MUTATIONAL ANALYSIS OF STRUCTURE–FUNCTION INTERACTIONS WITHIN SELECTED SITES ON THE *ESCHERICHIA COLI* RIBOSOME

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other institution, and all sources of information have been acknowledged by complete references.

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Jaroslav M. Belotserkovsky

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Abstract

Mutations were sought in *Escherichia coli* ribosomal RNA and ribosomal proteins that confer dependence to the antibiotic streptomycin, using both newly available as well as well-established genetic systems. I found that a classical ribosomal mutant, Sm-D3, was streptomycin dependent and had an additional mutation in another ribosomal component – protein L7/L12. The double mutant had an 8-fold lower streptomycin requirement as compared to Sm-D3 with a wild-type *rplL*. This supported a functional involvement of L7/L12 in the decoding center of the ribosome.

Acknowledgements

Massive respect and gratitude goes out to my most knowledgeable supervisor – Professor Eric Dabbs. Lots of love to my parents who carried me through the turbulent currents of student life and gained a few more grey hairs in the process. Infinite thanks to Professor Leif Isaksson for having me in Sweden, and Ernesto Gonzalez de Valdivia who helped me plenty with the β-gal work. To Christopher Arnot, for being a good friend and colleague through all the years at Wits.

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Abbreviations

Amp	ampicillin
Amp-R	ampicillin resistance
AP	alkaline phosphatase
bp	base pairs
°C	degrees Celsius
Cm	chloramphenicol
Cm-R	chloramphenicol resistance
Cm-S	chloramphenicol sensitivity
CsCl	cesium chloride
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide triphosphate
EMS	methanesulphonic acid ethyl ester
EtBr	ethidium bromide
g	grams
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobases
LA	Luria Bertani agar
LB	Luria Bertani broth
LC	Luria Bertani broth/agar supplemented with calcium chloride
M	molar
mA	milliAmper
mg	milligram
ml	milliliter
min	minutes
mM	millimole
µg	microgram
µl	microlitre
Nal	nalidixic acid
ng	nanogram

NTG	N-methyl-N'-nitro-N-nitroso-guanidine
OD	optical density
ONPG	*o*-nitrophenyl-β-galactosidase
ORF	open reading frame
PCR	Polymerase Chain Reaction
RF-2	release factor 2
Rif	rifampicin
Rif-R	rifampicin resistance
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	seconds
Sm	streptomycin
Sm-D	streptomycin dependence
Sm-I	streptomycin independence
Sm-R	streptomycin resistance
Sp	spectinomycin
Sp-S	spectinomycin sensitive
TBE	Tris boric acid EDTA
TE	Tris EDTA
Tet	tetracycline
Tet-R	tetracycline resistance
T_m	melting temperature

1. Introduction

1.1 The ribosome

The ribosome is a macromolecular complex on which the second step in gene expression takes place. Due to its central role in translation, it has been highly conserved in evolution.

The prokaryotic ribosome (70S) is made of two subunits – referred to as large (50S) and small (30S) (Figure 1). In *Escherichia coli* the small subunit consists of 21 proteins and a 16S rRNA molecule of 1542 nucleotides in length. The large subunit comprises of 31 proteins with two rRNA components – the 5S and 23S of 120 and 1542 nucleotides in length respectively (Yusupov *et al.,* 2001).

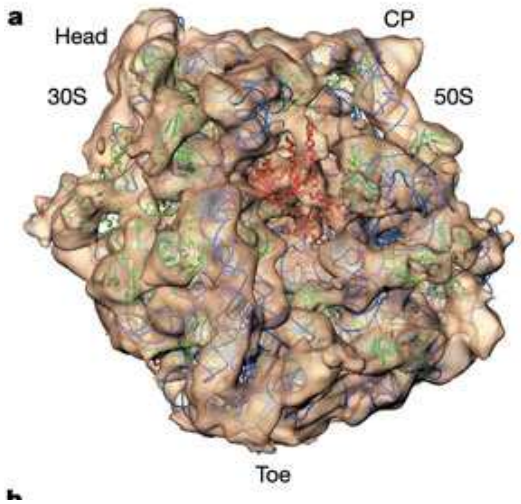


Figure 1. Structure of *E. coli* 70S ribosome in complex with release factor 2 (in red) as determined by angular reconstitution to 14 Angstroms (from Klaholz *et al.,* 2003).

Despite its structural complexity, the core function of the ribosome is the synthesis of a protein chain by translation of messenger RNA using the charged adaptor RNA (Spirin and Gavrilova, 1969). In addition it has become increasingly clear that the RNA component of the ribosome is the most important catalytically, which means it is a ribozyme. In particular, the coupling of amino acids to form peptides (peptidyl-transferase reaction) is catalyzed by domain V of the 23S rRNA, known as the peptidyltransferase centre (Vester and Garrett, 1988; Douthwaite, 1992).

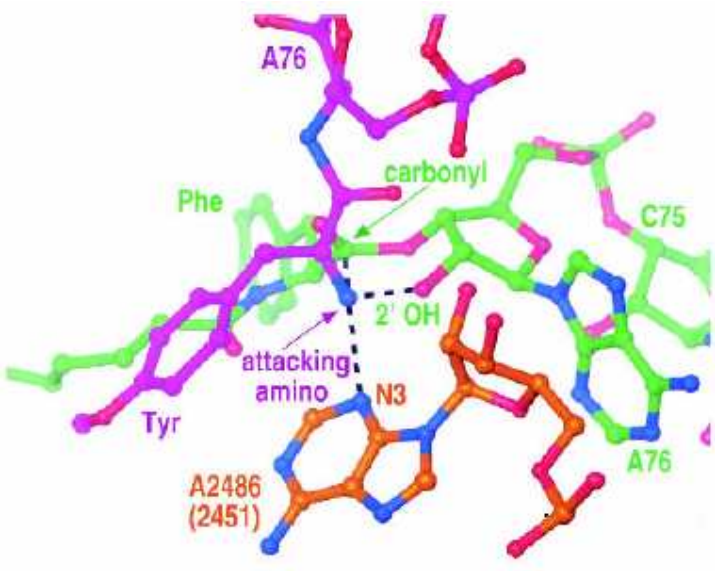


Figure 2. A graphical model of the peptidyl transferase centre of *Haloarcula marismortui* with substrates bound to the A (in purple) and P sites (in green). The functionally important nucleotide of 23S rRNA A2486 (*E. coli* numbering A2451) (in orange) as well as possible hydrogen bonds (broken lines) are indicated (from Moore and Steitz, 2003).

The ribosome can be divided into functional domains. These include binding sites for mRNA, tRNA, as well as protein factors. The decoding function of the ribosome – mRNA binding and tRNA selection, is primarily governed by the small subunit. This site on the prokaryotic ribosome is the focus of this study. Hence, a more detailed discussion follows on the specifics of this aspect of the translation process.

1.2 16S rRNA

In all organisms, translation initiation sets the reading frame of translation. The start codon of messenger RNA specifically interacts with the methionyl-tRNA in the peptidyl donor centre of the small subunit. In addition, in most eubacteria, a second specificity determinant is present. This is a polypurine tract 5′ of the start codon on mRNA. This sequence interacts complementarily with a 3′ end of 16S rRNA known as the Shine-Dalgarno sequence (Ganoza *et al,* 2002). Additionally, other conserved regions on the 16S rRNA, such as nucleotides 1471-1480 and 458-466 have been proposed to bind sequences 5′ or 3′ of mRNA start codon (Sprengart *et al,* 1996).

The 16S rRNA contains a conserved sequence located in the 1400-1500 region. A number of experimental observations have demonstrated that the “heart” of the decoding centre is encompassed by the nucleotides C1399-C1409 and G1492-G1504. Evidently that the major groove of the 1400-1500 region houses the tRNA and mRNA of the translation complex (Easterwood and Harvey, 1995).

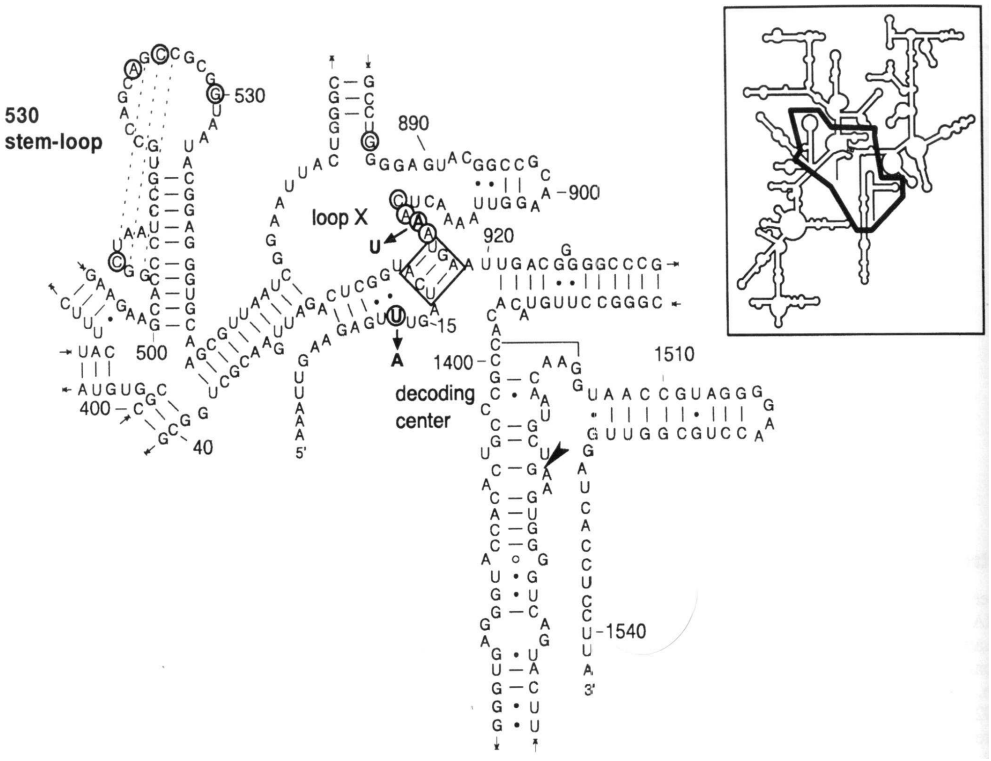


Figure 3. A portion of *E. coli* 16S rRNA showing secondary structure and the functionally important 1400-1500 region as well as the 530 loop (from Brinkier-Gringas *et al.,* 1995).

The majority of antibiotics that perturb translational accuracy interact with the small subunit, mostly with sites on 16S rRNA. In many cases, single mutations in RNA confer resistance to these antibiotics relating to a fact that they interact directly with 16S rRNA. Additionally, chemical probing experiments have demonstrated that hyper- or hypo-accurate mutations in ribosomal proteins S12 and S4 respectively affect tertiary conformation of 16S rRNA, indicating that these phenotypes could partly be due to altered RNA structure (Powers and Noller, 1994).

Biochemical studies also show that selected nucleotides on 16S and 23S rRNA interact, either directly or indirectly, with tRNA in the A, P and E sites on the ribosome. tRNA bound in the A site specifically protects nucleotides in 16S rRNA from chemical probes. These sites are clustered in the 1400-1500 region as well as the 530 stem-loop structure (Moazed and Noller, 1990). The 1400-1500 region is located in the small ribosomal subunit cleft, thought to be involved in codon-anticodon interaction (Scheinman *et al,* 1992). On the other hand, the 530 stem-loop structure is located near ribosomal proteins S4, S5 and S12 (Oakes and Lake, 1990).

1.3 Streptomycin

Streptomycin belongs to the aminoglycosidic group of drugs. It is a carbohydrate with strong basic properties which acts by specifically inhibiting the decoding apparatus of the 30S subunit. At low concentrations this drug causes translational errors – misreading of the template mRNA. This is illustrated in the fact that there is stimulation of binding of aminoacyl-tRNA's that do not correspond to codons on the template mRNA. For example, polyU mRNA normally binds phenylalanyl-tRNA, and only very weakly binds isoleucyl-tRNA. In the presence of streptomycin, the affinity for isoleucyl-tRNA is increased, along with leucyl-tRNA and seryl-tRNA (Spirin and Gavrilova, 1969). At higher concentration, streptomycin completely abolishes protein synthesis resulting in cell death without lysis.

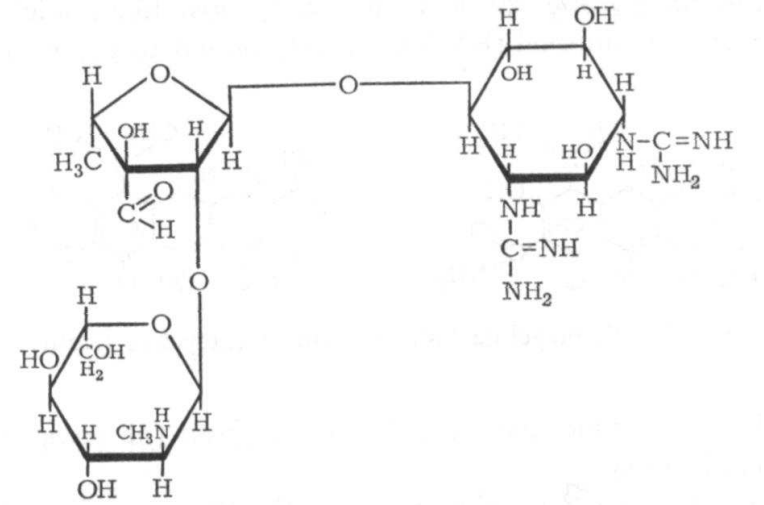


Figure 4. Molecular structure of streptomycin (from Spirin and Gavrilova, 1969).

1.4 Interaction of 16S rRNA, ribosomal protein S12 and streptomycin

Classically, mutations in the ribosomal protein S12 confer resistance and/ or dependence to streptomycin. Streptomycin resistance mutations in S12 are clustered in two regions: T6 and T15. Interestingly, streptomycin dependent mutations are found in the same regions of S12 as resistance mutations. This points to these two small regions as functionally important (Wittmann and Wittmann-Leibold, 1974).

The presence of multiple ribosomal RNA operons in most eubacteria (there are seven in *E. coli*) hampers the mutational analysis of structure-function relations within rRNA. However, the existence of a limited number of rRNA operons in *Mycobacterium smegmatis*, as well as the engineered *Escherichia coli* strains with single rRNA operons made possible the detection of streptomycin resistance and dependence mutations within 16S rRNA (Springer *et al.,* 2001). Most streptomycin resistance mutations in *E. coli* have been mapped to the 912-915 region on 16S rRNA. This region positioned within the 30S decoding centre is widely regarded as the streptomycin binding site, however, other regions within

this molecule have also been implicated, such as position 13 as well as the 530 stem-loop structure. Interestingly, a streptomycin dependence mutation has been isolated within the 530 stem-loop structure in *M. smegmatis* (Honore *et al.,* 1995). Thus various sites on 16S rRNA are involved in streptomycin binding, but do not necessarily interact with it directly, instead control conformational changes associated with its binding (Brakier-Gingras *et al.,* 1995).

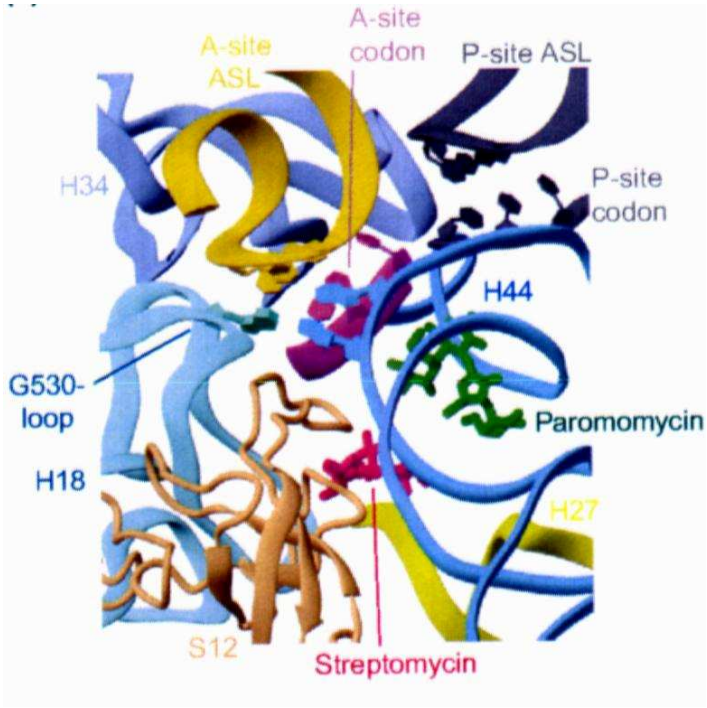


Figure 5. A close up of the induced fit model of 30S subunit showing the decoding centre and binding sites of streptomycin and paromomycin. Relevant functional elements are indicated (from Ogle *et al.,* 2003).

