Project 1: An IncP-1β plasmid present in *Pseudomonas aeruginosa* isolated from the Burns Unit of Queen Elizabeth Hospital, Birmingham

and

Project 2: Acid detection by the EvgS/A two component system

by

Nikhil Aggarwal

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Project 1: An IncP-1β plasmid present in *Pseudomonas aeruginosa* isolated from the Burns Unit of Queen Elizabeth Hospital, Birmingham

Abstract

To monitor the spread of infection by *Pseudomonas aeruginosa* a number of samples were collected from the Burns Unit of Queen Elizabeth Hospital, Birmingham and subjected to High Throughput Sequencing (HTS). The samples were from patients, water supply and environmental. BLAST searches of the data obtained from HTS revealed the presence of an IncP-1β plasmid in 13 isolates from a single room. Although the isolates had the same genomic sequence, none of plasmid positive samples were from the patients which may suggests the adverse effect of the plasmid on the virulence of *P. aeruginosa*. The presence of the plasmid was confirmed by experiments and the sequence of the plasmid was analysed. It was found that the plasmid has a chlorite dismutase gene inserted in its transposon, in lieu of an antibiotic resistance gene. Sequence alignment showed a number of mutations unique to the plasmid present in otherwise highly conserved regions. To assess the phenotype conferred by the plasmid on its host, isogenic strains with and without the plasmid were created. Although the plasmid does not influence the biofilm formation activity of *P. aeruginosa* it was observed that this particular plasmid had a special preference for it clinically isolated host. The presence of the plasmid also made *P. aeruginosa* more susceptible to commonly used antibiotics.

Acknowledgement

Table of Contents

List of Figures

List of Tables

Introduction

1.1 Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative bacterium often found to inhabit various niches such as soil, water, surface of plants and animals, humans, sewages and hospitals. It is rod-shaped, motile and a facultative aerobe. It commonly uses oxygen as the final electron acceptor during metabolism but can also survive in absence of oxygen by utilizing nitrate as the terminal electron acceptor. *P. aeruginosa* has minimal nutritional requirements and can grow in distilled water obtained in hospitals (Favero et. al., 1971). The optimal temperature for its growth is 37°C but it is known to grow at high temperatures of up to 42°C. It has an extremely versatile metabolic network that enables it to grow on different types of organic compounds (Stover et. al., 2000). In addition, it is resistant to commonly used antiseptics and antibiotics. These factors contribute to the ubiquity of *P. aeruginosa*.

1.1.1 Pathogenicity of P. aeruginosa

P. aeruginosa is an opportunistic pathogen mainly infecting individuals with suppressed immune system (Lang *et. al.,* 2004; Kipnis *et. al.,* 2006). This pathogen was isolated from infections by pharmacist Carle Gessard in 1882. In his study entitled 'On the blue and green coloration of bandages', Gessard refers to the characteristic pigment, pyocyanin, secreted by *P. aeruginosa*.

P. aeruginosa is a nosocomial pathogen often infecting patients suffering from AIDS or undergoing cancer treatment. It also infects patients with damaged tissues such as burn victims and cystic fibrosis (CF) patients. According to Centre for Disease and Control (CDC) *P. aeruginosa* is the fifth most common pathogen obtained from hospitals and is responsible for 10% of all nosocomial infection (Emori and Gaynes, 1993; Matar *et. al.,* 2005). The most common infections caused by *P. aeruginosa* are- pneumonia, bacteraemia, urinary tract infections and ulcerative keratitis (Morita *et. al.,* 2013). Given the ubiquity of this microorganism, the number of diseases and cases of infection associated with it is significantly low.

The pathogenicity of *P. aeruginosa* is associated with an arsenal of highly regulated virulence factors. These factors are either cell surface determinants (flagella, pili, and lipopolysaccharides) or secreted proteins (proteases, elastases, pyocyanin, exotoxins and extracellular polysaccharides) (Lyczak *et. al.,* 2000). Cell surface determinants, especially pili, promote the adhesion of *P. aeruginosa* to epithelial cells, which marks the initiation of the infection (Hahn, 1997; Harvey *et. al.,* 2009). After adhesion, *P. aeruginosa* secretes virulence factors such as proteases, exotoxins and elastases, which damage the host tissue and allows the bacteria to invade and disseminate. This results in onset of acute infection that is characterized by extensive tissue damage and inflammatory response. In case of chronic infection, there is an up-regulation of extracellular polysaccharides and down-regulation of acute virulence factors (Goodman *et. al.,* 2004). This allows the establishment of persistent and antibiotic resistant

biofilms (Drenkard and Ausubel, 2002). The transition from acute to chronic infection (or vice versa) involves changes in the phenotype, metabolism and regulation of specific genes. This transition is controlled by the cell-to-cell signalling system dependent on the cell density called quorum sensing (QS). QS is involved in regulation of key pathways in various bacterial species and is usually a target for antimicrobial agents.

1.1.2 Efflux pumps and resistance to antibiotics

P. aeruginosa is intrinsically resistant to commonly used antibiotics such as β- lactams, aminoglycosides and fluoroquinolones (Lister *et. al.,* 2009). This resistance is because of the low permeability of the outer membrane due to limited number of OprF porins, which form large sized channels, due to which many of the antibiotics are unable to diffuse into the cell (although this is highly debatable; Pumbwe *et. al.,* 1996). In addition, a number of multi-drug resistant (MDR) efflux pumps such as MexAB-OprM, MexCD-OprJ and MexEF-OprN are present in *P. aeruginosa*, which, along with exporting antibiotics, also pump out detergents, inhibitors, dyes and disinfectants (Gotoh *et. al.,* 1995; Poole, 2001). Apart from intrinsic resistance, *P. aeruginosa* can also acquire resistance via plasmids, transposons or genetic mutations. Mobile genetic material such as plasmids and transposons are known to carry antibiotic resistance cassettes and can be acquired by transformation, transduction or conjugation. *P. aeruginosa*, when grown in stressful environmental conditions, such as in presence of DNA damaging agents, can develop mutations in specific genes, which may lead to an increase in antibiotic resistance. For example, a mutation in *nfx*B leads to derepression of MexCD-OprJ efflux pump resulting in increased antibiotic resistance (Stickland *et. al.,* 2010). Another mechanism by which *P. aeruginosa* can become antibiotic resistant is by biofilm formation. This mechanism is especially important in case of infection by *P. aeruginosa* as formation of biofilm protects it from the host immune response and drug regime.

1.1.3 Biofilms

Biofilm is a community of micro-organisms attached to a surface and covered by a matrix of polysaccharides. These communities can be homogenous (made up of single type of bacteria) or heterogeneous (made up of different types of bacteria). Majority of bacteria transit from a planktonic state to a sessile state because of environmental cues and cell-to-cell signalling. The cells attach to the surface via structures such as pili and undergo physiological changes to adapt to this form of life. Thus, the bacterial cells in a biofilm exhibit a different phenotype than free-living planktonic cells.

P. aeruginosa is able to infect humans and cause chronic infections because of its ability to form biofilm in lungs of CF patients (Hoiby *et. al.,* 2010). It also forms biofilm on medical devices such as catheters, prosthetic heart valves and contact lenses (Donlan and Costerton, 2002). *P. aeruginosa* initiates biofilm formation by employing the flagella and type-IV pili to attach to the surface. After attachment, *P.* aeruginosa release three different polysaccharides,

alginate, PeI and PsI that form the matrix of the biofilm (Ma *et. al.,* 2009). Other components of the matrix include extra-chromosomal DNA (Allesen-Holm *et. al.,* 2006) and proteins. This matrix is known to protect *P. aeruginosa* from antibiotics and host defence mechanisms (Costerton *et. al.,* 1999). Apart from the matrix, the physiological adaptations that the cell undergoes to form biofilm may result in higher expression of efflux pumps and antibiotic degrading enzymes, which may lead to higher antibiotic resistance than planktonic cells.

1.1.4 Quorum sensing in P. aeruginosa
Quorum sensing (QS) is the mechanism by which bacteria responds to changes in environmental conditions by modulating the expression of various factors, which enable them to thrive in the new environment. Gram-negative bacteria such as *P. aeruginosa* usually produce acyl-homoserine lactones (AHLs) and 2-alkyl-4-quinoles (AQs) which act as QS signals and enable cell-to-cell signalling. *P. aeruginosa* has two QS pathways- *las* and *rhl* systems. The autoinducers of these two systems- *las*I and *rhl*I encode for *N*-(3-oxododecanoyl)-l-homoserine lactone (3O-C_{12}-HSL) and *N*-butanoyl-l-homoserine lactone (C_4-HSL) respectively. These autoinducers interact with their respective regulators- *las*R and *rhl*R and together control the transcription of nearly 10% of the genes of *P. aeruginosa* (Schuster *et. al.,* 2006). These two systems are interconnected with the 3O-C_{12}-HSL-lasR complex regulating the expression of *rhl*R *(Latifi et. al.,* 1996). The *las* and *rhl* systems control the expression of various virulence factors such as elastases, proteases, exotoxins and pyocyanins. They also regulate biofilm formation as *las*I mutants were found to form abnormal biofilm that was highly susceptible to SDS (Davies *et. al.,* 1998). QS also affects efflux pumps, metabolism, iron chelation, motility and host immune response (Williams and Camara, 2009).

1.2 Role of plasmids in bacteria
Plasmids are the member of the accessory genetic pool found in all types of environment such as soil, marine and clinical niches. They are capable of transferring between different types of bacteria (by conjugation), and even cross animal kingdoms, which is termed as horizontal gene transfer (HGT). Plasmids usually carry genes that provide a selection advantage to their hosts in a particular environment and have mechanisms for autonomous replication in bacterial cells. In majority of cases, these genes tend to be antibiotic resistance genes or genes that confer heavy metal resistance to their hosts. In some cases, anthropogenic pollutant degrading enzymes have also been found to be present on plasmid in contaminated soil (Heuer and Smalla, 2012). Certain plasmids also encode for factors that make their hosts more virulent. For example, the 0.2 Mb plasmid pTi found in plant pathogen *Agrobacterium tumefaciens* has genes for the type IV secretion system, which mediates the integration of the plasmid into the plant host chromosome (Morton *et. al.,* 2014).

Plasmids have mechanisms which ensure their own survival in a bacterial population- active partitioning, post segregational killing (*psk*) system and multimeric resolutions system. *psk*

system is often regarded as the selfish part of the plasmid as it preys on the resources of the hosts to ensure its own survival. On the other hand, many altruistic characteristics of the plasmid are known such as resistance genes carried by the plasmid, HGT and biofilm formation, although Kado (1998) considers these altruistic traits to be selfish too as ensuring the survival of the host ensures the survival of the plasmid. Therefore, the true nature of the plasmids is an open debate.

Role of plasmids in biofilm formation remains ambiguous till date. Although there are a number of studies which report the increase in biofilm formation when the hosts carry conjugative plasmids (Ghigo, 2001), there are other studies which show that presence of conjugative plasmids may inhibit biofilm formation in some species (Roder *et. al* 2013). Mating pair formation (Mpf) apparatus encoded by conjugative plasmids is usually implicated in increase in biofilm formation. Mpf promotes cell-cell and cell-surface interaction resulting in higher biofilm formation activity of the host.

Apart from biofilm formation, plasmids are involved in HGT. As mentioned before, plasmids can transfer from one bacterial species to another and even across kingdoms by transformation, transduction or conjugation. Plasmids, especially conjugative plasmids carry accessory genes that confer different phenotypes to their hosts. Uptakes of these plasmids provide ready-made genes to the hosts enabling them to adapt to novel niches. Therefore, plasmid driven HGT promotes evolutionary as well as ecological innovations.

1.3 IncP-1 plasmids

Incompatibility (IncP) plasmids are broad host range, low copy number plasmids typically found in nature in various Gram-negative bacterial species such as *Enterobacteriaceae, Pseudomonas spp., Rhizobium spp. Vibrio Cholera* etc. (Thomas and Smith, 1987). Plasmids, which have the same system for replication and stable inheritance are incompatible with each other and are grouped together in the same incompatibility group. IncP (in *E. coli*) or IncP-1 (in *Pseudomonas*) plasmids are typically found in wastewater from industries, contaminated soil, freshwater and hospitals. IncP-1 plasmids have been further divided into multiple groups, the major ones being α and β groups. Members of these two groups closely resemble each other and share a similar backbone. IncP-1α plasmids carry one or more transposons with wide variety of antibiotic resistance genes. The first IncP plasmid to be isolated was an IncP-1α from Birmingham hospital, U.K. in 1969. Nearly all the members of the IncP-1α group carry kanamycin and tetracycline resistance genes. IncP-1β plasmids also carry transposons with antibiotic resistance genes and degradative cassettes but, unlike the IncP-1α group, no common resistance gene is observed in this group. RK2 and R751 are the archetypes of IncP-1α and IncP-1β groups and have been fully sequenced (Pansegrau *et. al.* 1994; Thorsted *et. al.,* 1998).

The backbone of IncP-1 plasmids comprises of three main regions- *tra*-1 involved in conjugal transfer of plasmids, *tra*-2 or *trb* involved in mating pair formation and central control region (*ccr*) which has genes required for replication, stable inheritance and regulating *tra*-1 and *trb*. Restriction map of IncP-1α and β plasmids have shown that there are very few restriction

sites in the backbone indicating that it is not susceptible to restriction when transferred from one bacterial species to another. Majority of restriction sites are situated in between *tra* and *trb* regions and between *ori*V and *trf*A due to which the transposons are usually found to be present in these hotspots.

1.3.1 Replication of IncP-1 plasmids

IncP-1 plasmids replicate by the binding of replication initiation protein *trf*A to the vegetative replication of origin *ori*V. Both *trf*A and *ori*V are located in the *ccr* region of IncP-1 plasmids. The minimal region of *ori*V required for replication is 393 bp in length and comprises of iterons of 17bp repeated five times and at a distance of 22-23 bp. Following the iterons are the neighbouring A+T rich and G+T rich regions. *trf*A gene has a translational start in its open reading frame and thus encodes for two products- *trf*A1 and *trf*A2, which bind to the iterons in the *ori*V and initiate replication. Apart from this replication, *trf*A is also involved in controlling the copy number of the plasmids by a handcuffing model (Blasina *et. al.,* 1996).

1.3.2 Conjugative transfer of IncP-1 plasmids

As mentioned above, *trb* and *tra* regions are involved in transferring plasmids via conjugation. *trb* region encodes for proteins, which form the mating pair formation (mpf) apparatus that facilitates the transfer of plasmid from donor to recipient by rolling circle replication. *tra* region is responsible for encoding relaxosome and initiation of rolling circle replication. The proteins encoded by the *tra* region prepare the DNA to be transferred and initiate the replication by nicking the plasmid at the origin of transfer *ori*T.

1.3.3 Stable inheritance of IncP-1 plasmids

Along with replication and conjugal transfer, IncP-1 plasmids have several mechanisms by which they ensure their stable maintenance in a broad range of hosts. Low copy number plasmids such as IncP-1 plasmids are known to encode for proteins involved in active partitioning which is similar to their hosts. Par systems (which are the best studied partitioning systems) has three components- a DNA binding protein (ParB), an ATPase (ParA) and a centromere-like site to which ParB binds. After this binding, energy is provided by ATPase, which drives the segregation process. In IncP-1 plasmids, KorB and IncC are equivalent to ParB and ParA respectively and thus are involved in stable inheritance of IncP-1 plasmids.

It is another system of stable inheritance that is present on IncP-1 plasmids is the post-segregational killing (*psk*) system. This system comprises of a long lived toxin and a short lived anti-toxin. Both of these components remain in the cytoplasm due to which plasmid free segregants quickly die because of deprivation of anti-toxins, which leaves the toxins behind in the cells. In IncP-1α plasmids, ParD acts as the anti-toxin to the toxicity of ParE.

It is interesting to note that although IncP-1α plasmids have active partitioning and post-segregational killing system but IncP-1β plasmids only rely on the active partitioning system for

their stable inheritance.

IncP-1 plasmids encode a number of overlapping regulatory circuits, which control the replication, conjugal transfer and stable inheritance of the plasmids. These circuits provide tight but flexible control and are a unique feature of IncP-1 plasmids.

1.4 Background and Aims of this study

To study the spread of infection by *P. aeruginosa* in the Burns Unit of Queen Elizabeth hospital, Birmingham, 150 samples were taken and subjected to High Throughput Sequencing (HTS). The samples were collected over a span of 8-10 months and were from patients (wound swabs), water supply from shower, tap and hose and environmental samples from trolley, shower head, drain and sink located in particular rooms. From the data obtained by HTS, a phylogenetic map of all the *P. aeruginosa* isolates was created. It was observed that all the isolates had essentially the same genomic sequence, with the most diverged strain having four SNPs (single nucleotide polymorphisms) as compared to the parental strain. BLAST searches of the sequences obtained via HTS showed the presence of two IncP-1β plasmids in the isolated strains. The plasmids were named pPaeBURNS1 and pPaeBURNS2, after their bacterial host (Pae) and the location of the plasmid (Burns Unit). pPaeBURNS1 was present in thirteen isolates from a single room and pPaeBURNS2 was present in two isolates from another room. The isolates with pPaeBURNS1 were present in water as well as environmental samples whereas the 2 isolates with pPaeBURNS2 were from water samples. Interestingly, none of the plasmid positive isolates were from the patients. The plasmid free strains that were isolated from patients were found to be resistant to imipenem and meropenem whereas plasmid positive strains were susceptible to these antibiotics (Josh Quick and Nick Loman, pers. comm.)

The aim of this study is to study pPaeBURNS1 and investigate the phenotype that this plasmid confers on its clinically isolated host *P. aeruginosa* 943. The sequence of pPaeBURNS1 will be annotated and compared with other IncP-1β plasmids to determine any unique features present on the plasmid. A curing strategy, developed by Hale *et. al.* (2010) will be adopted to create isogenic strains with and without pPaeBURNS1. All the *P. aeruginosa* strains carrying the pPaeBURNS1 were isolated from the same room in the Burns Unit; a hypothesis to explain this confinement is that these isolates are being dispersed from a biofilm comprising of plasmid positive *P. aeruginosa* strains. Therefore, the effect of pPaeBURNS1 on the biofilm formation activity of *P. aeruginosa* 943 will be assessed by doing a biofilm assay with crystal violet staining. This will be compared to the effect of other IncP-1 plasmids, such as RK2 and R751, on the biofilm formation activity of different hosts like *P. putida, P. fluoresences* and *E. coli.* Furthermore, as the plasmid negative isolates were found to be resistant to imipenem and meropenem, in contrast to the plasmid positive strains, the role of pPaeBURNS1 in conferring antibiotic resistance on *P. aeruginsa* 943 will be investigated. MIC of *P.* aeruginosa 943 with and without pPaeBURNS1 for different antibiotics will be determined. Finally, the stability of

pPaeBURNS1 in *P. aeruginosa* 943 will be measured for strain grown in both LB-broth and M9 minimal media.

Materials and Methods

2.1 Bacterial strains and growth conditions

The *Pseudomonas* strains used in this study are- hospital acquired *Pseudomonas aeruginosa* 943 and wild type PAO1161, *Pseudomonas putida* KT2440 and *Pseudomonas fluorescens* SBW25. *E. coli* strains used are - S17-1 (*recA pro hsd*R RP4-2-Tc::Mu-Km::Tn7 λpir) (Simon et.al. 1983), C600 (F⁻ *ton*A21 *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *gln*V44 *rfb*C1 *fhu*A1 λ⁻), MV10 (C600 *trp*E5 Nal^R), HB101 (F⁻ mcrB mrr hsdS20[r_B⁻ m_B⁻] recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20[Sm^R] glnV44 λ⁻) and RU2537 (F+ *pro*-22 *met*-63 *recA*56 *nalA:: Tn*1723). *P. aeruginosa* and *E.coli* strains were grown at 37°C with shaking at 200 rpm whereas *P. putida* and *P. fluorescens* were grown at 30°C with shaking at 200 rpm. The *Pseudomonas* strains were grown in LB-broth or M9 minimal media. *E. coli* strains were grown only in LB-broth.

