* (Fig. 6.6) were analysed *in vitro* for their ability to interact with the StrR-binding site located upstream of the *strB1p*. Two plasmids containing the StrR-binding sites were used for the shift assays (provided by Dr. Sven Thamm). The pRBD13 has a 290 bp *EcoRI* fragment insert that contains the full palindromic StrR-binding site. The pB1p/2 plasmid has a 156 bp *EcoRI/HindIII* insert that contains the half palindromic StrR-binding site (Fig. 6.7). Consistent with previous studies (Retzlaff and Distler, 1995; Thamm and Distler, 1997) the interaction of StrR with these sites is specific. StrR which is present in the cell-free extracts of *E. coli* binds to full and half palindrome-containing StrR binding site in the presence of 50 µg of competitor DNA. No gel retardation of the labelled fragments, either full or half binding sites, was detected using a crude extract of *E. coli* harbouring no StrR (pET vector only).

As previously demonstrated (Thamm, 1999) StrR binds to a full palindromic site as a tetramer and to the half palindromic site as a dimer (Fig. 6.8A). However, less amount of protein can produce exactly the opposite oligomerisation pattern using the two binding targets (Fig. 6.8B). Similarly, C-terminal truncated StrRs showed a binding mixture of tetramer and dimer with the full binding site and only a dimer with the half binding site. StrR199 lacking the H-T-H motif could not bind to any target sequences (Fig. 6.9).

The N-terminal truncated StrRs did not bind to any target sites except NstrR2. The latter protein could give a retarded band only with the full palindromic site but not with the half binding site (Fig. 6.10).

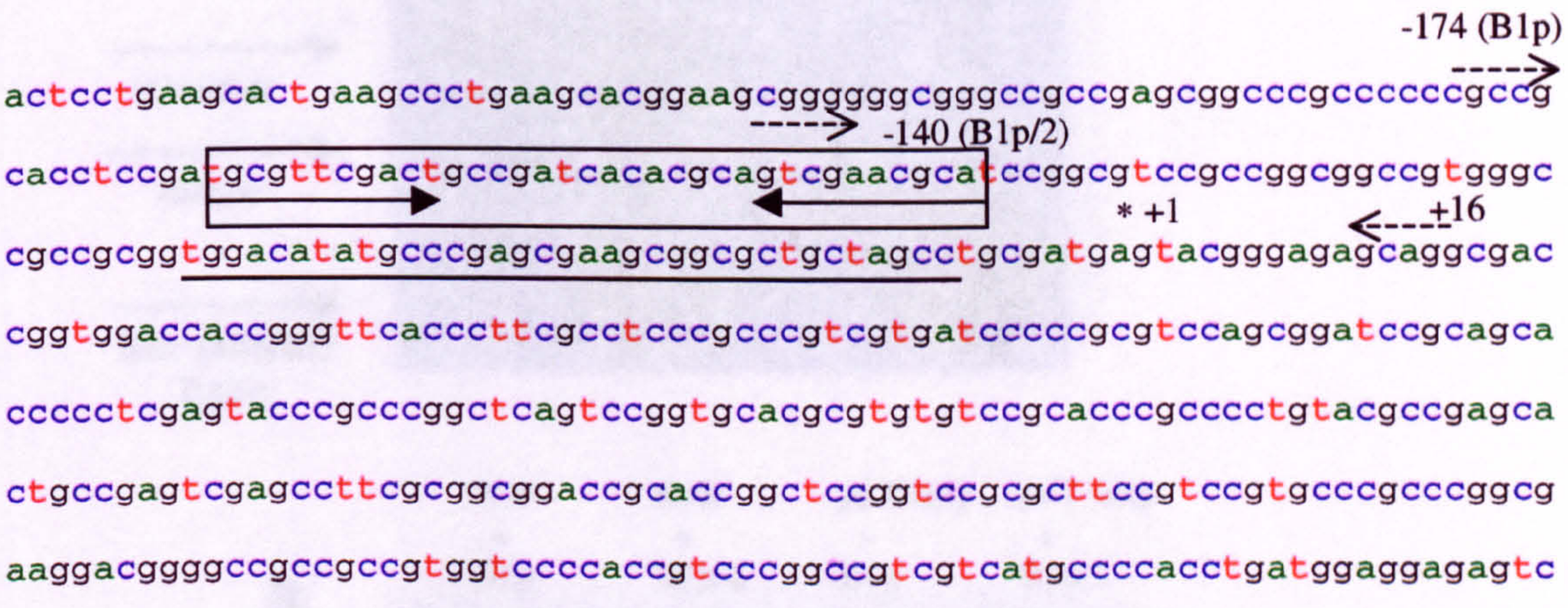


Figure 6.7: Sequence of the intergenic *strA-B1* region. StrR binding site consisting of two inverted repeats (black arrows) is boxed. The *strB1* promoter is underlined. The region between the dashed arrows shows the 290 bp fragment containing the full binding site (B1p) and the 156 bp fragment containing the half binding site (B1p/2). These fragments were used for DNA retardation assays. The sequence is numbered with respect to the transcriptional start point of *strB1* indicated by an asterisk.

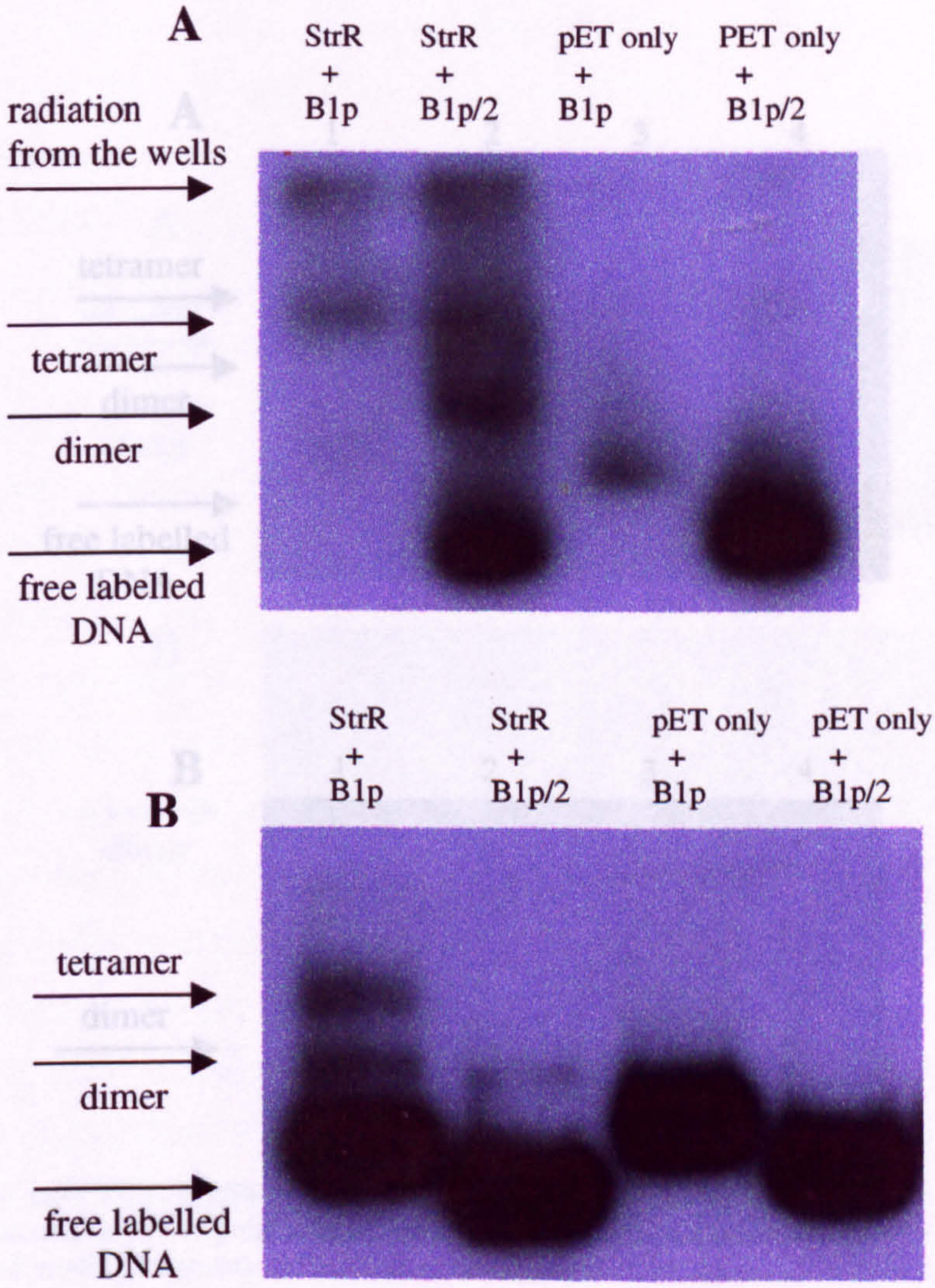


Figure 6.8: Different oligomerisation pattern for DNA binding of StrR expressed in *E. coli*. Autoradiogram of gel retardation assays using the *E. coli* cell-free extracts. Approximately 10 µg (A) or 2 µg (B) of total protein and 3 ng of end-labelled binding fragments (B1p, B1p/2) were used for a standard DNA binding assay.

