CHAPTER 4

Multicopy *relA* and *rumA* as conferring low level of multiple antibiotic tolerance in *Escherichia coli*.

"There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria can not react by in some way acquiring 'fastness'(resistance." - Sir Alexander Fleming, 1946

4.1 Introduction

Intrinsic resistance, natural resistance to antimicrobial agents possessed by majority population of the bacterial species to antibiotics, is a collective function of permeability of bacterial cell wall and efflux activities of different pump proteins in the cell (Nikaido, 2001, Piddock, 2006). In Gram-negative bacteria, Lipopolysaccharides (LPS) present in the outer membrane act as major permeability barrier. A second barrier effect is exerted by several types of efflux proteins. Additionally, in *E. coli*, function of the outer membrane porin proteins has been implicated in barrier effect of the outer membrane (Webber & Piddock, 2003). In the Gram-positive bacteria, the LPS protection is absent and the intrinsic resistance is mostly physiological (Wright, 2007). Alterations in the fatty acid composition (Guerin-Mechin *et al.*, 1999), phospholipids (Rodionov & Ishiguro, 1995), growth phase of the cells (Kolter *et al.*, 1993, Viducic *et al.*, 2006), and certain genetic mutations either sensitizes or elevates innate resistance of the organism (Girgis *et al.*, 2009, Hu & Coates, 2005, Tamae *et al.*, 2008). Our understanding of the innate resistance determinants is beginning to grow with other bacterial systems being studied (Schurek *et al.*, 2008, Breidenstein *et al.*, 2008, Fajardo *et al.*, 2008, Gomez & Neyfakh, 2006, Alvarez-Ortega *et al.*).

We describe here requirement of the pair of genes, *relA rumA* for a new phenotype - elevated tolerance to multiple antibiotics. The two genes relation to antibiotic resistance is not farfetched. RelA catalyses synthesis of (p)ppGpp from ATP and GTP in response to nutrient starvation and certain other stresses; (Potrykus & Cashel, 2008, Jain *et al.*, 2006). Null mutation/overexpression of *relA* is associated with altered antibiotic resistance/tolerance (Greenway & England, 1999b), (Rodionov & Ishiguro, 1995, Viducic *et al.*, 2006, Wu *et al.*, Hu & Coates, 2005). The other gene, *rumA*, encodes U1939 23S rRNA methyltransferase (MeT), and one of the two uracil methyltransferases in *E. coli*; the second being *rumB* (which codes for U747 23S rRNA

methyltransferase). The two U residues in 23S rRNA are highly conserved. RumA unlike many other MeTs (Long, 2009), has been found to be unimportant for antibiotic resistance (Persaud et al, 2010). Essentiality of *rumA* has earlier been addressed, though the deletion mutation encompasses *rumA relA* genes (Atherly, 1979, Persaud *et al.*, 2010).

In this chapter, our study was focused on understanding the mechanism of multicopy expression of two genes imparting multiple antibiotic tolerance phenotype. As described below, the clone containing the two genes was independently isolated on more than one occasion, from independent *E. coli* genomic libraries and invariably contained complete *rumA* gene. Given that methyl transferases involved in modification of rRNA and tRNA have been shown to second function (Gutgsell *et al.*, 2001), and that multiple copy expression of a gene product reveals its latent activities not otherwise obvious in the single copy state (Patrick *et al.*, 2007), we worked on the assumption that *rumA* in multiple copy is responsible for the low level antibiotic tolerance.

4.2 Materials and methods

4.2.1 Growth Conditions

Bacterial cells were normally grown in LB broth with shaking and in LA plates at 37°C. When necessary, media were supplemented with kanamycin (50µg/ml), ampicillin (50-100µg/ml). DNA manipulations were carried out according to protocols described in (Sambrook, 1989).

4.2.2 Bacterial Strains and Plasmids

Strains and plasmids used in this chapter are listed in Table 4.1

Table 4. 1: *Escherichia coli* strains/Plasmids used in this study

***E. coli* strains/Plasmids**	**Relative Genotype/Description**	**Reference**
DH5α	*supE44 hsdR17 recA1 endA1gyrA96 thi-1*Δ(*lacIZYA argF*) *U169 relA1*?	lab collection
MC4100 KP	*F⁻ araD139 (argF-lac)U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5301 rbsR*	lab collection
pBlueScriptKS	Cloning Vector Amp^r ColE1 replicon	Stratagene,USA
pBAD18Kan	Cloning Vector Kan^r ColE1 replicon	(Guzman *et al.*, 1995)
pBBR1MCS2	Cloning vector Kan^r p15A replicon	(Kovach *et al.*, 1995)