1.5 Ribosomal protein L7/L12

L7/L12 is one of the more extensively studied proteins in the ribosome. This is in part due to its central locality within a highly flexible protuberance on the ribosome – the stalk (Gudkov, 1997). The various conformations of the stalk are

reflective of its functional roles essential for binding of tRNA as well as translocation of peptidyl-tRNA from the A-site to P-site (Bocharov *et al.,* 2004). The monomer of L7/L12 is an acidic protein of 12 kDa. L7 is the N-terminal acetylated form of L12. L7/L12 is the only ribosomal protein present in more than one copy on the ribosome. Each monomer is present in four copies, as two dimers. The two dimer complex is in turn associated with other ribosomal parts through an intimate interaction with protein L10 on the large 50S subunit (Ostberberg *et al.,* 1977). The pentameric structure comprising L10 and L7/L12 tetramer are collectively known, in *E. coli*, as the L8 complex (Petterson *et al.,* 1976).

1.6 Functional domains of L7/L12

L12 can be divided into at least three functional domains. The C-terminal domain (CTD) is primarily responsible for the GTPase activity and association with translation factors (Kischa *et al.,* 1971). An N-terminal domain (NTD) is involved in dimerization as well as L10 binding (Gudkov *et al.,* 1995). Finally, the intervening hinge region facilitates the independent movement of the terminal domains. The overall body of evidence suggests that the hinge region confers a great deal of flexibility. In addition, L7/L12 can assume at least two different conformations, and its flexible nature is largely responsible for its function (Hamman *et al.,* 1996a,b).

1.7 Map location of *rplL* and the chromosome of *λrif^{d}18* transducing phage

Much work has been done to elucidate the genes involved in transcriptional and translational components of *E. coli.* In addition, the mapping of such genes has been well documented and refined, firstly through classical genetic experiments as well as the more recent sequencing of the *E. coli* genome. The defective transducing phage *λrif^{d}18* has been particularly instrumental in the studies of the gene cluster around the 88 minutes on the chromosome. This cluster includes

several components of translational machinery – 16S, 23S and 5S rRNA, the spacer tRNA (including *tgtB, thrU, tyrU, glyT* and *thrT*), EF-Tu, as well as the 50S ribosomal proteins L11, L1, L10 and L7/L12. In addition, the genes coding for two RNA polymerase subunits β and β', are also present in this cluster (Lindahl *et al.,* 1977).

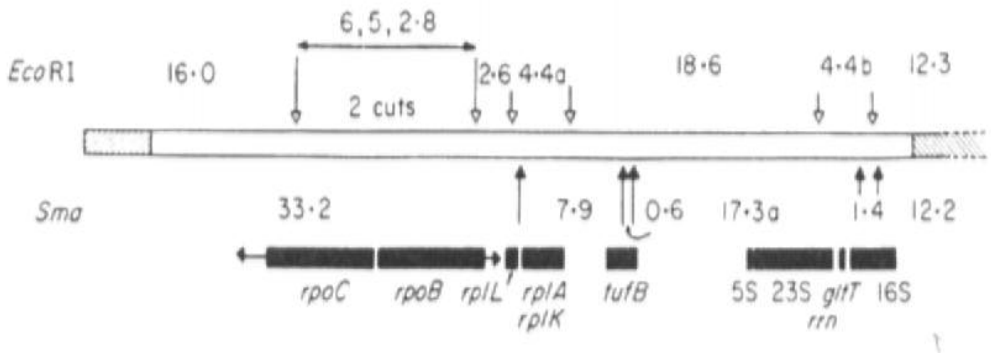


Figure 6. Genetic map of a portion of the *λrif*^{d}*18* chromosome. Selected restriction endonuclease recognition sites are indicated with arrows. Distances between these are in %-λ units (from Lindahl *et al.,* 1977).

1.8 Interaction of L7/L12 with elongation factors and S12

It has now been well established that L7/L12 dimer is an essential feature in the ribosome involved in elongation factor binding and translocation events (Bocharov *et al.,* 2004). There is sufficient evidence that the L7/L12 stalk is a movable ribosome module taking direct part in EF-Tu and EF-G associated events, and in particular, EF-G mediated translocation. In addition, it has been found that changes induced in the L7/L12 stalk, following EF-G·GTP binding and subsequent GTP cleavage, alter the conformations of both the large and small ribosomal subunits (Spirin, 2002). This is suggestive that GTP cleavage affects long range conformational shifts within the ribosome during the translocation

process. Furthermore, it is clear that the large distance between the decoding site and GTP hydrolysis centre, points to a ribosomal conformational change as a means of coupling the two mechanisms. It seems that tRNA binding induces a domain closure in the 30S subunit, leading to subsequent tRNA selection. A number of mutations in S12 result in a "restrictive" phenotype – those that increase fidelity of protein synthesis. It has been observed that such mutations result in decreased rates of GTP hydrolysis by EF-Tu and enhanced accuracy during initial tRNA selection and subsequent proofreading (Bilgin *et al.,* 1992). Since L7/L12 stalk is rather distant from the tRNA ternary complex, but is known to participate in EF-Tu GTPase activity, long range conformational changes, and possibly modulation of 23S rRNA conformations might provide a mechanism of action (Ogle *et al.,* 2003). Finally, it appears that the L7/L12 stalk, S12 and the decoding centre of the ribosome are a linked functional unit, whose activity is not due to direct interactions, but alterations and modulation of various parts of the ribosome.

1.9 Aims of the study

With the increasing need for a more detailed understanding of the functional interactions within important ribosomal regions, such as decoding and peptidyl transferase centers, and despite the current progress achieved with visualization techniques like crystallography and topography, the basic experimental approach of genetic characterization of antibiotic resistance mutations and relevant second site suppressors can yield a vast amount of insightful information.

The aim of this study was the elucidation of some of the complex structure-function relations within the 30S decoding centre, specifically involving the streptomycin binding site. To this extent, it is of interest to obtain mutations either in the 16S rRNA or ribosomal proteins (most likely S12) that confer streptomycin dependence in *Escherichia coli.* In order to obtain selectable mutations in 16S rRNA, an *E. coli* mutant was used in which all 7 chromosomal rRNA operons are deleted, and only one rRNA operon borne on a plasmid is present. Mutants were generated and screened using available techniques such as chemical mutagenesis and counter-selection.

Streptomycin dependent mutants were then selected for streptomycin independence, thus allowing for second site suppressor mutations to be mapped and characterized using standard molecular techniques. In addition, suppressor mutations were sought at specific loci of interest, such as ribosomal protein L7/L12 and release factors.

2. Materials and Methods

2.1. *Escherichia coli* strains

The cultures listed in Table 1 were kept in 33% glycerol at -70°C for long term storage. For short term storage, streaked cultures on Luria Bertani (LA) agar were kept sealed in parafilm at 4°C. To obtain liquid cultures, single colonies of streaked cultures were used to inoculate 5ml of Luria Bertani (LB) broth, with subsequent incubation at 37°C on a rotary drum overnight.

Table 1. *Escherichia coli* strains used in this study

Strain	Characteristics	Source
MM294-4	*endA1, hsdr17, gyrA*	E. Dabbs
NF910	$\lambda rif^{d}18$, $\lambda c_1 857s7$	N. Fiil
SQ170	Δ7 *rRNA,* pKK3535, ptRNA67	C. Squires
D7	Δ7 *rRNA,* pTS1192U, ptRNA67	C. Squires
YB101	Sp-S derivative of SQ170	This study
YB102	Rif-R derivative of YB101	This study
NF915	*argH, his, leu, thi, thr, rel+*	N. Fiil
NF916	*argH, his, leu, thi, thr, relA*	N. Fiil
CAG18456	λ^{-}, *cysG0, zhf-3084::Tn10, rph-1*	CGSC*
KO1418	*Δ(codB-lacI)3,relA1?, bglA677::Tn10, spoT1?, bglB676::λlacZ, bglGo-67, thi-1*	CGSC
CAG18500	λ^{-}, *rph-1, thiC::Tn10*	CGSC
CAG18500 Rif-R	Rif-R derivative of CAG18500	This study
LL103	Spontaneous Sm independent revertant of Sm-D strain VT, *rplL-15*	E. Dabbs

LL103 Rif-R, Tet-R	Rif-R derivative of LL103, *thiC::Tn10*	This study
XL1-Blue	*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F' *proAB, lacI^{q}ZΔM15::Tn10*	Stratagene
Sm-D2	Sm-D of NF915	This study
Sm-D3	Sm-D of NF915	This study
Sm-D4	Sm-D of NF915	This study
Sm-D5	Sm-D of NF916	This study
Sm-D6	Sm-D of NF916	This study
Sm-D7	Sm-D of NF916	This study

* *E. coli* Genetic Stock Centre.

2.2 Phage

P1 transducing phage lysates were stored at 4°C with 100µl of chloroform added to prevent bacterial contamination. In the case of lambda phages, suspensions were stored in SM buffer.

Table 2. Phage used in this study

Phage	**Characteristics**	**Source**
P1	Mediates generalized trasnduction in *E. coli*	C. Squires
ϕ18456	CAG18456 derived P1	This study
ϕ18500	CAG18500 derived P1	This study
ϕ18500rifR	CAG18500 Rif-R derived P1	This study
ϕ1418	CAG1418 derived P1	This study
ϕ103rifR	LL103 Rif-R derived P1	This study
λcI857	Lambda c_I857s7	Roche
λrif^{d}18	NF910 derived λ	This study

2.3 Plasmids

E. coli vectors were maintained in host strains at -70°C. Purified vectors were stored in sterile distilled water at -20°C.

Table 3. Plasmids used in this study

Plasmid	Size	Characteristics	Source
pKK3535	11.864 kb	*E. coli* low copy number vector derived from pBR322 carrying *rrnB* operon, Amp-R	J. Brosius
pTS1192U	~12 kb	Derived from pKK3535, Sp-R, Cm-R	P. Sergiev
pCY104	8.9 kb	*E. coli-Nocardia* shuttle vector	G. Heiss
pACYC184	4.245 kb	Low copy number vector, Tet-R, Cm-R	Roche
pUC18	2.686 kb	*E. coli* high copy number vector, Amp-R	Roche
pUC18-L12	3.081 kb	pUC18 construct with a cloned *rplL* ORF in *Bam*HI and *Hind*III restriction sites	This study
pCMS71	4.2 kb	Derivative of pCMS27 with *lacZ* cloning cassette	C. M. Stenström

2.4 *E. coli* small-scale plasmid preparations

E. coli cells were grown in 1ml of LB, supplemented with Sm and Amp to a final concentration of 200µg/ml, at 37°C on a shaker overnight. Cells were harvested by pelleting in a microfuge for 30 sec and resuspended in 80µl of solution 1. 160µl of solution 2 was added, mixed by inversion, and left to stand at room temperature for 15 min. Thereafter, 120µl of pre-chilled solution 3 was added and mixed with vigorous shaking. This was left to stand in ice-water slurry for 5 min, and microfuged in the cold room for a further 5 min. The supernatant was decanted into a sterile Eppendorf tube and the pellet discarded. The supernatant was warmed up in a 37°C water bath and 220µl of isopropanol was added, mixed gently by inversion and microfuged for 5 min at room temperature. The supernatant was discarded and the Eppendorf tube blotted on a piece of paper towel. This was followed by the addition of 150µl of 96% ethanol, microfugation for 30 sec, supernatant again carefully discarded, and the Eppendorf tube blotted on a paper towel. The pellet was dried in a vacuum for 20 min, resuspended in 150µl of sterile distilled water and stored at -20°C.

2.5 DNA manipulations

2.5.1 DNA digestion with restriction endonucleases

Restriction enzymes were obtained from New England BioLabs, Amersham, Promega or Roche, and used according to the manufacturer's specification. DNA to be digested was thawed from prior storage in a 37°C water bath. Aliquots of DNA (usually 13.5µl) were then mixed with a 1/9 volume from a 10 X stock solution of the appropriate restriction endonuclease buffer. This was mixed gently by tapping, to ensure uniform buffer distribution, and microfuged briefly. 0.3µl of the appropriate restriction endonuclease was then added, contents mixed and re-spun. Digestion was carried out at 37°C, or at other temperatures as specified by the manufacturer, overnight, or for 4-5 hours if the restriction endonuclease

exhibited ‘star activity’. In the case of double digestions, a buffer was selected in which both the restriction endonucleases exhibited suitable activity. If ligations were performed following digestion, the restriction endonucleases and buffers were removed by phenol and chloroform extraction, DNA precipitated with sodium chloride (NaCl) and ethanol, dried in vacuum and resuspended in sterile distilled water.

2.5.2 Agarose gel electrophoresis

0.4% and 0.8% stock solutions of agarose were prepared in 0.5 X TBE. During preparation, the agarose stock solutions were autoclaved (121°C for 20 min), thereafter stored at room temperature. When the agarose solutions were required for use, the stock was melted in a microwave, 20ml of molten agarose was poured into a gel molder with fitted well-former at 4°C, and allowed to polymerize for 30 min. The well-former was removed and the polymerized gel was placed into an electrophoresis unit. 200ml of electrophoresis buffer was then added such that the gel was submerged. Before loading, the DNA samples were mixed with 2µl tracking dye. Either λII or λIII molecular weight markers were also included in all gel electrophoresis runs. Electrophoresis was carried out at room temperature, and the parameters were set such that the voltage was 80-120V and the current 21-30mA. The tracking dye was monitored until it reached the bottom of the gel. At this point, the gels were viewed and photographed with the use of Pharmacia Image VDS system. The sizes of DNA fragments on the gel were obtained either from visual estimation as compared to the standard molecular weight markers, or from a standard curve.

2.5.3 Low-gelling agarose electrophoresis

20ml of 1% low-gelling agarose was prepared in the same way as for the conventional agarose gel. The gel was run at 4°C in a pre-chilled electrophoresis

tank and a pre-chilled electrophoresis buffer at a voltage of 100-120V and a current of 21-25mA. The gel was visualized under UV light (366nm), DNA fragments of interest excised with a scalpel and placed in sterile Eppendorf tubes. Low-gelling agarose, containing the DNA fragments of interest were melted at 60°C for 30 min and DNA was extracted as per section 2.5.4 and precipitated as per section 2.5.5.

2.5.4 Phenol and chloroform DNA extraction

TE buffer was added to the DNA sample, to a final volume of 300µl. One volume of TE-saturated phenol was added to the tube, mixed by inversion and microfuged at room temperature for 5 min. This was done to separate the organic and aqueous phases. The aqueous phase was then carefully pipetted into a sterile Eppendorf tube. In the case of DNA extraction from low gelling agarose, the addition of 1/3 volume phenol and microfugation was repeated three times. Subsequently, 1/3 volume of chloroform was added to the tube, mixed by inversion and microfuged for a further 30 sec. The top phase was then again pipetted into a sterile Eppendorf, and the DNA precipitated with NaCl and ethanol.

2.5.5 NaCl and ethanol DNA precipitation

Precipitation of DNA was achieved by addition of 1/10 volume of 1M NaCl and 2.5 volumes of 96% ethanol. This was then microfuged for 20 min at 4°C. The supernatant was discarded, and the Eppendorf tube blotted on a paper towel to remove excess ethanol. Thereafter, the pellet was dried in vacuum for 20 min, and DNA resuspended in sterile distilled water.