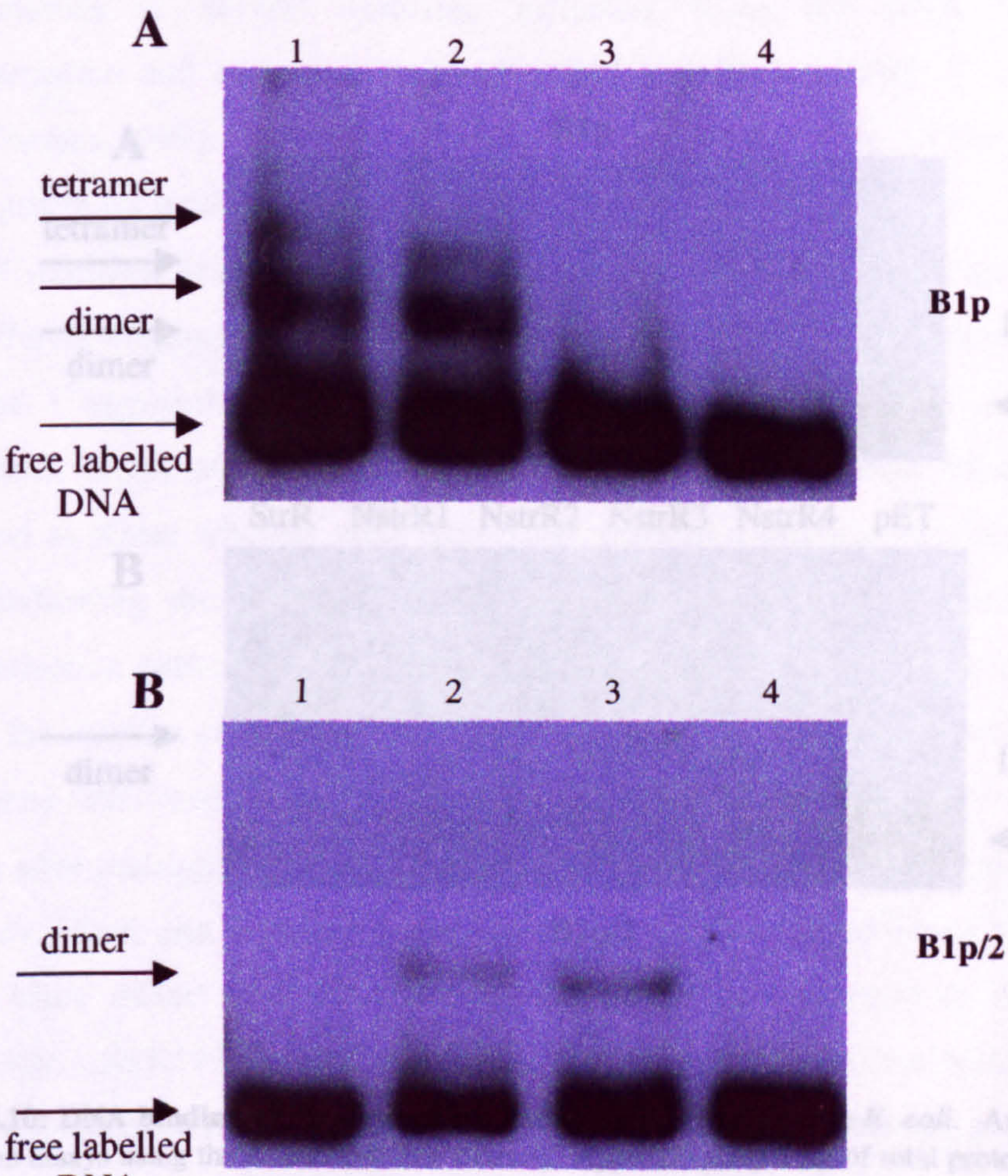


Figure 6.9: DNA binding of C-terminal truncated StrRs expressed in *E. coli*. Autoradiogram of gel retardation assays using the *E. coli* cell-free extracts containing the following StrR proteins: (A) StrR299 (lane 1), StrR231 (lane 2), StrR199 (lane 3), pET only (lane 4). (B) StrR299 (lane 1), StrR231 (lane 2), StrR199 (lane 3), pET only (lane 4). Approximately 2 µg of total protein and 3 ng of end-labelled binding fragments (B1p, B1p/2) were used for a standard DNA binding assay.

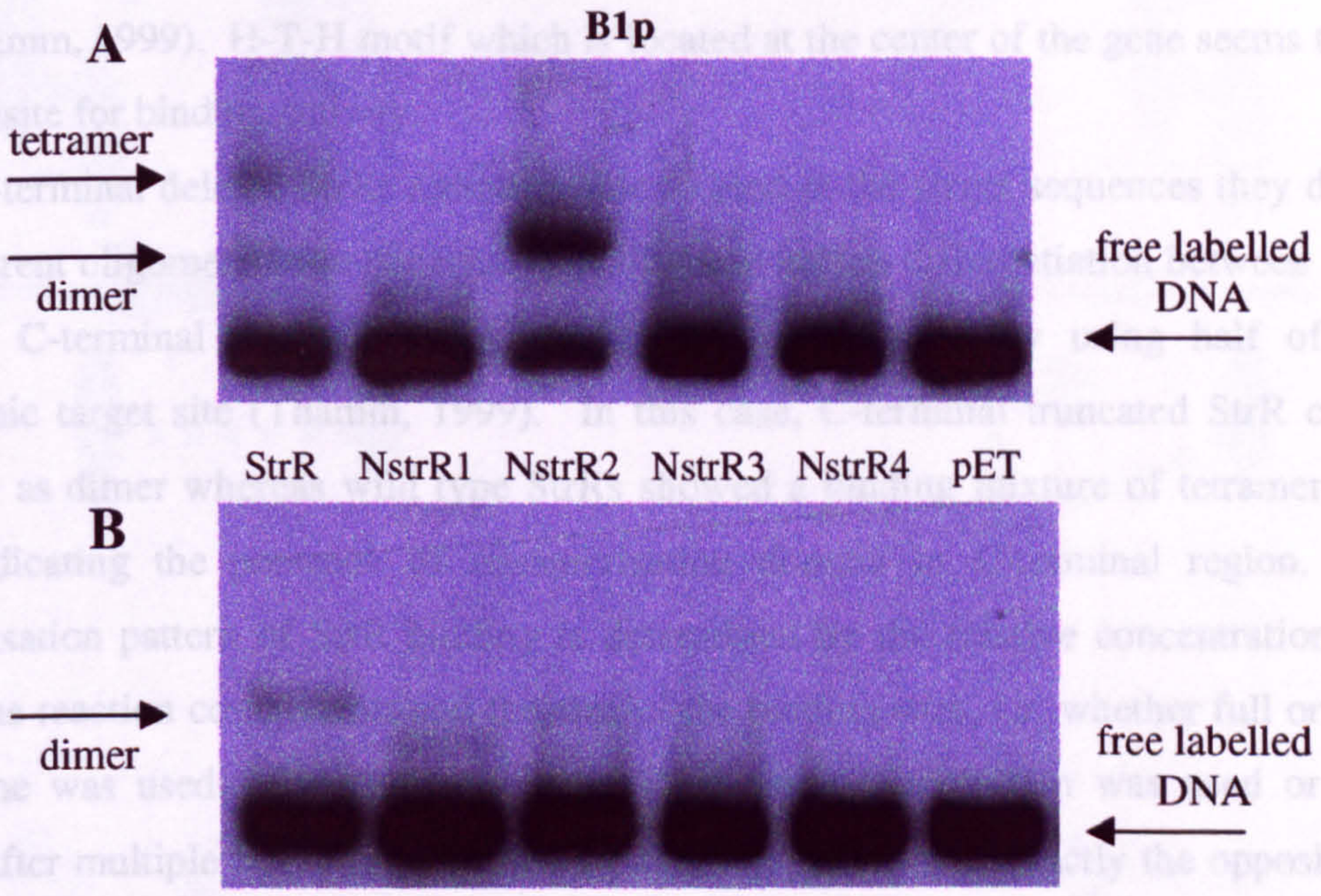


Figure 6.10: DNA binding of N-terminal truncated StrRs expressed in *E. coli*. Autoradiogram of gel retardation assays using the *E. coli* cell-free extracts. Approximately 2 µg of total protein and 3 ng of end-labelled binding fragments (B1p, B1p/2) were used for a standard DNA binding assay.

6.3 Discussion

Previous studies using full-length StrR and C-truncated derivatives showed that StrR consists of several domains, including those for DNA binding activity, oligomerisation and enzyme activity (Retzlaff and Distler, 1995; Thamm and Distler, 1997; Thamm, 1999). H-T-H motif which is located at the center of the gene seems to be a prerequisite for binding activity.

C-terminal deleted StrRs although able to bind at the target sequences they do so with different oligomerisation patterns. The oligomerisation differentiation between wild type and C-terminal mutated StrRs, could only be detected by using half of the palindromic target site (Thamm, 1999). In this case, C-terminal truncated StrR could only bind as dimer whereas wild type StrRs showed a binding mixture of tetramer and dimer indicating the presence of tetramerisation domain in C-terminal region. The oligomerisation pattern of StrR binding is determined by the relative concentrations of each of the reaction components and the size of the binding sites, i.e. whether full or half palindrome was used. Consequently, when less amount of protein was used or cell extracts after multiple freeze/thaw cycles the binding pattern was exactly the opposite in this study. StrR and C-terminal derivatives gave two retarded bands (a tetramer and a dimer) when mixed with full binding site and only one band (a dimer) with half palindrome. StrR199 derivative lacking H-T-H motif could not bind to any binding target indicating the importance of this domain for the DNA-binding property of StrR *in vitro*.

