Characterization of EvgAS: two component signal transduction system in *Escherichia coli*

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By
Prachi P. Pathak

DECLARATION

The work presented in this thesis was carried out in the School of Biosciences at the University of Birmingham, U.K during the period September 2012 to February 2013. The work in this thesis is original except where acknowledged by references.

No part of the work is being, or has been submitted for a degree, diploma or any other qualification at any other University.

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Prachi P. Pathak

ABSTRACT

The ability of bacteria to live in a diversity of host environments is governed by sensors and response regulators in order to respond to a wide array of stressors. *Escherichia coli* encounter many stresses, during passage through mammalian gut, including acid stress. The AR2, glutamate-dependent acid resistance system, is controlled by a regulatory network which responds to stress caused by acid in *E. coli*. There are three known "Two component systems" (TCS) involved in AR2 network which are EvgAS, PhoPQ and RcsB. Till today, the target genes under the control of EvgAS are not very well characterized.

Here, we have generated mutants and mapped them in gene encoding EvgS, the sensor kinase of EvgAS TCS. Using LacZ assay and acid resistance assay we characterized their contribution to acid resistance in *E. coli* K12.

Furthermore, our studies focus on expression of multi-drug resistance linked to EvgAS, two component signaling system. In order to understand this link, active efflux studies have been carried out for EvgS mutants with different genetic background. This suggests that the EvgAS system has significant role the expression of genes that confirms resistance to the drugs. These findings provides a platform for better understanding of mechanism underlying the occurrence of multi drug resistance.

Keywords: *Escherichia coli*, Two-component systems, EvgAS, Acid resistance, Drug efflux system.

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CHAPTER 1

1.1 Introduction

Escherichia coli is a member of the Enterobacteriaceae family, which inhabit the gut of warm blooded mammals. Depending upon strain, they either establish commensal association with host by supply of vitamin K2 or can contain virulence factors capable of causing severe illness (Bently *et al.*, 1982).

Micro-organisms that inhabit the pH neutral environment of the mammalian gut must first pass through the acidic environment of the stomach. The stomach can have a pH as low as 1.5 during starvation (Smith, 2003). Despite the lack of any clear correlation between acid resistance and pathogenicity acid resistance is a clinically important phenotype. This is because the greater an organism's acid resistance, the lower the infective dose of that organism (Foster, 2004). Once in the gut, *E. coli* must also cope with acid shock from volatile fatty acids produced by fermentation. *E. coli* is considered to be a neutrophile and yet can withstand acid shock levels equivalent to acidophiles. These attributes are unusual; therefore *E. coli* acid resistance mechanisms are an interesting subject to research.

1.2 *E. coli* acid resistance

In 1995, Lin *et al* were the first to show evidence for survival of *E. coli* at very low pH i.e 2.0, which was found to be dependent on the media which was used for growth and for acid challenge. The cultures which were grown and further tested in minimal glucose media without amino acids were killed due to acid stress. Yet cultures that were grown and tested in media rich in amino acids were able to survive at pH 2.5 for several hours, thus can be described as acid resistant. This extraordinary difference in phenotype forced the scientists to study the three acid-resistance (AR) systems. AR1 or oxidative acid resistance, the glutamate dependent (AR2) and arginine dependent (AR3) respectively. AR4 which is based on the

lysine decarboxylase, was found to be quite unsuccessful in enabling *E. coli* to survive prolonged periods of acid stress (Iyer *et al.,* 2003). Recent work on AR4 has indicated that it has a more crucial role helping the cell to persist endogenous stress caused by phosphate starvation. The defense provided by AR2 was significantly higher than the defense conferred by the other AR mechanisms at extreme pH. As a consequence of this superior protective capability AR2 is the most studied of the acid resistance systems (Lin et al., 1996). Recently in addition to the mechanisms of each of the amino acid dependent systems which have been characterized in detail, genes located on an Acid Fitness Island (AFI) have been associated in acid resistance (Hommais *et al*., 2004).

1.3 The Glutamate dependent acid resistance system (AR2) of *E. coli*

AR2 is the best characterized acid stress system and is acknowledged as the glutamate dependent mechanism (GAD) mechanism. AR2 includes three enzymes which consist of two decaboxylative enzymes GadA, GadB, and a γ-amino butyric acid (GABA) antiporter. The glutamate decarboxylase (GAD) activity of *E. coli*, had already been extensively characterized by the time of the discovery of AR2 (Smith *et al.*, 1992) and in addition, the sequences determined, the genetic loci had been mapped, and the biochemical properties established (De Biase *et al.,* 1996). As mentioned earlier, *E. coli* possesses two forms of GAD, which is encoded by two genes, *gadA* and *gadB,* located far apart on the chromosome (Smith *et al*., 1992). The two genes are thought to have arisen by gene duplication at an early stage in the evolution of *E. coli* (Bergholz *et al.,* 2007). It is currently unclear whether functional differences arise between the two copies of this gene. In *E. coli* K-12 just five amino acid differences exist between GadB and GadA. It is this region that has been revealed to be crucial for pH dependent localization of GadB to the inner membrane (Capitani *et al.,* 2003).

1.4 Mechanism of AR2 function

During acid challenge, glutamate is transported into the cell from the extra-cellular media through the GadC antiporter. Within an *E. coli* cell-encountering an extreme acid stress, GadB undergoes a conformational change to an active form as cytoplasmic pH drops to its pH optima between pH 3.8 to 4.6 (Capitani *et al.,* 2003). Chloride ions, present in gastric secretions, increase activation of GAD activity by serving to de-repress GadB auto-inhibition (Gut *et al.,* 2006). Overlying roles of the ClC H+/Cl- antiporters encoded by *eriC* and *mriT* may be significantly important for this activation (Iyer *et al.*, 2002; Richard and Foster, 2004; Accardi and Miller, 2004). In turn GAD is employed to the cell membrane where it decarboxylates glutamate to gamma amino butyric acid (GABA) (Capitani *et al.,* 2003). This reaction consumes an intracellular proton. GABA is then removed from the cell by GadC in return for fresh glutamate. AR2 activity seems to contribute to pH homeostasis, since after fermentative growth the internal pH of *E. coli* was found drop to pH 3.5 in the absence of glutamate, whereas it was measured at pH 4.2 in its presence (Richard and Foster, 2004). The AR2 mechanism is also reported to reverse membrane polarity from inside negative to inside positive, a scheme identified to be driven by acidophiles (Richard and Foster, 2004). It is presently not clear how effectively AR2 can function in either regard. Glutamate has a functional side chain with a pK_a of 4.1. Thus, during acid challenge (at pH 2.5), this side chain will be more than 50% protonated, whereas when it arrives, the less acidic cytoplasm through the GadC antiporter a substantial portion of these protons will dissociate causing a reduction in cytoplasmic pH before GadA/B activity integrates these into a GABA product. Less than 50% of GABA molecules are predicted to carry a positive charge inside the cell (Figure 1.1).

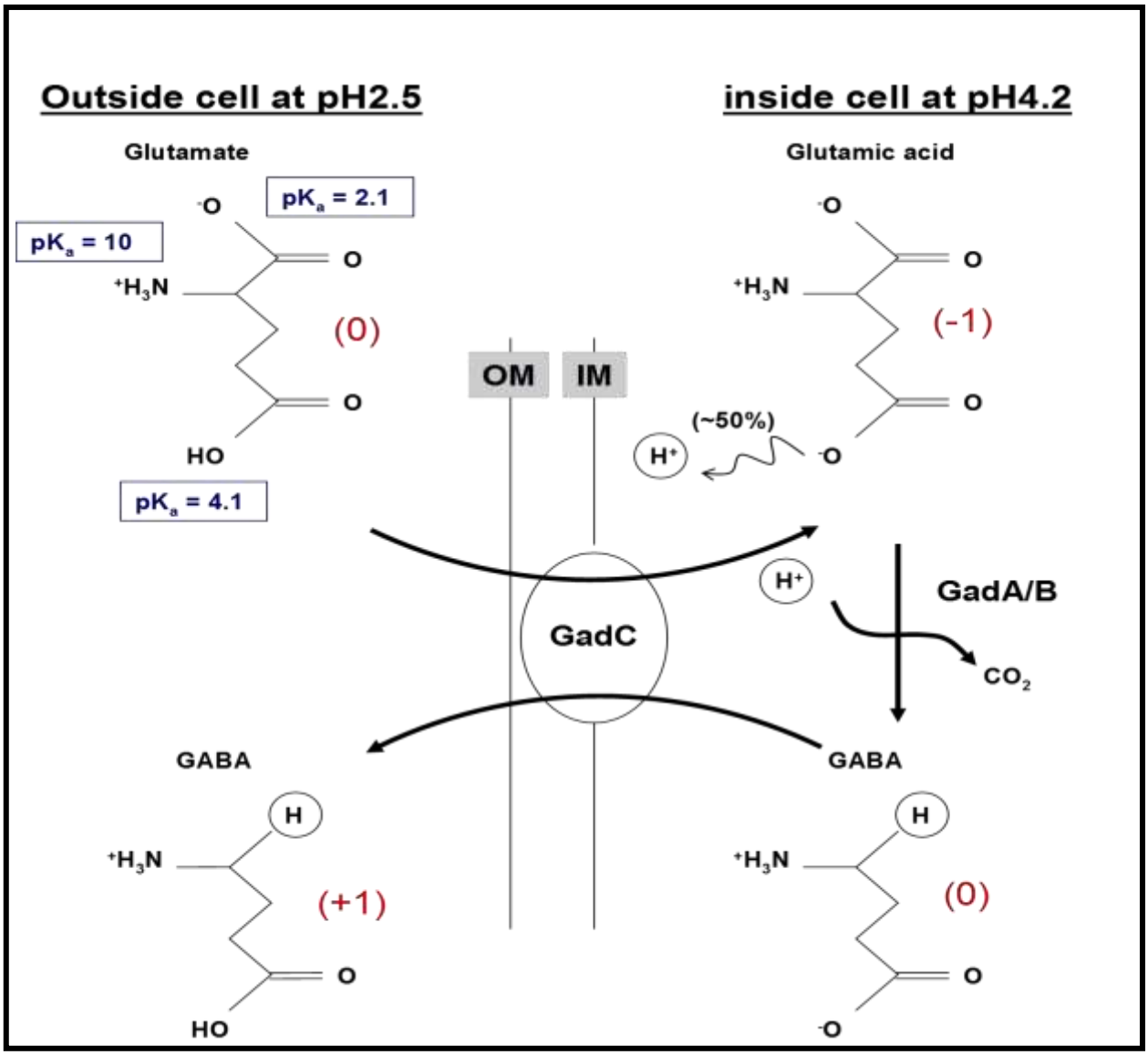


Figure 1.1 Mechanism of AR2 function during extreme acid stress at pH2.5. pK_a values of ionisable groups on glutamate are indicated in blue boxes. Predicted charge of the molecules is indicted in red font in brackets. IM = inner membrane. OM = outer membrane. Modified from Foster (2004).

Furthermore, during extreme acid challenge, the pH in the cytoplasm drops to 3.6 (measured without any induced acid resistance mechanisms), the acidification of the cytoplasm lowers the pH within the optimum range of the glutamate decarboxylase (3.8 to 4.6) (Capitani *et al*., 2003). However, the optimum pH for arginine and lysine decarboxylases is 5.25 and 5.5 respectively. Therefore, this environment could render the arginine and lysine decarboxylases ineffective (Blethen et al 1968; Lemonnier and Lane 2003). Moreover, when the arginine and glutamate systems are active they raise the cytoplasmic pH from pH 3.6 to 4.7 and 4.6 respectively (Richard and Foster 2004). It is thus recommended that *E. coli* is endeavouring to repel extracellular protons by reversing the membrane potential from a negative inside, relative to outside, to a positive inside relative to outside (Richard and Foster 2004). This is a approach employed by acidophiles and could elucidate why the pH of the cytoplasm remains below 5 even when glutamate or argenine are present in the media (Richard and Foster 2004).

1.5 Characterization of the EvgAS two-component system as an AR regulator

Bacteria have capability to sense environmental signals and respond accordingly, which requires sensors and response regulators. The gap between stimulus and gene regulation is usually bridged by two component system (TCSs). TCSs are composed of a histidine kinase and a response regulator. The key feature of two-component Systems is nothing but the typical organization of the sensors and of the response regulators (Mascher *et al*., 2006). The variability in functional domains and their architecture in diverse configurations made several kinds of phosphorelay circuits. In many cases, the activation occurs through multistep His-Asp phosphorelay cascades (Fig 1.2). In numerous cases, some protein partners have role in controlling the phosphorylation state of the response regulator, the ’on-off’ switch of the biological response which ultimately proliferates complexity of their regulation (Marie *etal*., 2001).

EvgAS was originally characterized as a homologue of the *B. pertussis* virulence regulators BvgAS. Recently whole genome sequencing experiments resulted in the identification of several constitutive mutations leading to acid resistance in EvgS. All of these mutations are positioned in PAS domain. Each mutation conforming activation of AR2 network (Bock and Gross, 2002).

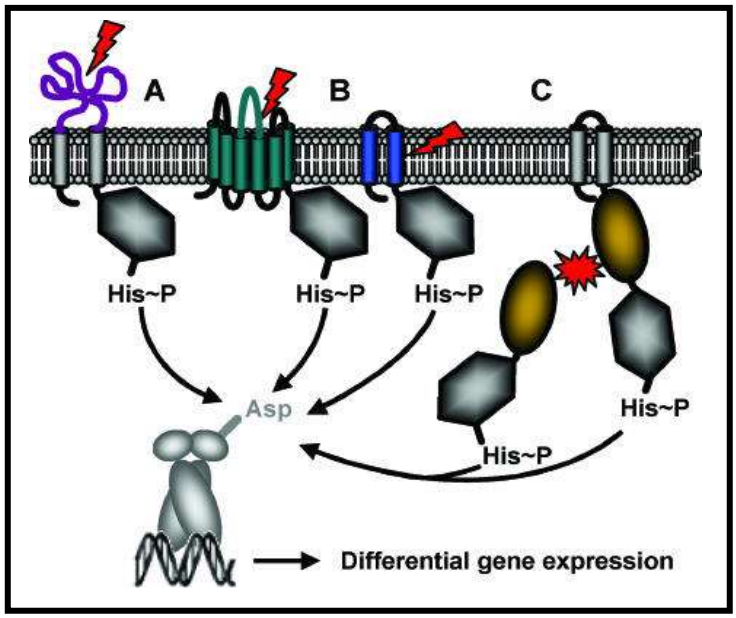


Fig 1.2 Mechanisms of stimulus perception

Schematic representation of the three different mechanisms of stimulus perception
(A) HKs that sense periplasm
(B) HKs that sense transmembrane region.
(C) HKs that sense cytoplasm.

The red arrow/red star represents stimulus
(Adapted from Mascher *et al., 2006*)

Extensive studies suggested that EvgAS is a regulator of multidrug efflux pump EmrKY. EvgAS was implicated in acid resistance in 2002 by Masuda and Church when they observed increased acid resistance of strains over expressing the response regulator EvgA as compared to that of wild type. It was found that EvgA over-expression up-regulate many genes which has key role in acid resistance, including the AR2 and AFI genes (Masuda and Church, 2002; Nishino *et al*, 2003). The up-regulated genes were analogous to those found when the transcriptome of a constitutively active EvgS mutant strain was compared to that of a wild type strain (Kato *et al*, 2000; Eguchi *et al*, 2003). Successive mutagenesis of the up-regulated genes showed that only YdeO, YdeP and YhiE (now GadE) reduced the endurance of the

artificially induced acid resistance phenotype, and correlates them in acid resistance (Masuda and Church, 2002). Concisely, GadE is a member of the LysR family of transcriptional regulators, YdeO belongs to AraC/XylS super-family of transcriptional regulators, and YdeP is a nothing but putative oxidoreductase which has homology to the alpha subunit of *E. coli* formate dehydrogenase H (Schell et al, 1993; Senda et al, 2005; Masuda and Church, 2003). When over-expressed, these genes can independently induce an acid resistant phenotype (Nishino *et al*, 2003). YdeP over-expression had the largest impact on acid resistance, and GadE had the lowest impact. However, the effect of over-expression of YdeP on acid resistance is less than that of EvgA over-expression. The role of YdeP in acid resistance has not as of yet been researched any further. In contrast, the role of transcriptional activators, EvgA and YdeO, has been widely characterized (Ma *et al.*, 2004).

1.6 Activity of active efflux in *E. coli.*

Resistance to antibiotics is widely known concept observed in wide range of bacterial species even those are harmful to human being. There is a miscellaneous mechanism found to subsidize the inherent and acquired resistance against antibiotics. The reason being resistant to many drugs in case of gram negative bacteria including *E. coli* is their typical cell structure and multidrug efflux pumps activity (Mark and Nick, 2009). Any antibiotic must access its target site(s) so as to become effective and this needs penetration through the cell envelope. It is found that, gram-negative bacteria, due to their double membrane structure, pose challenge for antibiotic accumulation. In order to target, an antibiotic has to cross the outer membrane which is rich in lipopolysaccharide and also should penetrate the inner bilayer of phospholipid, before inflowing the cell. Major inherent resistance to most of the existing antimicrobial compounds of Gram-negative bacteria in comparison with Gram-positive bacteria is mostly due to the poorer permeability of Gram-negative bacteria having double

membrane structure and the activity of multidrug efflux pumps (Babayan *et al.*, 2004). To understand the reason behind evolving bacteria into multidrug resistant, study of accumulation of antibiotics and their contribution in active efflux is required (Babayan *et al.*, 2004). Multidrug efflux pumps have broad spectrum of substrate which can be used to check the activity by measuring accumulation of antibiotic/fluroscent substrates including Bis-benzimide which can certainly be considered as markers of efflux activity. Variation in pump activity between strains indicates amount of drug accumulation into the cell and consequently alters fluorescence activity (Xu et al., 2003).

1.7 Aim and Objectives

The literature Review has described the mechanism of acid resistance in *E. coli*. Earlier studies in this lab had created *evgS* mutants shown to be acid resistant. Our study was to make more mutants by easier and novel method, and to characterize them to understand the mechanism of action of the EvgS protein, and which parts of it are important for function, as well as enabling study of the genes which it regulates.

The objective of this work was to understand the genetic changes in previously known acid sensitive strains which have turned into acid resistant on lab based experiments.
In order to achieve this, following sub objectives were completed.

- Random mutagenesis of EvgAS
- Characterization of mutants

Another objective was to understand link between EvgAS two-component system with the expression of multidrug resistance.

In order to achieve this, following sub objective was completed.