2.2 Growth media

Solid media was obtained by addition of 1.5% agar. M9 minimal media was prepared as follows:-

NaH_2PO_4	6.7 g
KH_2PO_4	3.0 g
NH_4Cl	1.0 g
NaCl	0.5 g

These salts were dissolved in 1 L distilled water, pH adjusted to 7.4 and autoclaved at 121°C. To this solution the following were added:-

40% glucose	2 mL
100mM $CaCl_2$	400 μL
1M $MgSO_4.7H_2O$	400 μL

2.3 Plasmids used in this study

Table 2.1 Plasmids used in this study

Plasmid	Size (kb)	Replicon	Selective Marker	Properties	Reference
RK2	60.1	IncP-1α	Ap^R Km^R Tc^R	Wild type, naturally occurring	Ingram *et. al.,* 1973
R751	53.3	IncP-1β	Tp^R	Wild type, naturally occurring	Jobanputra and Data, 1974

pAKE604	7.2	pMB1	Ap^R Km^R	*ori*T, *lacZ*, *sacB*	El-Sayed *et. al.,* 2001
pJH10	14.5	IncQ	Tc^R Sm^R	*ori*T, *tac*p, *lac*I^q	El-Sayed *et. al.,* 2003
pJH10.1	14.7	IncQ	Tc^R Sm^R	*kor*C inserted downstream of *tac*p in pJH10	This study
pCURE11	9.4	pMB1	Ap^R Km^R	*ori*T$_{RK2}$, *ori*V$_{RK2}$, *Inc*C$_{RK2}$, *par*D$_{RK2}$	Hale *et. al.,* 2010
pCURE11.0	21.7	IncQ, pMB1	Ap^R Km^R Tc^R Sm^R	pJH10 and pAKE604 ligated at *Eco*R1	This study
pCURE11.1	23.9	IncQ, pMB1	Ap^R Km^R Tc^R Sm^R	pJH10 and pCURE11 ligated at *Eco*R1	This study
pCURE12	24.1	IncQ, pMB1	Ap^R Km^R Tc^R Sm^R	pJH10.1 and pCURE11 ligated at *Bam*H1	This study

Tc: tetracycline; Sm: Streptomycin; Ap: Ampicillin; Km: Kanamycin; Tp: Trimethoprim

2.4 Oligonucleotides used in this study

Table 2.2 Primers used in this study

Name	Sequence (5' - 3')	Plasmid template	Region
korCF	ATTGAATTCATGACGAACGACGCCAATATC	pPaeBURNS1	*kor*C
korCR	TAGTCTAGATACCGTAATCCGACGCTGAC	pPaeBURNS1	*kor*C
UpF	ATTGAATTCGGCAGATCCACACACAGCAC	pPaeBURNS1	upstream of *cdm*
DownR	TCCGGATCCGTGTTCGGTATTTTTGCCGC	pPaeBURNS1	downstream of *cdm*

cdm: chlorite dismutase gene

Primers were prepared by AltaBioscience, Birmingham, UK and stored at -20°C after suspending them in sterile distilled water.

2.5 DNA isolation and manipulation
2.5.1 Plasmid DNA extraction
Plasmid was extracted from 5 mL of cultures grown overnight in LB-broth at appropriate conditions using Accuprep Plasmid Mini Extraction Kit from Bioneer. The manufacturer's instructions were followed to extract the plasmid and elution was done twice using 25 µL of

elution buffer. After elution, the plasmid was incubated at 70°C for 30 minutes and then stored at -20°C.

2.5.2 DNA digestion and ligation

To digest the DNA appropriate restriction enzymes from New England Biolabs (NEB) were used. 10 µL of the DNA (for example the eluted plasmid in section 2.5.1) was mixed with 2.5 µL of 10X buffer. Restriction enzyme (0.5 µL) was added to the mix and the volume was made up to 25 µL by sterilized distilled water. This reaction mix was incubated at 37°C for 1 hour. Heat inactivation of the restriction enzyme, if possible, was done by incubating the reaction mix at 80°C for 20 minutes. If heat inactivation was not possible DNA was purified from the mix by using the GE Healthcare GFX PCR Purification Kit. Higher quantity of DNA was used in case of digestion of low copy number plasmids.

Ligation was done by using a total of 11 µL of digested DNA (in a ratio depending upon the concentration and size of the fragments) and mixing with 5 µL of 5X ligation buffer and 1 µL of ligase enzyme (from NEB). The volume was made up to 25 µL by sterilized distilled water and the reaction mix was incubated for 16-18 hours at room temperature. Both digestion and ligation were confirmed by performing gel electrophoresis. 10 µL of ligation mix was used to transform competent bacterial cells.

2.5.3 Agarose gel electrophoresis and extraction

Agarose gel was prepared by dissolving 0.9% w/v agarose (AGTC Bioproducts, UK) in 1X TAE buffer. Ethidium bromide was added to the agarose solution to facilitate the visualization of DNA on the gel. The solution was poured into a cast with a gel comb and cooled until solid. DNA sample (10 µL) with loading dye was loaded into the wells of the gel and run for 40-45 minutes at 120V. The DNA bands were visualized by Bio-rad Gel Doc™ XR+ system.

Gel extraction was performed using the GE Healthcare GFX Gel Purification Kit following the manufacturer's instructions.

2.5.4 Polymerize Chain Reaction (PCR)

PCR was done to amplify DNA fragments or confirm the presence of the plasmid (by amplifying a genetic region on the plasmid). The following program was used to perform PCR:-

Step 1	Denaturation	94°C	5 min
Step 2	Denaturation	94°C	30 s
Step 3	Annealing	Tm-5°C	30 s
Step 4	Extension	72°C	90 s
Repeat Step 2-4 25 times			
Step 5	Extension	72°C	10 min

Depending upon the polymerase used to perform PCR different reaction mixes were prepared.

Composition of PCR mix for Invitrogen DNA Taq polymerase:-

dNTPs (2.5 mM)	4.0 μL
Forward primer (100 μM)	0.3 μL
Reverse primer (100 μM)	0.3 μL
50 % glycerol	10 μL
50 mM MgCl$_2$	1.0 μL
Buffer (by Invitrogen)	5.0 μL
Taq polymerase	0.6 μL
DNA template	5.0 μL

Volume is made up to 50 µL by sterile distilled water.

Composition of PCR mix for Bioline VELOCITY DNA Taq Polymerase: - This is a high-fidelity polymerase and is preferred to amplify fragments when constructing plasmids.

dNTPs (2.5 mM)	5.0 μL
Forward primer (15 μM)	2.0 μL
Reverse primer (15 μM)	2.0 μL
DMSO	1.5 μL
5X Hi-fi buffer (by Bioline)	10 μL
VELOCITY Taq polymerase	0.5 μL
DNA template	1.0 μL

Volume is made up to 50 µL by sterile distilled water.

The PCR product was purified by running the sample on an agarose gel and purifying the required DNA fragment by GE Healthcare GFX Gel Purification Kit.

2.6 Preparation of E. coli competent cells
Overnight culture of *E. coli* was diluted 1:100 fold and grown in 25 mL of LB-broth at 37°C with shaking at 200 rpm until OD$_{600}$ reached 0.4-0.6. The cells were harvested by centrifugation at 5,000 x g for 7 minutes at 4°C. The pelleted cells were re-suspended in 10 mL of pre-chilled 100 mM CaCl$_2$ and incubated on ice for 20 minutes. After incubation, the cells were again harvested at 5,000 x g for 7 minutes at 4°C and re-suspended gently in 2.5 mL of 100 mM CaCl$_2$. These cells were stored at 4°C and remain competent for two weeks.

2.7 Transformation of E.coli cells

To transform *E.coli* cells, 3-5 µL of DNA was added to 100 µL of competent cells as prepared above. In case of ligation, 10 µL of the mix was used to transform the cells. The DNA-cell mix was incubated on ice for 30 minutes. Following incubation, heat shock was given to the cells by incubating them at 42°C for 2 minutes. After heat shock, the cells were cooled on ice for 5 minutes. LB-broth (1 mL) was added to the mix and the cells were grown at 37°C for 1 hour and plated onto selective L-agar plates. For the negative control, no DNA was added to the competent cells.

2.8 Conjugation experiments

Cultures of donor and recipient were grown overnight at appropriate conditions (with antibiotics if required). 1 mL each of donor and recipient was mixed together and injected onto a filter paper. The filtered media was discarded and the filter disc with the cells was placed on a non-selective L-agar plate with the cells facing up. These cells were incubated at 30°C or 37°C for 5-6 hours to facilitate transfer of genetic material from donor to recipient. Following incubation, the bacterial cells on the filter disc were re-suspended in 1 mL of LB-broth or 0.85% saline. The cell suspension was diluted (1:1, 1:10 and 1:100) and 100 µL was plated onto L-agar or M9-agar plated with antibiotics to select for transconjugants. When colonies appear on the plate, PCR was performed (if possible) to confirm the presence of the genetic material in the recipient bacterial strain.

2.9 Displacing pPaeBURNS1 from Pseudomonas aeruginosa 943

To displace pPaeBURNS1 the pCURE strategy (Hale *et. al.,* 2010) was adopted. The pCURE plasmid was transferred to *P. aeruginosa* 943 by conjugation with *E. coli* S17-1 carrying the plasmid. Transconjugants were selected by plating the mix of donor and recipient onto M9-agar plates with kanamycin (500 µg/ml). M9 media will prevent the growth of *E. coli* S17-1 and kanamycin will prevent the growth of *P. aeruginosa* 943 without the pCURE plasmid. These transconjugants were subjected to PCR analysis (using UpF and DownR primers) to confirm the loss of pPaeBURNS1. The transconjugants that have lost the pPaeBURNS1 were inoculated into LB-broth and grown overnight without selection. This will facilitate the loss of pCURE plasmid. This overnight culture was diluted 1:1000 fold and plated onto L-agar plates with 5% sucrose solution. The colonies on these plates were screened for kanamycin resistance. Colonies that were kanamycin sensitive were grown overnight in LB-broth and stored at -80°C by diluting the culture 1:1 fold in 50% glycerol in LB-broth.

2.10 Biofilm Assay

Overnight culture of bacteria was diluted 1:100 in LB-broth and 200 µL was inoculated into the wells of the Co-star 96-well flat bottom plate. The plate was covered with lid and covered with cling film to retain the moisture in it. This plate was incubated at room temperature for 24 hours.

After incubation, OD_{600} was measured using BMG LabTech Spectrostar Nano microplate reader to determine the growth yield of the bacteria. The wells were then washed with sterile distilled water 2-3 times to remove spent media and cells that are not adhered to the plate. Following this washing step, 200 µL of 1% crystal violet was added to each well and incubated for 15-20 minutes at room temperature. After incubation, the wells were again washed with sterile distilled water to remove any unbound dye. The bound dye was solubilized by adding 200 µL of 70 % ethanol and incubated for 15-20 minutes at room temperature. OD_{600} was measured to determine the amount of biofilm formed. The experiment was done with eight repeats.

2.11 Stability test for plasmid
To determine the stability of the plasmid, cultures of the bacterial strain were grown overnight with selection for the plasmid. This culture was diluted 1:1000 fold and grown in LB-broth or M9 minimal media without selection for 12 hours at appropriate conditions. After every 12 hours the culture was diluted 1:1000 fold into fresh media and grown. This was repeated for five consecutive days. After every transfer to fresh media, the culture was diluted $1:10^8$ fold and 100 µL was plated onto L-agar or M9-agar plated without selection. These plates were incubated for 16 hours at appropriate temperature. From these plates, 35-45 colonies were picked and patched onto L-agar plate with selection for the plasmid and incubated for 16 hours. The stability of the plasmid was expressed as the percentage of colonies the have grown on selective plate to the number of colonies that were patched on it.

2.12 Determination of MIC
Minimum inhibitory concentration (MIC) of different antibiotics for *P.* aeruginosa 943, with and without pPaeBURNS1, was determined following the broth dilution method (similar to the one described by Andrews (2001)). Cultures of bacterial strains were grown overnight at appropriate growth conditions. OD_{600} of the strains was measured by diluting the cultures 1:10 fold. Depending on the value of $OD_{600,}$ the volume of the inoculum was calculated. A 96-well microtiter plate was inoculated with 200 µL of LB-broth or M9 minimal media. Fresh stocks of antibiotics were prepared with a concentration of 25 mg/ml. In case of sodium chlorite, a stock of 100 mM was prepared. Antibiotics were added to each well and serially diluted to give a wide range of concentrations. Chloramphenicol, nalidixic acid and kanamycin stocks were diluted to 32- 512 µg/ml, tetracycline to 8- 128 µg/ml, streptomycin to 64- 1024 µg/ml and sodium chlorite to 0.25- 4 mM. Overnight cultures were inoculated into each well such that they are 1:100 fold diluted. The microtiter plate was covered with its lid and wrapped in a cling film to trap the moisture. The plate was incubated at 37 °C for 24 hours at 200 rpm. After incubation, turbidity of each well was observed. The least concentration of antibiotic at which no turbidity is observed in the well was chosen as the MIC. The experiment was done with two repeats.

2.13 Statistical analysis

To determine the statistical significance of the dataset, a t-test was done using SIGMAPLOT. p-value was chosen to be 0.05 with the null hypothesis being the two data sets are statistically not different from each other. Therefore, datasets having p- value < 0.05 were statistically different.

Results

3.1 Annotation and analysis of pPaeBURNS1

The sequence of pPaeBURNS1 was annotated using ARTEMIS software and was analysed. The plasmid has a size of 50,381 bp with an IncP-1β backbone. The overall G+C content is 65%, which is typical of IncP-1 plasmids. Figure 1 shows the genetic map of pPaeBURNS1 with all the open reading frames marked and the regions involved in conjugative transfer (*tra* and t*rb*), central control region and a transposon shown in different colours. pPaeBURNS1 shows overall 99% similarity, with a coverage of 93% (determined by BLAST), to R751 (Thorsted *et. al.,* 1998) which is the archetype of IncP-1β plasmids. The dissimilarity in pPaeBURNS1 and R751 corresponds to the two transposons present in R751 - Tn4321 present between *trf*A and *ori*V and Tn402 present between *tra* and *trb* regions. In case of pPaeBURNS1, there is a single transposon present between *tra* and *trb* regions. This transposon carries a chlorite dismutase gene, unlike other IncP-1 plasmids, which usually carry antibiotic or heavy metal resistance genes. Chlorite dismutase (*cld*) is a heme-based enzyme involved in disproportionation of chlorite (ClO_2^-) into chloride (Cl^-) and oxygen. Chlorite is a toxic chemical used heavily for bleaching in pulp and paper industries and as a disinfectant. Thus, *cld* is the only gene present on pPaeBURNS1 that may provide some selection advantage to its host *P. aeruginosa* 943.
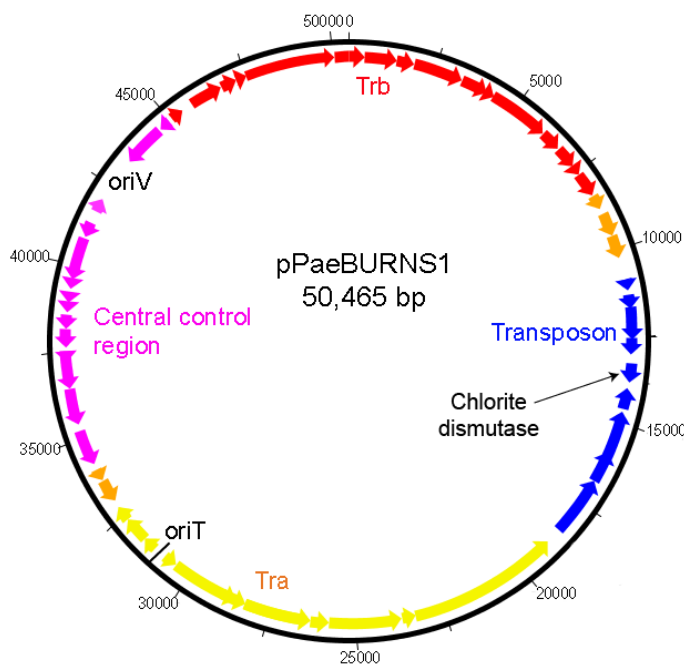

Figure 1: Genetic map of pPaeBURNS1. *tra, trb, ccr* and the transposon are shown in different colours. Location of the chlorite dismutase gene in the transposon is also marked.

Protein	Function	Length of protein in pPaeBURNS1 (aa)	Number of mutations unique to pPaeBURNS1	Conserved aa (%)	Number of mutations in other plasmids
upf54.4	Unknown	217	2	99.1	0
upf54.8	Unknown	115	1	99.1	0
KfrA	Regulation	343	10	96.8	1
KorB	Partitioning and regulation	349	5	98	2
IncC	Partitioning and regulation	358	5	98.6	0
KorA	Regulation	100	1	99	0
KleF	Plasmid Maintenance?	176	10	93.7	1
KleE	Plasmid Maintenance?	137	6	95.6	0
KleB	Plasmid Maintenance?	71	6	91.5	0
KleA	Plasmid Maintenance?	78	4	94.9	0
KorC	Regulation	85	1	97.6	1
KlcB*	Plasmid Maintenance?	404	28	93	0
KlcA	Plasmid Maintenance?	142	3	97.9	0
TrfA	Replication initiation	406	7	97.8	2
ssb	ssDNA binding	113	2	98.2	0
TraI	DNA relaxase	746	2	98.5	9
TraK	*ori*T binding	133	10	92.5	0
TraL	Conjugative transfer	241	1	99.6	0

Table 3.1: Comparison of translated sequence of ORFs in pPaeBURNS1 with other IncP-1β plasmids. * represents the comparison of pKV29, pAKD-1 and pPaeBURNS1 only. ? represents unconfirmed function.

To determine whether there are any mutations unique to pPaeBURNS1 a BLAST search was done using the DNA sequence of the plasmid, and the hits with the highest score were selected. The hits chosen were – pKV29 (organism- Delftia sp. KV29, source- wastewater treatment plant, Accession- JN648090.1), pAKD-1 (organism- uncultured bacterium, source- agricultural soil, Accession- JN106164.1) and pMBUI8 (organism- uncultured bacterium, source- creek water, Accession- KC170279.1). Apart from these three plasmids, pADP-1 (organism- Pseudomonas spp., Accession- U66917.2) was also chosen to compare pPaeBURNS1 with another IncP-1β plasmid found in *Pseudomonas* spp. pADP-1 has a 98% similarity to pPaeBURNS1 with a coverage of 78%. The amino acid sequence of the individual proteins of the hits was aligned with that of pPaeBURNS1, using Clustal Omega and the number of mutations unique to pPaeBURNS1 was reported in Table 3.1. This comparison was done only on the proteins of the IncP-1β backbone and not on the accessory elements such as transposons. The number of
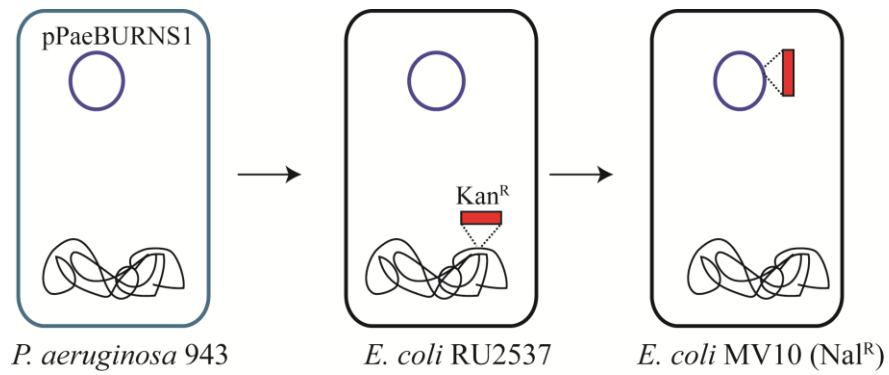
Figure 2: Schematic of the strategy adopted to tag pPaeBURNS1 by Tn1723 (Kan[R]).

mutations found on other IncP-1β plasmids (except for pPaeBURNS1) was also reported to determine the variability in the sequence observed in these proteins. Majority of the mutations were present in proteins located in the *ccr* region of the plasmid. All the genes of the *kil*E locus-*kle*A, *kle*B, *kle*E and *kle*F had mutations. The function of these genes is not yet known but they are required for stable maintenance of IncP-1 plasmids in *P. aeruginosa* (Wilson *et. al.,* 1997). Interestingly, fewer unique mutations were present in *kil*E locus of pADP-1, inspite of the fact that it is also an IncP-1β plasmid isolated from *Pseudomonas* spp., similar to pPaeBURNS1. Apart from *kil*E locus, the number of mutation in *klc*B was considerably high. The amino acid sequence of *klc*B in pADP-1 and pMBUI8 was significantly different from that of the other three plasmids; therefore, the number of mutation reported in this case was only by comparing the sequence of pKV29, pAKD-1 and pPaeBURNS1. As in the case of *kil*E locus, the role of *klc*B is also unknown. In the *tra* region only TraI, TraK and TraL had mutations with TraK having the highest number. *trb* region showed high sequence conservation across different IncP-1β plasmids. As seen in Table 3.1, pPaeBURNS1 carries a higher number of unique mutations as compared to other IncP-1β plasmids. This may indicate towards some unique phenotypes exhibited by pPaeBURNS1.

3.2 Tagging pPaeBURNS1 with an antibiotic resistance gene by conjugation

As mentioned before, pPaeBURNS1 does not carry any antibiotic resistance gene unlike other IncP-1 plasmids. The absence of selectable marker on the plasmid makes it difficult to select for plasmid positive strain and to determine various properties of the plasmid such as stability and transfer rate. As the plasmid is self-transmissible and has a broad host range, we did a conjugation experiment to insert a transposon carrying an antibiotic resistance gene into pPaeBURNS1. The scheme of the experiment is shown in Figure 2. *P. aeruginosa* 943 was conjugated with *E. coli* RU2537 on a solid surface. IncP-1 plasmids have a short and rigid pilus due to which the conjugative rate is higher on a solid surface than in liquid medium. *E. coli* RU2537 has a transposon Tn1723 inserted in its genome. Tn1723 was derived from Tn1722, and carries a kanamycin (Kan) resistance gene (Shingler and Thomas, 1984). As a result of

conjugation, pPaeBURNS1 will transfer to *E. coli* RU2537 and at low frequencies, Tn1723 jumps onto the plasmid. As the copy number of plasmid is more than that of the host chromosome, the number of copies of Tn1723 in RU2537 with the pPaeBURNS1 will be more as compared to the strain without the plasmid. This will make the plasmid positive strain more resistant to Kan than the plasmid negative strain, enabling its selection. After selecting for *E. coli* RU2537 (pPaeBURNS1::Tn1723), this strain was further conjugated with *E. coli* MV10. The MV10 strain has a mutation in its DNA gyrase making it resistant to nalidixic acid (Nal). Thus, with the transfer of pPaeBURNS1::Tn1723 from RU2537 to MV10, the latter will become Nal^R and Kan^R enabling its selection. The presence of the plasmid was further confirmed by PCR analysis using UpF and DownR primers (Table 2.2).