Based on the previous results, Thamm (1999) created a model for the mechanism of StrR binding to *str/sts* promoter sites (Fig. 6.11). It was assumed that elsewhere in the gene, there is a domain responsible for the dimerisation of the protein. In this study, it was attempted to detect such a domain in the N-terminus of *strR* gene. N-terminal truncated StrRs at certain sites but including the H-T-H binding motif were constructed and overexpressed in *E. coli*. The heterologous expression of these StrR derivatives was not so strong as the wild type StrR. In contrast, overexpression of the same C-terminal mutated StrRs as they were used in previous study (Thamm and Distler, 1997) was as

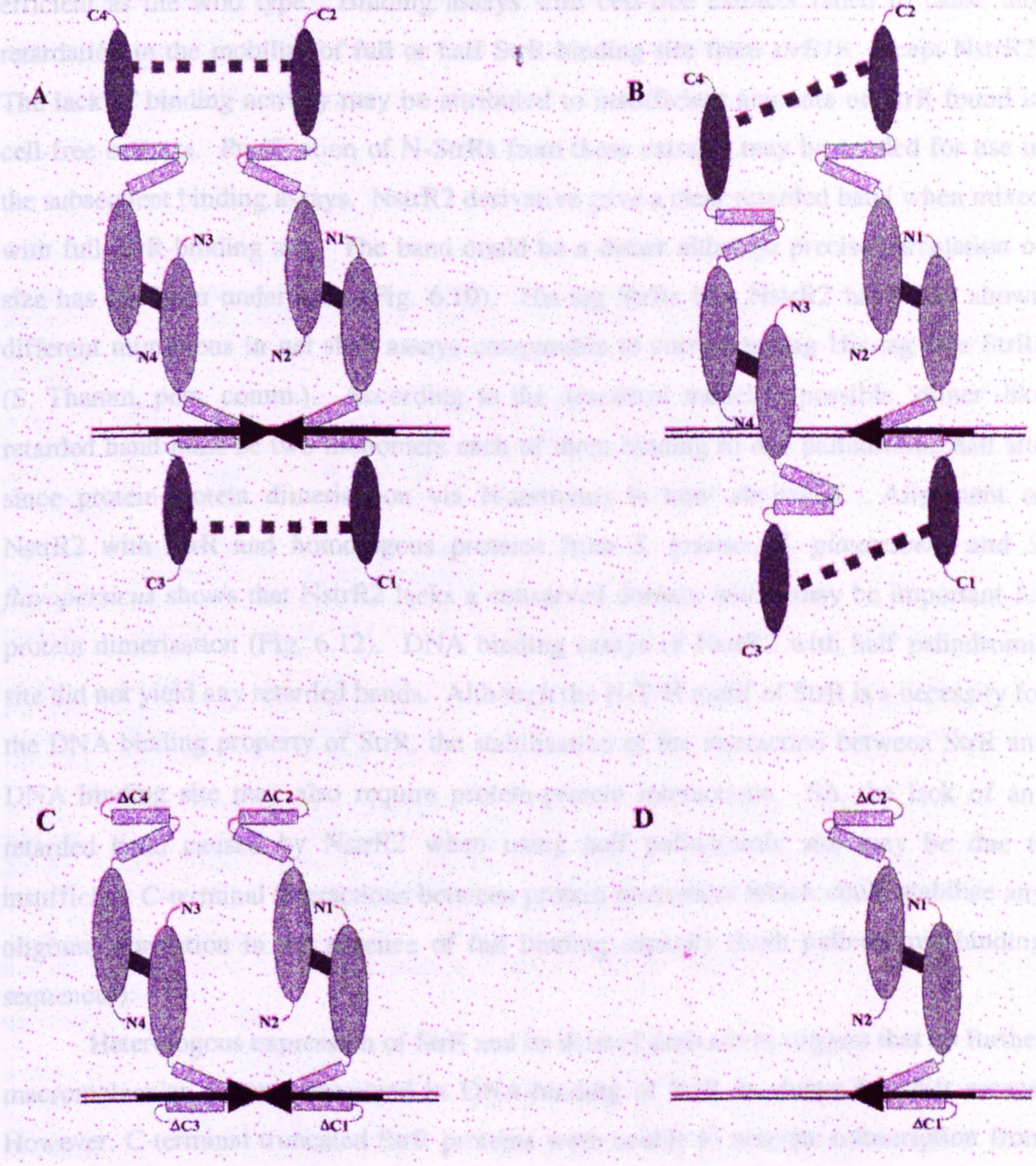


Figure 6.11: Model for DNA binding and oligomerisation of StrR. Several domains of StrR protein are important for efficient binding and stable oligomerisation. C-terminal regions (C1-C4), H-T-H motif and N-terminal regions (N1-N4) are indicated. C-terminal deletion of StrR are also indicated (ΔC1-ΔC4). In this model, H-T-H motif is a prerequisite for DNA binding property and N-terminus is responsible for dimerisation whereas C-terminus is responsible for tetramerisation. (A) Formation of StrR tetramer with the full palindromic site. (B) Formation of StrR tetramer is possible via C-terminal interactions in the presence of half palindromic site. (C) Formation of C-terminal truncated StrR pseudotetramer with full palindromic site. Two dimers each of them binding to one palindromic half site unable to interact with their C-terminals. (D) Formation of C-terminal truncated StrR dimer with half palindromic site. The absence of the other half binding site and the unavailability of the C-terminal regions do nor result in a stable positioning of the other StrR dimer. (Reproduced from Thamm, 1999).

efficient as the wild type. Binding assays with cell-free extracts failed to cause any retardation in the mobility of full or half StrR-binding site from *strB1p*, except NstrR2. The lack of binding activity may be attributed to insufficient amounts of StrR found in cell-free extracts. Purification of N-StrRs from these extracts may be needed for use in the subsequent binding assays. NstrR2 derivative gave a clear retarded band when mixed with full StrR-binding site. The band could be a dimer although precise calculation of size has not been undertaken (Fig. 6.10). His-tag StrRs like NstrR2 have also shown different migrations in gel shift assays comparable to corresponding His-tag free StrRs (S. Thamm, pers. comm.). According to the described model, a possible 'dimer'-like retarded band must be two monomers each of them binding to one palindromic half site since protein-protein dimerisation via N-terminus is now abolished. Alignment of NstrR2 with StrR and homologous proteins from *S. griseus*, *S. glaucescens* and *S. flavopersicus* shows that NstrR2 lacks a conserved domain which may be important for protein dimerisation (Fig. 6.12). DNA binding assays of NstrR2 with half palindromic site did not yield any retarded bands. Although the H-T-H motif of StrR is a necessity for the DNA binding property of StrR, the stabilisation of the interaction between StrR and DNA binding site may also require protein-protein interactions. So, the lack of any retarded band caused by NstrR2 when using half palindromic site may be due to insufficient C-terminal interactions between protein monomers which could stabilise any oligomer formation in the absence of full binding capacity (both palindromic binding sequences).

Heterologous expression of StrR and its deleted derivatives suggest that no further macromolecular factor is involved in DNA-binding of StrR as shown by shift assays. However, C-terminal truncated StrR proteins were unable to activate transcription from strR-dependent promoter *strB1p* (Thamm and Distler, 1997). Therefore, the C-terminal domain of StrR seemed to be necessary for its function as transcriptional activator most probably through interaction with parts of RNA polymerase holoenzyme. However, it was not tested whether N-terminal sequences may have any co-operative involvement in this transcriptional activation process.

In the case of *strB1p*, the StrR-binding site is located between positions –81 and –109 upstream of the *strB1p* transcription initiation site (Retzlaff and Distler, 1995). The spacing between StrR-binding sites and –35 and –10 hexamers indicates that bending of