- Drug efflux studies using Bis-benzimide (Hoescht H33342) accumulation assay

Characterization of the evolved strains could increase our understanding of acid resistance in *E. coli*. Acid resistance is regulated by a complex network; mutations in this network could give rise to an acid resistant phenotype. Studying the accumulation and tolerance of such mutations in regulatory networks could provide insights into the robustness of bacterial networks.

CHAPTER 2

2.1 Bacterial Strains, Plasmids and Primers

The bacterial strains used in this study are listed in Table 2.1. Mutant strains were constructed as described in section 2.5.The plasmids used in this study are listed in Table 2.2. The primers used for plasmid construction and for sequencing are listed in table 2.3 and 2.4 respectively.

Table 2.1.1 Bacterial strains used in this study.

Strain	Relevant genotype	Source and/or reference
DH5α	*E. coli* DH5α (F-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk-, mk+), *phoA*, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1)	Lab stock
MG1655	*E. coli* K-12 MG1655 (F-, lambda-, *ilvG*-, *rfb*-50, *rph*-1)	Blattner *et al.*, 1997
XL-1 red	*endA1 gyrA96 thi-1hsdR17 supE44 relA1lac mutD5 mutS mutt* Tn*10(Tet^r)*^{a}	Aglient Technologies,UK
BW25113	*BW25113 (lacI qrrnBT14 ΔlacZWJ16 hsdR514 Δara BAD AH33 ΔrhaBADLD78*	Datsenko *et al.*,2000

Table 2.1.2 Plasmids used in this study

Plasmid	Description	Source and /or reference
pBAD/Evgs-his	cloning the evgS gene into pBAD-TOPO using the NcoI and PmeI sites	Yoko Eguchi and Ryataro Utsumi
pBAD/Evgs-his/ ΔCla	ClaI digest of the pBAD.evgS.his and pBAD, and re-ligation of these to close the gap.	Rob Marshall, Student from Lab
pBAD/Evgs-his/ ΔSwa-Psi	SwaI PsiI double digest of the pBAD.evgS.his plasmid, and a blunt-end ligation to close the gap	Yandong Xia, M.sc Student from Lab

pydePtet	Derived from pLUXydePp by insertion of pBR322 tetR gene fragment including ribosome binding site,downstream of the promoter *ydePp*	Made by Rachel Chandler, an undergraduate student from lab
pevgAS	Derived from pZC320 by insertion of evgAS operon of E.Coli MG1655	Matt by Matt Johnson, PhD student
pCP20	Gene mutagenesis plasmid; ApR and CmR; ts replicon curable at 43°C; encodes FLP recombinase for FLP mediated excision of DNA between FRT sites	Cherepanov *et al.*,1995

Table 2.1.3 Primers used in this study

Primer Name	**Sequence (5'-3')**	**Purpose**
acrA Forward Primer	AATGCCAGTAGATTGCACCG	PCR Screening: MG1655/pBAD24/ ΔacrA
acrA Reverse Primer	GCAATCGTAGGATATTGCGC	PCR Screening: MG1655/pBAD24/ ΔacrA
Kanamycin Forward Primer	CCTGCAAAGTAAACTGGATG	PCR Screening: MG1655/pBAD24/ ΔacrA
Kanamycin Reverse Primer	CATGCTCTTCGTGCAGATCA	PCR Screening: MG1655/pBAD24/ ΔacrA
evgS FP+1081	GAGGATGGGATATAATAC	Gene Cloning: MG1655/pBAD/EvgS-his mutant
evgS RP +2461	GCAAAGGACTTGTAATGG	Gene Cloning: MG1655/pBAD/EvgS-his mutant

Table 2.1.4 Primers used for sequencing

Primer Name	**Sequence (5'-3')**	**Purpose**
evgS-110_FP	CAGCAACAAAAGCATTGCC	Sequencing of *evgS* gene.
evgS+79_RP	CGTCTGCTTCTTCTAATGTAGC	Sequencing of *evgS* gene.
evgS FP+1081	GAGGATGGGATATAATAC	Sequencing of *evgS* gene.
evgS_FP-1281	GGAGGTTGAATGGATACAGG	Sequencing of *evgS* gene.
evgS_RP+1330	CGCGACGTAAAGTGTTCC	Sequencing of *evgS* gene.
evgS_FP+2278	CACTCCTCGGCTTAATTG	Sequencing of *evgS* gene.
evgS RP +2461	GCAAAGGACTTGTAATGG	Sequencing of *evgS* gene.
evgS_RP+3679	GAGGACCGCTTAAACAAG	Sequencing of *evgS* gene.

2.2. Growth conditions

(A) *E. coli* was grown at 37°C, unless stated and with aeration at 180 rpm in shaking incubator. LB media (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v salt; pH 7) was used for standard growing conditions. LB agar (LB broth + 1.5% w/v bacto agar; pH 7) was used where plating of bacteria was required. Both LB and LB agar were made up in distilled water and sterilized by autoclaving.

(B) For P1 Transduction, Lennox Broth 0.5% w/v tryptone, 0.25% w/v yeast extract, and 0.25% w/v salt; pH 7) was used for growing conditions. Lennox Broth + 1.25% Nutrient

Agar; pH 7 was autoclaved prior to use, when sufficiently cooled, 40% glucose (1mL per 200mL of LB) and 0.1M $CaCl_2$ (4 mL per 200 mL of LB) was added which was used where plating of bacteria was required. Soft agar overlay tubes" (LB + 0.75% bacto agar; autoclaved 20 minutes; maintained in 3ml as single use aliquot which was placed in the waterbath at $45^{O}C$ prior to use).This was used to make bacterial lawns for P1 phage infection.

(C) M9 media based on M9 salts; pH 7 (Table 2.3) was used in P1 Transduction protocol for washing steps.

Appropriate selection of resistance tagged strains and plasmids, antibiotics were added to growth media as follows., ampicillin (Ap), 100µg/ml; kanamycin (Km), 50µg/ml; tetracycline (Tet) 6µg/ml.

Table 2.3 Composition of M9 media.

Compound	Chemical formula	working concentration
di-Sodium hydrogen orthophosphate	Na_2HPO4	42.3mM
Potassium dihydrogen orthophosphate	KH_2PO4	22.1mM
Sodium chloride	NaCl	8.56mM
Ammonium chloride	NH_4Cl	18.7mM
D-Glucose (anhydrous)	$C_6H_{12}O_6$	0.4% w/v; 22.2mM
Magnesium sulphate	MgSO4	2mM
Calcium chloride1	$CaCl_2$	0.1mM

2.4 General Molecular Biology Techniques

2.4.1 Plasmid Preparation

This method is used to isolate the plasmid DNA from bacterial cell overnight culture containing appropriate antibiotic. The cells were obtained by centrifugation at 12,000 rpm for 5 min. The cell pellet was suspended in 200 µl of solution I (Glucose 50 mM, EDTA 10 mM, Tris 25 mM, pH 8) and incubated at RT for 5 min. Subsequently, the cells were lysed by adding 400 µl of freshly prepared solution II (1%SDS, 0.2 m NaOH) and gently mixed by inversion. To this 300 µl of solution III (3M Sodium Acetate, pH 5.2) was added, mixed thoroughly by inversion and incubated at RT for 5 min. The mixture was then centrifuged at 13,000 rpm for 5 min. The supernatant was collected and centrifuged again at 13,000 rpm for 5 min at to get clear supernatant. The DNA was precipitated with 600 µl of isopropanol and centrifuged at 13,000 rpm for 15 min and washed with 70% ethanol at 13,000 rpm for 10 min. The pellet was then air dried and dissolved in TE/autoclaved distilled water that is free of DNase contamination.

2.4.2 Preparation of competent cells

One ml appropriate overnight culture was diluted to 100ml fresh LB media in a 500ml conical flask. This was grown at 37°C (or as appropriate if temperature selective plasmid) with shaking (180rpm) until the culture reached an OD (A600nm) of 0.3-.0.35. It was followed by incubation on ice for 30 min. The cultures were then pelleted (8,000 rpm, 4°C, 10 minutes) the supernatant removed by decanting then the pellet was re-suspended in 0.1v/v ice cold TSS (0.5 ml DMSO, 1gm PEG-3350,0.2 ML 1M MgSO4, pH 6.5.All dissolved in 10 ml LB).It was split into 100µl aliquots for the use of transformation.

2.4.3 Plasmid Transformation

(A) An aliquot of competent cells was thawed on ice and plasmid DNA (~50-70 ng) was added to it and the contents were mixed properly by swirling the eppendorf gently. The mixture was kept on ice for 30 minutes. 0.9 ml TSR (LB supplemented with 20 mM Glucose was added to it and incubated at 37°C for 1 hour in a shaker incubator. The transformed cells were then spin at 6000 rpm for 5 minutes. Supernatant was decanted, with the exception of 100uL and resuspended the pellet in this 100uL and plate onto selective agar.

(B) If XL-1 Red competent cells were used for transformation, 25mM β-Mercaptoethanol was added into the 100µl of competent cells prior to use. An aliquot of competent cells was thawed on ice and Plasmid DNA (~50-70 ng) was added to it and the contents were mixed properly by swirling the eppendorf gently. The mixture was kept on ice for 30 minutes. This was followed by heat shock treatment at 42°C for 45sec.Then it was incubated on ice for 2 minutes. 0.9 ml LB was added to it and incubated at 37°C for 1 hour in a shaker incubator. The transformed cells were then spinning at 6000 rpm for 5 minutes. Supernatant was decanted, with the exception of 100uL and resuspended the pellet in this 100uL and plate onto selective agar (Ampicillin in this case).

2.4.4 Preparation of electrocompetent cells

Four ml overnight culture was scaled up to 400ml fresh LB media in a 1L conical flask. This was grown at 37°C (or as appropriate if temperature selective plasmid) with shaking (200rpm) until the culture reached an OD (A600nm) of 0.6. The Heat Shock at 42°C was given to the culture in shaking condition, 180 rpm for 30 minutes. Further culture was shifted on ice and heat shock at 4°C was given by keeping the culture at 180 rpm. The cultures were then pelleted (4000 rpm, 4°C, 30 minutes).The supernatant removed by decanting and then

the pellets re-suspended in 100 ml 20% glycerol (filter sterilized) and 1 mM MOPS (20.9% W/V,pH 7.5). The cells were then re-spun and re-suspended further two times. After the third re-suspension the cells were spun down, the supernatant carefully removed and finally the cells re-suspended in 2mL of ice cold 20% glycerol and 1mM MOPS. In all cases aliquots were used for electroporation on the same day.

2.4.5 Electroporation

3-4 µl of DNA was added to 80 µl of electrocompetent cell aliquots and mixed by flicking. Electroporation cuvettes (Geneflow, Fradley, UK; 1mm gap) were pre-chilled prior to use. The competent cell/DNA mix was pipetted into the electroporation cuvette. The samples were then electroporated at 1800V (1mm) 2200 V (2mm) (Geneflow electroporator). 1 ml LB was added to it and it was shifted to 15 ml Falcon tubes and incubated at 37°C for 1 hour in a shaker incubator. The transformed cells were then spin at 6000 rpm for 5 minutes. Supernatant was decanted, with the exception of 100uL and resuspended the pellet in this 100uL and plate onto selective agar.

*In the case of pCP20 transformants, select for ampicillin was used for selection and culture was incubated overnight at 30^{o}C.

2.5 Random Mutagenesis of EvgS

pBAD EvgS-His was transformed into *E. coli* XL-1 Red competent cells, as described in section 2.4.3 (B). This strain is particularly suitable for generating random mutations within a gene as previous studies state that XL-1 Red is deficient in three of the primary DNA repair pathways and it has no selectable/ screenable phenotype. The transformed plate was divided into 8 sections in order to prepare the transformants plasmid using alkaline lysis method as described in section 2.4.1.The obtained 8 plasmids were further transformed into DH5α -

pydePtet competent cells as described in section 2.4.3 (A). Selection of each plasmid was done by 2 different types of antibiotic combination as described below.

1. LB Agar containing Ap, Km and Tet: To check for mutants.
2. LB Agar containing Amp and Kan: To check efficiency of transformants.

Ampicillin (Ap), 100µg/ml; kanamycin (Km), 50µg/ml; Tetracycline (Tet) 6µg/ml.

The colonies obtained on Amp/Kan/Tet plate was taken forward in order to prepare the plasmids, which were further transformed into pydep-lacZ cells and plated on amp/kan plate in order to get putative mutants. Further, below studies were carried out for putative mutants.

- Sequencing and data analysis
- Check for lacZ activity
- Acid Resistance Assay
- Acid Resistance V/S LacZ activity

2.6 Sequencing and data analysis

Plasmid DNA was sequenced using specific primers in the University of Birmingham Functional Genomics Laboratory, Plasmid to Profile sequencing (Birmingham, UK). Analysis was done to confirm the single amino acid change in the given sequence. This was carried out with the help of basic bioinformatics tools including Expasy Translate, Protein BLAST etc. (Section 3.1).

2.7 LacZ activity

EvgS putative mutants obtained were used for this assay along with active evgS mutants that had previously isolated in the lab as a positive control and pBAD24 (vector only) as a negative control. These were inoculated into 5ml LB broth containing ampicillin and

kanamycin at 37°C for 3-4 hours in a shaker incubator till the OD_{600} reaches to 0.35-0.4. For each culture OD600 was measured separately. At this point,0.1 ml of culture was mixed with 0.9 ml of Z buffer (0.5M Na2HPO4,0.5M NaH2PO4,1M KCl, 1M MgSO4 0.27% β - Mercaptoethanol). Cells were lysed using 0.1 % SDS and 40 µl CHCl3 followed by vortexing for 8-10 sec. Reaction was started by adding 0.2 ml ONPG followed by incubation at 30°C till getting the yellow color of reaction mixture. Incubation time was measured for the calculation of LacZ activity. Reaction was stopped by adding 0.5ml 1M Na2CO3. 1 ml reaction mixture was taken and spin at 10,000 rpm for 10 minutes. Supernatant was taken in fresh cuvette in order to measure the absorbance at 420 nm. LacZ activity was measured using the formula described below. The Graph was plotted accordingly (Fig 3.2). LacZ activity= OD420/ (OD600*Volume of reaction mixture in ml*Incubation time)

2.8 Acid Resistance Assay

Strains of EvgS mutants.to be assayed were inoculated in 5 ml LB, (containing appropriate antibiotic) overnight at 37°C, 180 rpm. 500 µl cultures from each tube was diluted into fresh 5 ml LB. Diluted cells were grown for 2 hours 37°C,180 rpm. At the end, the cells were in exponential phase, where acid resistance was measured.

A 96 well plate was used to dilute the cultures into pH 2.5and pH 7.0 LB medium. The plate was setup by adding 180 µl of LB, pH 2.5 into 6 wells in a row. The same was repeated for LB, pH 7.0.Then the plate was prewarmed at 37°C for 20 minutes prior to start the assay. 10 fold serial dilutions were carried out using the cells at exponential phase for both the rows. The 10^{-2} to 10^{-6} dilutions were immediately spotted onto square LB plates (containing appropriate antibiotic) using multi tip pipette. Once spotted, the plates were tilted to spread the spots into the line down the plate (Fig 2.1). Plates were incubated at 37°C overnight.

The survival of each strain was expressed as percentage, which was calculated by dividing the number of colonies from pH 2.5 dilutions by the number of colonies from pH 7.0 dilutions and multiplied by 100.The most concentrated dilution that could be counted was used. The graph for % survival at pH 2.5 with respect to pH 7.0 for each mutant was plotted (Fig 3.3).

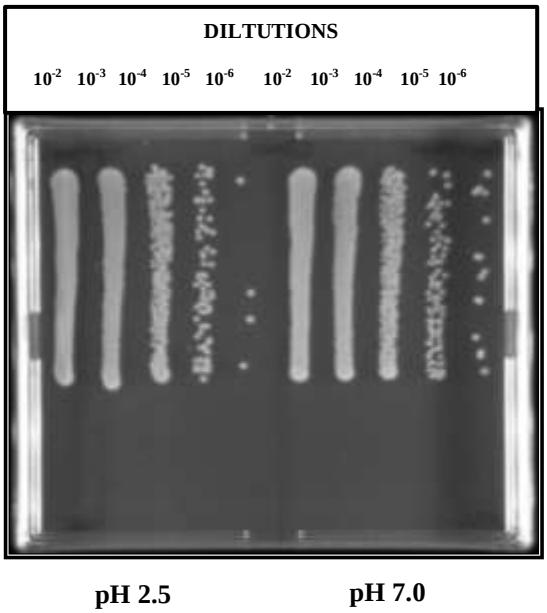


Figure 2.1 Example of an acid resistance assay.

The figure shows the colonies from an acid resistance assay. The dilution factors are shown in to top of the figure and the pH values are at the bottom. Colonies were counted by eye.

2.9 Acid Resistance V/S LacZ activity

The graph was plotted in order to analyze the correlation between % Acid Resistance and LacZ activity of the EvgS mutants (Fig 3.4).

2.10 Cloning: A) PBAD EvgS his mutant B) PBAD EvgS his mutant ΔSwa-Psi

2.10.1 PCR amplification

EvgS mutants were used as a template for amplification. Primers, FP +1081 and RP +246 were used for PCR amplification. (Refer Table 2.3). Gradient PCR machine was used to

amplify the gene and standardize the annealing temperature. Gradient was kept between 40°-50°C. The amplified products were checked on 1% agarose gel and the size confirmed by loading a 1kb DNA ladder. For further PCR reaction 45ºC was kept as annealing temperature as it was found to be appropriate temperature from gradient PCR.

2.10.2 Restriction digestion of vector and inserts

Once the amplified genes were obtained, restriction digestion was carried out for the vectors pBAD/EvgS-his/ΔCla and pBAD/Evg-his/Δcla-ΔSwa-Psi (which was obtained from lab, Previous unpublished work) With ClaI. Three independent EvgS mutants,A,B and C were also cut with the same restriction enzymes to generate sticky ends so that both the vector and the insert can be ligated under appropriate conditions.

2.10.3 Ligation

The digested vector with gene insert (In molar ratio 1:3) were then ligated with the help of T4 DNA ligase in a reaction mix containing ligation buffer in final concentration of 1X. The ligation was carried out at 16°C for 16 hours as the optimal activity of the enzyme was at 16°C.

2.10.4 Transformation and further studies

The ligated product then transformed into pydep-lacZ cells and plated on amp plate for overnight incubation. Following studies were carried out.

- Plasmid Preparation followed by restriction digestion with SalI for all 6 transformants (Fig 3.5-A)
- LacZ activity for all 8 transformants as described in Section 2.7 (Fig 3.5-B).