2.6 Transformation of bacteria

2.6.1 DNA transformation of *E. coli*

E. coli culture was grown in 5ml LB on a rotary shaker at 37°C overnight. 200µl of this pre-culture was then used to inoculate 20ml of pre-warmed LB containing 0.5% of glucose in a 250ml flask. This was grown on a shaker at 37°C for 1.75 hours or until $OD_{(590)}$ 0.2-0.4. Thereafter, the culture was rapidly chilled in ice-water slurry for 5 min, and transferred to a pre-chilled JA-20 tube, which was centrifuged (5 min at 10000 rpm). The supernatant was discarded and the pellet resuspended in 10ml of pre-chilled calcium-chloride transformation buffer. This was left to stand in ice-water slurry for 15 min. Another centrifugation step followed (5 min at 10000 rpm), the supernatant discarded and the pellet again gently resuspended in 1.33ml of calcium-chloride transformation buffer. The preparation was then left to stand in ice-water slurry for 2-24 hours. DNA samples were then aliquoted into sterile Eppendorf tubes (usually 2µl of either large-scale or small-scale DNA preparations, and 20µl of ligation mixtures), and chilled in ice-water slurry for 10 min. 50µl competent cells were then added to each tube, tapped gently, and air was bubbled with the use of a P200 micropipette. This was left to stand in ice-water slurry for a further 15 min to allow for diffusion of DNA. Heat shocking was done in a pre-warmed 42°C water bath for 90 sec. 0.5ml of pre-warmed LB was then added to each tube, followed by incubation, with tops of Eppendorf tubes open, at 37°C for one hour to allow for phenotypic expression of the resistance genes. The cell suspensions were then spread on dried LA plates containing the appropriate antibiotic and incubated at 37°C overnight.

2.6.2 Electroporation of *E. coli*

1ml of stationary-phase culture, grown in LB supplemented with the appropriate antibiotic, was aliquoted into an Eppendorf tube and pelleted by microfuging for 30 sec. The supernatant was discarded and the pellet resuspended in 1ml sterile

distilled water. Microfugation again followed for 30 sec and the supernatant discarded. The washing step was carried out three times to remove charged ions from solution. The cells were again resuspended in 1ml sterile distilled water. 100µl of cells were then aliquoted into pre-chilled BioRad 0.2cm electroporation cuvettes. These were allowed to stand in ice-water slurry for 10 min to allow the cells to cool down. 10µl of DNA (from small-scale DNA preparation) was then added and allowed to stand for a further 10 min in ice-water slurry. The cells were electroporated using a BioRad GenePulser I with the following settings: voltage 2.5kV, capacitance 25µF, resistance of 200Ω. Following electroporation, the cells were transferred to a sterile Eppendorf containing 1ml LB. These were incubated at 37°C for 1 hour to allow for phenotypic expression of the resistance gene, and then spread on LA plates supplemented with the appropriate antibiotic. The plates were then incubated at 37°C overnight.

2.7 *E.coli* Transductions

2.7.1 Preparation of P1 lysate

Donor *E. coli* culture was grown overnight in 5ml LB on a rotary shaker overnight. 2.5ml of fresh pre-warmed LB was then aliquoted into 2 small (5ml) glass tubes each. 50µl of overnight donor culture, as well as 20µl of 1M $CaCl_2$ was added to each tube, then vortexed briefly to mix. To one of the tubes, 50µl of seeding lysate (phage titre = $1x10^9$ pfu/ml) was added (the other tube served as a 'no phage' control), vortexed briefly, and allowed to stand at room temperature for 30 min to permit phage attachment to cells. Subsequently, 2ml of warm (60°C) sloppy agar was added to each tube, rolled between the palms of the hands and spread on pre-chilled (4°C) LA + 10mM $CaCl_2$ (LC) plates. These were allowed to set at room temperature, and incubated for 6-8 hours at 37°C. The two plates were then compared to detect a clearing in the agar as a result of cell lysis due to phage infection. The top layer (sloppy agar) of the phage containing plate was then transferred to a JA-20 centrifuge tube, and 100µl of chloroform added to lyse

the remaining cells. The tube was then vortexed briefly and centrifuged in a Beckman JA-20 rotor (10 min at 15000 rpm). Subsequently, the supernatant was collected in a sterile small 5ml bottle, another 100µl aliquot of chloroform added, and the bottle kept in ice for 3-4 days. Thereafter, the lysate was stored at 4°C.

2.7.2 Lysate titre estimation of P1 phage

Donor strains of *E. coli* were grown in 5ml LB on a rotary shaker at 37°C overnight. 2ml of LB was aliquoted into 5 small glass (5ml) tubes each. A dilution series of the prepared phage lysate was set up spanning the following range: 10^{-2}; 10^{-4}; 10^{-6}; 10^{-8}. The fifth tube served as the 'no phage' control. To each tube, 50µl of cell culture, 20µl of 1M $CaCl_2$, and 2ml of warm (60°C) sloppy agar was added, rolled between the palms of the hands, and plated on LC plates. The plates were then incubated at 37°C for 6-8 hours, until phage plaques became visible. The estimation of phage titre was done as follows: the plate with between 30 and 300 plaques was counted, and the dilution taken into account. The phage titre was then: the number of plaques multiplied by the dilution factor divided by the volume plated (2ml).

2.7.3 P1-mediated transduction

100µl of stationary phase *E. coli* recipient culture grown in LC medium (LB + 10mM $CaCl_2$), and in the cases of Sm-D strains supplemented with Sm 100-200µg/ml, was aliquoted into two Eppendorf tubes. The tubes were microfuged for 30 sec and the supernatant discarded. Cells were then resuspended in 1ml of fresh LC. To one of the tubes, 50-100µl of phage lysate was added, the other tube served as a 'no phage' control. Both tubes were mixed by inversion and allowed to stand for 30 min at 37°C for phage attachment to take place. Following this, the tubes were microfuged for 30 sec, the supernatant discarded, and the cells resuspended in 1ml of sterile distilled water. Microfugation again followed for 30

sec, and the cells resuspended in residual volume (~50µl) of water. The suspension was spread on LA plates supplemented with Tet to a final concentration of 10-40µg/ml, and in the cases of Sm-D strains, with Sm 100-200µg/ml. The plates were incubated at 37°C overnight.

2.8 λ Phage manipulation

2.8.1 Small scale reparation of λ phage

Overnight culture of *E. coli* λ lysogen was washed with water and 100µl was used to inoculate a 100ml flask containing 10ml LB medium. The culture was shaken at 30°C until an $OD_{(590)}$ 0.4-0.6 was reached. Culture was rapidly transferred to a 44°C water bath for 10 min for heat induction of the lysogen. Thereafter, the culture incubated on a shaker at 37°C for 1-3 hours. 1ml aliquots were microfuged and resuspended in 0.5ml LM (LB + 10mM $MgCl_2$) medium. 20µl of chloroform was then added and microfuged for 5 sec to lyse the cells. Lysates were stored at 4°C.

2.8.2 Large scale preparation of λ phage

E. coli λ lysogen starter culture was in 5ml LB on rotary shaker at 25°C overnight. 2ml of culture was diluted in 200ml LB in 2L flask and shaken at 30°C until an $OD_{(590)}$ of between 0.4-0.6 was reached. The flask was then rapidly transferred to a 44°C water bath and incubated with agitation for 20 min. This was followed by incubation at 37°C on a shaker for a further 4 hours. The cells were pelleted in Beckman JA-10 rotor (20 min at 6000 rpm) and resuspended in 10ml of SM buffer. 1ml of chloroform was then added to the suspension, and incubated on a shaker at 37°C for 10 min. Thereafter, 20µl of DNase I (1mg/ml) was added to the suspension and incubated for a further 10 min on a 37°C shaker. The lysates were

stored overnight at 4°C. An additional DNase treatment was done, before the lysate was centrifuged in Beckman JA-10 rotor (15 min at 10000 rpm). The supernatant was transferred to a clean bottle using a Pasteur pipette avoiding the bottom chloroform phase. 50% w/v of CsCl was added to the lysate and dissolved. A CsCl block gradient in Beckmann Quick seal tubes was prepared as follows: 3.5ml of the lysate suspension in CsCl was added to the tube using a long-neck Pasteur pipette. This was followed by 0.5ml each of CsCl solutions in SM buffer of specific densities 1.45, 1.5 and 1.7 g/ml, in such a way that each solution was added to the bottom of the tubes sequentially. Tubes were balanced and sealed. Centrifugation was carried out in a Beckmann VTi65.2 rotor (1 hour at 25000 rpm). The distinct blue band containing phage particles was extracted from the tube using a hypodermic needle.

2.8.3 DNA extraction from λ phage

The CsCl fraction containing λ phage was dialyzed twice against 250ml of λ dialysis buffer. The dialyzed suspension was then transferred to a sterile Eppendorf tube. 0.5M EDTA (pH 8.0) was added to a final concentration of 20mM. This was followed by the addition of a small amount (on a tip of a toothpick) of proteinase K and 10% v/w solution of SDS to a final concentration of 0.5%. The tube was incubated at 56°C for 1 hour. λ phage DNA was then extracted using the standard phenol-chloroform DNA extraction protocol, and precipitated with NaCl and ethanol.

2.9 Mutagenesis of *E. coli*

2.9.1 EMS mutagenesis of *E. coli*

100µl of overnight *E .coli* culture was used to inoculate 10ml of fresh LB in a 100ml flask. This was grown on a shaker at 37°C for 1-3 hours, to mid-logarithmic phase. 1ml of culture was then aliquoted into a sterile Eppendorf tube, microfuged for 30 sec, supernatant discarded, and the cells resuspended in 1ml of phosphate buffer (pH = 7.0). Methanesulphonic acid ethyl ester (EMS) was added to the tube to a concentration of 1-2% (v/v). The tubes were incubated for 1-2 hours at 37°C. Thereafter, the tubes were microfuged for 30 sec, the supernatant discarded, and the cells resuspended in 1ml of phosphate buffer. The washing step was done twice. Cells were resuspended in residual volume of phosphate buffer, and a dilution series was plated on LA plates under-laid with Sm to a final concentration of 200µg/ml.

2.9.2 NTG mutagenesis of *E. coli*

A solid phase culture of *E. coli* grown on an LA plate was scraped using a sterile toothpick and resuspended in 1ml Tris-maleate buffer (pH 4.8). NTG solution was prepared by dissolving 1.5mg of NTG powder in 1ml Tris-maleate buffer by heating in microwave oven for 4-5 sec. 100µl of this solution was added to the resuspended cells and incubated at 37°C for 10-15 min. Cells were microfuged and pallet washed twice with phosphate buffer (pH7.0) to inactivate the NTG. The pellet was resuspended in 10ml of LB, and in the case of SQ170, supplemented with Amp to a final concentration of 100µg/ml.

2.10 Counter-selection

100µl of NTG mutagenized overnight culture was aliquoted into an Eppendorf tube. This was microfuged for 30 sec, the supernatant discarded, and the cells resuspended in 1ml of sterile distilled water. The washing step was done twice to remove residual antibiotic. Cells were resuspended in residual volume of water and added to 10ml of LB in a 100ml flask, which was incubated on a shaker at 37°C for 2 hours. Subsequently, Amp or Nal was added to a final concentration of 50µg/ml and 100µg/ml respectively, and incubated for 1-2 hours. 100µl of cells were then aliquoted into an Eppendorf, microfuged for 30 sec, supernatant discarded, cells resuspended in 1ml of sterile distilled water. Washing step was repeated twice. The cells were used as inocula for 10ml of LB supplemented with Amp (in the case of SQ170) to a final concentration of 200µg/ml, and incubated overnight at 37°C. The counter selection procedure was again repeated at least twice. Washed cells were serially diluted 10-fold, 100-fold and 1000-fold and plated on LA plates supplemented with Amp to a final concentration of 200µg/ml. Plates were incubated at 37°C. Colonies thus obtained, were patched onto LA supplemented with Amp to a final concentration of 200µg/ml and incubated at 37°C.

2.11 Determination of reversion frequency of Sm-D mutants

5ml of overnight LB culture in 100ml flasks, supplemented with Sm to a final concentration 200µg/ml, were grown overnight at 37°C on a shaker. 1ml of culture was pelleted and washed in water twice. 100µl aliquots were spread onto LA plates and incubated for 1-4 days at 37°C. The reversion frequency was then calculated based on the number of colonies present on LA plates and the number of CFU/ml for each mutant in the original culture.

2.12 Plate patching technique

This technique was employed to detect particular phenotypes of *E. coli* under varying conditions antibiotic concentration. Individual colonies of *E. coli* were picked up using sterile toothpicks and streaked on LA and LA plates supplemented with an antibiotic. The streaking was done in a manner such that each individual colony could be identified on different LA plates. To achieve this, a numbered pattern was placed under each LA plate, and a streak was made on a particular corresponding number. The LA plates were then incubated for various durations of time (1-2 days) at 37°C. The phenotype was assessed based on the ability of the streaked cells to form confluent growth.

2.13 Spot tests

Spot tests were performed to determine various mutant phenotypes of *E. coli* under varying conditions of temperature and antibiotic presence or concentration. For this purpose, a replicator was used. Each well was filled with ~200µl of sterile distilled water. Individual colonies were then inoculated into each well, and a flame-sterilized replicator was used to replicate the bacterial suspensions onto appropriate plates. These were incubated under varying conditions, for varying durations of time (1-2 days). Phenotypes were assessed based on the ability of cells to form confluent growth.

2.14 β-Galactosidase assay

A modified version of the β-galactosidase assay by Miller J. H., was used. Host *E. coli* strains were transformed with the plasmid carrying the reporter *lacZ* gene. These were grown to stationary phase in LB supplemented with Sm 200µg/ml in the case of Sm-D mutants, and Amp 200µg/ml. Cultures were used to inoculate 3ml of the same medium at a 100 X dilution and grown to an exponentially

growing phase at an $OD_{(590nm)}$ of 0.4-0.5 without IPTG induction. Thereafter, all the proceeding steps were carried out on ice. Cells were harvested by diluting 100µl of culture in 900µl of Z-buffer with previously added chloroform (3 drops) and SDS (1 drop). Lysis of cells was done with vigorous vortexing for 15 sec. A chromogenic substrate *o*-nitrophenyl-*β*-galactosidase (ONPG) was used to assay *β*-galactosidase activity. All measurements were done using an iEMS Multiscan Microplate Photometer (Labsystems).

2.15 Polymerase Chain Reaction (PCR)

PCR was done in a Perkin Elmer Geneamp 2400 thermal cycler. Taq polymerase was used as a master-mix reaction with dNTP's and a standardized concentration of $MgCl_2$ from Fermentas. The following thermal parameters were used based on primer melting temperature (T_m): an initial denaturation step for 5 min at 94ºC; a cycle denaturation step for 1 min at 94ºC; an annealing step for 45 sec at 53ºC; and an extension step for 45 sec at 72ºC. This cycle was repeated 35 times. A final extension step was done for 7 min at 72ºC. Colony PCR was preformed to amplify wild-type chromosomal *rplL*. A toothpick was used to pick off a single colony from an LA plate and used to inoculate the PCR reaction mixture directly. Following PCR, a small aliquot was used to detect the presence of the desired product on a 1% agarose gel.

3. Results

3.1 Selection of streptomycin dependent (Sm-D) mutants of strain SQ170

E. coli strain SQ170 is deficient in all the seven rRNA operons on the chromosome and carries a single copy of the *rrnB* operon (containing 16S, 23S and 5S rRNA) on a plasmid pKK3535. Additionally, it has a second compatible plasmid ptRNA67 containing tRNA genes as well as a Sp-R determinant. This plasmid is essential since SQ170 lacks a number of chromosomally encoded tRNA genes that were deleted along with rRNA operons. It became obvious that prior to any mutagenesis and subsequent selection of Sm-D mutants of SQ170, the Sp-R determinant in this strain had to be removed. This is because both Sm and Sp are aminoglycosidic antibiotics, and potential resistance had to be avoided.