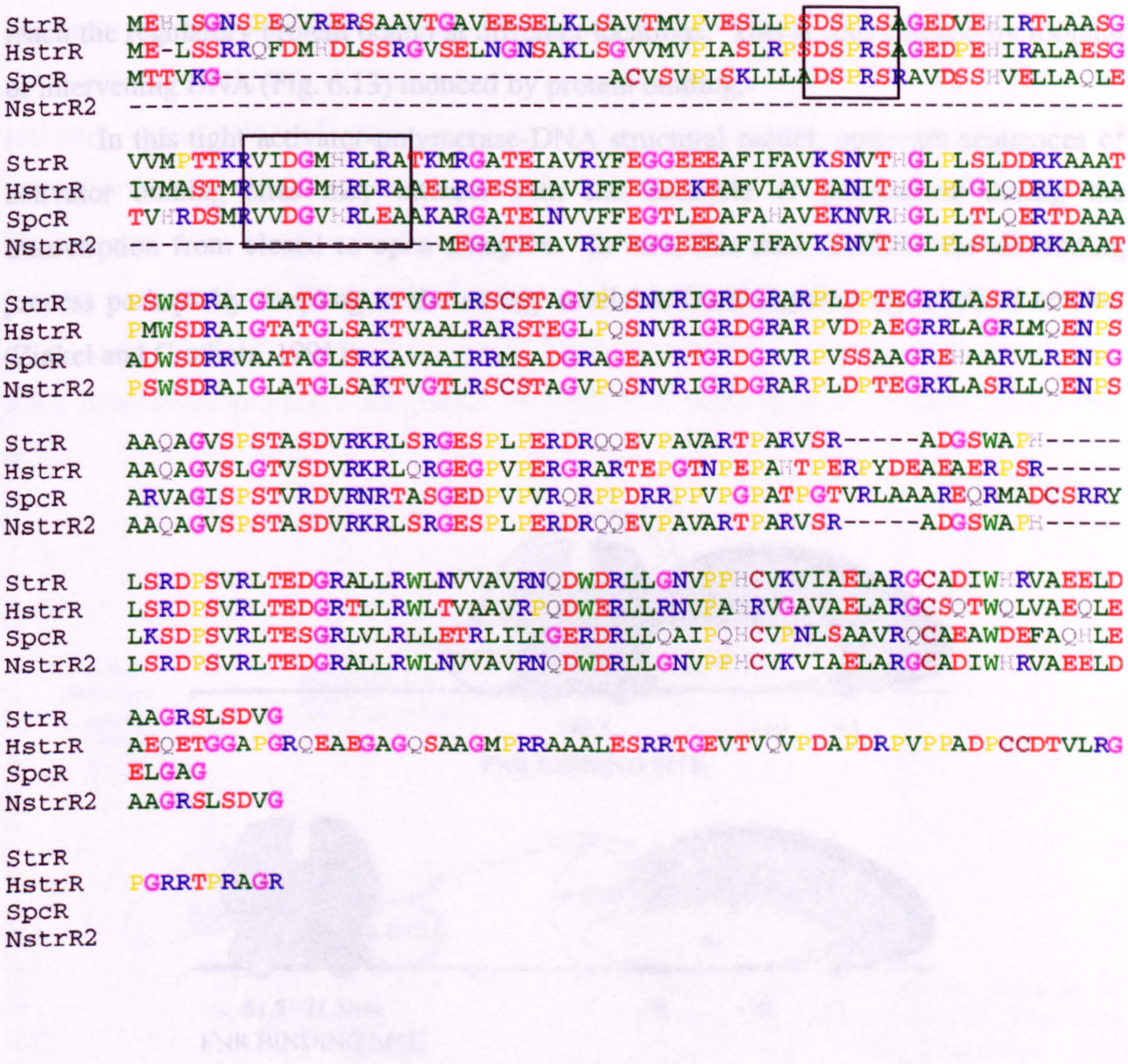


Figure 6.12: Alignment of StrR (*S. griseus*) and the homologous proteins HstrR (*S. glaucescens*), SpcR (*S. flavopersicus*) and one N-terminus truncated StrR (NstrR2). Two conserved regions that may be involved in StrR dimerisation and that are deleted from NstrR2 are boxed. Alignments were performed using ClustalW and BioEdit programmes.

the DNA is possibly involved in activating the transcription of strB1p. At promoters where regulatory proteins bind further upstream –35 and –10, like CAP and FNR protein in *gal/lac* operon and *melR* promoter respectively (Zhou *et al.*, 1994; Wing *et al.*, 1995), the C-terminal domain of the RNA polymerase α subunit interacts with the regulatory protein. The flexible linker joining the C-terminal domain and N-terminal domain of the RNA polymerase α subunit (Blatter *et al.*, 1994) may allow the C-terminal domain to reach the regulatory protein bound at different locations. This is also assisted by looping of intervening DNA (Fig. 6.13) induced by protein binding.

In this tight activator-polymerase-DNA structural model, upstream sequences of activator binding sites may interact with the backside of polymerase during the transcription from closed to open complex. In turn, this may facilitate the unwinding process perhaps by coupling to the energy available from negative superhelical tension (Zinkel and Crothers, 1991).

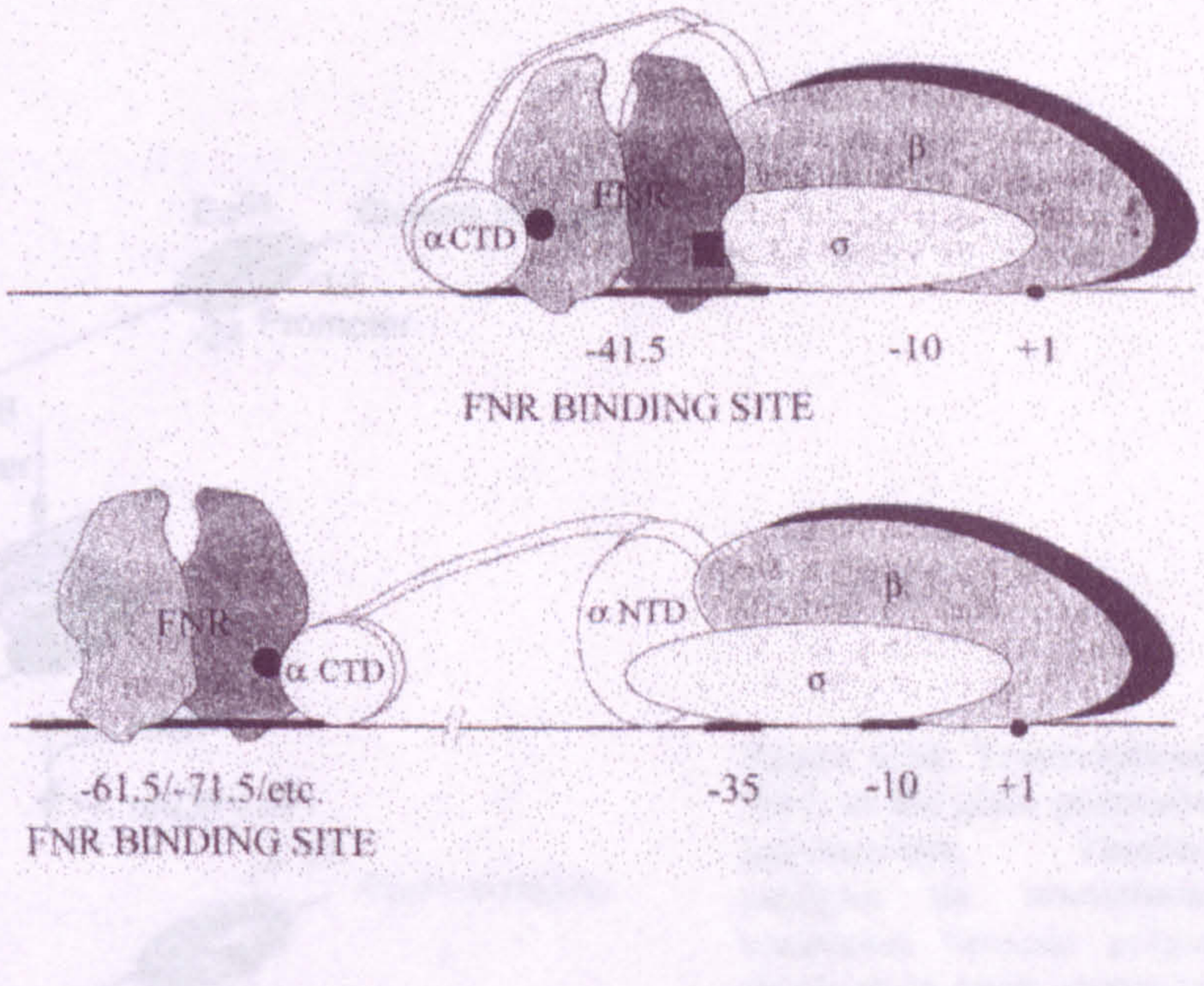


Figure 6.13: Binding of α-subunit of RNA polymerase at different positions at different promoters. The C-terminal of α-subunit of RNA polymerase (αCTD) is connected with a flexible linker that enables it to make different interactions at different promoters, i.e., off DNA, on DNA immediately upstream of the –35 element or on DNA further upstream like in the case of FNR-dependent promoter. (Reproduced from Wing *et al.*, 1995).

Thus both DNA curvature and protein-protein interaction may be important for transcription activation. However, in the described experiments by Thamm and Distler (1997), the importance of upstream sequences from StrR-binding site with *strB1p* towards transcription initiation was not addressed. On the other hand, no bacterial regulator protein recognises a binding site with similar dimensions and geometry (Retzlaff and Distler, 1995) and the spacing between StrR-binding site and promoter hexamers is longer than that of the above promoters.

This resembles better to the situation of nitrogen regulatory protein C (NtrC) that activates transcription of *glnA* gene. It binds upstream activating sequences (UAS) having properties of eukaryotic transcriptional enhancers (Wedel *et al.*, 1990). NtrC-binding sites serve to tether NtrC near to *glnA* promoter, leading to DNA loop formation, thereby increasing the frequency of collisions between NtrC and polymerase promoter complexes (Fig. 6.14). In the same sense, StrR may activate transcription by contacting RNA polymerase-promoter complexes directly, with such contact mediated by a DNA loop.