To check whether insertion of Tn1723 has not disrupted the self-transmissibility of pPaeBURNS1, *E. coli* MV10 (pPaeBURNS1::Tn1723) was further conjugated with *E. coli* HB101 that is streptomycin (Str) resistant. Transfer of pPaeBURNS1::Tn1723 from MV10 to HB101 makes the latter Str^R and Kan^R that was used for selection of transconjugants. The presence of the plasmid was again confirmed by PCR.

3.3 Displacing pPaeBURNS1 from P. aeruginosa 943 strain

Plasmids usually confer certain phenotypes on their hosts such as resistance to antibiotics, increased virulence etc. To determine these different phenotypes it is necessary to create isogenic strains of the host with and without the plasmids. Hale *et. al.* (2010) have developed an efficient strategy to displace different types of plasmids from their hosts. This involves designing a pCURE plasmid carrying genes that encode for proteins that will interfere in the stable maintenance of the plasmid to be displaced. To displace IncP-1 plasmids pCURE11 was designed. This plasmid has a pAKE604 backbone with Kan^R and ampicillin (Amp) resistance genes. It also has a *sac*B gene encoding levan sucrase which hydrolysis sucrose to form levans, which is toxic to Gram-negative bacteria. Thus, *sac*B can be used as a counter-selection for cells that have lost the pCURE11 plasmid. It also carries *ori*T$_{RK2}$, which will enable the self-transfer of pCURE11 when present in *E. coli* S17-1 carrying the *tra* genes. To enable pCURE11 to displace IncP-1 plasmids *par*D, *inc*C-*kor*A and iterons of *ori*V of RK2 were inserted into pCURE11. The replication of IncP-1 plasmids initiates by binding of replication initiation protein TrfA to the iterons in *ori*V. Thus, presence of *ori*V on pCURE11 will enable titration of TrfA away from the IncP-1 plasmid inhibiting its replication. In addition, TrfA-*ori*V complexes will associate with each other and prevent replication via handcuffing model (Blasina *et. al.,* 1996). ParD is the anti-toxin to the post-segregational killing system present on RK2, thus, preventing the harmful effect of toxin when IncP-1 plasmid is lost. KorA is a transcriptional repressor of *trf*A and IncC is known to destabilize RK2 when present in trans (due to incompatibility) (Meyer and Hinds, 1982). Hale *et. al.* have shown that pCURE11 can also be used to displace IncP-1β plasmids too and thus this strategy can be used to displace pPaeBURNS1 from *P. aeruginosa* 943.

pCURE11 has a pMB1 replicon which has a narrow host range due to which the plasmid
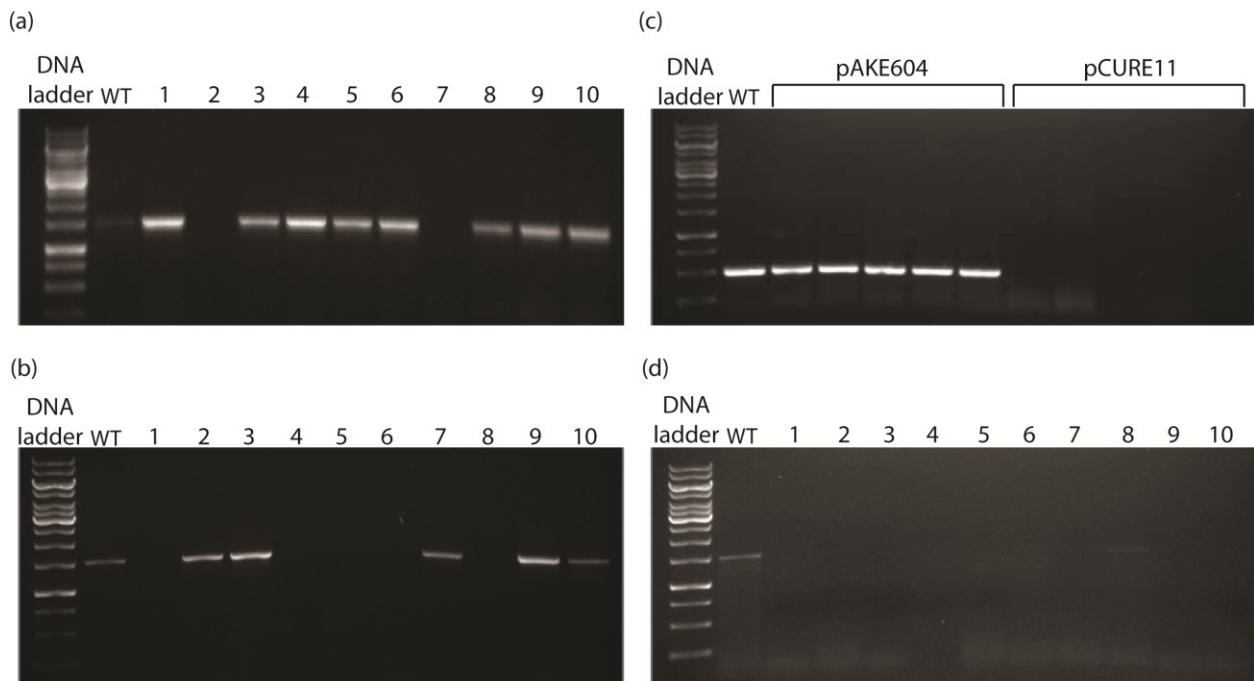
Figure 3: PCR analysis of curing of *P. aeruginosa* 943 (pPaeBURNS1) by (a) pCURE11.0 (b) pCURE11.1 and (d) pCURE12. (c) PCR analysis of curing of *E. coli* MV10 (pPaeBURNS1::Tn1723) by pAKE604 or pCURE11. The bands in lane 1-10 represent the presence of pPaeBURNS1. WT refers to *P. aeruginosa* 943 (pPaeBURNS1).

will not be able to replicate in *P. aeruginosa*. Therefore, pCURE11 was ligated to pJH10, which has a broad host range IncQ replicon. This hybrid, called pCURE11.1, was transferred into the *P. aeruginosa* 943 strain by conjugation. Recipients of the pCURE plasmid were selected by Kan (500 µg/ml) in M9 minimal media. As a negative control, pJH10 was also ligated with pAKE604 and the hybrid was called pCURE11.0. This plasmid was also transferred to *P. aeruginosa* 943 strain and selected, as in the case of pCURE11.1. PCR analysis of 10 colonies each on the selection plates was done using UpF and DownR to check for the presence of pPaeBURNS1. Figure 3a and 3b show the image of the gel electrophoresis done on PCR products in case of pCURE11.0 and pCURE11.1 respectively. As expected, pCURE11.0 was not able to displace pPaeBURNS1 as 8 out of 10 colonies still had the plasmid. However, in case of pCURE11.1, 50% of the colonies (5 out of 10) still had the plasmid. This shows that curing or displacement of pPaeBURNS1 by pCURE11.1 is not efficient.

The inefficient displacement of pPaeBURNS1 by pCURE11.1 can be because of a unique feature of pPaeBURNS1 not found in other IncP-1β plasmids. Another possible reason is that pCURE11 is unable to displace IncP-1 plasmids in hosts other than *E. coli,* such as *P*. aeruginosa. To further investigate this, we transferred pCURE11 into *E*.coli MV10 (Nal^R) carrying pPaeBURNS1::Tn1723 to see if pCURE11 can displace pPaeBURNS1 in *E. coli*. From Figure 3d, it can be seen that pCURE11 was able to displace pPaeBURNS1 in *E.* coli efficiently. This

indicates that the inefficient displacement observed with pCURE11.1 was because of *P. aeruginosa* host. As a negative control, pAKE604 was also transferred into *E. coli* MV10 (pPaeBURNS1::Tn1723) and no displacement of pPaeBURNS1 was observed. Wilson *et. al.* (1997) have shown that the *kil*E locus, present on IncP-1 plasmids, is required for their stable maintenance in *P. aeruginosa* but not in *E. coli. kil*E locus comprises of *kle*A-F in RK2 and *kle*A, *kle*B, *kle*E and *kle*F in R751 and is located in the *ccr* region of IncP-1 plasmids. In both IncP-1α and IncP-1β, the expression level of *kil*E locus is down regulated by two transcriptional repressors- KorA and KorC. As mentioned above, KorA is already present in pCURE11. Therefore, we PCR amplified *kor*C from pPaeBURNS1 with EcoR1 and Xba1 restriction sites at the 5' and 3' end respectively. This fragment was inserted downstream of the inducible *tac*p in pJH10 to give pJH10.1. The modified pJH10- pJH10.1 was ligated to pCURE11 to give pCURE12. As in the case of other pCURE plasmids, pCURE12 was also transferred into *P. aeruginosa* 943 by conjugation. The transconjugants were selected by plating on M9 minimal media plates supplemented with Kan (500 μg/ml) and IPTG (0.5mM). Again, PCR analysis was done to check for loss of pPaeBURNS1. As seen in Figure 3d, 9 out of 10 colonies had lost the plasmid. This shows that pCURE strategy can be used to displace IncP-1 plasmids from *P. aeruginosa* strain efficiently by inserting *kor*C into the pCURE plasmid.

To displace pCURE12 from the *P. aeruginosa* 943 strain that has lost pPaeBURNS1, colonies were picked from selection plates, grown overnight in LB without selection and checked for sucrose^R and Kan^S. This phenotype will be exhibited by the bacterial cells that have lost the pCURE12 plasmid thus resulting in isogenic strain of plasmid free *P. aeruginosa* 943.

3.4 Effect of IncP-1 plasmids on the biofilm formation activity of E. coli and Pseudomonas spp.

All the 11 *P. aeruginosa* isolates with pPaeBURNS1 were found in a single room in the Burns Unit of QE hospital. Such a high number of samples in the same room might indicate towards a biofilm at a point, perhaps the showerhead, from where the plasmid positive strains are being dispersed throughout the room. To check if the presence of pPaeBURNS1 has any effect on the biofilm formation activity of *P. aeruginosa* 943 a microtiter assay with crystal violet staining was done to compare the amount of biofilm formed by isogenic strains with and without the plasmid. In addition, to determine the effect of other IncP-1 plasmids on the biofilm formation activity RK2, R751 and pPaeBURNS1::Tn1723 were transferred, via conjugation, into the cured *P. aeruginosa* 943 strain. Figure 4a shows the amount of biofilm formed by *P. aeruginosa* 943 when it is hosting different plasmids. It can be seen that the cured *P. aeruginosa* 943 strain and the strain with IncP-1 plasmids form nearly the same amount of biofilm as the wild type *P. aeruginosa* 943 (pPaeBURNS1). Growth yield of the bacterial strains was also recorded, before staining the cells with crystal violet, to confirm that the difference in the amount of biofilm formed is not due to slow growth. From Figure 4b, except for the strain with RK2, all the other
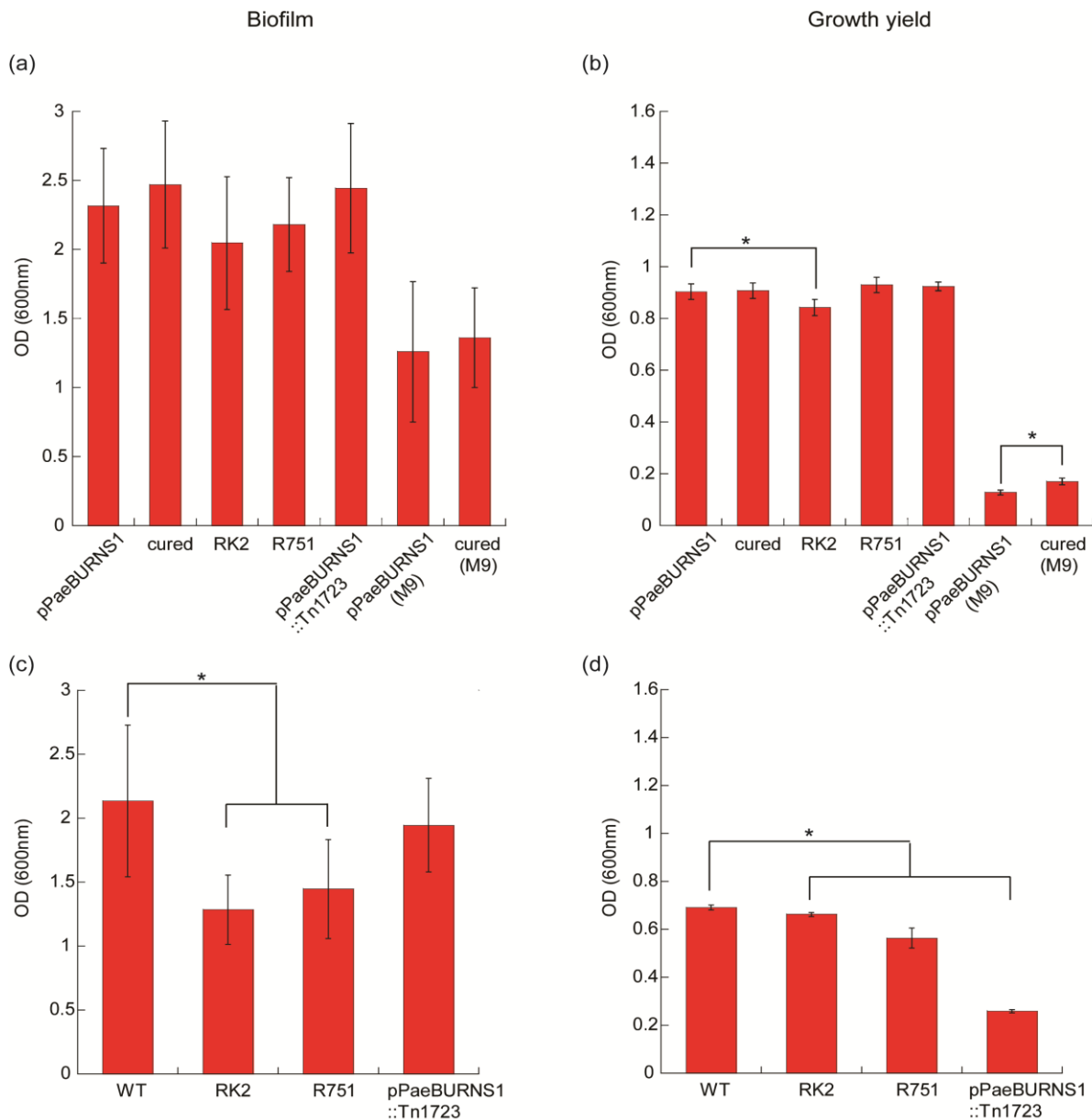
Figure 4: (a), (c) Amount of biofilm formed by *P. aeruginosa* 943 and PAO1161, respectively, carrying different plasmids. (b), (d) Growth yield of *P. aeruginosa* 943 and PAO1161, respectively, carrying different plasmids. M9 written in (a) and (b) represents growth yield of strains in M9 minimal media. * represents statistically significant data (p- value < 0.05). WT refers to plasmid-free strains. Error bars represent the standard error of the mean from eight biological repeats of the experiment.

strains have similar amount of growth. The strain with RK2 shows inhibited growth, which is significantly less compared to the strain with the pPaeBURNS1 plasmid. A similar trend is also observed when the wild-type plasmid positive strain and the cured strain are grown in M9 minimal media. The cured strain has slightly elevated growth yield (Figure 4b) but the amount of biofilm formed (Figure 4a) is nearly the same.
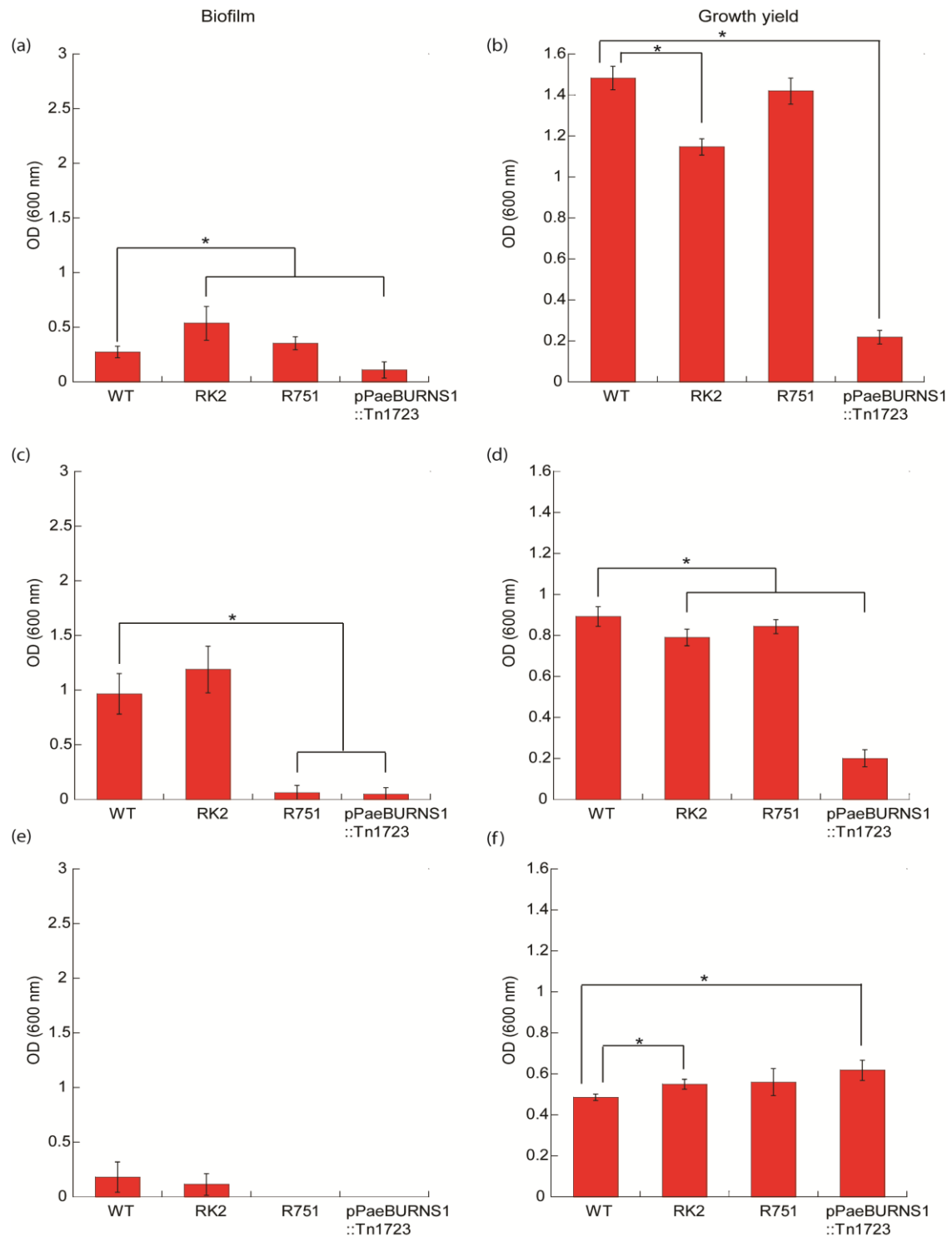
Figure 5: (a), (c), and (e) Amount of biofilm formed by *P. putida* KT2440, *P. fluorescens* SBW25 and *E. coli* C600 respectively, carrying different plasmids. (b), (d) and (f) Growth yield of *P. putida* KT2440, *P. fluorescens* SBW25 and *E. coli* C600, respectively, carrying different plasmids. * represents statistically significant data (p- value < 0.05). WT refers to plasmid-free strains. Error bars represent the standard error of the mean from eight biological repeats of the experiment.

	LB		M9	
	WT	Cured	WT	Cured
Chloramphenicol	256 µg/ml	512 µg/ml	128 µg/ml	256 µg/ml
Kanamycin	256 µg/ml	512 µg/ml	512 µg/ml	512 µg/ml
Naladixic acid	512 µg/ml	>512 µg/ml	64 µg/ml	128 µg/ml
Streptomycin	128 µg/ml	128 µg/ml	64 µg/ml	128 µg/ml
Tetracycline	32 µg/ml	32 µg/ml	32 µg/ml	32 µg/ml
Sodium chlorite	>4 mM	2 mM	2 mM	0.5 mM

Table 3.2: MIC values of different antibiotics for *P. aeruginosa* 943 with the plasmid (WT) and without the plasmid (cured) both grown in LB as well as M9 minimal media.