pTE1	pBBR1MCS2 containing 7164 bp of *BamH*I genomic insert bearing full length *relA*, *rumA*, *barA*, and *gudD*	This study
pTE1ΔBgl	Removal of 310 bp of *Bgl*II DNA from pTE1 by digestion by RE *Bgl*II and intramolecular ligation.	This study
pKC1	Spontaneous *relA*$^-$ mutant of pTE1 plasmid	This study
pTE2	pBBR1MCS2 plasmid containing *rumA* (1301 bp) + 586 bp upstream DNA and 1341 bp long *relA* DNA.	This study
pTE3	pBBR1MCS2 plasmid containing *rumA* (1301 bp) + 516 bp upstream DNA and 1368 bp long *relA* DNA.	This study
pTE4	Full length *relA* gene (2289 bp) cloned at *EcoR*I - *Kpn*I sites of pBAD18Kan.	This study
pTE1ΔAse	pTE1 derivative containing *relA* gene and upstream *relA*P1 & *relA*P2 promoters.	This study
pTE5	1529 bp *rumA* amplicon + 178 bp upstream *barA* DNA cloned at *EcoR*I – *Sac*I-*BamH*I of pBBR1MCS2.	This study
pTE6	3794 bp long contiguous segment bearing *relA* and *rumA* genes cloned at *EcoR*I - *Kpn*I fragment in pBAD18Kan.	This study
pTE7	2907 bp long contiguous segment bearing *relA* (NTD) and *rumA* genes cloned at *EcoR*I - *Pst*I fragment in pBAD18Kan.	This study
pTE8	3794 bp G251E mutant *relA* and *rumA* genes cloned in *EcoR*I-*Kpn*I of pBAD18Kan.	This study
pTE9	3794 bp C389P mutant *rumA* and *relA* genes cloned at *EcoR*I - *Kpn*I of pBAD18Kan.	This study

relA and *rumA* genes were each PCR amplified from pTE1 using the primers listed in Table 4.2.

Table 4. 2: List of Primers used in this work

Primer Name	**Primer sequence 5'→3'**	**Tm of primers (°C)**
RumAF1	TTA*GAATTCGGATCC*AGTTGACGCTGCA	65.1
RumAF3	AAT*GGATCC*TGCTGCGTAGTGGGAA	64.6
RumARPst1	AAT*CTGCAG*GACCAGACCTGCCGAA	66.3
RumARSac1	AAT*GAGCTCGGATCC*GACCAGACCTGCCGAA	72.2
RelAF	CTG*GAATTC*GCAGGTCTGGTCCCTA	66.3
RelAR	CGT*GGTACC*GAGCAAATTTCGGCCTA	66.4
C389ASDM1	TATCCCCGAACCCTGCAACGTTGGCTC	69.5
C389ASDM2	CAACGTTGCAGGGTTCGGGGATACATAAAC	68.1
G251E-5'[a]	GCGGAAGTGTATGAGCGTCCGAAACACATC	63.9
G251E-3'[a]	GATGTGTTTCGGACGCTCATACACTTCCGC	63.9

a (Gropp *et al.*, 2001)

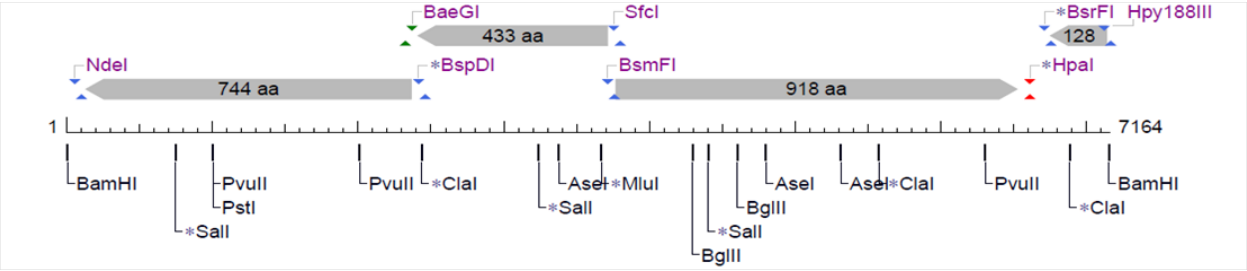


Figure 4. 1: Restriction map of pTE1 plasmid

4.2.3 Construction of plasmids used in this study

(i) Deletion of 310 bp of pTE1 DNA between the two *Bgl*II restriction sites present 588 and 898 bp upstream of *rumA* and into the coding region of *barA* generated 6854 bp plasmid pTE1ΔBgl.