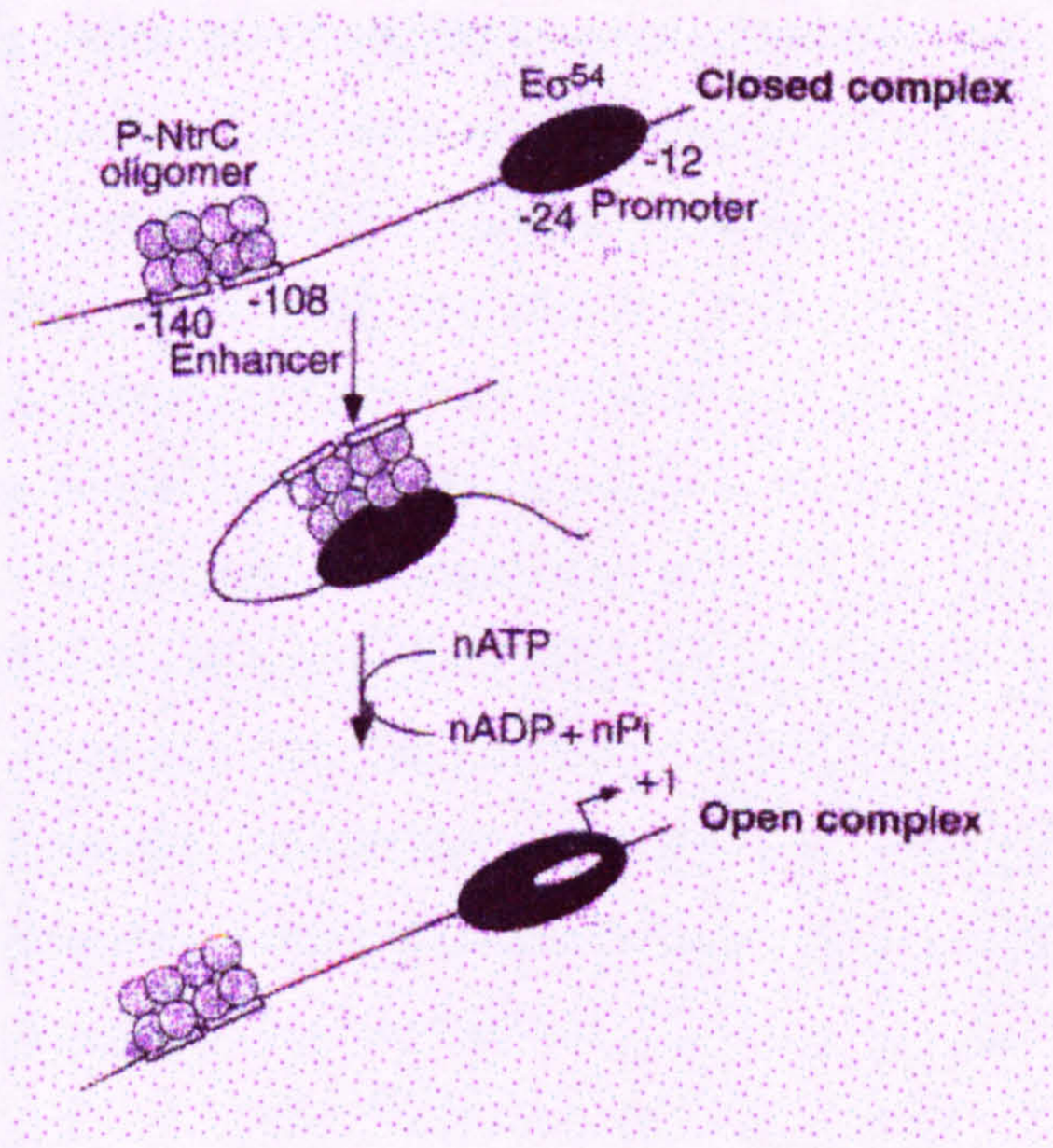


Figure 6.14: Trancriptional activation of NtrC at the *glnA* promoter of *Salmonella typhimurium*. Phosphorylated NtrC catalyses the isomerisation of closed complexes between polymerase and the promoter to open complexes, in which the DNA around the transcriptional start site is locally denatured and the correct strand can be used as template. (Reproduced from Wyman *et al.*, 1997).

In this interaction sufficient concentration of StrR specified by tetramerisation may catalyse the isomerisation of closed complexes between the polymerase and the promoter to open complexes. The spacing requirement may allow RNA polymerase and StrR activator to bind simultaneously to DNA, eliminating regulatory models that require competition between these proteins (Matthews, 1992).

Chapter 7

General Discussion

7.1 General Discussion

Comparative genomics revealed that HGT is not infrequent but may actually be quite common (Martin, 1999; Doolittle, 1999a). In fact, as an evolutionary strategy HGT may extend beyond inter-prokaryotic interaction like antibiotic resistance, antibiotic production and pathogenicity and may even apply to eukaryotes (de la Cruz and Davies, 2000). The occurrence of HGT calls in question some seldom-explained beliefs such as the importance of gene duplication in genome evolution (Doolittle, 1999b). Increasing molecular evidence underscores the importance of gene duplication but this may not be the only explanation. Instead, gene conversion and HGT can explain adequately the same observations of sequence similarities among species (Gogarten and Olendzenski, 1999). Intensive gene transfer may also blur the phylogenetic definition of common ancestry of species (Woese, 1998) and of pathways.

The actual occurrence and the high frequency of HGT as indicated by comparative genomics is in agreement with hypotheses supporting its involvement in antibiotic evolution (Piepersberg, 1997). The incongruency between the phylogeny of producers and phylogenetic relationships based on antibiotic biosynthesis genes, the presence of all antibiotic genes in clusters, the fact that antibiotic clusters are often flanked by IS elements, the strain-specific and not species-specific distribution of antibiotic production phenotypes, the homology that antibiotic genes share between different clusters, the genetic instability of antibiotic cluster and thetr location on chromosomal loci with high recombination frequency, argue for the generation of antibiotic pathways by horizontal gene transfer. Other possible suggestions (e.g. parallel evolution, multiple loss of pathways) suffer mainly by the genome size limitation. Even though at the first place of biochemical evolution, gene duplication might be the primary mechanism for generation of sufficient biosynthetic information and the partially developed and initially simplified gene clusters, horizontal gene transfer might be the powerful alternative for efficient and fast diversification and multiplicity of end-products thereafter.

A previous study (Wiener *et al.*, 1998) implicated the transfer of the streptomycin resistance gene *strA*, and one biosynthetic gene *strB1*, from a *S. griseus*-like donor to at least two streptomycetes isolated from Brazilian soil (ASB37 and ASSF15) (Huddleston *et al.*, 1997). The evidence for transfer was based on incongruent phylogenies derived from comparative analyses of 16S rRNA and *strA* gene sequences. The results from this study have shown that in addition to *strAB1* genes, the pathway specific regulator *strR* was nearly identical in a set of phylogenetically diverse streptomycetes, including *S. griseus* and six isolates from a region of Brazil.

Phylogenetic characterisation of these strains by 16S rDNA sequence analysis showed that they broadly fell into two groups; four isolates formed a cluster with *S. griseus* but two strains were shown to group with the non-streptomycin producing well-characterised type strain *S. coelicolor*. Subsequent analysis of the housekeeping gene *trpB* in these strains supported this grouping and further resolved the *S. griseus* cluster indicating the usefulness of the latter gene as a tool for phylogenetic analysis.

Two of these isolates (ASSF13 and ASSF22) were shown to be synonyms of the type strain of *S. griseus* from sequence analysis of 16S rRNA and tryprophan synthase gene. These strains produce streptomycin, possess all the streptomycin genes tested so far, and most likely possess the entire *str* cluster. The remaining four strains (ASSF15, ASB27, ASB33 and ASB37) were shown not to be closely related to *S. griseus* or other streptomycin related producers, and yet all possessed at least two additional genes from the cluster. Analysis of these isolates suggests that they possess various parts of the streptomycin cluster. ASSF15 and ASB37 possess *strRAB1* but do not appear to have any other gene from the cluster, nor do they produce streptomycin. ASB33 does produce streptomycin and has all the genes examined so far. This could be explained by transfer of the entire gene cluster as a functional unit into this isolate. Analysis of a more phylogenetically informative gene (*trpB*) has shown that ASB27 is very closely related to ASB33, and both are different from *S. griseus*, yet ASB27 was missing many genes (including *strR*) from the cluster. A possible explanation for this result is that part of the cluster was lost from ASB27.

By using Western analysis and RT-PCR it has been shown that at least *strA* and *strB1* were silent in the strains having part of the cluster. In the natural environment, the

strains could be found in niches where antibiotic production is at a low enough level for the strains to be resistant and thus have an advantage for carrying *strA*. They could also acquire the other two genes *strB1, strR* because they are adjacent to the resistant gene. Alternatively these three genes may have been involved in the diversification of the streptomycin pathway where the initial expression of genes may not be a prerequisite. The high degree of identity shown by these genes at nucleotide level in relation to their counterparts in the parental strain *S. griseus* indicates that the transfer event was a recent one. Post-transfer modification may be required for the optimisation of the expression of these genes. Interestingly, the pathways of streptomycin-related aminoglycosides contain *strRAB1* genes of striking similarity between different pathways that led to the suggestion that an ancestral production gene cluster for streptomycin-like aminoglycosides could have developed into another variant by divergent evolution after degeneration and/or modification (Piepersberg, 1997).

Evolution of antibiotic biosynthetic pathways may be achieved by continuous mixing of subsets of gene clusters and the formation of new ones. Certain core secondary metabolic pathways arisen early in evolution could have been maintained in reservoir organisms that served as donors for gene transfer. Widely distributed pathways with a common biochemistry may prove to have a relatively ancient core component on to which the more recent processing reactions responsible for the species specificity of the product have been grafted (Vining, 1992b). The organisation of the antibiotic genes in clusters seems to have evolved in order to facilitate the gene transfer step (Lawrence, 1999). For example, a number of *str* genes encoding for the 6-deoxyhexose metabolism (*strD*, *strE* and *strM*) have homologs in the cluster encoding for avermectin production which can be explained by past gene transfer events and subsequent divergence (Stockmann and Piepersberg, 1992).

Attempts to analyse the integration site of str*RAB1* subcluster in ASB37 Brazilian isolate did not yield any conclusive results. The region proved to be highly unstable and difficult to clone. Genes for dispensable properties like antibiotic production are often found on very unstable region close to the streptomycete chromosomal extremities, which undergoes very large deletions and amplifications. Instability has been observed previously for sporulation and pigment production in the genetically unstable *S. rimosus*

(Gravius *et al.*, 1993). Birch *et al.* (1991) showed that *S. glaucescens* was found to undergo large DNA deletions which often led to loss of hydroxystreptomycin production.