To determine how IncP-1 plasmids affect the growth yield and biofilm activity of different bacterial hosts, RK2, R751 and pPaeBURNS1::Tn1723 were transferred into *P. aeruginosa* PAO1161 that is a derivative of *P. aeruginosa* PAO1. In addition, these plasmids were transferred into other *Pseudomonas* spp. such as *P. putida* KT2440, *P. fluorescens* SBW25. These strains were chosen because of their natural occurrence in the environment. Apart from the *Pseudomonas* spp., the IncP-1 plasmids were also transformed into *E. coli* C600. Similar to *P. aeruginosa* 943, the growth yield and the amount of biofilm formed by these strains was measured. From Figure 4d, 5b and 5d, it can be seen that presence of IncP-1 plasmids causes the *Pseudomonas* spp. strains to grow slowly. The most drastic effect is observed in case of *P. putida* KT2440 and *P. fluorescens* SBW25 when they carry pPaeBURNS1::Tn1723. In *E. coli* C600, presence of the plasmids increases the growth yield of the bacterial strain, with the highest yield in case of pPaeBURNS1::Tn1723 (Figure 5f). Figure 4c, 5a and 5b show the effect of IncP-1 plasmids on the biofilm activity of their *Pseudomonas* hosts. In *P. aeruginosa* PAO1161, the amount of biofilm decreases when it carries IncP-1 plasmids as compared to the plasmid free strain. Presence of RK2 causes the strain to form the least biofilm, but in case of pPaeBURNS1::Tn1723, no difference is observed. A totally opposite trend is observed in *P. putida* KT2440 (Figure 5a), *P. fluorescens* SBW25 (Figure 5c) and *E. coli* C600 (Figure 5e). In these strains, presence of RK2 either increases or has no effect on the amount of biofilm formed but presence of R751 and pPaeBURNS1::Tn123 nearly abolishes the biofilm formation activity. These results may indicate that different IncP-1 plasmids may prefer specific bacterial hosts and exhibit different phenotype in different hosts.

3.5 Effect of pPaeBURNS1 on the antibiotic resistance of P. aeruginosa 943

From the data obtained through High Throughput Sequencing (HTS), the *P. aeruginosa* strains that were isolated from wound swabs and do not carry any plasmid were found to be resistant to imipenem and meropenem. However, the strains, such as *P. aeruginosa* 943 carrying pPaeBURNS1 that were isolated from environmental samples, were susceptible to these

antibiotics. This suggests that pPaeBURNS1 is affecting the antibiotic resistance of its hosts. To ascertain whether this is true for commonly used antibiotics, we determined the minimal inhibitory concentration (MIC) of *P. aeruginosa* 943 (pPaeBURNS1) and the isogenic strain without the plasmid. MICs were determined in LB as well as M9 minimal medium and are presented in Table 3.2. The antibiotics that were chosen belonged to different groups-aminoglycoside (kanamycin), fluoroquinolones (nalidixic acid), ansamycins (streptomycin), tetracycline and chloramphenicol. High MIC values were observed, in case of all the antibiotics tested, for both the plasmid positive and the plasmid negative strain. However, the former exhibited either the same or higher susceptibility (lower MIC values) to different antibiotics than the cured strain. This effect was observed in rich as well as minimal media. MIC of sodium chlorite was also determined to see whether the chlorite dismutase gene present on the plasmid is functional. The plasmid positive strain showed higher resistance to sodium chlorite than the plasmid negative strain, which indicates towards the chlorite dismutase gene being functional.

3.6 Stability of pPaeBURNS1 in P. aeruginosa 943 and E. coli C600

To test the stability of pPaeBURNS1::Tn1723 in *P. aeruginosa* 943, the plasmid positive strains were grown without selection as described in Materials and Methods. *P. aeruginosa* 943 (pPaeBURNS1::Tn1723) was grown in both LB and M9 minimal media and the stability of the plasmid was assessed. As seen in Figure 6, the plasmid is extremely stable up to 48 hours of non-selective growth in LB as well as minimal media. Thereafter, the plasmid is rapidly lost when the
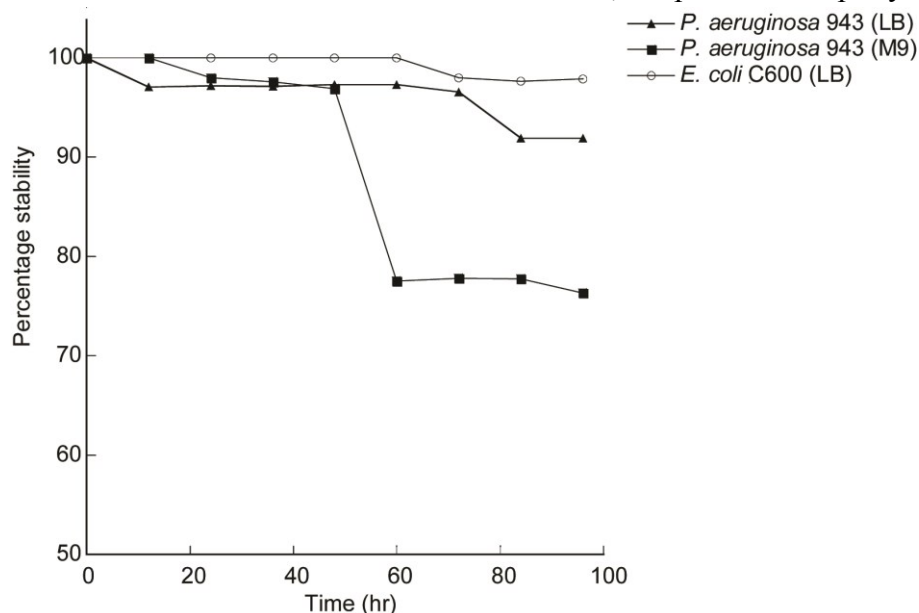
Figure 6: Stability of pPaeBURNS1::Tn1723 in *P. aeruginosa* 943 grown in LB (▲), M9 minimal media (■) and in *E. coli* C600 grown in LB (○).

strain is grown in minimal media (75% stability after 96 hours as compared to 95% stability for cells grown in LB). This shows that the type of growth media affects the stability of pPaeBURNS1 in *P. aeruginosa* 943.

The stability of the plasmid was also assessed in other bacteria such as *E. coli* C600 to see if pPaeBURNS1 prefers its *P. aeruginosa* host. Surprisingly, the plasmid showed higher stability in *E. coli* C600 as compared to *P. aeruginosa* 943 with 97% stability observed even after 96 hours of non-selective growth (Figure 6).

Discussion

pPaeBURNS1 is a 50 kb medium sized plasmid belonging to the IncP-1β group of self-transmissible and broad host range plasmids. A single transposon is inserted between the *tra* and *trb* regions on the plasmid. As compared to other IncP-1β plasmids, which carry antibiotic or heavy metal resistance genes, pPaeBURNS1 carries a chlorite dismutase gene (present in its transposon) which encodes for an enzyme involved in converting toxic chlorite into innocuous chloride and oxygen. Chlorite dismutase (Cld) is usually found in perchlorate-reducing bacteria (PRB) which respire in contaminated soil and water by reducing perchlorate into oxygen using perchlorate reductase and Cld (Bender *et. al.,* 2010). Chlorite is a toxic chemical used for bleaching in pulp and paper industries and as a component of fertilizers and cleaning solutions. It is a known contaminant of drinking water usually formed as a by-product of chlorination done to purify water (Yang *et. al.,* 2013) and due to environmental discharge by the above-mentioned industries. Thus, presence of *cld* on pPaeBURNS1 may provide resistance to this lethal chemical. Apart from providing resistance, Cld may also enable *P. aeruginosa* to grow in anaerobic niches as disproportionation of chlorite can provide oxygen for aerobic respiration. Frank *et. al.* (2008) have also mentioned the possibility of bacteria carrying the chlorite dismutase gene to live in a symbiotic relationship with PRBs under anaerobic conditions. The chlorite produced by PRBs by reduction of perchlorates can be supplied to the bacterium with *cld*, which can be further reduced into oxygen and enable the bacterium to thrive aerobically. Interestingly, Yoon *et. al.* (2002) have shown that *P. aeruginosa* forms more robust biofilms under anaerobic conditions than aerobic conditions. This suggests that in the presence of chlorite, *cld* may decrease the biofilm formation activity of *P. aeruginosa* 943. However, to analyse this effect functionality and activity of Cld needs to be assessed, although the difference in MIC of *P. aeruginosa* 943 with and without pPaeBURNS1 (Table 3.2) does indicate towards Cld being active.

The translated sequence of the various ORFs present in pPaeBURNS1 was compared to other IncP-1β plasmids- pAKD-1, pADP-1, pMBUI8 and pKV29. Aligning the sequence by Clustal Omega revealed a number of mutations unique to pPaeBURNS1. Majority of the mutation were in the *ccr* region of the plasmid and others in the *tra* region. A high number of mutations in the *ccr* may be suggestive of the different working of regulatory circuits in pPaeBURNS1 than in other IncP-1β plasmids. Out of the 104 unique mutations observed in pPaeBURNS1, seven mutations were present in the replication initiation protein TrfA. This protein recruits host proteins DnaB and DNA polymerase III to initiate the replication of IncP-1 plasmids (Jiang *et. al.,* 2003; Kongsuwan *et. al.,* 2006). Sota *et. al.* (2010) have shown that the IncP-1 plasmids shift their host range by developing single mutations in the N-terminal region of TrfA. These mutations result in increased stability in a new host but a loss in the stability in a previous host. In case of pPaeBURNS1, six mutations out of the seven present in TrfA are located in the N-terminal region of the protein. From this it can be hypothesized that

pPaeBURNS1 was initially unstable in *P. aeruginosa* 943 but has improved its stability by developing a number of mutations in the TrfA region. Modelling the structure of TrfA of pPaeBURNS1 is necessary to see whether these mutations change the structure of the protein, which may result in difference in its function. In the study by Sota *et. al.*, the mutations were observed when the bacteria, with the unstable plasmid, was grown under selective conditions for the plasmid. The same may also be true for *P. aeruginosa* 943 with the selection for pPaeBURNS1 being provided by chlorite. This is further confirmed by the result of the stability test shown in Figure 6. The figure shows a decrease in the stability of pPaeBURNS1 in *P. aeruginosa* 943 when grown in minimal media as compared to the strain grown in rich media. *P. aeruginosa* 943 was isolated from the water supply of the QE hospital and thus it is possible that in the absence of selection by chlorite, such low nutritional conditions would have caused the plasmid to be rapidly lost. The cluster of plasmid positive samples isolated from the same room indicates towards high chlorite selection in that particular room, which seems unlikely as the different rooms in the Burns Unit would have the same environmental conditions.

Apart from TrfA, a number of mutations were also present in the *klc*K and *kil*E region of pPaeBURNS1. *klc*K comprises of *klc*A and *klc*B and *kil*E comprises of *kle*A, *kle*B, *kle*E and *kle*F. These two operons are not involved in the replication or the conjugative transfer of IncP-1 plasmids but are considered to be required for the stability of the plasmid (Wilson *et. al.,* 1997; Thorsted *et. al.,* 1998) especially in *P. aeruginosa.* In pPaeBURNS1 these regions show a conservation of 91- 95% only when compared to other IncP-1β plasmids and thus it is possible that these mutations affect the stability of pPAeBURNS1 in *P. aeruginosa* 943. The number of mutations in other IncP-1 plasmids reported in Table 3.1 show that the amino acid sequence is fairly conserved among other IncP-1 plasmids and that pPaeBURNS1 has unusually higher number of mutations. The other IncP-1 plasmids have different source and host organism but still show a high conservation of the backbone. pPaeBURNS1, although obtained from *Pseudomonas* spp. as pADP-1, is significantly different which may indicate that the IncP-1β backbone adapts not only to new bacterial species but also to particular strains in the species. Thus, IncP-1β plasmids may not be as broad in their host range as previously considered. To validate this, further examination of different IncP-1β plasmids isolated from same species is required (similar to the study by Gelder *et. al.,* 2007 where they have used a single IncP-1β plasmid and observed its stability in different strains of the same species and genus). It is possible that not all the mutations observed in pPaeBURNS1 will be the result of host adaptation. The fact that the backbone of IncP-1 plasmids is considered to comprise of segments from other IncP-1 plasmids (Norberg *et.al.* 2011), and that some segments of pPaeBURNS1 show only 91- 95% similarity (in BLAST searches) may indicate towards other IncP-1 plasmids which have yet not been discovered.

To determine the phenotype conferred by pPaeBURNS1 on its host *P. aeruginosa* 943 isogenic strains with and without the plasmid were created. To displace pPaeBURNS1, a pCURE plasmid was created which have the genes inserted in it to facilitate the displacement of IncP-1

plasmids- *ori*V, *inc*C, *and parD*. This plasmid did not efficiently displace pPaeBURNS1 until *kor*C, which is a transcriptional repressor of kilE locus, was inserted. Inserting *kor*C increased the efficiency of the pCURE plasmid and thus pPaeBURNS1 was displaced from *P. aeruginosa* 943 without stressing the cells.

Next, to see whether pPaeBURNS1 has any effect on the biofilm formation activity of its host a standard microtiter assay was done to compare the amount of biofilm formed by the plasmid positive and plasmid negative *P. aeruginosa* 943. As seen in Figure 4a, there was no significant difference in the amount of biofilm formed by the two strains. A similar result was also observed when the strains were grown in M9 minimal media (Figure 4a). Although this result indicates that pPaeBURNS1 does not influence the biofilm formation activity of *P. aeruginosa* 943, it is also possible that in a biofilm, the cells rapidly lose the plasmid. *P. aeruginosa* is known to exhibit different physiological features in a biofilm as compared to planktonic cells (Sauer *et. al.,* 2002). These changes may result in the loss of the plasmid, which is otherwise somewhat stably maintained in planktonic cells (Figure 6). Before staining the cells with crystal violet, the absorbance of the cells was measured to see the effect of the plasmid on the growth of its host. Although no difference was observed in the growth yield of the two strains in LB, the cured strains had grown more in M9 minimal media than the plasmid positive strain.

To observe the effect of pPaeBURNS1 on other *Pseudomonas* spp. and *E. coli* the tagged plasmid (Figure 2) was transferred into *P. aeruginosa* PAO1161, *P. putida* KT2440, *P. fluorescens* SBW25 by conjugation with *E. coli* MV10 (pPaeBURNS1::Tn1723). The tagged plasmid was also transformed into *E. coli* C600. As in the case of *P. aeruginosa* 943, growth yield and amount of biofilm was recorded. As seen in Figure 4 and 5 the growth yield of all the *Pseudomonas* strains used was adversely affected. In contrast to this adverse effect, the growth yield of *E. coli* C600 increased in the presence of the plasmid (Figure 5f). The decrease in case of *Pseudomonas* strains, especially in *P. aeruginosa* PAO1161 further reaffirms our hypothesis that IncP-1 plasmids evolve to be stably maintained in a particular strain. The inhibited growth in these strains may be attributed to the copy number of pPaeBURNS1::Tn1723 or to the formation of persistor cells. Haugan *et. al.* (1995) have shown that some mutations in the *trf*A region of IncP-1 plasmids increase the copy number of the plasmid. These copy-up mutants may be toxic to hosts, which cannot tolerate the increase in the copy number. They have also shown that the tolerable copy number is different in different hosts and can be quite low in some bacterial species. This might explain why pPaeBURNS1::Tn1723 inhibits the growth of *Pseudomonas* strains but not *E. coli*. The copy number of the tagged plasmid may be quite high in the *Pseudomonas* strains resulting in decrease in their growth. Another possible explanation of this effect on growth is the formation of persistor cells as described by Poole (2012). Persistors are slow growing bacteria that arise randomly or due to environmental cues, such as stress conditions. Poole (2012) has mentioned the role of toxin-antitoxin (TA) system that may cause formation of persistors. The toxin released by this system is a bacteriostatic agent and thus effects the growth of bacterial cell. Thus, the presence of the tagged plasmid may cause the activation of

TA system in the *Pseudomonas* strains limiting their growth. The activation of TA systems is known to induce biofilm formation (Wang and Wood, 2011) which is observed in case of *P. aeruginosa* PAO1161 (Figure 4). The same phenomena is not observed in *P. putida* KT2440 and *P. fluorescens* SBW25 which might suggest a different mechanism, perhaps the increase in copy number of the plasmid, by which growth in these strains is affected. Another interesting observation from the results of the biofilm experiment is the effect of RK2 on different *Pseudomonas* spp. In case of *P. aeruginosa* 943 and PAO1161, the presence of RK2 decreases the growth yield as well as the amount of biofilm formed. However, in case of *P. putida* and *P. fluorescens* RK2 decrease the growth yield but the amount of biofilm formed increases as compared to the plasmid free strain. This shows that the phenotype exhibited by an IncP-1 plasmid depends on both the plasmid as well as the host, and cannot be generalized. An important thing to note is that the transposon (Tn1723) inserted into pPaeBURNS1, to tag the plasmid, may cause the plasmid to exhibit a different phenotype as compared to the untagged plasmid, such as a higher growth burden on its host or a change in its stability in different hosts. Also, all the strains, (except for *E. coli* C600) carrying the tagged pPaeBURNS1, were created by conjugation with *E. coli* MV10. The effect of plasmid donor on the host range of IncP-1 plasmids is well-documented (Gelder *et. al.,* 2005), and should be considered in this case.

After analysing the effect of IncP-1 plasmids on the biofilm formation activity of different hosts, we determined the effect of pPaeBURNS1 on the antibiotic resistance of *P. aeruginosa* 943. Table 3.2 shows that the plasmid positive strain was either more or equally susceptible (based on MIC values) to antibiotics tested as compared to the cured strain. This is in accordance with the fact that the plasmid free *P. aeruginosa* strain isolated from wound swabs of the burn victims was resistant to imipenem and meropenem, as compared to the plasmid positive strains (Josh Quick and Nick Loman, pers. comm.). *P. aeruginosa* shows resistant to many antibiotics due to a number of multi-drug resistant (MDR) efflux pumps that are employed to pump out antibiotics from the cell. Plasmids such as IncP-1 have been known to influence the expression of such pumps. Transcriptome analysis of *Pseudomonas* host with and without pCAR-1 (an IncP-7 plasmid) was done by Shintani *et. al.* (2010). They have shown that *P. putida* KT2440 (pCAR-1) has an up-regulated MexEF-OprN efflux pump as compared to the plasmid free strain. There are other studies which provide evidence of "crosstalk" between the plasmid and the host chromosome (Harr and Schlotterer, 2006; Miyakoshi *et. al.,* 2007). Thus, it is possible that the lower MIC values observed for *P. aeruginosa* 943 (pPaeBURNS1) is due to the plasmid down regulating an important component of the resistance mechanism of *P. aeruginosa*, such as a MDR efflux pump. This may also be the reason for its low virulence. Burn victims will usually be on a drug regime to prevent bacterial infection and therefore, the plasmid positive *P. aeruginosa*, being more susceptible to antibiotics, may not be able to infect the patients. As mentioned in Section 2.12, the MIC values were determined by broth dilution method using LB-broth or M9 minimal media. However, for MIC determination, there is a need for following standardised methods, such as those outlined by the British Society for Antimicrobial

Chemotherapy (BSAC). Also, only a single two-fold dilution difference was observed in the MIC values for the plasmid positive and the plasmid negative strain, which is usually considered as within the error of the method used (Prakash *et. al.,* 2008; Turnidge *et. al.*, 2006). Therefore, further experimentation is required, following standardised methods, to determine the MIC values for the two strains accurately.

Overall, there are some indications of pPaeBURNS1 conferring some phenotype on its *P. aeruginosa* host. The initial studies done on pPaeBURNS1 and reported here will pave the path for exploring the role of the plasmid in *P. aeruginosa* and divergence and evolution of IncP-1 plasmids in different hosts. Future work will include transcriptome analysis of *P. aeruginosa* 943 (pPaeBURNS1) and will be compared to that of the cured strain to determine if there is a crosstalk between the plasmid and the host chromosome. In addition, functionality of chlorite dismutase (present on pPaeBURNS1) will be confirmed by a suitable enzyme assay. Finally, as none of the plasmid positive *P. aeruginosa* strains were isolated from the patients, the virulence of *P. aeruginosa* 943 (pPaeBURNS1) will be compared to that of the cured strain, using epithelial cell lines.

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Project 2: Acid detection by the EvgS/A two-component system

Abstract

EvgS is a histidine sensor kinase and part of the EvgS/A two-component system involved in detecting low pH and regulating the expression of the glutamate-dependent acid resistance system. Although the exact mechanism of acid detection is unknown, it is likely that the periplasmic domains of EvgS mediate acid detection and transduce the signal to the cytoplasmic domains. As the optimum pH ($5.5 - 5.7$) detected by EvgS is close to the pKa of side chain of histidine residues, His-Gln and His-Ala mutations were introduced into EvgS to determine if any of the eight His residues, in the first periplasmic domain, play a role in acid detection. EvgS protein with either H63A or H226Q mutation exhibited complete loss of activity, although the former protein was not localized to the membrane. Site-directed mutagenesis was employed to randomly mutate His-63 and His-226 to different amino acids to decipher the exact role of these His residues. In the absence of specific free amino acids from the solution, no change in the activity of EvgS was observed at pH 5.6, suggesting that free amino acids do not play a role in activating EvgS. However, activity of EvgS was found to vary with the change in the concentration of KCl in the solution. Finally, affinity chromatography was used to purify the periplasmic domains of EvgS, which can be used for crystallization and other biophysical analysis.