3.2 Selection of spectinomycin sensitive (Sp-S) SQ170 mutants

Following NTG mutagenesis and three consecutive rounds of counter-selection using Nal as a bactericidal agent, a dilution series (10^{-1}; 10^{-2}; 10^{-3}) was plated on Amp containing LA plates. Selected colonies were patched on LA-Amp only and LA-Amp/Sp plates. Of the 111 colonies tested, 25 showed a Sp-S phenotype (these colonies failed to grow on Sp supplemented plates). It was necessary to identify Sp-S clones that showed no resistance to Sm. 18 Sp-S colonies were selected to perform a spot test to determine Sm-R phenotype on increasing concentrations of Sm containing LA plates.

Clones 2, 10 and 18 were ideal candidates for further work since these showed the 'cleanest' Sm-S phenotype and failed to produce any spontaneous resistance mutants. These clones, along with clones 2, 4, 6 and 12 were used to test for spontaneous resistance genesis by plating 100µl of washed stationary phase culture on Sm supplemented LA plates. All clones except 10 produced a small number of spontaneous resistance mutants. Thus it was decided that clone 10 would be most useful for generating Sm-D mutants. This clone was named YB101.

3.3 Selection of YB101 Sm-D mutants

YB101 was subjected to EMS mutagenesis and plated on LA plates under-laid with Sm. Selected colonies were patched on LA with Amp only and LA supplemented with Amp/Sm plates to detect Sm-D mutants. Out of 104 colonies screened, 101 exhibited an Sm-D phenotype (~97%), the rest were Sm-R clones. 19 Sm-D clones, as well as 1 resistant clone were selected and used in a spot-test to determine Sm-D phenotype.

Table 4. Spot-test to determine Sm-D phenotype of selected YB101 derived mutants

YB101	**LA-N supplemented with antibiotic (µg/ml):**						
mutant	Sm0	Sm5	Sm10	Sm20	Sm40	Sm80	Sm160
1*	+++	+++	+++	+++	+++	+++	+++
2	-	-	-	-	-(2°)	++	+++
3	-	--+	-(2°)	-++(2°)	++	+++	+++
4	-	-	-	-	-+(2°)	+++	+++
5	-	-	-	-	-+(2°)	+++	+++
6	-	-	-	-(2°)	-+(2°)	+++	+++
7	-	--+	--+	-+(2°)	-+(2°)	+++	+++
8	--+	--+(2°)	-+(2°)	+	++	+++	+++
9	-	-(2°)	-(2°)	-+(2°)	+	+++	+++
10	-	-	-	-	-+	++	+++
11	--+	--+	-+(2°)	-+	+	++	+++
12	--+	-+(2°)	-+(2°)	-+(2°)	+(2°)	+++	+++
13#							
14	-	-	-	-	-	-	+++
15	--+	--+	--+(2°)	--+(2°)	+	++	+++
16	--+	--+	-+(2°)	-+(2°)	+	++	+++
17	--+	--+(2°)	--+(2°)	-+(2°)	+	++	+++
18	-+	-+	+	+	+	++	+++
19	-+	-+(2°)	+	+	+	++	+++
20	-	-(2°)	-+(2°)	+(2°)	+	++	+++

Bacterial growth is indicated by (+) or (-), where (+++) is maximal confluent growth (++) is intermediate confluent growth, (+) single colonies (-+) is sparse single colonies, (--+) is very sparse growth, while (-) is complete absence of growth. (2°) indicates the presence of secondary colonies.

* Sm-R control.

\# mutant failed to produce any growth at 37°C.

It was desirable to select Sm-D mutants that showed varied requirements for streptomycin: YB101 mutants 2, 4, 6, 9, 10, 14, 16 and 20 were selected for further work.

Of interest was to obtain YB101 mutants that carried Sm-D mutations within the rRNA operon (i.e. plasmid-borne). To test whether any of the YB101 Sm-D mutants carried such mutations, a simple strategy was employed. This involved transformation of YB101 with plasmid DNA derived from all selected Sm-D mutants. Selection of transformed cells was done on LA-N supplemented with Amp only and Amp/Sm.

Transformation of YB101 failed to produce a significant number of colonies with varying amounts of competent cells and plasmid DNA. It appeared that the recipient YB101 strain was competent as indicated by presence of transformants with the positive control pCY104 plasmid that conferred resistance to Cm (results not shown). However, the presence of large colonies on a number of Sm supplemented plates, including the no DNA control plate suggested that these could be spontaneous Sm-R clones and not genuine transformants. This was confirmed by performing a patch on LA-N supplemented with Amp only and Amp/Sm. It became obvious that there was a general problem with transformability of the Sm-D phenotype from candidate Sm-D mutants into recipient YB101. In order to overcome this, a number of variables were changed. These included:

1. In order to minimize contamination during transformation, YB101 was made resistant to Rif 200μg/ml – new clone called YB102.
2. Compared transformation using electroporation and $CaCl_2$ method. Calibrated with *E. coli* MM294-4 and pUC18. It was concluded that transformation efficiency using electroporation was 4 times more efficient.
3. To increase the purity and quantity of plasmid DNA, used an Amersham GFX™ Micro Plasmid Kit to prepare DNA from selected YB101 Sm-D mutants 2, 4, 6, 9 as well as DNA from a pool of all Sm-D clones.

Table 5. Transformation of YB102 with plasmid DNA from an Amersham GFX™ Micro Plasmid Kit using optimized electroporation conditions

YB102 transformed with DNA:	**Number of colonies on LA-N Rif200/Amp200 (µg/ml):**
None	0
pCY104#	37
YB101(2)	0
YB101(4)	0
YB101(6)	3
YB101(9)	8
YB101(Pool)	14

in the case of pCY104 transformation, selection was on LA-N supplemented with Cm100/ Amp 200 (µg/ml).

Despite the recurrent problem of low transformation efficiency, colonies were assayed for Sm-D using a spot test (not shown). The results of the spot test indicated Sm-D transformability was not achieved in any case. Furthermore, a slow growing Sm-R phenotype was observed for all transformants throughout the Sm concentration range. On the basis of this, it was hypothesized that in all selected Sm-D mutants of YB101, there were at least two Sm-D conferring mutations. One plasmid-borne mutation that imparted an Sm-R phenotype in one of the rRNA genes, and a second genomic dependence imparting mutation (possibly in S12). Alternatively, a mixed population of ribosomes in the recipient YB101 strain contributed to the observed phenotype. In this case, a merodiploid condition was present, whereby more than one rRNA plasmid borne operon was actively expressed. To resolve this matter, a marker rescue experiment was performed. Sm-D mutants YB101(6) and YB101(9) were transduced with P1 phage derived from strain CAG18456 that carries a Tn10 near *rpsL*.

Table 6. Transduction of YB101(6) and YB101(9) with Φ18456

Sm-D mutant	**Number of colonies on LA-N supplemented with antibiotic (µg/ml):**	
	Tet50	Tet50/Sm200
YB101(6) - Φ	0	0
YB101(6) + Φ	0	178
YB101(9) - Φ	0	0
YB101(9) + Φ	0	234

Φ indicates presence or absence of P1 phage.

Although Sm-D phenotype was not rescued as indicated by the absence of colonies on the Tet only plates, it was of interest to screen available transductants for Sm-I or pseudo-dependence. A number of randomly selected colonies of YB101(6) and YB101(9) transductants from the Sm/Tet plate were patched on LA-N supplemented with Tet only and LA-N with Sm/Tet.

Table 7. Patching of selected YB101(6) and YB101(9) transductants

Sm-D mutant with Tn10 near *rpsL*	**Number of patches showing growth on LA-N supplemented with antibiotic (µg/ml):**		**co-transduction of Tn10 and Sm-D conferring mutation(s)**
	Tet50	Tet50/Sm200	
YB101(6)	90*	96	94%
YB101(9)	75*	99	76%

* Patches on tetracycline only plate showed considerably less growth (rated +) as compared to patches from the tetracycline and streptomycin plate (rated +++).

Transduction experiments suggested that YB101 derived Sm-D mutants indeed carried a mutation in ribosomal protein S12 that conferred a degree of streptomycin dependence. However, one could not rule out the possibility that more than one genetic locus was responsible for the observed phenotype.

3.4 Testing of transformability of Sm-D or Sm-R phenotype of YB101 mutants in the Δ7 *E. coli* strain containing the rRNA plasmid pTS1192U

To settle the question, whether any YB101 derived Sm-D mutants carried an rRNA mutation responsible for the observed phenotype, plasmid DNA from mutants was used to transform an *E. coli* lacking in all seven chromosomal rRNA operons, and containing an rRNA plasmid pTS1192U. Since the host plasmid has a Cm-R marker, one was able to screen on medium containing Amp, in this way, selecting for transcription of the mutant plasmid DNA. Competent *E. coli* host strain was prepared as per $CaCl_2$ method and transformed with 10µl pool of mutant plasmid DNA derived from all of the Sm-D mutants. Selection was done on LA plates supplemented with Amp only, Amp/Sm20 (µg/ml) and finally, Amp/Sm200 (µg/ml).

Table 8. Transformation of Δ7 *E. coli* strain with pool of mutant plasmid DNA derived from Sm-D mutants

Sample	**Number of colonies on LA supplemented with antibiotic (µg/ml):**		
	Amp200	Amp200/Sm20	Amp200/Sm200
No DNA control	0	0	0
Pool of plasmid DNA	34	0	0

Transformation results revealed that the neither Sm-R nor Sm-D phenotype was transformable with plasmid DNA derived from a pool of Sm-D mutants. Thus, it was concluded that the Sm-D phenotype was not due to mutations in the rRNA.

3.5 Site directed mutagenesis of pKK3535

As an alternative strategy to obtain an Sm-D mutant in 16S rRNA, site-directed mutagenesis was performed on isolated pKK3535 plasmid. This work was inspired by the existence of an Sm-D strain isolated from *Mycobacterium tuberculosis.* This strain carries a cytosine insertion mutation in the well conserved 530 loop of 16S rRNA which renders it dependent to high levels of Sm (more than 50μg/ml) (Honore *et al.,* 1995). The existence of an *E. coli* system with a single set of rRNA genes made possible the construction of an analogous mutation in the 530 loop of 16S rRNA. To do this, pKK3535 plasmid DNA was mutagenized with the aid of a Stratagene QuickChange® II XL Site-Directed mutagenesis kit. Mutagenic primers were designed according to Stratagene recommendations, with modifications as per Zheng *et al* (2004).

Table 9. Mutagenic primers used in the construction of a 16S rRNA mutant

Primer (5'-3')	**T_m (°C)**	**%GC**
530Smd_Frwd: ccgtgcca gccagccgcgg taatacggag	83.05	68
530Smd_Reverse: ccgcggctggc tggcacgg agttagcc	83.17	74

The site of cytosine insertion is highlighted in red.
T_m was calculated as per formula supplied with the Stratagene mutagenesis kit.
Primer-primer melting temperature was 75.2 °C.

Amplification was carried out in a PC-960G Gradient Thermal Cycler. The reaction parameters were according to Stratagene specifications with extension time of 30 minutes. 5μl of the reaction product was used to transform XL10-Gold® highly competent cells. Selection was done on LA plates supplemented with Amp. After overnight incubation, 12 colonies were present. Plasmid DNA was extracted from 3 randomly selected colonies and used to transform Δ7 *E. coli* strain D7. Transformation was done in duplicate, with selection on LA supplemented with Amp only and Amp/Sm50 (μg/ml).

Table 10. Transformation of D7 with site-directed mutagenized pKK3535

Mutagenized pKK3535 plasmid	**Number of colonies on LA supplemented with antibiotic (µg/ml):**	
	Amp200	Amp200/Sm50
Sample 1	3	0
Sample 2	1	0
Sample 3	4	0

Incubation of LA plates was done for 36 hours at 37ºC. All colonies on Amp containing plate were severely retarded in growth.

Colonies were patched out on LA supplemented with Amp only and Amp/Sm50 (µg/ml). Following incubation at 37ºC for 18 hours, no growth was observed on the Sm containing plate. This suggested that the mutagenized construct failed to produce the desired phenotype in the recipient D7 strain. This was either due to unsuccessful mutagenesis (which could be verified by sequencing), or the insertion of cytosine in the 530 loop of 16S rRNA does not produce the analogous Sm-D phenotype seen in *M. tuberculosis.*

3.6 Selection of Sm-D mutants of NF915/ NF916

Following EMS mutagenesis *E. coli* cultures NF915 and NF916 were serially diluted 10^{-1}; 10^{-2}; 10^{-3} and 10^{-4} and spread on an LA-N plates. Thereafter, an underlay technique was employed with Sm.

Table 11. Dilution series of mutagenized NF915 and NF916 strains on Sm containing LA-N plates

***E. coli* strain**	**LA-N under-laid with Sm200 (µg/ml)**			
	Number of colonies at dilution:			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
NF915	103	6	0	0
NF916	19	2	0	0

95 colonies were selected from 10^{-1} and 10^{-2} dilution plates for both NF915 and NF916 and patched on LA-N only and LA-N supplemented with Sm. After overnight incubation at 37°C, out of 95 colonies, 9 displayed a distinct Sm-R phenotype, while the rest were either true Sm-D or pseudo-dependent mutants (frequency ~90%).

6 dependent mutants were chosen randomly, 3 from NF915 and 3 from NF916 parental strains and a spot test performed to detect the lower limit of Sm-D for the selected mutants on varying amounts of Sm containing LA-N plates. An Sm-R control was also included.

Table 12. Spot test to detect lower limit of streptomycin dependence in selected Sm-D mutants*

Sm-D mutants	**LA-N supplanted with antibiotic (µg/ml):**					
	Sm0	Sm5	Sm10	Sm20	Sm40	Sm80
NF915(a)#	+++	+++	+++	+++	+++	+++
Sm-D2	-	-	-+	++	++	+++
Sm-D3	-(2°)	++	++	++	++	+++
Sm-D4	-	-(2°)	-(2°)	++	++	+++
Sm-D5	-	-	-(2°)	-(2°)	-+	++
Sm-D6	-	-	-	-(2°)	-+	++
Sm-D7	-	-	-(2°)	++	++	+++

* each spot was tested in duplicate, the same phenotype was observed.

\# Sm-R control strain derived from NF915 parental strain.

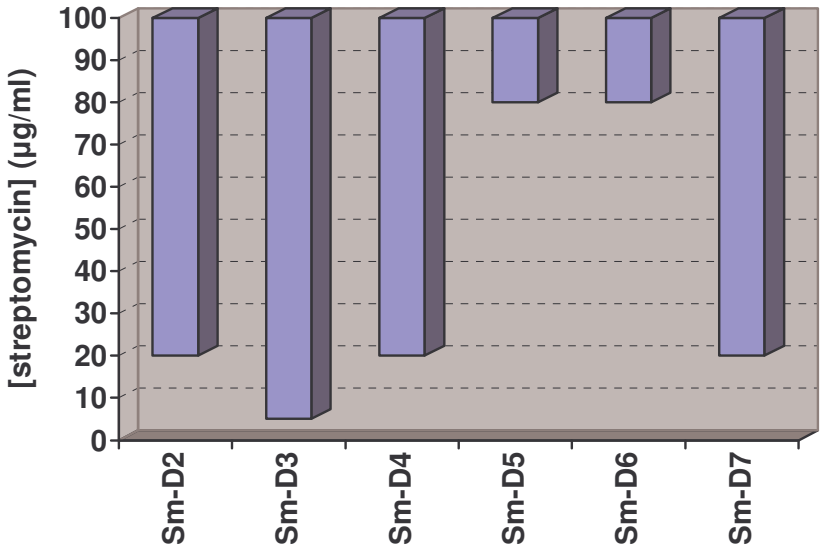


Figure 7. Histogram showing selected Sm-D mutants and their lower limits of dependence at various concentrations of streptomycin.

3.7 Selection of suppressor mutations near the *prfB* locus in selected Sm-D mutants

In order to detect suppressor mutations in the Release Factor 2 (RF-2), the following strategy was developed. An *E. coli* strain KO1418 containing a Tn10 marker near the *prfB* region was a donor for P1 transduction of selected Sm-D mutants derived from NF915 and NF916 parental strains. Transductants were selected on LA-N supplemented with Sm/Tet. It was of interest to identify those transductants that carried the Tn10 marker near *prfB* and still retained the original Sm-D phenotype in each case. To verify this, a spot test was carried out with a number of transductants for each Sm-D mutant on increasing concentrations of Sm. The original Sm-D mutant was included in each case to serve as a standard control against which the Tn10 carrying clones would be compared. Tn10 carrying clones that most closely matched the original Sm-D phenotype were selected for reversion experiments.