Genes located near the ends of linear chromosomes are probably more mobile than centrally located genes, because they will be more frequently transferred after single cross-over recombination or replicative transposition between linear chromosomes and plasmids (Volff and Altenbuchner, 1998). Many of the Brazilian isolates contain related linear plasmids which could be involved in the transfer of the streptomycin resistance and biosynthesis genes (Egan, 1998). Most *Streptomyces* plasmids are highly self-transmissible by conjugation and also mobilise chromosomal genes in a seemingly random manner. Linear plasmids have been demonstrated to encode resistance genes (Ravel *et al.*, 1998), genes with catabolic functions (Kalkus *et al.*, 1991) and biosynthetic genes (Kinashi and Shimaji, 1987). Interactions between linear molecules are possible by homologous or illegitimate recombination. Exchange of ends between a linear plasmid and a linear chromosome in *S. rimosus* led to the formation of a linear plasmid having one chromosomal end and the chromosomal oxytetracycline biosynthesis cluster (Gravius *et al.*, 1994) which can then be transferred by conjugation in other strains. Exchange of chromosomal ends can also be mediated by replicative transposition. SLP2 linear plasmid has one end identical to the terminal inverted repeats of the *S. lividans* chromosome which includes the transposable element Tn*4811*.

Spectinomycin biosynthetic cluster contains genes with striking similarity to streptomycin genes and sequences homologous to IS*112* element indicating that transposition and divergent evolution can yield simpler but still effective compounds (Lyutzkanova *et al.*, 1997). Transposition is also involved in the mobilisation of virulence determinants among streptomycetes. A pathogenicity factor is flanked by a new insertion element IS*1629* which is responsible for the dissemination of the pathogenicity determinant from *S. scabies* to unrelated species *S. acidiscabies* and *S. turgidiscabies* (Healy *et al.*, 1999).

Insertion sequences and transposons as well as genes for secondary metabolism and degradation of polymers are found closer to *Streptomyces* chromosomal telomeres. Conserved housekeeping genes are located far away from chromosomal ends of *S. coelicolor* that allows the exchange of these regions without affecting the viability of the

organism. This underlines an evolutionary gradient (Volff and Altenbuchner, 2000) on the *Streptomyces* chromosome with the most rearrangement taking place near the chromosomal ends rather than the centre of the chromosome. The genetic instability of this region may create the huge diversity of strains and secondary metabolites in response to changing environments.

Such genomic rearrangements can also lead to changes in gene expression. In particular, better understanding of the regulation of streptomycin production by the pathway specific regulator StrR in *S. griseus*, it may reveal the selective pressure that drives the horizontal transfer of this gene and the functionally dependent genes *strAB1* in the diverse Brazilian isolates. Previous studies showed that StrR is a trancriptional regulator of several genes –including *strB1*- in the streptomycin cluster by binding on their promoters (Retzlaff and Distler, 1995). StrR consists of structural and functional domains (Thamm and Distler, 1997; Thamm, 1999)). In particular, C-terminal of the protein contains a tetramerisation and transcriptional activation domains while DNA binding motif is located at the centre of the gene. It was speculated that an expected dimerisation domain should be located at the N-terminal of the StrR.

Four N-terminal deleted forms of StrR was generated and expressed in *E. coli* hosts. Using DNA binding assays, cell-free extracts from the strain harbouring NstrR2 protein (258 aa) resulted in a retardation of the mobility of the StrR binding site from *strB1p* promoter. All the other overexpressed proteins did not cause any retardation with the same radiolabelled DNA fragment. Mixing of NstrR2 and the full StrR palindromic site yielded a retarded band that run as a pseudodimer. In the possible absence of the dimerisation domain, such interaction will result in two StrR monomers each of them binding to one inverted repeat that comprises the full palindromic site.

DNA binding assays using NstrR2 and the half palindromic site did not yield any retarded band. According to the described model (Thamm, 1999) a mixture of dimer and monomer should be expected. In addition to helix-turn-helix motif, other protein-protein interactions may be necessary for the efficient binding of the oligomers when half binding site is used. The lack of retarded oligomers caused by NstrR2 and the half palindromic site may be due to the insufficient C-terminal interactions between protein monomers which could stabilise any binding. Alignment of the NstrR2 and full length

StrRs from streptomycin related compounds producers revealed that two conserved regions were deleted which may be involved in the dimerisation of StrR.

Although no other macromolecules are needed for the StrR binding activity, since the distance between the StrR binding site and the corresponding activated promoter is rather long, DNA curvature may be required for the interaction of the StrR with the RNA polymerase in the course of transcriptional activation. The dimension and the geometry of StrR binding site is unusual for bacterial regulator (Retzlaff and Distler, 1995). Thus the transcriptional activation mechanism by StrR may resemble better to the regulation mechanism of the glutamine synthase gene (*glnA*) by the nitrogen regulatory protein C (NtrC). The long interval between the regulatory binding sites and the downstream promoter as well as the many oligomers required for efficient protein binding and the transcriptional activation are common features in both regulatory mechanisms. StrR binding site may tether StrR towards the *str* gene promoters forming a DNA loop. In this structure four StrR monomers may be required for increased number of collision between StrR and polymerase leading to the isomerisation of closed to open complexes between the RNA polymerase and the promoter.

7.2 Future Work

Two *S. coelicolor* strains, ASSF15 and ASB37, have been shown to have acquired the identical resistance gene *strA* and the flanking regulatory and biosynthetic genes *strRB1* from the well characterised streptomycin producer *S. griseus*. Coinciding with the completion of the *S coelicolor* A3(2) genome sequence, it has been apparent that this strain has acquired a number of genes with a striking example being the vancomycin inducible resistance cluster. It will be interesting to isolate a number of *S. coelicolor* strains to question the consistency of gene flow to that particular microbial population. These isolates could be analysed using subtractive hybridisation with the laboratory strain *S. coelicolor* A3(2) for the detection of additional gene insertion events. Confirmation of the insertion site of *strRAB1* genes in ASB37 and full characterisation of the cloned flanking regions is important for the understanding of the selection pressure and the

mechanism of such transfer events. This putative insertion site in ASB37 showing high homology with SCF76 cosmid from *S. coelicolor* A3(2) contains a *adpA* homologue from A-factor cascade. It will be interesting to see whether this copy or the higher homologous one found in StC105 cosmid could bind to the upstream *strRp* promoter regions and subsequently activate the transcription of *strR* gene using promoter-probe vectors. Comparative genomics and use of the flanking regions for hybridisation studies can be informative about whether chromosomal region such as SCF76 cosmid or others are 'hot-spots' for both introgression and gene expression. *S. coelicolor* A3(2) genome sequencing project revealed the exclusive location of genes related to adaptive functions close to the chromosomal ends, a region known to be also recombinogenic and rich to mobile elements. A possible location of gene insertions on common chromosomal fragments as could be analysed by comparative pulsed field gel electrophoresis between *S. coelicolor* strains and other streptomycetes can indicate the mechanism of such gene transfer events, i.e. site-specific recombination. It is shown that the *strRA* genes in ASSF15 and ASB37 are not expressed. Detection by PCR of silent antibiotic genes can test the proposed hypothesis in this study of involvement of silent gene transfers in the evolution of antibiotic pathways and sequence analysis can elucidate the nature of these genes, i.e. biosynthetic and chromosomally located genes. It has been shown that tetracycline can stimulate resistance gene transfer as well as select for maintenance of strains that acquire the resistance gene (Salyers *et al.*, 1998). It will be interesting to test whether antibiotics can also stimulate the expression of the transferred silent genes. Recently, Hong *et al.* (2001) discovered a signal transduction system that allows *S. coelicolor* to sense and respond to changes in the integrity of its cell envelope. Specifically, vancomycin and other antibiotics can stimulate a two component regulatory system that transfers from the membrane a signal that is responsible for the transcription of genes with cell envelope-related functions. Therefore, addition of sub-lethal levels of streptomycin can assess its ability to induce *strRAB1* expression in ASSF15 and ASB37.

In this study, it was attempted to elucidate the binding properties of the streptomycin pathway-specific transcriptional activator, StrR. Following previous studies (Thamm and Distler, 1997; Thamm, 1999), N'-terminal deleted StrR derivatives were used to identify putative dimerisation domains. Determination of size of the retarded

band caused by the cell extracts containing NstrR2 protein can be achieved using the calibration method described by Orchard and May (1993). In addition, purification of his-tag N'-terminal forms of StrR may be useful for efficient DNA binding assays.