Acknowledgement

Table of Contents

List of Figures

List of Tables

Introduction

1.1 Acid resistance in Escherichia coli

Enteric bacterial species, both pathogenic and commensal, normally found in the digestive tract of humans and animals, must be able to survive the acidic conditions of the stomach where pH~ 2 is observed. This low pH usually acts as a natural barrier against microbial infection as acidic pH has adverse effects on protein folding, thus damaging the biological processes inside the cell (Hong *et. al.,* 2012). However, various bacterial species have developed intricate systems, which prevent the acidification of their intracellular environment, and thus, ensure their survival in such inhospitable conditions. *Helicobacter pylori* majorly rely on a urease dependent system that hydrolyses urea to ammonia and carbon dioxide, both of which are involved in maintaining the cytoplasmic and periplasmic pH near neutrality (Scott *et. al.*, 2002). In case of *Salmonella enterica* serovar Typhimurium, there is an acid tolerance response (ATR) by which bacteria, exposed to mild acidic conditions of pH 4.5, express acid shock proteins (ASPs) which enable its survival in subsequent extreme acidic conditions of pH 3 (Foster, 1999). Similar ATR is also present in *H. pylori* (Karita and Blaser, 1998) and *Vibrio cholerae* (Merrell and Camilli, 1999). In *E. coli,* acid resistance is mainly because of the three decarboxylase systems that utilize protons for decarboxylation of different amino acid substrates and are described in detail below. These decarboxylase systems are similar to the ATR observed in other enteric bacteria, such as, *Lactococcus lactis, Brucella abortus* and *Shigella flexneri* (Foster, 2000 and Hong *et. al.* 2012). Recently, Lund *et. al.* (2014) have reviewed the acid resistance mechanisms in both Gram-positive and Gram-negative bacteria.

1.1.1 Acid resistance (AR) systems in E. coli

As mentioned above, *E. coli* has four decarboxylase systems- AR1, AR2, AR3 and AR4. These systems mediate tolerance to different acid levels in different growth phase.

AR1 is an oxidative resistance system involving stationary phase sigma factor RpoS, CRP (cAMP receptor protein) and F_0/F_1 ATPase (Richard and Foster, 2004). When *E. coli* is grown in complex media and at pH 5.5, in the absence of glucose, to stationary phase AR1 is activated and provides resistance during acid challenge at pH 2.5 in minimal media. This resistance is not observed when *E. coli* is initially grown at pH 8. The exact mechanism by which AR1 protects the cell is still unclear. Castanie-Cornet *et. al.*(1999) have shown that amino acids, glutamate and glutamine, act as activators of AR1 and that, this system can provide protection even in *crp* mutants or glucose-repressed cells, if glutamate is present in excess. In addition, there is some indication of an inhibitor which is expressed at pH 8 and not at pH 5.5 which may prevent the activation (and not the synthesis) of the system.

The other three AR systems present in *E coli* comprise of one or more decarboxylase and an antiporter. The functionality of these ARs is dependent upon a particular amino acid substrate, such as, glutamate for AR2, arginine for AR3 and lysine for AR4. In all the three ARs, decarboxylases (isozymes GadA and GadB in AR2, AdiA in AR3 and CadA in AR4) act to replace the α-carboxyl group of their amino acid substrate with a proton acquired from the cytoplasm (Foster, 2004), thus maintaining the intracellular pH. The end products of these reactions are γ-amino butyric acid (GABA) for AR2, agmatine for AR3, cadaverine for AR4 and carbon dioxide. Following this reaction, the antiporters- GadC of AR2, AdiC of AR3 and CadB of AR4 expel the end products in exchange for new amino acid substrates. Among these ARs, the glutamate-dependent system (AR2) is the most effective, providing resistance at pH of 2.5 (Diez-Gonzalez & Karaibrahimoglu, 2004). It is induced at pH 5 in both log-phase and stationary phase cells (Castanie-Cornet and Foster, 2001). AR3 and AR4 are induced at pH of 5 and 5.5, respectively, under anaerobic conditions (Meng and Bennett, 1992). AR3 is able to provide protection at pH 2.5 whereas AR4 provides protection at a comparatively milder pH (Foster, 2004). This difference can be associated with the pH optima of the decarboxylases. During acid shock, the internal pH of the cells is maintained by the activity of the decarboxylase (to siphon off protons). If the internal pH of the cell tends to rise above the optimum pH of the decarboxylase, the activity of the decarboxylase decreases resulting in a concomitant decrease in internal pH. In case of AR4, the optimum pH of decarboxylase CadA is higher than the decarboxylases of other AR systems (Gale and Epps, 1944). Therefore, when cells are exposed to extreme acidic conditions (such as at pH 2.5), the initial proton intrusion drives the internal pH of the cells far below the required optima for CadA and thus, AR4 is unable to provide acid resistance at pH 2.5. It is important to note that the different AR system maintain the internal pH of the cells at different levels to ensure optimum activity of their decarboxylases.

AR systems are regulated by complex reiterative circuits enabling the induction of a particular system in different environmental conditions. The regulatory circuits of AR2 are the best studied, with at-least 11 regulators affecting the induction of AR2 (Figure 1). The central activator is GadE, which activates the transcription of the *gadA* and *gadBC* operons (Ma *et. al.*, 2003a). Other regulators such as RpoS, EvgAS, CRP, HNS, GadX, GadW etc. feed into GadE (Masuda and Church, 2003; De Biase *et. al.* 2003 and Ma *et. al.* 2003b). These regulators allow the induction of *gadA/BC* in acidified minimal media in the log as well as stationary phase. However, in complex media induction occurs in the stationary phase cells only (Foster, 2004). In case of AR3, *adiA* is located upstream of *adiC* and are separated by *adiY* which encodes for an AraC-like regulator. When overexpressed, AdiY activates the expression of *adiA* and *adiC* (Stim-Herndon *et. al.* 1996). CysB, a member of the LysR family of regulators, is also a regulator of AR3 (Shi and Bennett, 1994). Finally, the *cadAB* operon of AR4 is regulated by the protein expressed by *cadC*, located upstream of the *cadAB* operon (Watson *et. al.*, 1992). CadC senses the external pH directly via its periplasmic domain, where a disulfide bridge is known to
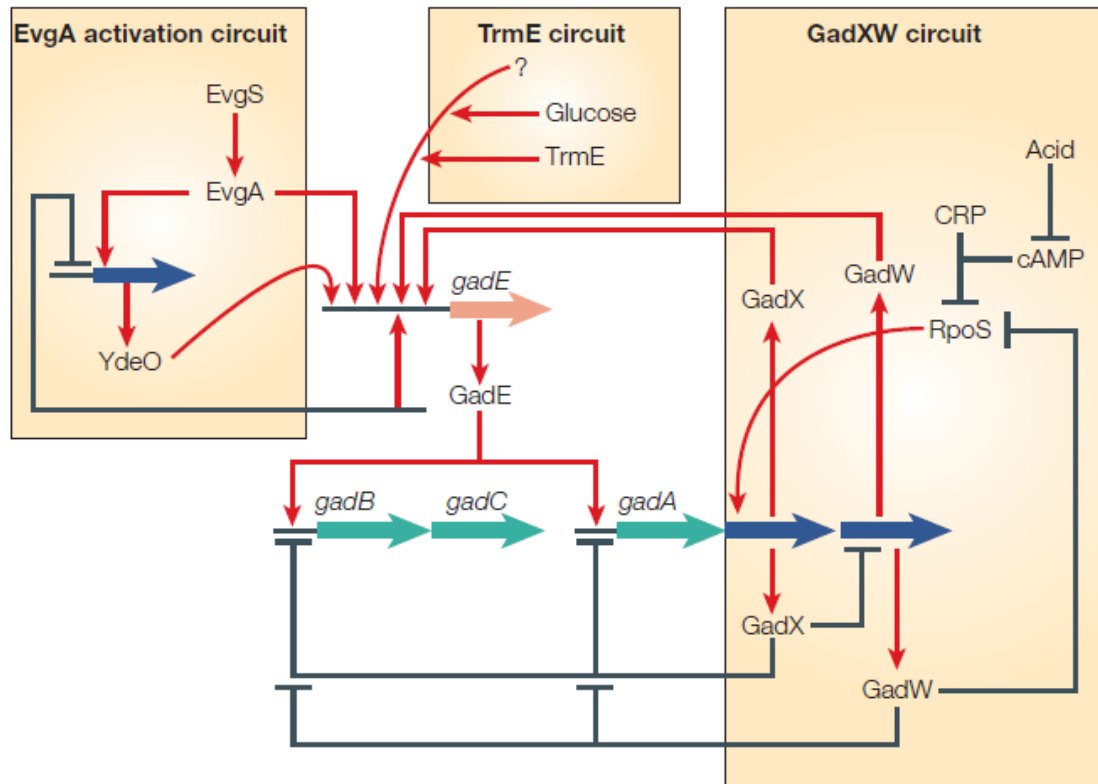
Figure 1: Regulatory circuits of glutamate-dependent acid resistance system (AR2). (Adapted from Foster, 2004)

play a role (Tetsch, 2011), and excess lysine, indirectly via interaction with the lysine permease LysP (Tetsch, 2008), and integrates this signal into the induction of the *cadAB* operon.

1.1.2 Other mechanisms of acid resistance in E. coli

Apart from the AR systems, other mechanisms exist which provide protection to *E. coli* under acid stress. Periplasmic chaperones such as HdeA, HdeB and Hsp31 (which is also a heat - inducible chaperone) are implicated to play a role in acid resistance in *E. coli*. HdeA and HdeB are encoded by the *hdeAB* (HNS-dependent expression A and B) operon, and provide protection by preventing the aggregation of periplasmic proteins at low pH. Both HdeA and HdeB exist in a dimeric form at neutral pH and attain a monomeric form with hydrophobic regions exposed when pH falls to 2 and 3, respectively. These exposed hydrophobic regions allow HdeA and HdeB to bind to substrate proteins and prevent their aggregation (Hong *et. al.*, 2005 and Kern *et. al.* 2007). It is interesting to note that the expression of *hdeAB* is regulated by YdeO and GadX, which also regulate the AR2 system (Masuda and Church, 2003). Although the exact mechanism by which Hsp31 provide protection against acid stress is still unclear, Mujacic and Baneyx (2007) have shown that Hsp31 may act as a cytoplasmic counterpart of HdeA and HdeB preventing aggregation of protein in stationary phase cells exposed to low pH.

Choi *et. al.* (2000) have shown that Dps (DNA-binding protein from starved cells), involved in DNA repair, also contributes to acid resistance in *E. coli*, either by protecting DNA from the harmful effect of low pH or by influencing the expression of some other gene involved in acid resistance.

1.2 Two Component Systems

Two-component system (TCS) is a signalling mechanism found in bacteria and certain eukaryotic organisms, and plays a pivotal role in detecting environmental cues. It consists of two proteins, a sensor kinase and a response regulator. A typical two-component system is shown Figure 2a. The sensor kinase comprises of an N-terminal input domain and a C-terminal transmitter domain while the response regulator comprises of an N-terminal receiver domain and a C-terminal output domain. The signal/cue, which is usually a phosphoryl group, is detected by the N-terminal domain of the sensor kinase and relayed to the response regulator via the C-terminal end. The response regulator receives the signal via its N-terminal domain. The phosphorylation state of the receiver domain regulates the activity of the C-terminal domain, which is usually a transcriptional regulator. This transcriptional regulator induces the expression of specific genes required for bacteria to adapt to the signal (Appleby *et. al*, 1996).
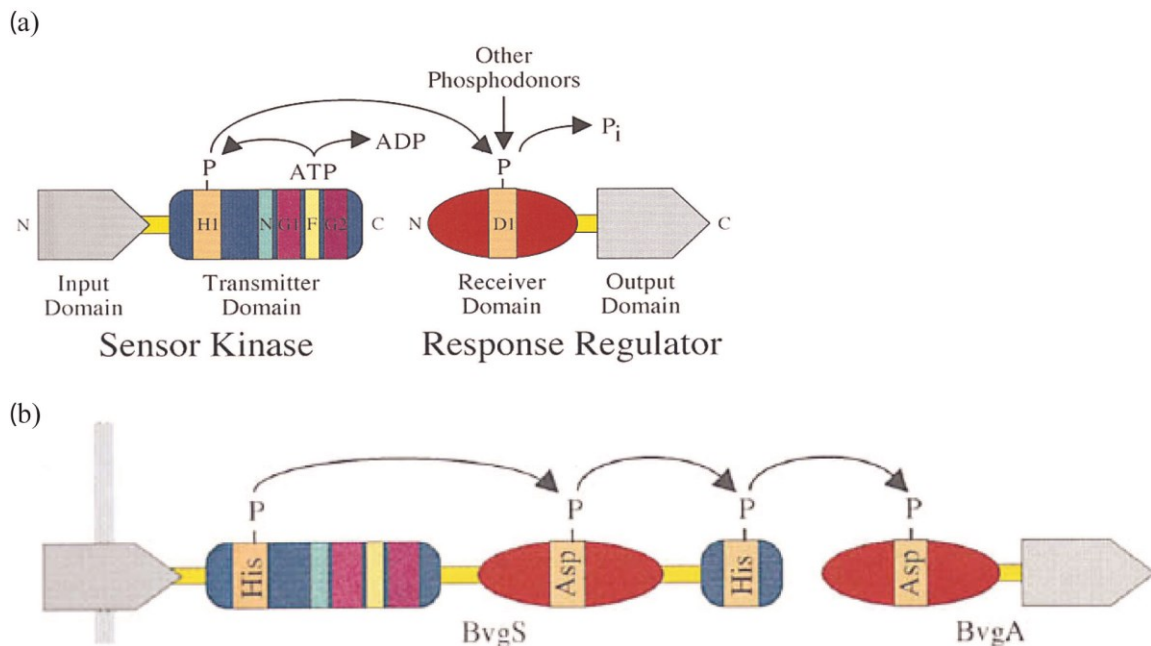
(a)

(b)

Figure 2: (a) Architecture of a typical two-component system (b) Architecture of BvgS/A two-component system (Adapted from Appleby *et. al*, 1996)

(a) EvgS
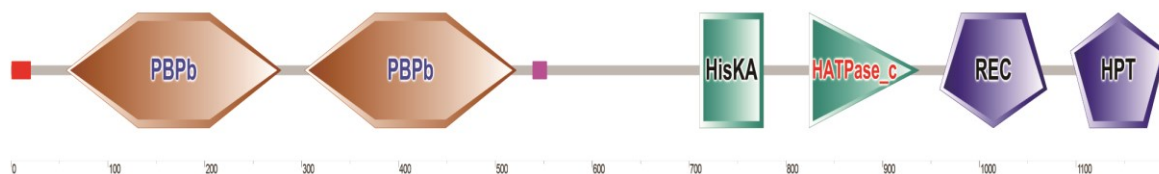
(b) BvgS
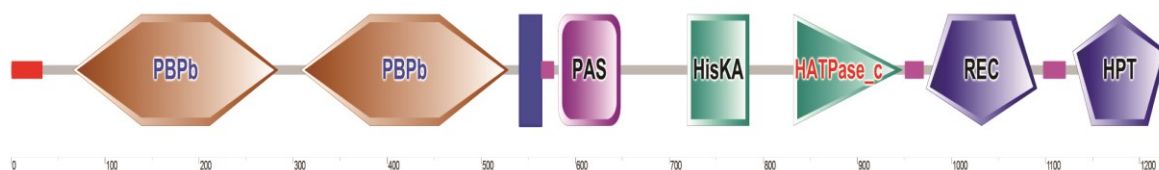
Figure 3: Domain architecture of EvgS and BvgS as obtained from SMART. The red box at the N-terminal end represents signal peptide and the blue box in between PBP and PAS domain is the transmembrane region

1.3 EvgS/A two component system

As shown in Figure 1, AR2 is under the control of different regulatory circuits. At the top of one of these circuits is the EvgS/A two-component system, comprising of a sensor kinase EvgS and a response regulator EvgA. EvgS/A is considered to be an unorthodox system because of the multiple domains present in the sensor kinase which are involved in a phosphorelay. Figure 2b shows the architecture of BvgS/A two component system (found in *Bordetella pertussis*) which closely resembles that of EvgS/A, although both systems do not have any functional similarity.

1.3.1 Architecture of EvgS

EvgS is a transmembrane protein 1197 amino acids in length and having a molecular weight of 134 kDa. The environmental signal detected by EvgS is considered to be low pH. Figure 3 shows the architecture of the sensor kinase EvgS obtained from SMART online server (Letunic *et. al.* 2011), along with a close homolog, BvgS. It comprises of two bulky periplasmic-solute binding protein (PBP) domains and five cytoplasmic domains- linker domain, His kinase A (HisKA) domain, His kinase-like ATPase (HATPase_c) domain, cheY-homologous receiver (REC) and His phosphotransfer (HPt) domain. The linker domain is a Per/ArnT/Sim (PAS) domain, although it is not recognised by the SMART server (Johnson *et. al.,* 2014). Typically, the solute binding domains, such as the two PBP domains, are flanked by transmembrane helices on either side (Mascher *et. al.*, 2006). These domains usually have a venus flytrap conformation with a solute-binding cavity, and occur in transporters of small molecules such as amino acids and opine (Lund *et. al.,* 2014 and Tam and Saier, 1993). However, EvgS has a single transmembrane helix after the second PBP (as shown in Figure 3) and is not known to play any role in transporting solutes. The five cytoplasmic domains are involved in a phosphorelay between a histidine residue

47

of one domain to the aspartate residue of another domain and so on. At the end, the phosphoryl group transfers from the His residue of the HPt domain to the Asp residue of the receiver domain of EvgA. This HPt domain is known to mediate the specificity of EvgS for its own response regulator (Perraud *et. al.*, 1998). Apart from phosphorelay, PAS domains are also known to function as sensors of light, oxygen and redox potential (Taylor and Zhulin, 1999) but it is not clear if the PAS domains does have such a role in EvgS. Through different studies, a number of constitutive mutations in EvgS are known which result in it being active even at neutral pH (Johnson *et. al.*, 2014 and Kato *et. al.*, 2000). These mutations map into the PAS domain of EvgS. Also, Moglich *et. al.* (2009) have shown that PAS domains are involved in dimerization of sensor kinases. Combining this with the position of the constitutive mutations in EvgS, Johnson *et. al.* (2014) have proposed a model for the activity of EvgS, according to which, two EvgS monomers are held tightly together in an inactive form. On receiving the signal, the dimer weakens and leads to activation of EvgS.

1.3.2 Signal detection by EvgS and regulatory function of EvgS/A system

DNA microarray analysis done by Masuda and Church (2002) on EvgA-overexpressing and EvgA-lacking *E. coli* strains revealed that in exponentially growing cells at pH 7, EvgA up-regulated the activity of 37 genes including the acid resistance genes *gadABC* and *hdeAB*, thus conferring acid resistance to the cells. Following this, Ma *et. al.* (2004) have shown that EvgA activates the expression of *gadE*, either directly or indirectly, by up-regulating YdeO that in turn activates *gadE*. The EvgA regulator is required for activation of *gadE* only at low pH of 5.5 in minimal media supplemented with salts and glucose. It is not required in stationary phase cells or cells growing in rich media such as LB (Ma *et. al.*, 2004). Burton *et. al.* (2010) have further shown that the promoters activated by EvgA, *b1500-ydeO*p, *gadE*p and *gadB*p, show an optimum activity between pH 5.5 and 5.7. These studies indicate that EvgA is activated at low pH and thus, it is likely that the environmental signal that EvgS detects is low pH ~5.5. However, it must be emphasized that no direct evidence of this is available and the exact mechanism by which EvgS detects low pH is still unknown. During growth at pH 5.5, the cytoplasmic pH of the cells is only slightly affected whereas the periplasmic pH equals that of external pH (Wilks and Slonczewski, 2007). This indicates that EvgS may detect low pH through its periplasmic domains. Johnson *et. al.* (2014) have shown that deleting the first PBP of EvgS (protein still found in membrane fractions) results in EvgS being unable to detect low pH thus further confirming that the periplasmic domains are involved in low pH detection.

Apart from up-regulating acid resistance genes, EvgA also up-regulates efflux pumps, such as, *emrKY, tolC, acrAB, mdfA* and *yhiUV* (Masuda and Church, 2002 and Eguchi *et. al.*, 2003), thus providing resistance against deoxycholate, ethidium bromide, SDS, crystal violet, erythromycin etc. YdeP and YdeO, which are up regulated by EvgA and are involved in mediating acid resistance by activating *gadE*, are also known to suppress the expression of type

III secretion genes in enteropathogenic *E. coli* (Nadler *et. al.*, 2006). Thus, EvgA indirectly affects the pathogenesis of *E. coli.*

1.4 Role of histidine residues in proteins

Due to its unique structural features, histidine plays many different roles in molecular interactions. The side chain of a histidine residue is an aromatic imidazole ring with a pKa of approximately 6, observed for the free amino acid in the solution. Thus, at pH < 6, histidine exists in a protonated form, with the positive charge equally distributed between the two nitrogen atoms of the imidazole ring. This ability of histidine makes it ideal for pH detection in various acid responsive proteins. Many studies have implicated histidine residues to be responsible for pH sensing (Weeks and Sachs, 2001; Perez and Groisman, 2007 and Fretz *et. al.* 2008). For example, Muller *et. al.* (2007) have shown that a histidine residue is involved in pH sensing by a sensor kinase ArsS of *H. pylori*. The proteins investigated in these studies detected low pH in the range of pH 5 – 6, similar to EvgS.

Apart from acid sensing, the imidazole ring of histidine may also be involved in forming coordination complex with metal cations and thus, histidine residues are usually present in the active site of metallo-enzymes (Lindskog, 1997 and Barondeau *et. al.*, 2004).

1.5 Background and Aims of this study

EvgS is a histidine sensor kinase, which is considered to detect low pH of 5.5-5.7 and initiates a cascade that leads to up-regulation of acid resistance genes such as *gadABC* and *hdeAB*. Although, the exact mechanism by which EvgS senses low pH is not known, it is likely that this is mediated by either one or both of the periplasmic domains of EvgS. This is because in cells growing at pH of 5.5, the cytoplasmic pH is only slightly affected whereas the periplasmic pH equals the external pH. The optimum pH at which the genes downstream of EvgS/A are activated is around 5.6. This is close to the pKa of the side chain of histidine residues. Thus, it is possible that EvgS is able to detect low pH via protonation of its histidine residues. It has also been shown that deleting the first periplasmic domain of EvgS results in the loss of its ability to detect low pH, although the protein is still present in the membrane (Johnson *et. al.*, 2014). This makes the histidine residues of the first periplasmic domain of EvgS the most likely candidates for playing a role in the activation of EvgS.