Stationary phase cultures of Sm-D mutants with Tn10 marker near *prfB* were plated on LA-N plates supplemented with Tet. Spontaneous Sm-I (reversion) mutants were pooled (from plates containing more than a 100 colonies) and used as donors for P1 transduction of corresponding original Sm-D mutants. In this way, a marker rescue experiment, whereby simultaneous selection for Tet-R (Tn10) and Sm-I, was done to detect suppressor mutations near *prfB*. For this purpose, only mutants Sm-D2, Sm-D3 and Sm-D4 were useful. This is because the NF916 derived Sm-D mutants failed to produce a reasonable number of revertants even when large volumes of stationary phase cultures were used (up to 200µl). Transductants were selected on LA-N supplemented with Tet only and LA-N supplemented with Tet/Sm.

Table 13. Transduction of Sm-D mutants with phage from corresponding Sm-I revertant donors

Sm-D mutant transduced with phage:	**Number of colonies on LA-N supplemented with antibiotic (µg/ml):**	
	Tet50/Sm200	Tet50
Sm-D2 - Φ	0	0
Sm-D2 + Φ	61	0
Sm-D3 - Φ	0	0
Sm-D3 + Φ	33	0
Sm-D4 - Φ	0	0
Sm-D4 + Φ	22	0

Φ indicates P1 phage derived from corresponding Sm-I donors for each Sm-D mutant.

Due to the absence of any colonies on LA-N Tet plates, in all cases, it was concluded that no suppressor mutations were present near the *prfB* locus. This experiment was repeated with varied amounts of phage and cells, in each case the same result was observed.

3.8 Selection of suppressor mutations near the *rpoB* locus in selected Sm-D mutants

The initial strategy for obtaining reversion mutations near *rpoB* of NF915 and NF916 derived Sm-D mutants was similar to the above. However, in the course of transducing Sm-D mutants with P1 phage derived from an *E. coli* strain CAG18500-Rif-R carrying a Tn10 marker near *rpoB,* it was noted that in the case of Sm-D3, two distinct phenotypic classes of Sm-D clones were obtained when tested on increasing concentrations of Sm containing LA-N plates. This was not seen with any of the other NF915 or NF916 derived Sm-D mutants.

Table 14. Spot-test to determine Sm-D phenotype of Sm-D3 clones with a Tn10 marker near *rpoB*

Spot	**LA-N supplemented with antibiotic (µg/ml):**							
	Sm0	Sm5	Sm10	Sm20	Sm40	Sm80	Sm160	Sm200/ Rif200
Sm-D3*	-	+	+	++	++	++	+++	-
Sm-D3*	-	+	+	++	++	++	+++	-
Sm-D3*	-	+	+	++	++	++	+++	-
Sm-D3*	-	+	+	++	++	++	+++	-
1	-	-(2°)	-(2°)	-+	++	++	+++	++
2	-	+	+	++	++	++	+++	-
3	-	-(2°)	-(2°)	-+	+	++	+++	++
4	-	-(2°)	-(2°)	-+	++	++	+++	++
5	-	-(2°)	-(2°)	-+	++	++	+++	++
6	-	-(2°)	-(2°)	-+	++	++	+++	++
7	-	-(2°)	-(2°)	-+	+	++	+++	++
8	-	-(2°)	-(2°)	-+	++	++	+++	++
9	-	-(2°)	-(2°)	-+	++	++	+++	++
10	-	-(2°)	-(2°)	-+	+	++	+++	++
11	-	-(2°)	-(2°)	-+	++	++	+++	++
12	-	-	-(2°)	-+	+	++	+++	++
13	-	-(2°)	-(2°)	-+	++	++	+++	-
14	-	-(2°)	-(2°)	-+	++	++	+++	++
15	-	-(2°)	-(2°)	-+	+	++	+++	++
16	-	-(2°)	-(2°)	-+	++	++	+++	++

* indicates original Sm-D3 mutant.

Spots 1 to 16 are Sm-D3 clones transduced with Φ18500rifR.

Table 14 indicates that there were at least two phenotypic classes of Sm-D3 clones with a Tn10 marker near *rpoB*. One class had a minimum requirement of Sm 5 (μg/ml) (as is seen with the original Sm-D3 mutant). The second class was evident when the *rpoB* locus was replaced with that derived from CAG18500 Rif-R strain. This class exhibited a higher dependence for Sm 40 (μg/ml). It was hypothesized that Sm-D mutant Sm-D3 carries two functional mutations with respect to streptomycin dependence, one conferred classical dependence, the other near the *rpoB*, conferred a suppression of dependence. It was of interest to determine the co-transduction frequencies of Tn10, Rif-R and the dependence suppression (Sm-Sup) loci. To do this, Sm-D3 was transduced with P1 phage derived from 18500 Rif-R strain. Transductants were patched on LA-N supplemented with Tet/Sm5 (μg/ml); LA-N supplemented Sm40 (μg/ml); and LA-N supplemented with Sm200/Rif200 (μg/ml).

Table 15. Co-transduction frequencies of Tn10, Rif-R and Sm-Sup in Sm-D3

Sm-D3 transduced with Φ18500rifR	**LA-N supplemented with antibiotic (μg/ml):**		
	Sm40/ Tet30	Sm5/ Tet30	Sm200/ Rif200
Number of patches showing growth	108	38*	80
Relevant markers tested	Tn10	Sm-Sup	Rif-R
% co-transduction of markers with Tn10		64	74

* 10 of 38 patches from Sm5/Tet30 plate were Rif-R.

% co-transduction was calculated as follows: number of total transductants – number colonies present on the selected marker plate / total number of transductants X 100.

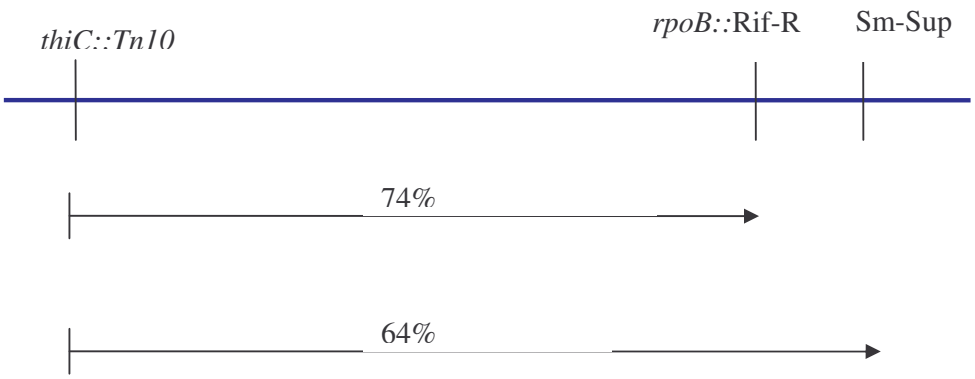


Figure 8. P1 linkage map of the markers *thiC::Tn10*, *rpoB::*Rif-R and Sm-Sup based on co-transduction frequencies in Table 15. The degree of linkage between the markers Rif-R and Sm-Sup indicated that this order of genes was most likely. Map units are given in % co-transduction.

3.9 Identifying if the LL103 streptomycin dependence inhibition phenotype is active in Sm-D3

E. coli mutant LL103 has been previously characterized (Nomura *et al.,* 2003) and shown to have a Sm-D suppression mutation in the L7/L12 ribosomal protein. It was of interest to examine whether this suppression would also be active in the Sm-D3 mutant. A marker rescue strategy was employed. LL103 was made resistant to Rif by plating stationary phase culture on Rif containing LA-N plates. A single Rif-R mutant was selected and used for transduction with Φ18500. Transductants were selected on Tet. This was done to introduce the Tn10 into a region near *rpoB*. In order to ensure that the suppression mutation in L7/L12 was not replaced, LL103 was first made resistant to Rif before introducing the Tn10. Since the map order of markers was *thiC::Tn10*, *rpoB::*Rif-R, and L7/L12, it was reasoned that the L7/L12 mutation would not be replaced when selecting for a clone that was both Tet-R and Rif-R.

Transductants were patched on LA-N supplemented with Tet only and LA-N supplemented with Tet/Rif. Result of patching indicated that there was ~84% linkage of Tn10 and Rif-R loci in LL103. A single LL103 clone was selected that was both Rif-R and had a Tn10 near *rpoB* region (Tet-R). This clone was a donor for P1 transduction of Sm-D3. Transductants were selected on Tet/Sm containing LA-N plates.

Table 16. Spot-test to determine phenotype of Sm-D3 transduced with Φ103rifR

Spot	**LA-N supplemented with antibiotic (µg/ml):**							
	Sm0	Sm5	Sm10	Sm20	Sm40	Sm80	Sm160	Sm200/Rif 200
Sm-D3*	-	++	++	+++	+++	+++	+++	-
Sm-D3*	-	++	++	+++	+++	+++	+++	-
Sm-D3*	-	++	++	+++	+++	+++	+++	-
Sm-D3*	-	++	++	+++	+++	+++	+++	-
1	-	--+	-+	+++	+++	+++	+++	+++
2	-	--+	-+	+++	+++	+++	+++	+++
3	-	--+	+	+++	+++	+++	+++	+++
4	-	--+	+	++	+++	+++	+++	+++
5	-	++	++	+++	+++	+++	+++	-
6	-	--+	-+	+++	+++	+++	+++	+++
7	-	--+	++	+++	+++	+++	+++	+++
8	-	++	++	+++	+++	+++	+++	-
9	-	--+	-+	++	+++	+++	+++	+++
10	-	--+	-+	++	+++	+++	+++	+++
11	-	+	++	+++	+++	+++	+++	+++
12	-	+	++	+++	+++	+++	+++	+++
13	-	--+	-+	++	+++	+++	+++	+++

* indicates original Sm-D mutant.

It was concluded that the streptomycin dependence suppression phenotype of LL103 was not expressed in Sm-D3.

3.10 Complementation of Sm-Sup mutation in Sm-D3

The initial strategy for complementation of Sm-Sup in Sm-D3 was as follows: transducing *λrif^{d}18* phage (Kirschbaum and Konrad, 1973) derived from an *E. coli* strain NF910 carries genes located at ~88 minutes on the *E. coli* chromosome. This chromosomal region includes genes coding for ribosomal RNA, subunits β and β' of RNA polymerase as well as a number of ribosomal proteins (Lindahl *et al.*, 1977). Based on linkage information the Sm-Sup mutation is located in the chromosomal region described above. Thus, *λrif^{d}18* is able to complement this mutation. For such purpose it was of interest to construct a plasmid vector that carries selected ribosomal protein genes derived from *λrif^{d}18* transducing phage DNA. This construct was used for complementation studies. In addition, a low copy number vector was needed since over-expression of ribosomal proteins has been shown to be moderately toxic to the cell (Fredrick *et al.,* 2000). It was decided to use a low copy number vector pACYC184 (see Appendix D). In order to complement any mutations within ribosomal protein L7/L12, a vector construct needed to carry genes *rplL* as well as the upstream *rplJ* coding for ribosomal protein L10. This is because L7/L12 is co-transcribed from a common promoter located upstream of *rplJ* (Little *et al.,* 1981). Based on sequence information of *E. coli* K-12, it was decided to use a combination of restriction enzymes of *Nco*I and *Rca*I to subclone a region of DNA derived from *λrif^{d}18* into a unique *Nco*I restriction site in pACYC184. The combination of these enzymes would produce a 2.1kb DNA fragment carrying the genes *rplL*, *rplJ* as well as any upstream promoter sequences required for co-transcription of this gene cluster. Upon large scale isolation of *λrif^{d}18* DNA derived from an *E. coli* strain NF910, restriction digests were performed with the above restriction endonucleases.

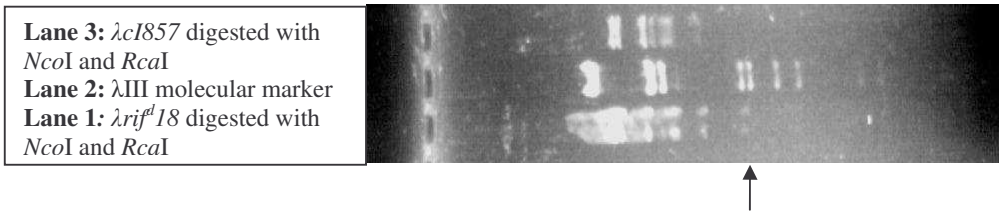


Figure 9. 0.8% agarose gel showing double digestions of *λrif*^{d}*18* and *λcI857* DNA with *Nco*I and *Rca*I. Arrow points to expected band of ~2.0kb in size. This band was not present in wild type *λcI857* DNA.

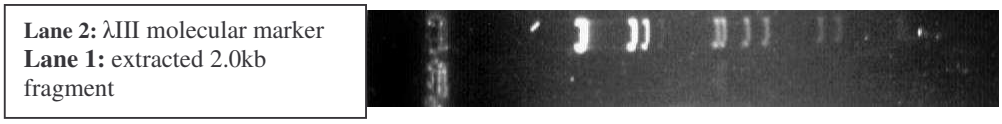


Figure 10. 0.8 % agarose gel showing phenol-chloroform extracted 2.0kb DNA fragment from low gelling agarose.

The selected 2.0kb DNA fragment derived from *λrif*^{d}*18* and expected to carry the desired gene cluster was sub-cloned into a unique *Nco*I restriction site located within the Cm-R marker on pACYC184. *Nco*I and *Rca*I produce compatible sticky ends, thus in addition to an *Nco*I restriction site, a hybrid restriction site *Nco*I/*Rca*I was expected to form in the resulting construct. Following digestion, AP treatment and ligations, *E. coli* MM294-4 cells were transformed using $CaCl_2$ method. Due to a lack of positive selection markers in pACYC184, screening for clones carrying inserts was a two step process. First, transformed cells were selected for Tet-R. Thereafter, transformants were screened for Cm-S by patching on LA-N supplemented with Tet only and LA-N supplemented with Tet/Cm. Of 54 clones screened, 3 were Cm-S. Plasmid DNA was prepared from these clones and digested with *Nco*I.

Lane 5: undigested plasmid
Lane 4: λIII molecular marker
Lanes 1-3: plasmid DNA from chloramphenicol sensitive clones digested with *Nco*I

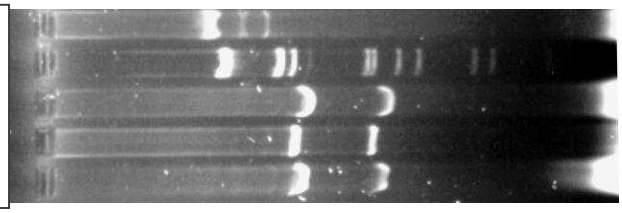


Figure 11. 0.8% agarose gel showing plasmid DNA from selected Cm-S clones digested with *Nco*I.

Upon digestion with *Nco*I the expected DNA construct should only produce one band migrating at 6.3kb. However, in all 3 instances, 2 DNA fragments were observed. One corresponded in size to the linearized pACYC184 and the other corresponded to the 2.0kb insert. Thus, it was concluded that the cloned insert was not one expected to carry the desired gene cluster. This led to the development of an alternative strategy to complement the Sm-Sup mutation.

3.11 Direct genomic PCR of wild-type *rplL*

In light of failure of previous efforts to complement the functional Sm-Sup mutation in Sm-D3, a novel approach was developed. Here, direct PCR, using wild type *E. coli* genome as the template, was used to amplify and clone the ORF of *rplL* into an appropriate plasmid vector. Several issues had to be considered prior to this. Since *rplL* is not transcribed off its own promoter, the cloned fragment had to be cloned into a vector with a suitable promoter such as the *lac* promoter. A high copy number plasmid vector pUC18 was chosen for this purpose. In addition, it was desirable to achieve low level expression of L7/L12 due to its toxicity in *E. coli.* To surmount this potential problem, the PCR product was inserted into the poly-linker of pUC18 such that start codon of *rplL* overlapped a stop codon from a residual *lac*Z gene. This was expected to provide a degree of translational down-regulation (Toivonen *et al.,* 1999).