2.11 Phage P1 transduction

BW25113/ ΔacrA which was a donor bacteria was grown in 1 mL of Lennox Broth and 2% 0.1M $CaCl_2$ overnight at 180rpm, 37^{o}C.The culture was diluted with fresh LB and it was returned on shaking incubator at 37^{O}C and 180 rpm for approximately 5 hours.

The P1 stock was diluted to 10^{-5} using 10^{-3}, 10^{-4}, 10^{-5} stocks using Lennox Broth in sterile glassware. It was Stored in the fridge till required

When the donor bacteria was thick 0.1mL of P1 $10^{-3/4/5}$ (only 1 stock) was added to the soft agar overlay tubes in the waterbath (Section 2.2-B), 0.1mL of donor bug was added, vortex and poured directly onto Lennox Agar plates (Section 2.2-B).This was repeated for all 3 dilutions ($10^{-3/4/5}$) of P1 stock. Plates were incubated at 37^{O}C, with the lids uppermost (i.e not inverted), incubated towards the end of the day to prevent overgrowing of bacteria. Overnight culture of recipient bacteria (that to be infected with P1, here *E. coli* MG1655) in 1mL of LB was prepared and incubated at 37^{O}C, 180rpm.On the following day, all the plates were examined and plate that shows a 'lacey plaque' effect were selected. To harvest the plate, glass homogeniser was prepared and stored on ice, to keep chilled. 1mL of chloroform was added to the homogeniser and returned to ice. To harvest the selected plate, 2mL of Lennox Broth was added to the surface and carefully removed the upper layer using a glass spreader and transferred to a glass homogeniser. It was homogenised until the mixture was smooth, whilst being stored on ice. The homogenised mixture was transferred to thick walled protein tubes (thick walled test tubes) and centrifuged at 2870rpm for 15 minutes. Supernatant was transferred to sterile glassware, containing 1mL chloroform, vortex and stored at 4^{O}C – This was the P1 stock required for transducing the recipient bacteria. P1 stock was diluted to 10^{-1} and 10^{-2} using LB. The recipient bacteria overnight culture (1mL) was transferred into 20mL of LB and returned to the incubator until thick (approximately 5 hours).Further it was

centrifuged at 2870rpm for 3 minutes. Pellet was resuspended in 0.5mL of LB. To the tubes following was added;

1. 0.1mL concentrated P1 stock
2. 0.1mL 10-1 P1 Stock
3. 0.1mL 10-2 P1 Stock
4. Nothing – Negative Control

The tubes (not shaking) were incubated in a waterbath for 20 minutes 1mL of 1x M9 media (Section 2.2-C) was added and centrifuged at 2870rpm for 3 minutes. Pellet was resuspended in 4mL of M9 media, centrifuged at 2870rpm for 3 minutes, supernant was disguarded. Above step was repeated until the cells have been washed 3-4 times, to remove any excess P1 stock. Pellet was resuspended in 2mL of LB and placed in the shaking incubator at 37oC, 180 rpm for 1 hour, to allow the cells to recover. Centrifuged as above and pellet was resuspended in 0.2mL of 1x M9 media, and plated onto selective agar plates for overnight incubation. On the following day, the plates containing recipient bacteria and various P1 stocks were examined and colonies were selected for screening using colony PCR. The reactions were then run in a thermal cycler set to the following programme.

Steps	Temp	Time	
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	30 cycles
Annealing	56°C	30 sec	
Extension	72°C	1.5 min	
Final Extension	72°C	5 min	

The 1% agarose gel was run in order to confirm the transfer of mutation into MG1655

2.12 Removal of kanamycin resistance cassette

Once transfer of mutation into MG1655 was confirmed, In order to remove kanamycin cassette within, the electrocompetent cells were prepared from P1 MG1655 strain (Section 2.4.4) The gene mutagenesis plasmid, pCP20 was prepared (Section 2.4.1).As this is temperature sensitive plasmid, overnight incubation (prior to start the miniprep) was done at 30°C. The Pcp20 plasmid was then transformed into competent cells made from strain of MG1655 containing the arcA:: kan marker (which had been introduced by P1 transduction) using electroporation method. (Section 2.4.5) and the transformants were plated onto LBA containing ampicillin 100ug/mL and incubated overnight at 30oC.On the next day, 10 colonies were picked and inoculated into 5mL LBB (containing no antibiotics) and was Incubated at 42^{o}C, 180 rpm for at least 6 hours. The culture was streaked in order to get the single colonies onto LBA Only plates – Again containing no antibiotics and incubated overnight at 37^{o}C.On the following day. Total 25 colonies were Picked across all the plates and patched the samples onto LBA only (all should grow), LBA containing ampicillin (to check the loss of pCP20) and LBA containing Kanamycin. The plates were incubated at 37^{o}C overnight. The plates were examined, only colonies which have grown on the LBA only and not on the plates containing either antibiotic have been selected. Colony PCR was performed (Section 2.11) to confirm the absence of the antibiotic resistance cassette (Fig 3.6-B). This was then taken forward for efflux assays.

2.13 Efflux assay using Hoescht 33342

2.13.1 Strains preparation

MG1655/pBAD24/ΔacrA, after getting rid of Kanamycin cassette was then processed to prepare the competent cells (section 2.4.2).Following list of plasmids with MG1655/pBAD24 background were transformed (section 2.4.3-A) into above mentioned competent cells in

order to get the new strains with ΔacrA background. Thus total 16 strains (each strain with MG1655/pBAD24 and MG1655/pBAD24/ΔacrA background) then processed for efflux assay.

Strains used were for this experiment were as follows:

1. pBAD 24 (Vector only)
2. pBAD-EvgS –his
3. pBAD-EvgS/ΔCla
4. pBAD-EvgS/ΔSwa-Psi
5. Constitative EvgS Mutant A
6. Constitative EvgS Mutant B
7. Constitative EvgS Mutant C
8. Constitative EvgS Mutant D

2.13.2 Bis-benzimide (Hoescht H33342) accumulation assay

The strains mentioned above were inoculated in 5ml LB containing appropriate antibiotic and incubated overnight at 37^{o}C.Cells were harvested at 10,000 rpm for 10 minutes at RT. Pellet was resuspended in 3 ml of PBS (0.1 M pH 7.2). Appropriate dilutions were made using PBS for each sample so as to reach OD_{600} of to 0.1.

2.13.3 Hoescht stock preparation:

Bis-benzimide (Hoescht 33342) was dissolved in sterile water to give 25 ml of 1 mM H33342 (13.35 mg/ml) in a volumetric flask. This stock was then diluted further to 25 µM in 10 ml of sterile water in a volumetric flask for further use.

2.13.4 Efflux Assay

The diluted suspensions of each strain were added (180 µl each) in triplicates to a black plastic 96 well plate. 200 µl of PBS was added into the 4 wells which were acted as a blank. Before placing microtitre tray in Fluostar machine, following things were checked including

configuration of plate reader, position of optic leads, value of excitation and emission wavelengths, volumes of bis benzimide for addition and setting of "gain" at 1460.Once this is done, the 96 well plate was placed in the machine tray .The stock of 25 µM H33342 was placed into the FLuoSTAR machine and program was set so that, 20 µl of H33342 would be injected to all the wells containing 180 µl of sample at cycle number 3.The fluorescence was measured with excitation and emission (355nm and 465nm) every min for 1 hour at 37^{o}C.The Graphs were plotted and data was analyzed (Fig 3.7 A-G).

CHAPTER 3

3.1 Random Mutagenesis of EvgS

Previous work in the Lund laboratory showed that selection for high levels of acid resistance in exponential phase resulted in strains that contained mutants in the PAS domain of the sensor kinase, EvgS. These showed constitutive activation of the genes of the acid resistance pathway (Johnson, 2011). These mutants have the potential to increase our understanding of the mechanism of action of the EvgS protein, and which parts of it are important for function, as well as enabling study of the genes which it regulates. However, the method of using selection for acid resistance is relatively slow. In order to obtain more mutants in the *evgS* gene, a novel selection has been set up in the lab where the evgA-regulated promoter of the *ydeP* gene was fused to a tetracycline gene, and it was confirmed that strains carrying this fusion were only tetracycline resistant when they also contained a constitutively active *evgS* mutant. Thus, new constitutive mutants in EvgS can be found by mutating a plasmid carrying the evgS gene, transforming it into strains carrying the pydeP-tet fusion, and screening for tetracylcine resistance. The plasmid pBADevgAS was mutagensised by passage through the XL-red mutator strain, extracting from the strain, and transformed into the strain carrying the pydeP-tet plasmid. Cells were plated onto tetracycline at 6 ug/ml. 9 colonies were found that were resistant to tetracycline. Plasmid DNA of putative mutant strains were sequenced using specific primers in the University of Birmingham Functional Genomics Laboratory.

The region from EvgS (Escherichia coli str. K-12 MG1655) is shown (Chart 3.1).

The mutations are shown with the mutated amino-acid above the wild type one. The DNA sequence shown is the WT sequence.

Mutants found by me are in highlighted in blue color. Additionally, previously established mutants by Matthew Johnson (Ph.D student from Dr. Lund's laboratory) are also shown in yellow. Mutants found by Ryutaro Utsumi's group in Japan are in green. The PAS domain is shown in **bold**.

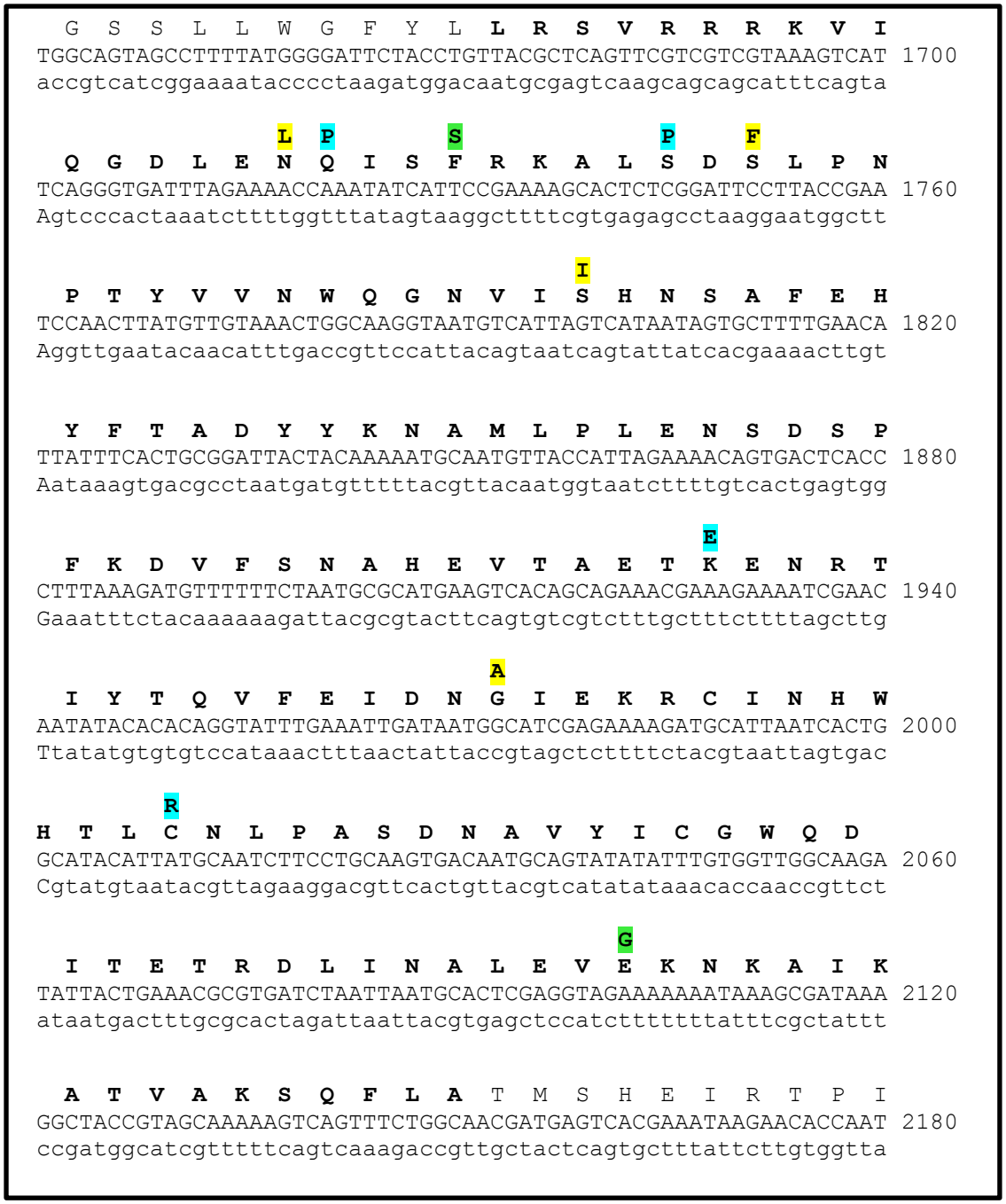


Chart 3.1

The region containing mutations from EvgS (Escherichia coli str. K-12 MG1655)

The list of primers used for sequencing the whole *evgS* gene is provided in table 2.4 from the section, materials and methods. The important observation to be noted here as, after sequencing the whole *evgS* gene in each case and found no other mutants.

The description of mutations, their position and is described. (Table 3.2)

As far as reason for repetition of the mutation/s is considered, there can be 2 possibilities,

First, it may be that several independent mutations have indeed occurred at the same position. Although the chances against this are high, they may still be found if there are only a very few mutations allowing a particular phenotype to occur. Second, it may be that the colonies containing the mutation are descended from the same original mutant. Our experimental design does make the second possibility quite possible, therefore if to get more independent mutants, several repeats of the original mutagenesis experiment which are completely independent of each other can be carried out.

It is also observed that, these mutations in EvgS are located in the PAS domain. Previous studies showed that *E. coli* has 3 main acid resistance mechanism and each mechanism requires different amino acid to function. (Lin *et al*., 1996).Thus finding and understanding the contribution of these mutants to the acid resistance phenotype is required to characterise them. In order to check this hypothesis, same strains were taken forward for LacZ activity and acid resistance assay.

	Serial number (same mutation also found in)	Mutation	Position	No. Of times, this mutation found
A	-	Q→P (Glutamine to Proline)	574	1
B	E,F,G,H,I	S→P (Serine to Proline)	582	6
C	-	K→E (Lysine to Glutamic acid)	643	1
D	J	C→R (Cysteine to Arginine)	671	2

Table 3.1 The description of mutations

The table explains mutated amino acid and it's position in EvgS (Escherichia coli str. K-12 MG1655).

3.2 LacZ activity of EvgS mutants

It is known that, in order to understand gene regulation in bacteria, the promoter of interest is either linked on plasmid/phage vector or to a reporter gene such as *lacZ*. The promoter activity of such *lacZ*-based vectors is assessed by measurements of β-galactosidase. This particular enzyme is very stable and thus easy to assay (Sung *et.al*., 1998). In our study, the plasmid pBADevgAS was mutagensised by passage through the XL-red mutator strain, extracting from the strain, and transformed into the strain carrying the pydeP-tet plasmid. pydeP is fused with reporter gene *lacZ*. pydep is strongly upregulated by evgA, therefore, this this promoter is a good choice for this assay.

In order to check the effect of EvgS mutations on the activity of AR2 network, strains were assayed for lacZ activity as described in materials and methods. The already studied and showing lacZ activity mutant was taken as positive control, whilst pBAD 24 (Vector only) was taken as negative control. The Fig 3.2 explains the pattern of LacZ activity for EvgS mutants. Each assay was done in triplicates and standard deviation was calculated (Fig 3.2).

The level of lacZ activity for different mutant differs; this is may be due to the structural location of each mutant in the PAS domain. Consequently each mutation can alter possible signaling in two component system at different level which can leads to difference in lacZ activity.

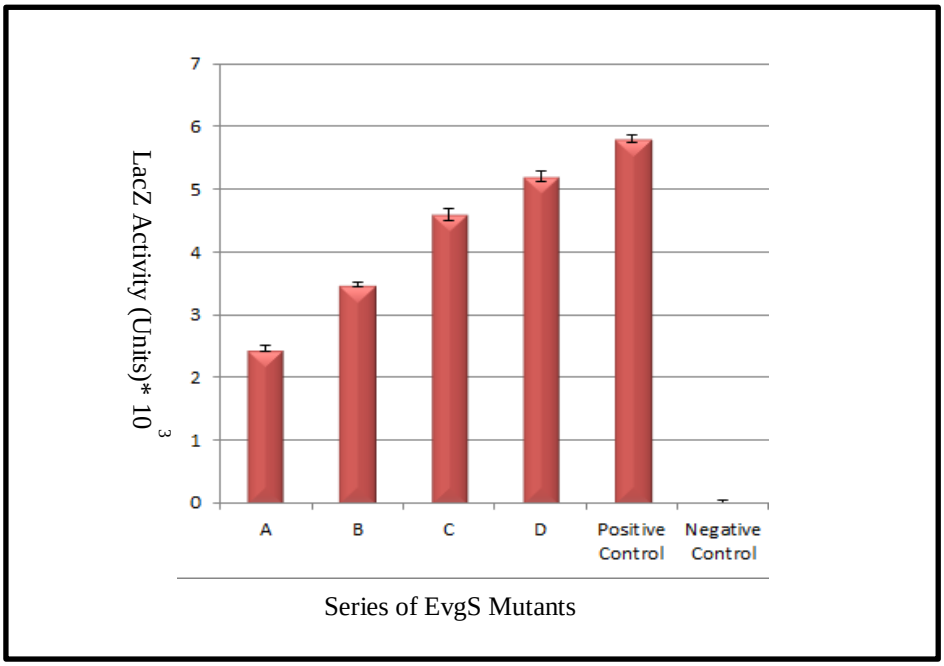


Figure 3.2 LacZ activity of EvgS mutants

To check the effect of EvgS mutations on the activity of AR2 network, strains were assayed for lacZ activity. The already studied and showing lacZ activity mutant was taken as positive control and pBAD 24 (Vector only) was taken as negative control. The level of lacZ activity is different for each mutant. All of the data points and error bars represent the average and standard deviation of 3 independent biological repeats respectively.

3.3 Acid resistance assay of EvgS mutants

Characterization of the lab based evolved strains could increase our understanding of acid resistance in *E. coli.* Acid resistance is regulated by a complex network; mutations in this network could give rise to an acid resistant phenotype. Studying the accumulation and tolerance of such mutations in regulatory networks could provide insights into the robustness and evolvability of bacterial networks.

As mentioned earlier, EvgS mutants would be expected to contribute into acid resistance phenotype. In order to robust the hypothesis, these mutants were taken forward for acid resistance assay as described in methods.