The aim of this study was to determine whether the eight histidine residues present in the first periplasmic domain of EvgS are required for EvgS to detect low pH. Site-directed mutagenesis was used to mutate the His residues to Gln or Ala and the activity of the corresponding EvgS mutants measured. Modelling the structure of the first periplasmic domain of EvgS has shown that it shares a high structural similarity with amino acid transporters. Therefore, role of free amino acids in the activation of EvgS was also investigated. Eguchi and Utsumi (2014) have shown that alkali metals Na^+ and K^+, in addition to low pH, are essential for

activation of EvgS. We further investigated the role of K^+ and determined the activity of EvgS by varying both pH and concentration of K^+. Finally, periplasmic domains of EvgS were purified by using affinity chromatography. The purified domains can be used further for crystallization and biophysical analysis.

Materials and Methods

2.1 Bacterial strains and growth conditions

The *E. coli* strains used in this study are - MG1655 (F⁻ λ⁻ ilvG- rfb-50 rph-1), MG1655 Δ*evgS*::aph *ydeP*p-lacZ, BL21 star (F⁻ ompT gal dcm hsdS$_B$ (r$_B^-$ m$_B^-$) *rne*131 (DE3)), C41 (F⁻ ompT gal dcm hsdS$_B$ (r$_B^-$ m$_B^-$) (DE3)). All the strains were grown in LB-broth, M9 minimal media. MG1655 Δ*evgS*::aph *ydeP*p-lacZ was also grown in 2X YT media. Growth conditions followed were- 37^{0}C with shaking at 180 r.p.m. unless stated otherwise.

2.2 Growth media and antibiotics

Solid media was obtained by addition of 1.5% agar. M9 minimal media was prepared as follows:-

NaH$_2$PO$_4$	6.0 g
KH$_2$PO$_4$	3.0 g
NH$_4$Cl	1.0 g
NaCl	0.5 g

These salts were dissolved in 1 L distilled water, pH adjusted to 7.0 or 5.6 and autoclaved at 121°C. To this solution, the following were added:-

20% glucose	20 mL
1M CaCl$_2$	100 μL
1M MgSO$_4$.7H$_2$O	2 mL
20% cas- amino acids	10 mL

2X-YT media was prepared as follows:-

Bacto tryptone	16 g
Yeast Extract	10 g
NaCl	5 g

These salts were dissolved in 1 L distilled water, pH adjusted to 7.0 and autoclaved at 121°C.

Kanamycin and carbenicillin were used in this study at a final concentration of 50 μg/mL and 100 μg/mL respectively. Stock solutions of both antibiotics were prepared in sterile distilled water.

2.3 Plasmids used in this study

Table 2.1 Plasmids used in this study

Plasmid	Size (kb)	Selective Marker	Properties
pBADevgS	7.5	Ap^R	*evgS* inserted downstream of pBAD promoter
pET41c	5	Km^R	Vector for expression of C-terminal 8X-His tagged proteins under the control of T7 RNA polymerase
pBAD24	4.5	Ap^R	Negative control for pBADevgS

Ap: Ampicillin; Km: Kanamycin

2.4 Oligonucleotides used in this study

Table 2.2 Primers used for site-directed mutagenesis

Name	Sequence (5' - 3')	Plasmid template*	Mutation
NAH106Q_F	CGGGAATACGCAGATCAGCAAAAAG CAATGGACGCG	pBADevgS	H106Q
NAH106Q_R	CGCGTCCATTGCTTTTTGCTGATCTGC GTATTCCCG	pBADevgS	
RMH131Q_F	CCGGCGCTGGTAACCACCCTTCAGGA CTCAATGCGACCG	pBADevgS	H153Q
RMH131Q_R	CGGTCGCATTGAGTCCTGAAGGGTGG TTACCAGCGCCGG	pBADevgS	
RM11His204_F	GCAGTATGATTTCCCGCTATTTCACTC AGTCCTTAAATGTAGTG	pBADevgS	H226Q
RM11His204_R	CACTACATTTAAGGACTGAGTGAAAT AGCGGGAAATCATACTGC	pBADevgS	
H63A_1	CGTAGCCGTTTGGGACTTAGCTACTG CAATCACAAGATTT	pBADevgS	H63A
H63A_2	AAATCTTGTGATTGCAGTAGCTAAGT CCCAAACGGCTACG	pBADevgS	
H72A_1	CGTTGCTGCGAATCGGTAGCCAACAA CGTAGCCGTTTG	pBADevgS	H72A
H72A_2	CAAACGGCTACGTTGTTGGCTACCGA TTCGCAGCAACG	pBADevgS	

H106A_1	GTCCATTGCTTTTTGAGCATCTGCGTATTCCCGGAGTG	pBADevgS	H106A
H106A_2	CACTCCGGGAATACGCAGATGCTCAAAAAGCAATGGAC	pBADevgS	
H124A_1	GCGGCGAAGTAACTAAAGCTGATAACACTATATCGACTTC	pBADevgS	H124A
H124A_2	GAAGTCGATATAGTGTTATCAGCTTTAGTTACTTCGCCGC	pBADevgS	
H153A_1	GGTCGCATTGAGTCGGCAAGGGTGGTTACCAGCG	pBADevgS	H153A
H153A_2	CGCTGGTAACCACCCTTGCCGACTCAATGCGACC	pBADevgS	
H180A_1	GCTTTTGGAAATGATTGAGCAATTACCTCGTCTGGGGGGTAA	pBADevgS	H180A
H180A_2	TTACCCCCCAGACGAGGTAATTGCTCAATCATTTCCAAAAGC	pBADevgS	
H205A_1	CTACCAATAAAGTAATCATTGGCCCCAGCTGAGACGGATGC	pBADevgS	H205A
H205A_2	GCATCCGTCTCAGCTGGGGCCAATGATTACTTTATTGGTAG	pBADevgS	
H226A_1	TTTCACTACATTTAAGGAGGCAGTGAAATAGCGGGAAATC	pBADevgS	H226A
H226A_2	GATTTCCCGCTATTTCACTGCCTCCTTAAATGTAGTGAAA	pBADevgS	
NAevgSH63N_F	CGTAGCCGTTTGGGACTTNNNTACTGCAATCACAAGATTT	pBADevgS	H63X**
NAevgSH63N_R	AAATCTTGTGATTGCAGTANNNAAGTCCCAAACGGCTACG	pBADevgS	
NAevgSH226N_F	TTTCACTACATTTAAGGANNNAGTGAAATAGCGGGAAATC	pBADevgS	H226X**
NAevgSH226N_R	GATTTCCCGCTATTTCACTNNNTCCTTAAATGTAGTGAAA	pBADevgS	
g2143t	TTGTAAACTGGCAAGGTAATGTCATTATTCATAATAGTGCTTTTGAAC	pBADevgS	S600I

g2143_antisense	GTTCAAAAGCACTATTATGAATAATG ACATTACCTTGCCAGTTTACAA	pBADevgS	

* Any plasmid carrying *evgS* can be used, ** X represents random amino acid.

Table 2.3 Primers for sequencing *evgS*

Name	Sequence (5' - 3')	Plasmid template	Remarks
RMevgS_R940	GGGGCGAGTAACGATTTTC C	pBADevgS	Can be used to sequence the His mutations in the first periplasmic domain
evgS+1330_R	CCTTGTGAAATGCAGCGC	pBADevgS	
evgS+1281_F	GGAGGTTGAATGGATACA GG	pBADevgS	Can be used to sequence the S600I mutation
evgS+2278_F	CACTCCTCGGCTTAATTG	pBADevgS	
NAevgS_2906F	CCAACAGGCTATTACTCAA ACG	pBADevgS	

Table 2.4 Primers for gene cloning

Name	Sequence (5' - 3')	Plasmid template	Remarks
NACpET41_ F	AAGGAGATATACATATGG ACGAAGATTACATCGAAT ATC	pET-41c	Used to clone the insert into the *NdeI-HindIII* digested pET-41c
NACpET41_ R	CGCGTGGCACAAGCTTTC CCCATAAAAGGCTACTG	pET-41c	
NAssCpET41 _F	AAGGAGATATACATATGA AGTTTTTACCCTATATTTT CTTC	pET-41c	Same as above with the signal peptide present in the insert

Primers were prepared by AltaBioscience, Birmingham, UK or Life Technologies and stored at -20°C after suspending them in sterile distilled water.

2.5 DNA isolation and manipulation
2.5.1 Plasmid DNA extraction
Plasmid was extracted from 5 mL of cultures grown overnight in LB-broth using Qiaprep Spin Miniprep Kit (Qiagen) or GeneJET Plasmid Miniprep Kit (Thermo Scientific). The manufacturer's instructions were followed to extract the plasmid and elution was done using 50 µL of elution buffer. After elution, the plasmid was stored at -20°C.

2.5.2 DNA digestion
To digest the DNA appropriate restriction enzymes from New England Biolabs (NEB) were used. 10 µL of the DNA (for example the eluted plasmid in 2.5.1) was mixed with 2.5 µL of 10X buffer. Restriction enzyme (0.5 µL) was added to the mix and the volume was made up to 25 µL by sterilized distilled water. This reaction mix was incubated at 37°C for 1 hour. Heat inactivation of the restriction enzyme was done by incubating the reaction mix at 80°C for 20 minutes.

2.5.3 Agarose gel electrophoresis and extraction
Agarose gel was prepared by dissolving 1% w/v agarose (Bioline) in 1X TAE buffer. Midori Green Advance DNA stain (Nippon Genetics Europe) was added to the agarose solution to facilitate the visualization of DNA on the gel. The solution was poured into a cast with a gel comb and cooled until solid. DNA sample with 5X loading dye (Bioline) was loaded into the wells of the gel and run for 30-40 minutes at 120V. The DNA bands were visualized by G:Box system from Syngene.

2.5.4 Polymerize Chain Reaction (PCR)
Colony PCR was done using My Taq Red Mix from Bioline. PCR reaction was set-up as follows:-

Template DNA	50 ng
Primer 1 (20 µM)	1 µL
Primer 2 (20 µM)	1 µL
My Taq Red mix (2X)	25 µL
distilled water	upto 50 µL

The following program was used to perform PCR:-

Step 1	Denaturation	95°C	1:30 min
Step 2	Denaturation	95°C	50 s
Step 3	Annealing	56°C	50 s
Step 4	Extension	72°C	30 s/kb of plasmid length

Repeat Step 2-4 30 times
Step 5   Extension   72°C   1 min

2.5.5 DNA sequencing

DNA samples were sequenced by the Functional Genomics Facility at the School of Biosciences, University of Birmingham, UK. The sample was prepared by adding 200-500 ng of DNA, 3.2 pmoles of primer to a final volume of 10 µL made up by water.

2.6 Preparation of E. coli competent cells

Overnight culture of *E. coli* was diluted 1:100 fold and grown in 25 mL of LB-broth at 37°C with shaking at 180 rpm until OD_{600} reached 0.4-0.6. The cells were harvested by centrifugation at 6,000 r.p.m for 7 minutes at 4°C. The pelleted cells were resuspended in 10 mL of pre-chilled 100 mM $CaCl_2$ and incubated on ice for 20 minutes. After incubation, the cells were again harvested at 6,000 for 7 minutes at 4°C and resuspended gently in 1.5 mL of 100 mM $CaCl_2$. These cells were aliquoted into smaller volumes and mixed with glycerol (final concentration of 20%) and stored at -80°C.

2.7 Transformation of E.coli cells

To transform *E.coli* cells, 3-5 µL of DNA was added to 100 µL of thawed competent cells as prepared above. The DNA-cells mix was incubated on ice for 30 minutes. Following incubation, heat shock was given to the cells by incubating them at 42°C for 60-90 seconds. After heat shock, the cells were cooled on ice for 5 minutes. LB-broth (800 µL) was added to the mix and the cells were grown at 37°C for 1 hour and plated onto selective L-agar plates. For the negative control, no DNA was added to the competent cells.

2.8 Site-directed mutagenesis

Site-directed mutagenesis (SDM) was done using QuikChange Lightning kit from Agilent Technologies following the manufacturer's instructions. Briefly, primers for SDM were designed using the Primer Design Program provided by Agilent Technologies. For mutant strand synthesis, PCR was performed on 75-100 ng of template DNA using 125 ng of each primer. The PCR program followed is given below:-

Step 1   Denaturation   95°C   2 min
Step 2   Denaturation   95°C   50 s
Step 3   Annealing   55°C   50 s
Step 4   Extension   65°C   30 s/kb of plasmid length
Repeat Step 2-4 18 times
Step 5   Extension   65°C   5 min

Dpn I digestion was carried out for 8-10 minutes instead of the recommended 5 minutes to achieve lower background. The digested mix (1.5 μL) was transformed into 45 μL of XL-10 gold ultracompetent cells following the manufacturer's protocol and 100 μL was plated onto LB-agar plates with the appropriate antibiotics. Colonies obtained were screened for SDM by DNA sequencing following plasmid extraction.

2.9 β-galactosidase assay

Overnight cultures of *E coli* MG1655 Δ*evgS ydeP*p-*lacZ* carrying the pBADevgs or pBAD24 plasmid were diluted to an initial OD_{600} = 0.005 or 0.05 into 5 mL of M9 minimal media at the required pH. KCl (125 mM) and arabinose (0.02%) was added to the diluted cultures. These cultures were grown for 60 min at 37°C with shaking and OD_{600} was recorded. Permeabilization solution for cell lysis was prepared by adding 20 μL of 0.1 % SDS and 40 μL of chloroform to 700 μL of buffer Z (composition given below). To this solution, 300 μL of minimal media cultures and 200 μL of ONPG (4 mg/mL) was added and incubated for 30 min at 30°C. Following incubation, 250 μL of 2M Na_2CO_3 was added to stop the reaction. From this solution 1 mL was transferred to a fresh microfuge tube (taking care to leave the chloroform ring) and centrifuged for 3 minutes at 13,00 r.p.m to remove any cell debris. OD420 was recorded and the activity was calculated in Miller units as follows:-

Composition of buffer Z:-
0.5 M Na2HPO4	12 mL
0.5 M NaH2PO4	8 mL
1 M KCl	1 mL
1 M MgSO4	100 μL
β-mercaptoethanol	270 μL

Total volume is made upto 100 mL by adding distilled water.

2.10 Preparing membrane fractions

Overnight cultures of *E coli* MG1655 Δ*evgS ydeP*p-*lacZ* carrying the pBADEvgS plasmid were diluted 1:100 fold into 100 mL of YT media and grown until OD_{600}~1. Cells were pelleted down by centrifuging the cultures for 10 min at 4°C and 8,000 r.p.m. The pellet was re-suspended in 16 mL of buffer M (composition given below) and again centrifuged for 10 min at 4°C and 8,000 r.p.m. The pellet was again resuspended in 8 mL of buffer M and sonicated for 15 min with 30 s bursts followed by 15 s of rest. The sonicated samples were centrifuged for 90 min at 4°C and 20,000 r.p.m. using JA-20 rotor. The pellet obtained was re-suspended in 4 mL of buffer M and again centrifuged for 60 min at 4°C and 20,000 r.p.m. Finally, the pellet was resuspended in 1.4 mL of buffer M. To analyse the membrane fractions on SDS-PAGE, 7.5 μL or 3.75 μL of sample was dissolved in 2X loading buffer and loaded onto the gel.

Composition of buffer M:-

TEA (pH 7.4)	50 mM
EDTA (pH 8)	1 mM
DTT	1 mM
Protease inhibitor tablet (Roche)	1 tablet per 100 mL of buffer

2.11 SDS-PAGE
Composition of two polyacrylamide gels:-

Components	Resolving gel (12%)	Stacking gel
Sterile distilled water	3.2 mL	1.4 mL
30% acrylamide/bis-acrylamide	4 mL	340 μL
1.5 M Tris pH 8.8	2.6 mL	-
1 M Tris pH 6.8	-	260 μL
10% SDS	100 μL	20 μL
10% APS	100 μL	20 μL
TEMED	6 μL	2 μL

SDS: Sodium Dodecyl Sulphate; APS: Ammonium Persulphate

2X loading buffer: - 100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 0.2% Bromophenol blue, 200mM β-mercaptoethanol

10X SDS running buffer: - 30 g Tris, 144 g glycine, 10 g SDS in 1L of water (diluted to 1X before use)

Staining solution: - 50% methanol, 10% glacial acetic acid, 0.1% Coomassie Brilliant Blue R-250

De-staining solution: - 30% methanol, 10 % glacial acetic acid

The samples were prepared by boiling for 5 minutes followed by a brief spin. The gels were run at 125 V till the tracking dye reached the end of the gel. Following this, the gels were stained using the staining solution for 1 hour and then de-stained overnight.

2.12 Gene cloning
To insert the fragment encoding for the periplasmic domains of EvgS into pET-41c, In-Fusion HD Cloning kit from Clonetech Laboratories was used and the manufacturer's instructions were

followed. Briefly, PCR was performed to amplify the insert using primers - NACpET41_F, NACpET41_R and NAssCpET41_F. NACpET41_F and NACpET41_R were used to amplify aa 22-554 of evgS and NAssCpET41_F and NACpET41_R were used to amplify aa 1- 554 of evgS using pBADevgS as the template. These primers were designed using the primer design tool from Clonetech Laboratories. Components of the PCR mix were:-

CloneAmp HiFi PCR mix (part of the In-Fusion kit)12.5 μL
Primer 1   5 pmoles
Primer 2   5 pmoles
Template   50 ng
Sterile distilled water   upto 25 μL

The PCR program followed was:-
Step 1   Denaturation   98°C   1 min
Step 2   Denaturation   98°C   50 s
Step 3   Annealing   55°C   50 s
Step 4   Extension   72°C   5 s/kb of plasmid length
Repeat Step 2-4 30 times
Step 5   Extension   72°C   1 min

pET-41c was linearized by digestion with *NdeI* and *HindIII*. Following this, the PCR mix and digested vector were purified by agarose gel extraction using the NucleoSpin Gel and PCR cleanup kit provided along with the In-Fusion kit. Insert:vector ratio was 2:1 and cloning was performed as recommended by the manufacturer. Colony PCR was done to check for the presence of the fragment and the correct insertion of fragment into the vector was confirmed by DNA sequencing.

2.13 Small scale protein expression and solubility
To check protein expression, overnight cultures of BL21 star (DE3) and C41 (DE3) carrying pET-41c with the insert were diluted 1:100 fold into 20 mL of LB-broth and grown at 37°C until $OD_{600} \sim 0.45$. At this point, a sample of the culture was aliquoted equivalent to 1 mL of culture at $OD_{600} = 0.5$. This is the uninduced sample. Following this, IPTG (final concentration of 100 μM) was added to the remaining culture which was grown further. Culture was aliquoted (as before) after every 60 minutes of growth for the next 3 hours. The aliquoted culture was centrifuged for 1 min at 13,000 r.p.m and washed with 1 mL of 0.85% saline or PBS. The cells were again pelleted and resuspended in 75 μL of water to which 75 μL of 2X loading buffer was added and run on polyacrylamide to check for expression of protein.

To check for solubility of protein, overnight cultures of BL21 star (DE3) and C41 (DE3) carrying pET-41c with the insert were diluted 1:100 fold into LB-broth and grown at either 37°C or 25°C until $OD_{600}\sim0.45$. Following this, IPTG (final concentration of 100 μM or 50 μM) was added to the cultures. The cultures that were grown at 37°C were further grown at 37°C or 30°C after induction and the cultures grown at 25°C were further grown at 23°C or 18°C after induction. In each case, a non-induced culture was also grown for comparison. Incubation time after induction was 5-6 hours in case of cultures grown at 30°C and 16 hours for cultures grown at 23°C or 18°C. After induction, cultures were aliquoted equivalent to 10 mL of culture at OD_{600} = 1. Cells were pelleted down and washed with 750 μL of PBS. These cells were sonicated in a microfuge tube for 5 min with 30 s burst and 15 s rest followed by centrifugation at 13,000 r.p.m for 5 minutes. The pellet was resuspended in 750 μL of water. Loading buffer (2X) was added to both the supernatant and the resuspended pellet and 25 μL was loaded onto the polyacrylamide gel for analysis.

2.14 Protein purification

Overnight culture of Bl21 star (DE3) carrying the pET-41c with the insert was diluted 1:100 fold into 400 mL of LB-broth and grown at 25C until OD600~0.45. Expression of the protein was induced by adding IPTG to a final concentration of 100 μM and culture was grown at 18C for 16 hours. Following this, the cells were pelleted down by centrifugation for 10 min at 8,000 r.p.m and then re-suspended in 25 mL of lysis buffer (composition given below). The cells were sonicated for 30 min with 30 s burst and 15 s. A sample (1 mL) was aliquoted from the cell lysate for analysis on polyacrylamide gel. The cell lysate was centrifuged for 10 min at 10,000 r.p.m at 4C and 1 mL of the supernatant was aliquoted.