(ii) 2289 bp *relA* gene amplicon generated by RelAF- RelAR primers was cloned at *EcoR*I - *Kpn*I of vector pBAD18Kan (pTE4).

(iii) pTE1ΔAse clone was generated by removal of AseI fragment, which retains, as a result, *relA* gene and upstream promoter *relA*P1 & *relA*P2.

(iv) 1529 bp *rumA* amplicon generated using RumAF1-RumARSacI primers was cloned in to the broad host range vector pBBR1MCS2 (Kovach *et al.*, 1995) at *EcoR*I-*Sac*I (pTE5) sites.

(v) 3794 bp *rumA+relA* amplicon generated using RumAF1 and RelAF primers was cloned at *EcoR*I - *Kpn*I site of pBAD18Kan to generate pTE6.

(vi) 2907 bp *rumA+relA* amplicon generated using RumAF1 and RelAF primers was cloned at *EcoR*I - *Pst*I site of pBAD18Kan to generate pTE7.

4.2.4 Site directed mutagenesis of *relA*

The mutagenic primer pair used for introduction of Gly251Glu change is already described (Gropp *et al.*, 2001). The primer pairs G251E-3'-RumAF1 and G251E-5'- RelAR were used in two separate PCR reactions in the presence of pTE1 template to produce respectively 2298 bp and 1516 bp long fragments. Each of the two DNAs were mixed, assembled and amplified in PCR to produce 3794 bp amplicon using RumAF1 and RelAR (Figure 4.5). The mutant product was cloned in pBAD18Kan plasmid at *EcoR*I – *Kpn*I sites to give rise to plasmid pTE8. In this

cloning, the effect of catalytic mutation in *relA* gene was tested in the context with wild type *rumA* gene for antibiotic resistance.

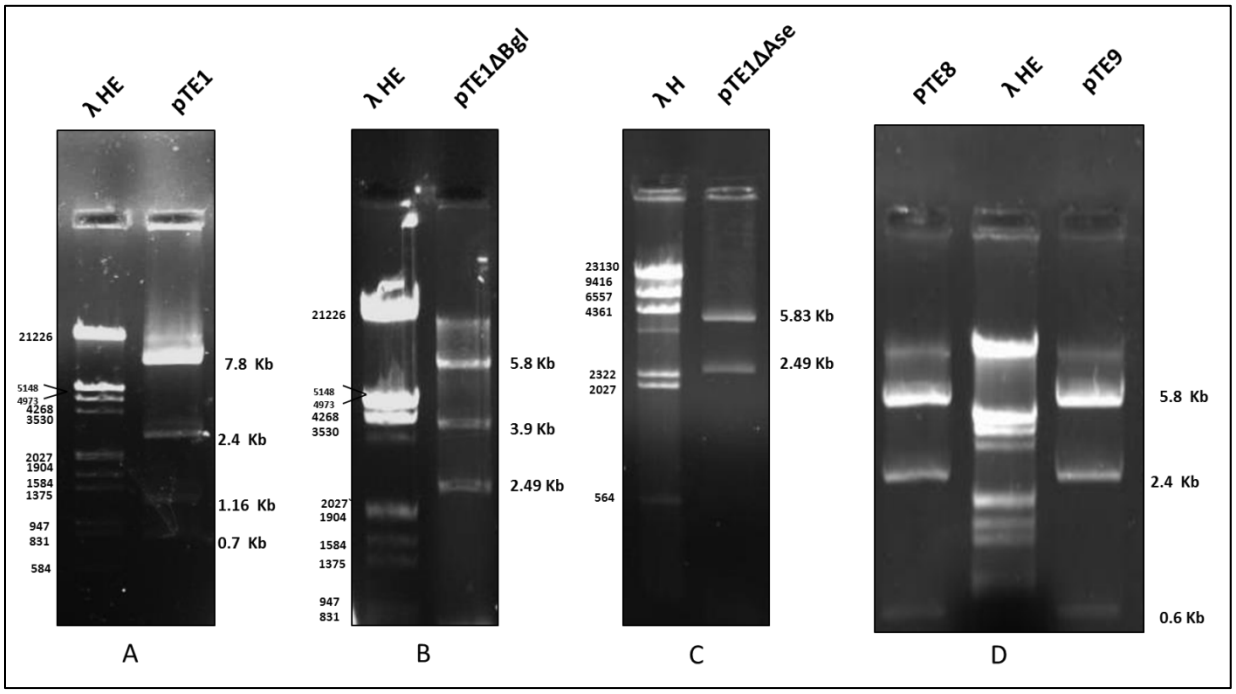
4.2.5 Site directed mutagenesis of Cys389 in the catalytic domain of *rumA*

Cys389pro mutation was incorporated in the *rumA* DNA essentially by the same method as that used for obtaining mutant *relA* gene.