Figure 12. *rplL* construct in the poly-linker of pUC18. An engineered stop codon is indicated with *. An overlapping, out of frame *rplL* start codon is underlined. Direction of transcription is shown with arrows. Amino acid residues are in blue and red for *lacZ* remnant and *rplL* respectively.

In order to clone the *rplL* ORF into the desired orientation and position in pUC18, several changes needed to be engineered into PCR oligos for amplification of *rplL*. These included restriction recognition sites for *Bam*HI and *Hind*III as well as an overlapping stop codon.

L7/Forward primer

```
    5′ CAGGATCCATTTTAATGTCTATCAC 3′
       ||||| | |||| ||||||||||||
TGATATTCAGGAACAATTTAAATGTCTATCACTAAAGATCAAATCATTGAAGCAGTTGCAGCTATGTCTGTAA
TGGACGTTGTAGAACTGATCTCTGCAATGGAAGAAAAATTCGGTGTTTCCGCTGCTGCTGCTGTAGCTGTAGC
TGCTGGCCCGGTTGAAGCTGCTGAAGAAAAAACTGAATTCGACGTAATTCTGAAAGCTGCTGGCGCTAACAAA
GTTGCTGTTATCAAAGCAGTACGTGGCGCAACTGGCCTGGGTCTGAAAGAAGCTAAAGACCTGGTAGAATCTG
CACCGGCTGCTCTGAAAGAAGGCGTGAGCAAAGACGACGCAGAAGCACTGAAAAAAGCTCTGGAAGAAGCTGG
CGCTGAAGTTGAAGTTAAATAAGCCAACCCTTCCGGTTGCAGCCTGAGAAAT
                  |||||||||| | |||||||
              3′ TTATTCGGTTCGAAAGGCCA 5′
```

L7/Reverse primer

Figure 13. *rplL* wild type sequence and PCR oligos used for amplification. Changes engineered into oligo primers are indicated in blue. Underlined sequences are recognition sites for restriction endonucleases, *Bam*HI in L7/Forward primer and *Hind*III L7/Reverse primer. Start and stop codons of the *rpl*L ORF are shown in red. Complementary alignment between oligos and *rplL* sequences are indicated with solid lines.

Direct colony PCR was performed with the designed oligo primers. *E. coli* NF915 was used as the source of wild type ribosomes. A PCR product of 395bp in length was expected.

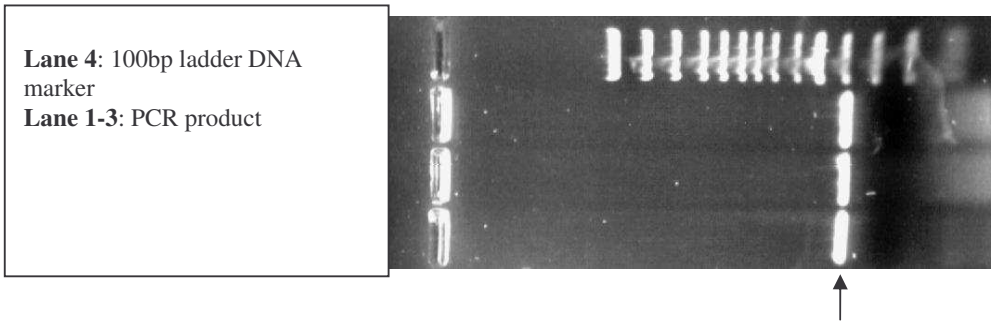


Figure 14. 1.0% agarose gel showing PCR product. Arrow indicates PCR product corresponding to ~400bp in length. Lanes 1 to 3 are standard PCR reaction mixtures with increasing concentration of $MgCl_2$ from 2.0mM to 2.8mM.

PCR product was phenol-chloroform extracted directly from PCR mixture and double digested with *Bam*HI and *Hind*III overnight. pUC18 plasmid vector was digested with the same restriction endonucleases and treated with AP overnight in the appropriate buffer. Phenol-chloroform extraction was then repeated for both the PCR product and pUC18 to prepare DNA for T4 ligation.

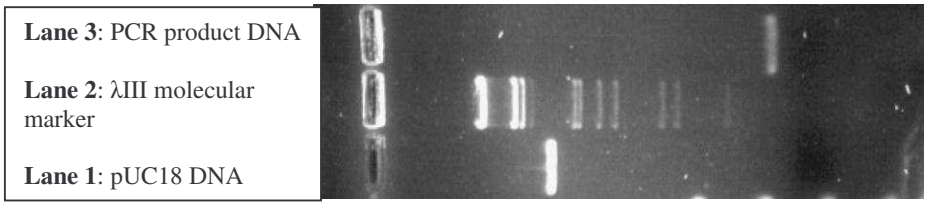


Figure 15. 1.0% agarose gel showing extracted PCR product and pUC18 DNA after *Bam*HI and *Hind*III double digestions.

PCR product and pUC18 DNA was ligated with T4 DNA ligase overnight. Ligations were used for transformation of competent *E. coli* XL-1 Blue cells. Selection for transformed cells was done on LA supplemented with Amp.

Table 17. Transformation of *E. coli* XL-1 Blue with DNA ligations

Ligation mixture	**Number of colonies on LA supplemented with Amp200/Nal 50 (μg/ml):**
No DNA control	0
pUC18 only	3
pUC18 and PCR product	41

5 randomly selected colonies were picked off the pUC18 and PCR product plate and used for small scale plasmid DNA preparation. Aliquots were double digested with *Bam*HI and *Hind*III overnight to confirm the presence of desired insert.

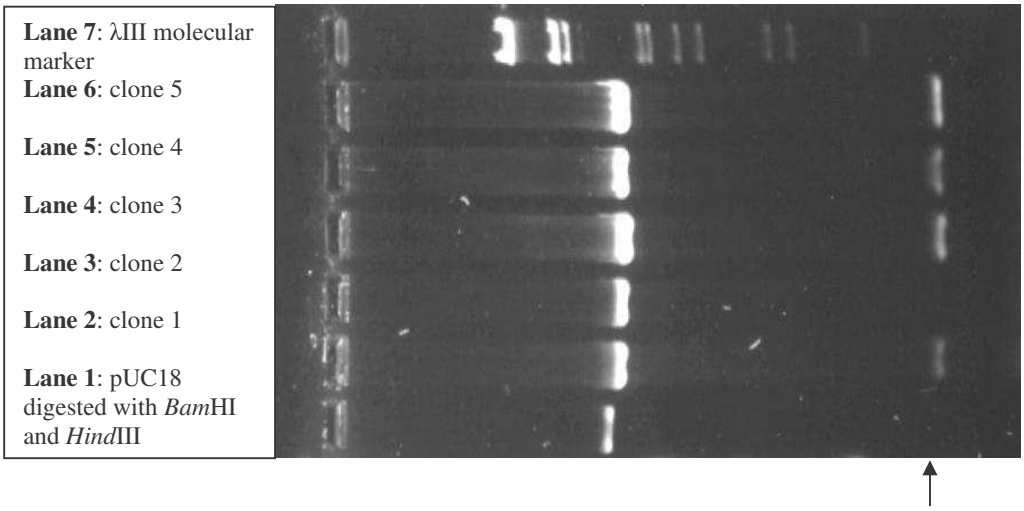


Figure 16. 1.0% agarose gel showing *Bam*HI and H*ind*III double digested DNA from selected clones. Arrow points to inserts of ~400bp in length released upon double digestions with *Bam*HI and *Hind*III from clones 1, 3, 4 and 5.

pUC18-L12 constructs 1, 4 and 5 were subsequently used to transform competent Sm-D3 cells using the $CaCl_2$ method. Transformants were selected on LA-N plates supplemented with Amp/Sm. pUC18 and no DNA controls were also included. In each case, more than a 1000 colonies appeared on each selection plate except for the no DNA control. 4 colonies were picked at random from each plate, including the pUC18 positive control and used for a spot-test to determine the Sm-D phenotype.

1. LA-N Amp200/ Sm5 **2**. LA-N Amp200/ Sm40

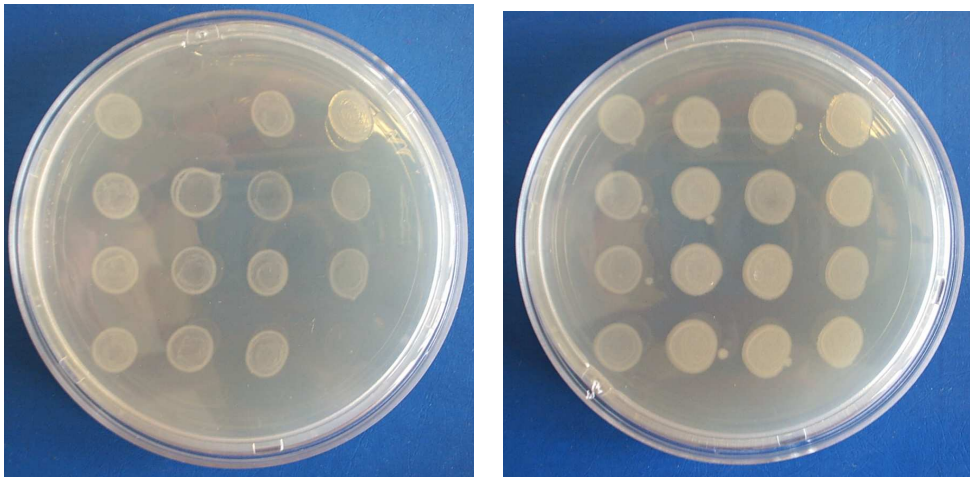


Figure 17. Spot-test to determine Sm-D phenotype of Sm-D3 transformed with constructs pUC18–L12 (1, 4 and 5). Spots are numbered from left to right. Spots 1, 5, 9, 13 are Sm-D3 clones transformed with pUC18 controls. Spots 2, 6, 10, 14 are Sm-D3 clones transformed with construct pUC18-L12(5). Spots 3, 7, 11, 15 are Sm-D3 clones transformed with pUC18-L12(4). Spots 4, 8, 12, 16 are Sm-D3 clones transformed with pUC18-L12(1). The spot-test was done in duplicate with and without the addition of 1.0mM IPTG, this had no effect on the observed phenotype. No growth was observed for any spots on the LA-N supplemented with Amp only plate (not shown).

Figure 17 indicates that two Sm-D3 clones, one carrying pUC18-L12(5) and the other, pUC18-L12(1) exhibited reduced growth on low concentration of Sm5 (μg/ml). It can be concluded that the construct pUC18-L12 was successful in complementing the Sm-Sup mutation. Thus, this mutation was placed within the *rplL* ORF. However, since not all transformed clones showed this phenotype, it was hypothesized that complementation was due to homologous recombination and not expression of the L7/L12 protein from the construct.

3.12 Testing level of expression of β-galactosidase system in the Sm-D3 mutant background

The available β-galactosidase reporter system is used as a measure of levels of gene expression. In addition it has been demonstrated that certain codons at position +2 downstream of the start codon have low efficiencies with regard to translation initiation (Stenström *et al.,* 2001). Using constructs developed by Stenström, (see Appendix E) it was possible to assay β-galactosidase expression efficiency in an Sm-D background.

Sm-D3 was transformed with a number of constructs corresponding to high and low efficiency codons at +2 position using the $CaCl_2$ method. Selection was carried out on LA supplemented with Amp/Sm. Single colonies were used to start cultures for the subsequent assay.

Table 18. The effect of selected +2 codons on translational efficiency in Sm-D3 as compared to wild-type background

Background	**Relative expression of codons tested at position +2**						
	AAA	AGA	AGG	CGA	CGC	CGG	CGU
w.t.	1.0	0.67	0.11	0.27	0.22	0.07	0.32
Sm-D3	0.51	0.60	0.08	0.12	0.13	0.03	0.17

Assay values are given as relative expression whereby 1.0 is equivalent of 234±7.6 (X 10^{-3}) β-galactosidase units. Each codon was sampled 4 times. The values for the wild-type (w.t.) background were as per Stenström *et al*., (2001).

The generated assay data indicated that the measured level of expression was generally lower in Sm-D3 as compared to wild-type. For codons CGA, CGC, CGG, CGU and AAA the level of expression was ~2-fold lower.

3.13 Testing selected +2 low and high efficiency codons in Sm-D mutants derived from NF915 and NF916 strains

Sm-D mutants derived from NF915 and NF916 parental strains were assayed using the β-galactosidase reporter system. Assayed codons were restricted to a high efficiency AAA (coding for lysine) and a low efficiency CGG (coding for arginine). Sm-D mutants 2, 4, 5, 6 and 7 were transformed with selected β-galactosidase reporter system constructs. Selection was done on LA plates supplemented with Amp/Sm.

Table 19. Expression of high and low efficiency codons in Sm-D mutants derived from NF915 and NF916

Background	**Relative expression of codons tested at position +2**	
	AAA	CGG
w.t.	1.0	0.07
Sm-D2	0.56	0.06
Sm-D4	0.53	0.05
Sm-D5	0.29	0.04
Sm-D6	0.42	0.04
Sm-D7	0.47	0.02

Each codon was sampled 4 times. The values for the wild-type (w.t.) background were as per Stenström *et al*., (2001).

Based on experimental evidence, it was concluded that all Sm-D mutants assayed exhibited a reduced level of expression regardless of the codon type tested at position +2 as compared to wild-type. This is in agreement with the previously observed decreased efficiency of translation associated with hyper-accuracy (Ruusala *et al.,* 1984).

4. Discussion

4.1 Isolation of Sm-D mutants in rRNA

With the recent development of an *E. coli* system that has a single rRNA operon located on a plasmid (Asai *et al.,* 1999), a vast gap in the understanding of the function of ribosomal RNA can be overcome. Previous efforts to characterize the structure-function relationships in rRNA have been based on visualization techniques. However, such an approach has limitations when one wishes to consider a closer picture of specific interactions within, and between ribosomal components. In addition, the contribution of research into antibiotics that target bacterial ribosomes has been very fruitful for furthering the understanding functional interactions in the ribosome. Thus it became possible, for the first time, to generate mutations in rRNA that confer antibiotic resistance or dependence in *E. coli*, and to characterize these mutations with respect to second site suppressors. Of particular interest was the development of antibiotic dependent mutants. This is because the isolation of second site suppressor mutations would be a technically simple procedure of direct selection.

In light of the above, a strategy was developed for the generation of Sm-D mutants in ribosomal RNA of *E. coli.* This involved the use of the previously described SQ170 strain deficient in all seven chromosomal rRNA operons. Mutants of this strain would be generated by high throughput chemical mutagenesis, and selected directly for the desired phenotype of streptomycin dependence. Furthermore, it was hypothetically possible to select for rRNA mutants by re-transformation of mutated plasmids into the original host strain, since only one plasmid-borne rRNA operon was present in this background.

Before the implementation of this strategy, it became immediately apparent that the presence of a Sp-R determinant in the SQ170 strain could affect further work with Sm since both of these antibiotics belong to the aminoglycosidic group. That is, one needed to avoid possible cross-reactivity with respect to antibiotic

resistance. To this extent, NTG mutagenesis was preformed to select for Sp-S clones. In retrospect this was not the ideal means of eliminating the Sp-R marker since this particular form of chemical mutagenesis is severely destructive. In addition, mutations generated by this method are localized to the DNA replication fork (Isaksson personal communication), thus a heterogeneous pool of plasmids would be generated in each clone. Despite of this, a number of candidate Sp-S clones were identified through counter-selection. The clone that showed the cleanest phenotype with respect to reversion and streptomycin cross-reactivity was selected and termed YB101.

This clone was subsequently subjected to a second round of chemical mutagenesis to select for Sm-D mutants. EMS was employed for this purpose. The rational for using this agent was the fact that predominantly transition type mutations are obtained. The reasoning was that subtle mutations in rRNA are required to achieve the desired phenotype. A large number of Sm-D mutants were generated in this manner. To distinguish between classical ribosomal protein mutants that give rise to streptomycin dependence (S12), and those that were plasmid-borne, an obvious screening involving re-transformation of isolated mutant plasmid DNA into the original host strain followed. Despite the recurrent problems of low transformation efficacy, it became clear that none of the isolated Sm-D clones from the SQ170 parental strain gave rise to a selectable Sm-D phenotype upon re-transformation.