For protein purification, HisTrap FF 1 mL column (from GE Healthcare Lifesciences) was used. The buffers and the samples were applied to the column via a syringe. The tip of the syringe was connected to the column, via a connector, by making a drop-to-drop connection. The flow-rate was maintained at approximately 1 drop/s. Before charging the column with Ni^{2+}, 10 mL of water was passed through the column to clean it. Following this, 5 mL of 40 mM $NiSO_4$ was applied to the column to recharge it and then 10 mL of water was again passed to remove any excess Ni^{2+}. The column was equilibrated by passing 5 mL of lysis buffer. The sample was then applied to the column in batches of 5 mL and the flow-through was collected for analysis later on. The lysis buffer (5 mL) was again passed through the column followed by washing with 25 mL of wash buffer. The flow-through was collected in batches of 5 mL. Finally, the protein of interest was eluted by passing 25 mL of elution buffer with the flow-through collected in batches of 5 mL. All the fractions were analysed by SDS-PAGE. To prepare the sample for analysis, 6 μL of the fraction was mixed with 6 μL of 2X SDS loading buffer.

Composition of buffers used:-
Lysis buffer- 30 mM Tris

300 mM NaCl
10 mM imidazole
1 protease inhibitor tablet per 100 mL of buffer

Wash buffer- 30 mM Tris
300 mM NaCl
40 mM imidazole
1 protease inhibitor tablet per 100 mL of buffer

Elution buffer- 30 mM Tris
300 mM NaCl
300 mM imidazole
1 protease inhibitor tablet per 100 mL of buffer

The pH of the buffers was adjusted to 7.4 which were then filtered by a 0.22 µM filter prior to use.

Results

3.1 Site-directed mutagenesis of histidine residues of EvgS

To determine if the eight His residues- His-63, His-72, His-106, His-124, His-153, His-180, His-205 and His-226, located in the first periplasmic domain of EvgS, play a role in its ability to detect low pH, site-directed mutagenesis was employed. Systematically at all the eight positions, His was mutated to Gln or Ala and the activity of EvgS was measured. Both Gln and Ala will be unprotonated at pH 5.6 and thus, this mutagenesis experiment will enable us to determine the His residues that are protonated at pH 5.6. Structurally, His-Gln is a conservative mutation whereas His-Ala is non-conservative mutation. Therefore, a His-Ala mutation will also aid us in determining if any of the His residues play a role in the structural stability of the protein. To determine the activity of the EvgS mutants, *E.coli* MG1655 Δ*evgS*::*aph ydeP*p-*lacZ* was used. *evgS* was complemented by transforming this strain with the pBADevgS plasmid. *ydeP*p is one of the many promoters activated by EvgA and thus, can act as an indirect reporter for the activity of EvgS. Expression of *evgS* was induced by arabinose, in cells grown at pH 5.6, and the β-galactosidase activity was measured to assess the effect of a mutation on the activity of EvgS. The β-galactosidase activity of *E. coli* MG1655 Δ*evgS*::*aph ydeP*p-*lacZ* (pBAD24) was also measured to account for the background activity due to the strain and the leakiness of *ydeP*p, and was deducted from the activity of EvgS mutants.

H63Q, H72Q, H124Q, H180Q and H205Q mutants have been made previously in our lab and do not exhibit any change in the activity compared to wild-type EvgS (unpublished data). Figure 4a shows the β-galactosidase activity of the other His-Gln and His-Ala mutants compared to the wild-type EvgS, for cells grown at pH 5.6. EvgS mutants carrying either H153A or H180A mutation did not show any change in the activity of EvgS. H72A and H205A mutants showed negligible change. For H153Q, H106A and H124A single mutants, a slight reduction (20-25%) in the activity of EvgS was observed as compared to the wild-type and for H106Q a slight increase in the activity was observed. In case of His-63 and His-226, H63Q and H226A single mutations did not result in any change in the activity of EvgS. However, introducing H63A and H226Q mutations resulted in a complete loss of the activity of EvgS. This result is a bit surprising because, as mentioned above, His-Gln is considered to be a more conservative mutation as compared to His-Ala and thus, one would expect a more radical change in the His-Ala mutant as compared to His-Gln (as is observed in case of His-63). As neither Gln nor Ala can be protonated at pH 5.6, similar activity is expected for both types of mutants. Any difference observed, as seen in case of H226Q and H226A mutants, is likely to be a result of a change in the structure of the protein.

To determine if the loss in the activity observed in case of H63A and H226Q is because of the incorrect folding or degradation of EvgS (and not because it has lost the ability to detect low pH), another point mutation, S600I, was introduced into the His mutants. The S600I mutation
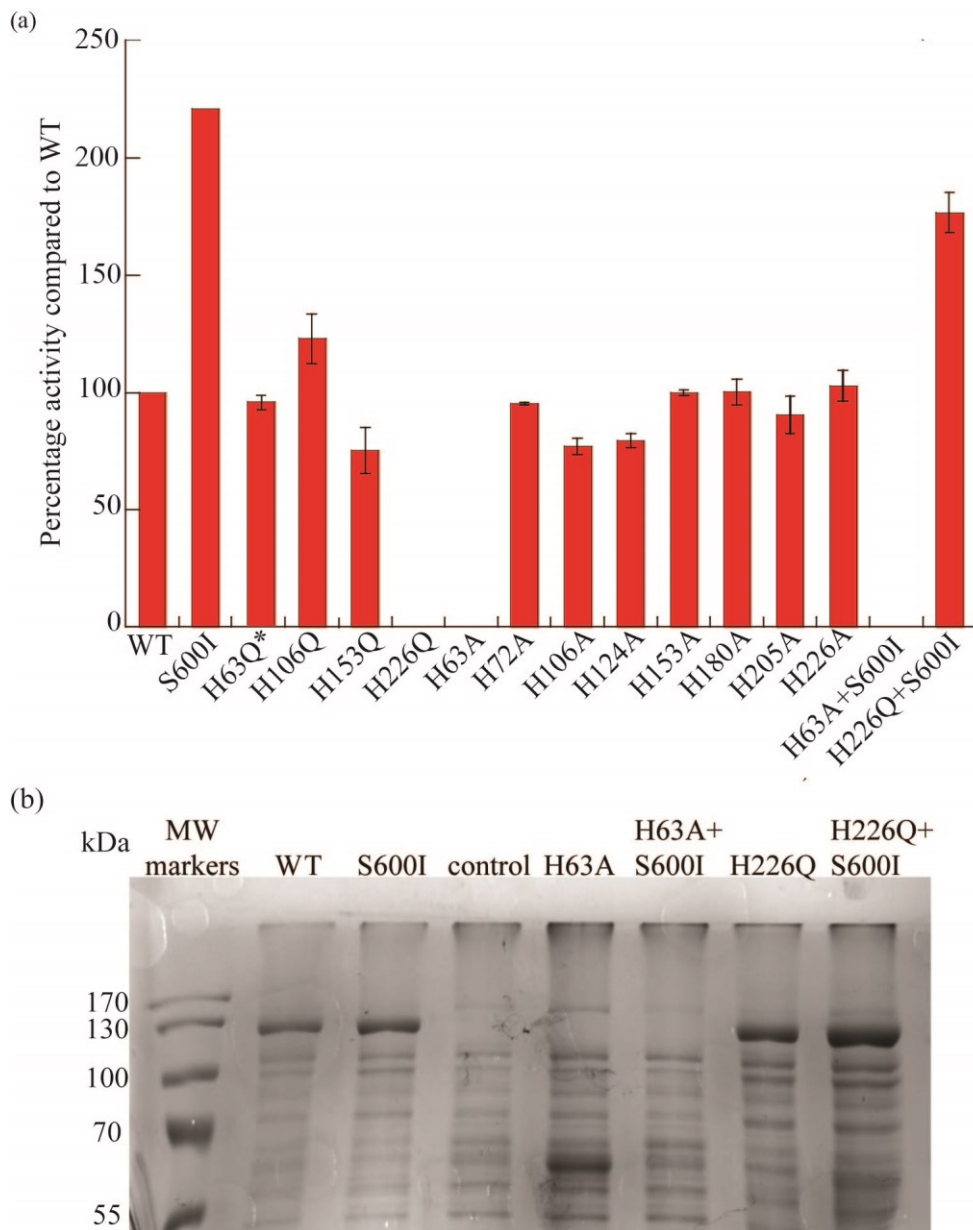
Figure 4: (a) Percentage β-galactosidase activity of EvgS mutants compared to wild-type at pH 5.6. The mutants are arranged in ascending order of the position of His residue. All the His-Gln mutants are positioned first, followed by His-Ala mutants and then the double mutants. * represents the mutant previously made. The background activity due to the *E. coli* strain and the leakiness of *ydeP*p has been deducted from all the samples. Error bars represent the standard error of the mean from three independent experiments. (b) Membrane fractions of wild-type EvgS and different mutants. EvgS has a molecular weight of 134 kDa. Control refers to the sample without the induction of EvgS by arabinose.

maps into the PAS domain of EvgS and is one of the constitutively active mutations discovered by Johnson *et. al.* (2014). As seen in Figure 4a, introducing S600I mutation into H63A mutant did not restore its activity. However, in case of H226Q, on introducing S600I, the activity of the double mutant was restored and was similar to the S600I single mutant. This result indicates towards H63A mutant being incorrectly folded or degraded due to which it is unable to constitutively activate when S600I mutation is introduced, unlike the H226Q mutant. Membrane fractions of the single mutants, H63A and H226Q, and the double mutants, H63A+S600I and H226Q+S600I, were prepared to further confirm the presence of the protein in the membrane. Figure 4b shows that H226Q and H226+S600I were present in the membrane fractions but not H63A and H63A+S600I. Thus, among all the His mutations made, only the H226Q single mutation results in a loss of activity of EvgS with the protein still present in the membrane.

3.2 Site-directed random mutagenesis of His-63 and His-226

His-63 and His-226 when mutated to Ala and Gln, respectively, resulted in the loss of activity of EvgS. These His residues were mutated to other amino acids randomly to decipher the role that they play in the activation of EvgS. Figure 5a and b shows the β-galactosidase activity of H63X and H226X (where X is a random amino acid) compared to the wild-type EvgS, for cells grown at pH 5.6. In case of His-63, H63D and H63F did not show any activity, similar to H63A. H63L, H63T, H63P and H63N exhibit activity significantly lower (60-80%) than the wild-type and H63C and H63V exhibited activity similar to H63Q and wild-type EvgS.

For His-226, H226P and H226W did not show any activity similar to H22Q and H226V, H226N and H226T exhibited activity more than 80% lower than the wild -type (Figure 5b). Hence, among the mutants made, only H226A showed activity similar to wild-type EvgS. His-226 was not mutated to all other amino acids and thus it is possible that apart from His and Ala residues, some other amino acid may exhibit activity similar to wild-type EvgS.

As before, for the mutants that do not show any activity (H63D, H63F, H226P and H226W) membrane fractions were prepared to determine if the protein is present in the membrane. From Figure 5c it can be seen that H63D and H63F are absent whereas H226P and H226W are present in the membrane. Membrane fractions were also prepared for H63L and H226V to determine if the low activity observed for these mutants is because of less amount of protein reaching the membrane. Comparing the band intensity of H63L with H63Q and H226V with H226A (in Figure 5c), it can be inferred that there is no change in the amount of protein reaching the membrane and thus is not responsible for the low activity observed for these mutants.

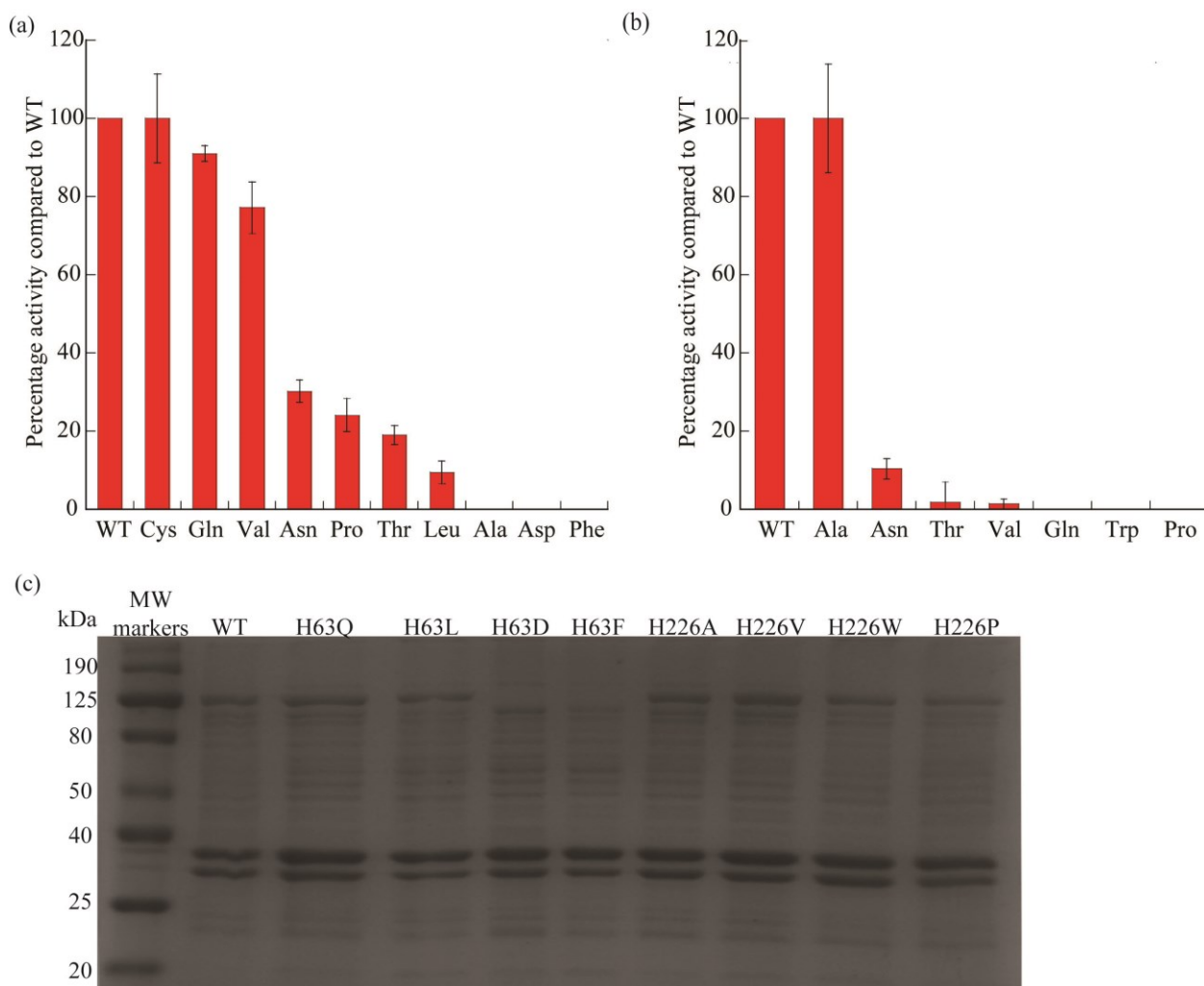**Figure 5:** (a) Percentage β-galactosidase activity of His-63 mutants compared to wild-type EvgS at pH 5.6. (b) Percentage β-galactosidase activity of His-226 mutants compared to wild-type EvgS at pH 5.6. The X-axis represents the amino acids to which the His residues in each case were mutated. Error bars represent the standard error of the mean from three independent experiments. (c) Membrane fractions of wild-type EvgS and different mutants. EvgS has a molecular weight of 134 kDa.

3.3 Role of amino acids in the activity of EvgS

The modelled structure of the first periplasmic domain of EvgS shows structural similarity to amino acid ABC transporters. Figure 6a shows the structural alignment of the first periplasmic-solute binding protein (PBP) domain of EvgS with glutamine-binding protein (PDB ID-1WDN), lysine-arginine-ornithine (LAO) binding protein (PDB ID- 2LAO) and histidine-binding protein (PDB ID- 1HSL). Owing to this structural similarity, it is possible that an amino acid may act as a ligand, binding to the first PBP of EvgS and, along with low pH, may play a role in activating EvgS. An interesting ligand will be glutamate, as this would indicate that glutamate is required for activation of EvgS as well as the glutamate-dependent acid resistance system that is regulated
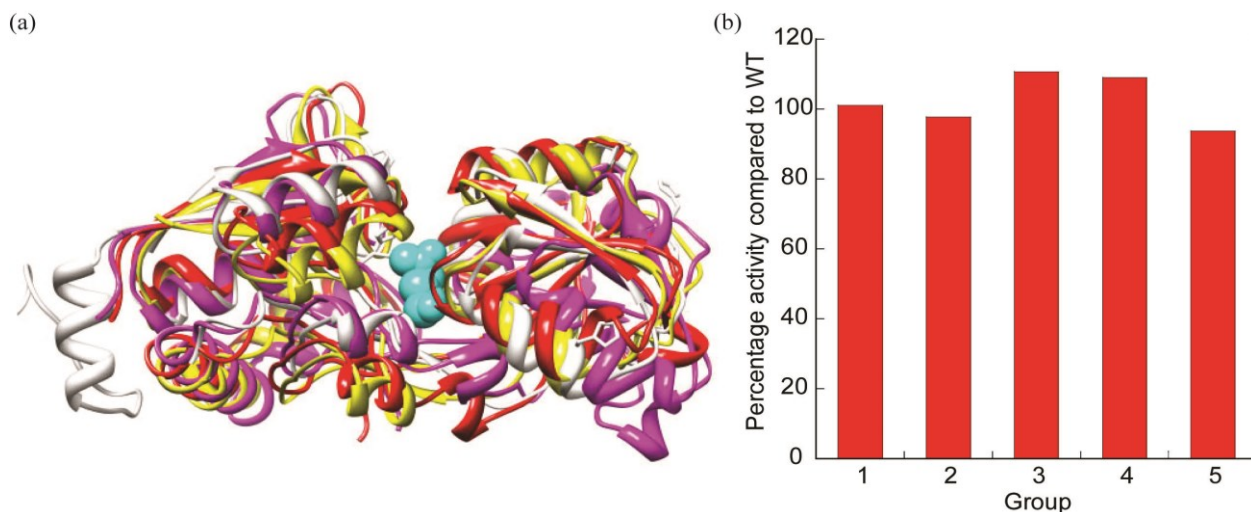
Figure 6: (a) Structural alignment of the first PBP of EvgS (white) with ABC transporters (coloured; see text for details). The ligand of the ABC transporters is shown as blue spheres. (b) Percentage β-galactosidase activity observed for EvgS in absence of groups of amino acids compared to wild-type (activity in presence of all amino acids). See text for details about groups of amino acids.

by the EvgS/A system. To determine if this is indeed the case, the 20 amino acids were grouped together into five groups based on their structure. The groups from 1 to 5, respectively, were-glutamine, asparagine, aspartate and glutamate; lysine, histidine and arginine; tryptophan, tyrosine and phenylalanine; glycine, alanine, valine, leucine, isoleucine and methionine; serine, threonine, cysteine and proline. *E. coli* MG1655 Δ*evgS*::*aph ydeP*p-*lacZ* (pBADevgS) was grown in minimal media at pH 5.6 in which each group of amino acids was systematically left out and β-galactosidase activity was measured. For all the five groups, no change in the β-galactosidase activity was observed (Figure 6b) indicating that it is unlikely for amino acids to be involved in activating EvgS.

3.4 Role of KCl in the activity of EvgS

It has been shown recently that the alkali metals Na^+ and K^+ are necessary for EvgS to detect low pH (Eguchi and Utsumi, 2014). However, in our hands, β-galactosidase activity of the wild-type EvgS, in cells grown in the absence of KCl (and at pH 5.6) is still observed, although it is lower compared to that of in cells grown with 125 mM KCl (Figure 7b). It is worthwhile to mention that, the composition of the salts in the minimal media used by us and Eguchi and Utsumi was the same. The difference was the concentration of supplements and that we added MOPS and MES to the minimal media. Since, neither the supplements nor MOPS and MES have Na^+ or K^+; these supplements are unlikely to be responsible for the difference in the activity of EvgS
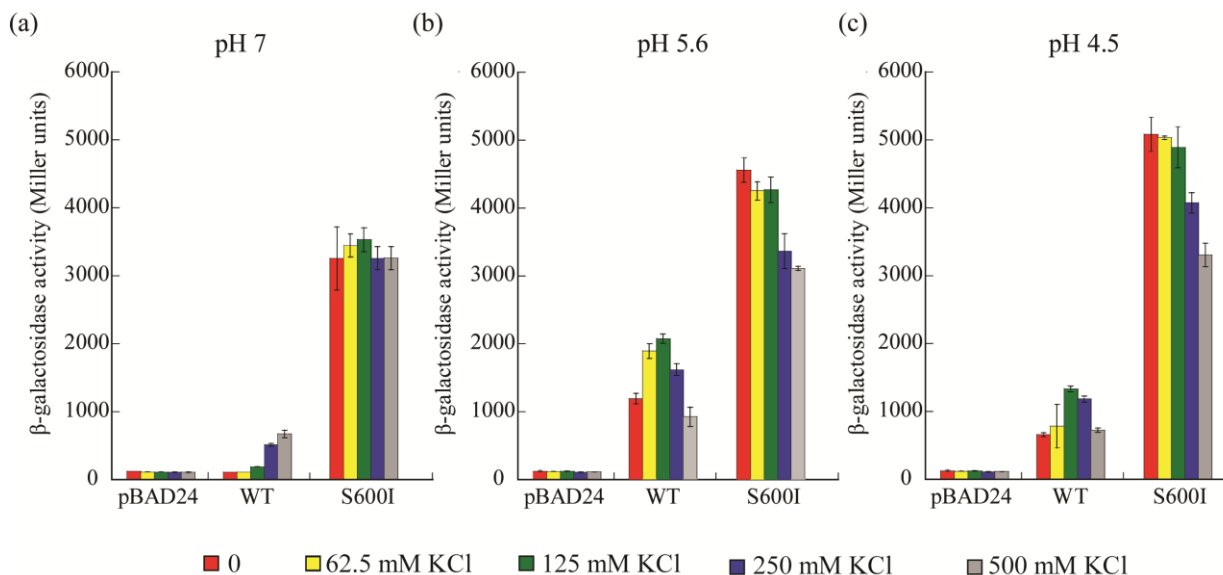
Figure 7: β-galactosidase activity of wild-type and S600I mutated EvgS at (a) pH 7, (b) pH 5.6 and (c) pH 4.5 with varying concentration of KCl. pBAD24 does not carry *evgS* and is a negative control. Error bars represent the standard error of the mean from three independent experiments.

observed in absence of KCl. However, it is still worthwhile to test the exact conditions of the β-galactosidase assay as performed by Eguchi and Utsumi. To further investigate the role of KCl in the activity of EvgS, β-galactosidase assays were done with the wild-type EvgS and the constitutively active mutant, S600I, grown with varying concentration of KCl (0 - 500 mM) at pH 7, 5.6 and 4.5. pBAD24, which does not carry the *evgS* gene, was taken as the control. At pH 7, the activity of the wild-type EvgS increased with increasing concentration of KCl, with a 6-fold increase in activity from 0 to 500 mM of KCl. For S600I, negligible change in the activity was observed with varying concentration of KCl (Figure 7a). At pH 5.6 and 4.5, the activity of the wild-type EvgS initially increased from 0-125 mM KCl, and then decreased with further increase in the concentration of KCl (Figure 7b and 7c). In case of S600I, at pH 5.6 and 4.5, the activity decreased with increasing concentration of KCl. The decrease in the activity was 1.5 fold from 0 to 500 mM KCl. It is interesting to note that at high concentration of KCl, the activity of wild-type EvgS tended to be similar irrespective of the pH level.