Mutagenic primers C389P-SDM1 and RelAR generates 2452 bp long fragment, whereas C389P-SDM2 and RumAF1 produces 1374 bp amplicon. Each of the products was mixed, assembled and amplified by PCR using RumAF1 and RelAR primers for production of 3794 bp full length mutant *rumA* and wild type *relA* genes. The cloning was carried out in pBAD18Kan at *Eco*RI - *Kpn*I to yield plasmid pTE9.

4.2.6 Confirmation of constructs

The plasmids constructed in this study have been confirmed by restriction digestion and restriction pattern was observed on 0.8% agarose gel (Figure 4.2). The mutation in each of the construct was confirmed by DNA sequencing .



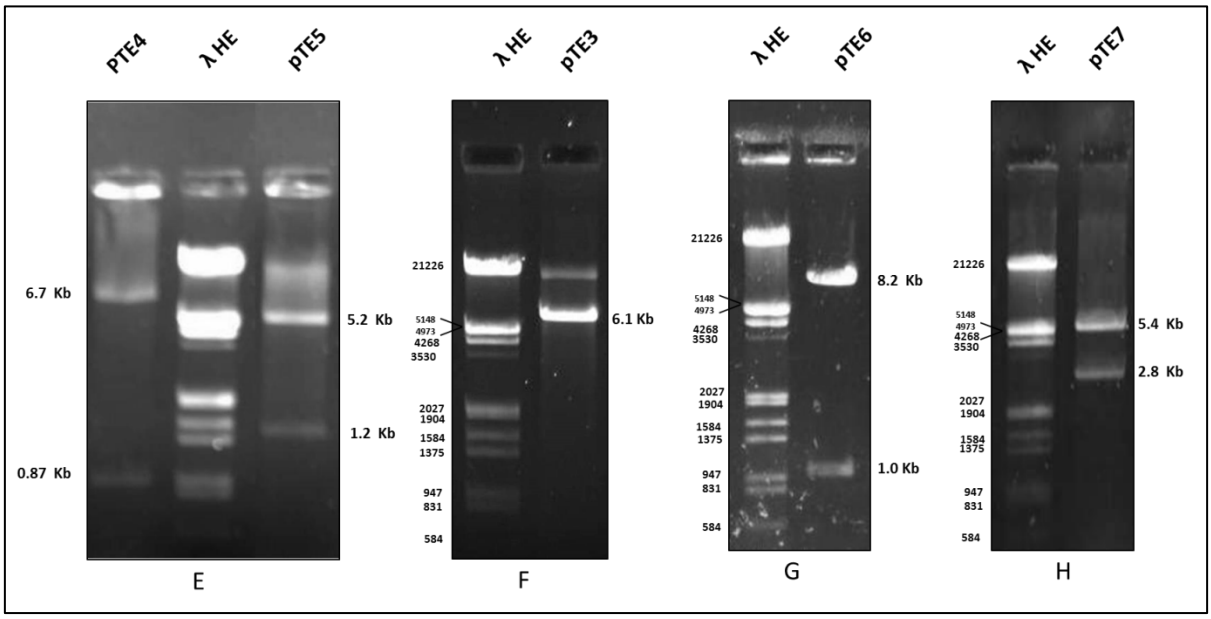


Figure 4. 2: Confirmation of *relA* and/or *rumA* clones by different restriction enzymes.
(A) pTE1 (*relA*$^+$, *rumA*$^+$, *barA*$^+$ in pBBR1MCS2), **(B)** pTE1ΔBgl (*relA*$^+$, *rumA*$^+$ and *barA*ΔBgl in pBBR1MCS2), **(C)** pTE1ΔAse (*relA*$^+$ in pBBR1MCS2), **(D)** pTE8 (*relA*$^-$*rumA*$^+$ in pBAD18 Kan) and pTE9 (*relA*$^+$ and *rumA*$^-$ in pBAD18 Kan) each digested by *Sal*I. **(E)** pTE4 (*relA*$^+$ in pBAD18Kan) digested with *Pst*I and pTE5 (*rumA*$^+$ in pBBR1MCS2) digested with *Cla*I**. (F)** pTE3 (*relA* upto 1368 bp, *rumA*$^+$ and 1/3 of *barA* in PBBR1MCS2) analyzed by *Pst*I. **(G)** pTE6 (*relA*$^+$ and *rumA*$^+$ in pBAD18Kan) digested with *Pvu*II**. (H)** pTE7 (1368 bp of *relA* and *rumA* in pBAD18Kan) confirmed by *EcoR*I-*Pst*I. λH and λHE were used as marker.

4.2.7 Antibiotic tolerance phenotype

Tolerance of DH5α cells harboring different DNA constructs was assayed on solid LA agar medium containing various concentrations of antibiotics. The growth in the presence of antibiotic was recorded after 24-36 hrs of incubation at 37°C.

4.3 Results and Discussion

4.3.1 Isolation of genomic clones containing *relA rumA* DNA