% Survival at pH 2.5 with respect to pH 7.0 is shown (fig 3.3). The EvgS mutant which is already studied and showing acid resistance was taken as positive control, whilst pBAD24 (Vector only) was taken as negative control. The Fig 3.3 B explains the pattern of % survival at pH 2.5 with respect to pH 7.0 for EvgS mutants. Each assay was done in triplicates and standard deviation was calculated.

Since pH 7.0 shows highest survival in all bacterial strains, pH 7 was assigned 100% survival.

The data about % survival at pH 2.5, implicated that previously known acid sensitive strains have turned into acid resistant on lab based experiments. Thus it is shown that, the EvgS mutations could be confirming acid resistance phenotype.

Finding and understanding the mutations that confer this increased resistance could enhance our understanding of acid resistance regulation in *E. coli.*

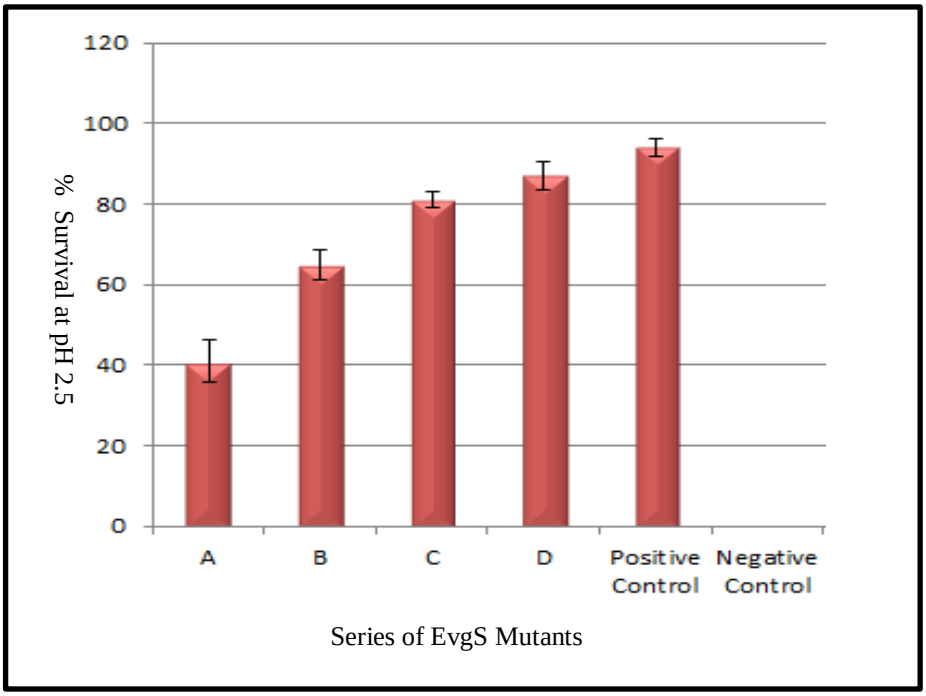


Figure 3.3 % Survival at pH 2.5

The figure describes % Survival at pH 2.5 with respect to pH 7 for EvgS mutants. The already studied and showing lacZ activity mutant was taken as positive control and pBAD 24 (Vector only) was taken as negative control. survival rate is different for each mutant All of the data points and error bars represent the average and standard deviation of 3 independent biological repeats respectively.

3.4 Correlation between AR assay with LacZ activity

In order to analyze the correlation between % Acid Resistance and LacZ activity of the EvgS mutants, the linier graph was plotted. To check this, only 4 independent mutants (i.e A, B, C and D) are taken for the consideration as, rests of the mutants are same as one of these 4 mutants.

R^2 Value from Figure3.4 suggests a very good correlation between lacZ activity values and % survival at pH 2.5 of the EvgS mutants. This strongly supports the hypothesis about the EvgS mutants that shows acid resistance phenotype.

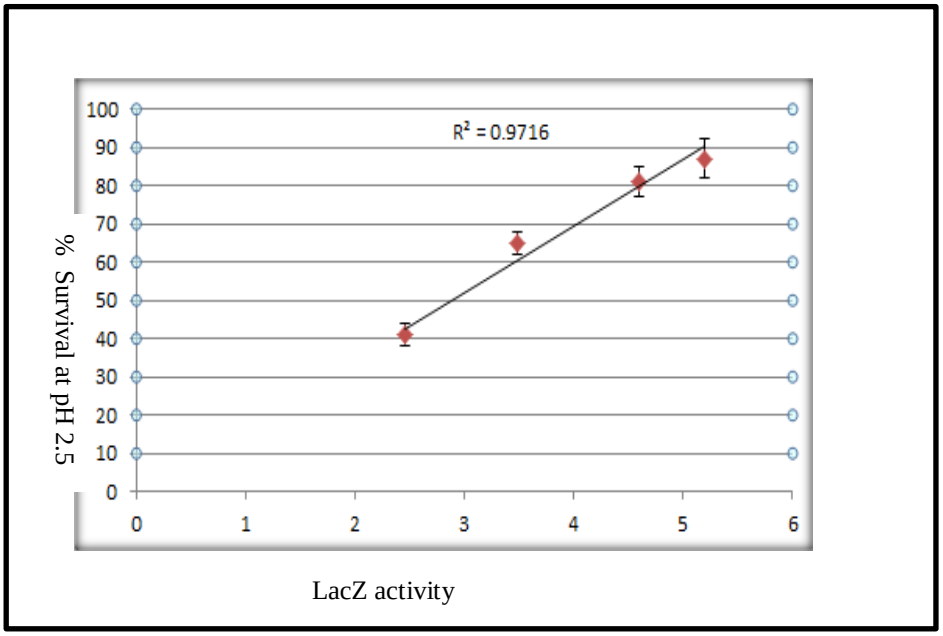


Figure 3.4 Survival at pH 2.5 V/S Lac Z activity

In order to analyze the correlation between % Acid Resistance and lacZ activity of the EvgS mutants, the linier graph was plotted. R^2 Value suggests a very good correlation between lacZ activity and % survival at pH 2.5 of the EvgS mutants. All of the data points and error bars represent the average and standard deviation of 3 independent biological repeats respectively.

3.5 Screening for Clone of A) pBAD EvgS his mutant B) pBAD EvgS his mutant ΔSwa/Psi

Previous study in Lund laboratory showed that, the SwaI/PsiI deletion from the otherwise wildtype gene shows no lacZ activity at either pH 7.5 or pH 5.7. Therefore it requires combination with constitutively activating mutations in order to check whether the deletion disrupts the ability of protein to detect acid. In order to check this hypothesis, we have cloned pBAD EvgS his mutant and pBAD EvgS his mutant ΔSwaI/PsIi. Three Constitive mutants , A,B and C are used as insert in both the cases.

In order to screen the clones, restriction digestion of ligated product of pBAD EvgS-his mutant, and EvgS-his mutant/ΔSwaI-PsiI with SalI was performed as described in materials and methods. The (fig. 3.5 A) explains the insert size for respective clones. Undigested plasmids were taken as control. Once the cloning for each constitutive mutants were confirmed, these strains then taken forward to calculate the lacZ activity in order to check role of SwaI/PsiI deletion in lacZ activity.

The fig (3.5 B) suggests that that there is always a slight drop in lacZ activity with the ΔSwaI/PsiI mutation as compared to full length pBAD EvgS his , however, activity is still clearly very much higher than in the negative control. As region SwaI/PsiI with different genetic background shows lacZ activity which suggests that deletion of region may have affected the activity of the protein to detect acid when it is combined with constitutively active mutants.

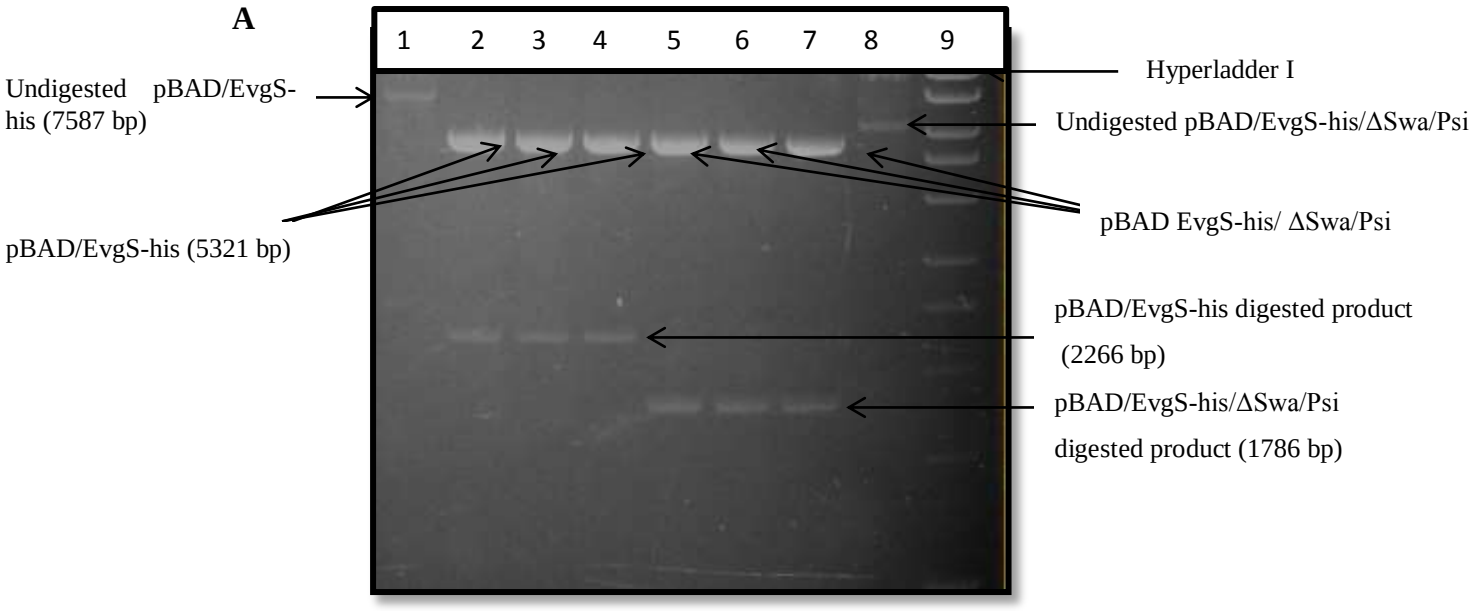


Figure 3.5 (A) Screening for Clone of A) pBAD EvgS his mutant B) pBAD EvgS his mutant ΔSwa/Psi

In order to check role of SwaI/PsiI deletion on lacZ activity, pBAD EvgS his mutants and pBAD EvgS his mutants ΔSwaI/PsiI were cloned. Three constitive mutants, A, B and C are used as insert in both the cases.

Lane 1: Undigested pBAD/EvgS-his, Lane 2-4: Restriction Digestion with SalI for pBAD EvgS his mutants, A, B and C respectively, Lane 5-7: Restriction Digestion with SalI for pBAD EvgS his mutants ΔSwaI-PsiI, A, B and C respectively, Lane 8: Undigested pBAD EvgSHis ΔSwa/Psi, Lane 9: Hyperladder I.

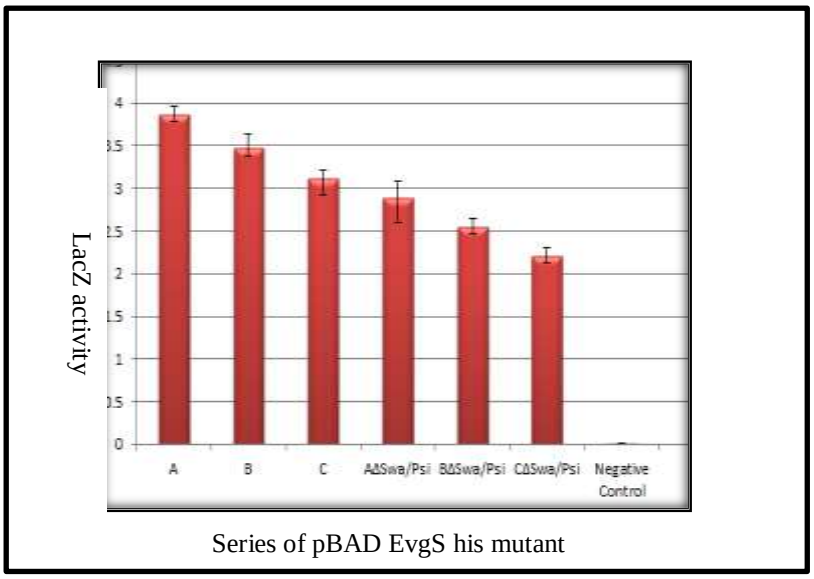


Figure 3.5 (B) LacZ activity of pBAD EvgS his mutant B) pBAD EvgS his mutant ΔSwa/Psi

To check role of SwaI/PsiI deletion on lacZ activity the above mentioned strains were taken forward to evaluate the lacZ activity. The figure suggests that that there is always a slight drop in lacZ activity with the ΔSwaI/PsiI mutatant as compared to full length pBAD EvgS his , however, activity is still clearly very much higher than in the negative control.

3.6 P1 Transduction

In 2003, Eguchi group showed that, a constitutively active mutant of EvgS conferred multi-drug resistance (MDR) to an *acrA* knockout *Escherichia coli*. This linked two-component system EvgAS with the expression of the MDR system. In order to check the hypothesis regarding link between EvgAS system with respect to MDR, we have constructed some strains with different genetic background.

The MG1655/pBAD24/ΔacrA strain was obtained from BW25113/ΔacrA::kan by phase P1 transduction as described in methods. In order to confirm the mutation transfer, colony PCR (Fig 3.6-A) was carried out with following combination of primers. Primers 1 and 2, Primers 1 and 4, Primers 3 and 2. Refer schematic representation, Fig 3.6 B.

The change in band size in lane 2, 3 and 4 in figure 3.6-A. is due to various products obtained from PCR with different set of primers, resulting in amplification of each respective region. Lane 7 and 8 does not show any band, as kanamycin markers were used in this particular PCR which could not amplify the specific region as the MG1655/pBAD24 strain does not have kanamycin cassette within.

The gel picture suggests that the mutation along with kanamycin cassette is transduced into MG1655 strain in order to obtain MG1655/pBAD24/ΔacrA:: kan. Once transfer of mutation was confirmed, kanamycin cassette was removed as described in methods, which was then confirmed by colony PCR with appropriate set of primers (Fig 3.6-C). MG1655/pBAD24 was taken as control. MG1655/pBAD24/ΔacrA::kan was loaded as a negative control. The controls were used in order to confirm the result about removal of kanamycin cassette.

Once the strain, MG1655/pBAD24/ΔacrA was obtained then these candidates were taken forward for efflux assays, as described in methods. This was done to understand the relationship between the two-component system EvgAS and the expression of the MDR system.

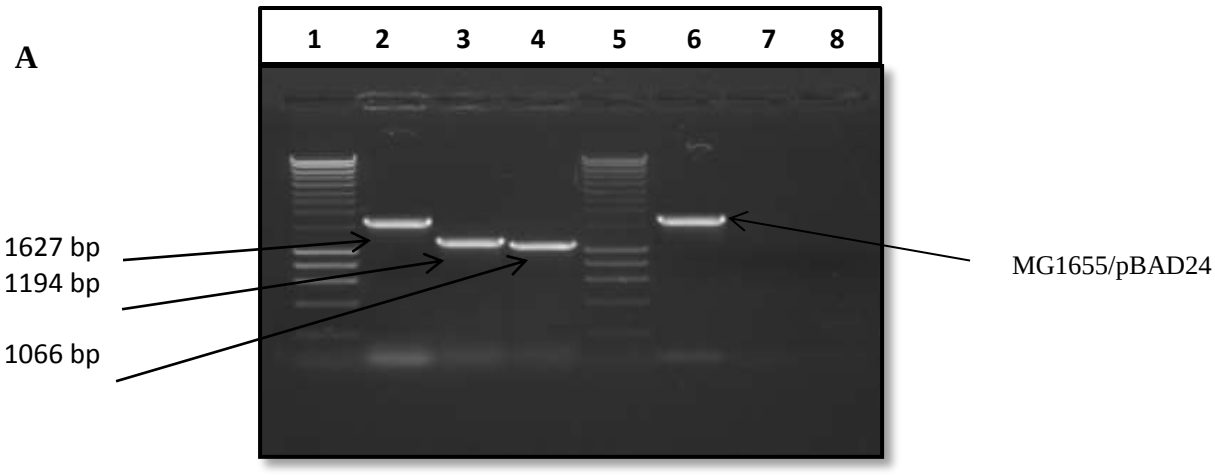


Figure 3.6 (A) Colony PCR: P1 Transduction.

The MG1655/pBAD24/ΔacrA strain was obtained by P1 transduction and confirmed by colony PCR using different sets of primers. Lane 1, 5: Hyperladder 1, Lane 2-4: MG1655/pBAD24/ΔacrA using sets of primers 1+ 2, 1+4 and 3+2 respectively. Lane 6-8: MG1655/pBAD24 PCR using Primer 1+ 2, 1+4 and 3+2 respectively. Refer Schematic representation of acrA::Kan (Fig 3.6-B)

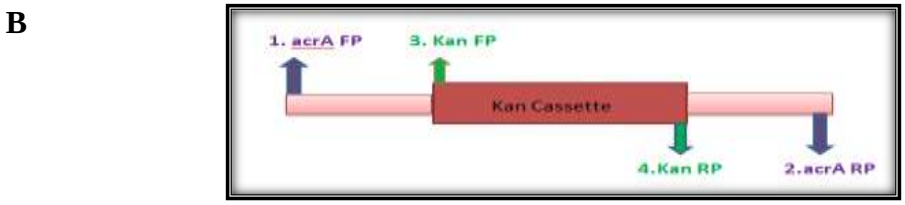


Figure 3.6 (B) Schematic representation of acrA::Kan

Schematic representation of MG1655/pBAD24/ΔacrA strain is shown.