Several testable hypotheses were put forward to explain the lack of transformability of the Sm-D phenotype. Initially, it was thought that direct selection of streptomycin dependence would be possible. In this scenario, one was able to simultaneously select for Amp and Sm, driving the selection of transcription in favor of the incoming plasmid in the host strain. Thus, it was assumed that the lack of transformability of Sm-D from incoming mutated plasmids reflects the fact that none of the obtained Sm-D mutants carried a plasmid-borne mutation. Alternatively, and perhaps unlikely, all of the Sm-D mutants carried at least two mutations responsible for the observed phenotype.

One chromosomal (presumably S12) and a second, in the plasmid encoded rRNA. This hypothesis was tested by a marker rescue experiment whereby the host *rplL* was replaced with a wild-type through P1 mediated transduction. The results seem to indicate that the tested Sm-D strains indeed carry chromosomal Sm-D or Sm-R mutation. The distinction between these two was unclear since direct marker rescue was not successful (on Tet only). Co-transduction frequencies were determined by patching out colonies from the plate containing both Tet and Sm, suggesting that these clones were still Sm-D. However, patching revealed that more than 76% of transductants were able to grow on Tet only (although considerably slower). Thus, the question of plasmid-borne Sm-D or Sm-R transformability was still unresolved.

To shed light on the seemingly ambiguous result obtained with marker rescue experiments, it was desirable to transform mutant plasmids obtained from selected SQ170 derived Sm-D mutants into a host strain with a different selectable marker. This became available in the form of D7, a strain derived from SQ170 but harboring a chloramphenicol determinant on the rRNA plasmid (pTS1192U). Literature indicates that when transforming mutated plasmids into the original host strain, a selection condition has to be in favor of the incoming plasmid in order to displace the resident plasmid (Vila-Sanjurjo and Dahlberg, 2001). Thus, it was possible to select simultaneously for Amp and Sm, in hope that this will drive the selection of transcription for the incoming plasmid and displace the resident one.

The results of this experiment showed that, in addition to the previously encountered problem of low transformation efficacy, neither the Sm-D nor the Sm-R phenotype were transformable. A conclusion can be drawn from this. Either there was a failure to obtain any type of functional Sm-D mutants within rRNA, or there is an inherit problem of selection of such plasmid-borne mutants in a new host. A possible reason for this has been addressed by Vila-Sanjurjo *et al.,* (1999). It was noted that when an incoming plasmid rendered the ribosomes non-functional, the incoming and the resident plasmids would coexist in the same

cells. A similar condition could have arisen in the above case. Here, the incoming plasmid carrying an Sm-D mutation would not be able to displace the resident wild type due to the decreased selection pressure associated with the reduced growth rate of a severely restrictive hyper-accurate phenotype. This would result in a heterogeneous population of ribosomes in the host.

Interestingly, it has been reported by Frattali *et al.,* (1990) that a 16S rRNA mutation U912 can result in a low level Sm-R phenotype, but only with serial sub-culturing on increasing levels of Sm. Despite the fact that these experiments were done in MM294 (which has all seven chromosomal rRNA operons) a parallel could be drawn with respect to the above. Such sub-culturing could progressively select for the incoming plasmid while retarding transcription of the resident rRNA. In light of this, it would be of interest to further examine and characterize YB102 transformants carrying mutant plasmid DNA from YB101(6), YB101(9) and YB101(pool). Serial sub-culturing of these clones on Amp/Sm could drive selection in favor of the incoming plasmids.

To give further insight into the mechanism of rRNA encoded Sm-D mutations and associated problems of selection, it was decided to construct a mutant pKK3535 plasmid with a cytosine insertion in the highly conserved 530 stem-loop. Such a mutation has been previously characterized and found to be associated with high level of streptomycin dependence in *M. tuberculosis* (Honore *et al.,* 1995). In light of the high degree of sequence identity in the 530 loop, it was reasoned that an analogous mutation in the *E. coli* strain with a single rRNA operon would produce a similar Sm-D phenotype. Following site-directed mutagenesis, the mutant plasmid was transformed into the host D7. Selection for Sm-D or Sm-R failed to produce any results. A number of possible reasons exist to explain this; (1), the problems associated with displacement of the resident plasmid previously discussed could have hindered selection of the desired phenotype; (2), in spite of the high degree of conservation within the 530 stem-loop, the described mutation in *M. tuberculosis* may not have the same phenotype in *E. coli*; (3), least likely, there was experimental failure in the mutagenesis of pKK3535, thus the tested

clones were simply wild-type. This could be verified by sequencing. Due to time constraints, further work with this mutation was limited.

It can be added that assuming mutagenesis was successful, and the mutant plasmid has indeed displaced the resident one, the effects of a cytosine insertion should be investigated on a broader scale. This would entail studies with growth rates, efficiency of translation and conditional lethality.

Future work with ribosomal RNA mutations associated with antibiotic resistance or dependence should primarily focus on the development of a stable system in *E. coli* whereby more than one selectable marker can be employed to displace resident plasmids and drive transcription in favor of the incoming one. In addition, more than one type mutagenesis should be used when generating plasmid-borne rRNA mutants. Alternatives to chemical mutagens could include the use of mutator strains. This will provide for an increased diversity of mutation types such as insertions and deletions. Finally, one can adopt a different approach to the issue of functional interactions between rRNA and ribosomal proteins. Here, a pool of mutagenized rRNA plasmids can be used to rescue conditional lethal mutants harboring known mutations in ribosomal proteins. This methodology can potentially bypass plasmid displacement problems.

4.2 Isolation and characterization of Sm-D mutants derived from *E. coli* strains NF915 and NF916

The classical investigation of ribosomal protein mutants and related antibiotic resistances has yielded a great deal of insight into the various functional interactions in the ribosome. This approach is technically accessible and well documented.

A number of Sm-D mutants were derived from *E. coli* strains NF915 and NF916 through chemical mutagenesis and direct selection. Mutants that were useful for

further work were selected based on phenotypic differences such as colony morphology and level of dependence to Sm. It was assumed that any Sm-D phenotype was the result of mutations in S12. Of interest was to get second site suppressor mutations that completely reversed Sm-D to Sm-I. The initial strategy to obtain second site suppressors involved a simple marker rescue experiment whereby Sm-D mutants carrying a Tn10 near a locus of interest were selected for independence to Sm. Revertants were used in P1 mediated transduction experiments to rescue Sm-D mutants with simultaneous selection on Tet. In this way, a number of loci were tested.

The first subject of interest was the locus *prfB* coding for RF-2. Cryo-electron microscopy indicates that the RF-2 termination complex interacts directly with the decoding center on the ribosome. Contacts include helix 18 and 44 of 16S rRNA as well as S12 (Klaholz *et al.,* 2003). Additionally, another protein factor, EF-Tu has been found to interact with the decoding center and reverse streptomycin dependence (Zuurmond *et al.,* 1998). Based on this, it was reasoned that such close association with the decoding center could have implications on the binding of streptomycin to Sm-D ribosomes. Sm-D mutants derived from NF915 were screened in the manner described above for second site suppressor mutations near the *prfB* locus. It is worth noting that Sm-D mutants derived from NF916 were less amendable to Sm-D reversion when selected for Sm-I. This could be a function of the genetic difference between NF915 and NF916.

Results of screening experiments suggested that no second site suppressors were present near *prfB*. A number of possibilities exist to explain this. First, even though the technical approach was sound, simply not enough spontaneous revertants were pooled. This can be considered if the frequency of hypothetical second site suppressors of Sm-D in RF-2 is extremely low. Second, literature indicated that S12 mutations that result in Sm-D phenotype are hyper-accurate, compensating for the error-prone effects of streptomycin. In addition, second site suppressors that result in Sm-I are typically involved in tRNA selection and proofreading associated with 912 region of 16S rRNA (Lodmell and Dahlberg,

1997). It can be argued that since RF-2 does not directly affect tRNA selection during initial proof-reading, but has direct contacts to S12, the only likely mechanism of Sm-D suppression would be distortion of S12. Such distortion might not be accessible to RF-2 since this requires specific interactions.

To resolve this matter, one can consider a more direct approach of selective mutagenesis of *prfB* through site-directed mutagenesis or gene replacement (Zeef and Bosch, 1993). In this way, RF-2 mutants can be preferentially selected and screened using previously described techniques. Interestingly it has been found that Sm-D5 mutant exhibited a decreased requirement for Sm when rescued by a Tn10 near *prfA* (Dabbs personal communication). This might reflect the possibility of isolating RF mutants in *E. coli* that can reverse the Sm-D phenotype.

The second genetic locus of interest examined for possible second site suppressor mutations was *rpoB*. This region was of particular interest since several large ribosomal subunit proteins are located in this region. These include L1, L10, L11, L7/L12 as well as protein factor EF-Tu. In light of the previously reported isolation and characterization of a Sm-D suppressor in L7/L12 (Nomura *et al.,* 2003), the *rpoB* gene cluster presented a good starting point for the search of other Sm-D suppressors.

To isolate Sm-D suppressors near *rpoB,* a similar marker rescue methodology was employed as described above. Following transduction of Sm-D3 with Φ18500rifR, it was noted that two distinct phenotypic classes were present. Thus, it appeared that a mutation in the *rpoB* gene cluster reduced the requirement to streptomycin of Sm-D3. The mutation was roughly mapped to be in L7/L12 or more distal L10. As a matter of interest, a question was posed whether the characterized L7/L12 suppressor of Sm-D from LL103 also was functional in Sm-D3 background. Although there appeared to be a slight decrease in streptomycin requirement, the LL103 derived L7/L12 failed to reverse Sm-D phenotype of Sm-D3. Whether the observed decrease in dependence was significant, or a result of variations in experimental conditions, this was not explored any further.

Efforts to fine-map the Sm-Sup mutation near *rpoB* began by focusing on the transducing phage *λrif^{d}18*. Literature indicates that this phage can be usefull in the construction of plasmids harboring genes near *rpoB* (Bernardi and Bernardi, 1979). However, in the processes of sub-cloning *rplL* technical difficulties were encountered. It was found, through restriction analysis and cloning, that DNA isolated from *λrif^{d}18* was not that expected when compared to previously published data. This was probably reflective of the general difficulties in the process of isolation of *λrif^{d}18* from NF910 and subsequent DNA purification from this phage (Pica and Calef, 1968; Lindahl *et al.,* 1977).

As a more direct approach, complementation of Sm-Sup was achieved by cloning a PCR fragment of wild-type *rplL* into pUC18 cloning vector. Initially, it was desirable to obtain a moderate level of expression from the cloned *rplL* gene. To this extent, several criteria were fulfilled during cloning, including placing an overlapping stop codon near the *rplL* AUG as per Toivonen *et al.,* (1999). However, it was noted that not all Sm-D3 clones transformed with the construct pUC18-L12 were able to complement the Sm-Sup mutation even under IPTG inducing conditions. This was explained as follows: since Sm-D3 is not recombination deficient, complementation of Sm-Sup was due to recombination of plasmid-borne wild type *rplL* and the chromosomal mutant, and not due to expression of L7/L12 from construct (Faustoferri *et al.,* 1998; Ryden *et al.,* 1986). This hypothesis can be tested by constructing a Rec minus derivative of Sm-D3 through P1 mediated transduction, followed by screening for Sm-Sup complementation (Dabbs personal communication). In addition, expression of L7/L12 from the pUC18-L12 construct can be monitored with optimized IPTG induction conditions on SDS-PAGE gels.

Complementation experiments confirmed the location of Sm-Sup mutation in L7/L12. This was not surprising since a number of L7/L12 mutants have been isolated that suppress Sm-D phenotypes and the involvement of L7/L12 in translational accuracy has been documented (Kirsebom and Isaksson, 1985). This is in accordance with the currently accepted model of the role of S12 in the

decoding centre of the ribosome. It can be hypothesized that a hyper-accurate mutation in S12 resulting in reduced translational efficiency is "balanced out" by the presence of a L7/L12 mutation that leads to increased misreading. An analogous condition has been identified by Kirsebom and Isaksson, (1985).

Finally, one can speculate that mutations that counteract hyper-accuracy associated with Sm-D phenotypes have the selective advantage of increased growth rate due to increased translation efficiency.

4.3 Testing translation initiation efficiency in Sm-D3

To answer the question, whether Sm-D3 mutant has increased translation efficiency due to a compensatory L7/L12 mutation, this mutant was assayed with the β-galactosidase reporter system using constructs developed by Stenström *et al*., (2001). Since these constructs have a well established expression profile in wild-type *E. coli,* it was possible to compare translation initiation efficiencies in the Sm-D3 background. The existence of mRNA codons downstream of the AUG that give poor gene expression (Gonzalez de Valdivia and Isaksson, 2004), prompted a search for ribosomal mutants that can enhance translation initiation efficiencies of these codons. The current hypothesis to explain poor gene expression of such codons is associated with peptidyl-tRNA drop-off from the A-site before translocation due to an increased affinity of tRNA anti-codons at these sites (Gonzalez de Valdivia personal communication). This suggests that such drop-off occurs after initial tRNA selection and proof-reading mediated by 30S domain closure but before translocation. In this case, one would expect to find that neither Sm-D hyper-accurate, nor *ram* error-prone ribosomes affect these codons since the drop-off event is downstream of tRNA selection and proofreading.

The result of β-galactosidase assay using both high and low efficiency codons indicated that there was generally a decreased level of gene expression regardless of the type of codon tested. This was true not only for Sm-D3 but also for all

NF915 and NF916 derived Sm-D mutants. A number of conclusions can be made: (1), observations were in accordance to the well documented decrease in gene expression of Sm-D ribosomes associated with hyper-accuracy; (2), the hypothesis proposed to explain low efficiency codons associated with peptidyl-tRNA drop-off is supported by the obtained results since Sm-D ribosomes were not expected to affect reactions downstream of 30S subunit closure; (3), it can be said that the level of gene expression tested with specific codons downstream of AUG in Sm-D3 background was comparable to that observed with other Sm-D mutants. This indicates that the L7/L12 Sm-Sup mutation does not play a significant role in efficiency of translation in Sm-D3 in relation to other Sm-D mutants. However, in order to say anything about the absolute effect of Sm-Sup in Sm-D3, one has to compare both Sm-D3 and Sm-D3 Sm-Sup minus gene expression levels. Due to time constraints this was not assayed.

Future studies must deal with sequencing of the Sm-Sup mutation in L7/L12. This mutation is of interest since it does not completely reverse Sm-D to Sm-I. Importantly, the L7/L12 mutant needs to be characterized biochemically. This would shed more light on this flexible ribosomal protein whose precise role is not fully understood. In addition, ribosomal mutants that rescue poor efficiency codons should be identified. This would assist the elucidation of the nature of poor gene expression associated with certain codons, and have practical applications.

5. Conclusions

In the process of selection of Sm-D mutants in rRNA, it became evident that the proposed approach is potentially useful for identifying and characterizing specific rRNA loci that have important functions in the ribosome. Additionally a number of specific technical issues were identified that can be addressed in future studies.

Classical experiments involving generation of antibiotic resistance mutants in ribosomes, and second site suppressors, have confirmed this approach as fruitful and instrumental in elucidating the complex structure-function relations in ribosomal components.