We have isolated *E. coli* genomic DNA fragment (7164 bp), cloned in the *BamH*I site of the vector pBBR1MCS2 (pTE1) to confer ~2 fold enhanced tolerance to antibiotics like erythromycin, tetracycline, chloramphenicol, rifampicin, mitomycin and daunomycin (Table 4.3). Sequencing revealed the insert in pTE1 to contain following four complete genes - *relA*, ppGpp synthetase I; *rumA*, 23S rRNA m^5U1939 Methyltransferase; *barA*, two component sensor

kinase; and *gudD*, glucarate dehydratase I and incomplete *chpKA* gene for toxin-antitoxin module (Figure 4.3; top).

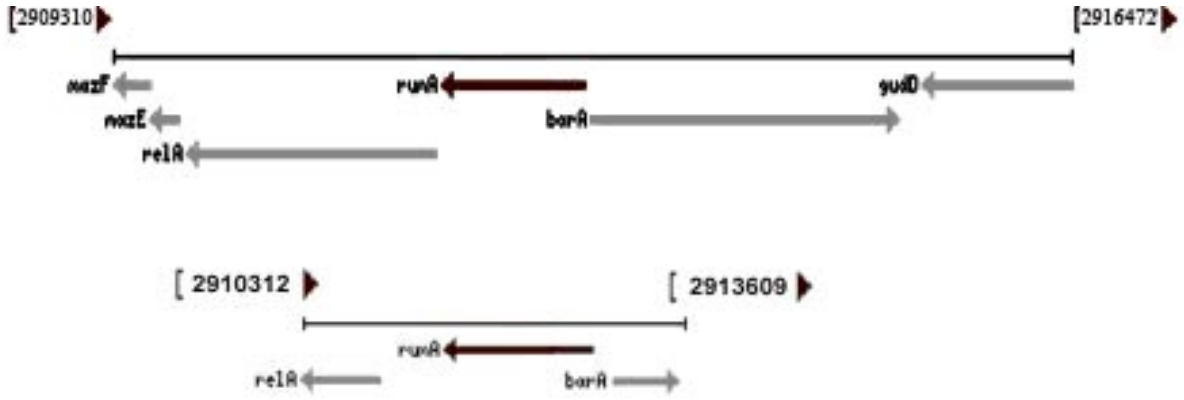


Figure 4. 3: Genomic Nucleotide Coordinates of the *relA rumA* DNA in pTE1 (top) and in pTE2/pTE3 (bottom). pTE2 and pTE3 are two independent clones differing from each other in a small way in that pTE3 contains 1368 bp of *relA* DNA compared to 1341 bp in pTE2.

Subsequently, two independent clones, pTE2 and pTE3 were isolated. Out of the four genes, these two clones contained complete *rumA* and two-thirds *relA* N- terminal domain (NTD). The 586/516 bp *barA* DNA present upstream of *rumA* in pTE2 and pTE3 respectively is without functional significance (Figure 4.3; bottom).

4.3.2 Multiple copies of *relA rumA* confer tolerance to several antibiotics

Since only 1/3 of *barA* is present in pTE2/pTE3; it can be safely assumed to have no role in antibiotic tolerance phenotype. One more evidence for similar assumption is that deletion of DNA between two *Bgl*II restriction sites in *barA* region of pTE1ΔBgl plasmid was able to retain multiple antibiotic tolerance. On the basis of these two evidences, we have focused on two genes *relA rumA* and made the construct with either single gene or both the genes together to find out the function of each in antibiotic tolerance.