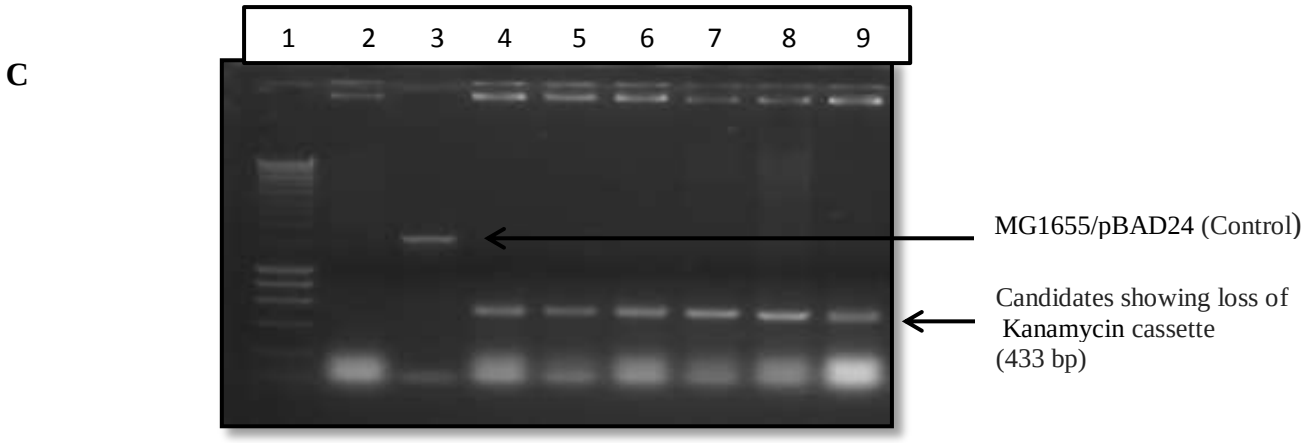


Figure 3.6 (C) Colony PCR: Removal of Kanamycin cassette

Removal of kanamycin cassette was confirmed by colony PCR.

Lane1: Hyperladder I, Lane2: MG1655/pBAD24/ΔacrA:: kan, Lane3: WT MG1655 contro,.Lane4-9: Candidates showing loss of Kanamycin cassette.

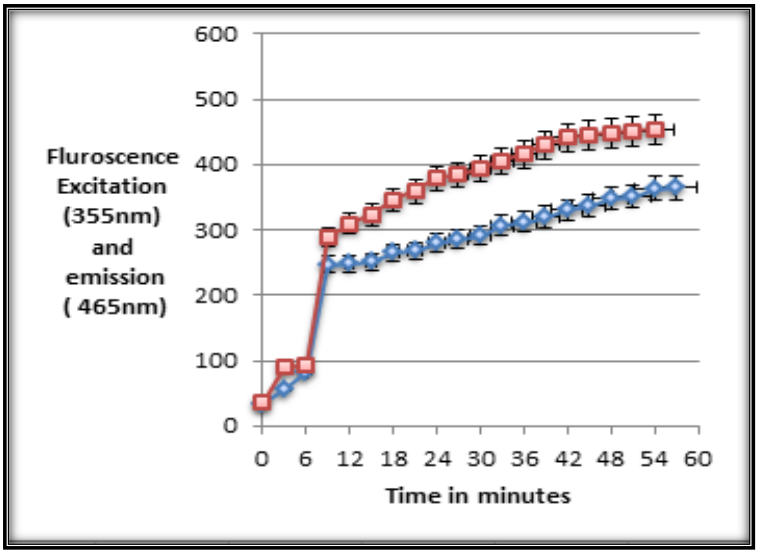
3.7 Efflux Assay

Membrane transporters or efflux pumps have been found in both prokaryotes and eukaryotes. Notably, some of these extrusion transport apparatus allow cells to achieve cellular self-defense mechanisms and resistance to noxious compounds, leading to multi-drug resistance (MDR) (Xu *et al*., 2003). H33342, used in our studies is a substrate for efflux pumps such as AcrAB-TolC. AcrA has a role as periplasmic adaptor protein (PAP) in most of the resistance nodulation division (RND) tripartite efflux pumps. AcrAB-TolC is considered one of the most important efflux pumps. This system discusses about innate multiple antibiotic resistance (Jessica *etal.*, 2009).

Previous study confirmed constitutively active mutant of EvgS (histidine kinase sensor) as multi-drug resistance (MDR) when it was deficient in acrA in *Escherichia coli*. This linked two-component system EvgAS with expression of the MDR system (Eguchi *et al*., 2003).

The objective of this experiment is to compare the fluorescence intensity of 2.5 µM bis-benzimide (Hoescht H33342) by various strains with reduced susceptibility to antibiotics. The strains with MG1655/ΔarcA/pBAD24 background were processed for efflux assay along with respective MG1655/pBAD24 control strains. Graphs were plotted containing fluroscence against time for each strain having respective controls for each. Comparative analysis of bis-benzimide accumulation into the cell was done (fig 3.7 A-H). A Change in molecular flux of H33342 reflects permeability or efflux activity. Bis-benzimide was used as a fluorescent marker. These dyes accumulate within cells and intercalate with DNA and thus change fluorescence wavelength optima, (Excitation 350 nm, Emission 461 nm) therefore this fluorescence is considered as marker of intracellular accumulation of drugs.

A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Time in minutes
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MG1655/pBAD24

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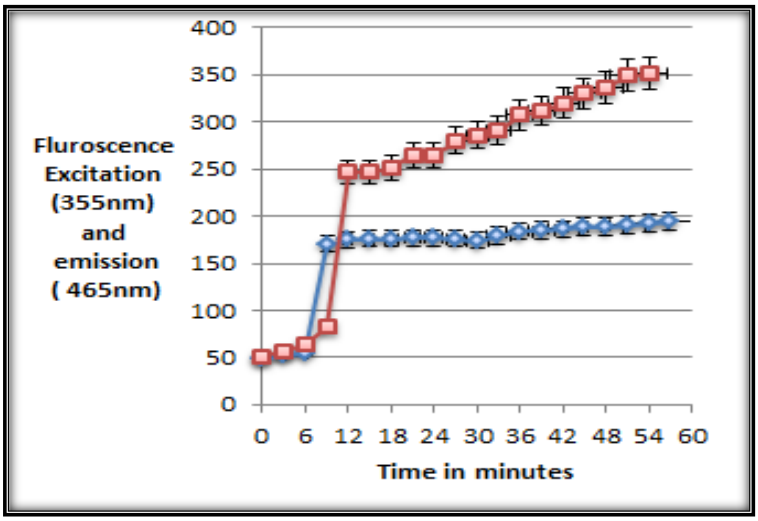
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MG1655/pBAD-EvgS-his

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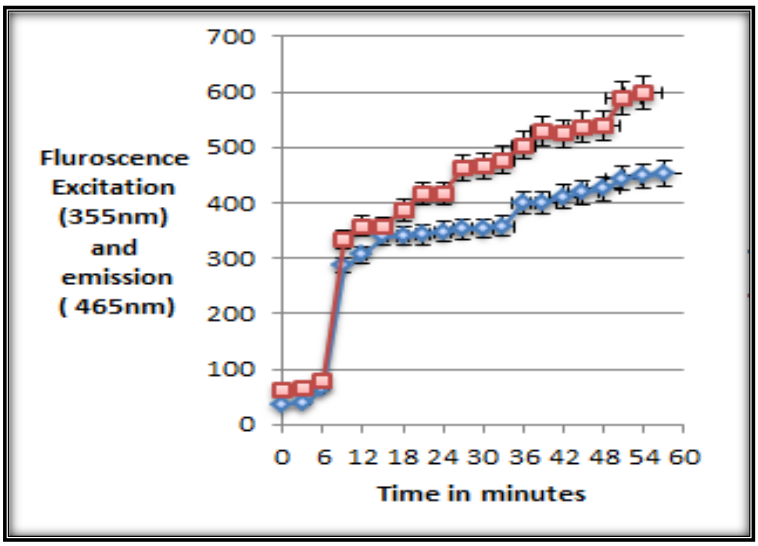
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MG1655/pBAD-EvgS-his/ΔClaI

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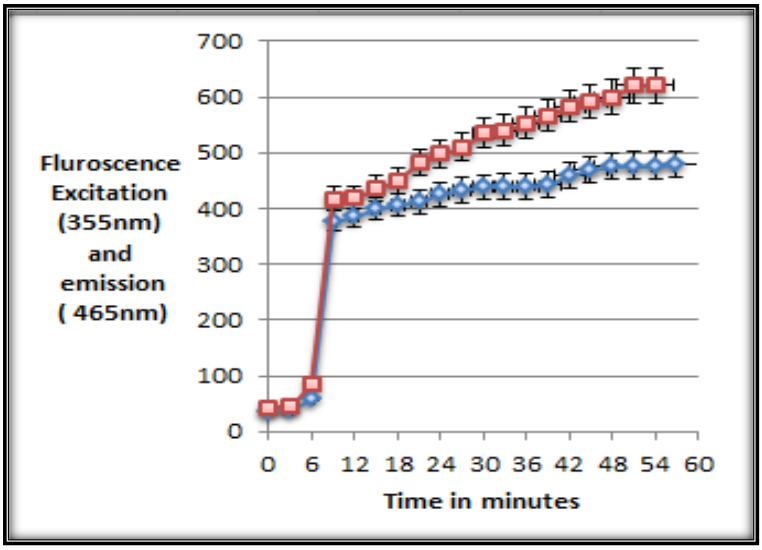
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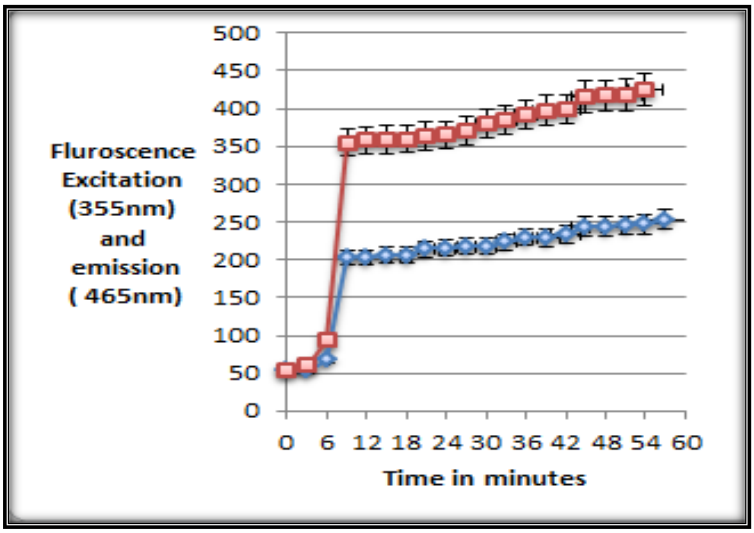
MG1655/pBAD-EvgS-his/ΔSwaI/PsiI

E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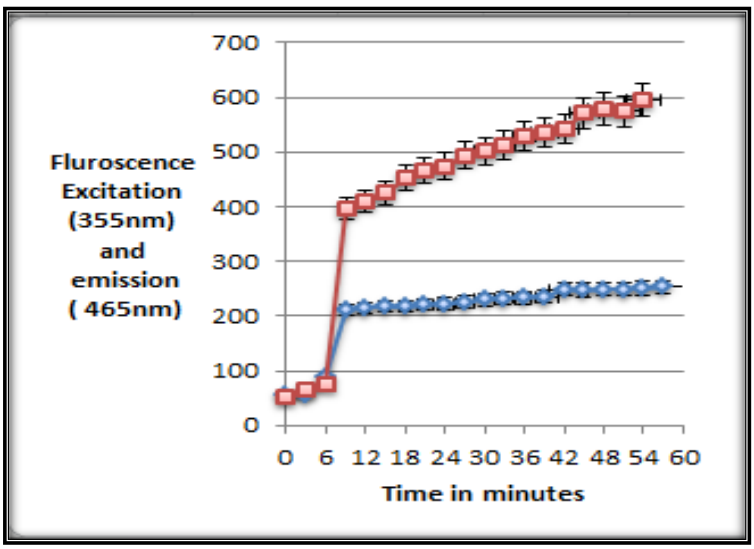
Time in minutes
MG1655/ΔarcA/pBAD-EvgS/Mutant A
MG1655/pBAD-EvgS/Mutant A

F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Time in minutes
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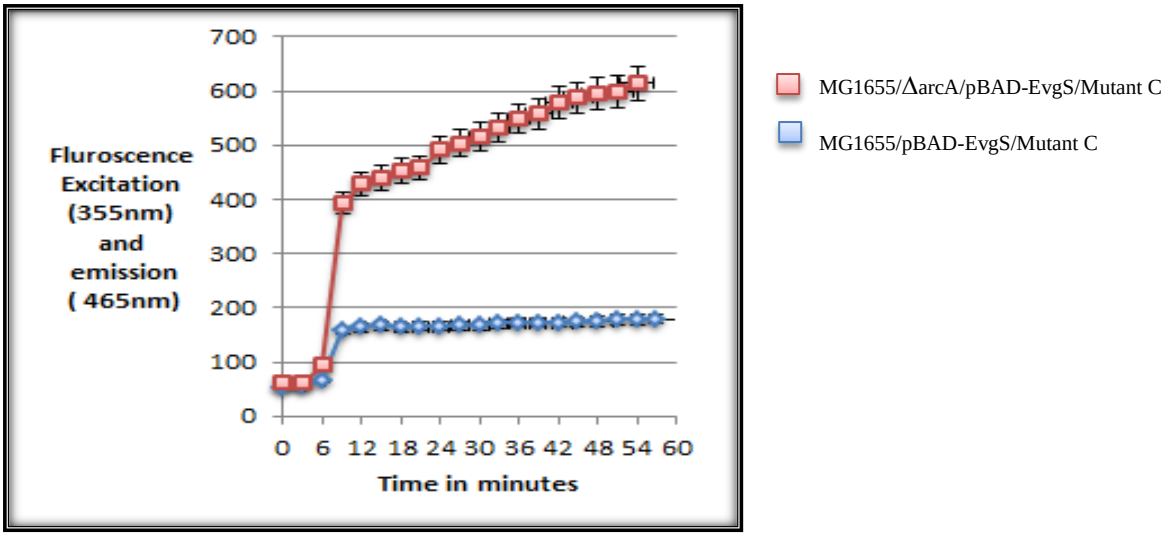
MG1655/pBAD-EvgS/Mutant B

G



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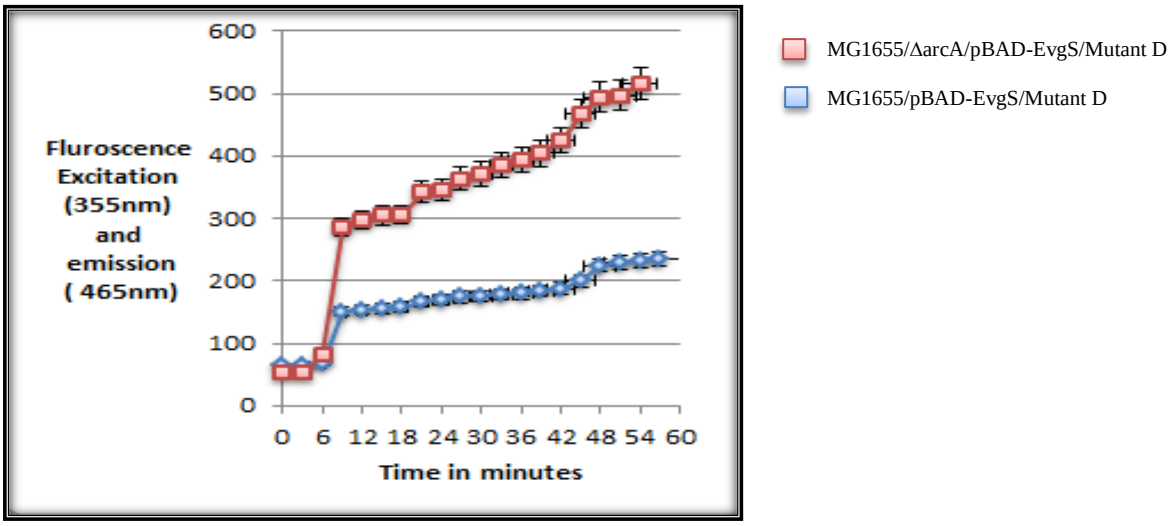

Figure 3.7 A-H Efflux Assay using bis-benzimide as a substrate.

Comparative analysis of bis-benzimide accumulation into the cell was done. Accumulation of bis-benzimide by the strains with MG1655/ΔarcA/pBAD24 background was processed for efflux assay along with respective MG1655/pBAD24 control strains. The figures (A-H) suggest that, in each case, ΔacrA mutants accumulate more bis-benzimide as compared to respective control strains.

As mentioned, the fluorescence activity is always linked to differences in pump activity between strains. The graphs plotted (Fig 3.7 A-H) suggest that accumulation of bis-benzimide by strains with MG1655/pBAD background is less than respective ΔacrA mutant. This is because these ΔacrA mutants lacking components of the major AcrAB-TolC efflux system. Thus they accumulate more bis-benzimide due to their lack of efflux activiy and allow cells to achieve cellular self-defense mechanisms and resistance to noxious compounds, leading to multi-drug resistance (MDR). This supports previous findings confirming the role of EvgAS system in expression of genes that confirms resistance to the drugs.

CHAPTER 4

The research into the mechanism of response to acid stress in *E. coli* has revealed a very complicated regulatory network, to which, this study has supplementary contributions. Our studies regarding mutagenesis and phenotypic assays have been useful for better understanding of the acid resistance phenotype.

The results presented in this report suggest that, the mutations characterized by genome sequencing included *evgS* mutations that were found in all evolved strains. EvgS is the HK of the EvgAS TCS that has been shown to respond to mild acid shock and activate acid resistance genes. The *evgS* mutations were found to be located in PAS domain of the EvgS protein. The *evgS* mutations were characterized and it is found that these mutations confirmed high level of acid resistance and AR2 promoter activity, which proposed that *evgS* mutations have altered the structure and consequently function of EvgS protein, therefore caused it to become constitutively on. The AR2 network, when it is normally inactive, seen to be activated by EvgS which was dissected by analyzing acid resistance in an EvgS strain in different knockout background. The results indicated that EvgS mutations confer an acid resistance phenotype and activation of AR2 network. The mutations characterized by this study could provide insights into the function of EvgS protein.

Another part of our study was to understand the connection between the EvgAS system and multi-drug resistance (MDR) expression using efflux assay. Our results demonstrates that AcrA, the most important tripartite efflux pumps is a vital component of the efflux process and without that proficient efflux activity is significantly reduced. This supports previous hypothesis that the AcrA has an active role in the pump activity.

Additional exploration of this network will elucidate the physiological function of the EvgAS system and recognize the environmental signal to which EvgS responds. This particular study will provides platform in order to understand the mechanism of multi drug resistance in *E. coli.*

CHAPTER 5

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Molecular mechanisms of lipid biosynthesis and transport in mycobacteria

UNIVERSITY OF BIRMINGHAM

Project Thesis
Submitted To
School of Bioscience, University of Birmingham
In Partial Fulfillment
Of the Requirements for the Award of the Degree Of
MRes in Molecular and Cellular Biology

By
Prachi P. Pathak

DECLARATION

The work presented in this thesis was carried out in the School of Biosciences at the University of Birmingham, U.K during the period March 2013 to August 2013. The work in this thesis is original except where acknowledged by references.