Chapter 8

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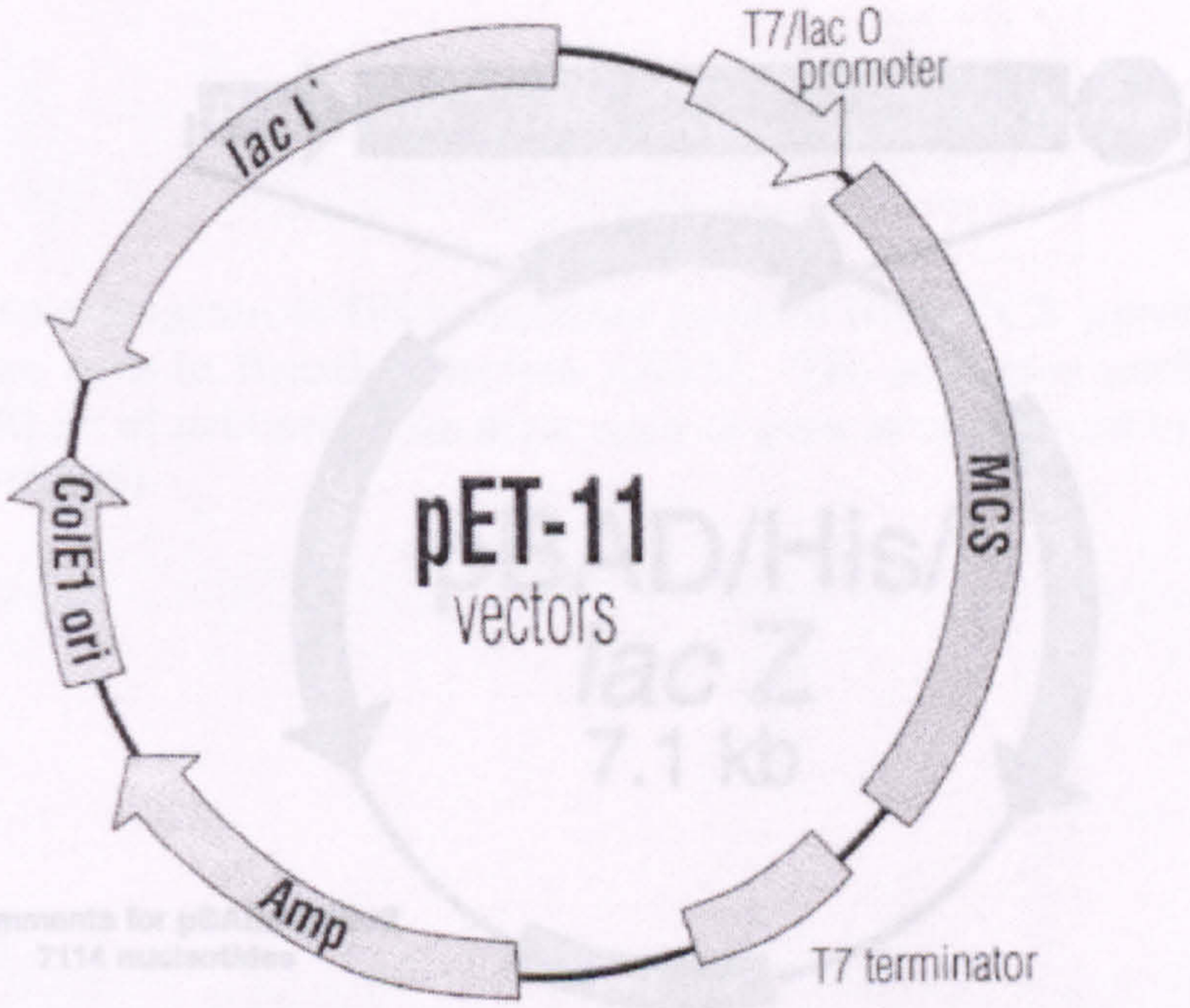
Chapter 9

Appendices

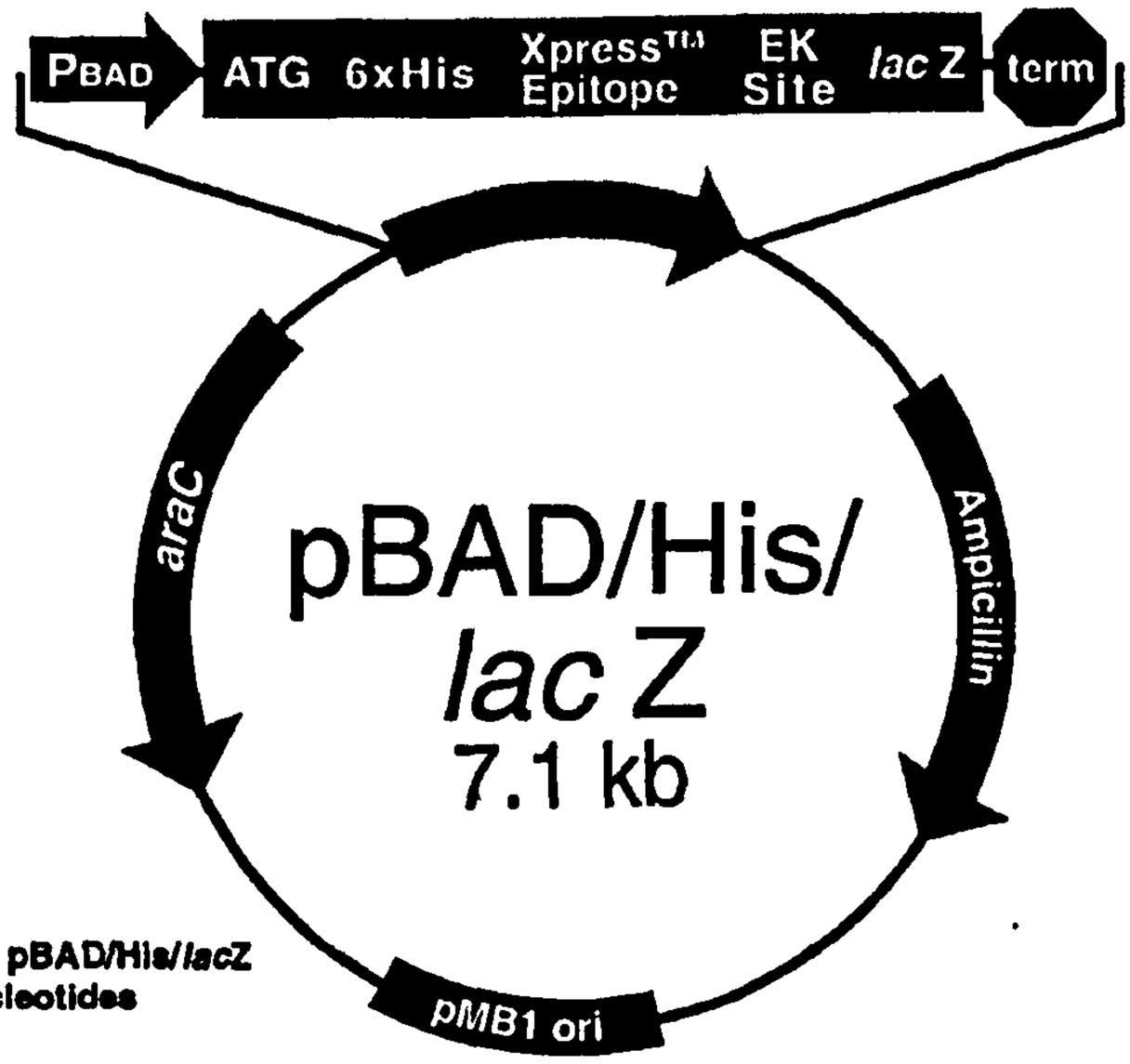
Appendices

Appendix 1



Appendix 1: pET11a vector for the cloning and expression of N'-terminal truncated StrRs (Stratagene).