We recapitulated the multiple antibiotic resistance phenotype (Table 4.3) with the construction of two plasmids pTE6 and pTE7 in which entire *rumA* and *relA* ORFs were present (pTE6) or entire *rumA* and two thirds of *relA* ORF upto the *Pst*I site (1368 bp downstream of *relA* start codon, essentially containing 456 amino acids out of 745 amino acids) is present in pTE7 (Figure 4.3).

RelA' polypeptide (456 aa) is an unstable truncated form of RelA protein that synthesizes (p)ppGpp in a growth phase independent manner (Metzger *et al.*, 1989).

Single gene of *rumA* or *relA* present in pBBR1MCS2 (pTE5) or pBAD18Kan (pTE4) render the transformant cells as sensitive to the antibiotics as the cells carrying the empty vector (Figure 4.4).

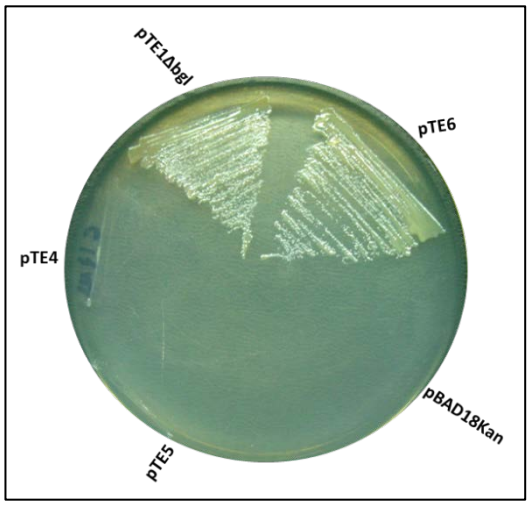


Figure 4. 4: Recapitulation of multiple tolerance phenotype of *relA* and *rumA* DNA. Representative LA plate supplemented with tetracycline (4µg/ml) indicating growth of E.coli DH5α transformants containing indicated plasmids (Also see table 4.3).

The results of cloning experiment strongly suggested that both *relA* and *rumA* are required and sufficient for phenotypes of multiple antibiotic tolerance.

4.3.3 RelA catalytic function is required for antibiotic tolerance

Isolation of clones pTE2 and pTE3 which contain respectively, 1341 and 1368 bp of 5' portion of *relA* DNA, indicates that C-terminal of RelA is dispensable for antibiotic tolerance phenotype. C-terminal of RelA has got regulatory role in that, it inhibits RelA's catalytic activity under nutrient excess conditions and is responsible for starvation mediated activation of N-terminal for (p)ppGpp synthesis through ribosomal protein, L11. Deletion of C-terminal portion relieves the inhibition on catalysis and causes growth phase and L11-independent constitutive synthesis of (p)ppGpp (Yang & Ishiguro, 2001).

Gly251Glu substitution in RelA's N-terminal domain has been described to drastically reduce (p)ppGpp synthesis (Gropp *et al.*, 2001). Using mutagenic primers G251E-5' and G251E-3' (Gropp *et al.*, 2001), catalytic mutant of *relA* was constructed (Figure 4.5). The mutation was

confirmed by sequencing, and also by inability of the *relA* mutant plasmid (pTE8) to complement *relA1* mutation in MC4100KP on 3-AT starvation plate (data not shown).