No part of the work is being, or has been submitted for a degree, diploma or any other qualification at any other University.

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Last but not the least I would like to thank my family and my classmates who have been supportive during my studies.

Prachi P. Pathak

ABSTRACT

Mycobacterium smegmatis is fast growing and nonpathogenic bacterium which has cell wall structure similar to the *Mycobacterium tuberculosis*, which is a causative agent of tuberculosis (TB) in humans. In order to identify and study the genes which are involved in the synthesis of mycobacterial cell wall components, *M. smegmatis* is extensively been used as a model organism mainly due to availability of their genome sequences.

Most of the antitubercular drugs target the cell wall containing mycolyl-arabinogalactan-peptidoglycan complex which is essential in mycobacterial sp such as *M. tuberculosis*. Mycolic acids are essential components of the mycobacterial cell wall. In this study, we confirmed that gene encoding permease can be deleted in *M. smegmatis* without affecting cell viability. Deletion/knockout of *MSMEG4721,* ortholog of *M. t*uberculosis *Rv2508* significantly modified the culture characteristics when compared with wild type. Mutant with altered phenotype were further complemented with their respective orthologues in mycobacteria in order to characterize their functions. In this study we have identified and characterized this particular mutant which is found to be involved in the process of mycolic acid reduction.

Altogether, the identification and characterization of such cell wall biosynthetic drug targets is promising research subject especially with the occurrence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains. The findings in this study not only help to understand complexities of mycobacterial cell wall biosynthesis but also denote potential new drug target for tuberculosis.

Keywords: Tuberculosis, *Mycobacterium smegmatis*, Mycolic acid biosynthesis, *MSMEG4721, Rv2508.*

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ABBREVIATIONS

AG	Arabinogalactan
AIDS	Acquired immune-deficiency virus
Ap	Ampicilin
BCG	Bacille Calmette-Guerin
EMB	Ethambutol
G	Grams
H	Hour
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HmB	Hygromycin-B
Km	Kanamycin
LAM	Lipoarabinomanan
LB	Luria-Bertani
LM	Lipomanan
M	Molar
MDR	Multi-drug resistant
Mg	Milligram
mM	Millimolar
MS	Mass Spectroscopy
NMR	Nuclear magnetic resonance
OD	Optical density
PCR	Polymerase chain reaction
PG	Peptidoglycan

TB	Tuberculosis
TSB	Tryptic-Soy Broth
v/v	Volume/volume
w/v	Weight per volume
µg	Microgram
µl	Microliter
µM	Micromolar

CHAPTER 1

1.1 Tuberculosis

The mycobacteria causing tuberculosis (TB) is commonly found infectious disease in humans, which can cause severe illness or even death throughout the world. The World Health Organization (WHO) estimated that in spite of vaccination of Bacille -Calmette Guerin (BCG), nearly one-third of the world population is infected by *Mycobacterium tuberculosis.* The situation has even worsened because of the presence of some most considering factors like, limited response to chemotherapy, HIV co-infection, changing efficiency of BCG vaccine and most importantly occurrence of multidrugresistant TB (MDR-TB) (Culliton *et al.,* 1992, Butler *et al.,* 2000). Moreover, in 2011, at least once case of extremely-drug resistant TB (XDR-TB) had been reported by 55 countries worldwide (WHO 2011).

The current increase in number of TB cases and especially occurrence of drug resistant mycobacteria indicate crucial requirement of novel anti-TB drugs. Existing anti- TB drugs cannot effectively kill the *M. tuberculosis* as the organism becomes persistent due to long duration of TB treatment. Recent developments in understanding the biological mechanism of the organism and the accessibility of the genome sequence give a chance to explore a wide spectrum of new targets for anti-TB drugs. It is proposed that the application of microarray and proteomics as genomic tools, if combined with recent approaches, such as structure-based drug design and discovery of new molecular scaffolds will surely lead to the development of new drugs that will certainly achieve effective control.

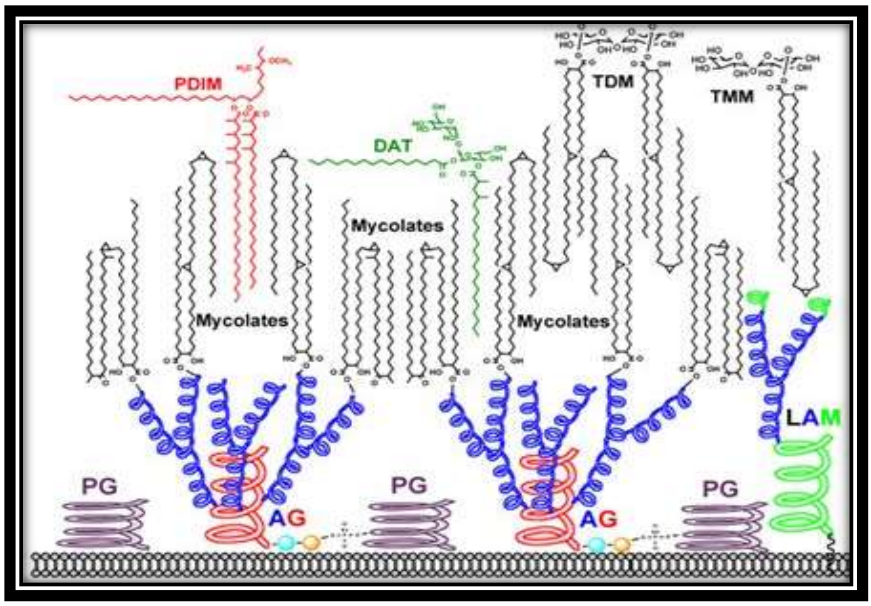
1.2 Mycobacterial cell wall

The complex structure of mycobacterial cell wall plays a vital role in drug resistance and virulence (Karakousis *et al*, 2004). The compositional complexity of mycobacterial cell wall (Fig 1.1) distinguishes *mycobacterium* species from the majority of the other prokaryotes. It is classified as Gram positive bacterium, however, their envelope share notable features with gram negative cell wall such as outer permeability barrier acting as pseudo-outer membrane (Bernan *et al.*, 1995).

The structural features of the cell wall envelope can be divided into three major components,

1. Plasma membrane.
2. The mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Besera & Bernan, 1997), consisting of three covalently linked macromolecules, peptidoglycan (PG), arabinogalactan (AG) and hydrophobic mycolic acids which decorate the non-reducing terminus of arabinan domains.
3. Lipoarabinimanan (LAM), lipomanan (LM), non-covalently bound lipids and glycolipids.

Such a unique arrangement in the outer envelope lowers the permeability to hydrophobic small molecule and renders resistant to many drugs (Brennen and Nikaido, 1995). Besides their functional role, these glycoconjugates are also responsible for the modulation of host response during infection (Chatterjee & Khoo, 1998, Maeda et al., 2003). Most of the genetic elements involved in their biosynthesis are essential for the survival of *Mycobacterium tuberculosis* therefore they represent potential drug targets.



I

Fig 1.1 Diagramatic illustration of mycobacterial cell wall. Adapted from Minnikin *et al* (2002).

The cell envelope consists of a cell wall core, plasma membrane, a capsule and an outer membrane. Lipoarabinimanan (LAM), mycolyl-arabinogalactan-peptidoglycan (mAGP), phthiocerol dimycocerosates (PDIM), peptidoglycan (PG), sulfated tetra-acyl trehalose (SL), diacyl trehalose (DAT), penta diacyl trehalose (PAT), trehalose monomycolate (TMM), trehalose dimycolate (TDM), N-acetyl muramic acid (NAM), N-acetyl glucosamine (NAG) and arabinogalactan (AG).

1.3 Mycobacterial cell wall: As a drug target

In case of MDR and XDR strains of *Mycobacterium tuberculosis*, the antibacterial compounds which can act on actively growing bacterium may help toward the treatment of TB (Bhowruth *et al*., 2007). In this context, cell wall inhibitors are widely used targets of chemotherapy. Agents such as INH and EMB which are inhibitors of mycolic acids and arabinogalactan (AG) biosynthesis respectively have proved highly successful against combating the disease (Bhatt *et al.*, 2007, Bhowruth *et al*., 2008). However due to development of drug resistant strains, certainly, the identification of novel drug targets and ultimately development of active compounds against them is an urgent requisite. In this respect the biosynthetic mechanism of mycobacteroial cell wall signifies an striking drug target (Bhatt et *al*., 2007).

1.4 Mycolic acid synthesis

Mycolic acids are integral part of mycobacterial cell wall, existing in either free form, bound to trehalose or associated with surface sugars making it complex mesh like structure. The significance of mycolic acids for mycobacteria has been discussed above. It is for this reason the biosynthesis of mycolates has been seen as a probable target for drug development.

Mycolates are produced by two distinct systems, Type I and type II fatty acid synthases (FAS-I and FAS-II) in *Mycobacterium sp* (Fig 1.1).The biogenesis of mycolic acid takes place in 4 stages (Brennan and Nikaido, 1995).

1. Synthesis of C24-C26 straight chain saturated fatty acids to produce C-1 and C-2 atoms and the α-alkyl chain.
2. Synthesis of meromycolic acids (C40-C60 acids) to deliver a main carbon backbone.
3. Modification of this backbone to produce other functional groups.
4. Condensation reaction to produce premycolic acid which on reduction produces mature mycolic acid.

The Mycolic acid synthesis is accomplished by concerted effort of FAS-I and FAS-II system. A particular gene fab (Rv2524c), encodes the multifunctional FAS-I system of *M. tuberculosis.* The gene expressing a protein forms homodimer, having domains of enoyl reductase, acyltransferase,, malonyl/palmitoyl transferase, dehydratase,,6-keto acyl reductase, 6 -ketoacyl synthase and acyl carrier protein, generating short chain fatty acyl-coA derivative for further elongation by FAS-II and to hexacosanoyl-S-coA for final condensation step (Takayama *et al,*2005).

FAS-II consists of group of enzymes elongating the fatty acyl-coA substrates into C-56 mero mycolate. In the first cycle, C16 acyl-AcpM is directly reduced by a 6-ketoacyl-AcpM reductase, FabG1 (MabA, Rv 1483) reduction via an enoyl-AcpM reductase. InhA (Rv 1484) (Gurvitz *et al* 2008).After the first cycle, MtfabH is replaced by KasA/KasB (Rv 2245/46) (Marrakchi *et al.,* 2002) which is followed by dehydration catalyzed by a 6-hydroxyacyl-AcpM dehydratase (Rv 0636) (Brown *et al*., 2007). KasA and KasB, the two discrete beta-ketoacyl synthases catalyze the condensation of the acyl-AcpM and malonyl-AcpM which results in a further increment of two carbon units (Fig 1.1) (Bhatt *et al.,* 2005).After about 20 cycles FAS-II produces a meromycolic acid (C56) which then undergoes condensation reaction to form a pre-mycolic acid (Bhatt *et al.,* 2007).

The acetyl-CoA carboxylase,AccD4 and AccD 5,carboxylate C_{26}-S_CoA after its synthesis by FAS-I and affords 2-carboxyl-C_{26}-CoA.Simulteniously a fatty acyl-AMP ligase,Fad32(Rv3801c) converts meromycolic acid derived from FAS-II to meromycolyl-AMP(Trivedi *et al*., 2004). The 2-carboxyl- C_{26}-CoA and mature meromycolyl-AMP undergo the final Claisen type condensation is catalyzed by polyketide synthase-13,Pk13(Rv 3800) (Gande *et al.*, 2004). Similar to FAS-I,Pks-13 also has multidomain structure with two phosphopantothein (PBP) domains, thioesterase (TE), acyl transferase (AT) and ketoaacyl synthase (KS), domain. The condenasation reaction results in the formation of 3-Oxo-C_{78}- mycolate which then reduced by a reductase (Rv 2509) and generates mature C_{78}- mycolic acid. (Bhatt *et al.*, 2008). The further step is not yet known, however it is hypothesized that the mature mycolic acid then reacts with trehalose 6 phosphate to yield phosphorylated trehalose monomycolate (TMM-P) (Shimkata & Minatogawa,2000) which is transported outside the cell by an ABC transporter after its dephosphorylation by a phosphatase through a mycolic acid carrier (Takayama *et al.,*2005). Further, mycolic acid is transferred to the arabinogalactan-peptidoglycan complex as well as

TDM and TMM by the antigen 85 complex in order to produce the arabinogalactan-mycolate and trehalose dimycolate respectively (Fig 1.1) (Takayama *et al.*, 2005).

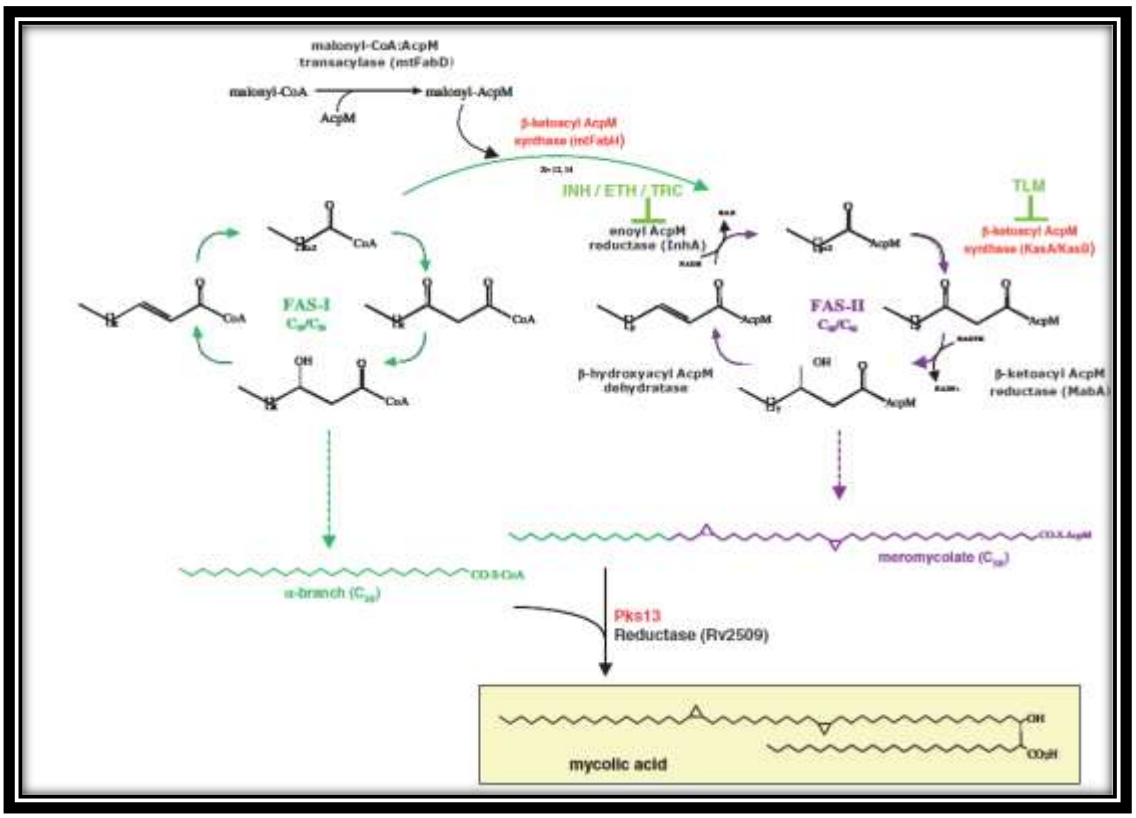


Fig.1.2 Pathway for Mycolic acid biosynthesis in *M. tuberculosis* (Adapted from Bhatt *et al.*, 2007)

Malonyl Co-A to malonyl-AcpM coversion is carried out by mFabD. Further, it is ligated by mtFabH to FAS-I derived C_{14}-Co-A.The FAS-II system continues to process C_{16} acyl-AcpM product producing meromycolates (C_{56}), followed by meromycolate precursors ligation to a FAS-I synthesized C_{26} fatty acid that constitutes the α branch of the final mycolic acid. Mycolic acids are then formed by the condensation of the α-branch and the meromycolate with the help of polyketide synthase Pks 13.

1.5 Background of the project

As described earlier, mycolates are a key element of the complex mycobacterial cell wall and consequently, disruption of mycolates is deleterious to cell survival. Many genes that have role in mycolate biosynthesis are thus shown to be essential and for this reason the enzymes involved in mycolate biosynthesis are seen as potential drug candidates.

In order to study cell wall biosynthetic pathways, *M. smegmatis* is been used as a substitute for *M. tuberculosis* as it is fast-growing, non-pathogenic, and most importantly this particular strain allows knockout of some genes which are essential in *M. tuberculosis* (Bhatt *et al.,* 2008). Thus, *M. smegmatis* is considered to be important organism in order to study cell wall biosynthesis genes. Moreover, despite of difference in mero-chain modifications between these two species, enzymes responsible for mycolate biosynthesis are identical (Bhatt *et al.,* 2008).

Many antituberculosis drugs, such as ethionamide, isoniazid, and thiolactomycin, target enzymes of mycolic acid biosynthetic pathway as this particular pathway is essential for mycobacterial survival. Previous reports from Dr. A. Bhatt's labs suggests the role of *Rv2509* in mycobacterial mycolic acid biosynthesis by generating a deletion mutant of it's homologue *MSMEG4722* in *M. smegmatis*. They deleted the gene encoding a reductase which was shown to be involved in the mycolic acid reduction in *Mycobacterium smegmatis.* Deletion of this particular gene did not affect the cell viability but altered culture characteristics and antibiotic sensitivity, emphasizing the potential of *Rv2509* as a "secondary" drug target (Bhatt *et al.,* 2008). This study probed the role of the extended region surrounding Rv2509.

Taking the above study as a platform, we hypothesized that *MSMEG4721*, which is the homologue of *2508c* in *M. tuberculosis* and it is in the same cluster as of *MSMEG4722* may have some significant role in mycolic acid biosynthesis. This hypothesis with respect to molecular mechanism of mycolic acid biosynthesis in mycobacteria has been tested by construction of a deletion knockout of *MSMEG4721* followed by characterization of the mutant strain.

1.6 Aim and objectives

Mycobacterial diseases are especially problematic to treat owing to the lipid-rich cell wall architecture. The mycobacterial cell wall is important for it's growth and survival. Due to development of MDR and XDR-TB cases leading to discover of novel drug targets and specific antimycobacterial compounds against them. (Telenti *et al.*, 1997).

The literature review has described the mechanism of mycolic acid biosynthesis in mycobacteria. The aim of the project was to study molecular mechanism of lipid biosynthesis in mycobacteria by making deletion mutant of *MSMEG4721* in *M. smegmatis* mc^2 155 which is in the same gene cluster as *MSMEG4722*, a gene encoding a reductase which had already shown to be involved in the final step of mycolic acid biosynthesis.