Appendix A: Media and Solutions

Media

LB (Luria Bertani broth)

1.0g tryptone

0.5g yeast extract

0.5g NaCl

Distilled water to 100ml

LA (Luria Bertani agar)

1.0g tryptone

0.5g yeast extract

0.5g NaCl

1.5g technical agar

Distilled water to 100ml

LA (A-N)

1g tryptone

0.5g yeast extract

1.5g technical agar

10ml 10 X (A-N) stock

Distilled water to 100ml

LC plates

As LA + 10mM $CaCl_2$

½ Agar (sloppy agar)

As LA except 0.75g technical agar

10 X A-N buffer

91.7g $K_2HPO_4.3H_2O$

26.8g KH_2PO_4

5.0g Na_3(citrate)

1.0g $MgSO_4$

Distilled water to 1000ml

Plasmid Preparation Solutions

Solution 1

50mM glucose

25mM Tris-HCl pH 8.0

10mM EDTA pH 8.0

Solution 2

0.2M NaOH

1.0% SDS

Solution 3

60ml 5M CH_3COOK solution

11.5ml CH_3COOH

28.5ml distilled water

pH 4.8

Solutions Used for Agarose Gel Electrophoresis

5 X TBE

54.0g Tris base

27.5g H_3BO_3

20ml 0.5 EDTA pH 8.0

Distilled water to 1000ml

Agarose gel stock solutions

0.8g, 1.6g or 2.0g agarose

20ml 5 X TBE

Distilled water to 200ml

Running Buffer

0.5 X TBE

0.1µg/ml EtBr

Molecular Weight Markers

5µl molecular weight marker

10µl buffer H

85µl sterile water

20µl tracking dye

***E. coli* Transformation Solutions**

Transformation Buffer

100mM $CaCl_2$

10mM Tris-HCl pH 7.5

λ phage preparation solutions

SM buffer

100mM NaCl

1mM $MgSO_4$

20mM Tris-HCl (pH7.5)

0.01% gelatin

λ dialysis buffer

10mM NaCl

50mM Tris-HCl (pH 8.0)

10mM $MgCl_2$

β-Galactosidase assay

Z buffer

Per liter:

16.1g $Na_2HPO_4 \cdot 7H_2O$

5.5g $NaH_2PO_4 \cdot H_2O$

0.75g KCl

0.246g $MgSO_4 \cdot 7H_20$

2.7ml β-mercaptoethanol

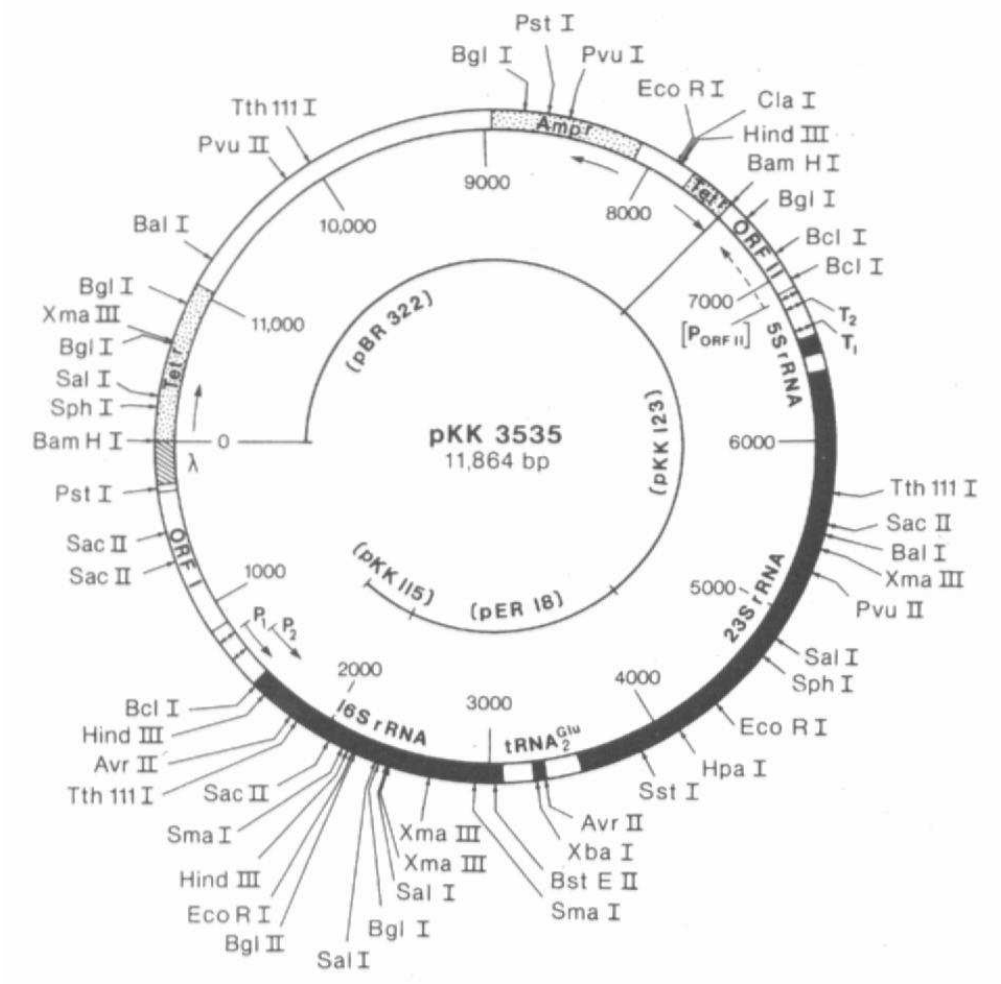
Do not autoclave

Adjust pH to 7.0

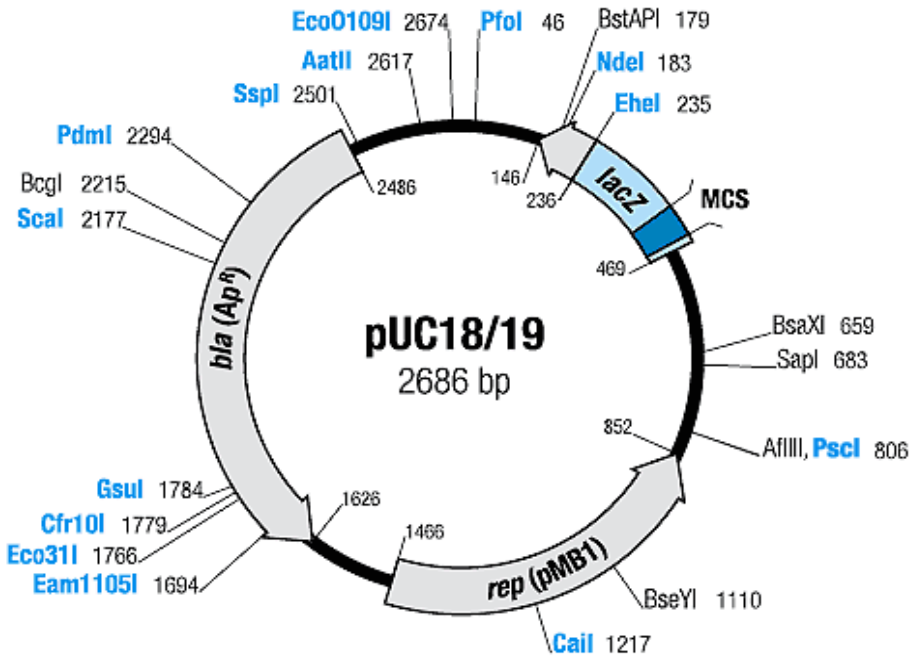
Stock Solutions of Antibiotics

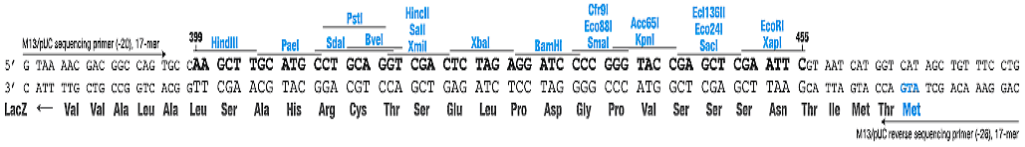
Antibiotic	Concentration	Solvent
Ampicillin	100mg/ml	7:3 ethanol:water
Chloramphenicol	10mg/ml	ethanol
Nalidixic acid	10mg/ml	ethanol
Rifampicin	10mg/ml	methanol
Spectinomycin	10mg/ml	water
Streptomycin	100mg/ml	water
Tetracycline	10mg/ml	methanol

Appendix B: restriction map of pKK3535

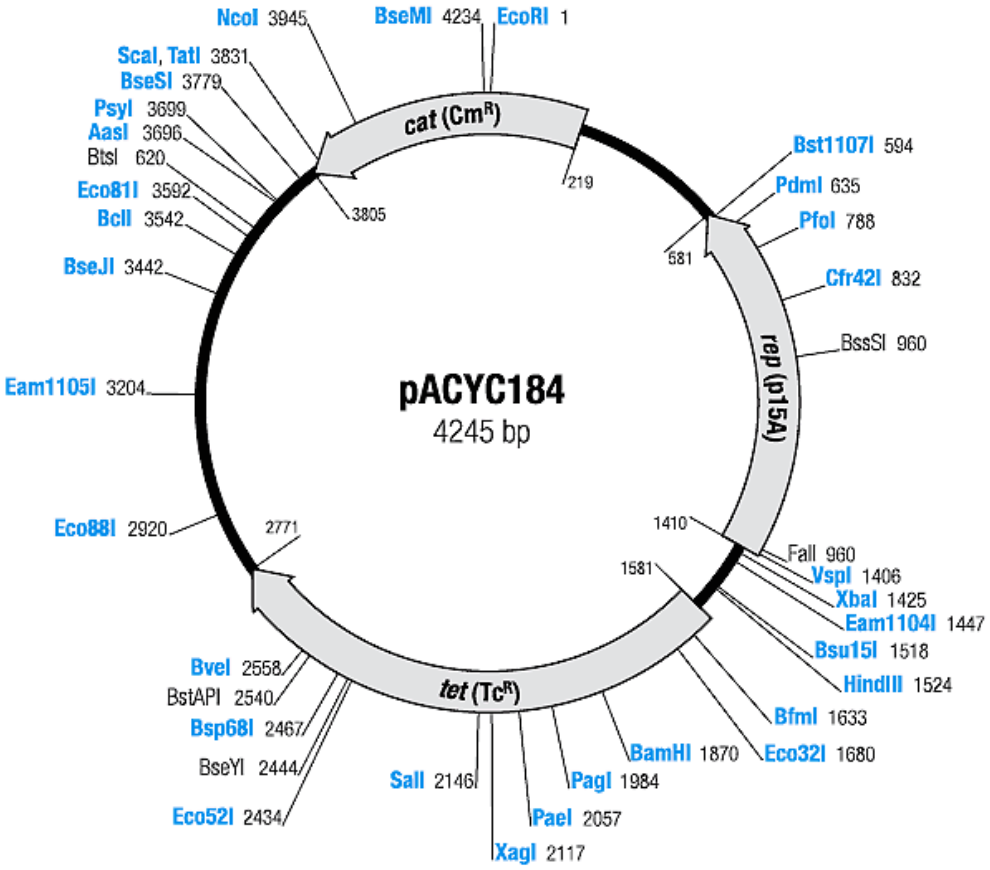


Appendix C: restriction map of pUC18 and multiple cloning site (MCS)

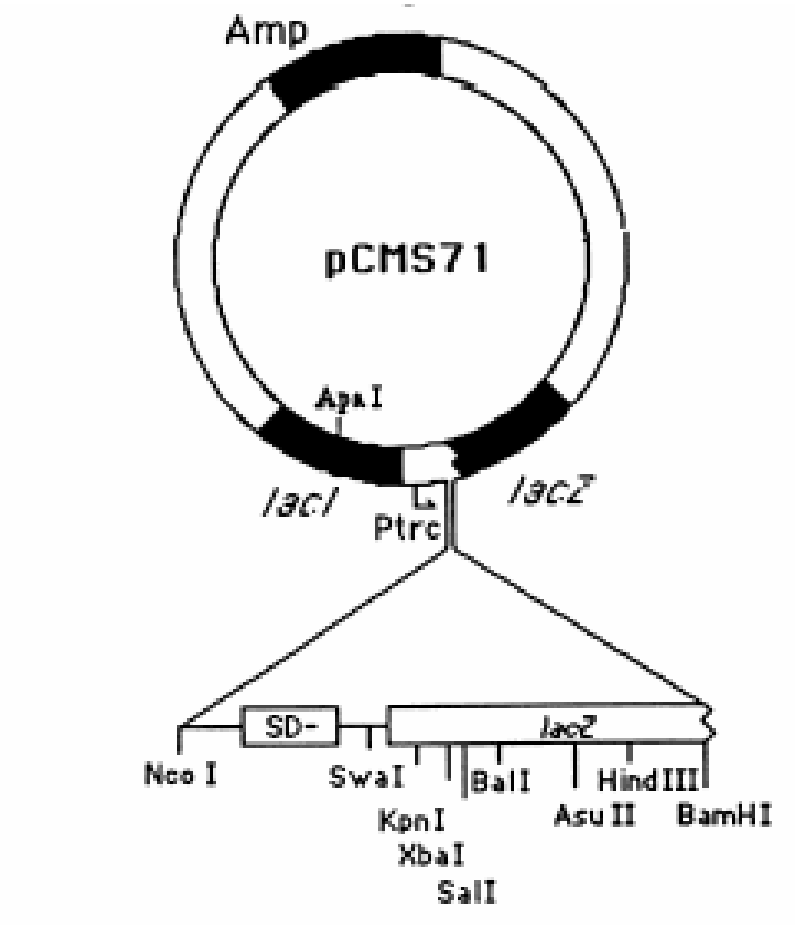




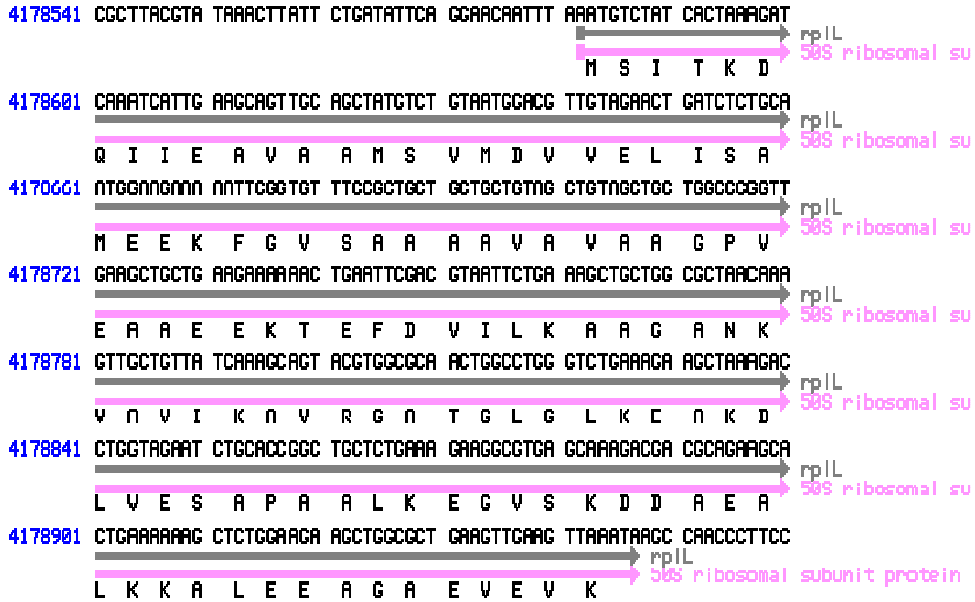
Appendix D: restriction map of pACYC184



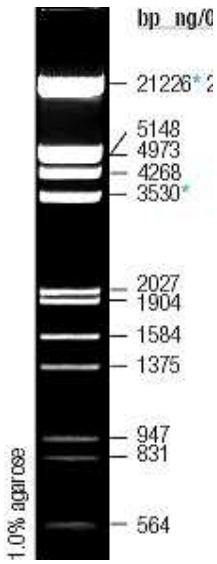
Appendix E: restriction map of pCMS71



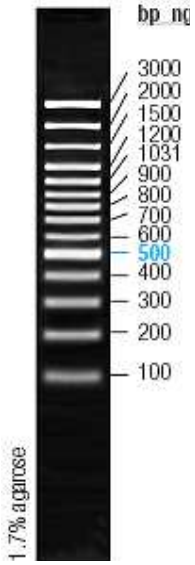
Appendix F: open reading frame (ORF) of *rplL*



Appendix G: Molecular weight DNA markers



λIII molecular marker



GeneRuler 100bp DNA ladder

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