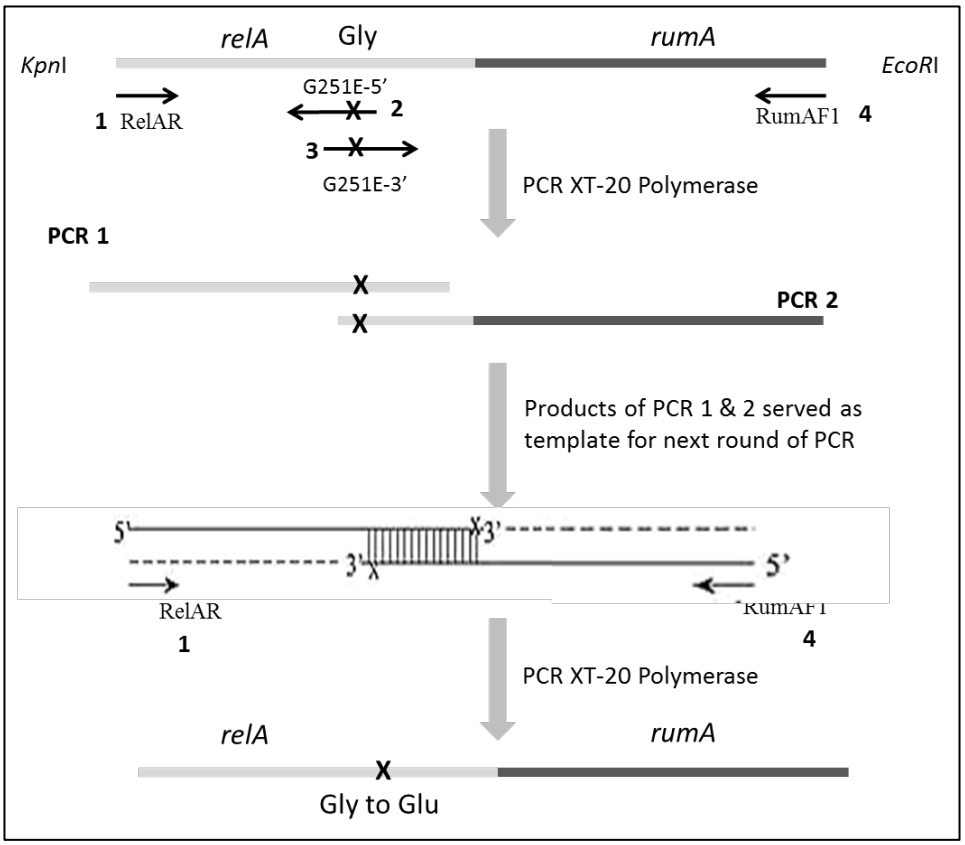


Figure 4. 5: Schematic diagram of *relA* SDM mutagenesis method.

Indeed, the catalytic function of RelA is indispensable for the phenotype of antibiotic tolerance, for Gly251Glu mutation completely abrogated the phenotype when tested in combination with wild type *rumA* (pTE8) (Figure 4.6, Table 4.3). The results indicate that (p)ppGpp synthesis by RelA is necessary for antibiotic resistant phenotype.

An Independent evidence that *relA* is required for antibiotic tolerance is provided in the result that pKC1 fails to produce the tolerance phenotype (Figure 4.6, Table 4.3). A spontaneous mutation was found in *relA* gene of pTE1ΔBgl (pKC1) DNA. This mutant has -2 frameshift change in the *relA* gene (Appendix I), which also abolishes RelA protein's ability to complement *relA* mutation in MC4100 (*relA1*).

4.3.4 Multiple copies of *relA* with its promoters confer resistance to several antibiotics

relA gene with its promoter pTE1ΔAse also reconstituted the multiple antibiotic tolerance phenotype upon introduction into DH5α (Figure 4.6, Table 4.3).

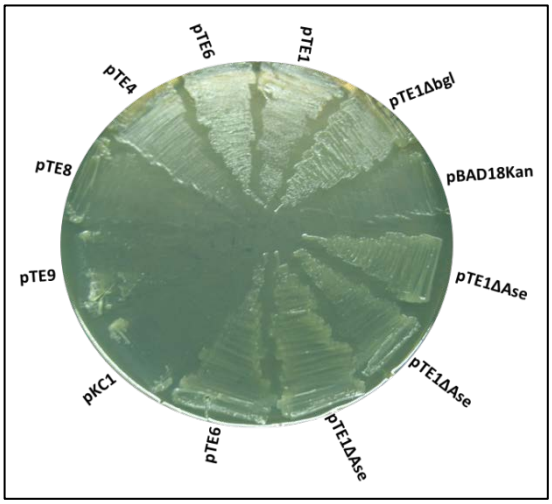


Figure 4. 6: A multiple antibiotic tolerance requires functional *relA*. Representative LA plate containing tetracycline (4 µg/ml) shows growth of DH5α transformants with different constructs of *relA* DNA.

The above result is confirmed also by mutating *rumA* sequence by SDM (see below), however the results were inconclusive due to inadvertent mutation (CTG to CCG/ Glu to Arg Mutation) in *relA* DNA generated during PCR (Appendix IIb).