To check this, following objectives were planned to understand the role of *MSMEG4721.*

- To construct deletion mutant of *MSMEG 4721*.
- Characterization of mutant: To understand the role of *MSMEG 4721* in mycolic acid reduction.
- Complementing the gene in order to restore WT *M. smegmatis* mc^2 155 conditions.

Characterization of the lab based genetically modified strain would certainly help to understand the importance of this particular gene involved in mycolic acid biosynthesis.

CHAPTER 2

2.1 Plasmids, Bacterial Strains, and Phages Used in This Study

	Description	**Reference**
Plasmids		
pMV261	*E. coli-Mycobacterium* shuttle plasmid vector with*hsp60* promoter and Kan^R cassette (*aph*)	Stover *et al.* (1991)
pMV261-*MSMEG471*	*MSMEG4721* cloned in pMV261	This work
pMV261-*Rv2508*	*Rv2508* cloned in pMV261	This work
pΔ*MSMEG4721*	Derivative of p0004s designed for allelic exchange of*M. smegmatis MSMEG4721*	This work
Bacterial strains		
mc^2155	Electroporation-proficient *ept* mutant of *M. megmatis*strain mc^26	Snapper *et al*. (1990)
Δ*MSMEG4721*	Deletion mutant of mc^2155 in which *MSMEG4721* is replaced by *hyg*	This work
Δ*MSMEG4721*-C	Complemented strain of Δ*MSMEG4721* containing pMV261-*MSMEG4721*	This work
Δ*MSMEG4721*-CRv	Complemented strain of Δ*MSMEG4721* containing pMV261-*Rv2508*	This work
Phages		
phΔ*MSMEG*4721	Derivative of phAE159 obtained by cloning pΔ*MSMEG4721* into its unique PacI site	Obtained from lab.

2.2 Chemical, reagents and growth condition

All solvents and chemicals were ordered from Biorad (CA, USA), Fischer chemicals, UK and Sigma-Aldrich (Dorset, UK). Enzymes were purchased from Sigma-Aldrich (Dorset, UK), Roche (Lewes, UK) or New England Biolabs (Boston, USA) and were the highest grade available. All chemicals and solvents were AnalR grade.

2.3 Bacterial growth condition

E. coli was grown at 37°C, unless stated and with aeration at 180rpm in shaking incubator. Luria Bertani broth (LB : 0.5% w/v yeast extract, 1% w/v tryptone, 1% w/v salt; pH 7) was used for standard growing conditions. LB agar (LB broth + 1.5% w/v bacto agar; pH 7) was used where plating of bacteria was required. Both LB and LB agar were made up in distilled water and sterilized by autoclaving.

M .smegmatis strains were grown in Tryptic soy broth (TSB, Difco), containing 0.05% Tween 80 (TSBT). *M .smegmatis a*nd mycobacteriophase were also propagated on Middlebrook 7H9 broth (Difco), with 0.2% glycerol and 0.05% Tween 80. Nutrient agar was made by adding 1.5% agar to above mentioned broth. The concentration of antibiotics for *M .smegmatis* were 25 µg/ml for kanamycin and 100 µg/ml for hygromycin-B.

2.4 General Molecular Biology Techniques

2.4.1 Plasmid Preparation *E.coli* strains.

This method is used to isolate the plasmid DNA from bacterial cell overnight culture containing appropriate antibiotic. The plasmid was isolated using Quagen plasmid mini kit as per manufacturer's instruction.

2.4.2 Preparation of Competent cells for *E.coli* strains.

One ml of appropriate overnight culture was scaled up to 100 ml fresh LB media in a 250 ml conical flask. 20mM $MgSO_4$ was added to it. This was grown at 37°C with shaking (180rpm) until the culture reached an OD (A_{600nm}) of 0.4 to 0.5. It was followed by incubation on ice for 30 minutes. The cultures were then pelleted (4000 rpm, 4°C, 10 minutes) and the supernatant was removed by decanting then pellet was re-suspended in 40 ml of ice cold TFB1 (30 mM Potassium acetate,10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% Glycerol, pH was adjusted to 5.8 with 1M acetic acid). The cells were incubated on ice for 5 minutes and then pelleted (4500 g, 4°C, 10 minutes). The supernatant removed by decanting and the pellet was re-suspended in 4 ml of TFB2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% Glycerol). It was then split into 200 µl aliquots for the use of transformation.

2.4.3 Plasmid Transformation

An aliquot of competent cells was thawed on ice and Plasmid DNA (~50-70 ng) was added to it and the contents were mixed properly by swirling the eppendorf gently. The mixture was kept on ice for 10 minutes. Heat shock was given at 42^oC for 45 seconds. 0.9 ml LB was added to it and incubated at 37°C for 1 hour in a shaker incubator. The transformed cells were then spin at 6000 rpm for 5 minutes, supernatant was decanted, with the exception of 100µL and resuspended the pellet in this 100uL and plated onto selective agar.

2.4.4 Genomic DNA preparation of *M. smegmatis.*

Once 10 ml *M. smegmatis* culture reached an OD (A_{600nm}) of 0.8-1, it was pelleted down at 4500 rpm for 15 minutes at room temperature. Supernatant was discarded. 500 µl of P1 buffer (Quagen mini kit) containing 50 mg/ml freshly added lysozyme. It was then mixed and incubated at 37 ^{o}C for 4 hours. 100 µl 10% SDS and mixed gently. Further, 50 µl 10 mg/ml Proteinase K was added freshly and incubated at RT for 1 hour. To that, 200 µl 5 M NaCl was added and mixed gently. Equal volume of chloroform was added and mixed by inverting which was followed by spin at 13,000 rpm for 10 minutes. Supernatant was transferred to new tube. Chloroform step was repeated again. Further, 0.7 volume of isopropanol was added and mixed by inverting. Tubes were incubated for 30 minutes at RT, followed by spin at 13,000 rpm for 15 minutes. Pellet was then washed with ethanol. Further pellet was air dried and dissolved in 30 µl of elution buffer in order to obtain genomic DNA.

2.4.5 Preparation of electrocompetent *M. smegmatis* cells

1 ml of overnight culture was scaled up to 100 ml fresh TSB media in a 500 ml conical flask. This was grown at 37°C with shaking (180rpm) until the culture reached an OD (A600nm) of 0.6. Further culture was shifted on ice .The culture was then pelleted (4000 rpm, 4°C, 30 minutes). The supernatant removed by decanting and then the pellet was washed 3 times with 10% Glycerol. (Filter sterilized). Finally the pellet was re-suspended in 1 ml of 10% glycerol. Aliquots were then used for electroporation

2.4.6 Electroporation

10 µl of DNA was added to 100 µl of electrocompetent cell aliquots and mixed by flicking. Electroporation cuvettes were pre-chilled prior to use. The competent cell/DNA mix was pipetted into the electroporation cuvette. The samples were then electroporated using appropriate voltage as described earlier (snapper *et al.,* 1990). 1 ml TSB was added to it and it was shifted to 15 ml Falcon tube and incubated at 37°C for 1 hour in a shaker incubator. The transformed cells were then spun at 8000 rpm for 5 minutes. Supernatant was decanted, with the exception of 100 µl and resuspended the pellet in this 100 µl and plated onto selective agar.

2.4.7 Gene Cloning

PCR amplification

Gene of interest was used as a template for amplification. Specially designed primers were used for PCR amplification. Gradient PCR machine was used to amplify the gene and standardize the annealing temperature. Gradient was kept between 65°-75°C. The amplified products were checked on 1% agarose gel and the size confirmed by loading a 1kb DNA ladder. The temperature which was found appropriate was used to amplify the gene.

Restriction digestion of vector and inserts

Once the amplified genes were obtained, restriction digestion was carried out for the vector and gene insert. The specific restriction enzymes were used to cut both the vector and the genes at specific sites and generate overhangs so that both the vector and the insert can be ligated under appropriate conditions.

Ligation

The digested vector with gene insert (In molar ratio 1:3) were then ligated with the help of T4 DNA ligase in a reaction mix containing ligation buffer in final concentration of 1X. The ligation was carried out at RT for 10 minutes. Ligated product along with control (vector alone) was then transformed and plated on selective agar and kept at 37 ^{o}C overnight as described earlier.

Clone screening

The colonies obtained during ligation were then inoculated in liquid media along with appropriate antibiotic in order to obtain plasmid as described earlier. Plasmid was then digested with appropriate restriction enzymes and product was then run on 1% agarose gel along with control. Probable positive clones were then confirmed by sequencing.

2.5 Construction of *MSMEG*Δ*4721*

The phasmid DNA of the knockout phage phΔ*MSMEG4721* was already constructed at the start of this project. The recovered phasmid was further transformed into *M. smegmatis* strain via electroporation and then cells were grown at 30^{o}C for 3 days to yield phase particles. Generation of high titre phase particles involved 200 µl of 0.8 $OD_{600\ nm.}$ Culture of *M. smegmatis* mixed into 5 ml molten7H9 soft agar and overlaid on basal layer of 7H9 agar. The phase stock was serially diluted (10^{-1}-10^{-10}) with MP buffer (50mM Tris.HCl pH 7.8, 150mM $MgSO_{4,}$ 2mM $CaCl_2$). Each dilution was spotted onto solidified overlay of *M .smegmatis.* Plates were incubated at 30^{o}C for 3 days, after which plaque forming units were counted and phase titre was calculated (pfu=number of pfu in spot*dilution factor*100). Original phase stock was diluted to yield approximately 500 plaques per plate and mixed with 200 µl of *M. smegmatis* in molten 7H9 agar, which was

overlaid on solid 7H9 agar. . Plates were incubated at 30^{o}C for 3 days, resulting in "lacy pattern". The high titre phase was then collected and filtered through 0.45 µM filter.

In order to perform specialized transduction, *M .smegmatis* cultures were grown to 0.8 $OD_{600\ nm}$ in 50 ml of TSB with 0.05%, Tween 80. Harvested cells were then washed three times with 50 ml MP buffer and finally resuspended in 5 ml MP buffer.0.5 ml of cells was then mixed with 0.5 ml of optimum high titre lysate (10^{10} pfu/ml) and incubated overnight at 37^{o}C,followed by plating onto TSB agar plate with hygromycin B (100µg/ml) . Plates were incubated at 37^{o}C for 1-2 weeks in order to obtain single isolated colonies. Deletion of *MSMEG4721* was confirmed by southern blot.

2.6 Southern blot analysis

Southern blot analysis was used to confirm gene knock-out mutants and was performed as specified in Roche Applied Science kit protocol. PCR products of 1kb representing the left and right flanking regions of *MSMEG4721* were used as probes. SacI digested genomic DNA form *M .smegmatis* WT $mc^{2}155$ and *M .smegmatis* Δ*4721* strains were subjected to the gel electrophoresis to separate the fragment. Following gel electrophoresis, the gel was depurinated (0.25 M HCl, 15 min) until the purple dye on gel turned yellow. The gel was then washed and put into denaturation solution for 15 minutes (1.5 M NaCl, 0.5 M NaOH) till the dye turned dark blue. The gel was washed and returned to neutralization solution (0.5 M Tris-HCl, pH 7.2, 1 M NaCl). The gel was subsequently transferred to positively charged nitrocellulose membrane via capillary action with 20xSSC (3 M NaCl, 0.3M sodium citrate, pH 7.0). DNA on membrane was covalently bound by crosslinking. The membrane was finally rinsed with distilled water and air dried. Probe preparation involved boiling at 105^{o}C, snap freezing and labeling with digoxigenin-dUTP, using DIG High prime DNA labeling and detection kit (Roche), as described in

manufacturer's protocol. The membrane was exposed to the probe at 65 ^{o}C overnight, followed by washing and finally immunodetected with antibody for 30 min at 37^{o}C. The hybridized probes were visualized with chemiluminescence.

2.7 Construction of complemented strains

MSMEG4721 gene was PCR amplified from parental *M. smegmatis* mc^{2}155 genomic DNA using the specific primers followed by cloning into the *E. coli-Mycobacterium* shuttle plasmid pMV261 with the primer incorporated HindIII and EcoRI sites, to obtain recombinant plasmid, pMV261-*MSMEG4721*. Similarly, the plasmid *pMV261-Rv2508* was also constructed with Rv2508, which was PCR amplified from *M. tuberculosis* H_{37}Rv genomic DNA with specific primers. The complemented strains Δ*MSMEG4721-C* and Δ*MSMEG4721-CRv* were obtained by selecting on kanamycin. Colonies were screened by restriction digestion. Positive clones then were transformed by electroporation with pMV261-*MSMEG4722* and pMV261-*Rv2508.* The complemented strains then were further used for lipid extraction along with Δ*MSMEG4721* and WT mc^{2}155 strains.

2.8 Apolar lipid extraction from mycobacterial cell wall

The strains Δ*MSMEG4721,* Δ*MSMEG4721-C* and Δ*MSMEG4721-CRv* along with WT mc^{2}155 were grown in 5 ml TSB at 37^{o}C for 2 days. The cultures were labeled with [^{14}C]-acetate (1µl/ml of culture). Next day, cells were pelleted down and used for lipid extraction. To the pellet 2ml of Methanol: 0.3% NaCl (10:1 v/v) and 1 ml of petroleum ether (60-80) was added. It was mixed on a rotator for 30 min, centrifuged at 4000 rpm for 5 min. The upper layer was transferred in a pre-weighed separate tube 'B'. 2ml of petroleum ether was added into lower layer (tuber 'A'),

mixed for 30 min on a rotator and then centrifuged for 5 min at 4000 rpm. Upper layer was transferred to tube 'B'. Tubes were dried on heating block (50^{o}C) under nitrogen in order to get apolar lipids. The dried pellet (Aploar Lipids) was resuspended in Chloroform: Methanol (2:1 v/v) solution. 5 µl was used to take radioactive counts.

2.9 Polar lipid extraction from mycobacterial cell wall

Further, into Tube 'A', 2.3ml of Chloroform: Methanol: 0.3% NaCl (9:10:3) was added to the pellet. Mixed for 60 min on the rotator and spun it for 3 min at 3500 rpm. Supernatant was transferred into tube 'C'.750 µl of Chloroform: Methanol: 0.3% NaCl (5:10:4) was added to the pellet of tube 'A'. Mixed for 30 min on a rotator, spun at 3500 rpm for 3 min. Supernatant was pooled in tube 'C'. This step was repeated once more to get Non-extractable Lipids (Tube 'A'). To the pooled extract, 1.3ml of Chloroform + 1.3ml of 0.3% NaCl was added, mixed for 5 min on a rotator and centrifuged for 3 min at 3500 rpm. The lower layer was collected and transferred to tube 'D' using long glass Pasteur pipette. The extract in tube 'D' was dried on heating block (50^{o}C) under nitrogen in order to get dry polar lipids. The dried pellet (Pola**r** Lipids) was resuspended in Chloroform: Methanol (2:1 v/v) solution to get Polar lipids.

2.10 TLC of apolar lipids

Equal count of the resulting solution of apolar lipid was subjected to TLC .Plates used for this experiment were silica gel plates (5735 silica gel 60F_{254}; Merck, Darmstadt, Germany). The samples were subjected to 2D-TLC System B .Direction 1 was Petroleum ether: Acetone (98:2 v/v).Direction 2 was Toluene: Acetone (98:2 v/v). The analysis of apolar lipids was additionally done with 2D-TLC System c .Direction 1 was Chloroform: Methanol (96:4 v/v). Direction 2 was

Toluene: Acetone (80:20 v/v). Autoradiograms were obtained by overnight exposure of Kodak X-Omat AR film to the plates to reveal [^{14}C]-labeled FAMEs and MAMEs. Data was analysed.

2.11 Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) extraction and visualization by TLC

For extraction of MAMEs, both the whole-cell pellets and delipidated cells were subjected to alkaline hydrolysis.this was done by 5% aqueous 2 ml of TBAH treatment at 95°C overnigh. Further, 4 ml CH_2Cl_2, 500 µl CH_3I, 2 ml water was added in to it which was then followed by mixing for 30 min. The aqueous phase (upper layer) was discarded following centrifugation, and the organic phase (lower layer) was washed thrice with water and evaporated under N_2 to dry. The resulting FAMEs and MAMEs were dissolved in 4 ml of diethyl ether, followed by sonication for 20 minutes. The residue (insoluble portion) was removed by centrifugation, and the ether solution evaporated to dryness and dissolved in 200 µl of $CHCl_3$:CH_3OH (2:1). 5 µl was used to take radioactive counts. Equal count of the resulting solution of FAMEs and MAMEs was subjected to TLC with silica gel plates and developed in petroleum ether-acetone (95:5). Autoradiograms having [^{14}C]-labeled FAMEs and MAMEs were obtained by exposingplates to to Kodak X-Omat AR film for 10-12 hours. Data was then analyzed.

CHAPTER 3

The literature review has described the mechanism of mycolic acid biosynthesis in mycobacteria. As described in methods, we made deletion mutant of *MSMEG4721* in *M. smegmatis* mc^2 155 which is in the same gene cluster as *MSMEG4722*, a gene encoding a reductase shown to be involved in the final step of mycolic acid reduction. The results will describe the functional link between these two genes.

3.1 Construction and growth of mutant Δ*MSMEG* 4721

To understand the role of *MSMEG4721 (*Homologue of Rv2508) in mycolic biosynthesis, we deleted *MSMEG4721* in *M. smegmatis* mc^2155 by specialized transduction (Bardarov *et al.*, 2002) (Figure 3.1-A). The ability to generate a knockout of this particular gene indicated that *MSMEG4721* was nonessential for the viability of *M. smegmatis* mc^2155. Deletion of *MSMEG4721* was confirmed by southern blot as described in methods. There were two SacI restriction sites in the wild type and three in the mutant due to presence of hyg^R gene marker (Figure 3.1 A). Following SacI digestion, one fragment was observed for wild-type and two fragments for *M. smegmatis*Δ*4721* (Figure 3.1-B).

Deletion of *MSMEG472*1 had significant change on the colony morphology in comparison with *M. smegmatis* mc^2155 when grown on tryptic soy broth (TSB) agar. Precisely, the colonies of the parental, wild-type strain mc^2155 were glossy, and the mutant strain Δ*MSMEG4721* had dry surface than that of wilt type strain (Figure 3.3). To check the hypothesis, whether an observed phenotype in the mutant strain is exclusively due to loss of *MSMEG4721*, we have cloned Δ*MSMEG4721-C* and Δ*MSMEG4721-CRv* and colonies were screened as described in methods. The figure 3.2 explains the insert size for respective clones. The respective phenotype on complementation of Δ*MMSMEG4721* could restore wild type characteristics demonstrating

that the experiential phenotypes in the mutant strain were only due to the loss of *MSMEG4721* (Figure 3.3 B).