3.5 Expression, solubility and purification of the periplasmic domains of EvgS

EvgS is a transmembrane protein with a molecular weight of nearly 134 kDa. It is known that membrane proteins are difficult to purify as they tend to be insoluble (Trimpin and Brizzard, 2009). Therefore, we planned to express and purify only the two periplasmic domains of EvgS, for X-ray crystallography and other biophysical analysis, to determine the role of the domains and possibly the ligand/signal that activates EvgS. For this, the T7 expression system was utilized. The DNA fragment encoding for the periplasmic domains with or without the signal

peptide, that is, aa 22-554 and aa 1-554 respectively was amplified and inserted into the pET-41c vector (for C-terminal His tag). The vector, with the insert, was transformed into BL21 star (DE3) and C43 (DE3) and expression of the protein was checked as described in Materials and Methods. The expression was observed only in case of cytoplasmic expression of protein in BL21 star (DE3).

The solubility of the protein expressed in the cytoplasm of BL21 star (DE3), grown at 37°C, was checked. Expression of the protein was induced by addition of 100 μM IPTG. As seen in Figure 8a and b, the protein expressed was insoluble with most of it present only in the pelleted fraction. Therefore, to favour the solubility of protein, we modified the growth temperature of BL21 star (DE3) and the IPTG concentration for induction. Cells were grown at 30°C, 23°C and 18°C and IPTG concentration used was 100 μM and 50 μM. Figure 8c and d show that the protein was soluble at all the conditions tested. Following this, growth temperature of 18°C and IPTG concentration of 100 μM was chosen for expression and purification of his-tagged protein by affinity chromatography. Figure 9 shows that the protein eluted in the first fraction was nearly 90% pure.
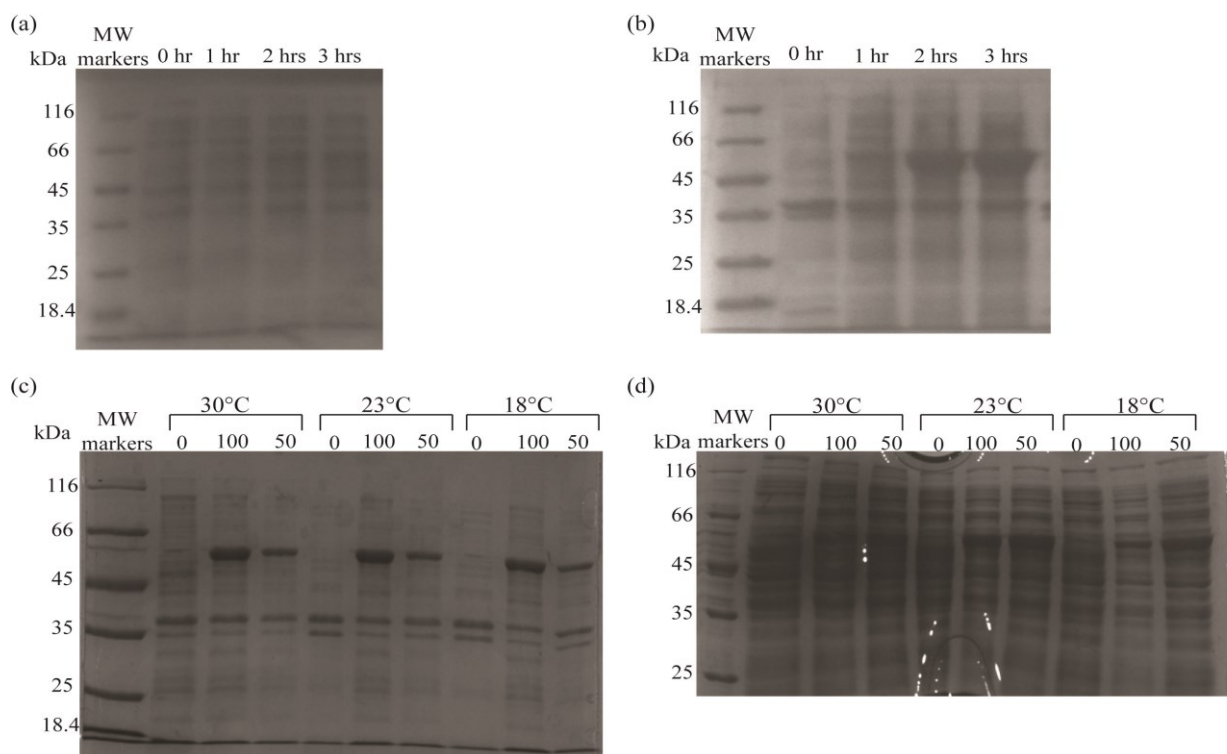
Figure 8: Testing the solubility of the periplasmic domains of EvgS (approximately 56 kDa) at different conditions. (a) and (b) Supernatant and pellet fractions, respectively, at growth temperature of 37°C and induction with 100 μM IPTG. (c) and (d) Supernatant and pellet fractions, respectively, at growth temperature of 30°C, 23°C and 18°C and induction with 50 and 100 μM IPTG.

Figure 9: SDS-PAGE to analyse the eluted fractions after expression and purification of the periplasmic domains of EvgS.

Discussion

EvgS is a histidine sensor kinase of a two-component system EvgS/A found in *Escherichia* and *Shigella*. As with most of the two-component systems, the ligand detected by EvgS is not known, although it is assumed th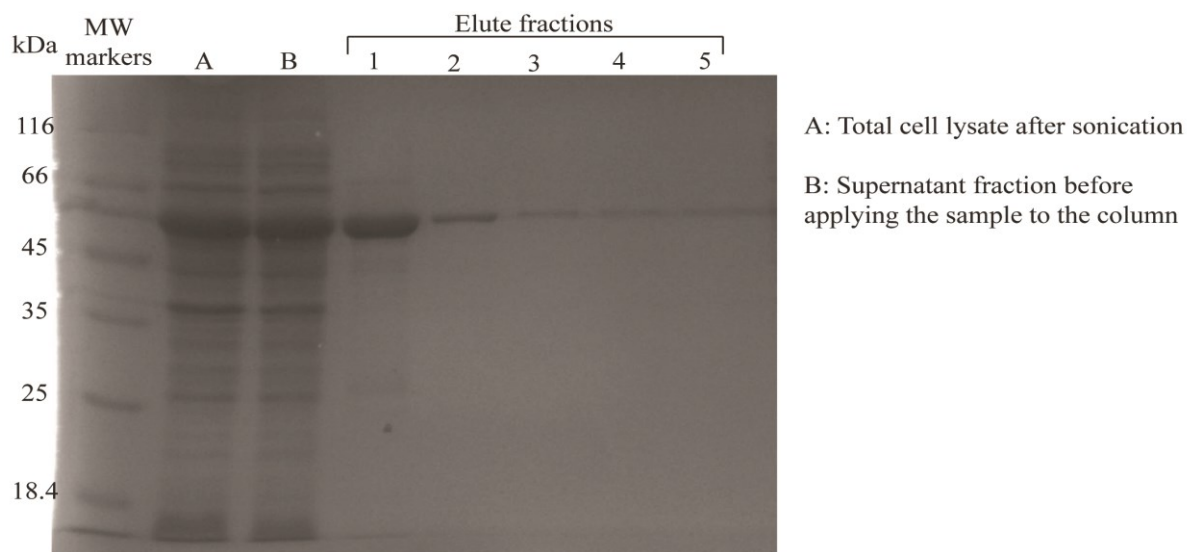at low pH activates EvgS and initiates a cascade which up-regulates acid resistance genes such as *gadABC* and *hdeAB*. EvgS is a transmembrane protein comprising of two periplasmic-solute binding protein (PBP) domains and five cytoplasmic domains. The cytoplasmic domains are involved in a phosphorelay and the function of the PBP domains is largely unknown. It is likely that the PBP domains detect low pH and relay the signal to the cytoplasmic domains. Recently, it has been reported that the linker domain, which is the cytoplasmic domain located immediately after the transmembrane helix, is also required for sensing low pH (Eguchi and Utsumi, 2014).

Burton *et. al.* (2010) have shown that EvgS exhibits optimum activity at pH 5.5-5.7. It is likely that the amino acids with a pKa (of their side chain) close to the optimum pH of EvgS may be involved in pH sensing. The pKa of the side chain of amino acids (in a protein) can vary depending upon its environment in the protein. Based on the electrostatic interaction and solvent accessibility, the pKa of amino acids can either increase or decrease (Srivastava *et al.* 2007). For example, Lu *et. al.* (2006) have shown that one of the Glu residues present in TraM has a pKa of ~7.7, instead of a pKa of 4.3 which is observed for the free amino acid in the solution. Among the charged amino acids, His has the pKa (~6.1) closest to the optimum pH range of EvgS. In case of Asp and Glu, which have a pKa of 3.9 and 4.3 respectively, there is a difference in their pKa and the optimum pH values of EvgS. However, as mentioned above, pKa values can vary, as observed in case of TraM. Thus, it is likely that His, Asp and Glu residues present in EvgS are involved in pH sensing. Lys and Arg residues, with a pKa of 10.5 and 12 respectively, will likely be protonated at both the neutral as well as the optimum pH of EvgS and thus are not expected to play a role in pH sensing. In this study, the role of eight His residues present in the first periplasmic domain, in activating EvgS, was determined. Figure 10a shows the modelled structure of the first PBP of EvgS showing all the eight His residues. Comparing Figure 10a and 6a, it can be seen that there is a cluster of His residues present in a pocket that corresponds to the amino acid binding pocket of ABC transporters (which share structural similarity with EvgS). This cluster of His comprise of His-63, His-106 and His-124 (Figure 10b), with His-63 and His-124 located on a loop whereas His-106 is at the beginning of an alpha-helix. Mutating these His residues, individually, to Ala resulted in a complete loss of activity of EvgS (in case of H63A) or a reduction in the activity (in case of H106A and H124A) (Figure 4a). For H63A, no protein was present in the membrane (Figure 4b), which may be because of incorrect folding or degradation of the mutated protein. This leads us to believe that His-63 is an essential amino acid for the
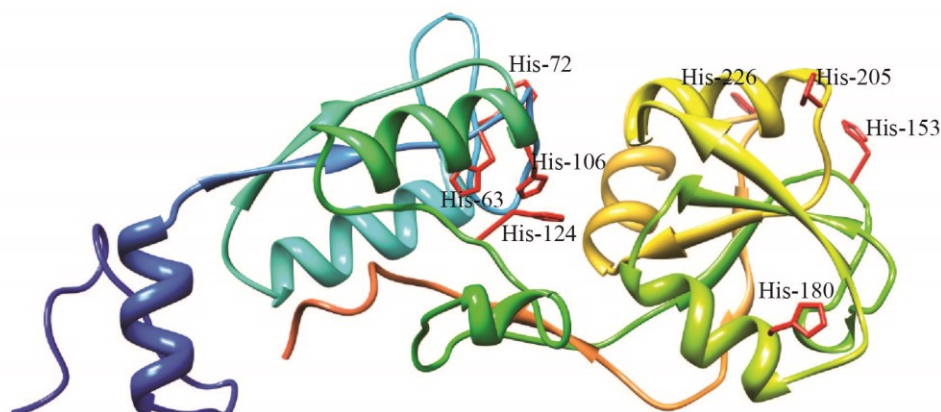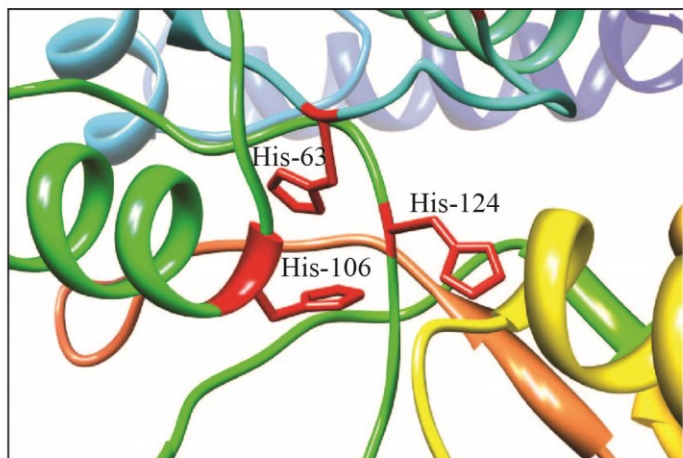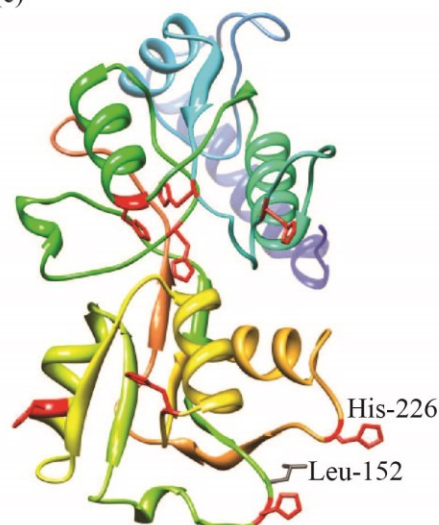
Figure 10: (a) Modelled structure of the first PBP of EvgS. The eight His residues are shown in red. (b) His cluster present in the first PBP of EvgS. (c) Structure of the first PBP of EvgS showing His-226 (in red) and Leu-152 (in grey).

correct folding of the first PBP domain of EvgS. When His-63 was mutated to Cys, no change in activity was observed (Figure 5a). The pKa of the side chain of Cys is ~8.0 and will likely be protonated at pH 5.6. It is possible that, due to this protonation, the EvgS mutant with Cys-63 exhibits the same activity as wild-type EvgS. When His-63 was mutated to other amino acids, such as Gln or Val, which are neutral at pH 5.6, the EvgS mutant exhibited reduced activity compared to the wild-type. As seen in Figure 5a, different mutants exhibited different levels of activity. A possible reason for this may be the formation of unstable protein due to the adverse effect of the mutation on the structure of the protein. However, this seems unlikely, as the amount

of protein formed for wild-type EvgS, H63Q and H63L mutants is the same (based on the band intensity in Figure 5c), although they exhibit different levels of activity. Apart from the His-63 mutants, H106A and H124A single mutants also showed reduced activity compared to the wild-type EvgS (Figure 4a). Based on this, it may be hypothesized that multiple His residues are involved in sensing acidic pH. Protonation of two or more His residues may lead to a conformational change, resulting from the electrostatic repulsion, which may act as a signal and transduced to the cytoplasmic domains. The location of the cluster favours this hypothesis as the protonation of His residues may have the same effect as the binding of the amino acid to its ABC transporter (in a structurally similar pocket). It is worthwhile to mention that the ABC transporters do not have a His cluster as is present in EvgS. Although, His-63 is positioned in between His-106 and His-124, it is possible that protonated His-106 and His-124 can repel each other, as His-124 is present on a loop that should be considerably flexible. Hence, it will be interesting to observe the activity of the EvgS mutant in which either two of these three His residues have been mutated, for example, H106A+H124A, H106Q+H124Q, H63Q+H106Q and H63Q+H124Q. Based on the hypothesis mentioned above, if any two His residues are required for EvgS to detect low pH, it would be expected that neither of these double mutants show activity. In addition, it is possible that mutating any of these three His residues to bulky amino acids, such as tryptophan, may result in a conformational change in EvgS, as a result of steric repulsion. This conformational change may possibly mimic the change due to the electrostatic repulsion of the protonated His residues and result in a constitutively active mutant of EvgS. Therefore, His-Trp mutagenesis may aid us in validating our hypothesis of multiple His residues playing a role in acid sensing.

Apart from this His cluster, mutating His-226 to Gln also abolished the activity of EvgS, although H226A mutation had no effect. Since the H226Q mutant was found in the membrane, it can be inferred that this His is also playing an important role in the activation of EvgS. From the structure of the first PBP of EvgS, it can been seen that His-226 is located on a loop which is on the surface of the protein (Figure 10c). As H226A showed activity similar to wild-type EvgS, it is unlikely that this His residue is involved in acid detection, as Ala is neutral at pH 5.6. Random mutagenesis of His-226 to other amino acids resulted in abolishment or significant reduction in the activity of EvgS, with the protein still found in the membrane (Figure 5b and c). This may indicate towards the structural, rather than functional, importance of this residue. Eguchi and Utsumi (2014) have reported a L152F mutation that leads to the inactivation of EvgS. Leu-152 is also located on a loop, close to His-226, on the surface of the protein. As both Leu-152 and His-226 are present near the C-terminal end of the first PBP, it is possible that the second PBP of EvgS may be located physically close to these residues and thus these residues are involved in transducing the signal from the first to the second PBP. The presence of these residues on flexible loops favours this hypothesis, however no direct evidence for this available at this point and further investigation is required. Determining the crystal structure of the periplasmic domains of EvgS will aid us in elucidating the exact role of these His residues.

Since the first PBP domain of EvgS shares structural similarity with ABC transporters, β-galactosidase assays were done with *E. coli* grown in absence of certain amino acids to ascertain the role that these amino acids might be playing in the activation of EvgS at pH 5.6. As seen in Figure 6b, the β-galactosidase activity is unaffected by the absence of amino acids and thus, they unlikely play any role in acid detection by EvgS. The PBP domain of EvgS is probably reminiscent of the ligand binding domain of the ABC transporters and most likely, had evolved to detect different cues or signals. Next, we investigated the role of KCl in the activation of EvgS (Figure 7). Eguchi and Utsumi (2014) have reported that no activity of EvgS is observed in the absence of K^+ which was not the case in our hands as we could still observe the activity of EvgS, although it was lower than in presence of 125 mM KCl (Figure 7b). Nevertheless, both studies indicate towards K^+ playing a role in the activation of EvgS. K^+ is known to be involved in cytoplasmic pH homeostasis via K^+/H^+ pumps, which maintain pH by the exchange of K^+ and H^+(Booth, 1985). Other alkali metals such as Na^+ are also involved in maintaining the cytoplasmic pH. Richard and Foster (2007) have shown that Na^+ modulates the activity of GadX and GadW, two AraC-like regulators of *gadE*, which in turn is a regulator of the AR2 system. Although the exact mechanism by which K^+ affects the activity of EvgS is unknown, one possibility is the allosteric effect of K^+ on EvgS. Many examples of allosteric effect of K^+ on the structure of a protein have been reported before (Page and Cera, 2006). Forming a coordination complex with K^+ may result in structural changes in EvgS that promotes the detection of low pH, although the high concentration of K^+ required for this effect makes this hypothesis unlikely as K^+ concentration of less than 1 mM is apt for structural changes to occur (Page and Cera, 2006). Another possibility is that EvgS, instead of detecting K^+ directly, is reacting to some consequence of high concentration of K^+. It has been shown that *E. coli* cells grown in minimal media and subjected to osmotic shock by high K^+ concentration accumulate glutamate as a counter-ion. This is then replaced by accumulation of other osmoprotectants such as trehalose or proline (Kempf and Bremer, 1998). Therefore, it is possible that one of these osmoprotectants is affecting the activity of EvgS.

Finally, we expressed and purified the two periplasmic domains of EvgS. Optimizing the conditions for expression of protein showed that growth temperature of 18-30°C with induction by IPTG at a final concentration of 50 or 100 μM resulted in the expression of a soluble protein. The protein will be analysed further and will aid us in validating the hypotheses stated above regarding the acid detection mechanism and role of K^+ on the activity of EvgS.

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