Appendix 2

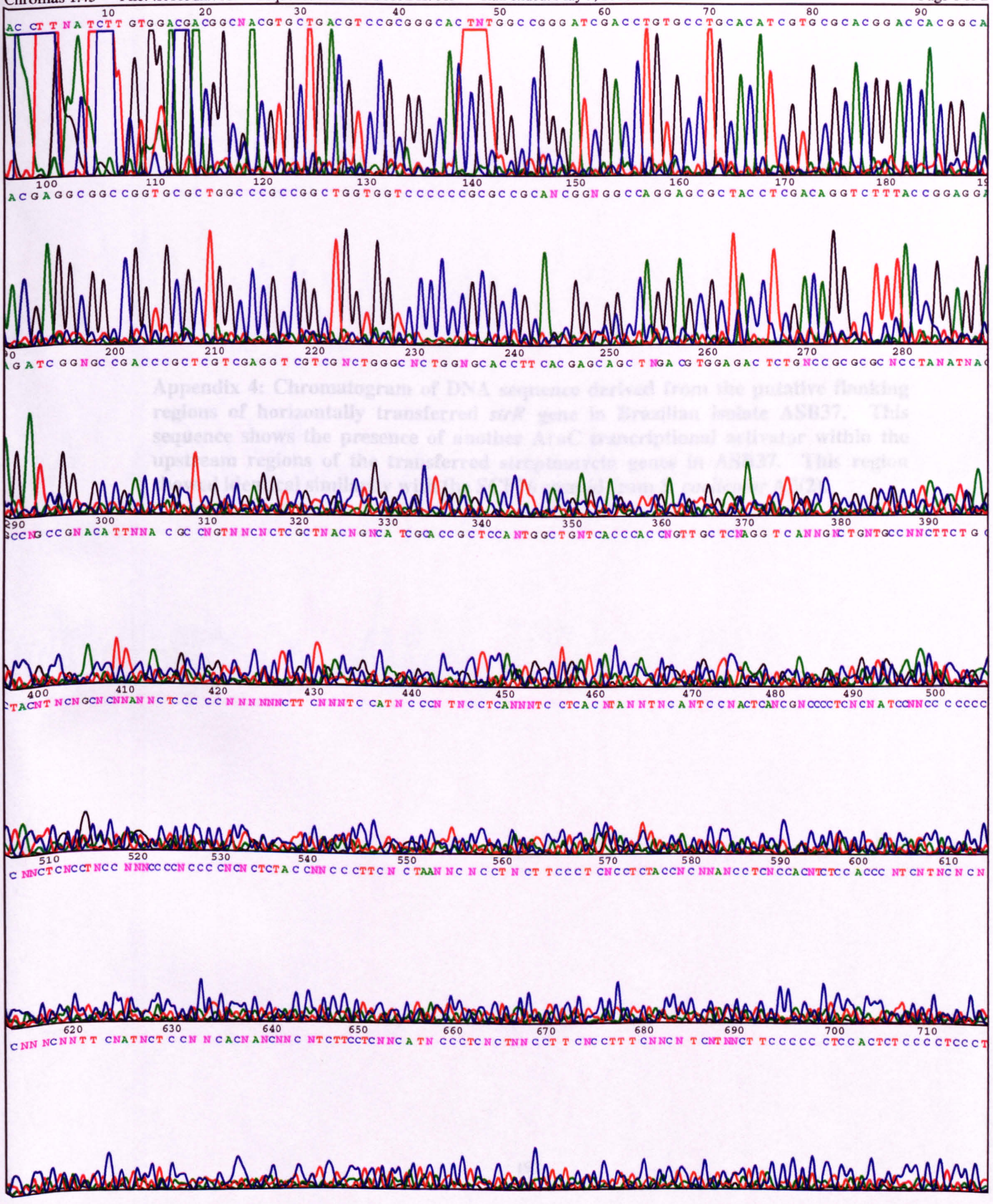


Comments for pBAD/His/*lacZ*
7114 nucleotides

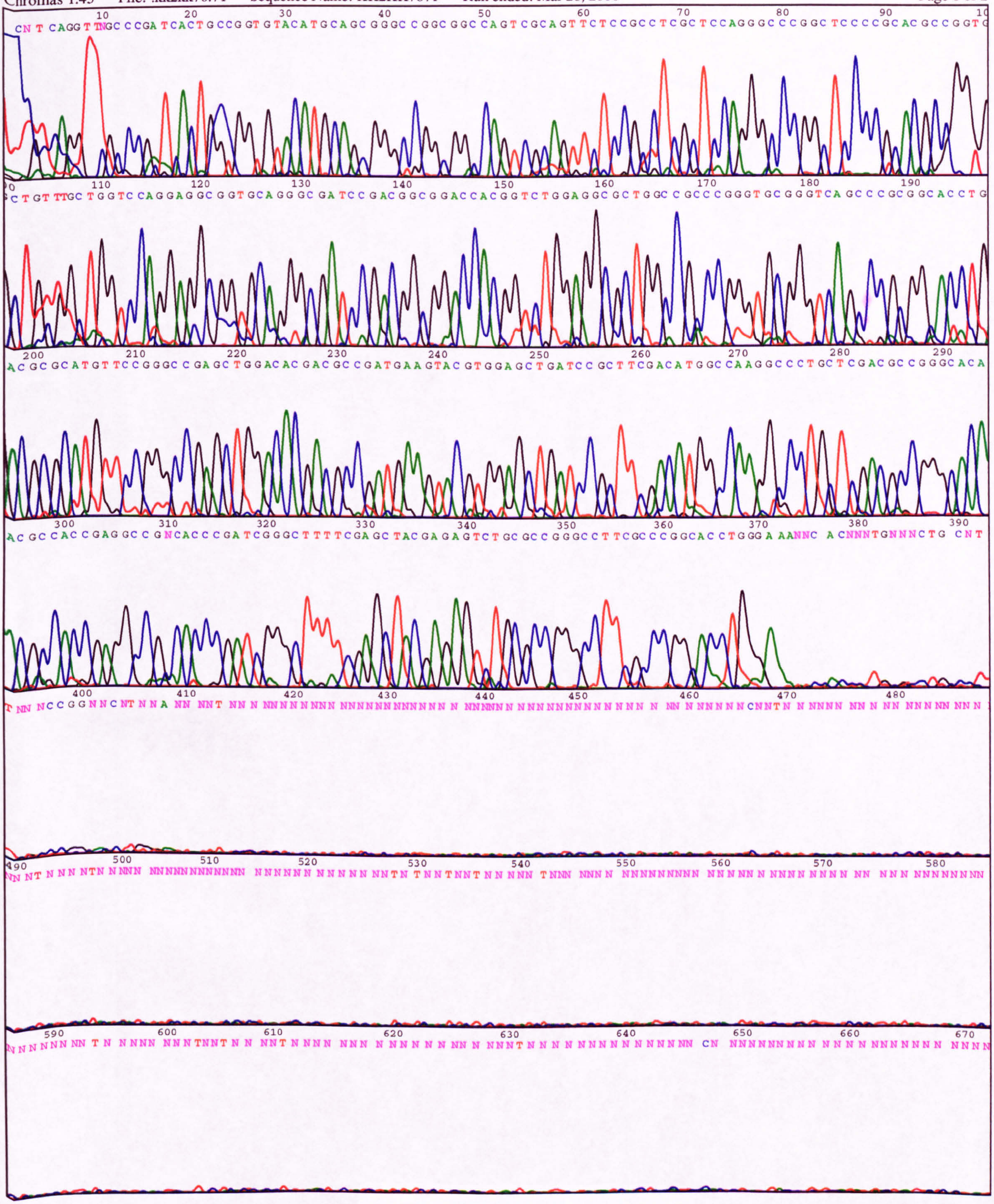
BAD promoter region: bases 2-250
pBAD Forward priming site: bases 208-227
Initiation ATG: bases 319-321
Polyhistidine tag: bases 331-348
Xpress™ epitope: bases 388-411
Enterokinase recognition site: bases 388-411
LacZ ORF: bases 418-3474
rrnB transcription termination region: bases 3564-3721
Ampicillin ORF: bases 4001-4861
pMB1 (pUC-derived) origin: bases 5006-5679
AraC ORF: bases 7088-6210

Appendix 2: pBAD-His vector for the expression of His-tag N'-terminal truncated NstrRs (Invitrogen).

Appendix 3: Chromatogram of DNA sequence derived from *PCR product of highly* homologous *adpA* gene in Brazilian isolate ASB37. The sequence performed using [AdpAF-AdpAR] set of primers. The same copy of gene is also found in *S. coelicolor* A3(2) (cosmid StC105).


10 20 30 40 50 60 70 80 90
AC CT T NA TCTT GTGGACGACGGCNACGTGCTGACGTCCGCGGGCAC TNTGGC CGGG ATCGACCTGTGCCTGCACATCGTGCGCACGGACCACGGCA
100 110 120 130 140 150 160 170 180
ACGAGGCGGCCGGTGCGCTGGC CCGCCGGCTGGTGGTCCCCCCCGCGCCGCANCGGNGGCCAGGAGCGCTACCTCGACAGGTCTTTACCGGAGGA
200 210 220 230 240 250 260 270 280
AGATC GGNGC CGACCCGCTCGTCGAGGT CGTCG NCTGGGC NC TGGNGCACCTT CAC GAGCAGC T NGA CG TGGAGAC TCTGNCCGCGCGC NCCTANATNAC
290 300 310 320 330 340 350 360 370 380 390
SCCNG CCGNACA TTNNA C GC CNGTN NCNCTC GCTNACNGNCA TCGCA CC G C T CCA NTGGC T GNTCACCCAC CNGTT GC TCNAGG T C ANNGNC T GNTGCCNNCTTCT G
400 410 420 430 440 450 460 470 480 490 500
CTACNT NCN GCN CNNAN N C TC CC C C N N N NNNCTT CNNNT C CAT N C CCN T N C CTCANNNTC CTCA C NTA N N T N C A N T C C N ACTCANCG NCCCCTCNCN A TCCNNCC C CCCC
510 520 530 540 550 560 570 580 590 600 610
C NNCTCNCCT NCC NNNCC CCN C C C CNCN CTCTA C CNN C C C CTTC N C TAAN N C N C C T N CT T C C C T CNCCT CTACCN C NNANCC TCNCCACNTCTCC ACCC NT CN T NC N C N
620 630 640 650 660 670 680 690 700 710
C NN NCNNT T CNATNCT C CN N C ACN ANCNN C NT CTTCCTCNNC A TN C C C TC N C TNN C C T T C NC CTT T CNNC N T CNT NNCT T C C C C C CTCC ACTCT C C C C TCC C T

Appendix 4: Chromatogram of DNA sequence derived from the putative flanking regions of horizontally transferred *strR* gene in Brazilian isolate ASB37. This sequence shows the presence of another AraC trancriptional activator within the upstream regions of the transferred streptomycin genes in ASB37. This region showed identical similarity with the SCF76 cosmid from *S. coelicolor* A3(2).


10 20 30 40 50 60 70 80 90
CN T CAGG T TNGC C CGA T CACT G CCG GT G TACA T GCA GC G GGC C GGC GGC CA GT CGCA GT T CT C CGCC T CGC T CCA GGGC C CGGC T CCCC GC AC GC C GGT C
110 120 130 140 150 160 170 180 190
C T GT TTGC T GGTC CA GGA GGC G GT GCA GG GC GA TCC GA CGGC G GA CCA CGGT CT GGA GGC GC T GGC C GCCC GGGT GC GGGT CA GC C C GC G GC A CC T G
200 210 220 230 240 250 260 270 280 290
AC GC GC A T GT T CC GGGC C GA GC T GGA CAC GA CGC C GA T GAA GTAC GT GGA GC T GA TCC GC T T CGA CA T GGC CAA GGC C C T GC T C GA CGC C GGGC A CA
300 310 320 330 340 350 360 370 380 390
AC GC C AC C GA GGC C G NCAC C C GA TCGGGC T T T T CGA GC TAC GA GA GTC T GC GC C GGGC C T T C GC C C GGCAC C T GGG A AANNC A CNNNTGNNNCTG CNT
400 410 420 430 440 450 460 470 480
T NN NCC GG NNC NT N N A NN NN T NN N NNNNNNNN NNNNNNNNNNNNN N NNNNNN N NN NNNNNNNNNNNN N NN N NNNNNCNNTN N NNNN NN N NN NNN NN NN N
490 500 510 520 530 540 550 560 570 580
NN NT N NN NTN NNNN NNNNNNNNNNNN NNNNNN N NNNN NNTN T NN T NN T NN NNN TNNN NNNN NNNNNNNNNN NNNNN N NNNN NNNN NN NNN NNNNNNNN
590 600 610 620 630 640 650 660 670
N N N NNN NN T N NNNN NN N TNNT N N NNT N NNN NNN N NNNNNNNNNNNN