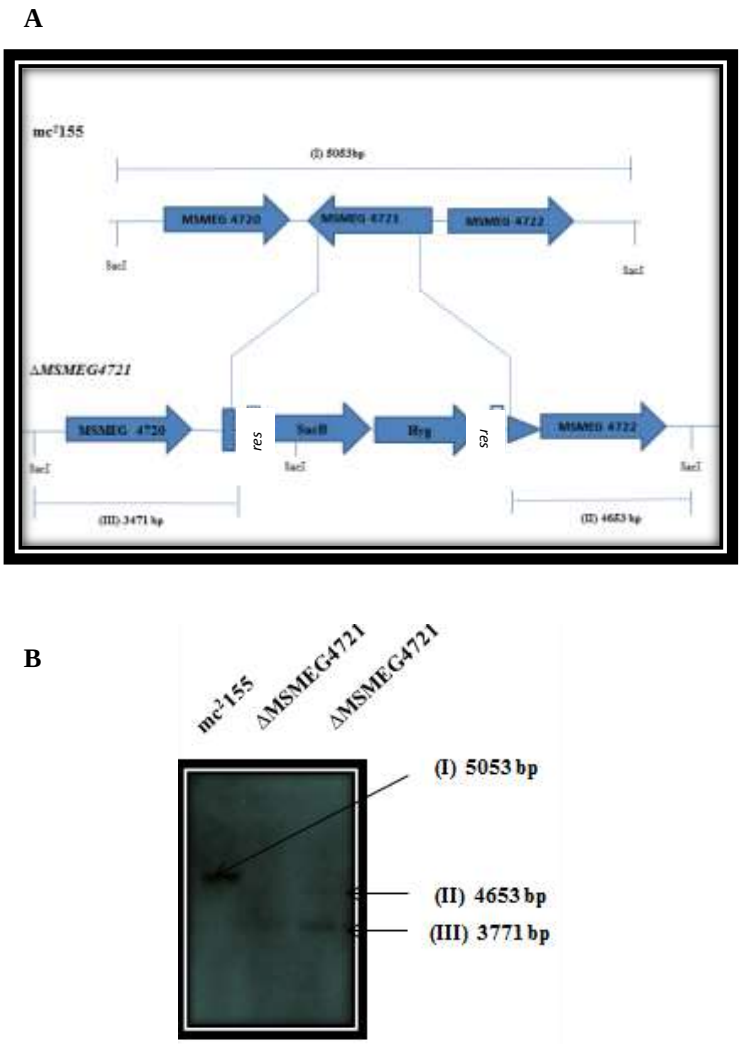


Figure 3.1 Construction of *MSMEG4721* knockout

A) Gene map of *MSMEG4721* region in the parental *M. smegmatis* strain mc^{2}155 and its resultant region in the Δ*MSMEG4721* mutant. *hyg*, hygromycin resistance gene from *Streptomyces hygroscopicus*; *sacB*, sucrose counterselectable gene from *Bacillus subtilis*. *res*, γδ resolvase site. Digoxigenin-labeled are indicated by thick lines with diamond-shaped ends.

B) SacI digested genomic DNA bands of strains of parental mc^{2}155 and *MSMEGΔ4721* reflected on Southern blot are indicated in roman numerals.

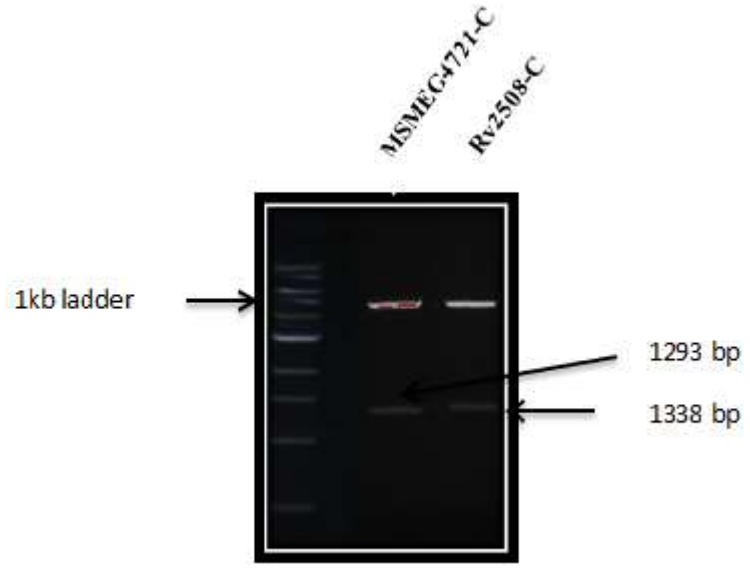


Figure 3.2 Clone screening

Clone screening of MSMEG4721-C and Rv 2508-C by restriction digestion using HindIII and EcoRI restriction enzymes. Expected band sizes were 1293bp and 1338 bp respectively.

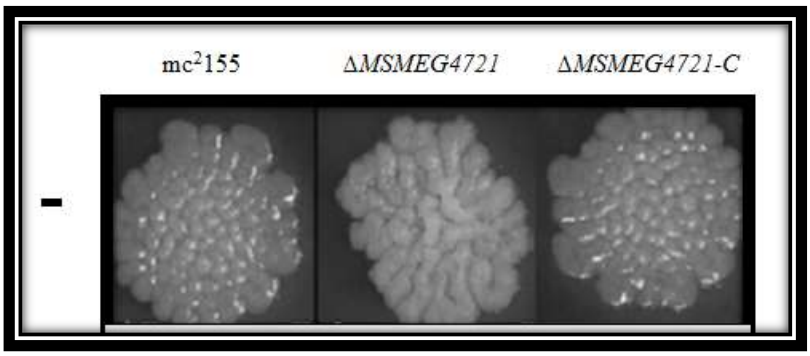


Figure 3.3 Colony morphology of wild-type (mc^{2}155), mutant (Δ*MSMEG4721*), and complemented (Δ*MSMEG4721*-C) strains.

Colonies of wild-type (mc^{2}155), mutant (Δ*MSMEG4721*), and complemented (Δ*MSMEG4721*-C) strains on TSB-agar without Tween-80.Colony growth was obtained by inoculating 5 µl of a broth culture of each strain on agar plate.

3.2 Characterization of mutant Δ*MSMEG4721*

As mentioned earlier, *MSMEG4722*, a gene encoding a reductase shown to be involved in the final step of mycolic acid reduction. (Bhatt *et al.*, 2008). In order to identify the functional link between *MSMEG4721 and MSMEG4722*, characterization of mutant Δ*MSMEG4721* was carried out.

The detected changes in the colony morphology of the Δ*MSMEG4721* mutant driven us to study mycolic acids in the respective mutant strain. The set protocol was used to release mycolic acids from mycobacteria. The procedure involves base hydrolysis of cells with the help of tetrabutyl ammonium hydroxide (TBAH). After this, phase-transfer catalyzed derivatization was done using methyl iodide resulting in the formation of mycolic acid methyl esters (MAMEs) (Dobson *et al.*, 1985). This was then analyzed by thin-layer chromatography (TLC). The [^{14}C]-labeled extracts obtained from base treatment and derivatization were analyzed by TLC. In the parental mc^{2}155 strain, presence of α, α′ and epoxy MAMEs were observed however all the three species were drastically reduced in the extract from the Δ*MSMEG4721* strain (Figure 3.4-A). Instead, there was accumulation of a product(s) found to be migrated above the fatty acid methyl esters (FAMEs) bands in the mutant strain. The similar outcome was found for extracts from delipidated cells that comprises of only cell wall bound mycolates (Figure 3.4-B), which indicates that both total and specifically cell wall-bound mycolates shows modifications in mycolate profiles in parental and mutant strain. Figure 3.4 also suggests that mycolic acid biosynthesis was restored in the mutant strain when it was complemented with *MSMEG4721 and Rv2508*. This indicates that *Rv2508 is* a functional homolog of *MSMEG4721* in *M. tuberculosis* (strains Δ*MSMEG4721*-C and Δ*MSMEG4721*-CRv, respectively (Figure 3.4-A).

As mentioned earlier, the significant reduction of α, α′ and epoxy MAMEs and the appearance of rapidly migrating species in extracts of the Δ*MSMEG47221* mutant suggest that these rapidly migrating species are the precursors of mycolates. The above experimental results thus suggests that the Δ*MSMEG4721 m*utant accumulate precursors of mycolic acids in the cell wall (Bhatt *etal.*, 2008). Thus *MSMEG4721 and Rv2508 are shown to be involved in mycolic acid reduction.*

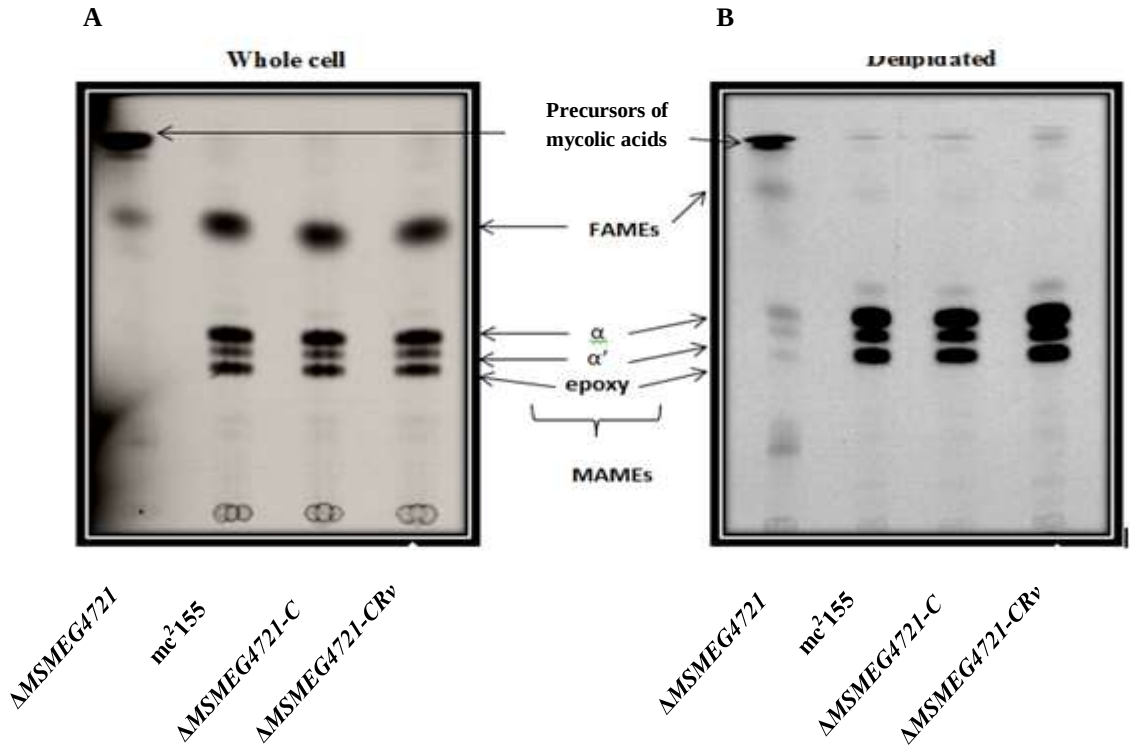


Figure 3.4 Visualization of Fatty acid methyl esters (FAMEs) and Mycolic Acid Methyl Esters (MAMEs) by TLC method.

TLC examination of FAMEs and MAMEs extracted from Δ*MSMEG4721*, mc^{2}155, Δ*MSMEG4721*-C, and Δ*MSMEG4721*-CRv strains. The speedily migrating species observed in Δ*MSMEG4721* are labeled as precursors of mycolates in total (Fig 3.4-A) and specifically cell wall-bound mycolates (Fig 3.4-B).

3.3 Lipid Analysis of Δ*MSMEG47221* mutant

[^{14}C]-acetate labeled apolar lipids were extracted from parental mc^{2}155, Δ*MSMEG4721*, and Δ*MSMEG4721*-C strains as described in methods. This was then studied by 2D-TLC. Moreover, the mutant strain was found to be accumulating nonpolar species as a result of complete loss of free mycolic acids. The respective lipid profile on complementation of Δ*MMSMEG4721* could restore wild type characteristics indicating that the observed profile in the mutant strain were exclusively due to the loss of *MSMEG4721* (Figures 3.5-B and 3.5-C).

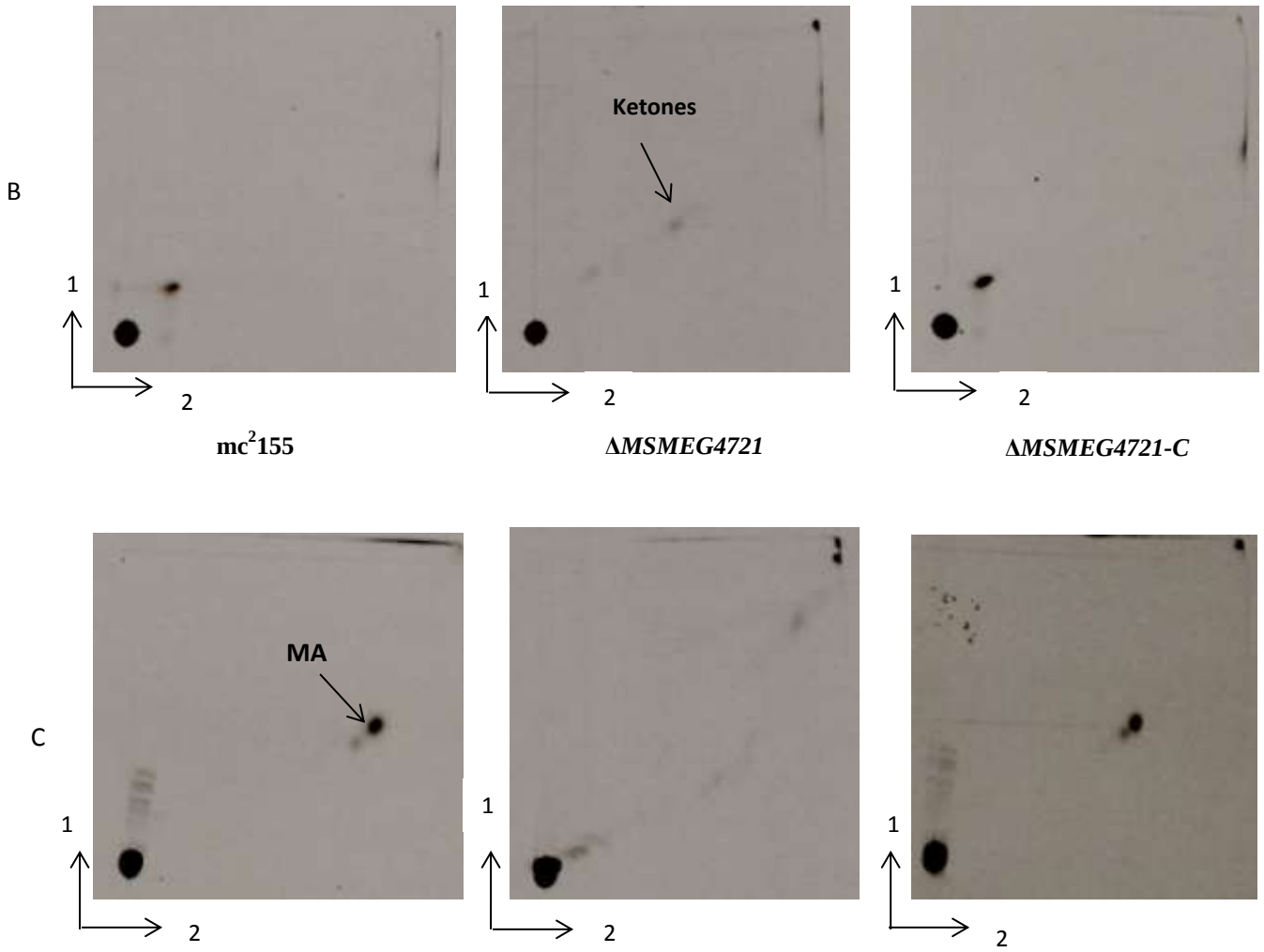


Figure 3.5 Lpids extracted from parental mc^{2}155 , Δ*MSMEG4721and* Δ*MSMEG472-C strains in system B and C*

System B: Direction 1: Petroleum ether: Acetone (98:2) v/v. Direction 2: Toluene: Acetone (98:2) v/v. Ketones are indicated by an arrow. System C: Direction 1: Chloroform: Methanol (96:4) v/v. Direction 2: Toluene: Acetone (80:20) v/v.MA : mycolic acids.

This particular experimental data support the above mentioned evidence that the Δ*MSMEG4721* *m*utant accumulate precursors of mycolic acids in the cell wall, thus it has role in mycolic acid reduction in *M. Smegmatis.*

CHAPTER 4

The lipid-rich cell wall of mycobacteria play important role in physical protection and it is also responsible for virulence. Several currently used anti-TB drugs including ethionamide and isoniazid target cell wall biosynthetic pathways (Banerjee *et al.*, 1994 and Kremer *et al.*, 2000). Thus better understanding of the cell wall assembly and it's biosynthesis will provide important clues for the development of novel anti-mycobacterial drug targets.

The central objective of the studies described in this thesis was to study cell wall biosynthesis in mycobacteria which mainly focus on the post-fatty acid synthase-II/Pks13 processing. To achieve this, we generated a viable null mutant of *MSMEG4721* which indicates that, it is nonessential gene. However our study has demonstrates that loss of function causes remarkable changes in the cell wall of *M. smegmatis.* Further characterization of this mutant suggests that the gene encoding permease is involved in mycolic acid reduction in *Mycobacterium smegmatis*. Moreover, characterization of *Rv2508,* the *Mycobacterium tuberculosis* homolog of *MSMEG4721,* suggests the potential of Rv2508 as a secondary drug target in *M. tuberculosis*.by showing it's role in mycolic acid reduction.

Combined outcomes based on previous studies in this lab and our current study suggests that the gene encoding reductase *MSMEG4722* and gene encoding permease *MSMEG4721*, both are involved in mycolic acid reduction. In future, a very well established method, 'mycobacterial protein fragment complementation' based on yeast-two hybrid system can be used in order to understand the link between these two genes.

To conclude, the identification and characterization of such ‘cell wall biosynthetic drug targets’ is an important step in order to treat tuberculosis especially when there is occurrence of MDR-TB and XDR-TB. Thus these studies put significant contribution in order to understand the complexities of mycobacterial cell wall biosynthesis and represent potential novel drug target against Tuberculosis.

CHAPTER 5

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