Cysteine389 is catalytic amino acid of RumA protein (Lee *et al.*, 2004, Lee *et al.*, 2005). To test if the catalytically active form of the protein is important for the phenotype, C→P substitution in the 389 position was carried out by PCR using mutagenic primers C389P-SDM1 and C389P-SDM2 (Table 4.2). The mutation was confirmed by sequencing. We have found in sequencing result that apart from the C→P mutation there was an unintended base substitution (Appendix IIa). The functionality of *relA* of this pTE9 construct when assayed after introduction into MC4100KP (*relA1*) was unable to support growth on 3-AT plate; it clearly means *relA* gene is nonfunctional (Appendix IIb). Therefore, multicopy expression of the combination of mutant *rumA* and *relA* genes (pTE9) was completely devoid of the phenotype of multiple antibiotic tolerance (Figure 4.6, Table 4.3) as expected.

Table 4. 3: MIC of various constructs for different antibiotics

Construct/gene	Tet	CAM	Dno	Ery
	µg/ml			
pBAD18Kan	**2.0**	**2.0**	**10**	**60**
pBBR1MCS2	**2.25**	**2.0**	**10**	**60**
pTE1/*relA rumA*	**4.0**	**4.0**	**20**	**120**
pTE1ΔBgl / *relA rumA*	**4.0**	**3.5**	**20**	**100**
pTE1ΔBgl (pKC1/pKC2)	**2.25**	**2.25**	**10**	**60**
pTE4/*relA*	**2.25**	**2.25**	**10**	**60**
pTE5/*rumA*	**2.25**	**2.25**	**10**	**60**
pTE6/*relA rumA*	**4.0**	**3.75**	**20**	**120**
pTE7/*relA rumA*	**3.5**	**3.2**	**20**	**100**
pTE1ΔAse	**3.5**	**3.5**	**20**	**100**
pTE8/*relA*G251E *rumA*	**2.0**	**2.0**	**7.5**	**50**
pTE9/*relA rumAC389A*	**2.0**	**2.0**	**7.5**	**50**

E. coli DH5α cells were transformed with indicated constructs and tested for tolerance to antibiotics listed in the table. Abbreviations used are as follow: Tet – tetracycline, CAM – chloramphenicol, Dno – daunomycin, Mit – mitomycin, Ery– erythromycin. The boxed portion represents clones that confer tolerance.

The antibiotic tolerance phenotype was unique to DH5α strain and could not be reproduced in MG1655. The transformants of MG1655 containing pTE1, pTE2 and pTE6 failed to grow on different antibiotics (data not shown).

4.4 Conclusion

We have isolated from 3 independent genomic libraries of *E coli* DH5α of clones that were able to form colonies in the presence of multiple antibiotics like tetracycline, chloramphenicol, rifampicin, mitomycin, erythromycin. In fact the clones were isolated from DH5α being plated on combination of antibiotics listed above. The clones being independent are evident from the fact that the insert DNA in each invariably contained *relA rumA* genes and in some, flanking genes as well. The antibiotic tolerance phenotype was reconstituted with DNA containing only *relA* and *rumA* genes. We also showed that *relA* DNA is important for the phenotype however full length of DNA of *relA rumA* conferred stronger growth phenotype than the *relA* alone construct (pTE1ΔAse). Our observation is that the strength of phenotype conferred by insert DNA of pTE in pBluescript plasmid was intense in comparison to that in pBBR1MCS2 (pTE1). Inability of pTE4 (*relA*$^+$ in pBAD18Kan) to permit growth of transformed DH5α cells to tolerate the level of antibiotics used in the study (it did not grow at the concentration at which pTE1, pTE2 and pTE6 transformants grew) can be ascribed to insufficient level of expression in LB medium with 0.2% arabinose. The result that the antibiotic tolerance phenotype was limited to the strain DH5α and could not be reproduced in MG1655 was highly unexpected. There are many phenotypes that are affected due to genetic differences between various strains of *E.coli* which affects the physiology and adaptation to different stress conditions (Freddolino *et al.*, 2012, Soupene *et al.*, 2003, Spira *et al.*, 2008, King *et al.*, 2004). ppGpp metabolism has been shown to be important in antimicrobial resistance. ppGpp^0 strains which are *relA*$^-$ *spoT*$^-$ are hypersensitive to several antibiotics (Greenway & England, 1999a). We seem to have the opposite phenotype of tolerance to antibacterial in presence of overexpression of *relA*. This could be an important factor contributing to intrinsic resistance given that genomic DNA amplification is quite rampant in bacteria (Romero & Palacios, 1997).Though the sequence of two genomes, MG1655 and DH5α is known it will be difficult to test the genetic differences that contribute to strain specificity.

4.5 References

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